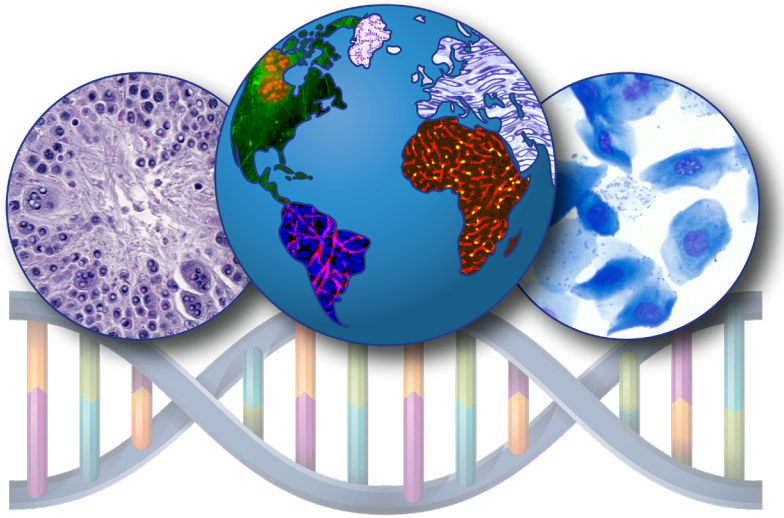
National Institute of
Environmental Health Sciences
Division of Translational Toxicology

Case Studies of Carbon Disulfide Central and Peripheral Neurotoxicity



Division of Translational Toxicology Global Toxicologic Pathology Training Program

National Institutes of Health • U.S. Department of Health and Human Services

Physical Properties of Carbon Disulfide (CS_2)

- CS_2 is an organic solvent
  - Easily explodes in air and catches fire
  - Rapidly evaporates at room temperature
  - The vapor is twice as heavy as air
- Pure CS_2 is a colorless liquid that is not very soluble in water and has a pleasant, sweet chloroform-like odor
- Impure, commercial grades of CS_2 are yellowish and have a foul odor like rotten eggs

Uses of CS_2

- Main use is in the manufacture of viscose rayon
- Other uses include
  - Fumigation in grain elevators, barges and airtight storage warehouses
  - Insecticide for fumigation of grains, fresh fruit and nursery stock and as a soil disinfectant against nematodes and insects
  - Solvent for fats, resins and for purification of single-walled carbon nanotubes
- Previously used to vulcanize rubber chemicals

Sources and Routes of Exposure

- Air
  - Inhalation of vapor is the primary route in both occupational and environmental exposures
  - Only workers in the viscose rayon industry are exposed to high enough concentrations to cause toxicity
  - Low amounts may be emitted naturally from volcanoes and marshes
  - The ocean is a major source
- Skin/eye contact
  - Direct contact with skin, eyes or mucous membranes may cause chemical burning
  - Only a hazard in the occupational setting
- Ingestion of contaminated drinking water
  - CS_2 can reach the waterways via wastewaters of viscose rayon plants

Kinetics and Distribution of CS_2

- In humans 10-30% of CS_2 absorbed by the body is exhaled and a further 70-90% undergoes biotransformation
- After absorption, CS_2 is transported by the blood
- Solubility in lipids and fats and its binding to amino acids and proteins govern its distribution in the body
- Fat solubility of CS_2 results in high concentrations in the brain and liver
- Metabolites of CS_2 are excreted in the urine

Biotransformation and Toxicokinetics of CS_2

Metabolism of CS_2 is performed by two main pathways

1. Metabolism via the microsomal cytochrome P-450 monooxygenase system into an unstable oxygen intermediate
2. Reaction with sulfhydryl groups of amino acids to generate highly polar dithiocarbamate metabolites (thiazolidine-2-thione-4-carboxylic acid)

- Increased amounts of thiazolidine-2-thione-4-carboxylic acid have been detected in the urine of workers and other individuals exposed to CS_2
- Dithiocarbamates have been shown to chelate metals (e.g., Zn++ and Cu++) and inhibit enzymes, and are the common metabolites formed in humans and animals
- Dithiocarbamates are metabolized to isothiocyanates that can covalently bind and cross-link with cytoskeletal proteins including neurofilaments, which may account for the giant axonal swellings in experimental studies

