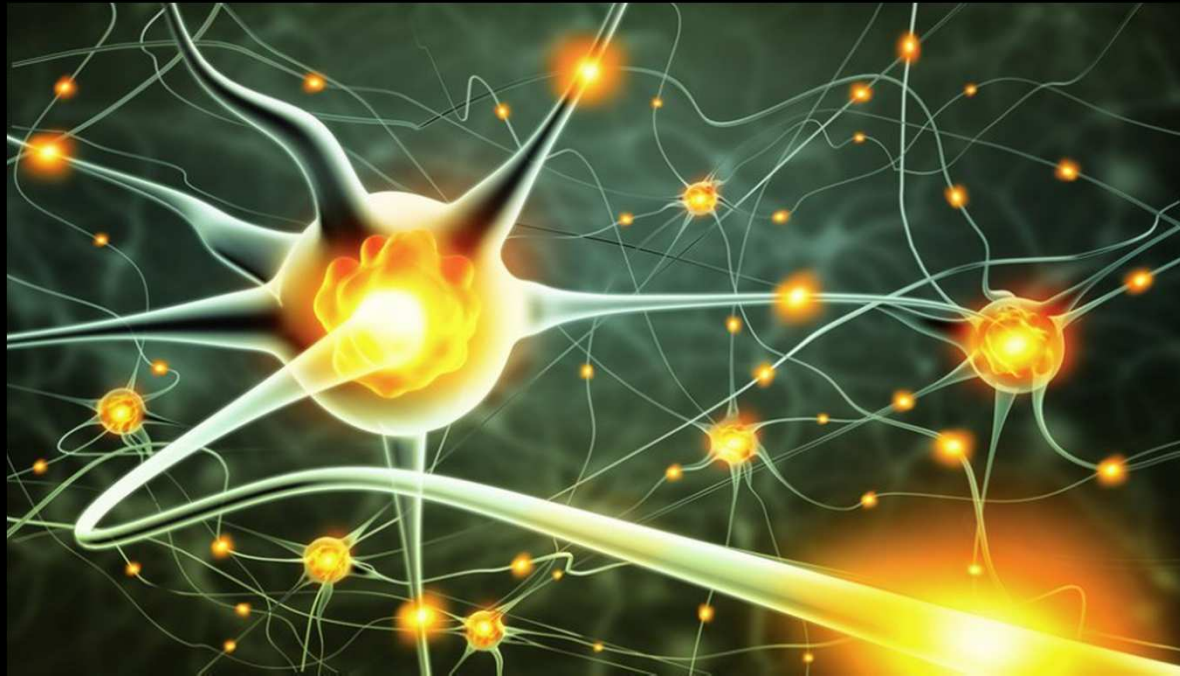


Techniques used in evaluation of neurotoxic potential of test compound/article in preclinical toxicity studies



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Disclaimer

The ideas and thoughts expressed in this presentation are purely that of the presenter and do not necessarily reflect the thinking of the organization or represented by the presenter.



INTRODUCTION

Recent emphasis on neurotoxicity testing by regulatory agencies, such as the US EPA and the OECD, and rapid growth in the area of neuropharmaceutical development has led to renewed interest in neuropathology procedures for industrial toxicologic pathology.



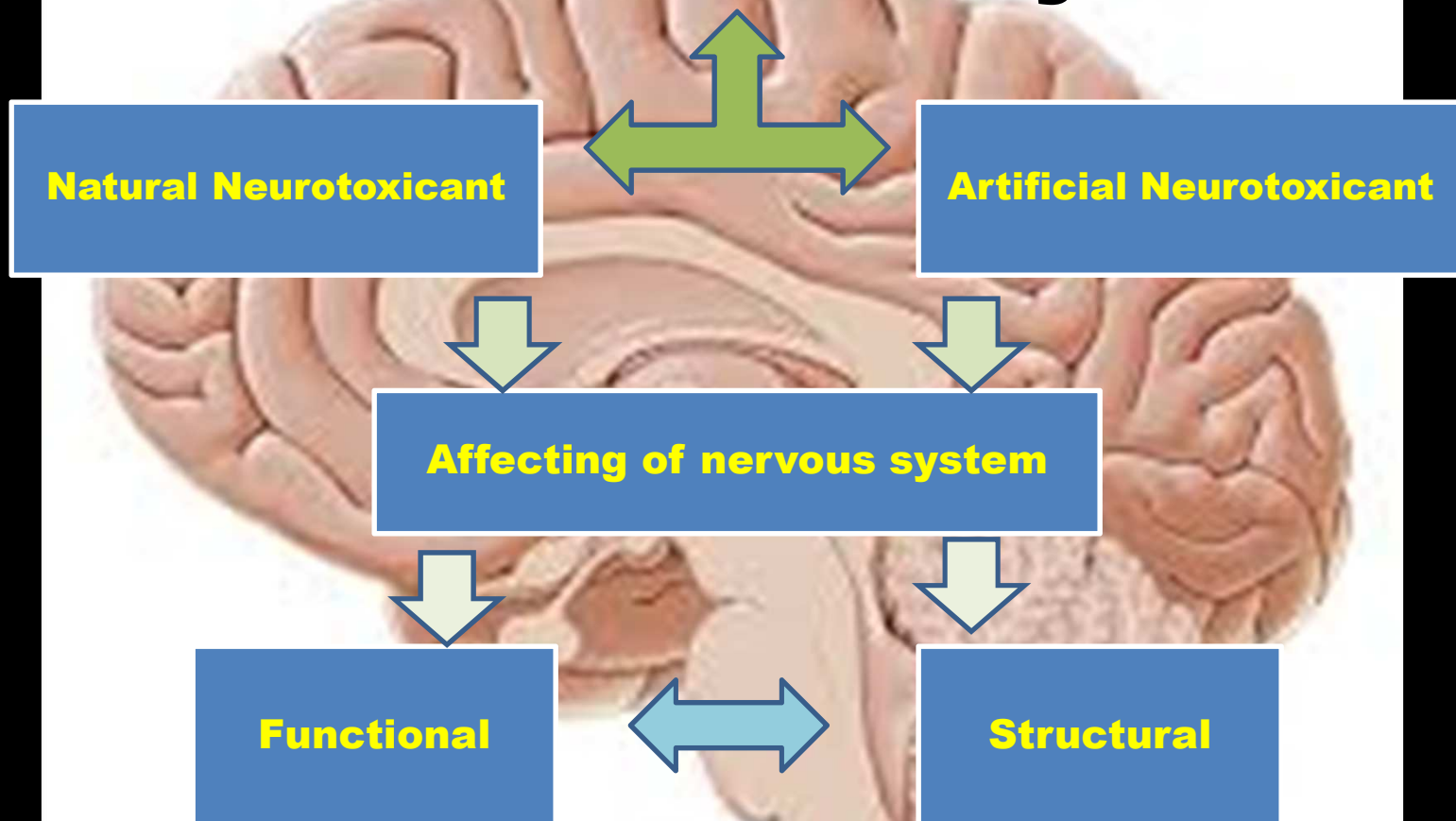


Aim of Neurotoxicity Studies

This neurotoxicity study, when used alone or in combination, provides information

- **Identify whether the nervous system is permanently or reversibly affected by the chemical tested**
- **Contribute to the characterization of the nervous system alterations associated with exposure to the chemical, and to understand the underlying mechanism**
- **Determine dose-and time-response relationships in order to estimate a no-observed adverse-effect level (which can be used to establish safety criteria for the chemical)**

Neurotoxicity



Behavioral and pathological assessments are complementary approaches

Pathologic

Reactive microglia

Reactive astrocytes

Alterations in Neurotransmitters

Changes in gene expression

Death of neurons, Astrocytes or microglia

Pathologic ONLY expressions

Overlapping expressions

Behavioral ONLY expressions

Behavioral

Motor incoordination

Sensory deficits

Altered states of arousal

Learning and memory impairment

Neurological dysfunction such as seizure, paralysis, tremor

Each approach has its strengths and challenges. Each is necessary and uniquely capable of detecting specific expressions of neurotoxicity

Guidelines



- **OECD 424: Neurotoxicity Study in Rodents**
- **OECD 418: Delayed Neurotoxicity of Organophosphorus Substances Following Acute Exposure**
- **OECD 419: Delayed Neurotoxicity of Organophosphorus Substances: 28-day Repeated Dose Study**
- **OECD 426 and US EPA OPPTS 870.6300 : Developmental Neurotoxicity Study**
- **US EPA OPPTS 870.6200: Neurotoxicity Screening Battery**
- **US EPA OPPTS 870.8600: Developmental Neurotoxicity Screen**
- **FDA Redbook 2000: IV.C.10 Neurotoxicity Studies: Toxicological Principles for the Safety Assessment of Food Ingredients**

Regulatory Guidelines Recommendations

- **EPA and OECD : Paraffin embedding acceptable for CNS samples**
- **EPA : Plastic embedding is required for PNS samples**
- **OECD : If signs of peripheral neuropathy, plastic-embedded PNS samples should be examined**
- **Best approach for nerve evaluation**
 - **a nerve cross section**
 - **fixed in osmium**
 - **embedded in plastic**
 - **sectioned very thin**
 - **stained with toluidine blue**



Tools to Study Neurotoxicity

➤ **Functional:**

- **NBO (Neuro behavioral Observation),**
- **FOB (Functional observational battery),**
- **Motor activities**
- **Learning and memory**

➤ **Structural:**

- **Histopathology**



Methods of Fixation

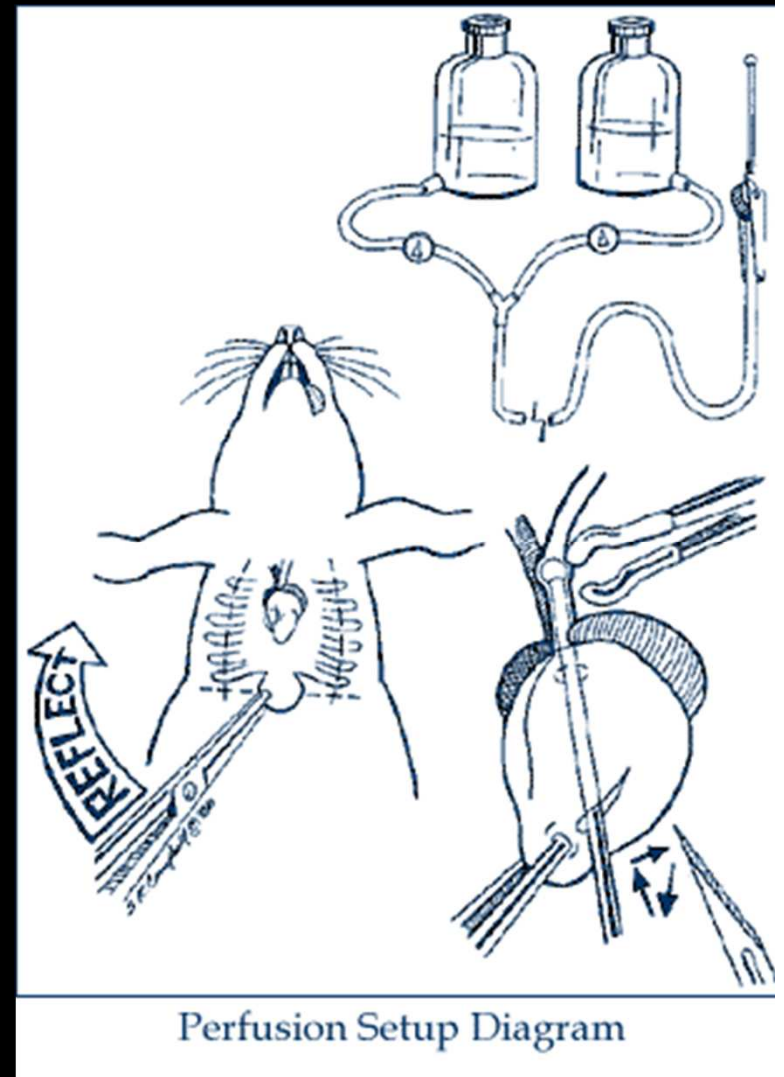
- **Routine**
- **Immersion**
 - **Quick**
 - **Cheap**
 - **Some artifacts**
 - **Can be used in most circumstances**

Methods of Fixation

- **Perfusion**

- Time consuming
- Expensive
- Optimal preservation of tissues
- Minimal artifacts

Note: Wait at least 24 hours before extracting brain, to allow brains to fully harden and to prevent the induction of artifacts during staining.



