Developmental Neurotoxicity Guidance on Interpretation

Neuropathology

Brad Bolon
GEMpath, Inc.
Longmont, Colorado, USA
bradgempath@aol.com

orcid.org/0000-0002-6065-1492

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Coauthors

Dr. Robert Garman	**Consultants in Veterinary Pathology, Inc.** **Murrysville, Pennsylvania, United States**
Dr. Wolfgang Kaufmann	**Independent Consultant** **Bad Duerkheim, Germany**

Objectives

- Pathologist guidance regarding the neuropathology portion of developmental neurotoxicity (DNT) testing
- Current practices in DNT neuropathology
  - Tissue acquisition and processing
  - Qualitative / semi-quantitative evaluation
  - Quantitative analysis
  - Interpretation principles
- Keys for successful neuropathology evaluation and interpretation

Aims of DNT Neuropathology

Define target regions and cells – especially

- Cerebral cortex (associative, motor, sensory)
- Hippocampus (learning, memory)
- Cerebellum (motor)

Robust neuronal differentiation as well as myelin and synapse production

Identify classes of lesions

- *Macroscopic findings*
  - Changes in region size or shape – neural tube defects
  - Missing or new structures
- *Microscopic alterations*
  - Cell degeneration / death
  - Cytoarchitectural anomalies – aberrant differentiation
  - Pattern disruptions – ectopia, migratory defects
  - Persistence of transitory structures – dysgenesis

Part I:

Neuropathology Guidance for Developmental Neurotoxicity Testing

Guidance for DNT Neuropathology

- **Organisation for Economic Co-operation and Development (OECD) (2007). Guideline for the Testing of Chemicals: 426, *Developmental Neurotoxicity Study***

- **OECD (2018). Guideline for the Testing of Chemicals: 443, *Extended One-Generation Reproductive Toxicity Study* (Cohort 2)**

- **U.S. Environmental Protection Agency (EPA) (1998). Health Effects Test Guidelines: OPPTS 870.6300, *Developmental Neurotoxicity Study***

Industrial Best Practices for Sampling

Toxicologic Pathology, 34:296–313, 2006

ISSN: 0192-6233 print / 1533-1601 online
DOI: 10.1080/01926230600713269

***Toxicologic Pathology 34*: 296-313, 2006**

A 'Best Practices' Approach to Neuropathologic Assessment in Developmental Neurotoxicity Testing—for Today

BRAD BOLON,[1] ROBERT GARMAN,[2] KARL JENSEN,[3] GEORG KRINKE,[4] BARRY STUART[5] (CHAIR), AND AN AD HOC WORKING GROUP OF THE STP SCIENTIFIC AND REGULATORY POLICY COMMITTEE

[1]*GEMpath Inc., Cedar City, Utah 84720, USA*
[2]*Consultants in Veterinary Pathology, Inc., Murrysville, Pennsylvania 15668, USA*
[3]*National Health and Environment Effects Research Laboratory, U.S. EPA, Research Triangle Park, North Carolina 27711, USA*
[4]*Independent Consultant, Frenkendorf BL 4402, Switzerland*
[5]*Bayer Crop Science LP, Stilwell, Kansas 66085, USA*

ABSTRACT

A key trait of developmental neurotoxicants is their ability to cause structural lesions in the immature nervous system. Thus, neuropathologic assessment is an essential element of developmental neurotoxicity (DNT) studies that are designed to evaluate chemically-induced risk to neural substrates in young humans. The guidelines for conventional DNT assays have been established by regulatory agencies to provide a flexible scaffold for conducting such studies; recent experience has launched new efforts to update these recommendations. The present document was produced by an ad hoc subcommittee of the Society of Toxicologic Pathology (STP) tasked with examining conventional methods used in DNT neuropathology in order to define the 'best practices' for dealing with the diverse requirements of both national (EPA) and international (OECD) regulatory bodies. Recommendations (including citations for relevant neurobiological and technical references) address all aspects of the DNT neuropathology examination: study design; tissue fixation, collection, processing, and staining; qualitative and quantitative evaluation; statistical analysis; proper control materials; study documentation; and personnel training. If followed, these proposals will allow pathologists to meet the need for a sound risk assessment (balanced to address both regulatory issues and scientific considerations) in this field today while providing direction for the research needed to further refine DNT neuropathology 'best practices' in the future.

Practical Techniques for Sampling

Toxicologic Pathology
2016, Vol. 44(1) 14-42

DOI: 10.1177/0192623315596858
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Recommended Methods for Brain Processing and Quantitative Analysis in Rodent Developmental Neurotoxicity Studies

Robert H. Garman[1], Abby A. Li[2], Wolfgang Kaufmann[3], Roland N. Auer[4], and Brad Bolon[5]

Toxicologic Pathology 44: 14-42, 2016

Abstract

Neuropathology methods in rodent developmental neurotoxicity (DNT) studies have evolved with experience and changing regulatory guidance. This article emphasizes principles and methods to promote more standardized DNT neuropathology evaluation, particularly procurement of highly homologous brain sections and collection of the most reproducible morphometric measurements. To minimize bias, brains from all animals at all dose levels should be processed from brain weighing through paraffin embedding at one time using a counterbalanced design. Morphometric measurements should be anchored by distinct neuroanatomic landmarks that can be identified reliably on the faced block or in unstained sections and which address the region-specific circuitry of the measured area. Common test article–related qualitative changes in the developing brain include abnormal cell numbers (yielding altered regional size), displaced cells (ectopia and heterotopia), and/or aberrant differentiation (indicated by defective myelination or synaptogenesis), but rarely glial or inflammatory reactions. Inclusion of digital images in the DNT pathology raw data provides confidence that the quantitative analysis was done on anatomically matched (i.e., highly homologous) sections. Interpreting DNT neuropathology data and their presumptive correlation with neurobehavioral data requires an integrative weight-of-evidence approach including consideration of maternal toxicity, body weight, brain weight, and the pattern of findings across brain regions, doses, sexes, and ages.

Comparison of Guidance: Timing

- Time point for neuropathology evaluation – early
  - EPA 870.6300 = PND 11 in guidance (now PND 22 in practice)
  - OECD 426 = between PND 11–22
  - OECD 443 = PND 21–22
  - Neuropathologists = depends on purpose
    - Histopathology (semi-quantitative) = PND 11 or optimally PND 22
    - Morphometry (quantitative) = PND 22
- Time point for neuropathology evaluation – late
  - EPA 870.6300 = sometime after behavioral testing ends (PND 60)
  - OECD 426 = around PND 70
  - OECD 443 = PND 75–90
  - Neuropathologists = PND 70–90

Toxicol Pathol 44: 14-42, 2016

Comparison of Guidance: Numbers

- No. of litters per dose for neuropathology
  - EPA 870.6300 = 12 required (20 recommended)
  - OECD 426 = 20
  - OECD 443 = 20 (≥12 litters for morphometry)
  - Neuropathologists = 20 recommended (≥12 litters for morphometry)

    NOTE: 1 pup per litter (to yield ≥6 per sex per dose per time point)
- Group size for neuropathology (per sex/dose/time point)
  - EPA 870.6300 = 6 required (10 recommended)
  - OECD 426 = 10
  - OECD 443 = 10 (≥6 for morphometry)
  - Neuropathologists = 10 recommended (≥6 for morphometry)

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Comparison of Guidance: Macroscopic (Gross) Evaluation

- Gross observations = yes (EPA 870.6300, OECD 426, OECD 443)
- Brain weights = yes (1 per litter for 20 litters to give 10/sex)
  - Fixation status
    - Fresh tissue – animals not slated for neuropathology
    - Fixed tissue – animals destined for neuropathology
  - Special instructions
    - EPA 870.6300 = none
    - OECD 426 = none
    - OECD 443 = counterbalance timing of weights across all dose groups
    - Neuropathologists = counterbalance timing across all dose groups
- Brain gross morphometry
  - Performed at institutional discretion (on images)
  - Not specified in either EPA or OECD guidance

Toxicol Pathol 44: 14-42, 2016

Gross Morphometry Options

- Linear or areal dimensions of major external domains
- Sites – neocortex, cerebellum

