Animal Models of Peripheral Neuropathy Due to Environmental Toxicants

Deepa B. Rao, Bernard S. Jortner, and Robert C. Sills

Abstract

Despite the progress in our understanding of pathogeneses and the identification of etiologies of peripheral neuropathy, idiopathic neuropathy remains common. Typically, attention to peripheral neuropathies resulting from exposure to environmental agents is limited relative to more commonly diagnosed causes of peripheral neuropathy (diabetes and chemotherapeutic agents). Given that there are more than 80,000 chemicals in commerce registered with the Environmental Protection Agency and that at least 1000 chemicals are known to have neurotoxic potential, very few chemicals have been established to affect the peripheral nervous system (mainly after occupational exposures). A wide spectrum of exposures, including pesticides, metals, solvents, nutritional sources, and pharmaceutical agents, has been related, both historically and recently, to environmental toxicant-induced peripheral neuropathy. A review of the literature shows that the toxicity and pathogeneses of chemicals adversely affecting the peripheral nervous system have been studied using animal models. This article includes an overview of five prototypical environmental agents known to cause peripheral neuropathy—namely, organophosphates, carbon disulfide, pyridoxine (Vitamin B_6), acrylamide, and hexacarbons (mainly n-hexane, 2,5-hexanedione, methyl n-butyl ketone). Also included is a brief introduction to the structural components of the peripheral nervous system and pointers on common methodologies for histopathologic evaluation of the peripheral nerves.

Key Words: acrylamide; carbon disulfide; environmental toxicant; hexacarbon; organophosphate; peripheral neuropathy; pyridoxine; vitamin B_6

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Introduction

Worldwide, the incidence of peripheral neuropathy in humans due to toxicants ranges across a broad spectrum that includes acute and accidental exposures, as well as chronic low-dose exposures in industrial or occupational settings, exposures in the ambient environment as a result of increased commercial use of chemicals, and less commonly, intentional exposures associated with recreational abuse, suicide, chemical warfare, and terrorist activities. Classes of toxic agents include pesticides, chemical and organic solvents, metals, chemical contaminants, nutritional ingredients, and pharmaceutical drugs (Spencer and Schaumburg 2000). Examples of specific toxicants affecting the peripheral nervous system (PNS) include, but are not limited to, organic solvents (n-hexane, trichloroethylene, methyl-n-butyl ketone, toluene, and styrene), industrial chemicals (carbon disulfide, dimethylaminoproprionitrile, and acrylamide), metals (arsenic, organic mercury, thallium, and lead), and pharmaceutical sources (vitamin B_6 or pyridoxine, immunosuppressants, and cancer chemotherapeutics). Additionally, organophosphate compounds are not only present as active ingredients in pesticides but have also been identified as contaminants in cooking oil and in illicit alcohol production (during the 1920s Prohibition era in the United States). More recently, emerging toxic neuropathies include an incidence of exposure to aerosolized porcine neural tissue that resulted in polyradiculo-neuropathy in swine abattoir workers (Holzbauer et al. 2010; Lachance et al. 2010). A list of well-known toxic agents inducing peripheral neuropathy in humans is summarized in Table 1.

The proportional incidence of peripheral neuropathy specifically due to environmental or industrial toxicants compared with other etiologies is unclear. Despite advances in the identification of etiologies of peripheral neuropathy, it is evident that in clinical settings idiopathic polyneuropathy remains common in humans (Weimer and Sachdev 2009). Given the vast range of neurotoxicants resulting in peripheral neuropathy, compounded with the inherent anatomic and physiologic complexity of the organization of the PNS, it is imperative that a thorough examination of the PNS be conducted during clinical evaluation in humans, in preclinical safety evaluation studies of potential pharmaceutical agents, and in the hazard identification of animal toxicity studies for screening of environmental chemicals. What follows is a brief