Common Artifacts

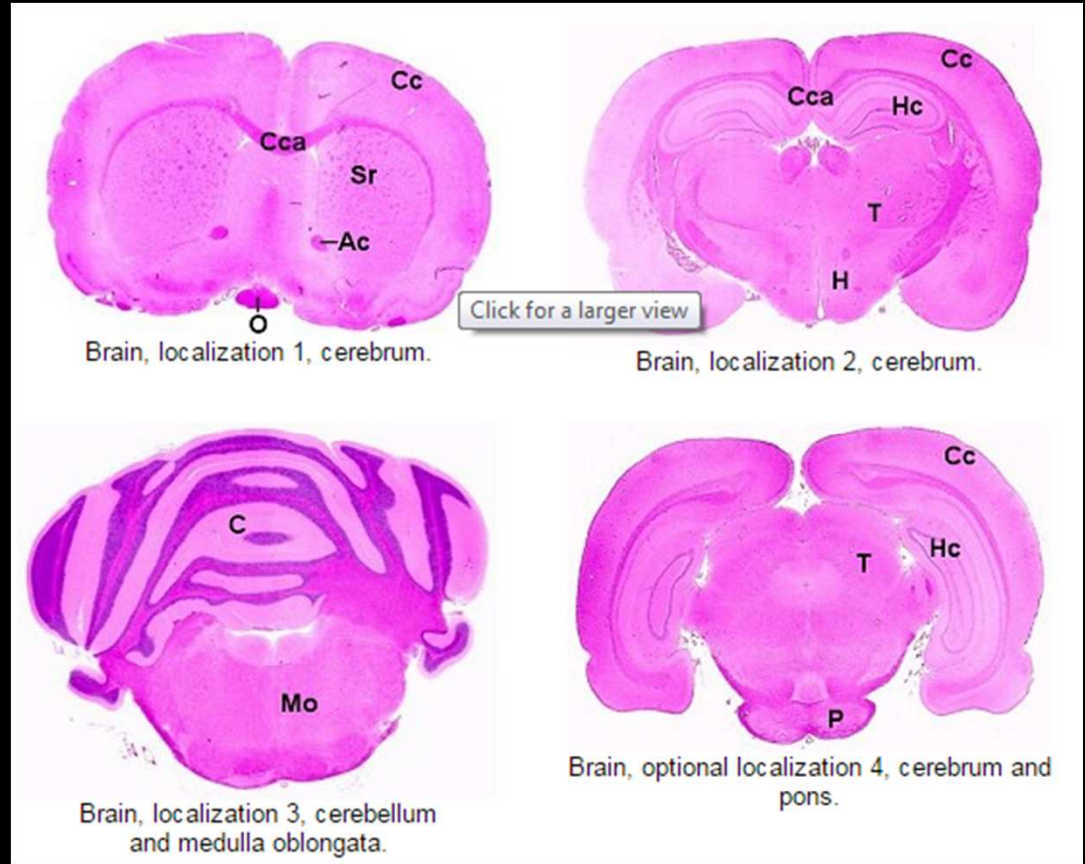
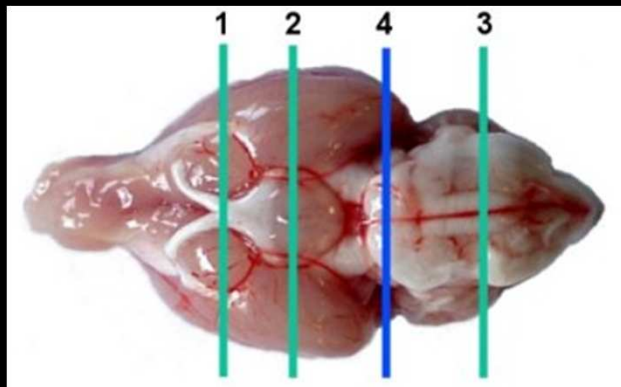
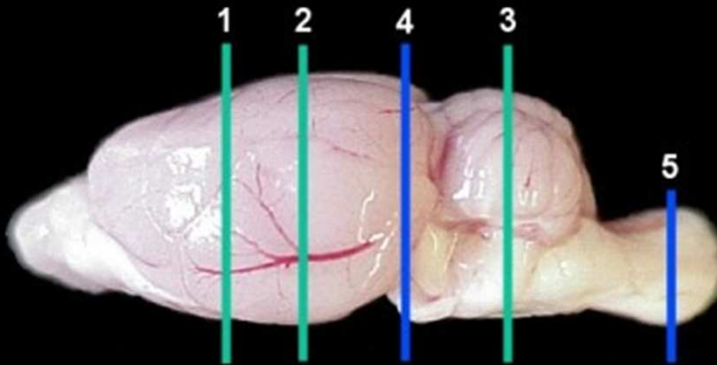
- **Dark neuron artifact**
- **Myelin Bubbles**
- **White Matter Vacuolation**

Tissue Collection and Embedding

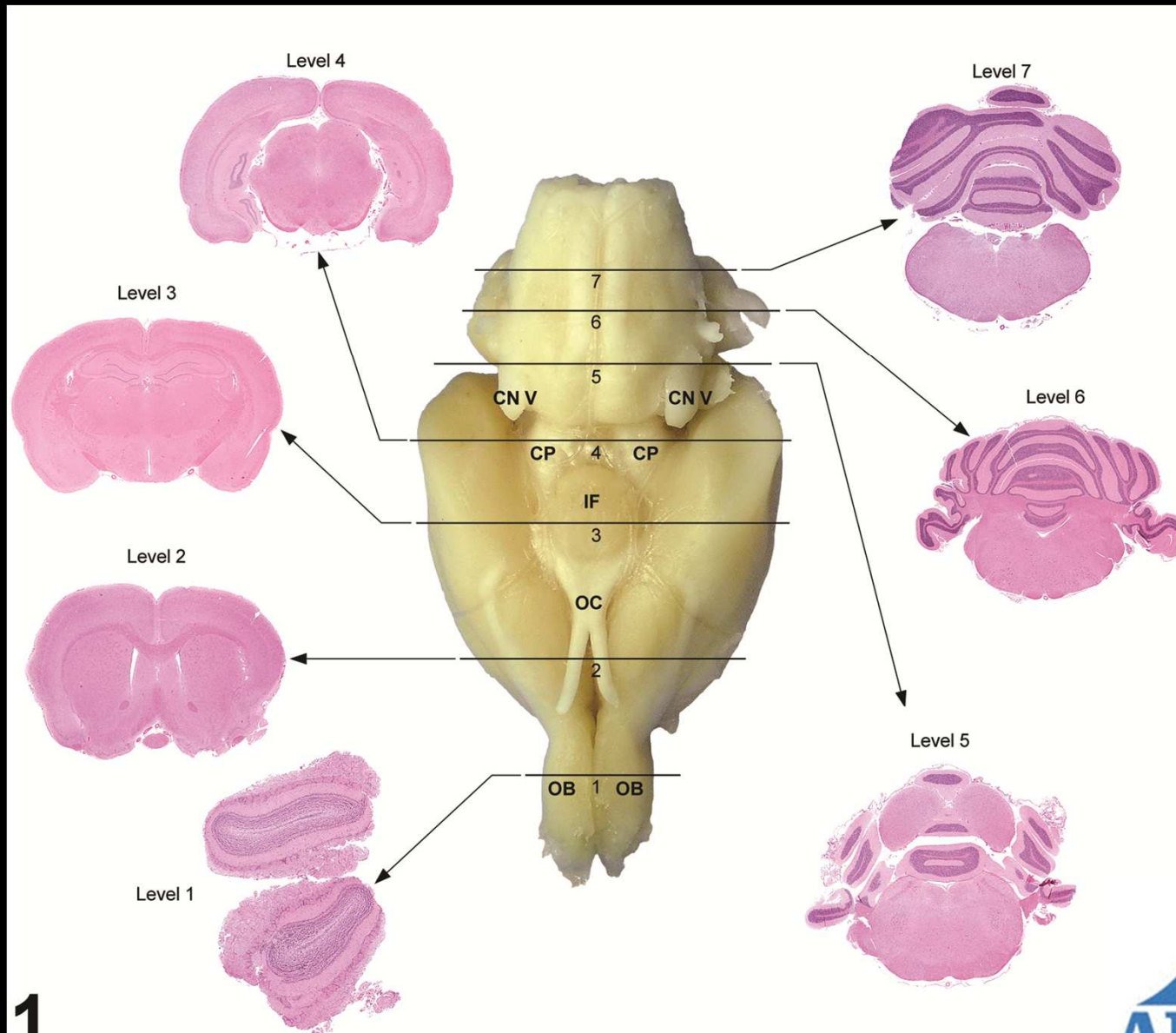
Tissue	Embedding
Brain Olfactory bulbs, Cerebrum, Basal ganglion, Hippocampus, Thalamus, Hypothalamus, Midbrain, Cerebellum, Pons, and Medulla	Paraffin
Spinal cord Cervical, Thoracic and Lumbar	Paraffin
Eye (with optic nerve)	Paraffin
Gastrocnemius muscle	Paraffin
Spinal nerves and Ganglion Dorsal root fibers and ganglia and ventral root fibers Trigeminal or Gasserian ganglia	Plastic/resin
Peripheral Nerves Sciatic, Fibular, Sural and Tibial nerves	Plastic/resin



Brain Trimming as per RITA

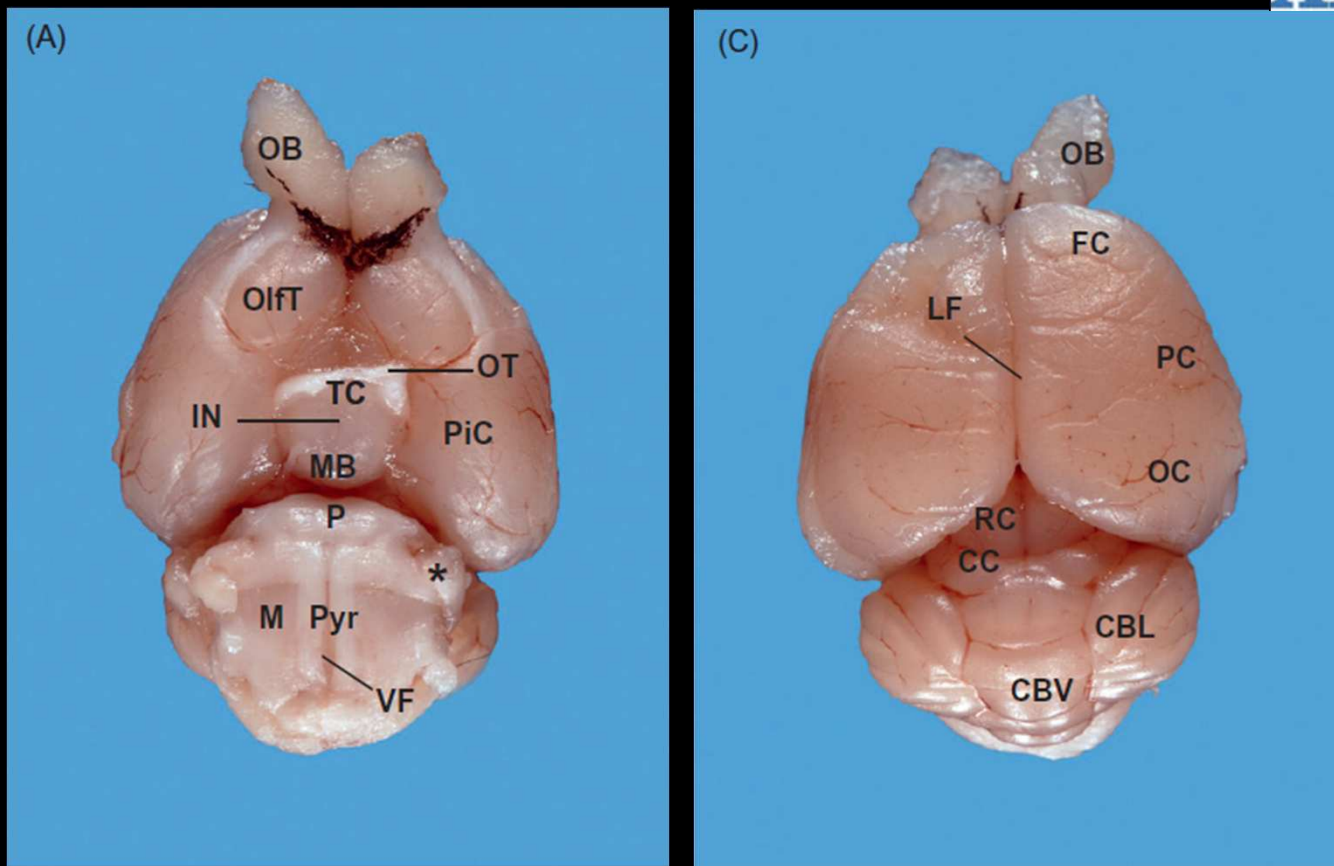


Seven transverse sections levels based on anatomic target landmarks

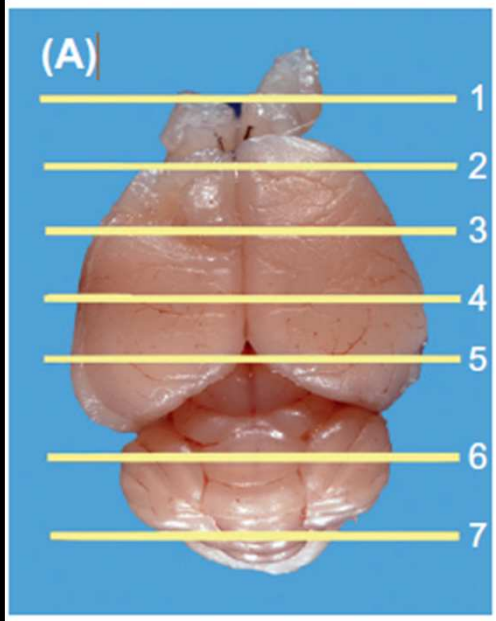


1

Mouse



Major gross features of the ventral (A and B) and dorsal (C and D) surfaces of fixed brains from an adult mouse (A and C) and human (B and D). The optic chiasm (OC) has been removed from the mouse brain, but the base of the optic tract (OT) is still visible. The ventral mouse brain shows meningeal melanosis (blackening) of the caudal olfactory bulbs, which is a relatively common spontaneous finding in C57BL/6 mice. Meninges have been removed from the human brain. CBL, cerebellum—lateral hemisphere; CBV, cerebellum—vermis; CC, caudal colliculus; FC, frontal cortex; FL, frontal lobe; IN, infundibulum; LF, longitudinal fissure; M, medulla oblongata; MB, mammillary bodies; OB, olfactory bulb; OC, occipital cortex; OCh, optic chiasm; OL, occipital lobe; OlfT, olfactory tubercle; ON, olfactory nerve; OT, optic tract; P, pons; PC, parietal cortex; PL, parietal lobe; PiC, piriform cortex; Pyr, pyramids; RC, rostral colliculus; TC, tuber cinereum; TL, temporal lobe; VF, ventral median fissure. The asterisk on the ventral surface of the mouse brain denotes the roots of cranial nerves V (trigeminal), VII (facial), and VIII (vestibulocochlear).

