# End-points to consider for dedicated neuropathology evaluation: Immunohistochemistry, Electron microscopy, Biomarkers, Neurofunctional assessment and Electrophysiology

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# Acknowledgments


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- Walt Bobrowski B.Sc., CEMT Pfizer Groton CT
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- Chang-Ning Liu PhD, Pfizer Groton CT
- Tim Coskran: MA, HTL

# Introduction Outline

- Preclinical safety assessments performed to evaluate neurotoxicity
- Immunohistochemistry
- Transmission electron microscopy (TEM)
- Biomarkers
- Neurofunctional assessment
- Electrophysiology
- Conclusion

# Preclinical safety assessments performed to evaluate neurotoxicity

- Comprehensive literature review
- Early target characterization
  - Protein and/or mRNA localization (e.g. PCR, IHC, ISH)
  - Genetically modified mouse models
  - In vitro/ex vivo and/or in vivo mechanistic studies
    - Isolated sciatic nerve preparation
    - Isolated neuromuscular junction
    - Brain slice preparation for seizure electrophysiology
    - Isolated retinal preparation
    - Cytotoxic endpoints: neuronal cultures (cerebral cortex, hippocampus, cell line (e.g. dopaminergic) plates)
    - Neurite outgrowth assays: central and peripheral
    - Electrophysiology

# Preclinical assessments performed to evaluate neurotoxicity

- Evaluation of the physiochemical properties to show risk of toxicity (***Hughes et al., 2008.)***
  - Higher lipid solubility
  - Low polarity
- Receptor binding assays to detect off-target pharmacology

# Preclinical studies performed to evaluate neurotoxicity

- **In vivo evaluation**
  - Exploratory toxicity studies
  - Neurobehavioral studies performed for drugs intended for CNS indications
    - Seizure
    - Cognition
    - Motivation/abuse potential
    - GI function
    - General behavior and pulmonary function
  - Pharmacokinetic studies
    - CSF vs. plasma vs. brain tissue drug concentrations in multiple species (rodents vs. large species)

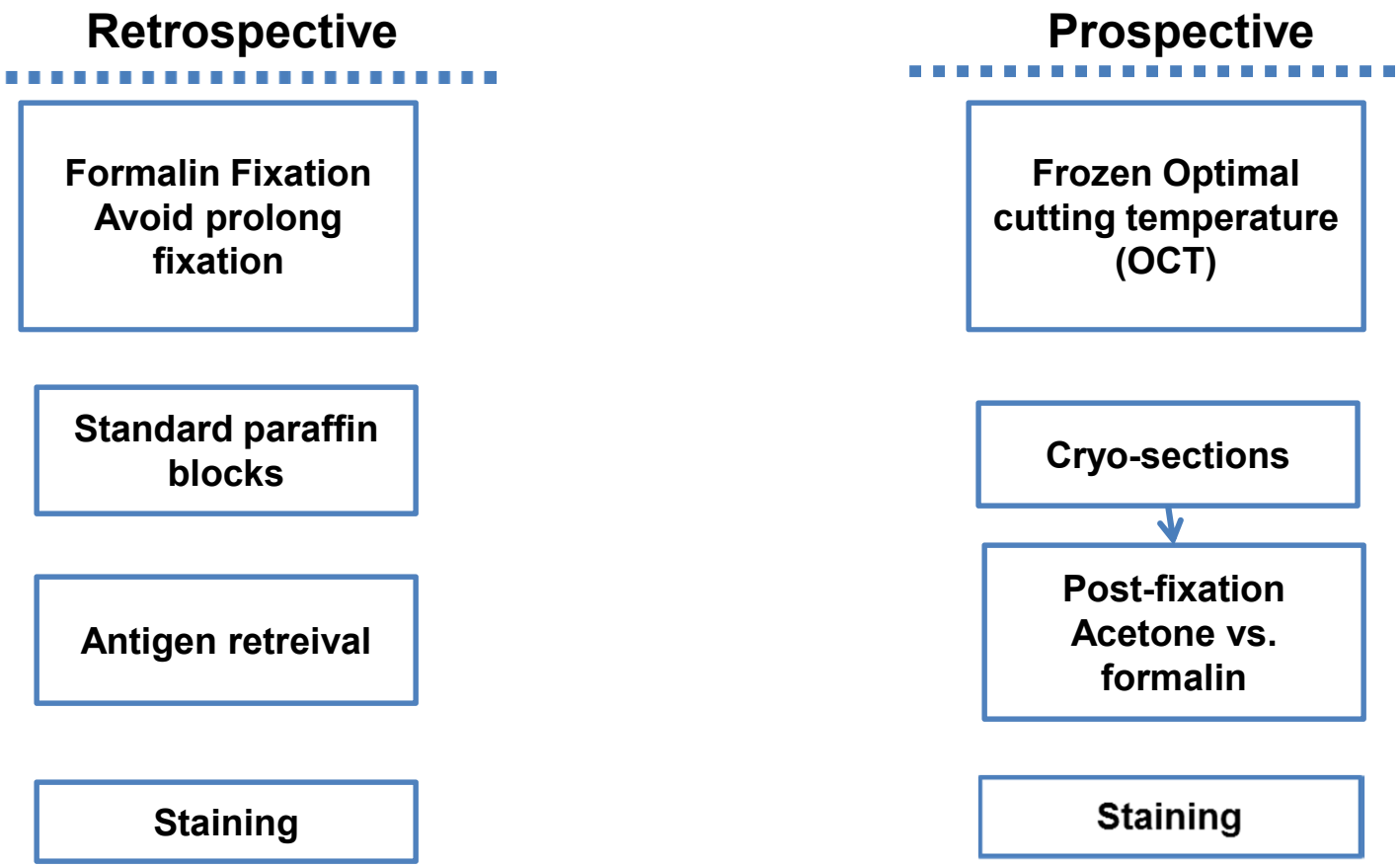
# Preclinical studies performed to evaluate neurotoxicity