Table 1 Examples of human exposures resulting in peripheral neuropathy

Substance	Location	Comments	Reference
Industrial chemical			
Acrylamide		Polyacrylamide production	(LoPachin et al. 2003)
	Canada	Grout worker	(Auld and Bedwell 1967; Kjuus et al. 2004)
Solvents			
n-Hexane	Iran	Shoemakers	(Neghab et al. 2012)
	United States	Furniture makers	(Herskowitz et al. 1971)
	India	Screen printers	(Puri et al. 2007)
Methyl n-butyl ketone	United States	Fabric plant workers	(Allen et al. 1975; Mendell et al. 1974)
	United States	Spray painters	(Mallov 1976)
	South Africa	Polymer factory workers	(Myers and Macun 1991)
Carbon disulfide	China Italy	Viscose rayon production workers	(Chu et al. 1995; Corsi et al. 1983)
2-t-Butylazo-2-hydroxy-5-methylhexane	United States	Workers in reinforced plastic bathtub manufacture	(Horan et al. 1985)
Organic solvent/mixtures (n-hexane, methyl ethyl ketone, toluene)	Germany United States	Glue sniffers and Huffer's neuropathy	(Altenkirch et al. 1977; Prockop et al. 1974; Towfighi et al. 1976)
Trichloroethylene	United States	Contaminated well water	(White et al. 1997)
Pesticides			
Organophosphates	Italy	Agricultural workers	(Lotti and Moretto 2005)
	Turkey	Suicides	(Ergun et al. 2009)
	Iran	Chemical warfare	(Holisaz et al. 2007)
	Japan	Terrorist activity (Sarin)	(Yokoyama et al. 1998)
	Morocco	Cooking oil (triorthocresylphosphate)	(Smith and Spalding 1959)
	South Africa	Cooking oil (triorthocresylphosphate)	(Susser and Stein 1957)
	Sri Lanka	Cooking oil (triorthocresylphosphate)	(Senanayake and Jeyaratnam 1981)
	United States	Beverage production (alcohol during Prohibition era) (triorthocresylphosphate)	(Aring 1942; Morgan and Penovich 1978; Woolf 1995)
Metals			
Mercury	Japan	Minamata disease	(Miyakawa et al. 1976; Ninomiya et al. 2005)
	Iraq	Pesticide-coated grain	(Bakir et al. 1973)
Arsenic	United States	Arsenic smelter workers	(Feldman et al. 1979)
Thallium	United Kingdom	Intentional ingestion of rodenticides	(Cavanagh et al. 1974)
Lead	India	Battery factory workers	(Shobha et al. 2009)
Pharmaceutical agents			
Thiocyanate	Nigeria	Cassava	(Osuntokun et al. 1970)
Vitamin B_6 (pyridoxine)	United States	Nutritional supplement	(Scott et al. 2008)
Clioquinol	India	Antifungal and antiprotozoal drug	(Wadia 1984)

Continued

Table 1 Continued

Substance	Location	Comments	Reference
Cancer chemotherapeutics	Australia	Platinum compounds, taxanes, vinca alkaloids, 5-fluorouracil	(Schloss et al. 2013; Zheng et al. 2012)
Cholesterol-lowering statins		3-hydroxy-3-methylglutaryl-Coenzyme A reductase inhibitors (HMG-CoA) (lovastatin, pravastatin, simavastin)	(Chong et al. 2004)
Other			
Aerosolized porcine neural tissue	United States	Swine abattoirs	(Holzbauer et al. 2010; Lachance et al. 2010)
Toxic oil syndrome	Spain	Rapeseed oil	(Altenkirch et al. 1988)

introduction of the structural components of the PNS relevant to toxic neuropathies, a short review of five prototypical PNS toxicants, and a brief overview of current approaches to histopathologic examination of peripheral nerves in animal studies.

Overview of the PNS

The challenge with assessing the effects of environmental toxicants on the PNS lies in that, both anatomically and functionally, the organization of the PNS is remarkably extensive. Moreover, environmental toxicants can affect any component or multiple components of the PNS. In general, the PNS includes the innervation of somatic (skeletal muscle) and visceral (autonomic) components of the nervous system by afferent (sensory) and efferent (motor) fibers. Somatic afferent fibers carry information from special organs for vision, hearing, and balance; from sensory receptors in skin for pain, touch, and temperature; and from receptors controlling proprioception in muscles and joints. Visceral afferents convey impulses (pain or reflex sensations) from the internal organs, glands, and blood vessels. Somatic afferents innervate striated muscle, whereas visceral efferents supply smooth muscles of blood vessels, glands, and the gut. Peripheral neurotoxicants affecting somatic sensory-motor fibers can impact sensory loss (changes in sensitivity to vibration, touch, and proprioception) and motor weakness in distal extremities (damage to nerves or neuromuscular junctions leading to muscle atrophy). Peripheral neurotoxicants affecting autonomic fibers can result in abnormal sweating, cardiovascular changes, or disturbances in the gastrointestinal and genitourinary systems.