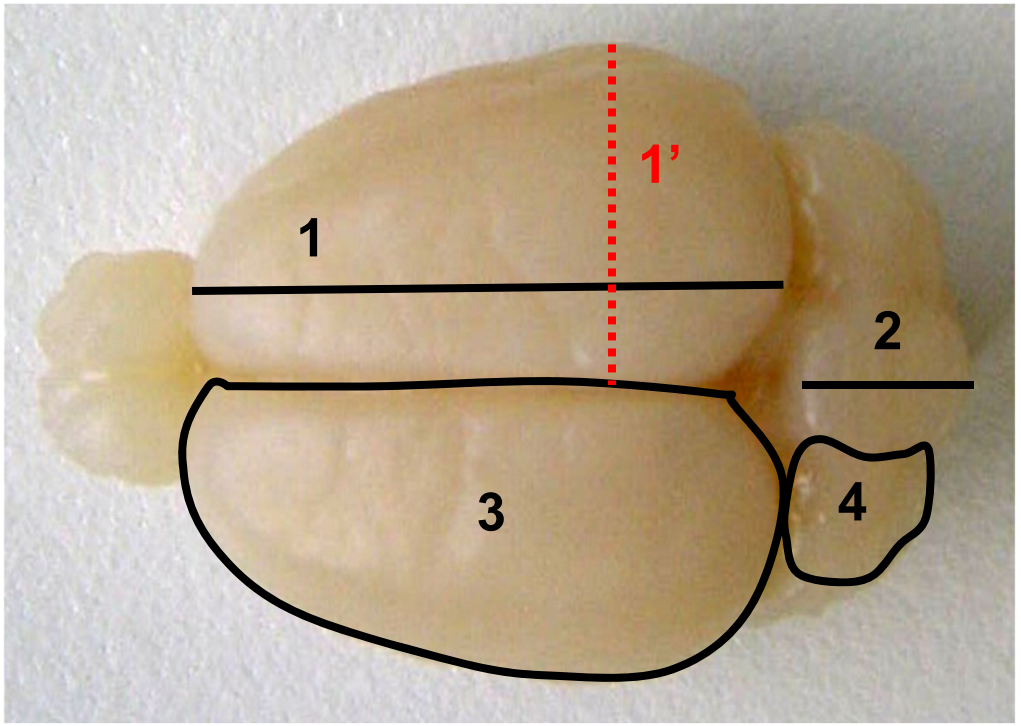
1 Cerebral length

1’ Cerebral width

2 Cerebellar length

3 Cerebral area

4 Neocerebellar area



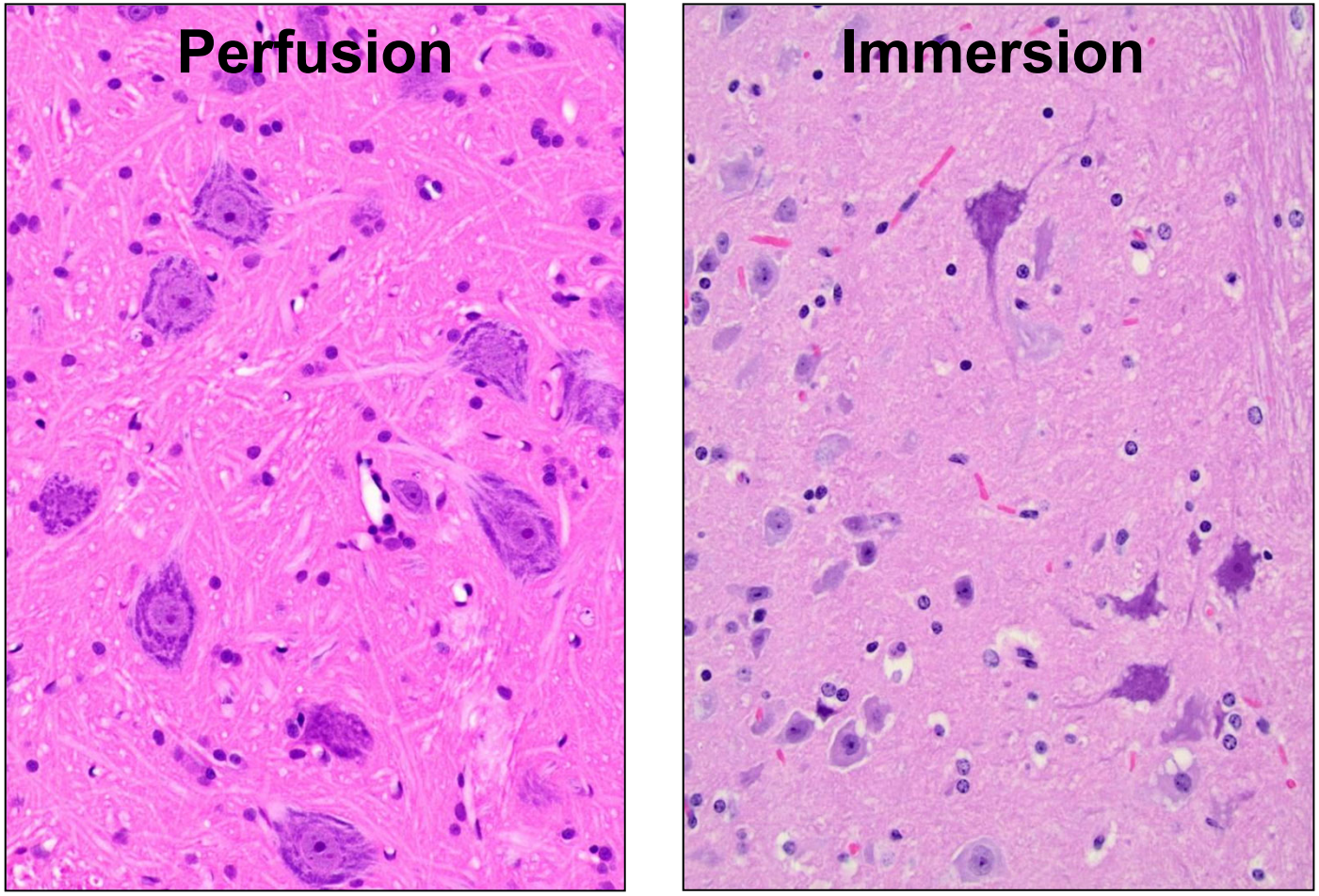
Toxicol Pathol 34: 296-313, 2006

Comparison of Guidance: Fixation

- **Fixative type (early and late time points)**
  - **EPA 870.6300 = aldehyde**
  - **OECD 426, OECD 443 = "appropriate aldehyde"**
  - **Neuropathologists = formaldehyde**
    - **neutral buffered 10% formalin (4% formaldehyde + 1% methanol [NBF])**
    - **methanol-free 4% formaldehyde (= "paraformaldehyde" [PFA])**
- **Fixation procedure**
  - **Early (PND 11 or PND 22)**
    - **EPA 870.6300 = immersion**
    - **OECD 426, OECD 443 = immersion or perfusion**
    - **Neuropathologists = immersion**
  - **Late (PND 60 to 70) = perfusion (EPA 870.6300, OECD 426, OECD 443)**

***Toxicol Pathol* 44: 14-42, 2016**

Impact of Fixation on Neural Features



Encyclopedia of Neurological Sciences, 2nd ed, Vol 2, 2014 ("Fixation and processing of CNS tissue")

Comparison of Guidance: Tissue Processing

- Up-front processing to embedded blocks for all dose groups
  - EPA 870.6300 = not specified
  - OECD 426 = not specified
  - OECD 443 = required
  - Neuropathologists = recommended (at least for brain blocks slated for morphometric analysis)
- Counterbalancing = all steps have samples from all dose groups
  - EPA 870.6300 = not specified
  - OECD 426 = required for perfusion, tissue processing, staining
  - OECD 443 = not specified
  - Neuropathologists = recommended for perfusion, tissue processing

Toxicol Pathol 44: 14-42, 2016

Comparison of Guidance: Tissue Processing

- **Embedding medium**
  - **EPA 870.6300 = paraffin for CNS, plastic for PNS (suggested for CNS)**
  - **OECD 426, OECD 443 = paraffin for CNS and PNS (or plastic if better resolution is needed)**
  - **Neuropathologists = paraffin for CNS and PNS (using plastic only if required by a regulatory agency)**
- **Staining procedures**
  - **Routine = H&E (EPA 870.6300, OECD 426, OECD 443)**
  - **Special stains = neuroaxonal and myelin (for CNS and PNS)**
    - **EPA 870.6300 = "if warranted"**
    - **OECD 426, OECD 443 = not specified**
    - **Neuropathologists = neuroaxonal and myelin stains recommended**