Acute Neurotoxicity: Human

- Acute inhalation exposure to CS_2 causes typical symptoms of narcosis including facial flushing, euphoria, tremor, and dazed behavior
  - Acute exposure to high concentrations of CS_2 leads to CNS dysfunction including confusion, memory impairment and hallucinations
  - Acute exposure to very high concentrations of CS_2 (200 to 500 ppm) during an accidental occupational release may cause CNS depression, unconsciousness, coma, respiratory paralysis and death
- The post-narcotic effects include headache, nausea, vomiting, excitability, and spasms

Chronic Neurotoxicity: Human

- Chronic low-level exposure to CS_2 causes a combination of neuropsychological abnormalities and peripheral neuropathy
- Psychological effects consist of personality changes, intellectual impairment, irritability, memory deficits, insomnia, bad dreams, decreased libido, constant fatigue, headache, dizziness, muscle pain, and depression
- Chronically exposed patients may also display CNS dysfunction with symptoms of Parkinsonism such as spasticity or hemiparesis
- Slowness of movement (bradykinesia), cogwheel rigidity (small, jerky ratchet-like movements) and tremor may also be present
- With increased or prolonged CS_2 exposure, a peripheral neuropathy develops involving dysfunction of many nerves (polyneuropathy)

CS_2 Toxic Peripheral Neuropathy: Human

- Toxic peripheral neuropathies are produced by xenobiotics that target various components of the peripheral nervous system
  - Represent a type of acquired polyneuropathy
  - Can be environmental, occupational, recreational or iatrogenic
  - Are generally dose-dependent, symmetrical, and reversible given adequate time
- CS_2 exposure produces a toxic peripheral neuropathy involving large, long axons
- Presents as sensory impairment with distal paresthesia, numbness or weakness in a ‘stocking and glove’ distribution (affects hands and feet)

Neuropathology: Human

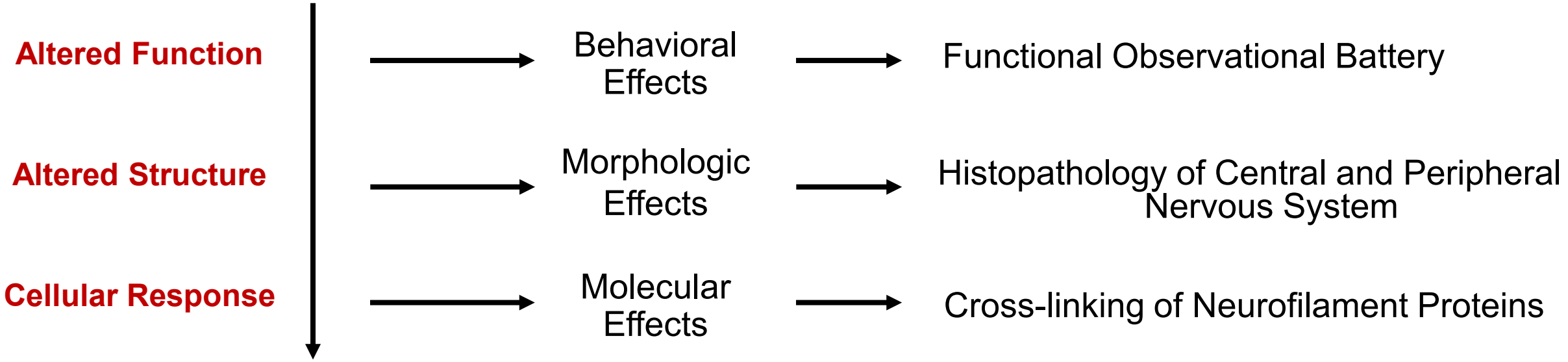
- The brain and spinal cord appear macroscopically normal and histologic changes are not well documented
- Peripheral neuropathy develops after exposure to levels of 100–150 ppm CS_2 for several months or to lesser levels for longer periods of time
  - Characterized histologically by axonal loss, giant axonal swellings, neurofilament accumulations and distal nerve fiber degeneration
  - Axonal swellings are caused by crosslinking and accumulation of cytoskeletal proteins including neurofilaments

NIEHS Rodent Neurotoxicity Studies of Carbon Disulfide

Study Design

Sills RC, Morgan DL, Harry GJ. Carbon disulfide neurotoxicity in rats: I. Introduction and study design. Neurotoxicology. 1998 Feb;19(1):83-7.

