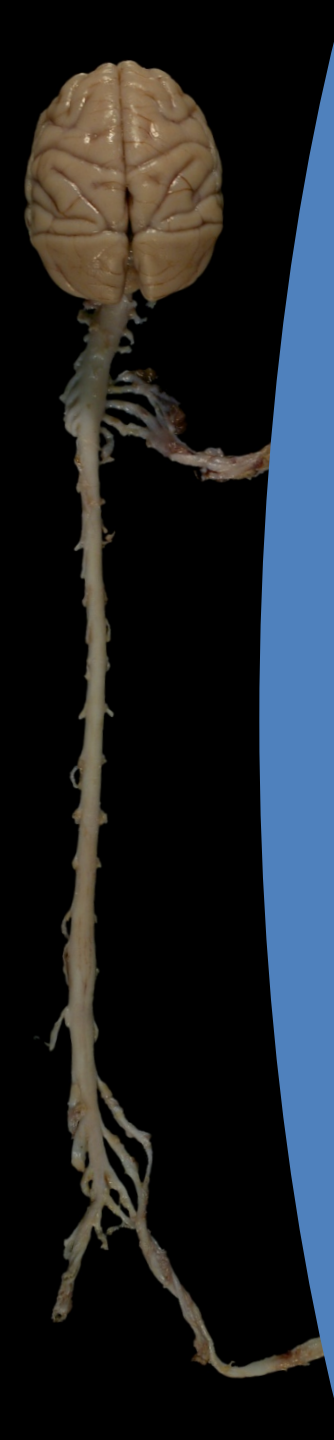

Brain and Peripheral Nerve System Processing, Staining, Common Artifacts and Unusual Normal Structures

Sixth Conference and Continued Education STPI meeting in Toxicologic Pathology of the Nervous and Musculoskeletal Systems

21-23 October 2016 Pune, India

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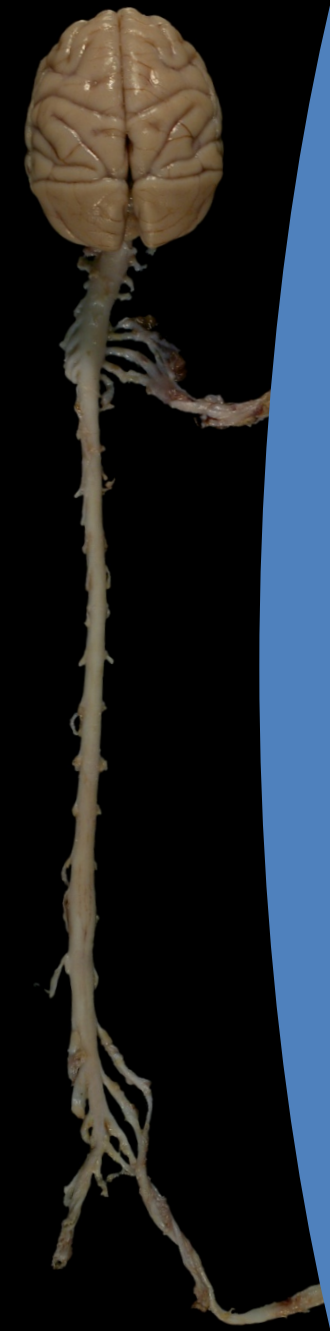
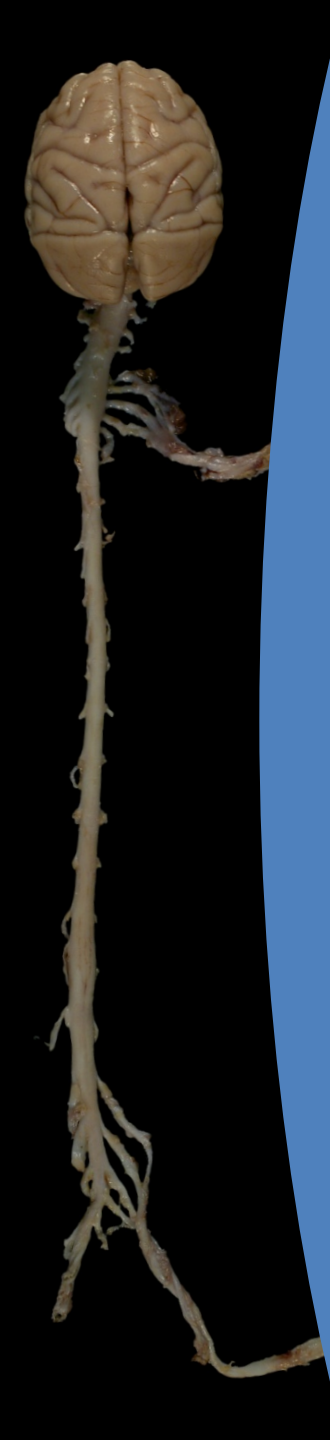


Disclaimer

- All procedures used to obtain images from animals in this presentation were approved by the animal care and use committee in accordance with federal regulations and the Guide for the Care and Use of Laboratory Animals

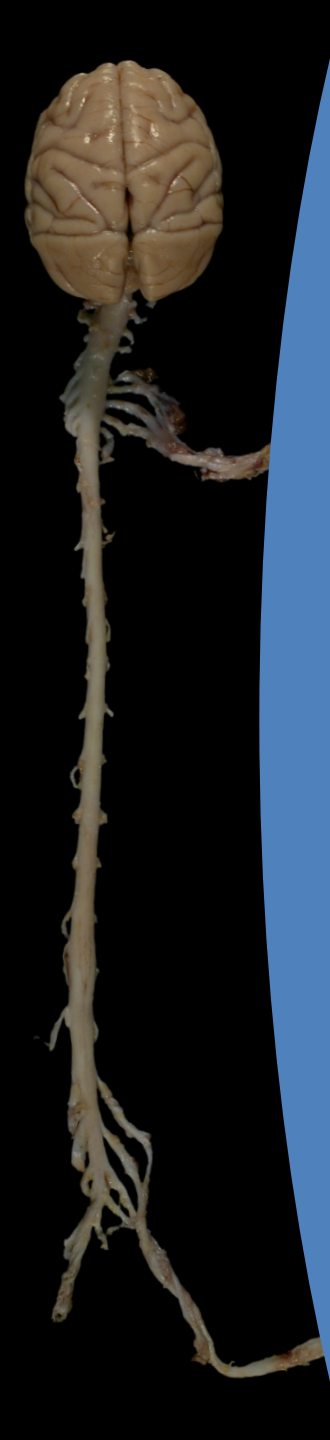
- Nervous system sampling protocols in this presentation have been endorsed by the Scientific and Regulatory Policy Committee of the Society of Toxicologic Pathology (SRPC)



STP Position Paper: Recommended Practices for Sampling and Processing the Nervous System (Brain, Spinal Cord, Nerve, and Eye) during Nonclinical General Toxicity Studies Toxicologic Pathology, 2013

STP Working Group on Nervous System Sampling:

Brad Bolon (chair), Robert H. Garman, Ingrid D. Pardo, Karl Jensen, Robert C. Sills, Aude Roulois, Ann E. Radovsky, Alys Bradley, Lydia Andrews-Jones, Mark Butt, Laura Gumprecht (co-chair)



Fixation and Tissue Collection

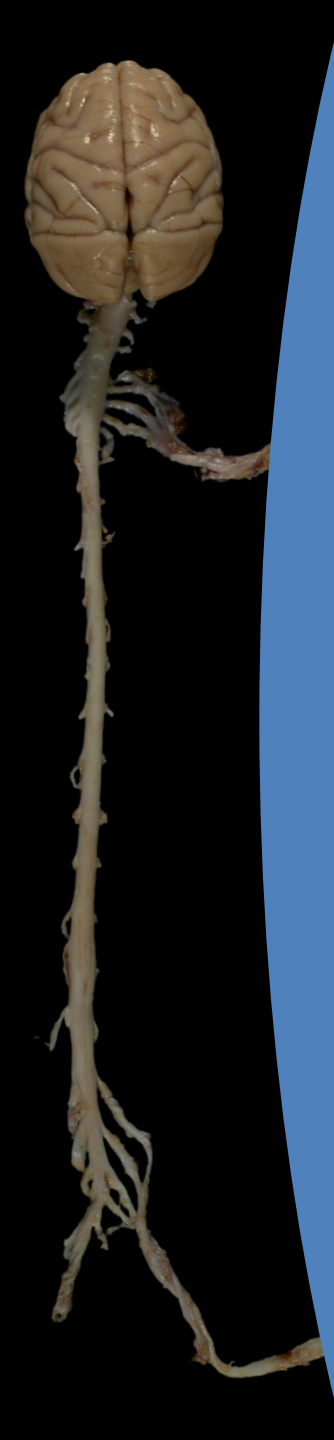
- Immersion fixation after organ removal for 18 hours (rodents) to 48 hours (non-rodents)

<u>Brain, spinal cord, and nerve</u>:

- Neutral buffered 10% formalin (NBF), with or without stabilizers
- Permits routine processing of neural tissues with other non-neural tissues

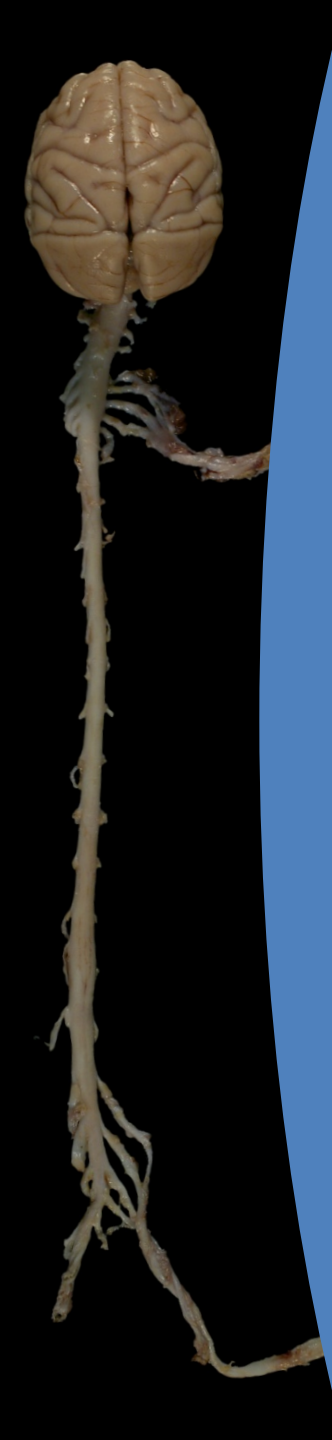
<u>Eye:</u>

- Bouin's: the RECOMMENDED fixation method
- Davidson's solution, or modified Davidson's solution is ACCEPTABLE fixation methods for eyes
  - intravitreal injection of NBF or NBF: glutaraldehyde mixtures
  - immersion in NBF, if and only if retinal artifacts can be minimized consistently



Brain Weights

- Fresh or fixed brain weight (as long as the same choice is employed throughout a given study)
- Care must be taken to ensure that all organs are removed trimmed in a comparable fashion (e.g., to include or exclude the olfactory bulbs)



Tissue Trimming

Brain:

- Relatively homologous levels acquired using defined external anatomic landmarks used for trimming and internal landmarks to select regions for analysis
- Level orientation generally coronal, but another plane may be selected at the discretion of the study pathologist

Spinal cord:

- Three levels (cranial cervical [approximately C_1-C_2], thoracic [approximately T_6-T_8], and lumbar intumescence [about L_4-L_5])
- Sections assessed in transverse and longitudinal / oblique orientations

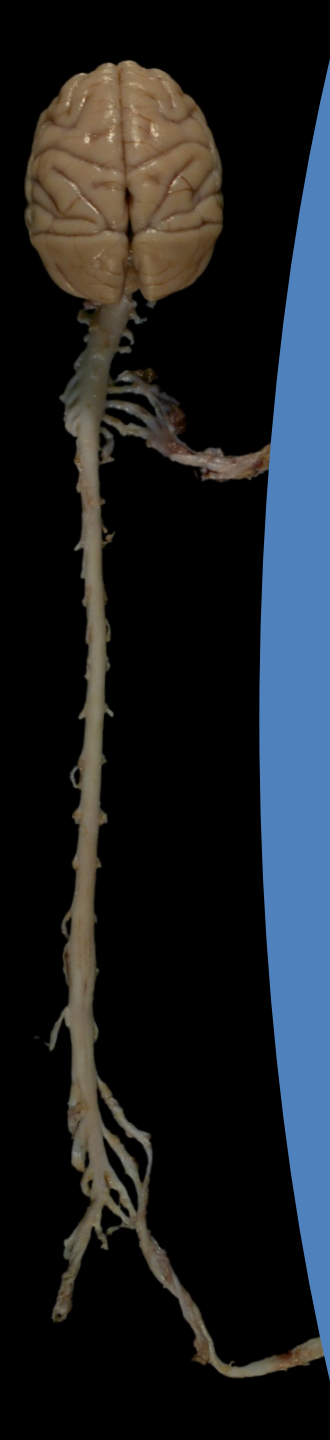
Tissue Trimming

Nerve:

- Longitudinal and transverse sections of the sciatic and/or tibial nerve trunk
- Bilateral collection recommended, but unilateral examination acceptable

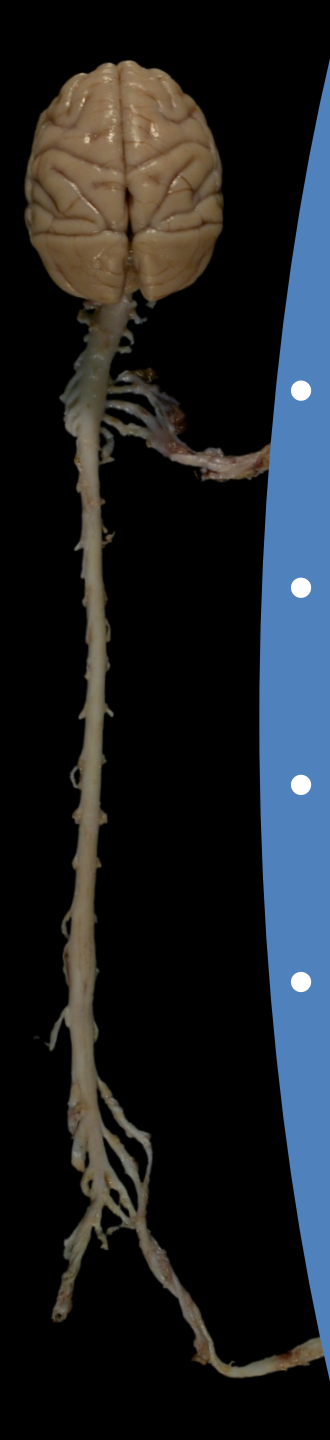
Eye and optic nerve:

- Axial section through the middle of the globe and nerve
- Bilateral examination recommended
- The chosen practices should be defined in a facility-specific standard operating procedure (SOP) or other reference document and used on all GLP-type general toxicity studies (e.g., 1-, 3-, 6-, 9-, and 12-month tests) in which neural tissues are to be examined



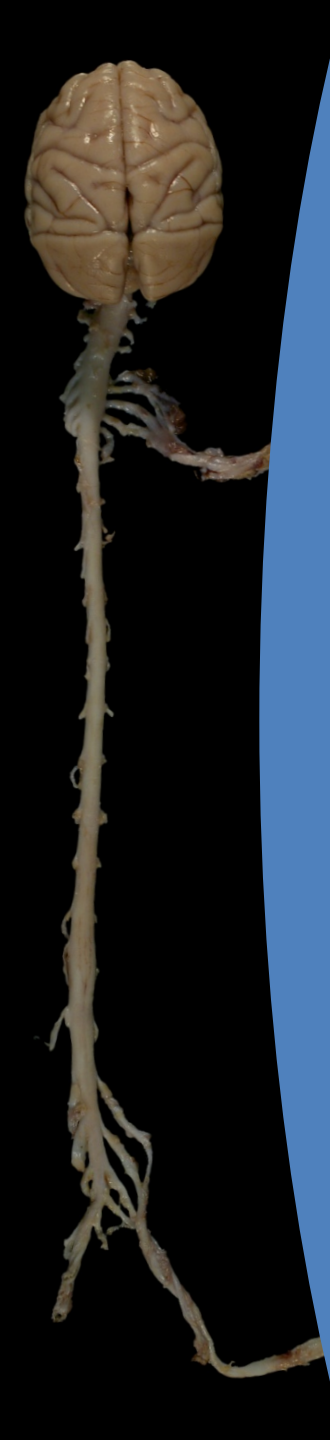
Tissue processing and staining

- **Tissue Processing:** standard paraffin embedding and standard section thickness (typically 4-8 mm)
- **Standard stain**: hematoxylin and eosin (H&E)
- **Special neurohistological procedures are employed in two instances:**
  - *Post hoc* at the discretion of the study pathologist.
  - Side-by-side with the H&E –stained material if presumptive evidence suggests that a neural structure may be affected
- For rodent carcinogenicity bioassays, the brain sampling protocol should be left to the discretion of the sponsoring institution



Histopathology Examination

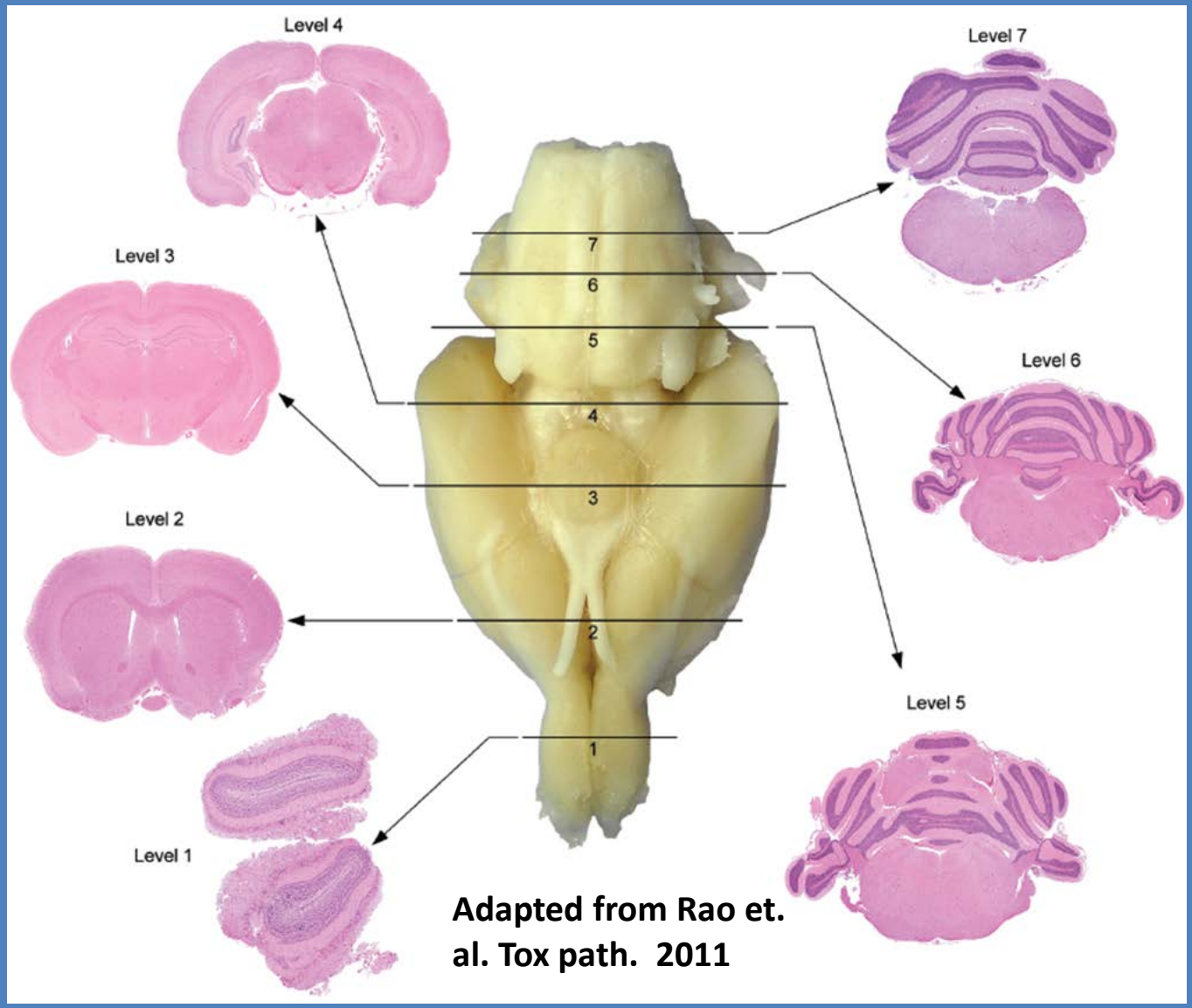
- qualitative analysis (no routine need for quantitative measurements)
- emphasis on structures to be examined rather than levels to be acquired
- levels should be fairly consistent for all animals in a given study
- study pathologist is aware of dose group identities, in-life findings, etc., (i.e., an uncoded [“unblinded”] analysis)



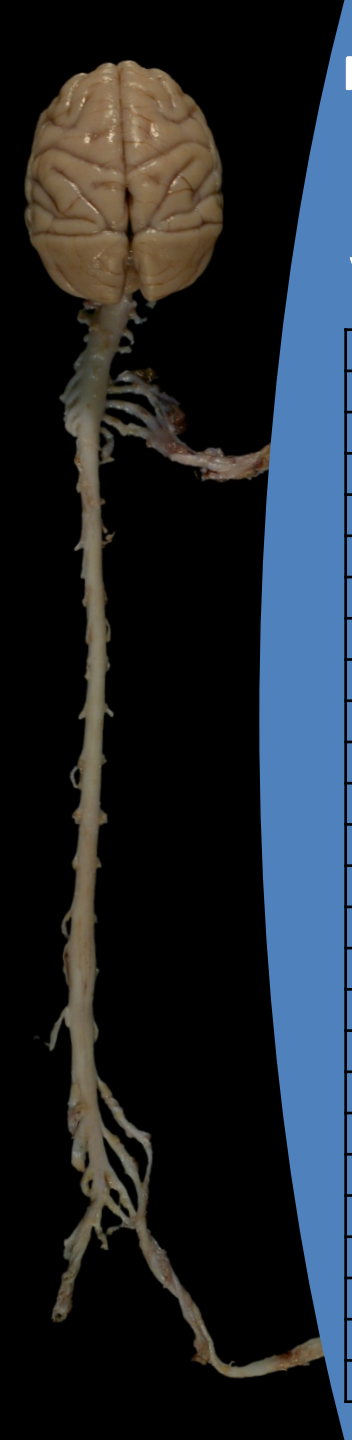
Reporting

- fixation, tissue processing, histological, and analytical practices are specified in some detail
- regions are designated using specific terms (e.g., cerebral cortex vs. forebrain)
- explicit reporting RECOMMENDED (e.g., a tabular listing of all regions assessed
- implicit reporting ACCEPTABLE
- listing regions examined in a separate SOP but not formally including it in the report
- citing literature regarding nervous system sampling procedures for the species in question

Ventral brain sectioning for the rat (a) and for the mouse (b).



Adapted from Rao et. al. Tox path. 2011

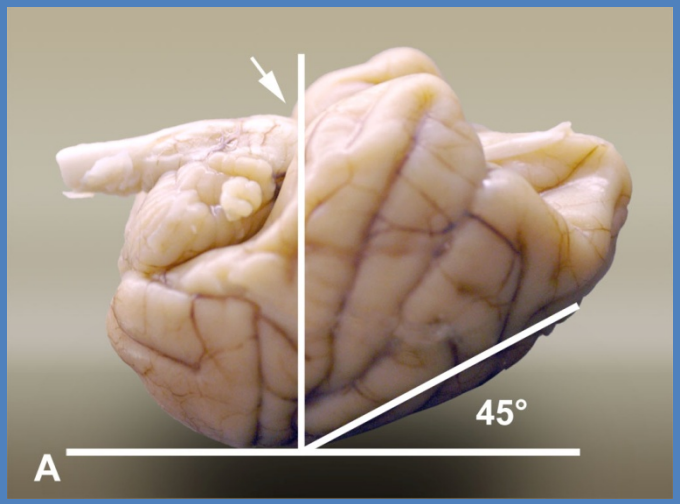


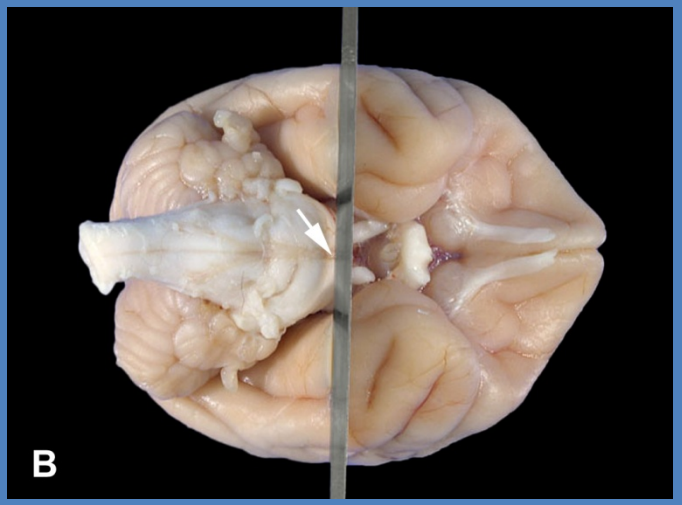
Major Landmarks for Level Orientation during Brain Sampling in GLP-type Nonclinical General Toxicity Studies in Adult Rodents

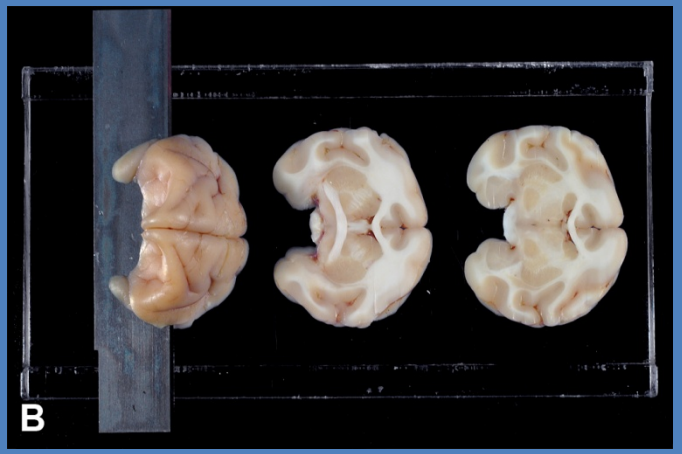
The table shows brain structures visible at given levels (using features visible when the organ is trimmed near the planes in rat brain Figure).

