PART 1

FUNDAMENTALS OF NEUROBIOLOGY

1

FUNDAMENTAL NEUROPATHOLOGY FOR PATHOLOGISTS AND TOXICOLOGISTS: AN INTRODUCTION

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THE IMPORTANCE OF NEUROTOXICOLOGICAL RESEARCH

Neurotoxicology is the study of the undesirable consequences that develop in the central nervous system (CNS) or peripheral nervous system (PNS) or both after an organism is exposed to a neurotoxic agent during development or adulthood. Such agents may be exogenous materials such as chemicals contaminating the external habitat (e.g., agrochemicals, pesticides, solvents) or introduced purposely into the internal environment (i.e., drugs); metals; or peptides/proteins (e.g., microbial toxins, biopharmaceuticals). Alternatively, neurotoxic agents may be produced endogenously (e.g., ammonia, unconjugated bilirubin) during the course of certain diseases. Thus, the nervous system is likely to experience constant exposure to a range of neurotoxic agents, although in many instances the level of exposure will be insignificant.

The potential scope of toxicant-induced neuropathology is immense. Each year in the United States, industries manufacture about 85,000 chemicals and register another 2000 to 3000 new compounds.[1] Approximately 3 to 5% of chemicals (between 2500 and 5000 entities) are estimated to be neurotoxic to some degree.[2] This estimate has serious implications for human, animal, and environmental health, because up to two-thirds of high-production-volume chemicals (those made yearly in quantities exceeding 1 million pounds) have never been tested sufficiently for neurotoxic potential.[3] The recognition that neurological dysfunction is a major occupational hazard for adults[4,5] and a common congenital occurrence in children[6] has engendered a wide-ranging global effort to identify and eliminate possible sources of neural damage—principally, sources of neurotoxicant exposure.

Neurotoxicity can present as aberrations in neural structure (i.e., toxicological neuropathology) or function (including altered behavior, biochemistry, cognition, or impulse conduction), or both.[7–11] All structural changes and any persistent functional deficits associated with xenobiotic exposure are judged to be neurotoxic because such effects cannot be countered by the meager regenerative capabilities of the CNS.[12] Reversible functional deficits linked to a recognized neurotoxicological mechanism (e.g., outright neurodegeneration or exaggerated neuropharmacological activity) or that might jeopardize occupational health (for adults) or scholastic performance (especially for children) are also considered to be neurotoxic manifestations. The current "best practice" in conducting risk assessments for potential neurotoxicants is to integrate all available structural and functional evidence in reaching a verdict.[9,13,14] Nevertheless, the permanence of toxicant-induced structural changes in the CNS typically leads regulators to place more emphasis on morphological data rather than on behavioral or biochemical alterations to determine reference doses for

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managing neurotoxic risk.[15] Therefore, a comprehensive toxicological neuropathology evaluation is and will remain a critical element of the risk assessment process for novel xenobiotics.[16]

The catastrophic outcome of neurotoxic damage to affected persons, and the strain placed on the resources (money, time) of their immediate caretakers and the societal entities that must often fund chronic health care, has led to the expanded use of neurotoxicity endpoints as major criteria for assessing the risks posed by exposure to xenobiotics.[17] This approach is a direct result of two factors. First and foremost, an unfortunate aspect of human history from ancient times through the twentieth century is that the neurotoxic effects of many agents [e.g., ethanol, *n*-hexane, lead, mercury, polychlorinated biphenyls (PCBs)] have been identified first in humans.[18] Second, exposure to potential neurotoxicants remains a common feature of human existence. Slightly less than a third of all high-volume industrial chemicals can elicit neurotoxic syndromes in the workplace.[19] Similarly, many drugs [antiepileptics (e.g., valproic acid), antineoplastics (e.g., vincristine)] can induce neurotoxic sequelae as an undesirable side effect.[18,20,21] Thus, a primary goal of current neurotoxicological research is to prospectively recognize the neurotoxic potential of novel compounds in laboratory animals rather than to discover it retrospectively after epidemics of neurotoxicity in humans.

THE EVOLUTION OF TOXICOLOGICAL NEUROPATHOLOGY

People have exhibited an interest in fundamental neuroscience for millennia (Table 1).[22,23] Initial neurobiology investigations concentrated on gross anatomical characterization of the CNS and its PNS projections as well as the clinical detection and treatment of diseases affecting the nervous system. Neurohistological evaluations were first undertaken in a piecemeal sense early in the eighteenth century, and more systematic assessments of discrete neural regions were begun in the 1840s. These early studies were organized as descriptive studies of the normal nervous system anatomy. The first neuropathology reports examined neuroanatomical alterations resulting from physical disruption (e.g., Wallerian degeneration in transected axons, first described in 1850) rather than toxicant-mediated neural damage. This emphasis reflected the close alliance between neuropathology and clinical neurology in the European (mainly German) medical schools in which neuropathological research was formalized in the modern era.

Human interest in toxicology also dates from antiquity.[24] The impact of widespread neurotoxicity on the advance of civilization became clear with the rise of industrialization in medieval and Renaissance Europe, when chronic exposure to lead and mercury represented a substantial occupational hazard to members of many professions (alchemists, goldsmiths, hatters, and millworkers, to name a few). Toxicological inquiry progressed in fits and starts during the nineteenth and early twentieth centuries before ultimately evolving into the hypothesis-driven applied science that exists today. The intermittent progress in toxicology stemmed from its expansive approach to experimentation; the field grew from a synthesis of most other basic biological and chemical disciplines, and at its inception the numbers of people with the time and money to excel in such diverse intellectual arenas were few.

Thus, toxicological neuropathology represents the modern-era intersection of three scientific fields: basic neurobiology, applied toxicology, and pathology. The rise of toxicological neuropathology was delayed until the first decades of the twentieth century because it required the advent of both technical advances in neuroanatomical handling and processing techniques (Table 1) and the availability of well-trained scientists versed in the fundamental concepts of all three disciplines. These prerequisites were clearly attained by 1906, as indicated by the publication in that year of a detailed neuropathological description of presenile neurodegeneration by Alois Alzheimer as well as the presentation of the Nobel Prize in Physiology or Medicine to Camillo Golgi and Santiago Ramón y Cajal for their studies of nervous system cytoarchitecture. The subsequent founders of toxicological neuropathology built on these accomplishments by developing significant expertise in morphological pathology, dedicating decades of research to defining the experimental conventions and procedures used in modern toxicological neuropathology investigations, and familiarizing ever greater numbers of colleagues with these conventions and procedures (via their numerous publications and many graduate students).

