NeuroScience Associates

NSA
NeuroScience Associates
NSALabs.com

2017 CATALOG
OF NEUROHISTOLOGY SERVICES

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Who are the clients of *NeuroScience Associates*?

The clients of NSA range in size and scope—from small academic research labs to major pharmaceutical companies. Organizations choose us to perform their neurohistology for a variety of reasons. Some clients completely outsource neurohistology to NSA, while others use us as an extension of their own capabilities. Our high throughput times allow time-conscious customers to rapidly respond to gaps in their internal capacity and meet critical deadlines. Clients often select NSA to perform the neurohistology for their biggest and most important studies. Regardless of the reason you choose NSA, we can assist you with your neurohistology needs and provide you with the most cost-effective, high-quality services available.

Read what our clients have to say about us:

"I have been a customer of NSA for several years and they have been critical for the success of several of our projects. Specifically, NSA provided high-quality sections for our stereology-based cell counting studies and saved us considerable time and money. Immunohistochemical studies we have completed on sections provided by NSA have been of outstanding quality and due to the MultiBrain® Technology and Large Format processing have led to significantly reduced variability in our data. NSA is responsive and collaborative and the staff are a pleasure to work with."

Dr. Elizabeth Head, Sanders-Brown Center on Aging, University of Kentucky

"By virtue of our collaboration with Dr. Robert Switzer and NeuroScience Associates, and the use of their MultiBrain® Technology, we have been able to map out, using disintegrative staining and immunohistochemical methods, a detailed time course of post-traumatic neurodegeneration in rodent models after traumatic brain injury. Consequently, we have demonstrated definitive neuroprotective effects of a novel and exciting neuroprotective compound. This work, carried out over the past four years, has resulted in three peer-reviewed papers. Dr. Switzer and his colleagues at NSA have consistently provided extremely high-quality work with a rapid turnaround and even assisted in the interpretation of results. Their creativity, exquisite attention to detail and professional manner has been a real joy. There is no way that my laboratory could have duplicated the work they have done for us. Even if we could, the cost-effectiveness and reliability of their histological services has made them the logical choice. We will continue to turn to them."

Dr. Ed Hall, Director, Spinal Cord & Brain Injury Research Center, University of Kentucky

"UCB started collaborating with NSA a few months ago, and I have already been very impressed by their MultiBrain sectioning technology and the high-quality of their immunostaining. This technology is particularly attractive for large studies including multiple experimental conditions. Because the variability of the immunohistochemistry procedure is reduced, it is possible to obtain reliable data and in a very short time frame. The technical staff from NSA have strong expertise in immunohistochemistry and are responsive to our requests. So far so good, we are happy with their quality and will continue working with them.

Georges Mariet-Coello, UCB Biopharma SPRL

"Having collaborated with NSA over the last few years ,I am glad to state the following: Interaction with NSA has always been very fruitful. The scientific input from NSA has been of very high standard and the company has always been very open-minded, so that novel approaches, proposed by either NSA or Lundbeck, to our studies have been implemented in the analysis phase of the different studies. This very interactive approach has proven intellectually very stimulative and has contributed significantly to the momentum of the project. The technical quality of the obtained sections and the analysis of the histology obviously is of first class, so I have a strong interest in continued collaboration with NSA."

Dr. Bjarke Ebert, Adjunct Professor in Molecular Pharmacology
Head of Electrophysiology, H. Lundbeck A/S

(More testimonials on the inside back cover)

NEUROSCIENCE ASSOCIATES

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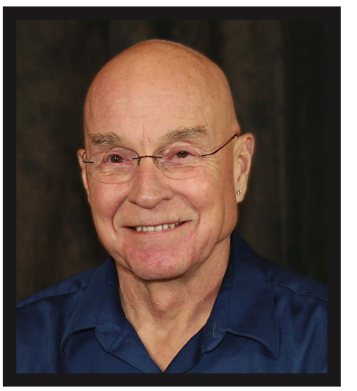
LETTER FROM THE PRESIDENT



It is likely that many readers of this catalog share the same passion for neuroscience that I have. My beginnings in comparative neuroanatomy seeded a fascination for the known and unknown of the brain, which is perhaps the "final frontier" of human anatomy. I have always enjoyed developing and learning new and innovative processes that can further advance this exciting field and as an explorer of this pioneering discipline, I continuously strive to learn more every day. The creation of NeuroScience Associates (NSA) has provided me with an opportunity to collaborate with others and contribute to the advancement of neuroscience.

A strong driving force in creating NSA was to make available to the neuroscience community the expertise that I had gained with the Disintegrative Degeneration stains to detect neurotoxicity as well as the high throughput capabilities that I had developed with MultiBrain® and Multicord® Technologies. In 1989, I began to provide these unique neurohistologic services and consultation through the creation of NeuroScience Associates. Over the past 28 years, the MultiBrain® process has proven to be an invaluable tool in helping our clients to reduce the throughput times for histologic processing. It has been very gratifying to witness the positive impact that NSA has had on neurologic research resulting from the mass production benefits of MultiBrain® Technology, the collaborative advice and guidance we provide and our expertise in the application of all varieties of neurohistology stains. I consider myself privileged to have had the opportunity to partake in and contribute to numerous advances and discoveries, and I look forward to being a part of many more breakthroughs.

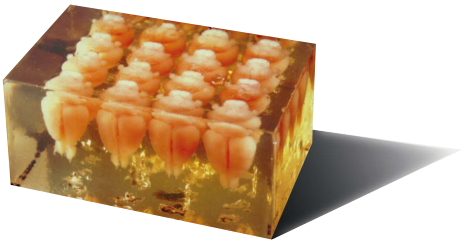
This catalog will describe the principles, technologies and mechanisms by which NSA empowers researchers to become more efficient and more productive and may enable them to entertain questions that would otherwise not be possible. I hope that you will find the information contained in this catalog informative, thought-provoking and ultimately useful. It is the objective of NeuroScience Associates to increase the productivity and enhance the success of our clients by providing superior histologic services in a cost-effective manner. I invite you to contact us today to discuss your scientific endeavors and determine how NSA can assist you with your histology and research needs.

Dr. Bob Switzer
President and Chief Scientific Officer

NeuroScience Associates, Inc.

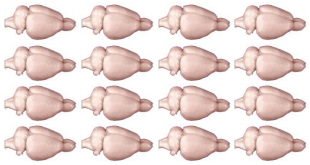
INTRODUCTION

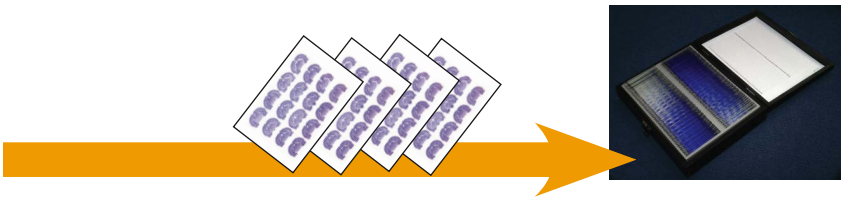
NeuroScience Associates (NSA) was founded in 1989 by Dr. Robert C. Switzer III, to provide premium quality mass production neurohistology services in support of both R&D and safety testing. Clients send brain or spinal cord tissue to NSA for neurohistologic processing. NSA performs MultiBrain® and MultiCord® embedding, sectioning and staining returning finished slides and free floating sections to our clients in as little as 2–4 weeks.



You send brains to NSA...

...NSA sends you finished slides and free-floating sections...





MultiBrain® / MultiCord® Technologies

NSA's proprietary MultiBrain® and MultiCord® Technologies were designed to deliver high throughput and reliable high-quality results. During the past 28 years, NSA has used MultiBrain® and MultiCord® Technologies to section over 100,000 neuronal tissues and stain over 8 million sections. The services of NSA enable our clients to reduce the neurohistology phase of R&D cycles from months/years to weeks, earning our reputation for delivering exceptional quality in a rapid, cost-effective manner.

Staining Expertise

As part of our commitment to excel in the art of neurohistology, we have developed expertise in the execution of a broad range of stains including basic traditional stains, our own specialty stains, and immunohistochemistry using commercially available or client-supplied antibodies. In our drive to remain on the cutting edge, we trial new antibodies on a daily basis.

Safety Testing Consultation and Execution

With NSA's wide breadth of staining capabilities, we are able to perform neurohistology to meet almost all client-defined and regulatory requirements, including full GLP compliance. Additionally, we have designed safety study protocol templates, ranging from low-cost neurotoxicity safety screens to a certification level. We utilize our vast safety testing experience to offer advice and study design, with regulatory approval-caliber protocols.

Imaging and Analysis

NeuroScience Associates offers imaging and analysis services to enhance your neurohistology. 10x and 20x digital imaging, 3-D reconstruction, particle analysis, densitometry, volumetric calculations, plaque load analysis, stereology, and pathologic analysis by a board certified pathologist are among our offerings.

NSA SERVICES: COMPREHENSIVE OVERVIEW

NSA provides Neurohistology Services, as well as a rapidly expanding repertoire of Supplemental Services that add tremendous value to our mass production neurohistology. Check our website for the latest information!

NEUROHISTOLOGY

SUPPLEMENTAL SERVICES

NSA NEUROTECHNOLOGIES™: EMBEDDING AND SECTIONING

MULTIBRAIN® TECHNOLOGY

MultiBrain® Technology is the cornerstone of the efficient, high-quality neurohistologic processing performed by NSA, the ONLY source for MultiBrain® services in the world.

NSA has revolutionized the execution of neurohistology services. With MultiBrain® Technology, up to 40 brains are embedded together in a solid gelatin matrix and processed as a single unit. This technique achieves uniformity of section thickness and staining quality across cases while providing "built-in" quality control, making subsequent analysis more efficient and efficacious.

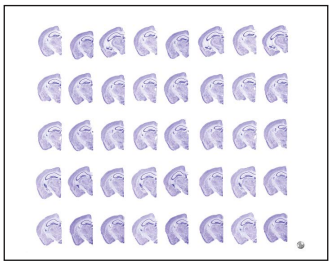
-----NSA is able to perform neurohistology up to 40X faster than traditional methods-----

Mass Production Neurohistology

With MultiBrain® Technology, the economic and qualitative benefits of "mass production" are now available in the neurohistologic field. We section, and we stain, up to 40 brains simultaneously.

MultiBrain® Features:

- Unified processing of multiple brains
- Uniform thickness of sections
- Uniform staining across all sections
- Variety of possible layouts, and orientations

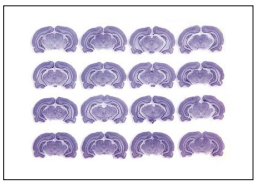


40 Mouse Brains
Coronal Nissl-Stained

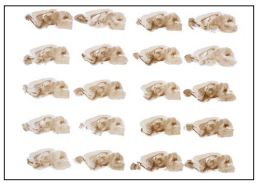
MultiBrain® Advantages:

- Accelerated histology up to 40X faster than traditional methods
- Simpler, more rapid comparative analysis
- Flexibility to retroactively stain adjacent sections
- Inherent quality control

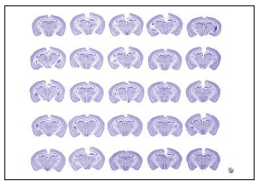
MultiBrain® principles can be applied to a variety of tissue types, orientations and numbers



16 Rat Brains
Coronal Nissl-Stained

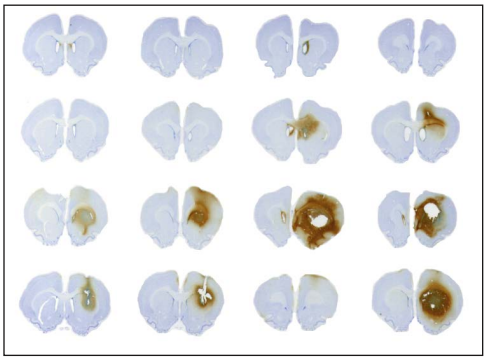


20 Mouse Brains
Sagittal Campbell-Switzer AD-Stained



25 Mouse Brains
Coronal Nissl-Stained

Analysis of slides is accelerated through MultiBrain® Technology



Control

Dose Level A

Dose Level B

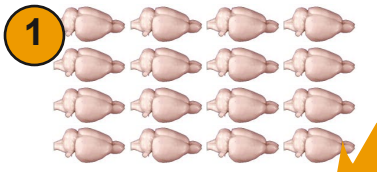
Dose Level C

16 rat brains were sectioned together in a single block, and stained with the blood brain barrier compromise stain. Note the differences that can be easily observed across groups and doses by having all of the sections on the same MultiBrain® slide.

MultiBrain® services enable researchers to more efficiently design experiments and significantly accelerate neuroanalysis. Making valid comparisons across treatment groups and controls has never been easier or faster!

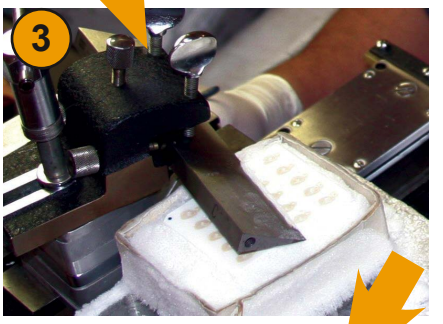
NSA NEUROTECHNOLOGIES™: EMBEDDING AND SECTIONING

MULTIBRAIN® TECHNOLOGY: HOW DOES IT WORK?

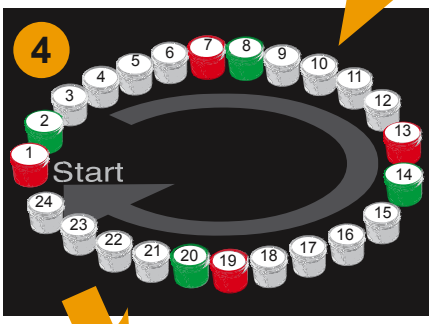


1 **Clients send brains to NSA.**
Ideally the brains are stored in preferred solution found on our website (under Client Resources > Tissue Prep > Shipping) and shipped overnight. NSA will perform the brain removal when requested.

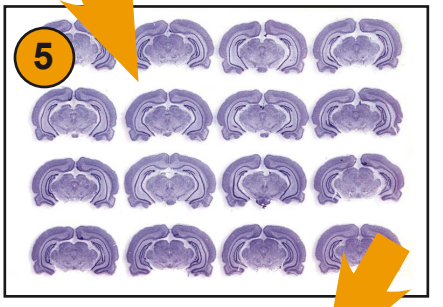
2 **Multiple brains are embedded into each block.**
NSA embeds up to 40 brains per block. The researcher can specify the location of each brain in the block using the appropriate "Block Map" template available on our website. This enables efficiencies in subsequent neuroanalysis. Specimens are encased by the gelatin matrix, not infiltrated; therefore, the matrix has no effect on staining.



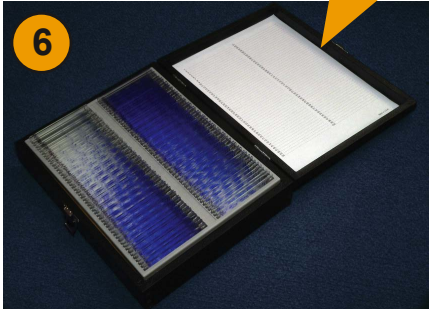
3 **The block is freeze-sectioned.**
Sectioning is performed with a sliding microtome, producing MultiBrain® sheets of sections, in thickness ranging 30–80µ based on the researcher's specifications.



4 **A resource of sections is created.**
With MultiBrain® processing, every free-floating sheet that is cut is collected into a series of 24 cups (NSA default) containing antigen preserve solution. Each succeeding sheet is placed into the next cup in the series of 24, and the process cycles back to cup 1 for the 25th section. Therefore, each cup contains 1 of every 24th cut section; adjacent cups contain adjacent serial sets of sections. The result is a valuable "resource of sections" that provides ample material for several initial stains as well as subsequent stains as needed.



5 **The designated sections are stained.**
For example, when processing mouse brains, we typically stain every sixth section with each stain selected by our client. To accomplish this, we would stain tissues from cups 1, 7, 13 and 19 with Stain "A". A second stain, Stain "B", could then be applied to the adjacent set of sections, in cups 2, 8, 14 and 20. The remaining cups are available, from which clients can request other stains, whether planned in advance or warranted based on results from the first set of stains. Clients may also request specific cup(s) be shipped to them for their own staining.



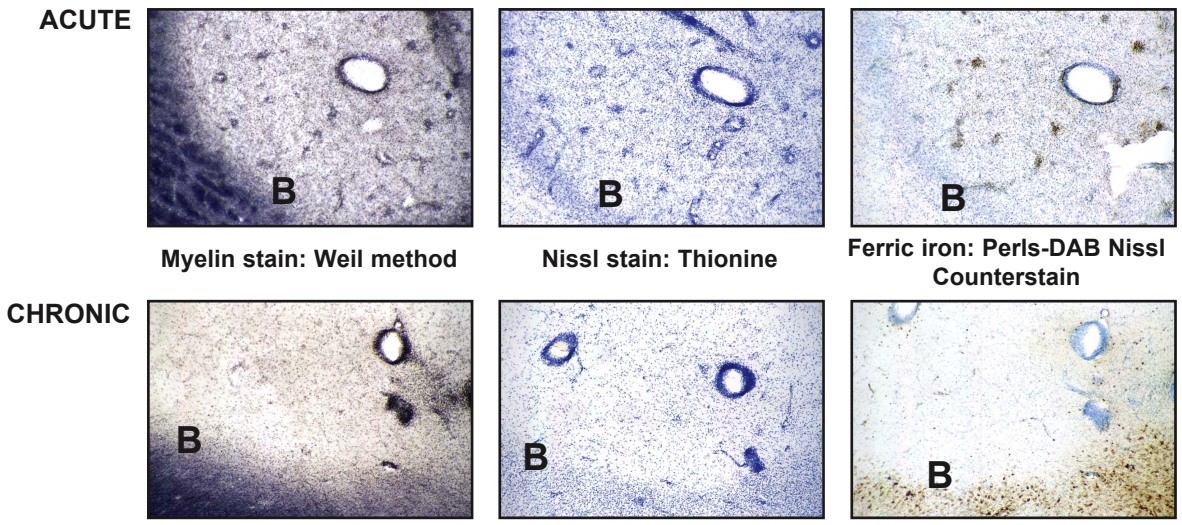
6 **Finished slides are shipped back to the client.**
The stained slides are anatomically ordered and labeled by NSA. The entire process from receipt of tissue at NSA to shipment of slides is usually about 3-4 weeks. Any remaining free-floating sections may be returned at the conclusion of the histology and/or stored at NSA for a fee.

NSA NEUROTECHNOLOGIES™: EMBEDDING AND SECTIONING

MULTIBRAIN® TECHNOLOGY

Resource of Sections Allows for Differential Staining on Adjacent Sections

Example: Tissue from a human multiple sclerosis brain



Myelin stain: Weil method | **Nissl stain: Thionine** | **Ferric iron: Perls-DAB Nissl Counterstain**

Near-adjacent sections from the same case of multiple sclerosis (MS) were stained for different features to allow comparisons of recently developed lesions (acute) versus long-standing lesions (chronic). The hallmark lesion of MS is the loss of myelin seen to the left and high cellularity around vessels (cuffing) seen in each of the photos. This cellularity around small vessels in the acute series is absent in the chronic. Iron positivity corresponds to small vessel cellularity in the center of the lesion of the acute and in the boundary (B) zones where reactive glia are present.

Frequently Asked Questions About MultiBrain® Technology:

Q. How is the identity of each brain preserved in the MultiBrain® Block?

A. At the time of embedding, only one brain at a time is outside of its container. Once the brain is in the gelatin matrix, its position relative to the other brains is permanent. The unambiguous orientation of the MultiBrain® Block Map is provided by the black MultiBrain® Reference Marker which is comprised of one or more black dots in a designated corner of each MultiBrain® sheet of sections.

Q. If the brains I want processed by NSA are already frozen, can they be processed successfully?

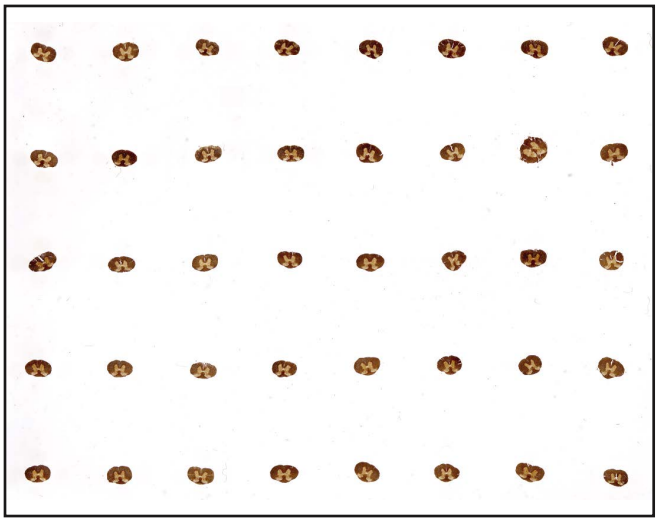
A. Yes, it is possible. If the tissue has not been cryoprotected (with sucrose or glycerol), there is the risk of freeze artifact (micro tears in the tissue, often rendering it useless for microscopy). If the tissue has been cryoprotected, then the chance for success is excellent. In either case, the risk for freeze artifact depends on the speed with which the tissue was frozen. Immersion in a slurry of 2-methyl butane (isopentane) and crushed dry ice is best. If the frozen brains were not fixed before freezing, they should be immersed in room temperature 10% phosphate-buffered formalin, with continuous stirring / agitation. Thawing and initial fixation are accomplished in one step. After overnight in the formalin, switch the tissue to buffer and the tissue is ready to be shipped to NSA for processing with MultiBrain® Technology. If the brains had been fixed, cryoprotected or not, immerse the brains in room temperature water or buffer for rapid thawing, with continuous stirring / agitation. The brains are typically able to be processed with MultiBrain® Technology successfully and the resulting sections should reveal scant or no freeze artifact.

NSA NEUROTECHNOLOGIES™: EMBEDDING AND SECTIONING

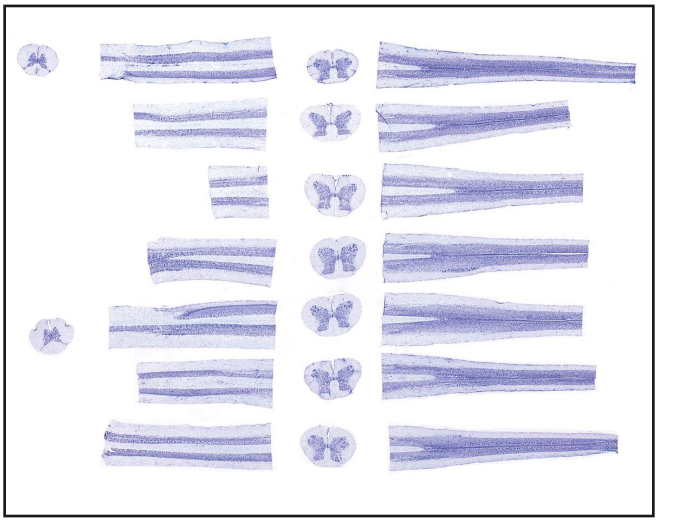
MULTICORD® TECHNOLOGY

Multiple Embedding of Spinal Cords

MultiCord® Technology uses the same revolutionary set of technologies as MultiBrain® Technology, with as many as 40 spinal cords embedded, sectioned and stained simultaneously in the transverse/coronal plane. When there is value in viewing the same cord in different planes, multiple segments and planes of a single spinal cord can be processed together and presented on a single slide. With this configuration, up to 8 spinal cords can be processed within the same MultiCord® block. The images below depict both the "traditional" transverse option for sectioning as well as an example of multiple plane sectioning that can be accomplished through MultiCord® Technology.



40 Rodent Spinal Cords Transverse / Coronal Myelin-Stained



7 Rhesus Monkey Spinal Cords Transverse / Coronal and Longitudinal Thionine-Stained Segments

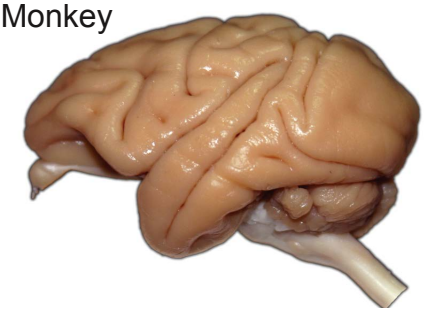
Rhesus spinal cord before (above) and after dividing (below)
Segments are oriented during embedding to allow alternate transverse / coronal and longitudinal sectioning and longitudinal sectioning.

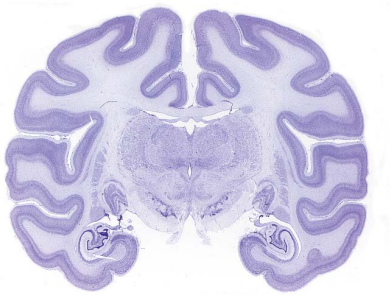
LARGE FORMAT™ TECHNOLOGY

The embedding and sectioning of large brains is achieved utilizing many of the principles of MultiBrain® Technology. This Large Format™ Technology enables NSA to section large intact brains continuously with no interruptions. Below are images of brains from relatively small species routinely processed by NSA on 2”x 3” slides, typically sectioned at 40µ, shown with Nissl staining.

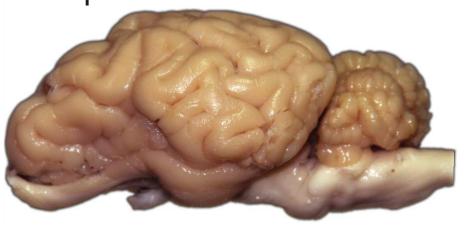
For larger brains, a specially designed stage and a modified hydraulically driven microtome are used, resulting in 3”x 5” slides for grizzly bear brains, and for human brain hemispheres. Large sections such as these are typically cut at 60–80µ and are mounted by NSA intact on a single slide.

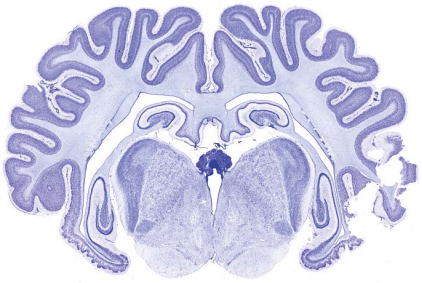
Monkey



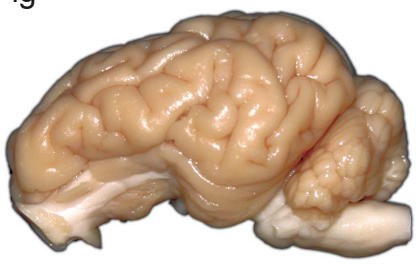


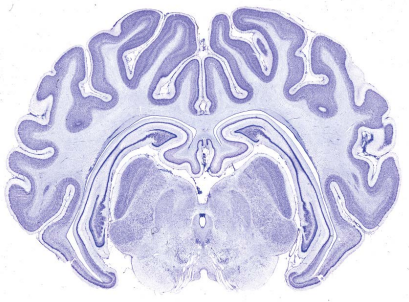
Sheep



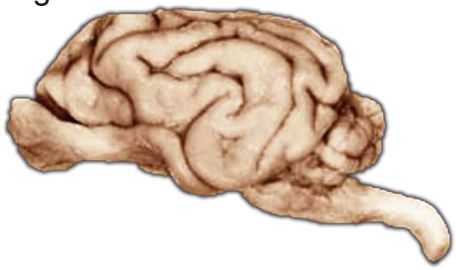


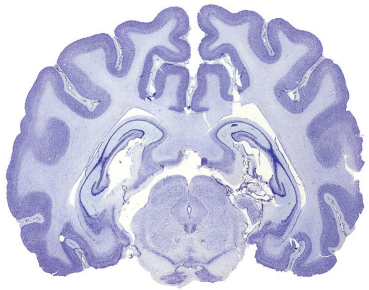
Pig





Dog

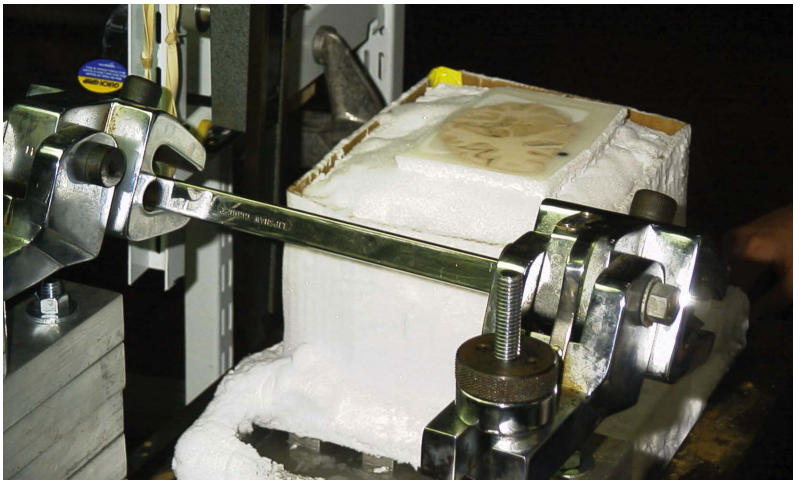




NSA NEUROTECHNOLOGIES™: EMBEDDING AND SECTIONING

LARGE FORMAT™ TECHNOLOGY
HUMAN BRAIN HEMISPHERES

Key preparation elements for successfully processing human and other large brains (see NSALabs.com for more specific, up-to-date information)

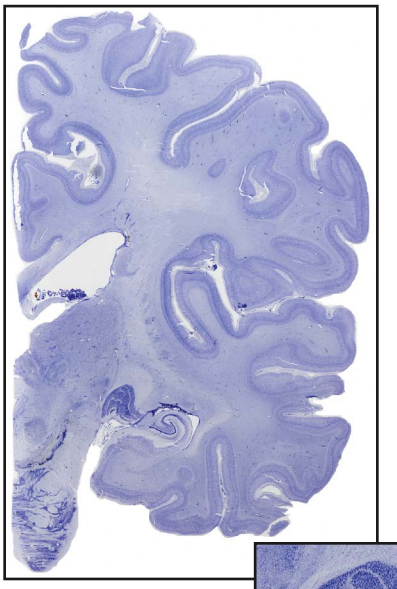


- Minimize the time from death until formaldehyde fixation.
- At brain removal, perfuse fix through the vasculature.
- Store the brain in a large volume (5–10X brain volume) of buffered formaldehyde and/or change regularly.
- NEVER FREEZE the tissue.

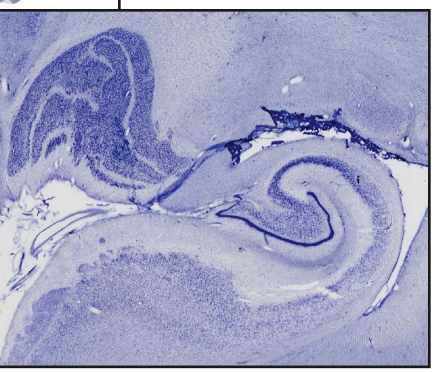
NSA works with tissue in a variety of conditions, but best results are achieved using the guidelines above. Contact NSA to discuss your specific needs.

Advantages:

- There is uniformity of staining and section thickness across the entire intact section of the human brain hemisphere.
- For stereology, only intact, adjacent sections yield sampling that is compliant with stereologic principles.
- The inclusion of adjacent structures on the same slide provides anatomical coherence and a comprehensive perspective of the brain, even when a small area of the brain is the focus.



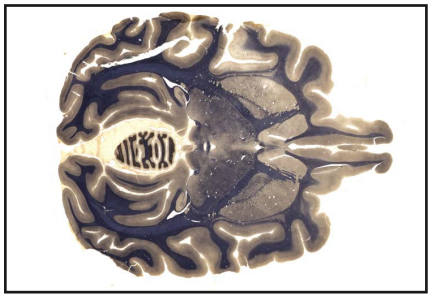
Pictured is a section from a human brain stained with thionine to reveal cell bodies. Magnification shown below.

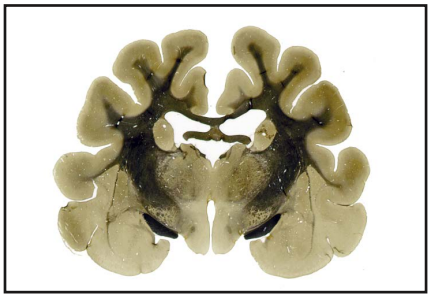


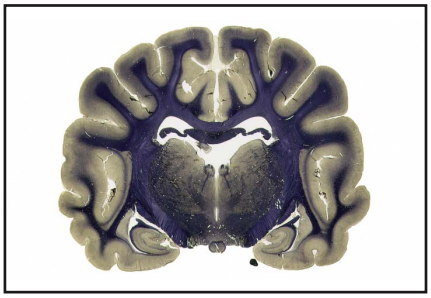
Tissue Resources for Researchers

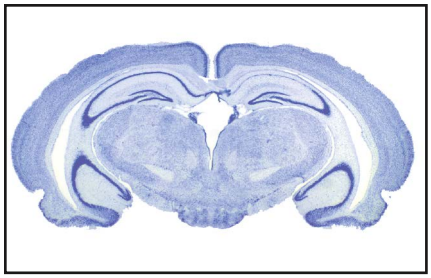
NSA can provide recommendations for organizations that bank human brain tissue with specific characteristics (disease, genetic traits, etc.). Some organizations offer specified tissue for purchase. Other organizations grant requested tissue to researchers that fulfill certain requirements, such as use for research or educational purposes.

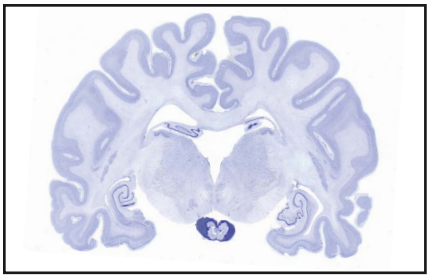
SPECIES PROCESSED UTILIZING NSA NEUROTECHNOLOGIES™

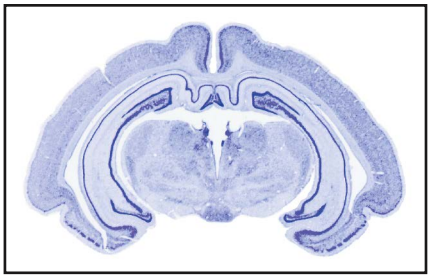

Baboon

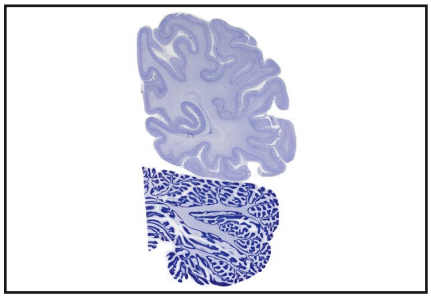

Cat

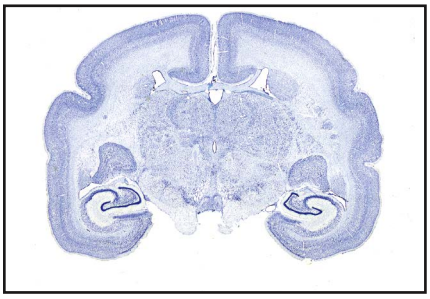

Dog

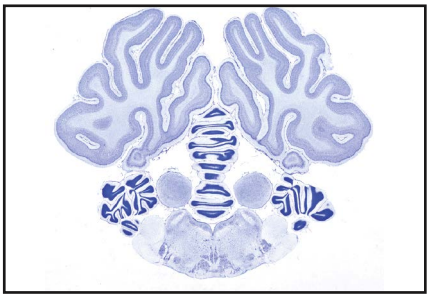

Gerbil

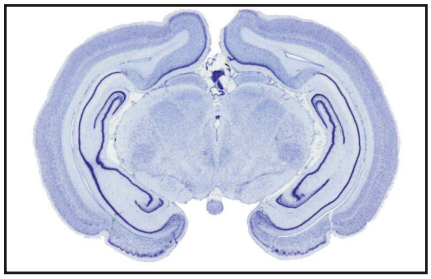

Grizzly Bear

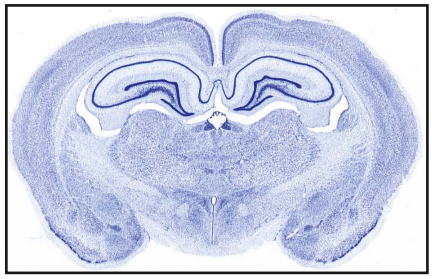

Guinea Pig

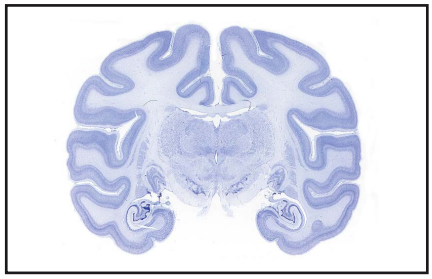

Human Hemisphere


Marmoset Monkey


Pig


Rabbit


Rat


Rhesus Monkey

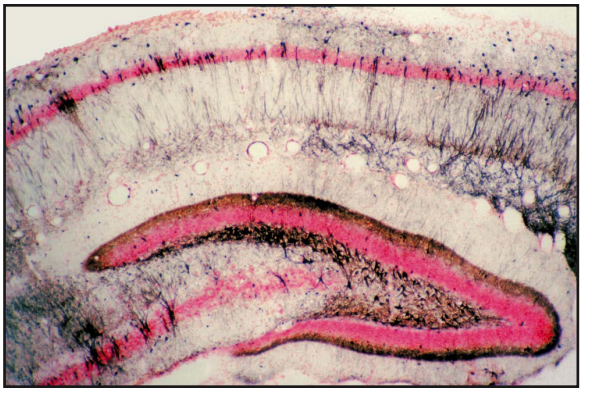
In addition to the species pictured above, NSA has also processed:

Armadillo	Dolphin	Manatee	Raccoon
African Green Monkey	Elephant Forebrain	Manta Ray	Sheep
Bear	Goat	Mouse	Snake
Chicken	Hammerhead Shark	Mud Puppy	Squid
Chinchilla	Hamster	Opossum	Vole
Cow	Lizard	Pigeon	Wood Rat

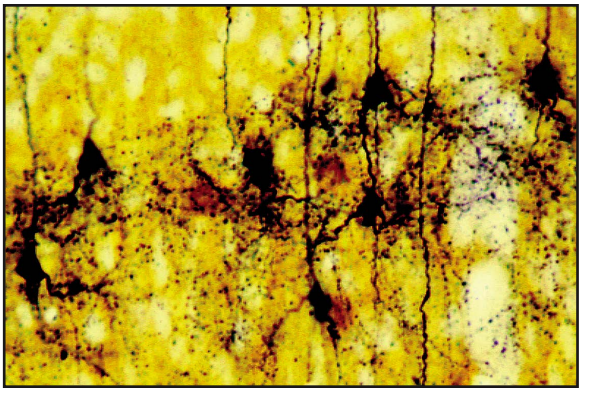
...and more!

NSA SERVICES

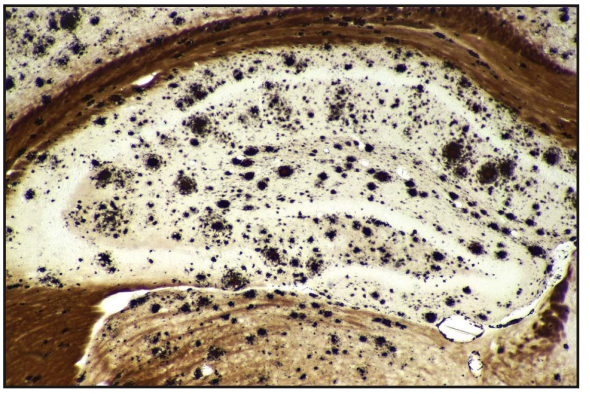
SPECIALTY STAINS



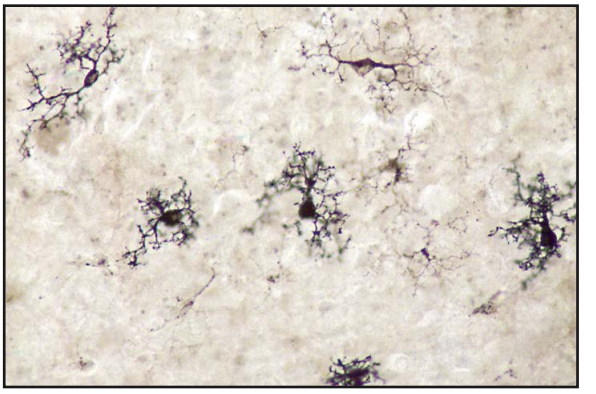
Amino Cupric Silver (Amino CuAg)
to reveal degeneration
*deOlmos JS et al. Neurotox and Teratol 16:545–561,1994
see pages 26, 47-48, 57, and 80-83 for more details



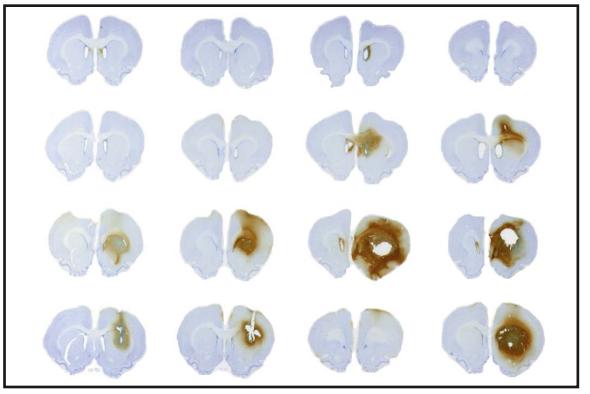
Cupric Silver (CuAg) method*
to reveal degeneration in fragile tissue
*deOlmos JS et. al. *Brain Res*.33: 523–529,1972
see pages 63, 80 and 84-85 for more details



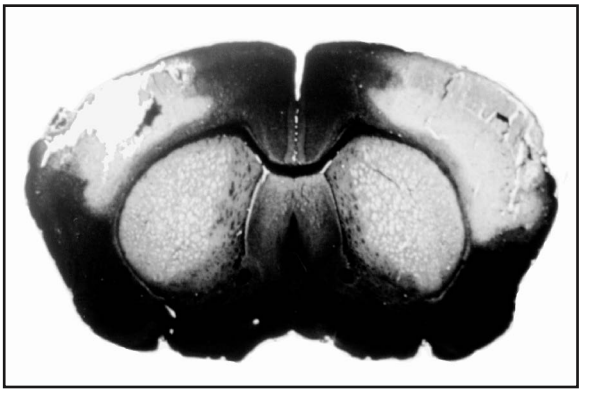
Campbell-Switzer Alzheimer
to reveal amyloid plaques and tau abnormalities of Alzheimer pathology
see pages 5, 27-29, 36-38 and 46-47 for more details



NSA's Reactive Microglia Stain
to reveal reactive microglia
see pages 46, and 78-79 for more details



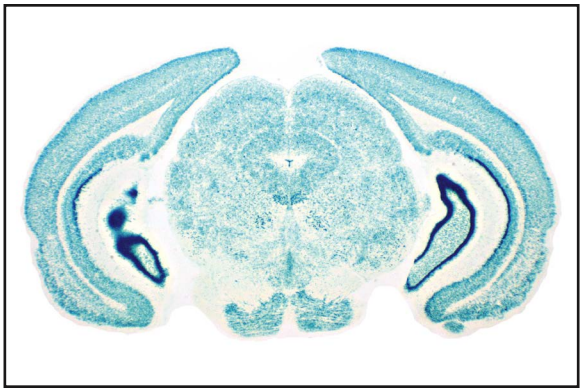
Blood Brain Barrier (BBB)
to reveal the locations of blood brain barrier compromise in the brain
see pages 5, 69-71 and 90 for more details



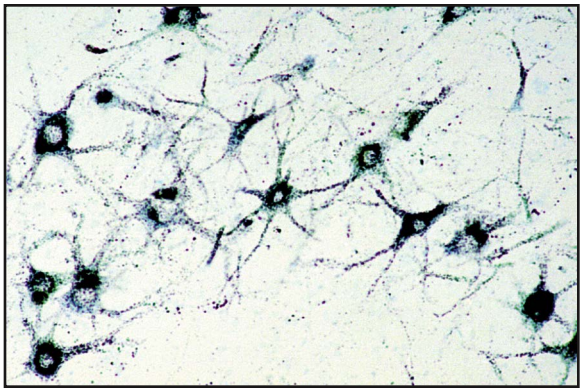
Ischemia Contrast
to reveal the volume of tissue affected following ischemia
see pages 25 and 65 for more details

NSA SERVICES

SPECIALTY / CLASSIC STAINS

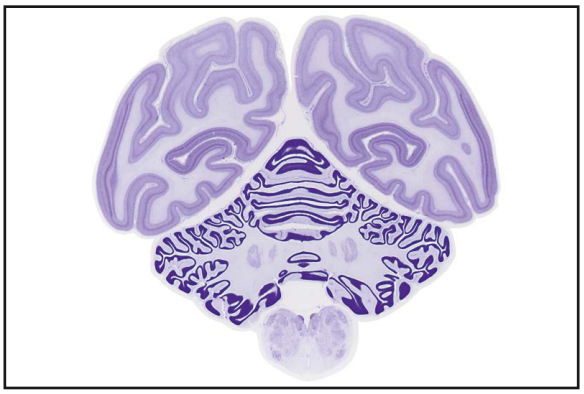


X-Gal
to reveal β-galactosidase
see pages 68 and 74 for more details

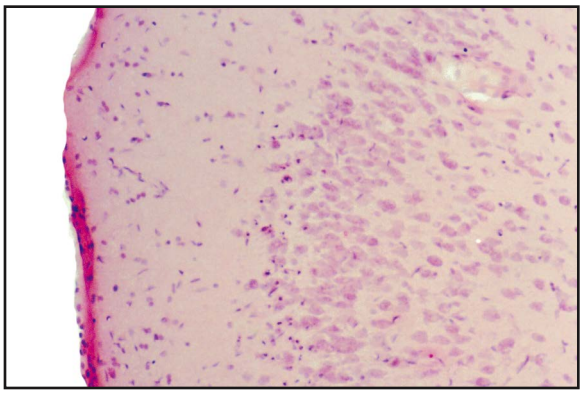


Autometallography
for the detection of metals
see page 67 for more details

CLASSIC STAINS (continued on following page)



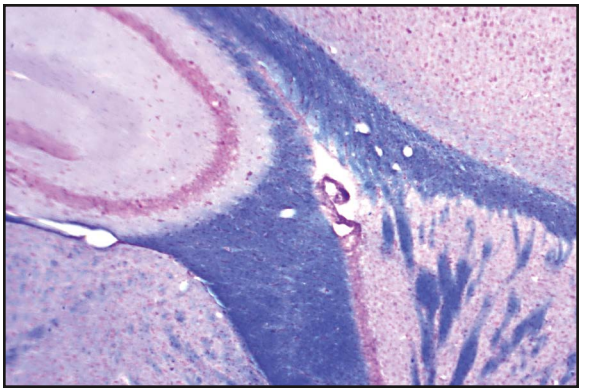
Thionine (Nissl)
to reveal cell bodies in tissue. This may be applied as a counterstain against suitable stains
see pages 7, 8, 10, 58, 63 and 84 for more details



H&E
to reveal the nuclei and cytoplasm of cell bodies
see pages 76 and 80-82 for more details

NSA SERVICES

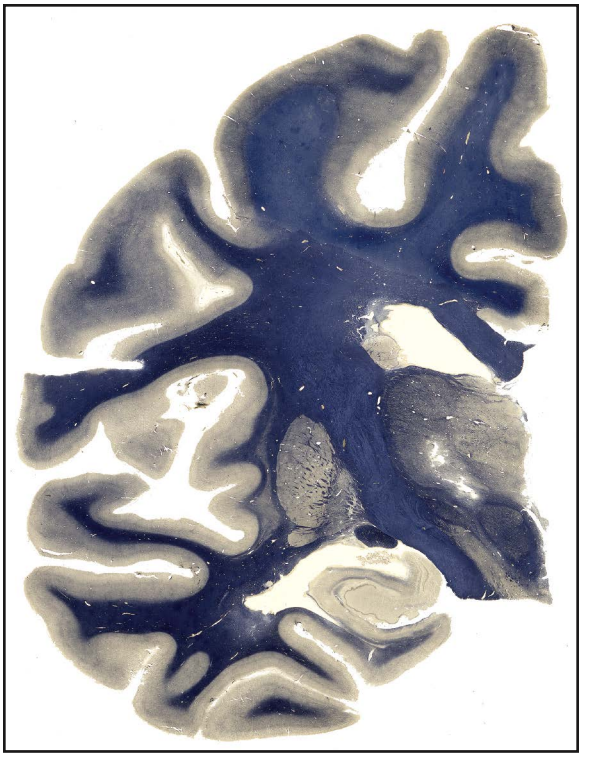
CLASSIC STAINS (continued)



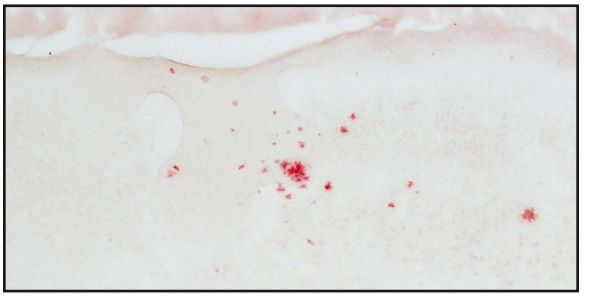
Solochrome
to reveal myelin
see page 55 for more details

AChE
to reveal Acetylcholinesterase
see page 66 for more details

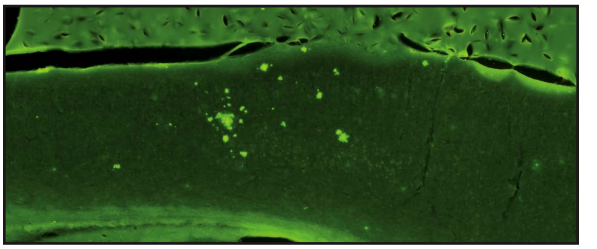
Perls for ferric Iron (with or without DAB)
to detect the presence of ferric iron in tissue (normally occurring, or from ruptured red blood cells)
see pages 7, 49, 58, 63 and 72 for more details



Weil-Myelin (human brain)
to reveal the myelinated axons
see pages 7-8, 31, 55, 57 and 58 for more details



Congo Red
to reveal dense fibrillar amyloid plaques
see pages 27, 36 and 37 for more details



Thioflavin S
to reveal fibrillar amyloid
see pages 36, 37 and 47 for more details

NSA SERVICES

IMMUNOHISTOCHEMISTRY / ANTIBODY STAINS

Immunohistochemistry (IHC) is used to detect the presence of specific biomolecules (antigens) in tissue sections by applying an antibody to that antigen. IHC has become one of the most common tools in neurohistologic research due to the potential of high specificity when staining a target feature. NSA uses TritonX-100 to fenestrate membranes, allowing antibodies to stain the entire thickness of the section (30–80µ), removing thickness as a factor in NSA's protocols.

