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www.toxsci.oxfordjournals.org

TOXICOLOGICAL SCIENCES, 148(2), 2015, 332–340

doi: 10.1093/toxsci/kfv188
Forum

FORUM

Translational Biomarkers of Neurotoxicity: A Health and Environmental Sciences Institute Perspective on the Way Forward

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ABSTRACT

Neurotoxicity has been linked to a number of common drugs and chemicals, yet efficient and accurate methods to detect it are lacking. There is a need for more sensitive and specific biomarkers of neurotoxicity that can help diagnose and predict neurotoxicity that are relevant across animal models and translational from nonclinical to clinical data. Fluid-based biomarkers such as those found in serum, plasma, urine, and cerebrospinal fluid (CSF) have great potential due to the relative ease of sampling compared with tissues. Increasing evidence supports the potential utility of fluid-based biomarkers of neurotoxicity such as microRNAs, F_2-isoprostanes, translocator protein, glial fibrillary acidic protein, ubiquitin C-terminal hydrolase L1, myelin basic protein, microtubule-associated protein-2, and total tau. However, some of these biomarkers such as those in CSF require invasive sampling or are specific to one disease such as Alzheimer's, while

others require further validation. Additionally, neuroimaging methodologies, including magnetic resonance imaging, magnetic resonance spectroscopy, and positron emission tomography, may also serve as potential biomarkers and have several advantages including being minimally invasive. The development of biomarkers of neurotoxicity is a goal shared by scientists across academia, government, and industry and is an ideal topic to be addressed via the Health and Environmental Sciences Institute (HESI) framework which provides a forum to collaborate on key challenging scientific topics. Here we utilize the HESI framework to propose a consensus on the relative potential of currently described biomarkers of neurotoxicity to assess utility of the selected biomarkers using a nonclinical model.

Key words: neurotoxicity; biomarker; imaging; CSF; neurotoxicity

Abbreviations:

ALS,	amyotrophic lateral sclerosis;
CNS,	central nervous system;
CSF,	cerebrospinal fluid;
ELISA,	enzyme-linked immunosorbent assay;
EPA,	Environmental Protection Agency;
F_2-IsoPs,	F_2-isoprostanes;
FDA,	Food and Drug Administration;
GFAP,	glial fibrillary acidic protein H & E, hematoxylin and eosin;
HESI,	Health and Environmental Sciences Institute;
MAP -2,	microtubule-associated protein-2;
MBP,	myelin basic protein miRNA, microRNA;
MRI,	magnetic resonance imaging;
MRS,	magnetic resonance spectroscopy;
MS,	multiple sclerosis;
NeuTox,	neurotoxicity;
PET,	positron emission tomography;
SBDP-145,	spectrin breakdown product-145;
TBI,	traumatic brain injury;
TMT,	trimethyltin;
TSPO,	translocator protein;
UCH-L1,	ubiquitin C-terminal hydrolase L1

Neurotoxicity, defined below, is an underlying feature of several debilitating diseases, to which a number of common drugs and chemicals have been linked (Spencer *et al.*, 2000). For example, inorganic metals, organometals, and pesticides are associated with damage to nervous tissue (Bachmann *et al.*, 1993; Dopp *et al.*, 2012; Keifer and Firestone, 2007; Liu *et al.*, 2014; Sadiq *et al.*, 2012; Stephenson *et al.*, 2014), as are drugs of abuse such as amphetamines and other psychostimulants (Ferris *et al.*, 2008; Gouzoulis-Mayfrank and Daumann, 2009).

Identifying neurotoxicity can improve outcomes in relevant diseases in a number of ways, including increasing our efficiency and accuracy of diagnosis and our ability to intervene with pharmaceutical treatments. Early identification of neurotoxicity enables early intervention, which improves outcomes. Utilization of biomarkers of neurotoxicity also allows for continual monitoring of disease states and drug efficacy and, thus, may improve disease management. From a therapeutic standpoint, detecting and predicting neurotoxicity in preclinical (testing phase before new drugs enter the clinic) and nonclinical (testing of nondrug entities at all phases or ongoing testing of drugs in parallel to clinical development) models can improve decision making during drug development. For drugs where the risk–benefit ratio favors further research and development, monitoring neurotoxicity during clinical trials can mitigate risks for human subjects (Figure 1).