In this regard, two scientists in particular served as major role models for the growth of toxicological neuropathology during the mid-twentieth century, ultimately influencing several generations of modern toxicological neuropathologists (including the careers of the two authors). One person was John B. Cavanagh, a British physician and professor who devised many of the routine morphological approaches used to evaluate toxicants (especially metals, pesticides, and solvents) associated with occupational neurotoxicity.[25–30] The other founder was Adalbert Koestner, a German veterinarian and professor at several U.S. universities whose interests ranged from the morphology and mechanisms of mutagen-induced neural neoplasms to the potential utility and safety of food additives and novel neurotherapeutics.[31–34] Modern investigations in toxicological neuropathology have since evolved to incorporate many other innovative neuropathology endpoints in addition to the traditional morphological techniques (Table 2). Nevertheless, the methods pioneered by these two

TABLE 1 Selected Historical Landmarks in the Evolution of Toxicological Neuropathology

Date	Event
ca. 1700 B.C.E.	First written record about the nervous system
ca. 1000 B.C.E.	First written treatise describing surgical treatments for some neurological disorders (Al-Zahrawi, also known as Abulcasis or Albucasis)
ca. 500 B.C.E.	First descriptions of nervous system dissection (cranial and sensory nerves) (Alcmaion of Crotona)
ca. 80	First description linking lead exposure to neurological disease (Dioscorides)
1549	Publication of *De Cerebri Morbis*, an early book devoted to neurological disease (Jason Pratensis)
1660–1700	First publications dedicated to neuroanatomy: *Cerebri Anatome* (Thomas Willis, 1664), *Neurographia Universalis* (Raymond Vieussens, 1684) and *The Anatomy of the Brain* (Humphrey Ridley, 1695)
1684	First record of a special preservation technique for neural tissue (boiling oil as a hardening agent, by Raymond Vieussens)
1717	First description of the nerve fiber in cross section (Anton van Leeuwenhoek)
1760	Initial demonstration that cerebellar damage affects motor coordination (Arne-Charles Lorry)
1766	Earliest scientific description of the cerebrospinal fluid (Albrecht von Haller)
1810–1825	First functional–structural correlates for many CNS regions are defined
1836	Neuron nucleus and nucleolus first differentiated by microscopy (Gabriel Gustav Valentin) Myelinated and unmyelinated axons are discerned (Robert Remak)
1837	Cerebellar neurons and their processes first investigated (Jan Purkinje)
1838	Myelin-forming cells in the peripheral nervous system described (Theodor Schwann)
1842	Spinal cord anatomy first studied in serial sections (Benedikt Stilling)
1844	First illustration provided of the six cerebrocortical layers (Robert Remak)
1850	Initial experimental investigation of axonal degeneration (Augustus Waller)
1859	The term *neuroglia* is coined (Rudolph Virchow)
1861	Functional localization in the cerebral cortex is described (Paul Broca)
1865	Axons and dendrites are first differentiated (Otto Friedrich Karl Deiters)
1873	First work on the silver nitrate method to enhance neuronal contrast (Camillo Golgi)
1878	Regular interruptions in the peripheral nerve myelin are first appreciated (Louis-Antoine Ranvier)
1884	Granular endoplasmic reticulum is discriminated in neurons (Franz Nissl)
1889	Nerve cells are proposed to be independent functional elements (Santiago Ramón y Cajal)
1891	The lumbar puncture (spinal tap) is developed (Heinrich Quinke) *Journal of Comparative Neurology* is founded
1897	Formaldehyde is employed as a brain fixative (Ferdinand Blum)
1906	First description of Alzheimer's disease (Alois Alzheimer) Nobel Prize in Physiology or Medicine awarded to Camillo Golgi and Santiago Ramón y Cajal for their work on neural cytoarchitecture
1921	Microglia described (Pío del Río-Hortega)
1929	Correlation between nerve fiber size and function is identified (Joseph Erlanger and Herbert Spencer Gasser)
1949	National Institute of Mental Health (NIMH) is launched at the U.S. National Institutes of Health (NIH)
1950	National Institute of Neurological Disorders and Stroke (NINDS) is established at the NIH
1959	Methylmercury from industrial effluent identified as the cause of a neurotoxicity epidemic in humans and feral cats living in villages lining Minamata Bay in Japan
1961	International Brain Research Organization (IBRO) is formed as an independent, nongovernmental organization
1964	Methylnitrosourea (MNU) identified as a relatively selective model neurocarcinogen in rats
1968	Neurotoxic potential of polychlorinated biphenyls (PCBs) is first recognized in Japan among people who have ingested rice oil that was contaminated during manufacturing
1969	Society for Neuroscience (SfN) is founded in the United States
1973	*Fetal alcohol syndrome* (FAS) is coined as the term for a distinct pattern of craniofacial (including brain), limb, and cardiovascular defects in children born to alcoholic mothers
1982	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) shown to be the etiology of Parkinsonism in young illicit drug users who used an improperly synthesized bootleg version of an opioid analgesic
1990	"Decade of the Brain" is declared in the United States by presidential proclamation

Source: Adapted in part from Chudler.[22]

TABLE 2 Morphological Techniques Used in the Modern Practice of Toxicological Neuropathology

Test	Type of Neuropathology Data
Gross evaluation	Identifies overt lesions within neural tissues (via subjective gross examination of surface and internal features) May provide a crude quantitative estimate of large-scale cell loss (via organ weights or linear or areal morphometric measurements)
Light microscopy	Identifies region-specific vulnerability and susceptible cell populations [routine stains such as H&E, Fluoro-Jade (for neuronal degeneration), and anti-GFAP (for reactive astrocytes in affected regions)] Characterizes the nature, location, and quantity of macromolecules, and provides insights into neurotoxic mechanisms [special histochemical, immunohistochemical, and molecular methods, especially if employed in conjunction with such specialized microscopy methods as laser capture microdissection (LCM)]
Electron microscopy	Identifies subcellular targets of neurotoxicity and provides an indication of the metabolic state of the nervous system (transmission electron microscopy) Addresses the subcellular distribution of xenobiotics (specialized autoradiographic and immunoelectron microscopy techniques, elemental composition analysis)
Noninvasive imaging	Allows in vivo assessment of neuroanatomic integrity [computed tomography (CT), magnetic resonance imaging (MRI) and microscopy (MRM), ultrasound (US)] Permits in vivo investigation of region-specific neurochemistry and function, offering insights into mechanisms of neurotoxicity [optical imaging, positron emission tomography (PET), single photon-emission computed tomography (SPECT)]

men and others are—and will remain—the foundation of toxicologic neuropathology investigations in the foreseeable future.

