PATHOLOGY TECHNIQUES IN RAT NEUROTOXICITY STUDIES

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Goals Of This Presentation

- Definition of Neurotoxicity
- Regulatory Guidelines
- Tissue fixation
- Tissue collection
- Common embedding techniques
- Conventional histochemical stains & Immuno-labels
- Transmission electron microscopy
- Important landmarks in different sections of brain
- Examples of findings in the nervous tissues
Neurotoxicity

- Various bio-pharmaceuticals drugs and environmental toxins have potential to cause neurotoxicity

- What is neurotoxicity?
  - an adverse effect on the structure and/or function of the nervous system
Regulatory Guidelines


- FDA (Food And Drug Administration) Redbook 2000: *IV..C.10 Neurotoxicity Studies: Toxicological Principles for the Safety Assessment of Food Ingredients*
Types of Neurotoxicity Studies

- Developmental neurotoxicity studies
- Delayed neurotoxicity studies
- General neurotoxicity studies
Purpose of Neurotoxicity Studies

- To look for adverse effect of xenobiotics on NS
- To characterize the scope of nervous system involvement
- To determine dose response kinetics
  - including NOAEL
Neurotoxicity Studies

Tools to screen potential neurotoxicity in rats

- *Functional observational battery (FOB)*
- *Study of motor activity*
- *Histopathology*
Types of Tissue Fixation

- Immersion fixation
  - Quicker
  - Cheaper
  - More artifacts
  - Can be used in most circumstances

- Intravascular perfusion fixation [OECD & EPA]
  - Time consuming
  - Expensive
  - Optimal preservation of tissues
  - Decreased artifacts
    - esp. neuropil vacuolation and dark neurons
  - Helpful in interpreting subtle effects
Most Common Tissue Fixatives

- 10% neutral buffered formalin
  - penetrates tissue quickly

- 4% formaldehyde (aka paraformaldehyde)
  - penetrates tissue quickly

- ≤ 4% glutaraldehyde
  - penetrates tissue slowly
  - enhanced cross-linking of proteins
  - better lipid preservation
    - myelin in nervous system is lipid
  - often preferred for transmission electron microscopy
Rat Intravascular Perfusion Apparatus

Container I – Initial Perfusate [heparinized saline sodium nitrite solution]

Container II – Primary Perfusate [50% Karnovsky’s fixative (paraformaldehyde+glutaraldehyde)]

III – Cannula (blunt bore needle) for insertion into the left ventricle of heart
Most Common Artifacts

Dark Neuron Artifact

Most Common Artifacts

Neuropil Vacuolation
### Tissue Collection and Embedding

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

- Better quality sections with improved cellular morphology
- Uniform thin and semi-thin sections can be prepared
- Ability to go as thin as one micron
- No heat and solvent artifacts as seen with paraffin
- Harder embedding provides support to tissue during sectioning
What regulators and experts suggest about plastic?

- Paraffin embedding acceptable for CNS samples (EPA and OECD)
- Plastic embedding is required for PNS samples (EPA)
- If signs of peripheral neuropathy, plastic-embedded PNS samples should be examined (OECD)

- Best approach for nerve evaluation
  - a nerve cross section
  - fixed in osmium
  - embedded in plastic
  - sectioned very thin
  - stained with toluidine blue

***Glycol methacrylate (GMA) is not compatible with osmium
***Osmium is the key to examine myelin
Matrix-guided Brain Trimming for Coronal Sections

89 to 100 % success in getting area of interest on the slide
Rat Brain Trim

Bolon et al., (2013). STP position paper: Recommended practices for sampling and processing for the nervous system (brain, spinal cord, nerve and eye) during nonclinical general toxicity studies. Toxicol Pathol

Rat Level 1 – Olfactory Bulb*

**ON:** Olfactory Nerve Layer  
**G1:** Glomerular Layer  
**EP1:** External Plexiform Layer  
**Mi:** Mitral Cell Layer  
**GrO:** Granular Cell Layer

*B Collection and evaluation of this section is protocol-specified depending on client requirement.

Bregma 6.70 mm (approximate)
Rat Level 2 – Forebrain

**Cg**: Cingulate Cortex
**FC**: Frontal Cortex
**PC**: Parietal Cortex
**Pir**: Piriform Cortex
**cg**: Cingulum
**gcc**: Genu Corpus Callosum
**ec**: External Capsule
**CPu**: Caudate Putamen
  *(basal ganglia)*
**SN**: Septal Nuclei
**Acb**: Accumbens Nucleus
**ac**: Anterior Commissure
**lo**: Lateral Olfactory Tract
**ICj**: Islands of Calleja

Bregma 1.20 to 0.20 mm (approximate)
Rat Level 3 – Cerebral Cortex

**RS**: Retrosplenial Cortex  
**M**: Motor Cortex  
**S**: Somatosensory  
**Au**: Auditory Cortex  
**Ect**: Ectorhinal Cortex  
**PRh**: Perirhinal Cortex  
**Ent**: Entorhinal Cortex  
**Pir**: Piriform Cortex  
**3V**: Third Ventricle