The ability to reliably identify neurotoxicity in preclinical animal models has been a challenge, especially during drug development, where central nervous system (CNS) toxicity is a major contributor to drug failure (Cook *et al.*, 2014). Thus, neurotoxicity raises significant issues both in monitoring and managing risk from existing drugs and chemicals and in guiding the development and use of new ones. The development of biomarkers of neurotoxicity is a goal shared by scientists across academia, government, and industry and as such is an ideal topic to be addressed via the Health and Environmental Sciences Institute (HESI) framework, which facilitates collaboration among scientists from the public and private sectors on key challenging scientific topics.

Detecting and managing neurotoxicants, such as lead, that may be present in the environment is another important objective and is a major focus for the Environmental Protection Agency (EPA) (Fowle and Sexton, 1992). Only around 200 chemicals out of more than 80 000 registered with the EPA have undergone extensive neurotoxicity testing, and many chemicals found in consumer goods are not required to undergo any neurodevelopmental testing (Miodovnik, 2011). The magnitude and potential severity of health risks related to environmentally induced neurotoxicity makes strengthening our basis for preventive intervention an important objective, facilitated by the development of biomarkers of neurotoxicity at the individual and population levels.

Given the significant impact of neurotoxicity on human health, there is a great need to characterize the biological signals that can identify neurotoxic agents and enable us to deploy methods to minimize the adverse impact they can have on health outcomes. Accordingly, there is an abundance of research on the utility of various biomarkers and many new candidates have emerged in recent years. Although there are a number of available definitions for neurotoxicity, we will utilize the EPA definition, which specifies neurotoxicity as “an adverse change in the structure or function of the central and/or peripheral nervous system following exposure to a chemical, physical, or biological agent” (EPA, 1998). Similarly, we define biomarkers as surrogate or predictive markers of physiological and morphological changes in cells and tissues resulting from exposure to chemicals.

IDENTIFYING NEUROTOXICITY

Traditionally, neurotoxicity has been assessed preclinically using composite datasets of functional assessments, such as behavioral and electrophysiological measures, coupled with histopathological assessment of neural tissues. There are, however, many shortcomings with this current approach. For instance, histopathological analyses typically suffer from limited spatial sampling and a reliance on hematoxylin and eosin assessments that lack both sensitivity and specificity and are generally not quantitative (Bolon *et al.*, 2013). Because histopathological assessments require invasive sampling, obtaining sufficient sample material can be

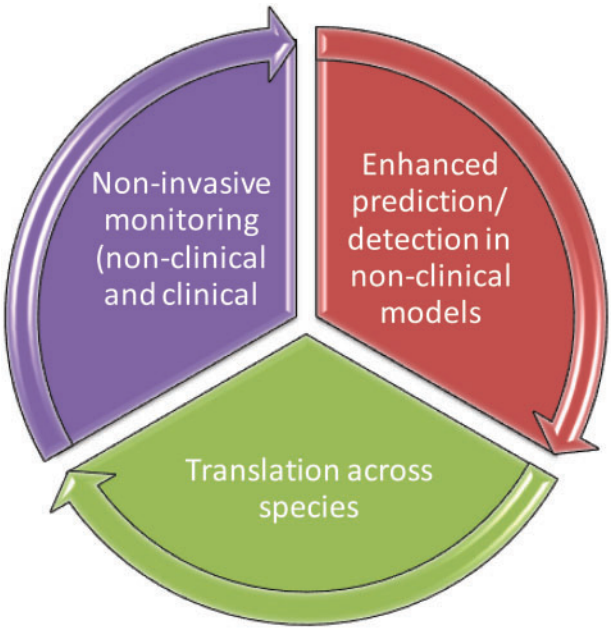

FIG. 1. A schematic depicting the interdependency among enhanced detection preclinically, enhanced capability in translation of nonclinical data to the clinic, and enhanced capability for noninvasive monitoring in the clinic.

problematic, making the approach impractical for subsequent clinical decision making.

Functional assessments of behavioral and electrophysiological endpoints may be insensitive because of reserve capacity, large variability, or lack of connection of the measurement to an understanding of the underlying pathology. Although there are good quality datasets acquired from nonclinical studies, utilizing these findings to interpret human risk can be problematic due to differences among species. The rapid expansion of genomic and proteomic technologies has led to the identification of thousands of plausible biochemical biomarkers that may be superior to current techniques for the detection and monitoring of neurotoxicity (Poste, 2011). Such biomarkers, if shown to be indicative of disease or toxicity, have the potential to vastly improve our ability for diagnosis and treatment (Auffray *et al.*, 2009).