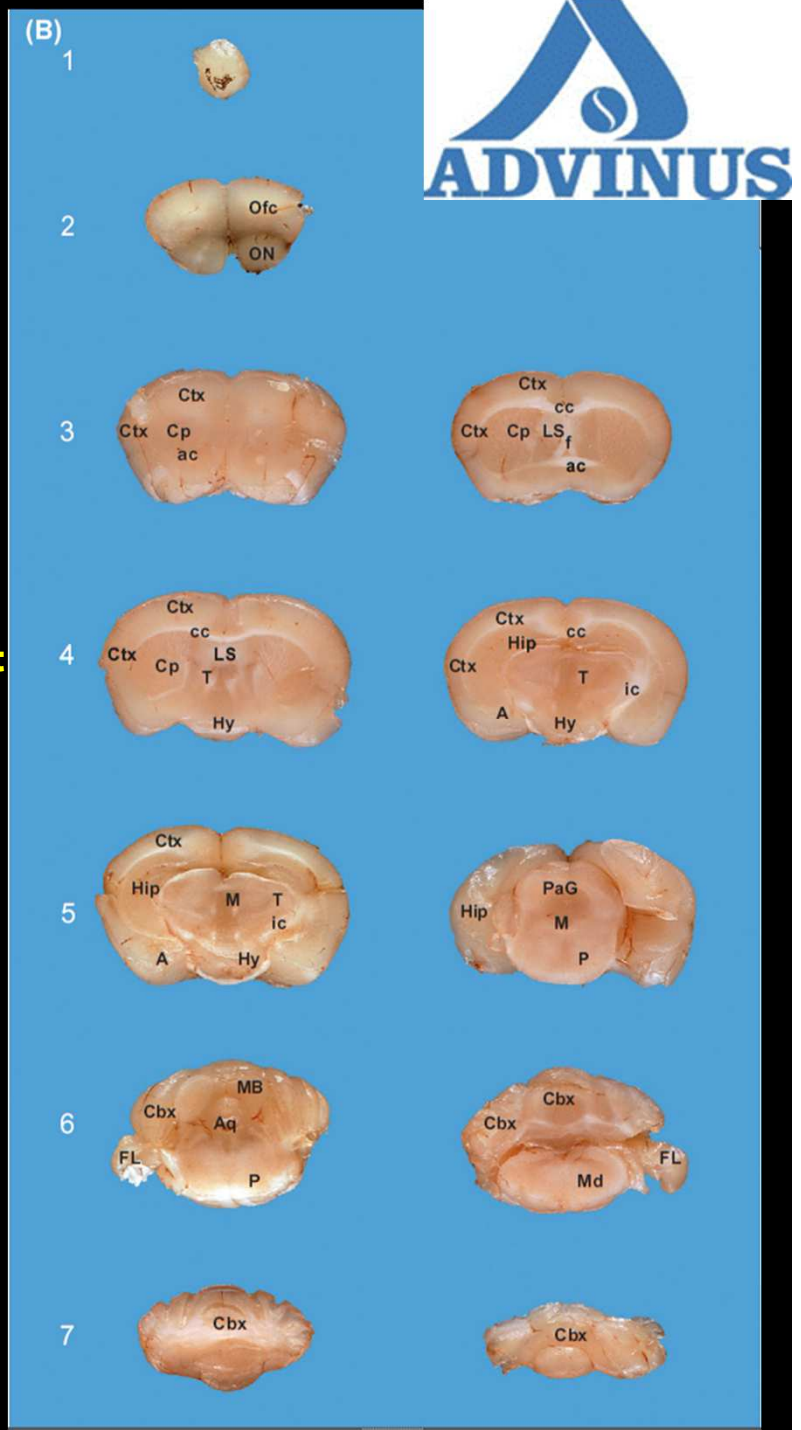


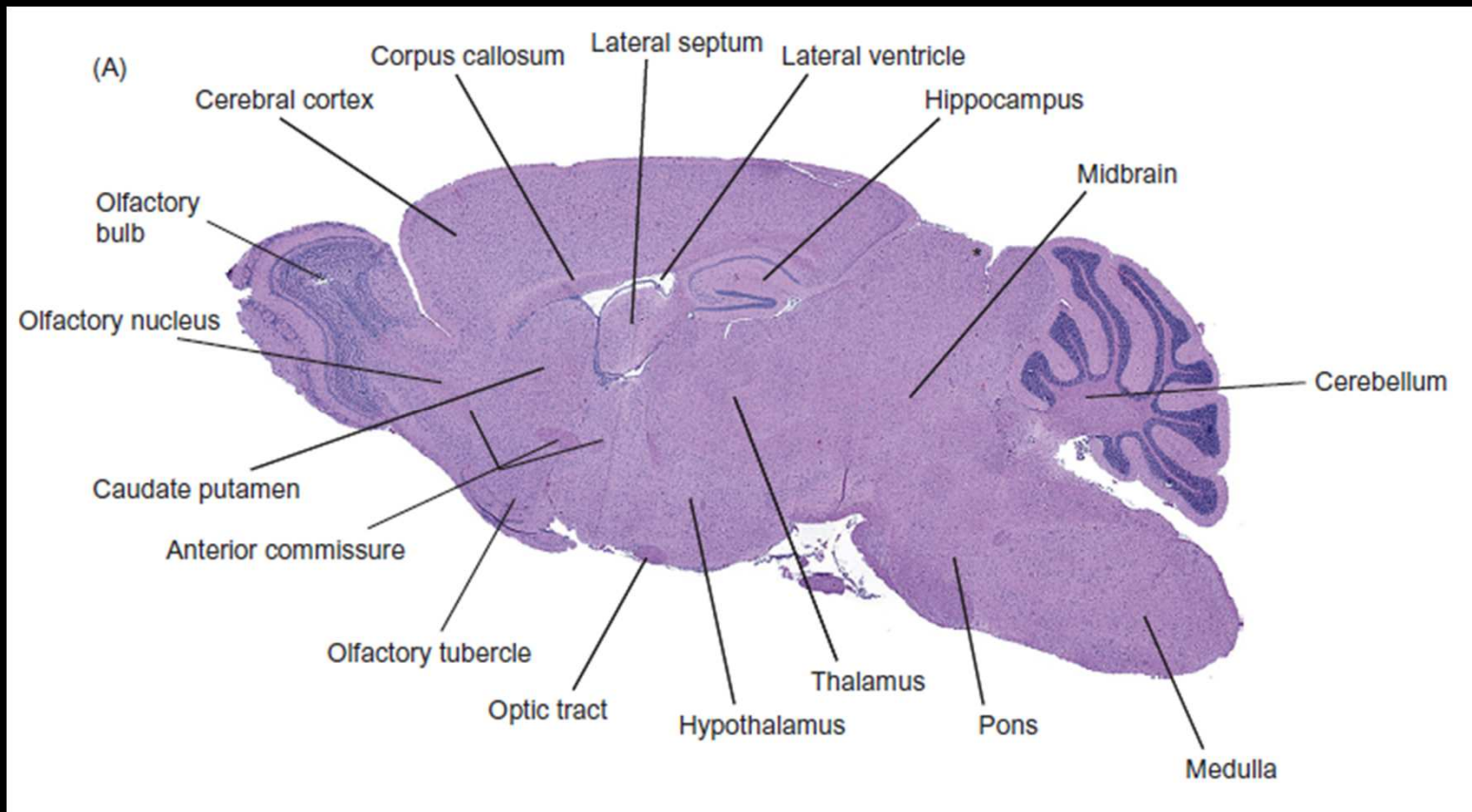
Gross images of coronal (cross) sections of an adult mouse brain.

The numbered levels shown in Panel A correspond to the labeled levels in Panel B.

The sections on the left column show the rostral side of each slice, whereas the right column shows the caudal side. Note that only one olfactory lobe is shown for Level 1.

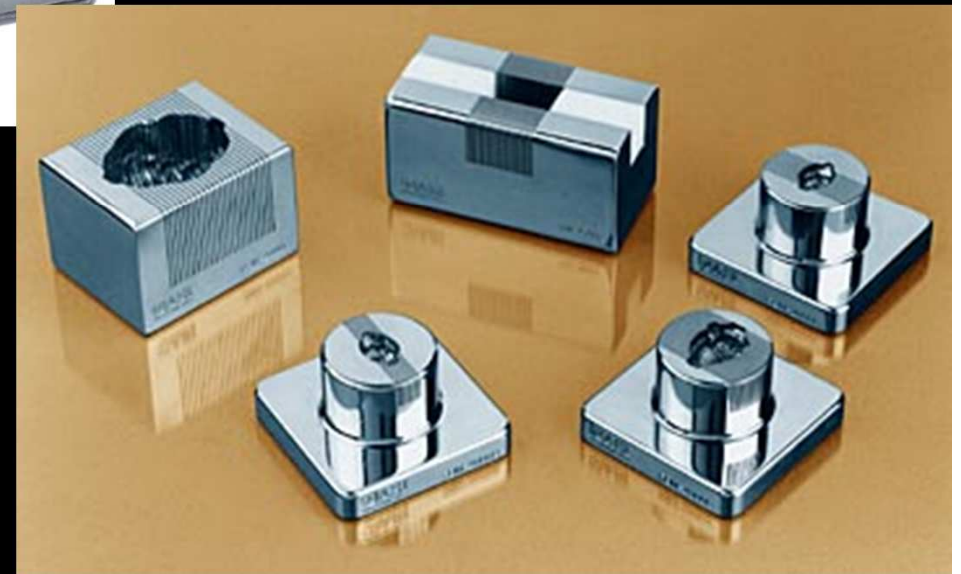
A, amygdala; ac, anterior commissure; Aq, mesencephalic aqueduct; Cbx, cerebellar cortex; cc, corpus callosum; Cp, caudate/putamen; Ctx, cerebral cortex (neocortex); f, fornix; FL, flocculus lobe of the cerebellum; Hip, hippocampus; Hy, hypothalamus; ic, internal capsule; LS, lateral septum; M, mesencephalon (midbrain); Md, medulla oblongata; Ofc, orbitofrontal cortices; ON, olfactory nucleus; P, pons; PaG, periaqueductal gray matter; T, thalamus.