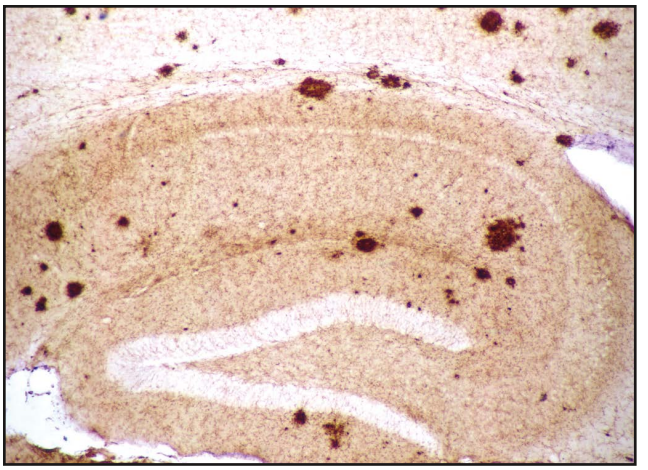
We offer high-quality immunohistochemistry services utilizing commercially available antibodies and client-supplied proprietary antibodies and routinely perform titration series to expand our repertoire.

Below is a list, as of this printing, of commercial antibodies that we have successfully applied. Following the list are representative images of some of our most commonly requested immunohistochemistry. Visit our website for an up-to-date listing. *(See index for photo samples throughout this catalog)*

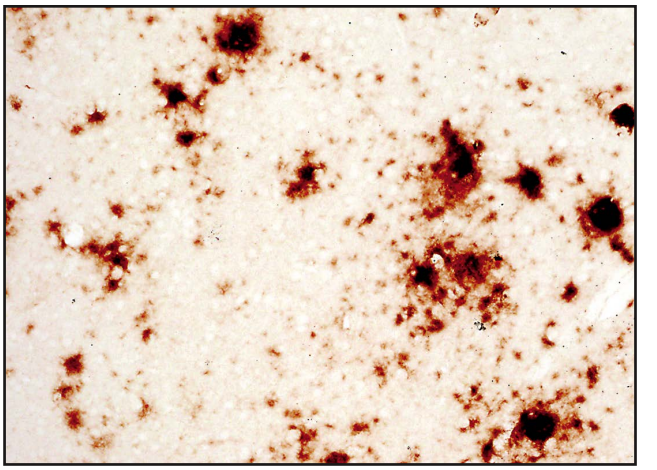
4H7H7 Huntington Disease Aggregates
6E10 β-Amyloid (1-17)
82E1 β-Amyloid (1-16)
ApoTag 4
APP
ARC
Aβ (1-40)
Aβ (1-42)
α-Synuclein
α-Synuclein (pSer129)
α-Synuclein-Human 4B12 (103-108)
α-Synuclein 211 (121-125)
BACE1
BDNF
β-galactosidase
c-Abl (pTyr412)
c-Fos
c-Jun (pSer63)
Calbindin
Calretinin
Caspase-3-activated
Caspase-9-activated
Cathepsin-D
CD11b
CD11c
CD163
CD1B3
CD3
CD4
CD45
CD45R
CD68
CD8
CGRP
ChAT
Claudin-1
c-Myc
CNPase (RIP)
CRF
d-serine
DARPP32
Doublecortin
eGFP
EGFR
EGR-1
eIF-alpha
EM-48
EPO
Ergothioneine Transporter
Ferritin
FGFR-1
FGFR-2
FLAG
Frataxin (Anti-Human)
GABA
GAD-65
GAD-67
GAP-43
GAP-43 (pSer96)
GAP-43 (pThr172)
GDNF
Gephryn
GFAP
GFP
Glutamate
GPEET
GSK-3β (pSer9)
HNE
Human Nuclear Protein (HuNu)
Huntingtin
Hypocretin (Orexin)
Iba1
IFN-gamma
IGF-1
IgG-2a
IgG-Cyno
IgG-Human
IgG-Human Fc fragment
IgG-Human(H+L)
IgG-Monkey
IgG-Mouse
IgG-Rat
IgG-Sheep
Ki-67
Kv3.1b
Laminin
LAMP-1
LC3B
Lef-1
LRRK2
MAG
MAP-2
MCA566
MHCII
NADPH
NCAM
Nestin
NeuN
Neuroligin-2
NF-1 (Neurofilament-200)
nNOS
Nogo-A
NS1 Glycoprotein [DN3]
Occludin
Olig-2
Opioid Receptor-Kappa
Opioid Receptor-mu
Orexin (hypocretin)
Parvalbumin
PBR1
PDGFRbeta
Phospho-Akt (Thr308)
Pin-1
Procyclin
PSA-NCAM
PSD-95
R10Z8E9
RGMa
RMO-14 (neurofilament-M)
S100 beta
Serotonin
Serotonin Transporter
SMA
SMI-31
SMI-311
SMI-312
SMI-32
SMI-71
SMI-94
SMI-99 myelin basic protein
SOD1
Somatostatin
Sphingosine kinase
STEM-101
STEM-121
STEM-123
Substance P
Synaptophysin
Tau 39E10 (189-205)
Tau 46 (403-441)
Tau 5 (210-230)
Tau AT100 (pSer212/pSer214)
Tau AT180 (pThr231; PHF-6)
Tau AT181 (pThr181)
Tau AT8 (pSer202,pThr205)
Tau CP-13 (pSer202)
Tau HT7 (159-163)
Tau MC1 (312-322)
Tau PHF-1 (pSer396,404)
Tau PHF-13 (pSer396)
Tau (pSer422)
Tau-ALZ50 (2-10 &312-342)
TDP-43
TNF-α
Tryptophan Hydroxylase
Tyrosine Hydroxylase
Ubiquitin
USP-14
VAChT
VEGF
YFP
Zona Occludens-1

NSA SERVICES

IMMUNOHISTOCHEMISTRY / ANTIBODY STAINS (continued)

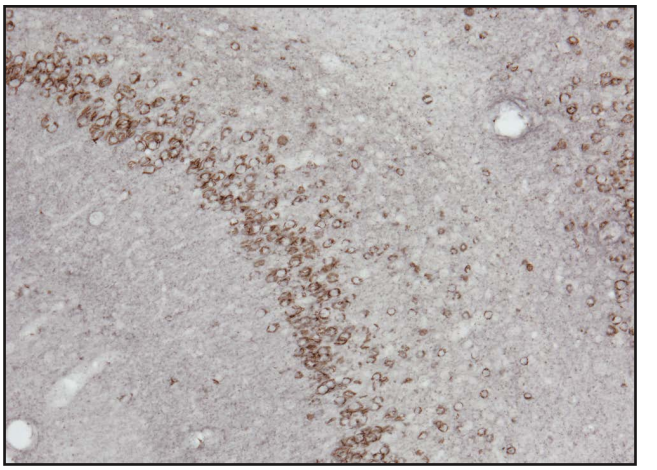


4G8 (Mouse Hippocampus)
PS-1/APP Transgenic

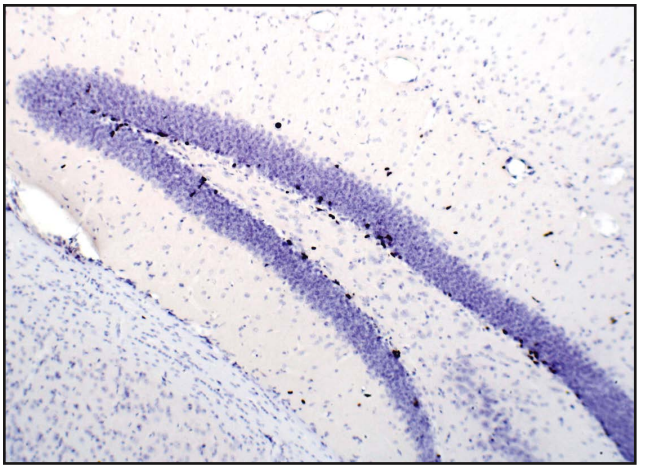


6E10 (Mouse Alzheimer Model)

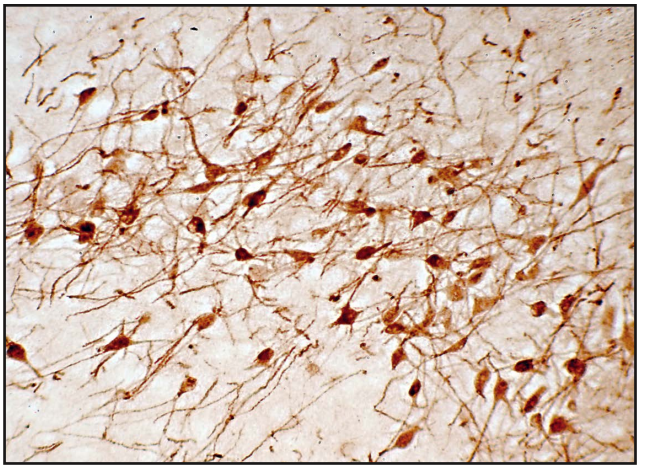
Alpha-Synuclein



AT100 (Mouse Hippocampus) 10x



BrdU

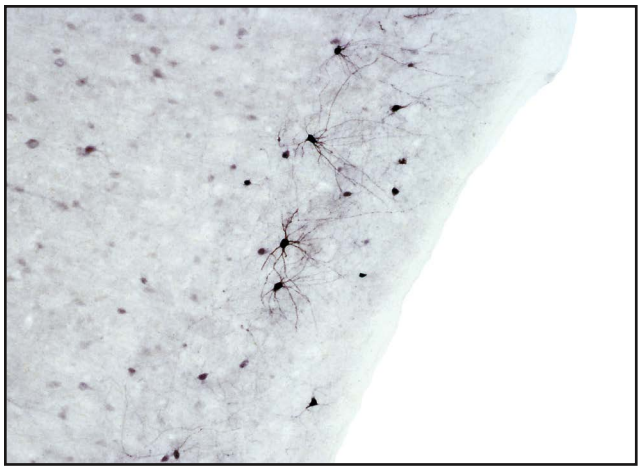


Calbindin

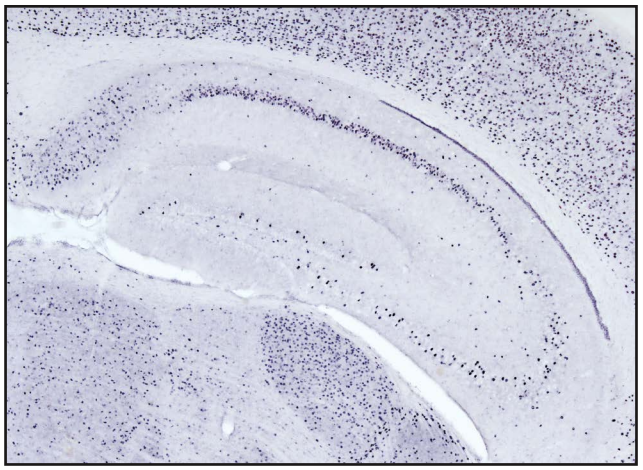
Didn't find what you're looking for? Check our website for a current listing or request a titration series for a new or custom antibody!

NSA SERVICES

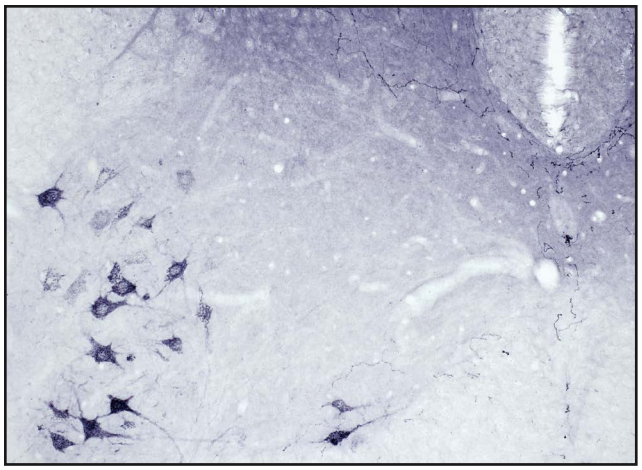
IMMUNOHISTOCHEMISTRY / ANTIBODY STAINS (continued)



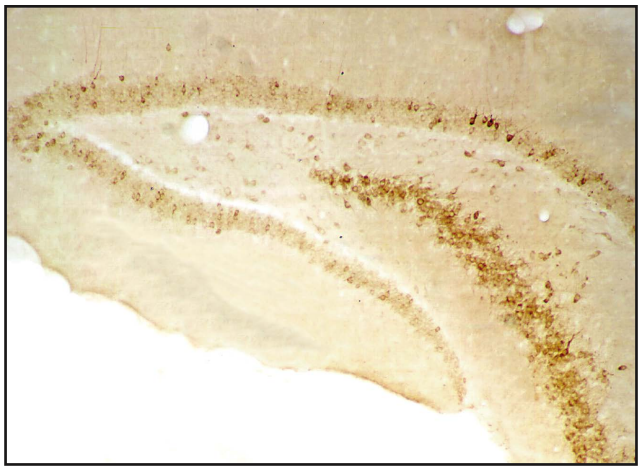
Calbindin (Sheep Cortex)



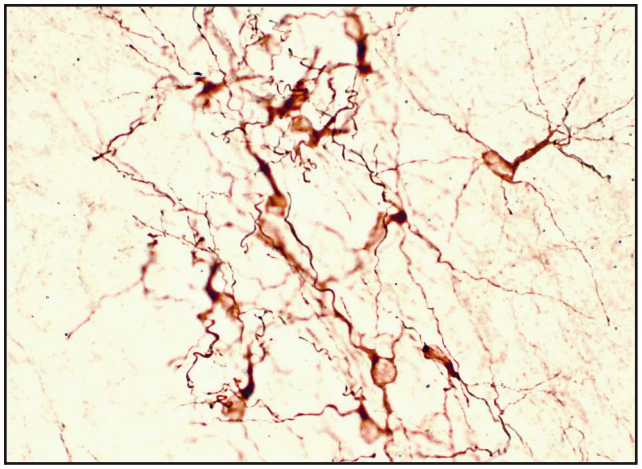
c-fos (Mouse Hippocampus)



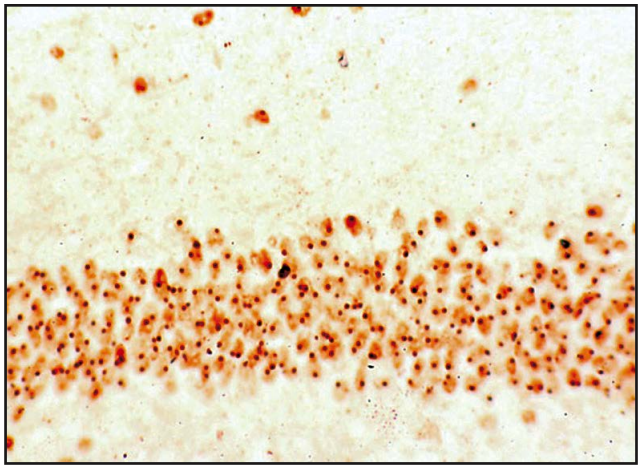
cGRP (Mouse Spinal Cord) 10x



COX-2 (Rat Hippocampus)



Doublecortin



EM48 Hippocampus

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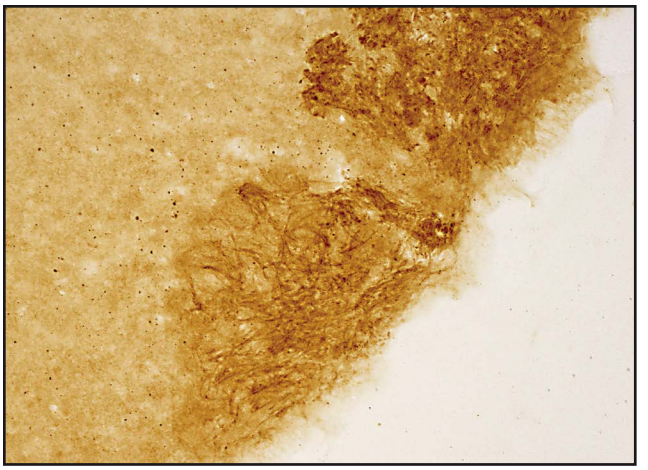
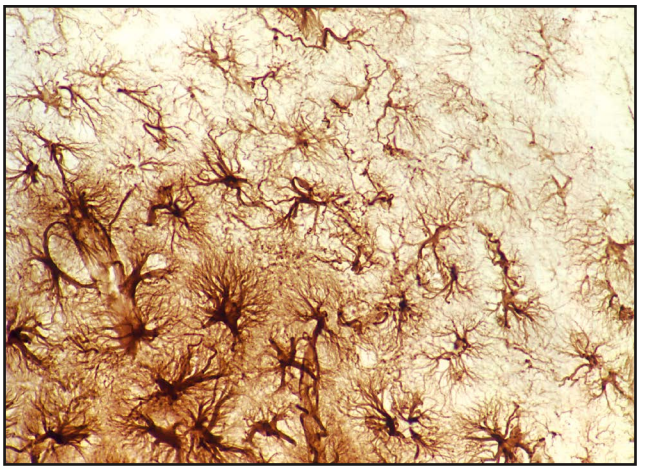
NSA SERVICES

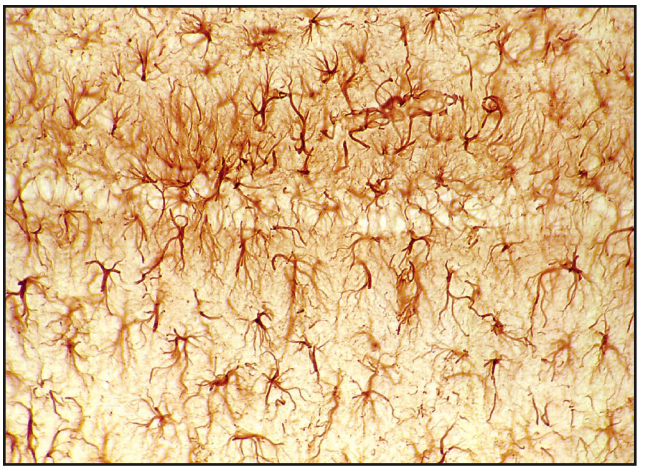
IMMUNOHISTOCHEMISTRY / ANTIBODY STAINS (continued)



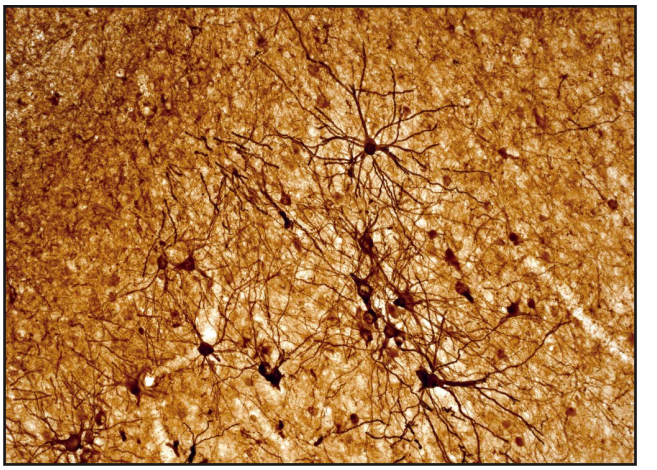
phospho GAP 43 pT172 (Rat Olfactory Bulb)



GFAP (Mouse Amygdala)
Idiopathic Astrocyte Reactivity



GFAP (Rat Hippocampus)
Trimethyl Tin Intoxication



GFP

GFP (Rat Cortex)

Iba1

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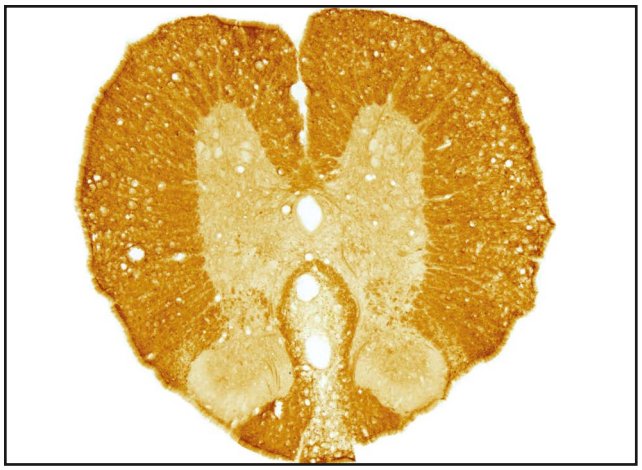
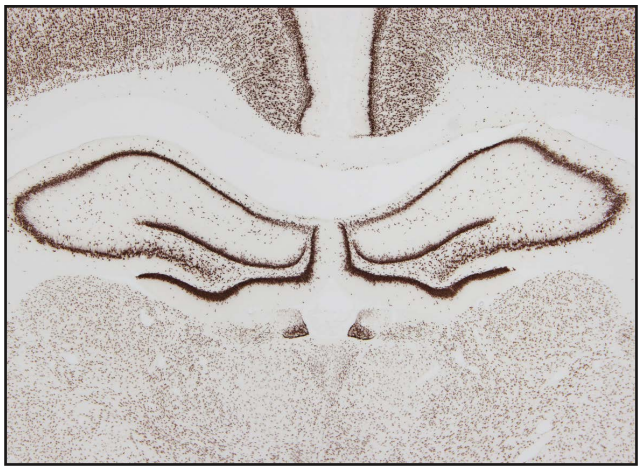
NSA SERVICES

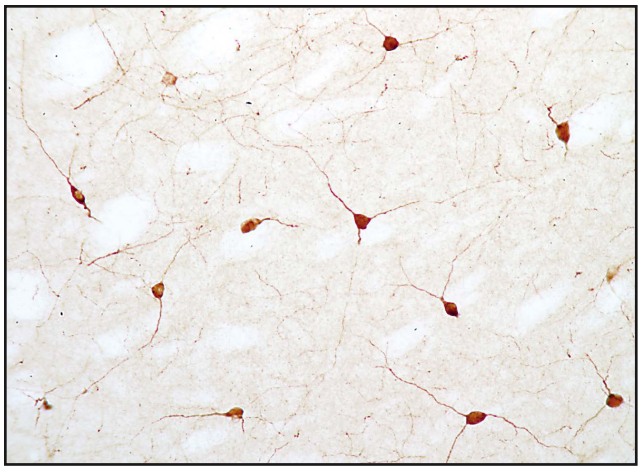
IMMUNOHISTOCHEMISTRY / ANTIBODY STAINS (continued)



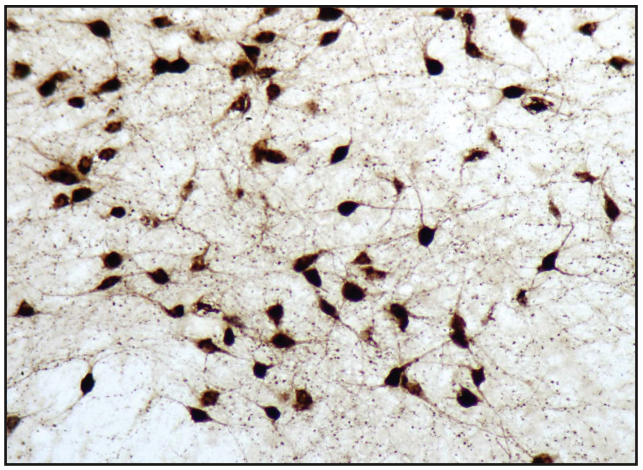
Myelin Basic Protein (Mouse Spinal Cord)



NeuN (Rat Hippocampus) 2x



nNOS



Orexin A (Rat Hypothalamus)

Phospho Tau Ser 396 (Mouse Brain)

Phospho Tau Serine 422 (Human Cortex)

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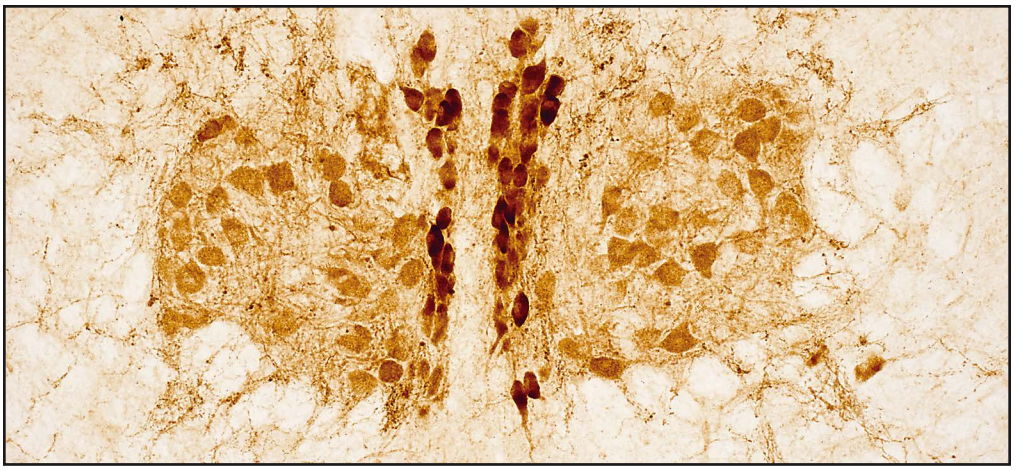
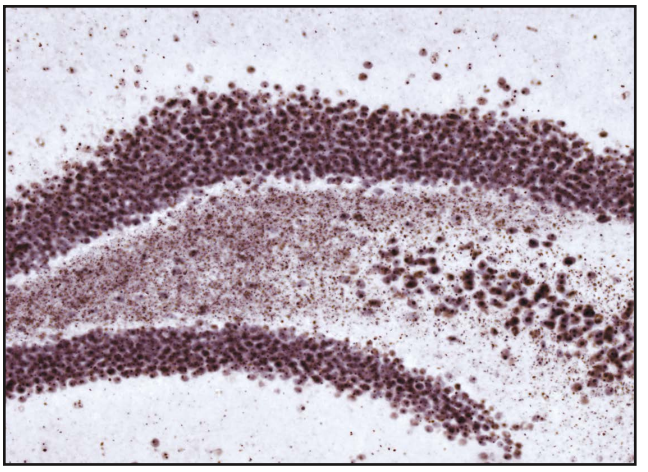
NSA SERVICES

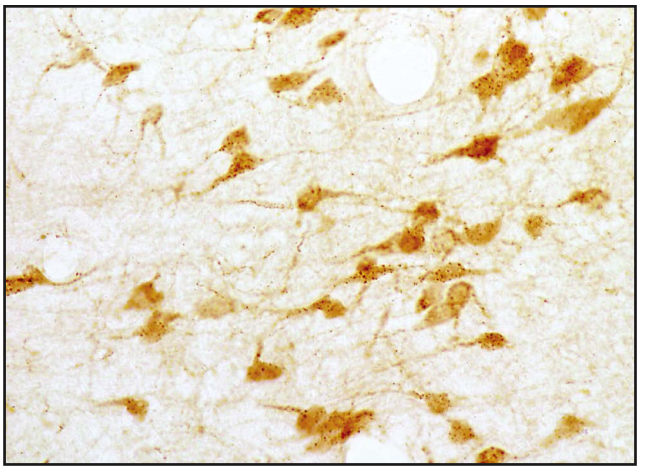
IMMUNOHISTOCHEMISTRY / ANTIBODY STAINS (continued)



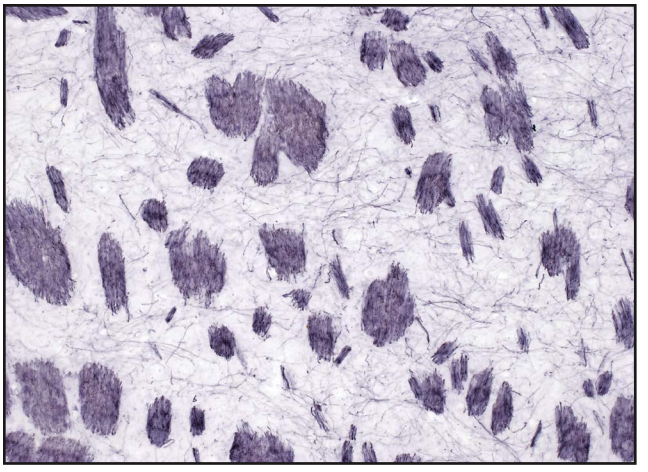
Procyclin in Mouse Brain
Edinger-Westphal & Oculomotor Nuclei



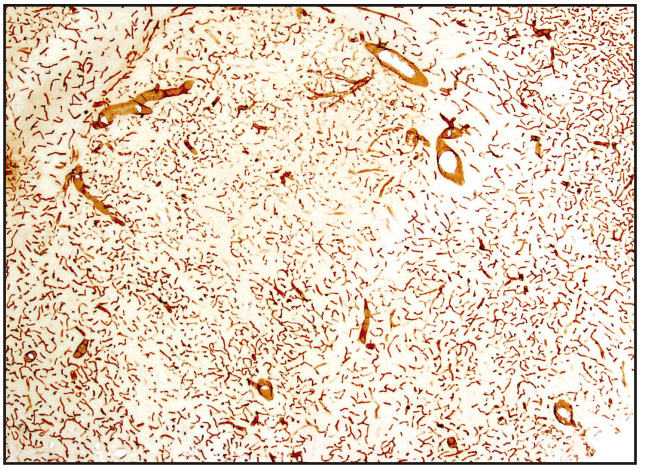
S830, Huntington Disease Mouse Model
Hippocampus, 20x



Serotonin



SMI-312 (Mouse Striatum)



SMI-71 (Stroke Rat)

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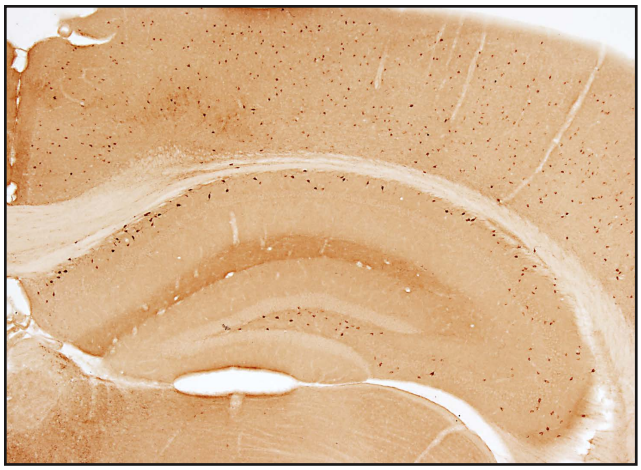
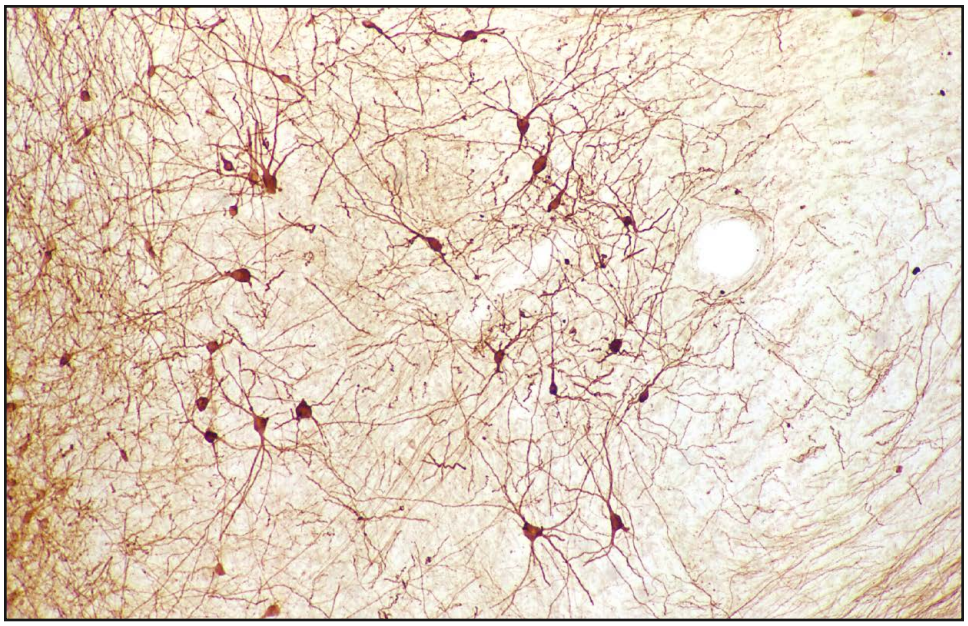
NSA SERVICES

IMMUNOHISTOCHEMISTRY / ANTIBODY STAINS (continued)



Somatostatin (Mouse Hippocampus)

TDP43 (Human Hippocampus AD) 4x



Tyrosine Hydroxylase (Rat Brain)

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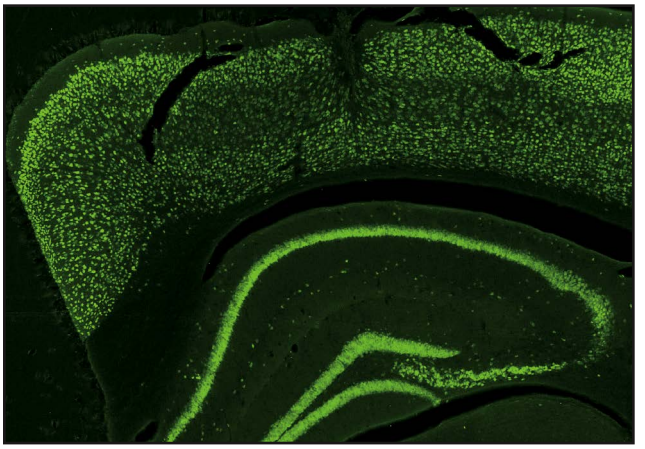
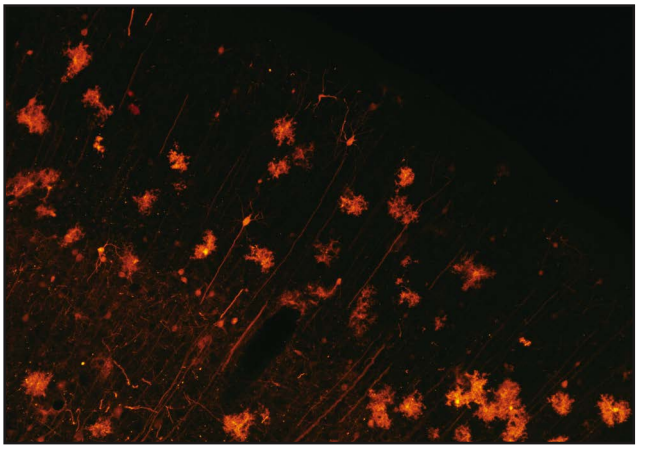
NSA SERVICES

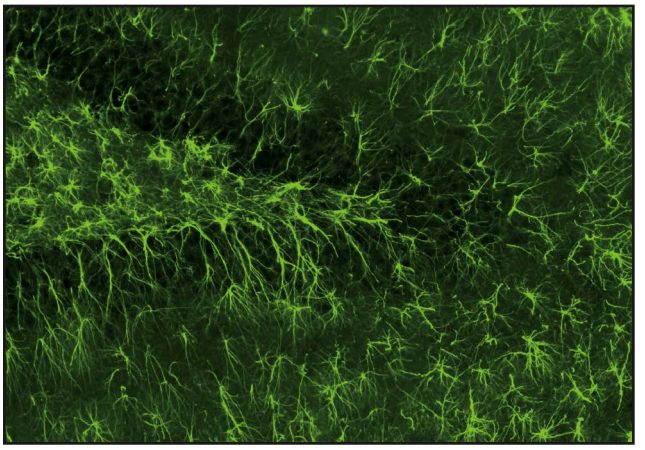
FLUORESCENT IMMUNOHISTOCHEMISTRY / ANTIBODY STAINS



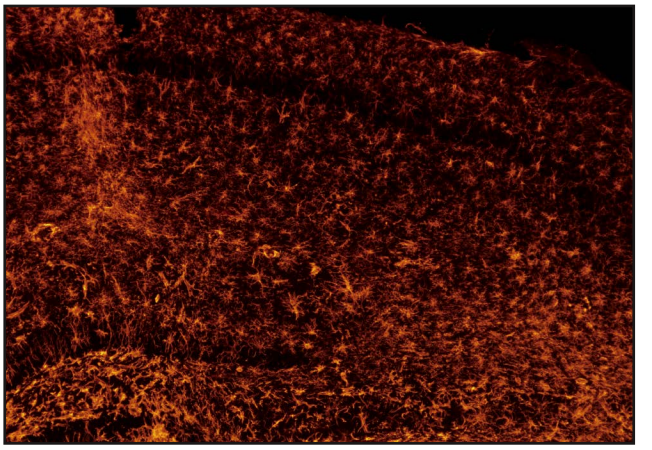
NeuN Mouse Hippocampus



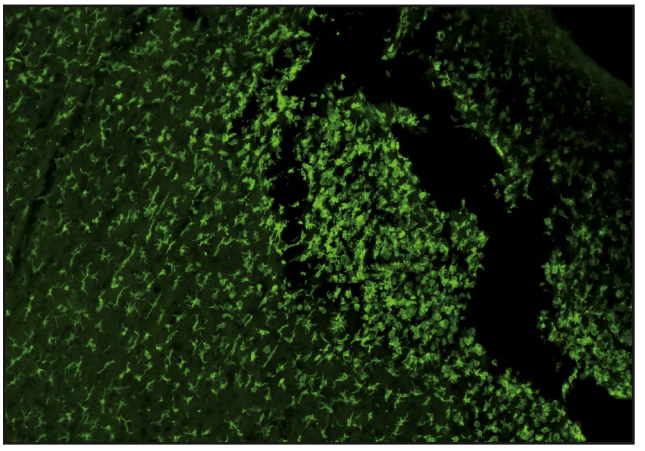
YFP Rat Cortex



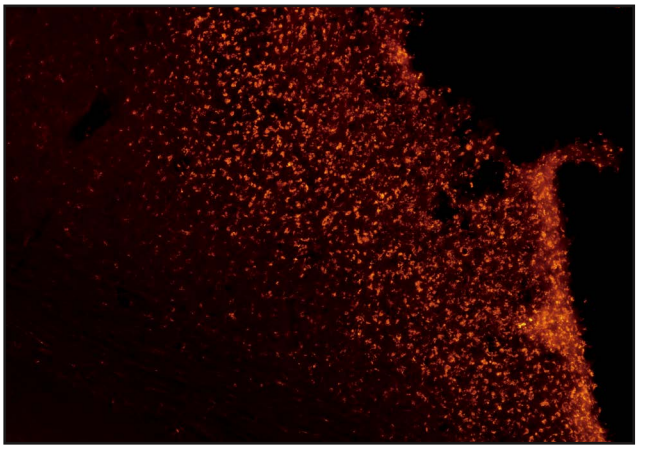
GFAP Rat Cortex



Iba1 Mouse Hippocampus



Iba1 Rat Cortex



CD68 ED1 Rat Cortex

Didn't find what you're looking for? Check our website for a current listing or request a titration series for a new or custom antibody!

NSA SERVICES: IMAGE ANALYSIS

QUANTITATIVE ANALYSIS BY NSA

Turn histology into answers with analysis. Once your tissues are processed by one of NSA's NeuroTechnologies™, the benefits of being stained at the same time and under the same conditions are reaped with minimized variation, increased confidence and lower analytic costs. NSA offers both Image Analysis and Stereological Analysis. We can help you plan a study to quantify most endpoints available with bright field or fluorescent microscopy.

IMAGE ANALYSIS:

The first step in analyzing a high resolution image is capturing the image. Our onsite TissueScope™ platform from Huron Digital Pathology performs state-of-the-art digital imaging. These seamless, razor sharp images captured with a 10x or 20x objective allow faster review and a wide range of analytical techniques to be applied.

By applying proven image processing techniques, NSA quantifies a range of changes in stained tissue with highly reproducible results, yielding data superior to subjective assessments. Below are descriptions of the more popular image analyses performed and a summary of the examples on the following pages.

Densitometry is the relative density and intensity of staining in 2 dimensional space, used to measure the signal of a given stain in a particular Area Of Interest (AOI/ROI). Typically all stained cellular elements are combined: cells, fibers, neuropil. Applications not described herein include assessing injection fields, enzyme staining, fiber density, AAV infection area.

Particle Counts assess the relative size, number, and distribution of stained cellular elements (2D), by focusing on isolated aspects of staining for features such as number of cells, size of deposits, and number of plaques. Densitometric analysis can be applied to particle counts to provide further information.

Volume Analysis is used to measure AOIs/ROIs and compare across animals lesions, brain structures, stained population of cells, etc.

Visualization Tools help to better understand what is going on in tissue, view in planes not available with singular plane histology, and view differences across animal groups

Examples of NSA Image Analysis described on the following pages:

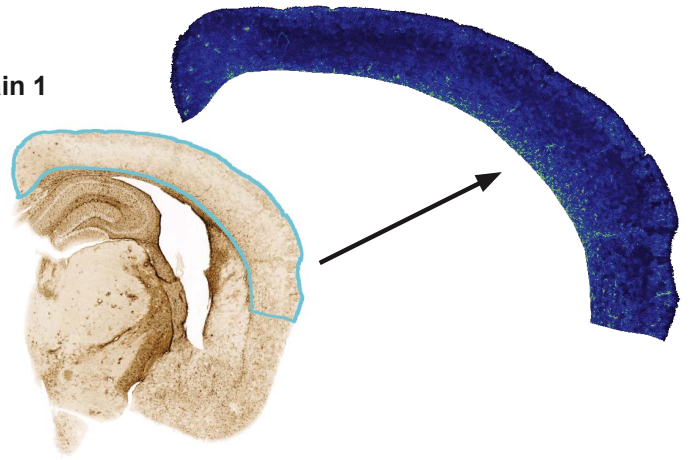
	Densitometry	Particle Counts	Volume	Visualization Tools	Page #
Intensity of Staining	√				**24**
% of Ischemic Area in Stroke Model	√				**25**
Quantifying Degenerated Cells vs Survivors		√			**26**
Alzheimer Plaque Burden: Particle Count and Densitometry	√	√			**27**
Alzheimer Plaque Quantification	√	√			**29**
Volumetric Calculations	√		√		**30**
3D Reconstructions			√	√	**31**
Volume Rendering			√	√	**32**

STEREOLOGY is a statistically rigorous means of quantifying morphological elements (2D and 3D) such as cell number, cell area/volume, nuclear area/volumes, process length, etc. *See page 33 for more details.*

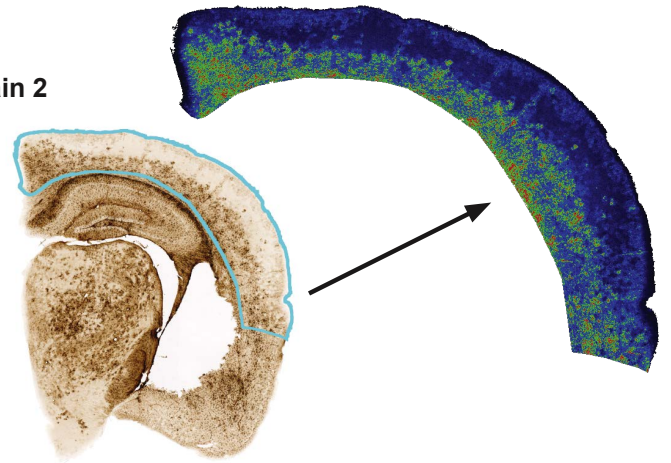
NSA SERVICES: IMAGE ANALYSIS

INTENSITY OF STAINING

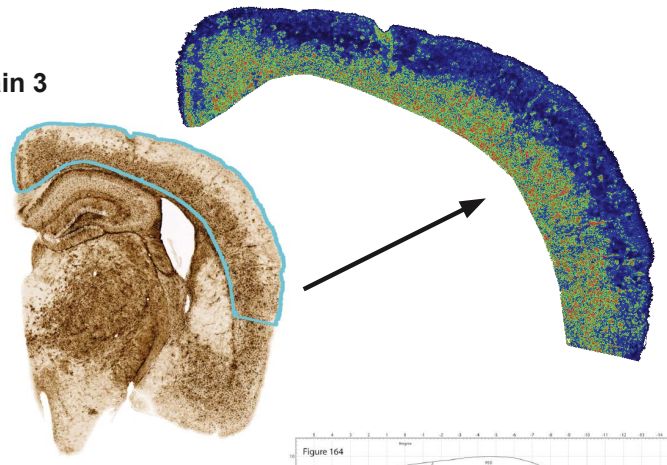
Brain 1



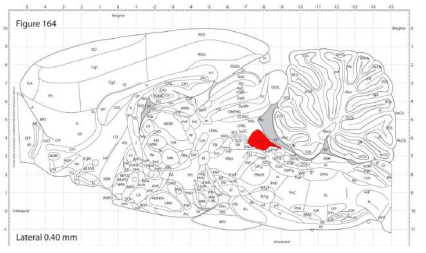
Brain 2



Brain 3



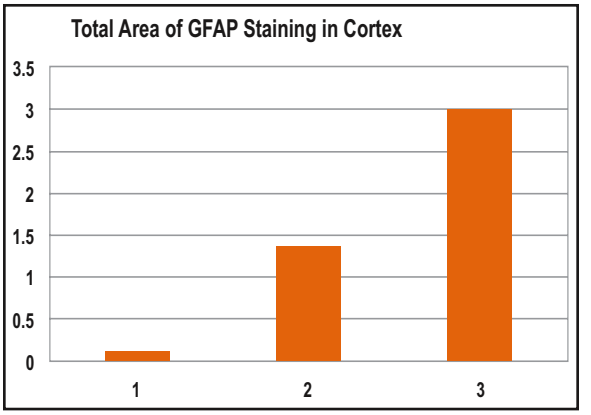
To further reduce technical variance, expert NSA neuroanatomists ensure consistent regions of interest (AOI/ROI).

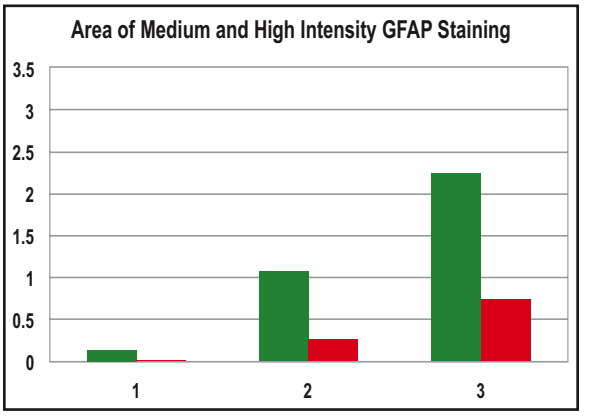


Neuroscience Associates has the capability to quantify antibody staining on tissue processed at our facilities. An example of GFAP quantification in mouse cortex is shown to the left, with corresponding data below.

A specific area of interest (AOI/ROI) is delineated followed by image processing steps to make the area more amenable for quantification. In this example, the total amount of GFAP staining can be measured as both percent area occupied of AOI/ROI and total area occupied in appropriate units (millimeters squared for example).

