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Evaluation of Large-Sized Brains for Neurotoxic Endpoints

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ABSTRACT

Sampling of large-sized brains (eg, dog, primate) for microscopic examination is frequently inadequate to detect localized neurotoxic injury. Furthermore, the examination of H&E-stained sections alone will often be insufficient for the detection of subtle neuropathogic alteration. It is imperative for any pathologist evaluating brain sections to have knowledge of microscopic neuroanatomy and to also have some understanding of basic neurochemistry. When a focus of degeneration is detected within the brain, the pathologist needs to ascertain not only the specific anatomic location of this focus but also the neuroanatomic regions that project to and receive output from the injured focus. Because of the complexity of brain circuitry and the fact that the brain contains many distinctive neuron populations, many more brain sections are required for adequate microscopic evaluation than for any other body organ. Deciding which and how many areas should be examined, microscopically, from a large size brain is often problematic. Although any sampling protocol will be influenced by what is known about the test chemical, it has been well established that certain regions of the brain (eg, hippocampus and other components of the limbic system, basal ganglia, Purkinje neurons) are more susceptible than others to a variety of physical, metabolic, and chemical insults. Knowledge of these regional sensitivities will assist in guiding the pathologist in the development of an adequate sampling protocol.

Keywords. Brain; cupric silver stain; dog; Fluoro-Jade stain; neuroanatomy; neuropathology; neurotoxicity.

INTRODUCTION

There is no single "best practice" for performing microscopic evaluations on large-sized brains such as those from dogs or nonhuman primates. For example, the stains to be used will be determined, in part, by the time course of the study and by those endpoints that may be of particular concern. Special stains may, in fact, be employed only after initial screening of hematoxylin and eosin-stained sections. However, decisions as to which brain regions are to be sampled for microscopic evaluation will generally come prior to any such initial screening. The sampling protocol may well be dictated by available knowledge of the test chemical's structure and/or mode of action. Certainly, the degree of brain sampling required to evaluate for potential neuropathologic effects of a highly polar nonlipophilic chemical that is not expected to have penetrated the blood brain barrier will differ from that required to adequately evaluate the safety of a neuropharmacologic agent known to demonstrate agonistic or antagonistic activity at neuronal receptor sites. Nevertheless, the heterogeneous nature of the brain with regard to metabolic and nutritional requirements as well as neurochemistry suggests that many more brain sections need to be obtained than would be prepared from any other large-sized but more homogeneous organ (eg, the liver or kidney). In fact, just as the body may be considered in terms of systems (such as urinary, hepatobiliary, digestive, respiratory)—all of which should be adequately sampled for microscopic evaluation—it is helpful to also think of the brain in terms of its multiple systems (eg, sensory, auditory, visual, motor, limbic, autonomic, etc.) in order to provide a rough guide to adequate sampling. Although the major emphasis of this article will be on sampling techniques, there will also be a brief discussion of tissue handling techniques, the importance of recognizing artifacts, and the use of selective special stains that are superior to H&E (hematoxylin and eosin) for revealing cellular degeneration within the central nervous system.

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METHODS AND DISCUSSION

Avoiding Artifact

It may seem trite, but is still worth mentioning that the principle concerns of the microscopist performing safety evaluations are to avoid both false positives and false negatives. Cytologic artifacts are exceedingly common in the central nervous system and may lead to either false positives (mistakenly interpreted as representing degeneration) or to false negatives (masking genuine degenerative changes). Two of the most frequent artifacts are basophilic ("dark") neurons and myelin artifact, both of which may be induced by improper tissue handling (4, 6). Even when tissues are fixed via perfusion (a requirement when certain stains for cellular degeneration are employed), such artifacts may be produced—usually as a result of handling tissues too soon after the perfusion has been completed. Judging from the number of peer-reviewed publications that the author has found in which artifact is erroneously reported as degeneration, overinterpretation of artifact continues to be a problem. Critical microscopic evaluations are best performed on perfusion-fixed brains, but a post-perfusion time interval of at least several hours should be allowed prior to removing brains from the cranial vault.

Special Stains

The H&E stain, while very useful for neuropathologic evaluations, is not the "gold standard" for detecting subtle degrees of cellular degeneration. While a plethora of special stains is available for demonstrating various components of the central nervous system, the stains that the author has found to be most helpful for revealing acute to subacute neuronal injury are Fluoro-Jade (including Fluoro-Jade B) and the silver

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degeneration stains (eg, amino cupric silver stain). There are advantages and disadvantages to each of these stains. The Fluoro-Jade stain may be performed on paraffin-embedded (or frozen) sections, while the silver degeneration stains require frozen sections. Although the Fluoro-Jade staining procedure is relatively simple to perform, the amino cupric silver technique requires extreme attention to detail if excessive non-specific staining is to be avoided. Advantages of the silver degeneration stains include their permanence and high resolution. The latter feature is particularly helpful when one is looking for very subtle degenerative changes such as the finely granular staining indicative of terminal degeneration. To avoid nonspecific staining, both the Fluoro-Jade and silver degeneration stains should be performed on appropriately perfusion-fixed tissues. Examples of the Fluoro-Jade B and amino cupric silver stains are presented in Figure 1, and additional information about these stains may be found in numerous publications (2, 13, 14, 17, 18).

Utilization of either the Fluoro-Jade stain or a silver degeneration stain is highly recommended for assisting the pathologist in detecting cell degeneration within brain sections and in establishing no-observed-effect levels. Depending upon the study type and/or the applicable regulatory guidelines, additional special stains may also be indicated. For example, a stain for neuronal processes such as one of the nondegenerative silver stains (eg, a Bielschowsky's or Bodian's stain) or a neurofilament stain may be important for assessing neuronal polarity in developmental neurotoxicity studies. Stains for myelin, for degenerating myelin, for reactive astrocytes, or for other cell types may be called for in certain situations. A discussion of the use of these stains is beyond the scope of this manuscript but may be obtained elsewhere (4).