Biologic- and Mechanistic-Based Paradigm for Evaluating Carbon Disulfide Neurotoxicity



Experimental Design for CS_2 Inhalation Exposures

- F344 rats (male and female) were exposed to either 0, 50, 500, or 800 ppm CS_2 by inhalation for six hours/day, five days per week, for 2, 4, 8, or 13 weeks
  - Exposures were a combination of nose-only and whole-body inhalation exposures
  - Exposure on the day prior to the final exposure was nose-only (to accommodate sample collection and testing), all others were whole body
- Control animals were exposed to conditioned air (HEPA-filtered, charcoal-scrubbed, temperature and humidity-controlled)
- Dose selection was based upon previous studies that demonstrated neurotoxicity in F344 rats at concentrations above 300 ppm and established a no observable effect level (NOEL) at 50 ppm

Experimental Design for Testing and Sample Collection

Time Point	Procedure
Week -2	Implant transponders Randomization
Week -1	Functional observational battery Electrophysiology
Weeks 1 to 13 (6 hrs/d, 5 d/wk)	CS_2 inhalation exposures
Weeks 1 to 13 (weekly)	Body weights Clinical observations
Weeks 2, 4, 8, 13	Collect urine and blood Functional observational battery Electrophysiology Collect tissues for analysis

Behavioral Study

Moser VC, Phillips PM, Morgan DL, Sills RC. Carbon disulfide neurotoxicity in rats: VII. Behavioral evaluations using a functional observational battery. Neurotoxicology. 1998 Feb;19(1):147-57.

Study Design

- A Functional Observational Battery (FOB) was used to assess neurological effects of CS_2 exposure
- The FOB consists of a standardized series of home-cage, handling and open-field behavioral evaluations
  - It is a series of subjective evaluations and semiquantitative measurements made within a relatively short time by a trained observer
  - Used to assess the rat's neuromuscular, sensorimotor, autonomic and integrative neurological functions
- FOB was conducted on all rats at the start of the study and again on the morning following the last exposure

Measures of the FOB Grouped into Functional Domains

Autonomic	Activity	Neuromuscular
Salivation	Rearing	Gait score
Lacrimation	Open-field activity	Forelimb grip strength
Pupil response	Home-cage activity	Hindlimb grip strength
Defecation		Landing foot splay
Urination		

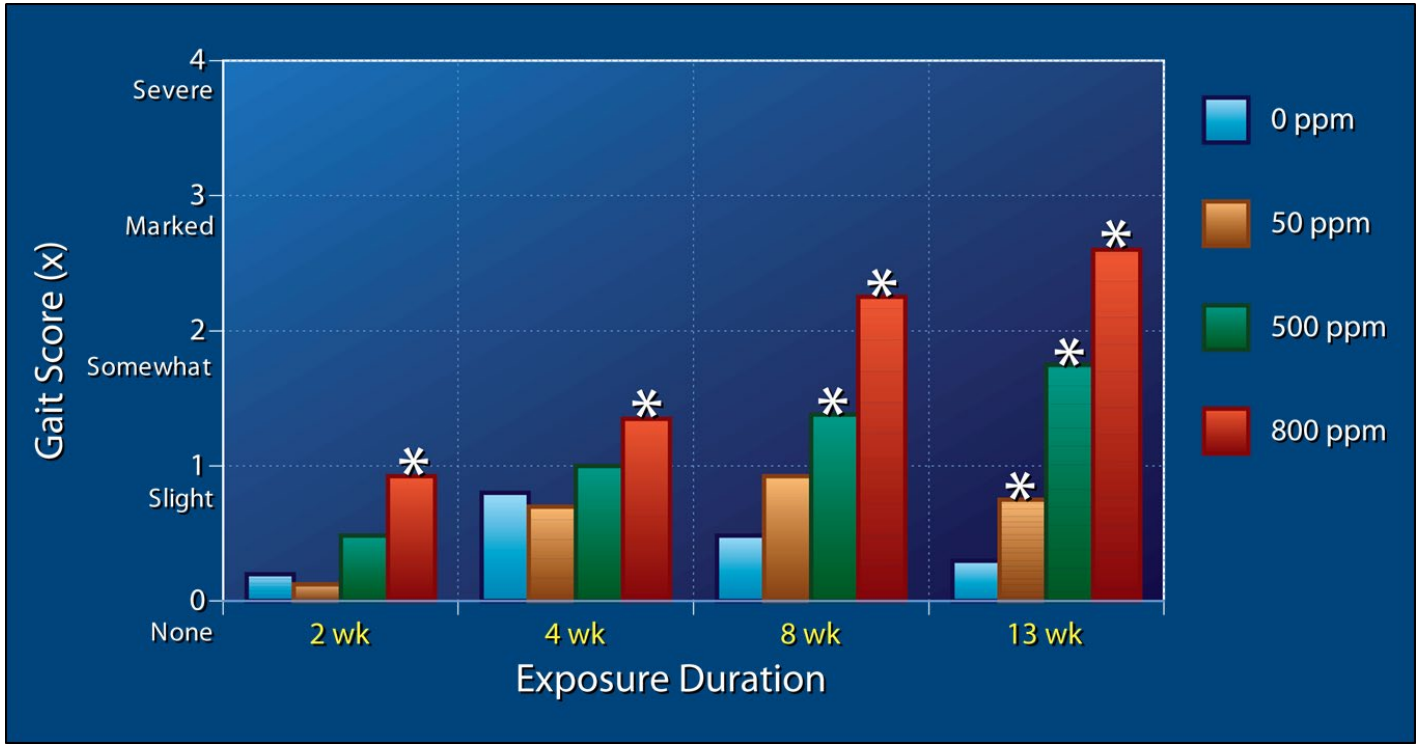
Reactivity	Sensorimotor	Convulsive
Arousal	Tail-pinch response	Tremorgenic score
Handling activity	Click response	Clonus
Removal activity	Touch response	Tonus
	Approach response	

