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Glial fibrillary acidic protein and related glial proteins as biomarkers of neurotoxicity

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A variety of '-omic' technologies are being increasingly applied in preclinical safety assessments. Such approaches, however, have not been implemented in neurotoxicity safety evaluations. Current regulatory guidelines for assessing neurotoxicity emphasise reliance on traditional histopathological stains and behavioural testing batteries. Although these methods may be sufficient to detect some neurotoxic effects, they lack both the sensitivity and specificity required for broad-scale neurotoxicity screening. The glial reaction to nervous system damage, often termed gliosis, represents a hallmark of all types of nervous system injury. As such, the development and implementation of gliosis biomarkers represents a broadly applicable approach for neurotoxicity safety assessment. Using a panel of known neurotoxic agents, the authors have shown that the astroglial protein, glial fibrillary acidic protein (GFAP), can serve as one such biomarker of neurotoxicity. Qualitative and quantitative analysis of GFAP has shown this biomarker to be a sensitive and specific indicator of the neurotoxic condition. The implementation of GFAP and related glial biomarkers in neurotoxicity screens may serve as the basis for further development of molecular signatures predictive of adverse effects on the nervous system

Keywords: biomarkers, drug development, drug safety assessment, drug toxicolgy, glial biomarkers, glial fibrillary acidic protein (GFAP), gliosis, molecular neurotoxicology, preclinical drug screening

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1. Introduction

Determining the toxic effects of drugs on the nervous system is hampered by a lack of knowledge as to what constitutes a neurotoxic response. Although intense current interest is focused on establishing the molecular and cellular basis of neurological diseases such as Alzheimer's and Parkinson's disease, or the early molecular signatures of the damaging effects of stroke and neurotrauma, relatively little effort has been directed towards a broader understanding of molecular and cellular events common to neurotoxic effects, (i.e., drug- or chemical-induced damage of the nervous system). Thus, whereas histological features and biochemical changes associated with specific neurological disease states can be identified in postmortem brain tissue from humans, or from brain tissue prepared from animal models of a given disease condition, broadly applicable biochemical markers for detecting all types of neurotoxic effects remain to be defined. This is not a surprising situation, given that the neural (i.e., neuronal and/or glial) targets of agents toxic to the nervous system are not only extremely diverse but they are also unpredictable, owing to the extreme molecular and cellular heterogeneity of the mammalian CNS [1]. Unfortunately, this neurobiologically based 'selective vulnerability' remains the central obstacle to the establishment of a molecular framework for defining end points linked to neurotoxic effects.

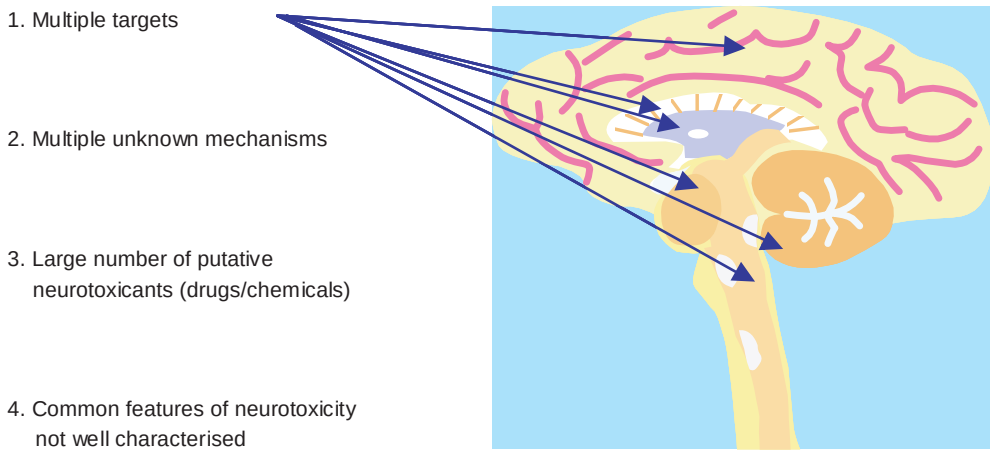

Figure 1. The neurotoxicity dilemma.

Indeed, our knowledge of the relationship between a specific set of molecular changes and subsequent damage to the nervous system is just now moving from a conceptual stage to experimental validation [2-9]. These issues frame the neurotoxicity dilemma (Figure 1). Thus, for a given chemical/drug exposure, there are multiple potential targets of neurotoxicity working through multiple and unknown mechanisms. Moreover, there are potentially large numbers and classes of putative neurotoxicants for which there is no known shared toxicological feature. Viewed in terms of a risk assessment paradigm, where hazard x exposure = risk, the scientific basis for hazard identification must be established to obtain a valid basis for assigning risk. Essentially, hazard identification remains the key issue facing contemporary neurotoxicology.