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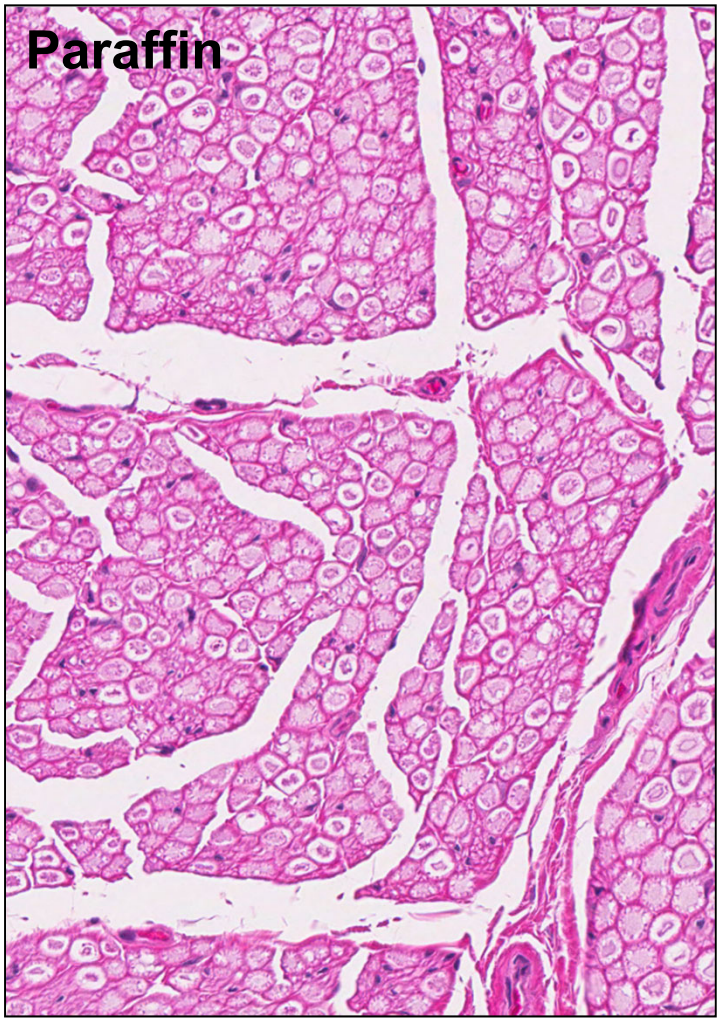
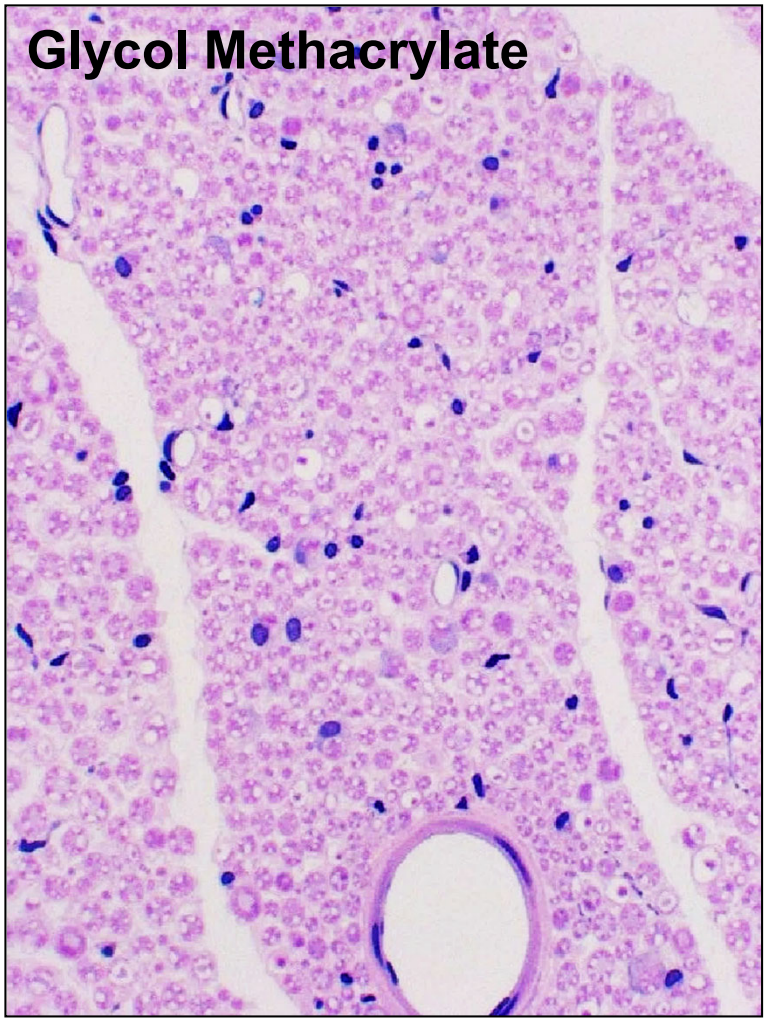
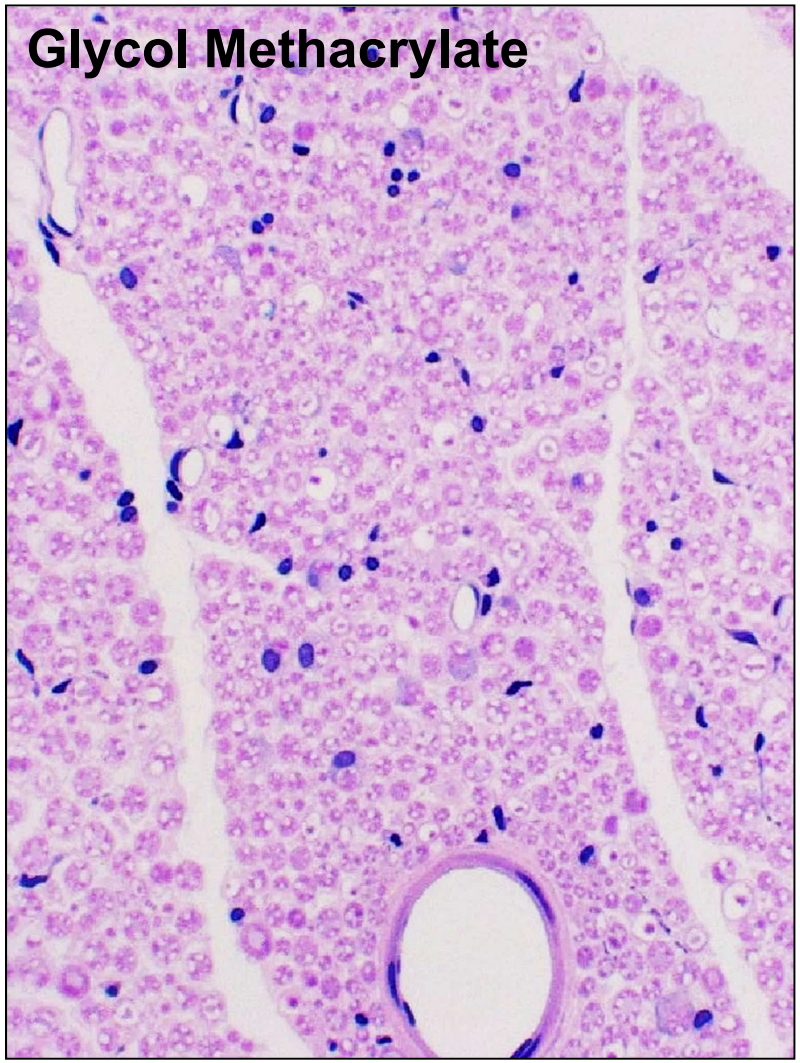
Paraffin = “Soft Plastic”

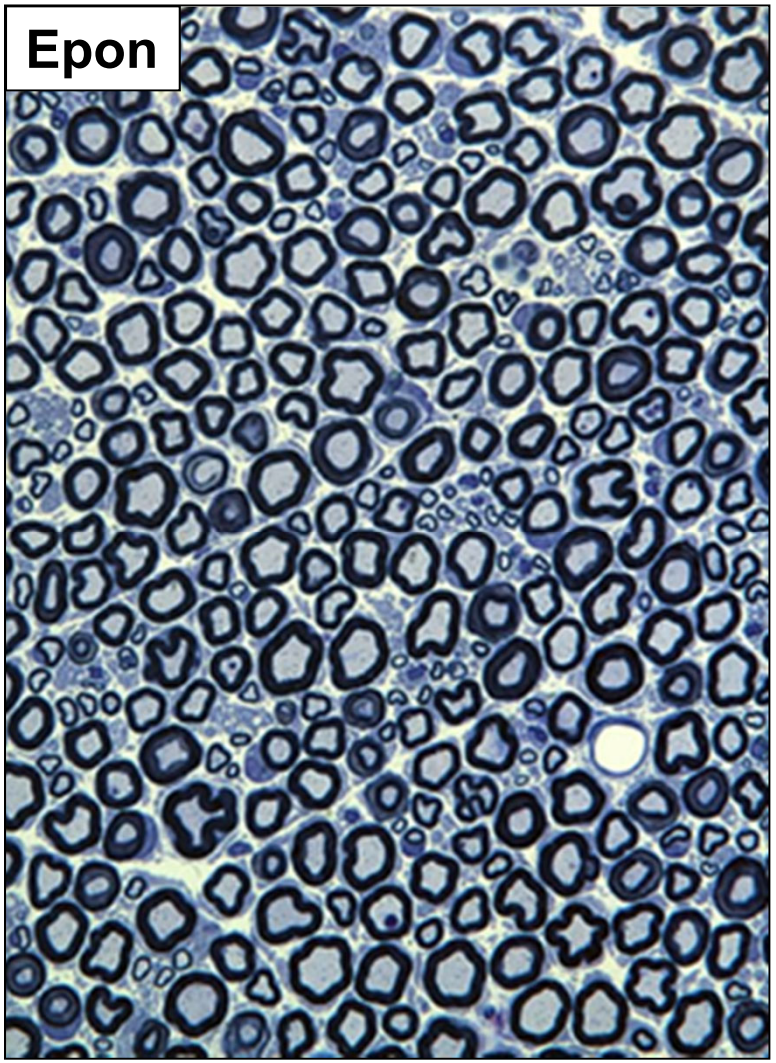




Toxicol Pathol 46: 372-402, 2018

“Soft Plastic” ≠ “Hard Plastic”



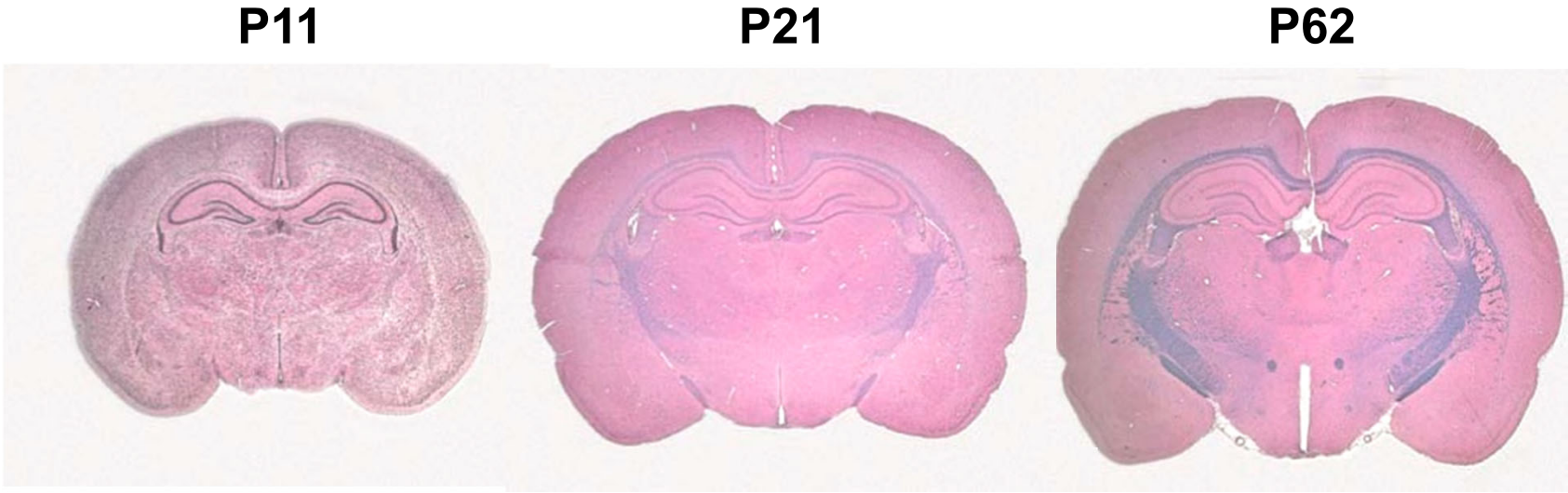


Toxicol Pathol 46: 372-402, 2018

Comparison of Guidance: Tissue Section Preparation

- **Sectioning**
  - Number – take several, assess one (with proper anatomy)
  - Thickness – depends on the embedding medium
    - Paraffin – 4 to 5 µm
    - Soft plastic – 2 µm
    - Hard plastic – 1 µm
- **Staining**
  - Cellular stains: if warranted by professional judgment (EPA)
    - Cell-specific
      - Neurons = cresyl violet (neurons)
      - Myelin = Luxol fast blue (myelin)
    - Neuroaxonal integrity – silver stains (Bielschowsky's)
    - Glial reaction – mainly P60 or older
      - Astrocytes = glial fibrillary acidic protein (GFAP)
      - Microglia = ionized calcium binding adaptor molecule 1 (Iba1)
  - Degeneration stains (rare): cleaved caspase 3, Fluoro Jade B