Sampling Issues

Although false negative studies may result from a failure to utilize stains more sensitive than H&E for revealing subtle degenerative change, false negatives may also result from inadequate sampling of those brain regions most susceptible to injury. This is due to the fact that histopathologic alterations in the brain tend to be regional rather than diffuse. The pathologist needs to avoid thinking of the brain as a single organ but, rather, as an aggregate of disparate regions serving diverse functions and with different levels of physiologic activity, metabolic needs, and chemical makeups. For example, brain regions that are generally considered to be most

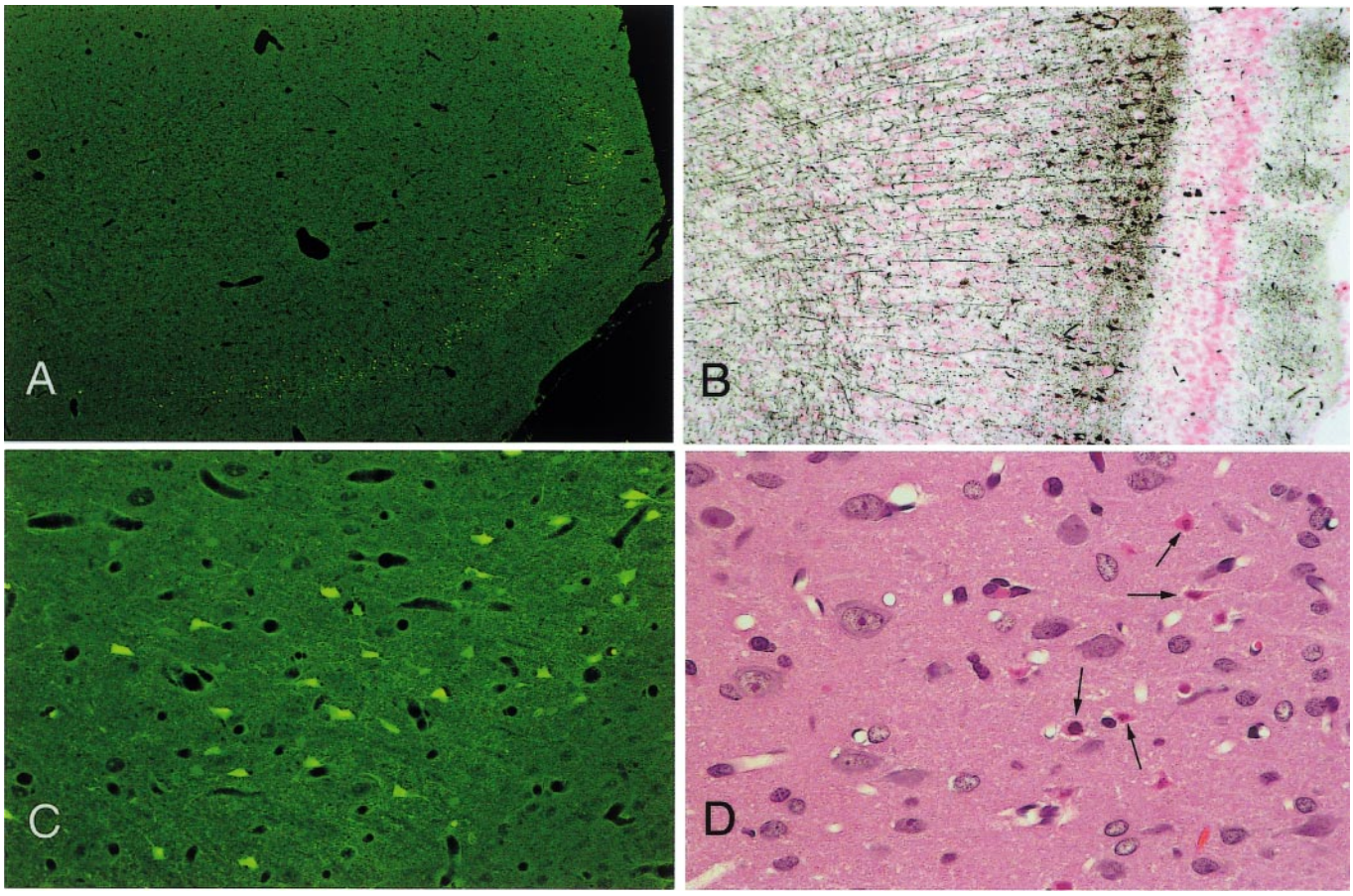

FIGURE 1.—This panel of 4 micrographs shows gradually increasing magnifications of the retrosplenial cortex of a rat injected seven days earlier with MK-801. The highest magnification (Panel D) shows an H&E-stained section containing scattered necrotic neurons (arrows), but these were difficult to visualize at lower magnifications. The Fluoro-Jade B stain (Panels A and C) reveal the brightly-stained necrotic neurons quite well, while the amino cupric silver stain (Panel B) shows exquisite detail of the necrotic pyramidal neurons in layers IV and V, as well as of their axons extending into deeper regions. Degenerating terminals in lamina I (right hand side of micrograph) are also stained with the cupric silver stain. Original magnifications: 1a = ×62; 1b = ×125; 1c = ×312; 1d = ×500.

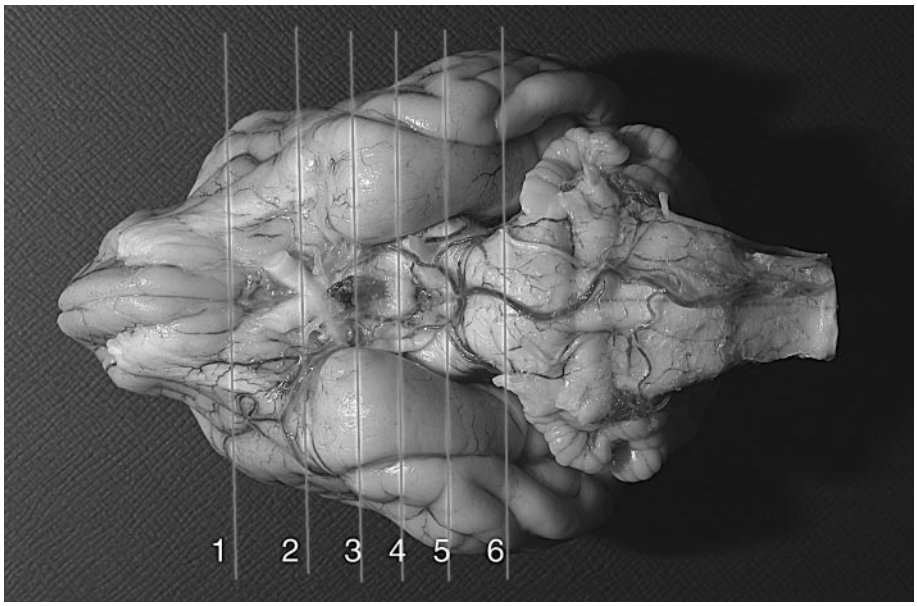

FIGURE 2.—Ventral aspect of a dog brain showing the levels of the first six slices. #1 = frontal pole; #2 = optic chiasm; #3 = infundibulum (hypophyseal region); #4 = mamillary bodies; #5 = base of third cranial nerve; #6 = anterior portion of pons. See Figure 3 for some additional cuts made from the dorsal surface to pass through the cerebellum and underlying brain stem.