2. The need for more sensitive and specific measures

Historically, neurotoxicity has been assessed by relying on classical histological observations performed by the pathologist and, more recently, by applying behavioural analysis methodologies of the experimental psychologist. Molecular approaches have not been implemented for hazard identification with respect to neurotoxic effects, despite the fact that a variety of molecular technologies are being introduced in other areas of preclinical safety assessment [10]. The authors' contention is that the introduction of molecular approaches to neurotoxicity assessment is overdue. This recommendation is not based on the desire to follow current trends; rather, it is based on the recognition that present methods for neurotoxicity screening may not be adequate for addressing the problem. For example, as the authors detail below, standard tools of the histopathologist, when applied to nervous tissue anatomy, suffer from a lack of sensitivity (i.e., brain damage is present but not detected). Behavioural analysis of neurotoxicity ('behavioural toxicology'), in contrast, suffers from a lack of specificity (i.e., a change from baseline can be detected but such effects often reflect physiological, pharmacological or non-nervous system actions of a compound in question [11,12]). Moreover, one cannot assume that behaviour, the final output of the nervous system, is a sensitive measure. The structural reserve of the nervous system can confer an enormous barrier to disruption in function. For example, motor or cognitive declines associated with the development of Parkinson's disease and Alzheimer's disease, respectively, do not manifest themselves until the disease has progressed for many years. Thus, even though behavioural test methods are currently used as a 'rodent neurological exam,' the authors note that these test batteries are no more sensitive than simple cage-side observation in terms of detecting apparent adverse effects on the nervous system [13]. Moreover, neurobehavioural assessments, in general, are less sensitive than general toxicology parameters in neurotoxicity hazard identification [14]. In summary, the drawbacks associated with existing morphological and behavioural approaches to neurotoxicity assessment are suggestive of the need for a more sensitive and specific means with which to detect and quantify neurotoxicity (i.e., adverse effects on the nervous system engendered by pharmaceutical and chemical exposures to broad classes of agent and mixtures). Clearly, this need can be addressed through the development and validation of sensitive and specific biomarkers of neurotoxicity.

3. Desirable features for biomarkers of neurotoxicity

Given the above considerations and requirements, one can begin to describe the desirable features for a biomarker of neurotoxicity (Box 1). First and foremost in the evaluation of drugs or chemicals, a neurotoxicity biomarker must reflect the adverse effect of a given agent on a neurobiological target and it must change in the predicted manner no matter what type or class of drug is being assessed. In the absence of prior information to direct a given toxicological evaluation to a specific

Box 1. Biomarkers of neurotoxicity: desirable features.

- Respond to diverse types of insults affecting any region
- Sensitive – low incidence of false-negatives
- Specific to the neurotoxic condition – low incidence of false-positives
- Simple to evaluate
- Quantitative

neural substrate in a specific brain region, as is most often the case, any site within the entire nervous system must be viewed as a potential target and the biomarker response must reveal it. A particular pharmacological profile or target of a drug should not be of particular concern due to the fact that drug-induced neurotoxic effects are not simply a dose-related extension of their pharmacological effects; therefore, a molecular or cellular target of neurotoxicity cannot be assumed.

The sensitivity of a neurotoxicity biomarker must be such that damage to any subcellular or cellular target in any region of the nervous system is revealed in order to avoid false-negatives. Classical histological stains are clearly not sufficient to fulfill the sensitivity requirement because they will not reveal evidence for neurotoxicity beyond overt neural cell loss or damage, the so-called red and dead response seen with haematoxylin and eosin staining [15]. Moreover, such cell loss or damage must be in layered structures of the brain to be obvious or, alternatively, decreases in cell loss must exceed 30 – 40% to be visible against the background of unaffected cells. Modern stereological approaches can help overcome problems associated with reliance on subjective detection of cell loss because stereology will yield true quantitative data [16,17]. In practice, however, one must first decide which brain area to analyse and no guideposts exist to obtain this information. Viewed in very practical 'real-world terms', detection of neural cell damage in brain tissue is plagued by a signal to noise problem. Small, but neurotoxicologically significant, damage may simply be masked by normal surrounding cells. Recent advances have been made to overcome this problem, including the development and implementation of two specialised staining procedures, as well as the authors' combined morphological and biochemical analysis of gliosis (see Section 4). The staining approaches, silver degeneration/disintegration staining [15,18] and Fluoro-Jade B degeneration [19,20] stains both have the advantage of revealing very discrete areas of damage by standing out as positive signals against a negative background. Although each of these neuropathological stains have slight individual disadvantages, their implementation in neurotoxicity assessment screens would move the neurohistopathology field dramatically forward by providing much needed increases in sensitivity.