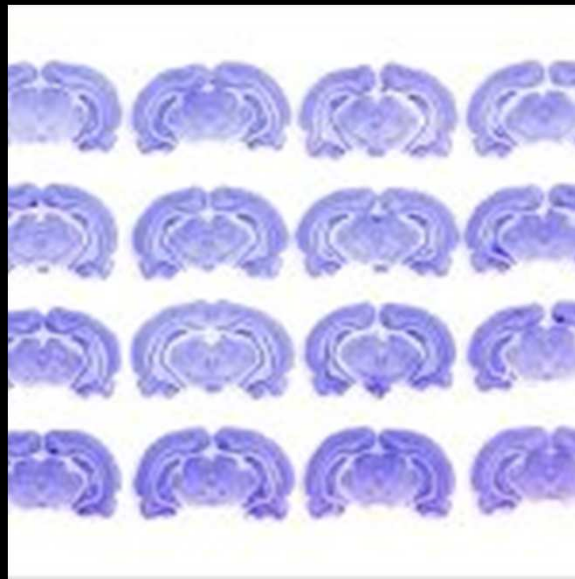
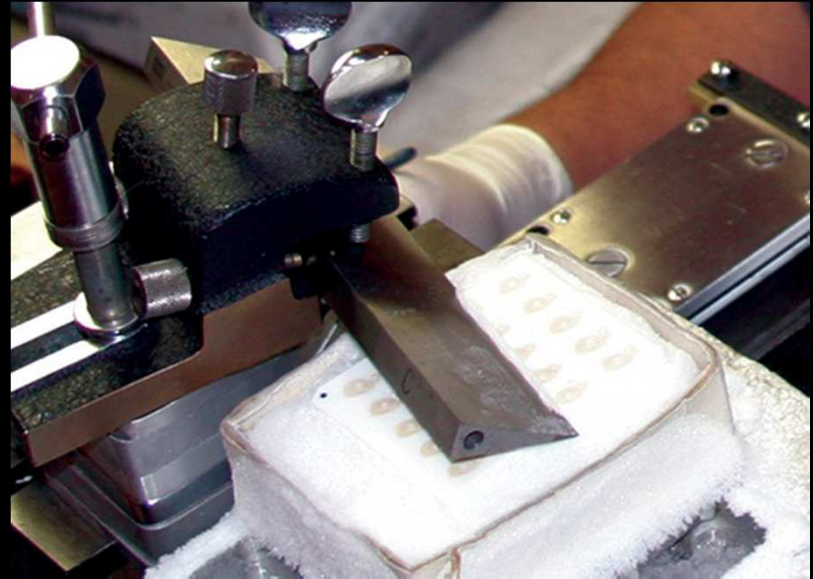


Parasagittal section of adult mouse brain

Brain Matrix for Coronal/Sagittal Sections



MULTIBRAIN TECHNOLOGY



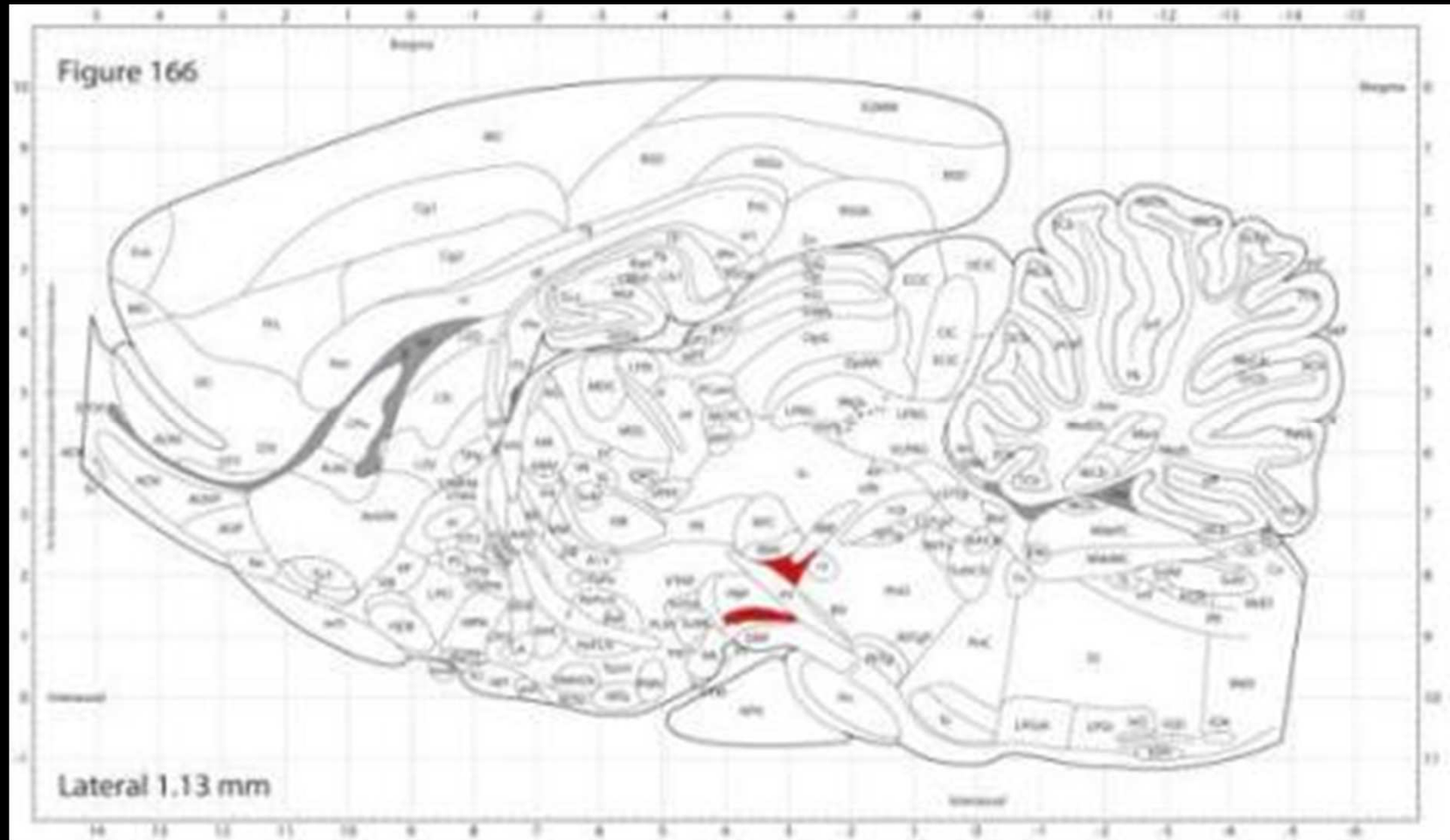


For any species, sampling the same number of levels provides comparable representation

Species	Brain Length (mm)	Sampling Interval (in mm)		
		Using 40 samples	Using 60 samples	Using 80 samples
Mouse	12	0.30	0.20	0.15
Rat	21	0.53	0.35	0.26
Monkey	65	1.63	1.08	0.81
Dog	75	1.88	1.25	0.94

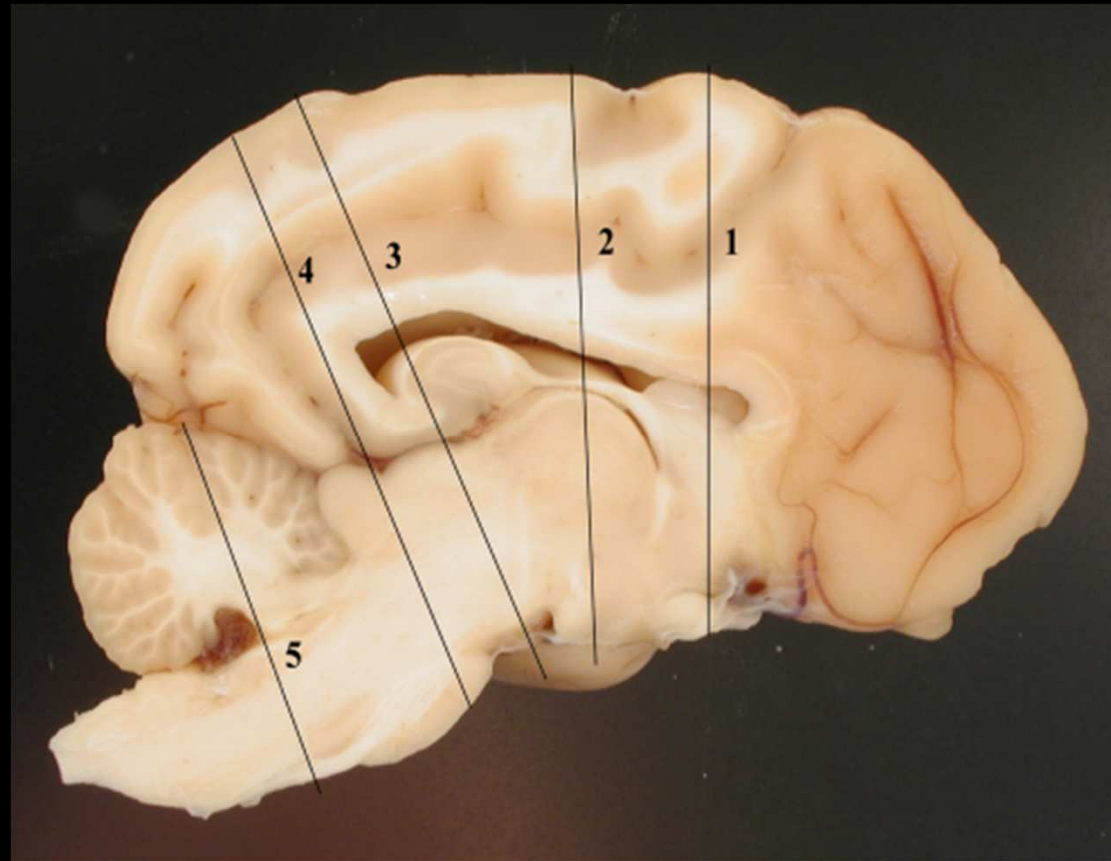
A sampling rate of 50-60 levels per brain offers a balance between a reasonable safety assessment and reasonable effort.

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine



Dog Brain Trimming

Fix the intact brain in 10% NBF in separate containers over absorbent cotton for 48-72 hours. Replace the NBF with fresh solution and fix further for 48 hours.



(Singer 1962)

- **Trim 5 transverse (coronal)** sections from each brain using the Singer dog brain atlas (Singer 1962), and divide so that approximately 7 histologic slides are prepared from each dog. The approximate levels of these 5 coronal sections are as follows:
- **Level of the optic chiasma.** Prepare a tissue block from the ventral half of the section.
- **Infundibulum** including the middle of the thalamus. Divide section in half and prepare tissue blocks from the ventral and dorsal halves.
- **Between the mammillary body and rostral edge of the pons,** including anterior midbrain and hippocampus. Divide section in half and prepare tissue blocks from the ventral and dorsal halves.
- **Rostral pons, including the posterior midbrain and possibly the hippocampus.** Divide section in half and prepare tissue blocks from the ventral half of the section.
- **Middle of cerebellum** including subjacent medulla oblongata. Prepare a tissue block that does not include overlying cerebral cortex.



Dog Brain Trimming



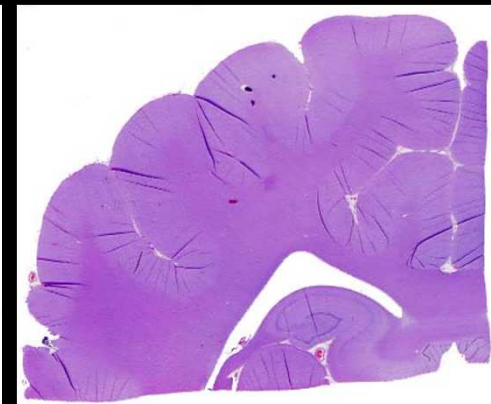
Level 1



Level 2d



Level 2v



Level 3d



Level 3v



Level 4



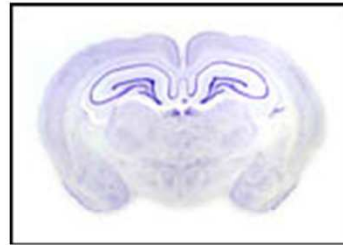
Level 5

(Singer 1962)