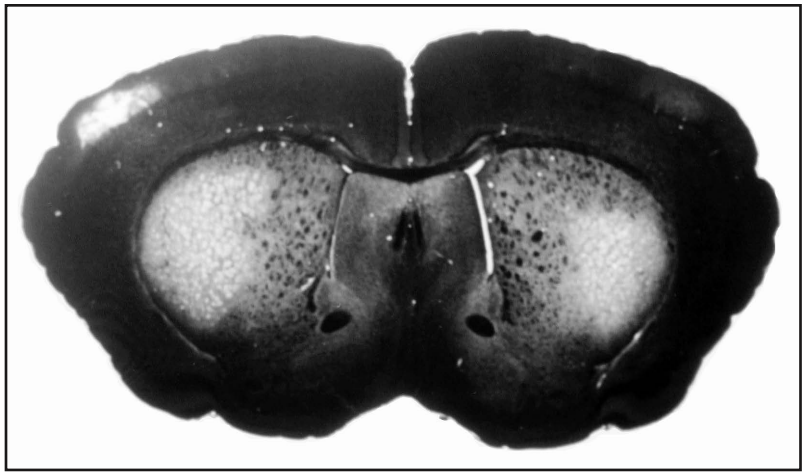
Due to the gradation of light to more intense staining, the total GFAP staining can be further divided into medium (shown as green) and high (shown as red) intensity staining totals in the given AOI/ROI. These can be measured as percent area occupied of the AOI/ROI or total area of medium or high intensity staining.





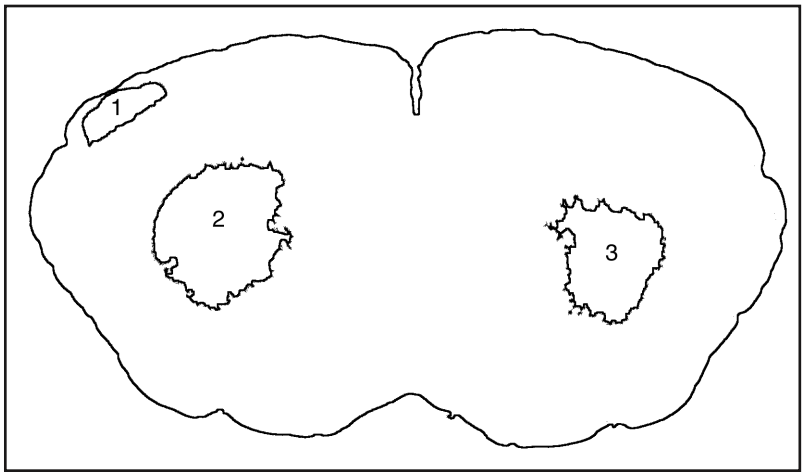
NSA SERVICES: IMAGE ANALYSIS

PERCENT OF ISCHEMIC AREA IN STROKE MODEL



Gerbil brain section rendered ischemic from bilateral carotid ligation. The Ischemia Contrast stain sharply delineates affected areas.

Once the image of the section is digitized, boundaries of the entire section and individual ischemic zones (1–3) are drawn, using density thresholding techniques with an NIH Image. Total volumes or an "index" of the volumes affected can be calculated from analysis on additional sections.



DATA:

	Area (sq. units)
Entire section	2.73
Area 1	0.02
Area 2	0.17
Area 3	0.11

% Ischemic area: 10.99%

NSA SERVICES: IMAGE ANALYSIS

QUANTIFYING DEGENERATED CELLS VS SURVIVORS

Knowing the exact degree of change between treatment groups improves therapy modeling, e.g. dose range selection in tolerance or efficacy studies. NSA quantifies the amount of damage and resistance/protection of susceptible cell populations, within any area of interest (AOI/ROI). Using this precise, reproducible approach yields data superior to subjective assessments, and informs the study design of more rigorous stereological methods.

Below, labeled healthy and dead neurons in rat cortex (NeuN and Amino Cupric Silver degeneration stains).

Original Image

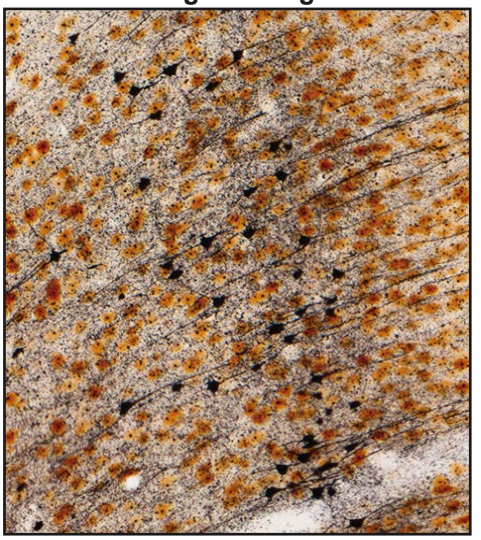
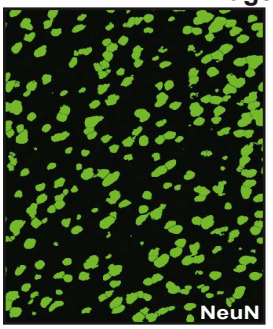
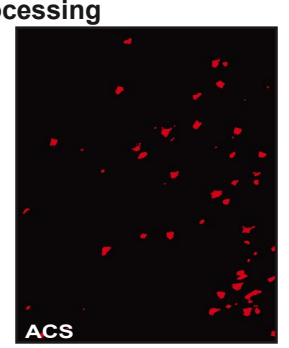
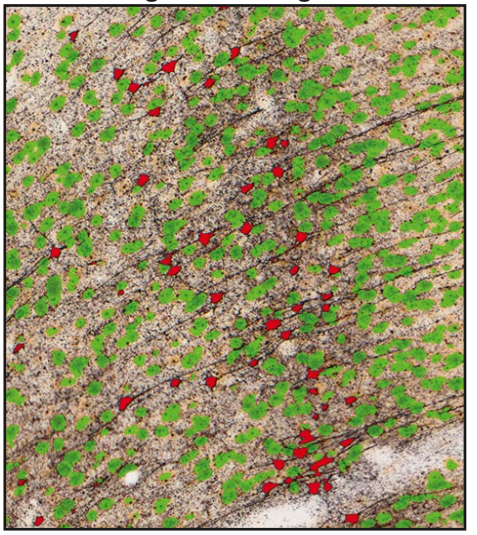


Image Processing



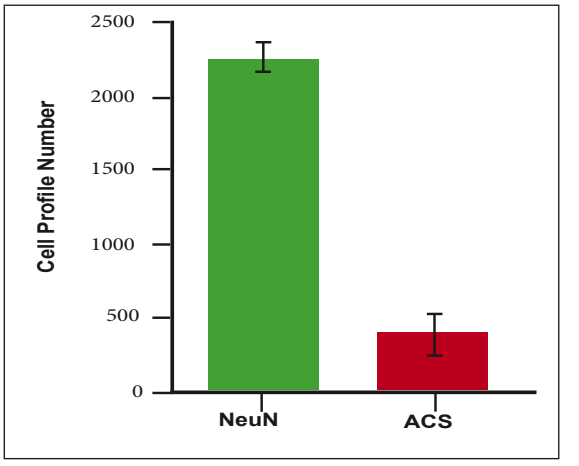


Segmented original



Results:

NeuN-positive and Degenerating cellular profiles in Rat Cingulate Cortex



Particle Analysis parameters may include:

1. Total profile number for healthy and degenerating cell populations
2. A real density of the damaged population
3. Binning by size class category

Stains amenable to quantification include most cell phenotype and degeneration markers.

Lower Variance, Increased Confidence

MultiBrain® and MultiCord® processing minimizes technical variance, thereby increasing your ability to detect treatment or biological effects.

Expert NSA neuroanatomists ensure consistent AOI/ROIs, further reducing technical variance.

Method: The original image (top) is converted into binary, false-color representations of the NeuN positive (green) and degenerating cell populations (red) for segmentation (bottom). The NeuN positive cells are varying shades of red/brown while degenerating neurons are black. This high-contrast staining allows for robust processing and segmentation.

NSA SERVICES: IMAGE ANALYSIS

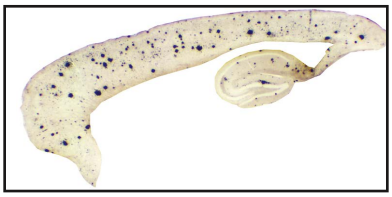
ALZHEIMER PLAQUE BURDEN: PARTICLE COUNT AND DENSITOMETRY

Mouse models of Alzheimer disease are designed to display neuritic plaques similar to those found in humans with the disease. Drugs that may potentially retard plaque development or prevent the creation of plaques are then tested on these mouse models. Part of the determination of the efficacy of a drug candidate is to measure the plaque load, the percent of cortex and/or hippocampus that is occupied by plaques. Other meaningful measures that should be considered when measuring efficacy include quantification of the total number of plaques and the frequency of plaque sizes. Below is an illustration of how NSA gathers the data.

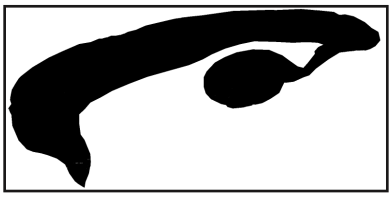
NSA Neurohistology: Neuritic plaques are revealed in freeze-cut, free-floating sections with the Campbell-Switzer staining method. Image analysis is performed on other stains, such as Aß 1-40, Aß 1-42, Thioflavin S, Congo Red, or any other stains that would reveal Alzheimer plaques.

NSA Analysis: High-resolution digital images of designated sections are captured. The percent of the area of cortex-hippocampus occupied by plaques is obtained through the following steps:

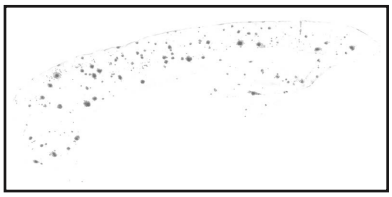
- Outline cortex and hippocampus and create a separate "traced" image.
- From the traced image, two other images are created in order to:
    1. Determine the total area of cortex-hippocampus.
    2. Determine the individual and total area of the plaques.



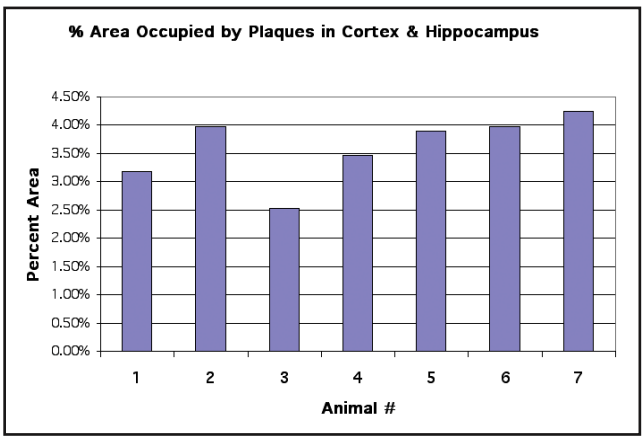
Traced image



The area of interest is rendered totally black to get total area.



Analyze Particles: Individual plaques are automatically outlined, and the area is calculated for each.

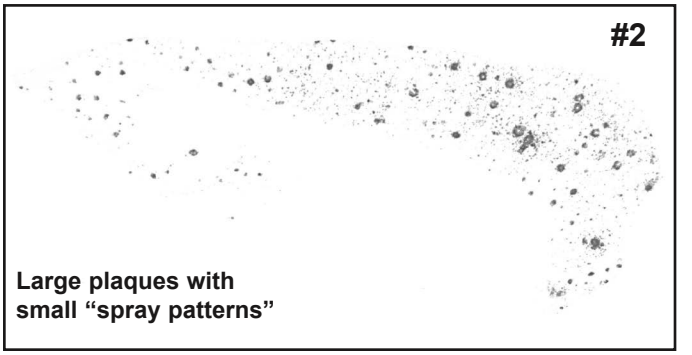


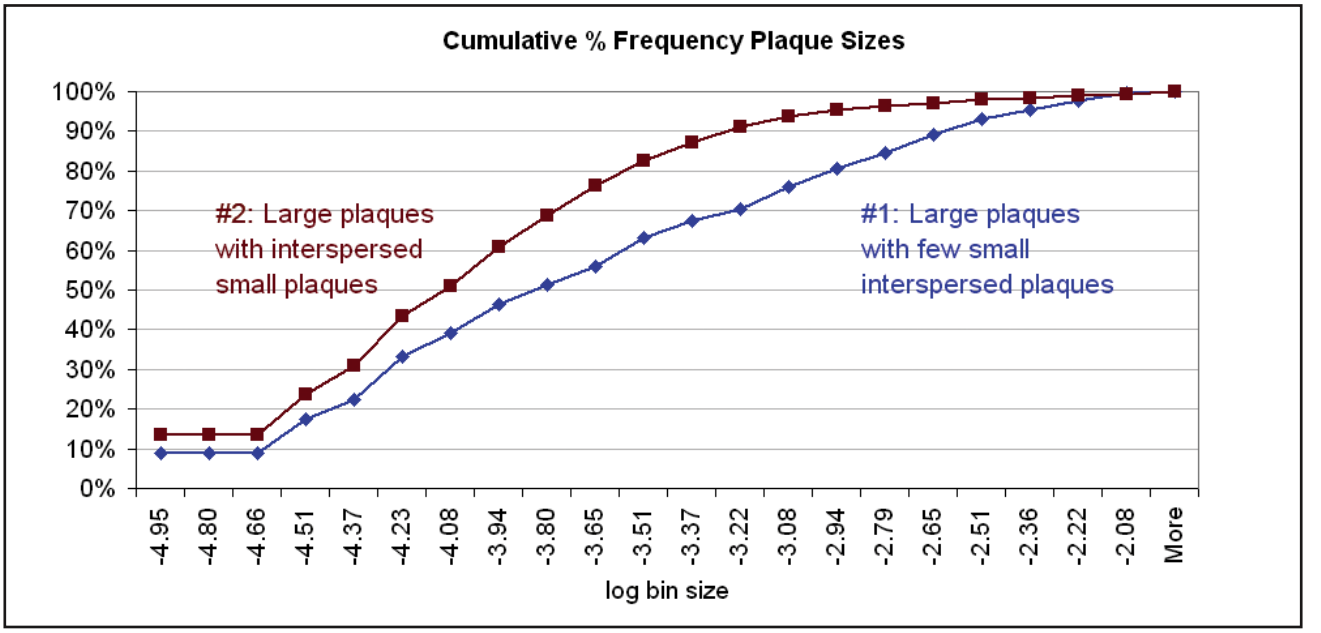
Compilation of data from several animals yields a histogram within which comparisons of different treatment groups and controls can be made.

NSA SERVICES: IMAGE ANALYSIS

ALZHEIMER PLAQUE BURDEN: PARTICLE COUNT AND DENSITOMETRY (continued)

In addition to percent area occupied and plaque density, visualizing the number of plaques of different sizes may prove valuable. In the two images below, case #1 appears to have numerous large plaques but not many small ones. In #2, however, many small plaques accompany numerous large plaques. This qualitative difference is graphically depicted by plotting the cumulative frequency of sizes for each as shown here, from which quantitative comparisons can be derived.

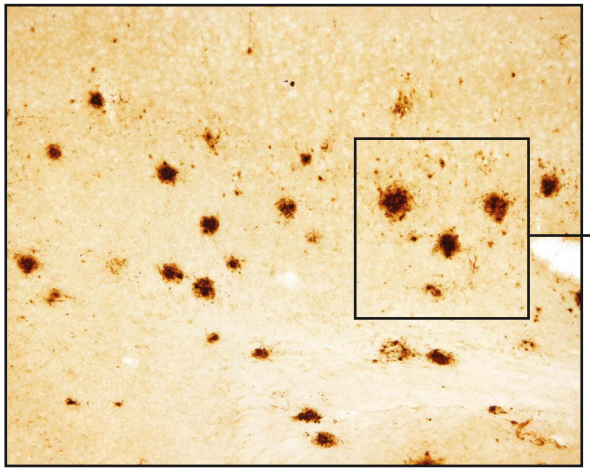




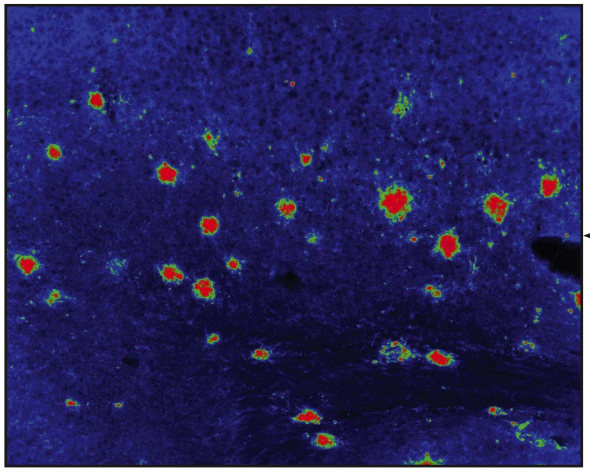
NSA SERVICES: IMAGE ANALYSIS

ALZHEIMER DISEASE PLAQUE QUANTIFICATION

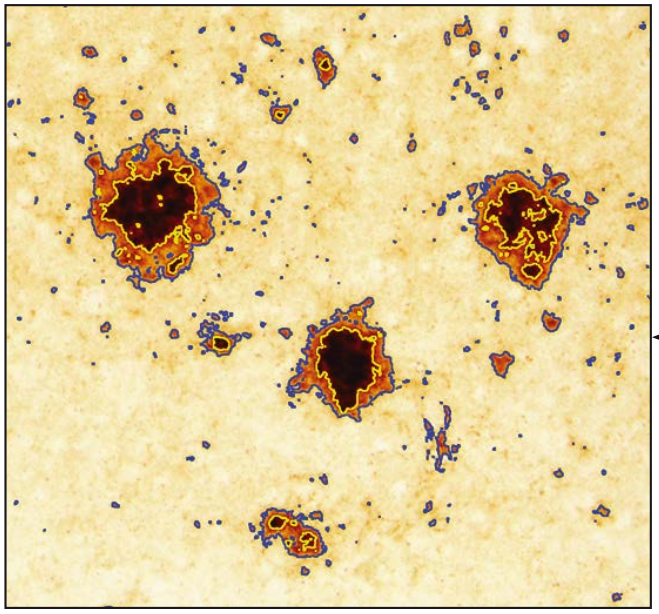
Original Image: A-β (1-42)



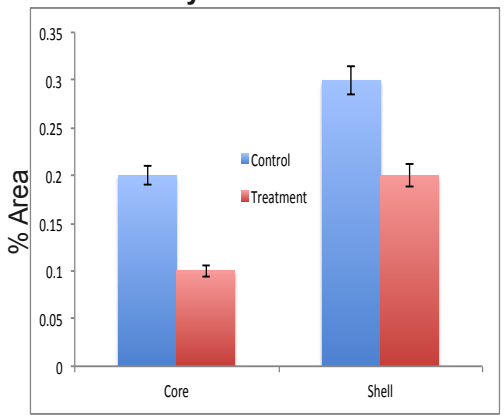
Processed image:



Segmented original:



Densitometry Results



Densitometric parameters may include:

1. Total number of plaques
2. (%) Area occupied by plaques
3. Binning by staining intensity/gradient
4. Binning by size class category

Stains amenable to quantification currently include Campbell-Switzer Alzheimer stain, A-ß(1-40), and A-ß(1-42).

Method: The original image (top) is processed to a false-color representation of the plaques (middle) for aggregate segmentation (bottom).

Using the gradation in staining intensity (green to red in the false color image), individual plaques are outlined (blue outline), and the plaque core may be isolated separately (yellow outline).

Lower Variance, Increased Confidence
MultiBrain® processing minimizes technical variance, thereby increasing your ability to detect treatment or biological effects.

NSA SERVICES: IMAGE ANALYSIS

VOLUMETRIC CALCULATIONS

NSA offers multiple methods for calculating volumes depending on the researcher's budget and data endpoint needs. These techniques include the ellipsoid method and Simpson's Rule method.

The ellipsoid method can be implemented if the area of interest (AOI/ROI) is roughly ellipsoidal in shape. By taking a handful of measurements, a volume estimate can be calculated with ease.

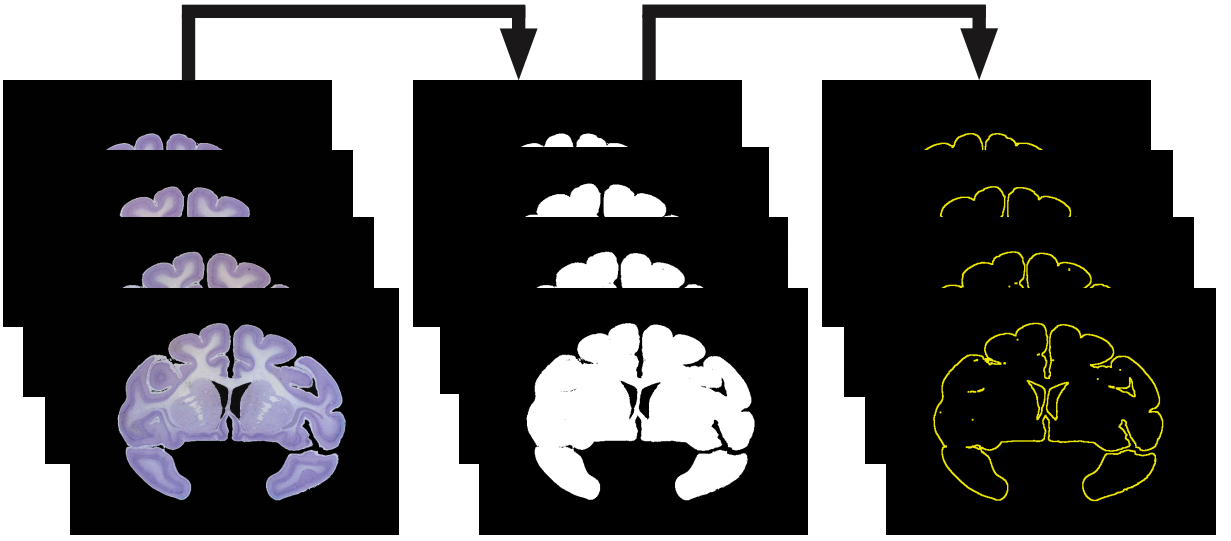
The following formula is used to calculate the volume of ellipsoid-shaped regions of interest:

$$V = \frac{4}{3}\pi \times \frac{1}{2}(a + b + c)$$

Where

V = Volume
a = Height of AOI/ROI
b = Width of AOI/ROI
c = Length of AOI/ROI

The more exact measure of volume calls for using an application such as Image J/FIJI to measure the areas of interest. Eleven (or more but an odd number of) uniformly spaced measures must be made to comply with the application of Simpson's rule. More levels yields greater accuracy. Simpson's rule is a power series used to approximate definite integrals as well as areas under curves.



Original images are taken through a series of processing steps (abbreviated above) to allow for thresholding and area of interest (AOI/ROI) selection. Stained images are converted to binary (black and white) by applying an appropriate thresholding filter. A selection is made from these binary images (shown by the yellow outline in the third column of images). It should be noted that the AOI/ROI selection method is dependent upon the AOI/ROI and the staining of tissue. Each analysis case presents unique challenges regarding tissue and staining, therefore different methods are implemented based on these factors. After AOI/ROI are defined, they are saved and measured (area measurements shown in the bottom right images). These measurements are then processed using Simpson's rule for approximating definite integrals (formula shown below).

Results

	Label	Area
1	Stack0001.tif:Level 1	715.36
2	Stack0001.tif:Level 2	820.34
3	Stack0001.tif:Level 3	885.92
4	Stack0003.tif:Level 4	880.59
5	Stack0004.tif:Level 5	922.19
6	Stack0001.tif:Level 6	958.18
7	Stack0006.tif:Level 7	1030.32
8	Stack0007.tif:Level 8	1074.40
9	Stack0008.tif:Level 9	1076.94
10	Stack0009.tif:Level 10	1143.04
11	Stack0010.tif:Level 11	1103.69
12	Stack0011.tif:Level 12	1157.26
13	Stack0012.tif:Level 13	1171.04
14	Stack0013.tif:Level 14	1192.71
15	Stack0014.tif:Level 15	1194.92
16	Stack0015.tif:Level 16	1165.95
17	Stack0016.tif:Level 17	1187.08
18	Stack0017.tif:Level 18	1212.10
19	Stack0018.tif:Level 19	1158.52

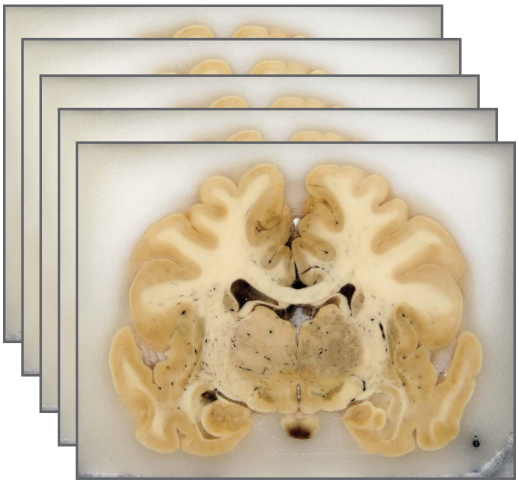
$$\int_a^b f(x)dx \approx (f(x_0) + 4f(x_1) + 2f(x_2) + 4f(x_3) + 2f(x_4) + \cdots$$

$$+2f(x_{n-2}) + 4f(x_{n-1}) + f(x_n))\frac{\Delta x}{3}$$

NSA SERVICES: IMAGE ANALYSIS

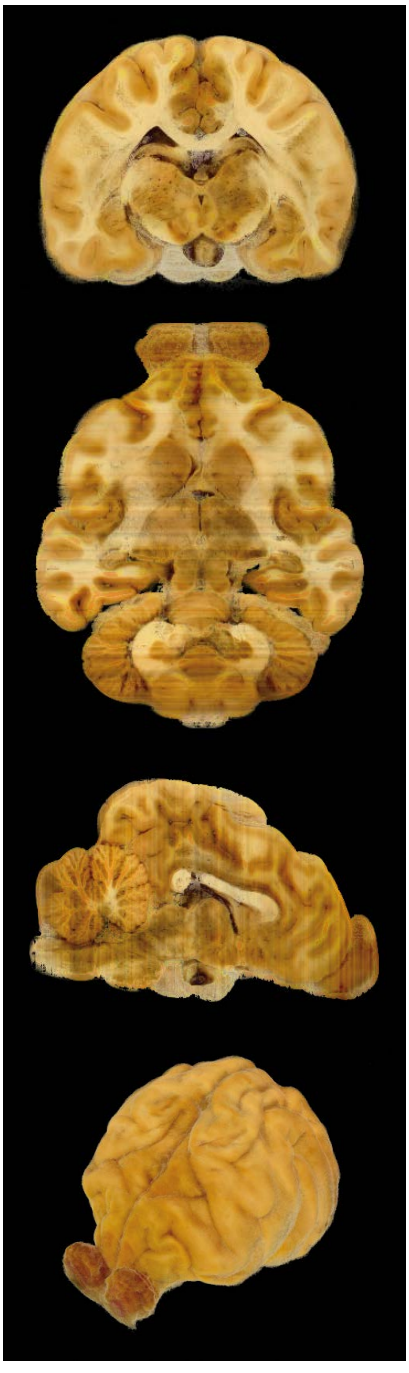
3D RECONSTRUCTIONS

While volume calculation can provide a quantitative endpoint to researchers, having a 3-dimensional volume or surface map rendering of the same tissue that accompanies this data can offer visualization that otherwise might be difficult to picture. This is especially helpful for viewing and comparing smaller portions of tissue in relation to the whole specimen (entire brain or hemisphere), such as tumor or lesion volume reconstructions or renderings of structures within the brain.



Block-face images (shown as an abbreviated series at the top left) are taken during the sectioning process. These images are combined to create a 3D volume rendering of the tissue that can be digitally sliced in any plane (coronal, sagittal, and horizontal shown below).

Block-face



Myelin Stain

In addition to reconstructing tissue from block-face images, stained tissue can also be used to create volume renderings. Stained tissue reconstructions require a higher than normal frequency of staining in order to render a volume with enough resolution for identifying regions of interest.

In the example shown to the right, coronal, sagittal, and horizontal planes for block-face images as well as Weil-Myelin stained tissue of the same grizzly bear brain are shown. The images at the bottom of each column represent volume renderings of the respective column of images.

By using the block-face images as a template, the Weil-Myelin stained tissue can be matched to corresponding block-face images using linear and non-linear transformations. This transformed stack of images can then be rendered as its own volume.

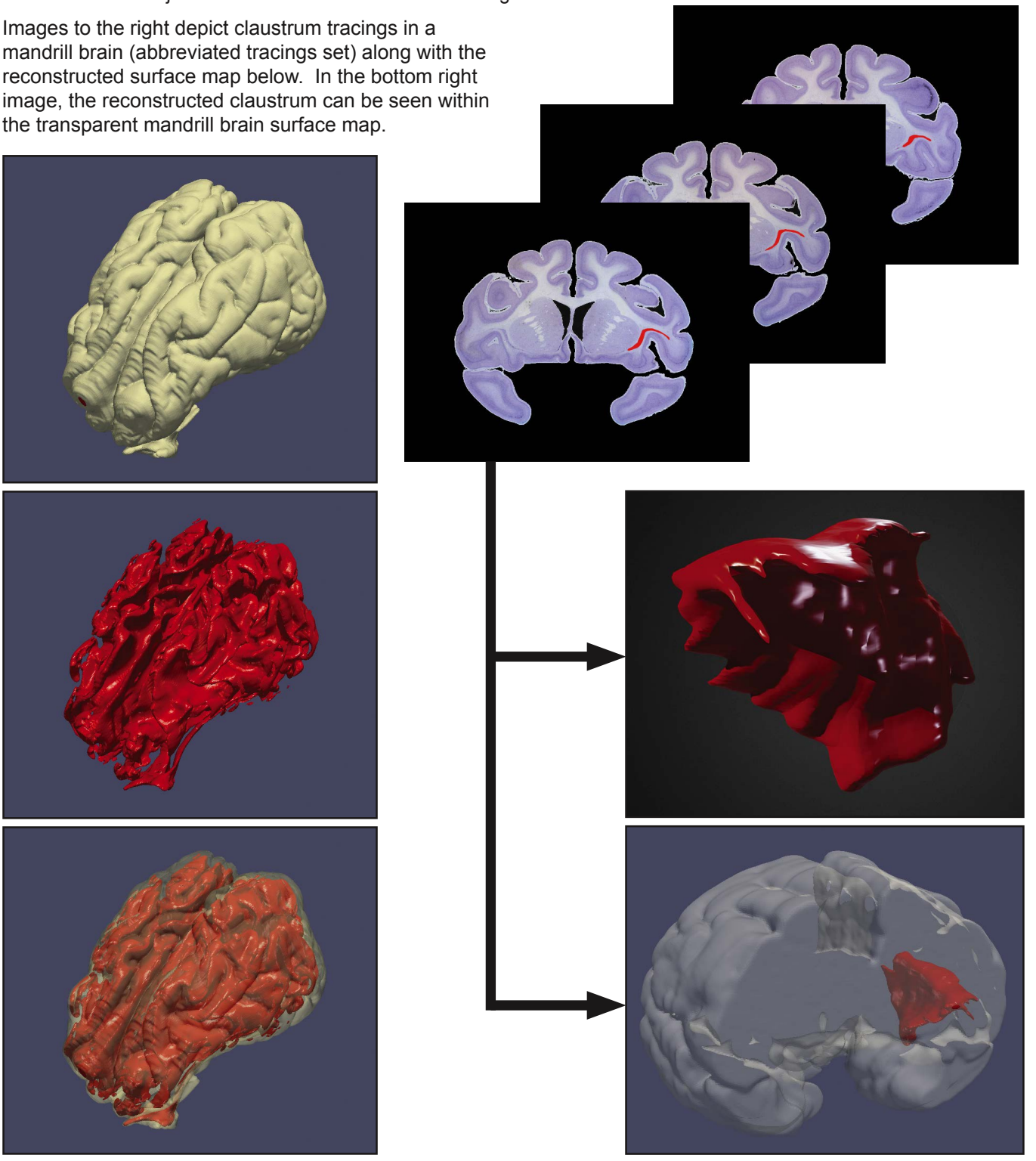
NSA SERVICES: IMAGE ANALYSIS

VOLUME RENDERING

In addition to whole brain or hemisphere reconstructions, segmentation of structures or cell populations within the brain can be performed to render even more informative volumes or surface maps. These renderings can be viewed as part of the whole specimen (striatum rendering within the whole brain volume, for example).This can be especially useful for visualizing and comparing lesions.

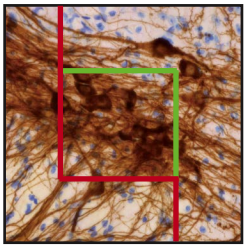
The images below (from top to bottom in the left column) show a surface map rendering of a pig brain, a surface map rendering of the white matter of the same pig brain, and finally a combination of the two renderings. The opacity of the whole brain was adjusted to view the white matter rendering inside.

Images to the right depict claustrum tracings in a mandrill brain (abbreviated tracings set) along with the reconstructed surface map below. In the bottom right image, the reconstructed claustrum can be seen within the transparent mandrill brain surface map.



NSA SERVICES: UNBIASED STEREOLOGY

ACCURATE ANATOMICAL QUANTIFICATION



TH Stain
with Counting Frame

Unbiased stereology provides the reference standard for quantitation of cytoarchitecture; such as **cell number, process length, cell volume, and structure volume**. In providing a statistically accurate assessment of these endpoints, unbiased stereology serves as the “gold standard” when benchmarking other methods, or as the definitive measure in critical studies.

While changes in these measures will capture many experimental effects, perhaps the greatest utility of stereology is the potential to detect the absence of cells following injury, impossible with other methods; i.e. in chronic studies where weeks pass after elements of damage have been removed or disappear.

Plan:

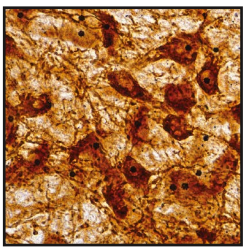
While the execution of stereological studies can seem daunting, it’s very efficient with appropriate care in study design. NSA will help you **identify and control your histology variables** so you can proceed with confidence. This includes the anatomical boundaries of your AOI/ROI, specimen number, section thickness, number of sections, and the number and area of sampling fields required to meet rigorous statistical requirements.

Process:

The fundamental advantage of MultiBrain® and MultiCord® technology for stereological studies follows from the analytical efficiency of the MultiBrain® layout (**up to 40X specimens per slide**), consistency of staining and reliable tissue thickness, and exhaustive sectioning. Additionally, by increasing throughput and lowering costs per section, a project can increase specimen number and **reduce the greatest source of variance** in counting; inter-specimen/biological variation.

Execute:

Count neurons faster with nucleolar staining using the razor sharp AgNOR silver stain**,** which increases decision speed while reducing fatigue error at the each critical step of identifying neurons. AgNOR may further reduce counting time by allowing lower power objective and/or numerical aperture. Similarly, quickly **identify and quantify specific cell types** and features with immunohistochemical labeling (e.g. tyrosine hydroxylase or NeuN), optimized for contrast to resolve cellular or subcellular boundaries. Also of note, **reduced slide/section sorting and searching** via MultiBrain® layout drastically improves efficiency.



AgNOR Stain

Outsource:

NSA partners closely with trusted contract service providers for exceptional quality and turnaround times.

APPLYING NSA SERVICES IN RESEARCH

DISEASE RESEARCH OVERVIEW AND TABLE OF STAINS

The table below depicts some of the most commonly used stains for detecting various categories of markers in Alzheimer (AD), Parkinson (PD), Multiple Sclerosis (MS), Amyotrophic Lateral Sclerosis (ALS), Huntington (HD) and Stroke research.

Stain	AD	PD	MS	ALS	HD	Stroke
4H7H7 Huntington Disease Aggregates					AG	
6E10 β-Amyloid (1-17)	A					
82E1 β-Amyloid (1-16)	A					
AChE	cN	cN	cN	cN	cN	cN
Amino CuAg	Dg	Dg	Dg	Dg	Dg	Dg
ApoTag 4	CD	CD	CD	CD	CD	CD
Aβ (1-40)	A					
Aβ (1-42)	A					
α-Synuclein		cN				
α-Synuclein (pSer129)		cN				
α-Synuclein-Human 4B12 (103-108)		cN				
α-Synuclein 211 (121-125)		cN				
Autometallography	MetD	MetD	MetD	MetD	MetD	MetD
c-Fos	act	act	act	act	act	act
Calbindin	cN	cN	cN	cN	cN	cN
Calretinin	cN	cN	cN	cN	cN	cN
Campbell-Switzer	A, pT					
Caspase-3-activated	CD	CD	CD	CD	CD	CD
Caspase-9-activated	CD	CD	CD	CD	CD	CD
CD11b	G	G	G	G	G	G
CD68	rG	rG	rG	rG	rG	rG
ChAT	cN	cN	cN	cN	cN	cN
Congo Red	A, pT					
CuAg	Dg	Dg	Dg	Dg	Dg	Dg
DARPP32		cN				
EM-48					AG	
Ferritin	cN	cN	cN	cN	cN	cN
GABA						cN
GFAP	Ast	Ast	Ast	Ast	Ast	Ast
Glutamate					cN	
H&E	cN	cN	cN	cN	cN	cN
Human Nuclear Protein (HuNu)	cN	cN	cN	cN	cN	cN
Huntingtin					cN	
Iba1	G	G	G	G	G	G
Ischemia Contrast			pInt			pInt
Ki-67	iN	iN	iN	iN	iN	iN
Nestin	rAst	rAst	rAst	rAst	rAst	rAst
NeuN	cN	cN	cN	cN	cN	cN
Nissl	cN	cN	cN	cN	cN	cN
Parvalbumin						cN
Perls	MetD	MetD	MetD	MetD	MetD	MetD
PSA-NCAM	iN	iN	iN	iN	iN	iN
Reactive Microglia	rG	rG	rG	rG	rG	rG
S100 beta	Ast	Ast	Ast	Ast	Ast	Ast
Serotonin	cN	cN	cN	cN	cN	cN
Serotonin Transporter	cN	cN	cN	cN	cN	cN
SMI-99 myelin basic protein	myl	myl	myl	myl	myl	myl
Solochrome	myl	myl	myl	myl	myl	myl
Somatostatin	cN	cN	cN	cN	cN	cN
Substance P	cN	cN	cN	cN	cN	cN
Synaptophysin	cN	cN	cN	cN	cN	cN
Tau 39E10 (189-205)	pT					
Tau 46 (403-441)	pT					
Tau 5 (210-230)	pT					
Tau AT100 (pSer212/pSer214)	pT					
Tau AT180 (pThr231; PHF-6)	pT					
Tau AT181 (pThr181)	pT					
Tau AT8 (pSer202,pThr205)	pT					
Tau CP-13 (pSer202)	pT					
Tau HT7 (159-163)	pT					
Tau MC1 (312-322)	pT					
Tau PHF-1 (pSer396,404)	pT					
Tau PHF-13 (pSer396)	pT					
Tau (pSer422)	pT					
Thioflavin S	pT	pT				
Tryptophan Hydroxylase		cN				
Tyrosine Hydroxylase		cN				
VAChT				cN		
Weil-Myelin	myl	myl	myl	myl	myl	myl

Code Legend:

A = amyloid
act = activity marker
AG = aggregates
Ast = astrocytes
CD = cell deat
cN = chemo-specific neuron
Dg = Degeneration
G = microglia
iN = immature Neurons
MetD = Metal Detection
myl = myelin
pInt = physical integrity
pT = phosphorylated Tau- tangles
rAst = reactive Astrocytes
rG= reactive marker

APPLYING NSA SERVICES IN RESEARCH

ALZHEIMER DISEASE

See Table of Stains appropriate for AD page 34

See pages 27-29 for a comprehensive explanation of our IMAGE ANALYSIS SERVICES and how they can benefit your ALZHEIMER DISEASE STUDY.

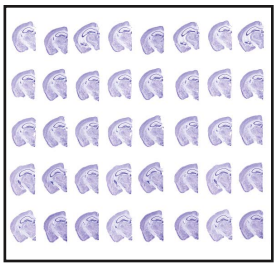
Overview

The hallmark pathological features of AD in humans are amyloid plaques and tau abnormalities. An increasing number of genetically engineered mouse models for AD research have been developed to exhibit classic Alzheimer features. The time line for development of disease features ranges from a few months to ~18 months. NSA has processed hundreds of human AD tissues and tens of thousands of brains from AD mouse models.

Mouse Tissue:

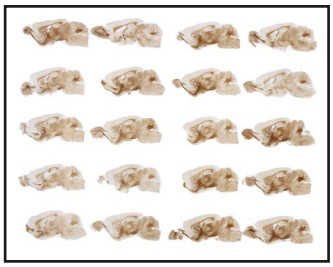
When processing mouse brain hemispheres coronally, NSA embeds up to 40 in each MultiBrain® Block (#1); when processing hemisphere or intact brains sagittally, 20 (#2) entire brains are embedded (25 if the area of interest is the cortex/cerebrum). The preference of the researcher determines which alignment is chosen.

#1 Mouse Brains Hemisphere Coronal



40 mouse brain hemispheres are co-embedded and appear on each MultiBrain® slide section.

#2 Mouse Brains Sagittal

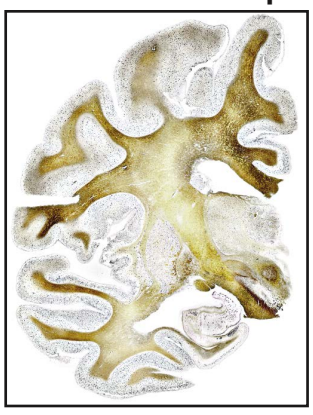


20 mouse brains are co-embedded and appear on each MultiBrain® slide section (25 mouse brains if the area of interest is only cortex / cerebrum).

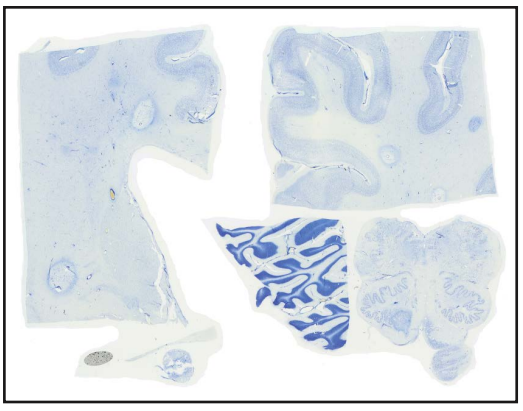
Human Tissue:

NSA processes large format sections, as seen below, providing a unique opportunity to assess large contiguous cross-sections of tissue. NSA also processes multiple smaller samples from one or more brains using MultiBrain® Technology. The standard NSA practice of encasing the brain tissue in gelatin provides a significant aid in the handling of tissue sections resulting in an improved final product.

Human Brain Hemisphere



Multiple Embedded Human Brain Tissues



APPLYING NSA SERVICES IN RESEARCH

ALZHEIMER DISEASE (continued)
AMYLOID PLAQUES AND TAU ABNORMALITIES

The hallmark features of AD are amyloid plaques and tau abnormalities. Amyloid-laden plaques exist as a diffuse form and as a more dense/mature form (congophilic). Amyloid is also found as deposits in vessels. Tau abnormalities are present in cell bodies and in the neuropil as “neuropil threads.”

The choice of which stain(s) to use is based on the specific needs of the study. The Campbell-Switzer Alzheimer pathology stain, developed by NSA, shows all of the hallmark features of Alzheimer pathology, while other antibody methods reveal limited, specific features. Please note that the Campbell-Switzer stain does not show this pathology on monkey tissue.

Strategic Approach to Amyloid Detection: Different approaches yield unique features
At specific times during an R&D cycle, different approaches to amyloid detection may be most useful. Each of the methods described has its own strengths and specialized advantage.

Campbell-Switzer Method: Dr. Bob Switzer and his former associate, Shannon Campbell, developed the Campbell-Switzer Alzheimer Pathology stain in the early 1980’s. Over the years, this unique stain has become a “work horse” tool for researchers, particularly in the earlier phases of R&D. Diffuse plaques are stained black and the denser fibrillar amyloid forms are stained amber. Tau abnormalities typically stain black.

Immunohistochemistry for Amyloid Plaque Detection: The antibody methods are intended to be very specific and reveal amyloid peptides that have been cleaved at a specific location. Based on a particular mouse model, treatment approach and other factors, a specific antibody is more useful than others in revealing a specific measure of efficacy.

Examples of different antibodies related to AD are shown in the next several pages.

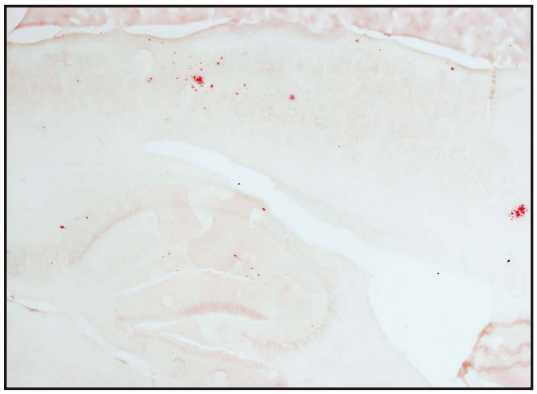
Congo Red: Congo Red specifically reveals the denser, fibrillar amyloid plaques (hence, congophilic plaques). While the fibrillar amyloid cores stain red, other entities in the sections can also be stained. To distinguish amyloid staining from other staining, the sections can be viewed with polarized microscopy. Fibrillar amyloid stained with Congo Red is birefringent, so when viewed with crossed polarizers a ‘cross’ will be observed with one arm being red and the other ‘apple green’—often termed a ‘maltese cross’. Paired helical filaments that form the tangles in neurons will also display this phenomenon. The birefringence occurs due to the congo red molecules binding in an orderly way on the beta pleated sheet-like configuration of the fibrillar amyloid and the paired helical filaments.

Thioflavin S: This stain, when bound to beta pleated sheet-like configurations of the amyloid and paired helical filaments, will fluoresce a yellow-green. This stain is a useful option if double staining with fluorescent markers are being used.

APPLYING NSA SERVICES IN RESEARCH

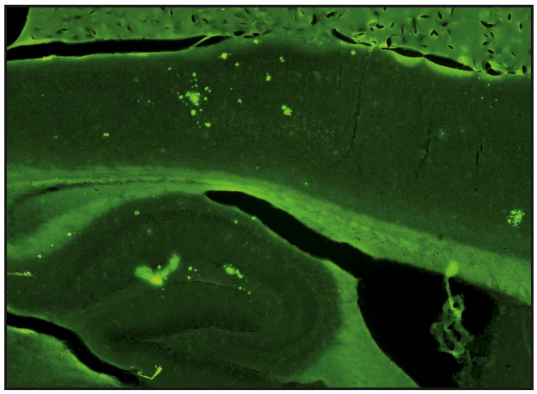
ALZHEIMER DISEASE (continued)
AMYLOID PLAQUES

The following images were acquired by applying five common techniques to adjacently cut sections of the same Tg2576 mouse brain:

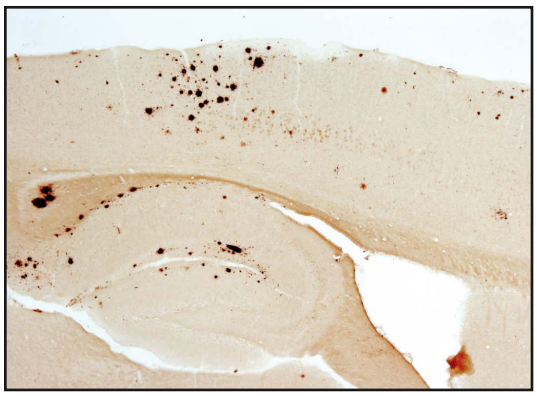


Congo Red Stain

Congo Red and Thioflavin S reveal only fibrillar amyloid (dense-core, congophilic) plaques. If the goal is to detect ONLY congophilic plaques, Congo Red and Thioflavin S are good staining choices.

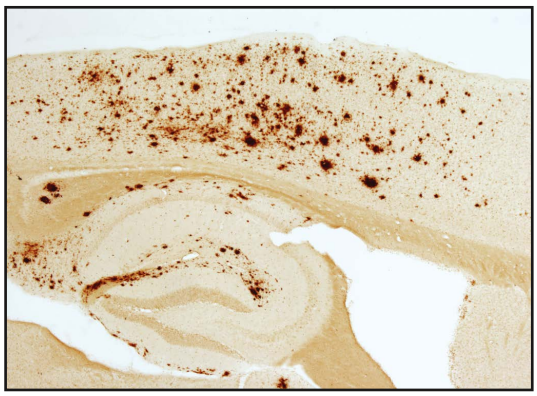


Thioflavin S Stain

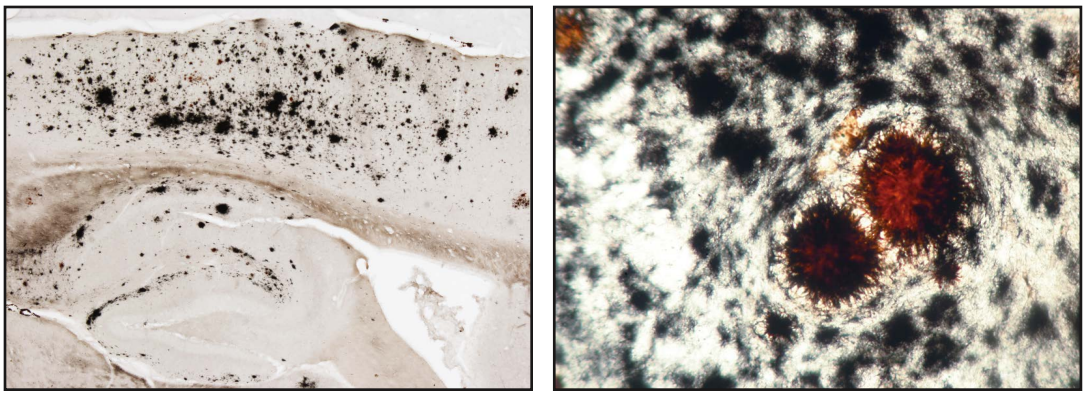


Aβ 1-40 IHC

Aβ 1-40 IHC displays a broader range of amyloid than the congophilic markers shown above. Aβ 1-42 IHC displays a broader range of features than Aβ 1-40.