Other Measures	Vestibular
Body weight	Ataxia score
Piloerection	Righting reflex
General appearance	
Posture	

Gait Abnormalities

- The predominant effects of CS_2 were on neuromuscular function preferentially affecting the hindlimbs
- Mild gait abnormalities were observed beginning at 2 weeks in male rats (800 ppm exposure group)
  - Uncoordinated placement of the hindlimbs and tip-toe walking
  - Progression to impaired hindlimb control by 13 weeks (all exposure groups)

Gait Abnormality – Males

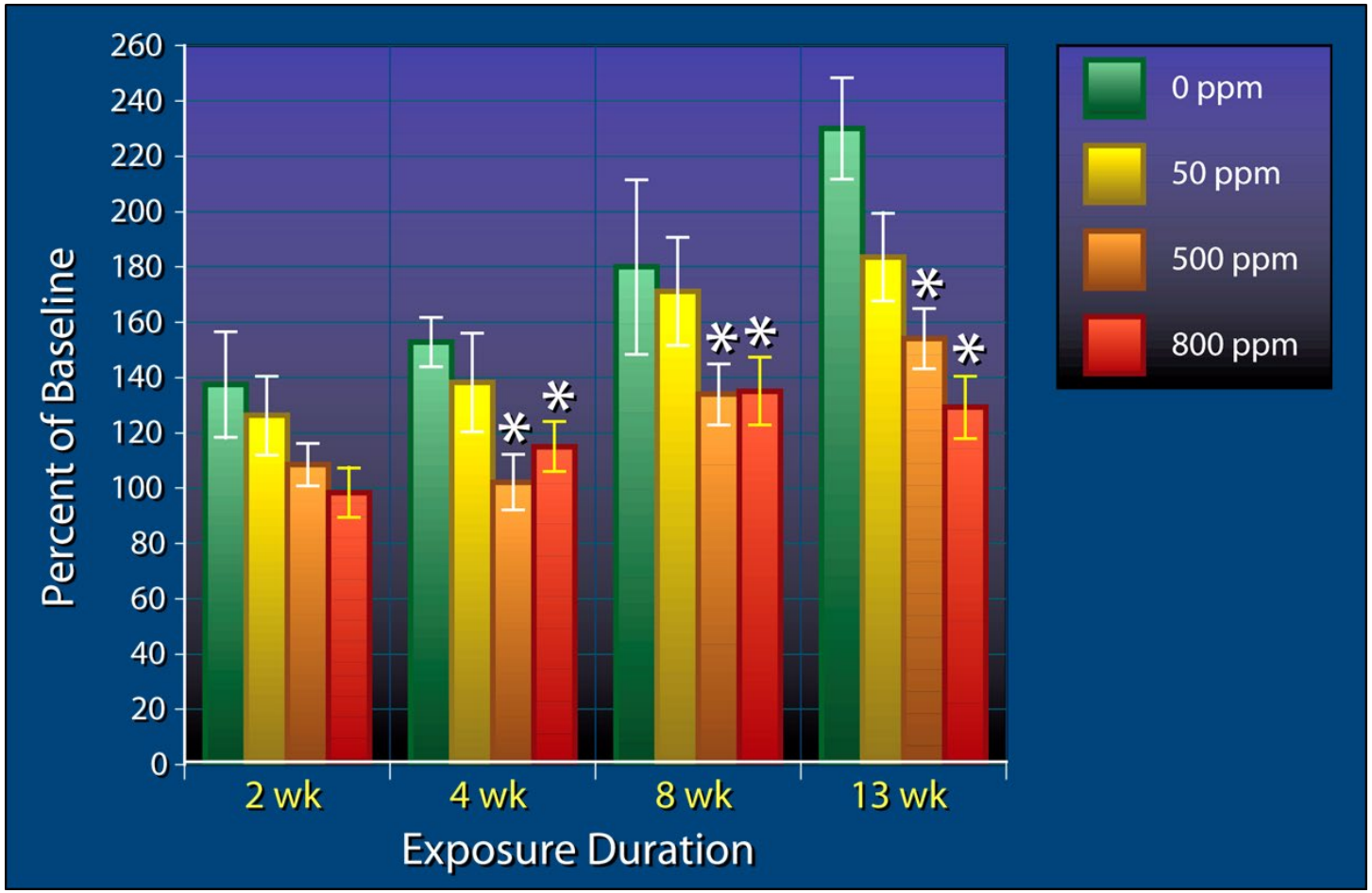