Levels (from Figure 1)							**Brain Structures** (listed from rostral to caudal)
1	2	3	4	5	6	7	
X							Olfactory Bulb
	X						Anterior Commissure
	X						Septal Nuclei
	X						Caudate / Putamen
	X	X	X				Cerebral Cortex (frontal, parietal, temporal, occipital)
	X	X					Corpus Callosum
	X	X					Internal Capsule
	X	X					External capsule
		X					Optic Tract
		X					Amygdala
		X	X				Hippocampus
		X					Thalamus
		X					Hypothalamus
		X	X				Cerebral Peduncles
			X				Midbrain, Rostral
				X			Midbrain, Caudal
				X			Pons
				X	X	X	Pyramids
				X	X	X	Cerebellum
					X		Deep cerebellar nuclei
			X	X	X	X	Reticular formation
					X	X	Trigeminal Nuclei & Tracts
						X	Medulla oblongata
		X				X	Choroid plexus

Brain sectioning NHP

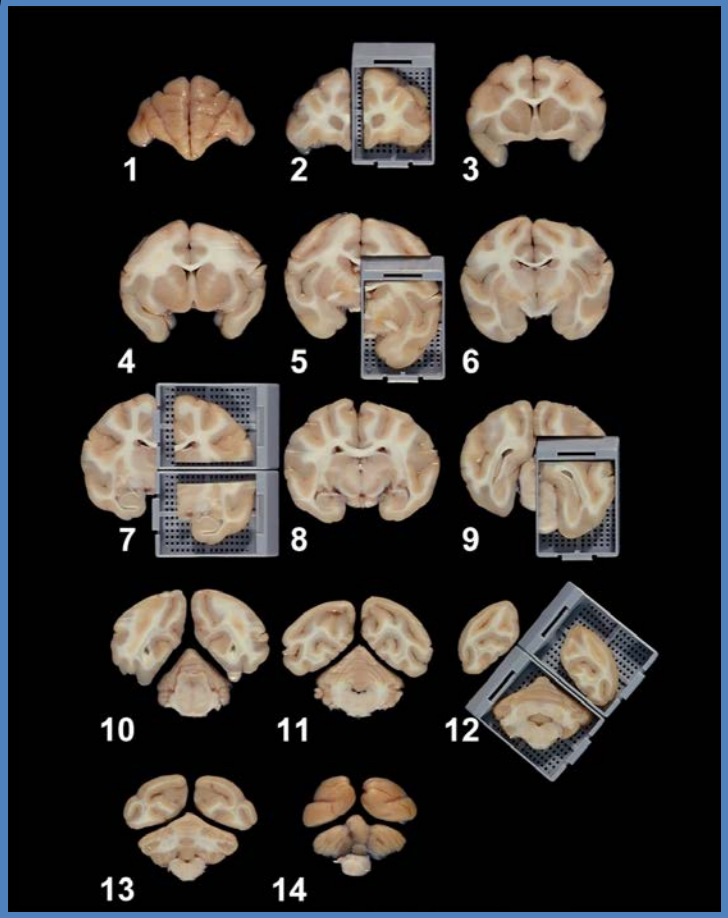


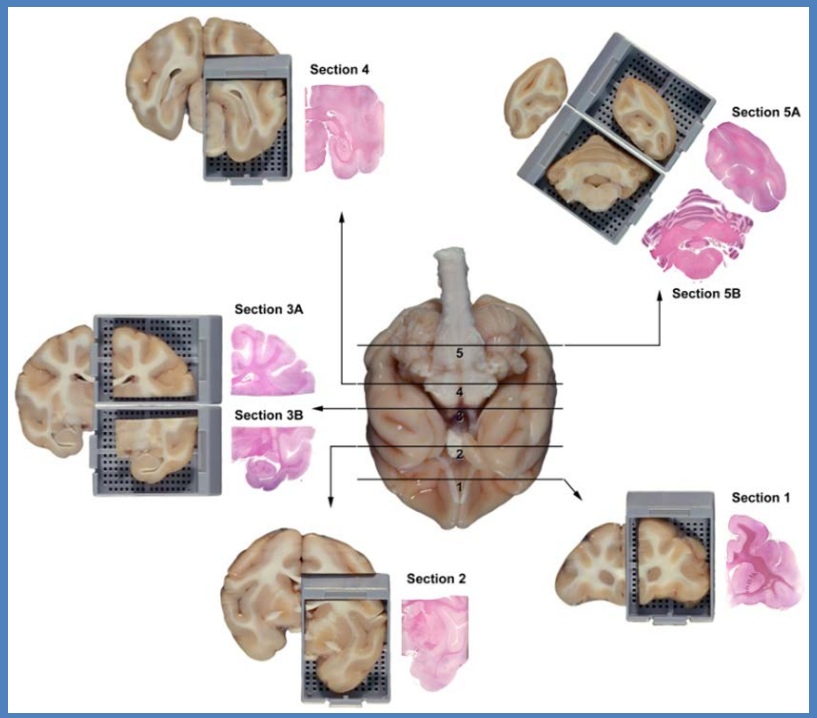




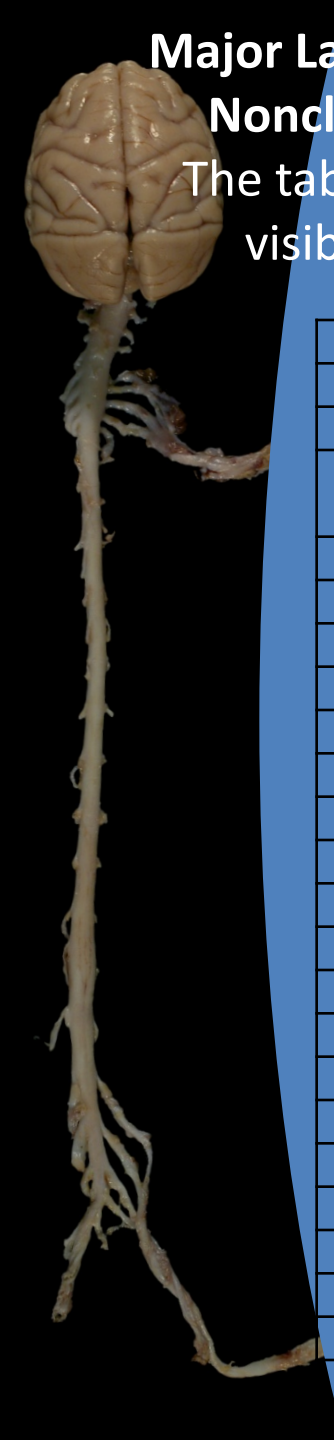
Pardo et al., Tox Path 2012

Brain sectioning NHP





Adapted from Pardo et al., Tox Path 2012

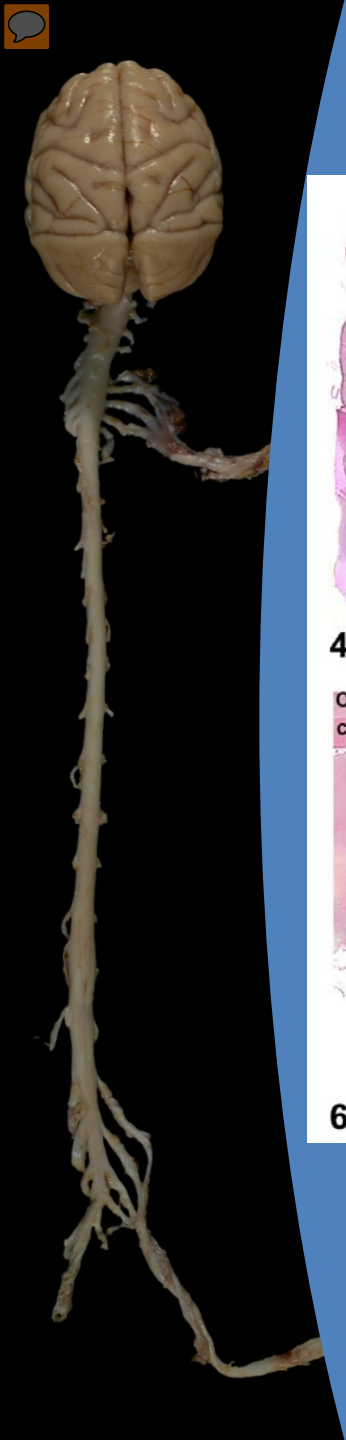


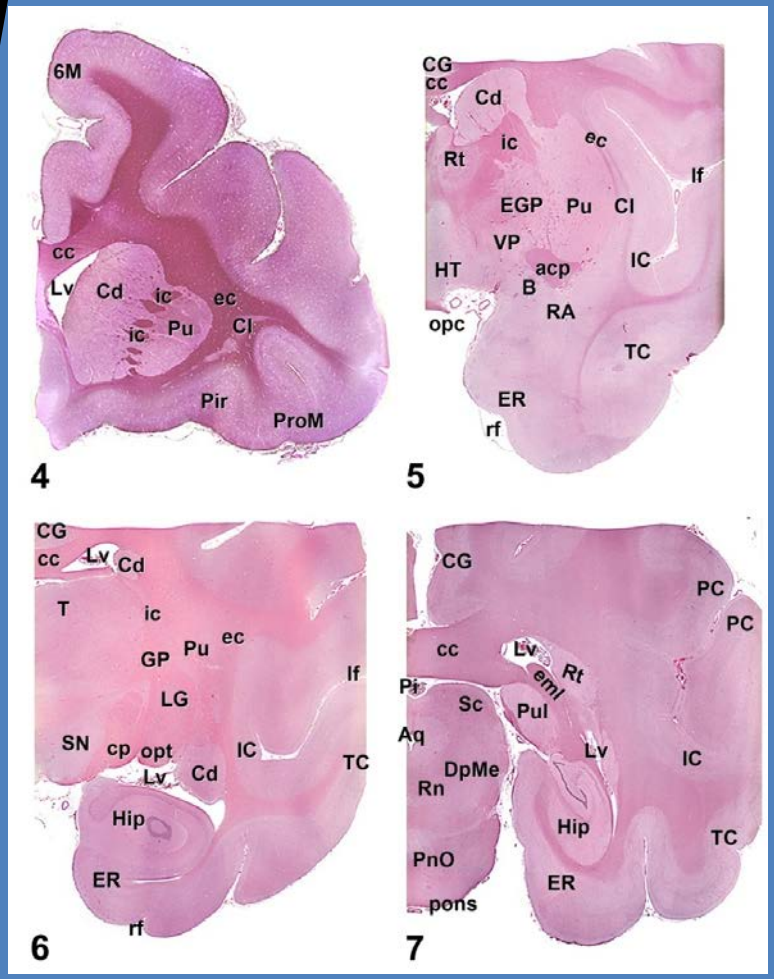
Major Landmarks for Level Orientation during Brain Sampling in GLP-type Nonclinical General Toxicity Studies in Adults of Non-Rodent Species

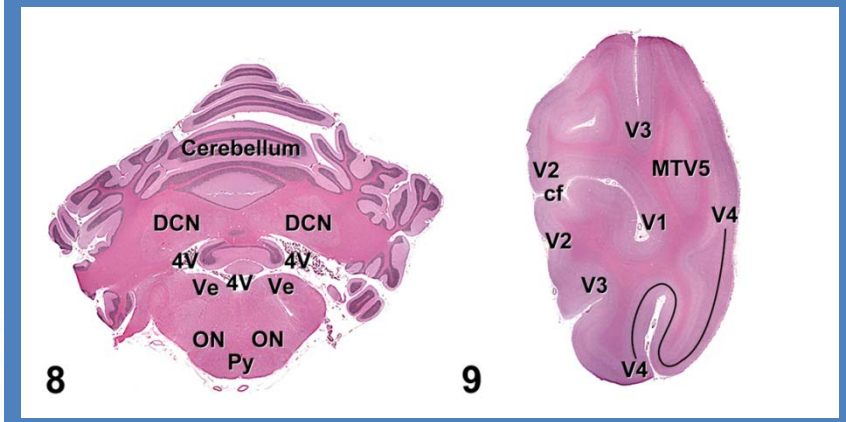
The table shows brain structures visible in given sections (using features visible when the organ is trimmed at the levels in the NHP Figure).

Sections (from Figure 2)							**Brain Structures** (listed from rostral to caudal)
1	2	3A	3B	4	5A	5B	
X	X	X	X				Caudate / Putamen
X	X	X	X	X	X		Cerebral Cortex (frontal, parietal, temporal, occipital)
X	X	X					Corpus Callosum
	X						Anterior Commissure
	X						Septal Nuclei
	X	X	X				Internal Capsule
	X	X	X				External capsule
	X		X				Hypothalamus
	X						Amygdala
		X	X	X			Thalamus
			X	X			Hippocampus
			X	X			Cerebral Peduncles
			X				Optic Tract
				X			Midbrain
				X			Pons
						X	Pyramids
						X	Cerebellum
				X		X	Reticular Formation
						X	Trigeminal Nuclei
						X	Medulla oblongata
	X	X	X	X		X	Choroid plexus

