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A 'Best Practices' Approach to Neuropathologic Assessment in Developmental Neurotoxicity Testing—for Today

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ABSTRACT

A key trait of developmental neurotoxicants is their ability to cause structural lesions in the immature nervous system. Thus, neuropathologic assessment is an essential element of developmental neurotoxicity (DNT) studies that are designed to evaluate chemically-induced risk to neural substrates in young humans. The guidelines for conventional DNT assays have been established by regulatory agencies to provide a flexible scaffold for conducting such studies; recent experience has launched new efforts to update these recommendations. The present document was produced by an ad hoc subcommittee of the Society of Toxicologic Pathology (STP) tasked with examining conventional methods used in DNT neuropathology in order to define the 'best practices' for dealing with the diverse requirements of both national (EPA) and international (OECD) regulatory bodies. Recommendations (including citations for relevant neurobiological and technical references) address all aspects of the DNT neuropathology examination: study design; tissue fixation, collection, processing, and staining; qualitative and quantitative evaluation; statistical analysis; proper control materials; study documentation; and personnel training. If followed, these proposals will allow pathologists to meet the need for a sound risk assessment (balanced to address both regulatory issues and scientific considerations) in this field today while providing direction for the research needed to further refine DNT neuropathology 'best practices' in the future.

Keywords. Developmental neurotoxicity; guidelines; neuropathology; regulatory; rodent.

INTRODUCTION—SCOPE OF NEURODEVELOPMENTAL RISK IN HUMAN POPULATIONS

Birth defects affect thousands worldwide each year, including an estimated 2% to 3% of congenital anomalies thought to arise from maternal exposure to chemical teratogens in the environment (Wilson, 1973). The developing nervous system is a common target; indeed, approximately half of the children born each year in the United States with anatomic and/or functional deficits at birth exhibit abnormalities of the nervous system (Bearer, 2001). While the etiology of congenital neural aberrations cannot be defined in most instances, the susceptibility of the developing human nervous system to environmental toxicants has been amply shown in the past century by catastrophic prenatal intoxications with ethanol, lead, methyl mercury, and polychlorinated biphenyls (Rice and Barone, 2000; Costa et al., 2004). These events share 2 chief features: a great societal price (hundreds of millions of dollars per year to treat complications of fetal alcohol syndrome alone) and an acute onset associated with extensive chemical and structural disruption of the immature nervous system. Following prenatal exposure, such agents exhibit a similar neurotoxic potential in various animal models as that observed in humans (Stanton and Spear, 1990; Scharden, 1998).

A cardinal feature of known developmental neurotoxicants is their capacity for inducing structural damage in the developing nervous system. One manifestation of developmental neuropathology is the induction of frank malformations. For example, 1 infant per 1000 live births is afflicted with a neural tube defect in most countries, although the incidence increases markedly (up to 8 per 1000 births) in regions with either genetically predisposed populations or substantial environmental contamination (Campbell et al., 1986; Anonymous, 1991). Another category of developmental neuropathology is microscopic neural disruption in conjunction with altered behavior (Purpura, 1974), such as occurs in 2.5% of mentally retarded Americans (Bearer, 2001). Thus, neuropathologic evaluation is an important component of assessing neurotoxic risk to the developing nervous system.

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The serious concerns raised by the known association between xenobiotic-induced neuroanatomical changes and measurable alterations in behavior, learning and memory have led to the advent of developmental neurotoxicity (DNT) animal studies as a means of assessing risk in vulnerable populations of young people. Such assays represent an important follow-up evaluation, to be performed only if evidence of functional or structural damage to the nervous system is suggested by findings in first-tier (general, developmental, neurotoxicity, or reproductive) toxicity studies; the absence of adult neurotoxicity cannot be assumed to indicate a lack of DNT potential as many neurotoxic agents induce adverse effects in developing animals that differ both qualitatively and quantitatively from the impact observed in mature animals (Kimmel et al., 1990; Crews et al., 2000). Debate about the scientific merits of various approaches in DNT testing is wide-ranging in the research community (Broxup et al., 1989; Reuhl, 1991; McMaster, 1993; Kaufmann, 2000; Cory-Slechta et al., 2001; Dorman et al., 2001; Garman et al., 2001; Mileson and Ference, 2001; Kaufmann, 2003). At present, general agreement regarding the components that should be included in a routine DNT evaluation—including the procedures to be used in the neuropathology arm—has not been achieved.

The Scientific and Regulatory Policy (SRPC) Committee of the Society of Toxicologic Pathology (STP) established a subcommittee (1) to evaluate current procedures for neuropathologic examination as performed during the course of conventional DNT studies and (2) to provide an overview of current best practices that will address the diverse regulatory requirements of the U.S. Environmental Protection Agency (EPA; EPA, 1998b) and the Organization for Economic Cooperation and Development (OECD; OECD, 2003), as these two institutions currently serve as the main drivers for such investigations. The recommendations given below for best practices when conducting a DNT neuropathology assessment (Table 1) are based on personal experiences of the subcommittee members tempered by recent commentary in the scientific literature, most notably discussions of the DNT neuropathology assessment by scientists in America (Garman et al., 2001) and Europe (Kaufmann, 2000, 2003). Where consensus has not yet crystallized on certain points, we have included several mainstream options and then discussed potential advantages and disadvantages of each. Given this divergence among techniques in different laboratories, additional research over some years will be required to define a final "best practices" approach for developmental neuropathology assessment. These current recommendations will facilitate this endeavor while still meeting the need for a sound approach to risk assessment today.

Design Considerations in DNT Studies

Species

Conventional DNT bioassays are performed using outbred rats. This choice is dictated by the well characterized nature of rat neurobiology (see, for example, Paxinos, 1995, 2004; Paxinos and Watson, 1997), the similarities between human and rat neural development (Bayer et al., 1993; Wood et al., 2003), and the ability to achieve a shorter study length and larger sample size (thereby increasing the power and lowering the cost) relative to tests conducted using larger mammals. Justification for using another species (e.g., mouse) might be made if prior studies indicate the rat to be an inappropriate species for DNT testing; however, we have not encountered this situation in our experience. Should another species be used, the timing of the treatment regimen and neuropathology assessment will likely need to be shifted due to the differing times of similar neurodevelopmental events among species (MacKenzie and Hoar, 1995).

Treatment Regimen

Regulatory documents propose two potential schedules for exposure. Both EPA (EPA, 1998b) and OECD (OECD, 2003) guidelines call for treatment to be initiated on gestational day (E) 6, the time at which rat embryos implant in the uterine wall (neurogenesis in rat embryos commences about E 9). Obviously, the test agent is administered to the pregnant dam, typically but not always by the oral route. Treatment is continued throughout gestation and then may be halted for the day of delivery (which is generally designated postnatal day [PND] 0). Subsequently, test agent is administered from PND 1 until PND 10 (i.e., throughout the phases of major neuronal differentiation and synaptogenesis) (EPA, 1998b) or until weaning (OECD, 2003). If feasible, exposure of the young animals is managed by continued maternal dosing with secretion of the test agent into the milk (typically confirmed in a prior pharmacokinetic study). If passage of test agent into the milk cannot be confirmed, the young animals may have to be dosed directly, usually by gavage and/or by eating the food (typically starting about PND 14).

Group Size

In our experience, the group size in conventional DNT studies is usually set at 20 litters, with representatives from 10 of these relegated to the neuropathy cohort. For this purpose, litters are generally standardized to 8 pups (4 of each sex, if possible) between on (EPA, 1998b; OECD, 2003) or shortly before (OECD, 2003) PND 4. One rat per litter is then chosen at each time point to serve as that litter's representative for the DNT neuropathology examination. The selection is random except that the final count for each treatment ideally should include equal numbers of males and females.

Age at Evaluation

The objective of the DNT study is to assess the potential for injury to the developing nervous system resulting from low-level xenobiotic exposure. Critical periods of human neural development span both gestation (embryonic and prenatal periods) and childhood (neonatal through adolescent stages) (Rodier, 1994; Rice and Barone, 2000). Neural maturation in rodents follows a similar sequence of events (Rodier, 1980; Bayer and Altman, 1995; Rice and Barone, 2000). Thus, the consensus regarding DNT studies is that assessment needs to include both juvenile and adult animals that have been exposed to a test agent throughout development. That said, opinions vary considerably regarding the relative importance of factors that must be considered when choosing the most appropriate ages for the DNT neuropathology examination.

In our experience, the adult time point in the typical DNT study falls between PND 60 to 75 (i.e., between 1 to 2 months

TABLE 1.—Summary of best practice proposals for conduct of the DNT neuropathology evaluation in rats.