The major challenge involves translating a potential biomarker into a tool with utility in preclinical and clinical decision making. Few biomarkers associated with nervous tissue damage have been validated for routine use in clinical practice, most likely because they fail to demonstrate predictive clinical value. As Poste (2011) points out, over 150 000 papers claim to have identified biomarkers yet there are only around 100 used clinically, highlighting the difficulties faced in translating scientific insights into medically relevant tools. There are many factors that contribute to these challenges. For example, diseases may involve changes in multiple molecular pathways (Schadt, 2009). Even when relevant pathways are defined, sample collection and handling are often not standardized, or the sample volume is not sufficient to provide the statistical power needed to satisfy regulatory requirements. These limitations can be tackled using the HESI collaborative approach to data generation and data sharing that also incorporate critical computational and modeling capabilities to facilitate data interpretation.

Biomarkers that are statistically sensitive and specific and that are either common across several different disease mechanisms or are highly specific to one disease would contribute significantly to the development of valuable tools for diagnosis and clinical decision making. Biomarkers that are measurable with minimally invasive techniques, such as biological fluid-based chemical markers and emerging imaging modalities, could provide the opportunity for noninvasive or minimally invasive longitudinal assessments, with wide utility both preclinically and clinically.

POTENTIAL BIOMARKERS

Fluid-based Biomarkers

Fluid-based biomarkers are those found in bodily fluids such as blood (including serum and plasma), urine, and cerebrospinal fluid (CSF). As far as neurotoxicity is concerned, biomarkers present in the CSF can be particularly valuable because of the colocalization of the CSF with the target tissues and the relative inaccessibility of the CSF to biomarkers indicative of changes in other tissues (Wan *et al.*, 2012). CSF biomarkers are also likely to be valuable in those diseases for which a change in CSF composition is part of the pathology, as is the case with multiple sclerosis (MS; Tumani *et al.*, 2009). Additionally, because the gene expression in neural cells is modified when cells are damaged, biofluids represent an opportunity for identifying alterations in cellular RNA (Guo *et al.*, 2013; Koh *et al.*, 2014). Accordingly, an increasing body of scientific literature provides evidence for the potential utility of fluid-based biomarkers of neurotoxicity.

A number of different protein-based biomarkers reflective of different disease mechanisms are listed in Table 1, along with their proposed endpoints and characteristics. These biomarkers are each indicative of specific types of neural damage associated with neurotoxicity. For instance, tau, which is a well-known indicator of Alzheimer's disease, is a biomarker of axonal injury and neurodegeneration for which enzyme-linked immunosorbent assays (ELISA) are already developed (Blennow *et al.*, 2012; Perrin *et al.*, 2009; Rosen and Zetterberg, 2013; Tumani *et al.*, 2009). Thus, using biomarkers like tau is feasible in the clinical and nonclinical setting.

Glial fibrillary acidic protein (GFAP) is a biomarker of astrogliosis, a cellular reaction which indicates both neuronal and glial damage. A GFAP ELISA assay is available (O'Callaghan, 1991) and it has been widely implemented to detect and quantify broad classes of neurotoxic compound (O'Callaghan and Sriram, 2005). Microtubule-associated protein (MAP-2) is another biomarker with an established ELISA assay. However, unlike GFAP, MAP-2 loss is a characteristic dendritic injury that typically occurs following traumatic brain injury (TBI) (Mondello *et al.*, 2012). Another way to detect TBI is to recognize the oxidative injury that occurs in this scenario, which can be accomplished using F_2-isoprostanes as markers. However, these biomarkers are not specific for neurotoxicity and, thus, do not necessarily provide a clear picture of neurotoxicity status nor provide dynamic predictive information (Bayir *et al.*, 2004; Milne and Morrow, 2006; Varma *et al.*, 2003). Additionally, there are challenges with specificity and sensitivity with all of these biomarkers that need to be overcome. It is unlikely that one or 2 biomarkers will provide the required specificity and sensitivity to distinguish neurotoxicity from disease or other types of neurotoxicological stress. Perhaps a fingerprint comprising several different biomarkers will ultimately be more informative.