Histologically, although the major components of the PNS are axons, myelin sheaths, and Schwann cells, ganglia contain neuronal cell bodies and satellite cells. Because the cell bodies of motor neurons lie within the spinal cord and brainstem and some peripheral sensory neurons have processes extending in spinal and brainstem tracts, a true distinction between the central nervous system and the PNS is superficial.

Environmental Agents Toxic to the PNS

Typically, environmental toxicants can cause neuronopathies (primarily impacting the neuronal cell bodies), axonopathies (affecting the axon), and myelinopathies (involvement of the myelin sheath or the Schwann cell). Neuronopathies have been reported after exposure to pyridoxine, arsenic, and thallium. Axonopathies are seen with organophosphates and carbon disulfide exposures, whereas myelinopathies can be induced by trichloroethylene. Knowledge and understanding of the pattern by which neural cells are affected has been gained from experimental and morphologic studies. In fact, in most of the human intoxications of the nervous system, knowledge of the structural changes is based almost exclusively on laboratory animal studies. A few select peripheral neurotoxicants taken from the list in Table 1 are reviewed in the following sections.

Organophosphorus Ester-Induced Delayed Neurotoxicity

Organophosphorus ester-induced delayed neurotoxicity (OPIDN) is an entity that has been recognized in man for more than 100 years (Ehrich and Jortner 2002, 2010). It is distinct from acute cholinergic organophosphate poisoning, although some, but not all, OPIDN-inducing agents also elicit acute toxicity. OPIDN is manifested by degeneration of distal regions of large, long axons and thus involves myelinated fibers in the peripheral (and central) nervous system. For the classical form of OPIDN (described here), neurotoxicants must have their phosphorus in a pentavalent state and include direct acting agents, such as mipafox, diisopropyl phosphofluoridate, and phenyl saligenin phosphate, and protoxicants, such as leptophos and tri-*ortho*-tolyl (cresyl) phosphate (Ehrich and Jortner 2010). As part of the name (i.e., "delayed") implies, there is a latent period between exposure to a toxic dose of the organophosphate and clinical recognition of the disease. Clinical signs in man include bilateral weakness progressing to flaccidity, tingling, loss of sensation, locomotor difficulties, and abnormal reflexes of the limbs,

especially distally (Ehrich and Jortner 2002). In the domestic chicken, the standard animal model, similar signs of weakness and incoordination, initially affecting the legs, are also noted (Cavanagh 1954). Peripheral nerve lesions in experimental studies in chickens include axonal swelling with associated granular degeneration of the cytoskeleton or aggregation of dark-stained membranous masses, altered mitochondria, and dense bodies (Figure 1). The altered axons are unable to maintain their myelin sheaths, and the altered fibers undergo phagocytosis and degradation within Schwann cells or macrophages (Figure 1), resembling changes seen in Wallerian degeneration. This evolves to formation of columns of Schwann cells (the so-called bands of Büngner), which provide sites for axonal regeneration (Jortner et al. 1989). As the clinical signs suggest, lesions are more severe distally but may progress proximally along the nerve trunk. There is associated similar but more slowly evolving bilateral myelinated fiber degeneration in long tracts of the central nervous system, but as expected, regeneration does not occur in these regions.