Procedure	Basic Approach	Extended Examination, with Optional Methods
Fixation and Tissue Collection	• 10% formalin • immersion for pups (PND 11) • immersion or perfusion for adolescents (PND 22) • perfusion for young adults (PND 60 to 75)	• special fixatives (e.g., EM-grade methanol-free formaldehyde, "universal fixative" [paraformaldehyde/glutaraldehyde], Bouin's solution)
Gross Morphometric Evaluation and Brain Weights	• postfixation brain weights at all ages • linear measurements of cerebral and cerebellar lengths (Figure 1)	• weights of unfixed brains (from satellite groups) • weights of microdissected brain regions, when macroscopic changes in brain size or shape are observed (satellite groups) • area measurements of cerebral hemispheres and neocerebellum (Figure 1)
Tissue Trimming and Embedding in Paraffin The brain is collected at all ages, other tissues typically only from young adults (PND 60 to 75). All brains (and ideally all tissues) from all dose groups are embedded up front to standardize processing conditions for sections destined for morphometric analysis	• brain: 6–10 homologous coronal (transverse) sections, using definite external anatomic structures as landmarks for trimming (Figure 2) and internal landmarks for sectioning (Figure 3), retaining identification of left and right sides • spinal cord: transverse and longitudinal sections of three major divisions: cervical (fiber tracts [C_1]), thoracic (middle), and lumbar (enlargement [L_4–L_5]) • dorsal root ganglion (DRG): one or more cervical (C_4–C_7) and one or more lumbar (L_4–L_5) ganglia, taken bilaterally and embedded as a single unit along with their corresponding dorsal and ventral spinal nerve roots (longitudinal orientation) • cranial nerve V (trigeminal [Gasserian]) ganglia • peripheral nerves (in paraffin, when permitted by specific guidelines; OECD, 2003): longitudinal and transverse sections of the sciatic, tibial, common peroneal (fibular), sural, and plantar nerves (all individually identified) • eye: longitudinal sections through the middle of the globe along the axis from rostral (cornea) to caudal (optic disc), including the optic nerve (cranial nerve II) • skeletal muscle: sections in transverse and longitudinal orientation through the proximal third (gastrocnemius) or middle (biceps femoris) of a muscle belly, or through the muscular portion of the diaphragm	• remove cerebellum to trim in mid-sagittal plane (for gross morphometry of the vermis) prior to making transverse sections of the remaining cerebellar hemispheres, cerebellar roof nuclei, and pons • transverse and longitudinal sections of cervical enlargement (C_4–C_7) • a montage of all DRG, assessed in two or more step sections • peripheral nerves (in plastic, when required; EPA, 1998b). See Embedding in Plastic (below). • additional areas of the autonomic nervous system (e.g., superior [cranial] cervical, nodose, stellate, or celiac ganglia) • innervated effector organs, using special stains to reveal the innervation/nerve endings • enzyme histochemical stains in flash-frozen tissue to identify muscle fiber types (satellite groups) • special stains to demonstrate nerve endings (e.g. motor end plates, sensory endings in muscle spindles)
Embedding in Plastic (for PNS tissues, when required by a specific guideline [EPA, 1998b])	• processed into soft plastic (e.g., glycol methacrylate), cut at 1–2 μm, and stained with an appropriate technique (e.g., H&E, toluidine blue, modified Lee's methylene blue–basic fuchsin, etc.)	• post-fixed in osmium tetroxide, processed into hard plastic (e.g., epoxy resin [epon]), cut at 1 μm, and stained with toluidine blue • electron microscopy on thin section (~600 nm) from the same epon-embedded block when necessary (e.g. functional evidence of peripheral neuropathy or suspected changes in subcellular organelles)
Stains and Markers	• general stain: hematoxylin and eosin (H&E) • combined neuronal/myelin stain (e.g., cresyl violet/luxol fast blue [LFB]) on sections used for morphometry to facilitate the evaluation	• axons: silver stains (Sevier-Munger's, Bielschowsky's, Bodian's); immunohistochemistry for phosphorylated neurofilaments • astrocytes: immunohistochemistry for glial fibrillary acidic protein (GFAP), S-100 protein • microglia: lectin binding, immunohistochemistry for macrophage markers • neurotransmitter systems: immunohistochemistry for transmitters or constituents in their metabolism • neuronal apoptosis: anti-caspase-3 • neuronal degeneration/necrosis: amino-cupric–silver, Fluoro-Jade
Microscopic Qualitative Examination	• brains of young adult (PND 60 to 75) and juvenile (PND 11 or 22) rats are assessed • comparison starts with control and high-dose groups • pathologist is aware of dose group identity	• if the high-dose group is affected, qualitative analysis of targeted neuroanatomic locations at all dose levels to (1) determine the dose-response and (2) define the NOEL/NOAEL • pathologist is unaware of dose group identity (i.e., coded slides are used)
Microscopic Quantitative Evaluation (Morphometry)	• brains of young adult (PND 60 to 75) and juvenile (PND 11 or 22) rats are assessed • linear measurements taken at several sites (such as those illustrated in Figure 5) • number of measurements for each organ not to exceed the group size (Muller et al., 1984; Hays, 1994) • comparison starts with control and high-dose groups • pathologist is aware of dose group identity	• additional linear, area, and/or counting measurements (global or regional) of brain if warranted by qualitative observations or the initial morphometric measurements • measurements of spinal cord white matter tracts, optic nerve, and/or peripheral nerves if warranted by other observations
Statistics	• Qualitative data (incidence and severity of gross and microscopic lesions): generally ordinal, so assessed appropriately using the Mann–Whitney *U*-test or Wilcoxon rank sum tests • Quantitative data (brain weight, gross and microscopic morphometric measurements): continuous data, so test for homogeneity of variance to determine whether groups should be compared by parametric (ANOVA) or nonparametric (Kruskal–Wallis ANOVA) tests	• absolute brain weight and morphometric values can be compared with relative values for each age and sex • multivariate analysis of measurements in conjunction with other factors (e.g., age, sex, treatment) may be more informative (Bailey et al., 2004)

(Continued on next page)

TABLE 1.—Summary of best practice proposals for conduct of the DNT neuropathology evaluation in rats. *(Continued)*

Procedure	Basic Approach	Extended Examination, with Optional Methods
Statistics (cont'd.)	• For morphometric data from paired structures, data from left and right sides should be first evaluated separately; if statistically equivalent, the data from both sides may be pooled and re-evaluated	
Reporting	• detailed methods section, including relevant descriptions of the fixation, tissue processing, histology, and pathology practices • inclusion of suitable concurrent control groups (negative ± positive); in the absence of a concurrent positive control, the validity of methods and skills of personnel may be demonstrated using a historic positive control performed under the same specific laboratory conditions. • include low-magnification images of entire sections showing sites for morphometric measurements • DNT studies with test agent of unknown neurotoxic potential: explicit text and/or tabular listing of all anatomic locations examined for each rat (in methods), with the results attained for the evaluation (normal, lesion [with its severity], or not examined) • DNT positive control studies with a known neurotoxic agent: implicit reporting, with text and/or tabular listings of all regions examined (in methods) and the lesion severity in affected target sites (in results)	• illustrations of characteristic findings, especially the results obtained using special stains • illustrations of examples for lesion severity scores • include low-magnification images of entire sections from selected (or ideally all) animals from all treatment groups to confirm that sections used for morphometry were homologous

after exposure ceases). This point represents a reasonable compromise between neurobiological (i.e., this age range is the earliest stage of adult-like neural maturity in the rat; Kaufmann, 2000) and fiscal (lower cost for shorter assays) considerations. The DNT neuropathology assessment in such 2-month-old adult rats parallels the conduct of conventional neurotoxicity studies in which animals are first treated as young adults and examined at an older age (usually 3 to 6 months). Thus, the major innovation of the DNT neuropathologic evaluation is oriented toward methods to assess the juvenile brain.

Early neuropathologic assessments are typically made between PND 11 (pups) to PND 22 (adolescents). A well recognized time point, and the choice that we recommend for now if existing information does not endorse a different age, is PND 22. This suggestion is based on several considerations. First, rats will have received the longest possible neurodevelopmental exposure (from E 6 through weaning). In addition, the PND 22 brain is sufficiently mature, with the exception of myelination and some neurotransmitter systems (Kaufmann, 2000), to be a suitable substrate for most routine methods used in neuropathologic evaluation. The larger size and firmer consistency of neural tissues in PND 22 rats renders the DNT neuropathology examination more convenient in a purely technical sense. Finally, brain size in rats is visibly more uniform at PND 22 relative to younger ages (Garman et al., 2001), so the choice of PND 22 will enhance the ability to perform meaningful quantitative measurements.

That said, earlier ages might be the more appropriate time for conducting the DNT neuropathology assessment in some circumstances. The first case is that in which extant guidelines require assessment at PND 11 (EPA, 1998b) and evaluation at PND 22 cannot be negotiated. The second instance is that in which functional signs or overt structural changes in neonates provide a biological rationale for examination at an earlier age (e.g., PND 11). Election of PND 11 will permit examination of the dynamic structural changes that occur in rapidly growing neural tissues, which contrasts with the more stable neuroanatomical features observed at PND 22. Indeed, several recent studies have suggested that rapid remodeling in rodent neural centers will obscure easily visible evidence of neurotoxicant-induced damage (e.g., frank cellular degeneration) unless the tissue is harvested and preserved within hours after early developmental exposure (Ikonomidou et al., 1999, 2000; Bittigau et al., 2002). However, the greater divergence in brain size (Garman et al., 2001) and the resulting disparities in neural microanatomy at this young age might obscure the ability to detect subtle dose-dependent differences related to xenobiotic exposure. Furthermore, younger brains are more friable than older organs (Garman et al., 2001), so greater technical skill is needed to ensure a successful dissection without inducing artifacts. These biological and technical factors will have to be carefully balanced when selecting the earliest age at which to perform the DNT neuropathology evaluation.

Fixation and Tissue Collection

In conventional DNT tests, our experience has been that all the tissues recommended for neuropathology evaluation in adult animals (OECD, 1997; EPA, 1998a) are also evaluated in young adult rats (PND 60 to 75). In contrast, common practice for younger animals (PND 11 to PND 22) in many laboratories is to collect all tissues but only evaluate the brain up front, or just to collect and evaluate the brain. This latter strategy is founded in a common interpretation of the requirements stated in the current DNT guidelines (EPA, 1998b).

Current regulatory guidelines (EPA, 1998b; OECD, 2003) for DNT studies recommend that optimal preservation of neural tissues from adolescent (PND 22) and young adult (PND 60 to 75) rats in DNT experiments is obtained by perfusion fixation followed by an additional period of immersion postfixation, just as is mandated for adult rats (OECD, 1997; EPA, 1998a). That said, some laboratories prefer to conduct large DNT studies in which neural tissues for each adult treatment group are fixed in equal numbers using perfusion/immersion or immersion alone. In our experience, we have

found that perfusion fixation of adolescent and young adult rats is indeed the best practice for preserving neural architecture for the DNT neuropathology evaluation.

Various options for perfusion fixation of neural tissues in adult rats (3 to 6 months of age) have been detailed in one recent review (Fix and Garman, 2000), and these techniques can be readily adapted to the younger rats examined in DNT studies. The first step may be to briefly flush the vascular system with buffer (at room temperature) containing sodium nitrate and/or heparin to prevent vasoconstriction (sodium nitrate only) and clotting (both agents). Such a pre-flush is essential for some fast-acting fixatives (e.g., glutaraldehyde) but may not be necessary for slower-acting agents (formalin). The fixative is infused using either a perfusion pump (typically maintaining an infusion pressure between 120 to 150 mm Hg [the systolic pressure of an adult rat; Fix and Garman, 2000]) or a gravity-based apparatus (with the fluid reservoir generally suspended about 80 to 120 cm above the level of the rat). The flow rate can also be regulated by the choice of infusion needle; younger rats require a smaller gauge (21 to 25) than do adult animals (19 to 21) (comment provided by an expert reviewer). Some laboratories prefer that perfusion solutions be chilled, while others use fluids maintained at room temperature; our experience has been that both facilitate acceptable preservation, although extremely cold perfusates can engender some perivascular artifacts in brain (e.g., neuropil pallor, accentuated formation of dark neurons). Successful perfusion is as much art as science, so the most important indications that the proper flow rate has been achieved and maintained in neural tissues are blanching of internal organs and stiffening of the distal limbs. An adequate duration (7 to 15 minutes) and volume of perfusate (125 to 250 ml) is important to ensure optimal fixation of neural tissues.