Histologic sections of the brain in NHP

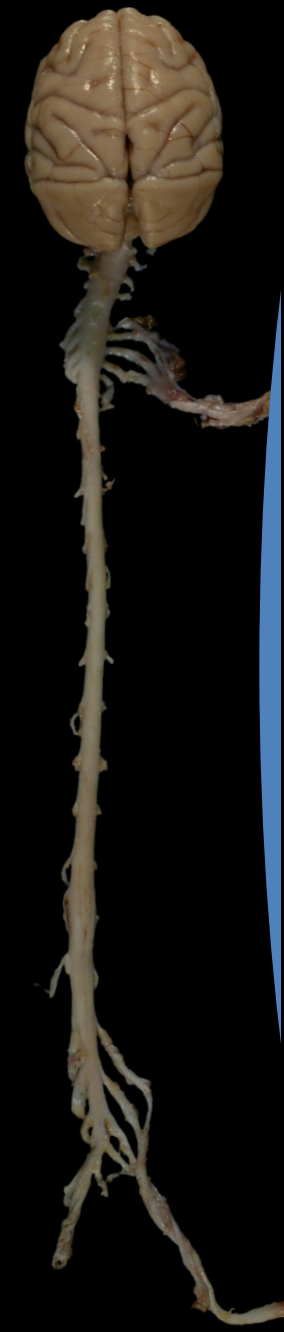


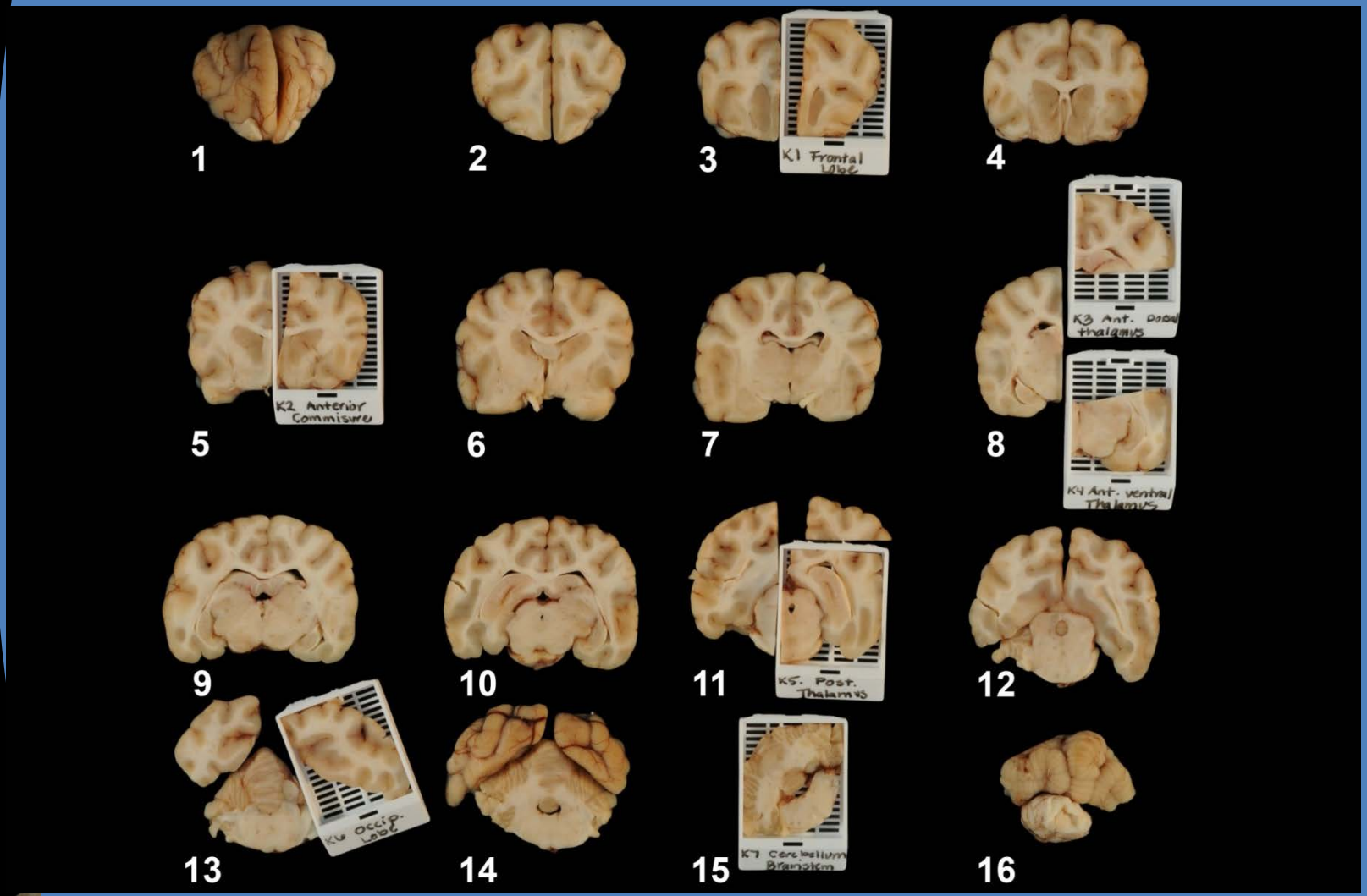




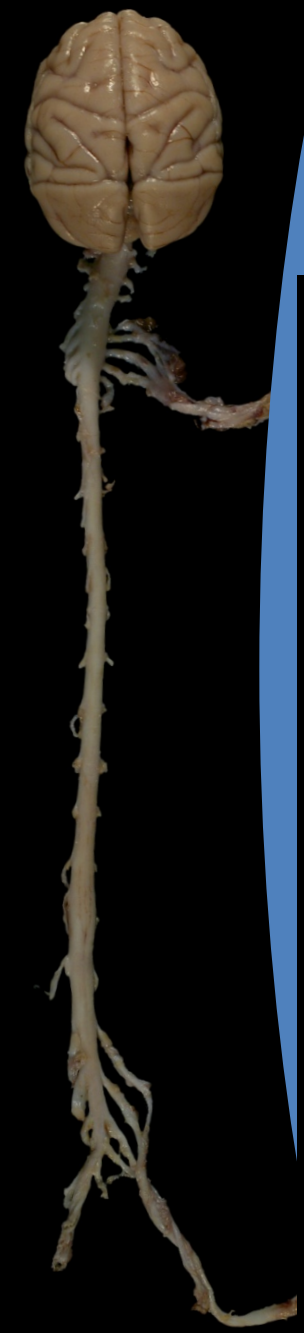
Pardo et al., Tox Path 2012

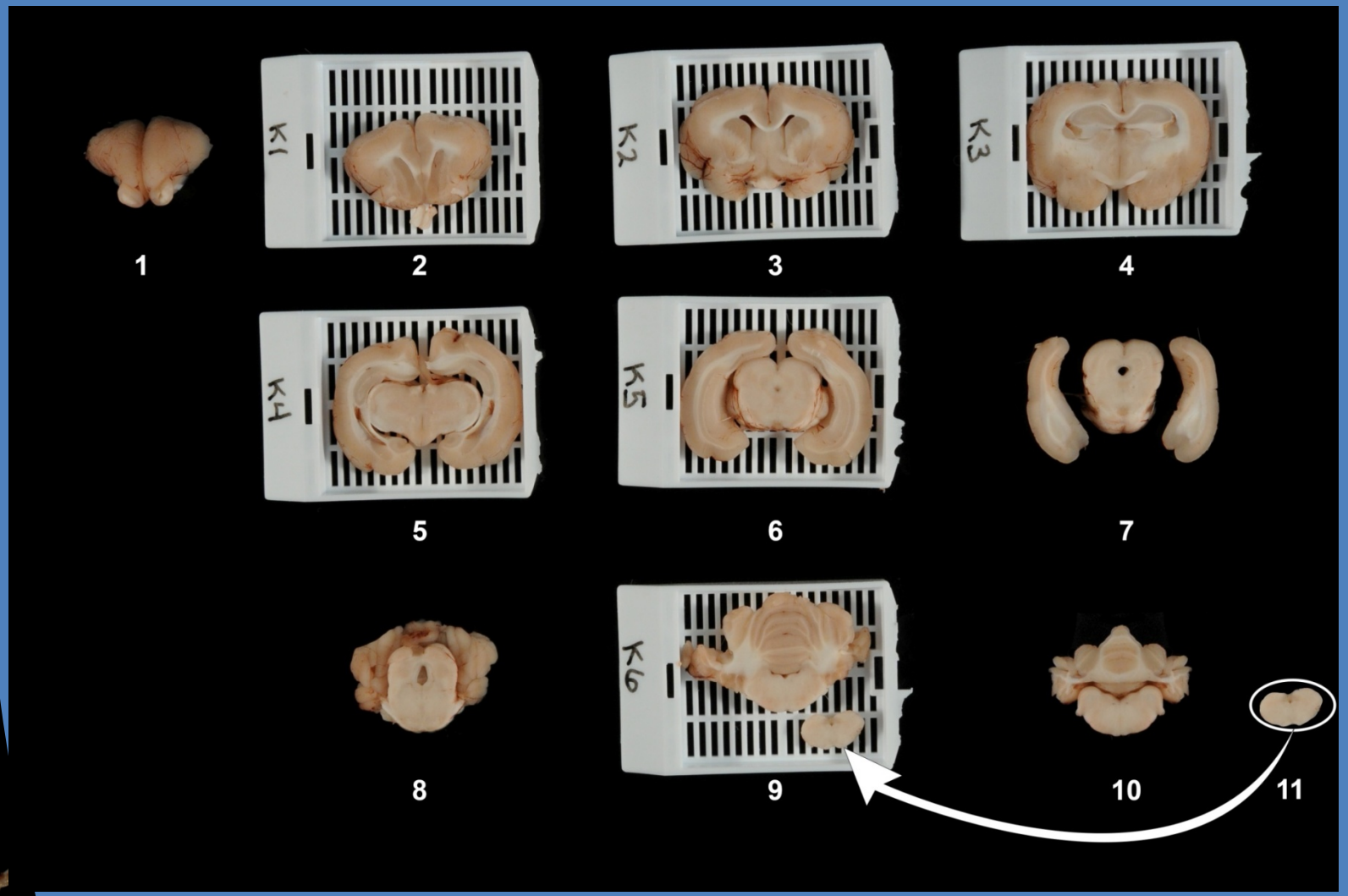
Dog Brain Sectioning



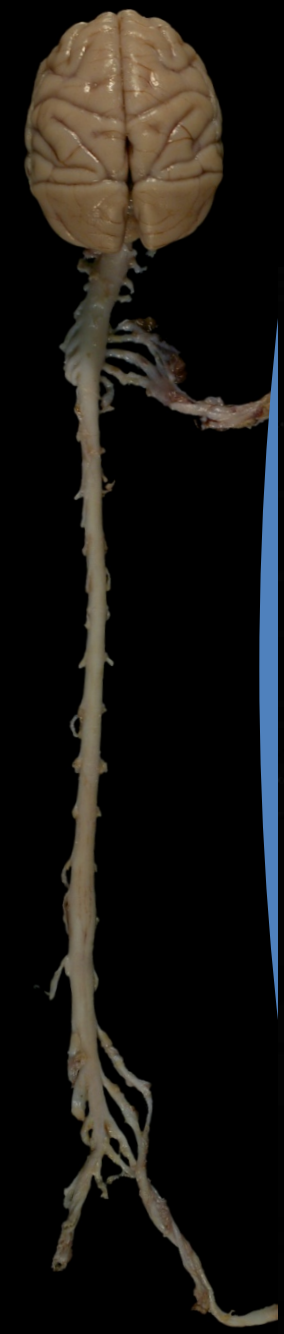


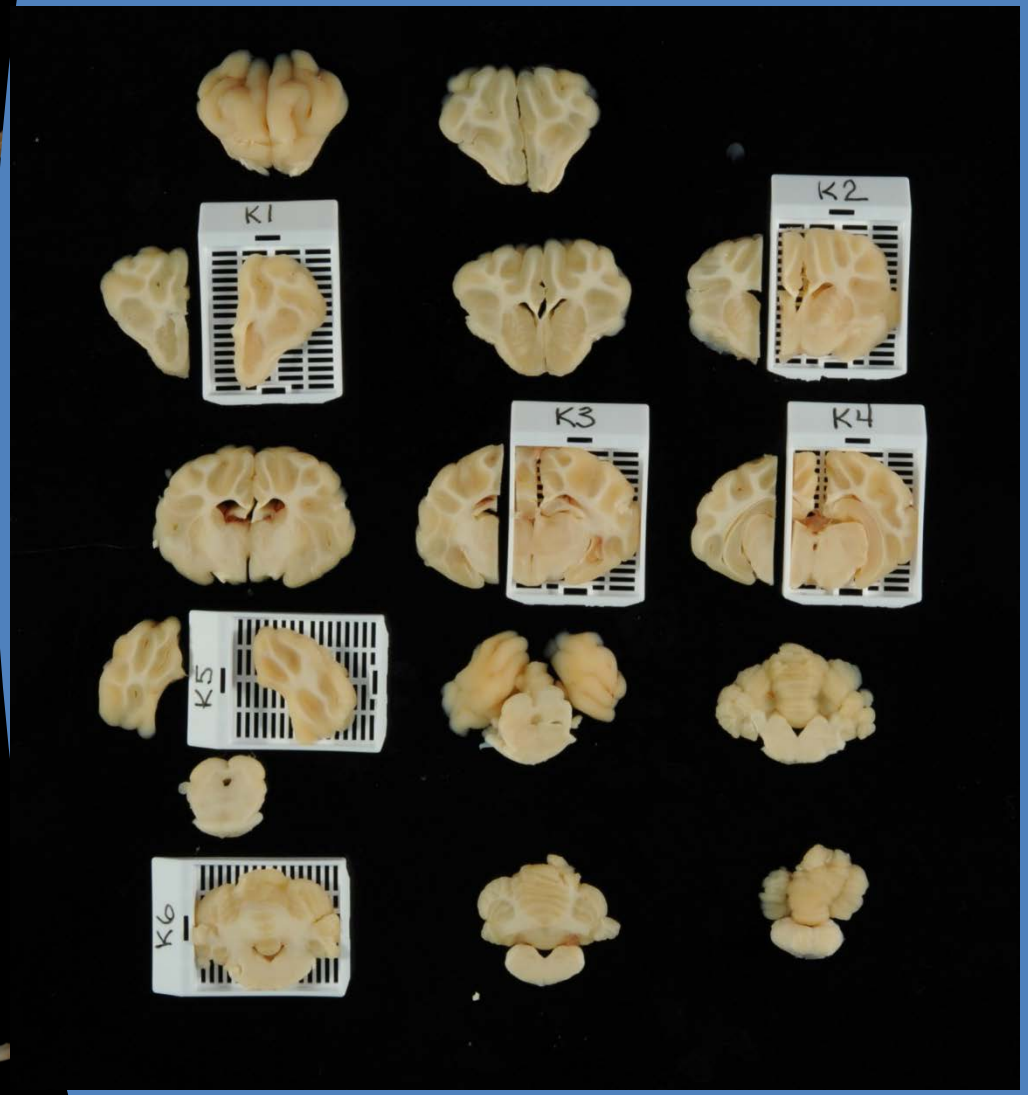
Rabbit Brain Sectioning

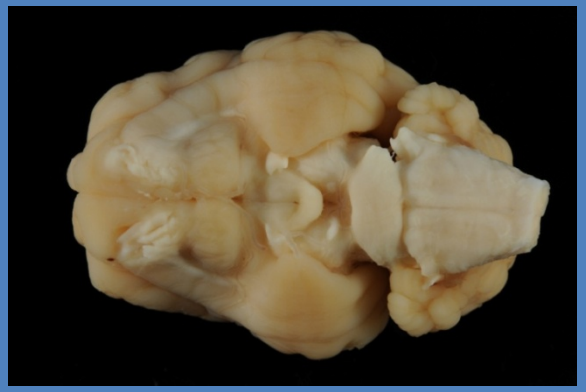


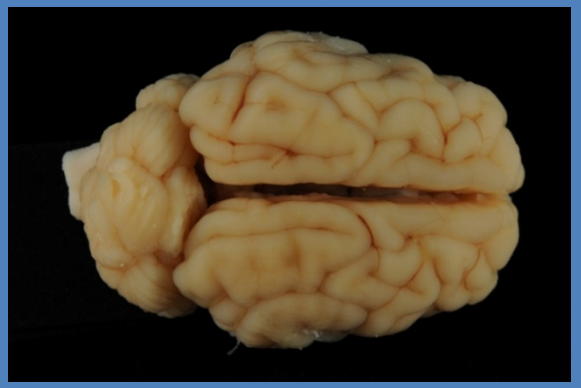


Minipig Brain Sectioning

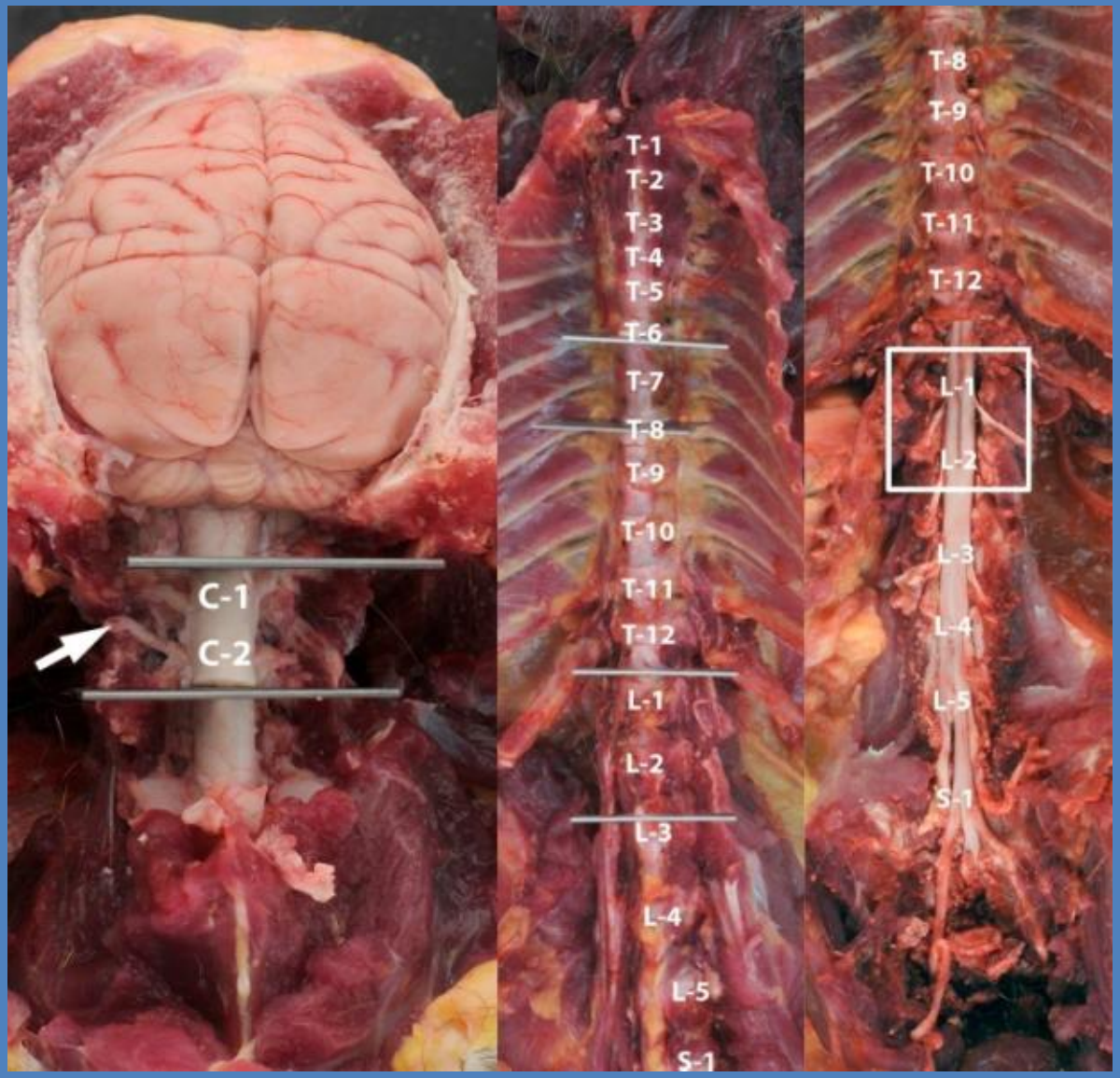






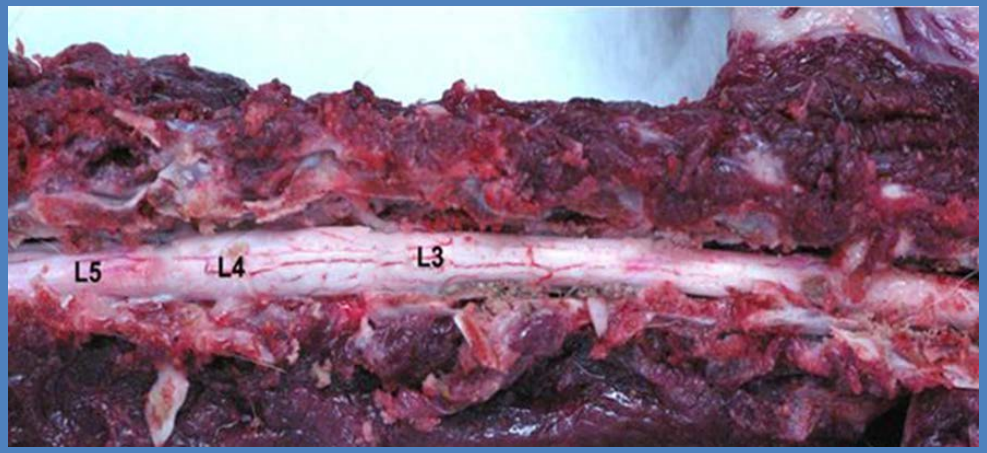


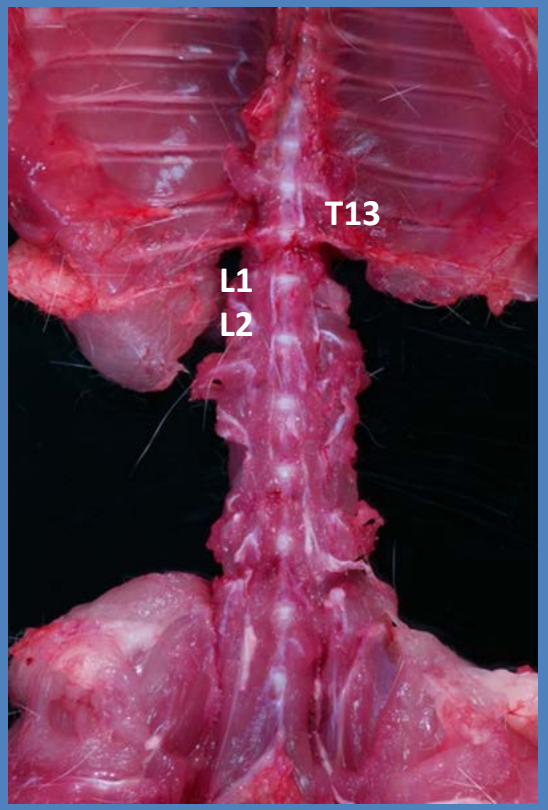
Monkey spinal cord sections



Bolon et. al., Tox Path 2013

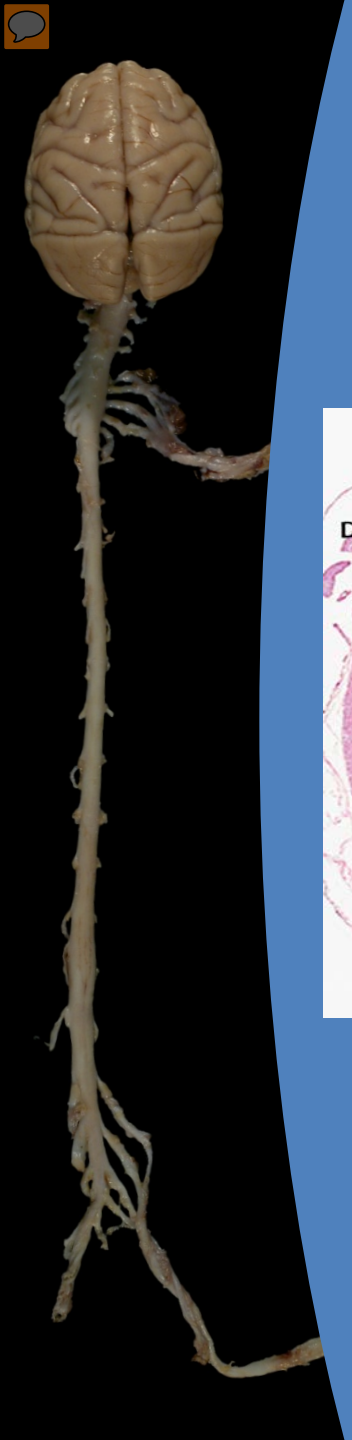
Spinal cord dog and rodent





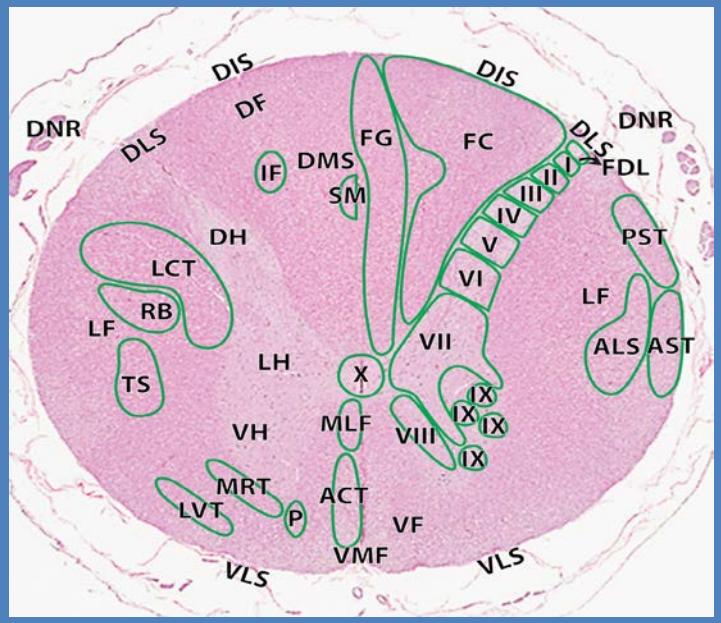
Sectioning the spinal cord NHP transverse and longitudinal (or oblique)

Pardo et. al., Tox Path 2012



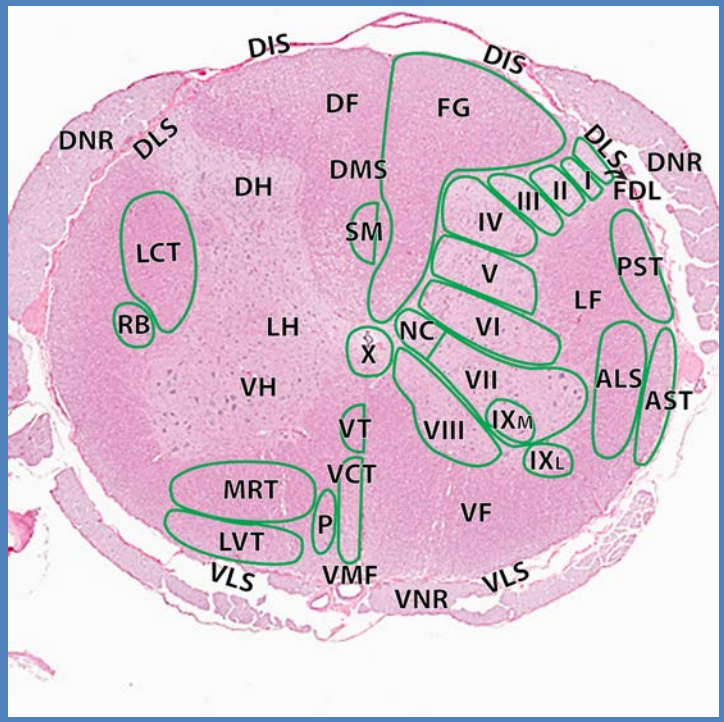
Spinal cord NHP

Cervical cord (C1-C2)

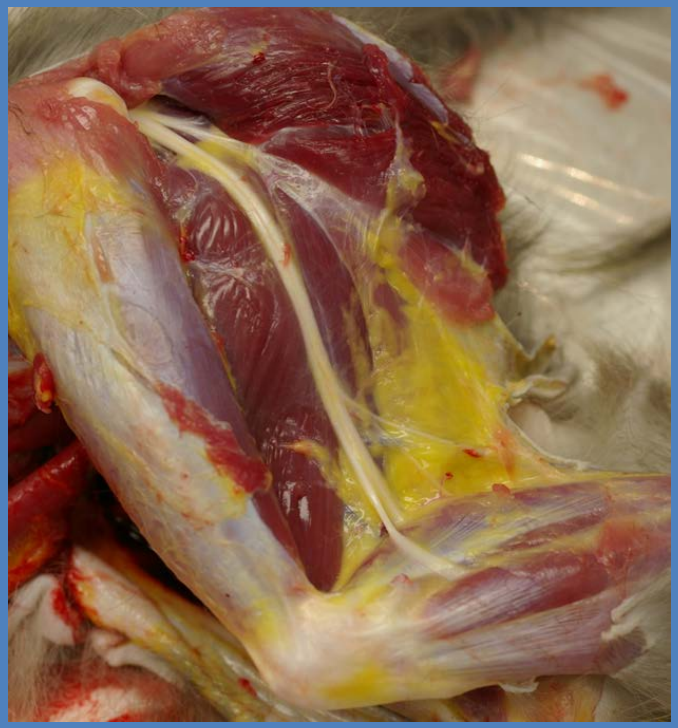


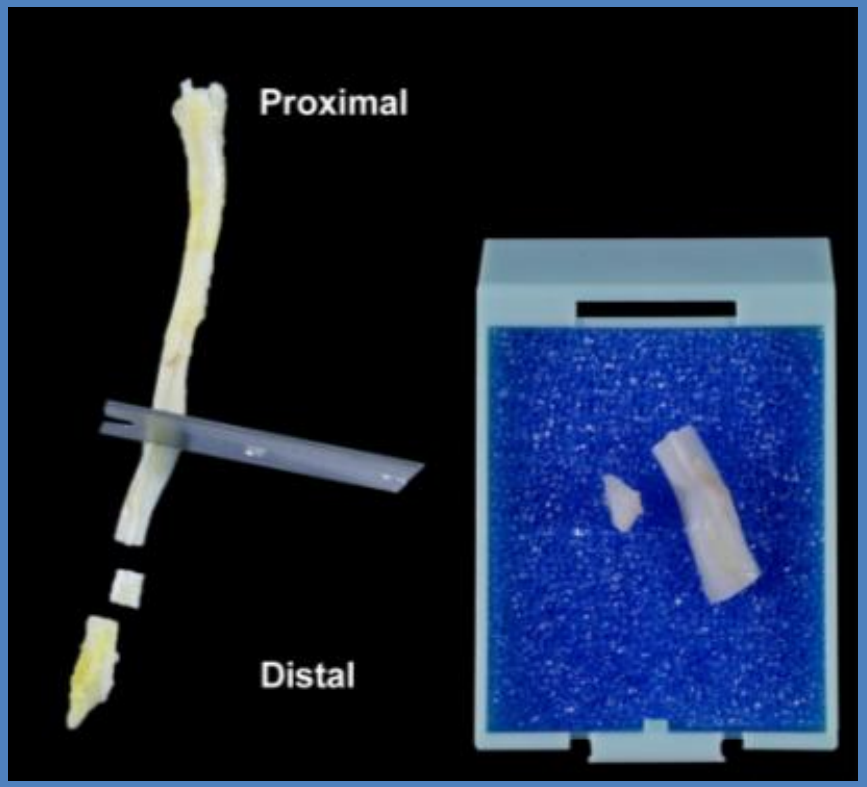
Pardo et. al., Tox Path 2012

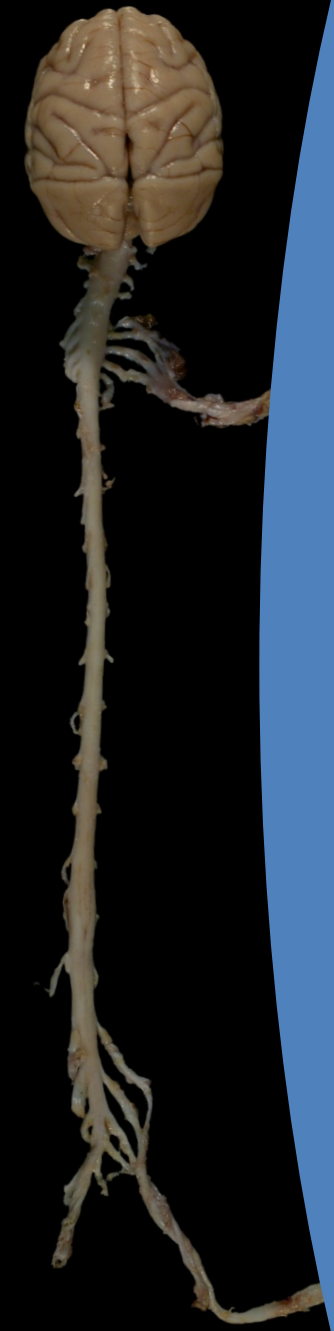
Lumbar cord NHP (L4-L5)



Sectioning the sciatic nerve NHP







Common artifacts and Unusual Normal Structures

Nice references

Toxicologic Pathology, 40: 87S-157S, 2012

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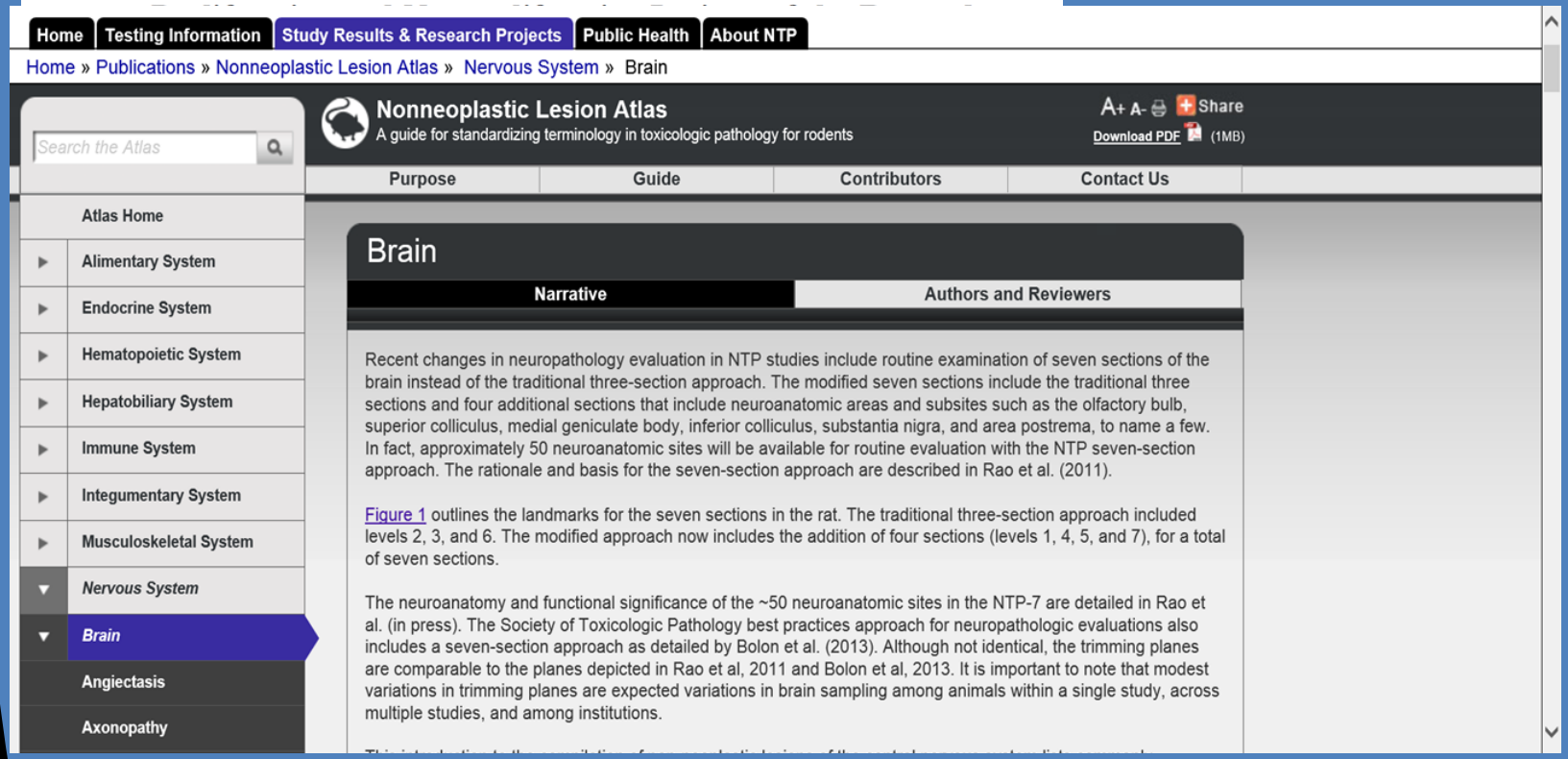
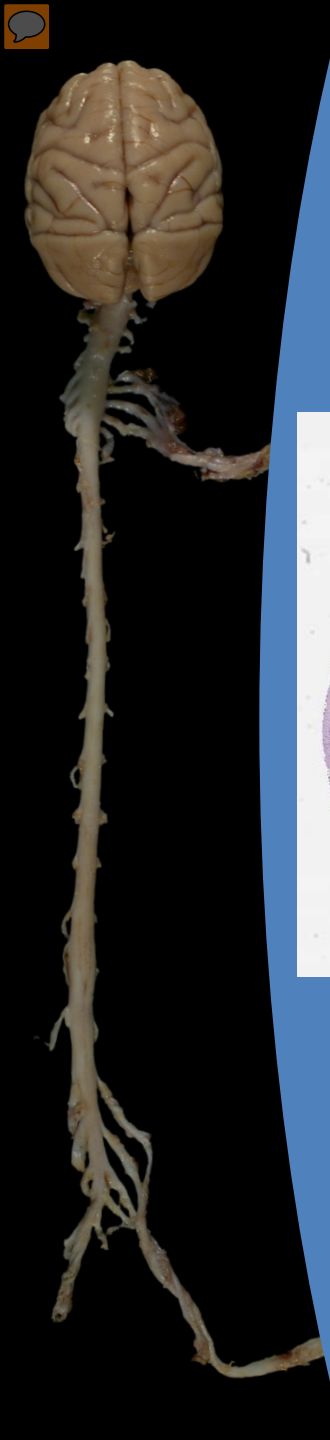
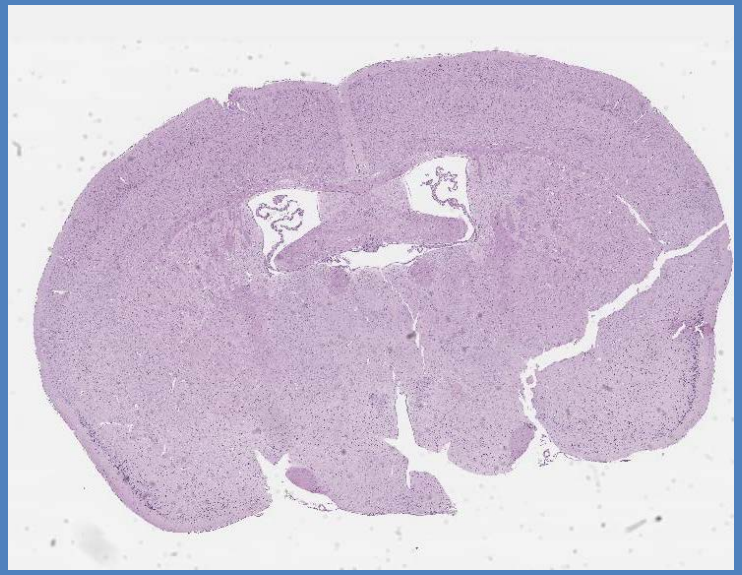
DOI: 10.1177/0192623312439125

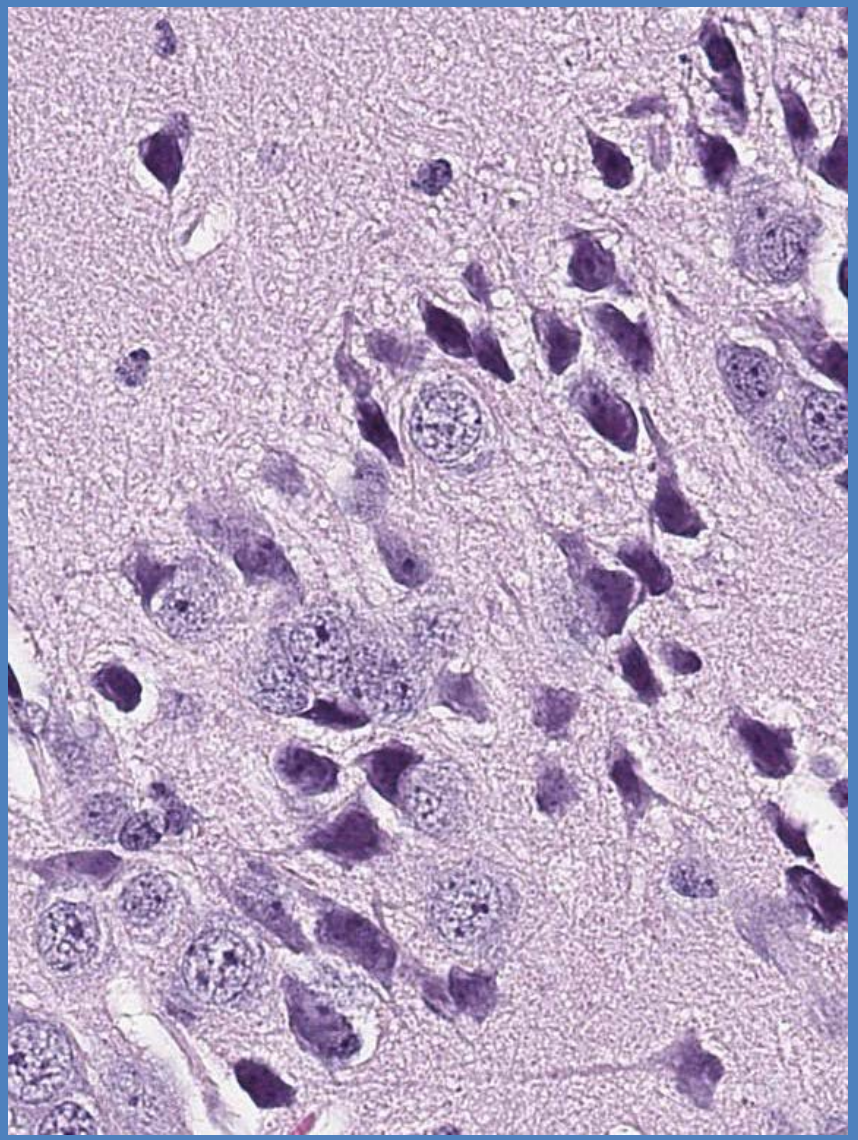


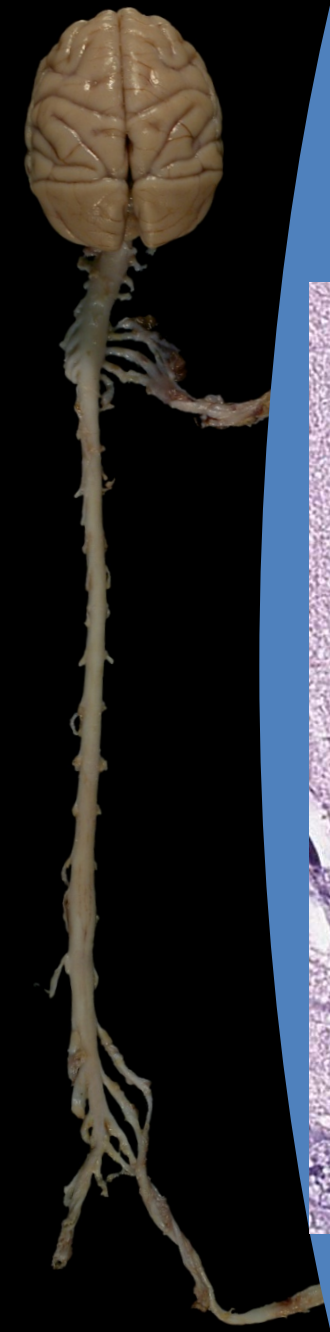
Nonneoplastic Lesion Atlas
A guide for standardizing terminology in toxicologic pathology for rodents