Ubiquitin C-terminal hydrolase L1 is a biomarker that indicates injury to the cell body and can be used to identify this kind of damage in cases of severe TBI, ischemia, or hemorrhage (Brophy *et al.*, 2011; Lewis *et al.*, 2010; Liu *et al.*, 2010). When the axon is injured, rather than the cell body, neurofilaments are

TABLE 1. Potential Biomarkers of Neurotoxicity

Fluid Based - Direct analysis of plasma, serum, urine, or CSF – longitudinal and minimally invasive		
Biomarker	Endpoint	Comments
F_2-IsoPs (F_2-iso prostanes)	Indirect measurement of oxidative injury	Used clinically as biomarker of exposure Not specific for neurotoxicity
GFAP (glial fibrillary acidic protein)	Biomarker of all types of neural (neuronal and glial) damage	ELISA already developed GFAP is a sensitive and specific marker of astrogliosis (indicative of all types of CNS damage)
MAP-2 (microtubule-associated protein)	Biomarker of dendritic injury	ELISA already developed
MBP (myelin basic protein)	Biomarker of myelin disruption	Immunoassay developed, but not widely used
Microtubule-associated protein tau (total tau, phosphorylated tau, and cleaved tau)	Biomarker of neurodegeneration/axonal injury	ELISAs developed
Neurofilament (light chain and phosphorylated heavy chain)	Biomarkers of axonal injury	ELISA exists
Spectrin breakdown product (SBDP-145)	Found in the CSF as a biomarker for neurodegeneration (apoptosis and necrosis)	Recently reported
TSPO (translocator protein)	Biomarker of activated glia	Has been validated in a variety of preclinical models of neurotoxicity including preclinical and clinical imaging
UCH-L1 (ubiquitin C-terminal hydrolase)	Biomarker of cell body injury	Immunoassay developed
Imaging - less invasive, longitudinal analysis in living animals, high-resolution in postmortem fixed animals		
MRI T_2 relaxation	Detects edema, hemorrhage, water redistribution, cellular disruption, cellular density, infiltration, blood flow changes, and temperature changes	Data obtained using T2 relaxation is quantitative Correlation to pathology can be achieved via digital analysis
MicroPET	Molecular level view of biochemical, physiological, pathological, and pharmacological processes in vivo	Tags for specific neurotransmitter receptor systems can be used Resolution less than MRI needs specific short-lived radiolabeled ligand to probe the function of interest

Abbreviations: CFS, cerebrospinal fluid; CNS, central nervous system; ELSIA, enzyme-linked immunosorbent assay; MRI, magnetic resonance imaging; PET, positron emission tomography.

better biomarkers. They can be used to identify MS, and an ELISA for them already exists (Teunissen and Khalil, 2012). MS can also be detected with myelin basic protein, which is an indicator of disruption of myelin, a well-known hallmark of MS. However, this biomarker is not only present in MS but also in other cases of neural damage, such as TBI, thus representing a potentially useful biomarker (Belogurov *et al.*, 2008; Berger *et al.*, 2006, 2007).

Other relevant biomarkers include translocator protein (TSPO), which is indicative of activated glia and can be imaged using positron emission tomography (PET) ligands in animal models and humans. TSPO is often elevated in neurological and psychiatric disorders and may signal neuroinflammation (Kreisl *et al.*, 2013; Rupprecht *et al.*, 2010). Spectrin degradation products signal cell death activation, including both apoptosis and necrosis and are found in cases of TBI (Berger *et al.*, 2012). Spectrin breakdown products (SBDPs) like SBDP-145 are biomarkers of neurodegeneration. Recent data have shown that SBDP-145 in CSF correlates with the severity of neurodegeneration in rats treated with neurotoxic agents (Pritt *et al.*, 2014). Monitoring the production of these and similar molecules may prove useful in tracking aspects of neurotoxicity.

Caution is required when considering any biomarker for neurotoxicity when data may need to be disentangled from disease states such as Alzheimer's, Parkinson's, or head injury. Rather, these markers cannot be used as a standalone diagnostic or prognostic tool. They must be coupled with additional information, including patient exposure history, body burden of chemicals, and individual genetics.