Aβ 1-42 IHC

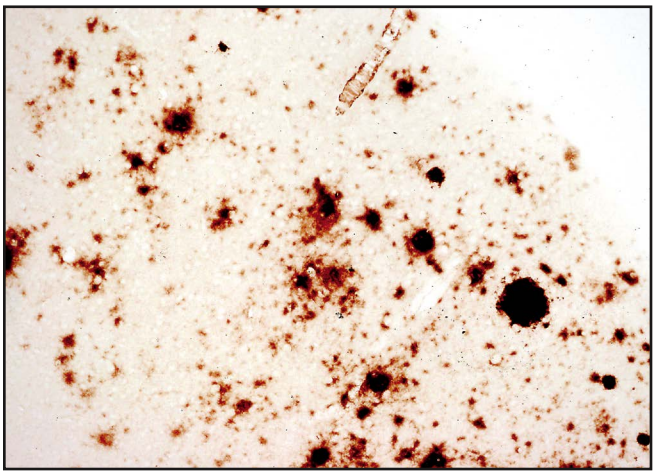


Campbell-Switzer Alzheimer Pathology Stain

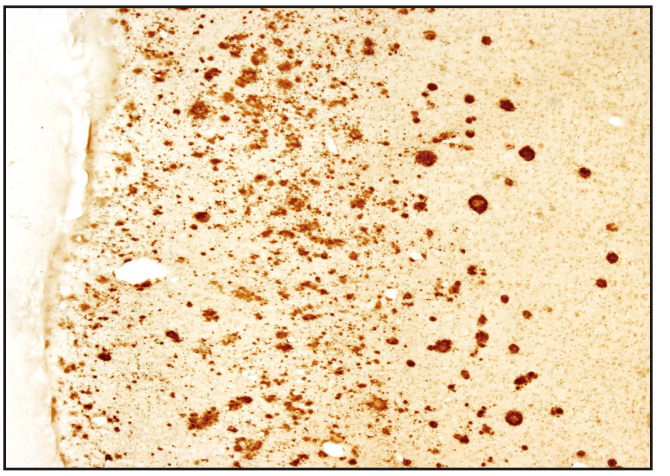
Alternatively, the Campbell-Switzer method stains the broadest range of amyloid while allowing for differentiation between congophilic and diffuse plaques: The Campbell-Switzer method appears to reveal a similar level of amyloid as Aβ 1-42 IHC. However, as can be seen in the close-up, the congophilic plaques stain a different color (amber) vs. the diffuse plaques (black), whereas with the Aβ 1-42 IHC method, both diffuse and congophilic appear the same color. The high-contrast images resulting from this stain are ideally suited for densitometric analysis of plaque loads (more details on pages 27-29).

APPLYING NSA SERVICES IN RESEARCH

ALZHEIMER DISEASE (continued)
AMYLOID PLAQUES AND TAU ABNORMALITIES

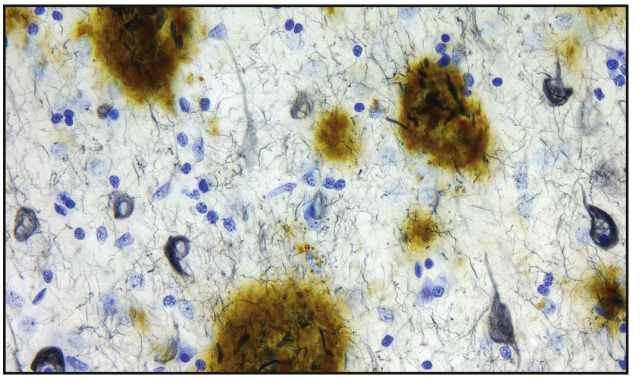


6E10 Mouse Alzheimer Model



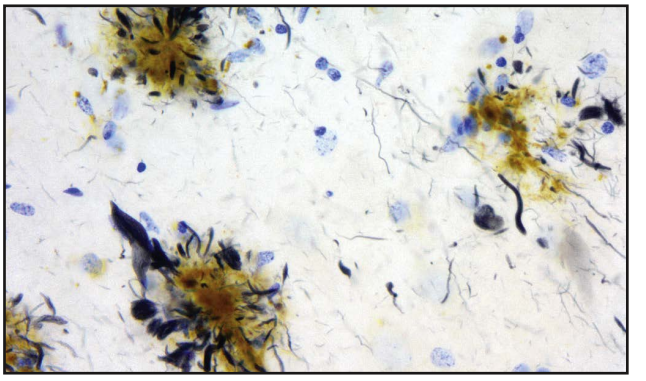
Aβ 1-42 Human Alzheimer

Aβ 1-28 IHC



Early-onset, familial Alzheimer disease in temporal lobe cortex

The pathology is characterized by few neurons with tangles (black circular to tear-drop shapes) in hippocampus (right) while abundant in temporal lobe.

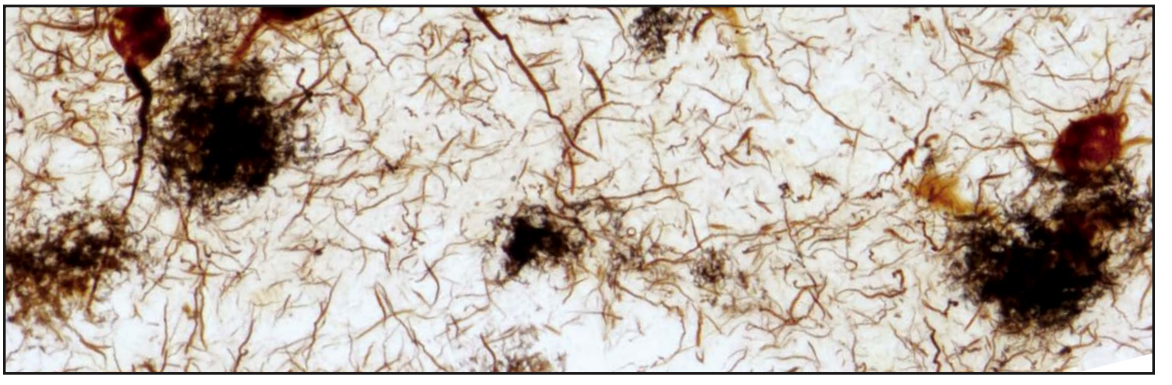


Neuritic plaques (amber-brown) revealed by Aß 1-28 immunohistochemistry in the hippocampus

Blue = Nissl substance (RNA) in cell bodies of neurons and glia. Black = Tau abnormalities and neuropil threads; Gallyas silver method.

Aβ 1-28 IHC Images courtesy of Dr. Alex Osmand, University of Tennessee, Knoxville

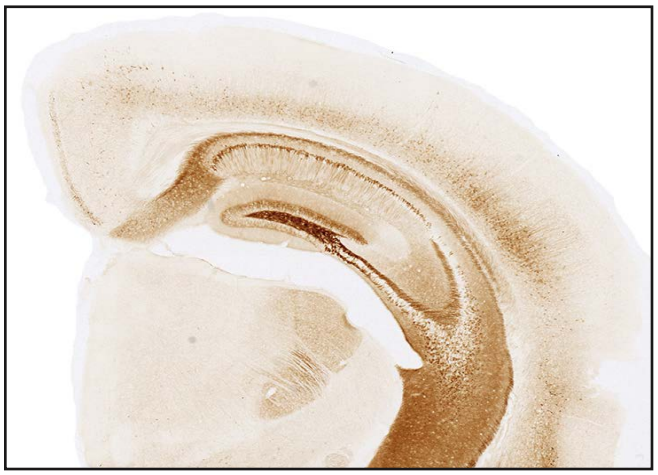
Campbell-Switzer Alzheimer Pathology Stain (Human)



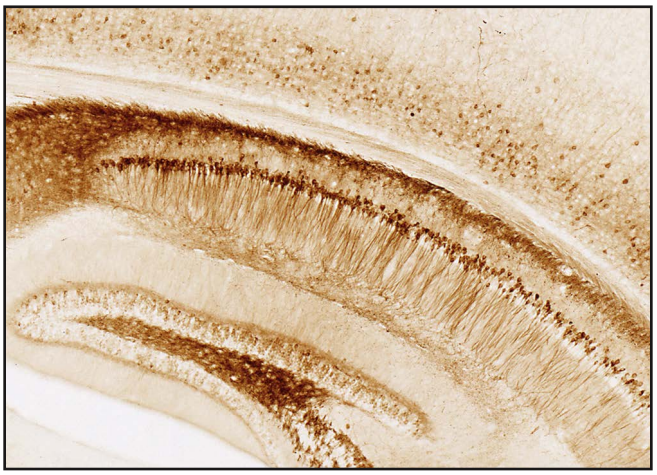
Neuritic Plaques and Tau abnormalities in Striatum

APPLYING NSA SERVICES IN RESEARCH

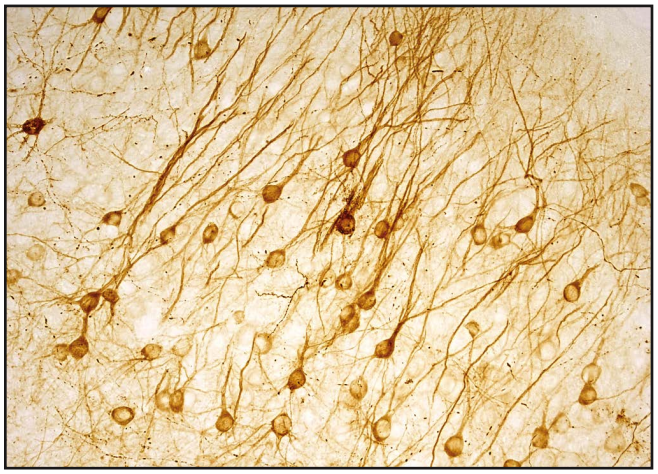
ALZHEIMER DISEASE (continued)
TAU ABNORMALITIES



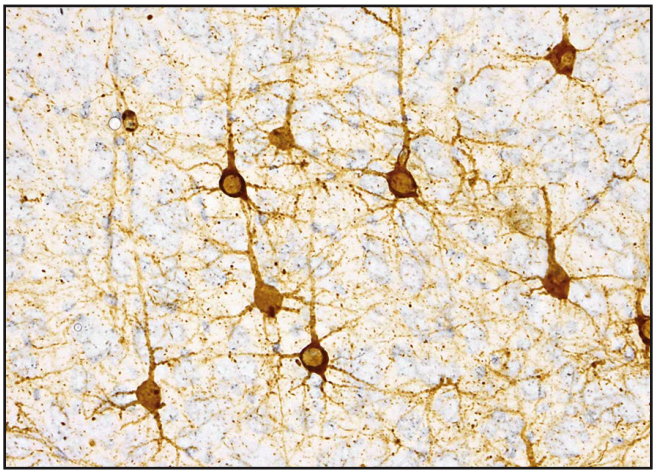
Anti-Tau HT7 (Epitope 158-163)
Mouse P301L AD Model



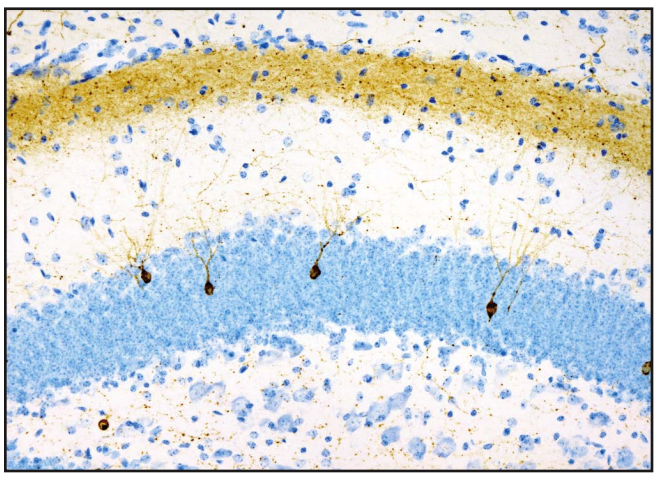
Anti-Tau HT7 (Epitope 158-163)
Mouse P301L AD Model, 10x



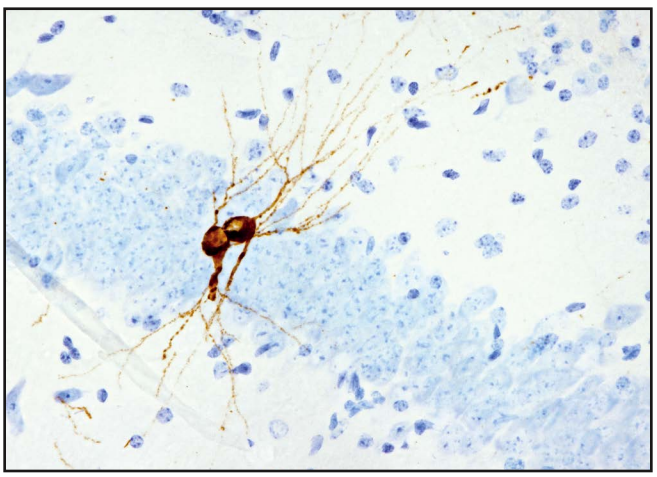
AT8 (pSer202 + Thr205)
Surface Cells, P301L Mouse Cortex, 20x



AT8 (pSer202 + Thr205)
Unspecified AD Mouse Model, Cortex, 20x



AT8 (pSer202 + Thr205)
Unspecified AD Mouse Model, Hippocampus, 20x

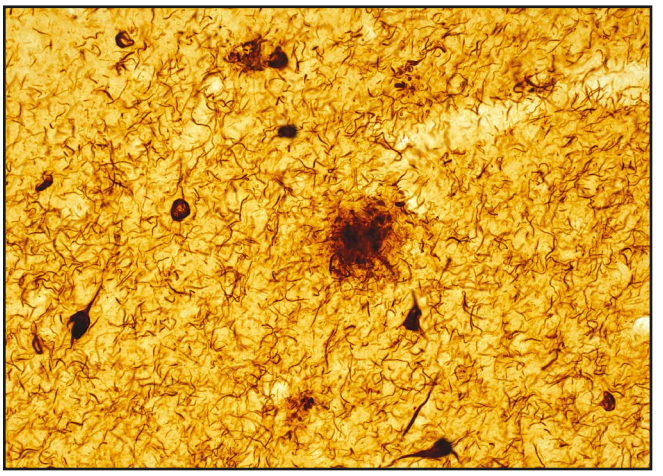


AT8 (pSer202 + Thr205)
Unspecified AD Mouse Model, Hippocampus, 40x

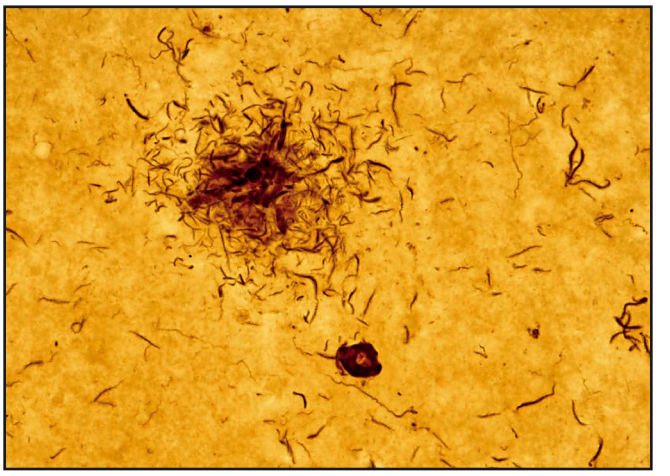
APPLYING NSA SERVICES IN RESEARCH

ALZHEIMER DISEASE

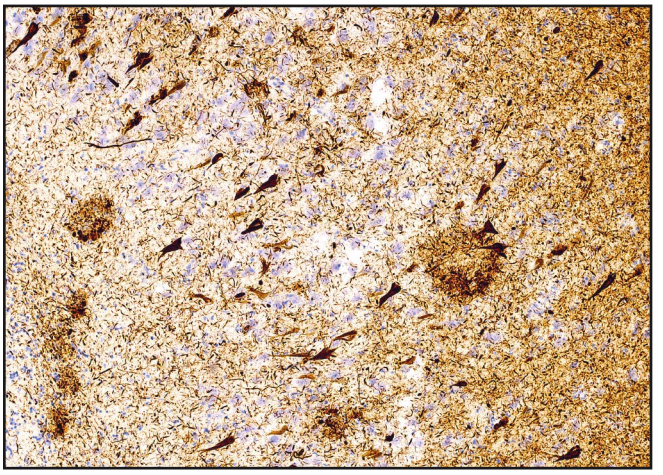
TAU ABNORMALITIES (continued)



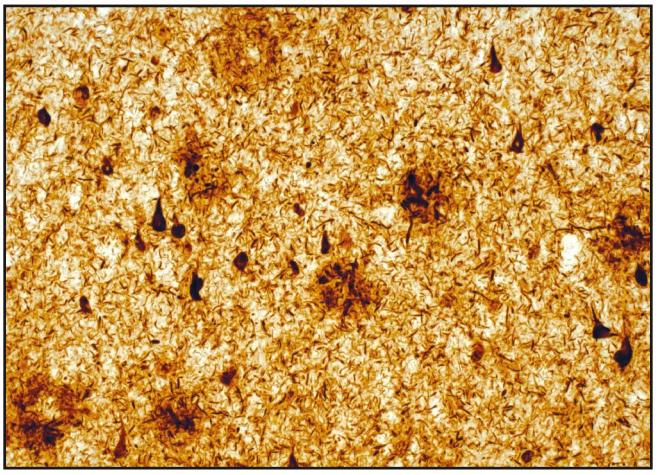
Anti-Human Tau (Epitope 243-441)
Human AD Cortex, 20x



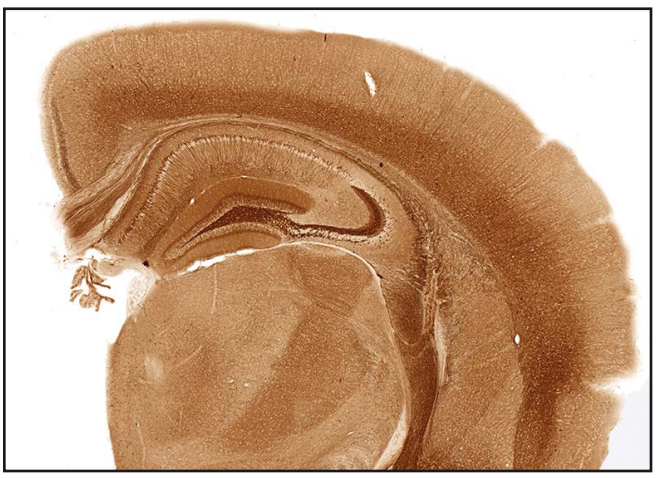
Anti-Human Tau (Epitope 243-441)
Magnified to show neuritic plaque and Tau abnormalities, 40x



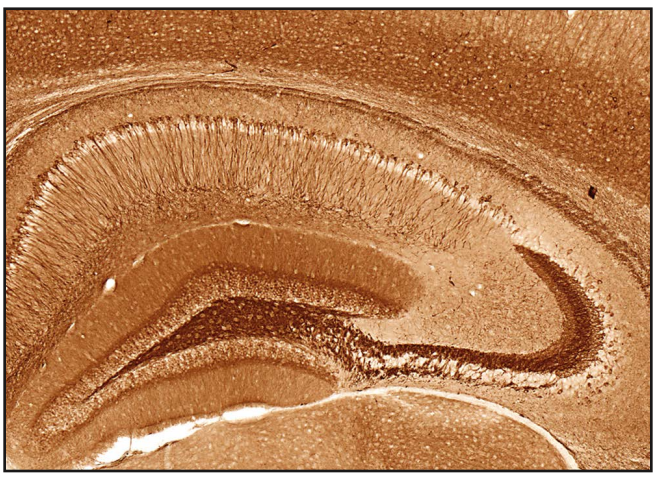
Tau 46 (Epitope 404-441)
Human AD Cortex, 10x



Tau 46 (Epitope 404-441)
Human AD Cortex, Magnified to show neuritic plaque and Tau abnormalities, 20x



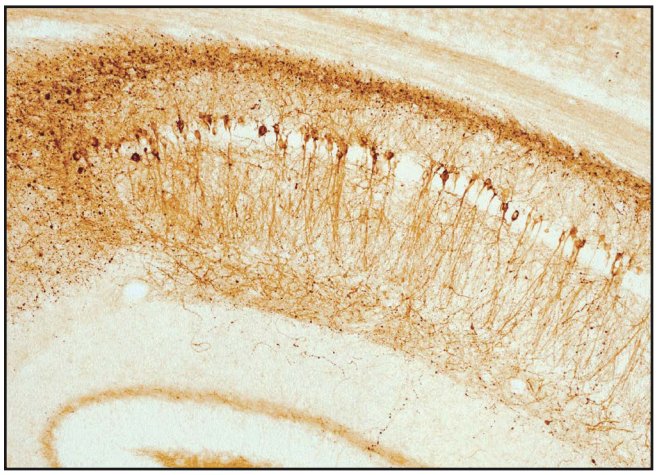
TAU 46 (Epitope 404-441)
Mouse P301L AD Model, Hemisphere



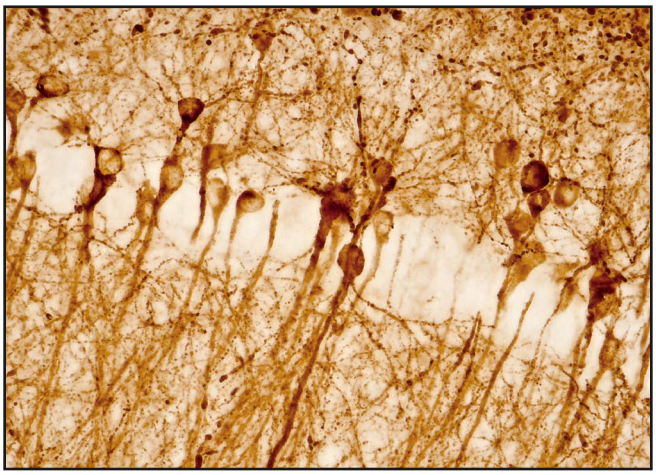
Tau 46 (Epitope 404-441)
Mouse P301L AD Model, Hippocampus

APPLYING NSA SERVICES IN RESEARCH

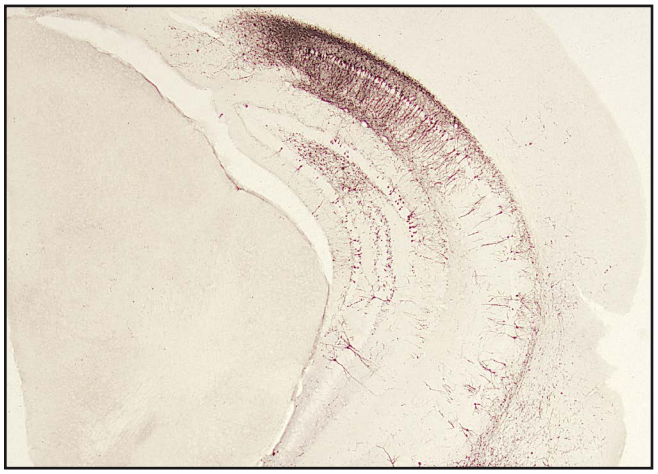
ALZHEIMER DISEASE
TAU ABNORMALITIES (continued)



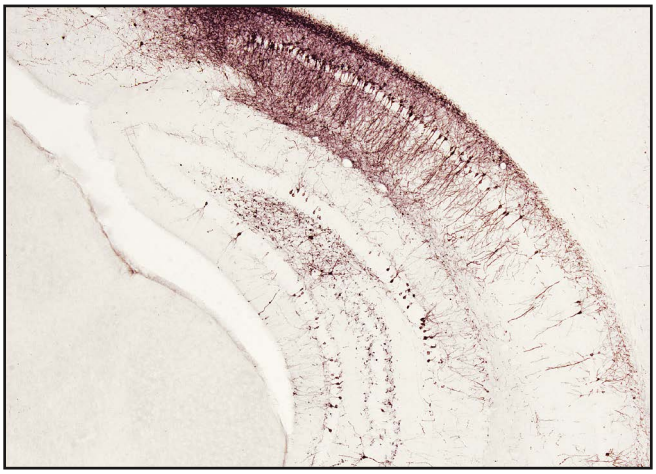
Tau pSer396
p301L Mouse Model, Cortex and Hippocampus, 10x



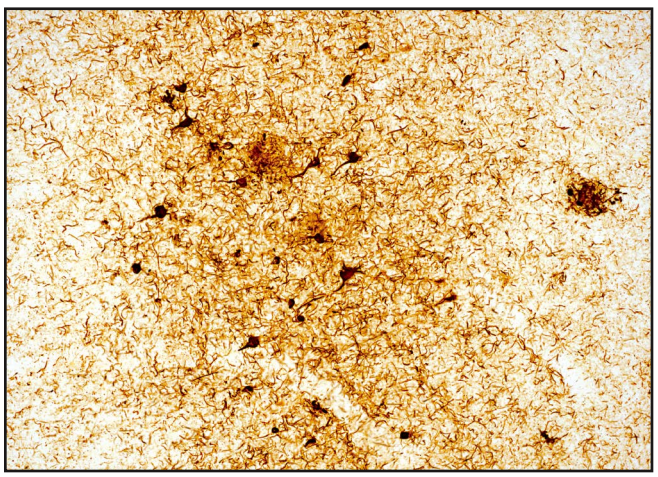
Tau pSer396
p301L Mouse Model, Hippocampus, 40x



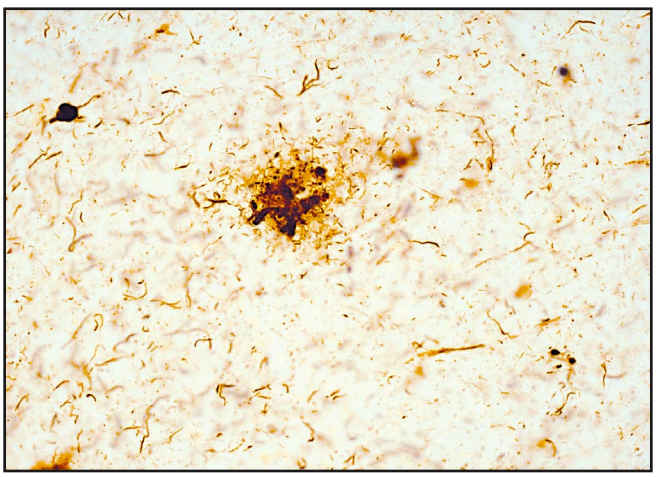
Tau pSer422
p301L Mouse Model, Cortex and Hippocampus, 2x



Tau pSer422
p301L Mouse Model, Cortex and Hippocampus, 4x



Tau pSer422
Human Cortex, 10x



Tau pSer422
Human AD Cortex, 20x

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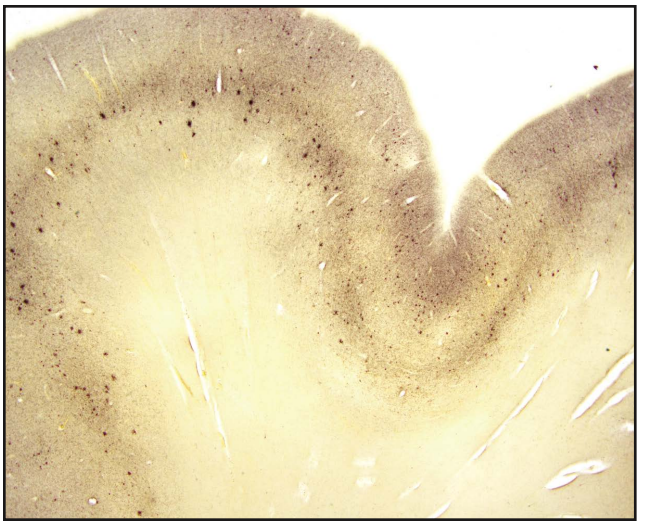
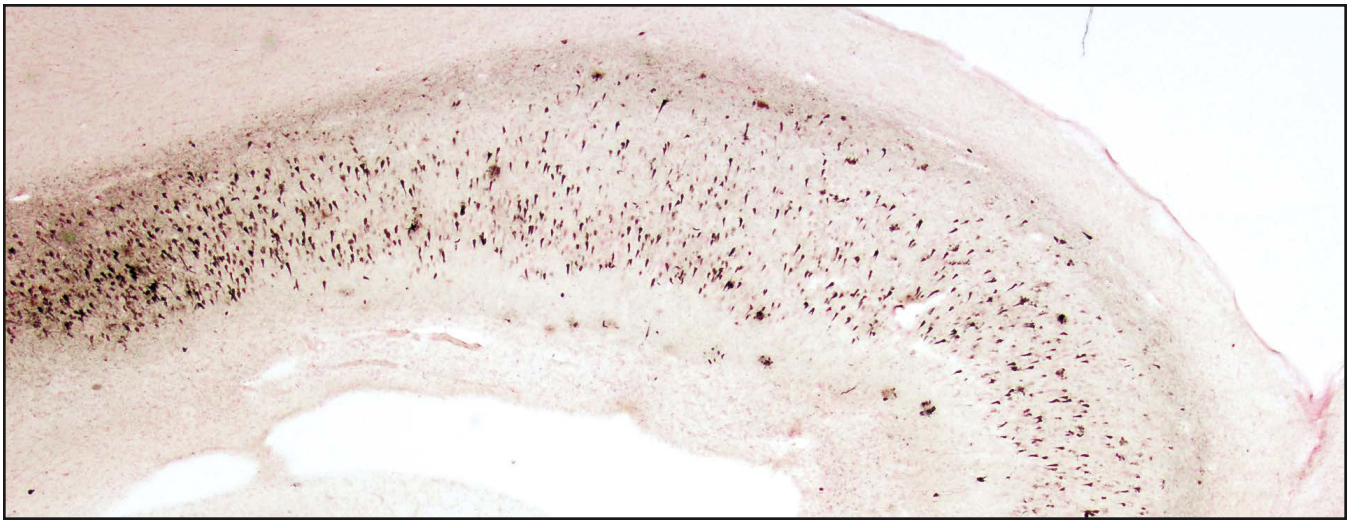
ALZHEIMER DISEASE
TAU ABNORMALITIES (continued)



Tau pThr181
Human AD Cortex, 1x

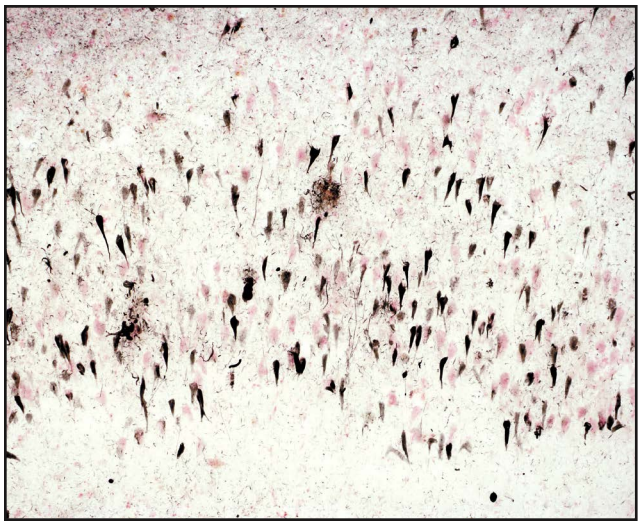
Tau pThr181
Human Cortex, 10x



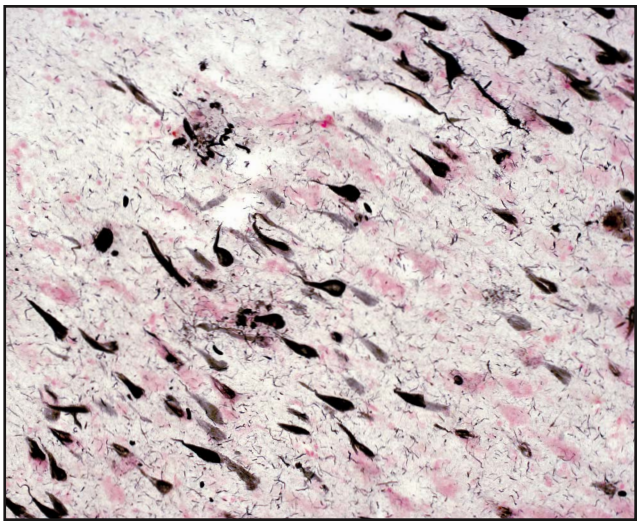
Gallyas Silver Method for revealing Tau abnormalities
Human AD Hippocampus 2x

APPLYING NSA SERVICES IN RESEARCH

ALZHEIMER DISEASE
TAU ABNORMALITIES (continued)

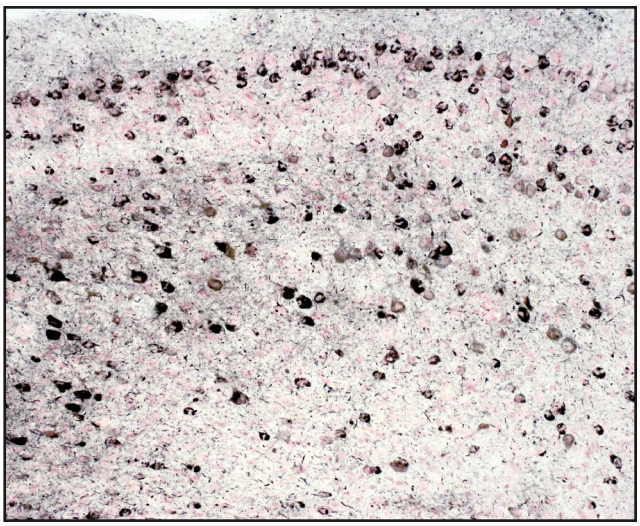


Gallyas Silver Method
Human AD Hippocampus 10x



Gallyas Silver Method
Human AD Hippocampus 20x

Gallyas Silver Method
Mouse Cortex 10x



Gallyas Silver Method
Mouse Cortex 20x

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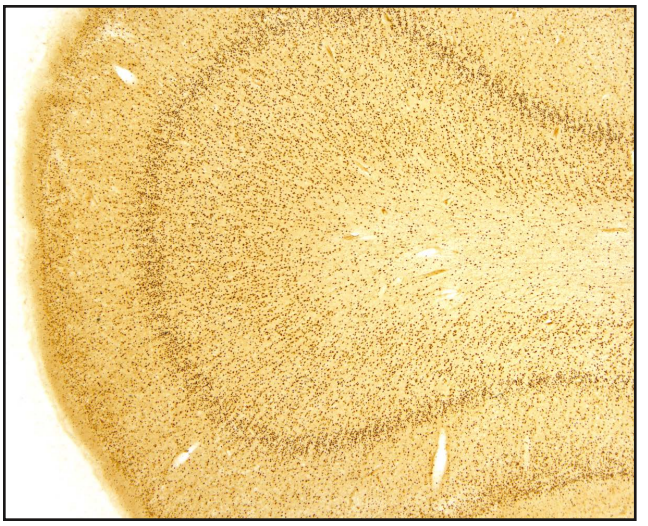
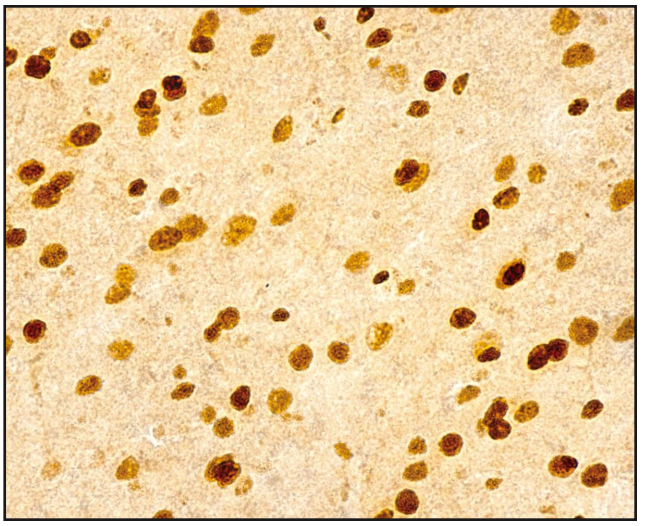
ALZHEIMER DISEASE (continued)
OLIGOMERS

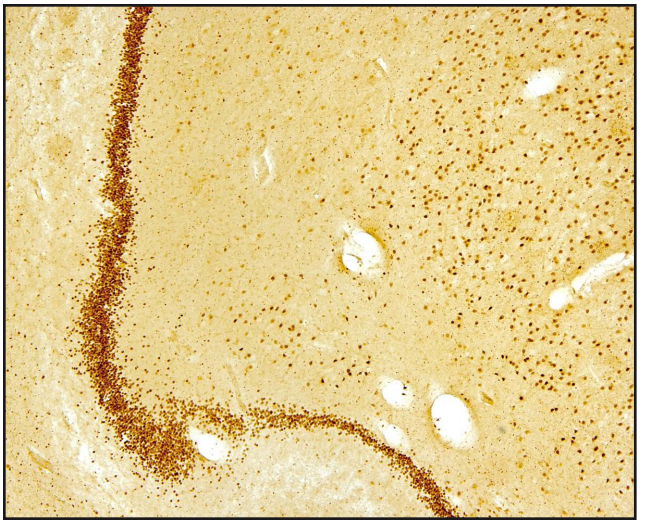
Beta Amyloid Oligomers



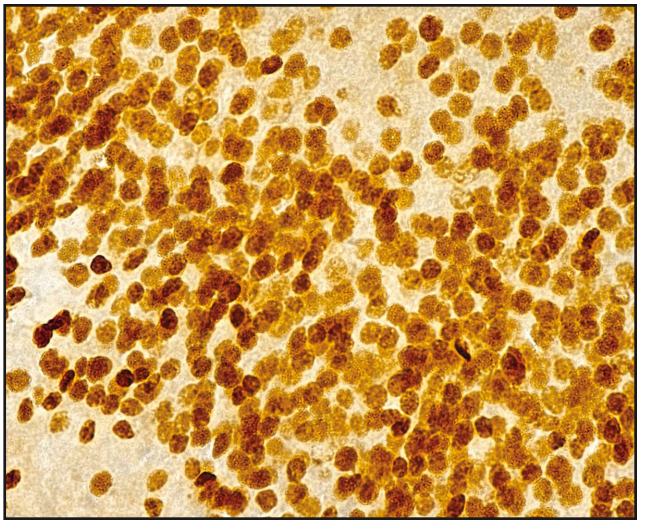
Amyloid Oligomer A11
Human AD Cortex, 2x



Amyloid Oligomer A11
Human AD Cortex, 40x



Amyloid Oligomer A11
Human AD Dentate-Gyrus, 4x

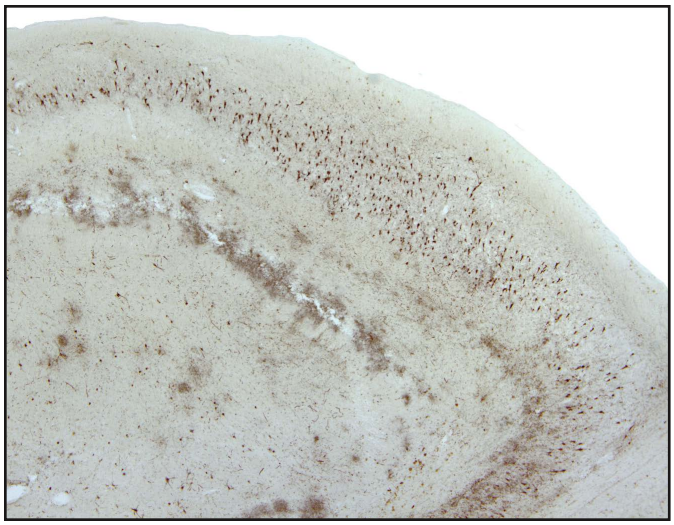


Amyloid Oligomer A11
Human AD Dentate-Gyrus, 40x

APPLYING NSA SERVICES IN RESEARCH

ALZHEIMER DISEASE (continued)
TAU OLIGOMERS

Tau Oligomers



Anti-Tau T22 Tau oligomers, Human AD cortex, 2x

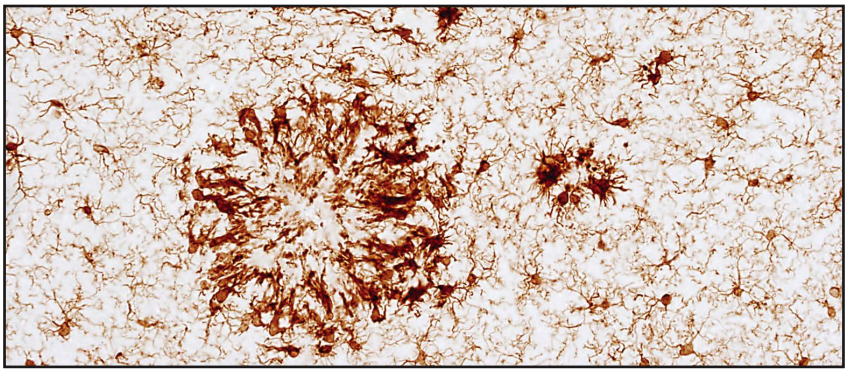
Anti-T22 Tau oligomers in human AD cortex shown at higher magnification in neuron cell bodies and dendrites. 20x

Anti-T22 Tau oligomers display neurites and tau abnormalities, Human AD cortex, 10x

APPLYING NSA SERVICES IN RESEARCH

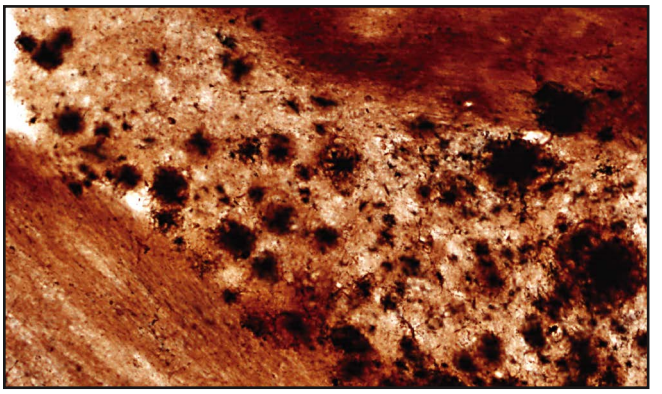
ALZHEIMER DISEASE (continued)
INFLAMMATION

Inflammation occurs in conjunction with the hallmark pathologic features of AD and can be detected by examining microglia (Iba1 IHC) or astrocytes (GFAP IHC).

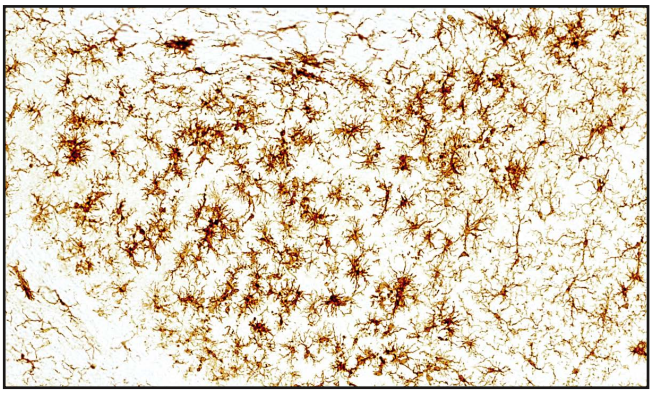


Iba1 Immunoreactive Microglia: Alzheimer Disease Mouse Model

Alzheimer Mouse Model Plaque Chemoarchitecture

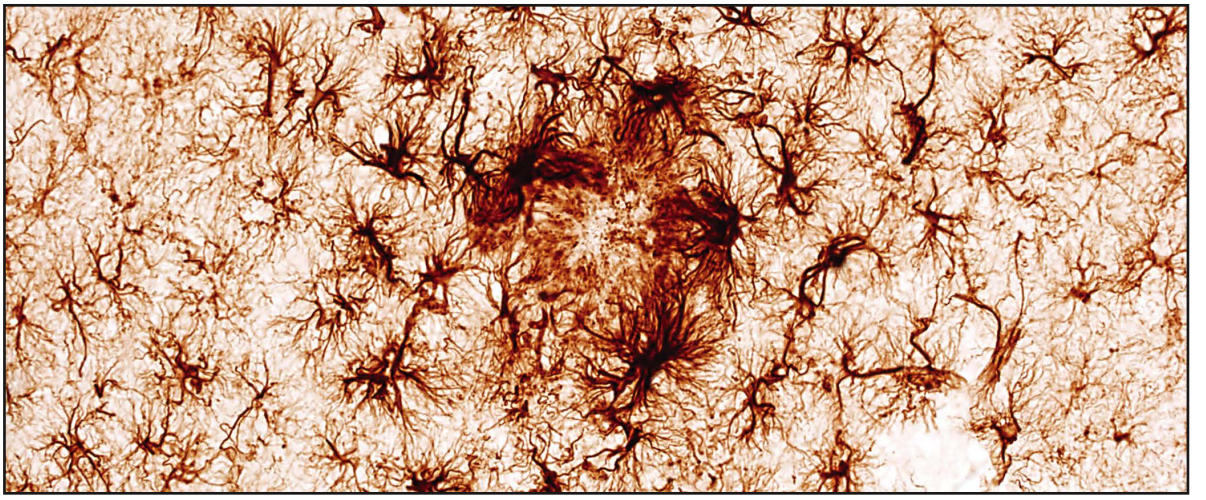


Campbell-Switzer Stain



Microglia-Iba1 Antibody Stain

The commonly high density of plaques found in the subiculum is shown. Co-located with the plaques, shown by the Campbell-Switzer method, are reactive microglia (Iba1 immunohistochemistry). The reactive microglia are more evenly distributed, rather than clustered tightly around particular plaques.

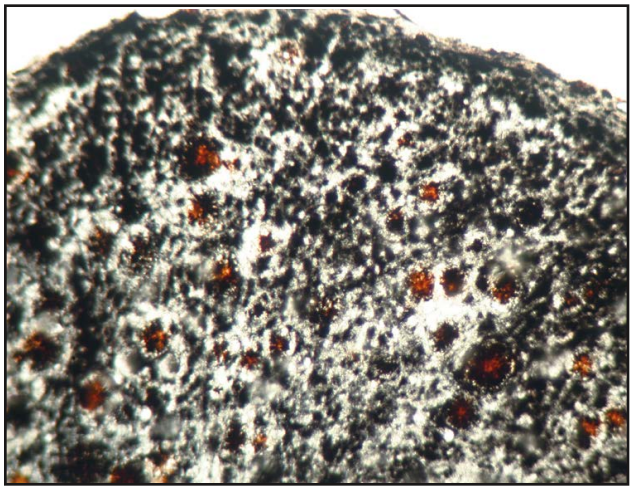


GFAP Immunoreactive Hypertrophic Astrocytes Around Neuritic Plaque

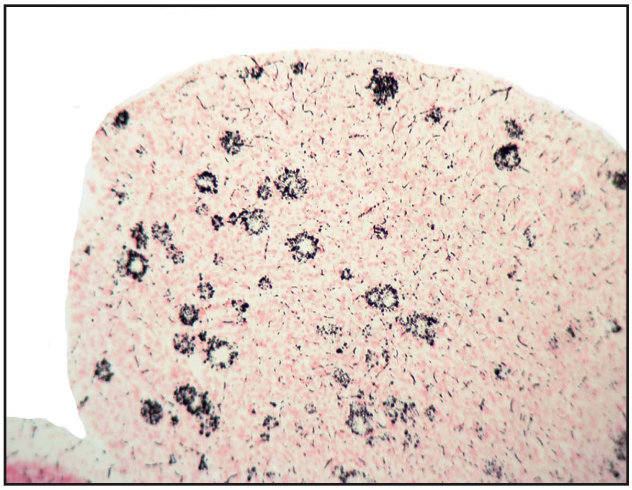
APPLYING NSA SERVICES IN RESEARCH

ALZHEIMER DISEASE (continued)
DISINTEGRATIVE DEBRIS

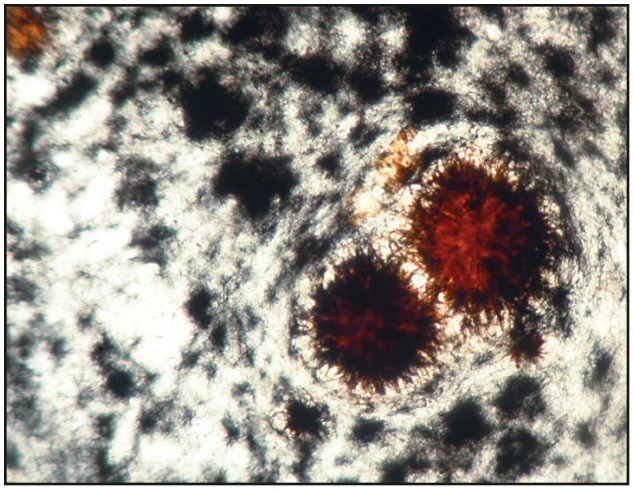
Sections of the brain of an old, >12 months, PS-1/APP mouse



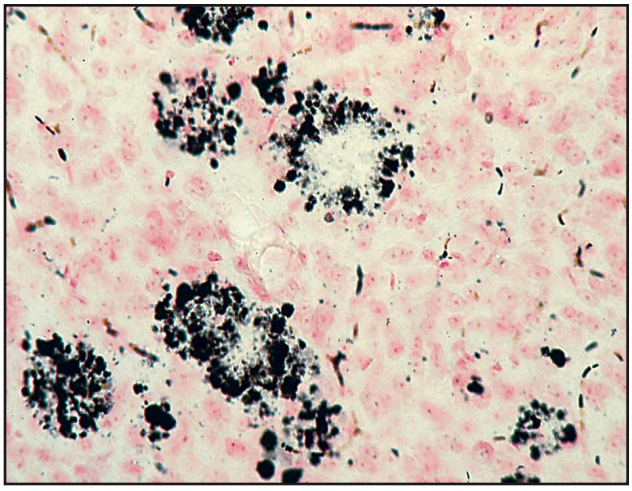
Campbell-Switzer Alzheimer Stain



AmCuAg Disintegrative Degeneration Stain



Numerous diffuse plaques (in black) and the fewer “mature” plaques (in amber) that contain fibrillar amyloid (congophilic, Thioflavin S positive) are depicted.