If a neurotoxicity biomarker is to have utility, specificity must go hand-in-hand with sensitivity. Thus, in addition to the need for sufficient sensitivity to overcome the problem of false-negatives, it is equally important for a biomarker not to be responsive to conditions that do not cause damage to the nervous system (false-positives). For example, it is difficult to envision the usefulness of a biomarker of neurotoxicity, no matter how sensitive it is to a given neurotoxic exposure, if the same biomarker responds to physiological changes or administration of pharmaceuticals at therapeutic dosages. Drugs designed to affect a neurotransmitter or its receptor might be viewed as 'neurotoxic' if decreases in these same transmitters or receptors were viewed as damage or loss to specific neuronal cell types. Although reversibility may be at issue here, with toxic effects presumed to be long-lasting, protracted down- or upregulation of receptors and neurotransmitters may be taken as desired signs of drug efficacy and represent a normal regulatory action of a drug on a specific neurotransmitter system. In this regard, it is difficult to see how behavioural testing batteries can be used to assess drug-induced neurotoxicity because these same tests, now recommended for neurotoxicity testing by the US Environmental Protection Agency [101], were originally designed and implemented for drug discovery in neuropharmacology. The issue of specificity with respect to neurotoxicity assessments will continue to be a problem as '-omic' approaches are implemented for developing biomarkers of neural-acting pharmaceuticals. Discovery leads that stem from such analyses may overlap with data obtained from molecular toxicity screens. Where similar effects on a given biomarker emerge from discovery and toxicity screens, interpretation of the relationship of such changes to 'efficacy' as opposed to 'toxicity' may become problematic and this issue is likely be a pressing problem in the near future [21].

The above outlined requirements for neurotoxicity biomarkers would appear to be near absolute; however, the authors have also listed simplicity and quantitative as desirable features. Under simplicity, one could infer 'high-throughput', but that would seem premature at this juncture because the extreme molecular and cellular heterogeneity of the developing and mature nervous system does not readily lend itself to high-throughput analysis. Contemporary risk assessment paradigms and reasonable concerns of regulatory agencies for the development of drug safety databases suggests the need for quantitative approaches, regardless of the particular nature of a given biomarker introduced for neurotoxicity testing.

4. Gliosis: a common feature of central nervous system damage

The awareness of the drawbacks of current approaches to neurotoxicity assessment led the authors to consider what features are desirable for a widely applicable biomarker of neurotoxicity. These deliberations, in turn, led us to embark on an examination of gliosis as a potentially common feature to unify all neurotoxic responses.

For at least a century, the neuropathology literature has documented that damage to the CNS results in conversion of microglia and astrocytes into their 'reactive' or 'activated'

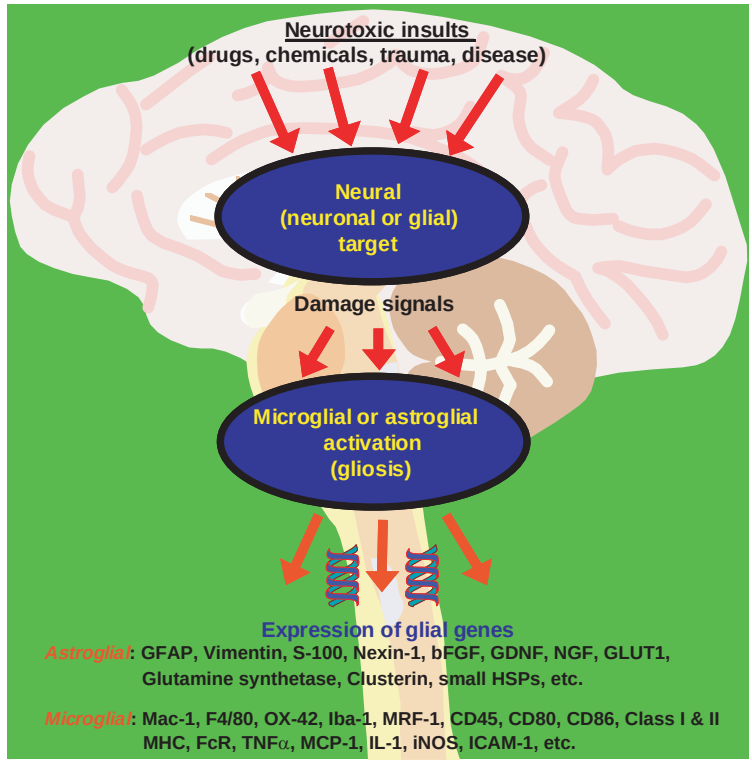

Figure 2. Cellular events associated with disease, injury or toxicant-induced gliosis.
bFGF: Basic fibroblast growth factor; FcR: Fc receptor; GDNF: Glial-cell line-derived neurotrophic factor; GFAP: Glial fibrillary acidic protein; GLUT: Glucose transporter; HSP: Heat-shock protein; ICAM: Intercellular cell-adhesion molecule; iNOS: Inducible nitric oxide synthase; MCP: Monocyte chemoattractant protein; MHC: Major histocompatibility complex; MRF: Microglial response factor; NGF: Nerve growth factor; TNF: Tumour necrosis factor.

