Methods to Identify and Characterize Developmental Neurotoxicity for Human Health Risk Assessment. II: Neuropathology

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Neuropathologic assessment of chemically induced developmental alterations in the nervous system for regulatory purposes is a multifactorial, complex process. This calls for careful qualitative and quantitative morphologic study of numerous brains at several developmental stages in rats. Quantitative evaluation may include such basic methods as determination of brain weight and dimensions as well as the progressively more complex approaches of linear, areal, or stereologic measurement of brain sections. Histologic evaluation employs routine stains (such as hematoxylin and eosin), which can be complemented by a variety of special and immunohistochemical procedures. These brain studies are augmented by morphologic assessment of selected peripheral nervous system structures. Studies of this nature require a high level of technical skill as well as special training on the part of the pathologist. The pathologist should have knowledge of normal microscopic neuroanatomy/neuronal circuitry and an understanding of basic principles of developmental neurobiology, such as familiarity with the patterns of physiologic or programmed cell death (apoptosis) expected at those developmental stages to be examined. Key words: brain development, fixation, histology, morphometry, neuropathology, neurotoxicity testing, peripheral neuropathy, special stains, stereology. — Environ Health Perspect 109(suppl 1):93–100 (2001). http://ehpnet1.niehs.nih.gov/docs/2001/suppl1-1/93-100garman/abstract.html

A developmental neurotoxicity study is used to assess neurologic development in laboratory animals following toxicant exposure. One important component of this evaluation is histopathology. Differences between species in the rates and complexities of biologic processes underlying neurologic development contribute significantly to the challenge of using an animal species, such as the rat, to predict the neurotoxic potential of a chemical in humans. Central to morphologic assessment of the developing brain is knowing what to look for and when to look. Corresponding time frames of rat and human development for key events in nervous system morphogenesis provide a global spatial–temporal map that together with an understanding of the pathogenesis of nervous system malformations can guide the pathologist in identifying and interpreting treatment-related effects (1,2). Two considerations are essential for identifying adverse effects: First, regressive events essential to normal development such as programmed cell death must be distinguished from treatment-related effects that may exhibit similar morphologic characteristics. Second, disruption of relatively early developmental events can alter growth and differentiation, thus producing alterations in the size of neuroanatomic structures, even in the absence of evidence of cell death or of associated tissue reaction. Quantitative assessment of morphologic parameters has been proposed as an important measure in developmental neurotoxicity testing (3). Given that few laboratories have had experience in the performance of the histopathology portion of developmental neurotoxicity studies (especially the morphometric components), we present an overview of current procedures and related background information.

Practical Issues

Personnel Qualifications

Conduct of the neuropathology component of a developmental neurotoxicity study requires special training for the pathologist and technical staff. The study pathologist should be experienced in neuropathology and should have sufficient knowledge of neuroanatomy to be able to recognize histologic sections for consistency of level and orientation. For example, the pathologist should be able to recognize readily whether a nonhomologous section represents a level anterior or posterior to the desired plane and should also be able to determine whether nonstandard sections should be measured or excluded from the morphometry data set. The pathologist should also have some knowledge of functional neuroanatomy and of the major neurotransmitter circuits. Finally, the pathologist should have sufficient experience examining developing rat brains to be able to recognize those normal regressive processes (e.g., programmed cell death) that might be misconstrued as pathologic. The technical staff should also have some knowledge of gross neuroanatomy. For example, prosectors must be able to recognize the neuroanatomic landmarks used for standardized slicing of brains. Furthermore, both prosectors and histotechnicians should be able to determine by gross examination which face of a brain slice is anterior and which is posterior, in the event that these slices flip over during slicing or processing. Finally, because slight deviations from standard procedures may have significant effects on the outcome of a study, all prosectors and histotechnicians should be meticulous in their attention to detail.

Ages and Numbers of Examined Rats

Various guidelines may require the examination of brains from postnatal day (PND) 11 or PND22 rats, as well as the brains of young adult rats (usually PND60) (4,5). Note that methods of classifying rat age vary from laboratory to laboratory. The first 24 hr after pairing of the adult male and female rat may be classified as embryonic day (E) 0, or E1, depending on the laboratory. Similarly, the day of birth may be considered PND0 or PND1. Selection of the most appropriate sampling times may relate to both the duration of exposure and the milestones of neuroanatomic development. Regardless of the provisions of the specific test guidelines, the...
most appropriate sampling time may be guided by knowledge of the potential for the chemical to interact with the brain during certain critical points or phases in its development. Distinct populations of neurons have relatively specific “birth dates” (7,8). For example, mitotic activity is still present within the external germinal (granular) layer of the cerebellum at PND11 as well as within the dentate gyrus of the hippocampus. Cessation of mitotic activity does not occur in the rat brain until PND16 (9). By PND21 the rat brain is essentially in its adult form, even though poorly myelinated and not of adult size. Therefore, examination of rat brains at PND21 may not necessarily have a significant advantage over examination of adult rat brains.

Although six animals per sex and treatment group have frequently been used for these studies, this number may be too limited for morphometric evaluations of PND11 rats because of the rapid growth that the rat brain is undergoing at this stage. A group size of 10 rats/sex has been found to be more appropriate by one of the authors (R.H.G.) based on personal experience and data power analysis. Even if the group size is increased over that recommended by current guidelines, brains from additional rats should be saved in fixative in case some brains are traumatized during removal and thus may need to be excluded from evaluation. In some situations, it may also be desirable to increase the group size to measure structures in different section planes or in different orientations (e.g., sagittal rather than coronal) to perform morphometry by more than one technique.