The precise mechanisms of neurotoxicity are not known, but there is a well-described association of inhibition and aging, a related intramolecular rearrangement, of the nervous system enzyme neurotoxic esterase (neuropathy target esterase, NTE) induced by such agents, and the subsequent development of OPIDN. The physiologic functions of NTE are unknown, as is its role in OPIDN-induced nerve fiber dege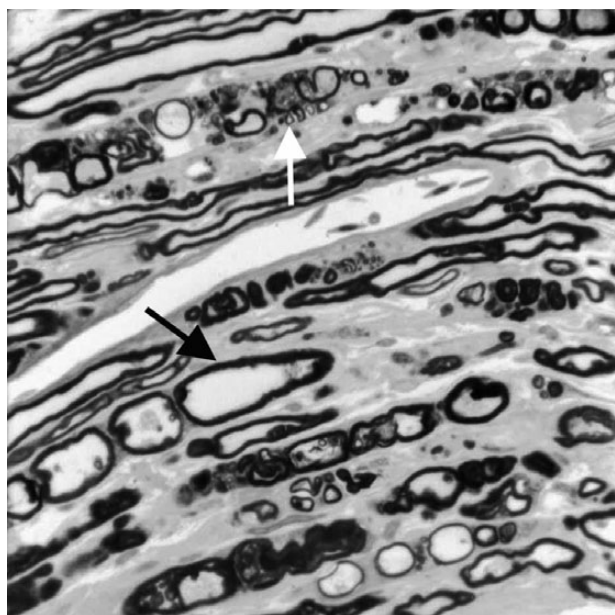neration (Ehrich and Jortner 2002). There are temporal and dose level–related aspects to the association of toxicant exposure, NTE inhibition, and subsequent OPIDN. As an example, using a single neurotoxic dose in the chicken leading to inhibition of at least 70% of the brain NTE by 48 hours after exposure is likely to be followed by OPIDN in a 7 to 10 day period, by which time NTE has largely regenerated. In addition to work with NTE, studies have suggested altered threshold activity of peripheral nerve, disturbed axonal transport, increased protease activity, and abnormal protein phosphorylation as mechanistic factors in the axonal degeneration of OPIDN (Abou-Donia 1995; Ehrich and Jortner 2002; El-Fawal et al. 1990; Pope et al. 1995; Richardson 1995). There appears to be an age-related issue in OPIDN, as mature chickens are more susceptible than chicks.

Carbon Disulfide

Carbon disulfide is used in the viscose production of cellophane and rayon, with approximately 76 million pounds released into the atmosphere annually in the United States during production. Exposure to carbon disulfide in humans results in tingling and numbness of the extremities and weakness of limbs. In rats exposed to carbon disulfide, neuromuscular deficits are more pronounced in the hindlimbs where there is decreased grip strength and gait alterations (Moser et al. 1998). In both human occupational settings and experimental animal models, exposure to carbon disulfide can result in prominent central and peripheral neuropathies with a predilection for long, large-diameter myelinated axons (Graham et al. 1995). The axonopathy is first observed in the long distal axons and is characterized by axonal swelling (Figure 2) with the accumulation of neurofilament proteins in distal and sensory and motor nerve tracts and is most often detected proximal to the nodes of Ranvier (Gottfried et al. 1985; Sills et al. 1998). The accumulation of neurofilaments
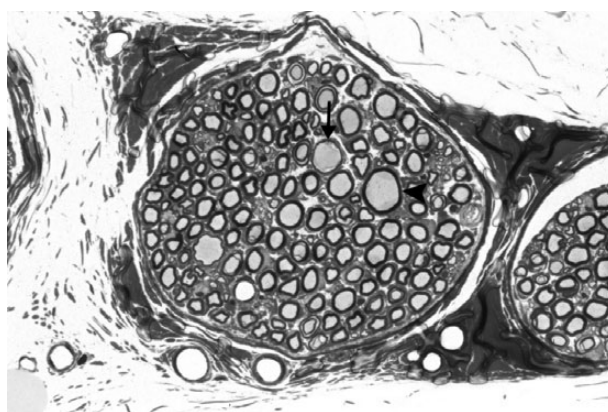

Figure 1 Tangential section of perfusion-fixed sciatic nerve embedded in epoxy resin of a chicken exposed to a neurotoxic dose (2.5 mg/kg) of phenyl saligenin phosphate (intramuscular) 15 days before it was killed. Several stages of myelinated fiber degeneration are seen, including a swollen pale stained axon (black arrow) and a fiber undergoing degradation (Wallerian-type) within a phagocyte (white arrow). Toluidine blue and safranin stain.