vulnerable to hypoxia include the hippocampus (particularly the dorsal portion), laminae III, V, and VI of the neocortex, the Purkinje neurons of the cerebellum, and the basal ganglia. Areas of particularly high glucose consumption, on the other hand, include the vestibular nuclei, the nucleus of the spinal trigeminal tract, the olivary nuclei (superior and inferior), inferior colliculi, cerebellar nuclei, certain thalamic nuclei, and lamina IV of the neocortex (16). Specific neural regions may be selectively damaged by chemicals that either target receptors on neurons in those areas or affect neurons that send afferent projections to the same. Some chemicals may, for example, gain entry into neurons or glial cells via specific receptors. A classic example of this mechanism is MPP^+ (*N-methyl-4-phenylpyridinium ion*), a neurotoxin that is taken up selectively via dopamine transporters and, therefore, creates lesions only in nigrostriatal neurons. [MPP^+ is formed when its precursor MPTP (*1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine*) is acted on by monoamine oxidase B present in astrocytes (16).] Many chemicals interact as agonists or antagonists with specific receptor sites on neurons and, by such interactions, may compromise the impacted cell's homeostatic state. Selected brain regions may

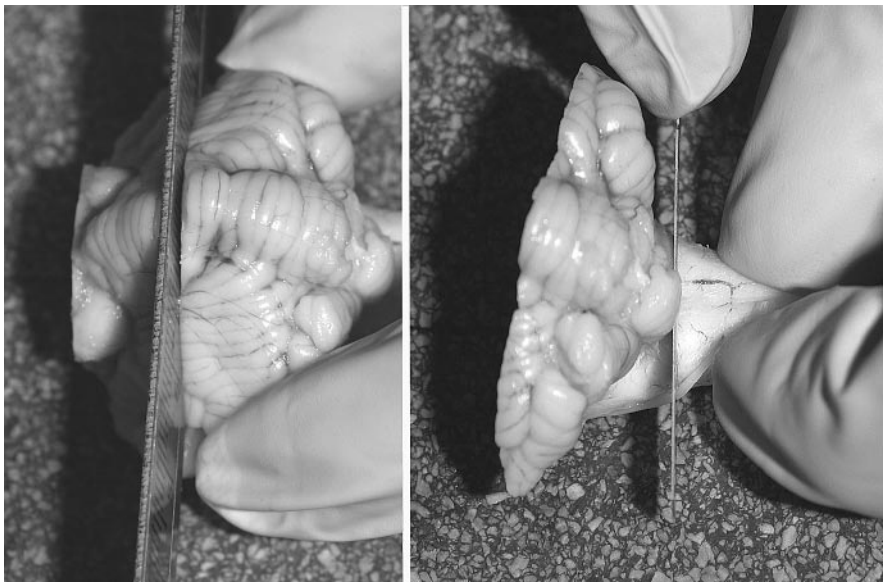

FIGURE 3.—Dorsal aspect of the cerebellum and medulla showing the levels of the first cut through the cerebellum (left-hand panel), second cut through the cerebellum, and cut through the medulla oblongata (right-hand panel).

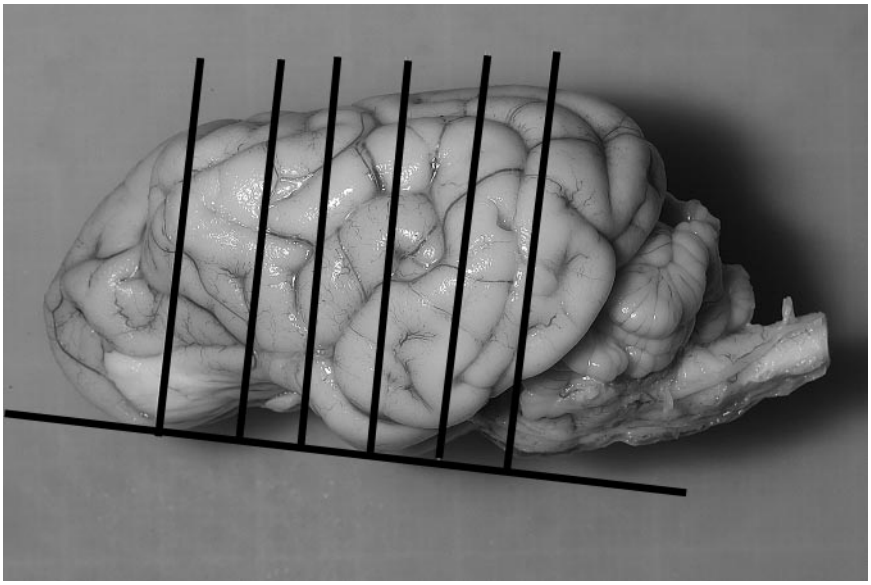

FIGURE 4.—Lateral view of the dog brain showing the approximate angles of the slices if the prosector makes the cuts starting at the dorsal surface.

be particularly susceptible to nutritional deficiencies and/or excesses—particularly of the B complex vitamins and, to a lesser extent, Vitamin A. Other regions may be particularly vulnerable to deficiencies or excesses of certain metals.

With mounting interest in neuropharmacologic agents that act as either agonists or antagonists at specific neurotransmitter receptor sites, it is important for the pathologist to have an appreciation for the delicate balance that may exist between excitatory (eg, glutamate) and inhibitory (eg, gamma amino butyric acid or "GABA") neurotransmitters in certain brain regions. For example, inappropriate activation of the NMDA (*N*-methyl-D-aspartate) glutamate receptor has the potential to result in an excitatory form of cell death. However, non-competitive blocking of the NMDA receptor may also result in neuron degeneration within selected brain regions (5). This degeneration may be the result of decreased inhibitory input to these regions when NMDA receptors are blocked on projecting GABAergic and/or noradrenergic neurons (11, 12). For pathologists concerned with the developing nervous system, it is important to also note that there is differential maturation of the neurotransmitter systems and their receptors. At certain stages of brain development, in fact, GABA is excitatory rather than inhibitory (19). While over-stimulation of neurons may result in cell death, chronic loss of trophic input may also result in the demise of neurons; this is particularly important during brain development. In the developing