In our experience, a reasonable fixative for perfusion is neutral-buffered 3.7% formaldehyde ("10% formalin"), which is available commercially or may be made by mixing one part of concentrated (37%) formaldehyde into 9 volumes of phosphate buffer. A consideration in using commercial formalin solutions is that high concentrations of methanol (included as a stabilizing agent) can induce artifacts in the neuropil, especially in specimens destined for ultrastructural analysis. Other fixatives, such as "electron microscopy-grade, methanol-free formalin" or mixtures of paraformaldehyde and glutaraldehyde in buffer should be considered if electron microscopy is anticipated, but such special solutions are not needed if the planned DNT neuropathologic assessment will be performed only by light microscopy.

Different pathologists employ various options for subsequent harvesting of the central nervous system (CNS) tissues. We recommend that the perfused carcass be placed in a cooler (~4°C) for several hours prior to removal of the calvaria (in order to minimize pressure artifacts in superficial neural tissues), after which the head should be separated from the carcass so that nonneural tissues can be removed from the carcass while the brain continues to fix in situ. The head and intact vertebral column are postfixed by immersion in additional fixative (typically for 24 to 48 h) to ensure that the elements of the CNS (brain, spinal cord, and dorsal root ganglia [DRG]) are well preserved before their removal. Some pathologists detach the calvaria at the time of initial necropsy to allow better fixative penetration, while others prefer to remove it only after the postfixation period to further reduce the potential for trauma-induced artifacts.

A consensus has not been reached regarding the need for perfusion fixation of the immature brain. While in situ perfusion fixation is optimal for adult nervous tissue, immersion fixation may prove adequate at PND 11 (and even at PND 22 in some cases). This technical difference is due to the smaller size, less extensive myelination, and higher water content of neural tissues in juvenile animals (Garman et al., 2001), all of which combine to allow more rapid penetration of fixative. If perfusion is performed at PND 11, the infusion pressure may need to be lowered to prevent vascular rupture and artifactual distortion of brain tissue (comment provided by an expert reviewer). Personnel familiar with collection of perfusion-fixed (hardened) neural tissue from adult animals may find it difficult to isolate similar structures in rat pups (PND 11) or even in adolescent rats (PND 22) due in part to the relative softness of the immature brain, even when appropriately fixed. Thus, it is critical that the brain be treated with special care in immature animals because the softness and fragility readily promote artifact formation (Garman et al., 2001). If perfusion fixation is not employed at these early ages, removal of the calvaria and immersion fixation in situ before extraction of the brain from the skull is preferred. Again, we recommend neutral buffered 10% formalin for fixing juvenile rat brains during routine DNT studies. If greater hardening of immature neural tissues is required, immersion post-fixation in situ using Bouin's solution may be employed. An advantage of Bouin's is that the solution decalcifies bone while preserving tissue, thereby allowing for *in situ* examination of the spinal cord and dorsal root ganglia (DRG). However, the use of Bouin's solution requires more care in processing times (no more than 48 h in fixative, followed by extensive washing in 70% ethanol); furthermore, the dehydrating proclivity of Bouin's can produce substantial brain shrinkage, which may lead to sizeable alterations in the brain weights and quantitative measurements. For these reasons, we do not recommend the use of Bouin's solution for brains allocated to morphometric evaluation.

Opinions vary among pathologists regarding the best method for collecting the spinal cord. The cord is fixed in situ (as described above). Some pathologists prefer to remove the cord from the vertebral column (typically approaching via a dorsal laminectomy) so that the three major divisions (cervical, thoracic, lumbar) may be identified and trimmed individually using external landmarks. This procedure also allows isolation of the DRG. Other pathologists advocate that the fixed vertebral column be decalcified and trimmed into transverse blocks by counting vertebra. The advantage to this latter technique is that the nerve roots connecting the cord to the DRG remain intact. However, if this method is used the technical staff must remember that the spinal cord segments in mammals occupy a position in the vertebral canal cranial to the location of their corresponding vertebral body (Habel, 1981). Thus, the lumbar enlargement (L_4 to L_5), which contains the large motor neuron bodies that innervate the hind limb, is usually situated inside vertebrae L_1–L_2 in young adult rats (Mitsumori and Boorman, 1990). The shift is less pronounced in juvenile rats, so that the lumbar enlargement is typically located inside vertebrae L_3–L_4 in PND 22 rats (Mitsumori and Boorman, 1990), and about

vertebrae L_4–L_5 in PND 11 animals (comment provided by an expert reviewer).

In our experience, in situ fixation in 10% formalin by either immersion or perfusion provides suitable preservation of elements in the peripheral nervous system (PNS) for routine DNT neuropathology evaluations. However, for certain purposes PNS tissues and other organs may require special fixation and collection methods to attain optimal preservation. Eyes, for example, may be fixed by immersion in Bouin's or Davidson's solutions to enhance hardening of the delicate retinal tissues. Nerves may be postfixed by immersion in osmium tetroxide (to preserve the lipid components of myelin sheaths in preparation for plastic embedding) if clinical signs or neurobehavioral abnormalities suggest the existence of lesions relegated to the PNS. An option for collection of peripheral nerves employed by some laboratories is to remove tissue from the carcass immediately after fixative perfusion and then staple it (near the transected ends) to a flat card for immersion in fresh fixative. In this way, the proximal and distal ends of the nerves can be identified on the card (an advantage when contemplating "teased fiber" preparations, where the nerve fibers are dissociated while maintaining their orientation) rather than by the more tedious procedure of tying a string around one end (Krinke et al., 2000). Subsequently, the untraumatized central portion of the fixed nerve is isolated for further processing. This technique may afford a more rapid penetration of fixative, but this potential advantage may be jeopardized by the tendency for structural artifacts to develop if the nerves are stretched during removal. Our recommendation is that formalin-fixed nerves (either by in situ immersion or perfusion followed by additional post-fixation after removal) should be collected and individually identified (as to both location and side).

Muscle tissue is included in the neuropathology assessment of adult neurotoxicity studies and should also be available for the DNT neuropathology assessment. Common sites for sampling skeletal muscle are the gastrocnemius muscles (a recommended site based on its known anatomic and physiological characteristics, such as the presence of intramuscular tibial nerve branches that are known to be highly sensitive to neurotoxicity; Krinke et al., 1979, 2001), the diaphragm, and the biceps femoris muscle. Evaluation of fixed muscle tissue allows agent-induced structural changes to be defined in both muscular and neural elements. Assessment of more subtle biochemical changes (e.g., alteration in the ratio of Type 1 [red, or slow twitch] to Type II [white, or fast twitch] muscle fibers) necessitates a flash-frozen muscle sample and enzyme histochemical procedures, and is therefore performed only under the special circumstance wherein a distinct muscle lesion has been previously demonstrated to exist.

A critical consideration in DNT studies is extended retention of neural tissues in fixative. In general, the initial neuropathology evaluation is limited to the control and high dose groups; specimens from intermediate dose groups are archived until a positive finding in the high-dose cohort actually necessitates their evaluation. The pathologist is faced with 2 options regarding neural tissues from these intermediate-dose groups: process them all into blocks up front (which incurs monetary costs which may not be necessary if no lesions are found in the high-dose rats) versus holding them in fixative (usually for months to years). Variations in how long neural tissues are retained in fixative can engender differential changes in the size of structures within the brain which may preclude meaningful comparisons of morphometric data (thereby necessitating a follow-up study—and further expense). Given the fast pace of product development today, we recommend that all CNS tissues and at least one of each paired PNS structure from all dose groups be processed into blocks as soon as possible to avoid the potential loss of time resulting from deterioration of neural tissues kept for long periods in fixative. A tolerable alternative to preserve the quality of morphometric samples while moderating up-front study costs would be to embed brains from all animals in all dose groups, but process other neural tissues only for the control and high-dose groups.

Gross Morphometric Evaluations and Brain Weights

Postfixation brain weights are obtained on both juveniles and adults in conventional DNT studies. Separate weights may be taken on additional unfixed brains available at termination. We recommend that brains destined for immersion fixation and subsequent neuropathology evaluation not be weighed prior to fixation due to the extended interval between removal and fixation (though this objection could be obviated by weighing brains in a tared volume of fixative). Obviously weights obtained from fresh and fixed tissue should not be pooled for statistical analysis, as fixation impacts brain weight unpredictably by removing water from the neuropil (e.g., Bouin's solution) and by filling the cavities of the ventricular system. Gross brain measurements, such as the length or area of the cerebrum and cerebellum (Figure 1), are most readily obtained prior to trimming. Values may be acquired using any apparatus subject to calibration (e.g., ruler, calipers, or digital imaging software). Monitoring the regional weight of brain portions during trimming has been advocated in some previous studies as a less time-consuming means of acquiring quantitative data regarding regional vulnerabilities to xenobiotics (Kaufmann, 2000; de Groot et al., 2005a). In the current setting, however, guidelines are generally interpreted to recommend that quantitative measurements must be gathered from histologic sections. We believe that this mandate removes the need for regional weights in the DNT neuropathology evaluation, which is a desirable development as it prevents the induction of structural distortions during dissection that might render qualitative neuropathology difficult and morphometric analysis all but impossible.