- **Regulatory studies**
  - Detailed evaluation of CNS clinical signs
  - Histopathology of brain, spinal cord, and peripheral nerves in general toxicity studies in 2 species
  - Single or multiple doses neurobehavioral safety pharmacology studies (rat or mouse)
    - Functional observation battery (FOB) and quantitative local motor activity (standard tests performed before or during GLP studies)
    - Abuse potential prior to phase 2 b
  - Mechanistic and/or additional histopathologic studies to characterize and explain findings in general toxicity studies
    - Electrophysiology
    - TEM
    - Biomarkers

# Immunohistochemistry (IHC)

- Application
  - Design into study where there is a known morphologic finding (s)
  - Cellular characterization: neurofilament, tubulin stains, synapsis, dendrites, phospholipidosis, mitochondria
  - Alterations in the blood brain barrier: albumin, astrocyte activation, endothelial damage, cell infiltrate characterization
  - Myelin changes

# IHC road map


Retrospective
Formalin Fixation
Avoid prolong fixation
Standard paraffin blocks
Antigen retreival
Staining
Prospective
Frozen Optimal cutting temperature (OCT)
Cryo-sections
Post-fixation
Acetone vs. formalin
Staining

# Common CNS IHC Antibodies

**Glial Cells**

**Astrocytes**: GFAP

**Oligodendrocytes:** CNPase, Myelin Basic Protein

**Microglia:** Iba1

**Axons:** Neurofilament 68, 160, 200

**Dendritic Marker:** MAP2

**Synaptic Markers**: Synaptophysin, Synapsin, SNAP25

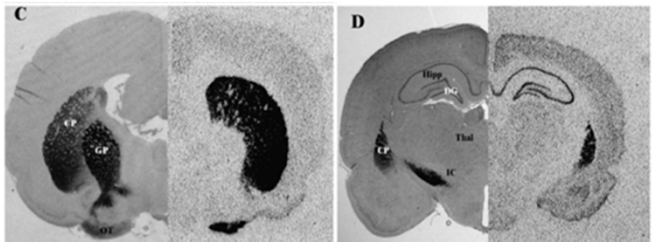
**Neuronal Cell Types:** Calbindin, parvalbumin, GABA, CHAT, tyrosine hydroxylase

1.IHC followed by LFB on the same slide works (even though the color of the IHC chromagen changes from brown to olive green, it remains specific and readable)
2.LFB followed by IHC on the same slide does not work (IHC protocol removes the blue of the LFB).

# IHC Case Study

# Evaluation of PDE10A IHC in Multiple Species

PDE10A IHC PDE10A ISH PDE10A IHC PDE10A ISH



PDE10A is predominantly located in the caudate putamen, globus pallidus, and substantia nigra

Combination of IHC and ISH was used to build confidence in IHC assay for PDE10A in rat brain

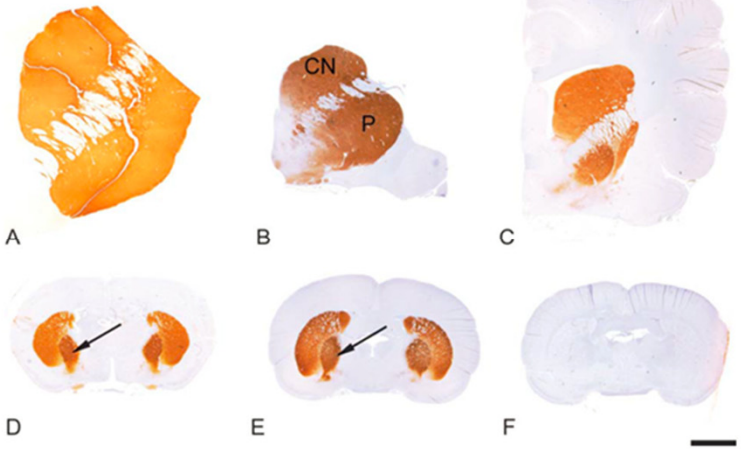
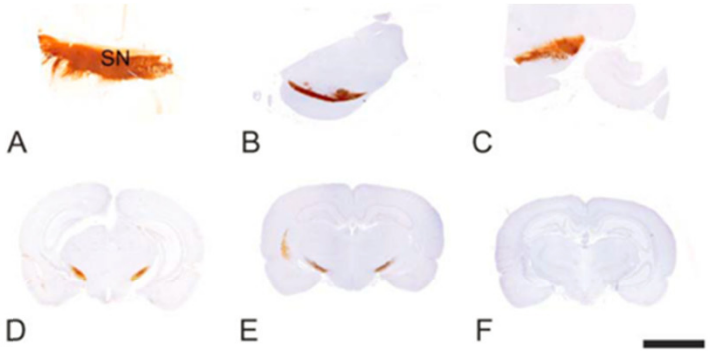



**Figure 1** Immunohistochemical staining of mammalian striatum with anti-PDE10A. **(A)** Human. **(B)** Cynomolgus macaque. **(C)** Dog. **(D)** Mouse. **(E)** Rat. PDE10A-ir is seen throughout the striatum in both the caudate nucleus and putamen of all species examined. Mouse **(D)** and rat **(E)** also show strong PDE10A staining of the globus pallidus as indicated by the arrows. **(F)** Rat brain stained with a mouse IgG1 isotype as a negative control. Black arrows indicate PDE10A-ir in the globus pallidus. CN, caudate nucleus; P, putamen. Bar = 10 mm.

**Figure 3** Immunohistochemical staining of mammalian substantia nigra with anti PDE10A. **(A)** Human. **(B)** Cynomolgus macaque. **(C)** Dog. **(D)** Mouse. **(E)** Rat. **(F)** Rat brain section stained with a mouse IgG1 isotype as a negative control. PDE10A-ir is present in the substantia nigra pars reticulata of all species examined. SN, substantia nigra. Bar = 10 mm.