Figure 2 Muscular branch of the posterior tibial nerve (perfusion fixed and embedded in epoxy resin) with multiple swollen axons (arrow head and arrow) within a nerve fascicle from a rat exposed to 800 ppm of carbon disulfide (by inhalation) for 13 weeks (6 hours/day for 5 days/week). Note the thinning of the myelin sheath in a swollen axon (arrow). Toluidine blue stain.
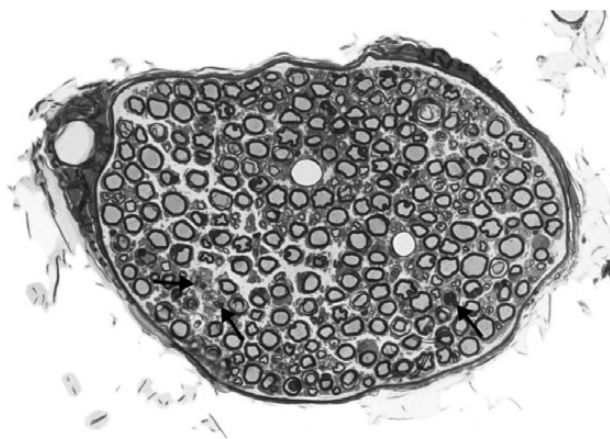
Figure 3 Muscular branch of the posterior tibial nerve (perfusion fixed and embedded in epoxy resin) illustrating axonal degeneration (arrows) within a nerve fascicle from a rat exposed to 800 ppm of carbon disulfide (by inhalation) for 13 weeks (6 hours/day for 5 days/week). Toluidine blue stain.

in the axoplasm of swollen myelinated axons displaces mitochondria and neurotubules to the periphery of the axon. The swollen axons may have thin myelin sheaths (Figure 2) that have a normal configuration but are composed of a reduced number of myelin lamellae. After continuous exposure to carbon disulfide, degeneration (Figure 3) and regeneration of axons in the PNS may occur. A common mechanism has been proposed for the axonal swelling and degeneration observed after exposure to chemicals that cause axonopathies such as carbon disulfide and 2,5-hexanedione. Carbon disulfide covalently modifies neurofilament proteins. Specifically, carbon disulfide has been shown to cause Lys-Lys thiourea cross-linking of neurofilament proteins that results in the accumulation of neurofilaments in the axon, which causes the characteristic lesion of axonal swelling (Valentine et al. 1997).

Hexacarbons (n-Hexane, Methyl n-Butyl Ketone, 2,5-Hexanedione)

2,5-Hexanedione is the active γ-diketone metabolite of n-hexane and methyl n-butyl ketone. The parent hexacarbons (n-hexane and methyl n-butyl ketone) have been implicated in a multitude of occupational and intentional inhalational abuse exposure settings (Spencer and Schaumburg 1980). In humans, teased myelinated nerve fiber preparations from nerve biopsies after occupational exposure to n-hexane illustrated paranodal giant axonal swellings accompanied by myelin retraction, and electron microscopy showed neurofilament accumulation in axonal swelling (Rizzuto et al. 1977). As with carbon disulfide, experimental studies in animals have shown axonal swellings containing neurofilament masses as the morphologic hallmarks of hexacarbon toxicity (Graham et al. 1982; Spencer and Schaumburg 1980). Specifically, increased numbers of malaligned neurofilaments

(Gottfried et al. 1985; Spencer and Schaumburg 1977) within areas of axonal swelling are seen in both hexanedione and carbon disulfide exposure. There are also decreased numbers of microtubules, sequestration of axoplasmic organelles, and expansion of Schwann cell cytoplasm (Gottfried et al. 1985; Spencer and Schaumburg 1977). As noted above, there may be a common molecular mechanism of toxicity between 2,5-hexanedione and carbon disulfide. In addition to axonal swelling, experimental animal studies have shown that axonal atrophy is an important pathologic change observed with various dosing paradigms (Lehning et al. 1995; LoPachin et al. 2000). Although pigeons, chickens, mice, guinea pigs, cats, dogs, and monkeys have been used in experimental studies to delineate mechanisms of peripheral neuropathy, hexacarbon neuropathy has been most thoroughly investigated in the rat (Spencer and Schaumburg 1980).