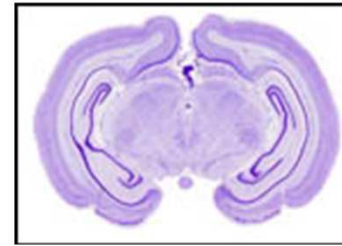
Different species brain



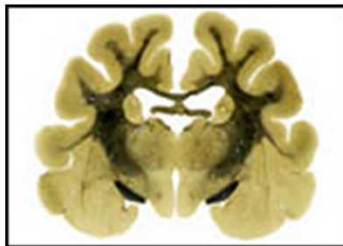
Neonate-Rat



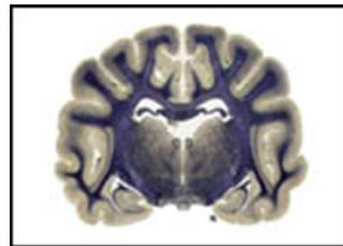
Rat



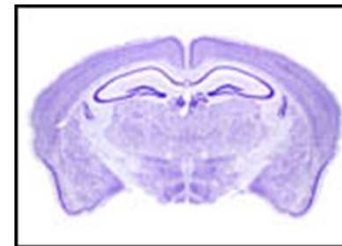
Rabbit



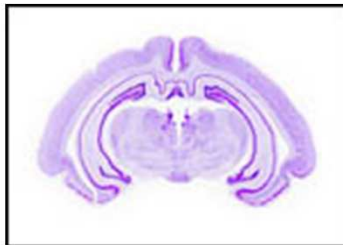
Cat



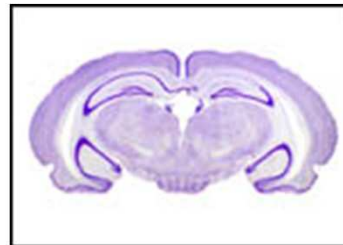
Dog



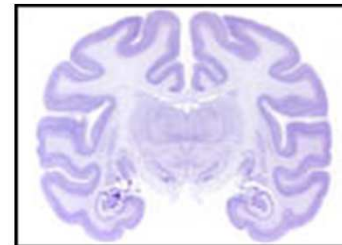
Mouse



Guinea Pig



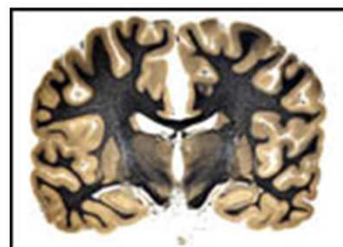
Gerbil



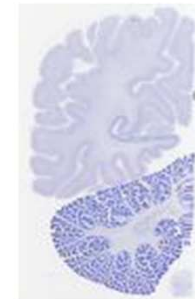
Monkey



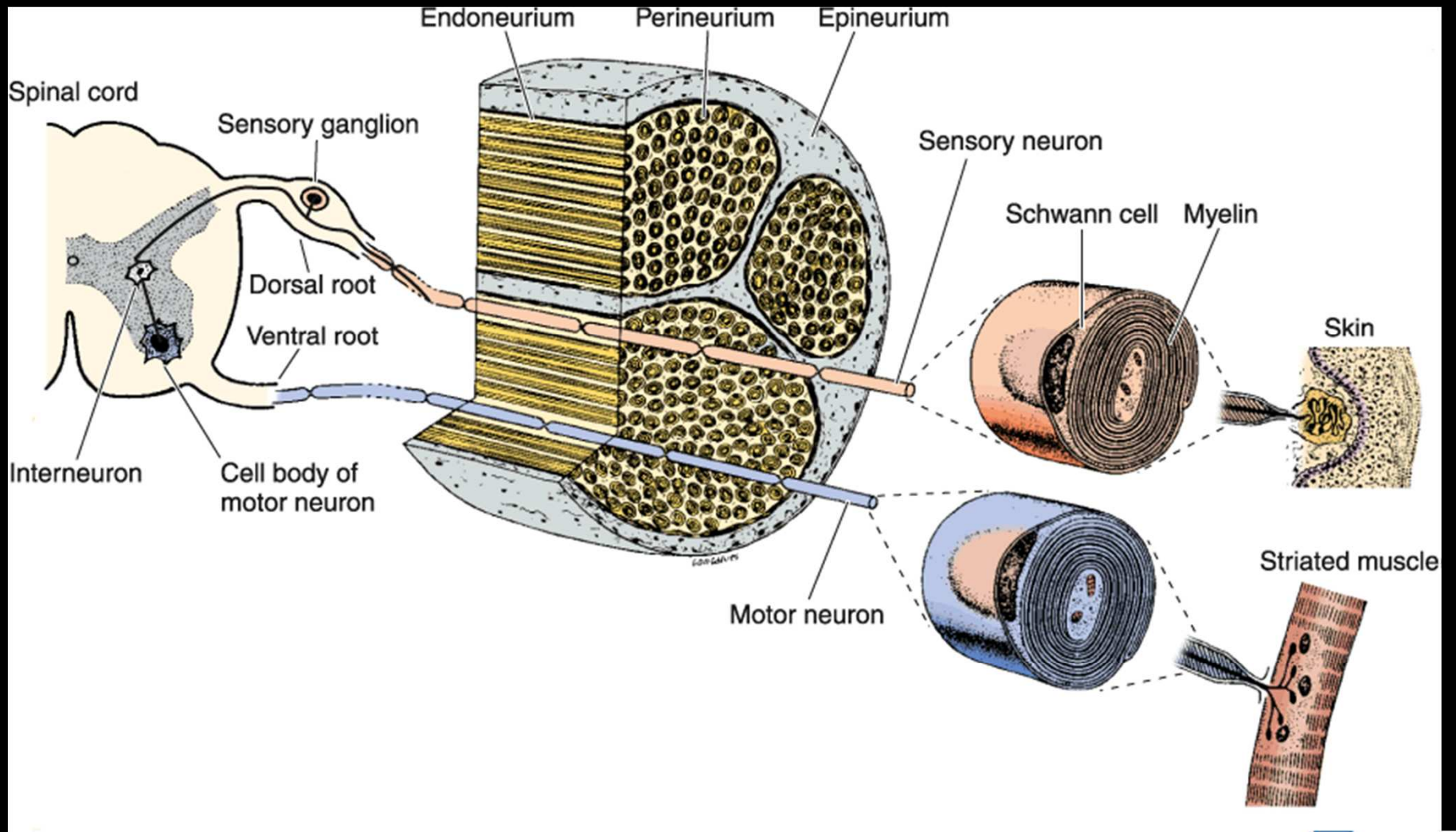
Baboon



Human

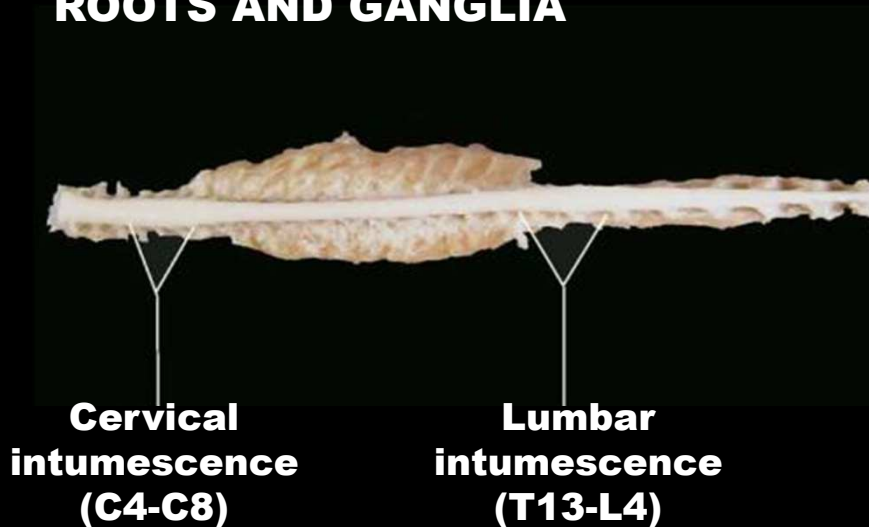


Human Hemisphere

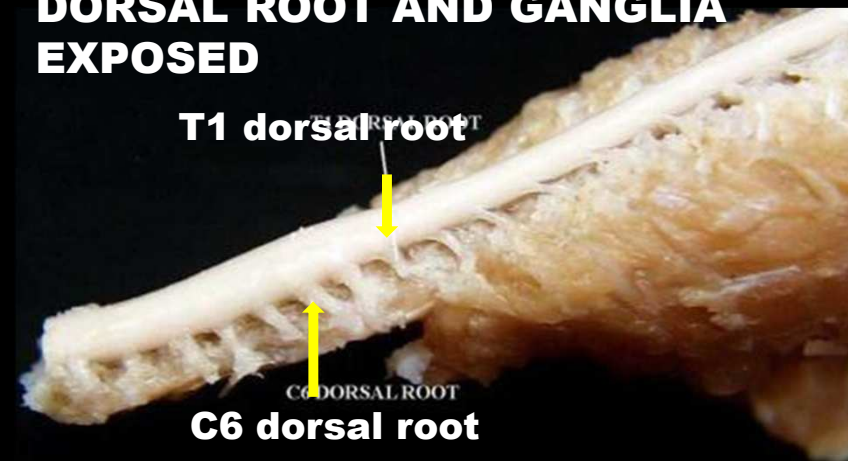


Rat Spinal Cord, Dorsal Nerve Roots and Dorsal Root Ganglia

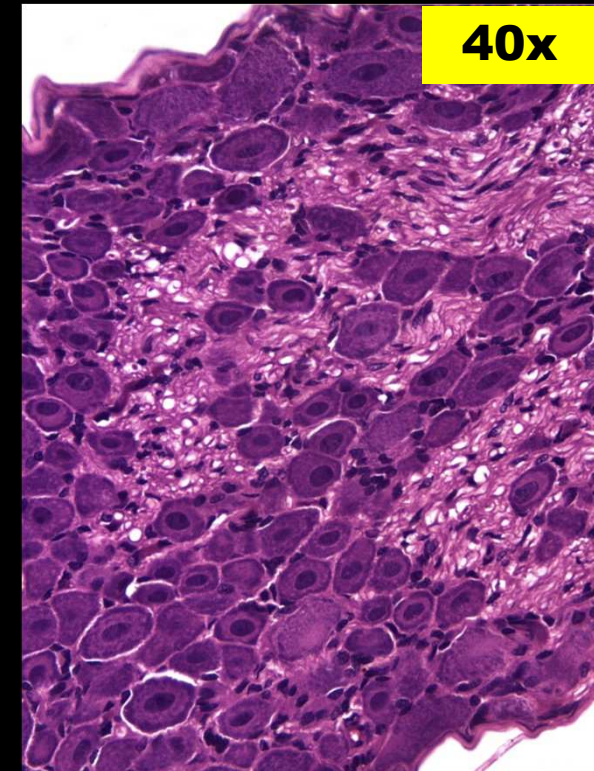
DORSAL VIEW OF ENTIRE SPINAL CORD WITH EXPOSED DORSAL ROOTS AND GANGLIA

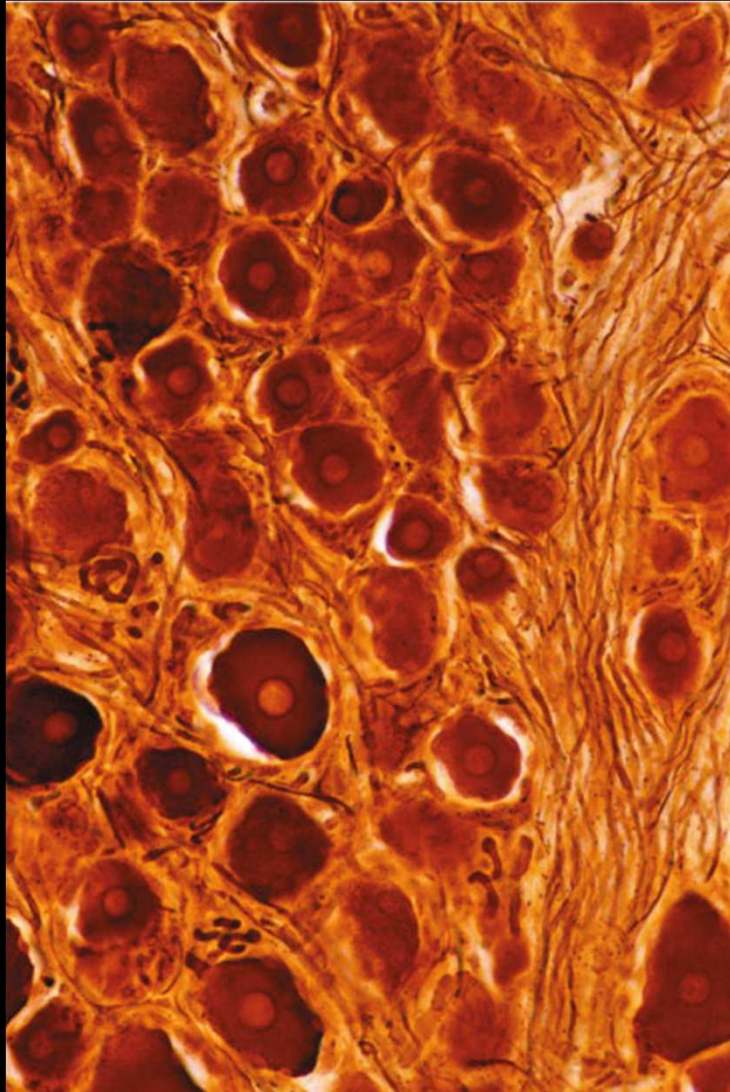


CERVICAL SPINAL CORD WITH LEFT DORSAL ROOT AND GANGLIA EXPOSED



Rat Spinal Cord, Dorsal Nerve Roots and Dorsal Root Ganglia

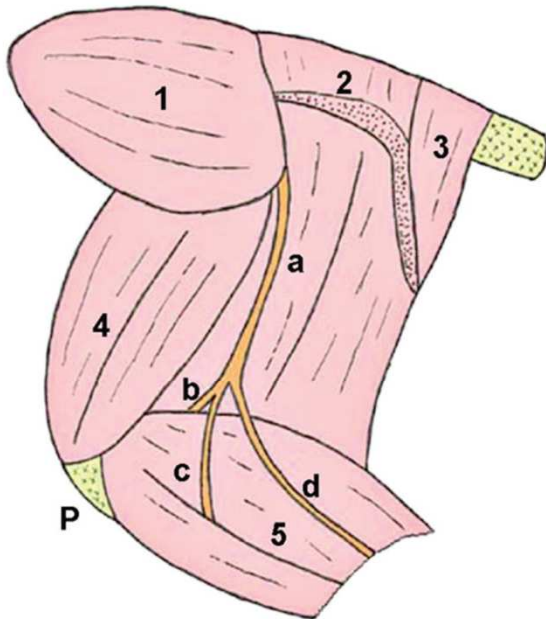




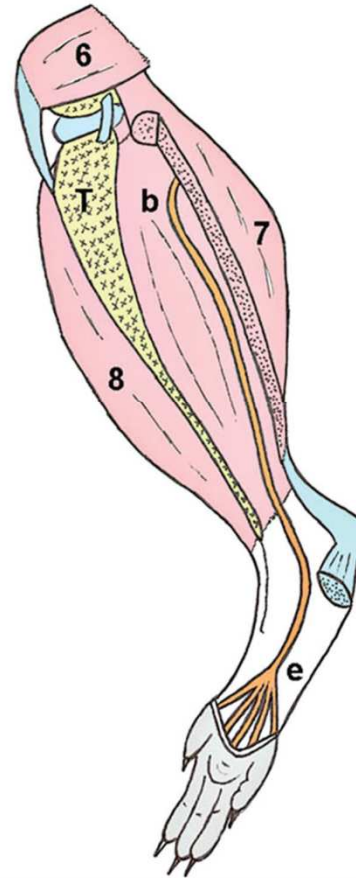
Silver-impregnated sensory ganglion consisting of pseudounipolar neurons.