Moments Matter



Progressive myelination in control Wistar rat brains

***Fundamental Neuropathology for Pathologists and Toxicologists: Principles and Techniques*, 2011;**
W. Kaufmann, pp. 339-363 (Figures 1-3)

Comparison of Guidance: Neuropathology Design

- Conventional (semi-quantitative) lesion scoring
  - Examination of "multiple representative sections"
  - Evaluation of "all major structures"
  - True for all guidance (EPA 870.6300, OECD 426, OECD 443)
- Morphometric analysis – on highly homologous sections
  - Key structures
    - Routine: neocortex (Neo), hippocampus (Hip), cerebellum (Cer)
    - Extra: striatum (Str), corpus callosum (CC)
  - Recommendations
    - EPA 870.6300 = "simple" linear for Neo, Hip, Cer
    - OECD 426, OECD 443 = "linear or areal" for "representative" regions
    - Neuropathologists = "simple" linear for Neo, Hip, Cer, Str, CC

Toxicol Pathol 44: 14-42, 2016

Comparison of Guidance: Spinal Cord Sampling

- **Guidelines differ on segments to sample**
- **Key recommendations**
  - **EPA 870.6300 = "multiple representative sections" (not specified)**
  - **OECD 426, OECD 443**
    - **Cervical segment required (level not specified)**
    - **Thoracic segment not specified**
    - **Lumbar segment required (level not specified)**

      NOTE: Cross and longitudinal (or oblique) sections should be viewed
  - **Neuropathologists = specific segments recommended**
    - **Cervical = $C_1 \pm C_{4-6}$**
    - **Thoracic = T_{6-8}**
    - **Lumbar = L_{4-5} (which is found inside vertebrae L_{1-2})**

      NOTE: Cross and longitudinal (or oblique) sections should be viewed

***Toxicol Pathol* 44: 14-42, 2016**

Comparison of Guidance: Sampling Ganglia and Nerves

- **Guidelines differ on organs to sample**
- **Key recommendations**
  - **EPA 870.6300 = "multiple representative" organs (not specified)**
  - **OECD 426, OECD 443**
    - **Dorsal root ganglia (DRG) = "representative"**
    - **Spinal nerve roots = dorsal and ventral (sites not specified)**
    - **Nerves = sciatic (proximal), tibial (proximal), tibial branches to muscle**

      NOTE: Both cross and longitudinal sections of nerve should be viewed
  - **Neuropathologists = specific segments recommended for collection**
    - **DRG = multiple cervical, thoracic, lumbar**
    - **Spinal nerve roots = many dorsal and ventral (cervical, thoracic, lumbar)**
    - **Nerves = multiple hind limb nerves (sciatic, tibial, fibular, ± sural)**

      NOTE: Both cross and longitudinal sections of nerve should be viewed

***Toxicol Pathol* 44: 14-42, 2016**

Comparison of Guidance: Need for "Blinded" Evaluation

- Guidance differs on use of coded ("blinded") microscopic analysis
- Key recommendations
  - EPA 870.6300 = required if lesions seen during initial open ("non-blinded") histopathologic evaluation
  - OECD 426, OECD 443 = recommended if lesions observed in initial open evaluation
  - Neuropathologists – depends on task
    - Routine (semi-quantitative) assessment
      - Initial evaluation = non-blinded evaluation recommended
      - *Post hoc* review = use blinded evaluation when needed to refine target organs or clarify potential dose-response effects
    - Morphometric (quantitative) analysis = blinded analysis recommended

Toxicol Pathol 44: 14, 2016

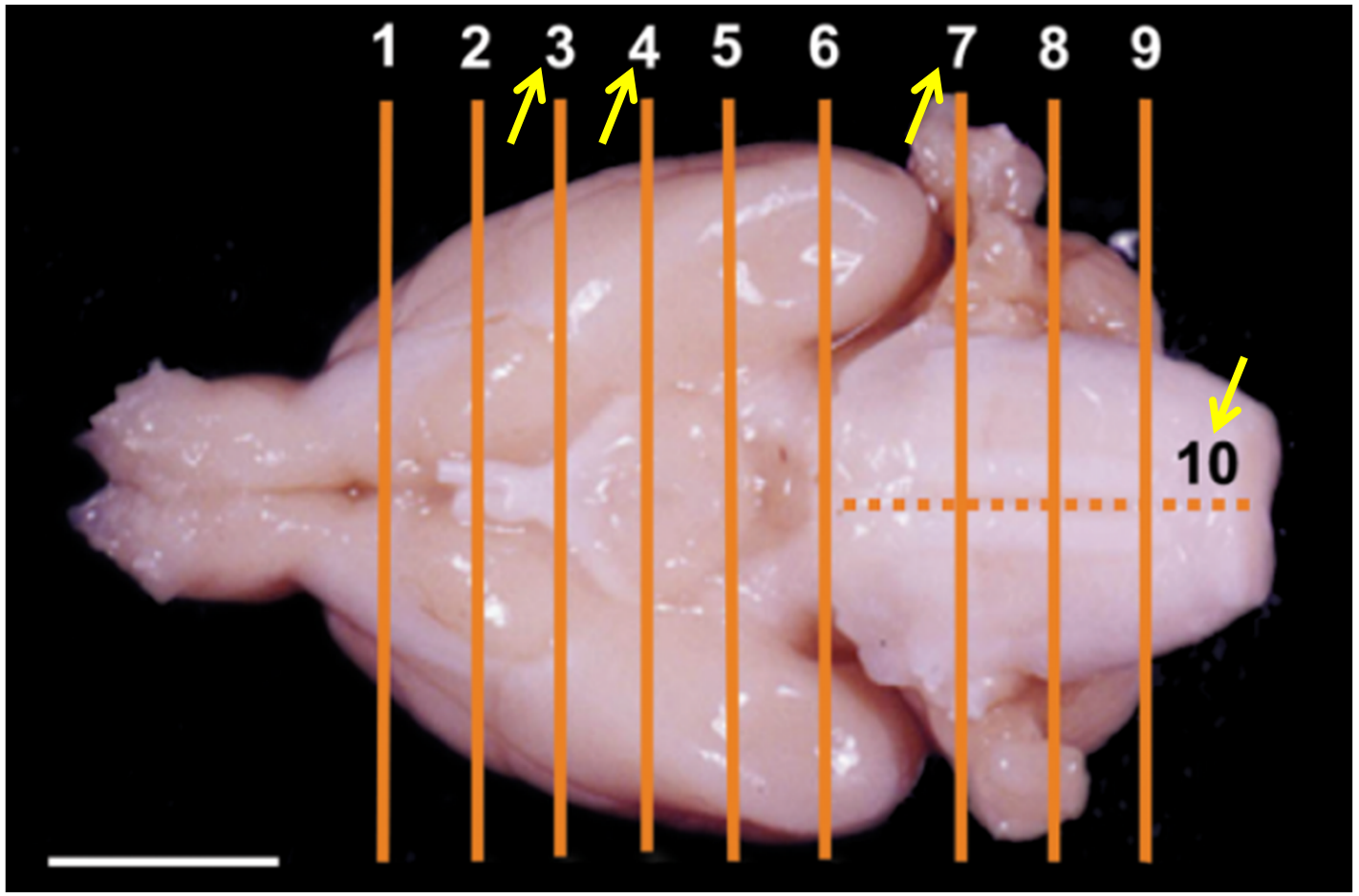
Comparison of Guidance: Reporting

- Guidance is less prescriptive in EPA 870.6300
- Major topics (combined from EPA 870.6300, OECD 426, OECD 443)
  - Methods
    - Detailed procedures (with automated devices and their calibration)
    - Description of balancing procedures
    - Proof of sensitivity (via concurrent and historical positive control data)
    - Justification for any decisions involving professional judgment
  - Results
    - Appropriate diagnostic nomenclature is required for clarity, data bridging
    - Correlation of neuropathologic to behavioral effects
    - Discussions of critical factors (e.g., biological vs. statistical significance, dose–response relationships—but NOT the determination of an NOAEL)
    - Essential images
      - Representative test article-related findings
      - Whole-section views to confirm high homology for morphometry

Part II:

Keys for Successful Neuropathology Evaluation and Interpretation

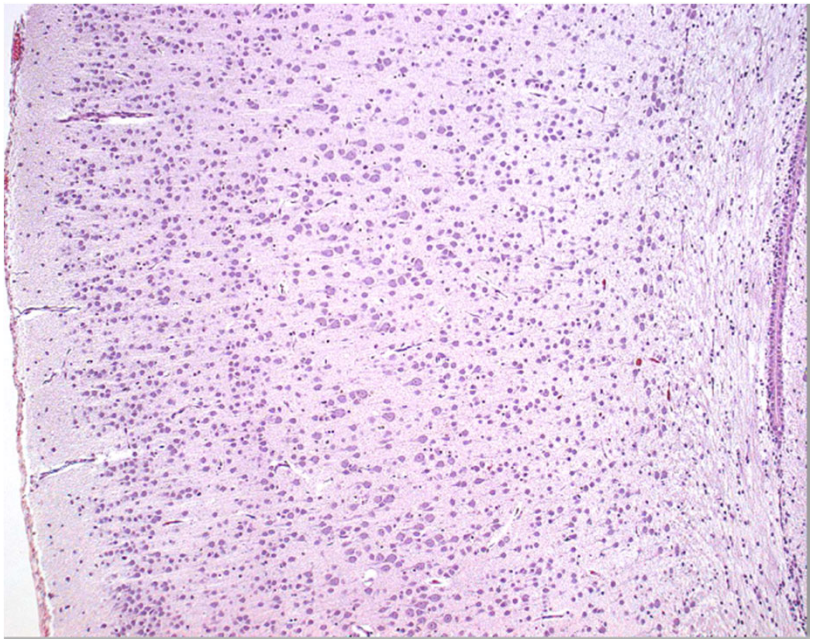
Brain Trimming for DNT Neuropathology



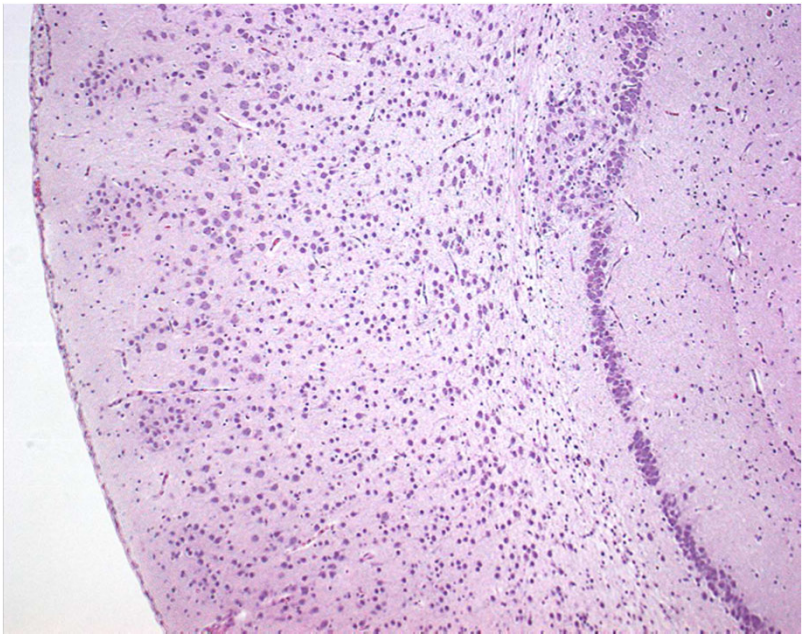
Toxicol Pathol 44: 14-42, 2016

Pattern: Altered Numbers

Vehicle Control



MAM-Treated

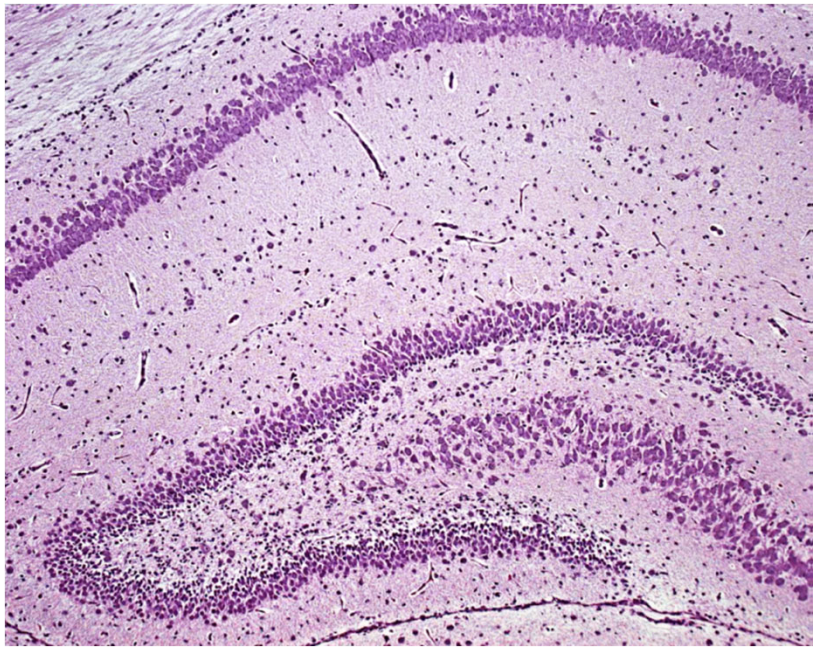


Section of a Wistar rat brain at P62 following maternal methylazoxymethanol (MAM) exposure at 30 mg/kg IP on E15. Relative to an age-matched control, the cerebral cortex is smaller and the cerebrocortical neurons in the parietal cortex are significantly reduced in number.

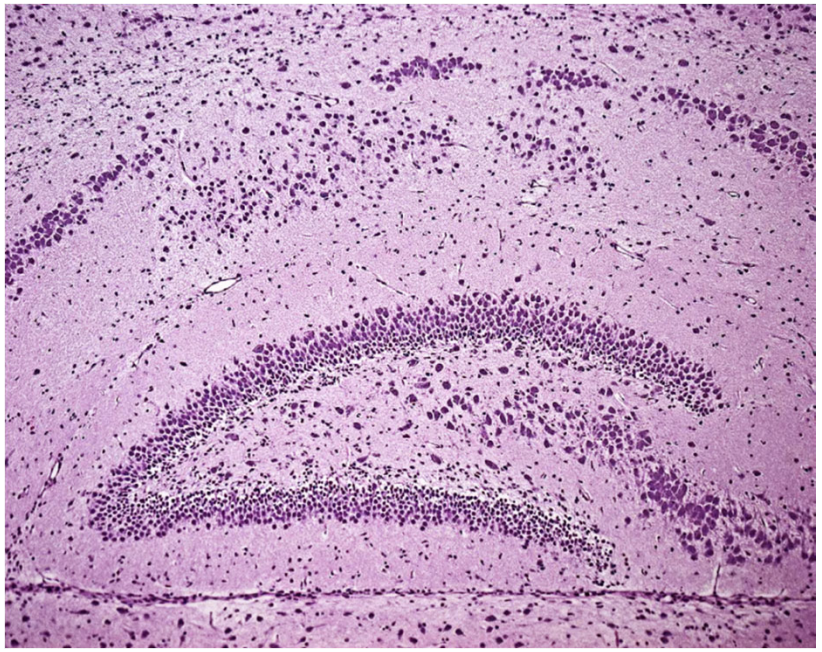
***Fundamental Neuropathology for Pathologists and Toxicologists: Principles and Techniques*, 2011; W. Kaufmann, pp. 339-363 (Figures 15 and 19)**

Pattern: Altered Organization

Vehicle Control



MAM-Treated

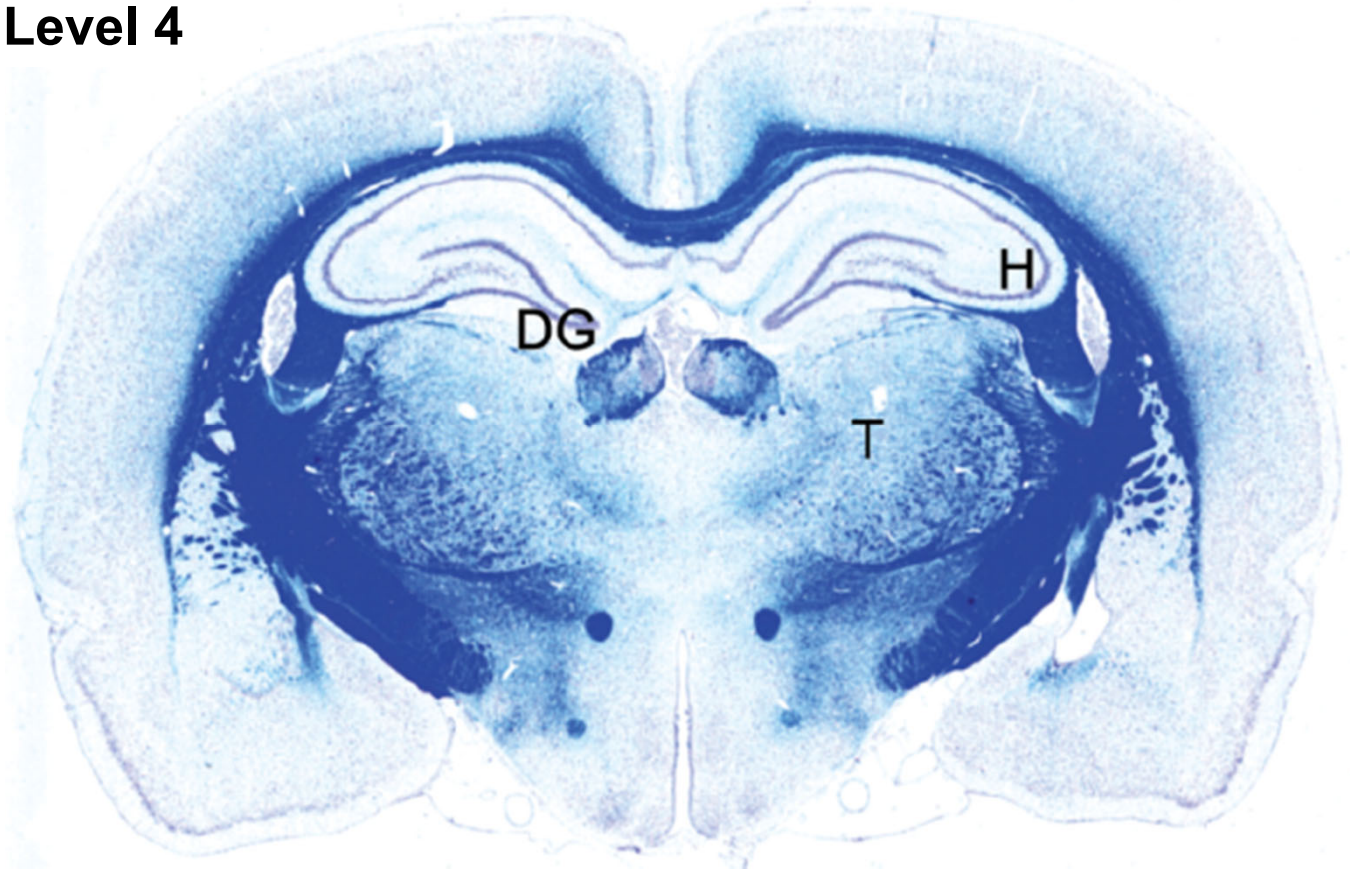


Section of a Wistar rat brain at P62 following maternal methylazoxymethanol (MAM) exposure at 30 mg/kg IP on E15. Relative to an age-matched control, heterotopiae (ectopiae) comprised of displaced neurons are widespread in the hippocampus.