Emerging Imaging Biomarkers

Neuroimaging has advantages for identification of potential biomarkers of neurotoxicity because it is less invasive than other procedures, longitudinal studies can be performed, and subjects can serve as their own controls. Two imaging techniques of potential utility in monitoring aspects of neurotoxicity are magnetic resonance imaging (MRI) and MicroPET (Hanig *et al.*, 2014; Liachenko *et al.*, 2015) (Table 1). MRI can provide information on toxicity to cells by detecting changes in relevant tissue characteristics such as cellular integrity, cell density, and water redistribution in vivo. There are several applicable MRI methods, but quantitative mapping of T_2 relaxation, despite some potential ambiguity in interpretation, is most useful in this context because it is relatively simple, can result in evenly distributed time course scans, and can produce quantitative metrics (Hanig *et al.*, 2014; Liachenko *et al.*, 2015). MicroPET imaging may also be useful because this method allows for functional imaging at the molecular level and, thus, can provide valuable insight into biochemical, physiological, and pathological processes during the expression of neurotoxicity in vivo (Chen and Guilarte, 2008; Pogge and Slikker, 2004; Zhang *et al.*, 2013). PET imaging requires positron emitting ligands that can be designed to track specific endpoints such as the cellular membrane disruption thought to be associated with apoptosis (via radiosynthesis labels DFNSH and Annexin V) or gliosis (radiolabel FEPPA). All these imaging methods are useful because they can provide data over multiple time points within the same subjects.

Biomarkers Considered but Not Included

Although there are a number of additional biomarkers for consideration, many have similar advantages and disadvantages as those considered by the authors, who acknowledge that the list is not exhaustive. Additionally, many well-established laboratories are currently pursuing the search for biomarkers specific to the nervous system. However, the majority of these efforts are for the early detection or to follow the progression of neurodegenerative disease and as such may have limited potential for detection of neurotoxicity induced by exposure to drugs or chemicals. The biomarkers being pursued are based on mechanistic hypotheses associated with neurodegenerative disease and for the most part are specific to a given disease. For example, the majority of biomarkers for Alzheimer's disease focus on the amyloid cascade hypothesis or hyperphosphorylated tau. The relevant biomarkers either image the neurofibrillary tangles or amyloid plaques, or quantitate the products of these hallmarks of the disease such as total tau, phospho-tau, and Aβ1-42, from fluid samples. Similarly, biomarker efforts directed toward Parkinson's disease focus on α-synuclein or markers relevant to the mitochondrial dysfunction mechanism of Parkinson's disease. This is also the approach taken with neurodegenerative diseases where the causative hypothesis has yet to be isolated to a specific biochemical pathway or protein within the pathway. In cases such as MS or amyotrophic lateral sclerosis (ALS), fluid-based biomarkers for inflammation or altered metabolic pathways may not be disease specific, but are also unlikely to be indicative of all forms of neurotoxicity. In contrast, advances in imaging that have been shown to be useful in following disease processes in ALS and MS may also have broader application to neuronal loss, generally.

TESTING THE PARADIGM

When monitoring for neurotoxicity that results from the administration of an exogenous chemical entity, the mechanistic basis of the toxicity is often not known. Hence, biomarkers that could be common but yet specific across all types of neurotoxicity would be of tremendous value. Structural damage to neurons and supporting cells would lead to the leakage of cell contents that may be detected in CSF and possibly blood. Evidence shows that microRNAs (miRNAs) control a large number of biological processes and appear in extracellular fluid once cellular membrane integrity is lost. The same is true for proteins, many of which are only expressed in specific cell types. Imaging has the advantage of being used in vivo not only to monitor the location and life cycle of a lesion, but also the functionality of specific brain regions. Supporting cells, including microglia and astrocytes, may also be sources of biomarkers specific to neurotoxicity. Microglia scavenge damaged neurons upon activation and astrocytes play a role in the repair and scarring process of the CNS following injury. The approach in this investigation is to include aspects of protein, miRNA, and neuroinflammatory signal detection, as well as imaging techniques as part of a broad approach to the assessment of neuronal toxicity, in general, rather than being specific to only a single neurodegenerative insult (Figure 2).

To test the utility of biomarkers that may be valuable in decision making, model neurotoxicants can be used to determine if a candidate biomarker can successfully identify the resulting toxicity in vivo. HESI is uniquely positioned to balance contributions from its member scientists from both the public and private sectors, who bring their expertise and experience to help design and conduct such a study. This approach pools knowledge and also drives shared responsibility for resources needed and ultimate outcome and adoption of results.