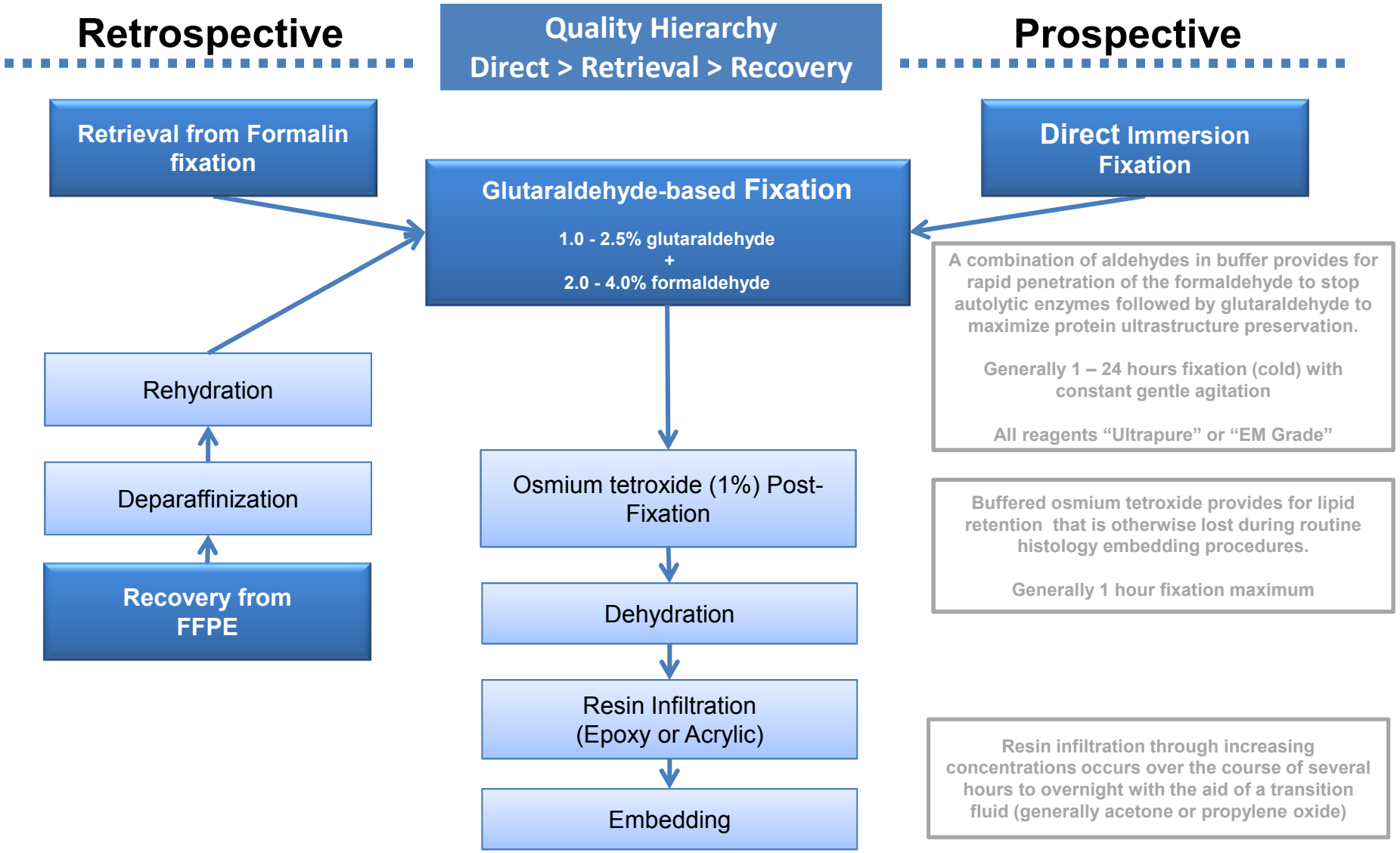



The same IHC assay was utilized to demonstrate similar PDE10A localization in the brain of multiple mammalian species

# Transmission Electron Microscopy

- Applications
  - Design into study where there is a known morphologic finding (s)
  - Confirm finding observation from light microscopic examination (most common)
  - Additional structural characterization “what is the substance”

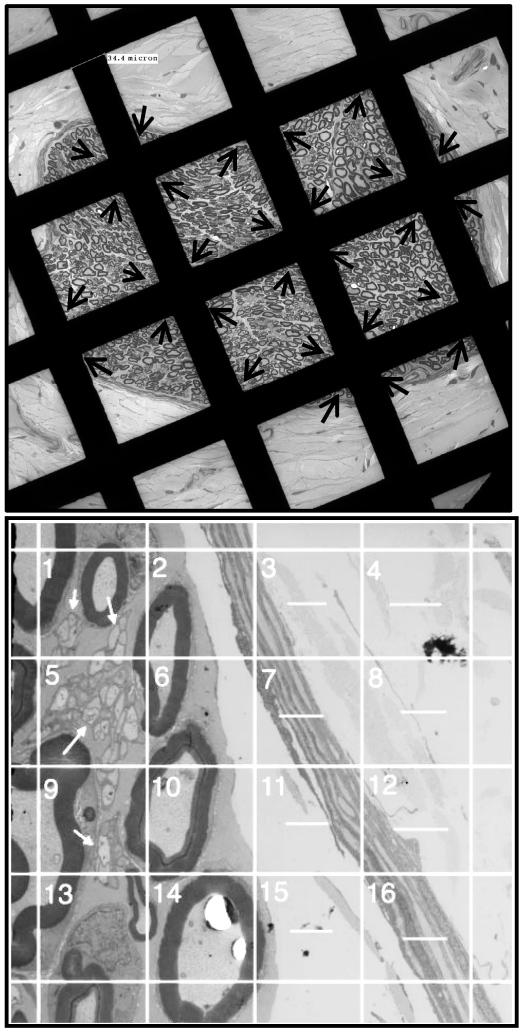
# TEM Road Map


Retrospective
Quality Hierarchy
Direct > Retrieval > Recovery
Prospective
Retrieval from Formalin fixation
Direct Immersion Fixation
Glutaraldehyde-based Fixation
1.0 - 2.5% glutaraldehyde
+
2.0 - 4.0% formaldehyde
A combination of aldehydes in buffer provides for rapid penetration of the formaldehyde to stop autolytic enzymes followed by glutaraldehyde to maximize protein ultrastructure preservation.
Generally 1 – 24 hours fixation (cold) with constant gentle agitation
All reagents "Ultrapure" or "EM Grade"
Rehydration
Deparaffinization
Recovery from FFPE
Osmium tetroxide (1%) Post-Fixation
Buffered osmium tetroxide provides for lipid retention that is otherwise lost during routine histology embedding procedures.
Generally 1 hour fixation maximum
Dehydration
Resin Infiltration (Epoxy or Acrylic)
Resin infiltration through increasing concentrations occurs over the course of several hours to overnight with the aid of a transition fluid (generally acetone or propylene oxide)
Embedding