REQUIREMENTS FOR PROFICIENCY IN TOXICOLOGICAL NEUROPATHOLOGY

More than for any of the other subdisciplines of toxicology or pathology, competent practitioners of toxicological neuropathology must have the appropriate educational and work-related experiences to succeed. Advanced theoretical and practical training in neurobiology and experience in neuropathology will appreciably enhance the pathologist's ability to recognize abnormalities in neural tissues.[35] Proficiency as a toxicological neuropathologist requires comprehension at multiple levels of biological organization (e.g., whole animal, cellular, biochemical, and molecular) and the ability to integrate this information with basic medical tenets to formulate differential diagnoses as well as to identify and characterize etiologies and mechanisms of neural disease. Acceptable "entry-level" proficiency in toxicological neuropathology requires that a person have expertise in (1) comparative and correlative aspects of normal neuroanatomy and neurophysiology, (2) causes and mechanisms of major background and neurotoxicant-induced diseases of humans and common laboratory animal species, and (3) principal techniques used for evaluating neuropathological changes (e.g., gross dissection, light and electron microscopy, immunocytochemistry, advanced in situ molecular methods, and morphometry). Therefore, the most direct means of acquiring sufficient expertise in toxicological neuropathology is to complete a clinical degree in either medicine or veterinary medicine and then pursue postgraduate training in either diagnostic pathology (e.g., a residency) or toxicological pathology (such as an advanced research degree or a clinical fellowship) in a program that specializes in nervous system investigations. Fundamental research in the field can also be done by Ph.D. biologists with in-depth training in a relevant discipline (e.g., comparative pathology, neurotoxicology) as long as the focus emphasizes an integrative strategy for nervous system assessment (i.e., investigating questions at the whole animal, organ, cellular, and biochemical/molecular levels, as necessary) rather than a reductionist approach (e.g., limited to cellular or molecular studies).

In current practice, however, general toxicological pathologists and toxicologists must often undertake their own instruction in toxicological neuropathology. Such exposure is usually acquired via self-study or through mentored on-the-job experience, and may be gained in several fashions. The most straightforward way is to study standard references in the field, and in allied biological disciplines (see Appendixes 2, 3, 4, and 5). Indeed, people engaged in toxicological neuropathology on a regular basis will require ready access to many of these references, particularly to neuroanatomy atlases (Appendix 2), in order to undertake meaningful analyses of neurotoxicant-induced lesions. Two other paths are to find Web sites (Appendix 4) or to read classical literature reports related to specific research questions. In the authors' experience, however, the two latter routes are suitable only if one has sufficient prior familiarity with the field for efficient and effective sifting of many possible citations to find those that are most useful. Thus, we recommend that generalists tasked with learning toxicological neuropathology spend the effort, money, and time to understand the relationship between various neural structures and functions (a correlative approach), and to do so across species (a comparative approach).[36]

FUNDAMENTAL PRINCIPLES OF TOXICOLOGICAL NEUROPATHOLOGY

The complexity of the nervous system is a key factor in its vulnerability to toxicant insult.[37] Moreover, these same structural and functional intricacies render even the simplest assessments quite challenging.[13,38] Success in research in toxicological neuropathology thus requires strict adherence to a few fundamental principles. In the remainder of the chapter we list these basic concepts and suggest some practical steps to implement them in toxicological neuropathology research. These principles and practices are described in much greater detail in later chapters.

Principle 1: Learn the lingo. As with many technical fields, neurotoxicological research has developed a jargon that is typically the unique domain of experts in the field. It goes without saying that a solid knowledge of this nomenclature is a mandatory prerequisite to achieving proficiency as either a toxicological neuropathologist or a neurotoxicologist.

A topic that has caused some confusion is the difference between the naming conventions for neural structures in humans (and nonhuman primates) and other animals. The misunderstanding arises from the dissimilar body orientations of these species. Primates are bipedal, with a nervous system arranged along a vertical (upright) axis, whereas other laboratory animals commonly employed in toxicological neuropathology research are quadrupeds having a horizontal nervous system axis. These divergent body carriages dictate different naming conventions for neural structures in primates and other vertebrates (Table 3). To avoid confusion, publications and reports that describe toxicological neuropathology findings should invoke the correct nomenclature for the species being investigated. A compromise that can be applied when naming neural structures in animals is to include the medical (*nomina anatomica*) term for the structure in parentheses behind the veterinary (*nomina anatomica veterinaria*) term. For example, the *superior cervical ganglion* in humans should be designated in animals as either the *cranial cervical ganglion* (the recognized term) or the *cranial cervical ganglion* (*superior cervical ganglion*). Descriptive anatomical terms should be used rather than eponyms (e.g., *mesencephalic aqueduct* in preference to *aqueduct of Sylvius*) when identifying neural structures to promote clarity in communication of neuropathology findings across all species.

TABLE 3 Species-Specific Directional Nomenclature for Designating Neural Structures

Direction	Biped (Humans, Nonhuman Primates)	Quadruped (Carnivores, Lagomorphs, Rodents)
Up	Superior	Dorsal
Down	Inferior	Ventral
Front	Cranial (outside the skull)	Cranial (outside the skull)
	Anterior (inside the skull)	Rostral (inside the skull)
Back	Posterior	Caudal

Principle 2: Responses are restricted. Neuropathological lesions resulting from neurotoxicant exposure have been implicated in acute[21,39] and delayed[18,40–43] neurodegeneration, neuronal heterotopia,[44] and neural neoplasia.[32,45,46] The same lesion generally is elicited by many structurally different neurotoxicants, because these agents often act via a common molecular mechanism (e.g., peripheral axonopathy as a consequence of cytoskeletal cross-linking following exposure to *n*-hexane or carbon disulfide[47]). Therefore, the pathologist who is able reliably to discern a few basic lesions (Table 4) in neural tissue is reasonably well equipped to participate in toxicological neuropathology assessment.

TABLE 4 Fundamental Structural Alterations in Neural Tissues from Toxicological Neuropathology Studies

Cell Type	Lesion Type	Preferred Method of Neuropathology Analysis[a]
Neuron	Cell death	Light microscopy of specially stained sections (Fluoro-Jade, silver impregnation)
	Cell loss	Light microscopy of specially processed sections [IHC for cell type–specific markers (e.g., enzymes or neurotransmitters)]
		Morphometric measurements of specific regions on tissue sections
		Stereological counts of specific cell populations
	Cell displacement (ectopia)	Light microscopy of routinely stained sections (H&E) or sections processed to reveal cell type–specific markers
	Abnormal neurite conformation	Light microscopy of specially stained sections (Fluoro-Jade, silver impregnation)
	Altered axonal size	Light microscopy of specially stained sections (IHC for cell type–specific cytoskeletal markers, silver stains)
Glia	Numerical changes	Light microscopy of specially processed sections (IHC for cell type–specific markers)
	Myelin amount/integrity	Light microscopy of specially stained sections (Luxol fast blue, IHC for cell type–specific markers)

[a] H&E, hematoxylin and eosin; IHC, immunohistochemistry.

Considerable care must be taken when investigating chronic neural diseases, as the damage to the principal target cell population may elicit secondary changes in other parts of the affected cells (e.g., central chromatolysis of the neuron cell body after transection of its axon) and/or in nearby groups of healthy cells (e.g., Schwann cells, which proliferate as a normal response to degeneration and dissolution of their associated axon).[48,49] The extent of the secondary repair processes may substantially exceed the reaction by the primary target cells, especially if the long-standing neural disease has already obliterated the target cells. The complete absence of a defined cell population may be obvious [e.g., selective loss of neurons in specific CA (cornu ammonis domains of the hippocampus)], but more often it is quite subtle and may easily be missed if more complex structures are evaluated by subjective estimates of cell number rather than objective quantification (e.g., reduction in neuronal numbers within the layers of the cerebral cortex). Special immunohistochemical procedures to detect markers specific for reactive astrocytes or activated microglia (Appendix 1) are often needed to detect neuronal lesions reliably, as expression of these glial markers is typically elevated in regions where neuronal degeneration has occurred.