Of all the many described in the literature, the neurotoxicant trimethyltin (TMT) has advantages as a prototypic compound, including that relevant data are available on dose response, time course, and site of action. TMT causes frank

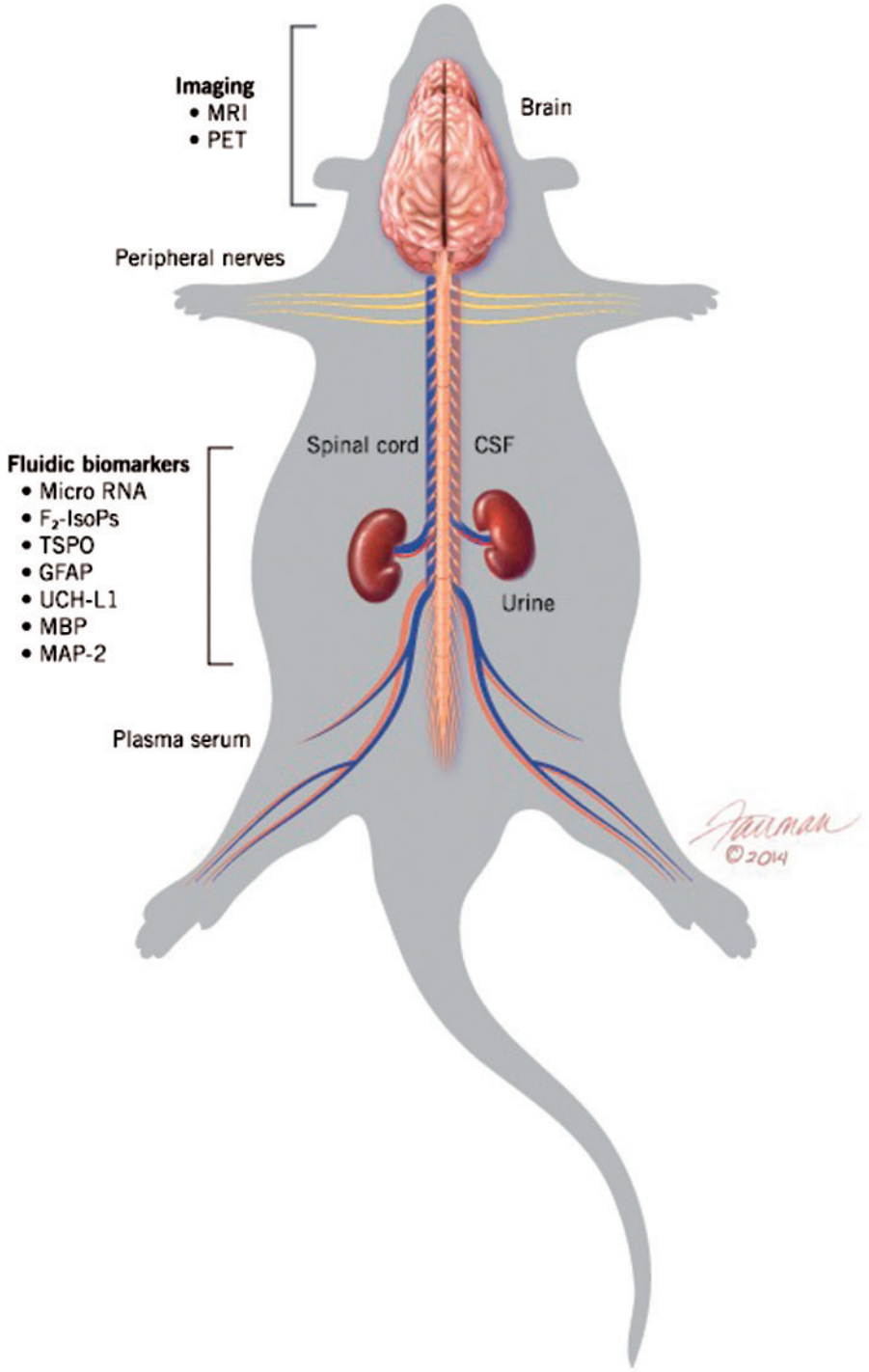

FIG. 2. Schematic depicting proposed biomarkers and their tissue origins. Abbreviations: CSF, cerebrospinal fluid; F_2-IsoPs, F_2-isoprostanes; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein-2; MBP, myelin basic protein; MRI, magnetic resonance imaging; PET, positron emission tomography; TSPO, translocator protein; UCH-L1,ubiquitin C-terminal hydrolase L1.

neuropathology, which remains the gold standard anchor of neurotoxicity (Balaban *et al.*, 1988; Heikkila *et al.*, 1984). Studies show that TMT causes changes in neural miRNA expression that are associated with neurological symptoms, suggesting that biomarkers that identify altered miRNA may be clinically valuable (Ogata *et al.*, 2015).