Peripheral Nerves and Skeletal Muscles

Lateral View of Hindlimb



Medial View of Hindlimb



✓ **Location of peripheral nerves to be evaluated for DNT neuropathology.**

✓ **Muscles:**

- 1 = gluteus medius;
- 2 = biceps femoris;
- 3 = semitendinosus;
- 4 = quadriceps femoris;
- 5 = gastrocnemius lateralis;
- 6 = rectus femoris;
- 7 = gastrocnemius medialis;
- 8 = tibialis cranialis.

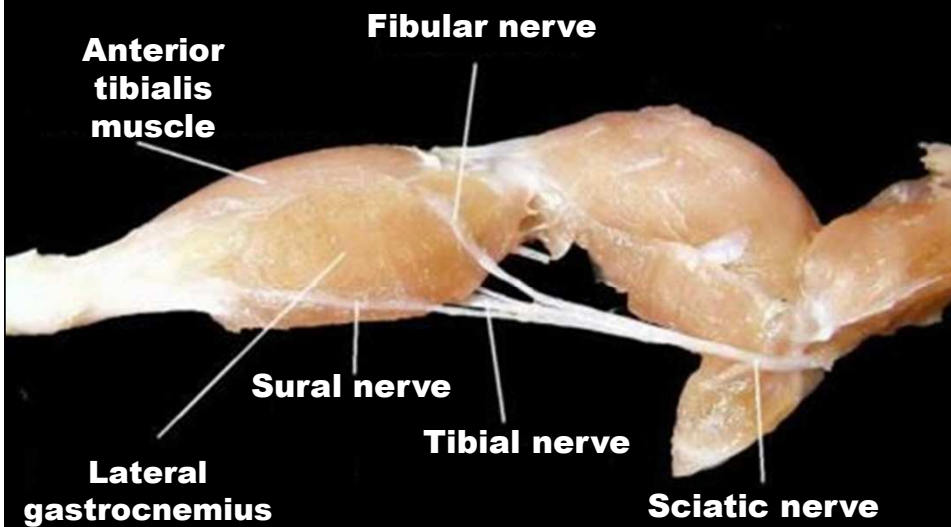
• **Nerves:**

- a = sciatic;
- b = tibial;
- c = common peroneal (fibular);
- d = lateral sural;
- e = plantar.

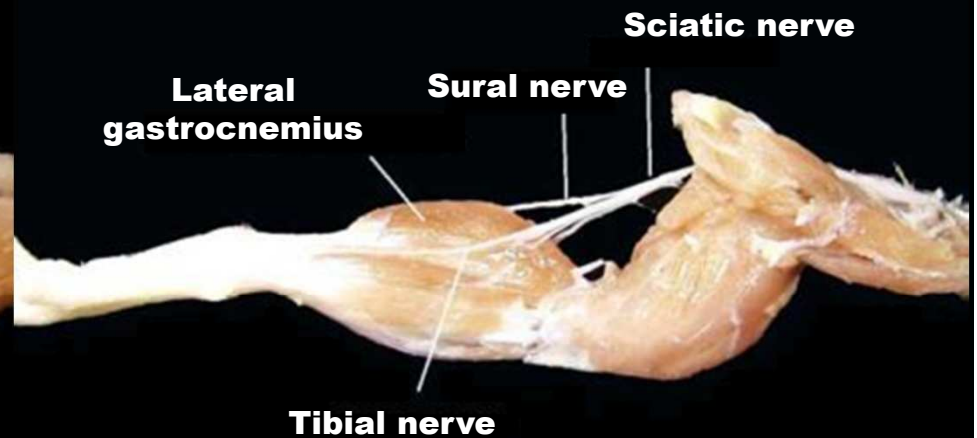
▪ **Bones:**

- P = patella;
- T = tibia.

Lateral view of the left hind limb with nerves exposed



Medial view of the left hind limb with nerves exposed



Histologic Techniques (Stain/Methods) for CNS and PNS

Stain/Methods	Stain/Methods
Hematoxylin and eosin (H&E)	Luxol fast blue
Hematoxylin van Gieson	Loyez
Reticulin stains	Osmium
Masson's trichrome	Periodic acid-Schiff (PAS)
Alcian blue	Polarized light (frozen section)
Toluidine blue	Marchi's
Oil red O	Toluidine blue and carbol fuschin.
Palmgren's or Bodian's (silver stains)	Osmium tetroxide stained
Sudan black B	Nissl method
Gold method	Gold/toluidine
Immunochemistry	Fluoro-Jade
resin sections	Electron microscopy
Teased fibers	Many more....



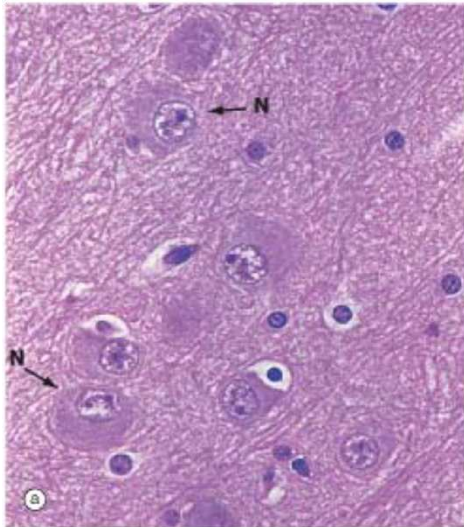
Common stains and their endpoints

STAIN	Degeneration				Perturbation	
	Loss of Neuronal Soma	Loss of Axons	Loss of Dendrites	Loss of Axon Terminals	Perturbed Astrocytes	Activated Microglia
GFAP					X	
Nestin					X	
Iba1						X
H&E	X					
Nissl	X					
TUNEL	X					
FluoroJade	X	X	X	X		
CuAg Methods	X	X	X	X		

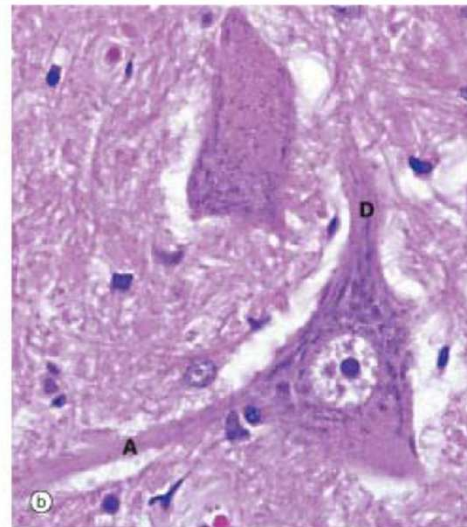
Establishing the boundary of the acceptable threshold of neurotoxicity will dictate which stain(s) must be included in a routine neuropathologic assessment



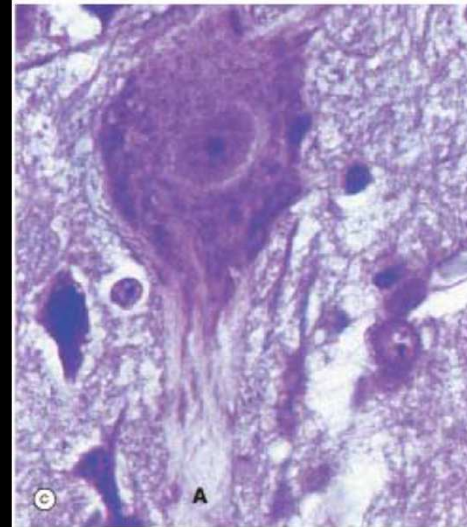
Neurons and methods of study with light microscopy