# Transmission Electron Microscopy Case Studies

# Case Study #1

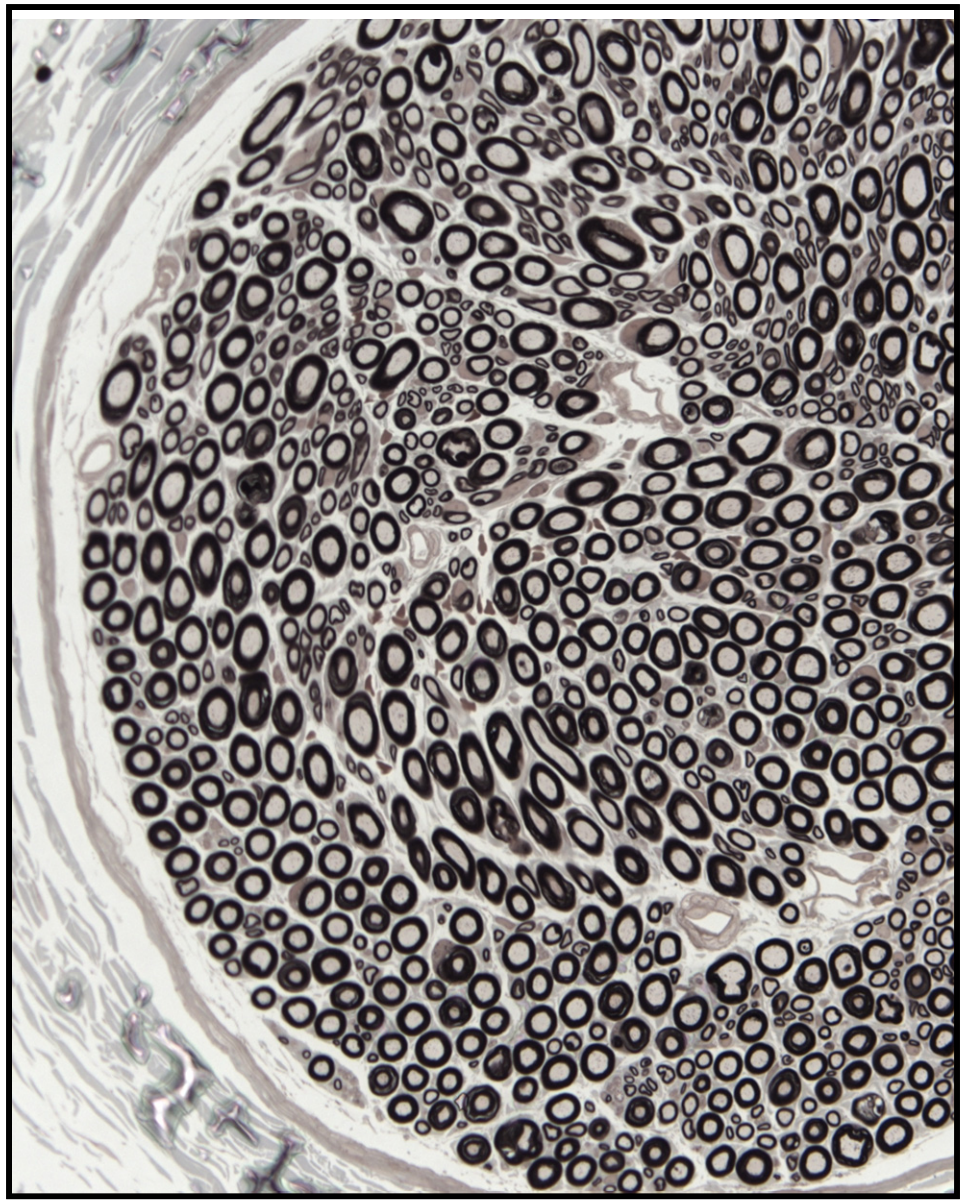
- Morphologic, Stereologic, and Morphometric Evaluation of the Nervous System in Young Cynomolgus Monkeys (Macaca fascicularis)
- TEM analysis supported a comprehensive neuropathologic evaluation of young nonhuman primates from a developmental toxicity study of tanezumab, a nerve growth factor (NGF) inhibitor
- Analysis of myelinated nerve fibers in sural nerves (cross-sectional area, total myelinated nerve fibers (MNFs), average MNF radius, average axon radius, ratio of axon diameter to a single MNF) was conducted using LM on 0.5µm resin sections in a non-biased manner
- Analysis of unmyelinated nerve fibers in sural was conducted using TEM images in a non-biased manner
- Stereologic principals were applied
- Helped remove clinical hold




TOXICOLOGICAL SCIENCES, 142(2), 2014, 463–476

# Case Study #2

- Application of special stain, paraphenylenediamine (“PPD), for potential evaluation of myelinopathies
- Post-fixation  of tissues with osmium tetroxide preserves lipids that would otherwise be removed during routine histologic processing
- Embedment in resin (epoxy) rather than paraffin enhances structural integrity
- Thick sections (1.5µM) prepared for light microscopy (LM) are stained with methanolic paraphenylenediamine revealing lipid-rich axonal sheaths
- Clarity of presentation makes this application amenable to quantitative analysis.