***Fundamental Neuropathology for Pathologists and Toxicologists: Principles and Techniques*, 2011; W. Kaufmann, pp. 339-363 (Figures 16 and 20)**

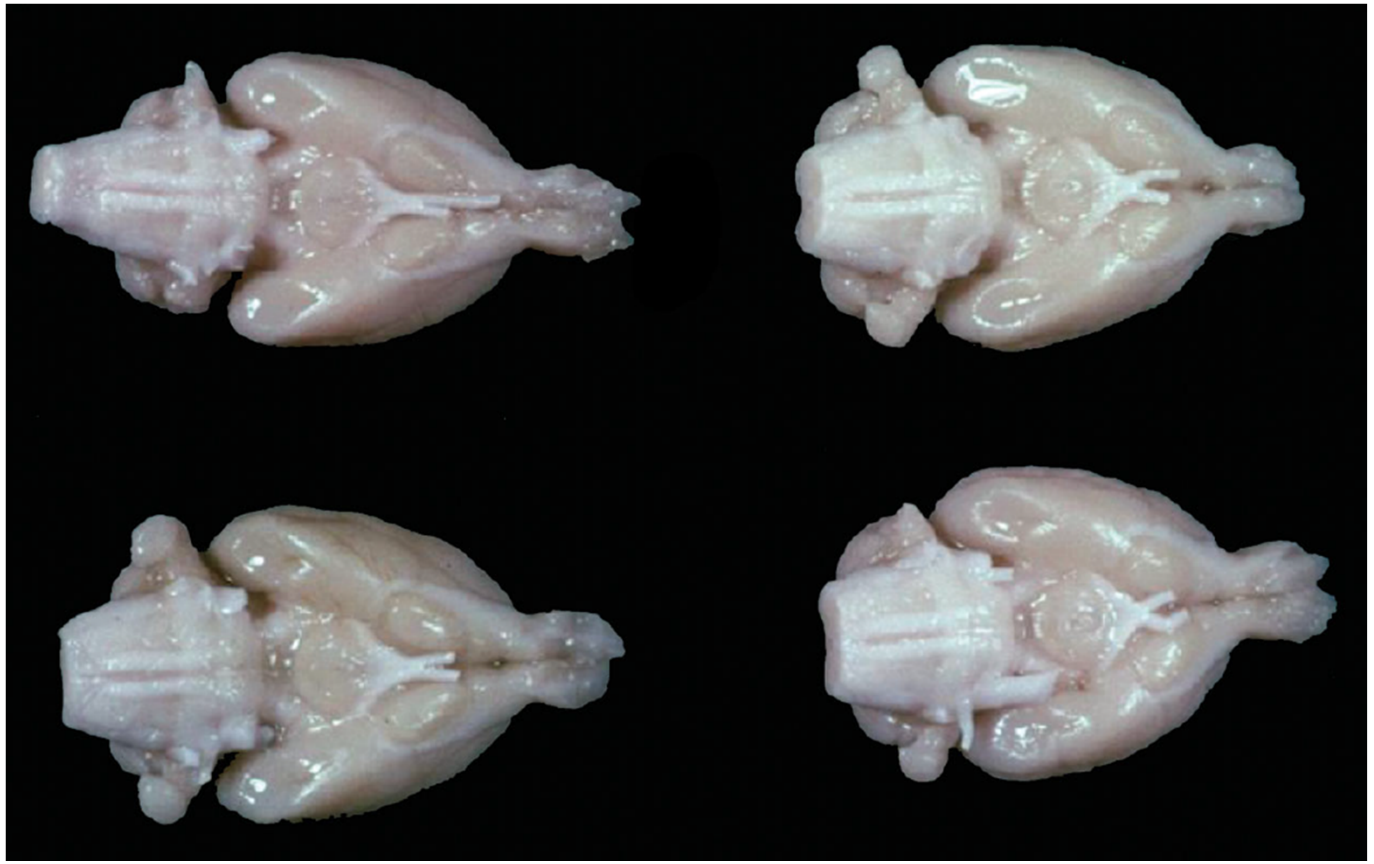
Symmetry is Essential for Morphometric Measurements