Dark Neuron

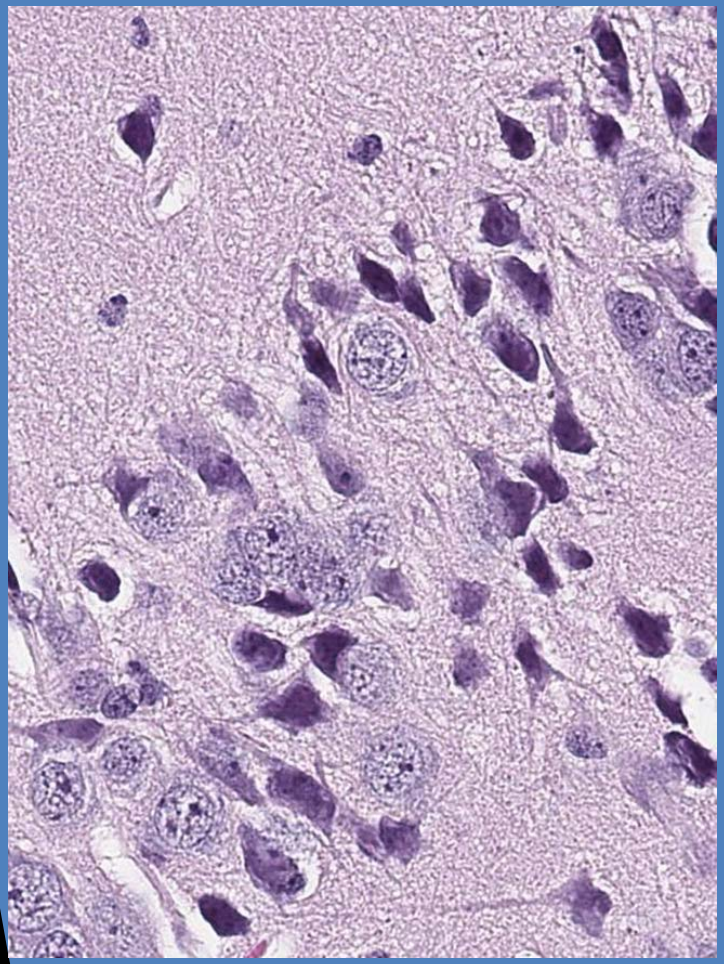


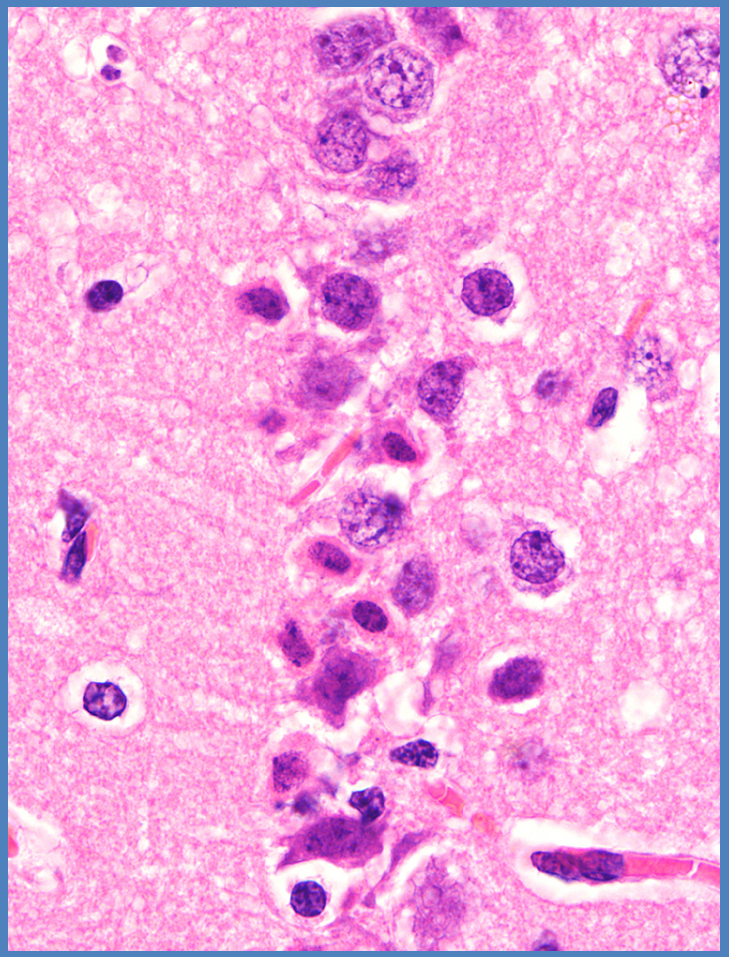




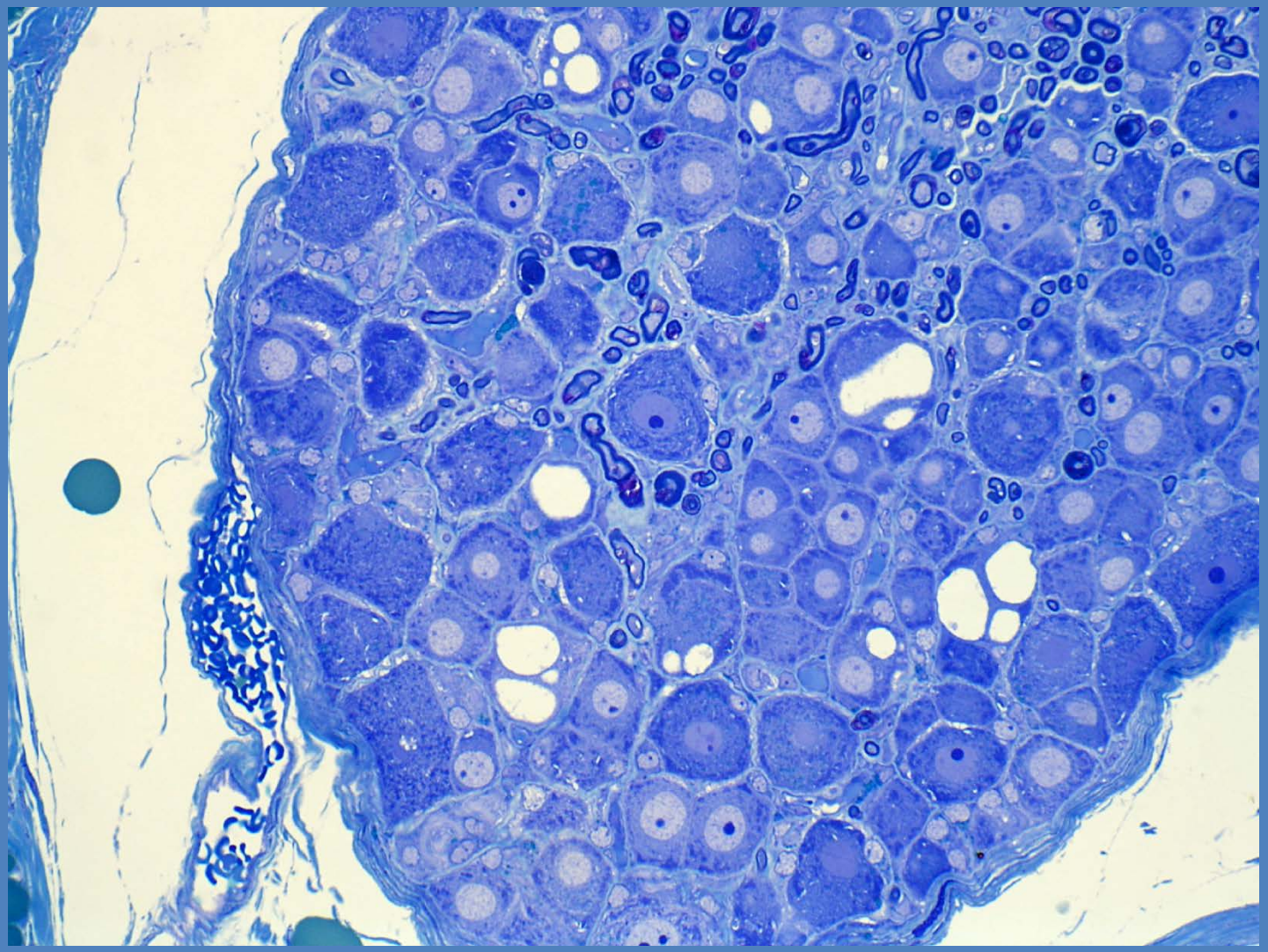


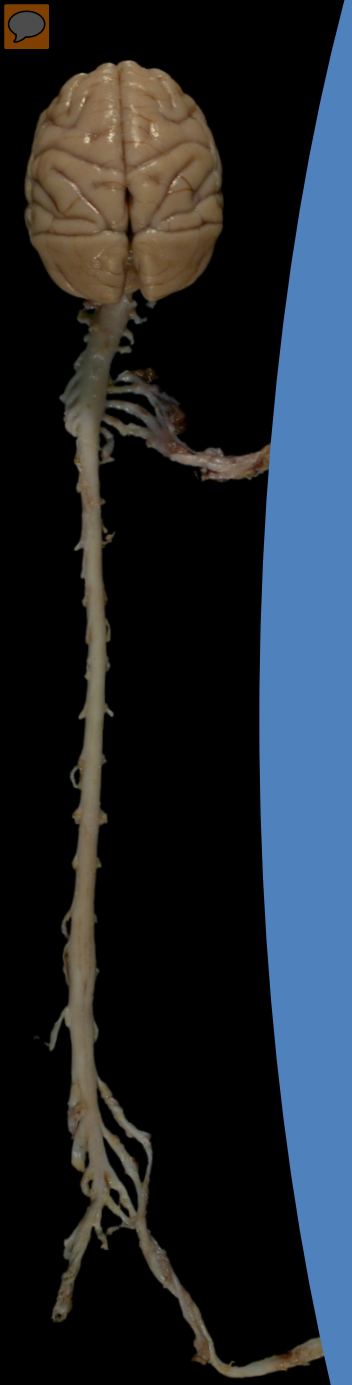
Dark Neurons vs. Necrotic Neurons H&E stain



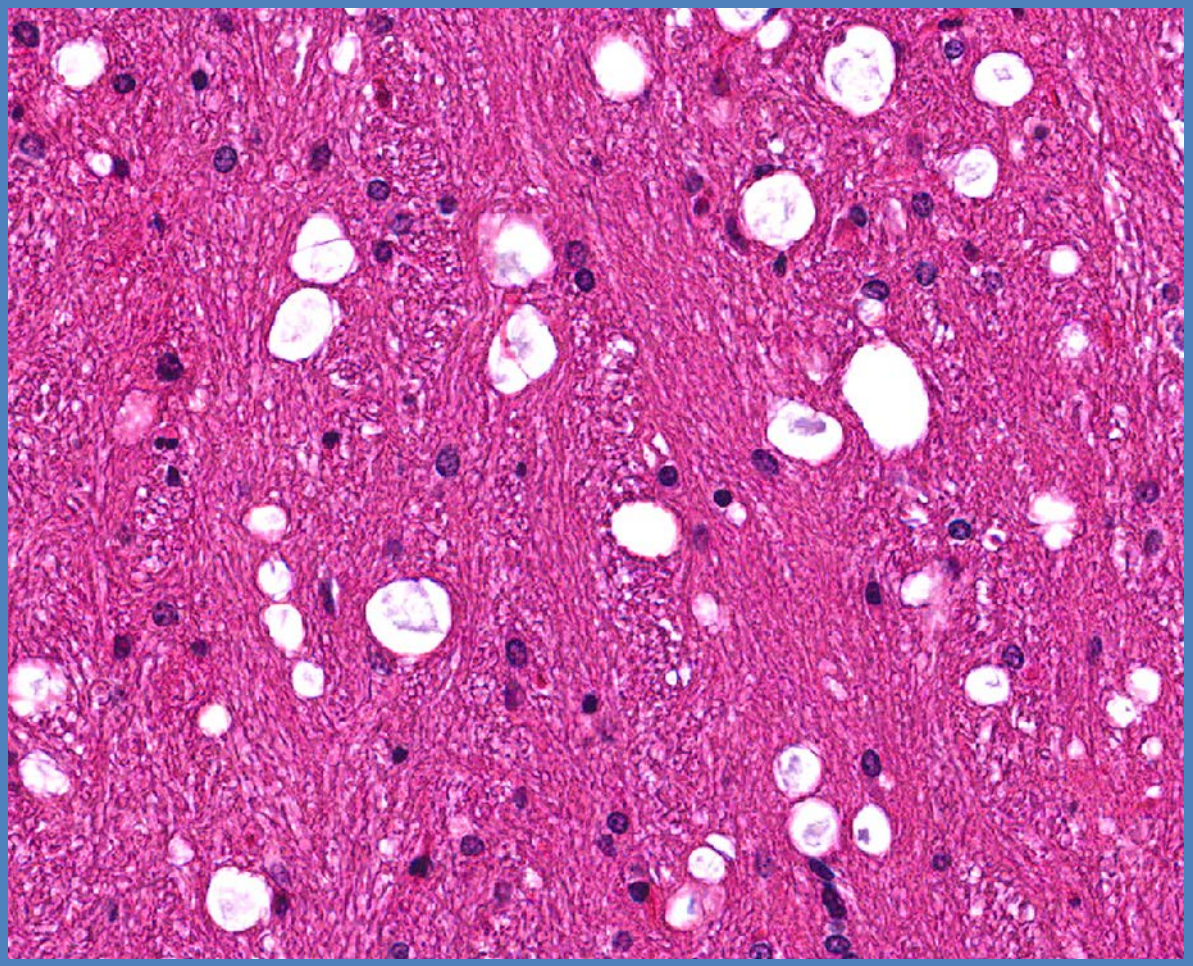


Neuronal Vacuolation
Dorsal root ganglion T-blue



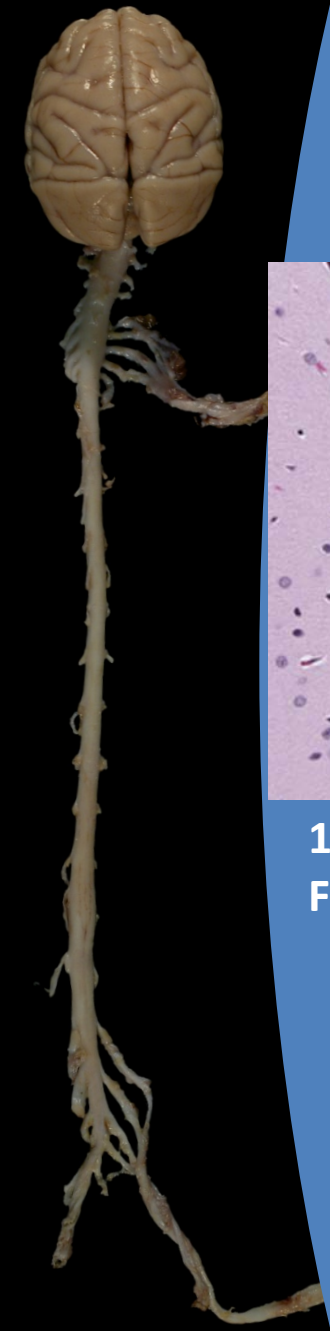


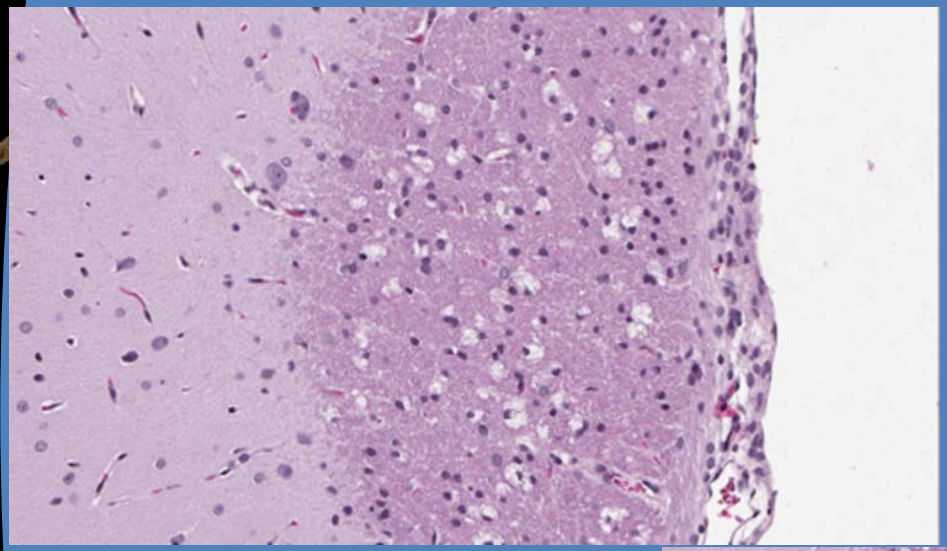
White Matter Vacuolation



Buscaino bodies, mucocytes or metachromatic bodies

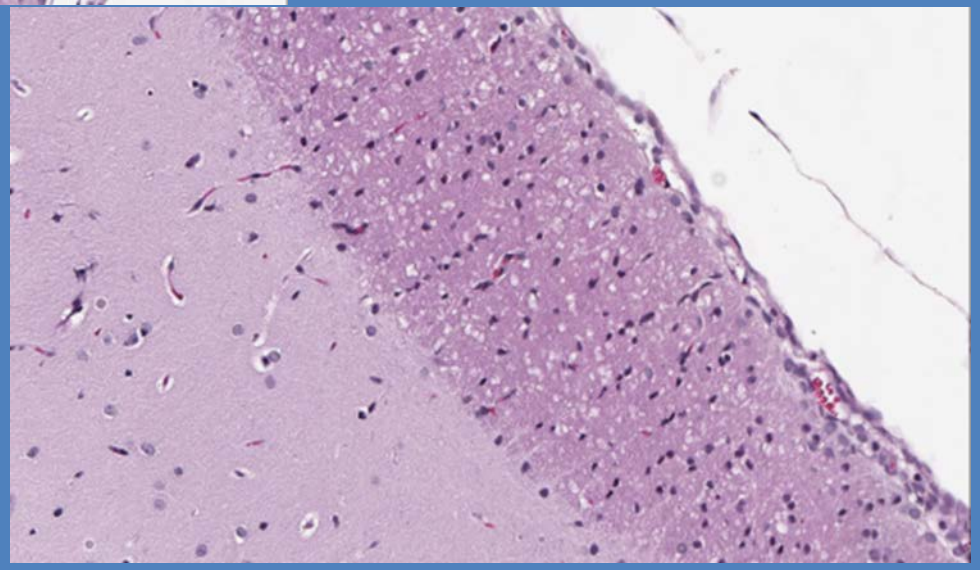
Immersion Fixed Brain (25 days)



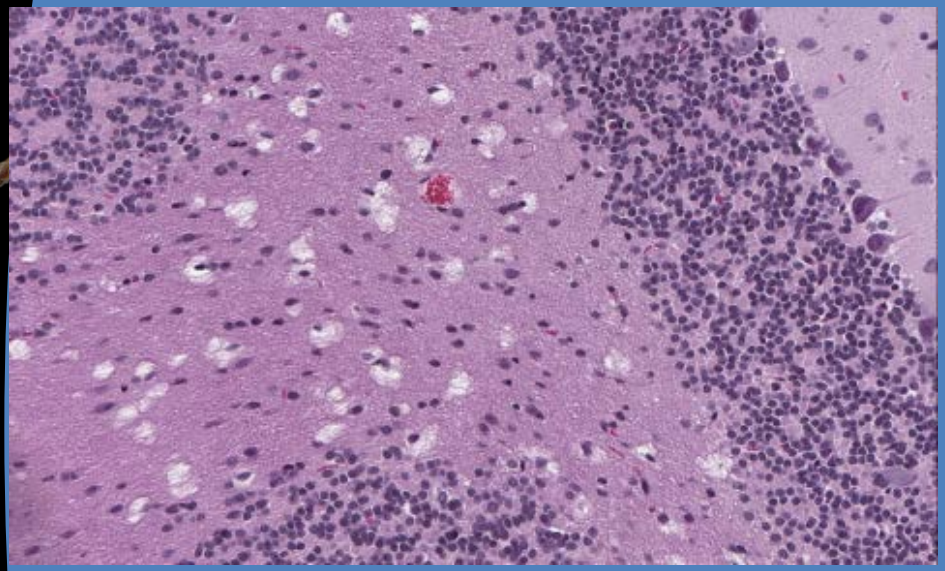


10% Neutral Buffer Formalin (NBF)

4% Paraformaldehyde (PFA)

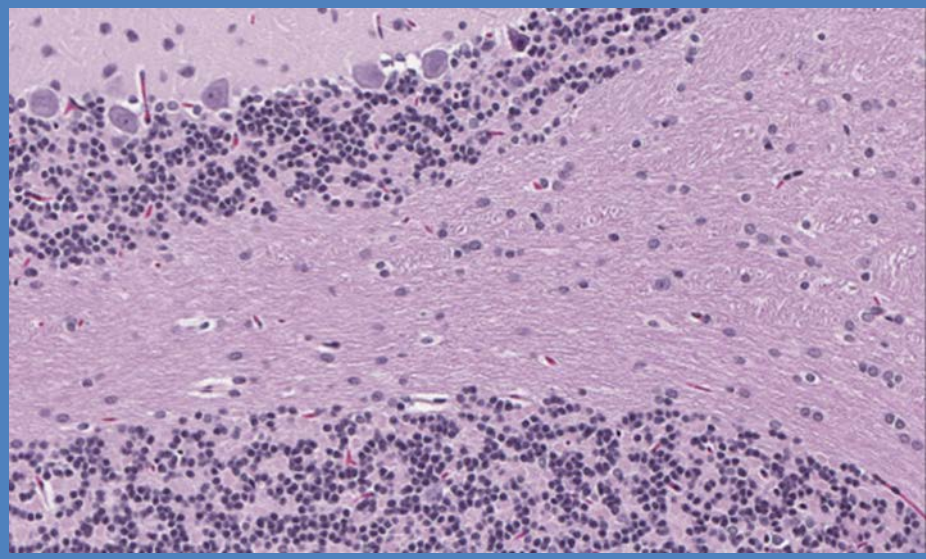


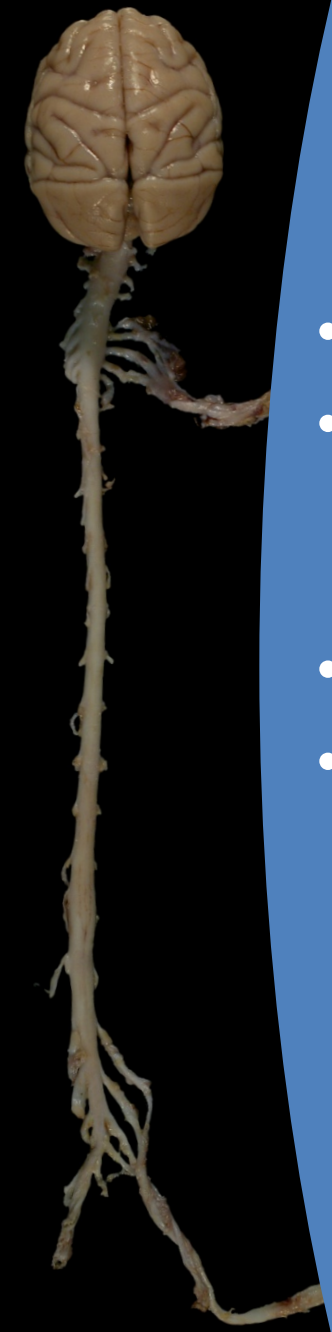
Brain fixed ex-situ vs. in-situ 10% NBF



Brain removed from skull

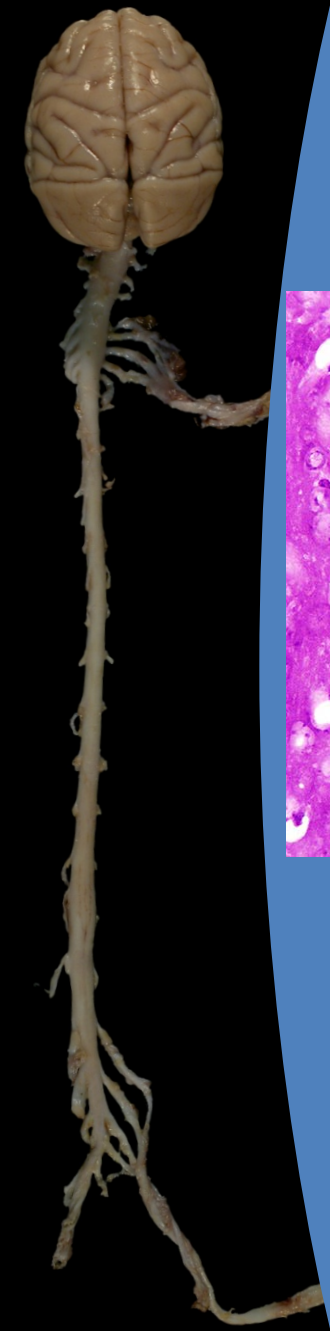
In-situ (calvarium intact)