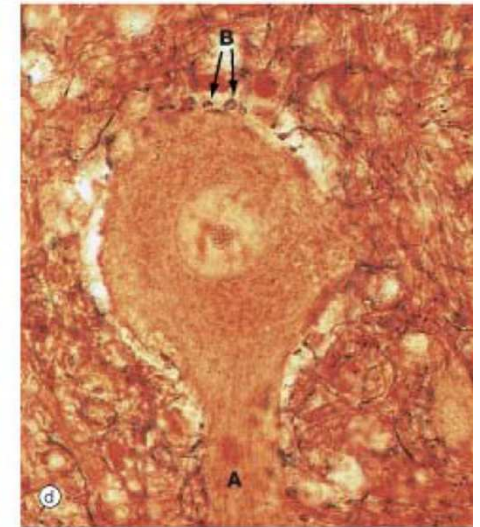
H & E
×480



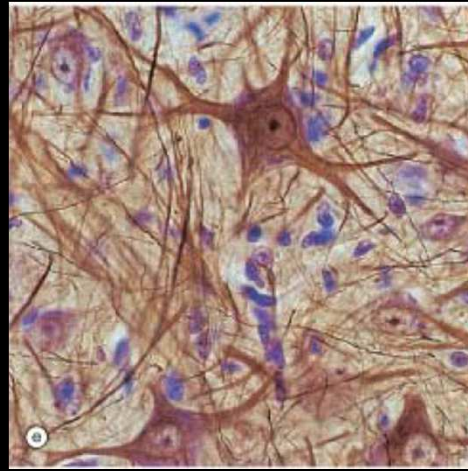
H & E
×1200



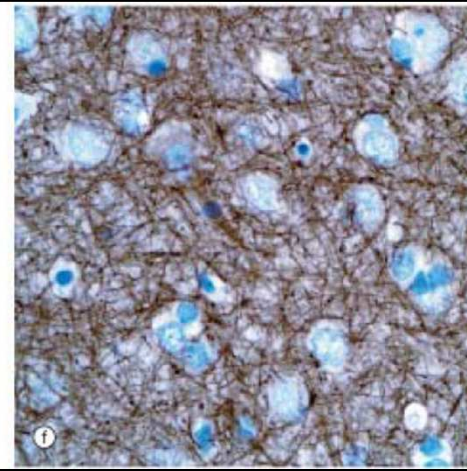
Nissl method
×1200



Gold method
×1200



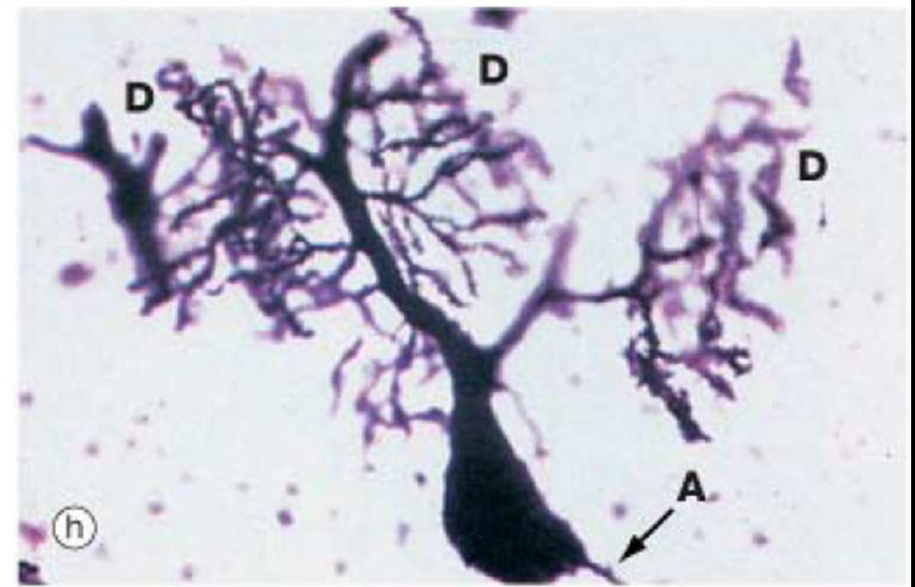
**Gold/toluidine
blue ×600**



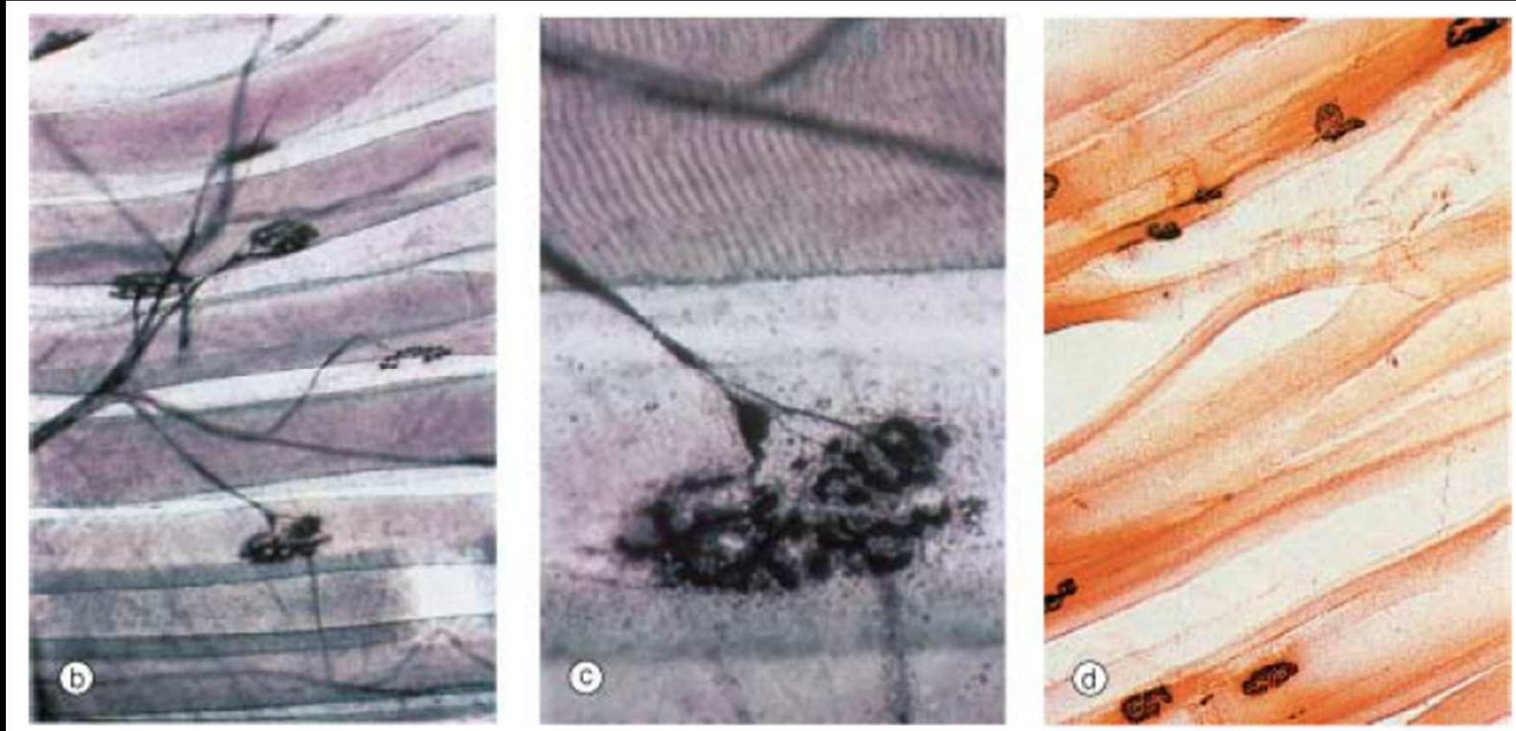
**Immunocytochemistry neurofilament
protein ×480**



Spread preparation, gold method ×320



Golgi-Cox ×320

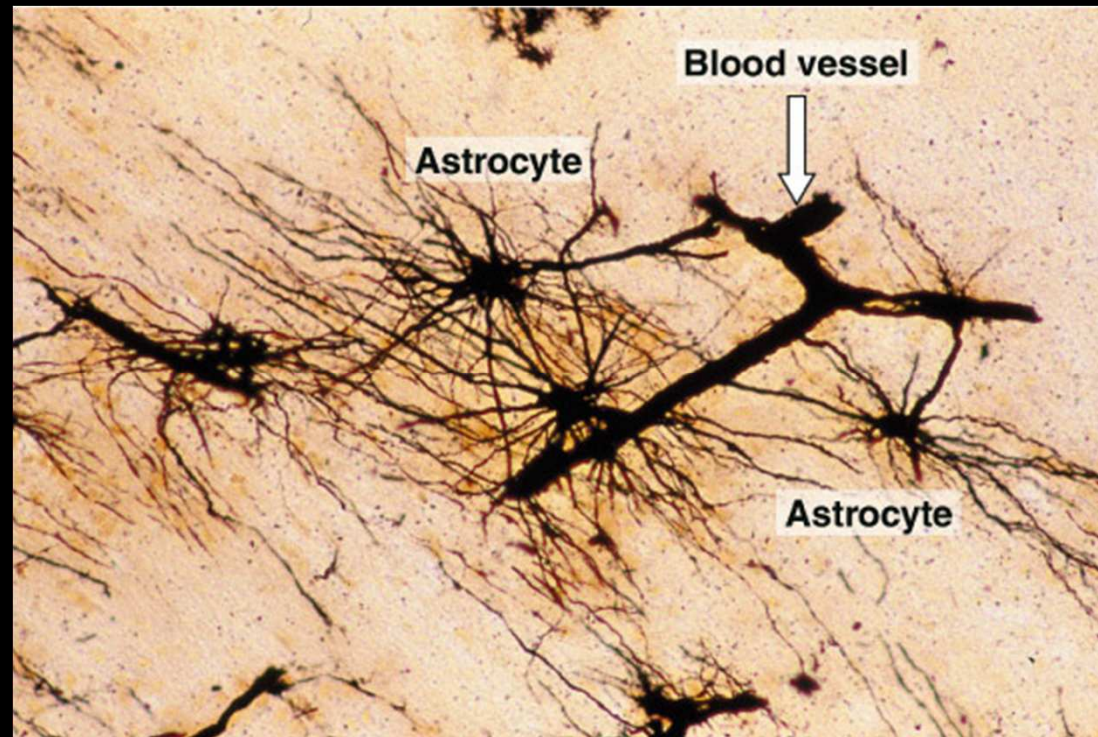


Motor end plates

(b) Teased preparation, gold method ×320

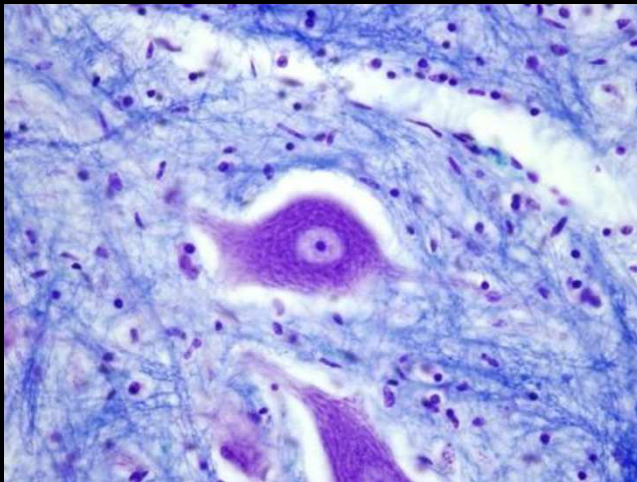
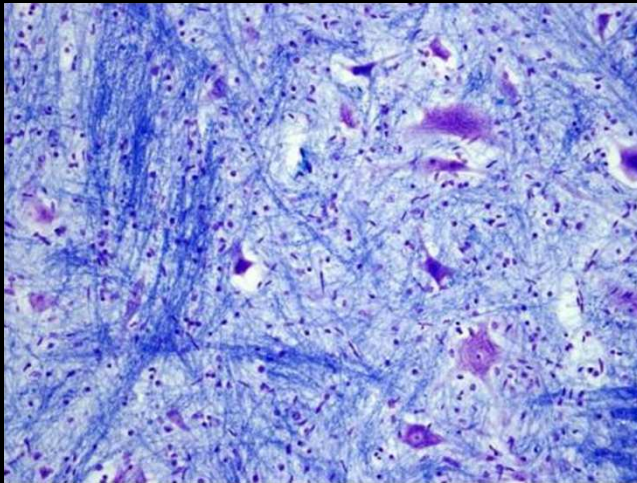
(c) Teased preparation, gold method ×800

(d) Histochemical method for acetylcholinesterase ×320



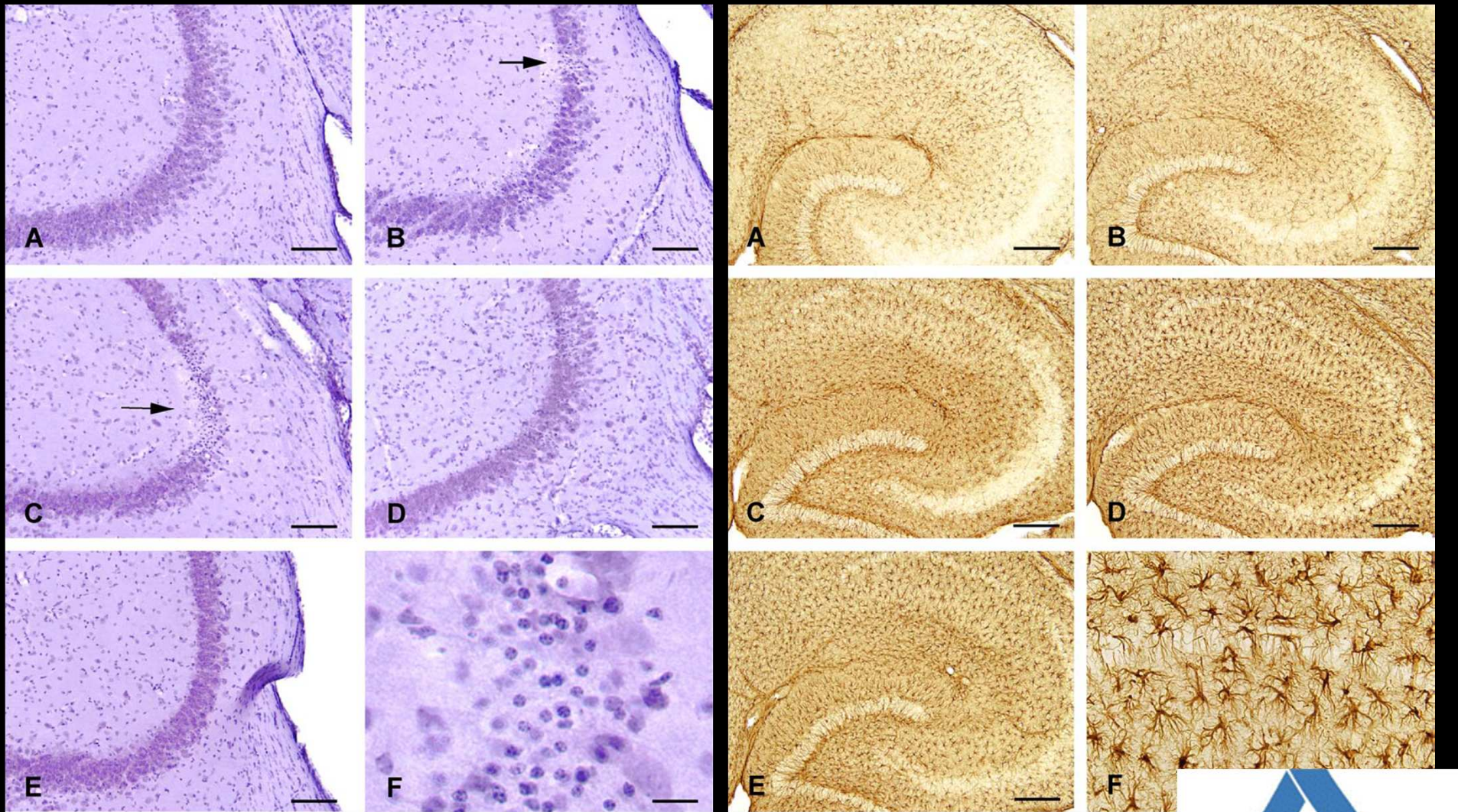
Brain section prepared with Rio Hortega silver stain showing fibrous astrocytes with their processes ending on the external surface of blood vessels.