Tissue Fixation and Handling

Perfusion fixation should be employed for the adult (PND60) rats, whereas immersion fixation is usually adequate for the juvenile (PND11) rats. The calvaria should be removed from the nonperfused PND11 rats at the time of necropsy and the brains subsequently fixed in situ to minimize artifact. The brains of PND11 rats are very soft, however, and are easily traumatized during removal of the calvaria.

Brain weights for both juvenile and adult rats should be taken postfixation. In removing brains for weighing and subsequent dissection, it is important to keep the olfactory bulbs intact and also to standardize the level at which the medulla is transected. The medulla is usually cut just posterior to the occiput (i.e., just anterior to the atlas). Because fixation represents a chemical process that takes at least several hours, perfusion-fixed PND60 rat brains should be left in situ for additional immersion fixation rather than being removed immediately after perfusion. The fixative to be used and the length of time that tissues are held in fixative may be dictated by anticipated histologic procedures, such as those involving special stains. For example, the staining quality of many immunoperoxidase stains is adversely affected by prolonged fixation or by fixation in certain fixatives such as Bouin’s. For a more detailed discussion of neuropathology techniques, see Fix and Garman (10).

PND11 rat brains have high water content and are nonmyelinated. Therefore, they are very soft and easily traumatized. Some anatomic structures that might be used as gross dissection guidelines, such as the optic chiasm, are difficult to visualize at this young age. The cerebellum of PND11 brains is incompletely formed, making it difficult to achieve highly standardized coronal sections of this structure. Standardized sagittal sections of the cerebella (or the entire brain) may be easier to obtain. Use of magnification will aid in identifying neuroanatomic landmarks on PND11 rat brains. Brains of rats at PND11 will be characterized by greater variations in size than brains from older or adult rats due to rapid growth of the brains at this stage coupled with slight variations (in hours) in length of gestation and preculling of litter size (Figure 1).

Brains from juvenile rats may be placed in Bouin’s fixative to enhance their firmness, although fixation in Bouin’s will cause brain shrinkage and should be controlled carefully. For example, experience (in the first author’s laboratory) using Bouin’s fixative after formalin fixation caused approximately 23% decrease in brain weight and 7% decrease in anterior-to-posterior brain length measurement for PND12 rats. If brains are placed in Bouin’s fixative before formalin fixation, they should be transferred through at least two changes of alcohol and then placed in formalin. Brains should not be left in alcohol solutions because this may induce myelin vacuolation (11–13). The dehydration times on the tissue processor must be shorter for the PND11 rat brains than for adult rat brains; otherwise, the PND11 brain slices may become excessively dehydrated.

Nervous system tissues from representative numbers of animals from each of the control and high-dose treatment groups are examined microscopically in most studies [e.g., (4,14)]. If treatment-related microscopic changes or intergroup morphometric differences are noted, additional evaluations are then performed on tissues from the other treatment groups. Animal tissues from the intermediate and low-dose treatment groups are generally held in formalin, and if morphometric evaluations are to be performed one must consider the potential effects of long-term formalin fixation on tissue volume. Evidence shows that prolonged formalin fixation may enhance brain shrinkage (15,16) and that brain shrinkage can result from paraffin embedding (17). Therefore, to ensure consistency, brain slices should be prepared, processed, and embedded at approximately the same time for all animals on which morphometric evaluations may be performed. Representative numbers of brains from both control and treated animals also should be processed in the same batch to prevent intergroup differences in shrinkage related to the freshness of the alcohol and paraffin solvent solutions in the processor.

Dissection Procedures for Brains

Pathologists may prefer to evaluate coronal (transverse) sections of rat brains because this orientation is most familiar to them; however, sagittal sections may be most appropriate for tracing brain stem neural pathways or for measuring distances between certain subcortical anatomic structures. Additionally, anterior-to-posterior dimensions for certain structures, such as the developing cerebellar cortex, may be quite important. Anterior-posterior (AP) measurements of the cerebrum and cerebellum may be obtained from intact brains with a Vernier caliper before slicing the brain if coronal slices are to be prepared. Brain widths and heights may also be taken with a Vernier caliper before slicing. However, these dimensions are less critical to obtain on the whole brain because they can be obtained subsequently from the tissue sections. Some pathologists may prefer to perform a mid-sagittal section through the cerebellum rather than standardized coronal sections through various levels of the cerebrum and brainstem. If a sagittal section is to be taken of the cerebellum, the width measurement of the cerebellum (rather than its AP dimension) might be obtained with a Vernier caliper before brain slicing.