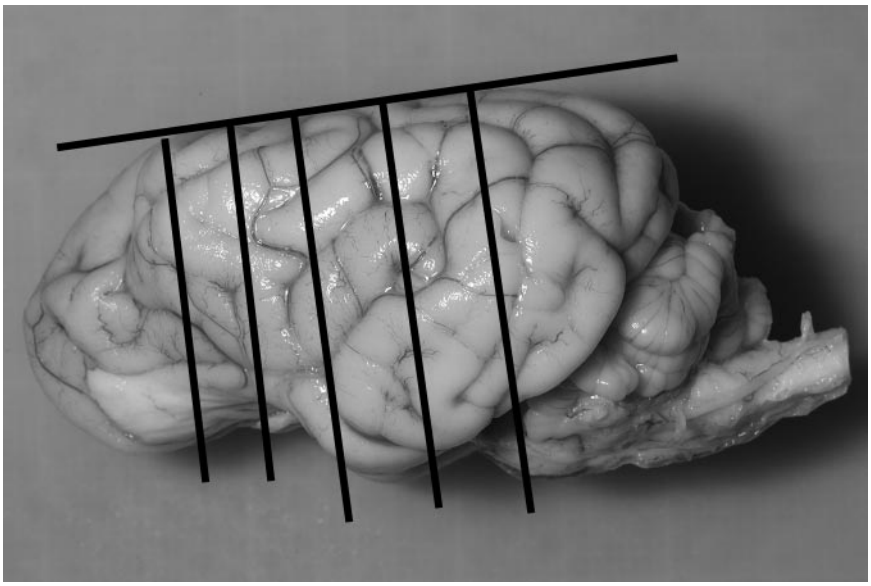

FIGURE 5.—Lateral view of the dog brain showing the approximate angles of the slices if the prosector cuts starting at the ventral surface (as shown in Figure 6).

FIGURE 6.—One method of achieving slices of uniform thickness is to rest the brain between two knife supports (in this case, three 2 × 3 inch glass slides that are one mm in thickness and that are glued together). This is a useful technique but not the one preferred by the author.

brain, for example, glutamate acting at NMDA receptors may control neuron survival. A blockade of NMDA receptors at critical time points in brain development may, therefore, trigger widespread neuronal apoptosis (7).

When a focus of degeneration is detected within the brain, the pathologist needs to ascertain not only the specific anatomic location of the damaged focus but also what neuroanatomic regions project to this region and to what regions the damaged focus projects. Additional sections may then be required to examine these other regions as well. The pathologist should also determine what neurotransmitters and neurotransmitter receptors are most prominent in these interconnected regions. Because of the complexity of brain circuitry and the fact that the brain contains many distinctive neuron populations, many more brain sections are required for an adequate assessment than for any other organ. For a laboratory rodent, multiple slices (10–12 coronal sections) through the entire brain may be embedded within 2 tissue blocks, thus providing a relatively thorough microscopic examination at minimal cost. (For neurotoxicity studies in rodents, the entire brain should be sliced and embedded—not merely the "standard three sections" frequently examined in

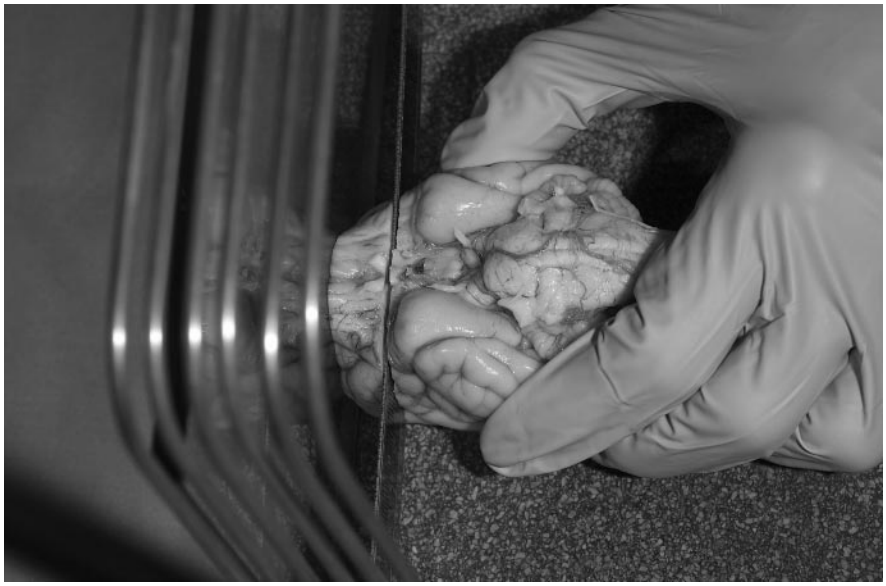

FIGURE 7.—The author's preferred trimming method is to use a cutting board with metal guides to keep the knife perpendicular to the long axis of the brain and cutting board. A glass plate at the left side of the board assists in achieving parallel sections. In this photo, the knife is passing through the optic chiasm (Cut #2 in Figure 6). (Manufacturer: Lipshaw, now ThermoShandon, Pittsburgh, PA).

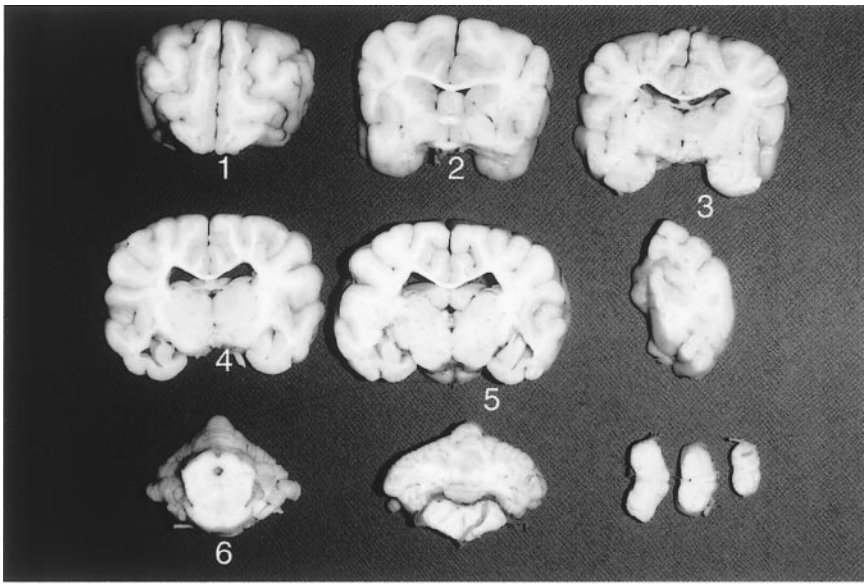

FIGURE 8.—Eleven brain slices are shown. Those numbered 1–6 correspond to the cuts shown in Figure 2. The occipital pole section (to the right of the #5 slice in this figure) results when Cut #6 is made through the pons. Additional sections in this photo include a slice through the middle of the cerebellum (as in Figure 3) and three levels of the brain stem shown at the lower right.

chronic rodent toxicity studies.) However, deciding which regions should be examined microscopically from large-sized brains (dogs or nonhuman primates) is problematic. Any sampling protocol will undoubtedly be influenced by what is known about the test chemical. Nevertheless, certain regions of the brain are more susceptible than others to a variety of physical, metabolic, and chemical insults. Therefore, these regions should be included in most evaluations.