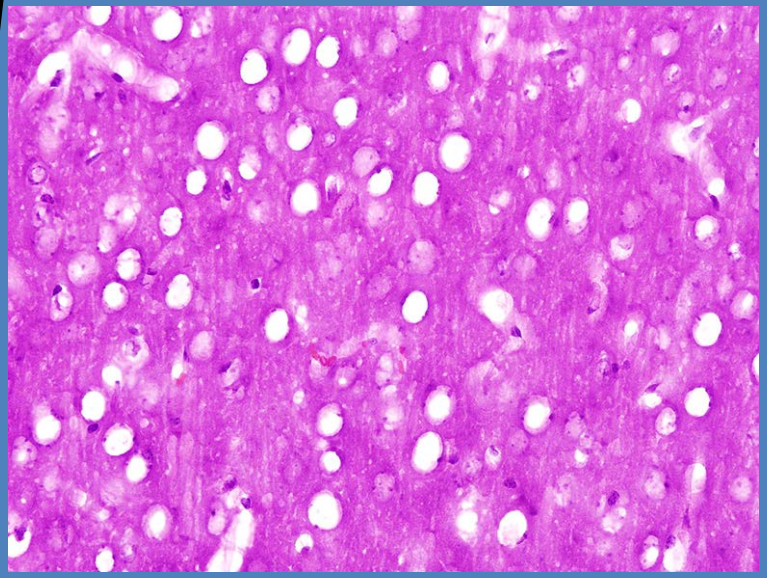


Recommendations

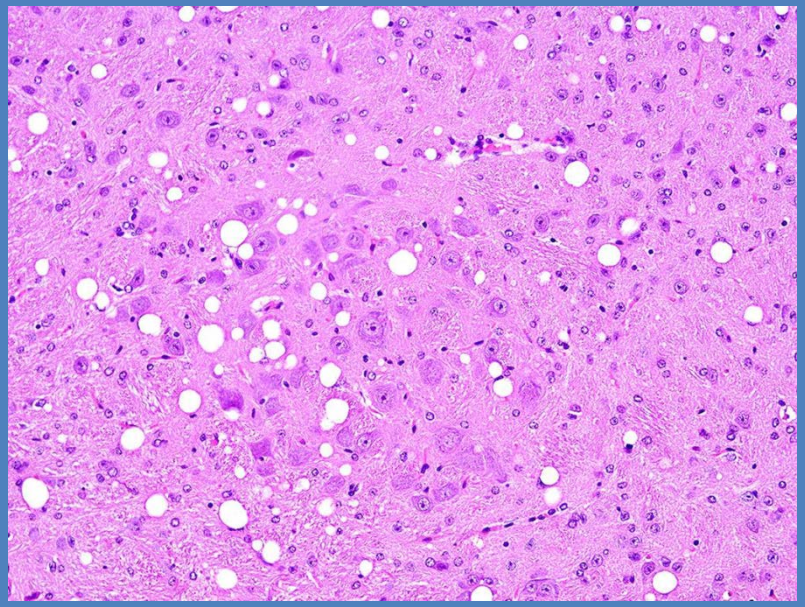
- Handle brain very gentle during necropsy
- Avoid leaving the brain in fixative for a long term (>15 days) before its processed for light microscopic evaluation
- Process all brains together under same conditions
- Leaving the brain in the calvarium appeared to reduce artificial vacuolation



More vacuolation

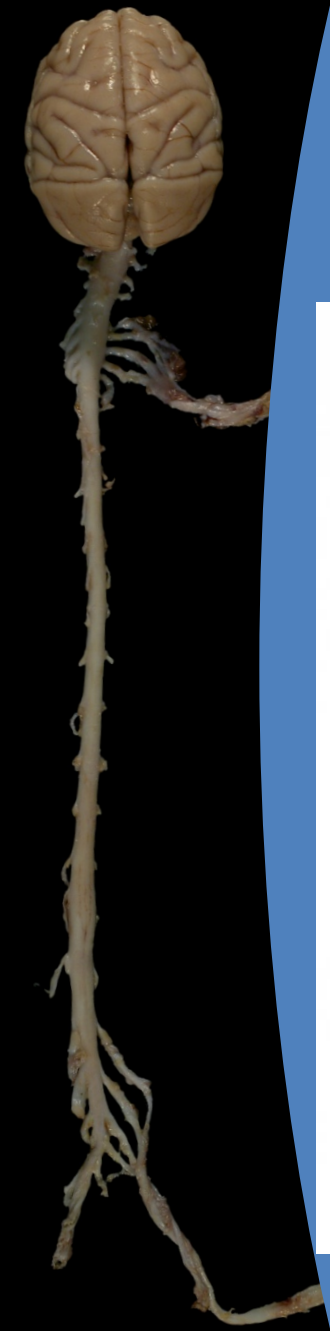


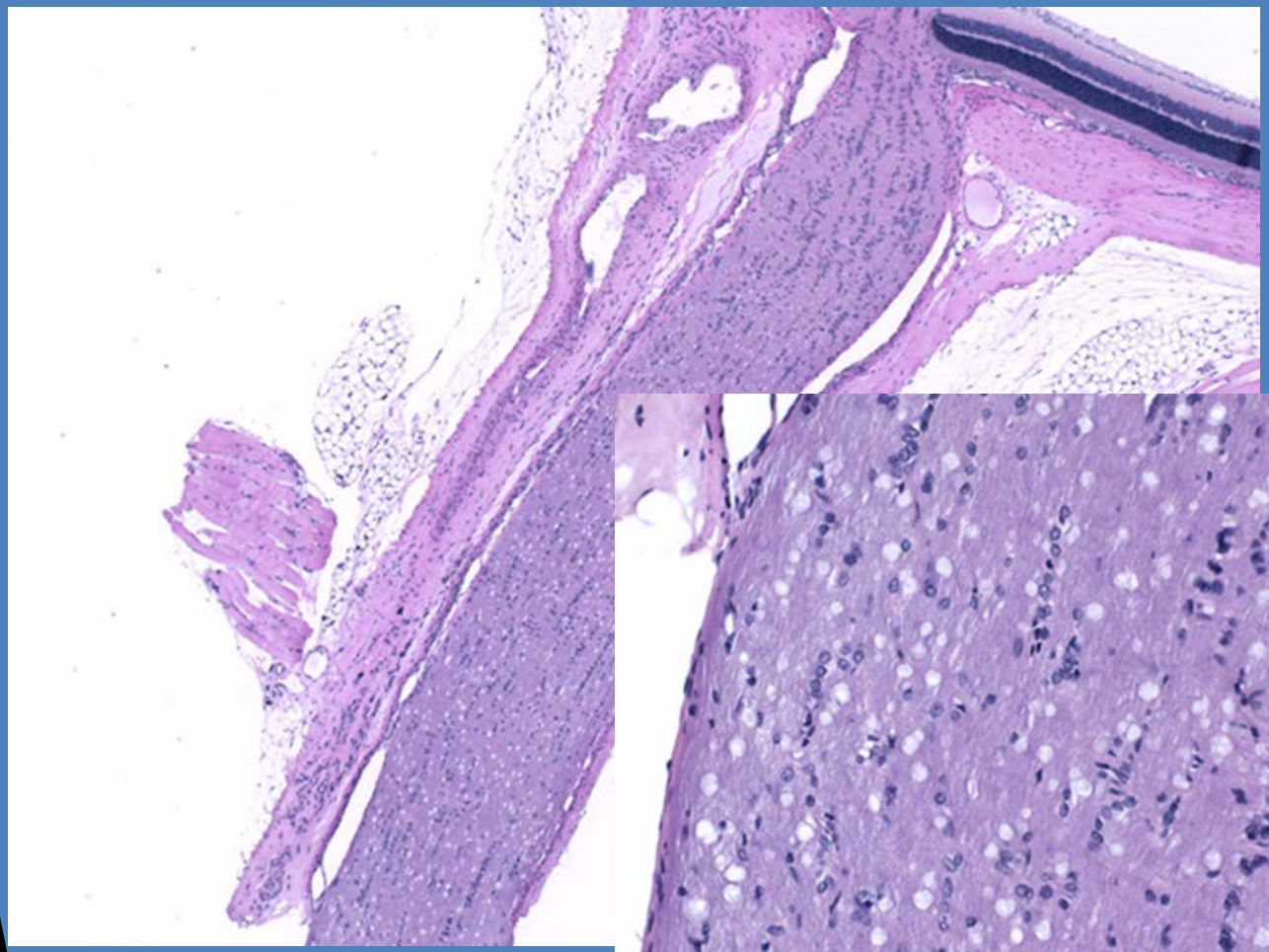
Vacuolation caused by frozen in Sucrose Solution



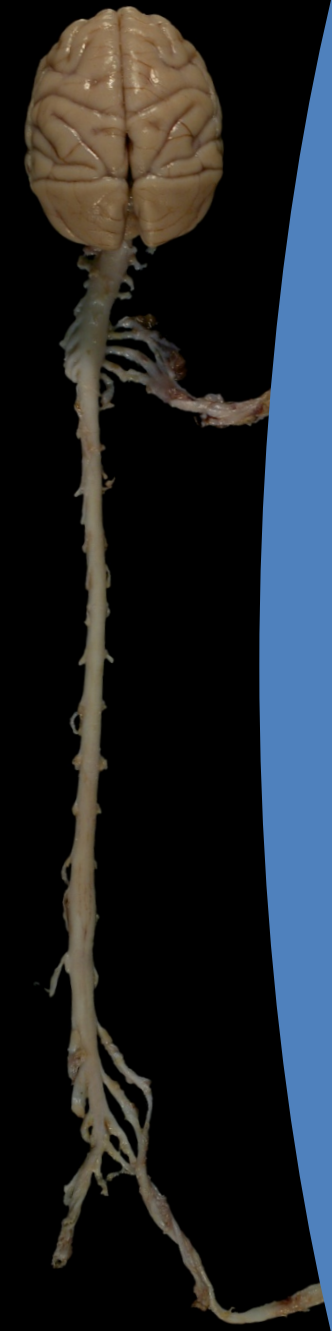
Vacuolation caused by Davidson's Fixation (contains 33% ethanol for 7 days)

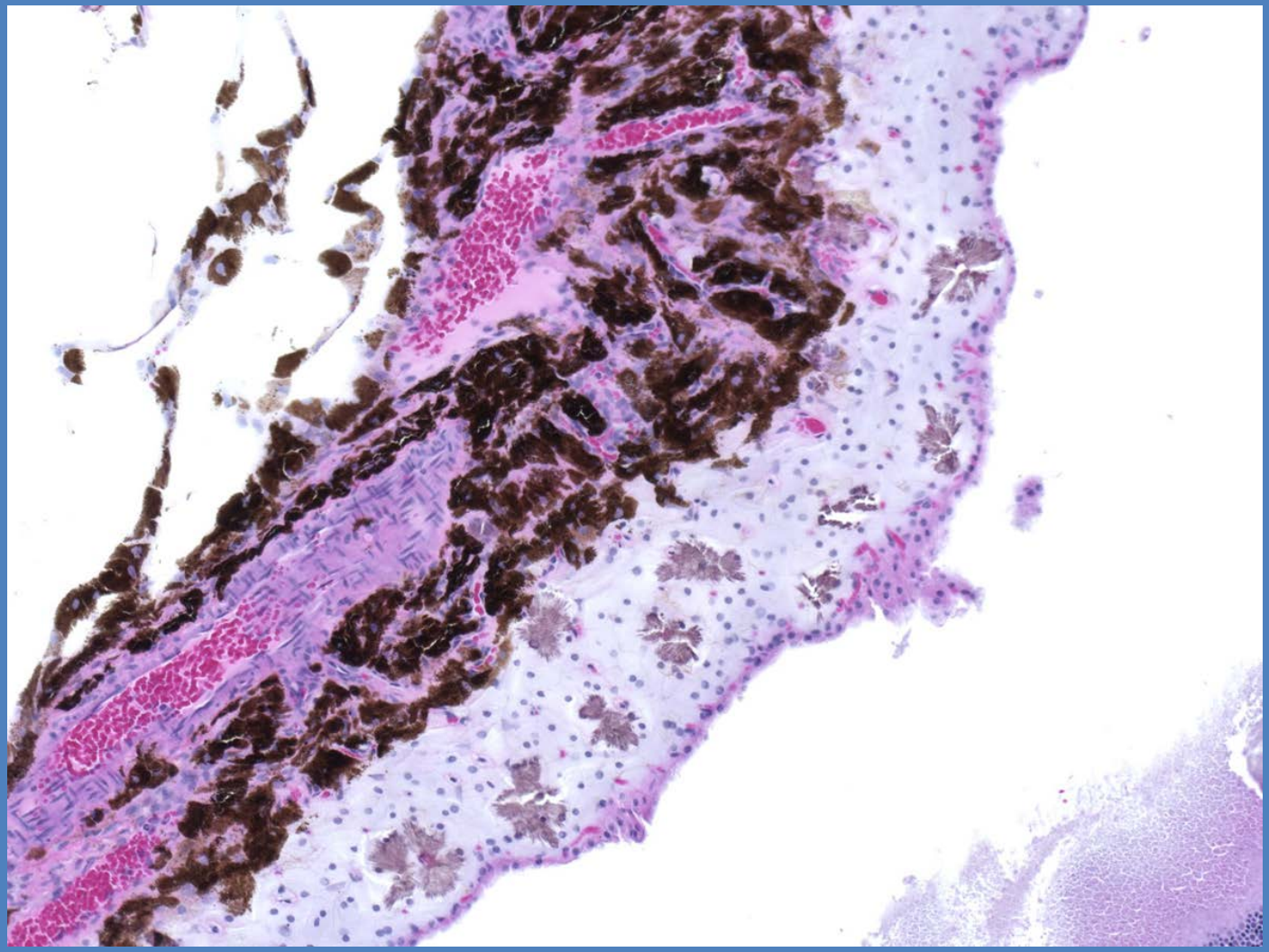
Vacuolation of the optic nerve Davidson's Fixation

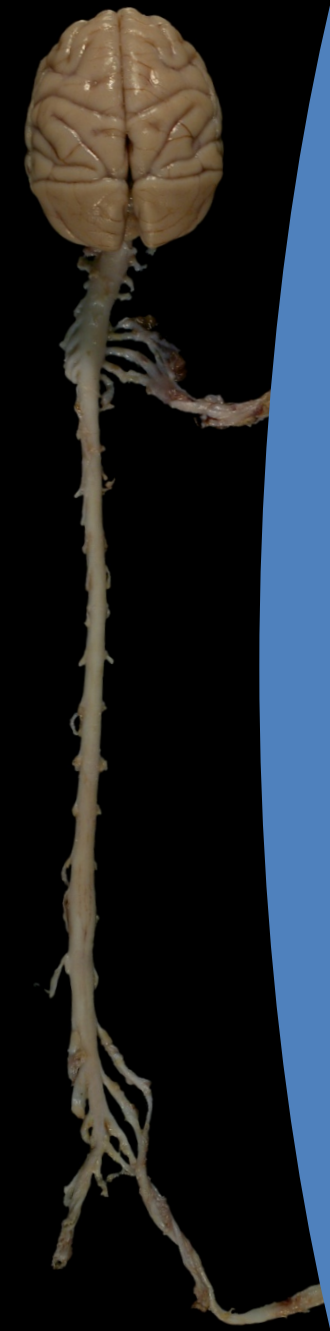




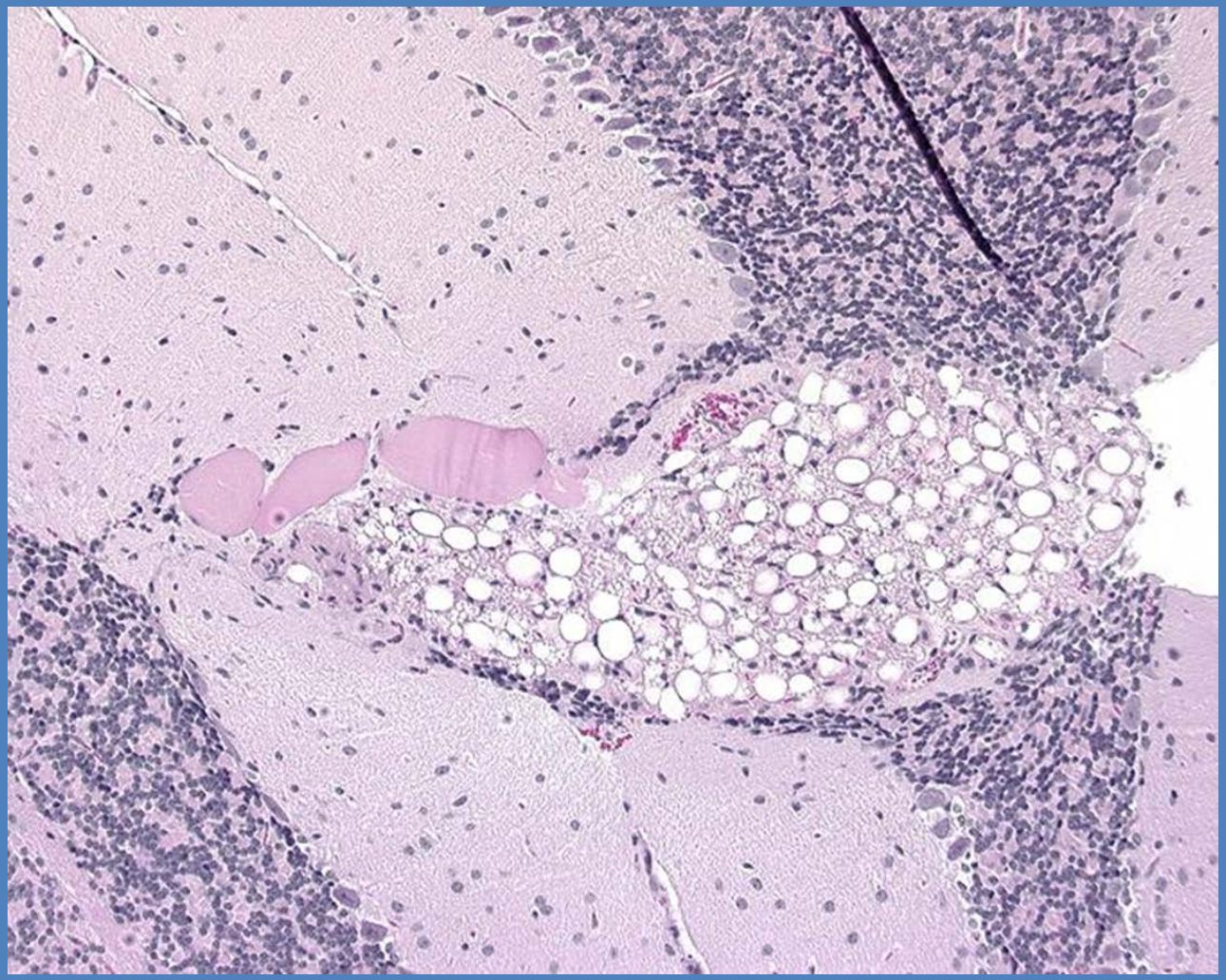
Deposition of crystal in the tapetum lucidum caused by buffer in the fixative