Brain matrix molds/miter boxes may be helpful for obtaining vertical slices of even thickness. However, these molds are of limited value for obtaining homologous sections from either adult or juvenile rat brains.
Multiple molds would be required because brain size varies at PND11 (Figure 1), and these molds may traumatize the PND11 brains during slicing. Even when the molds are of the correct size (e.g., for adults), the knife grooves in the molds may not line up correctly with the desired gross anatomic structures. Sections from these molds therefore will not be as standardized as those obtained by using specific neuroanatomic landmarks as guides. However, free slicing of brains outside of a mold requires practice and experience. Placing a mirror on the opposite side of the brain may assist in keeping the knife axis perpendicular to the long axis of the brain. The same individual should slice all of the brains on a study for a particular sex and age of rat to ensure consistency. Encasing each brain in an agar medium as described by Duffell et al. (18) is an alternative technique that may help some proponents achieve parallel vertical cuts. On the other hand, the agar may make it more difficult to see some of the small gross neuroanatomic landmarks present on PND11 rat brains. The prosector should understand that “facing in” of the tissue blocks by the histotechnician will cause some tissue loss. Each gross cut made in the brain, therefore, should be slightly anterior to the desired level (assuming that the anterior face of the slice is the one to be sectioned). Brain blocks also must be sectioned in a highly standardized manner. Generally, the initial full-face section is obtained from the block, after which multiple step sections are prepared (at least two or three step sections initially), sectioning deeper into the block as necessary. The histotechnician must recognize the desired tissue level by observing the sections as they float out on the water bath or by looking at unstained sections under the microscope. Furthermore, embedding must be performed in a highly standardized manner and the plane of sectioning must not be changed. Most of the useful anatomic landmarks for guiding dissection are present on the ventral aspect of the brain, except the cerebellum. Specific neuroanatomic landmarks for brain slicing may include structures such as the optic chiasm, the infundibulum, and the mammillary body. Note, however, that the level of the optic chiasm may vary significantly between rats, particularly in juvenile animals. The measured sections must be homologous among individual rats when linear measurements are performed to satisfy the morphometry component of a developmental neurotoxicity study. In contrast, certain procedures may call for randomly selected sections (see discussion under “Morphometry”).

**Histologic Stains**

Special morphologic staining procedures can and should be considered for use in developmental neurotoxicity studies. The addition of special histochemical or immunocytochemical procedures is scientifically necessary in many cases to adequately identify and characterize toxicant-related effects. Special approaches may provide additional information about morphologic changes detected on standard slides stained with hematoxylin and eosin or may allow the pathologist to detect effects not readily evident in these slides. Although special stains are not required under current regulations (4), some guidelines suggest that both myelin stains and silver stains be performed on the adult rats (14).

Various special stains traditionally have been used to evaluate nervous tissue components. Procedures for these can be found in general reference texts on histology methods (19–21). Further, newer methodologies are emerging from the neuroscience literature, and a selection of these is represented in Table 1. Any of these stains might be applied and appropriately interpreted in developmental neurotoxicity studies, although the immature brain does not have a mature complement of structures stained by these procedures. As a result, the characteristics of a particular stain will vary, depending on the age of the animal. Most of the special stains presented in Table 1 are appropriate for paraffin-embedded tissues; but some (such as the black-gold stain and some silver degeneration stains) are used best on either frozen or vibratome sections.

The literature is fairly limited, unfortunately, on the application of special staining procedures in the evaluation of developmental brain injury induced by neurotoxicants. Study reports that do exist typically lack a period of gestational exposure and vary from study to study regarding the dosage regimen, i.e., the age of initial exposure and length of treatment. Many approaches are available to characterize aspects of developmental neurobiology, but these methods have not been used widely to study the effects of neurotoxicants on developmental processes.

**Stains for Neurons and Myelin**

The cresyl echt violet (Nissl) stain demonstrates many details in the nerve cell body, particularly in larger neurons. This technique stains all nuclei in the section (neurons plus astroglia). Immunohistochemistry is particularly useful to detect changes in glial markers and to study the effects of neurotoxicant exposure on microglia. Special stains and ancillary procedures for consideration in developmental neurotoxicity studies are shown in Table 1.

**Table 1.** Special stains and ancillary procedures for consideration in developmental neurotoxicity studies.

| Tissue component and neuron-specific proteins | Procedure | Comments | References |
|----------------------------------------------|-----------|----------|------------|
| Neurotransmitters | Neurochemistry or immunohistochemistry | May provide topographic orientation to other biochemical data | Virgili et al., 1990(33) Axt and Mulliver, 1991(34) Kalia et al., 2000(35) |
| Astrocytes | Immunohistochemistry for GFAP | Increases in GFAP content occur in association with the astrocytic hypertrophy | Goodlett et al., 1993(44) Freeman et al., 1994(47) Harry et al., 1996(48) |
| Microglia | Lectin histochemistry | Detects morphologic transformation from the resting to phagocytic state | Moriya and Streit, 1991(54) Fix et al., 1996(26) |
| Immunohistochemistry for MHCs and complement receptors | Upregulation of immunologic antigens may occur before morphologic transformation to the phagocytic state | Milligan et al., 1991(55) Moriya and Streit, 1991(54) Wilson and Mulliver, 1999(150) McFarlane et al., 1995(52) Otho et al., 1995(53) |

GFAP, glial fibrillary acidic protein. □ Review article or text. ○ Does not specifically address neonatal development.
all others) and is therefore useful as an indicator of overall cellularity. In addition, this stain highlights the intensely basophilic endoplasmic reticulum (Nissl substance) in the perinuclear cytoplasm of larger neurons. Loss of Nissl staining can be associated with damage to the axon, particularly as seen with various types of physical injury. Cresyl echt violet staining may be combined with a myelin stain such as luxol fast blue in one procedure (19–21).

Luxol fast blue stain reacts with lipoproteins that are heavily concentrated in myelin, providing delineation of the perinuclear myelin sheath. This stain provides excellent histologic differentiation between gray and white matter and is often a good stain for morphometric evaluations in some techniques. Myelination is actively ongoing during the postnatal period. Therefore, stains that identify myelin layers around axons will not appear as intense in juvenile rats until myelination is complete. Little or no central myelin is present within the brains of PND11 rats, so a myelin stain is not very useful at this stage. A myelin stain combined with a nuclear stain for cellularity, such as Nissl, may still be useful in the evaluation of neonatal rat brains.