Principle 3: Some sectors are selectively sensitive. Certain neural structures are much more susceptible to injury induced by many etiological agents, including neurotoxicants. Perhaps the most important attribute of toxicological neuropathologists and neurotoxicologists is their knowledge of the basic lesion patterns that can develop following neurotoxicant exposure.

"Hot spots" for neurotoxic damage can arise from many different factors.[37] One mechanism of enhanced regional susceptibility is the intricacy of the neural circuitry in a given structure. The more complex interconnections and dense synaptic beds that are characteristic of the cerebral cortex, hippocampus, and cerebellum render these regions quite sensitive to neurotoxic insult, particularly in periods of rapid cell proliferation during development.[50,51] Another factor leading to differential vulnerability of various neuron populations is the markedly high metabolic rate of the brain. This organ consumes disproportionate shares of the total cardiac output and blood-borne oxygen supply (approximately 15% and 20%, respectively) even though the brain mass represents only about 2% of the total body mass.[52] This tremendous metabolic rate makes the brain as a whole especially vulnerable to neurotoxicants that disrupt intracellular energy production.[53] That said, zonal variations in basal metabolic rate among neuronal populations predispose certain brain regions [especially gray matter (nuclei) of the thalamus, mammillary bodies, periaqueductal and periventricular brain stem, and cerebellar vermis] to toxicant-induced injury, above and beyond the level of vulnerability for the bulk of the brain.[25,26] An important ancillary consideration is that dependence on a high rate of oxidative metabolism rate is a property also shared by the heart. Toxicants that injure the brain often injure the heart, and vice versa, so that in many instances (e.g., cyanide toxicity) it is difficult to distinguish a primary neurotoxic event from neural damage that results from primary cardiac toxicity. A third factor contributing to augmented regional vulnerability is the existence of cell type–specific neurochemical machinery. A biochemical example of such compartmentalization is the selective sensitivity of dopaminergic neurons to toxicants such as 6-hydroxydopamine[54] and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP),[55] both of which result in selective degeneration of dopaminergic neurons. A related biochemical route leading to higher susceptibility results from variations in chemical composition among cells; the extensive lipid content of neural cell membranes, especially myelin, provides an abundant target for the oxidizing actions of certain neurotoxicants.[56] Enhanced regional vulnerability may also reflect disparities in local blood flow and repair mechanisms. The efficiency of most biochemical, metabolic, and reparative processes decreases with age, which can further magnify zonal differences in neural tissue sensitivity to neurotoxicants.

Principle 4: What gets wrecked depends on when it gets whacked. As in other organs, toxicant-induced damage in the nervous system occurs only if the agent reaches a target cell population at a time when those cells are vulnerable. Such critical periods of sensitivity to toxicant exposure have been well documented in the developing nervous system following exposure to many different chemicals. For example, grossly evident malformations of the neuraxis happen in mouse embryos only if exposure occurs during neurulation,[57–59] which is the stage at which the cranial neuropore closes to form the brain primordium. As the brain continues to evolve during late gestation, each nucleus has one or two other critical periods for neuron production; a brief toxicant exposure during region-specific neurogenesis can thus decimate a structure engaged in its peak effort at neuronal production while causing minimal or no disruption in nearby quiescent regions.[60–62] Critical periods for some neuronal populations and processes extend well after birth,[61,63] including neuronal and glial expansion and migration, axonogenesis, synaptogenesis, and myelin formation over the first several years of postnatal life in human infants.[64–66]

Principle 5: When assessing acute lesions in neurons, "red and dead" is the real deal. In our experience, the majority of neurotoxicant-induced lesions in neurons are the outcome of primary degeneration. The main evidence for such a process is often the presence of dead and dying neurons, usually in clusters or dispersed throughout a given brain region. These degenerating cells exhibit a characteristic constellation of changes dominated by cytoplasmic hypereosinophilia in conjunction with either pyknosis (condensation and shrinkage) or karyorrhexis (fragmentation) of the

nucleus. Such disintegrating neurons are typically termed *acidophilic neurons*, *eosinophilic neurons*, or "*red dead*" *neurons* (Chapter 13, Figure 2C and D).

This change must be distinguished from *dark neuron* artifact (Chapter 13, Figure 2A and B). Dark neurons indisputably embody the most common CNS artifact encountered by neuropathologists. Unfortunately, dark neuron artifacts have often mistakenly been judged by inexperienced pathologists, toxicologists, and neuroscientists to be evidence of neurodegeneration, and has been reported as such in the neuroscience and neurotoxicology literature.[67] Such reports have misidentified artifacts as neurotoxic injury, with subsequent unnecessary regulatory and public health alarm. Dark neuron artifact is usually observed with larger cells, such as the pyramidal neurons in the cerebral cortex and motor neurons in the spinal cord, and is characterized by darkly stained cytoplasm (especially intense in the apical dendrite) and nucleoplasm and shrunken cell bodies. If dark neurons represent the only visible alteration in sections of neural tissue, it is generally safe to interpret the change as artifactual. The main exception to this rule is for studies designed specifically to detect hyperacute neuron damage, as the earliest evidence of incipient neurodegeneration is transient cytoplasmic basophilia (Chapter 13), but even here later time points can be used to verify the nonartifactual nature of the alteration. Any practicing neuropathologist knows that dark neurons are readily produced by even mild trauma to the tissue before fixation is achieved, possibly as a consequence of localized ischemia, hypoglycemia, and excitatory neurotoxicity.[68] The simplest prospective way to avoid misinterpretation of dark neurons is to ensure that the neural tissues are fixed properly before they are handled and processed (Chapter 10). A post hoc means of distinguishing genuine lesions from dark neuron artifact is to process a serial section of each sample to reveal reactive astrocytes or activated microglia using immunohistochemical markers (Appendix 1), either or both of which may collect in areas where true neurodegeneration has transpired.

Principle 6: Make "special" stains part of your routine. The workhorse stain for screening most organs for toxicant-induced lesions is hematoxylin and eosin (H&E). This stain works well in the brain but is not suitable for detecting the entire spectrum of neurotoxic lesions that is ordinarily induced in neural tissue. The most readily recognized lesion in H&E-stained neural sections are neoplasms, but such sizable masses are an infrequent consequence of toxicant exposure. The more common toxicant-induced neural lesions—especially neuronal degeneration, myelin disruption, and glial hypertrophy/hyperplasia—may be recognized on H&E-stained sections, but the low contrast between the affected cells and the adjacent neuropil makes such evaluations relatively laborious and prone to false-negative errors.