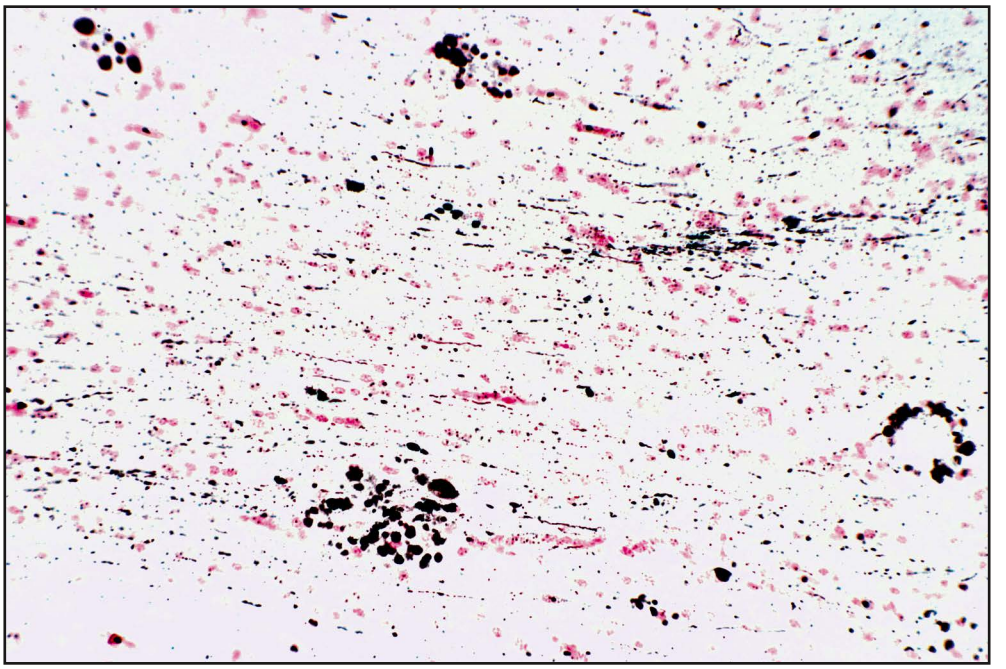
The degeneration stain above reveals degenerative globular profiles at the perimeter of the mature (amber) plaques but not with the diffuse plaques. These profiles have been identified as dystrophic neurites (Brendza et al. J Comp Neurol 456: 375–383, 2003). These may also be engorged glia processes.

APPLYING NSA SERVICES IN RESEARCH

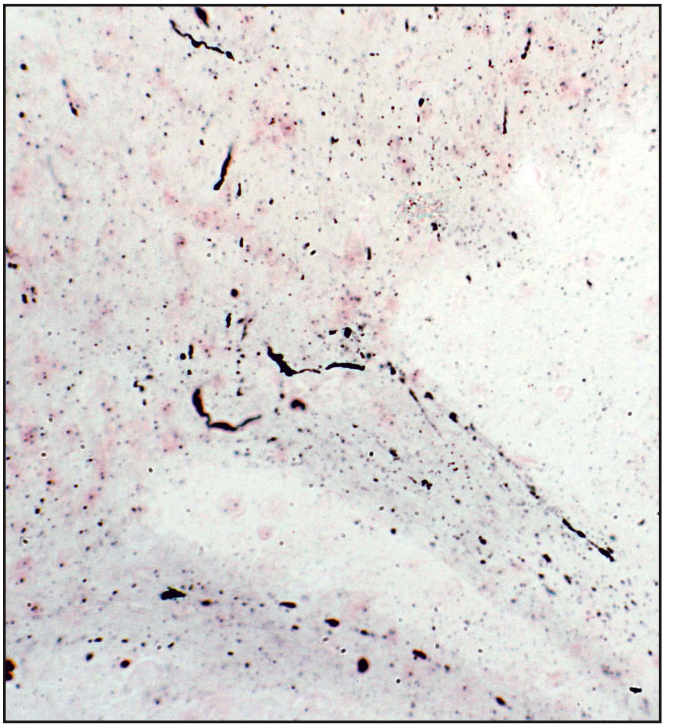
ALZHEIMER DISEASE

DISINTEGRATIVE DEBRIS (continued)

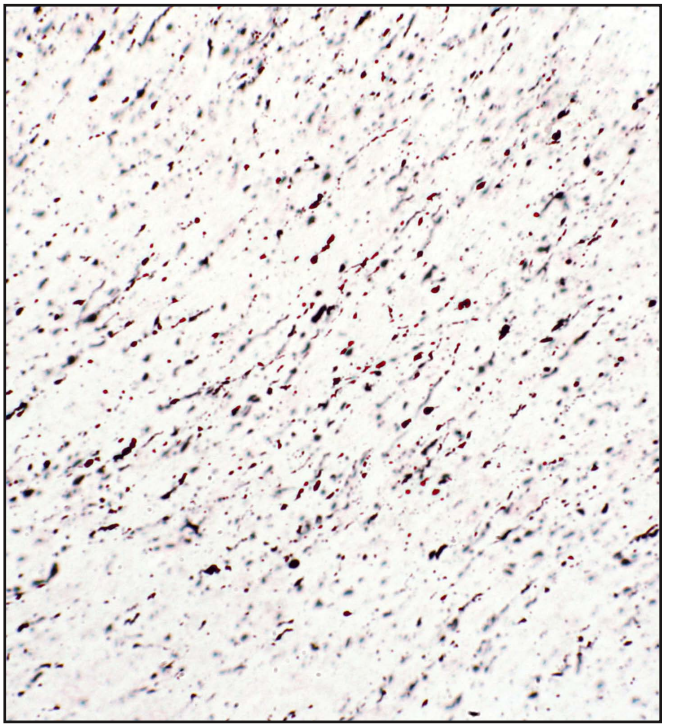
AmCuAg Disintegrative Degeneration Stain
Reveals Axon Degeneration in APP mouse



Degenerated Axons Amidst Mature Plaques in Olfactory Peduncle



Degenerated Axons in the Corpus Callosum and Striatal Fiber Bundles

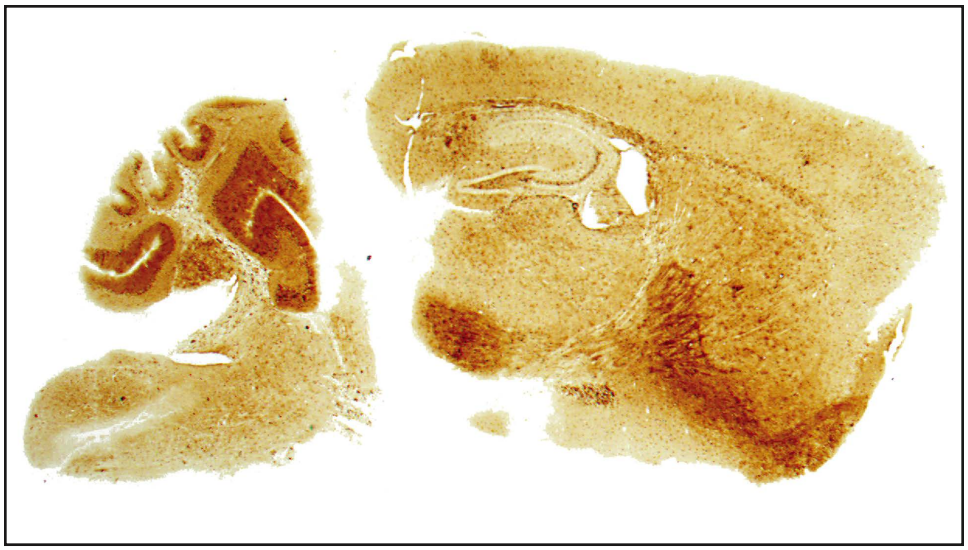


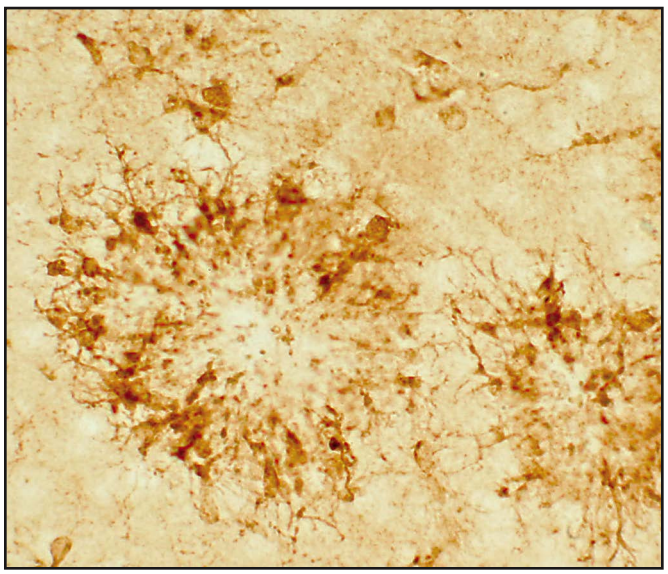
Degenerated Axons in Optic Tract

APPLYING NSA SERVICES IN RESEARCH

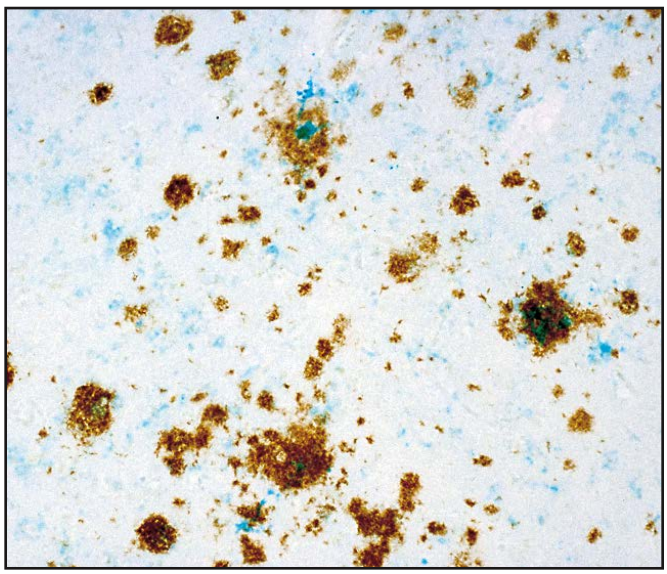
ALZHEIMER DISEASE (continued)
IRON INCREASE

Ferritin Immunoreactivity in a Mouse Model of Alzheimer Disease





Immunoreactivity of an antibody against ferritin (Sigma) in the cortex of a mouse. Ferritin synthesis is triggered by the presence of iron and may act as a persistent indicator of earlier as well as current levels of iron. Staining with Perls-DAB for ferric iron does not yield the same image.



**AD Human Cortex: Aβ 1-42 (brown)
Perls Stain for Ferric Iron (blue)**

APPLYING NSA SERVICES IN RESEARCH

AMYOTROPHIC LATERAL SCLEROSIS (ALS)

See Table of Stains appropriate for ALS page 34

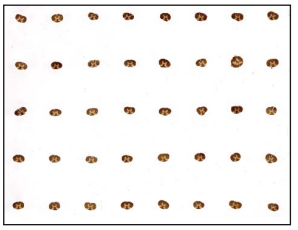
Overview

Also referred to as Lou Gehrig's Disease, Amyotrophic Lateral Sclerosis (ALS) is a progressive, neurodegenerative disease affecting nerve cells in the brain and spinal cord. Motor neurons reach from the brain to the spinal cord, and from the spinal cord to the muscles throughout the body. The progressive degeneration of the motor neurons in ALS eventually leads to their death, resulting in a loss of the brain's ability to initiate and control muscle movement.

Rodent Spinal Cords:

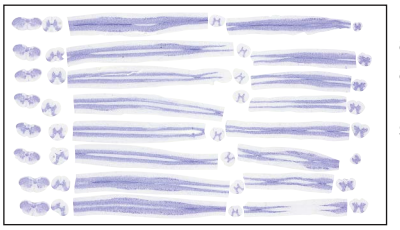
Spinal cord tissue of animal models is commonly used in ALS research. There are several approaches NSA employs in processing spinal cord tissue.

40 Rodent Cords
Transverse/Coronal



Up to 40 spinal cords are co-embedded and appear on each MultiBrain® slide section.

8 Rodent Cords
Transverse / Longitudinal

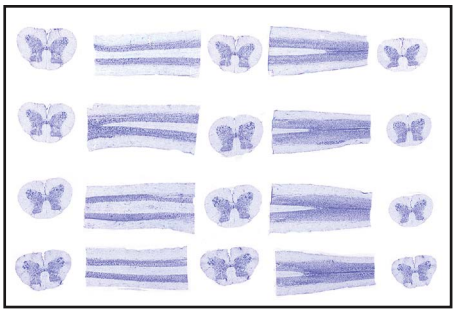


Up to 8 spinal cords are co-embedded and appear on each MultiBrain® slide section.

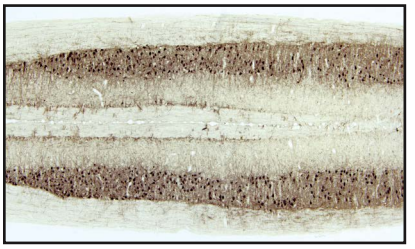
Human tissue:

NSA processes large format sections, as seen below, providing a unique opportunity to assess large contiguous cross-sections of tissue. NSA also processes multiple smaller samples from one or more spinal cords using MultiBrain® Technology. The standard NSA practice of encasing the spinal cord tissue in gelatin provides a significant aid in the handling of tissue sections resulting in an improved final product.

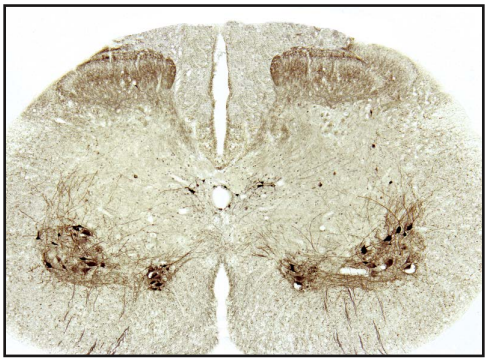
Multiple-Embedded Human Spinal Cords



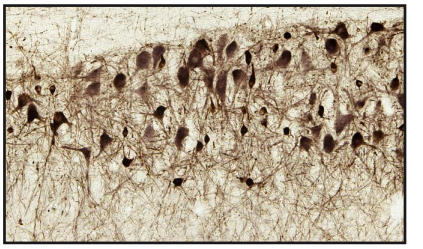
ChAT Motor Neuron Marker



ChAT IHC shown in longitudinal plane of mouse spinal cord, 2x.



ChAT IHC shown in coronal mouse spinal cord to detect motor neuron cells.

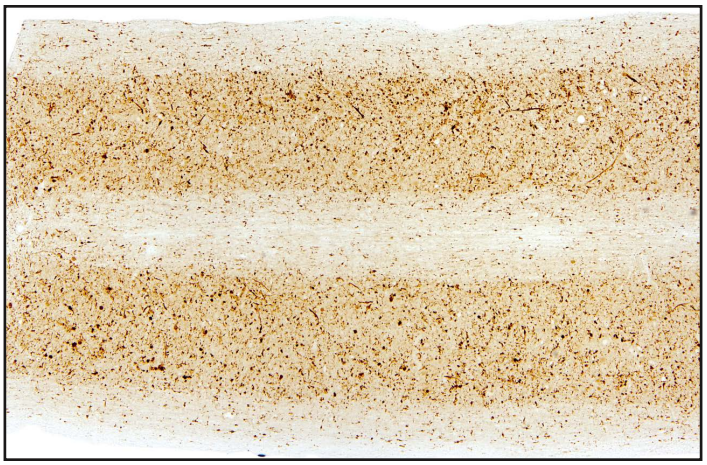


ChAT IHC in mouse spinal cord magnified to 10x to show details.

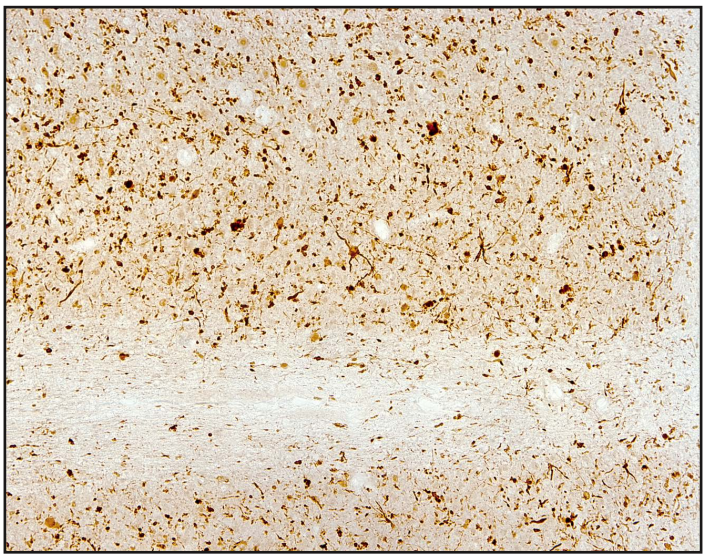
APPLYING NSA SERVICES IN RESEARCH

AMYOTROPHIC LATERAL SCLEROSIS (ALS) (continued)

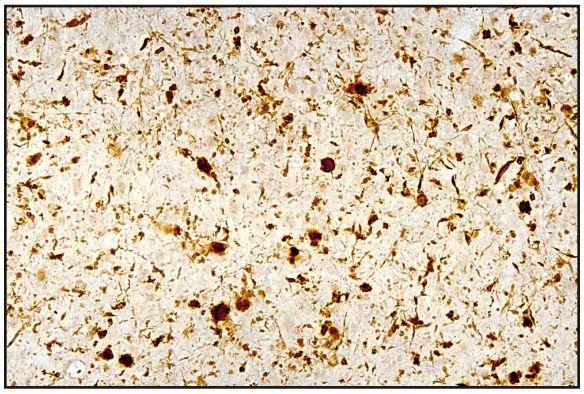
Ubiquitin in SOD1 Mouse Model



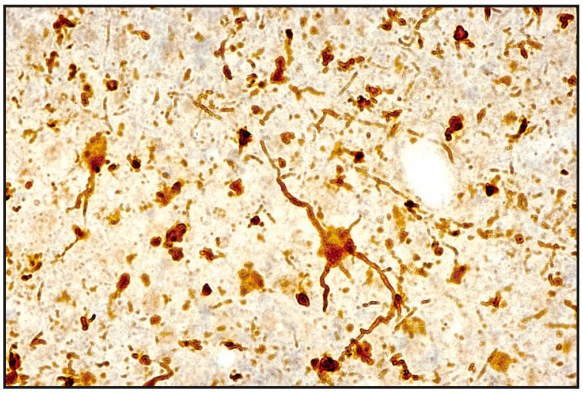
Ubiquitin can help identify abnormal accumulations of this protein inside the cells, indicating a disease process. mouse spinal cord, horizontal



Ubiquitin, mouse sagittal spinal cord, 10x



Ubiquitin, mouse sagittal spinal cord, 20x



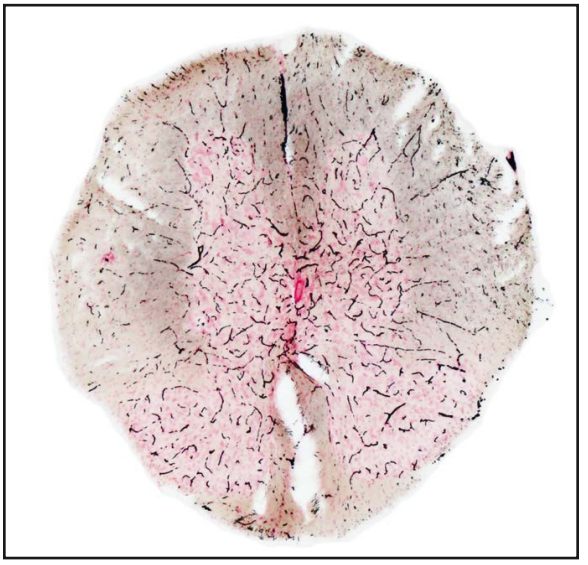
Ubiquitin, mouse spinal cord, 40x

APPLYING NSA SERVICES IN RESEARCH

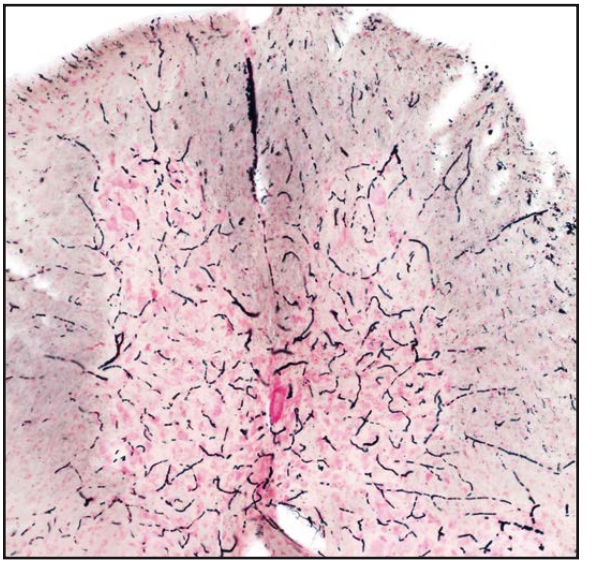
AMYOTROPHIC LATERAL SCLEROSIS (ALS) (continued)

Amino Cupric Silver Stain

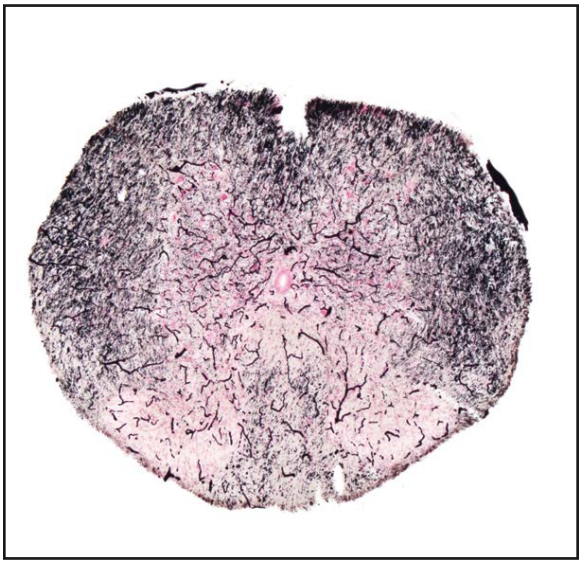
Amino Cupric Silver stain in SOD ALS mouse spinal cord model is shown below. The black “squiggles” are red blood cells within vessels and the fine black lines indicate bundles of degenerated axons.



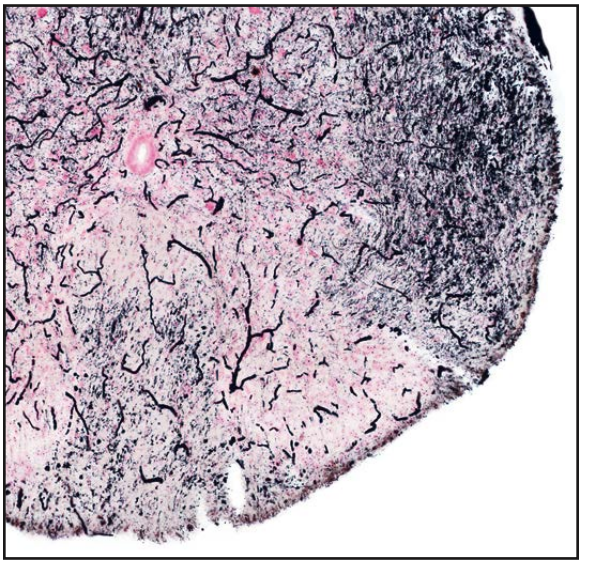
90 day old SOD1 mouse model, 4x



90 day old SOD1 mouse model, 10x



120 day old SOD1 mouse model, 4x



120 day old SOD1 mouse sagittal spinal cord model, 10x

APPLYING NSA SERVICES IN RESEARCH

HUNTINGTON DISEASE

See Table of Stains appropriate for HD page 34

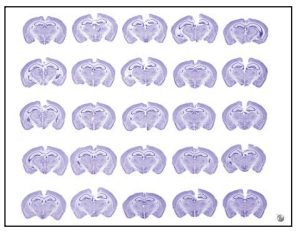
Overview

The pathological features of Huntington Disease that are most often studied through neurohistology include de-myelination, axonal degeneration and inflammation involving an immune reaction including high cellularity around vessels. NSA has processed both human and animal model brains and spinal cords in support of Huntington Disease research.

Rodent Brains:

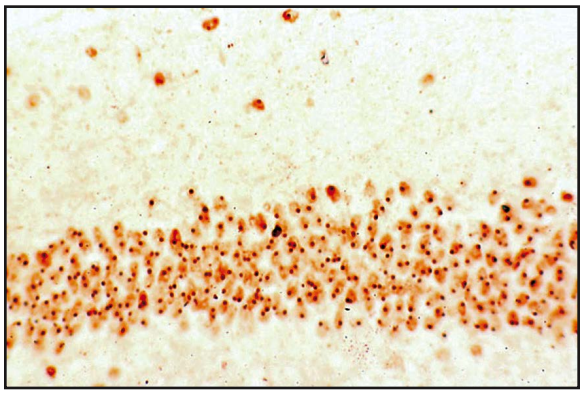
Brain tissue of animal models is commonly used in Huntington Disease research. There are several approaches NSA employs in processing brain tissue.

25 Mouse Brains Coronal

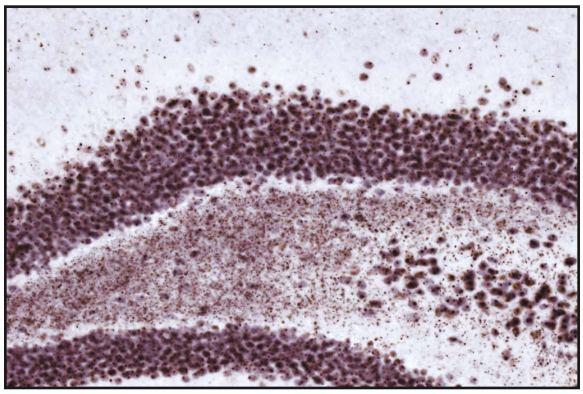


Up to 25 mouse brains are co-embedded and appear on each MultiBrain® slide section.

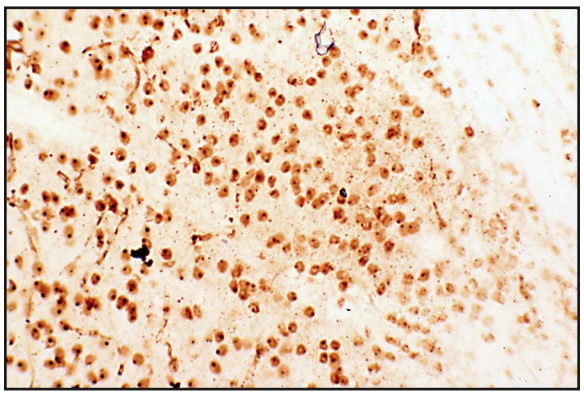
EM48 and S830 Show the Aggregates of Huntington Disease



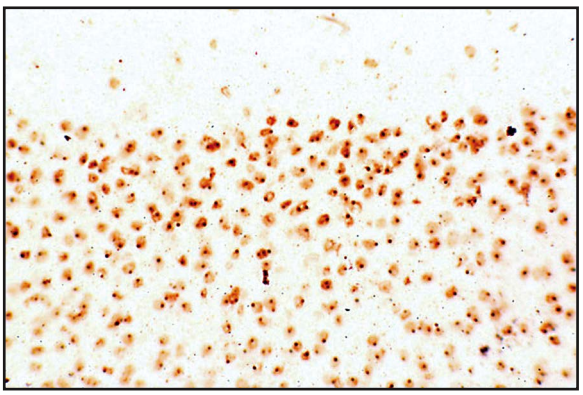
EM48 Hippocampus



S830, Huntington Disease Mouse Model Hippocampus, 20x



EM48 Striatum



EM48 Cortex

APPLYING NSA SERVICES IN RESEARCH

MULTIPLE SCLEROSIS

See Table of Stains appropriate for MS page 34

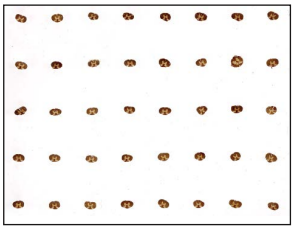
Overview

The pathological features of Multiple Sclerosis (MS) that are most often studied through neurohistology include de-myelination, axonal degeneration and inflammation involving an immune reaction including high cellularity around vessels. NSA has processed both human and animal model brains and spinal cords in support of MS research.

Rodent Spinal Cords:

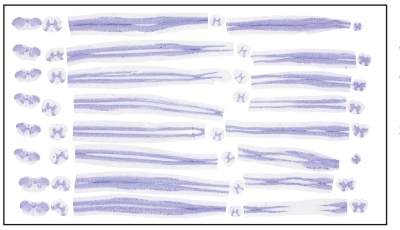
Spinal cord tissue of animal models is commonly used in MS research. There are several approaches NSA employs in processing spinal cord tissue.

40 Rodent Cords Transverse/Coronal



Up to 40 spinal cords are co-embedded and appear on each MultiBrain® slide section.

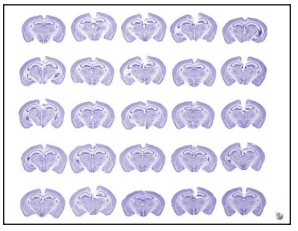
8 Rodent Cords Transverse / Longitudinal



Up to 8 spinal cords are co-embedded and appear on each MultiBrain® slide section.

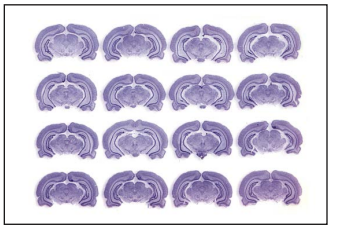
Rodent Brains:

25 Mouse Brains Coronal



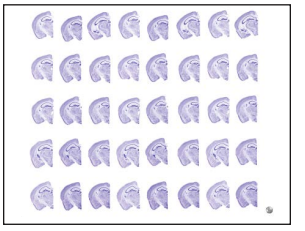
Up to 25 mouse brains are co-embedded and appear on each MultiBrain® slide section.

16 Rat Brains Coronal



Up to 16 rat brains are co-embedded and appear on each MultiBrain® slide section.

40 Mouse or 32 Rat Brain Hemispheres Coronal

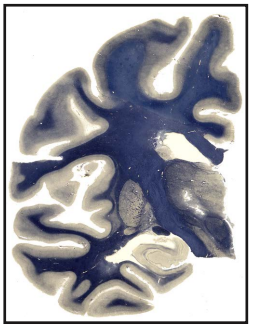


Up to 40 mouse brains are co-embedded and appear on each MultiBrain® slide section.

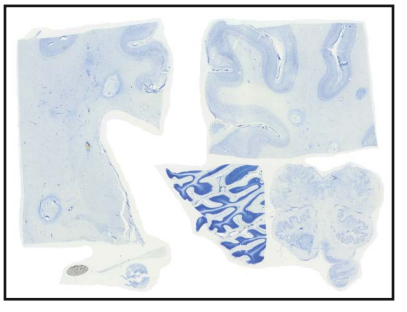
Human tissue:

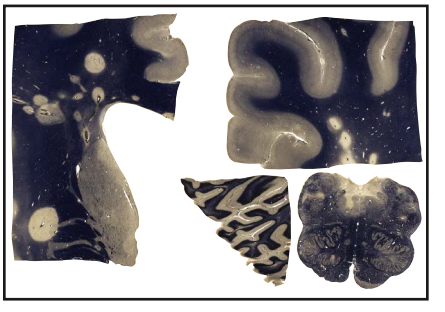
NSA processes large format sections, as seen below, providing a unique opportunity to assess large contiguous cross-sections of tissue. NSA also processes multiple smaller samples from one or more brains using MultiBrain® Technology. The standard NSA practice of encasing the brain tissue in gelatin provides a significant aid in the handling of tissue sections resulting in an improved final product.

Human Brain Hemisphere



Multiple-Embedded Human Brain Tissues

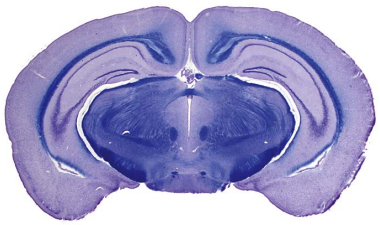




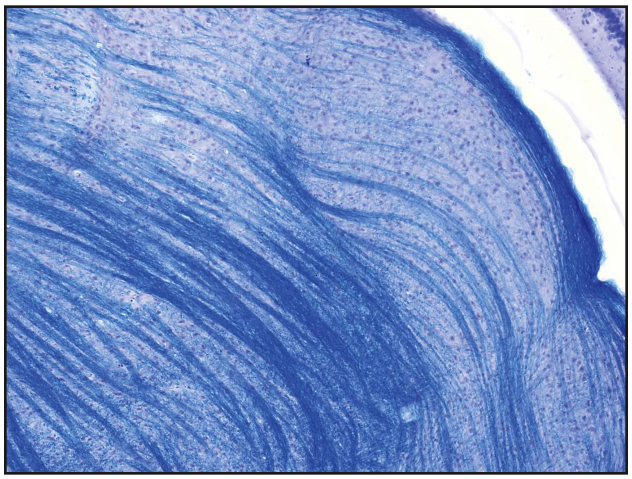
APPLYING NSA SERVICES IN RESEARCH

MULTIPLE SCLEROSIS (continued)
DE-MYELINATION

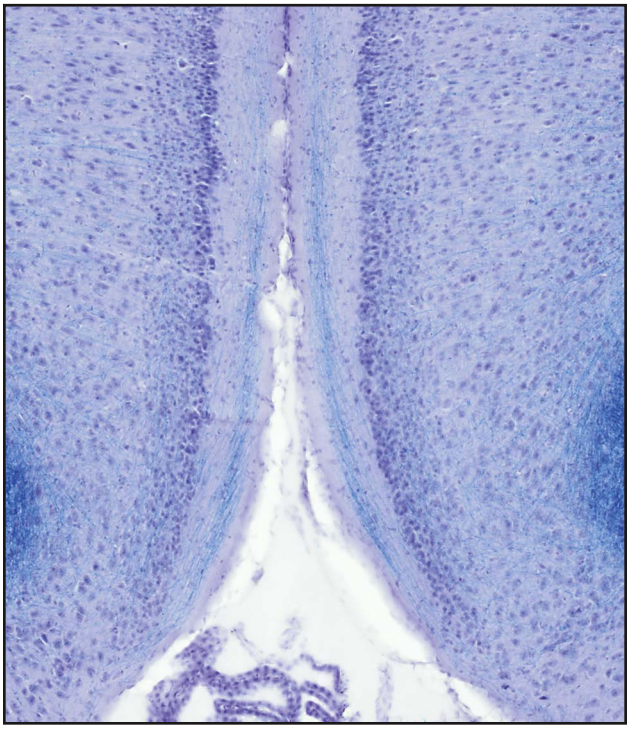
A variety of methods can be used to reveal myelin, which effectively exposes areas of de-myelination. The most common techniques employed by NSA include the Weil-Myelin method and Solochrome method.



Solochrome

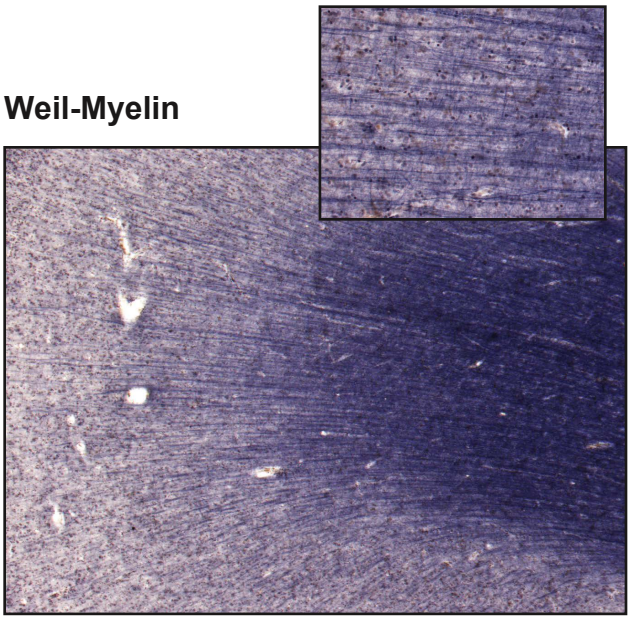


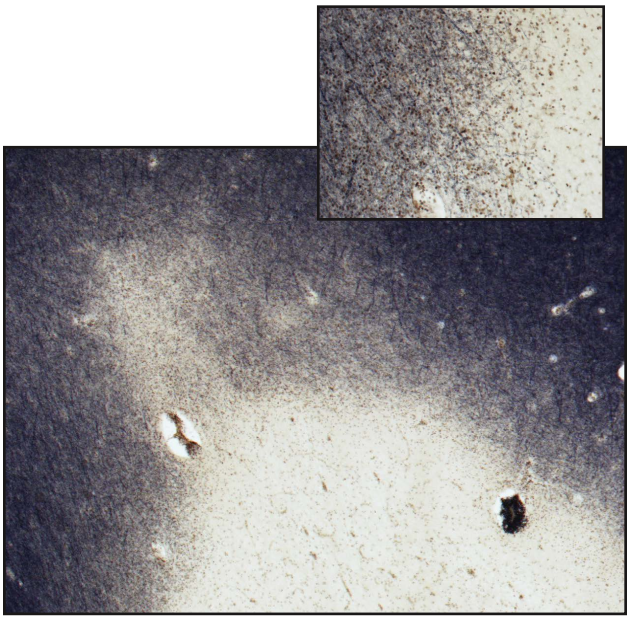
Thalamus



Cingulate

Weil-Myelin



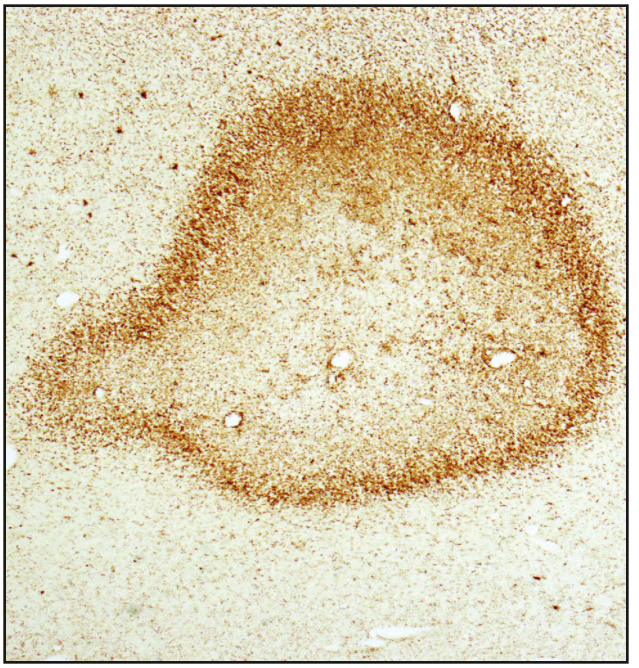


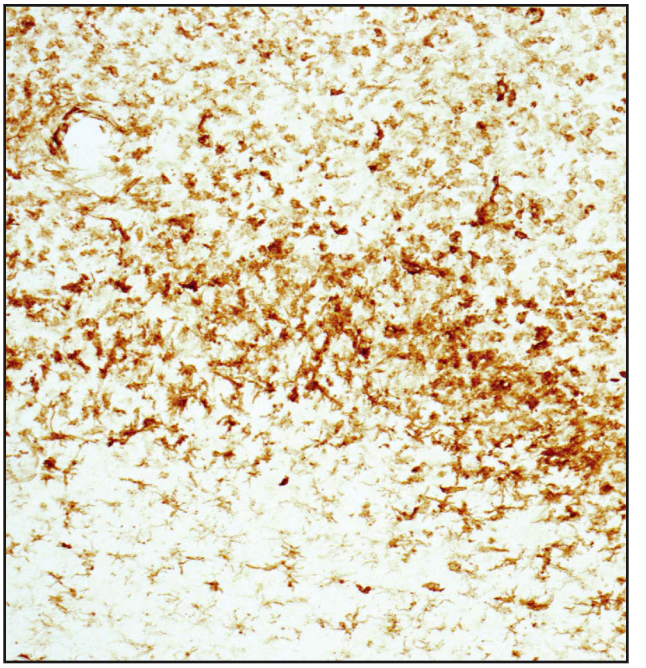
APPLYING NSA SERVICES IN RESEARCH

MULTIPLE SCLEROSIS (continued)
INFLAMMATION

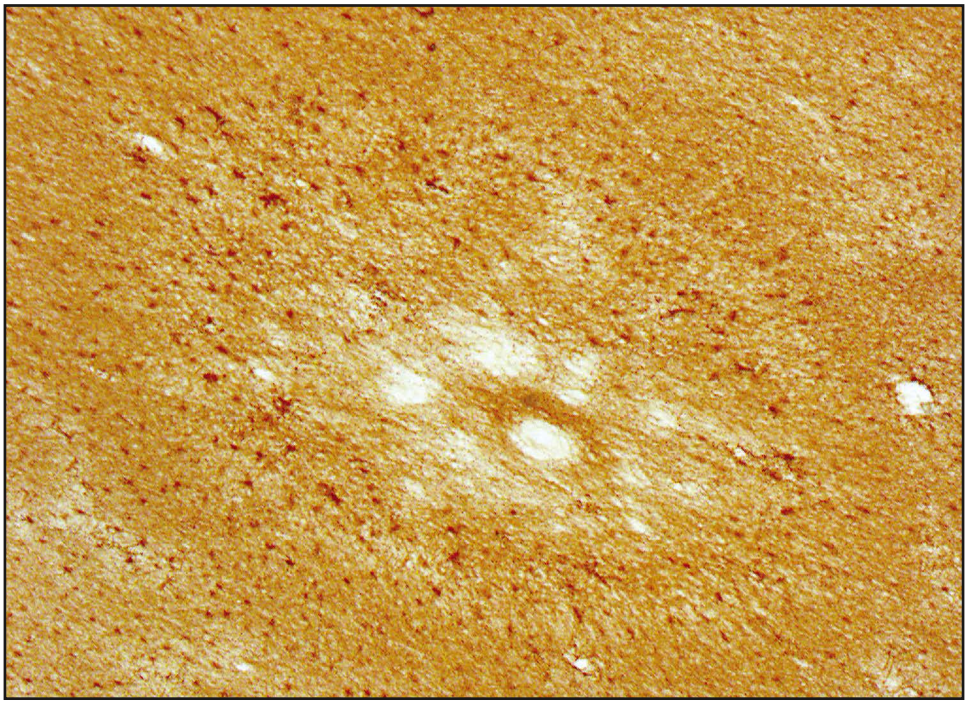
As with other disease models, inflammation markers such as Iba1 and GFAP can be useful indicators of perturbations in conjunction with other MS pathology, as seen below.

Iba1





GFAP



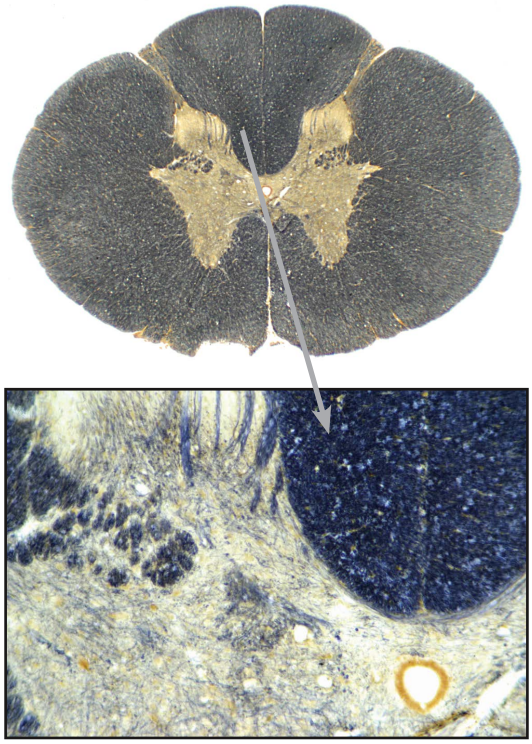
Plaques in White Matter

APPLYING NSA SERVICES IN RESEARCH

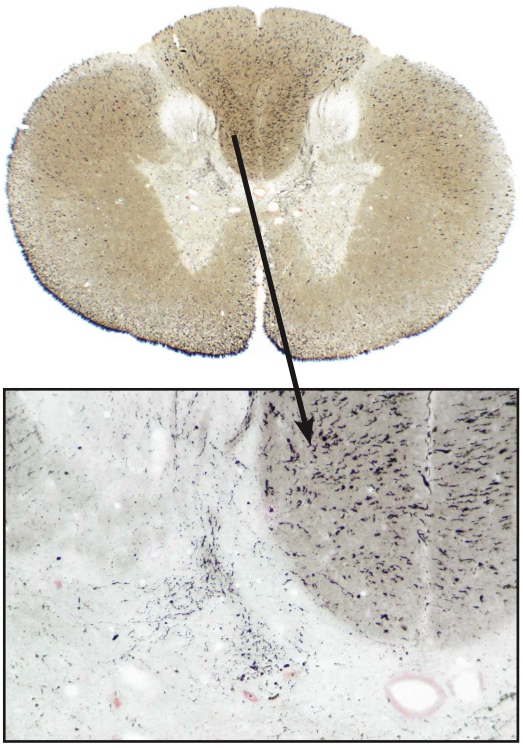
MULTIPLE SCLEROSIS (continued)
AXONAL DEGENERATION

MS Monkey Spinal Cord: Adjacent Stained Sections

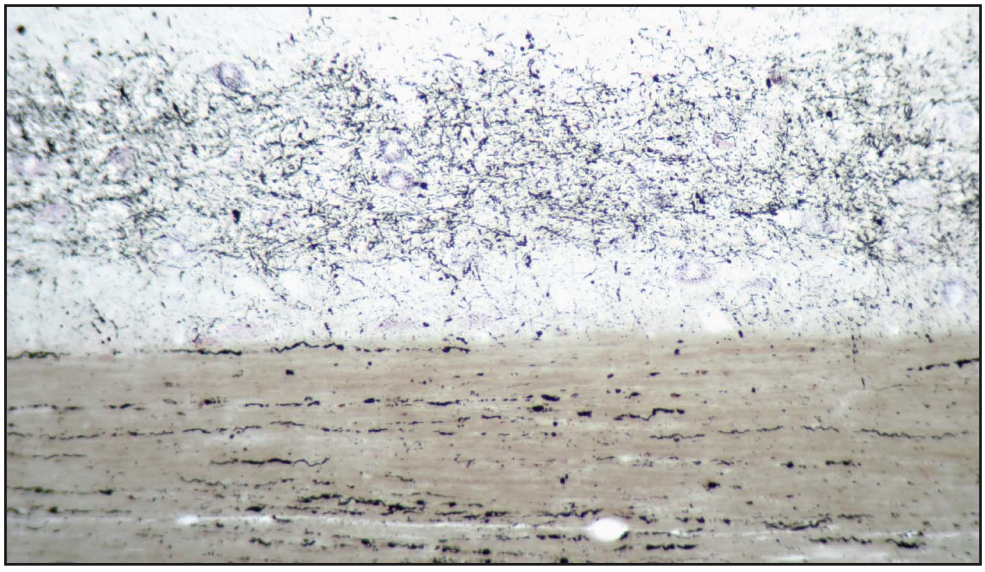
Myelin Stain
Weil Method



Neurodegeneration Stain
Amino-Cupric Silver



Degenerated axons (black) are visible in the upper right, but are not in the same location in the myelin-stained image to the left.