Pyridoxine (Vitamin B$_6$) Neuropathy

Although not a classical environmental toxicant, pyridoxine (vitamin B$_6$) is included here because it illustrates neuronopathy, an important form of toxic neuropathy. Pyridoxine is a dietary requirement and is a coenzyme in many biologic reactions, but megadoses of this vitamin are neurotoxic, eliciting bilateral sensory neuropathy in humans and experimental animals. Examples of such excessive dosages are daily oral exposures of 7 to 10 grams for 2 months in humans (Schaumburg 2000) or 100 to 300 mg/kg for 12 weeks or 600 to 1200 mg/kg intraperitoneally for 6 to 10 days in rats (Xu et al. 1989). Such toxic exposures lead to neuronopathy, a condition in which the primary insult is in the neuronal cell body (Krinke and Fitzgerald 1988). For pyridoxine, such injury is manifested by injury to the soma of larger neurons of primary sensory ganglia, such as dorsal root ganglia. This process may lead to progressive injury to the neuron, resulting in necrosis (Figure 4). A consequence is the inability of the injured neuronal cell body to maintain its long axon. Thus, there is axonal degeneration progressing to myelinated fiber degeneration, which is more severe distally (Jortner 2000; Krinke and Fitzgerald 1988). In severe or prolonged intoxication, proximal fiber levels are affected, such as dorsal spinal roots (Figure 4). Central axonal projections from affected ganglionic neurons, such as in the gracile fasciculus, are also affected. If injury is sublethal, cessation of pyridoxine exposure may result in clinical improvement, likely an expression of axonal repair or regeneration, with the latter being restricted to peripheral nerves (Schaumburg 2000; Windebank et al. 1985). If neurons are severely injured or necrotic, then such axonal restoration is not possible. The mechanism of pyridoxine neuronopathy is not fully understood, but it is thought that high circulating levels of pyridoxine might have a direct toxic effect on neurons of the peripheral sensory ganglia (Jortner 2000), which may relate to the presence of a lower ("leaky") blood–nerve barrier in such regions (Olsson 1984). The more complete blood–brain barrier in the central nervous system, along with a saturable pyridoxine
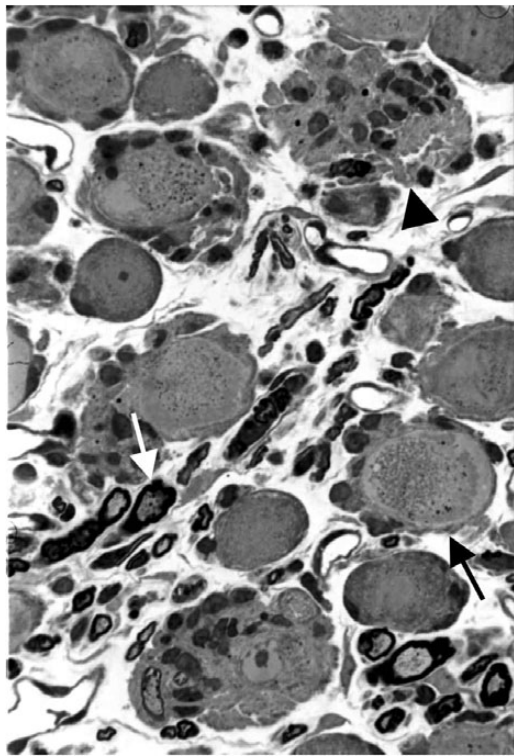
Figure 4 Dorsal root ganglion of a rat (perfusion fixed and embedded in epoxy resin) exposed to a toxic dose (600 mg/kg) of pyridoxine administered intraperitoneally twice a day for 4 days and killed 2 days after the last exposure. Degradative change is seen in the soma of a neuron (black arrow), which progresses to necrosis manifest by satellite cell phagocytosis of an affected neuron cell body (neuronophagia) (arrowhead). There also is axonal degeneration of a myelinated dorsal root fiber within the ganglion (white arrow). Toluidine blue and safranin stain.

transport system, protects neurons in those regions from high blood levels of pyridoxine (Schaumburg 2000).