The cure for this difficulty is to expand the menu of routine procedures that are used to screen neural tissues for neurotoxic lesions to include certain special stains. For most hypothesis-driven animal studies, the H&E-stained section should automatically be accompanied by serial sections processed to reveal degenerating neurons (e.g., Fluoro-Jade; Chapter 11) and reactive astrocytes [e.g., antiglial fibrillary acidic protein (GFAP); Chapters 10 and 21]. Inclusion of these two additional methods as a matter of course when conducting prospective neurotoxicity studies rather than waiting to request them based on the outcome of the examination using the H&E-stained section will substantially shorten the length of the analytical phase, because these two "special" procedures greatly simplify the neuropathologist's efforts to identify lesions, especially subtle ones. The choice regarding whether or not to include these additional stains in a diagnostic neuropathology setting can be left to the discretion of the pathologist.

Principle 7: Seeing is believing, but don't believe everything you see. Neuropathologists and neurotoxicologists have been educated to possess built-in biases to detect toxicant-induced alterations in cells. However, not all structural changes observed in neural tissues after exposure to potential neurotoxicants during a carefully controlled neurotoxicity study are the result of exposure to that agent.

We have seen several spurious causes of neurological dysfunction in toxicant-treated individuals which had nothing to do with the test agent. One example is spinal cord trauma and paralysis in incompletely restrained rabbits, which can kick so hard when handled that they fracture their vertebral column. A second instance is the incidental occurrence at necropsy of widespread neuronal necrosis in the cerebral cortex, hippocampus, and thalamus of some transgenic mice generated on the FVB genetic background. This spontaneous lesion has been attributed to intermittent seizure activity[69] rather than toxicant exposure, as the identical finding is evident in untreated control animals that have the same genetic background; the high susceptibility of FVB mice to chemically induced seizures indicates that great care will be required to confirm that neurodegenerative changes of this nature are truly related to toxicant exposure rather than to background neural overactivity. Finally, we have observed rodents treated with a known neurotoxicant to develop disorientation and ataxia as a sequel to acute bacterial meningitis. The point of these anecdotes is that the toxicological neuropathologist cannot set aside fundamental diagnostic skills when analyzing neural tissues from toxicant-exposed individuals.

Principle 8: Don't limit yourself to the pathology perspective. Although toxicant-induced neuroanatomical changes are often emphasized by regulators in managing neurotoxic risk,[15] reliance solely on neuropathological changes to identify neurotoxicants can be misleading. Some well-known neurotoxicants induce profound functional

changes in the absence of recognizable structural alterations.[9,10] Some classic instances include chlorinated hydrocarbons (e.g., dieldrin), pyrethroids, and strychnine, all of which incite excessive synaptic excitation but no neuropathology, as well as barbiturates, lithium, and organic solvents (e.g., xylene), which cause neuronal depression in the absence of neuromorphological changes. On the other hand, clinical observation of functional deficits can signal the presence of subtle structural lesions. An example is the ability of early reductions in hindlimb grip strength and later progression to paralysis to indicate the presence of a distal axonopathy.

Furthermore, neurological signs in a toxicant-exposed individual are not necessarily evidence of direct neurotoxicity. Anorexia and associated weight loss in rodents are associated with many behavioral changes, including increased motor activity and escape behaviors, decreased hindlimb grip strength, and cognitive learning deficits.[70–72] In like manner, chemically induced injury to some extraneural organs (especially the kidney and liver) can lead to the induction of secondary neurological dysfunction via the accretion of unprocessed neurotoxic waste products. The classic example of this scenario is hepatic encephalopathy, in which severe liver damage permits ammonia accumulation in the blood and brain and ultimately disrupts many CNS metabolic pathways (especially in astrocytes) and glutamatergic excitatory neurotransmission.[73,74] Similarly, renal failure leads to uremic encephalopathy following increased circulating levels of many amino acids and protein metabolites. These examples again underscore the importance of integrating all available structural and functional evidence in reaching a conclusion regarding the risk posed by a potential neurotoxicant.[9,13,14,16]

Principle 9: Carry on with care. In practice, screening studies for neurotoxicity generally administer high doses of test agent to a small-animal species (typically, rats) over relatively short periods of time, assuming that the data gained in the exercise can be extrapolated from high doses to low and from animals to humans. This approach has worked reasonably well but is obviously not perfect, as a number of neurotoxicants have been detected first by epidemic intoxications in humans.[18]

Efforts at extrapolation among species are complicated by the large divergence in responsiveness following neurotoxicant exposure. For example, MPTP depletes nigrostriatal dopaminergic cells in humans and nonhuman primates, eliminates nigrostriatal synaptic terminals in mice, but has a minimal impact on comparable structures in the rat.[39] Species differences in MPTP neurotoxicity reflect variations in the rate and sites at which it is converted to its toxic metabolite, MPP^+ (1-methyl-4-phenylpyridine), by monoamine oxidase type B (MAO-B); in rats, the enzyme is localized in brain microvessels to exclude the highly polar MPP^+ from the neuropil, whereas in primates it is concentrated in astrocytes and acts as a bioactivator of MPTP.[39] Dopaminergic neurons containing a higher quantity of neuromelanin (NM) also may be more susceptible to MPP^+ neurotoxicity, as the NM is thought to serve as a depot for extended release of the toxic metabolite within the target cells.[75,76] Similarly, differential responses between various rodent strains can affect the outcome of neurotoxicity screening studies.[77–79] For example, following amphetamine exposure, both Long–Evans and Sprague–Dawley rats have been shown to develop a comparable dose-dependent reduction of nigrostriatal dopaminergic terminals as well as damage to pyramidal cells and the somatosensory cortex, but only Long–Evans rats exhibit dense axonal degeneration and occasional degenerating cells in the frontal motor cortex.[80] Such differences in neurotoxicity between species and strains probably stem from many factors, including divergent pharmacokinetic profiles, dissimilar genetic backgrounds, unique neurophysiological processes (e.g., binding or uptake sites for toxicants, neural connectivity, naturally occurring neuroprotective agents, enhanced repair processes), or such environmental elements as husbandry and degree of stress. Discrimination among these factors requires a thorough understanding of each agent's mechanism of neurotoxic action. Interspecies extrapolation is especially difficult if xenobiotic-induced neural responses differ among animals of different strains, genders, and ages.[9,81–86]

Principle 10: Garbage in, garbage out. The prime directive for toxicologic neuropathology investigations is to have a preset plan, and to follow it. Professionals in this field follow a standard study design (e.g., Chapter 21; see also the article by Bolon et al.[38] for potential design considerations) that they use for conducting all routine screening studies, which they can then adapt for any specialized follow-up studies that may be required. Standardization of experimental methodology is essential so that the neuropathologist can become familiar with normal neuroanatomical features and neurobiological variations within and among various species and strains, and the technical staff can develop confidence in their prosection and processing skills via repeated practice.

Adequate assessment of neural tissues requires distinctive harvesting and processing techniques[87–89] to preserve structural detail and to avoid confusing artifacts.[90] Precise regional dissection,[91,92] special stains,[89,93–96] intricate morphological measurements,[97–101] and/or noninvasive imaging methods[102–109] may prove useful if biochemical and molecular (i.e., functional) information is to be evaluated in the context of its neuroanatomical localization. A major advantage of noninvasive imaging is that it allows researchers to conduct time-course experiments and more targeted neuroanatomical investigations with fewer animals, as the ability to screen for neuroanatomical changes in vivo helps in selecting subjects that actually have lesions.