The current use of neurotoxicants in the identification and description of relevant biomarkers has been limited for a number of reasons, including that the effects on tissues is not easily detectable using traditional staining techniques. TMT administration produces a characteristic pattern of early behavioral depression followed by persistent increases in activity, which may be harder to detect than overt toxic signs of tremors (McMillan *et al.*, 1986; Moser, 1996). As a starting point for the proposed studies, TMT seems, therefore, to offer utility as a prototypical neurotoxicant in rats because its damage to the

hippocampus is more obvious and the neuropathology induced by TMT is best observed in rats (Donnan *et al.*, 1986). Because the rat is the typical species of choice for regulatory decision making, the rat was chosen for the proposed studies.

Repeated assessments of blood, CSF, and urine for candidate biochemical markers coupled with targeted MRI and magnetic resonance spectroscopy should generate a useful profile of fluid and imaging biomarkers indicative of the neurotoxicity induced by the prototype compound, TMT. These observations will be most useful if linked to existing knowledge via correlated traditional histopathological analyses. Monitoring motor activity in treated animals will offer an opportunity to compare functional effects of TMT to assess sensitivity and specificity of potential biomarkers with regards to behavioral changes.

TMT data suggest that sensitivity will be paramount when assessing potential biomarkers of neurotoxicity: They should be sensitive enough to provide a low incidence of false negatives, specific enough to the neurotoxic condition to provide a low incidence of false positives, but simple enough such that they can be easily evaluated and quantified (O'Callaghan and Sriram, 2005).

In interpreting the data, care must be taken to establish specificity for each of the markers alone and in combination. The reliance on a single or battery of proteins to delineate disease may be more suitable in a controlled drug discovery setting or one evaluating potential neurotoxic agents using animal models. In these situations the exposure scenario is known and the pathology and biomarker utility can be correlated. For the future, it will be useful to perform concurrent evaluations of particular diseases in order to address the specificity of the marker for neurotoxicology. For example, GFAP may be elevated in an animal exposed to a neurotoxicant and also in an animal with TBI; thus GFAP alone is neither specific nor selective as a standalone marker. However, a fingerprint using a combination of markers may provide the specificity and sensitivity required.

CONCLUSION

New biomarkers that can be used to predict, detect, and monitor neurotoxicity could prove invaluable in environmental, preclinical, nonclinical, and clinical decision making. Subtle neurotoxic events can easily be missed using current methodologies, and translation from animal data to human risk assessment is challenging. Identifying and monitoring neuronal damage using minimally invasive biomarkers could facilitate detection of damage earlier than with current methods. Better biomarkers may also provide molecular target information, providing the opportunity to build mechanistic information into the preclinical picture for the development of future therapeutics.

Imaging and omics-based approaches such as in vivo MRI, PET, and genetic and protein fluid-based biomarker assessments offer a way forward, anchored to traditional histopathology endpoints. Prototype neurotoxicants such as TMT provide tools with which to assess the utility of these endpoints as biomarkers with the hope that they will have wide applicability. In summary, the HESI approach has provided a collaborative framework to select and assess translational Biomarkers of Neurotoxicity by pooling of knowledge and resources across academia, industry, and government.

ACKNOWLEDGMENTS

The authors would like to recognize additional contributors from the HESI Committee on Translational Biomarkers of Neurotoxicity: Marion Ehrich, Virginia-Maryland Regional College of Veterinary Medicine; Warren Glaab, Merck & Co., Inc.; Ron Tjalkens, Colorado State; Ambuja Bale, US EPA; Russ Bialecki, AstraZeneca; Wayne Buck, AbbVie; Keri Cannon, Pfizer; Lois Freed, US FDA; Reina Fuji, Genentech, Inc.; Allan Johnson, Duke University Medical Center; Donna Lee, Genentech, Inc.; Robert Martone, Covance; Chris Morris, Newcastle University; Hiroshi Onodera, PMDA; Deepa Rao, NIEHS; Will Redfern, AstraZeneca; Gary Russo, US EPA; Yuko Sekino, National Institute of Health Sciences; Beatriz Silva Lima, Lisbon University Faculty of Pharmacy; Christopher Somps, Pfizer; Greet Teuns, Janssen; Christopher Toscano, US FDA.

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