Tissue Trimming and Sectioning

The DNT neuropathology guidelines are adapted from the comparable documents set forth for adult neurotoxicity studies (EPA, 1998a; OECD, 1997). Multiple coronal (transverse) brain sections of each rat are produced in a routine DNT study. Most laboratories perform this assessment on intact coronal sections, but some facilities prefer to split the brain along the midline and process transverse slices from each hemisphere independently. Some differences of opinion exist among pathologists regarding the number of levels to examine. The main consideration is that the DNT neuropathology analysis should sample at least the major structures correlated to known functional and neurochemical domains (Table 2); in our experience, 6 to 10 levels are necessary to adequately evaluate the rat brain. In some instances it may be

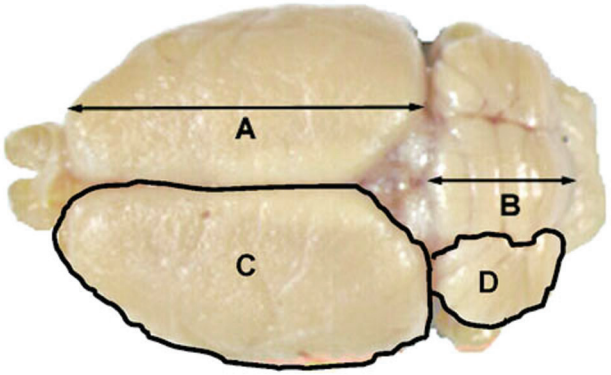

FIGURE 1.—Quantitative measurement of gross anatomic features in the developing brain. Collection of morphometric data from the developing brain can be accomplished readily and rapidly using simple linear (A, B) or area (C, D) measurements of the dorsal brain surface at the macroscopic level. (A) Cerebral length (from the rostral border just lateral to the olfactory bulb to the most caudal border). (B) Cerebellar length (over the midline of the vermis). (C) Cerebral area. (D) Neocerebellar area (i.e., the most evolutionarily advanced region, located lateral to the vermis). Other common measurements (not shown) include brain length (at the midline, from the rostral cerebral margin to the caudal cerebellar border) and cerebral width (widest distance across both hemispheres) (Rodier, 1978). Dorsal view of intact brain from a young adult rat.

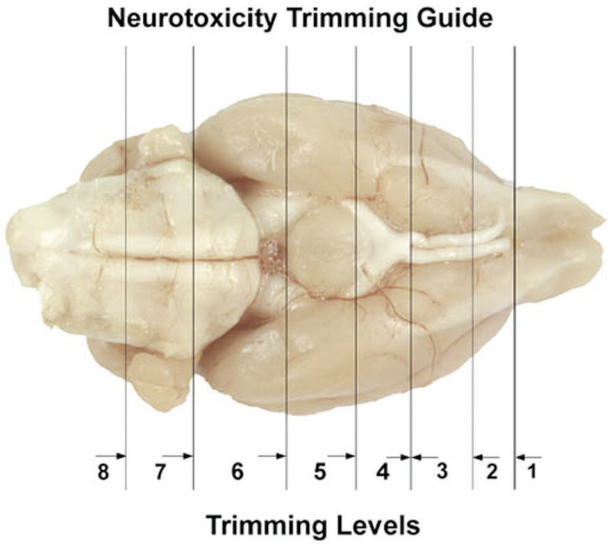

FIGURE 2.—External landmarks for trimming the immature brain. Representation of structural landmarks on the ventral surface of the young adult rat brain used to produce homologous coronal sections for qualitative and quantitative DNT neuropathology assessment. The lines show possible locations for placement of transverse cuts, and the arrows indicate which face should be sectioned (i.e., which side should be placed down in the tissue cassette); the numbers provide labels for the sections shown in Figure 3.

advantageous to remove the cerebellum to obtain mid-sagittal sections of the vermis before making transverse sections of the remaining cerebellar hemispheres, deep cerebellar nuclei and pons (Duffell et al., 2000; Kaufmann, 2000). In particular, such mid-sagittal cerebellar sections provide another means of obtaining homologous cerebellar sections for quantitative morphometry, and they also permit a better assessment of vermis lobulation and the terminus of the nerve fibers carried in the spinocerebellar tract (in the rostral lobules; Voogd, 1995).

Trimming the brain to obtain homologous sections among control and treated animals is a critical consideration in the DNT neuropathology investigation (Garman et al., 2001). In our experience, homologous coronal sections are best obtained by using definitive anatomic landmarks, especially those visible externally on the ventral aspect of the brain (Figure 2). For example, the optic chiasm serves as a landmark for the region of the decussation of the rostral (anterior) commissure. The variable brain sizes of age-matched juvenile rodents (Garman et al., 2001) indicate that commercially available brain matrices (designed to deliver slices of standard thickness [2 to 3 mm] with a consistent vertical orientation) will not automatically deliver an equivalent anatomic localization among immature animals. An alternate means of attaining consistent coronal slices for brains of different sizes

TABLE 2.—Recommended minimal tissue battery for the best practice DNT neuropathology evaluation.

System	Site	Rationale/Comment
CNS—Brain	Cerebral cortex	Motor and sensory divisions
	Striatum	Motor center with high acetylcholine and dopamine content
	Hippocampus	Major center for processing memory
	Corpus callosum	Principal inter-hemispheric myelinated tract
	Thalamus	Sensory relay area
	Hypothalamus	Center for autonomic and neuroendocrine control
	Midbrain (mesencephalon)	Tectum (sensory centers) and tegmentum (motor centers)
	Cerebellum	Motor coordination
	Pons	Motor coordination
CNS—Spinal Cord	Cervical	C_1—cord level containing largest quantity of fiber tracts
	Thoracic	Middle (as thoracic cord structure is similar at all levels)
	Lumbar	L_4 and/or L_5—level providing the largest contribution to hindlimb nerves
PNS—Dorsal Root Ganglia (DRG)	Cervical	C_4 to C_7—these DRG participate in forming forelimb nerves
	Lumbar	L_4 and/or L_5—the largest DRG contributing to hindlimb nerves
PNS—Nerve	Sciatic	Proximal peripheral nerve (middle portion)
	Tibial (internal popliteal)	Mid-level peripheral nerve from medial side of hindlimb
	Common peroneal (external popliteal or fibular)	Mid-level peripheral nerve from lateral side of hindlimb
	Sural	Most commonly sampled peripheral nerve in humans
	Plantar	Distal peripheral nerve
Receptor Organs	Eye/optic nerve	
Effector Organs	Skeletal muscle	

We suggest that a smallest acceptable DNT neuropathology assessment should include the following sites (listed from rostral to caudal and then dorsal to ventral, starting with the CNS).

would be a guillotine, though to our knowledge such neuroanatomical research instruments are seldom if ever used in a routine DNT neuropathology evaluation. Therefore, for conventional DNT studies we recommend free hand trimming as the method of choice for parsing the brain. This proposal is founded on the fact that final selection of sections for the DNT neuropathology examination (and particularly those destined for morphometry) is dictated by the presence of internal landmarks (e.g., those shown in Figure 3) that can only be appreciated during step-sectioning of embedded tissue—which would be true even if trimming was performed with a brain matrix. For small rat brains (especially at PND 22 or earlier), the sections adjacent to those selected for initial evaluation should be gathered on slides and retained without staining in case they are needed to achieve a closer degree of homology (particularly for morphometry) or for examination of additional neural markers using special stains.

Because the interconnected nature of brain circuits may lead to degeneration within specific pathways at multiple levels, some mechanism must be utilized to identify the sides (right and left) in each brain section. Options include cutting a shallow groove in one side of the brain, marking one lateral surface with an insoluble dye, or following a highly standardized procedure that maintains the orientation during

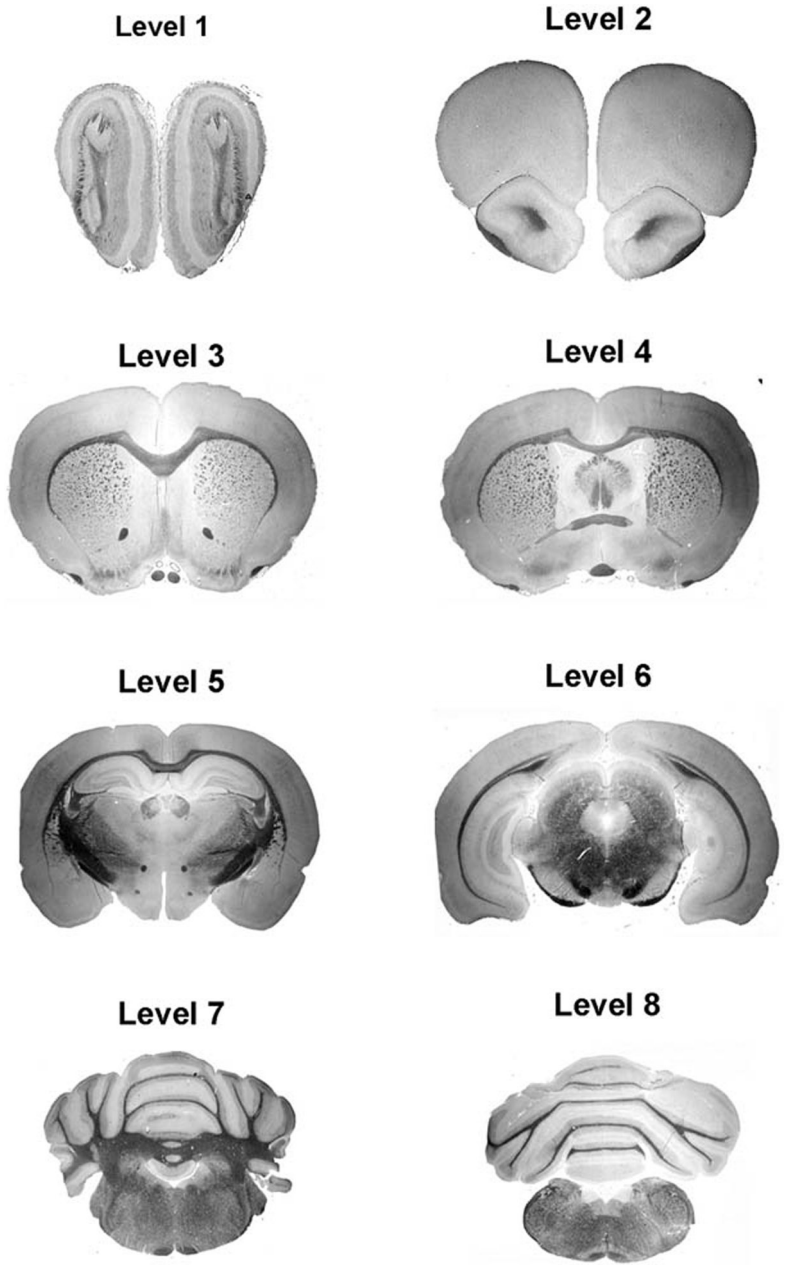

FIGURE 3.—Anatomical features of selected levels from young adult rats (PND 60). Representative examples of some important internal anatomic landmarks in coronal histologic sections of the young adult rat brain resulting from gross trimming (using external landmarks at the levels defined in Figure 2) followed by careful histologic sectioning to reveal the illustrated internal features. Stain: cresyl violet/luxol fast blue; thickness: 6 μm. Note: Always maintain left and right orientation when evaluating brain sections.

embedding and slide preparation (such as the use of multiwell cassettes or sponges to prevent flipping of brain slices during processing). We recommend using one of the latter two options as they do not damage the brain.