form. Microglia and astrocytes constitute subtypes of glial cells, glia and neurons being the main cellular constituents of the nervous system. Injury-induced transformation of microglia and astrocytes into their 'activated' phenotype often is referred to as 'reactive' gliosis or simply, gliosis. Trauma, ischaemia, infectious and neurological diseases and, more recently, chemical exposures, are all known to have the capacity to induce gliosis. Despite the century-old recognition of gliosis as a response to nervous system damage, only recently have biochemical features of this cellular response been documented. Thus, evaluation of gliosis is hardly a novel approach for assessing brain damage but it is one for which a 'biomarker' component is just now being explored. What is now known, is that early in the time course of a nervous system cell type response to injury, microglia and astroglia become activated, elaborating their cellular processes and increasing their expression of glial genes (Figure 2). It is not yet clear as to the functional significance of gliosis; however, recognition that it is a rapid and universal response to all types of brain insults argue in favour of a role for gliosis in repair and recovery [3,4,9,22,23]. From the standpoint of a neurotoxicological assessment strategy, the neurobiological significance of gliosis need not be known as long as it is a consistent response to neural injury. Moreover, mechanisms underlying this response also do not have to be known in order for biomarkers of gliosis to be developed and validated.

4.1 Glial fibrillary acidic protein as a key biomarker

If gliosis is a dominant and universal response to nervous system damage, what are the key biomarkers of this cellular reaction to injury? Of the many glial genes now known to be expressed during gliosis (see Figure 2); the oldest and most well documented is glial fibrillary acidic protein (GFAP). The astroglial component of gliosis is characterised at the electron microscopic level by the accumulation of glial filaments, of which GFAP is the major constituent. By definition, therefore, astrocytic gliosis is accompanied by an increase in GFAP. Indeed, the use of antibodies to GFAP in histological studies has firmly established the existence of reactive gliosis as a dominant response to many different types of brain injuries [3,9,23,24]. Although GFAP immunohistochemistry has not been applied in the context of first level neurotoxicity screens, where tissue sections from neurotoxicant exposed animals have been evaluated, enhanced expression of this protein has been seen in target-appropriate brain regions. Thus, based on the accumulated morphological data, the ideal 'biomarker' of all types of nervous system injuries is enhanced expression of GFAP.

4.2 Validation strategy

To establish GFAP and other microglia- and astroglia-associated genes as biomarkers of neurotoxicity a validation strategy was required. The general strategy pursued by the authors was

Table 1. 'Training set' neurotoxicants and their known targets (compounds known to induce gliosis).

Toxicant	Regional target	Cellular target	Subcellular target
Trimethyltin	Limbic structures	Neurons	Perikarya
Triethyltin	Limbic structures	Neurons	Perikarya
Kainic acid	Limbic structures	Neurons	Perikarya
Domoic acid	Limbic structures	Neurons	Perikarya
MPTP	Neostriatum	Dopaminergic neurons	Nerve terminals
Methamphetamine	Neostriatum	Dopaminergic neurons	Nerve terminals
6-Hydroxydopamine	Neostriatum	Dopaminergic neurons	Nerve terminals, perikarya
MDA	Neostriatum	Dopaminergic neurons	Nerve terminals
MDMA	Neostriatum	Dopaminergic neurons	Nerve terminals
Cadmium	Striatum	Neurons, glia	?
Methylmercury	Cortex, hippocampus	Neurons	Nerve terminals, perikarya
Methylazoxymethanol	Cortex, hippocampus	Neurons	Nerve terminals, perikarya
Bilirubin	Cerebellum	Purkinje neurons	Perikarya
Colchicine	Hippocampus	Dentate neurons	Perikarya
3-Acetyl pyridine	Inferior olive	Neurons	Perikarya
Iminodipropionitrile	Cortex, brain stem, olfactory bulb	Neurons	Glomerular layer of olfactory bulb, cortical axons
MK-801 (Dizocilpine)	Cortex	Neurons	Perikarya?
Ketamine	Cortex	Neurons	Perikarya?
5,7-Dihydroxytryptamine	Hippocampus	Serotonergic neurons	Nerve terminals, perikarya
2,6-Dichlorobenzonitrile	Olfactory bulb	Sensory neurons	Nerve terminals, perikarya