Treatment groups which were significantly different from their respective controls are indicated (*).

Hindlimb Grip Strength

- Grip strength was lowered in both forelimbs and hindlimbs starting at 4 weeks (500 and 800 ppm exposure groups)
  - The magnitude of this change was greater in the hindlimbs
  - Dose-response was seen only in male animals
- Other lesser effects included tremors, ataxia and changes in visual reactivity

Hindlimb Grip Strength – Males



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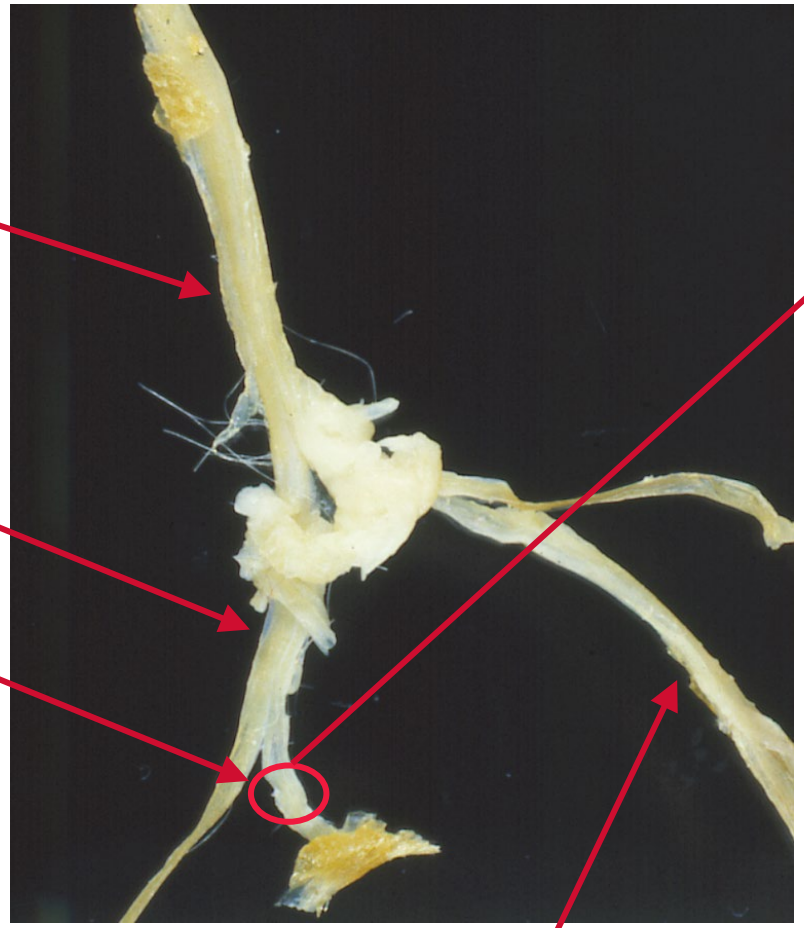
Morphology Study