Again, opinions differ among pathologists regarding the best method for examining the spinal cord. Many pathologists evaluate sections in both transverse and longitudinal orientation. The longitudinal section reveals all structures on one side of the cord and is usually taken in a para-sagittal plane 1 to 2 mm to one side of the midline. However, some pathologists prefer an oblique longitudinal orientation (made using a dorsoventral cut which angles across the midline). Still other pathologists, noting that the cord is organized radially about the central canal, only assess coronal sections. We suggest that the cord assessment include both transverse and longitudinal sections as axonal damage in the white matter often stands out to a greater degree in longitudinal orientation (where the axonal axes are visible for an extended distance) than may be visible in adjacent transverse sections. Regardless of section orientation, the cord is typically sampled at three or four sites representing all three divisions (cervical, thoracic, lumbar). Some pathologists choose the cranial cervical segment (C_1–C_2), which houses the terminal portions of the ascending sensory tracts in the dorsal funiculus; this site is known to be exquisitely sensitive to neurotoxic agents that injure the distal axon (Schaeppi and Krinke, 1985). Other pathologists also include the caudal portion of the cervical cord that encompasses the cervical enlargement (C_4–C_7), because the large motor neurons in the ventral gray matter of this region innervate the muscles of the forelimb. The thoracic cord may be sampled anywhere along its length. The lumbar cord sample is usually derived from the lumbar enlargement (L_4–L_5). As with the brain evaluation, consistent trimming is necessary to obtain homologous sections across all dose groups. All levels of spinal cord can be processed in the same cassette as their origin can be distinguished due to their unique morphologic appearances. Alternatively, slices for each cord level can be processed in separate cassettes.

Methods for evaluating the dorsal root ganglia (DRG) are also quite variable. Some pathologists process the spinal cord and DRG in situ, and then evaluate the neural tissues after decalcification of the vertebral column. The major advantage of this technique is that DRG as well as their connections can be evaluated in their intact state. The two main disadvantages are that extended decalcification might induce subtle artifacts in neuronal morphology and that few DRG are sampled due to the difficulty in obtaining sections of vertebral column that contain more than a few DRG. Other pathologists prefer to dissect numerous DRG, embed them in a single paraffin block, and then prepare at least two step sections (since the diverse size of ganglia prevents them from all being adequately sampled in a single section). Still others separate the ganglia from each region (cervical, thoracic, lumbar) or from each side (all regions on the left or right) and place them in a single block, while some harvest and step section (typically in a plastic medium) specific ganglia and their associated spinal nerve roots bilaterally. A common choice in this latter regard are L_4 and L_5 (the largest lumbar DRG, as they are the source of the greatest contribution to the rat sciatic nerve and are readily identified during necropsy; Aldskogius et al., 1988; Devor et al., 1985; Paul and Devor, 1987; Schmalbruch, 1987). Indeed, some pathologists only evaluate the lumbar DRG, reasoning that evaluation of cervical and thoracic DRG is meaningless because their peripheral nerve trunks are not analyzed. Given the divergence in present opinion, we cannot recommend at this time a single best practice for sampling DRG during the DNT neuropathology evaluation. However, while the examination of one cervical (taken from C_4 to C_7) and one lumbar (from L_4 or L_5) DRG should satisfy the minimal requirements stated in the regulatory guidelines, we recommend that multiple DRG be taken to ensure an adequate sample size and to attain a good plane of section through at least one.

The paired trigeminal (Gasserian [cranial nerve V]) ganglia, the other commonly sampled discrete collection of neuronal bodies, are usually processed in situ or harvested from the base of the cranium (flanking the pituitary gland). In situ processing requires decalcification of the basal skull, while isolated trigeminal ganglia are embedded in a single paraffin block. Trigeminal ganglia usually are evaluated in a single section.

The regulatory guidelines relating to the DNT neuropathology evaluation of the peripheral nervous system (PNS) allow some latitude in tissue sampling. In general, peripheral nerves in DNT studies are routinely taken from the hind limb and include the sciatic, tibial (internal popliteal), common peroneal (external popliteal, fibular), sural, and plantar nerves (Figure 4). In our experience, all these levels must be sampled due to inherent differences in the susceptibility of various regions of nerve from proximal to distal to toxicant-induced damage (Krinke et al., 1979, 2001). Some pathologists prefer to post-fix the nerves while they remain in the carcass, while others remove nerves immediately after perfusion fixation and staple them (through their ends) to a flat card to facilitate post-fixation. Regardless of when the tissue is harvested, care must be taken not to stretch the nerves so that trauma-induced artifacts are avoided. Nerves are embedded in both cross and longitudinal orientations. Some pathologists prefer to sample both the proximal and distal portions of the sciatic nerve, and embed them in separate blocks for ready identification. We have not observed that this latter practice is necessary for the routine DNT neuropathology analysis.

Eyes are placed in a cassette without trimming, and then embedded in such a fashion that the globe is sectioned through the long axis of the eye from rostral (cornea) to caudal (optic disc and, ideally, optic nerve).

Skeletal muscle fibers should be evaluated in transverse and longitudinal orientations. The sections should be taken consistently in all rats both within and between studies. The usual site is through the proximal third (gastrocnemius) or middle (biceps femoris) of a muscle belly, or through the muscular portion of the diaphragm.

Tissue Processing

Neural tissues from all treatment groups should be handled in the same time frame, particularly when morphometry will be performed, to avoid any systematic variation that may be associated with the processing variables (particularly the length of time in fixative). Thus, we recommend for each rat that the entire brain (including olfactory bulbs, which are a potential target for both ingested [Crews et al., 2000] and

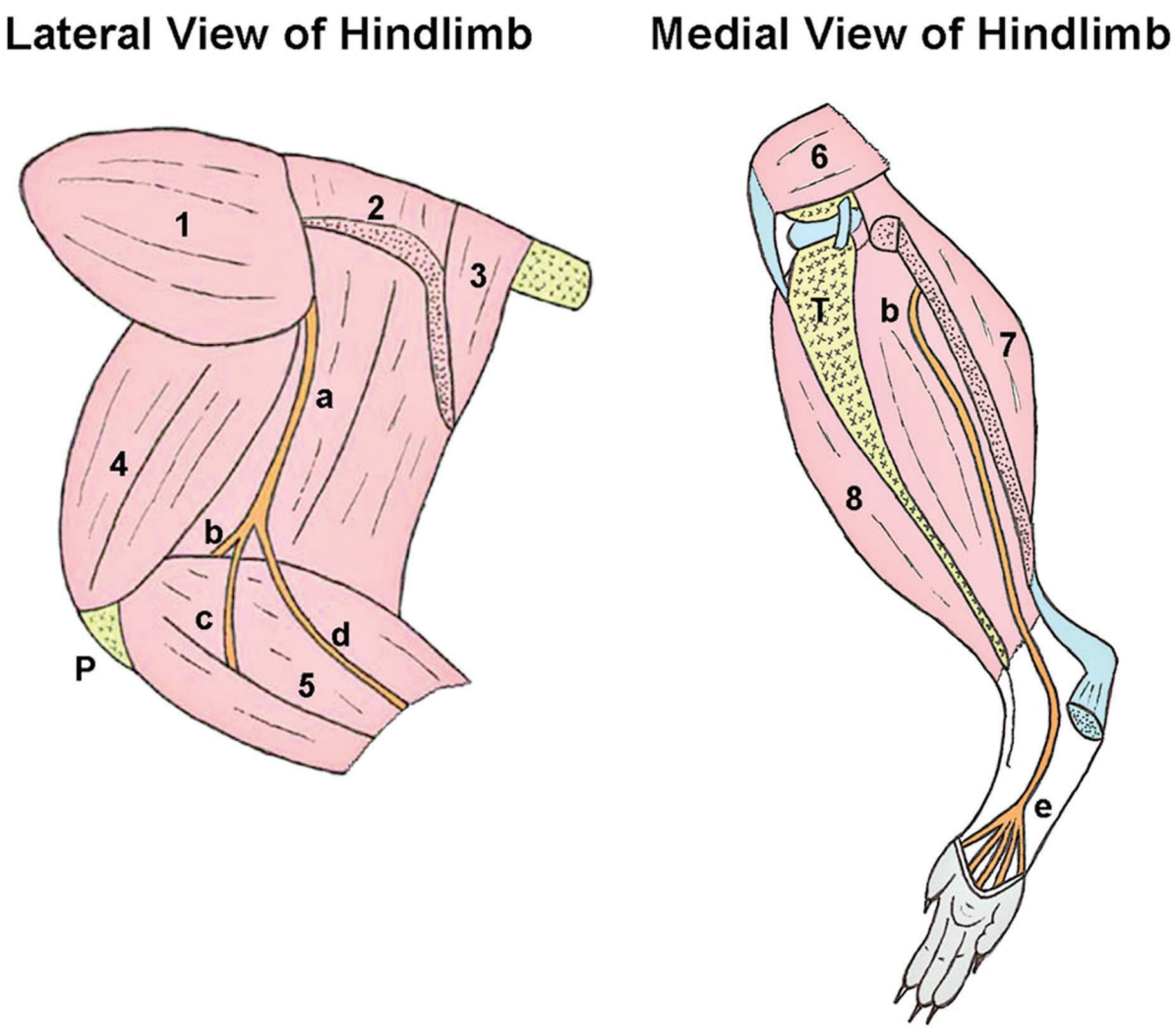

FIGURE 4.—Location of peripheral nerves to be evaluated for DNT neuropathology. Schematic diagram illustrating avenues to approach the peripheral nerve elements that should be considered for evaluation in a routine DNT neuropathology exam. (Adapted from Popesko et al., 2003 by permission of the Authors and Publisher). Muscles: 1 = gluteus medius; 2 = biceps femoris; 3 = semitendinosus; 4 = quadriceps femoris; 5 = gastrocnemius lateralis; 6 = rectus femoris; 7 = gastrocnemius medialis; 8 = tibialis cranialis. Nerves: a = sciatic; b = tibial; c = common peroneal (fibular); d = lateral sural; e = plantar. Bones: P = patella; T = tibia.

inhaled [Colin-Barenque et al., 1999] toxicants) as well as spinal cord, DRG, and PNS be processed up front as a routine practice, whether or not sections are to be taken during the initial evaluation. One good way to standardize the effects of fixation and processing is to include cassettes from animals in different treatment groups within each processing "run" so that any artifacts elicited by handling are balanced throughout all dose groups.