MDA: Methylenedioxymethamphetamine; MDMA: d-3,4-methylenedioxymethamphetamine; MK-801: 5-methyl-10,11-dihydroxy-5h-dibenzo (a,d)cyclohepten-5,10-imine; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

to use known neurotoxicants as tools (often referred to as the 'training set' in genomics) to damage the nervous system (Table 1). Importantly, to mimic real-world scenarios, the training set was constructed of diverse types of compounds that affected diverse areas of the nervous system. Thus, > 30 different neurotoxicants were purposely chosen, each of which preferentially damaged a specific region of the nervous system and, in a given region, specific cell types were targeted. Finally, a given compound often preferentially affected a specific subcellular element of a given cell type. Not only would this approach serve to validate the expected 'proof of concept' increases in GFAP, but it would provide a rich source of tissue which could subsequently be used to potentially discover novel biomarkers of neurotoxicity. Because the authors' overall goal was to develop a quantitative biomarker for neurotoxicity hazard identification, they chose not to rely solely on a qualitative immunohistochemical approach for examination of GFAP. This dictated the need to develop an assay for this protein. Two different GFAP assays have been developed in the authors' laboratory and specific protocols have been published [25,26]. Moreover, standard operating procedures for this assay are available upon request and performance of this assay in a contract research organisation setting can be fully good laboratory practice-compliant. A flow chart for tissue preparation and analysis of GFAP by immunoassay is presented in Figure 3.

In the context of preclinical safety assessment, histochemical and biochemical analysis of GFAP each have their advantages and disadvantages. Implementing neurotoxicity screening with GFAP immunohistochemistry is advantageous because it can easily be added to existing toxicity screening protocols. Thus, the current practice of preparing brain sections for classical histological stains can be incremented to include additional sections for GFAP immunohistochemistry. This can be achieved at relatively little additional cost in terms of time, labour and expense. As the authors have stressed above, however, the selective and unpredictable vulnerability of different regions of the nervous system to toxic insults dictates the need to cut sections throughout the rostral-caudal axis of the brain in order not to miss sites showing gliosis. This may entail the addition of more tissue sections than would normally be sampled in routine veterinary pathology examinations. The subjective results obtained from these additional sections, where positive (i.e., showing increased GFAP immunoreactivity), can then be combined with staining of other tissue sections containing the affected region to confirm neural damage with sensitive neuronal degeneration stains (silver staining, Fluoro-Jade B staining) and/or stains for microglial activation (e.g., Lectin

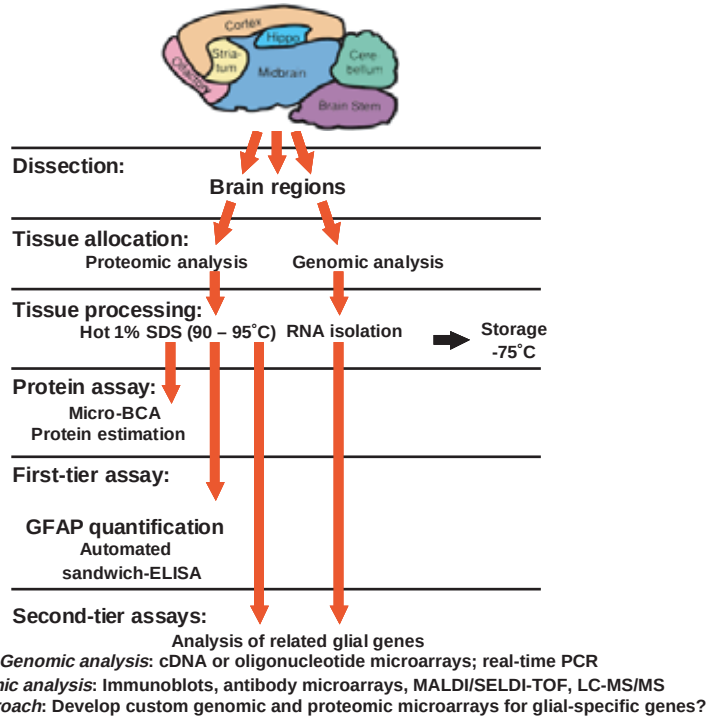

Figure 3. Sample preparation flow-chart for GFAP and second-tier assays.

BCA: Bicinchoninic acid; ELISA: Enzyme-linked immunosorbent assay; GFAP: Glial fibrillary acidic protein; LC-MS/MS: Liquid chromatography-mass spectrometry/mass spectrometry PCR: Polymerase chain reaction; MALDI: Matrix-assisted laser desorption ionization; SDS: Sodium dodecyl sulfate; SELDI: Surface enhanced laser desorption ionization.

Box 2. GFAP analysis as a biomarker of neurotoxicity: what has been learnt?