Luxol Fast Blue



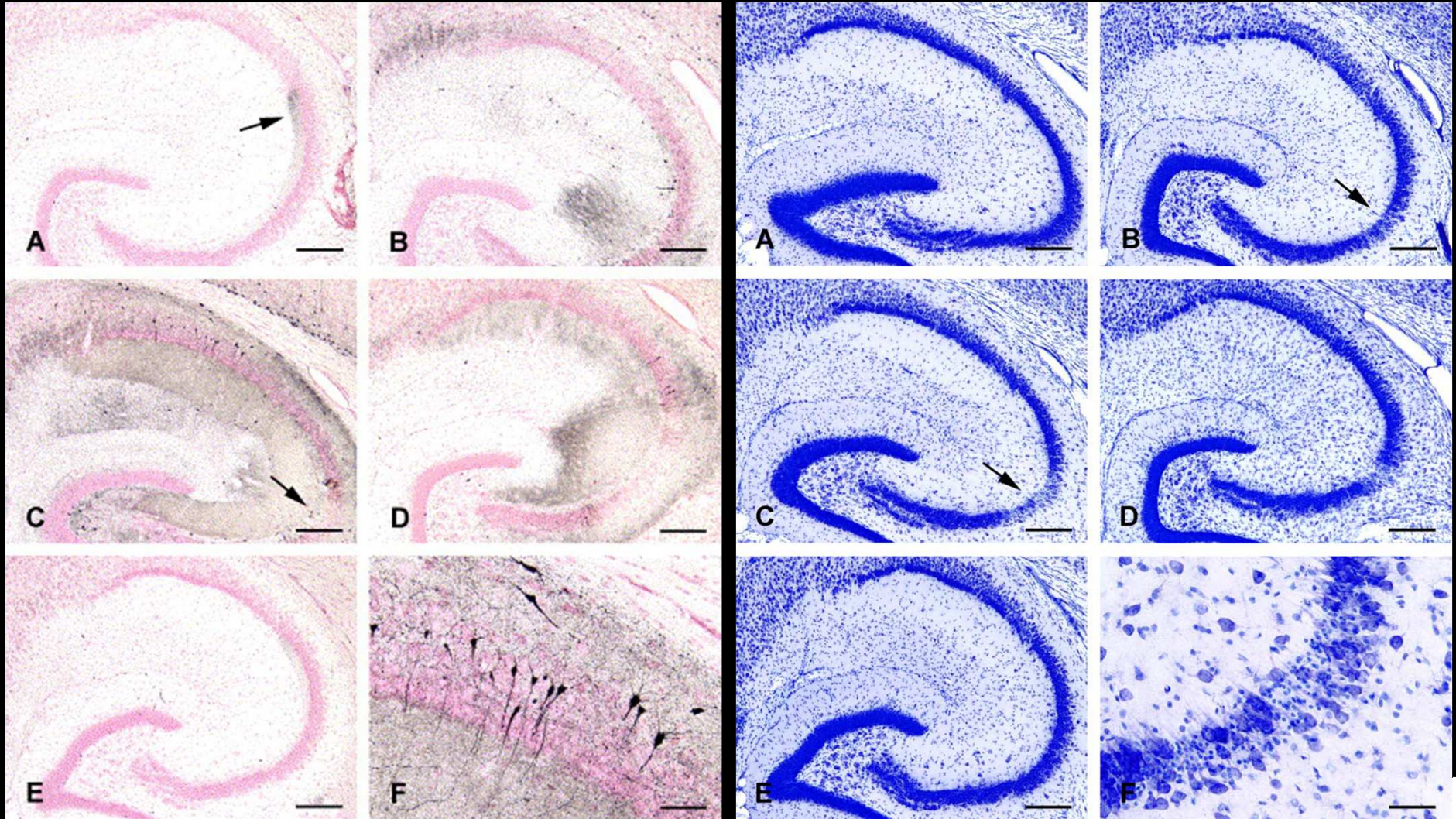
- **The stain works via an acid-base reaction with the base of the lipoprotein in myelin.**
- **Myelinated fibers appear blue. Counterstaining with a nissl stain reveals nerve cells in purple. (e.g. cresyl violet)**
- **Stains myelin.**
- **Does not stain axoplasm, cell bodies, terminals, or dendrites.**

H &E and GFAP immunostaining in hippocampus of mice (C57BL/6J) treated with saline or kainic acid



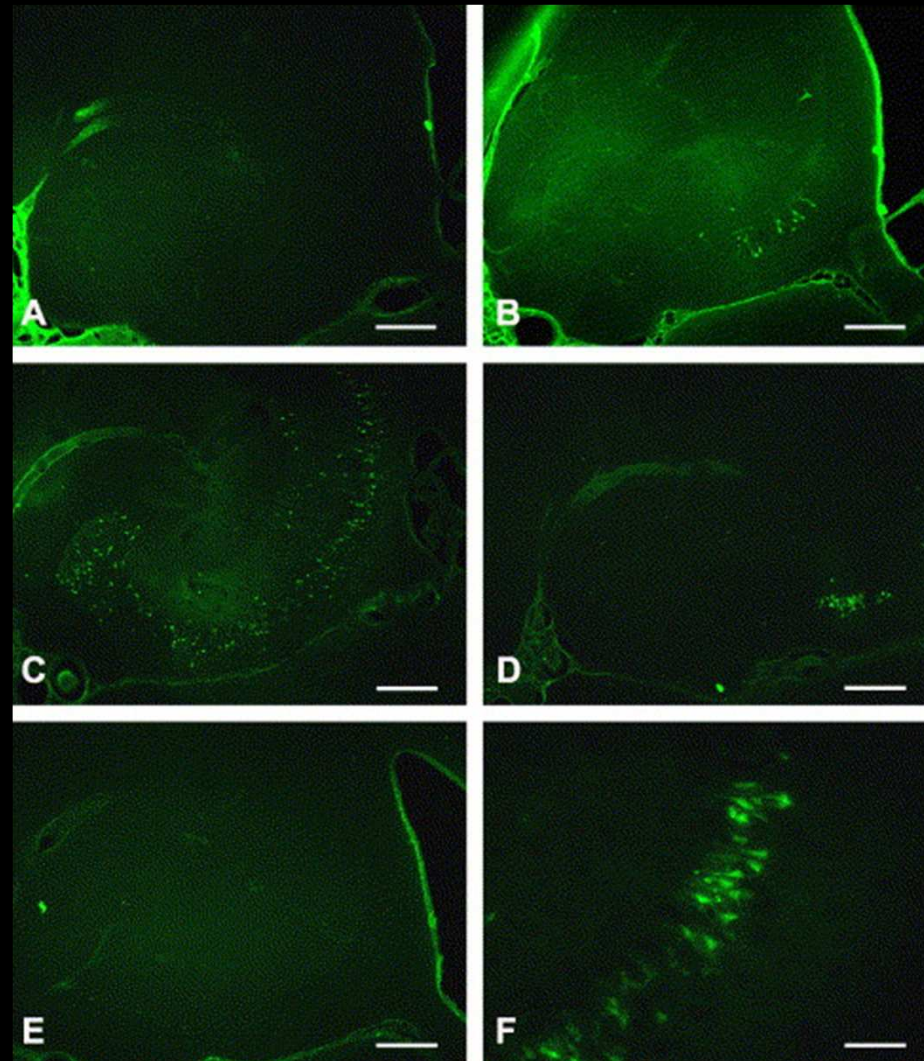
(Benkovic *et al.*, 2004)

CuAg and Nissl methods in mice (C57BL/6J) treated with saline or kainic acid



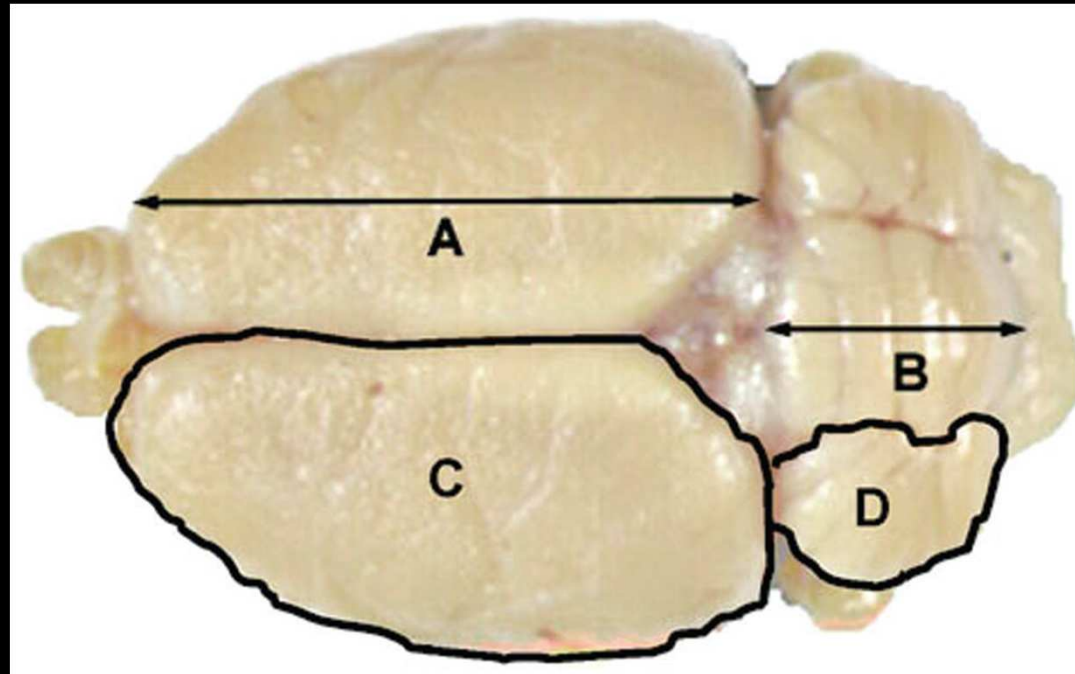
(Benkovic *et al.*, 2004)

FluoroJade Staining in mice (C57BL/6J) treated with saline or kainic acid



(Benkovic *et al.*, 2004)

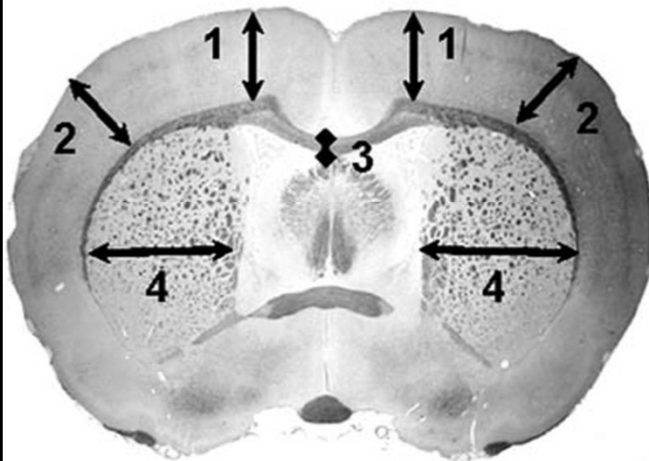
Morphometric Evaluations



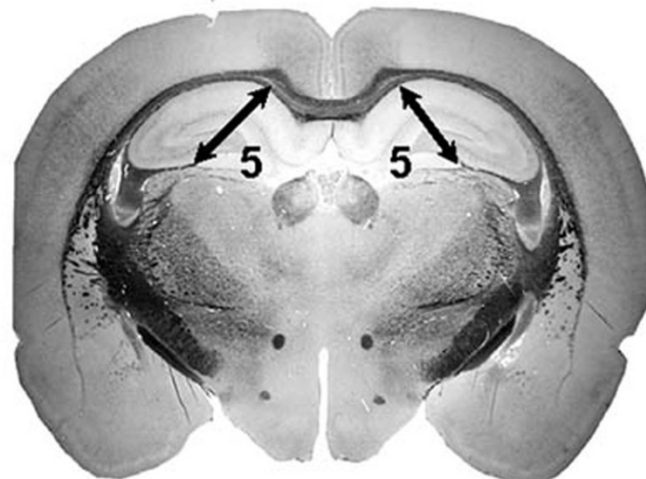
Quantitative measurement of gross anatomic features in the developing brain. Collection of morphometric data from the developing brain can be accomplished readily and rapidly using simple linear (A, B) or area (C, D) measurements of the dorsal brain surface at the macroscopic level. (A) Cerebral length (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.**

Morphometric Evaluations

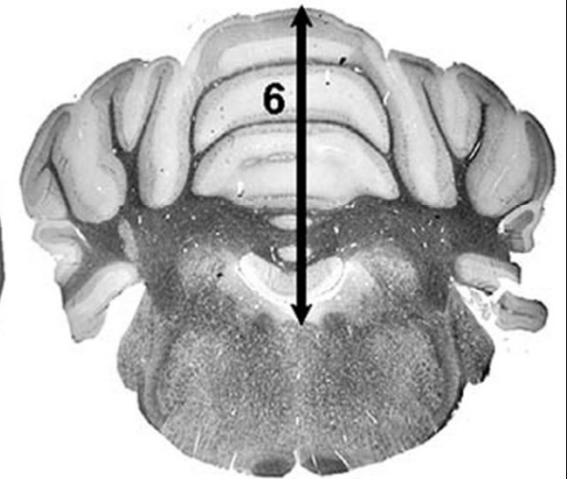
Level 4: Forebrain



Level 5: Midbrain



Level 7: Hindbrain



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 μ m. Note: Always maintain left and right orientation when evaluating brain sections.

1 = Cerebral Cortex (Frontal) Thickness

3 = Corpus Callosum Thickness

5 = Hippocampus Gyrus Thickness

2 = Cerebral Cortex (Parietal) Thickness

4 = Striatum Width

6 = Cerebellum Height

Other techniques

- **Immunohistochemistry**
- **Digital Imaging and Analysis**
- **Computerized tomography (CT scan)**
- **Magnetic Resonance Imaging (MRI)**
- **Positron Emission Tomography (PET)**
- **Recording Electrical Activity in the Brain (eg. electroencephalogram -EEG)**



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Thank You