The remainder of this manuscript will present one protocol for trimming a large brain, using the dog as a model species. [Note: The gross photos in this paper are from the brain of a foxhound weighing between 20 and 25 kg. In order to not obscure the appearance of the brain slices, only a small number of major neuroanatomic regions are labeled in these photos.] The described trimming protocol is designed to generate paraffin-embedded sections mounted on standard-sized (ie, 1×3 inch) microscope slides. [Note that using larger-sized slides (ie, 2×3 inches) and embedding full coronal sections or half-sections of brains is quite acceptable and also has the advantage of retaining many anatomic landmarks. However, many labs do not like this approach based on a variety of histotechnical, equipment (microtomes, automatic staining

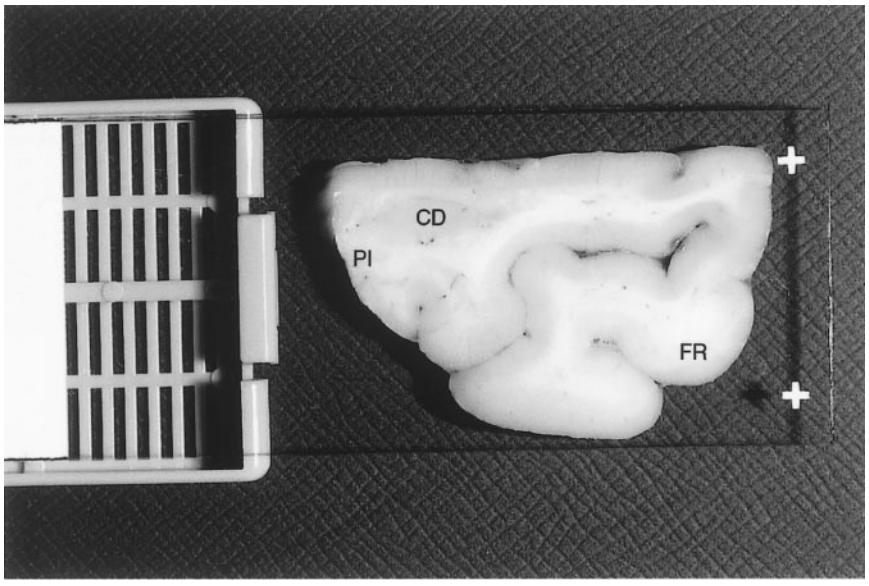

FIGURE 9.—Block No. 1—Section through the frontal pole (Cut #1 Figure 2). The superior edge (to the right) is trimmed off so that the section will fit into the cassette. This slice includes the frontal cortex (FR), the tip of the caudate nucleus (CD), and the piriform cortex (PI).

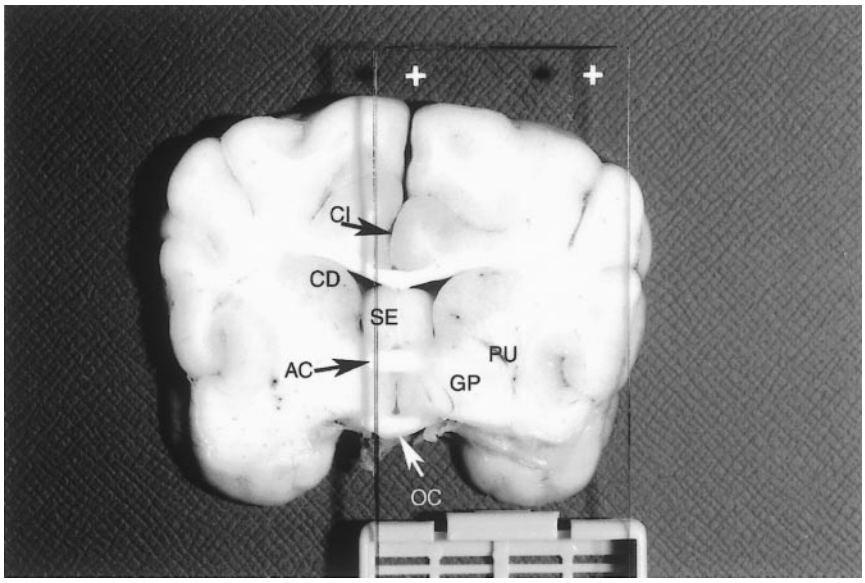

FIGURE 10.—Block No. 2—Section through the optic chiasm (OC) and the crossing of the anterior commissure (AC). This slice includes some of the basal ganglia such as the caudate (CD), putamen (PU), and globus pallidus (GP), the basal forebrain/preoptic region (just ventral to the anterior commissure), the septal nuclei (SE), and the cingulate cortex (CI). As with the slice shown in Figure 9, the superior edge is trimmed off so that the section will fit into its cassette. (See Figure 18.)

and cover-slipping machines) and/or archiving reasons.] It should be emphasized that the trimming procedure presented here represents a minimalistic approach to evaluating brains from animals treated with neuropharmacologic or potentially neurotoxic agents, and it should not be concluded that this sampling procedure is optimal or even sufficient for all neurotoxicity studies. This procedure might need to be modified depending upon the chemical class being tested. In particular, additional sections are always recommended if there is any concern that specific areas are being missed.

Methods of Slicing and Section Orientation

Pathologists are generally most familiar with the examination of coronal brain sections, and there is no need, in most studies, for another orientation. However, sagittal sections through certain brain regions such as the cerebellum and brainstem may be indicated in developmental neurotoxicity studies.