- All types of drug/chemical-induced damage to the CNS result in enhanced expression of GFAP
- Drugs at therapeutic doses do not affect GFAP
- Increases in GFAP are very rapid and are linked to the time course and location of damage
- Increases in GFAP occur at toxicant doses below those associated with overt cytopathology, as assessed by classical histology
- Increases in GFAP occur at toxicant doses below those associated with behavioural changes
- Although less validated, other biomarkers of gliosis (S-100, pSTAT3, Mac-1, F4/80, MCP-1) appear to serve as sensitive and broadly applicable biomarkers of neurotoxicity

GFAP: Glial fibrillary acidic protein; MCP: Monocyte chemoattractant protein; STAT: Signal transducer and activator of transcription.

staining). The disadvantage of using GFAP immunohistochemistry is that background immunostaining for this protein in specific brain areas (e.g., hippocampus, cerebellum) may mask small but neurotoxicologically significant increases in GFAP. Moreover, from a quantitative risk assessment point of view, GFAP immunoreactivity assessments are inherently qualitative and do not lend themselves to quantification. Implementing neurotoxicity screening with GFAP assays (mRNA and protein) has the inherent advantage of being quantitative, thus lending analysis of GFAP to quantitative risk assessment paradigms. In addition, direct comparisons of GFAP immunostaining procedures with GFAP assays show that the latter is more sensitive. Using GFAP assays to screen for neurotoxicity will add costs in terms of labour and time because an additional group of animals will have to be added and brain dissection procedures will need to be implemented to insure sampling of sufficient brain regions to detect areas of increased GFAP that would otherwise be masked by background levels. These disadvantages are offset by the opportunities for discovery and validation of additional mRNA and protein species that can be linked to neural damage (Figure 3). Effects that emerge from these analyses can then be compared to effects observed in drug discovery screens, with the ultimate goal of defining changes that are linked to toxicity from those that are linked to efficacy.

4.3 Results of validation exercise: what has been learnt?

A summary of the results of our exercise to validate GFAP and other glial biomarkers of neurotoxicity appears in Box 2. Using brains from experimental animals [3,4,9] or samples of postmortem human brain [27], combined with the implementation of the novel assay procedures described above, the authors have shown that the enhanced expression of GFAP mRNA and protein occurs after exposure to a

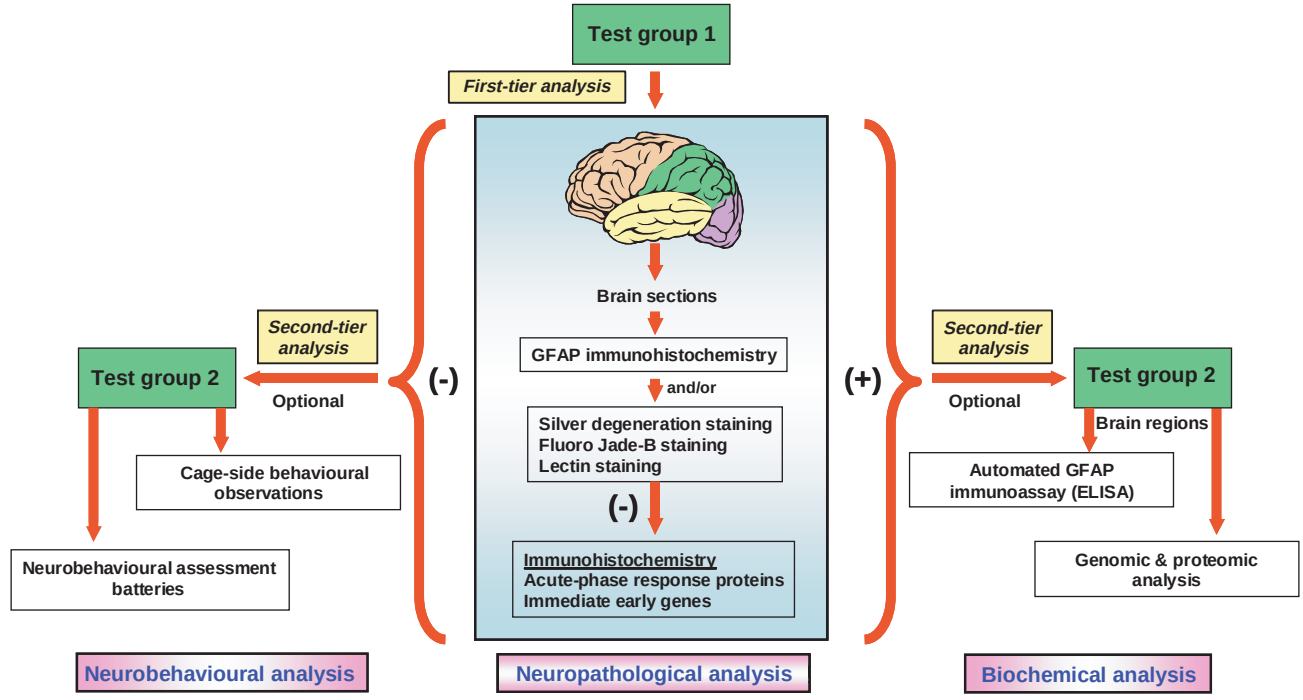

Figure 4. Screening for neurotoxicity. First-tier histochemical analysis of GFAP and associated second-tier assays. The initial step in screening for neurotoxicity would be to analyse GFAP by immunohistochemistry with/without inclusion of degeneration stains (first-tier analysis). (-): Failure to observe increased GFAP may necessitate analysis of some early response genes or neurobehavioral screening to rule out CNS effects independent of neural damage (second-tier analysis). (+): On the other hand, the increase in GFAP detected by immunohistochemistry may be further validated by an automated immunoassay to obtain quantitative data (second-tier analysis). Finally, the analysis could be further extended to screen for other glial biomarkers by genomic and proteomic assays.
ELISA: Enzyme-linked immunosorbent assay; GFAP: Glial fibrillary acidic protein.