# Biomarkers

- There are external and internal efforts to use biomarkers for neurotoxicity

# External Efforts to Use Biomarkers for neurotoxicity: HESI initiative

## A search for biomarkers of neurotoxicity: Trimethyltin as a prototypic neurotoxicant

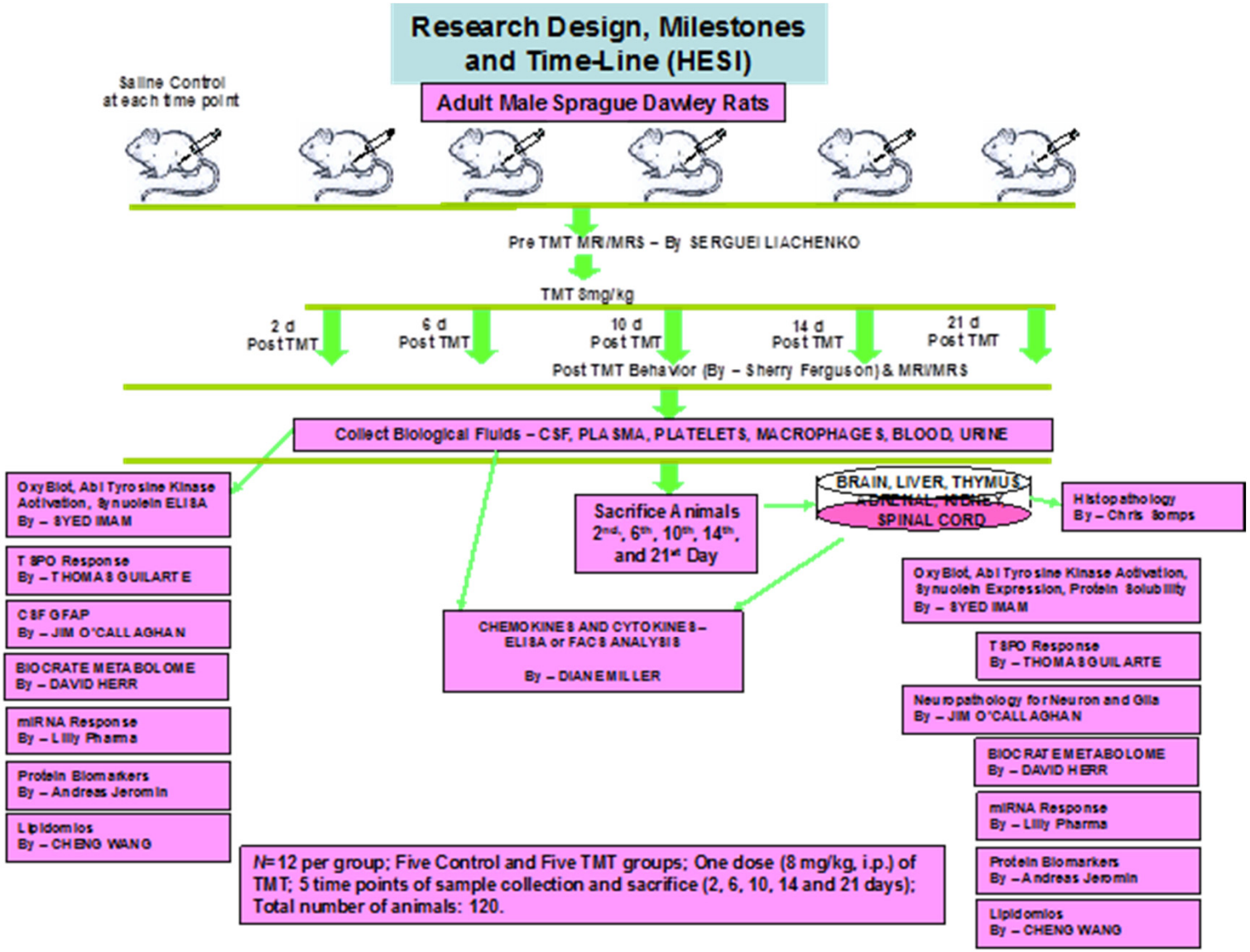
**Principal Investigators :** Merle G. Paule, Ph.D.**Co-investigators:** Syed Z. Imam, Ph.D, Serguei Liachenko, Ph.D., Jaivijay Ramu, M.S., Sumit Sarkar, Ph.D, Cheng Wang, M.D., Ph.D, Sherry Ferguson, Ph.D, Charles Law, B.S., Joseph P. Hanig, Ph.D., Jim O'Callaghan, Ph.D., Diane B. Miller, Ph.D., Ginger Moser, Ph.D., Ruth Roberts, Ph.D., Andreas Jeromin, Ph.D., Tomás R. Guilarte, PhD, David Calligaro, Ph.D., Christopher Somps, Ph.D., David Herr, Ph.D., Slikker, William, Jr., Ph.D

The ultimate goals of this working group are to identify the biological pathways relevant to the expression of neurotoxicity (central and peripheral) in order to develop specific, sensitive, reliable and easily measureable biomarkers.

# HESI Initiative

- Time-course assessments of blood, cerebral spinal fluid and urine to search for candidate biochemical markers of neuronal degeneration
  - micro RNAs, F2-isoprostanes (F2-IsoPs), translocator protein 18 KDa (TSPO), glial fibrillary acidic protein (GFAP), ubiquitin C-terminal hydrolase L1 (UCH-L1), myelin basic protein (MBP), microtubule-associated protein-2 (MAP-2), and total tau
- Functional (behavioral), imaging (magnetic resonance imaging, MRI) and neurochemical analysis (magnetic resonance spectroscopy, MRS)
- Histopathologic analyses to correlate the imaging and behavioral endpoints with the profile of observed fluid biomarkers