Level 4



***Toxicol Pathol* 44: 14-42, 2016**

Brain Trimming is Not Trivial

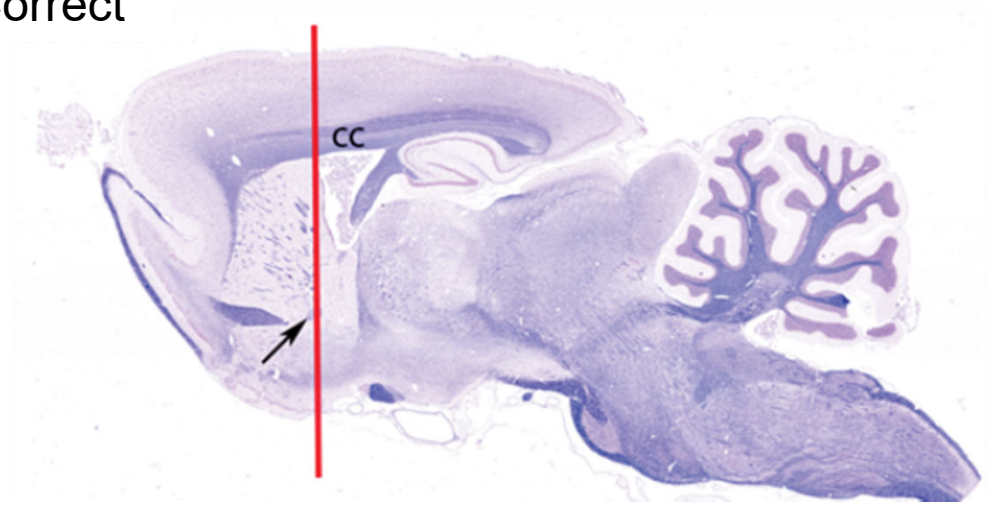


Four control adult rat brains at ~P70

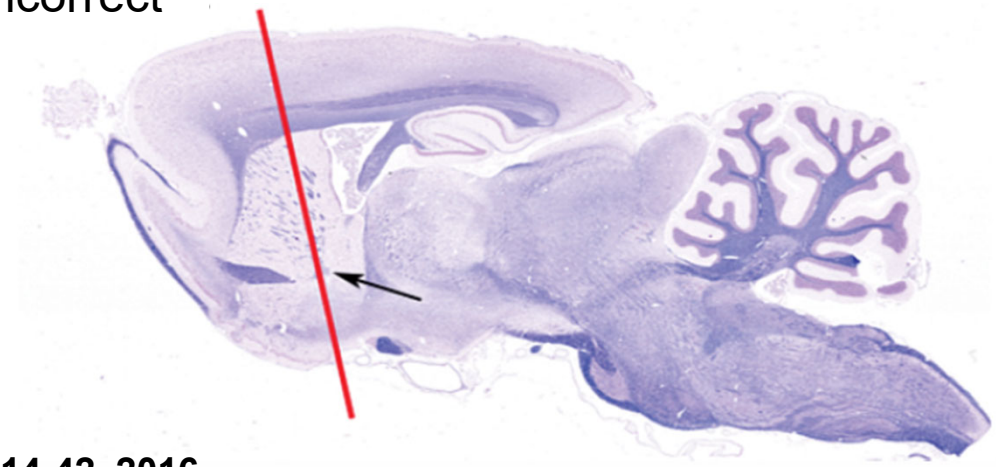
***Toxicol Pathol* 44: 14-42, 2016**

Carelessness has Consequences

Correct

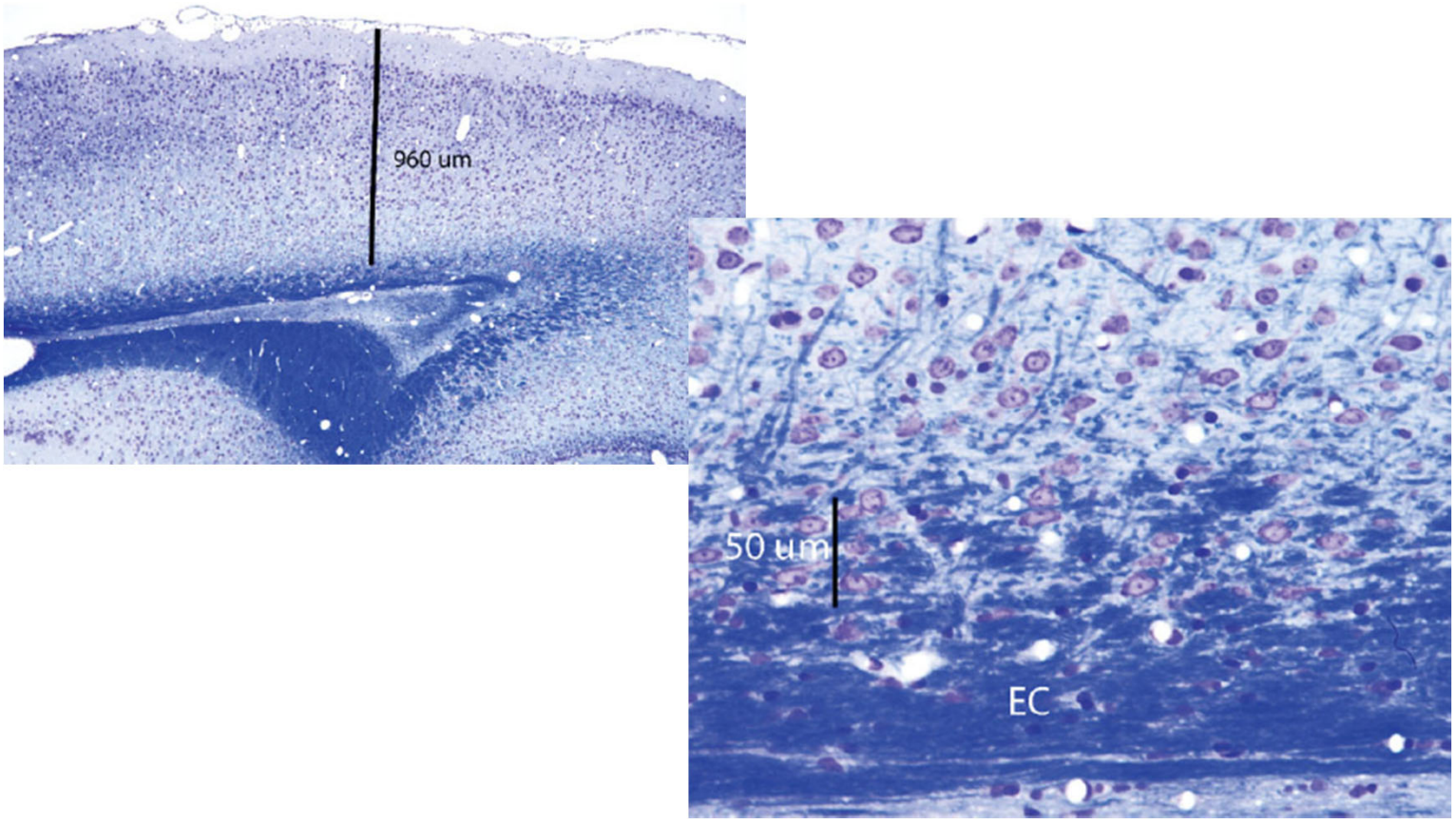


Incorrect



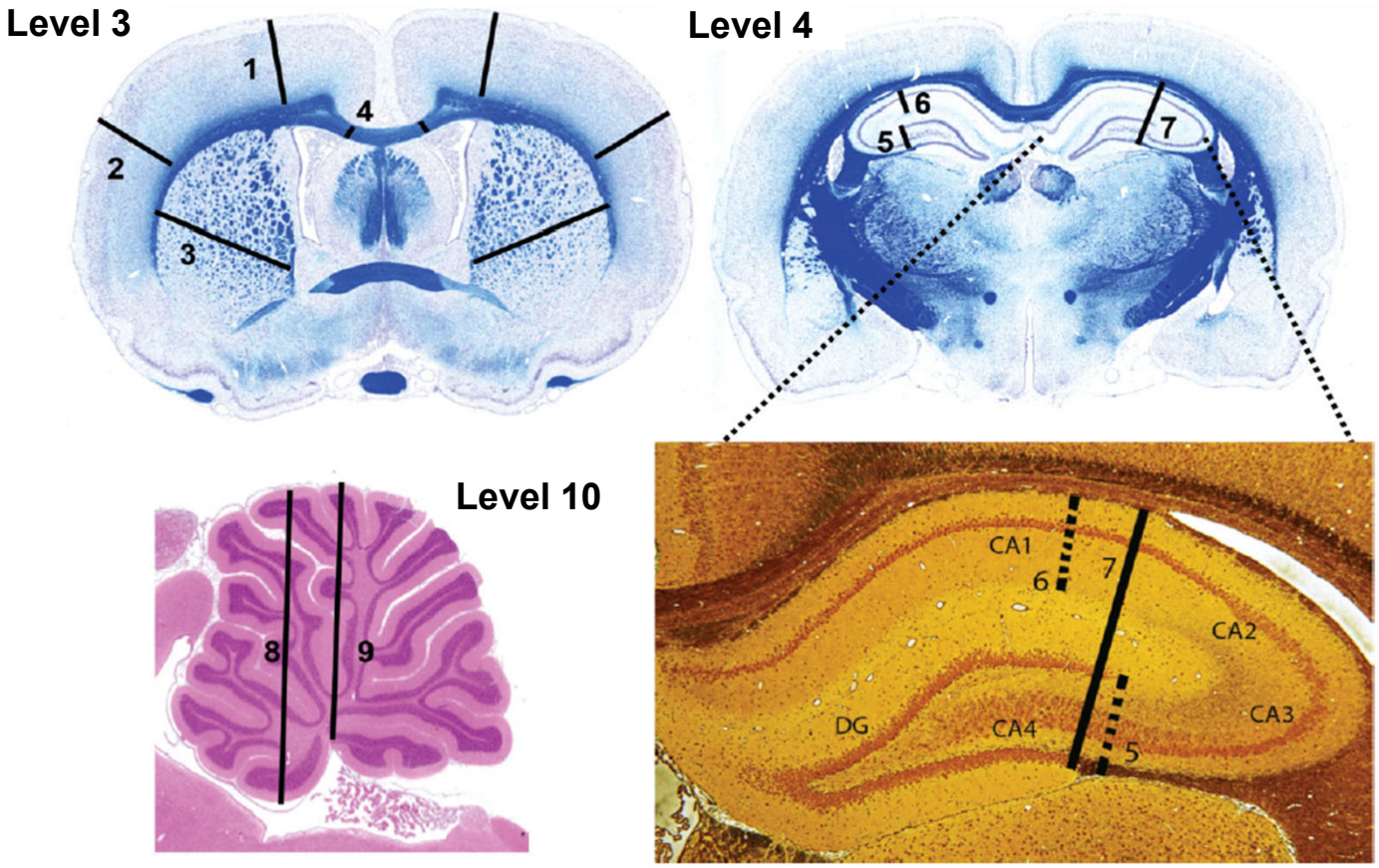
Toxicol Pathol **44: 14-42, 2016**

Morphometric Measurement Sites: Mooring Matters



***Toxicol Pathol* 44: 14-42, 2016**

Morphometric Measurement Sites: Neuropathologist Preferences



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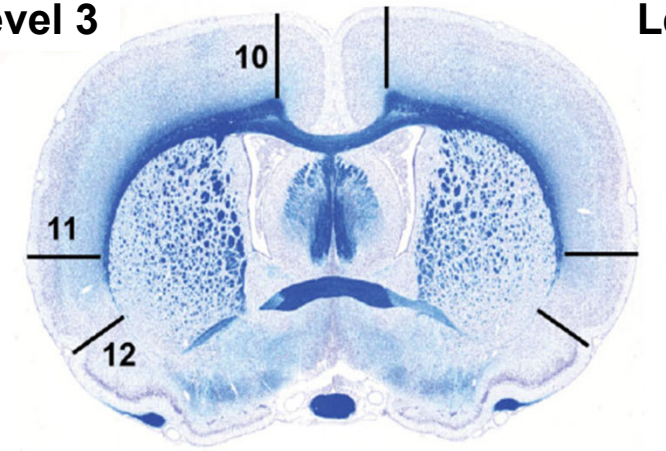
Morphometric Measurement Sites: Acceptable Alternatives

Level 3



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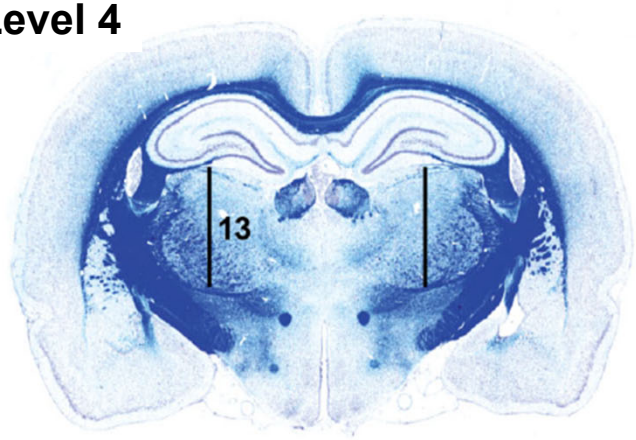

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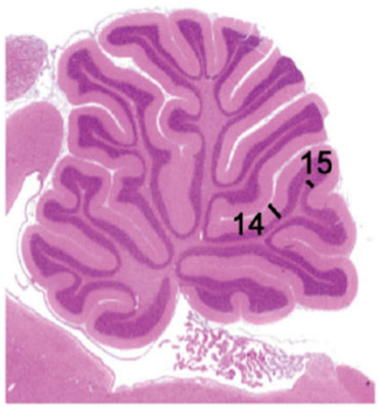
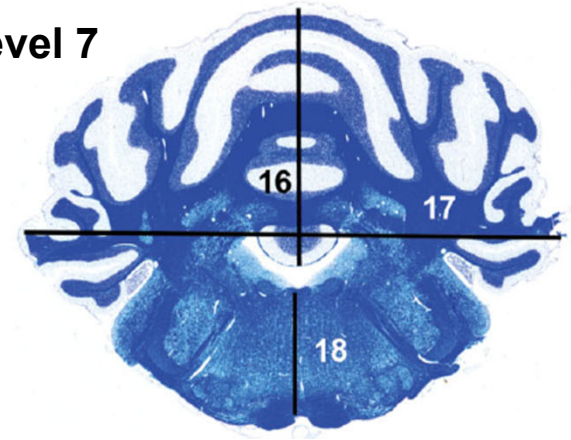