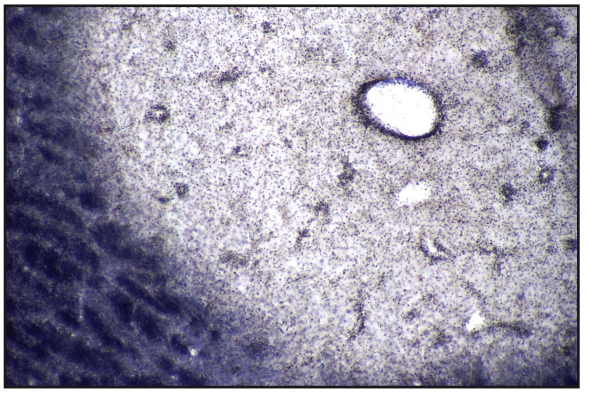
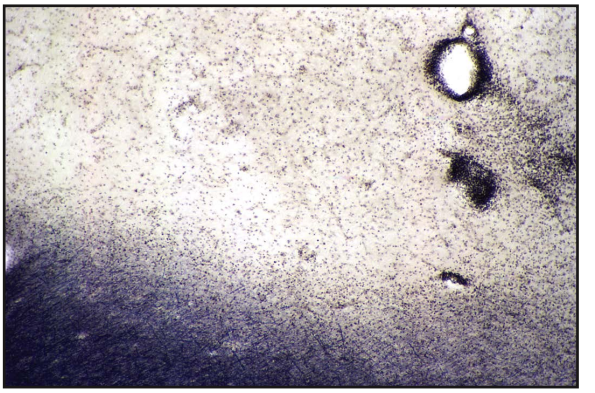
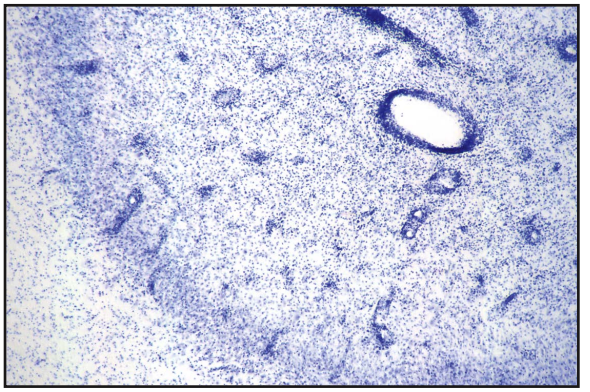
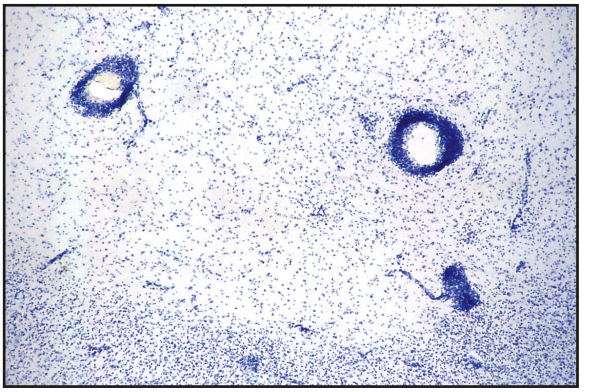
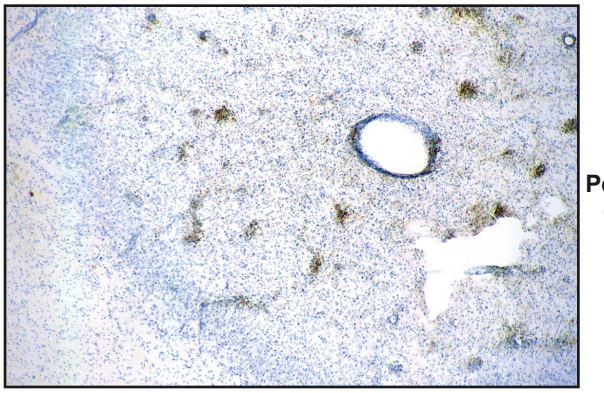
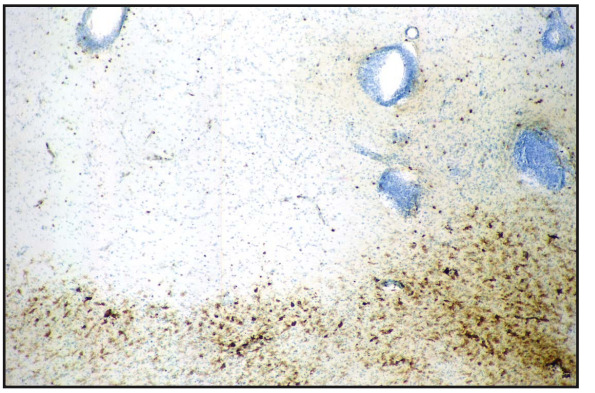


Longitudinal section (from the same cord shown above) that clearly reveals the degenerating pattern of axons. These images suggest that axon degeneration may precede the loss of myelin.

APPLYING NSA SERVICES IN RESEARCH

MULTIPLE SCLEROSIS (continued)
LESIONS AND CUFFING

The presence of high cellularity around vessels, a characteristic of MS pathology which is often called cuffing, as seen around the large vessels in these adjacent sections.

ACUTE		CHRONIC
	**Myelin stain: Weil Method**	
	**Nissl stain: Thionine**	
	**Ferric iron: Perls-DAB Nissl Counterstain**	

Different stains provide different perspectives into the architecture of the MS lesions. For example, the margin of the plaque in the chronic plaque contains cells (microglia) rich in iron, while the corresponding zone in the acute plaques does not.

APPLYING NSA SERVICES IN RESEARCH

PARKINSON DISEASE

See Table of Stains appropriate for PD page 34

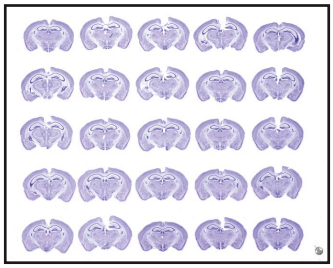
Overview

The primary pathological features of Parkinson Disease (PD) in humans are the loss of dopamine cells, the presence of Lewy bodies in existing cells and changes in the expression of alpha-synuclein. Disease models vary and NSA employs a wide range of tools suited to the traits of specific models.

Rodent Tissue:

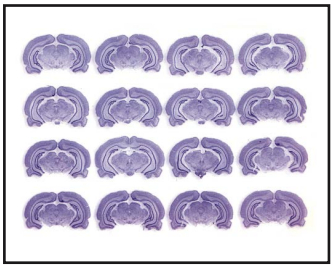
Rodent brains are the primary tissue processed in PD Research by NSA. NSA can process up to 40 tissues in each MultiBrain® Block.

25 Mouse Brains Coronal



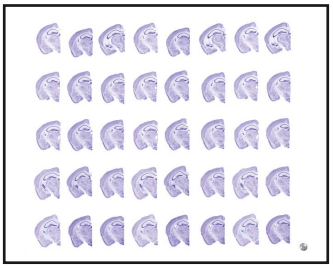
Up to 25 mouse brains are co-embedded and appear on each MultiBrain® slide section.

16 Rat Brains Coronal



Up to 16 rat brains are co-embedded and appear on each MultiBrain® slide section.

40 Mouse or 32 Rat Brain Hemispheres Coronal

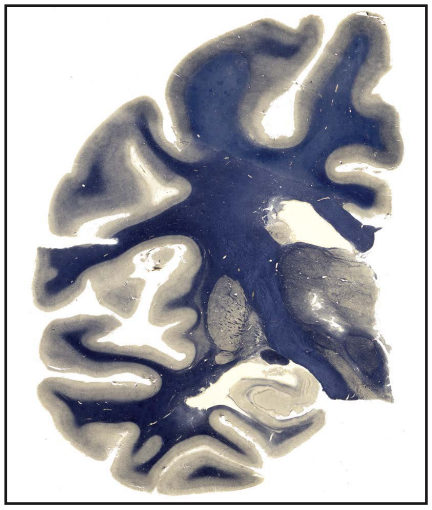


Up to 40 mouse brains are co-embedded and appear on each MultiBrain® slide section.

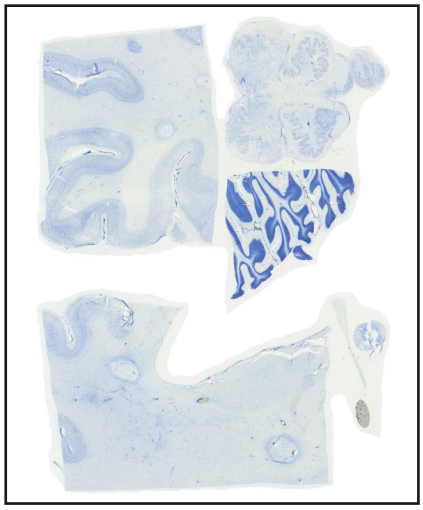
Human Tissue:

NSA processes large format sections, as seen below, providing a unique opportunity to assess large contiguous cross-sections of tissue. NSA also processes multiple smaller samples from one or more brains using MultiBrain® Technology. The standard NSA practice of encasing the brain tissue in gelatin provides a significant aid in the handling of tissue sections resulting in an improved final product.

Human Brain Hemisphere



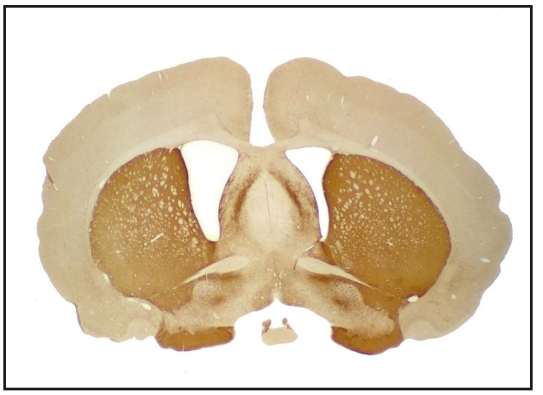
Multiple-Embedded Human Brain Tissues



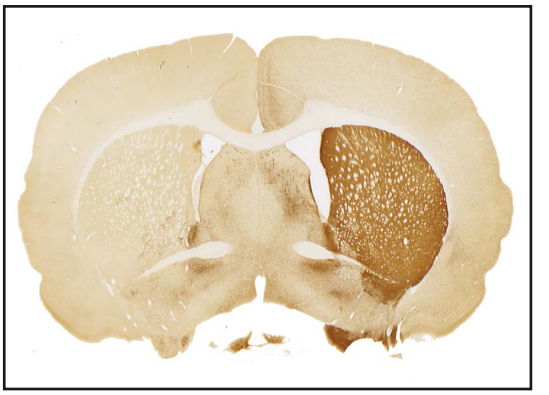
APPLYING NSA SERVICES IN RESEARCH

PARKINSON DISEASE (continued)
TYROSINE HYDROXYLASE IHC

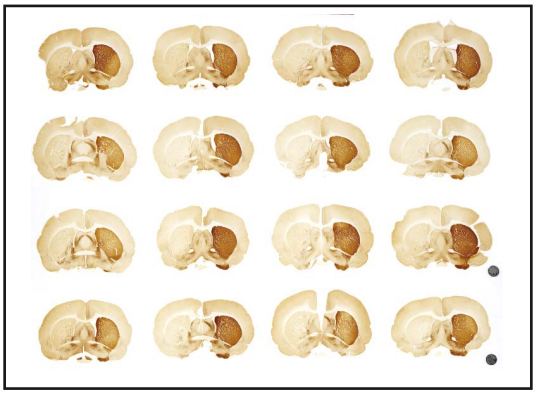
PD research models typically use a method for inducing the loss of dopamine cells, or create lesions that interrupt the axons projecting to striatum. Tyrosine Hydroxylase (TH) is a robust marker of elements of the dopamine system.



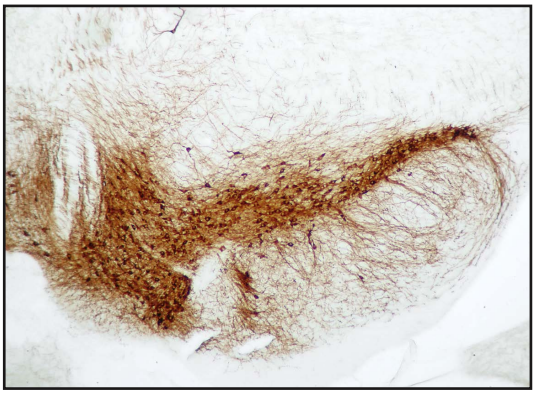
Rat brain normal expressing TH equally in both hemispheres.



Rat animal model treated to create the loss of the dopamine cells that produce TH.

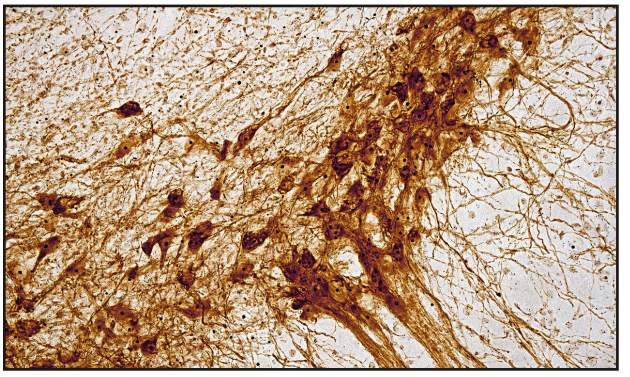


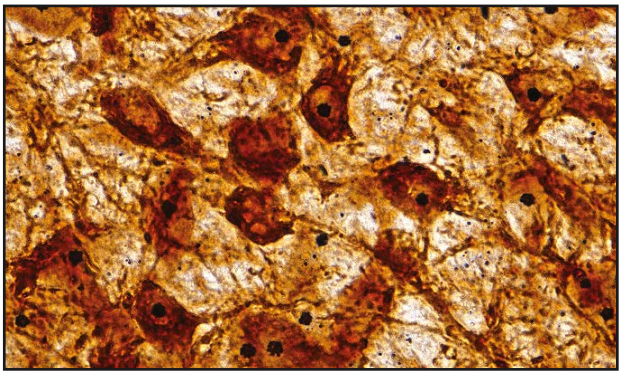
16 rat brains of a PD model on a MultiBrain® slide at the approximate same levels. All brains have lost TH staining in the left striatum.



TH-Stained Substantia Nigra

Tyrosine Hydroxylase IHC



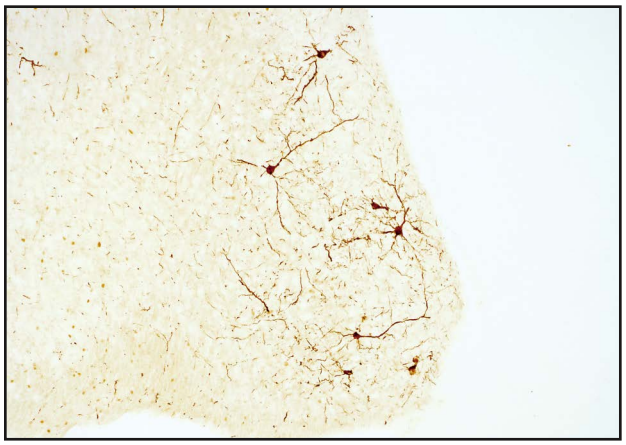


AgNOR-TH in Substantia Nigra

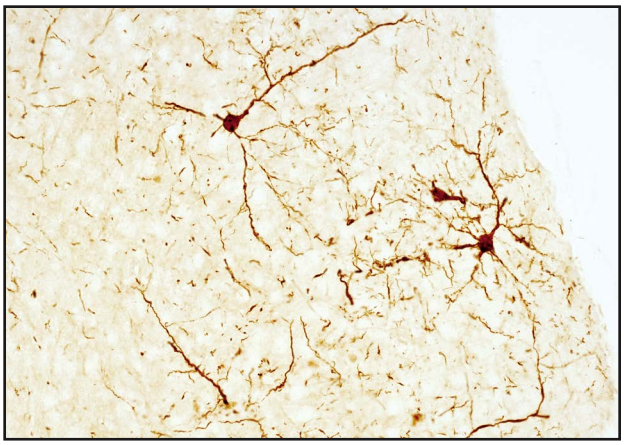
A silver nucleolar stain (AgNOR: Ag Nucleolar Organizing Region protocol used in oncology research), when paired with Tyrosine Hydroxylase IHC staining, is an excellent tool for use in stereology in Parkinson research.

APPLYING NSA SERVICES IN RESEARCH

PARKINSON DISEASE (continued)
ALPHA-SYNUCLEIN

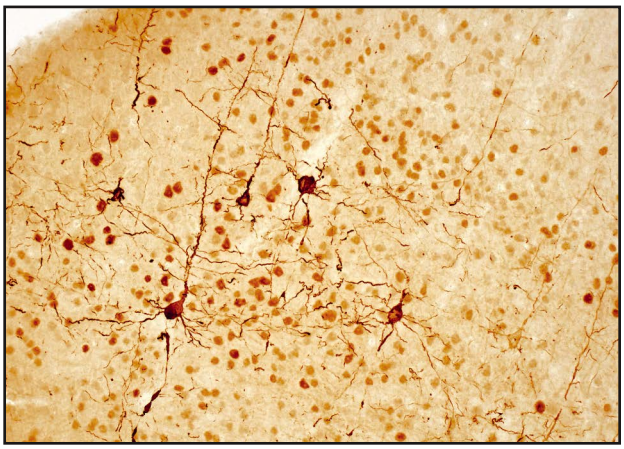


Alpha-Synuclein pSer129
Mouse Brain Cortex,10x

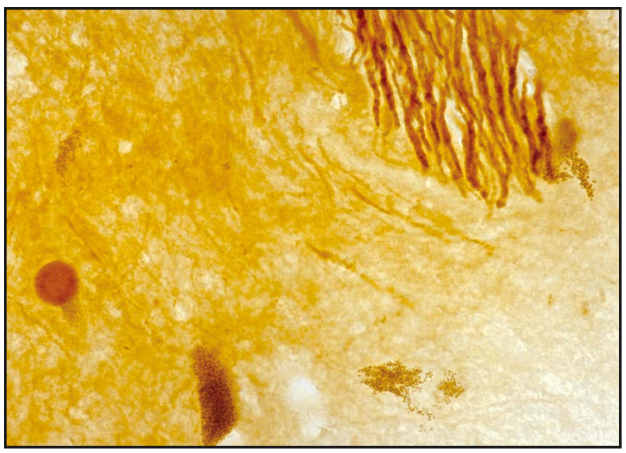


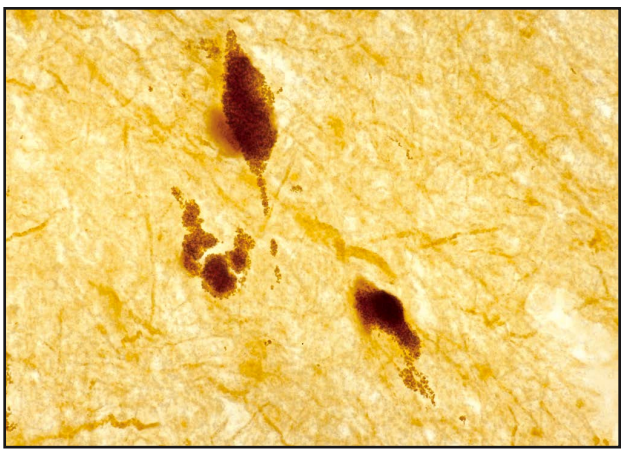
Alpha-Synuclein pSer129
Mouse Brain Cortex, 20x

Alpha-Synuclein pSer129
Synthetic A-Syn Fibril Injected
Mouse Brain Cortex, 20x



Alpha-Synuclein pSer129
A53T mouse cortex,
pyramidal cells

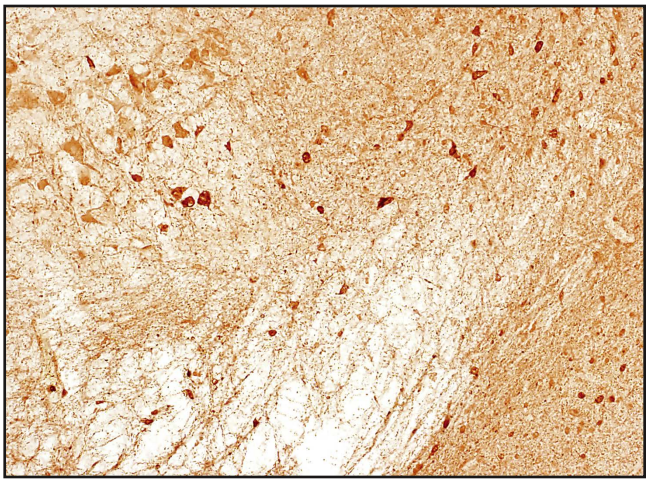




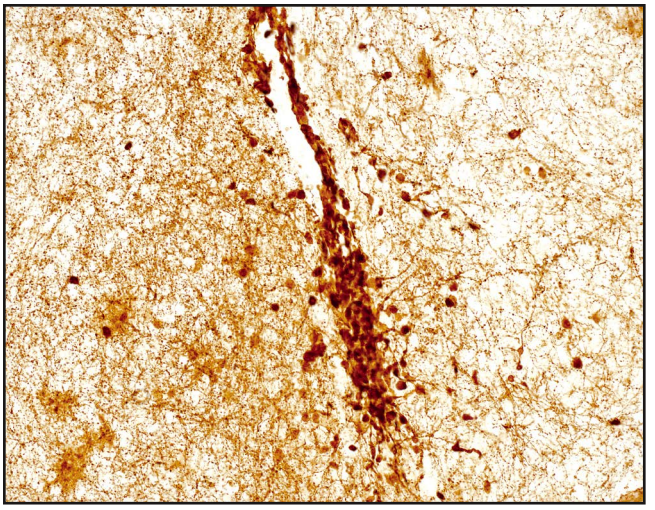
Alpha-Synuclein pSer129
Substantia nigra in a human PD brain, cytoplasmic and axonal aggregates, 60x

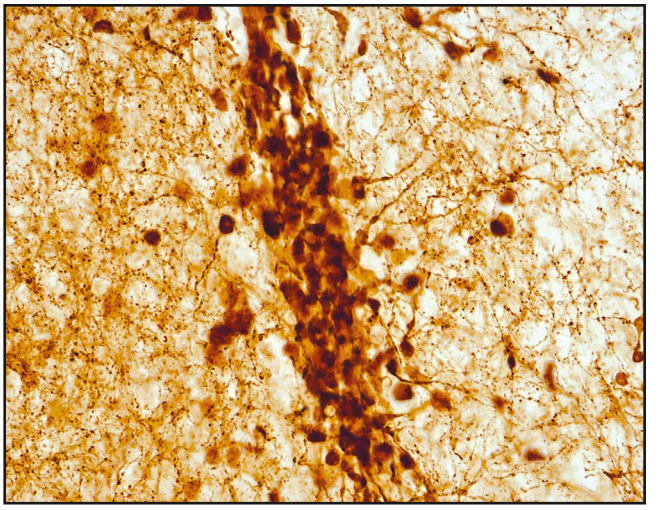
APPLYING NSA SERVICES IN RESEARCH

PARKINSON DISEASE
ALPHA-SYNUCLEIN (continued)



Placement of a vector containing the code for human alpha-synuclein in rat brains is taken up by cells and Alpha-Synuclein is expressed.





Transgenic mouse brain expressing human Alpha-Synuclein 211 (121-125), 20x (left) and 40x (right)

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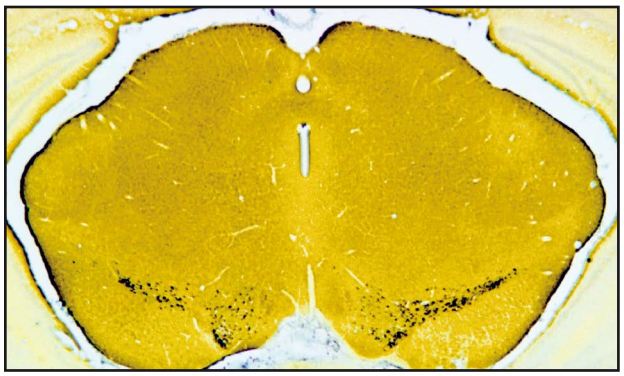
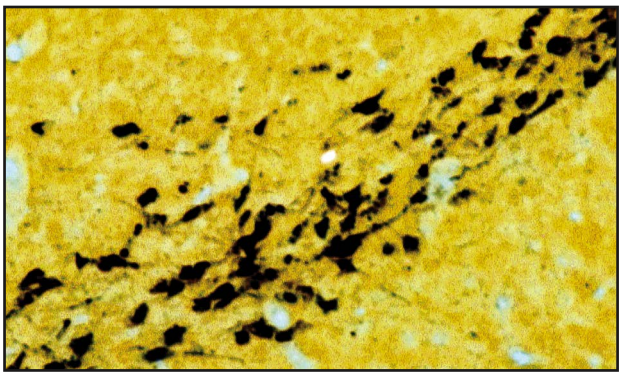
PARKINSON DISEASE (continued)
DISINTEGRATIVE DEGENERATION

In PD research, models that employ an acute method of destroying the TH-producing cells, the degeneration of those cells can be witnessed using a Disintegrative Degeneration stain: Cupric Silver deOlmos method.





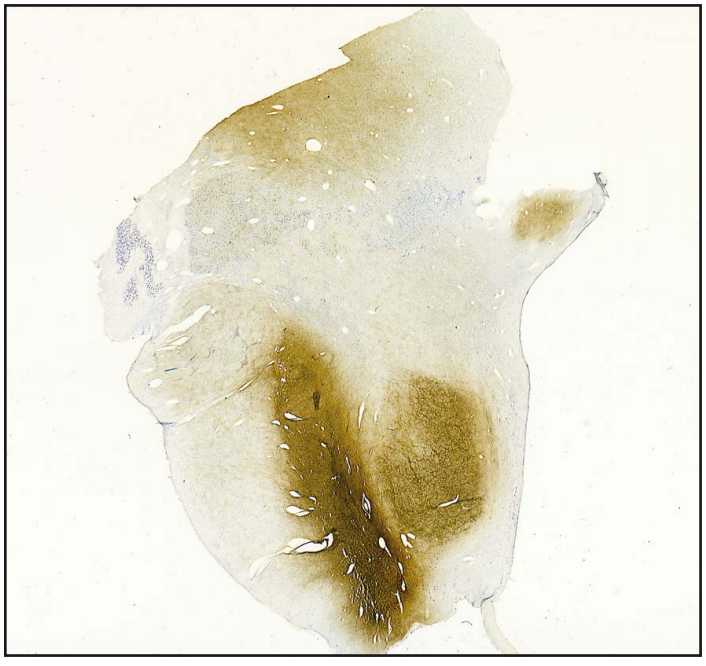
Degenerated neurons in substantia nigra in MPTP-treated BALB/C57 mouse

Inflammation

As with other disease models, inflammation markers such as GFAP and Iba1 can be useful indicators of perturbations in conjunction with other PD pathology.

Aberrant Iron in PD

Iron 'decompartmentalized' has been implicated in the pathologic process. Useful tools for probing this include Perls-DAB for ferric iron and Ferritin IHC for the iron storage molecule.



Perls-DAB reveals iron in the substantia nigra (left) and red nucleus (right) of this human tissue. This section was counterstained for nissl substance with Thionine (blue).

APPLYING NSA SERVICES IN RESEARCH

STROKE

See Table of Stains appropriate for Stroke page 34

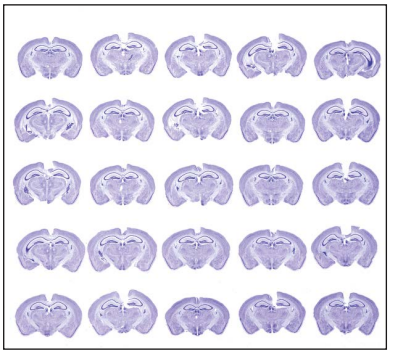
See Stroke Image Analysis page 25

Overview

Animal models for stroke attempt to induce a cerebral ischemia to mimic the effects of a stroke in humans, which tends to be more difficult to replicate in smaller rodents than in larger animals. Neurohistology for stroke research primarily focuses on methods to quantify the ischemic region and characterize the margin of surviving tissue.

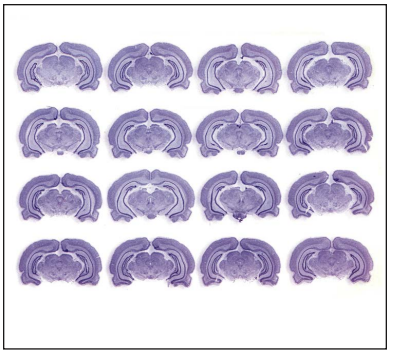
Animal Models:

25 Mouse Brains Coronal



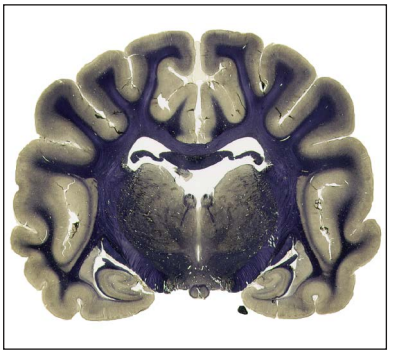
Up to 25 mouse brains are co-embedded and appear on each MultiBrain® slide section.

16 Rat Brains Coronal



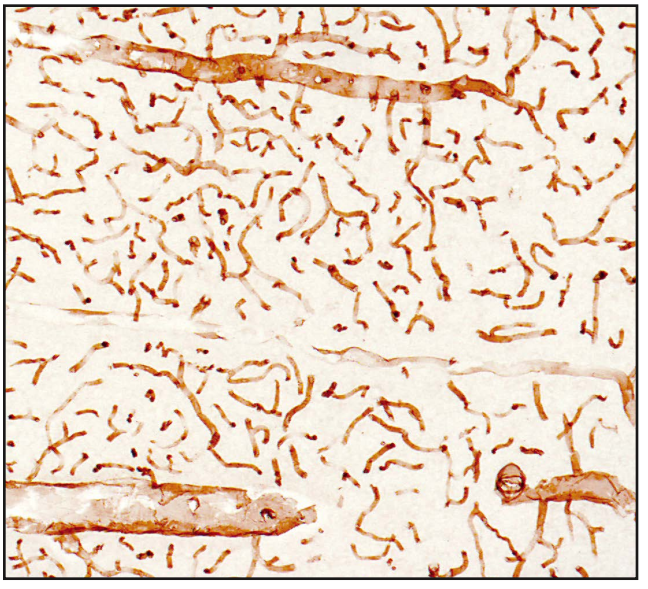
Up to 16 rat brains are co-embedded and appear on each MultiBrain® slide section.

Dog Brain

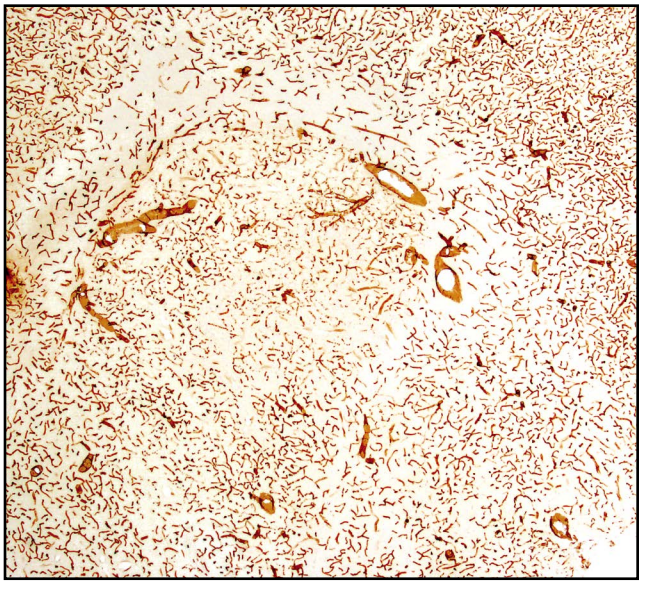


Large brains such as monkey, sheep, and dog are processed intact. If hemisected two hemispheres are co-embedded and appear on the same MultiBrain® slide section.

Blood Brain Barrier (BBB) Compromise



SMI-71 Endothelial Barrier Protein EBP Rat-normal vessels



SMI-71 Endothelial Barrier Protein EBP Rat stroke model reveals loss of EBP associated with Blood Brain Barrier Compromise

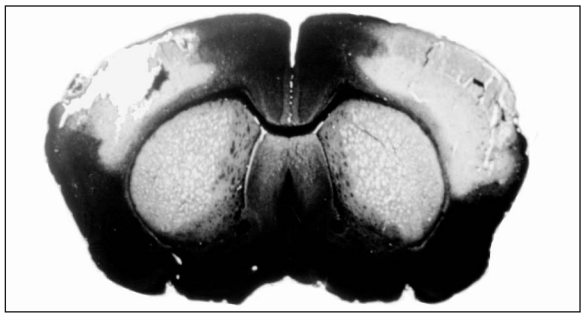
APPLYING NSA SERVICES IN RESEARCH

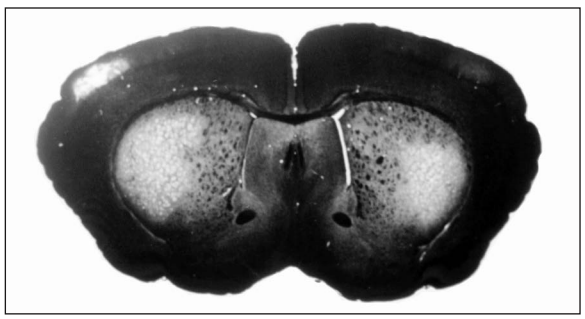
STROKE
ISCHEMIA

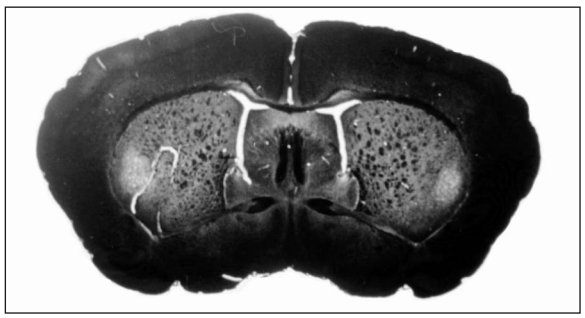
Protective effects of interventions after stroke are ideally based on clinical neurologic outcomes and subsequent neurohistology. In small animal stroke models, difficulties with long survival times and neurologic evaluation prompt many investigators to perform acute studies and determine protection by histologic evaluations immediately after inducing an infarction. Many stains have been used to identify infarct areas, but it is not clear if these areas include or could delineate the penumbra, i.e., the potentially salvageable tissue at the margin of the infarct. The triphenyltetrazolium chloride (TTC) method, for instance, is known to give variable infarct volumes depending on how long after the infarct the tissue is harvested. A fortuitous observation led to a staining method which delineates the penumbra while highlighting the infarcted volume in sharp contrast. Normal tissue is dense black, infarcted areas fail to stain and gradations of gray identify the penumbra zone. Section images are digitized and, based on optical density, the volumes of the infarct, penumbra and normal brain are calculated. Adjacent sections stained with other methods including H&E, thionine and GFAP IHC display less definition of affected areas.

Appearance of stroke in popular animal models with decreasing severity

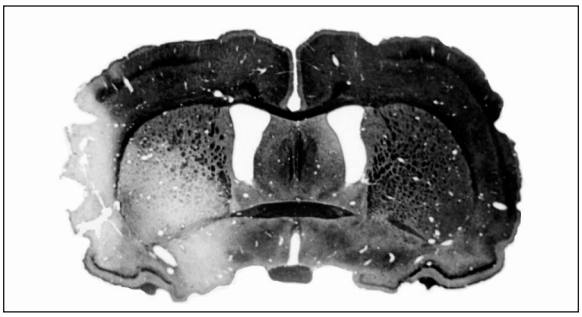
Gerbil
Ligation of Carotid Arteries

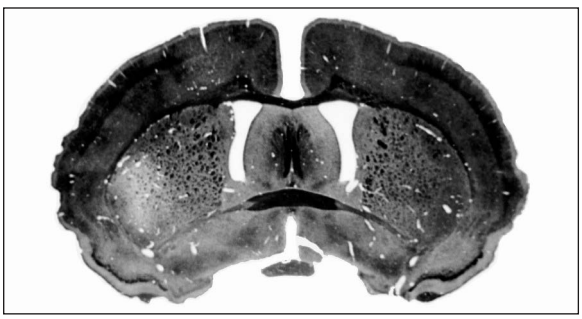


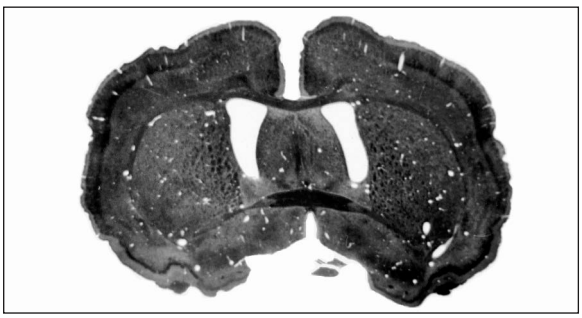




Rat
Middle Cerebral Artery Occlusion







APPLYING NSA SERVICES IN RESEARCH

ACETYLCHOLINESTERASE ENZYME DETECTION

Applications:

- Stain reveals acetylcholinesterase enzyme activity.

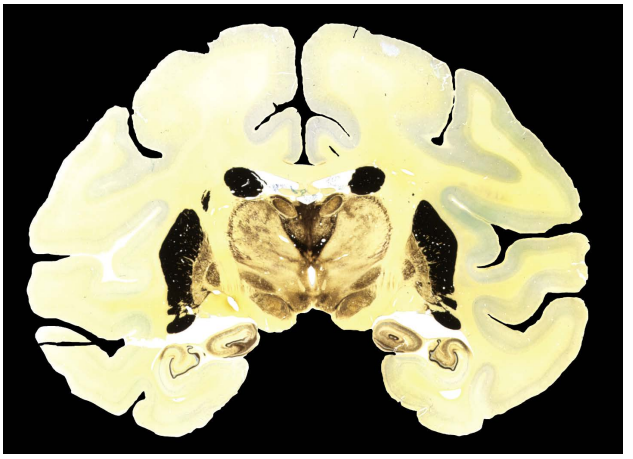
Advantages:

- Allows different brain cell groups to be distinguished from others.

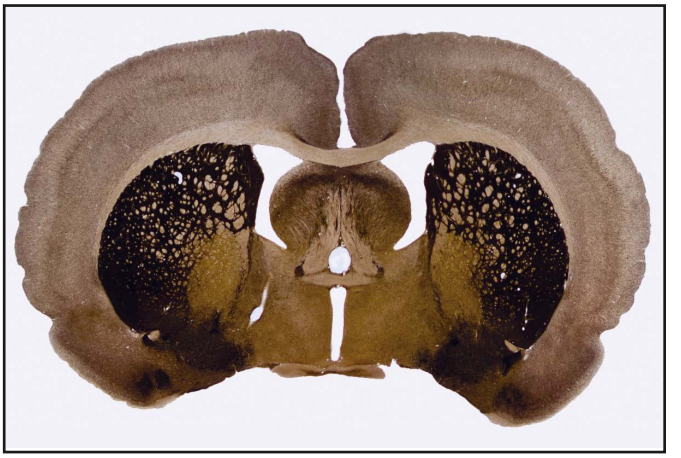
Specifications:

- Tissue must be sent within 48 hours after perfusion.
- After overnight in perfusion fix, then switch over to buffer and ship as soon as possible after that. This is an enzyme whose activity diminishes in time until the tissue is sectioned and stored in 'antigen-enzyme' preserve solution. Contact NSA to verify scheduling.
- Follow perfusion instructions for immunohistochemistry stains on our website.

Acetylcholinesterase (AChE) Enzyme Staining



Macaque monkey: AChE activity revealed with acetylthiocholine substrate and intensified with silver nitrate
(Hardy, H et al. *Neuroscience Letters* 3: 1/5, 1976)



Rat brain section stained for AChE with the Hedreen method

Rat brain cortex stained for AChE with the Hedreen method

APPLYING NSA SERVICES IN RESEARCH

METAL DETECTION: AUTOMETALLOGRAPHY

Applications:

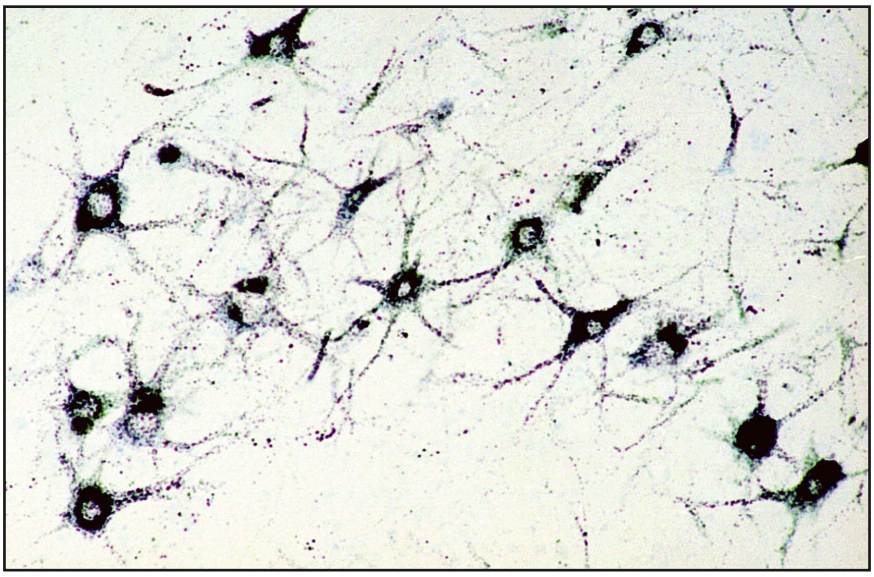
- Used for the detection of various metals in tissue, e.g., Bi, Cu, Hg, Au and Ag.

Advantages:

- A histologic means of detection for the location of certain metals.
- Complements the nonanatomical detection of metals by atomic absorption (A.A) or by ICP (Inductively Coupled Plasma) spectroscopy.

Autometallography is a histologic technique that exploits the presence of metal ions in tissue to act as a nucleation point around which silver ions are gathered to create a visible reaction product. The process is called “physical development.” The Timm’s silver sulfide method is the most well-known autometallography procedure and it favors the revelation of zinc but not other metals.

Gorm Danscher in Denmark has championed the application of other autometallography protocols for detecting various metals in tissue. Another contributor, F. Gallyas of Hungary, has developed a family of physical developers that broaden the range of metals detectable with autometallography.



Neurons in the hypoglossal nucleus of a mouse that was chronically administered bismuth sub-salicylate. The location of bismuth was revealed using an acidic physical developer described by Gallyas.

(Ross, J.F., Switzer, R.C. III, Poston, M.R., and Lawhorn, G.T. Distribution of bismuth in the brain after intraperitoneal dosing of bismuth subnitrate in mice: Implications for routes of entry of xenobiotic metals into the brain. *Brain Res* 725: 137–154, 1996)

APPLYING NSA SERVICES IN RESEARCH

ß-GALACTOSIDASE (ß-GAL) DETECTION

Applications:

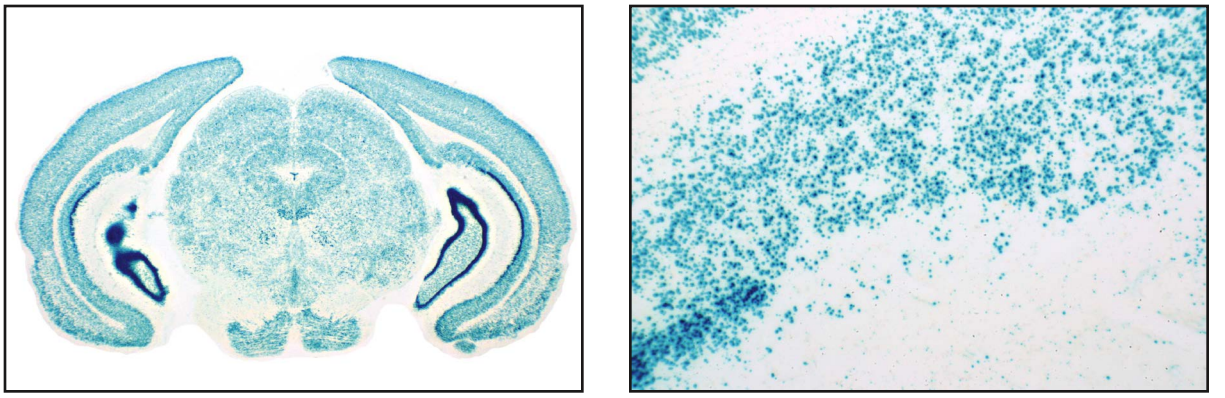
- The X-Gal enzyme stain and the ß-Gal IHC method are tools to reveal ß-Gal presence.
- These are used to identify the product of the lacZ gene, which is used as a marker for identifying cells that have been introduced into a host animal or transfected with a viral vector.

Advantages:

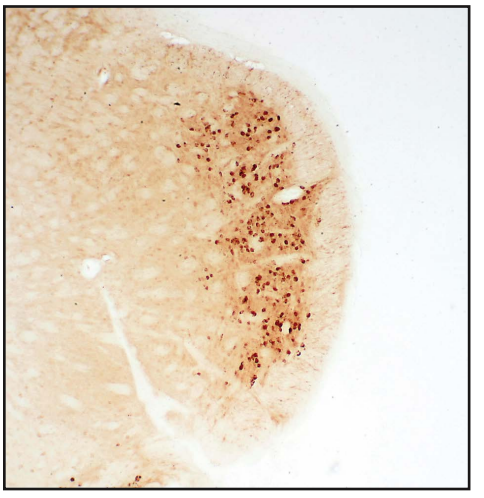
- The signal observable from the enzyme method is usually considered superior to that achieved with the IHC method.
- The enzyme method forms an intense blue precipitant in the presence of ß-Gal expression.
- Tissue must be sent shortly after perfusion, as this is an enzyme whose activity diminishes with time.
- Contact NSA to verify scheduling.

Specifications:

- Tissue must be sent within 48 hours after the animal is perfused.
- After perfusion and over night in fix, switch the brains to buffer (PBS) and then ship as soon as possible to NSA. This is an enzyme whose activity diminishes with time.
- Contact NSA to verify scheduling.



X-Gal Enzyme Histochemistry



β-Galactosidase IHC (β-Gal)

APPLYING NSA SERVICES IN RESEARCH

BLOOD BRAIN BARRIER (BBB) COMPROMISE DETECTION

Applications:

- Anti-IgG Stain reveals the locations of blood brain barrier compromise in the brain.

Advantages:

- Allows for visualization of BBB compromise that is not accompanied by a loss in cells.
- Does not require the injection of a foreign protein marker (i.e., horseradish peroxidase (HRP)) into the vasculature.

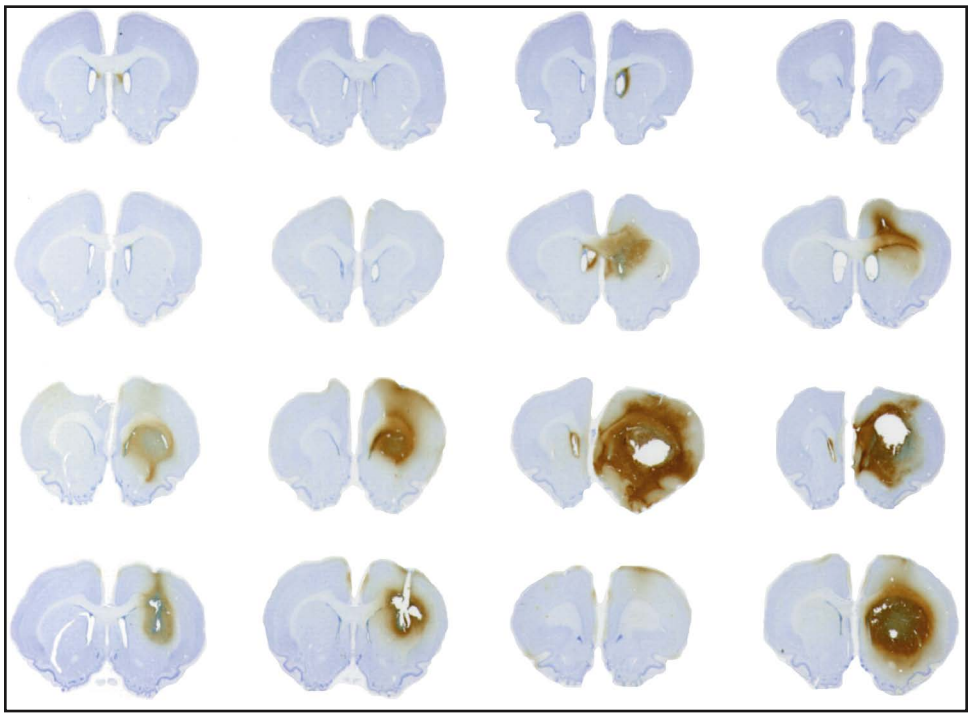
Specifications:

- Perfusion fixation is preferred with overnight in perfusion fix then transfer to PBS. CAUTION: sacrifice with CO2 suffocation and not immediate brain removal and placement in fix can allow serum proteins to extravasate into the brain parenchyma and yield a false positive of BBB compromise (see image bottom right on p 71).

The locations of BBB compromise can be revealed with immunohistochemistry by using an antibody against the animal's own IgG. Areas with "leaky" BBB allow serum proteins to pass into the brain parenchyma, e.g., IgG's and albumin.