Standard silver stains can be used to identify axons and some dendrites in tissue sections, whereas silver degeneration stains selectively bind to degenerating neurons. Standard silver methods stain both normal and degenerating nerve processes and require careful comparison between treated and control animal tissues for interpretation of effects. The staining principle is based on the impregnation of the nerve fiber by silver ions, which are then chemically reduced to visible metallic silver. There are several silver staining procedures named for those who developed them, including Bodian, Holmes, Sevier-Munger, and Bielschowsky (19–21).

Silver degeneration stains are a modification of the standard silver staining procedures mentioned above, but they have the ability to selectively stain degenerating components of injured neurons. In contrast to standard silver stains, which primarily reveal larger-sized neuronal processes such as axons, silver degeneration stains can potentially delineate degenerative effects within any portion of the entire neuron, i.e., dendrites, cell body, axons, and nerve terminals. These procedures have been used for the identification of apoptotic neurons during the early postnatal period (22) as well as for neurotoxicity evaluations (23,24). To date, silver degeneration stains have been applied mostly in adult neurotoxicity studies (25,26), but their potential for adding to the understanding of developmental neurotoxicity is beginning to be explored. For example, silver degeneration stains have been used recently to show that ethanol and other drugs that antagonize the N-methyl-D-aspartate receptor (the major excitatory receptor in the brain) dramatically increase the rate of apoptosis during the early postnatal period in rat pups (27,28). These new data support the emerging use of silver degeneration stains for developmental neurotoxicity studies.

Stains for Neurotransmitters and Neuron-Specific Protein Markers

Immunochemical staining techniques for specific brain neurotransmitters could be considered for use in developmental neurotoxicity studies, and several atlases are available to assist in understanding the regional distribution of these neurotransmitters in the adult rat brain (29–32). Investigation has shown that neurotransmitter system maturation tends to parallel the general establishment of functional activity in the brain during the process of synaptogenesis (33). In a strict sense, staining for changes in specific neurotransmitters is a biochemical not morphologic approach, and therefore may be most useful for further characterizing effects found with other morphometric procedures rather than as an initial procedure. In addition, neurotransmitter immunohistochemistry may augment the interpretation of other biochemical data. This approach has been applied to neurotoxicity evaluations that characterize the loss of serotonin immunoreactivity in adult rats after treatment with drugs known to act on this neurotransmitter system (34,35). These studies have shown not only the loss of serotonin immunoreactivity but also changes in the morphologic appearance of the serotonin nerve terminal network. Application of this approach should be performed in a topographically restricted manner because the time course of neurochemical system maturation varies in different brain regions because of the diversity of cell populations and differential growth (36).

Biochemical methods have been used in developmental neurotoxicity studies to assess levels of neurochemicals, including neuron-specific protein markers. Levels of neuronal proteins such as synaptophysin, neurofilament, and myelin basic protein have been shown in these assays to change in response to administration of various neurotoxicants during the developmental period (36–40). Development and use of immunohistochemical procedures to detect these or other similar neuronal protein markers might augment biochemical assessments and provide information on topographic aspects of toxicant effects on nervous system development.

Stains for Astrocytes

Astrocytes have been known for years as major glial cells in the brain with important physiologic and supportive activities (41). These cells undergo hypertrophy and produce more glial fibrillary acidic protein (GFAP), an astrocyte-specific intermediate filament marker, in response to injury. Many researchers consider an increased level of GFAP a definitive marker of brain injury, including damage induced by neurotoxins. GFAP content can be evaluated biochemically or immunohistochemically in tissue sections (26,42,43). Enzyme-linked immunosorbent assays for GFAP performed in developmental neurotoxicity studies have revealed alterations in GFAP content in response to such neurotoxics as polychlorinated biphenyls, methylmercury, methylazoxymethanol, 6-hydroxydopamine, tributyltin, and triethyltin (37–40). Given this information, it is reasonable to use GFAP immunohistochemistry as an indicator of injury in the developing rat brain.

Developmental neurotoxicity studies with ethanol, triethyltin, and lead have also used GFAP immunohistochemistry to demonstrate alterations in the timing of astrocyte differentiation, as well as increases and/or decreases in GFAP immunoreactivity (44,47,48). When conducting developmental neurotoxicity studies with altered levels of GFAP as an end point, one must consider several aspects of GFAP biology. The GFAP content of astrocytes varies considerably in different brain regions, with levels lower in gray matter and higher in white matter. In addition, GFAP is developmentally regulated during the early postnatal period, with mRNA levels increasing from birth to PND15 and declining to adult levels by PND25 (48).

Stains for Microglia

The cells in the brain with primary immunologic and phagocytic roles are the microglia. These cells can be demonstrated with a variety of histologic and histochemical techniques (49). In the adult brain, microglia exist as small resting cells with highly ramified processes extending from a central nucleus. Microglia are activated in response to injury. When activated, they increase their expression of various immunologic molecules [e.g., major histocompatibility antigens (MHCs), complement receptors] and transform morphologically to a phagocytic phenotype. Cytokine production and antigen presentation are associated with this activation and transformation. Changes in microglial morphology and immunology correlate with neurotoxicity in adult rats (26,50). The nature of these glial reactions may vary with different injurious stimuli.