Level 7

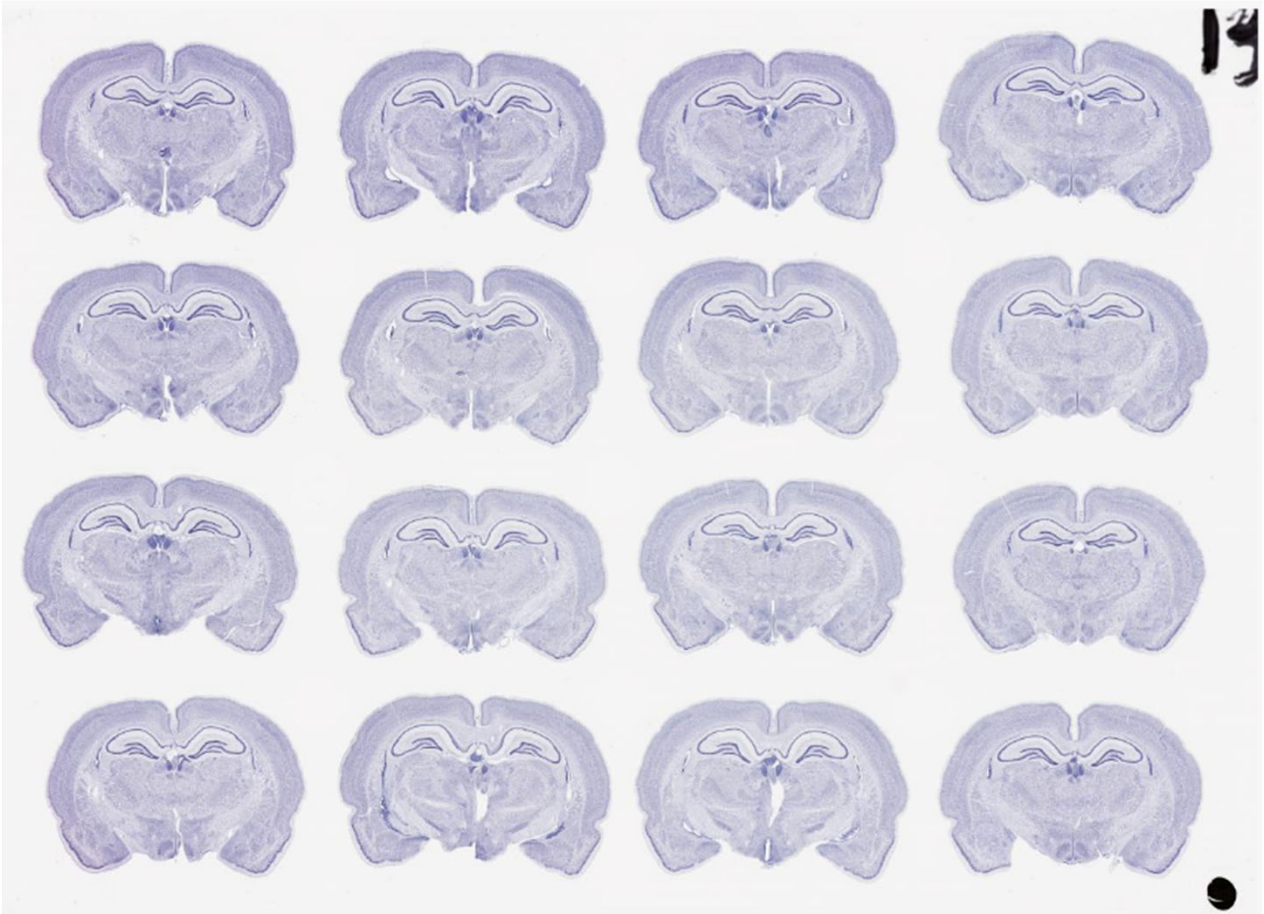


Toxicol Pathol 44: 14-42, 2016

Technology Can Improve Homology

Multibrain® preparation

(thionine stain for neurons on frozen sections)



NeuroScience Associates, Knoxville, Tennessee, USA
https://www.neuroscienceassociates.com/

Stereology for Quantitative Analysis in DNT Neuropathology?

- Rationale: even an experienced neuropathologist can find it difficult to detect cell deficits of <25%
- Stereology typically is deployed *post hoc* based on weight-of-evidence arguments from other pathology endpoints
- Advantages: sensitive, precise, and unbiased
- Disadvantages: labor-intensive and very slow
- Practical notes:
  - Tests: cell density (disector), volume (Cavalieri methods)
  - Regions to measure: major brain targets (alternating sides)
  - Process: serial physical sectioning of brain (4 hrs/rat)
  - Analysis: ~100 objects from 25 to 50 400x fields in 6 to 8 serial, 3-μm-thick disectors (pairs of adjacent sections)—about 1 hr/site or 1 to 4 hrs/rat—"blinded" approach to analysis

Toxicol Pathol 39: 289-293, 2011

Comparative Performance of Common DNT Neuropathology Endpoints

Parameter	Sensitivity	Speed	Quantitative?	Cost
Organ weights	High	Fast	Yes	Low
Macroscopic observations	Low	Fast	No	Low
Routine microscopic evaluation	Medium	Medium	Semi	Low
Special neurohistological stains	High	Medium	Semi	Medium
Morphometric measurements	High	Slow	Yes	Medium
Stereology	High	Slow	Yes	High

Pathologist Views on Data Interpretation 1

Clear Evidence of Developmental Neurotoxicity – 1 or 2 or 3, alone or in any combination

1) Dose-related presence (or increased frequencies) of qualitative anatomic lesions
2) Statistically significant, dose-related differences in the same linear measures for
    a) both PND 22 and PND 70 animals, especially if these treatment-related differences are more pronounced in the adults
    b) both male and female PND 70 rats
3) Statistically significant, dose-related differences in behavioral effects—especially if these are not characterized by complete recovery and cannot be explained by pharmacology

Possible Evidence of Developmental Neurotoxicity – combination of 1 with 2 and/or 3

1) Absence of treatment-related microscopic alterations in the nervous system
2) Statistically significant, dose-related differences in the same linear measures for male and/or female rats at PND 22 only
3 Behavioral effects that are inconsistent across dose and/or age, but that appear to be linked to morphologic changes (e.g., hippocampal lesions and learning and memory effects; cerebellar lesions and difficulty with balance)

Toxicol Pathol 44: 14-42, 2016 (Table 3)

Pathologist Views on Data Interpretation 2

Ambiguous Evidence of Developmental Neurotoxicity – combination of 1 with 2 or 3

1) Absence of treatment-related microscopic alterations in the nervous system
2) Statistically significant differences in linear measurements that appear to be:
    a) inconsistent across brain regions, age, sex or dose (e.g., present in low and/or mid dose but not in the high-dose group; isolated finding in one sex but not the other) or
    b) present in conjunction with progeny brain weight decreases that can be linked to a more general effect on growth (e.g., decreased total body weight in juveniles or dams and/or maternal care issues)
3) Behavioral effects that are inconsistent across dose, age and sex

No Evidence of Developmental Neurotoxicity

1) No evidence of any treatment-related microscopic alterations in the nervous system
2) No statistically significant differences in linear measurements
3) No evidence of treatment-related behavioral effects

Toxicol Pathol 44: 14-42, 2016 (Table 3)

Neuropathology Interpretation: Examples

- **Possible Evidence of Developmental Neurotoxicity**
  - **Low brain but not body weights in all high-dose rats at PND 22**
  - **No treatment-related microscopic findings in the nervous system**
  - **Statistically significant, dose-related differences bilaterally in linear measures for hippocampus in males and females at PND 22 only**
  - **Reduced auditory startle response in high-dose females at PND 22**
- **No Evidence of Developmental Neurotoxicity**
  - **Low brain and body weights in some high-dose rats at PND 11**
  - **No treatment-related microscopic findings in the nervous system**
  - **Inconsistent statistically significant differences in linear measures**
    - **Females – unilateral decrease in left hippocampus height at high dose**
    - **Males – unilateral decrease in right cerebral length at high dose**
  - **No treatment-related behavioral effects**

Limitations of DNT Neuropathology

- **Technical considerations**
  - **Throughput is slow (especially when performing quantitative assessments)**
  - **Expert staff (at all levels) are often hard to find**
  - **Current pathology endpoints are static measures**
- **Strategic concerns**
  - **Conventional studies yield little mechanistic data and do not define critical periods**
  - **Current practice cannot always confirm that neural changes result from chemical exposure rather than from confounding environmental or maternal influences**

Questions Unanswered by Current DNT Neuropathology Tests

- **What are the risks of exposure during adolescence?**
  - **Chemical exposure from E6 to P10 or P21 does not span the full adolescent phase in rodents (P11 to P30), a time of major synaptogenesis and gliogenesis**
  - **Human adolescents are exposed to neurotoxic mixtures**
- **What are we missing? Necropsy at P60 cannot assess:**
  - **Heightened susceptibility to challenges (later neurotoxicant exposures)**
  - **Premature senescence or enhanced degeneration with age**

Resources for DNT Neuropathology 1

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- Li AA, Sheets LP, Raffaele K, et al. Recommendations for harmonization of data collection and analysis of developmental neurotoxicity endpoints in regulatory guideline studies: Proceedings of workshops presented at Society of Toxicology and joint Teratology Society and Neurobehavioral Teratology Society meetings. *Neurotoxicol Teratol*. 2017; 63: 24-45
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