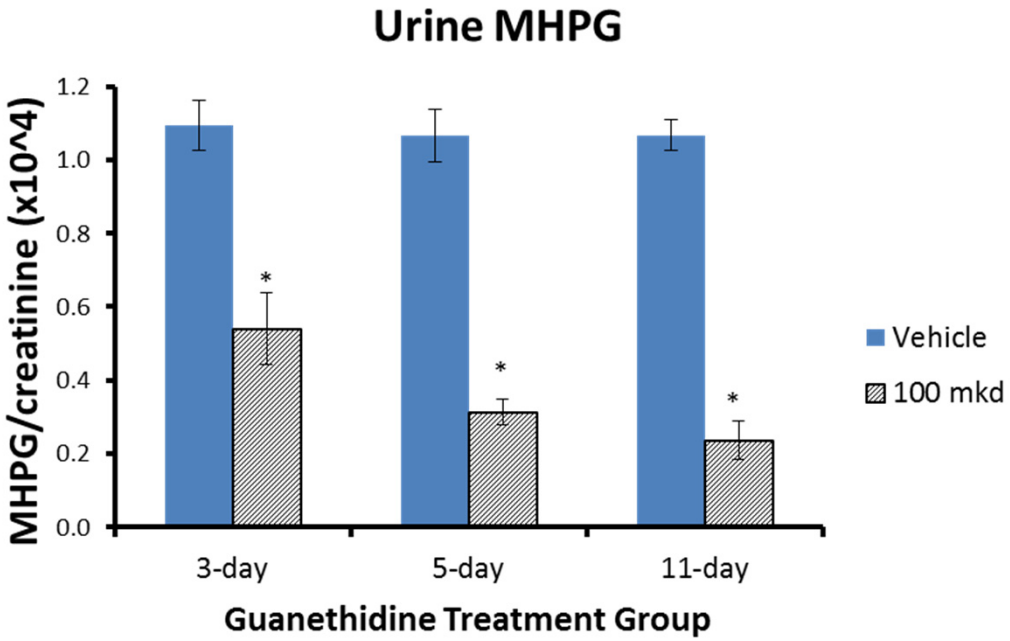
# HESI Study Design



# Internal Efforts to Use Biomarkers for neurotoxicity

Relation of neuron counts in the superior cervical ganglia and urinary MHPG in rats following guanethidine treatment

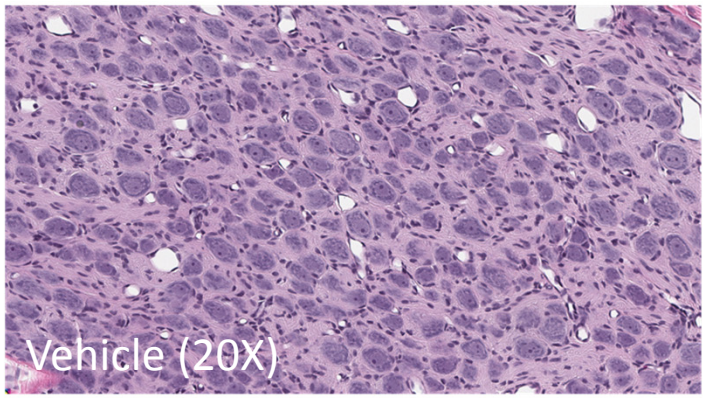
C. Somps, C. Okerberg, C-N. Liu, M Boucher, I. Pardo, et al.
Drug Safety R&D, Pfizer Inc., Groton, CT, USA

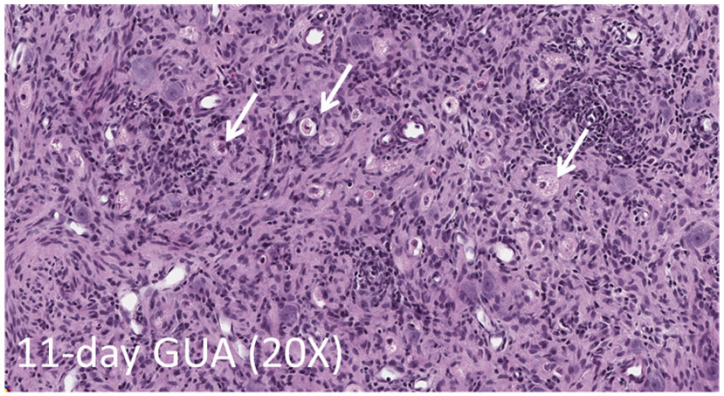


# Internal efforts to Use Biomarkers for Neurotoxicity

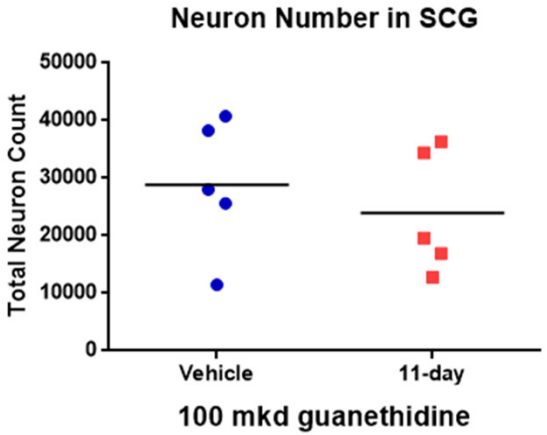
Relation of neuron counts in the superior cervical ganglia and urinary MHPG in rats following guanethidine treatment

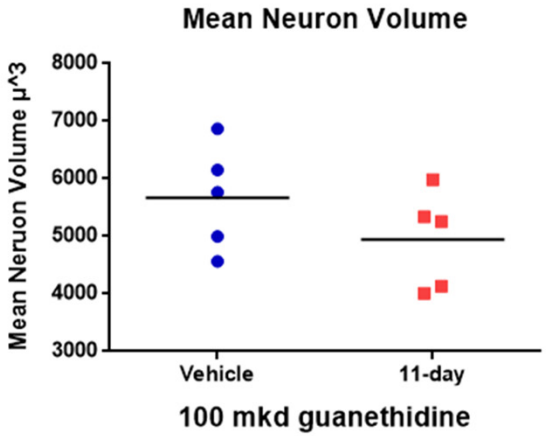
**H&E. Superior cervical ganglia**




**Stereology**



# Neurofunctional assessment

- **Neurofunctional assessments**
  - Functional Observational Battery (FOB):
    - Series of noninvasive observational and interactive measures
    - Provides multiple and overlapping assessments of the functional integrity of the animal
  - Locomotor activity
    - Quantitatively determines horizontal movements (ie, total distance traveled) and rearing
  - GLP neurofunctional assessments required prior to FIH for all small molecules