The first decision that the pathologist needs to make is whether to slice the brain starting from the dorsal (superior) or the ventral (inferior) aspect. To obtain relatively homologous sections, it is easier to use the ventral approach for

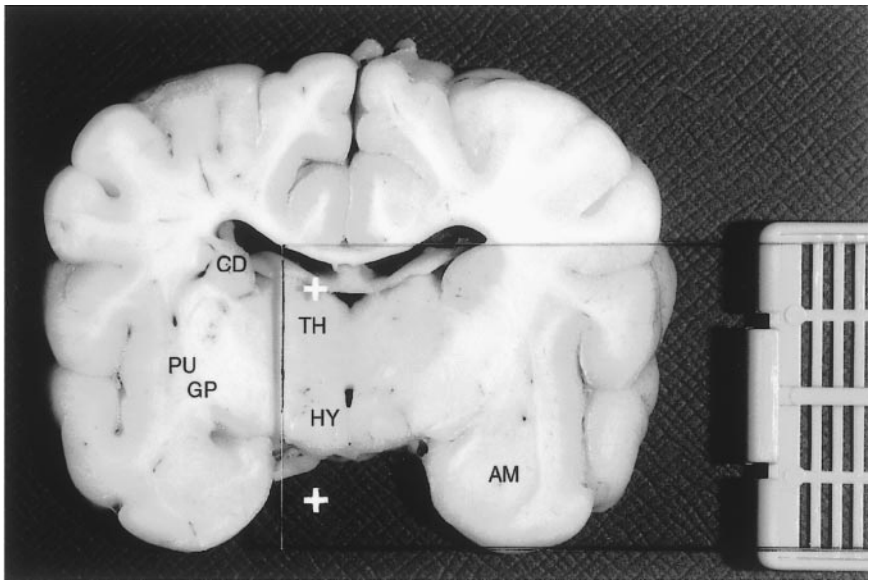

FIGURE 11.—Block No. 3—Slice made at the level of the infundibulum, with the portion taken for processing including the anterior thalamus (TH), hypothalamus (HY), amygdala (AM), and the posterior levels of some of the caudate (CD), putamen (PU), and globus pallidus (GP).

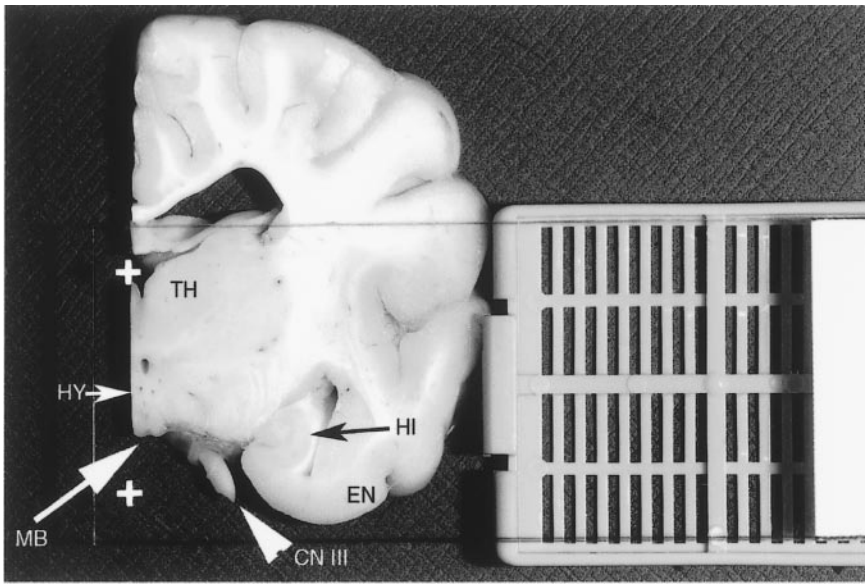

FIGURE 12.—Block No. 4—Hemi-slice taken through the mamillary bodies (MB) and just anterior to the oculomotor nerve (CN III). In addition to the mamillary bodies, the portion taken for processing includes the mid portion of the thalamus (TH), posterior hypothalamus (HY), anterior level of the ventral hippocampus (HI), and the adjacent entorhinal cortex (EN). (The oculomotor nerve was the landmark for the next cut.)

most slices because of the distinct anatomic landmarks that are present (Figure 2). It is also easier to train technicians to trim brains in a standardized fashion when using a ventral approach. However, due to the prominent contours of the cerebellum, it is easier for the prosector to slice the cerebellum starting from its superior aspect (Figure 3). Assuming that the brain is resting on a flat surface during slicing (as apposed to utilizing a stereotaxic approach with the brain left in situ), the decision as to whether to slice starting at the superior or inferior aspect of the brain will impact on the angle of the cuts (Figures 4 and 5). Although the precise angle of slicing is not critical, this angle may be important to the neophyte neuroanatomist lucky enough to have one of the out-of-print dog brain atlases (3, 8, 9, 15). (These atlases have slightly different section orientations.) If no such atlas is available, reading some comparative neuroanatomy literature (eg, Ref 1) coupled with the use of one or more of the numerous human and/or nonhuman primate atlases that are available (or even a rodent atlas in a pinch) will generally guide the pathologist in the recognition of at least the most

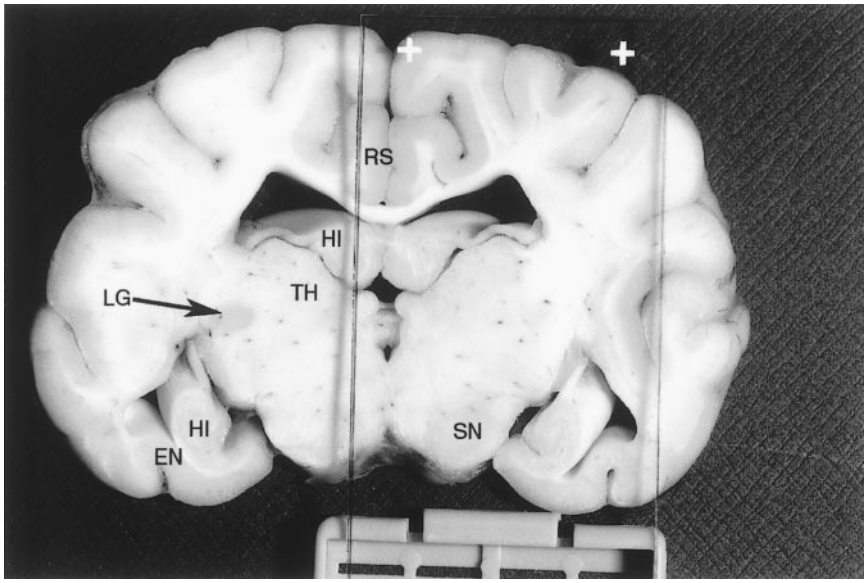

FIGURE 13.—Block No. 5—Slice taken from just posterior to the oculomotor nerve. The portion taken for sectioning includes the posterior thalamus (TH), the dorsal and ventral portions of the hippocampus (HI), the retrosplenial (RS) and entorhinal (EN) cortices, the anterior portion of the substantia nigra (SN) and the lateral geniculate body (LG).