BBB Compromise by Physical Trauma

In the MultiBrain® section below (derived from freeze-sectioning a MultiBrain® Block of 16 rat brains), forebrain sections are shown from rat brains that had been injected with a test article. Different amounts were injected, then the rats were sacrificed at different times following the injection. The result was different degrees of leaky BBB as indicated by the brown coloration (the blue is a Nissl counterstain). Note the halo of BBB immunostaining extends beyond the margin of the frank lesion.



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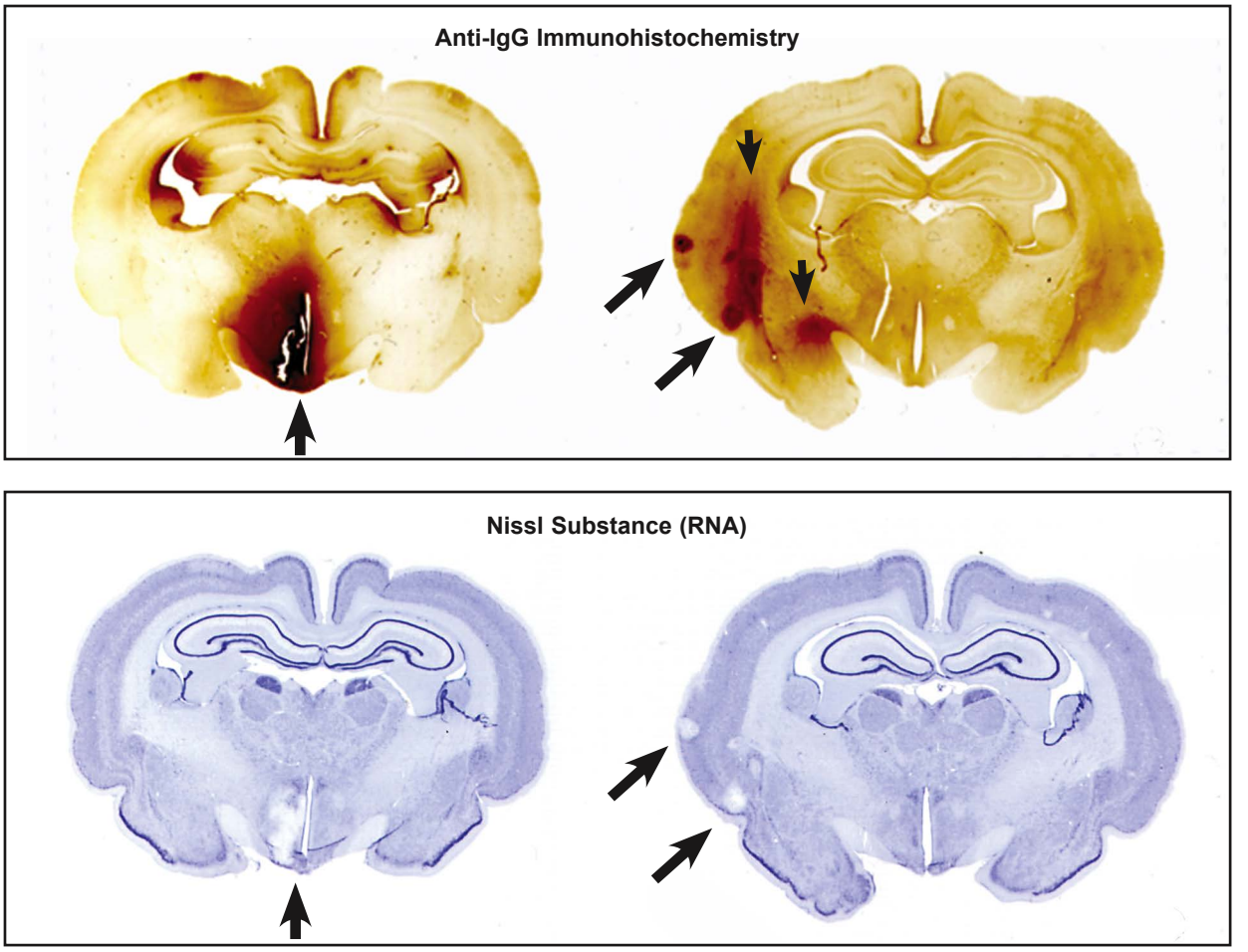
BLOOD BRAIN BARRIER (BBB) COMPROMISE DETECTION (continued)

The images in each panel below show sections from the brains of two different rabbits that were subjected to fine air emboli to create multiple infarctions. In the top panel, the locations of BBB compromise are seen as deep amber-colored areas. In the bottom panel, the lesion sites are seen by an absence of staining of cell bodies. The arrows illustrate some of the infarction sites in each of the differently stained, near adjacent sections. In the section on the left, the rather large halo of IgG staining goes beyond the lesion site near the third ventricle, as revealed with the Nissl stain below it. On the other hand, in the section on the right, the angled arrows show two distinct sites of IgG staining that correspond in size to the unstained patches in the Nissl section.

Other zones of IgG staining do not correspond to a loss of Nissl staining, perhaps illustrating BBB compromise which is unaccompanied by the loss of cells. For example, note the large area in the right section that encompasses the left external capsule, as well as the area adjacent to the left optic nerve (vertical arrows).



NSA staff hypothesizes that the BBB may not merely be open or shut but may be opened to varying degrees such that molecules of different sizes would accordingly penetrate different degrees. For a given time after BBB compromise, small serum proteins may be found at a greater radius from a given site than larger proteins, i.e., IgGs.

BLOOD BRAIN BARRIER (BBB) COMPROMISE DETECTION (continued)

CO_2 SACRIFICE

There is evidence that sacrifice by CO_2 causes the BBB to open during the sacrifice process. When conducting a BBB compromise study, the impact of this event could serve to give a false indication of brain penetration for a particular compound, as the compound may not have crossed the BBB under normal circumstances. We surmise that the extreme stress, induction of acidosis and lack of oxygen during the several minutes before death result in a breakdown of the BBB. If the brain is not removed immediately or soon after cessation of breathing, IgG serum proteins may enter the brain parenchyma. The images below depict the difference between a normal IgG antibody staining pattern of an anesthetized, perfused mouse versus abnormal IgG antibody staining seen from a mouse sacrificed by CO_2 suffocation. The brain was not removed immediately after death.

The appearance of IgG staining in normal (mouse) brain shows only some tincture in periventricular structures and other areas known to have leaky BBB, such as the median eminence and area postrema.

Brain from a mouse sacrificed by CO_2 suffocation and brain was not removed and placed in fix soon after death. Widespread IgG staining results due to extravasation of serum proteins including IgGs.

NSA RECOMMENDATION: If the intactness of the BBB is to be studied, the animals should be anesthetized with a barbiturate such as Nembutal, then perfused transcardially with a suitable aldehyde fix. Note that use of gaseous anesthetics such as Fluothane tends to prevent flushing of red blood cells from the brain vasculature.

References of potential interest:

Use of anti-IgG to detect BBB breakdown:
Richmond, J.D., et al. Induction of heme oxygenase-1 after hyperosmotic opening of the blood-brain barrier. *Br Research* 780: 108–118, 1998

References of interest within this paper:
37) Rapoport, S.I., et al. Quantitative aspects of reversible osmotic opening of the blood-brain barrier. *Am J Physiol* 238: R421–R431, 1980
45) Tanno, H., et. al. Breakdown of the blood-brain barrier after fluid percussive head injury in the rat. Part I. Distribution and time course of protein extravasation. *J Neurotrauma* 9: 21–32, 1992

Other serum proteins (albumin, fibrinogen, fibronectin):
Yu, W.R., et al. Systemic hypothermia following compression injury of rat spinal cord: reduction of plasma protein extravasation demonstrated by immunohistochemistry. *Acta Neuropath (Berl)* 98: 15–21, 1999

Entry of other agents:
Muldoon, L.L., et al. Comparison of intracerebral inoculation and osmotic blood-brain barrier disruption for delivery of adenovirus, herpes virus, and iron oxide particles to normal rat brain. *Am J Path* 147: 1840–1851, 1995

APPLYING NSA SERVICES IN RESEARCH

ELECTRODE TRACT DETECTION

Applications:

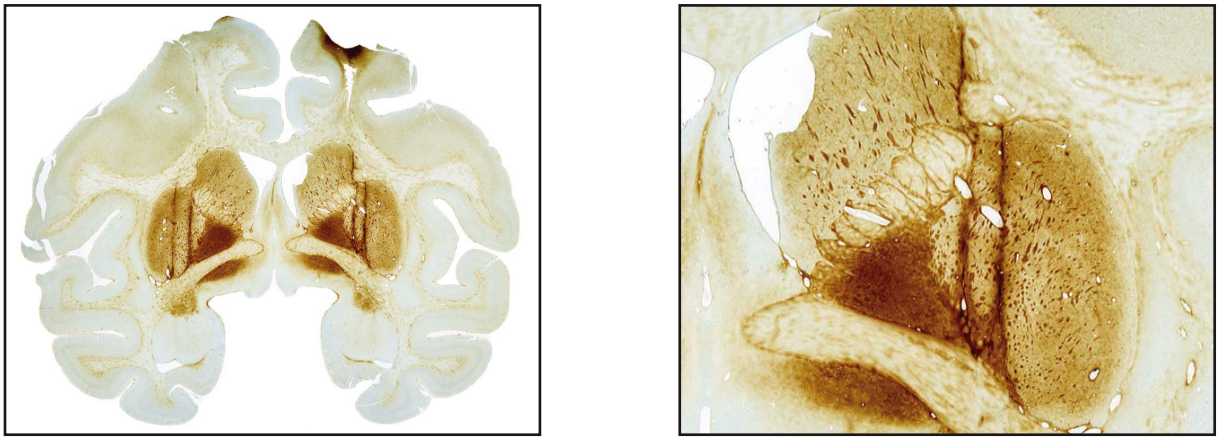
- Electrodes or injection are commonly made into the brain or spinal cord. Determining the locations is essential for the recording of brain electrical events and for injections of an experimental substance.

Advantages:

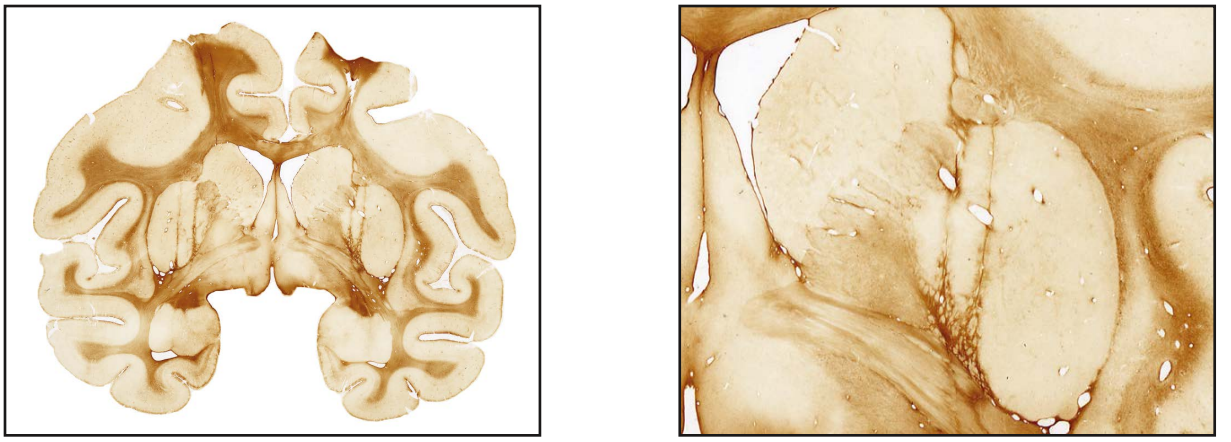
- The penetration into the brain or spinal cord by even the finest electrode or needle inadvertently nicks capillaries causes a small amount of blood to enter the parenchyma. The iron content of the red blood cells is soon released and remains in that location virtually permanently. Detection of the presence of the iron becomes an excellent means of revealing the tract and end points of electrodes and injection needles.In conjunction with the presence of the iron, the astrocytes in that locale are rendered in a persistent reactive state. Detection of this reactivity with staining with a GFAP antibody provides a means to cross check the iron date.

Specifications:

- Transcardial perfusion with fix and execution of the Perls reaction histochemical procedure (for ferric iron) ensures successful tract identification. Standard IHC methods for applying the GFAP antibody also allows for successful acquisition of data.



Perls-DAB Stain



GFAP Stain

APPLYING NSA SERVICES IN RESEARCH

STEM CELL DETECTION

Applications:

- Stem cells are introduced into a living host to often achieve changes in the host's composition. E.g. to replace missing/lost cells; replace cells with absent or faulty protein production.

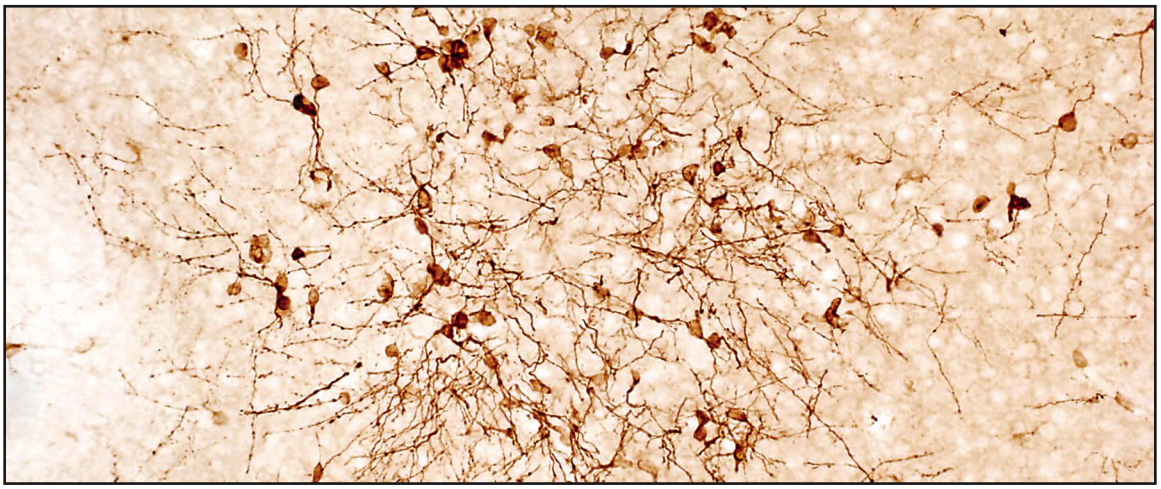
Advantages:

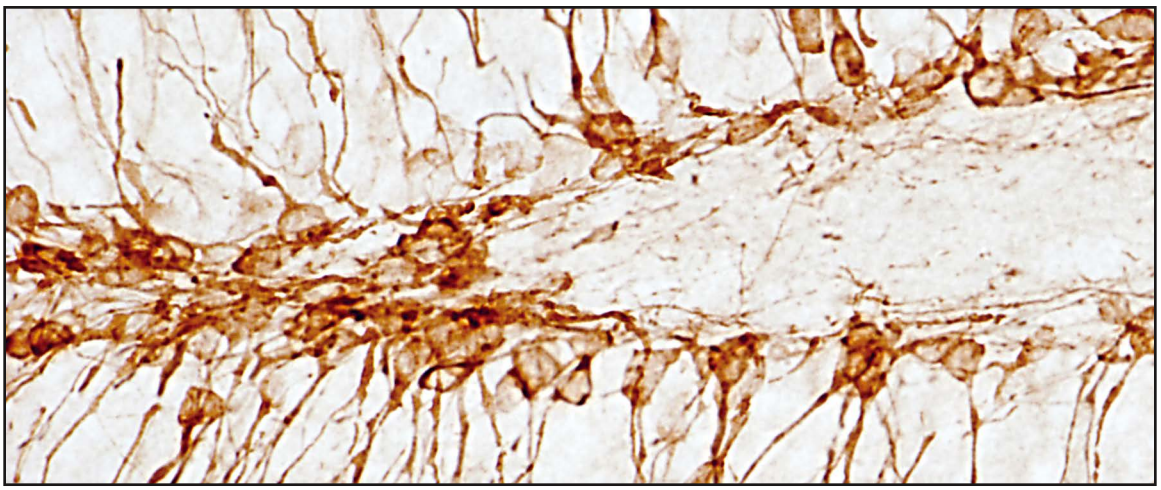
- The success of stem cell introduction is measured by a number of possible means but the initial goal is that the stem cells were placed in the correct location; that they survive and produce the changes intended. This assessment is accomplished by histologic examination of the hosts after different lapses in time after stem cell introduction.

Specifications:

- Optimal fixation of the host brains by transcardial perfusion optimizes the conditions to ensure successful identification of the stem cells in the host animal.

Identification of Juvenile Neurons: Doublecortin (dbx) IHC Reveals Neurons That Are <7 Days Old



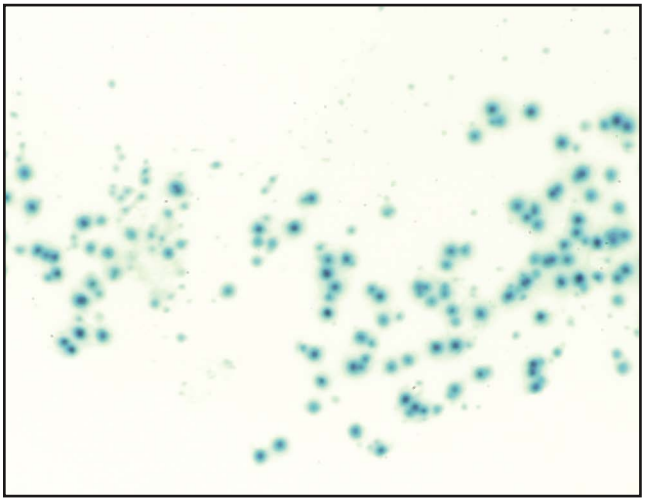


APPLYING NSA SERVICES IN RESEARCH

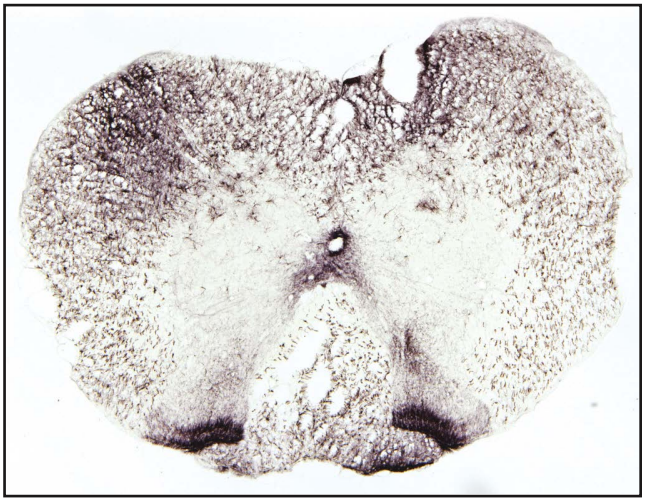
STEM CELL DETECTION (continued)

Stem Cell Location

A fundamental measure of efficacy for stem cell treatments is to determine whether the stem cells reached the desired location in the central nervous system. Various immunohistochemistry probes can be used to detect signature markers of the stem cells (such as a unique human protein). Also stem cells can be deliberately made to produce markers such as β-Galactosidase, green fluorescent protein or others so that subsequent staining is able to highlight the location of introduced stem cells.



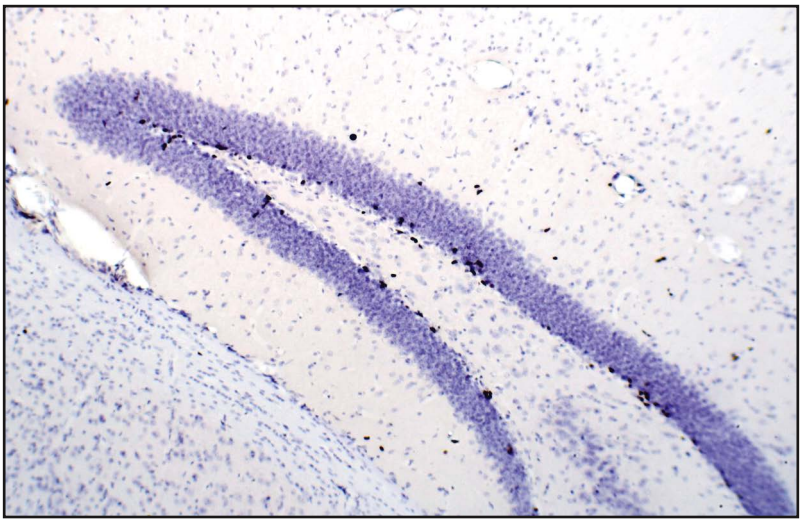
Cells were tagged with β-Galactosidase, revealed by X-Gal histochemistry, demonstrates a successful localization of the newly placed cells.



STEM 121 (human stem cells) in mouse spinal cord

Stem Cell Activity Example: Bromodeoxyuridine (BrdU) IHC

For some stem cell therapies, it is desirable for the cells to undergo a limited degree of division within the target organ. BrdU injection into the host animal (ip) before sacrifice (e.g. 6-12h) will allow the BrdU to be incorporated into newly formed DNA of dividing cells. BrdU masquerades for thmydine, which is only used in DNA synthesis. New cells created after the BrdU injection will contain BrdU which is detected and quantified with an antibody against BrdU.



The darkly stained cells (dots), mostly along the inner edge of the "arrowhead" of the dentate formation indicate DNA synthesis activity. In this manner, cell division can be witnessed and quantified. The purple staining is normal cell body background stained for anatomical reference.

NSA NEUROSAFETY™ TESTING

OVERVIEW

See NSALabs.com for our "Neurotoxicity Assessment Presentation 2015".
Go to NSALabs.com > Reference > Papers and Reference Materials and select the link.

NeuroScience Associates was founded in 1989 with the primary goal of delivering neurohistologic safety testing services to researchers worldwide. While NSA has expanded significantly in both size and breadth of offerings since that time and provides a full suite of neurohistology services including R&D discovery and efficacy of therapies, safety testing has continued to be a primary service for over 28 years. NSA's unique approaches to testing capitalize on the capabilities of contemporary neurohistologic techniques thereby increasing the reliability of results. Reductions in the number of stains, the number of animals, the amount of time necessary to achieve results, and the amount of time and money required to conduct the safety tests are among the many benefits of working with our staff of experts.

Safety testing can take many forms depending on the phase of research, the mechanism of the test article and the audience for which the testing is being performed. NSA recognizes these differences, works with clients to assure their specific needs are met, and accommodates the very specific needs of a client's predefined protocol, as well as requests from regulatory agencies such as FDA and EPA.

Approach

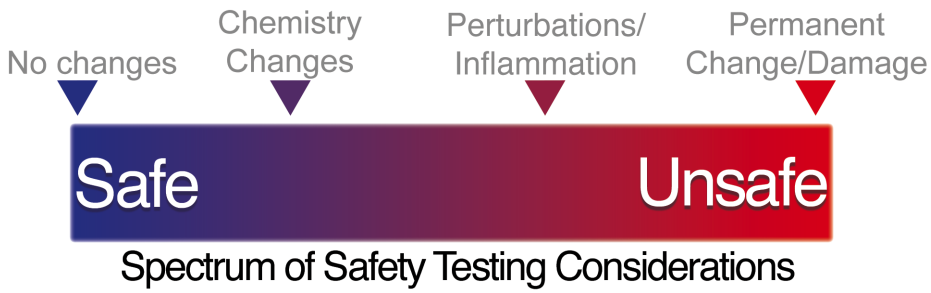
There is no single approach to every neurologic safety testing need. Rather, a toolset of approaches exists from which each safety assessment study can choose the tools most appropriate to the study.

Behavioral vs. Pathological Assessments

Behavioral and pathologic tests have their own unique strengths and challenges. These are complementary approaches in the realm of safety testing and should be employed in a manner suited to each.

Spectrum of Pathologic Endpoint Detection

Unfortunately, there is not a single endpoint that is both valid and comprehensive for meeting the needs of all pathologic safety evaluations. Rather, there is a spectrum of safety considerations in the pathology of the brain. At one end of the spectrum, there are no detectable changes to the Central Nervous System (CNS) (safe), and at the other end of the spectrum are permanent changes to the CNS (unsafe). Between these two extremes, for example, are detectable changes in chemistry, neurotransmitter responses and perturbations leading to an inflammatory response. In the illustration below, the farther a change falls to the right of the spectrum of changes, the more serious is the concern that change represents from a safety perspective. At the left end of the spectrum (i.e., chemistry changes), there are many potential pathologic endpoints from which to choose. Moving to the right (unsafe) side of the spectrum, the number of detection methods decreases and endpoints are more specific and definitive as a safety concern. While each safety study has different approaches, the best designs include evaluation of a selected point on this spectrum as well as assessments to the right of that point. Many designs focus on merely the most conclusive endpoint of permanent damage at the far right of the spectrum.



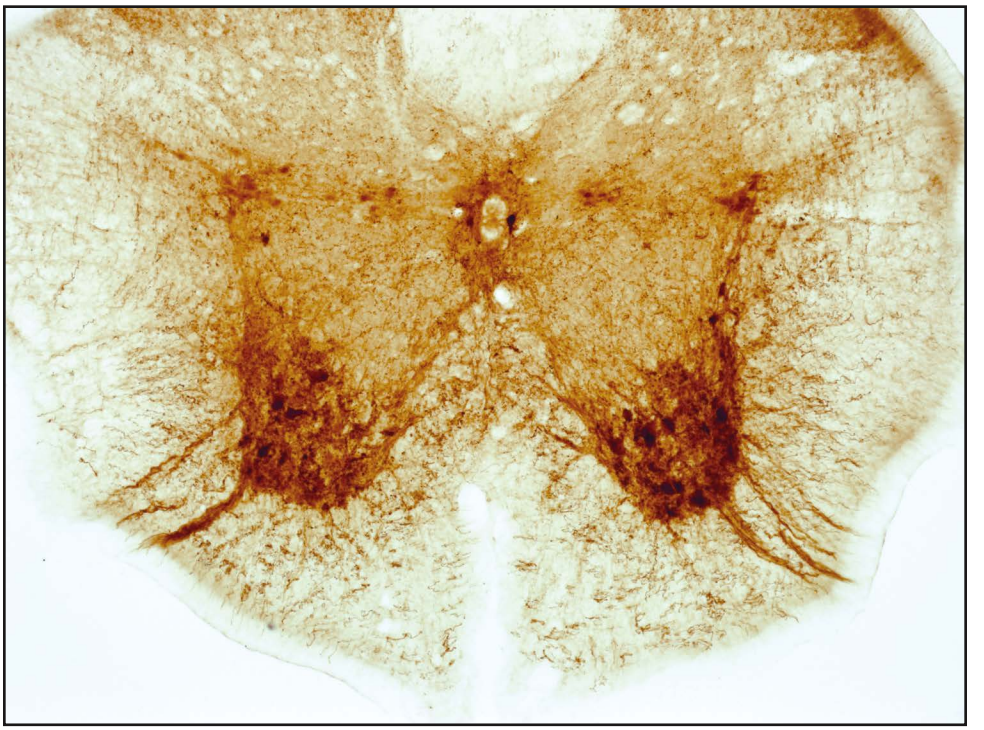
Spectrum of Safety Testing Considerations

A successful safety study must first determine the scope of changes to be evaluated, then choose the methods appropriate to evaluate those chosen endpoints.

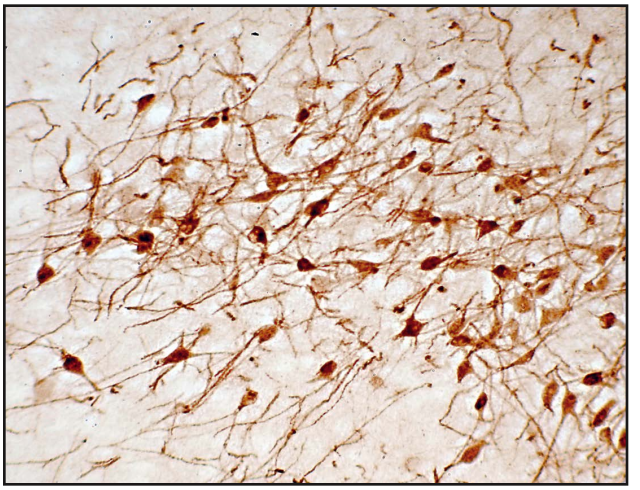
NSA NEUROSAFETY™ TESTING

CHEMISTRY OR OTHER CHANGES FROM NORMAL

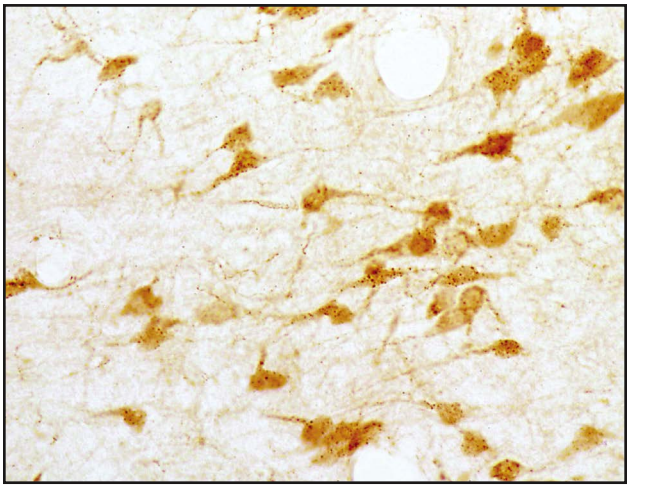
In its most literal form, neurotoxicity could be defined as any departure from normal in the CNS. However, all departures from normal or changes in CNS are not negative. In fact, many compounds deliberately cause changes to the CNS as therapy. Judgment is required as to what changes constitute a negative impact and should be evaluated in the scope of a safety study. Most often, compounds that rely on a specific mechanism of action are evaluated with respect to their impact on the brain relative to that mechanism to make sure intended effects are not accompanied by unintended ones. There are an incredible number of endpoints that could be evaluated to include a departure from normal. H&E is an example of one of the many tools used to evaluate general changes in the brain. Another common approach is to evaluate endpoints specific to the known mechanism of a compound specifically quantifying the impact on the brain. Examples of endpoints that are evaluated to reveal chemistry changes are shown in the images below:



ChAT



Calbindin



Serotonin

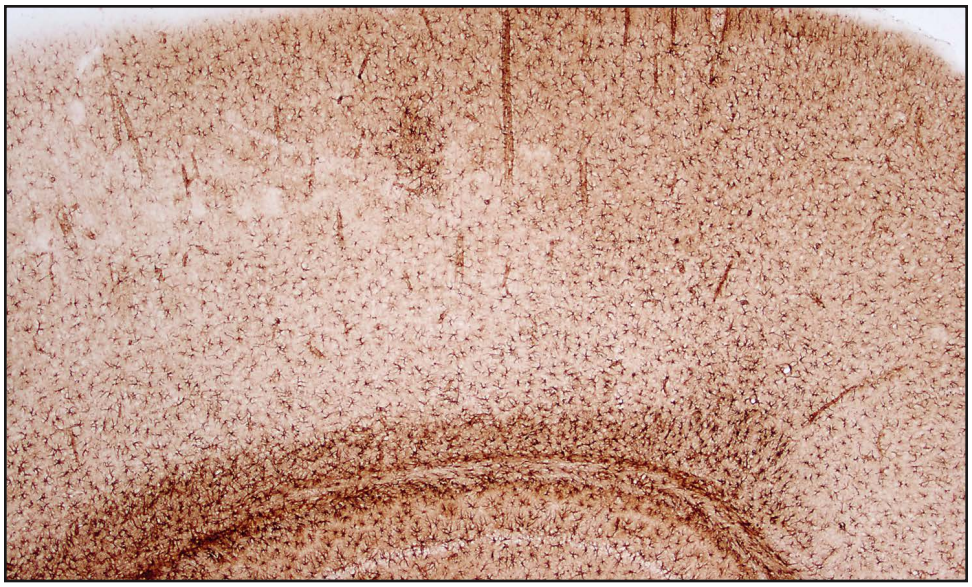
NSA NEUROSAFETY™ TESTING

PERTURBATIONS / INFLAMMATION

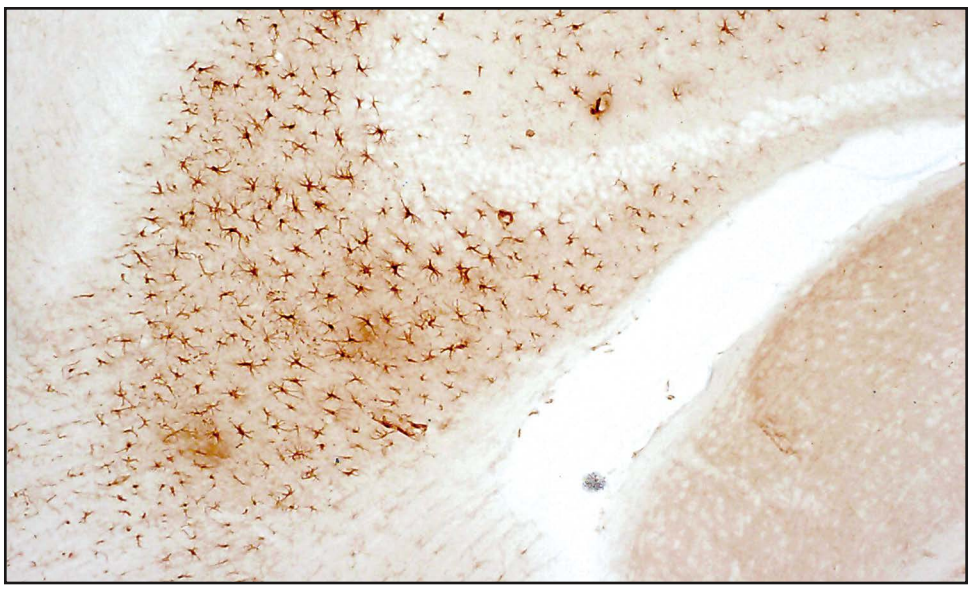
The detection of perturbations in the brain is often employed as a key tool in safety assessments. The presence of a perturbation signal is an undesirable finding in safety testing and represents a degree of harm has been caused to the brain. In some approaches, perturbations are used as an indicator to search for signs of permanent damage, serving as a “canary in a coal mine.” In the context of some studies, the detection of a perturbation in itself is enough to warrant a conclusive safety concern. A perturbation represents a stress to the health of the brain, but a perturbation signal alone does not indicate the infliction of permanent damage to the brain. Other endpoints discussed in the next section are able to differentiate an injury/perturbation that resolves versus an injury that leads to permanent damage.

Astrocyte Perturbations:

GFAP IHC reveals all astrocytes ‘resting’ or reactive and Nestin IHC reveals only reactive astrocytes.



GFAP



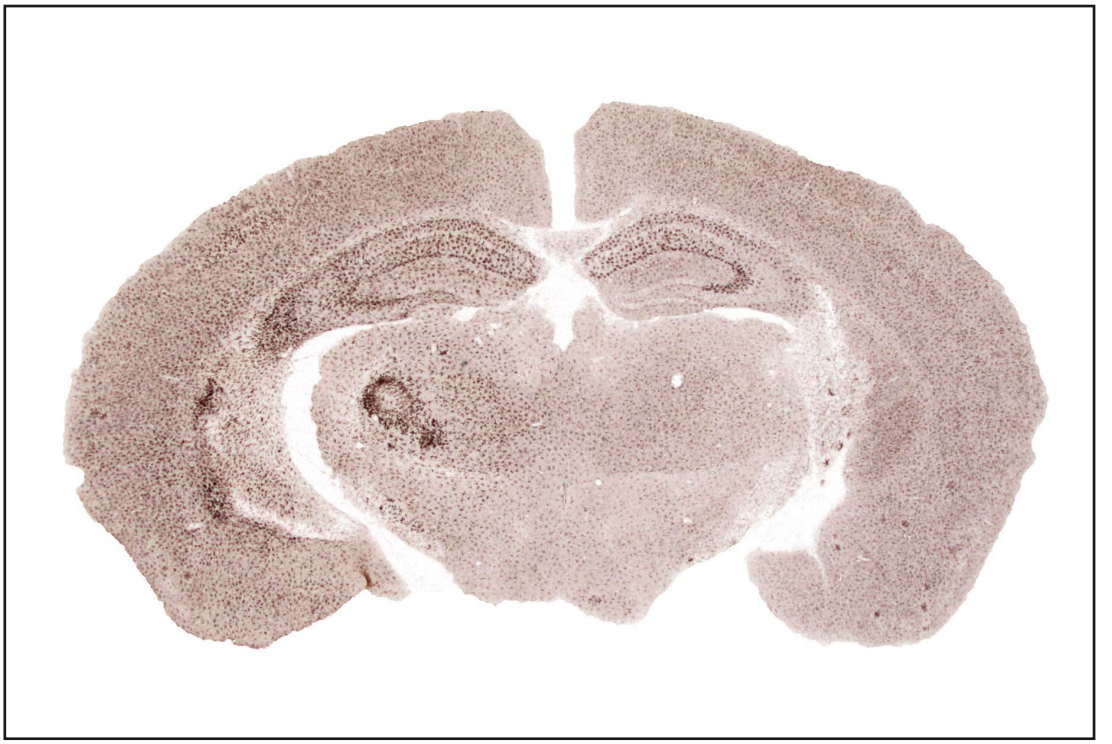
Nestin-Reactive Astrocytes

NSA NEUROSAFETY™ TESTING

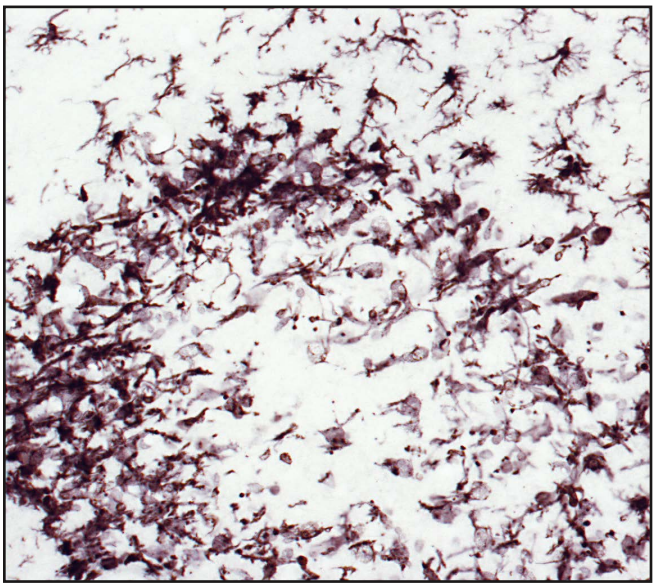
PERTURBATIONS / INFLAMMATION (continued)

Microglia Perturbations

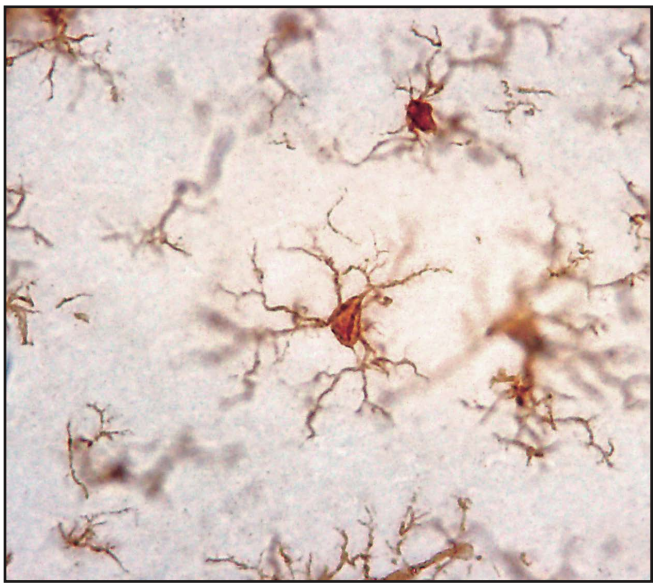
Iba1 IHC and NSA's internally developed Reactive Microglia stain are capable of revealing activated microglia. Reactivity in Iba1 stained sections is determined by hypertrophy of the cells. The Reactive Microglia stain shows cells that are in a reactive state due to an acute perturbation. Microglia in a reactive state due to a chronic perturbation are not visible with this method.



Iba1 IHC



Iba1 Hypertrophied microglia

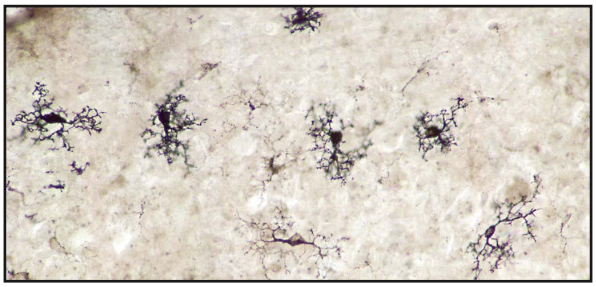


Iba1 Normal microglia

PERTURBATIONS / INFLAMMATION (continued)

NSA's Reactive Microglia Stain

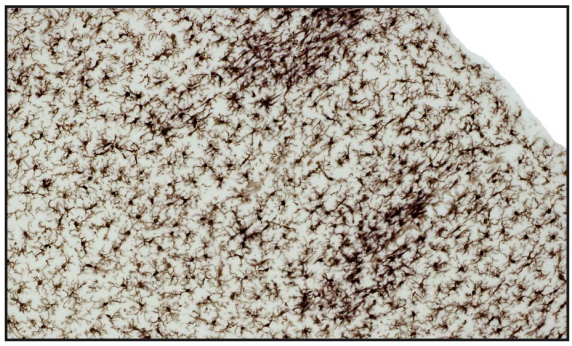
Resident microglia of the brain become reactive following various forms of insult, including chemical trauma, viral infection and physical trauma. The NSA protocol stains reactive microglia but not resting microglia. After staining sections from animals that had a broad range of insults, it was realized that this 'reactive microglia' protocol favors the staining of microglia where the injury was acutely induced. Microglia in animals with a chronic state of perturbation, such as in EAE (experimental allergic encephalitis), failed to stain---later, using the Iba1 antibody, staining revealed abundant microglia in a state of hypertrophy. Consequently, NSA's reactive microglia staining protocol should only be used in animals that have suffered acute injury. In acutely injured brains, NSA's reactive microglia stain reveals the same reactive features found by using the mouse and rat specific CD68 antibodies (FA11 and ED1, respectively) and the isoB4 lectin staining.



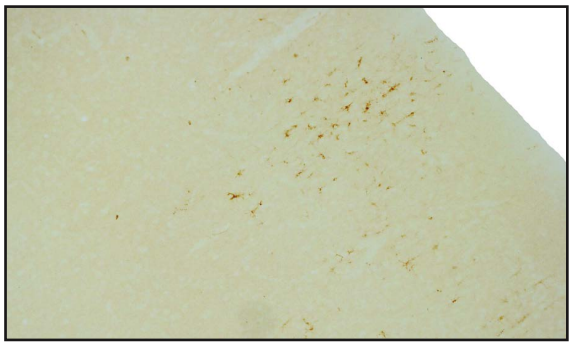
NSA's reactive microglia stain

Iba1 vs CD68

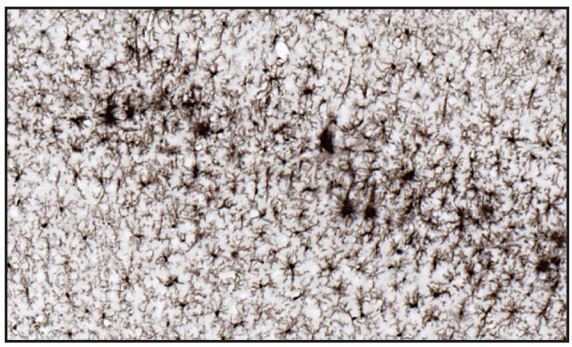
Not all hypertrophied microglia stain positive with CD68.



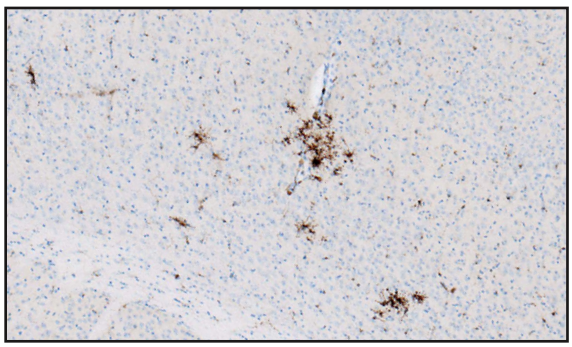
Iba1-hypertrophied microglia in rat cortex



CD68 clone ED-1 (rat)



Iba1-hypertrophied microglia in mouse cortex



CD68 clone FA-11 (mouse)

NSA NEUROSAFETY™ TESTING

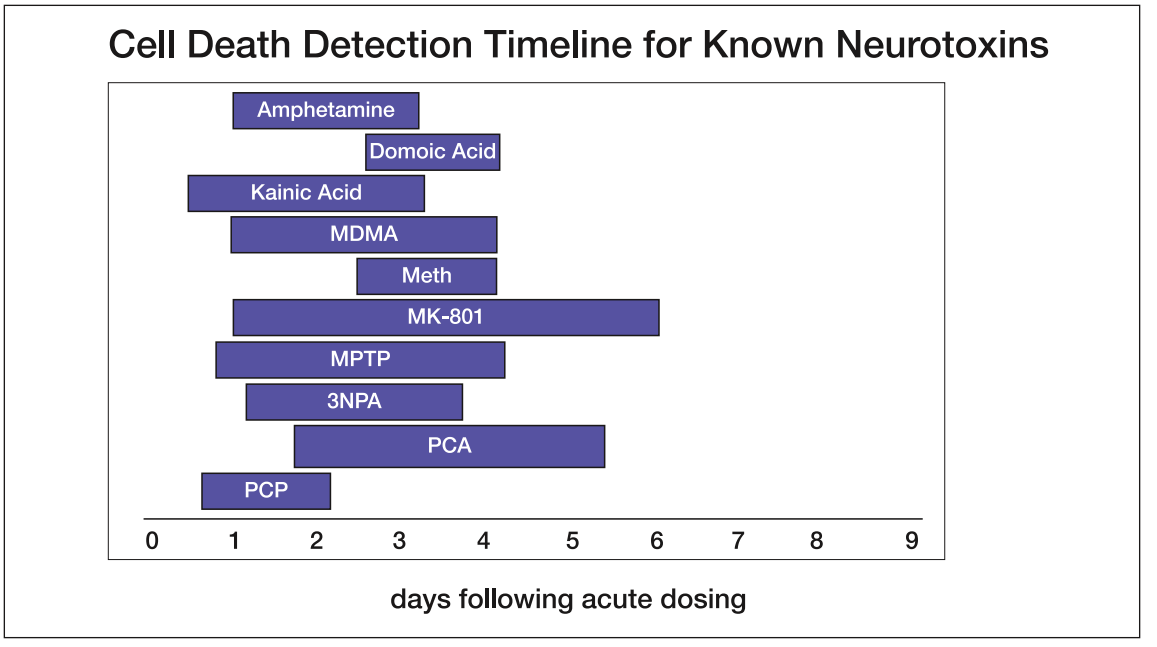
PERMANENT DAMAGE

Many alterations to the brain can be negative and potentially long-lasting, but not all can be considered permanent. Some injuries can be treated or will resolve after time if the source of the injury is removed (i.e., a harmful compound ceases to be administered), while other injuries lead to permanent implications. The most definitive and easy way to interpret evidence of permanent damage is the destruction of neurons. Even though the brain has amazing functional compensatory power, the loss of irreplaceable neurons creates a permanent deficit. All comprehensive safety evaluations at some point need to evaluate the risk of permanent damage and the mechanism of that evaluation is to observe neurodegeneration as it occurs during the cell death cycle.

There are timing and scope considerations in designing the safety study and in selecting a detection method.

Timing

Detection of neurodegeneration requires critical timing considerations. Depending on the detection method chosen, a dying cell can be viewed for only ~2–6 days from the time it begins to disintegrate, after which there is no debris remaining to observe. Therefore, the timing of sacrifice following exposure to a compound is a critical element of study design. The graphic below shows the timeline of the detectability of degenerating neurons for some known neurotoxins. The days indicate the amount of time following a single acute dose on day 0. The bars represent the most probable timing opportunities for detection.



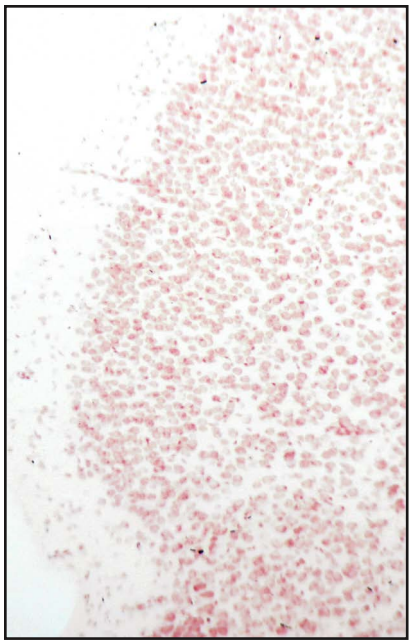
Scope of Elements

The neuron has an extended temporal and spatial footprint when the dendrites, axon terminals and axons are evaluated in addition to the neuron cell body itself. The class of stains called the disintegrative degeneration stains is able to detect disintegration of each of these elements, while other methods capable of detecting cell death only reveal pathology of the cell body itself. Disintegrative degeneration stains include various Fluoro-Jade and Cupric Silver methods. NSA routinely performs the disintegrative degeneration stains as well as methods such as H&E.