- **Abuse liability assessments**
  - Required for all CNS active drugs prior to Phase 3 clinical trials
  - Rodents or NHPs, depending on pharmacology of test article
  - Self administration, drug discrimination, and physical dependence
  - Functional Observational Battery (FOB):
    - Series of noninvasive observational and interactive measures
    - Provides multiple and overlapping assessments of the functional integrity of the animal

- **Electroencephalogram (EEG)**
  - Can assess sleep/wake cycles, central drug penetration, monitor for convulsions

- **Sensory function**
  - Hot plate (thermal sensitivity) or von Frey filament testing (mechanical sensitivity)

# Functional Observation Battery

## CNS Parameters Assessed

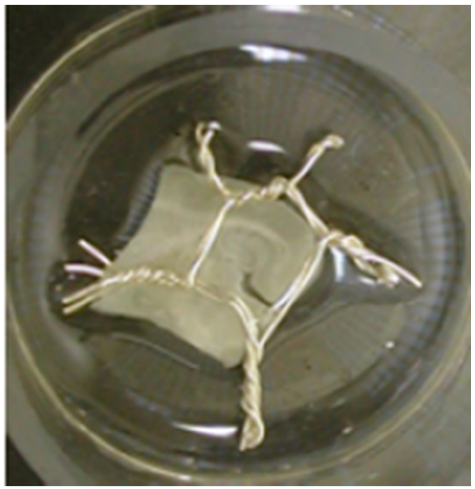
| | | | |
|---|---|---|---|
| General overt behaviors | Click response | Gait | Body temperature |
| Involuntary motor movements | Air righting reflex | Extensor reflex | Spontaneous locomotor activity |
| Bizarre behavior | Palpebral closure | Forelimb grip strength | |
| Respiration | Palpebral reflex | | |
| Ease of removal from home cage | Eye observations | | |
| Handling reactivity | Salivation | | |
| Arousal | Piloerection | | |
| Ambulatory activity | Diarrhea | | |
| | Pupil diameter | | |
| | Pupil response | | |

# Electrophysiology

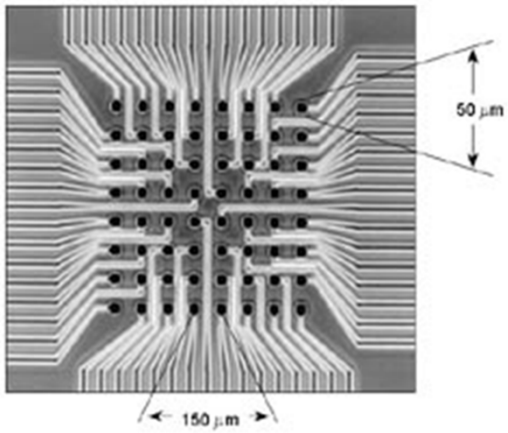
- In vitro: cell lines
  - Calcium
  - ATP depletion
  - Primary cell lines
- Ex vivo
  - Hippocampal (memory loss and epilepsy)
  - Nerve (mechanical deficits)
  - Nerve conducting velocity or compound action potential
- In vivo
  - Nerve conducting velocity or compound action potential
  - CatWalk
  - CoB