There are sufficient data on adult rat brains to consider morphologic and immunologic changes in microglia reasonable markers...
Neuropathology in developmental neurotoxicity

A certain degree of subjectivity is involved in deciding the boundaries of individual neuroanatomic regions and the exact start and end points of a linear measurement. It is important, therefore, to use a level of magnification that will provide a reasonable degree of resolution. The best resolution will be achieved with a microscope fitted with a calibrated ocular micrometer. The disadvantage of this approach is that there are no resulting images that include the exact location of the linear measurements taken. Although it may not be necessary to have a visual display of every linear measurement taken, it is highly advantageous to have images of the measured sections included within the raw data. To demonstrate section homology, these images should be taken at a sufficiently low magnification to allow a reviewer to establish the level of the section. These images should also be taken at a standardized magnification with sufficient information provided in the report or raw data to allow a reviewer to repeat the measurements directly on the images. The degree of standardization of section level can thus be established by a peer reviewer without the need to reexamine the glass slides. Images digitized and archived on a CD can then be viewed on a monitor, printed to hard copy, or subjected to additional analysis without the need to reevaluate the original glass slides. Whenever linear measurements are to be performed directly on photographic prints (either film-based or digitized), controls must be in place to ensure that the image magnifications are identical. Additionally, it is important to note that the rotation of an image axis in most computer software programs will shrink the image.

Various software programs are available for performing linear or area measurements, and one (Scion Image for either Windows or Macintosh platforms; Scion Corp., Frederick, MD) is available in the public domain (57). Areal measurements may be taken from the same sections used for the linear measurements, and these sections may also be used for the histopathologic evaluations. Image analysis software packages provide the most convenient method for performing areal measurements, but other non-computer–based approaches have been used for years (58).

Linear and/or areal measurements may be quite helpful for detecting certain developmental neurotoxic end points; however, some neuroanatomic regions are poorly demarcated and are difficult to measure by linear or areal techniques. Optical dissector stereology may be more sensitive for detecting certain types of cell loss and reduces the need for highly homologous sections. The reader is directed to the literature for a more comprehensive discussion of the principles of stereology (59–68). The concept of randomly selected...
sections (which represents part of the unbiased sampling concept) may confuse the reader perusing the literature on optical disector stereology. The sections that are randomly selected represent serial sections taken through a particular neuroanatomic structure rather than random sections taken throughout the brain. Thus, one must still obtain relatively homologous sections that pass through the same level of a particular neuroanatomic region. Stereology, sometimes called quantitative histology, may be used to determine cell numbers within a particular volume of tissue and infer cell densities. Two-dimensional counting of cell profiles, referred to as model-based stereology, is based on certain assumptions about cell geometry. Three-dimensional stereology, is based on certain assumptions about cell geometry and the in vivo preservation of cell density (70). The optical dissector method of unbiased stereology uses a high numerical aperture objective providing high magnification with a narrow depth of field to count cell profiles present in multiple optical planes within relatively thick (20–50 μm) sections. Plastic-embedded sections are generally 20–25 μm thick, whereas thicker paraffin sections are usually prepared to allow for subsequent shrinkage resulting from paraffin removal and subsequent staining. Optical dissection requires a microscope with a stage-calibrated for movement along the z axis. Random sampling involves a random or unbiased sampling of section levels within a given neuroanatomic region in the same tissue block of relatively large neuroanatomic areas. Multiple serial sections are prepared through the neuroanatomic area to be enumerated. The first section to be examined in this series is randomly selected and cells are counted within a given number of fields in that section. Subsequently, other slides in the series (e.g., every tenth section after the first randomly selected section) are similarly counted. Only those cells intersecting the lines of a standardized grid or counting frame are included in the total count; therefore, only a relatively small number of cells must be counted within the area of interest on a particular slide. The numbers of cells present within a given volume of tissue are then calculated using mathematical formulas. Stereologic procedures may be used to quantify numbers of dead or degenerating cells, a procedure that may be enhanced further by the application of a variety of special stains such as terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, cupric silver, or Fluoro-Jade (27, 28). Stereology also may be used to determine synaptic densities (70). Various staining procedures can also help enumerate other cell types such as astrocytes or microglial cells through optical dissector stereology (70).

Stereology takes significantly more time than linear or areal measurements. For example, a pathologist can take a dozen linear measurements with an ocular micrometer in approximately 15 min. Performing unbiased stereology on a dozen different areas of the brain would require at least several hours. Optical dissector methodology also uses relatively thick serial sections, thus requiring the use of extra animals (besides those used for histopathologic evaluations) and incurring additional histology costs.

In summary, unbiased stereology allows enumeration of cells and determination of cellular densities, whereas linear and/or areal measures provide only an indication of the overall size of the measured area. The greater time investment and the need for thick sections, however, make stereology recommended as a second-tier procedure. Optical dissector stereology procedures are likely the most sensitive indicator of postmigrational cell loss (e.g., neurotoxic insults in adult animals). However, linear measures may be as sensitive as stereology for detecting treatment-related differences following exposure to toxicants that affect early cell proliferation or migration. Regardless of the techniques used for morphometric analysis in a developmental neurotoxicity study, treatment-related alterations within the developing brain may be manifested as differences in the sizes of neuroanatomic structures or as alterations in cell or synaptic densities. In contrast, the adult brain may show degenerative or reactive changes such as eosinophilic neurons, reactive astrocytes, spongiosis, demyelination, or increased numbers of microglial cells.