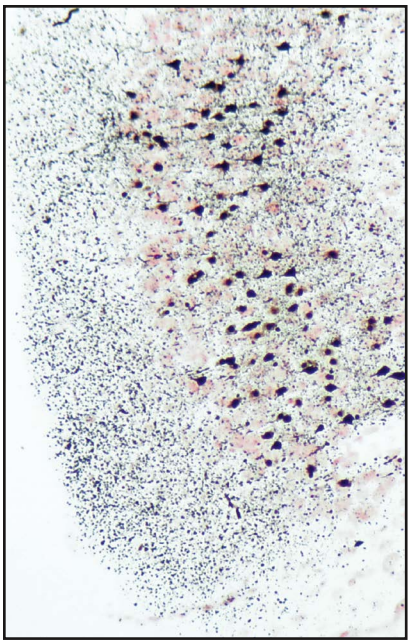
NSA NEUROSAFETY™ TESTING

PERMANENT DAMAGE / SCOPE OF ELEMENTS (continued)

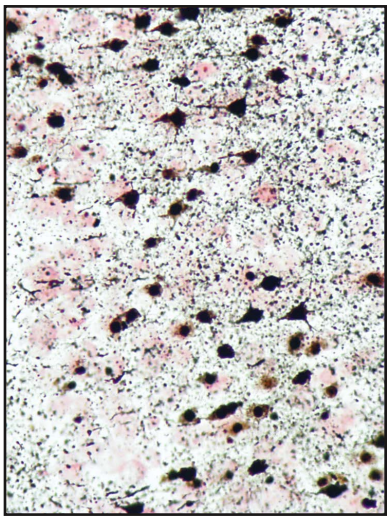
Features visible in a Full Scope Disintegrative Degeneration Stain (Amino CuAg) compared to a Cell Body–Only Stain (H&E)



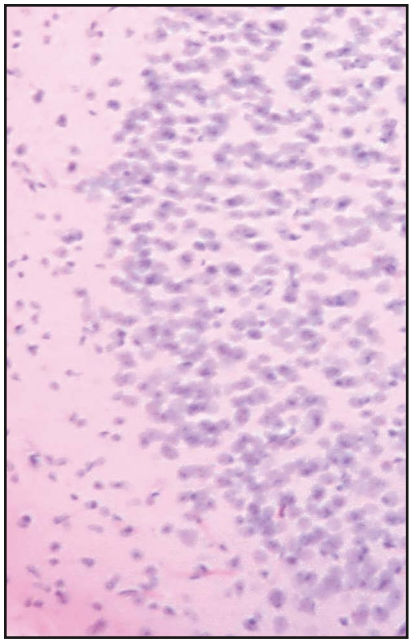
Amino CuAg Control



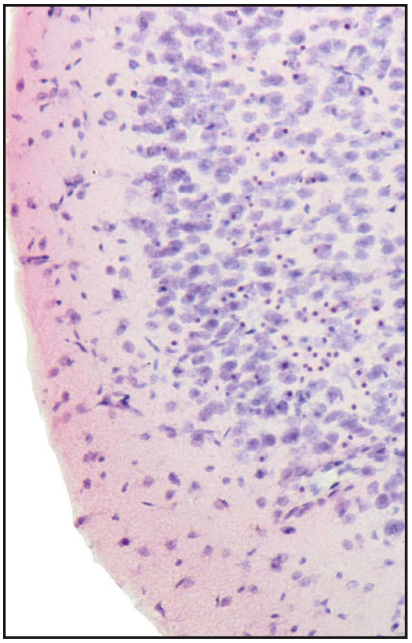
Amino CuAg Affected



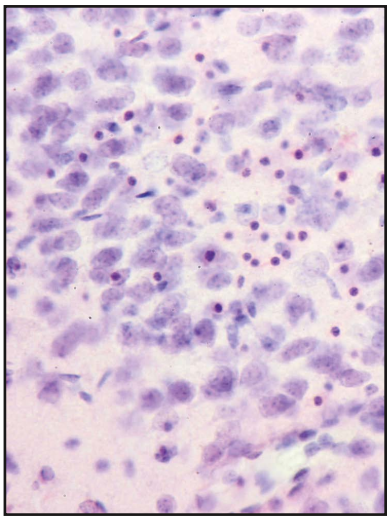
Amino CuAg Affected



H&E Control



H&E Affected



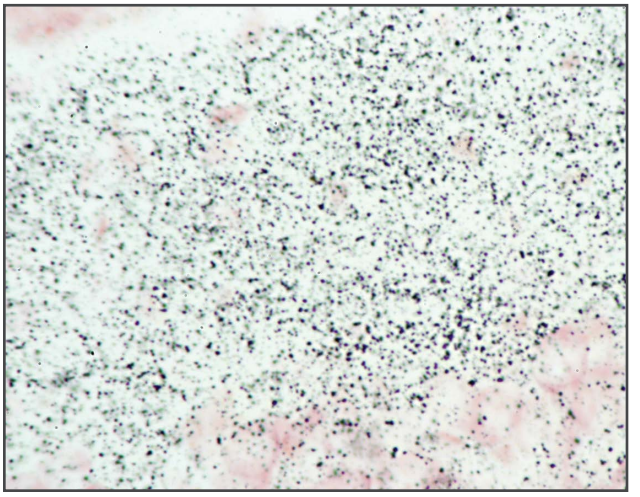
H&E Affected

In the Amino Cupric Silver–stained section, the disintegrating cell bodies are clearly visible, and the degenerating synaptic terminals, dendritic debris and axons are also conspicuous. In the H&E-stained section, the damage is far less obvious and the characteristic eosinophilia of the cytoplasm and pyknotic nuclei of affected cells requires higher magnification to be distinct, as shown on the right. While it is possible to witness cell death with either category of stain, the degeneration stains provide a higher contrast signal leading to cost and time efficiencies during analysis.

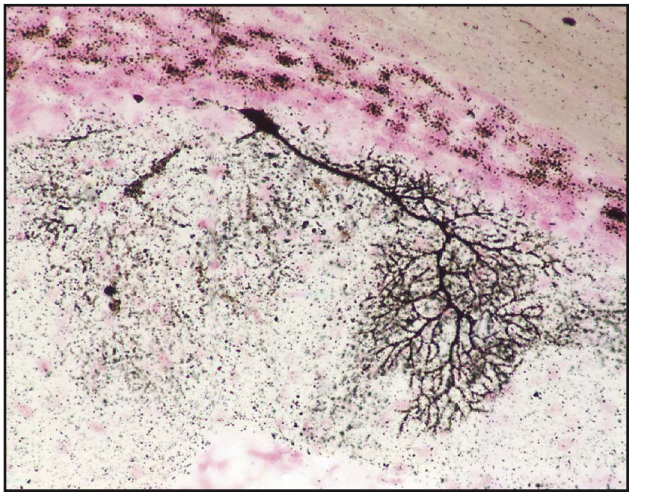
NSA NEUROSAFETY™ TESTING

PERMANENT DAMAGE / SCOPE OF ELEMENTS (continued)

Features visible in the Amino CuAg Disintegrative Degeneration–stained sections

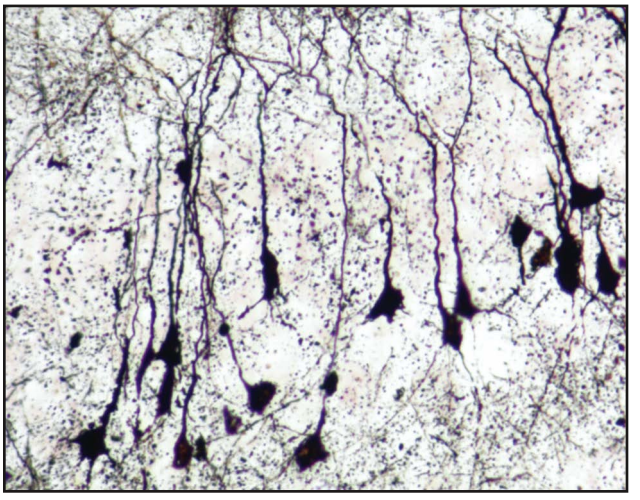


Degenerating Synaptic Terminals

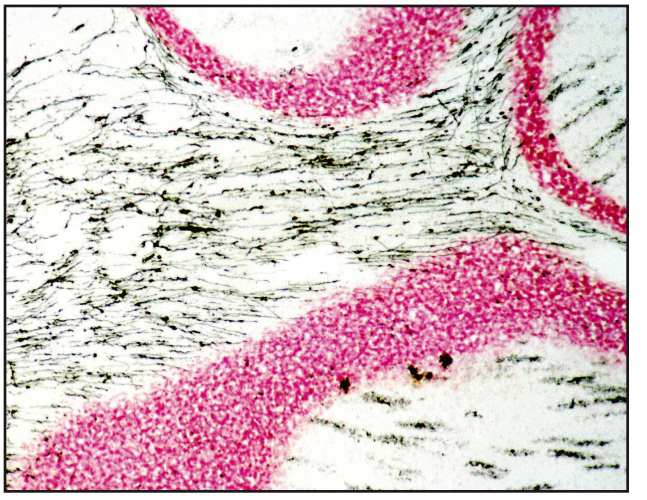


Degenerating Dendrites

Of all four degenerated elements shown on this page, only the cell bodies in the picture below (not including the dendrites) are visible with a cell body stain such as H&E.



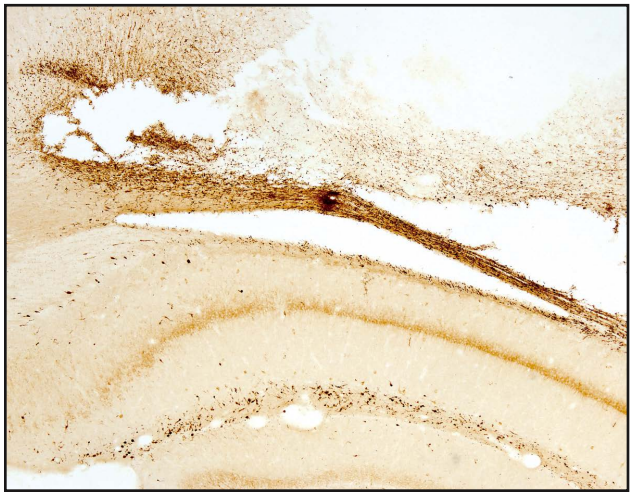
Degenerating Cell Bodies



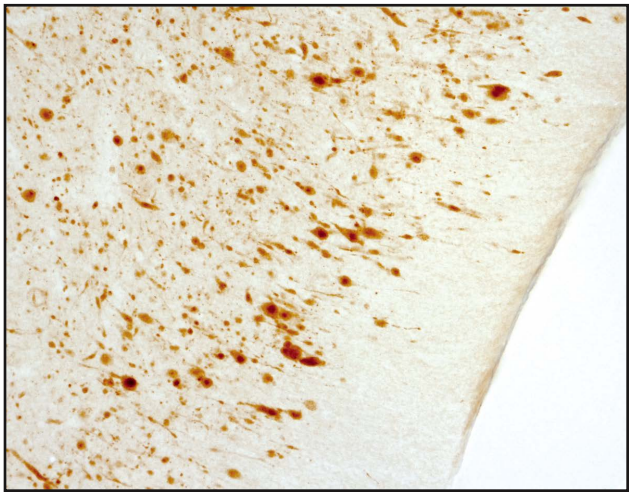
Degenerating Axons

PERMANENT DAMAGE / SCOPE OF ELEMENTS (continued)

Beta-Amyloid Precursor Protein (ß-APP)



Beta-Amyloid Precursor Protein (ß-APP), rat TBI model, corpus callosum and cortex



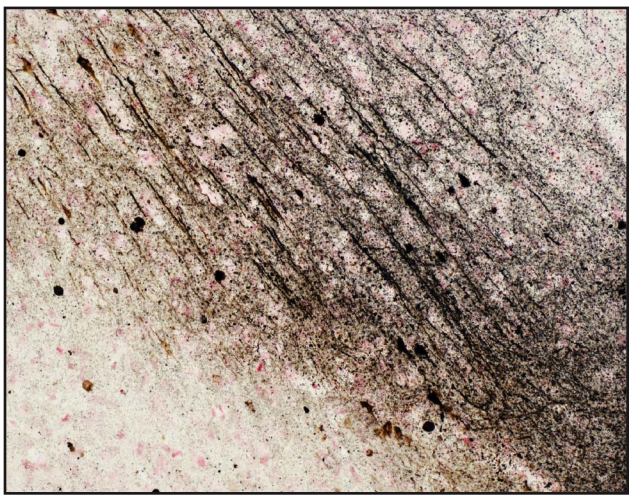
ß-APP, **rat hippocampus**

Beta-Amyloid Precursor Protein (ß-APP) has become a useful marker for axonal injury and commonly termed: 'diffuse axon injury'. ß-APP is carried out by fast anterograde transport vesicles to distal sites in the axon. Upon axonal injury, ß-APP pools in areas of impaired transport.

Alternative means of detecting axon damage use the disintegrative degeneration stains (most notably, the amino cupric silver (ACS) method of deOlmos). Adjacent sections stained with the ACS stain and for ß-APP reveal quite different degrees of staining: much more axonal injury/damage is shown by the ACS suggesting that the ß-APP staining is revealing a totally different form of damage or a subset of what is diplayed in the ACS stain.

Amino Cupric Silver Stain

Amino Cupric Silver Stain- rat TBI model, corpus callosum and cortex



Amino Cupric Silver Stain Rat Cortex

NSA NEUROSAFETY™ TESTING

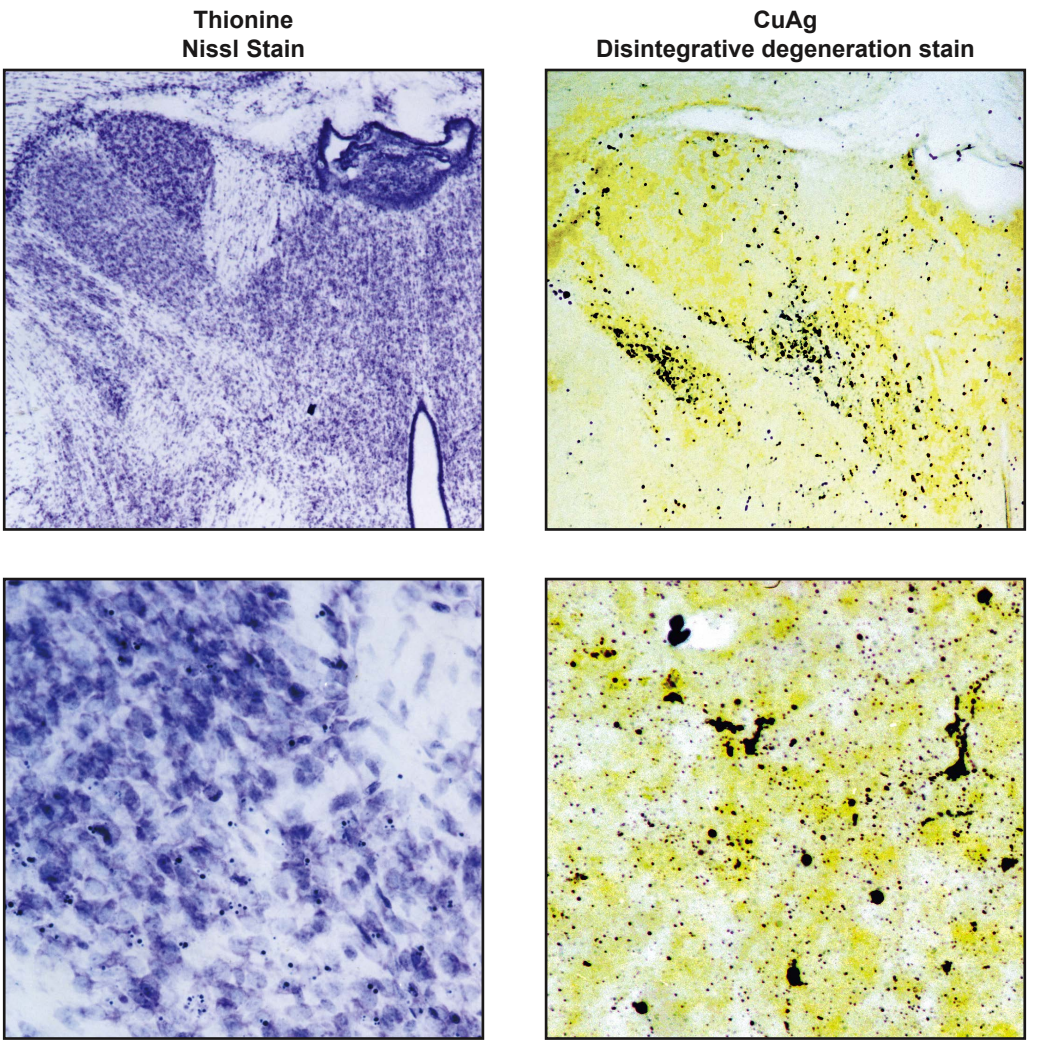
NEONATE AND JUVENILE

Developmental Neurotoxicity Assessment: Neonatal Programmed Cell Death

The assessment of permanent damage in neonates and juveniles is difficult due to the normal occurrence of programmed cell death in the developing brain, and timelines for disintegration (and the corresponding windows to view these) are shortened from days to hours. Patterns of degeneration must be compared to a baseline level to determine changes in cell death activity. Architecting a study design to account for the earlier and shorter observable opportunity of cell death becomes a critical consideration.

The disruption of the chronology of programmed cell death (apoptosis) in the developing brain is an increasing issue of concern. As an example, the Disintegrative degeneration stains, i.e. CuAg have been used to show how alcohol causes extensive cell death in neonate rats (Ikonomidou et al. *Science* 257: 1056, 2000).

Adjacent sections from neonate rat brain stained by NSA

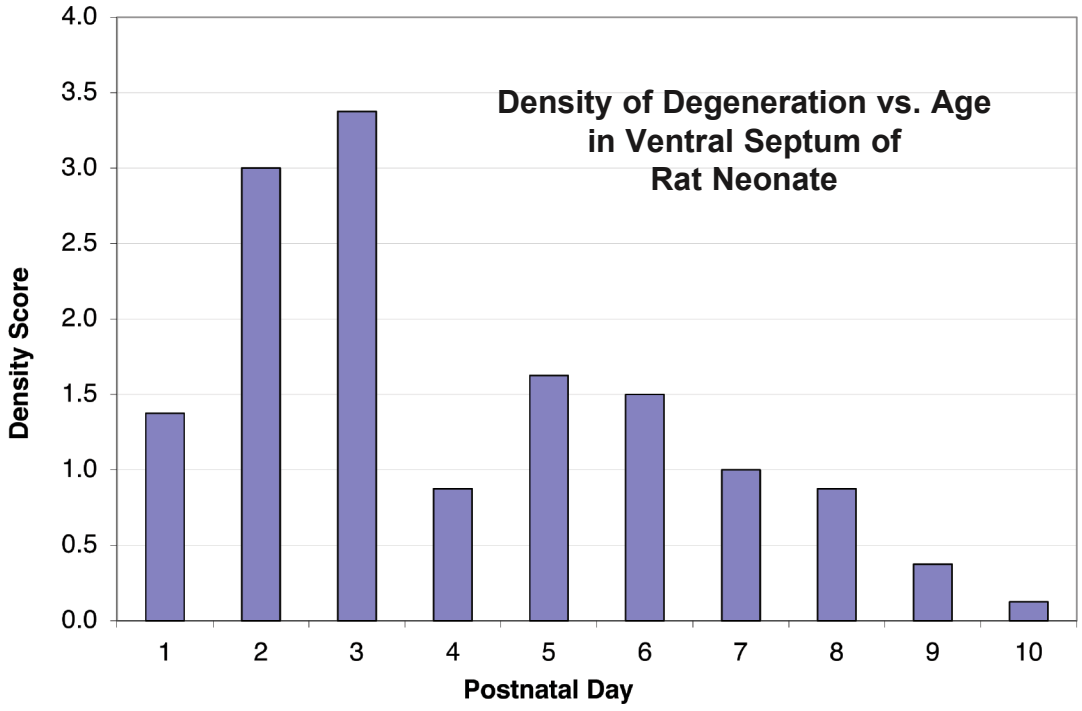


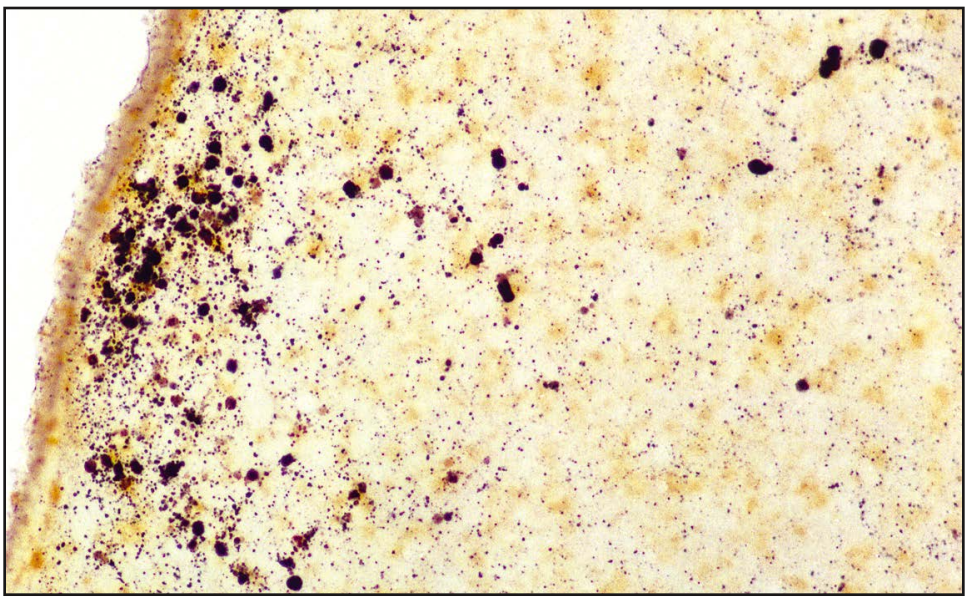
The top two images are from two adjacent sections of the anterior thalamus from a 3 day old rat in which abundant programmed cell death (apoptosis) is taking place. The upper left image is stained for nissl substance to show neuron cell bodies and the upper right with the cupric silver stain to show disintegrative degeneration-seen as black dots. The ease of detection of degeneration in the cupric silver stained section vs the cell body only nissl stain is quite clear. A more highly magnified view of both of these stains is shown in the lower two images. Numerous dark blue dots can be detected among normal cells in the nissl stained image which are the pyknotic nuclei cells undergoing apoptosis. In the lower right image the black irregular profiles of degenerating cells are quite conspicuous and easily detected.

NSA NEUROSAFETY™ TESTING

NEONATE AND JUVENILE (continued)

The temporal histogram below illustrates peak times of cell death in the ventral septum of rat brain. The tabulation of all neuronal populations will identify critical periods during which brain development might be perturbed.





Degeneration in the Lateral Septum; Postnatal Day 3

NSA NEUROSAFETY™ TESTING

PREDEFINED PROTOCOLS

NSA has generic protocols for meeting common safety testing needs, as well as offering assistance in the design and execution of custom safety testing protocols. NSA offers two general approaches to neuropathologic safety testing: “NeuroSafety™ Certification” and “Neurotoxicity Screening.” Additionally, NSA has developed a packaged certification protocol for meeting FDA requirements for N-methyl-D-aspartic acid (NMDA) receptor antagonists.

NeuroSafety™ Certification

Certification studies are GLP-compliant protocols designed specifically to evaluate compounds in order to meet regulatory requirements. NSA has predefined protocols that are designed to meet the needs of most clients. These study designs are then customized to meet specific regulatory requirements or to address specific safety concerns of a particular class of compound.

Neurotoxicity Screening

While NeuroSafety™ Certifications are performed as a requirement intended to prove the safety of a compound, Neurotoxicity Screens are conducted by NSA using the Amino CuAg Disintegrative degeneration stain, a strategic tool during the R&D cycle with the goal of identifying high-risk candidates early. Neurotoxicity screens are rapid, inexpensive tests conducted during preclinical development stages designed to offer valuable decision-making data. Eliminating non-viable compounds early offers a huge potential for R&D cost savings:

> **Janet Woodcock, Former FDA Director of Center for Drug Evaluation and Research (CDER) stated:**
> *“There is not enough research in finding new methods of understanding toxicology at the basic scientific level.... Improving any one of these issues could achieve a 10% improvement in predicting failures before clinical trials and could save $100 million in development costs per drug.”*

NSA’s Approach to Neurotoxicity Screening Saves Companies Time and Money

NSA’s screening protocols reduce development cycle times and regulatory approval timelines and enable more effective use of R&D resources. The protocols provide a new mechanism to “weed out” non-viable compounds. Resources may instead be directed toward modifying non-viable compounds, refocused on viable candidates or reassigned to new candidates.

Early Indicator for Neurotoxicity Screening

NSA has developed a ‘turn key’ protocol to determine at an early stage in drug development if candidate compounds are neurotoxic. Multiple compounds can be tested simultaneously. The protocol uses a minimum number of animals; a maximally tolerable dose; the amino cupric silver stain to detect degeneration, and a pathologist’s read and report that dichotomously indicates the presence of degeneration or not.
Contact NSA for more specific details and pricing.

NSA NEUROSAFETY™ TESTING

PREDEFINED PROTOCOLS (continued)

NMDA Receptor Antagonist Certification

The FDA has a testing requirement as specified in an FDA document entitled "**RECOMMENDATIONS FOR THE DEVELOPMENT OF NMDA RECEPTOR ANTAGONISTS**." This is based upon an Agency letter dated January 27, 1995, describing the FDA's current recommendations for development of NMDA receptor antagonists. The requirements in this document are based upon safety concerns discovered with MK-801, and the FDA now provides this document to sponsors that are submitting a compound for approval in this class of compounds. NSA has developed a GLP safety certification study design created specifically to meet the FDA's latest interpretation of these requirements.

Good Laboratory Practices (GLP)

NSA is proficient in performing services compliant with GLP for any of our services. GLP is defined as Good Laboratory Practices as promulgated by USFDA 21CFR Part 58, USEPA and OECD. NSA utilizes an independent auditor with over 25 years QA experience to provide services related to all GLP-regulated studies.

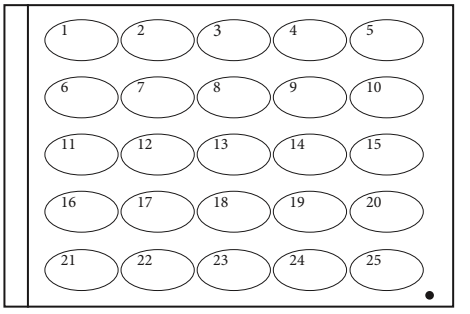
Services provided by the auditor include:

- Inspect facility periodically, provide GLP training to NSA staff, review Standard Operating Procedures (SOPs), review equipment records
- Review protocols, inspect work in progress, audit study data, audit study reports, provide QA Statement to Study Director
- Report inspection findings to NSA management and to Study Director and testing facility QA
- Host visiting QA site visits

See NSALabs.com for our
"Neurotoxicity Assessment Presentation 2015".
Go to NSALabs.com > Reference > Papers and Reference Materials and select the link.

CLIENT RESOURCES

OVERVIEW



Visit our website at NSALabs.com for up-to-date information referenced in this section as well as Frequently Asked Questions, papers, presentations, MultiBrain University™ webinar recordings, and much more!

CLIENT RESOURCES

PLANNING YOUR STUDY

Contact NSA Labs® during the planning phase of your study:

- Benefit from NSA's staff, powered by decades of neurohistology experience, who routinely assist clients with study design for a wide range of research interests. From dosing, to survival times, to animal counts, to stain selections, to analysis, our staff will help shape the details that can make the difference between a successful study and an unsuccessful study.
- Maximize the advantages of NSA's high throughput NeuroTechnologies™ including MultiBrain®, MultiCord® and Large Format™ Technologies.
- Gain valuable assistance in preparing grants.

TISSUE PREPARATION FOR OPTIMAL PROCESSING

NSA tissue preparation protocols are critical to maximize the results of MultiBrain® and MultiCord® processing. Review in detail the perfusion protocols and carefully select the appropriate perfusion solution. Consider the stain(s) that will be executed, including potential stains that may be executed at a later date (see resource of sections on page 6). If your project involves spinal cords, also follow the post-perfusion spinal cord preparation. Pre-evaluations of tissue will be performed by NSA prior to processing to ensure intended results.

Visit our website NSALabs.com and select **Client Resources**|**Tissue Preparation** to view the most up-to-date protocols including:

Perfusion Instructions, Pump Calibration

Post-Perfusion Spinal Cord Preparation

Fixation Method, Solutions*, and Timing

Post-Fixative Buffer Solutions, Storage and Timing

Shipping Preparation and Instructions of Tissue

*High-quality perfusion solutions yield high-quality results. Commercially available formaldehyde solutions are NOT fresh and usually have added ingredients to "stabilize" the solution for a long shelf-life.

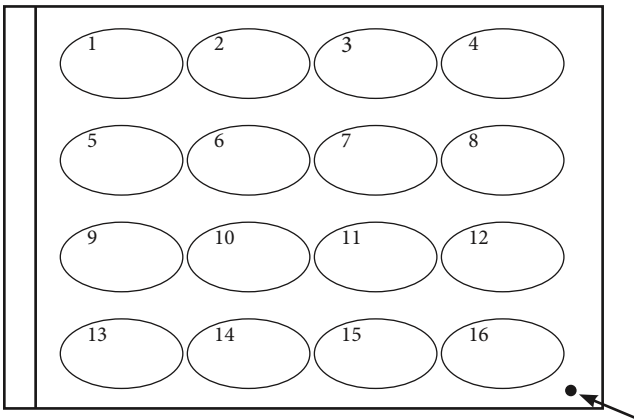
CLIENT RESOURCES

MULTIBRAIN® AND MULTICORD® BLOCK MAPS

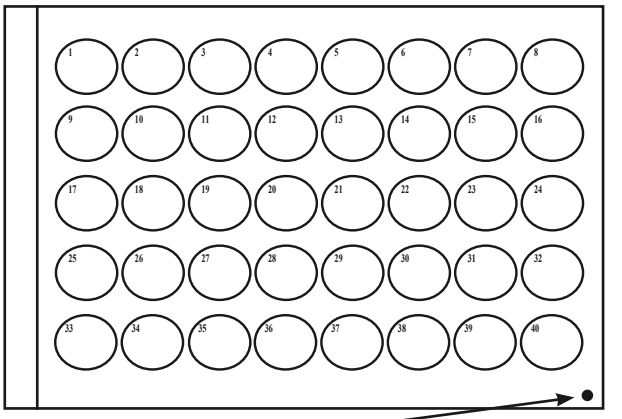
A Full Experiment in One Block

To specify the location of each tissue in the block, complete the appropriate map, email to histology@nsalabs.com, and include a copy with the tissue shipment. Visit our website and choose Client Resources to access the map best suited for your experiment. Below are examples of four common maps.

Rat Brain Block Map:
up to 16 coronally-embedded brains

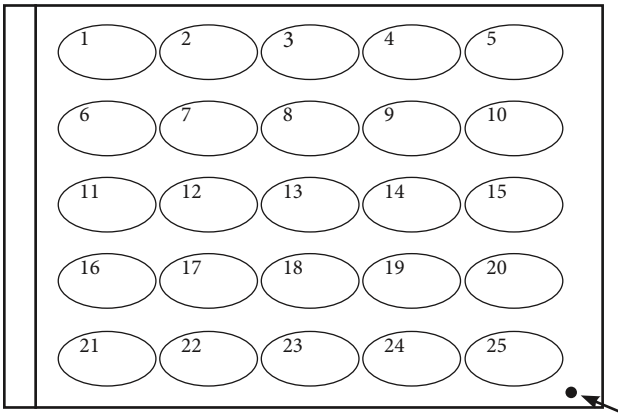


Rodent Spinal Cord Block Map:
up to 40 coronally-embedded cords

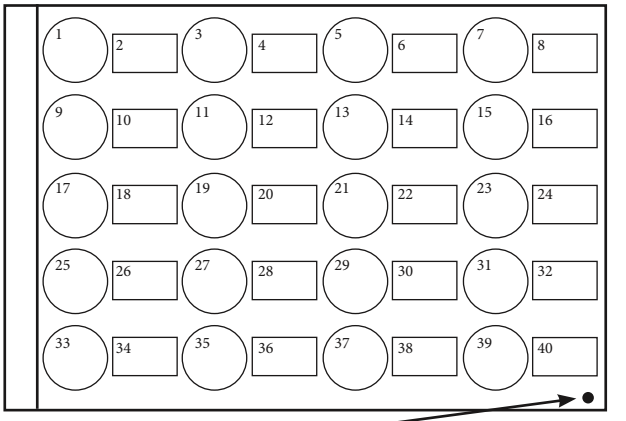


MultiBrain® Reference Markers

Mouse Brain Block Map:
up to 25 coronally-embedded brains



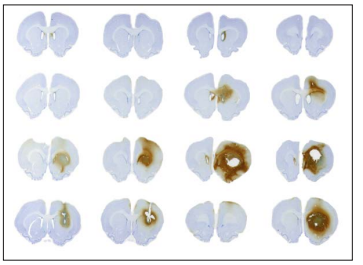
Rodent Spinal Cord Block Map:
up to 40 alternating coronal-horizontal embedded cord segments



MultiBrain® Reference Markers

These maps represent the relative location of individual brain or cord sections on slides. Specifying where each tissue will appear on the slides facilitates comparison across treatment groups and controls. The MultiBrain® Reference Marker preserves left-right orientation. If a Block Map is not provided, the brains/cords will be embedded left to right, in an ascending alphanumeric sequence based on client ID#'s.

Analysis of slides is accelerated through MultiBrain® Technology:



Control

Dose Level A

Dose Level B

Dose Level C

16 rat brains sectioned together in a single block, and stained with the blood brain barrier compromise stain. Note the differences that can be easily observed across groups and doses by having all of the sections on the same MultiBrain® slide.

CLIENT RESOURCES

QUOTE REQUESTS

Each study has a unique set of parameters, requiring a custom quote. Requests can be submitted on our website at NSALabs.com, via email at info@NSALabs.com, or by phone at 865-966-1266. The following information will be discussed:

- **TIME FRAME**: Less than a month; 1-6 months; 1-3 years; Over 3 Years
- **GRANT PROPOSAL**
- **GLP**
- **PROJECT DESCRIPTION** In general: experiments, goals, anything that could impact the quality of the histology
- **SPECIES**
- **TYPE OF TISSUE**: brains, cords
- **STAINING FREQUENCY**: Standard staining frequencies for brains are every 6th for mice, every 8th for rats, every 24th for monkeys and every 33rd for humans
- **ANIMAL COUNT**
- **BRAIN/CORD REMOVAL BY NSA**
- **TISSUE ORIENTATION**: coronal, sagittal, horizontal, other
- **SECTION THICKNESS**: typically 40µ for rat brains, 35µ for mice, 80µ for humans; no less than 25µ for any tissue; no less than 40µ for stereology
- **AREA OF INTEREST / REGION OF INTEREST (AOI/ROI):** Frequently Requested are:
  Brains: Entire
  Cerebrum (no olfactory bulb; no cerebellum)
  Hippocampus
  Striatum
  Striatum through substantia nigra
  Cords: Entire
  CTL (~10mm length each of cervical, thoracic, and lumbar)
  Dot-Dash (Alternating coronal-horizontal segments for area)
- **ANIMAL MODEL**: genotype, vendor and stock number.
- **POSITIVE CONTROL TISSUE** organ, animal, fixation, etc.
- **STAINS AND IMMUNOHISTOCHEMISTRY**
- **CHROMAGEN PREFERENCE FOR IMMUNOHISTOCHEMISTRY**:
  DAB (brown color)
  Nickel-DAB (blue-black color)
  Immunofluorescence: fluorophores (i.e. Alexa 488)
- **CELL POPULATION OF INTEREST**
- **CELL STRUCTURE OF INTEREST**: cytoplasm, nuclei, neuropil, synapsis, axons
- **INJECTION SITES**: Are there any? If so, in which location? include Bregma coordinates
- **SUPPLEMENTAL SERVICES**: Digital Image Capture, Image Analysis, Stereology, Pathology
- **LITERATURE RELATED TO YOUR PROJECT:** Provide links or email files to info@NSALabs.com.

CLIENT RESOURCES: SHIPPING

NSA RECOMMENDS FedEx FOR BOTH DOMESTIC AND INTERNATIONAL SHIPMENTS

SHIPPING ABCs

A. Pack the Tissue to assure the tissue arrives safely and in optimal condition for NSA processing:

1. Use enough solution in each container to minimize airspace. For each rodent tissue, use a minimum 25cc container. Shipping in PBS is safe for all cases, but is required for immunohistochemistry. Consult NSA with questions about which solution is best given your staining needs.
2. Do NOT freeze the tissue or refrigerate it during shipping. Keep the tissue in buffer (consult NSA depending on future staining requirements). Ship tissue at room temperature. **Freeze artifact will ruin tissue.** Do NOT include ANYTHING COLD in shipping container. If the tissue has already been frozen (cryoprotected or not), contact NSA for instructions on how to best handle the tissue in preparation for shipping.
3. It is not recommended to place the tissue in a cryoprotect solution prior to shipping. NSA will use a glycerol- and DMSO-based formulation to cryoprotect the tissue prior to sectioning.
4. Specimen container lids must have a seal on the inside.
5. Each specimen container should be indelibly and uniquely marked. Label both the container and container lid.
6. Wrap each specimen container with parafilm and place into zip-lock or heat-seal plastic bags, so that the solution cannot leak into the box. Line the box with a plastic bag.
7. Use styrofoam, bubble packing or other packing material to completely cushion and protect the specimen containers, especially if using glass containers!
8. If using glass containers, each container should be individually wrapped to prevent breakage.

B. Complete the appropriate MultiBrain® Block Map (see page 90)

C. Ship the specimens overnight when possible:

NeuroScience Associates
Attn: Lab Director
10915 Lake Ridge Drive
Knoxville, TN 37934 USA
865-675-2245

INTERNATIONAL SHIPPERS:

To satisfy USDA Guideline #1103, type the following on your organization's letterhead and place four copies with original signatures on the outside of the box along with the airbill:

1. The contents are fixed, normal rat (or species being shipped) brains (or spinal cords) contained in individual containers. *For example: Sixteen fixed, normal rat brains in a buffered, aqueous preservative, individually contained for tissue analysis. Non-toxic, non-infectious and non-contagious.*
2. They are derived from euthanized animals: a) that have not been exposed to, or inoculated with, any livestock or poultry disease agents exotic to the United States and b) they did not originate from a facility where work with exotic disease agents affecting livestock or avian species is conducted.
3. No antisera is present.
4. The contents are non-toxic, non-infectious and non-contagious.

Check with your shipping provider and/or your government to determine what paperwork and procedures are required based on current laws and the shipping origin.

CLIENT RESOURCES

FREE FLOATING TISSUE STORAGE AND HANDLING

Antigen Preserve Solution

All MultiBrain® and MultiCord® sections produced by NSA are placed into antigen preserve solution during sectioning and storage, maintaining the viability of this valuable resource of sections for decades. To prevent breakage of the MultiBrain® sections during transit, the containers MUST be fully topped off with this solution. Instructions for preparing antigen preserve solution can be found under Client Resources at NSALabs.com.

Mounting Instructions, Video and Solutions

The remaining resource of free floating sections are shipped back to you, for you to perform your own stains, or return to NSA at a later date for additional stains and supplementary services. Best practices for handling the sections, including a video, are available at NSALabs.com under Client Resources. You will also find instructions for our recommended mounting solutions used to mount the free floating sections onto slides.

HistoTools® Store

Visit our website at NSALabs.com under Client Resources to visit our HistoTools® Store for all of the products we offer to assist you with handling our free floating sections.

INDEX

Visit NSALabs.com for a downloadable catalog

INDEX (CONTINUED)

Testimonials from NSA clients (continued from front cover)

"I have collaborated with Dr. Switzer and NeuroScience Associates on a number of different projects that required varied and in some instances unique approaches to achieve desired results. As a pathologist, it is very clear that NSA's MultiBrain® and MultiCord® Technology allows morphologic evaluation of the central nervous system in a comprehensive manner that would be not be possible using other existing technologies. As it relates to toxicity testing in pharmaceutical development, this technology provides a substantial advantage for detecting lesions that would often be missed in standard evaluations of brain, such as are used in routine toxicity testing or even in specific neurotoxicity testing that doesn't use this technology. I have found Dr. Switzer's extensive experience and expertise and the technical excellence of the NSA staff to be invaluable in providing the creativity and precision needed to meet the demands of their clients."

Dr. Daryl Thake, Pathology Consultant, Midwest ToxPath Sciences, Inc.

"We have been collaborating with NSA for several years with excellent results. Our studies include crossing between different transgenic lines and the comparison between double transgenic mice to their single transgenic as well as non-transgenic littermates. The excellent blocking and sectioning of multiple brains enables us to treat all the brain sections in exactly the same conditions for the same length of time, resulting in dependable comparison between the mice. We plan to continue this collaboration for all our studies."

Dr. Efrat Levy, Nathan Kline Institute

"NeuroScience Associates Services is a remarkable service. In my experience, they go the extra mile, solving histology problems which other services would run from. I wholeheartedly recommend them to anyone who needs high quality histology for their research."

Dr. Mike Tyszka, Caltech Brain Imaging Center

"NSA was extremely helpful from my very first interactions and throughout the project. NSA's commitment to customer satisfaction is unparalleled, and they are happy to help with study design and will even find ways to help the customer get their pathology done in the most cost-effective manner. I have worked with several pathologists/ clinical labs and am very impressed with the quality of tissue preservation and immunostaining using MultiBrain® Technology. The work done by NSA is of the highest quality. I look forward to working with them in the future."

Dr. Andrew Steele, California Polytechnic State University

""What we appreciated most with NSA, besides their splendid work, was not only the expertise in but also the availability for discussions around different labeling methods and protocols. NSA seems to care about the outcome of the projects they help with and do not see their clients only as a source of income."

Dr. Martin M. Mortazavi, Neurosurgeon, Thousand Oaks, California

Testimonials from NSA clients (continued)

"The extraordinary technique of NSA to embed and serially section up to 25 mouse brains in a single block, such that all brains are in register, and then stain floating serial sections simultaneously in cups has provided our research with information about neural circuitry not otherwise possible.

"We image living mouse brains with MRI at high magnetic field, using genetic manipulation to create mouse models of human mental and neurological disorders. We were the first to co-register 3D MR images of multiple mouse brains (Tyzka et al 2006), and were able to correlate tractography from DTI analysis of aligned images with histologic sections from NSA, also of multiple animals and in 3-dimensions. Subsequently we have published seven more full-length peer-reviewed high impact papers in NeuroImage, PLOS ONE and Magnetic Resonance in Medicine. NSA is responsive and technically expert in immunohistology and histochemistry."

Dr. Elaine Bearer, University of New Mexico Health Sciences Center, Albuquerque

"In 2010, my young research program had adopted a brain injury model that had drifted from contemporary use. Publications on midline fluid percussion injury were few and far between, leaving little literature to validate our results. We sent 16 brains to Neuroscience Associates for silver staining, and added IBA-1 immunohistochemistry at the last minute because microglia and inflammation were becoming central to Neurotrauma pathophysiology. Before we were ready, two elegant boxes overflowing with masterpieces of sectioned, stained, and mounted slides arrived. Fast forward six years, we have now published 10 peer-reviewed manuscripts on these sections, having described rod microglia in the pathophysiology of TBI, shown the biomechanical forces of fluid percussion, and repeatedly provided evidence that neuropathology does not equate to neurodegeneration. Every new trainee in our research group uses these slides to orient themselves to rodent neuroanatomy and the unique pathophysiology uncovered by Neuroscience Associates. This Fall, new trainees will arrive and likely see something for the first time and organically grow another publishable study."

Dr. Jonathan Lifshitz, University of Kentucky Medical Center

"With NeuroScience Associates you not only get an on-time, reliable, high-quality histology service that you can absolutely depend on—you also get the intellectual interaction of a very experienced team of neuroscientists and neurohistologists. We consider the staff at NSA an extension of our project teams, frequently benefiting from their troubleshooting skills and involving them in the interpretation of data."

Dr. Lisa Shafer, TEVA Pharmaceuticals

"NeuroScience Associates has been an excellent partner through several research projects. Specifically, the NSA team and the MultiBrain® Technology were instrumental in our evaluation of several treatments for spinal cord injury in a non-human primate model. NSA provided sound scientific advice on stains and immunolabels, and performed high-quality sectioning, histology, and immunohistochemistry economically and on time. I look forward to working with NSA on future projects."

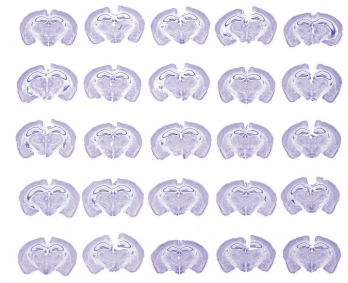
Dr. Rick Layer, Research and Development Manager, InVivo Therapeutics Corporation

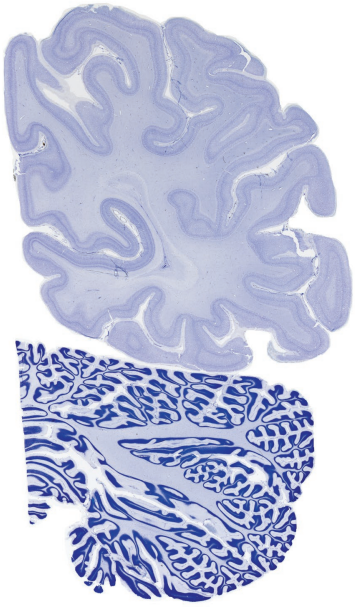
"We have been very happy with the fast and efficient services provided. NSA has a wide background of neurotoxicology experience to draw on for planning work and analyzing results. We hope to continue to use their services."

Dr. Michael Guarnieri, Johns Hopkins Medical Institute

(More testimonials on the inside front cover)

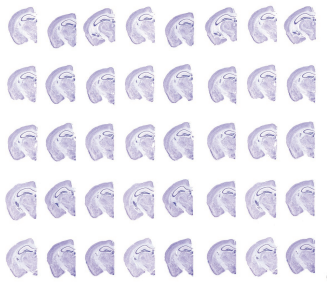
25 coronal mouse brains





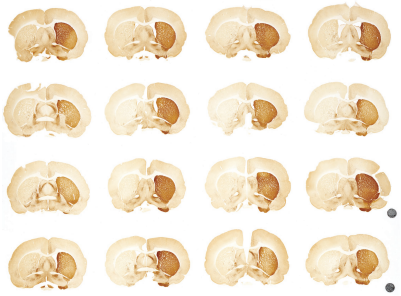
coronal human brain hemisphere

40 coronal mouse brain hemispheres

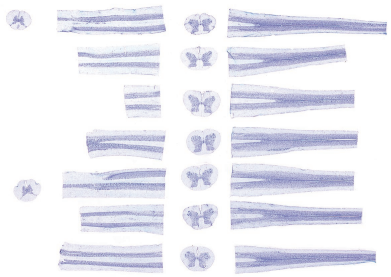


20 sagittal mouse brains

16 coronal rat brains



7 transverse/longitudinal rhesus monkey spinal cords



NEUROSCIENCE ASSOCIATES

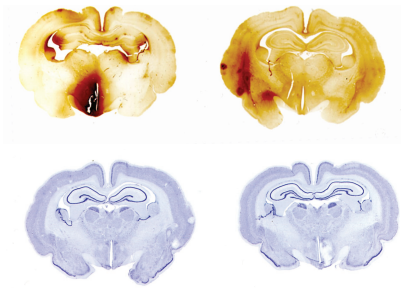
NSALabs.com

10915 Lake Ridge Drive

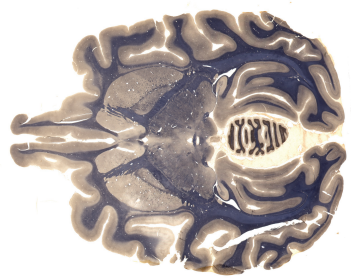
Knoxville, TN 37934

Main Line: (865) 966-1266

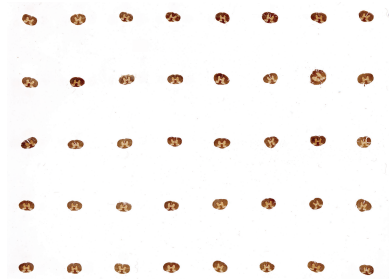
4 coronal rabbit brains



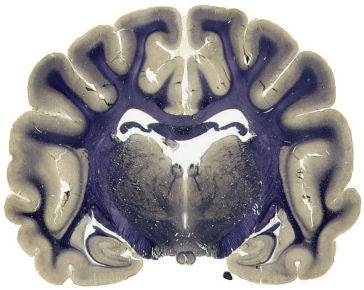
horizontal baboon brain



40 coronal mouse spinal cords



coronal dog brain



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