Most neural tissues and skeletal muscle are embedded in paraffin for routine DNT neuropathology assessment. However, the regulatory guidelines relating to the evaluation of ganglia and peripheral nerves diverge. The current EPA guidelines (EPA, 1998b) require that PNS tissues be embedded in plastic. In contrast, the proposed OECD guidelines (OECD, 2003) do not specify plastic embedding, which allows ganglia and peripheral nerves to be encased in paraffin when the tissues have been properly fixed and carefully handled. Historically, the use of a hard plastic medium (e.g., epoxy resin [epon]) was mandated because ultrastructural assessment of PNS was required as a part of standard rodent neuropathology evaluations. Initial experience indicated that hard plastic sections (cut at 1 μm thick) provided better resolution of cellular detail, particularly for subtle changes induced at lower doses of test agent, than did the standard 5- to 8-μm-thick paraffin sections. The inability to perform special stains on tissues encased in hard plastic has lead to the current practice of embedding PNS tissues in softer plastic media (e.g., glycol methacrylate). However, in our experience sections supported in soft plastic (usually cut at 2 μm) do not provide much—if any—more resolution than is afforded by paraffin sections. Obviously, PNS tissues must continue to be embedded in plastic where required by existing regulations. However, our "best practice" recommendation is that, where guidelines permit this option, PNS tissues be embedded in paraffin as it maintains the ability to use special stains in processed neural tissues and removes the need for staff

to work with the expensive neurotoxic reagents needed to polymerize plastics.

Qualitative Neuropathology Assessment

As in conventional neurotoxicity studies with adult rats, the foundation of the DNT neuropathology evaluation is light microscopic analysis of global and cellular structure in tissue sections (Figure 3). The assessment is conducted in a stepwise fashion, starting with control and high-dose groups. The qualitative evaluation is performed by finding lesions in sections stained with hematoxylin and eosin (H&E) or a special neurohistologic stain, developing a tiered ordinal scale with distinctive criteria for grading the changes (e.g., minimal, mild, moderate, marked, and severe), and then scoring the lesions in each animal. Neural lesions are generally graded in an uncoded ("unblinded") fashion (i.e., the pathologist is acquainted with the animal's treatment) at first, after which a coded assessment can be performed in all dose groups if warranted by the preliminary findings. We recommend that the minimally acceptable qualitative DNT neuropathology analysis should include a battery of structures in the CNS and PNS (Table 2).

Special histologic stains often are applied to serial, paraffin-embedded brain sections, especially for those levels at which morphometry is to be performed, to provide additional discriminating power. The DNT neuropathology assessment for both younger (PND 11 to 22) and older (PND 60 to 75) rats employs essentially the same special stains as the adult neurotoxicity study. However, the pathologist must understand that the results of some methods may diverge from the findings expected in adult neural tissues due to stage-specific events in development of the nervous system; examples include the region- and time-specific cycles in the production, removal, migration, and terminal differentiation of cells throughout prenatal and early postnatal development (Barone and Das, 2000) as well as the persistence of incompletely myelinated tracts into young adulthood (Kaufmann, 2000). Consequently, the regional staining patterns for each marker must be carefully evaluated in comparison with those of control tissues derived from age-matched rats.

Common neurohistologic stains for DNT neuropathology studies include cresyl violet (to evaluate cellularity and subcellular features of neurons), luxol fast blue (LFB, to assess myelin), and silver impregnation (e.g., Sevier-Munger's, Bielschowsky's or Bodian's methods, to evaluate axonal changes). Combined stains (e.g., cresyl violet/LFB or Bodian's/LFB) are often applied to the same section to allow simultaneous visualization of neuronal and glial elements. Other stains and markers for specific cell types (e.g., glial fibrillary acidic protein [GFAP] for astrocytes) or developmental processes (e.g., immunoperoxidase procedures to detect the proliferation or apoptotic markers) or degenerating neurons (e.g., Fluoro-Jade B; Scallet et al., 2004) may also have utility in the DNT neuropathology evaluation, but such methods must be validated for use on the immature nervous system. Several excellent reviews (Fix et al., 1996; Barone and Das, 2000; Fix and Garman, 2000) and research articles (Kaur et al., 1990; Streit, 1990; Gerloff et al., 1993; De Olmos et al., 1994; Zimmer et al., 1995; Schmued and Slikker 1999; Olney et al., 2000, 2002; Schmued and Hopkins, 2000; Krinke et al., 2001; Olney, 2002; Schmued et al., 2005) in the recent literature address such standard methods. Additional stains may be used to further characterize neural lesions, although the need for unfixed or specially processed tissue requires either a parallel treatment group (generally included only for the control and high-dose groups) or a follow-up experiment. Silver stains for neuronal degeneration (Felderhoff-Mueser et al., 2005) are a well-recognized example of such a procedure. A major consideration for all these techniques is to reduce the variability in tissue staining that arises from processing brain sections from different animals in separate processing runs. In our experience, such differences may be decreased using automated histostainers or by processing brains from multiple rats in the same block (taking care to keep straight the identity and orientation [left and right sides] for each sample) (Fix et al., 1996).

Ganglia and peripheral nerves are stained using comparable methods. Initial screening is typically performed on H&E-stained (for paraffin-embedded) or toluidine blue-stained (for plastic-embedded) sections. If warranted by in-life abnormalities consistent with a peripheral neuropathy, serial sections are stained using special methods to delineate both axon and myelin structure (such as Bodian's/LFB). In such cases, additional nerve segments that have been fixed but not processed may be postfixed in osmium tetroxide, embedded in hard plastic (e.g., epoxy resin [epon]), and stained with toluidine blue to explore fine structural details of PNS structure, such as degenerating axons or clusters of demyelinated ("naked") axons. Plastic sections are suitable for subsequent quantitative evaluation of nerve fiber characteristics using a light microscope, while epon-embedded tissue can be readily employed for transmission electron microscopy. The same methods used in older animals (Krinke et al., 2000) can also be employed to produce "teased fiber" preparations from juvenile and young adult rats. In this technique, Sudan black-stained nerves are dissociated in glycerin to reveal fine structural details of axonal and Schwann cell morphology in individual nerve fibers. The tedious nature of this procedure generally limits its use to those DNT studies in which functional changes or structural alterations in paraffin-embedded histopathologic sections provide a trigger for the more in-depth evaluation—and typically only in a few control and high-dose animals at that.

Eyes and skeletal muscle are evaluated for structural changes (atrophy, degeneration, etc.) in routine H&E sections. Evidence of biochemical lesions in skeletal muscle may warrant further evaluation with special stains like ATPase and NADH-TR to quantify specific fibers (Kremzier, 1984), but such enzyme histochemical procedures require unfixed frozen tissue and thus usually necessitate a satellite group or follow-up study to provide material.

In summary, our recommended staining battery for the initial DNT neuropathology evaluation includes application of H&E and a combined neuronal/myelin stain to serial paraffin sections of brain and spinal cord, H&E (on paraffin) or toluidine blue (on plastic) for nerve and ganglia sections, and H&E for the other paraffin-embedded tissues. If necessary, other special stains may be applied to the intervening sections that were acquired and stored without staining.

Quantitative Assessment (Morphometry)

Morphometric analysis of neural tissues (chiefly brain) as a component of the DNT neuropathology evaluation has been demonstrated to be a sensitive indicator of xenobiotic-induced neuroanatomical alterations (de Groot et al., 2005a; Raffaele et al., 2005). This practice is a required component for brain evaluations under the DNT guidelines for EPA (EPA, 1998b) and those proposed for OECD (OECD, 2003). The reason for this sensitivity is that many developmental neurotoxicants alter brain volume rather than elicit overt cytoarchitectural abnormalities (e.g., changes in the density or structure of neural cells), while qualitative histopathology is much more adept at defining anomalies in the latter category (Rodier, 1990; de Groot et al., 2005a). An increase in brain parenchyma (as indicated by larger global or regional brain size), which is typically associated with disrupted neural cell regression and disordered synaptogenesis during development (as seen in knockout mice; Kuida et al., 1996; Yoshida et al., 1998; Backman et al., 2001), can be just as devastating in this regard as a decrease in brain parenchyma, which usually reflects an excessive loss of neurons and synaptic connections (de Groot et al., 2005b).

The basic techniques whereby sections are prepared and evaluated are comparable for both juvenile and adult rats. The fundamental issue is to ensure the availability of homologous sections among animals from each treatment group at a given age. In our estimation, additional discriminating power might also be achieved if the same structures are evaluated in both juvenile and adult animals from the same study. The reason for this proposal is that alterations often will be more evident when the effects at a given site can be compared directly between a developmental stage characterized by dynamic shifts in cell numbers and connections (e.g., PND 11 to PND 22), and one in which the alterations have been accentuated by an intervening period of slow but steady growth (PND 60 or later). The greater consistency in brain size in adolescent rats at PND 22 compared to earlier time points (Garman et al., 2001) may be advantageous in reducing the inherent variability among animals that is apparent when making morphometric measurements. Regardless of the age assessed, our combined experience indicates that morphometric measurements are acquired most easily from sections stained with a combined neuronal/myelin method (e.g., cresyl violet/luxol fast blue) due to the heightened contrast among structures, though some laboratories do prefer H&E for linear morphometry (Duffell et al., 2000).

With sufficient care, morphometry of brain structures can produce a data set with small variance. This outcome requires great attention to detail in the preparation and evaluation of homologous histological sections to minimize systematic error. Such care is particularly important when making linear measurements (Figure 1), since a variety of technical factors can readily introduce such errors. Area (Figure 1) and volume measurements as well as cell counts (stereology) are less sensitive than are linear measurements to systematic error associated with histological processing and section homology, but these assessments require more planning and resources and typically should be reserved for studies in which there is sufficient a priori evidence to justify a specialized design.