Relative to other organs and body systems, the elements of the normal CNS are anatomically diverse, exhibiting major structural changes at both macroscopic and microscopic levels over very short distances and in all three dimensions. Thus, it may be difficult to discern the complete pattern of neural damage induced by a toxicant when the neuropathology screen is performed in two dimensions with only a few brain sections per animal. The complexity is magnified if the toxicant-induced lesions are very subtle[110,111] and the sections available for analysis are not taken at comparable levels for all individuals. The latter issue may be ameliorated by careful attention to detail during sampling[112] and the use of precision sectioning methods to assess multiple animals simultaneously.[13]

CONCLUDING REMARKS

Exposure to potential neurotoxicants in the home, the workplace, or the community is a fact of modern human life. Neurotoxicity epidemics have been induced in humans during recent decades by chemicals,[18] drugs,[39] and metals.[18] Thus, neurotoxicological research to identify and characterize the risk from existing and new compounds of unknown neurotoxic potential is a pressing need.

Toxicological neuropathology is a major consideration for neurotoxicity testing because structural effects are typically permanent in the CNS and at best slowly repaired in the PNS. Researchers who evaluate toxicological neuropathology endpoints must be thoroughly educated in multiple aspects of basic and applied neurobiology so that they can readily identify lesions and then correlate the neuroanatomical changes with biochemical and functional endpoints to provide an integrated, mechanistically based risk assessment. In most settings, toxicological neuropathologists and their neurotoxicologist colleagues are members of interdisciplinary teams rather than solo practitioners. This volume and the current chapter are designed to help neuropathologists and neurotoxicologists achieve a common baseline understanding of major principles and practices in toxicological neuropathology.

REFERENCES

1. Goldman LR. Linking research and policy to ensure children's environmental health. *Environ Health Perspect*. 1998;106 (Suppl 3):857–862.
2. Claudio L. An analysis of the U. S. Environmental Protection Agency neurotoxicity testing guidelines. *Regul Toxicol Pharmacol*. 1992;16:202–212.
3. Environmental Defense, Fund. *Toxic Ignorance: The Continuing Absence of Basic Health Testing for Top-Selling Chemicals in the United States*. Washington, DC: Environment Defense Fund; 1997.
4. Connelly JM, Malkin MG. Environmental risk factors for brain tumors. *Curr Neurol Neurosci Rep*. 2007;7:208–214.
5. Gobba F. Occupational exposure to chemicals and sensory organs: a neglected research field. *Neurotoxicology*. 2003; 24:675–691.
6. Bearer CF. Developmental neurotoxicity: illustration of principles. *Pediatr Clin N Am*. 2001;48:1199–1213.
7. Bouldin TW, Cavanaugh JB. Organophosphorous neuropathy: I. A teased-fiber study of the spatio-temporal spread of axonal degeneration. *Am J Pathol*. 1979;94:241–252.
8. Broxup B, et al. Correlation between behavioral and pathological changes in the evaluation of neurotoxicity. *Toxicol Appl Pharmacol*. 1989;101:510–520.
9. Dorman DC. An integrative approach to neurotoxicology. *Toxicol Pathol*. 2000;28:37–42.
10. Ray DE. Function in neurotoxicity: index of effect and also determinant of vulnerability. *Clin Exp Pharmacol Physiol*. 1997;24:857–860.
11. Spencer PS, Schaumburg HH. Classification of neurotoxic disease: a morphologic approach. In: Spencer PS, Schaumburg HH, eds. *Experimental and Clinical Neurotoxicology*. Baltimore: Williams & Wilkins; 1980:92–99.
12. Sette WF, MacPhail RC. Qualitative and quantitative issues in assessment of neurotoxic effects. In: Tilson HA, Mitchell CL, eds. *Neurotoxicology*. New York: Raven Press; 1992: 345–361.
13. Fix AS, et al. Integrated evaluation of central nervous system lesions: stains for neurons, astrocytes and microglia reveal the spatial and temporal features of MK-801-induced neuronal necrosis in the rat cerebral cortex. *Toxicol Pathol*. 1996;24:291–304.
14. Sette WF. Complexity of neurotoxicological assessment. *Neurotoxicol Teratol*. 1987;9:411–416.
15. Crofton KM, Jarabek AM. Neurotoxicity as the basis for chronic dose–response estimates. *Toxicologist*. 1995;15:34.
16. Eisenbrandt DL, et al. Evaluation of the neurotoxic potential of chemicals in animals. *Food Chem Toxicol*. 1994;32: 655–669.
17. McMaster SB. Developmental toxicity, reproductive toxicity, and neurotoxicity as regulatory endpoints. *Toxicol Lett*. 1993;68:225–230.
18. Costa LG, et al. Developmental neuropathology of environmental agents. *Annu Rev Pharmacol Toxicol*. 2004;44:87–110.
19. Campbell IC, Abdulla EM. Issues in in vitro neurotoxicity testing. *In Vitro Toxicol*. 1995;8:177–186.
20. Bittigau P, et al. Antiepileptic drugs and apoptotic neurodegeneration in the developing brain. *Proc Natl Acad Sci USA*. 2002;99:15089–15094.
21. Weiss B. Chemobrain: a translational challenge for neurotoxicology. *Neurotoxicology*. 2008;29:891–898.
22. Chudler EH. Milestones in Neuroscience Research. Available at: http://faculty.washington.edu/chudler/hist.html. Accessed Sept 11, 2009.
23. Henry JM. Neurons and Nobel Prizes: a centennial history of neuropathology. *Neurosurgery*. 1998;42:143–155 (discussion, 155–156).