# In vitro electrophysiology

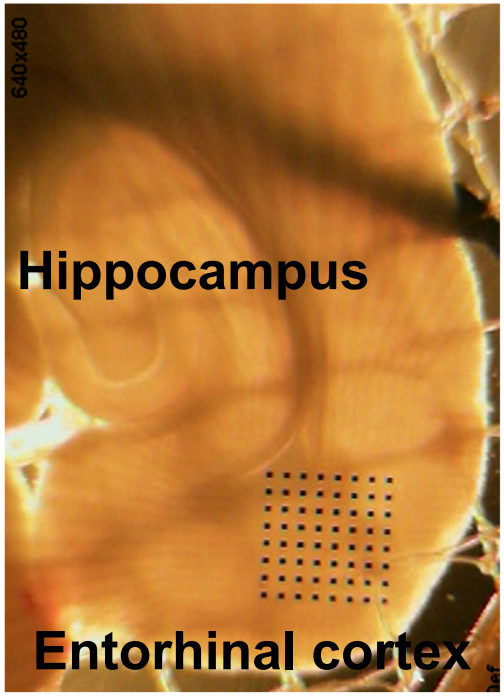
## Brain slice preparation for seizure

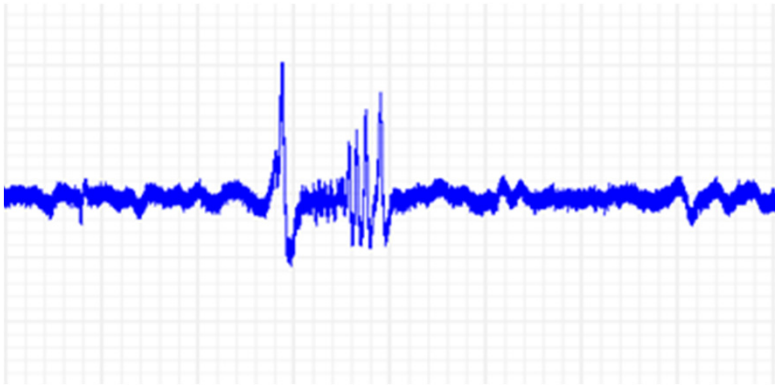


Brain slice




Multielectrode

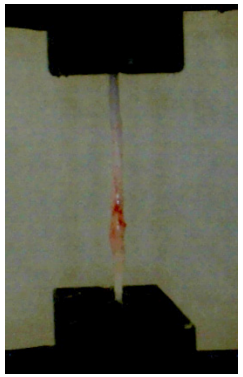


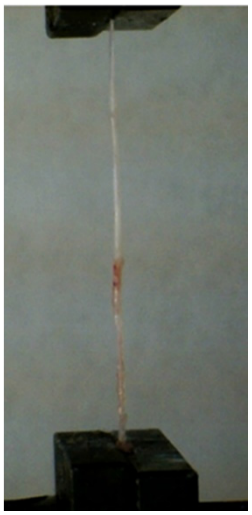
Baseline Epileptiform Discharges

# Ex-vivo electrophysiology

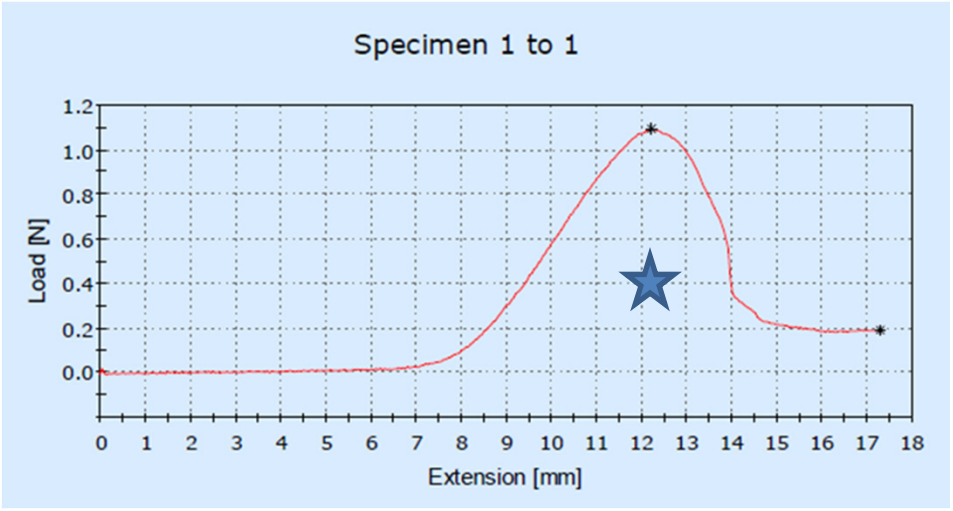
## Mechanical nerve damage

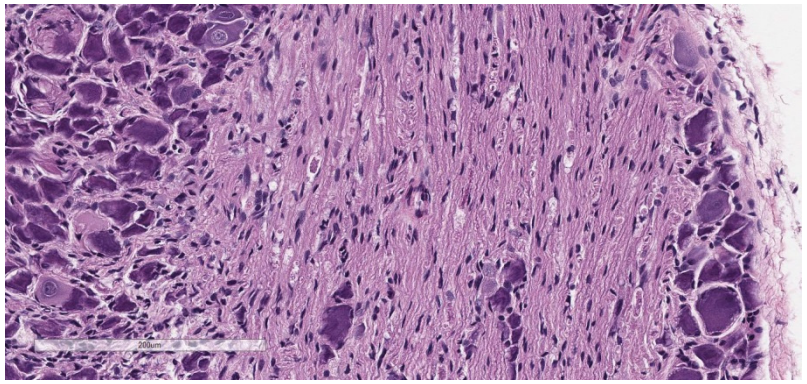


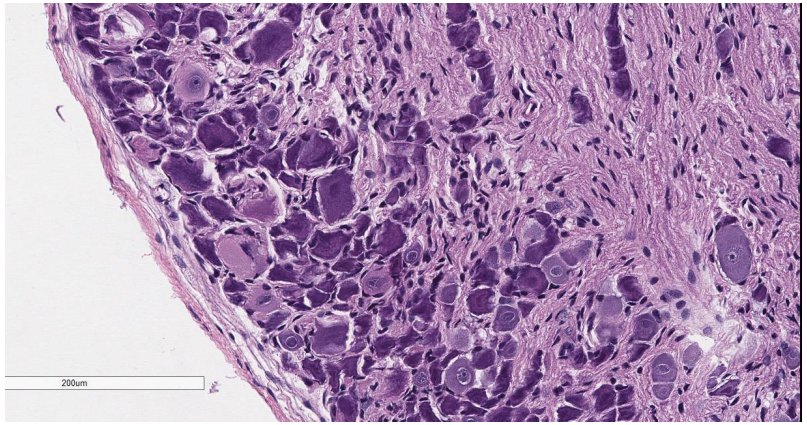
Before treatment

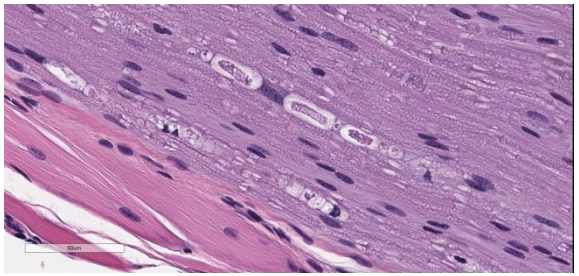


After treatment





# In vivo electrophysiology

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## Correlation and Dissociation of Electrophysiology and Histopathology in the Assessment of Toxic Neuropathy

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ABSTRACT

The evaluation of neurotoxic damage involves a unique set of challenges. Vulnerable structures, such as neocortex, hippocampus, spinal cord, and peripheral nerve are complex and sharply differentiated; deficits can result from insults to one or more element(s) in the system (e.g., myelin, axon, soma, synapse, or glia). In-life assessment of neurotoxic damage is complicated by the relative inaccessibility of structures in the brain and spinal cord, and recovery is severely limited. Histopathology and electrophysiology represent two of the most commonly used and valuable techniques in this field. This review outlines the strengths and limitations of these procedures and focuses on circumstances in which findings from these measures are dissociated. Electrophysiology is noninvasive and affords a longitudinal view of onset and progression of deficits; however, measures are generally weighted to large-diameter myelinated axons and to regions of primary sensory and motor processing. Histology is a highly validated biomarker, but it is restricted by sampling issues and is insensitive to some elements of neurotoxicity (e.g., altered channel function) associated with profound functional consequences. The central tenet of the discussion is that histology and electrophysiology offer complementary views of neurotoxic damage and, whenever possible, they should be used in concert.

*Keywords:* neurotoxicity; electrophysiology; histopathology; biomarkers; neuropathology.

INTRODUCTION

dementia (de la Monte et al. 2009), Parkinson's disease (Arif

# Conclusion

- End-points such as immunohistochemistry, electron microscopy, biomarkers, neurofunctional assessment and electrophysiology for neurologic evaluation provide useful information of the test article and/or preclinical clinical signs related to a target and should be used in conjunction with histopathologic evaluation