Bregma -3.30 mm (approximate)
Rat Level 3 – Thalamus

cc: Corpus Callosum
CA: Cornu Ammonis
DG: Dentate Gyrus
chp: Choroid Plexus
Hb: Habenular Nuclei
Am: Amygdala
TH: Thalamus
HT: Hypothalamus
ec: External Capsule
ic: Internal Capsule
f: Fornix
mt: Mammillothalamic Tract
opt: Optic Tract
rf: rhinal fissure

Bregma -3.30 mm (approximate)
Rat Level 4 – Ventral Hippocampus/Midbrain

**RS**: Retrosplenial Cortex  
**OC**: Visual (Occipital) Cortex  
**Au**: Auditory Cortex  
**TC**: Temporal Cortex  
**Ect**: Ectorhinal Cortex  
**Ent**: Entorhinal Cortex  
**rf**: Rhinal Fissure  
**SC**: Superior (Anterior) Colliculi  
**Aq**: Aqueduct (Sylvius)  
**MG**: Medial Geniculate  
**Ra**: Raphe Nucleus  
**Re**: Red Nucleus  
**SN**: Substantia Nigra  
**ml**: Medial Lemniscus  
**cp**: Cerebral Peduncles  
**IP**: Interpeduncular Nucleus

Bregma -5.06 to -6.04 mm (approximate)
Rat Level 5 – Midbrain

**IC**: Inferior (Posterior) Colliculi

**Me5**: Mesencephalic 5 Nucleus

**DTg**: Dorsal Tegmental Nucleus

**mcp**: Middle Cerebral Peduncle

**s5**: Sensory root of CN V

**py**: Pyramidal Tract

**PF**: Parafllocculus

Bregma -9.16 mm (approximate)
Rat Level 6 – Mid-Cerebellum

**PF**: Paraflocculus  
**Ver**: Vermis  
**AL**: Ansiform Lobe  
**LI**: Lingula  
**DC**: Cochlear Nuclei (CN 8)  
**icp**: Inferior Cerebellar Peduncle  
**sp5**: Spinal Trigeminal Tract  
**7**: Facial Nucleus (CN 7)  
**Ve**: Vestibular Nuclei  
**py**: Pyramidal Tract

Bregma -11.30 mm (approximate)
Rat Level 7 – Caudal Medulla Oblongata

10: Vagal Nucleus (CN 10)
12: Hypoglossal Nucleus (CN 12)*
ECu: External Cuneate Nucleus
Sol: Solitary Tract Nucleus
IO: Inferior Olivary Nucleus
sp5: Spinal Trigeminal Tract
Sp5I: Spinal Trigeminal Nucleus
RF: Reticular Formation
py: Pyramidal Tract
4V: Fourth Ventricle

* Relative location CN 12

Bregma -12.80 mm (approximate)
Rat Spinal Cord, Dorsal Nerve Roots and Dorsal Root Ganglia

- **Spinal cord**: 1 transverse and 1 longitudinal section (cervical, thoracic & lumbar)
- **Dorsal root ganglia**: 1 cervical & 1 lumbar
- **Trigeminal/Gasserian ganglia**
Peripheral Nerves and Skeletal Muscles

Nerves
a. Sciatica
b. Tibial
c. Common peroneal (fibular)
d. Lateral sural
e. Plantar

Digital (Not shown)

Bones
P Patella
T Tibia

Muscles
1. Gluteus medius
2. Biceps femoris
3. Semitendinosus
4. Quadriceps femoris
5. Gastrocnemius lateralis
6. Rectus femoris
7. Gastrocnemius medialis
8. Tibialis cranialis

Conventional Neurohistology Stains & Immuno-labels
Retrosplenial cortex of rat injected with MK-801

Bielschowsky’s silver stain

Cresyl violet/Luxol Fast Blue

Figure 2B and 2C from Brad Bolon et al. Toxicol Pathol 2008;36:871-889
Neuronal chromatolysis and vacuolation, dorsal root ganglia, Cresyl Violet, rat
Peripheral Nerve – H & E and Luxol Fast Blue Staining
Peripheral Nerve – Plastic embedding & Toluidine Blue Staining
Ultrastructural Study of Peripheral Neuropathy

Arrow-head: Loss and thinning of myelin
Arrow: Büngner bands

Dorsal Root Ganglia
Positive controls

**Trimethylnit**
- Brain
- Spinal cord
- Spinal nerves
- Peripheral nerves

**Acrylamide**
- Brain
- Peripheral nerves
Trimethyltin: Neuronal Necrosis in Hippocampus

Hippocampus

Dentate granule cells

Dentate hilus

CA1
Trimethyltin: Neuronal Necrosis in Piriform Cortex
Trimethyltin: Purkinje Cell Necrosis in Cerebellum
Acrylamide: Axonal Degeneration in Sural Nerve
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Questions/Comments?
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