24. Gallo MA. History and scope of toxicology. In: Klaassen CD, ed. *Casarett and Doull's Toxicology: The Basic Science of Poisons*. New York: McGraw-Hill; 1996:3–11.
25. Cavanagh JB. Lesion localisation: implications for the study of functional effects and mechanisms of action. *Toxicology*. 1988;49:131–136.
26. Cavanagh JB. Selective vulnerability in acute energy deprivation syndromes. *Neuropathol Appl Neurobiol*. 1993;19: 461–470.
27. Cavanagh JB, et al. Selective damage to the cerebellar vermis in chronic alcoholism: a contribution from neurotoxicology to an old problem of selective vulnerability. *Neuropathol Appl Neurobiol*. 1997;23:355–363.
28. Hawkes CH, et al. Motoneuron disease: a disorder secondary to solvent exposure? *Lancet*. 1989;1:73–76.
29. Tomiwa K, et al. The effects of cisplatin on rat spinal ganglia: a study by light and electron microscopy and by morphometry. *Acta Neuropathol*. 1986;69:295–308.
30. Wadsworth PF, et al. The topography, structure and incidence of mineralized bodies in the basal ganglia of the brain of cynomolgus monkeys (*Macaca fascicularis*). *Lab Anim*. 1995;29:276–281.
31. Butchko HH, et al. Aspartame: review of safety. *Regul Toxicol Pharmacol*. 2002;35:S1–S93.
32. Koestner A. The brain-tumour issue in long-term toxicity studies in rats. *Food Chem Toxicol*. 1986;24:139–143.
33. Koestner A. Characterization of *N*-nitrosourea-induced tumors of the nervous system: their prospective value for studies of neurocarcinogenesis and brain tumor therapy. *Toxicol Pathol*. 1990;18:186–192.
34. Yaeger MJ, et al. The use of nerve growth factor as a reverse transforming agent for the treatment of neurogenic tumors: in vivo results. *Acta Neuropathol*. 1992;83:624–629.
35. Katelaris A, et al. Brains at necropsy: to fix or not to fix? *J Clin Pathol*. 1994;47:718–720.
36. Bolon B. Comparative and correlative neuroanatomy for the toxicologic pathologist. *Toxicol Pathol*. 2000;28:6–27.
37. Bolon B, et al. Current pathology techniques symposium review: advances and issues in neuropathology. *Toxicol Pathol*. 2008;36:871–889.
38. Bolon B, et al. A "best practices" approach to neuropathologic assessment in developmental neurotoxicity testing—for today. *Toxicol Pathol*. 2006;34:296–313.
39. Kopin IJ. MPTP: an industrial chemical and contaminant of illicit narcotics stimulates a new era in research on Parkinson's disease. *Environ Health Perspect*. 1987;75:45–51.
40. Ekino S, et al. Minamata disease revisited: an update on the acute and chronic manifestations of methyl mercury poisoning. *J Neurol Sci*. 2007;262:131–144.
41. Jones DC, Miller GW. The effects of environmental neurotoxicants on the dopaminergic system: a possible role in drug addiction. *Biochem Pharmacol*. 2008;76:569–581.
42. Migliore L, Coppedè F. Environmental-induced oxidative stress in neurodegenerative disorders and aging. *Mutat Res*. 2009;674:73–84.
43. Rice D, Barone S Jr. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ Health Perspect*. 2000;108(Suppl 3):511–533.
44. Battaglia G, et al. Neurogenesis in cerebral heterotopia induced in rats by prenatal methylazoxymethanol treatment. *Cereb Cortex*. 2003;13:736–748.
45. Bassil KL, et al. Cancer health effects of pesticides: systematic review. *Can Fam Physician*. 2007;53:1704–1711.
46. Rice JM, Wilbourn JD. Tumors of the nervous system in carcinogenic hazard identification. *Toxicol Pathol*. 2000;28:202–214.
47. Graham DG, et al. Pathogenetic studies of hexane and carbon disulfide neurotoxicity. *Crit Rev Toxicol*. 1995;25:91–112.
48. Adams JH, Duchen LW. *Greenfield's Neuropathology*, 5th ed. Oxford, UK: Oxford University Press; 1992.
49. Summers BA, et al. *Veterinary Neuropathology*. St. Louis, MO: Mosby; 1995.
50. Barone SF, et al. Vulnerable processes of the nervous system development: a review of markers and methods. *Neurotoxicology*. 2000;21:15–36.
51. Rodier PM, et al. Morphologic effects of interference with cell proliferation in the early fetal period. *Neurobehav Toxicol*. 1979;1:129–135.
52. Heiss WD. Cerebral blood flow: physiology, pathophysiology and pharmacological effects. *Adv Otorhinolaryngol*. 1981; 27:26–39.
53. Nicklas WJ, et al. Mitochondrial mechanisms of neurotoxicity. *Ann NY Acad Sci*. 1992;648:28–36.
54. Kostrzewa RM, Brus R. Destruction of catecholamine-containing neurons by 6-hydroxydopa, an endogenous amine oxidase cofactor. *Amino Acids*. 1998;14:175–179.
55. Tomac A, et al. Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. *Nature*. 1995;373:335–339.
56. Juurlink BH, Paterson PG. Review of oxidative stress in brain and spinal cord injury: suggestions for pharmacological and nutritional management strategies. *J Spinal Cord Med*. 1988;21:309–334.
57. Bolon B, et al. Phase-specific developmental toxicity in mice following maternal methanol inhalation. *Fundam Appl Toxicol*. 1993;21:508–516.
58. Kotch LE, Sulik KK. Experimental fetal alcohol syndrome: proposed pathogenic basis for a variety of associated facial and brain anomalies. *Am J Med Genet*. 1992; 44:168–176.
59. Nau H, et al. Valproic acid-induced neural tube defects in mouse and human: aspects of chirality, alternative drug development, pharmacokinetics and possible mechanisms. *Pharmacol Toxicol*. 1991;69:310–321.
60. Altman J, Bayer SA. *Atlas of Prenatal Rat Brain Development*. Boca Raton, FL: CRC Press; 1995.
61. Rodier PM. Chronology of neuron development: animal studies and their clinical implications. *Dev Med Child Neurol*. 1980;22:525–545.