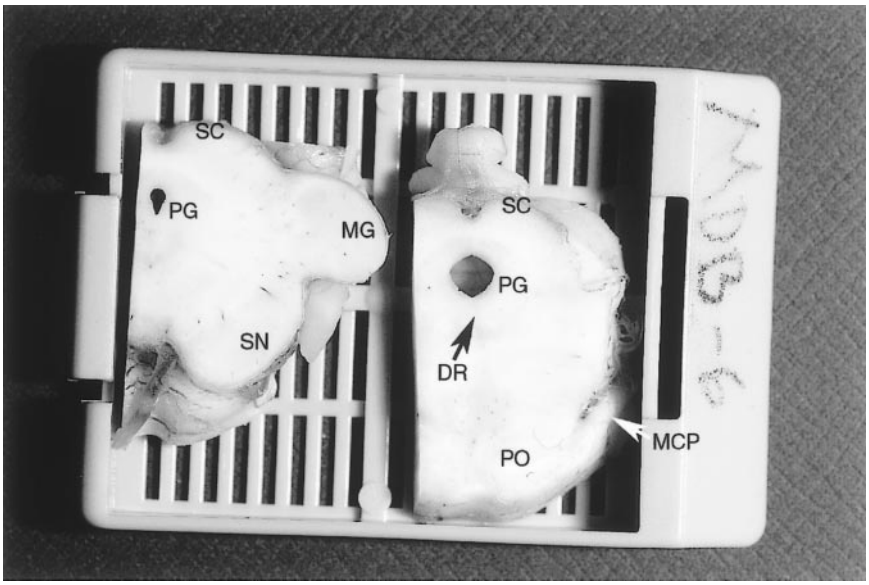

FIGURE 14.—Block No. 6—Portions of two slices through the midbrain region. The section on the left side is the more anterior of the two and contains the substantial nigra (SN), the medial geniculate body (MG), and the superior colliculus (SC). The section on the right includes the anterior pons (PO), superior colliculus (SC), and dorsal raphe nucleus DR). The periacqueductal gray matter (PG) and a variety of mesencephalic nuclei and prominent fiber tracts are also present in each section. MCP = middle cerebellar peduncle.

prominent neuroanatomic structures. Whereas highly homologous sections are not necessary when morphometric procedures are not to be performed (such as are required in certain developmental neurotoxicity studies), having relatively homologous sections will assist the pathologist in performing interanimal comparisons. However, having highly homologous sections from large-sized brains also means that certain smaller-sized neuroanatomic regions will be excluded from evaluation for all brains.

Methods of slicing large brains vary. If slices of standardized thickness are desired, the approach shown in Figure 6 may be followed. For this technique, an initial perpendicular slice is made at a specific neuroanatomic location (eg, through the optic chiasm) in order to create a flat cut surface. Then, the cut surface of the brain is placed downwards on a cutting board, and the knife is supported by two (or more) platforms that are the thickness of the desired brain slices. In Figure 6, the knife rests on two glued stacks of three 2×3 inch slides, each slide being 1 mm in thickness. The author's preference is to use a cutting board with knife guides (Figure 7) and to make the coronal slices at the specific landmarks shown in Figure 2, even though this results in slices of

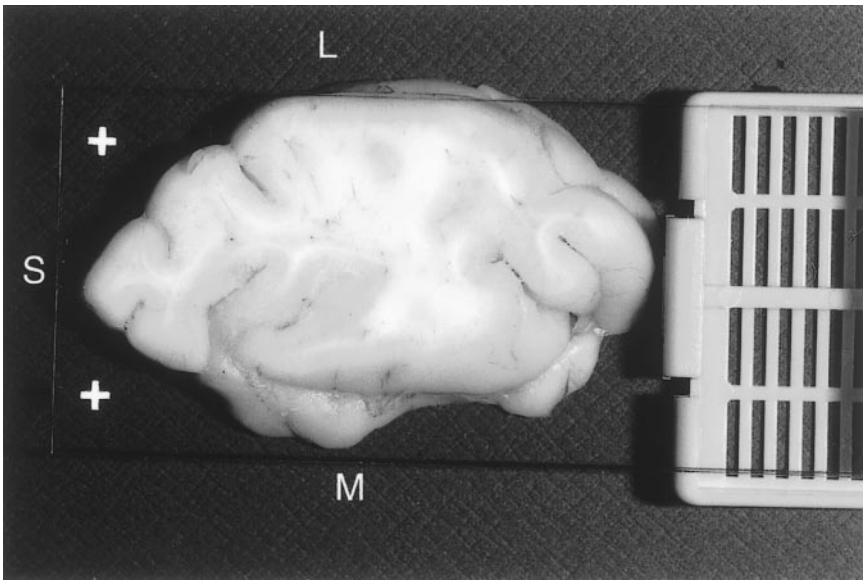

FIGURE 15.—Block No. 7—Slice through the occipital pole which includes the visual cortex. In contrast to the slices in Blocks No. 1 and 2, the ventral (rather than the dorsal) portion of this slice is trimmed off slightly so that it will fit into its cassette. S = superior; L = lateral; M = medial.

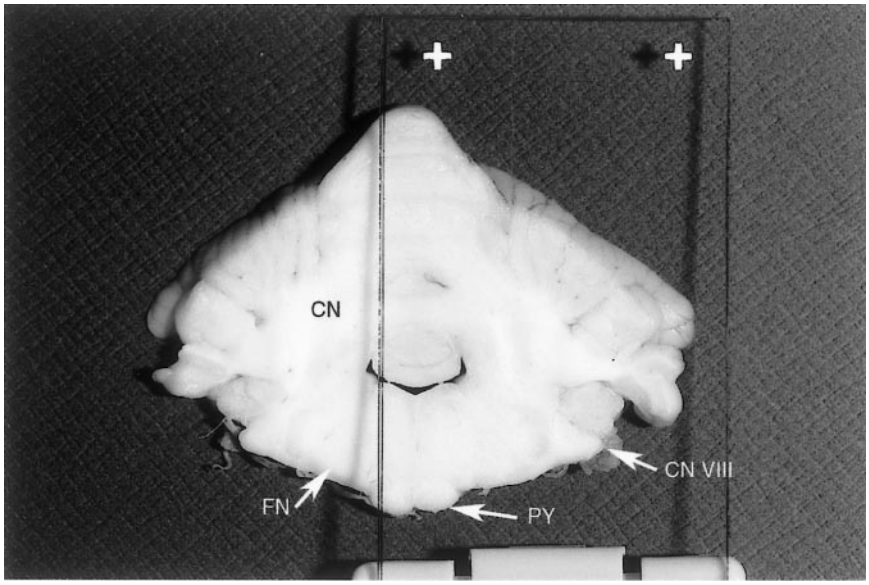

FIGURE 16.—Block No. 8—Slice taken at approximately the midpoint of the cerebellum. This includes the posterior pons as well as deep cerebellar white matter and portions of the deep cerebellar nuclei (CN). The facial nucleus (FN) is in this section, while a section slightly more anterior would contain the superior olivary nuclei. CN VIII = eighth cranial nerve; PY = pyramidal tract.

varying thickness that require additional trimming for proper thickness prior to processing.