Interpretation of Morphometry Data

The biologic significance of brain size variation encountered at PND11 may be difficult to interpret given the rapid brain development occurring at this time. This is confounded when treatment-related morphometric variations are seen in PND11 brains but not in adult rat brains in the same study. An additional concern is the potential effect on brain size as a result of maternal toxicity. Brain sparing refers to the observation that brain weight is relatively unaffected by changes in total body weight, whereas changes in the weights of many other organs tend to be proportional to changes in body weight. Brain sparing is typically seen in undernourished adult animals, whereas delayed brain development and thus smaller brains are seen in juvenile animals when caloric or nutrient restriction occurs during early postnatal development (72–76). Delays in rat brain development are easily demonstrable in the brains of rats from litters of different sizes, but are also seen in situations of decreased lactation, decreased maternal nutrition, or maternal neglect. In fact, the brains of undernourished pups may never achieve the developmental level of those of control group or optimally nourished rats. Differential sensitivity in the degrees of retardation of brain development may be expected from one area of the brain to another. For example, areas that mature after birth (e.g., cerebral cortex, cerebellum, and hippocampus) might be more affected by undernutrition than might subcortical structures that develop in utero. Subtle shifts in morphometric dimensions may indicate the presence of a treatment-related effect on brain development. However, one must consider the possibility that this effect may stem from nutrition rather than from developmental neurotoxicity. Pair-feeding studies may help differentiate the effects of undernutrition from those caused by a developmental neurotoxicant (77).

Brain-to-body-weight ratios of undernourished pups are generally equal to or slightly greater than ratios of adequately nourished rat pups, although the amount of milk present in a pup’s stomach may significantly influence measured body weight. Data have not been published on brain-to-body-weight ratios in developmental neurotoxicity studies to determine whether alterations in this ratio are predictive of neurotoxicity (i.e., vs. malnutrition). Nevertheless, we recommend that brain-to-body-weight ratios be recorded.

Peripheral Nervous System

Morphologic assessment of the peripheral nervous system of rats (peripheral nerves and ganglia, spinal nerve roots, and possibly other structures) is required under current U.S. EPA regulatory guidelines for adult rats in developmental neurotoxicity studies. Such assessment is not required for juvenile rats, even though peripheral nerves are easily accessible in juvenile rats and their myelinated nerve fibers traditionally have been used to provide qualitative and quantitative histologic data for diagnostic and investigative evaluation (4, 73). Routine evaluation of the peripheral nervous tissue of juvenile rats might add important neurodevelopmental information to that derived from study of the brain and warrants further consideration. Evaluation of properly prepared peripheral nerve tissue can provide qualitative and quantitative developmental data regarding axon size, axon numbers, and the relationship between the axons.
and supporting glia. Although some of this information may be gleaned from the study of sections of the CNS, the better histologic visualization of individual myelin sheaths and their associated axons within the peripheral nerve endonuclear matrix greatly enhances such morphometric assessments. Peripheral nerve assessment can provide a quantitative morphologic reflection of the integrity of complex developmental processes such as neuronal growth, axonal guidance, development and connection of nerve endings, Schwann cell evolution, migration and axonal association, and myelination (78, 79).

The current regulatory developmental neurotoxicity guidelines call for histologic assessment of sensory ganglia and of multiple levels of peripheral nerve in perfusion-fixed adult (PND60) rats (4, 80). As noted above, peripheral nerve from younger, nonperfused animals can be evaluated similarly. The sciatic nerve in these animals is accessible and animals can be evaluated similarly. The sciatic nerve from younger, nonperfused adult (PND60) rats (4, 73) can reveal progressive increases in the caliber of myelinated fibers as well as myelin sheaths and axonal diameters and axon distribution (73). Other nerve trunks are also available from the U.S. EPA guidelines (4, 80). Additional tissue levels of peripheral nervous system tissues are being developed regularly; histologic markers of neuronal or glial cells and neuronal populations are being used to assess morphologic alterations in the developing nervous system. The increasing resolution and availability of techniques such as magnetic resonance imaging may soon provide a superior methodology for evaluating treatment-related alterations in the brain (84). New immunologic staining procedures are being developed regularly; histologic markers of neuronal or glial cell injury, as well as of neurotransmitter levels, will likely prove vital to the pathologist in the evaluation of these studies as the traditional stains for myelin, glial cells, and nerve processes currently in use. As both computer-imaging programs and the specificity of stains improve, analysis of patterns of dendritic arborization and neurotransmitter localization may become as important for evaluating developmental neurotoxic action as current routine histopathologic evaluation. Although our focus here is morphology, there is tremendous potential for biochemical and molecular biological techniques to complement current morphologic techniques. Neuropathology will certainly continue to change rapidly, and many of the new techniques in investigative neuropathology will soon find their way into routine use.

Future Directions

The methods and techniques for pathologic evaluation described here are valuable tools used to assess morphologic alterations in the developing nervous system. The increasing resolution and availability of techniques such as magnetic resonance imaging may soon provide a superior methodology for evaluating treatment-related alterations in the brain (84). New immunologic staining procedures are being developed regularly; histologic markers of neuronal or glial cell injury, as well as of neurotransmitter levels, will likely prove vital to the pathologist in the evaluation of these studies as the traditional stains for myelin, glial cells, and nerve processes currently in use. As both computer-imaging programs and the specificity of stains improve, analysis of patterns of dendritic arborization and neurotransmitter localization may become as important for evaluating developmental neurotoxic action as current routine histopathologic evaluation. Although our focus here is morphology, there is tremendous potential for biochemical and molecular biological techniques to complement current morphologic techniques. Neuropathology will certainly continue to change rapidly, and many of the new techniques in investigative neuropathology will soon find their way into routine use.

REFERENCES AND NOTES

1. Bayer SA, Altman J, Russo RJ, Zhang X. Timetables of neurogenesis in the human brain based on experimentally determined patterns in the rat. Neurotoxicology 14(1):83-144 (1993).

2. Rice DC, Barone S. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Neurotoxicol Teratol 23(3):351-539 (2001).

3. Daudt L, Kwa W, Russell A, Wallinga D. Testing methods for developmental neurotoxicity of environmental chemicals. Toxicol Appl Pharmacol 161(1):11-23 (2000).