62. Rodier PM. Vulnerable periods and processes during central nervous system development. *Environ Health Perspect*. 1994;102(Suppl 2):121–124.
63. Dobbing J, Sands J. Comparative aspects of the brain growth spurt. *Early Hum Dev*. 1979;3:79–83.
64. Herschkowitz N, et al. Neurobiological bases of behavioral development in the first year. *Neuropediatrics*. 1997;28: 296–306.
65. Herschkowitz N, et al. Neurobiological bases of behavioral development in the second year. *Neuropediatrics*. 1999;30: 221–230.
66. Qiao D, et al. Developmental neurotoxicity of chlorpyrifos: What is the vulnerable period? *Environ Health Perspect*. 2002;110:1097–1103.
67. Jortner BS. The return of the dark neuron: a histological artifact complicating contemporary neurotoxicologic evaluation. *Neurotoxicology*. 2006;27:628–634 [see comment in *Neurotoxicology.* 2006;27:1126].
68. Kherani ZS, Auer RN. Pharmacologic analysis of the mechanism of dark neuron production in cerebral cortex. *Acta Neuropathol.* 2008;116:447–452.
69. Goelz MF, et al. Neuropathologic findings associated with seizures in FVB mice. *Lab Anim Sci.* 1998;48:34–37.
70. Albee RR, et al. Neurobehavioral effects of dietary restriction in rats. *Neurotoxicol Teratol.* 1987;9:203–211.
71. Dobbing J, Sands J. Vulnerability of developing brain: IX. The effect of nutritional growth retardation on the timing of the brain growth-spurt. *Biol Neonate*. 1971;19:363–378.
72. Gerber GJ, O'Shaughnessy D. Comparison of the behavioral effects of neurotoxic and systemically toxic agents: How discriminatory are behavioral tests of neurotoxicity? *Neurobehav Toxicol*. 1986;8:703–710.
73. Butterworth RF. Portal-systemic encephalopathy: a disorder of neuron-astrocytic metabolic trafficking. *Dev Neurosci.* 1993;15:313–319.
74. Felipo V, et al. Neurotoxicity of ammonia and glutamate: molecular mechanisms and prevention. *Neurotoxicology*. 1998;19:675–681.
75. Herrero MT, et al. Does neuromelanin contribute to the vulnerability of catecholaminergic neurons in monkeys intoxicated with MPTP? *Neuroscience*. 1993;56:499–511.
76. Zecca L, et al. The neuromelanin of human substantia nigra: structure, synthesis and molecular behaviour. *J Neural Transm*. 2003;Suppl:145–155.
77. Fink JS, Reis DJ. Genetic variations in midbrain dopamine cell number: parallel with differences in responses to dopaminergic agonists and in naturalistic behaviors mediated by central dopaminergic systems. *Brain Res*. 1981;222: 335–349.
78. Mohajeri MH, et al. The impact of genetic background on neurodegeneration and behavior in seizured mice. *Genes Brain Behav*. 2004;3:228–239.
79. Stöhr T, et al. Rat strain differences in open-field behavior and the locomotor stimulating and rewarding effects of amphetamine. *Pharmacol Biochem Behav*. 1998;59:813–818.
80. Ryan LJ, et al. Histological and ultrastructural evidence that D-amphetamine causes degeneration in neostriatum and frontal cortex of rats. *Brain Res*. 1990;518:67–77.
81. Bondy SC. Especial considerations for neurotoxicological research. *Crit Rev Toxicol.* 1985;14:381–402.
82. Claassen V. *Neglected Factors in Pharmacology and Neuroscience Research*. New York: Elsevier; 1994.
83. Dyer RS. Cross species extrapolation and hazard identification in neurotoxicology. *Neurobehav Toxicol Teratol.* 1984;6: 409–411.
84. Kacew S, Festing MFW. Role of rat strain in the differential sensitivity to pharmaceutical agents and naturally occurring substances. *J Toxicol Environ Health.* 1996;47:1–30.
85. Kacew S, et al. Strain as a determinant factor in the differential responsiveness of rats to chemicals. *Toxicol Pathol.* 1995;23:701–715.
86. Tipton KF, Singer TP. Advances in our understanding of the mechanisms of the neurotoxicity of MPTP and related compounds. *J Neurochem.* 1993;61:1191–1206.
87. Krinke GJ, et al. Optimal conduct of the neuropathology evaluation of organophosphorus induced delayed neuropathy in hens. *Exp Toxicol Pathol.* 1997;49:451–458.
88. Krinke GJ, et al. Teased-fiber technique for peripheral myelinated nerves: methodology and interpretation. *Toxicol Pathol.* 2000;28:113–121.
89. Switzer RC III. Application of silver degeneration stains to neurotoxicity testing. *Toxicol Pathol.* 2000;28:70–83.
90. Garman RH. Artifacts in routinely immersion-fixed nervous tissue. *Toxicol Pathol.* 1990;18:149–153.
91. Palkovits M, Brownstein MJ. *Maps and Guide to Microdissection of the Rat Brain*. New York: Elsevier; 1988.
92. Toga AW. Brain-mapping neurotoxicity and neuropathology. *Ann NY Acad Sci.* 1997;820:1–13.
93. Downing AE. Neuropathological histotechnology. In: Prophet EB, et al., eds. *Laboratory Methods in Histotechnology*. Washington, DC: American Registry of Pathology; 1992: 81–104.
94. Morgan KT, et al. Vascular leakage produced in the brains of mice by *Clostridium welchii* type D toxin. *J Comp Pathol*. 1975;85:461–466.
95. O'Callaghan J, Sriram K. Glial fibrillary acidic protein and related glial proteins as biomarkers of neurotoxicity. *Expert Opin Drug Saf.* 2005;4:433–442.
96. O'Callaghan JP. Quantitative features of reactive gliosis following toxicant-induced damage to the CNS. In: Johannessen JN, ed. *Markers of Neuronal Injury and Degeneration*. New York: New York Academy of Sciences; 1993: 195–210.
97. Broxup BR, et al. Quantitative techniques in neuropathology. *Toxicol Pathol.* 1990;18:105–114.
98. Coggeshall RE, Lekan HA. Methods for determining numbers of cells and synapses: a case for more uniform standards of review. *J Comp Neurol.* 1996;364:6–15.
99. Diemer NH. Quantitative morphological studies of neuropathological changes: Part 1. *Crit Rev Toxicol.* 1982;10:215–263.

100. Hyman BT, et al. Stereology: a practical primer for neuropathology. *J Neuropathol Exp Neurol.* 1998;57:305–310.

101. Murray JM. Neuropathology in depth: the role of confocal microscopy. *J Neuropathol Exp Neurol*. 1992;51: 475–487.

102. Heckl S, et al. Molecular imaging: bridging the gap between neuroradiology and neurohistology. *Histol Histopathol.* 2004; 19:651–668.

103. Hesselbarth D, et al. High resolution MRI and MRS: a feasibility study for the investigation of focal cerebral ischemia in mice. *NMR Biomed.* 1998;11:423–429.

104. Kornguth S, et al. Near-microscopic magnetic resonance imaging of the brains of phenylalanine hydroxylase–deficient mice, normal littermates, and of normal BALB/c mice at 9.4 tesla. *Neuroimage*. 1994;1:220–229.

105. Sharif NA, Eglen RM. Quantitative autoradiography: a tool to visualize and quantify receptors, enzymes, transporters, and second messenger systems. In: Sharif NA, ed. *Molecular Imaging in Neuroscience: A Practical Approach*. Oxford, UK: IRL Press; 1993: 71–138.

106. Sills RC, et al. Contribution of magnetic resonance microscopy in the 12-week neurotoxicity evaluation of carbonyl sulfide in Fischer 344 rats. *Toxicol Pathol*. 2004;32: 501–510.

107. Suehiro M, et al. A PET radiotracer for studying serotonin uptake sites: carbon-11-McN-5652Z. *J Nucl Med.* 1993;34: 120–127.

108. Willerman L, et al. Hemisphere size asymmetry predicts relative verbal and nonverbal intelligence differently in the sexes: an MRI study of structure–function relations. *Intelligence*. 1992;16:315–328.

109. Woodroofe MN, et al. In situ hybridization in neuropathology. *Neuropathol Appl Neurobiol*. 1994;20:562–572.

110. Balaban CD. Central neurotoxic effects of intraperitoneally administered 3-acetylpyridine, harmaline and niacinamide in Sprague–Dawley and Long–Evans rats: a critical review of central 3-acetylpyridine neurotoxicity. *Brain Res.* 1985;356: 21–42.

111. Fix AS, et al. Quantitative analysis of factors influencing neuronal necrosis induced by MK-801 in the rat posterior cingulate/retrosplenial cortex. *Brain Res*. 1995;696: 194–204.

112. Broxup B. Neuropathology as a screen for neurotoxicity assessment. *J Am Coll Toxicol*. 1991;10:689–695.