diverse variety of neurotoxic agents or neurological diseases. Importantly, increases in this biomarker of neurotoxicity occur very early in the time course of neural injury and at toxicant dosages or disease conditions below those that result in overt cytopathology or behavioural abnormalities. Moreover, the authors have shown that enhanced expression of GFAP and other glial biomarkers of neurotoxicity occur at the site of neural injury. Finally, the authors have demonstrated that agents/conditions that do not cause neural damage do not result in the enhanced expression of GFAP and related glial biomarkers. Thus, enhanced expression of GFAP and other biomarkers of gliosis represent sensitive and specific indices of toxicant- and disease-induced neural damage and satisfy all the desirable requirements for a biomarker of neurotoxicity (see section above). More recently, the link between astroglial activation and neural damage has been extended to a second glial cell type: microglia. Although our knowledge of gene-expression events related to microglial activation is limited, studies to date show that, as with astroglia, activated microglia serve as microsensors of all types of brain pathology, including that engendered by toxic exposures [28,29].

Taken together, the results of the authors' validation exercise indicate that adding glial biomarkers to current neurotoxicity screening approaches can add sensitivity and specificity at modest cost and, potentially, with the use of less animals. Therefore, the authors suggest that neurotoxicity screens include GFAP immunohistochemistry as an obligatory component at the first 'tier' (Figure 4). This could be performed utilising automated immunohistochemical procedures to insure reproducibility and to reduce the already modest time requirements and costs associated with such analyses. Sensitive neurodegeneration stains could also be included at this first-tier level or be used as follow-ups to positive results from the GFAP immunohistochemical evaluations. Positive results at this first level of neurotoxicity screening would be taken as sufficient enough evidence for an adverse effect to preclude additional behavioural, neuroanatomical or biochemical evaluations with commensurate savings in terms of time, cost and animal use. The qualitative effects observed in the first-tier could then be followed with the GFAP immunoassay, both as a confirmatory exercise and to provide quantitative data, albeit with the addition of another set of test animals. A representative data set obtained with this approach is shown in Figure 5. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a toxicant known to destroy dopamine-containing neurons, was administered to mice and brain sections were prepared at various postdosing time points. A separate set of mice was used to prepare samples for analysis of GFAP, either by real-time polymerase chain reaction or enzyme-linked immunosorbent assay (ELISA). The data show a marked increase in GFAP