Acrylamide Neuropathy

Acrylamide is a water-soluble vinyl monomer used to produce polyacrylamide (a polymeric form) that is used extensively in chemical industries, in molecular research laboratories, and as a by-product in certain food preparations (Spencer and Schaumburg 1974; Tareke et al. 2000). It is important to note that only the monomeric form is neurotoxic. With the initial recognition of skeletal muscle ataxia and weakness in humans exposed to acrylamide (Prineas 1969), the elucidation of PNS toxicity preceded the demonstration of central nervous system target sites in animal studies. Today, the hallmark morphologic effects of acrylamide are considered to include the progressive loss of cerebellar Purkinje cells (Cavanagh and Nolan 1982; Lehning et al. 2002) and, peripherally, the degeneration of sensory and somatomotor nerve terminals (LoPachin et al. 2003). Although animal studies demonstrate axonal degeneration resulting from

chronic and low-dose exposures (Crofton et al. 1996; Prineas 1969), it is interesting to note that even with high doses, the earliest neuropathologic features of acrylamide neurotoxicity include the PNS—specifically, degeneration at the nerve terminals (Lehning et al. 1998). It is now understood that the effects at the motor nerve terminals of hindlimb neuromuscular junctions exhibit degenerative alterations before changes in preterminal axons (DeGrandchamp and Lowndes 1990). Changes in the preterminal fibers subsequently progress to the intramuscular nerves and, finally, the main nerve tracts, suggesting a sequential "dying-back" process (Cavanagh 1964). The progressive changes in the distal nerve are seen only as a function of continued acrylamide exposure. Similar patterns of dying-back degeneration have been produced in rats, dogs, cats, and primates (Spencer and Schaumburg 1974). Elegant morphometric and ultrastructural studies of neuromuscular junctions after acrylamide exposure have shown swellings containing tubulovesicular profiles, neurofilaments, and degenerating mitochondria (DeGrandchamp and Lowndes 1990; DeGrandchamp et al. 1990). The molecular mechanism of toxicity remains to be determined. Relevant hypotheses include impaired membrane fusion processes mediating exocytosis and vesicle trafficking in the nerve terminals and Purkinje cells (centeral nervous system) as well as axoplasmic changes in ionic balance (Lehning et al. 1998).

Current Approaches to Histopathologic Evaluation of Peripheral Nerves in Animal Studies

There are several excellent references that address sampling, fixation, and tissue processing for peripheral nerves (Fix and Garman 2000; Jortner 2011; Kasukurthi et al. 2009; King 1999; Krinke et al. 2000; Lauria et al. 2005; Lauria et al. 2009). Ideally, sampling of tissues should include both proximal and distal levels for peripheral nerves, as well as peripheral ganglia such as the trigeminal ganglion or dorsal root ganglia (Jortner 2011), or autonomic ganglia in visceral organs. Although perfusion fixation is the preferred method, immersion fixation has also been used (Kasukurthi et al. 2009). Specific details on types of fixative and concentrations for immunohistochemical endpoints and/or electron microscopy have been published (Fix and Garman 2000). Although epoxy resin embedding is the preferred approach, paraffin embedding is still useful for light microscopic examination. Special procedures for histopathologic evaluation include morphometric assessments of individual teased nerve fiber preparations (Krinke et al. 2000), as well as routine crosssections of nerves (King 1999). Recent advances include a diagnostic assessment of cutaneous nerve fibers, such as in the rat footpad, to evaluate peripheral neuropathy (Lauria et al. 2005; Lauria et al. 2009). Typically, in routine toxicity screening studies, practice recommendations for the peripheral nerve include longitudinal and transverse sections of the sciatic and/or tibial nerve trunk. Although bilateral examinations

are encouraged, unilateral examinations are acceptable. In addition, bilateral examination of the eyes examined through the middle of the globe and nerve are routinely included (Bolon et al. 2013).

Conclusions

As noted in Table 1, there is a broad spectrum of chemicals that can cause peripheral neuropathy. Given that there are at least 80,000 chemicals in the United States registered for use with the US Environmental Protection Agency (US Environmental Protection Agency, Office of Pollution, Prevention, and Toxics 2010), it is important to note that at least 1000 chemicals are known to be neurotoxic in laboratory animal studies, of which 201 are known to be neurotoxic in humans (Grandjean and Landrigan 2006). At this time, it is unclear how many are PNS neurotoxicants, central nervous system neurotoxicants, or both. In conclusion, evaluation of the peripheral nerve during clinical examination in humans and routine screening in toxicity/safety animal studies is vital for complete evaluation of the nervous system. Therefore, contemporary and future directions in this field will benefit from including peripheral nerve evaluation during routine clinical examination in humans, preclinical safety evaluation studies of therapeutics in animals, and routine toxicity screening of environmental agents with unknown potential for toxicity in animal studies.

Acknowledgments

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