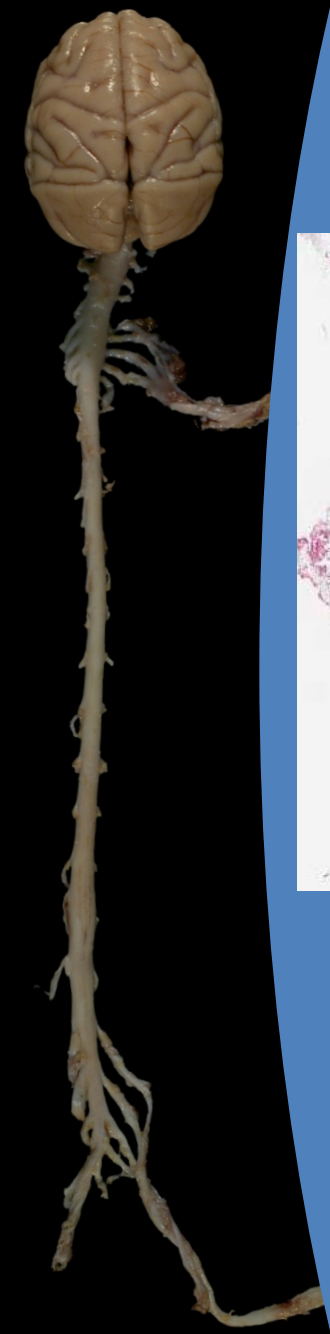


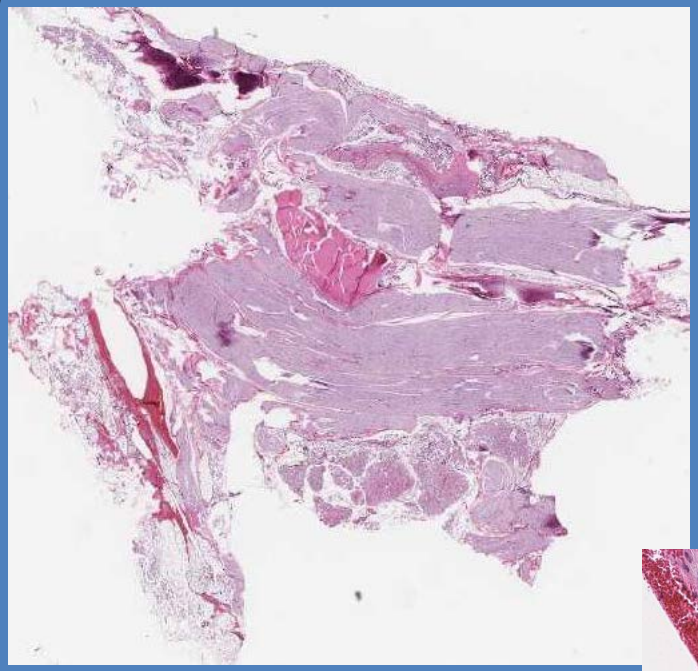


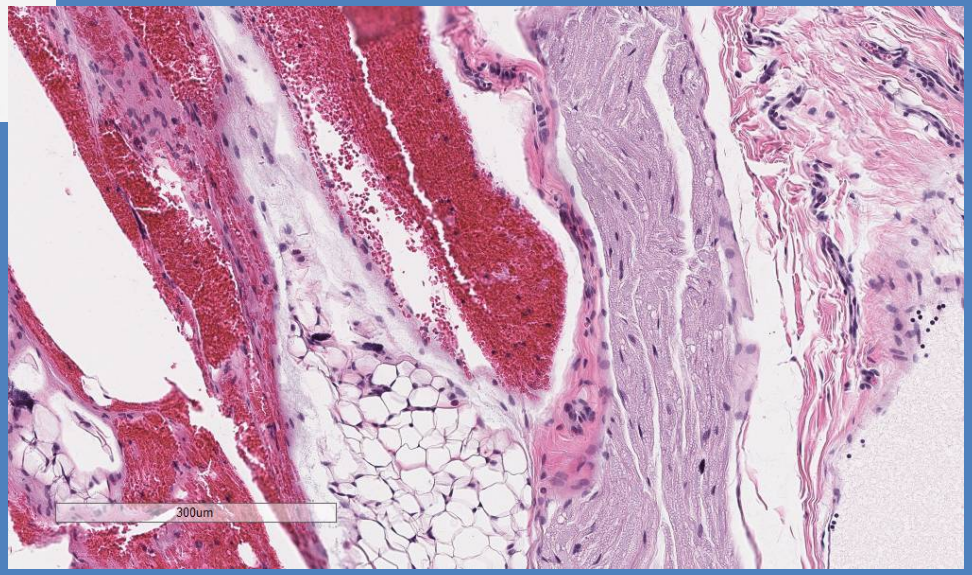
Introduction of Foreign Material



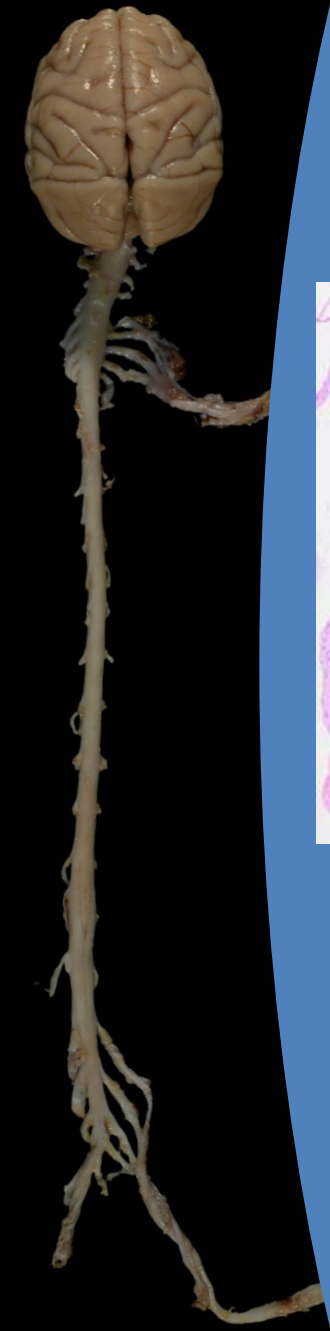
Hemorrhage in nerve

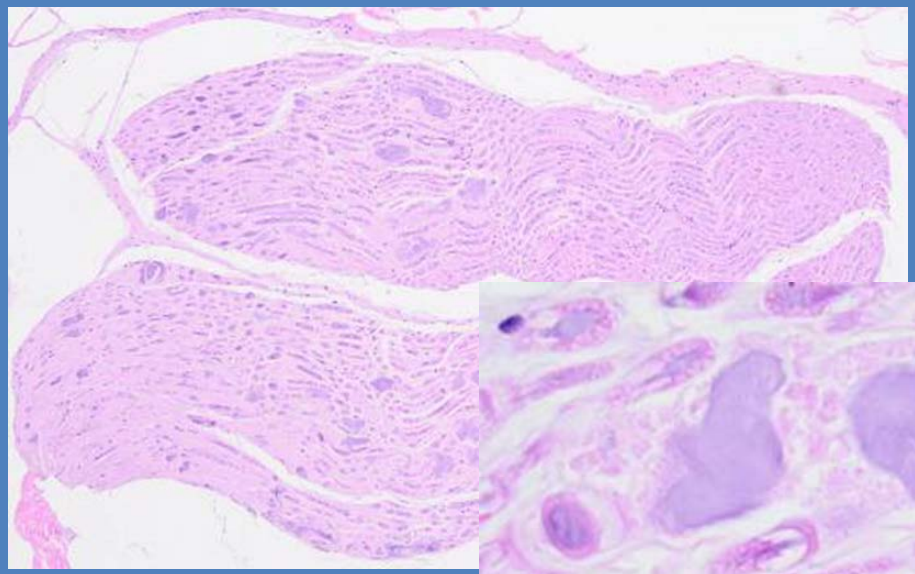


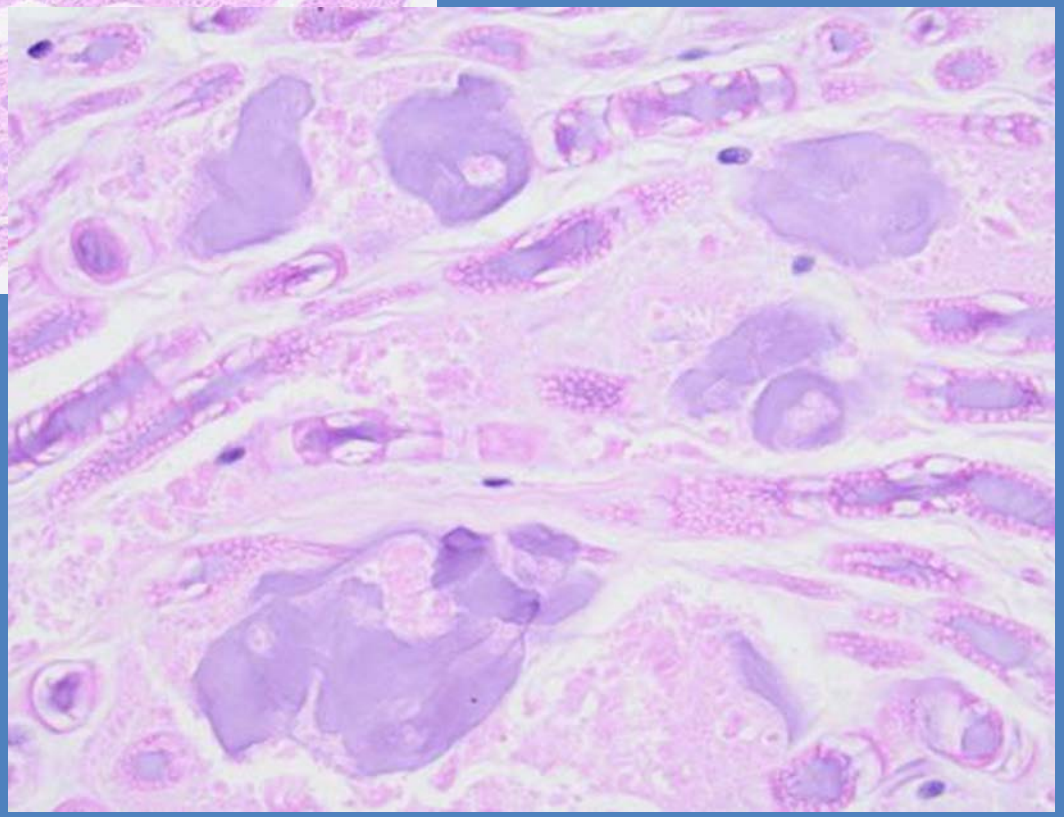




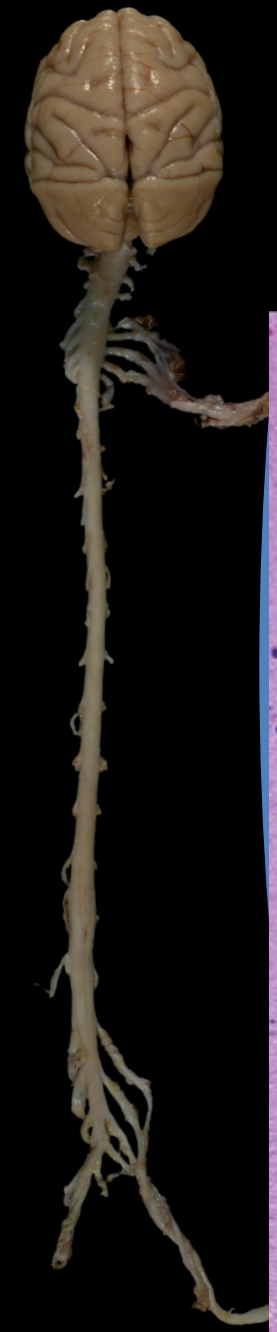
Pulling nerve during necropsy

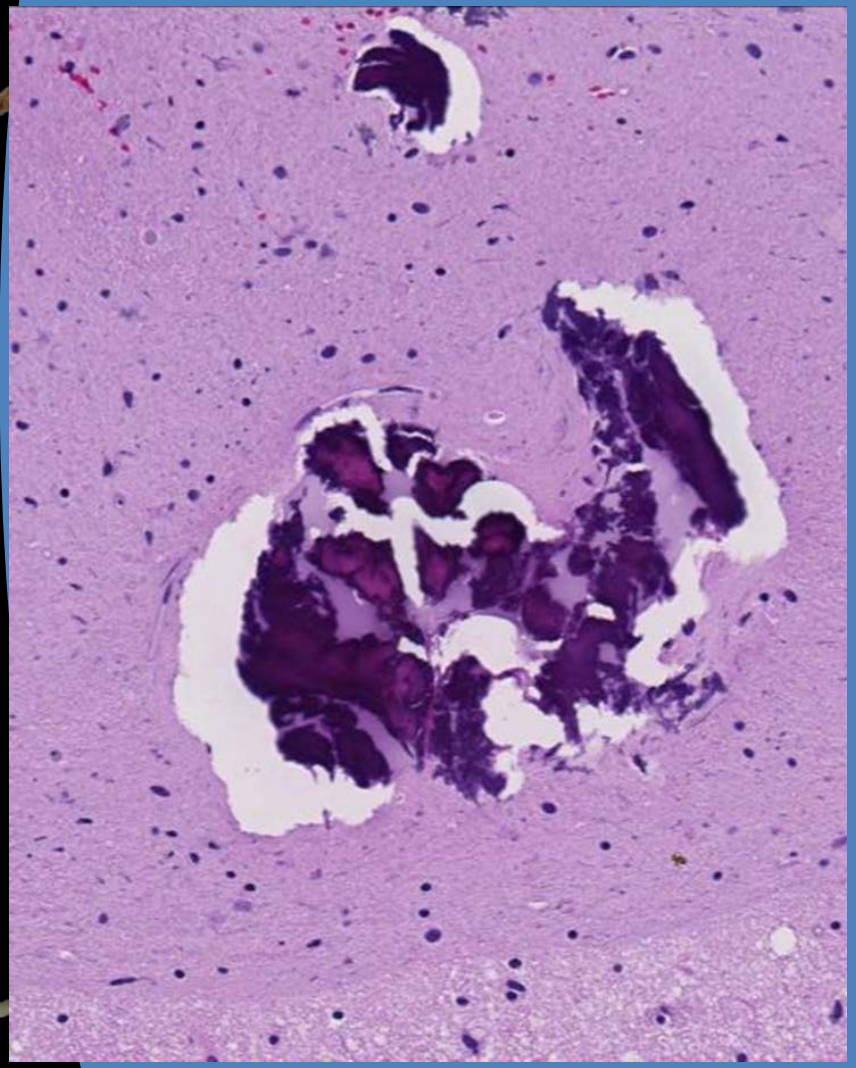


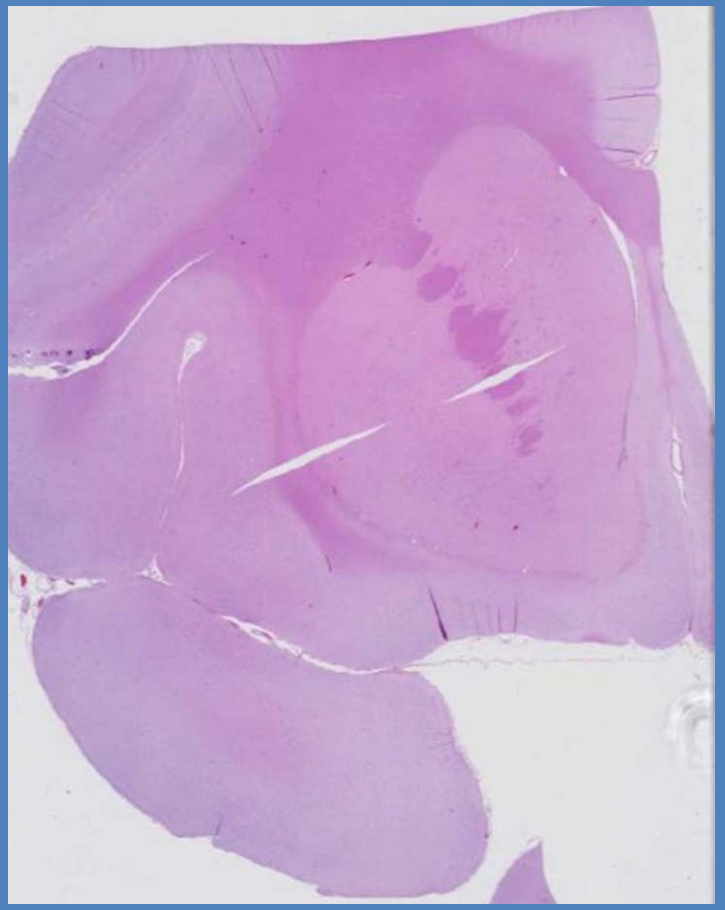




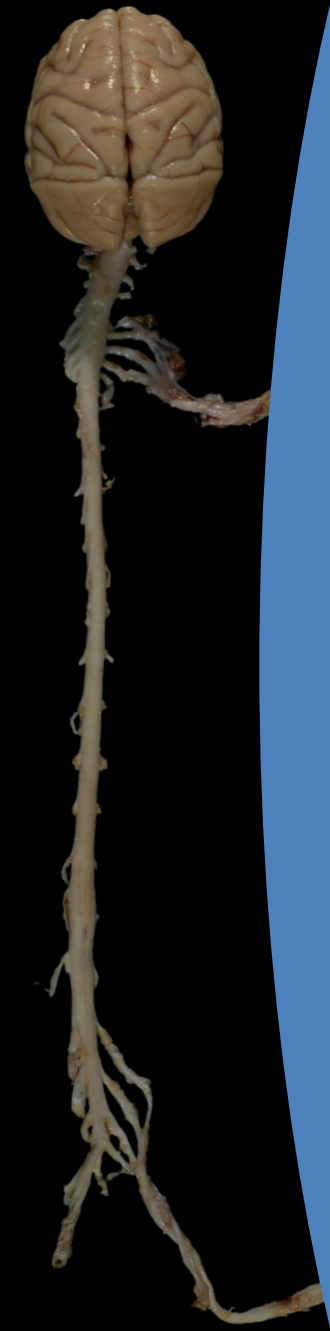
Introduction of bone material using the bone saw

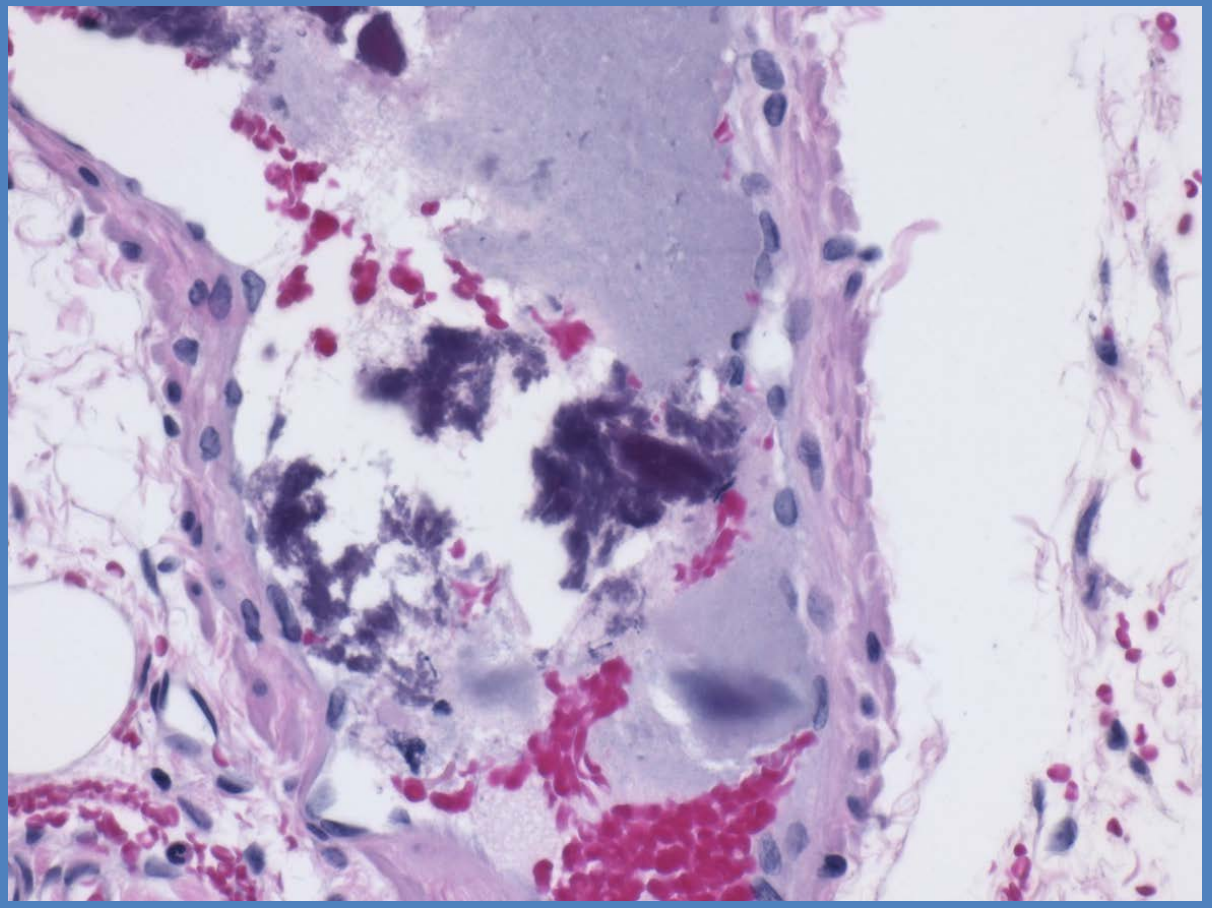




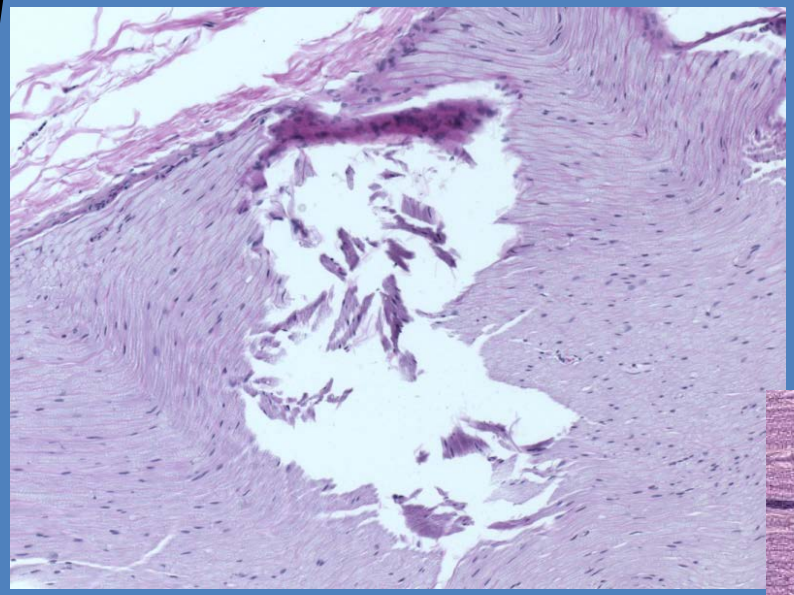


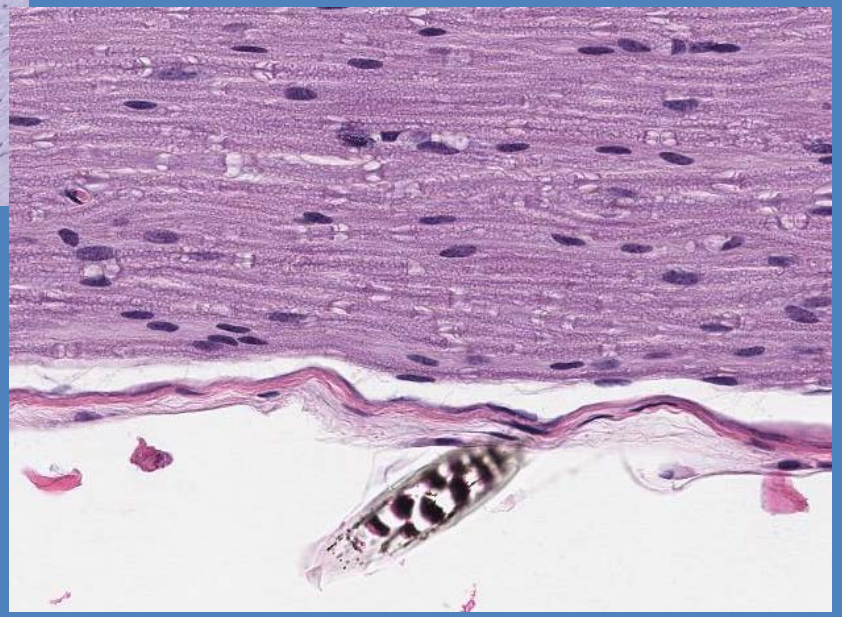
Emboli of bone material produced at necropsy

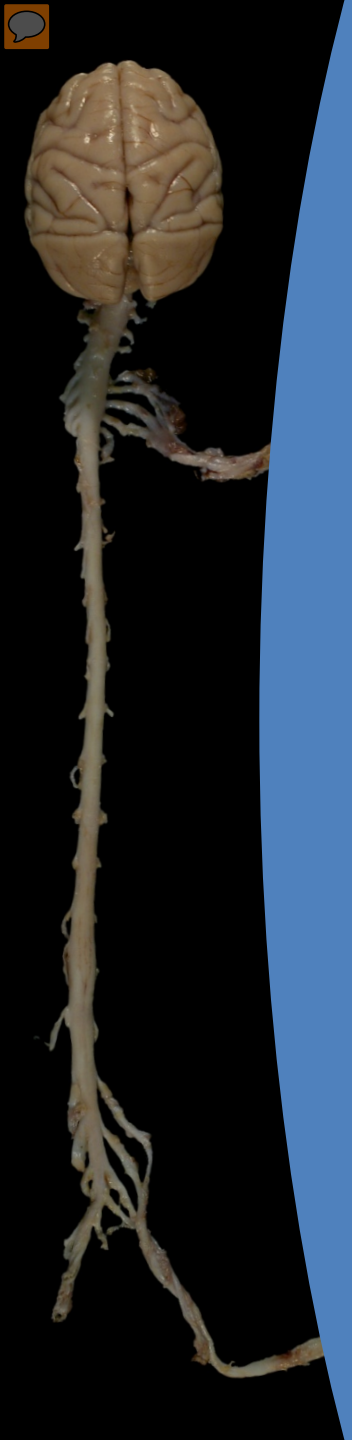




Microtome/water bath related-artifacts



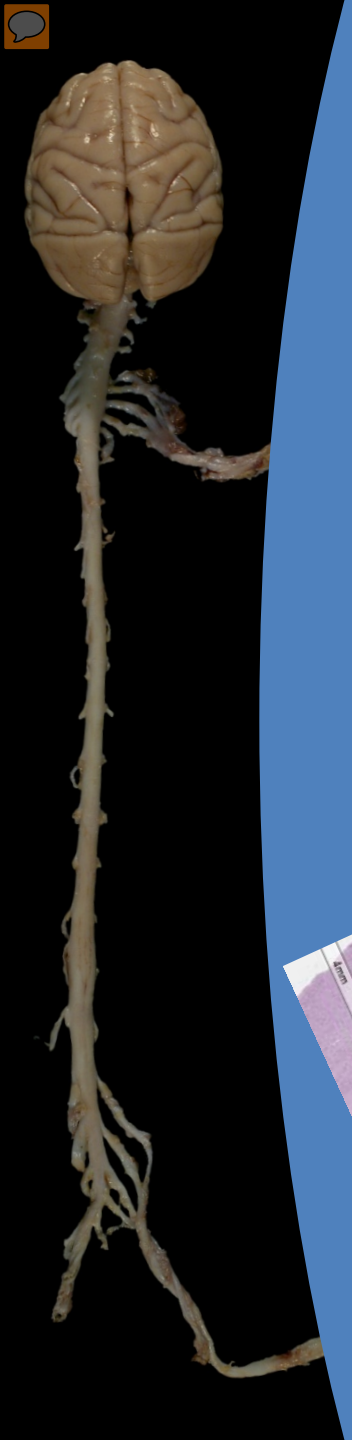


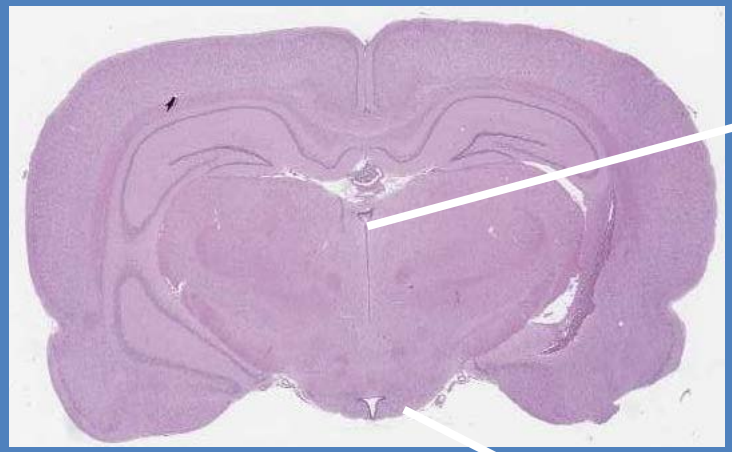


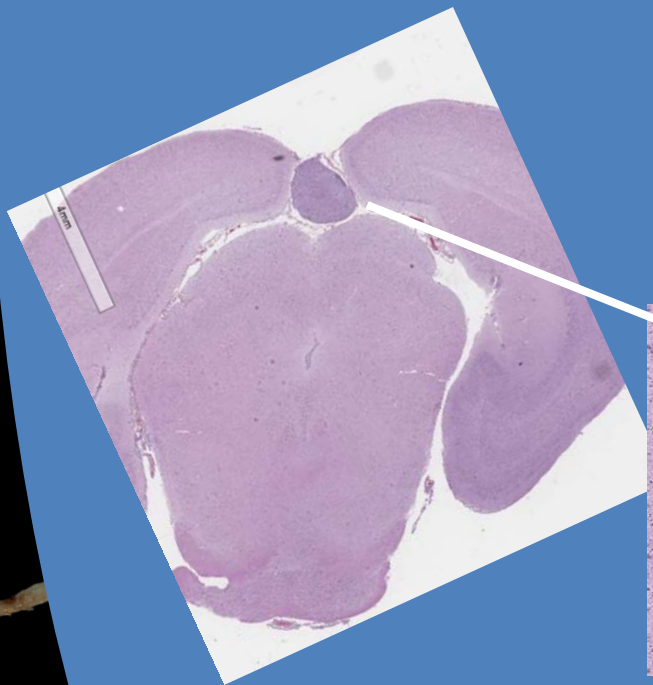
Circumventricular organs (CVOs)

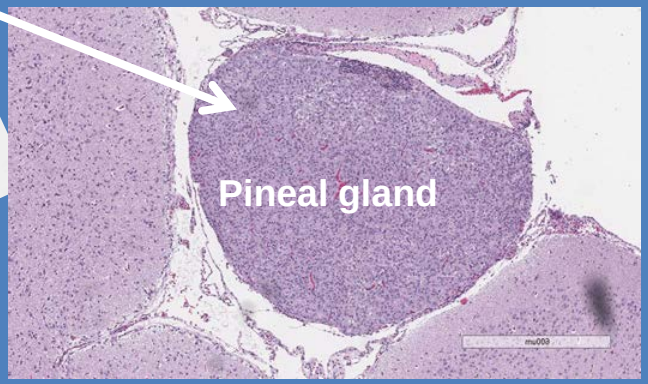
CVO	Location	Function
Subfornical organ	3V	Sensory: involved in the thirst response to increase osmolality (angiotensin II) in severe dehydration
Subcommissural organ	3 V	Secretory: spondin (normal development of Reissner's fiber and ventricular system)
Organum vasculosum of lamina terminalis	3V	Sensory: secretory Detects peptide concentrations in order to control fluid regulatory (homeostatic) responses
Median eminence (ME)	3 V	Secretory: Neurohormones (inactivation of tyrosine hydroxylase in ME is needed to induce a prolactin response in the pituitary gland
Pineal gland	3V	Secretory: Integrate circadian rhythms (melatonin)
Pituitary gland	3V	Secretory: ADH and oxytocin
Choroid plexus	Lateral, 3V and 4V	CSF
Area postrema	4V	Sensory: vomit reflex center

Circumventricular organs (CVOs)

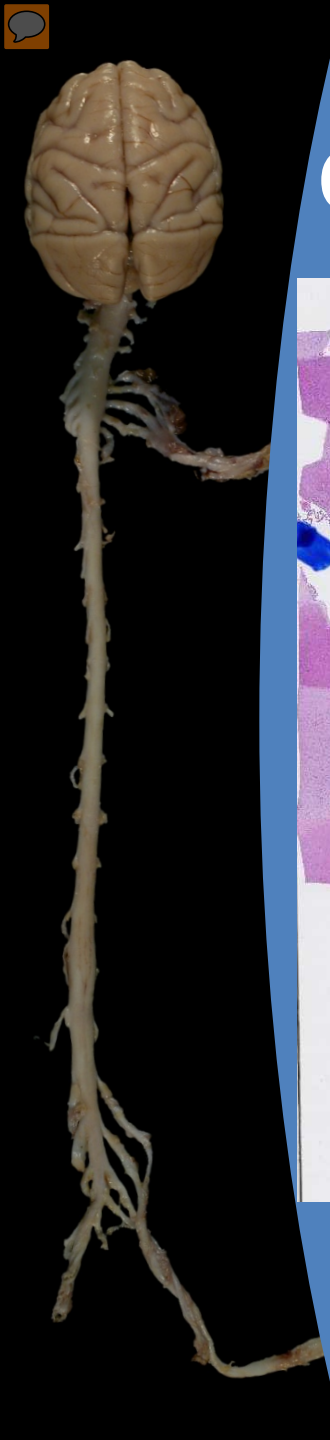


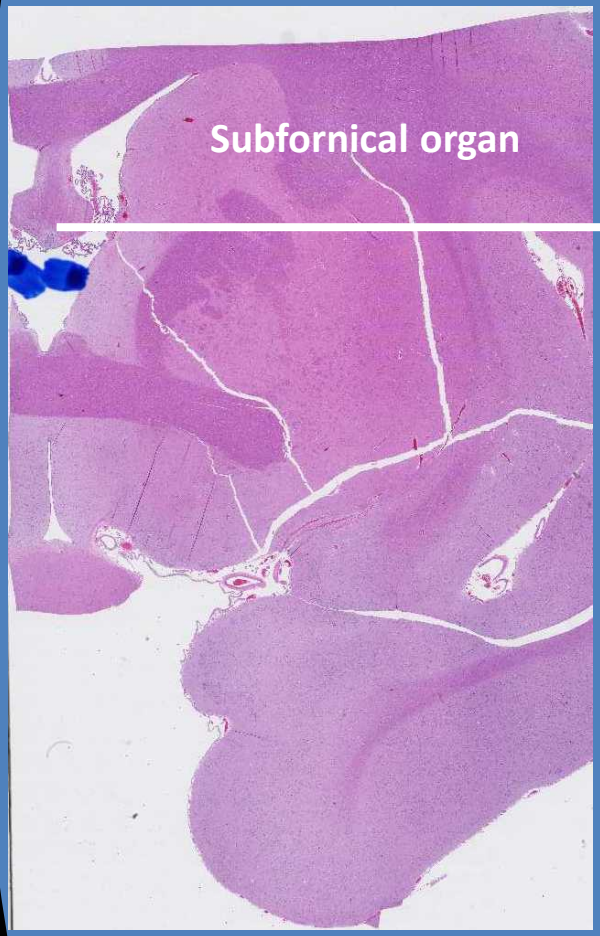


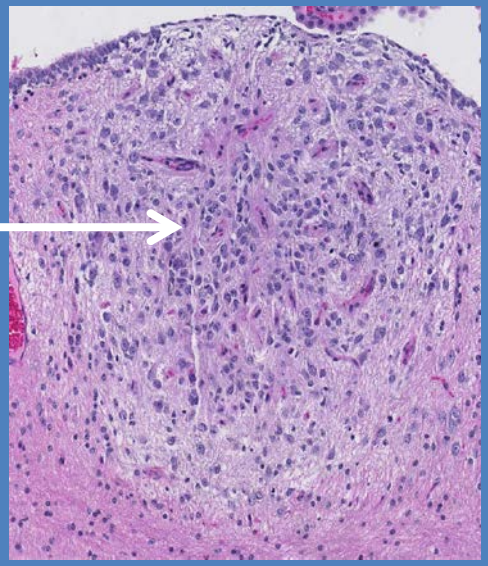


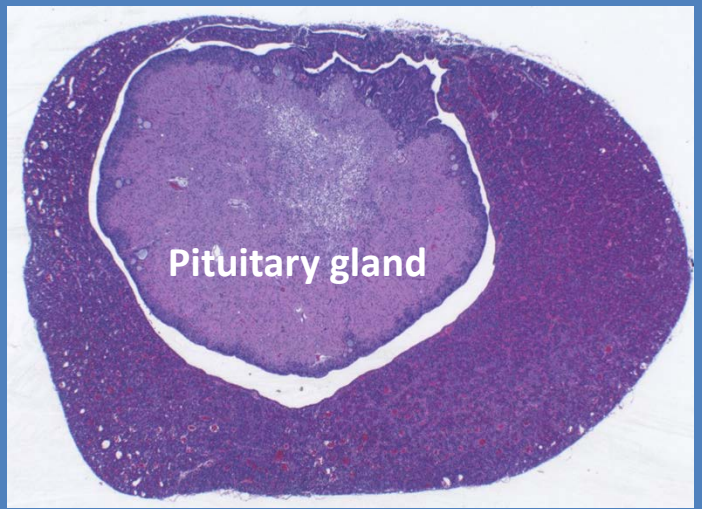


Circumventricular organs (CVOs)