4. U.S. EPA. OPPTS 870.6300, Developmental Neurotoxicity Study. EPA 712-C-98-239. U.S. Environmental Protection Agency. Washington, DC: U.S. Government Printing Office, 1988.

5. OECD. OECD Test Guideline Developmental Neurotoxicity Study (DRAFT). Paris: Organisation for Economic Co-operation and Development, 1998. 426.

6. Bennett GD, Frenelli RH. Periods of susceptibility to induced malformations of the developing mammalian brain. In: Handbook of Developmental Neuropathology (Slikker W, Chang LW, eds). New York: Academic Press, 1998:189-238.

7. Bayer SA, Altman J. Principles of neurogenesis, neuronal migration, and neural circuit formation. In: The Rat Nervous System, 3rd ed. Paxinos G, ed. New York: Academic Press, 1995:1079-1098.

8. Jensen KF, Catalano SM. Brain morphogenesis and developmental neurotoxicology. In: Handbook of Developmental Neuropathology (Slikker W, Chang LW, eds). New York: Academic Press, 1998:31-41.

9. Jacobsen M. The germinal cell, histogenics, and lineages of nerve cells. In: Developmental Neuropathology, 3rd edition (Lazoisten M, ed). New York: Plenum Press, 1991:41-93.

10. Fux AS, Garman RH. Practical aspects of neuropathology: a technical guide for working with the nervous system. Toxicol Pathol 28(1):122-131 (2000).

11. Chalaby MU. Artifact in tissues held in 70% ethanol. HISTO-LOGIC TECHNICAL BULLETIN FOR HISTOPATHOLOGISTS 3(3):3-6 (2000).

12. McLinn G. Vacuoles in the fiber tracts of rat CNS tissue. J Histotechnol 5:171 (1982).

13. Wells G, Wells M. Neuronal visualization in the brain: a reproducible histological processing artifact. J Comp Pathol 101:355-362 (1989).

14. U.S. Environmental Protection Agency Health Effects Guidelines OPPTS 870.6200, Neurosensory Screening Battery. Washington, DC: U.S. Government Printing Office, 1998.

15. Dam AM. Shrinkage of the brain during histological procedures with fixation in formaldehyde solutions of different concentrations. J Histotechnol 20:113-119 (1997).

16. Sadoski M, Moryn J, Belder B, Maciejewska B. Influence of fixation and histological procedure on the morphometric parameters of neuronal cells. Folia Morphol 54:219-226 (1995).

17. Guestier R, Schridde R. The shrinkage of the human brain stem during formalin fixation and embedding in paraffin. J Neurossci Methods 75:81-98 (1997).

18. Duffull SJ, Soames AR, Gunby S. Morphometric analysis of the developing rat brain. Toxicol Pathol 28(1):39-48 (2000).

19. Luna LG. Manual of Histologic Staining Techniques of the Armed Forces Institute of Pathology. New York: McGraw Hill Co., 1988.

20. Sheehan DC, Hrapchak BB. Theory and Practice of Histotechnology. St. Louis: MOCV/Modry Co., 1980.

21. Carson FL. Histotechnology: A Self-Instructional Text. Chicago: ASCP Press, 1997.

22. Yamamoto T, Iwashita M, Lara D, Isoda H. Identification of cells undergoing physiological neuronal death in the neonatal rat brain by the Fink–Heimer method. Brain Res. 374:419-424 (1986).

23. Beltramini CA, delMeso J, Gallays F, Holmer L, Zaborkis J. Silver staining as a tool for neurotoxic assessment. NIDA Research Monograph Series. Assessing Neuroxicity of Drugs of Abuse. Washington, DC: National Technical Information Service, 1993:101-126.

24. Switzer R. Morphologic approaches for evaluating nervous system injury: application of silver degeneration stains for neurotoxicity testing. Toxicol Pathol 28:70-83 (2000).

25. Jensen KF, Dim J, Hyakutake N, O’Callaghan JP, Miller DB, de Olmos JS. Mapping taurine-induced neuronal system damage with a cupric silver stain: a quantitative analysis of neural degeneration induced by 3,4-methylenedioxymethamphetamine. NIDA Research Monograph Series. Assessing Neuroxicity of Drugs of Abuse. Washington, DC: National Technical Information Service, 1993:133-153.

26. Fux AS, Pass JS, Stitzel SR, Switzer RC. Integrated evaluation of central nervous system lesions: stains for neurons, astrocytes, and microglia reveal the spatial and temporal features of MK-801-induced neuronal necrosis in the rat cerebral cortex. Toxicol Pathol 24:291-304 (1996).

27. Ikonomidou C, Bosch F, Miksa M, Bigsland P, Vlückier J, Dikranian K, Tenkova T, Stefoshka V, Turski L, Diney JW. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 290:70-74 (1999).
28. Ikonomidou C, Bitigau P, Ishimaru MA, Wozniak DF, Koch C, Gent K, Price MT, Stafinski V, Hörppa A, Terakawa T, et al. Ethanol-induced apoptotic degeneration and fetal alcohol syndrome. Science 287:1096–1100 (2000).

29. Panosso G, Watson C. The Rat Brain in Stereotaxic Coordinates. San Diego: Academic Press, 1998.

30. Paxinos G, Kus G, Ashwell KWS, Watson C. Chemoarchitectonic Atlas of the Rat Forebrain. San Diego:Academic Press, 1999.

31. Paxinos G, Carver P, Wang H-W, Yang PY. Chemoarchitectonic Atlas of the Rat Brainstem. San Diego:Academic Press, 1999.