The regions to be evaluated quantitatively for the DNT neuropathology evaluation should be representative of the major structural features of the developing brain (Figure 5). In our experience, linear measurements of the cerebral and cerebellar lengths (macroscopic) and cerebellar height (microscopic) and/or the thickness (microscopic) of neuronal layers

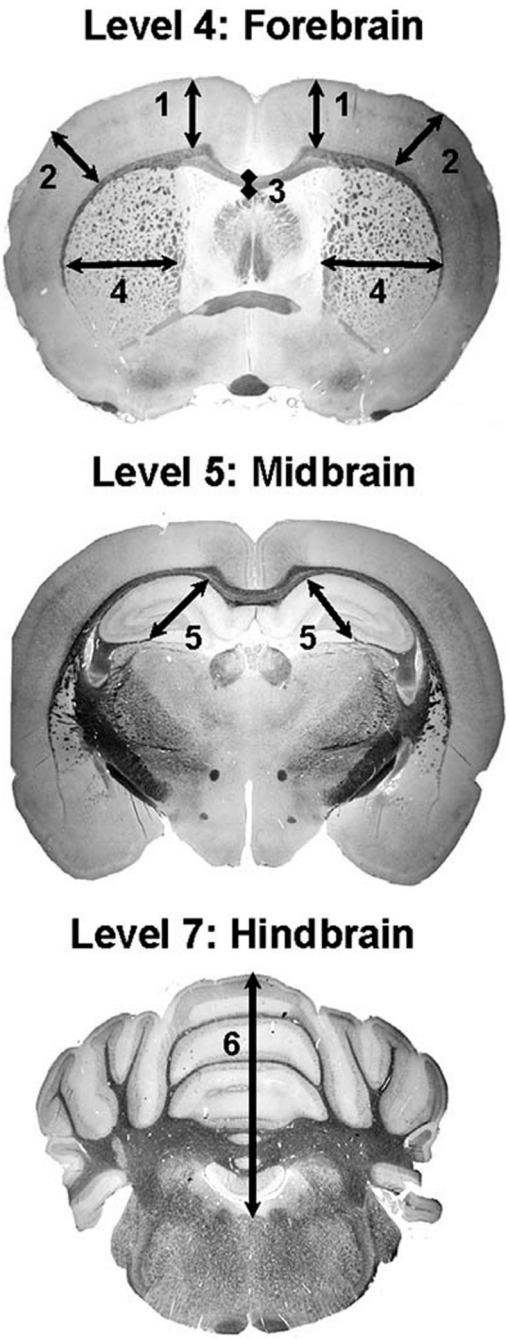

FIGURE 5.—Examples of quantitative linear measurements in adult rat brain. Representative examples of sites at which to obtain quantitative brain measurements in the young adult rat brain. Comparable sites may be evaluated in juvenile rats (pups [PND 11] or adolescents [PND 22]). In some cases it may be more appropriate to acquire linear data at other sites or at different orientations. Stain: cresyl violet/luxol fast blue; thickness: 6 μm. Note: Always maintain left and right orientation when evaluating brain sections. 1 = Cerebral Cortex (Frontal) Thickness, 2 = Cerebral Cortex (Parietal) Thickness, 3 = Corpus Callosum Thickness, 4 = Striatum Width, 5 = Hippocampus Gyrus Thickness, 6 = Cerebellum Height.

in (from rostral to caudal) the striatum, cerebral cortex (motor and sensory regions), hippocampus, and cerebellar molecular layer afford a reasonable means of providing a simple, cost-effective, and quantitative assessment of brain structure. Such linear measurements have been confirmed to be a sensitive means of evaluating brain size (de Groot et al., 2005a). That said, other groups advocate stereological procedures as the method of choice due to the difficulty in acquiring homologous sections suitable for linear measurements (Duffell et al., 2000); however, we note that the potentially greater sensitivity of stereology only applies to those situations in which neural cell numbers are affected, and will be of less utility where alterations in volume (e.g., reduced myelin or synapse production) rather than altered cell counts are suspected. The particular brain regions that we mentioned above are recommended specifically for quantitative assessment because of their intimate involvement in the neural differentiation and synaptogenesis (cerebral cortex , hippocampus, and cerebellum [Rodier and Gramann, 1979; Barone and Das, 2000]), high concentration of major neurotransmitters (e.g., acetylcholine and dopamine in the striatum [Paxinos, 2004]), and their different developmental profiles with respect to neuron proliferation (Altman and Bayer, 1995). Measurements can also be acquired for additional sites that are suspected to represent targets for a particular test agent, such as olfactory bulb for inhaled xenobiotics (Colin-Barenque et al., 1999). We also recommend that the thickness (microscopic) of the corpus callosum be acquired to evaluate the degree of brain myelination. This site is suitable as it is the major myelinated fiber tract that carries associative fibers between cerebral hemispheres, and because its large size allows for simpler production of homologous sections among animals relative to smaller white matter tracts (e.g., rostral [anterior] commissure). With respect to other CNS regions, studies of rats with genetic myelin defects have shown the optic nerve and ventral tracts of the cervical spinal cord to be sensitive sites for assessing myelin sufficiency (Kondo et al., 1991, 1992; Lunn et al., 1999). In contrast to the typical linear or areal measurements of brain structures, morphometric analysis of peripheral nerves in DNT studies usually involves the acquisition of absolute counts for particular structures (e.g., fiber caliber distribution or fiber counts).

Morphometric evaluation is performed starting with the control and high-dose groups. Some institutions use manual procedures (e.g., ocular reticle) while others employ semi-automated algorithms to acquire digital images. We believe that either means is appropriate in the hands of a skilled practitioner; morphometric software may facilitate the rapid acquisition of measurements (especially cell counts) but is not an absolute requirement for obtaining high-quality data. If the preliminary examination suggests the existence of a treatment-induced morphometric change, the pathologist should look at more animals per group and/or additional dose groups to obtain more reliable data with which to demonstrate a graded response. Homology is best judged using region-specific anatomic features. Low-magnification images of entire sections (especially coronal views of the brain) are especially useful for this purpose (Figures 3 and 5); suitable digital images of sections can be obtained by scanning the histological slides on a conventional flat-bed scanner or a 35-mm slide scanner modified to accept glass slides. Such images can be arranged on one or more pages for comparison and are an appropriate means of providing a visual record of the pathology raw data.

Several considerations must be addressed when designing the morphometric arm of the DNT neuropathology exam. First, the scale on which morphometric measurements are based (e.g., ocular reticle at a particular objective, or resolution of an electronic image) should fall within a range appropriate to observed variance of the measurement. The selected objective needs to have sufficient resolution to discriminate between subtle differences relating to the natural neuroanatomical variability among animals. Finally, a decision regarding whether or not morphometric data will be taken unilaterally or bilaterally is required prior to initiation of the DNT neuropathology examination. We recommend that measurements be acquired bilaterally, whether taken from complete coronal sections or from brains halved prior to processing.

Statistical Methods

The advanced nature of current commercial software allows the statistical analysis of DNT neuropathology data to be performed directly by the neuropathologist. Thus, the choice of an appropriate statistical test, which depends on both the nature of the data and the underlying assumptions required to employ the test, often rests with the pathologist. The basic statistical principles required to ensure the selection of an appropriate test by the neuropathologist are well detailed in a recent review (Gad, 2001). Nevertheless, consultation with a biostatistician is warranted in those instances where interacting variables are to be examined concurrently.

Qualitative results (e.g., incidence and severity of gross or histopathologic findings) are discontinuous data. Collections of such measurements are analyzed effectively using non-parametric tests, which are designed for use with data sets that are discontinuous or skewed (non-normal). For example, nominal data (i.e., a lesion exists or it does not) might be evaluated by either a Chi-Square test or Fisher's exact test, while ordinal data (i.e., a lesion is categorized as absent, minimal, mild, moderate, marked, or severe) might be analyzed using the Mann–Whitney U-test or the Wilcoxon rank sum test. In our experience, the neuropathology data sets acquired in conventional DNT studies are well suited to analysis using one of the latter 2 tests.

Quantitative measurements (brain weight and morphometry results) are continuous data. Assuming that the population of values is normal (unskewed), the preferred means of analyzing such data is a univariate parametric test due to the discriminating power offered by such methods. The homogeneity of the data is assessed first using a method such as Bartlett's test (used to compare the variances among 3 or more groups of continuous data). If homogeneity is established, the data are usually evaluated using a conventional 1-way analysis of variance (ANOVA), which is designed to compare normally distributed, continuous data from 3 or more treatment groups. Should a difference exist by ANOVA, a multiple range test (such as Dunnett's or Scheffe's tests, which compare the means of each treatment group to a designated control group) is employed to define the source of the divergence. In cases where the criteria for homogeneity are not met, then a

nonparametric 1-way ANOVA (e.g., Kruskal–Wallis) is used to compare the continuous data from 3 or more groups. While such a nonparametric approach is acceptable, the best power for detecting subtle changes is obtained using the parametric tests. Thus, we recommend that the neuropathologist assess the normality of quantitative data sets and homogeneity of variances during the collection phase, so that if these assumptions hold true a parametric ANOVA may be employed to analyze the quantitative DNT neuropathology data. A standard means of accomplishing this feat is to use balanced groups of sufficient size (typically N = 10/sex/group for DNT studies). Our final recommendation on this topic is that the bilateral morphometric measurements acquired from intact coronal sections or coronal hemi-sections be first evaluated for differences between left and right sides. In those cases where data from the left and right sides are statistically equivalent, the values for left and right side measurements for each site can be pooled and evaluated by ANOVA. While more cumbersome than immediately averaging the side-specific values, the advantage of this extra ANOVA is that it reduces the probability of false-positive results (Type I error, in which a statistically significant difference has no biological relevance). Obviously, if prior clinical data or the results of the qualitative histopathology exam demonstrate a unilateral neural lesion, the averaging of the values from left and right sides should be avoided.

The traditional approach to statistical analysis of toxicity test data is to analyze values separately for each sex and age. However, added value may be gained by using a factorial design to determine if interactions exist among various factors (e.g., treatment, dose, and sex) (Bailey et al., 2004). This capability may be particularly important for morphometric data where the statistical design can evaluate not only effects related to sex and age, but provide for a more robust analysis where there are multiple measurements acquired in a particular region (e.g., cerebral cortex). Absolute morphometric values can be compared with relative values based on brain weight or brain volume to evaluate the whether an alteration is either localized or related to a more general effect on brain growth. Treatment of brain weight or brain volume as a covariate may also be informative (Bailey et al., 2004).