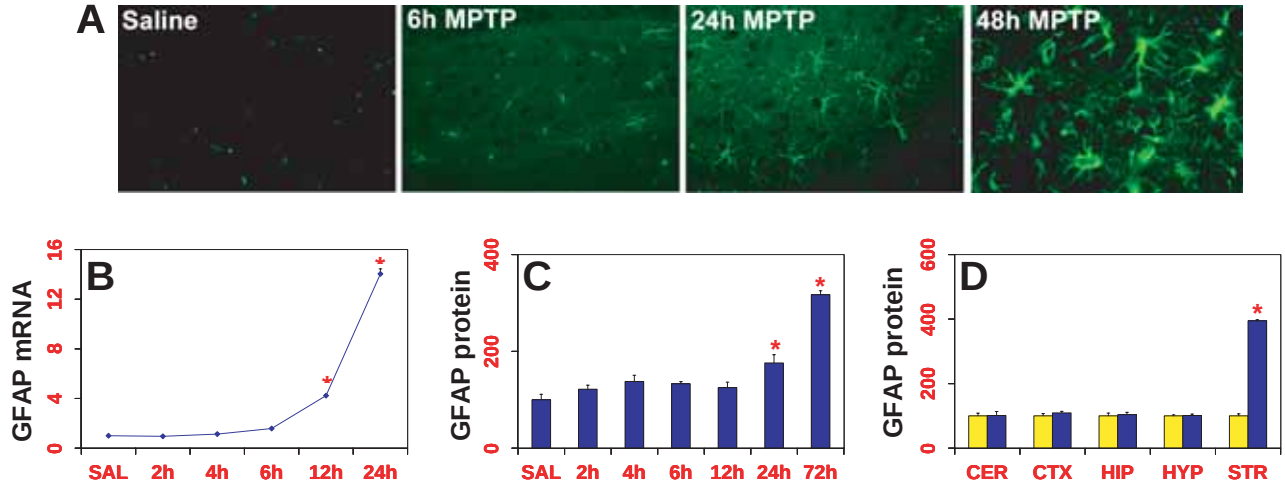

Figure 5. Time-course and location of GFAP-induction following treatment with MPTP. The dopaminergic neurotoxicant, MPTP, results in a rapid induction of astrogliosis in the affected region of mouse brain (striatum). (**A**) Immunohistochemical analysis of GFAP reveals a time-dependent astrogliosis following MPTP (12.5 mg/kg, s.c.). (**B, C**) Levels of GFAP mRNA and protein were measured in striatum by TaqMan® real-time PCR or sandwich ELISA and are represented as fold or percentage increase over corresponding saline treated controls, respectively. (**D**) GFAP protein levels in various brain regions of saline (yellow bar) or MPTP (blue bar) treated mice, 48 hours post-MPTP.

CER: Cerebellum; CTX: Cortex; ELISA: enzyme-linked immunosorbent assay; GFAP: Glial fibrillary acidic protein; HIP: Hippocampus; HYP: Hypothalamus; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SAL: Saline STR: Striatum.

immunostaining of astrocytes in the striatum over a 48 hour postdosing interval. These qualitative observations were associated with quantitative increases in GFAP expression, as evidenced by analysis of GFAP mRNA and protein levels in samples prepared from the target brain region (striatum). Non-target brain areas served as negative controls and elevations in GFAP were not seen in these regions. As with analysis of GFAP as a generic biomarker of MPTP-induced damage, silver degeneration staining also revealed damage in the target region, damage that was not apparent with conventional Nissl-based histopathology (data not shown) [2]. Also of note, was the authors' observation that motor activity decrements often associated with damage to dopaminergic neurons were not observed in mice at the dose of MPTP used in this experiment (data not shown) [30]. Thus, in a dosing model sufficient to elicit a nerve terminal damage-associated astroglial response, MPTP would be erroneously viewed as 'clean' using traditional histopathology and motor activity assessments.

As a neurotoxicity screening database emerges, based on the use of GFAP and related glial biomarkers at the first-tier, it may be possible to limit evaluations to these biomarkers even when the testing outcome is negative. Nevertheless, if GFAP or other qualitative assessments of neural damage are negative at the first-tier, the possibility remains that such compounds could have unwanted direct or indirect actions on the nervous system. To assess the potential for such effects, these compounds could be subjected to further evaluation with cage-side behavioural observations or behavioural assessment batteries. Moreover, additional tissue sections could be evaluated for neural activation using stains for expression of acute-phase response or immediate-early genes known to be associated with activation of neural circuits in the absence or presence of neural damage. The adverse nature of effects on the nervous system in the absence of evidence of damage could be defined based on factors such as dose and an acceptable side effect profile for a given therapeutic use.

5. Expert opinion

Decades of advancement in the neurosciences have resulted in numerous breakthroughs in our understanding of the molecular and cellular basis of neurological diseases that afflict millions of people [31]. Although some of the discoveries in basic neuroscience have been applied to further our knowledge of mechanisms underlying the toxic effects of specific agents on the nervous system, neurotoxicology has yet to yield biomarkers that can be broadly applied for the assessment of drug-induced neurotoxicity. Traditional stains used in pathology as well as behavioural test methods have been applied with some success to screen for neurotoxic effects. Nevertheless, the inherent limits on the sensitivity and specificity of these procedures dictate the need for novel approaches. The authors have proposed that development and implementation of biomarkers of gliosis, a universal cellular reaction to neural tissue injury, will provide the needed advances in sensitivity, specificity and quantification required for neurotoxicity assessment. The authors' results, focused on the use of GFAP as a key glial biomarker, have shown that GFAP and related

glial biomarkers serve as sensitive and specific biomarkers for the toxic effects of a variety of chemical agents on the nervous system. Combining immunohistological assessments of GFAP with traditional histopathology and sensitive degeneration stains represents an inexpensive route to obtain the needed advances in neurotoxicity assessment with respect to added sensitivity and specificity. Evaluation of GFAP and related biomarkers by immunoassay or quantitative real-time PCR will provide further enhancements in sensitivity and the quantitative properties that lend themselves to risk assessment analysis. With the advent of specialised genomic and proteomic microarrays that survey modules of gene expression patterns, it is likely that an inexpensive 'glial biomarker array/chip' can be developed to screen for neurotoxicity. Moreover, surveys with such a glial biomarker array can be combined with high-throughput screens used during drug discovery or drug efficacy evaluations [10]. The results of such screens will likely lead to a clear distinction, at the molecular level, of neurotoxic responses from those responses linked to desirable features of drug actions on the nervous system.

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