32. Tsakamatu M, Takatsuki K. Atlas of Neuroactive Substances and Their Receptors in the Rat. New York:Oxford University Press, 1998.

33. Virgil M, Barnabei D, Contestabile A. Regional maturation of neurotransmitter related and glial markers during postnatal development in the rat. Int J Dev Neurosci 8(2):159–168 (1990).

34. Axt KJ, Milloller MI. Immunocytochemical evidence for methamphetamine-induced astrocytic axon loss in the rat brain. Synapse 9:323–331 (1991).

35. Kalia M, O'Callaghan J, Miller DB, Kramer M. Comparative study of fluoxetine, sibutramine, sertraline and desipramine on the morphology of serotoninergic nerve terminals using serotonin immunocytochemistry. Brain Res 88(1):105–105 (2000).

36. Miller DB, O'Callaghan J. Biochemical, functional and morphological indicators of neurotoxicity: effects of acute administration of thymine to the developing rat. J Pharmacol Exp Ther 231(1):744–751 (1984).

37. O'Callaghan J, Miller DB. Acute exposure of the neonatal rat to thymine results in decreases in biochemical indicators of synaptogenesis and myelogenesis. J Pharmacol Exp Ther 240(1):394–402 (1987).

38. O'Callaghan J, Miller DB. Acute exposure of the neonatal rat to thymine results in persistent changes in neurotropic and gliotropic proteins. J Pharmacol Exp Ther 244(1):388–378 (1988).

39. O'Callaghan J, Miller DB. Assessment of chemically-induced alterations in brain development using assays of neuronal- and glia-localized proteins. Neurotoxicology 10(3):303–406 (1989).

40. Miron DC, Pug A, Wesolowski W, van den Berg KJ, Brouwer A. Persistent alterations in regional brain glial fibrillary acidic protein and synaptophysin levels following pre- and postnatal polychlorinated biphenyl exposure. Toxicol Appl Pharmacol 130:125–232 (1996).

41. Montgomery DL. Review: astrocytes: form, functions, and roles in disease. Vet Pathol 31:145–167 (1994).

42. O'Callaghan J, Jensen KF, Miller DB. Quantitative aspects of dopaminergic neurons. Neurotoxicology 15(1):149–159 (1994).

43. Freeman JH Jr, Barone S Jr, Stanton ME. Cognitive and neuropsychomotoric effects of methylazoxymethanol in utero in the rat. Brain Res 634:165–175 (1994).

44. Gary JG, Schmitt TJ, Gong Z, Brown H, Zawia N, Evans HL. Lead-induced alterations of glial fibrillary acidic protein (GFAP) in the developing rat brain. Toxicol Appl Pharmacol 139(1):84–93 (1996).

45. Streit W, Graeber M, Kreutzberg G. Functional plasticity of microglia: a review. Glia 8:183–197 (1988).

46. Wilson M, Milloller MI. Mucosal response to degeneration of serotoninergic axon terminals. Glia 11(1):18–34 (1994).

47. Milligan GE, Levitt P. Cunningham T-J. Brain macrophages and microglia respond differently to lesions of the developing and adult visual system. J Comp Neurol 314(1):136–146 (1991).

48. McFarland JL, Gilland E, Faena H, Hagenberg M. Microglia activation after neonatal hypoxic-ischemia. Brain Res Dev Brain Res 84(2):245–252 (1996).

49. Mihm M, Astariz H, Shimada M. Glial responses to hypoxic/ ischemic encephalopathy in neonatal rat cerebral cortex. Brain Res Dev Brain Res 84(2):249–259 (1996).

50. Morikota T, Streit WJ. Expression of immunomolecules on microglial cells following neonatal sciatic nerve injury. J Neuroimmunol 35:21–30 (1991).

51. Rodier PM, Graminan WJ. Morphologic effects of interference with cell proliferation in the early fetal period. Neurotox Toxicol 11:129–135 (1997).

52. Rodier PM. Structural-functional relationship in experimentally induced brain damage. Prog Brain Res 107:335–348 (1998).

53. Scion Image. Available: http://www.sciencemag.org (Cited December 2000).

54. Kutscher CL. A morphometric analysis of thymine-induced change in rat brain using the Thim technique. Brain Res Bull 28:519–527 (1992).

55. Coghlan RE, Lenahan M. Methods for determining numbers of cells and synapses: a case for more uniform standards of review. J Comp Neurol 364:6–15 (1997).

56. Geisinger Y, Gunderson HJG, Van Der Zee E, West MJ. Towards obtaining unbiased estimates of the total number of synapses in a brain region: problems of primary and secondary importance. J Neurosci 26(11):713–179 (1997).

57. Howard CV, Reed MG. Unbiased Stereology: Three-Dimensional Measurement in Microscopy. New York:Bio Scientific Publishers, 1998.

58. Hyman BT, Sotomayor-Tata J, Hrincey MJ. Stereology: a practical primer for neuropathology. J Neuropathol Exp Neurol 57(4):305–310 (1998).

59. Kiroto L, Anderson BB, Ladeloff D, Meller A. Total number of various cell types in rat cerebellar cortex estimated using an unbiased stereological method. Brain Res 609:268–269 (1992).

60. Peterson DA. Quantitative histology using confocal microscopy: implementation of unbiased stereological procedures. Methods 19:493–507 (1999).

61. Schmued L, Albertson C, Slikker WJ. Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. Brain Res 751:37–46 (1997).

62. Schmued L, Hopkins K. Fluoro-Jade: novel fluorochromes for detecting toxicant-induced neuronal degeneration. Toxicol Pathol 28:91–99 (2000).