Trimming of Brain Slices

Figure 8 shows the brain slices obtained by making the cuts depicted in Figures 2 and 3, along with two additional cuts through the posterior medulla/anterior cervical spinal cord. Because most of these slices are too large to fit into a cassette for a standard-sized block (ie, for a 1 × 3 inch microscope slide), they will require additional trimming. The portions selected for processing and microscopic evaluation are depicted in Figures 9–18. This trimming protocol results in nine tissue blocks that contain a total of 12 sections (Figure 18). Some of the key neuroanatomic regions present in each section are given in the captions for Figures 9–17. At a minimum, the sections on these 9 slides will include portions of the following regions:

1. Neocortex—Frontal, parietal, temporal, and occipital
2. Archicortex, paleocortex, and transitional cortex—Piriform, periamygdaloid, and entorhinal

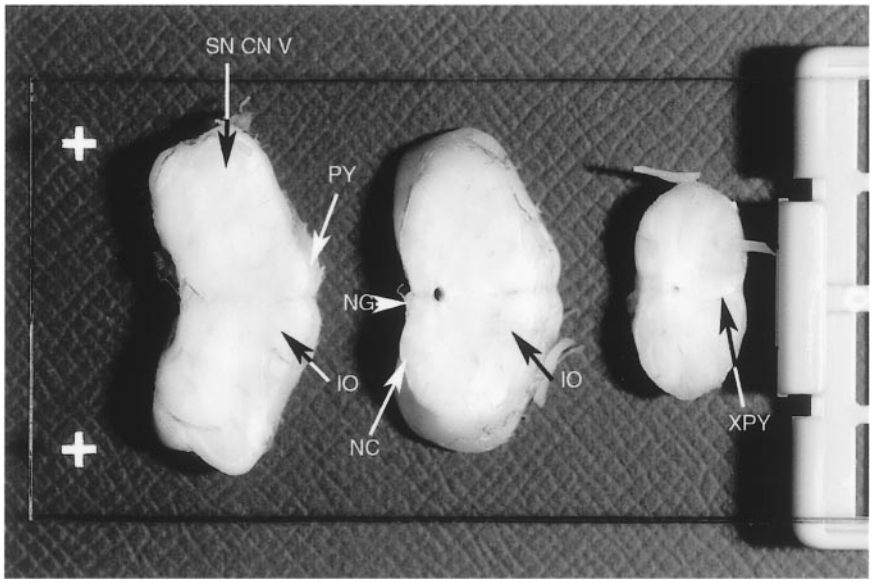

FIGURE 17.—Sections through two levels of the medulla oblongata (left and center) and at the junction of the medulla and the cervical spinal cord (right). Depending on the specific levels sampled, these sections will contain a variety of important neuron groupings such as the inferior olivary nuclei (IO), nucleus gracilis (NG), nucleus cuneatus (NC), and various reticular formation nuclei such as raphe nuclei (not labeled). Each level contains a different component of the nucleus of the spinal trigeminal tract (SN CN V), even though this is only indicated on one section. PY = pyramidal tract; XPY = decussation of the pyramidal tract.

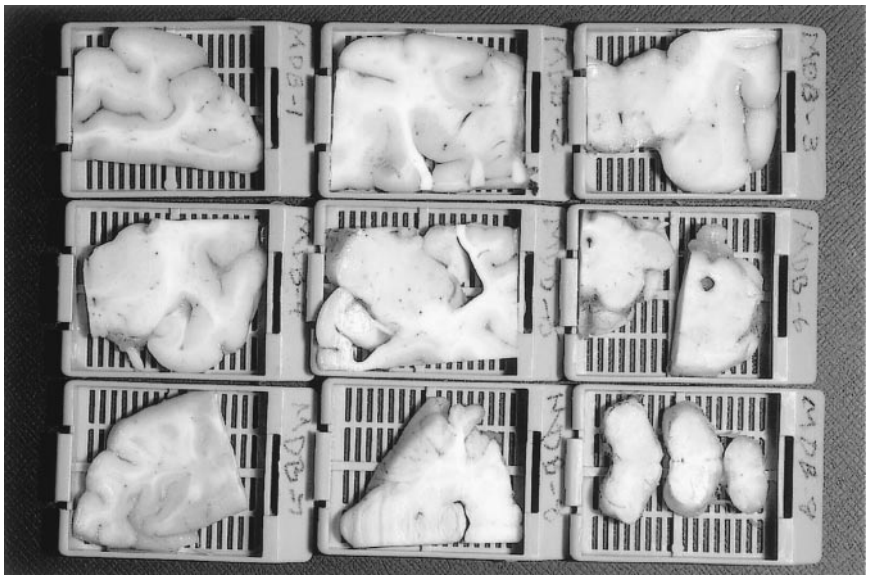

FIGURE 18.—The 6 cassettes resulting from this dissection procedure. The thickness of each slice has been reduced to approximately 3 mm for optimal processing. This trimming procedure results in nine slides with a total of 12 sections.

3. Basal ganglia—Caudate, putamen, globus pallidus, claustrum, and substantia nigra
4. Limbic system—Amygdala, septal nuclei, substantia innominata, mamillary body (of Hypothalamus), thalamus (anterior and dorsal nuclei), cingulate and retrosplenial cortices, hippocampus (dorsal and ventral portions)
5. Thalamus (anterior, middle and posterior levels) and hypothalamus
6. Midbrain regions—Substantia nigra, superior colliculus, lateral, and medial geniculate nuclei, pontine nuclei, and dorsal raphe nucleus
7. Cerebellum and pons
8. Medulla oblongata and anterior cervical cord

CONCLUDING REMARKS

Based on peer review work and queries to pathologists at a number of toxicology laboratories, the author has concluded that large-sized brains such as those from dogs frequently receive rather limited microscopic examinations. In-depth discussions of neurotoxic mechanisms and the known reasons for differential vulnerabilities of selected brain regions to various neurotoxicants could not be adequately presented, here, due to space limitations. However, an attempt has been made to at least introduce the following concepts: 1) the importance of recognizing and avoiding artifact in central nervous system tissue sections, 2) the value of utilizing special stains to detect cellular degeneration, and 3) the need to think of the brain not as a single organ but, rather, as a complex of interconnected systems made up of different cell populations with unique characteristics and vulnerabilities to injury. One sampling procedure for large-sized brains is presented, here, in order to stimulate the reader to look critically at trimming procedures currently present in his or her laboratory. Although this sampling procedure will generally be adequate, the microscopic examination of additional regions may be indicated in some studies.

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