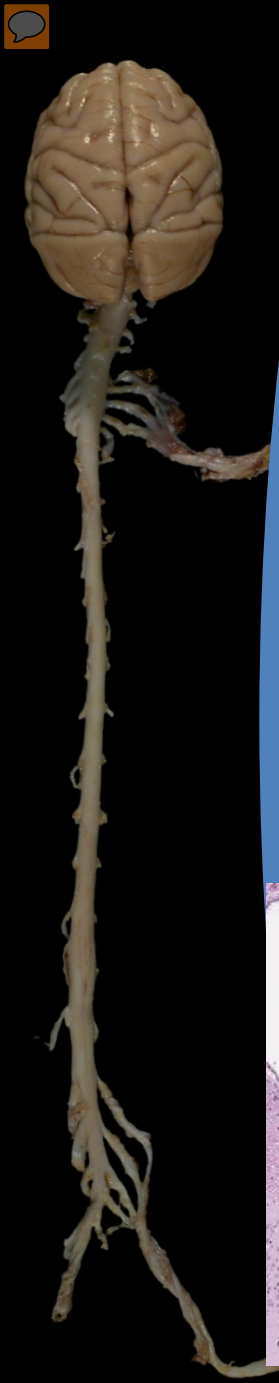


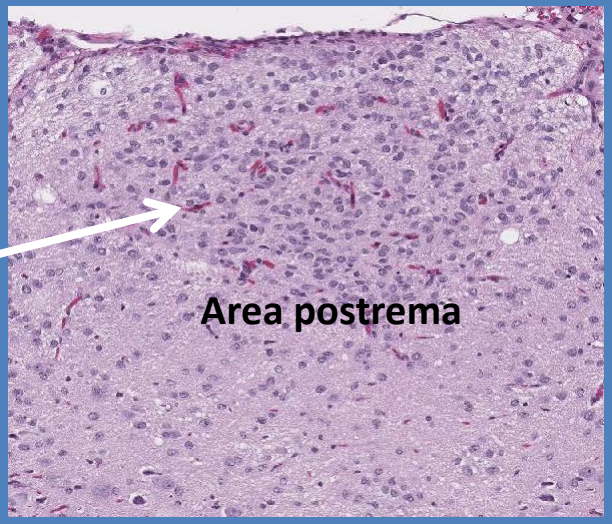


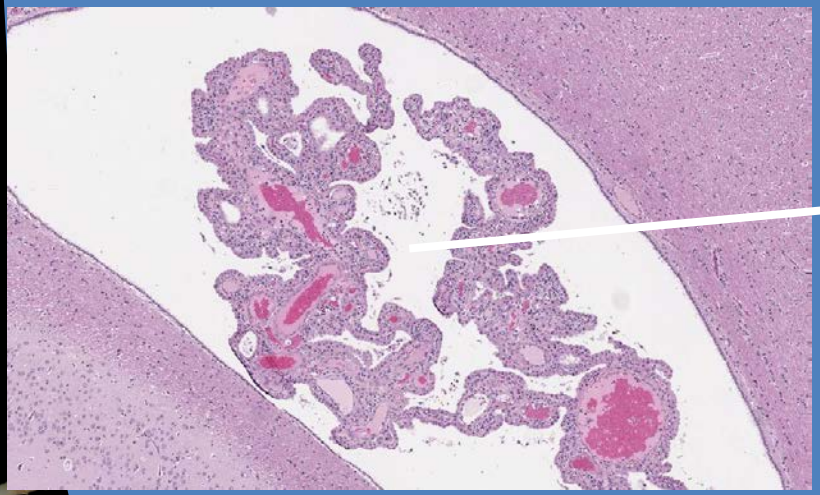




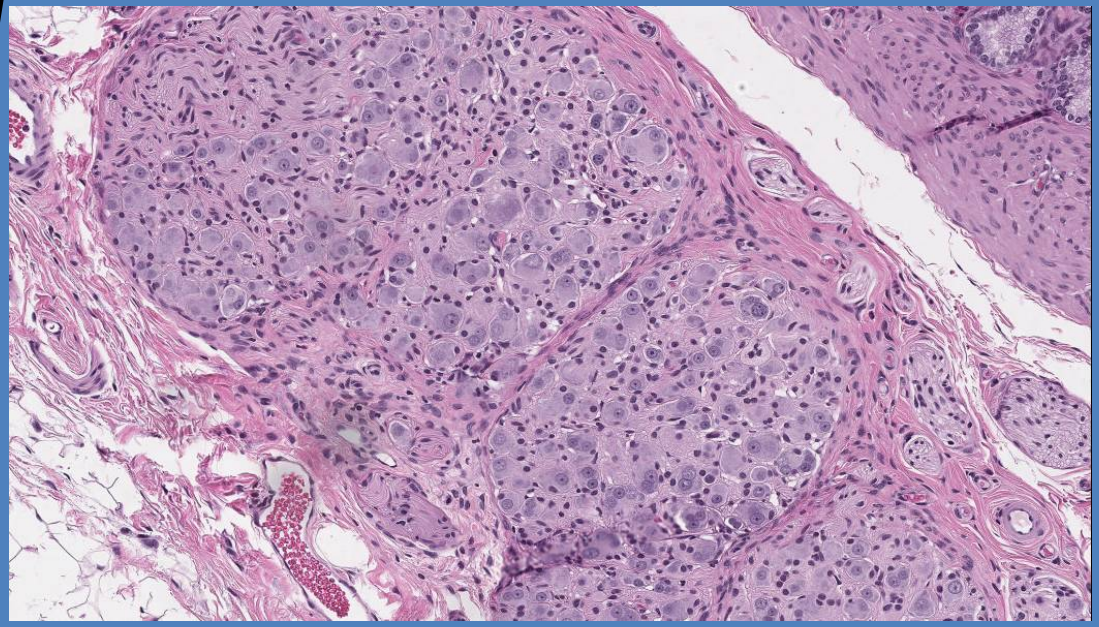
Circumventricular organs (CVOs)



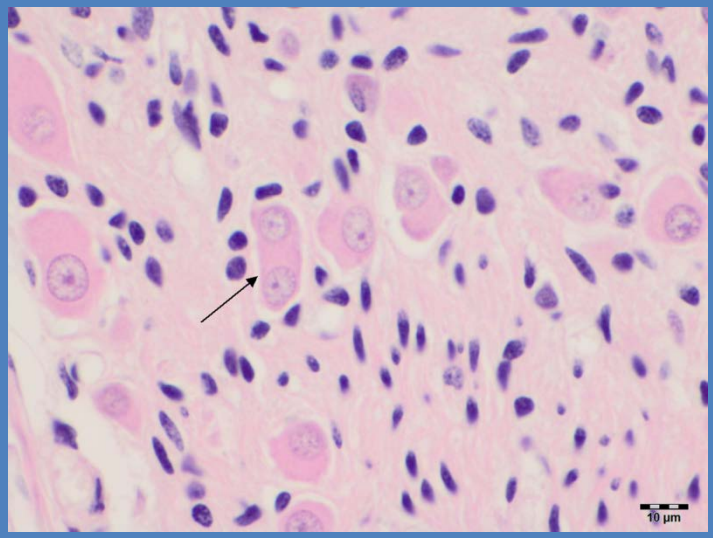




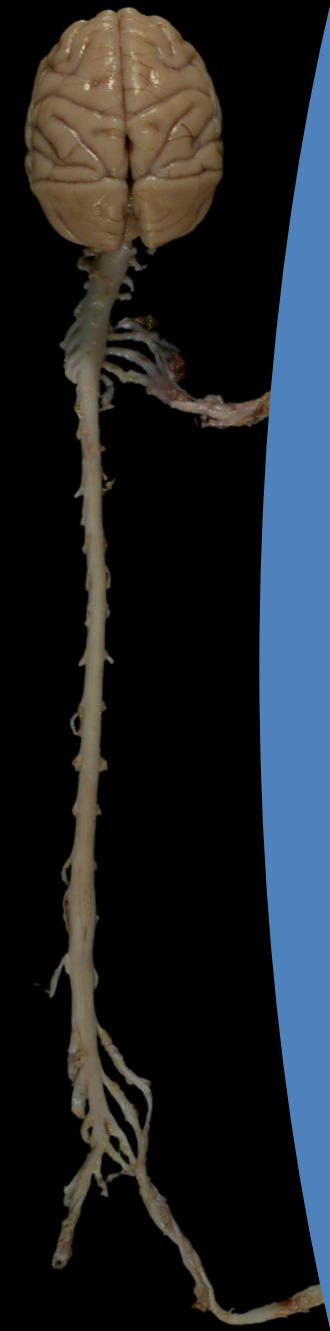
Binuclear neurons-ganglion

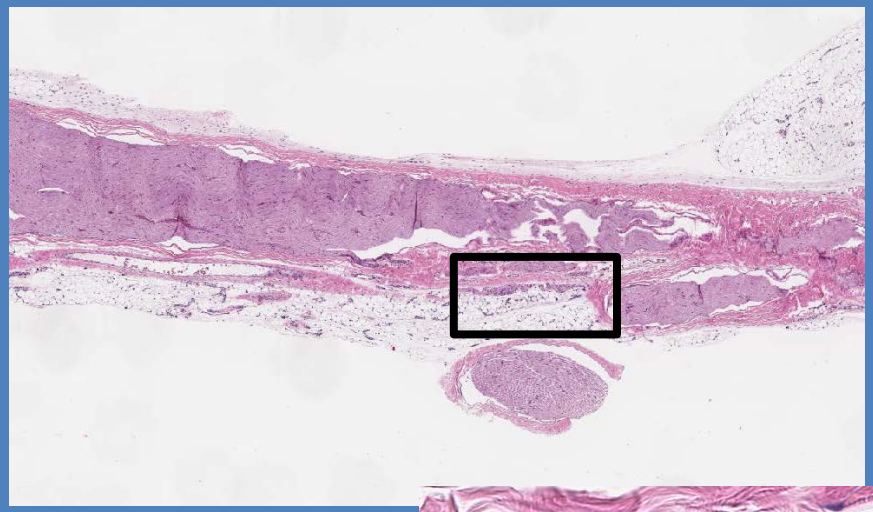


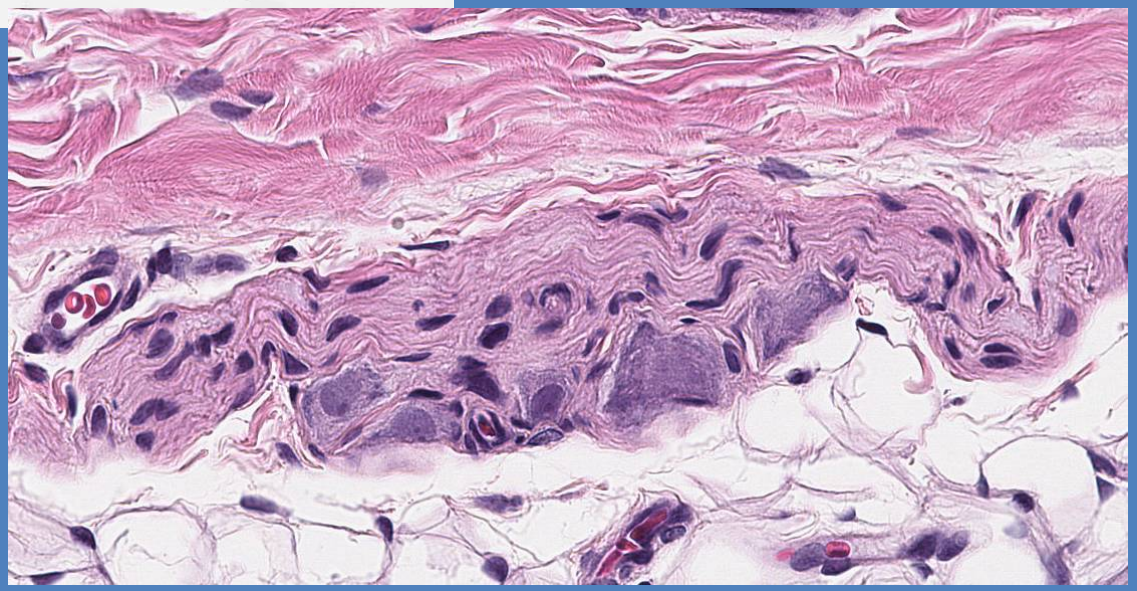
Parasympathetic ganglion-
Seminal vesicle

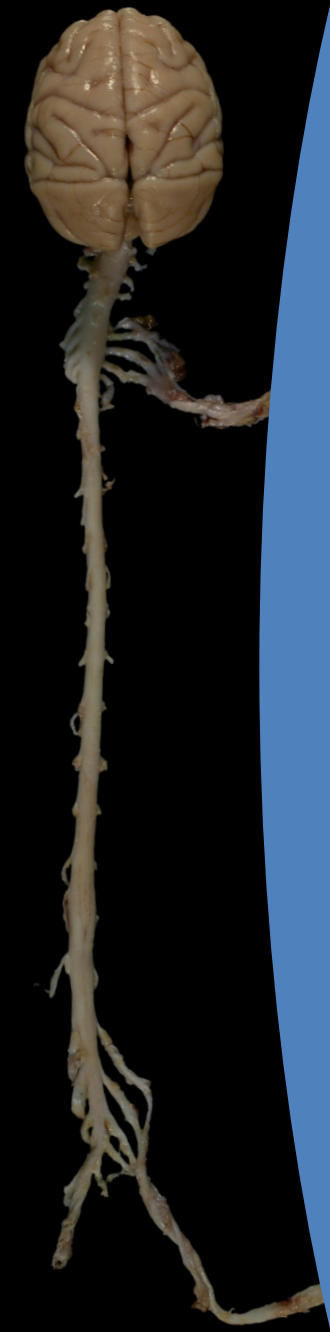


Neurons sciatic nerve

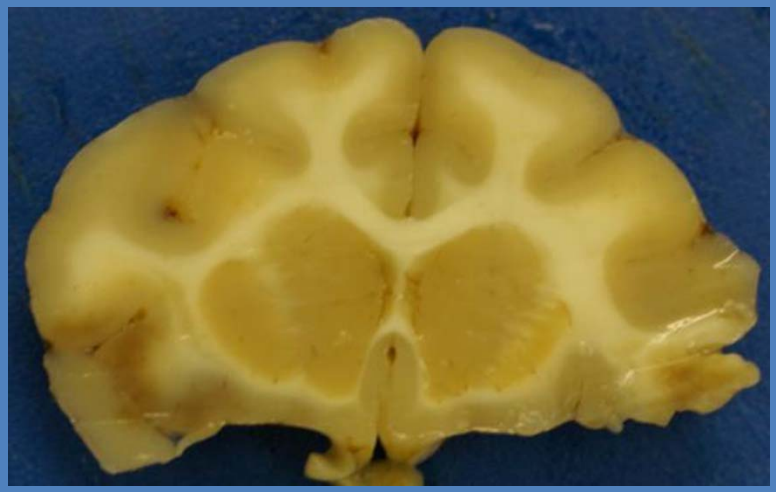


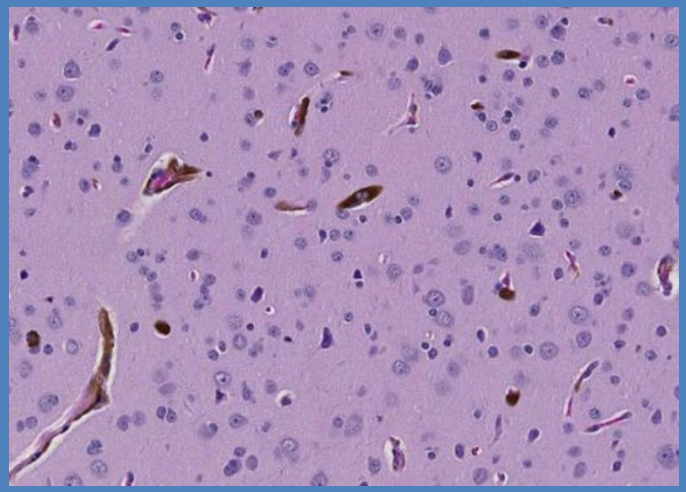


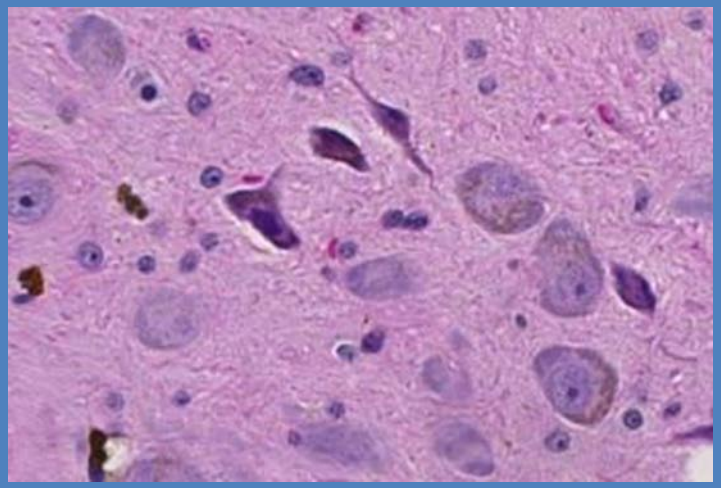


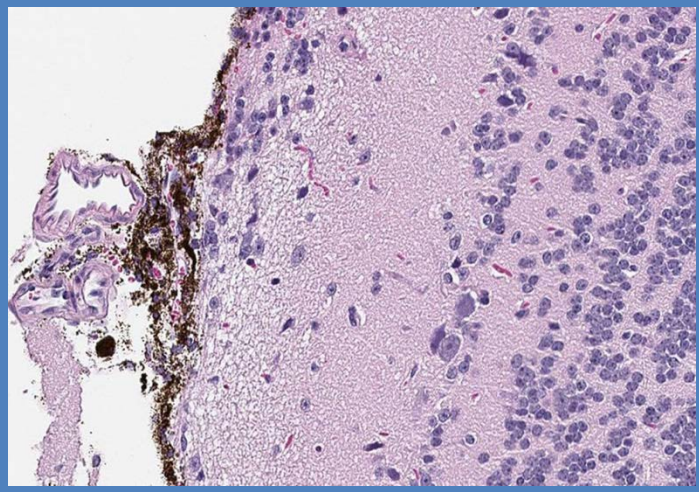


Melanin pigment

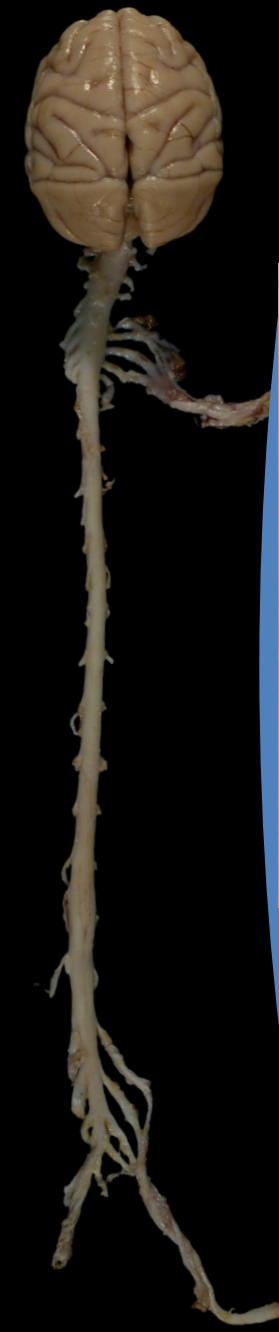


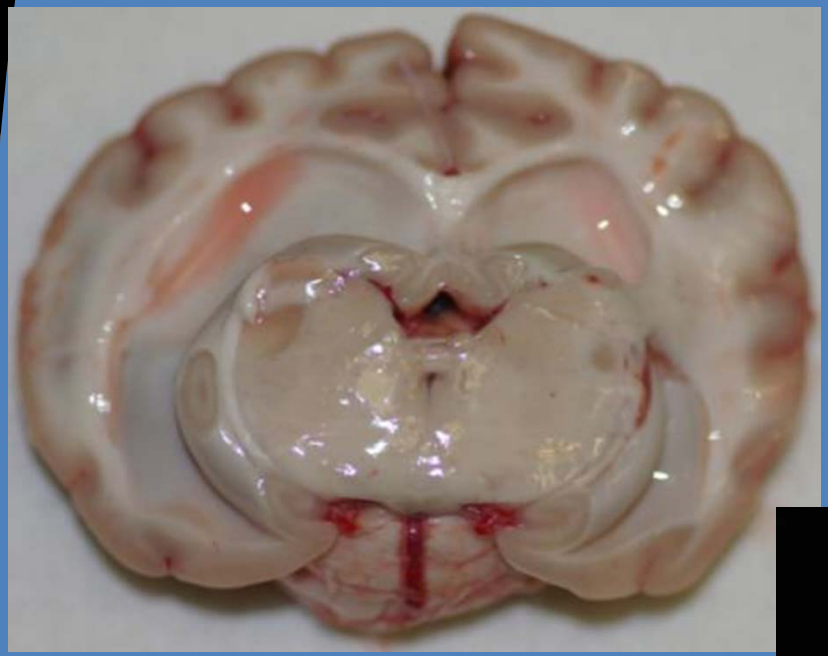


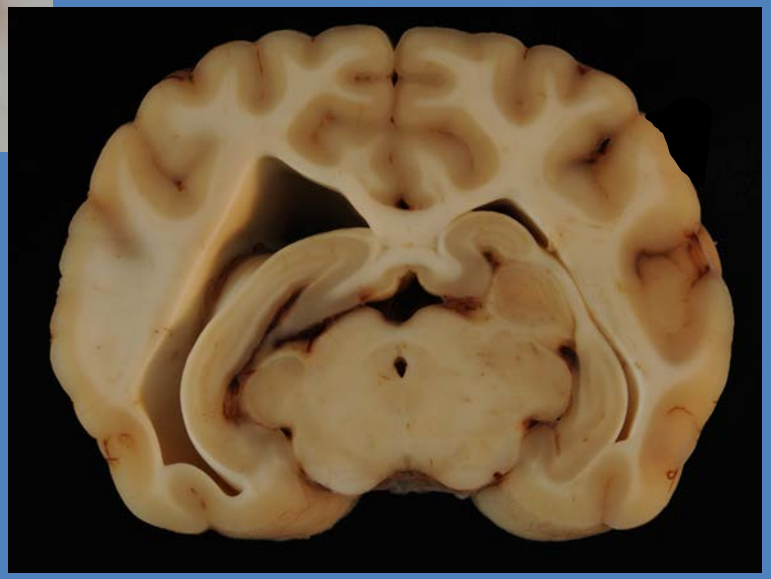


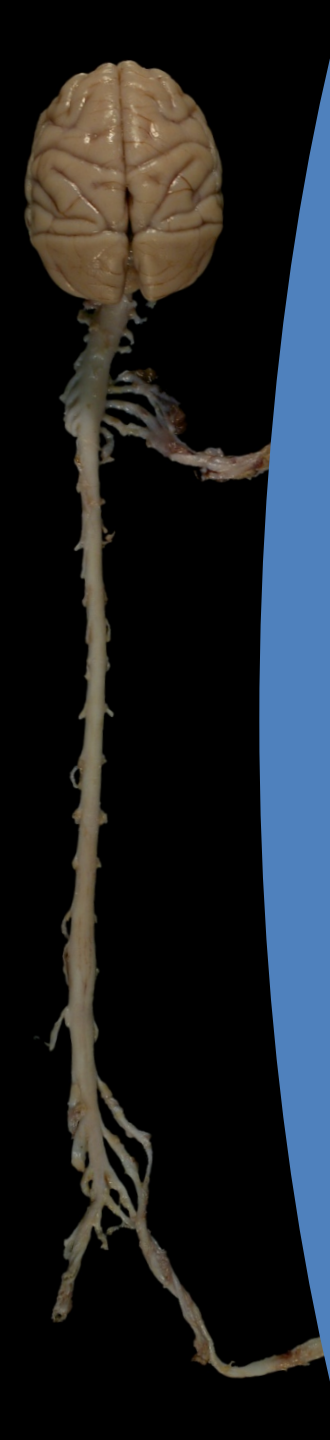


Ventriculomegaly in Beagle dogs





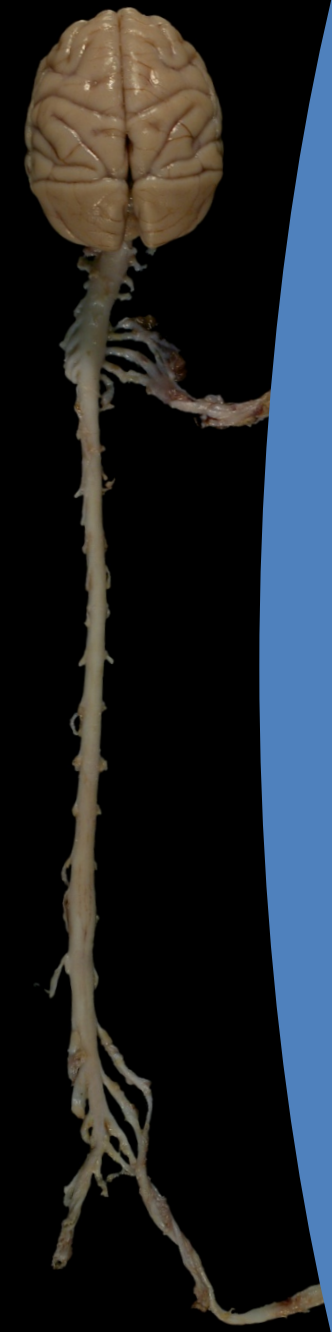




Conclusions

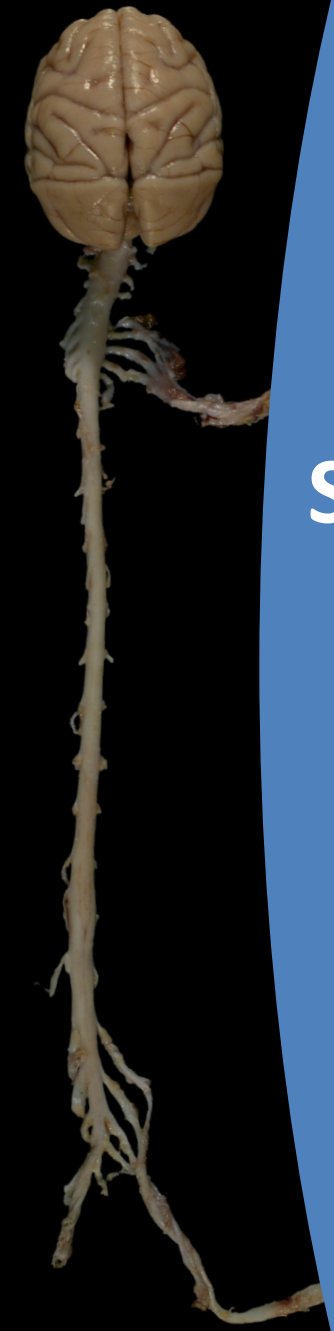
- Be familiar with the new recommendations to sample and process the nervous system and normal neural structures in preclincial species