Another statistical consideration is the number of morphometric measurements to take. If a neurotoxic effect is indicated by functional abnormalities and/or lesions seen in the qualitative histopathology analysis, many morphometric measurements may be used to quantify the extent of the previously defined alteration (e.g., Duffell et al., 2000). However, for a given sample size, increasing the number of measurements enhances the probability of finding a statistically significant but biologically irrelevant difference (i.e., false positive; Muller et al., 1984). Statistical corrections can be undertaken to minimize such false positives for a large number of measurements (Muller et al., 1984; Hays, 1994), but such corrections decrease the power to detect a real (treatment-related) difference. Both kinds of error can be minimized by ensuring that the number of measurements does not exceed the sample size. For this reason, our recommendation with respect to the number of morphometric values to collect in a standard first-tier DNT neuropathology examination (where the group size is generally 10) is to restrict the quantitative data set to measurements at up to nine sites (such as those shown in Figure 5).

Control Materials

The quality of the data and resulting conclusions for the DNT neuropathology evaluation depend on identifying and categorizing neuroanatomic lesions and assessing their dose-dependence. As with other toxicity studies, the DNT bioassay requires the appropriate selection of control materials to demonstrate proficiency in fulfilling these objectives. Two types of control material are required for this purpose.

The first is the use of negative (vehicle) control groups when conducting the study. A concurrent negative control cohort assesses the toxicity of the dosing solution and serves as the baseline against which data from the various treatment groups are analyzed for statistical significance. The use of historical negative control data can uphold the interpretation regarding the relevance of a neural alteration by revealing the kinds and incidences of spontaneous changes that are present within the rat strain used by any given laboratory.

The second kind of control material is tissue from a positive control group (either concurrent or historical), in which animals have been exposed to a treatment known to posses developmental neurotoxic properties. This cohort is used to confirm that the neuropathology methods selected to identify and classify neural lesions are suitable to assess developmental neurotoxicity. Particular considerations for the positive control agent are that the evaluation should conform as much as possible to the procedures used for assessing the test substance (e.g., comparable criteria for selecting tissues and sections) and that the control treatment should elicit neural effects corresponding to those of concern for the test agent. In the absence of information that provides an a priori basis for expecting particular kinds of alterations, a "prototypical developmental neurotoxicant" may be employed to demonstrate the ability of the selected neuropathology methods to detect the distribution and dose dependency of prototypical neuroanatomic lesions. Perusal of the recent rat DNT literature reveals no examples of published studies in which developmental neurotoxic agents were given at low doses according to the conventional DNT regimen (maternal dosing from E 6 to PND 10 or PND 21, ± excluding the day of birth). That said, the teratology field is rife with reports of disrupted neurogenesis following prenatal or early postnatal chemical exposure. In our opinion, anti-mitotic chemicals represent a suitable choice as positive control agents based on their capacity to produce predictable and reproducible cytoarchitectural changes in the developing brain; in particular, brief prenatal exposure to methylazoxymethanol (MAM) has been used successfully to explore the dose-dependent consequences to brain structure (Battaglia et al., 2003; de Groot, et al., 2005a, 2005b). Other potent and commonly used developmental neurotoxicants include ethanol and organic metals (e.g., lead, mercury, tin), but the great diversity of treatment protocols and neuropathology analyses described in this literature preclude the rational design of suitable DNT positive control studies at this time. Recent papers demonstrating that non-chemical treatments (e.g., hormonal shifts; Litteria, 1994) and mechanical manipulation (Maurissen and Marable, 2005) are effective means of

altering neural development deserve further investigation as a potentially useful alternative for procuring positive control materials.

THE DNT NEUROPATHOLOGY REPORT

The final product of the DNT neuropathology examination is a detailed final report. The typical report contains all the elements of a conventional pathology report (including individual animal and summary data tables for qualitative and quantitative measurements as well as the results of statistical calculations), but special care must be given to describing the specific neuropathology methods employed for the study in question. Such attention must be given not only to describing the quantitative procedures but also to defining how qualitative scores are assigned. For example, for paired structures pathologists might grade each side separately, combine the grades for each side into an "average" score, or choose the grade from the most severely affected side. In our estimation, all 3 means are suitable, as long as the method used in a given study is noted in the report.

Depending on the pathologist's preference or the flexibility of the software system for collating data, the DNT neuropathology report is generally constructed in 1 of 2 formats. The first contains a listing of the important structures examined in each region and reports only abnormal findings. This "implicit reporting" approach may be suitable for describing the lesions induced with known neurotoxic agents in positive control studies (Crofton et al., 2004). The second arrangement explicitly lists the major anatomic sites in each section as discrete locations and requires a notation as "normal," "abnormal," or "not examined" for each site. We recommend this latter approach as it credibly documents to regulatory reviewers the extent of the examination performed with novel test articles, even if they produce no neuropathologic effects. Photographs of representative macroscopic and microscopic lesions are also particularly suitable items to include in the final product.

In all reports, neuroanatomic nomenclature should be standardized. A helpful means of cataloging anatomic features is the use of appropriate brain atlases for the developing (Paxinos et al., 1994) and adult (Paxinos and Watson, 1997) rat brain. In addition, the pathologist should use accepted diagnostic nomenclature for describing toxicant-induced neural lesions in rats (McMartin et al., 1997; Solleveld et al., 1991). Word choices should reflect currently accepted veterinary nomenclature for quadruped animals rather than the homologous medical terms for the biped human.

In our experience, a common problem that occurs during regulatory review of DNT neuropathology reports concerns the comparability of measurements among animals, in particular the lack of data confirming that quantitative data were acquired in homologous sections. The absence of this data may engender a 3- to 6-month delay as the submitting company pulls archival materials to obtain such images. We note that this suspension would be obviated if low-magnification images of each section were gathered at the time when the slides were actually being evaluated. Such data could be submitted with the initial registration package (perhaps in an electronic medium rather than a printed hard copy), or retained for rapid delivery if requested by the regulatory agency. We suggest that this procedure, while not a current practice, would be a valuable addition to the procedures used in completing the standard DNT neuropathology evaluation.

PERSONNEL

The complexity of the DNT neuropathology examination mandates that the pathologist and support staff engaged in collecting and analyzing the data have specific training and relevant experience in conducting such studies. Particular needs for the pathologist include a good working knowledge of neural structure (especially the ability to recognize the proper orientation and sequence of sections in three dimensions), function, and chemistry as well as a thorough awareness of normal structural changes (e.g., cyclic expansion and regression of the cell-producing zones; Duffell et al., 2000) that take place during neural development. A fair degree of neuroanatomical knowledge is also required by the technical staff tasked with harvesting and sectioning neural tissues or making morphometric measurements. As with other toxicity studies, the standard means of assuring competence in technical staff will be a focused training program involving communication of DNT-specific standard operating procedures (SOPs) and laboratory rotations to practice particular skills (such as perfusion fixation and brain trimming).

Many resources on various aspects of rat neurobiology have been published in recent years, and these volumes will serve as important supplemental material for personnel tasked with performing the DNT neuropathology assessment. The texts of most relevance on a daily basis are likely to be the atlases for the developing (Altman and Bayer, 1995; Paxinos et al., 1994) and mature (Kruger et al., 1995; Paxinos and Watson, 1997) rat brain. More in-depth information for the supervising pathologist likely will include specific references on neuroanatomy (Bolon, 2000; Paxinos, 2004), neurochemistry (Siegel et al., 1994), and neurotoxicology (Chang and Slikker, 1995; Kaufmann, 2000; Slikker and Chang, 1998) as well as continual surveillance for relevant articles in scientific journals (especially such targeted forums as *Neurobehavioral Toxicology and Teratology* and *NeuroToxicology*). In particular, the papers in two past issues of *Toxicologic Pathology* (Vol. 18, No. 1, Pt. 2, 1990 and Vol. 28, No. 1, 2000) contain detailed up-to-date knowledge regarding many aspects of toxicologic neuropathology.

SUMMARY BEST PRACTICE RECOMMENDATIONS FOR DNT NEUROPATHOLOGY ASSESSMENT

The neuropathology component of the developmental neurotoxicity (DNT) study should use a staged analytical approach, considering the functional (clinical neurology, behavioral testing) and qualitative structural (macroscopic and microscopic lesions) data in conjunction with selected quantitative measurements as the first tier. More extensive morphometric procedures as well as specialized neurohistologic techniques may be warranted by the changes defined in one or more endpoints in the basic analysis. The DNT neuropathology examination should be conducted under the direct supervision of an experienced toxicologic pathologist familiar with comparative and correlative neurobiology among various mammalian species (particularly rodent, canine, and primate) and across the entire life span (development, adolescence, maturity, and senescence). Laboratory personnel tasked with necropsy, tissue processing, and histology tasks

should be similarly experienced in dealing with neural tissues. The qualitative evaluation should include examination of all major regions of the brain (to include at least the cerebral cortex [motor and sensory divisions], basal ganglia, hippocampus, thalamus, hypothalamus, cerebellum, and brainstem) and spinal cord (cervical, thoracic, and lumbar) as well as important components of the peripheral nervous system (dorsal root ganglia [DRG] and several peripheral nerves) and specialized sensory organs (e.g., eyes). Effector organs targeted by neuron processes (e.g., skeletal muscle) and neural secretions (e.g., endocrine organs) are also recommended for qualitative examination.

In all DNT studies, the neuropathology methods and results must be documented in exquisite detail, both to provide the highest quality report and to form a foundation for refining the DNT studies of the future. Why? Because we anticipate that the current DNT neuropathology evaluation will evolve over time as community experience and emerging technologies (e.g., real-time molecular measurements, noninvasive imaging, novel stereological algorithms) shape the interpretation of "best practices" in coming years. Furthermore, we anticipate that future DNT programs for at least some xenobiotics will be expanded to include additional levels of testing. Possible scenarios include taking rats challenged with neurotoxicants during development and then evaluating their capacity for neuroanatomical plasticity (Wallace et al., 2003) or predisposition to accelerated neurodegeneration during senescence (between 270 to 360 days), or even re-exposing them during 'middle age' (120 to 200 days of age) to test for increased vulnerability of the nervous system to subsequent exposures. For the foreseeable future, however, qualitative histopathology and limited morphometry of conventionally stained neural tissue from juvenile and young adult rats will remain the cornerstone for assessing the adverse impact of chemicals to the developing mammalian nervous system.

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