- Be aware that inappropriate handling of the nervous system during the harvesting and processing for histopathologic evaluation can induce artefacts



Acknowledgments

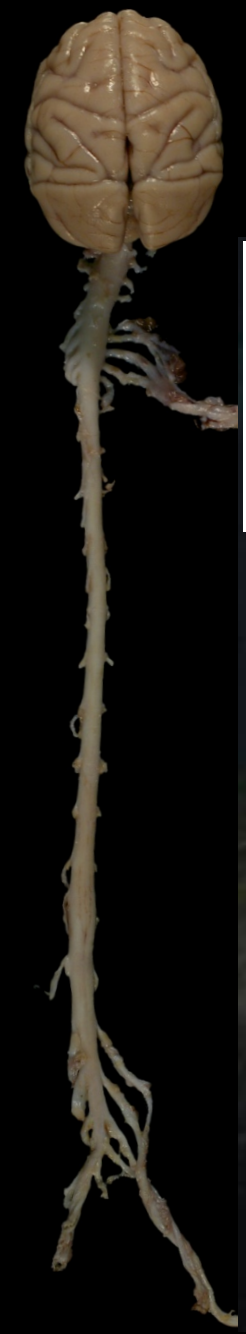
- Dan Morton (Pfizer, Cambridge, MA)
- Brad Bolon and other members of the STP Working Group on Nervous System Sampling
- Walt Bobrowski, B.Sc., CEMT (Pfizer, Groton, CT)

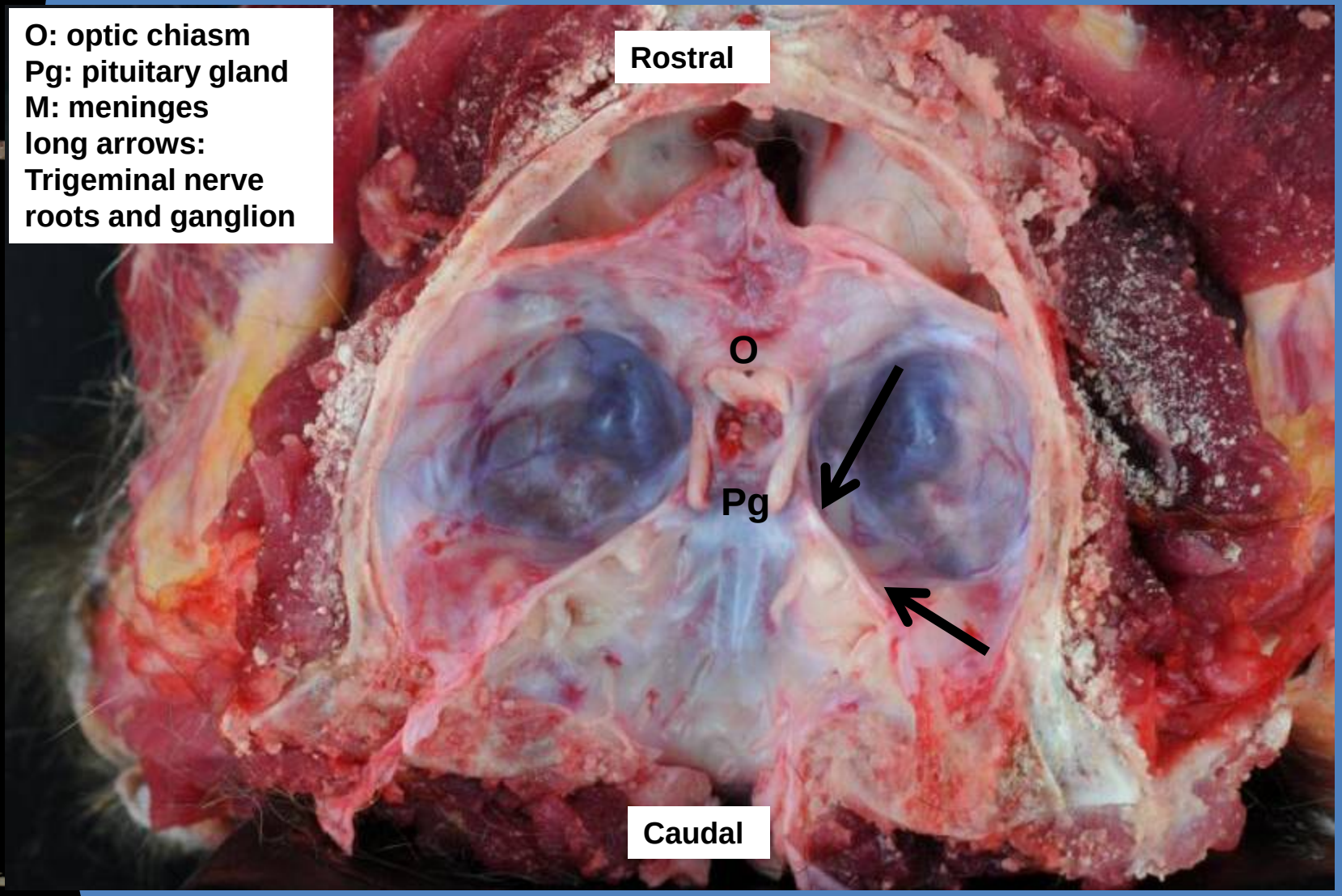


Special necropsy collection to derisk neurotoxicity

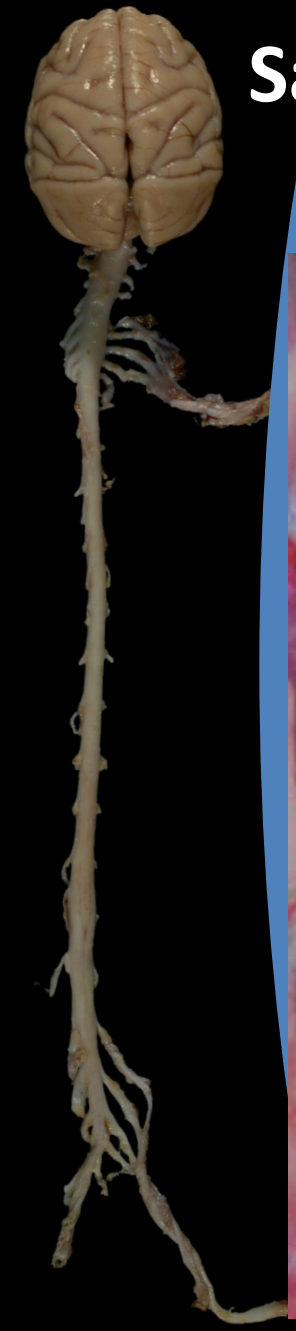
Non-human primate

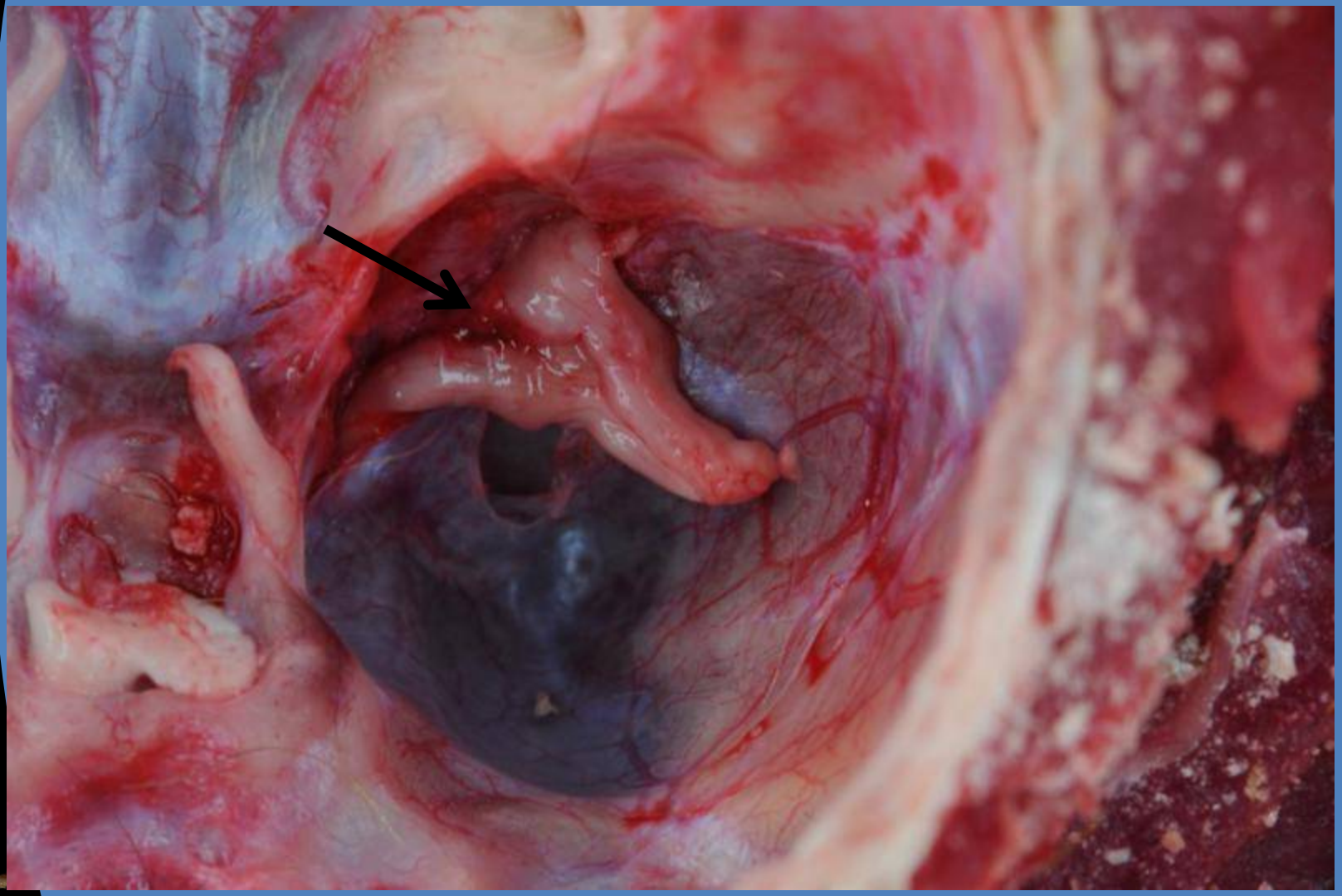
Sampling the trigeminal nerve roots and ganglion



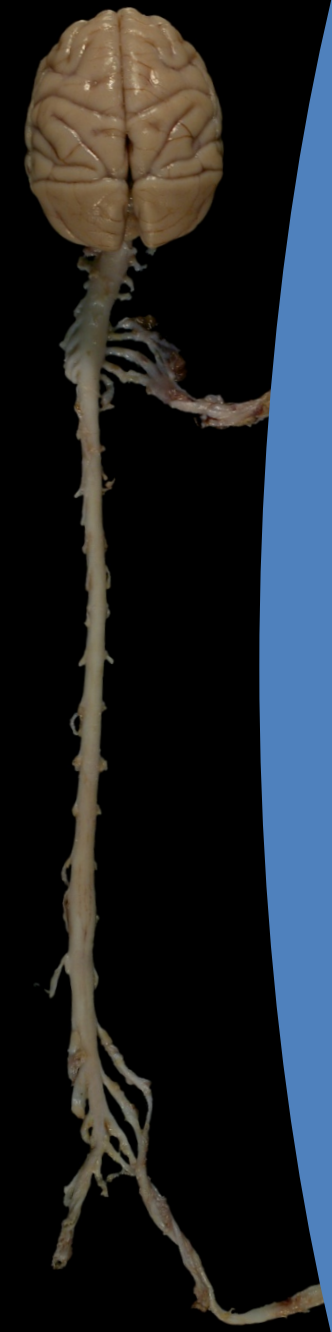


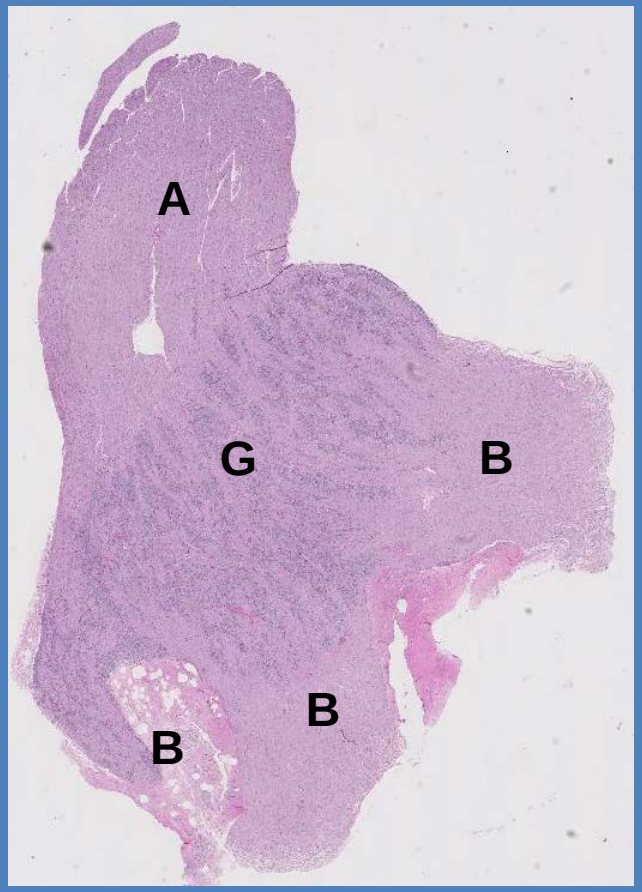
Sampling the trigeminal nerve roots and ganglion

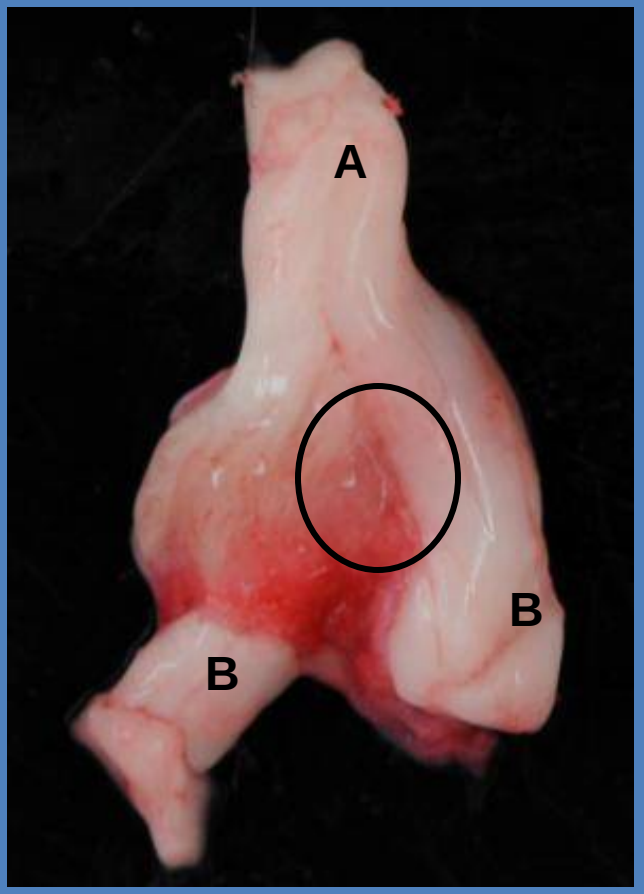




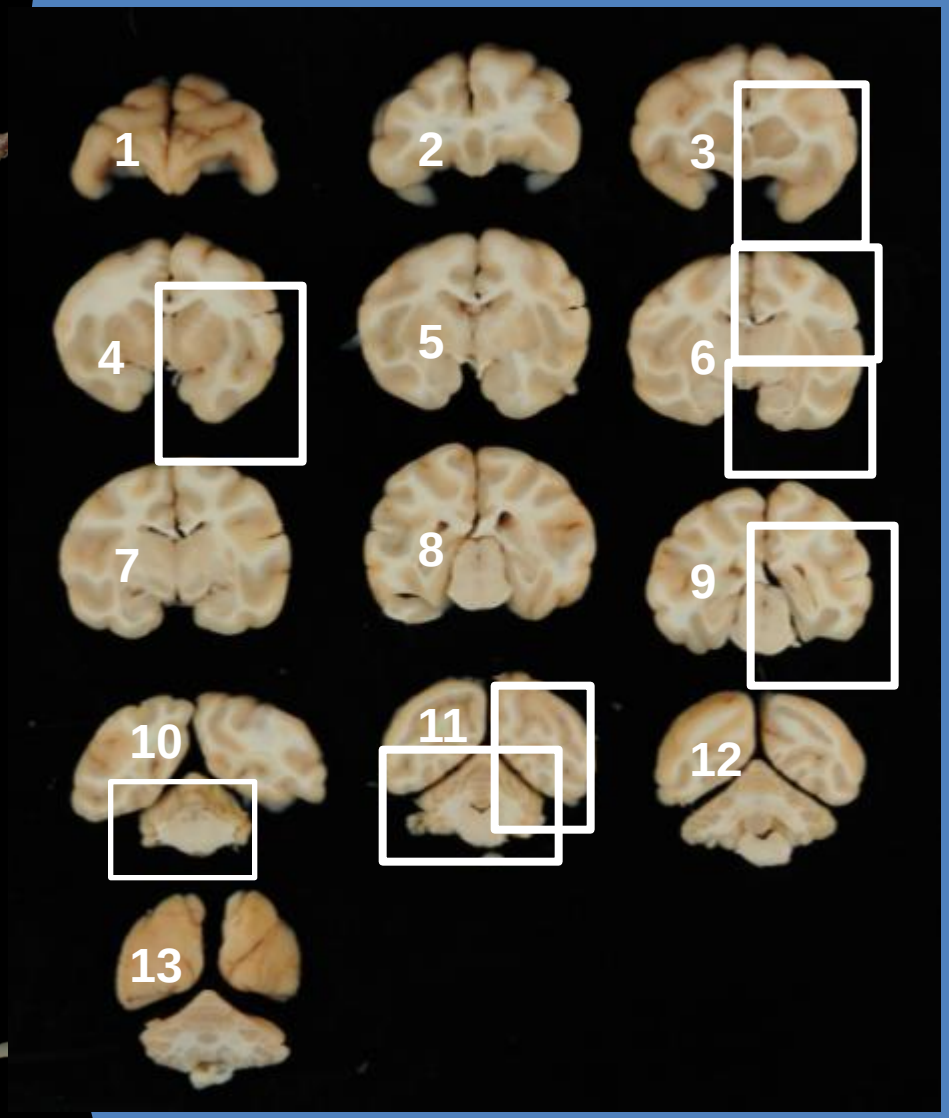
Sampling the trigeminal nerve roots and ganglion

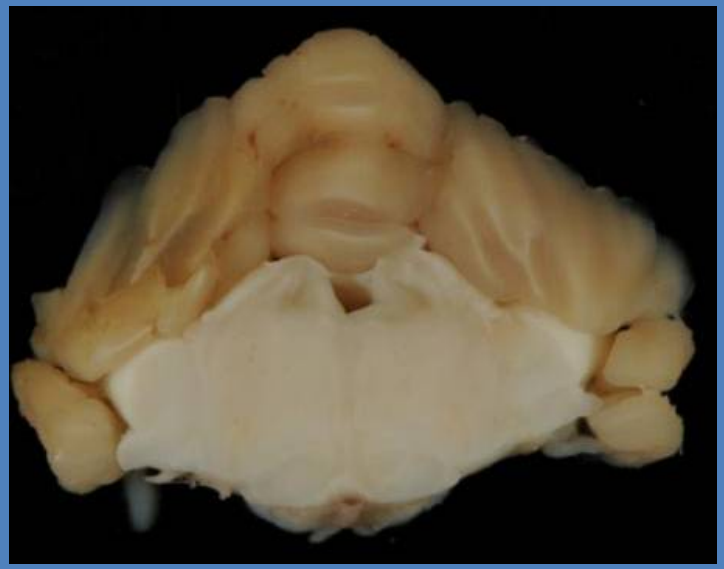


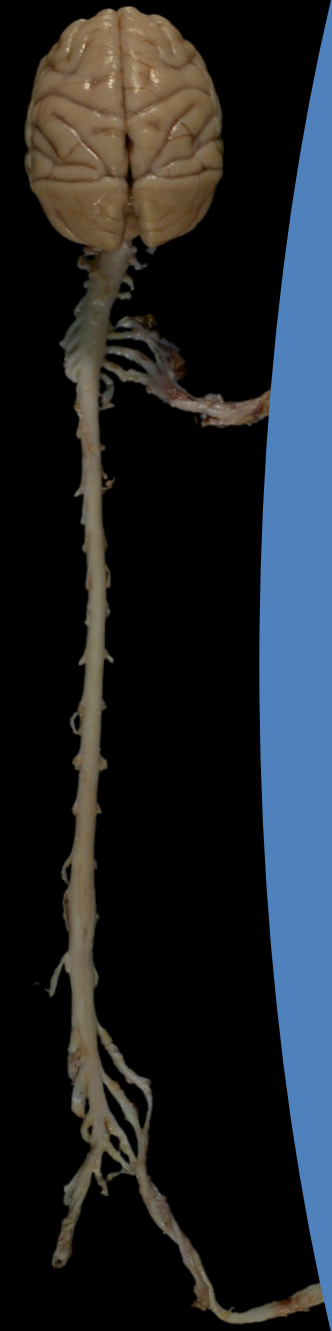




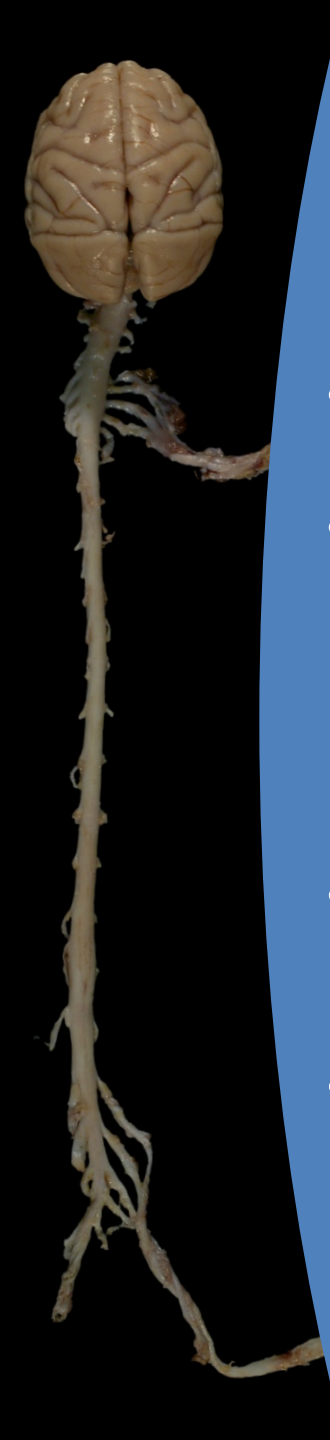
Sampling the trigeminal nerve roots and ganglion





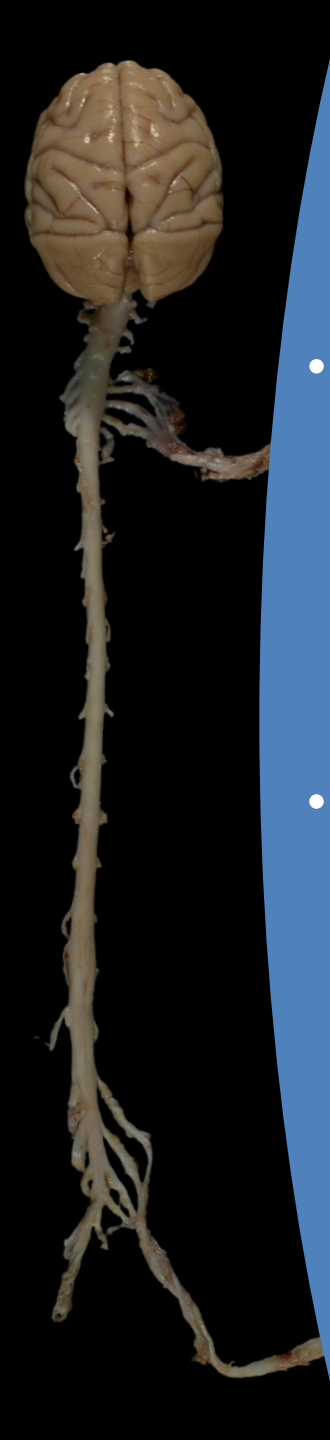


Backup slides



Abstract

- Brains should be weighed and trimmed similarly for all animals in a study.
- Certain structures should be sampled regularly: caudate/putamen, cerebellum, cerebral cortex, choroid plexus, eye (with optic nerve), hippocampus, hypothalamus, medulla oblongata, midbrain, nerve, olfactory bulb (rodents only), pons, spinal cord, and thalamus.
- Brain regions may be sampled bilaterally in rodents using 6 to 7 coronal sections, and unilaterally in nonrodents with 6 to 7 coronal hemisections.
- Spinal cord and nerves should be examined in transverse and longitudinal (or oblique) orientations.



Abstract

- Most Working Group members considered immersion fixation in formalin (for CNS or PNS) or a solution containing acetic acid (for eye), paraffin embedding, and initial evaluation limited to hematoxylin and eosin (H&E)-stained sections to be acceptable for routine microscopic evaluation during general toxicity studies; other neurohistological methods may be undertaken if needed to better characterize H&E findings.

- Initial microscopic analyses should be qualitative and done with foreknowledge of treatments and doses (i.e., "unblinded"). The pathology report should clearly communicate structures that were assessed and methodological details. Since neuropathologic assessment is only one aspect of general toxicity studies, institutions should retain flexibility in customizing their sampling, processing, analytical, and reporting procedures as long as major neural targets are evaluated systematically.