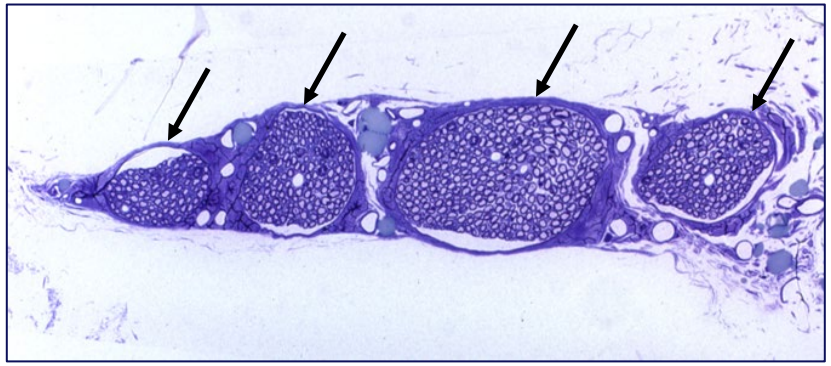
Sills RC, Harry GJ, Morgan DL, Valentine WM, Graham DG. Carbon disulfide neurotoxicity in rats: V. Morphology of axonal swelling in the muscular branch of the posterior tibial nerve and spinal cord. Neurotoxicology. 1998 Feb;19(1):117-27.

Study Design

- Nerves from the right hindlimb were processed for light microscopic and electron microscopic evaluations
- Central nervous system was sampled as follows
  - Multiple levels of the spinal cord including cervical levels C1 and C2 and lumbar levels L1 and L2 were dissected for light and electron microscopic analyses (Neuropathology evaluation in National Toxicology Program studies)
  - Other CNS tissues processed for routine histopathologic examination included whole brain (cerebrum, cerebellum and midbrain)

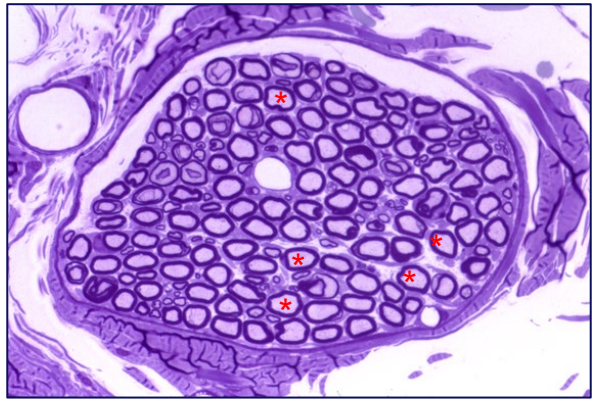
Morphological Evaluation of the Peripheral Nervous System





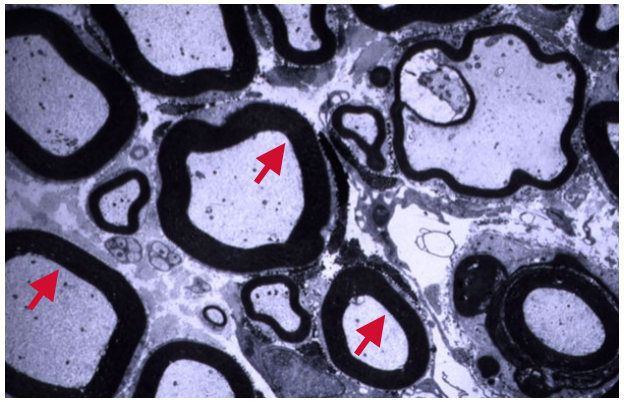
The muscular branch of the posterior tibial nerve consists of 4 nerve fascicles (arrows).

Light Microscopy



Section of nerve fascicle stained with toluidine blue. Myelinated nerve axons stain purple/blue (*).

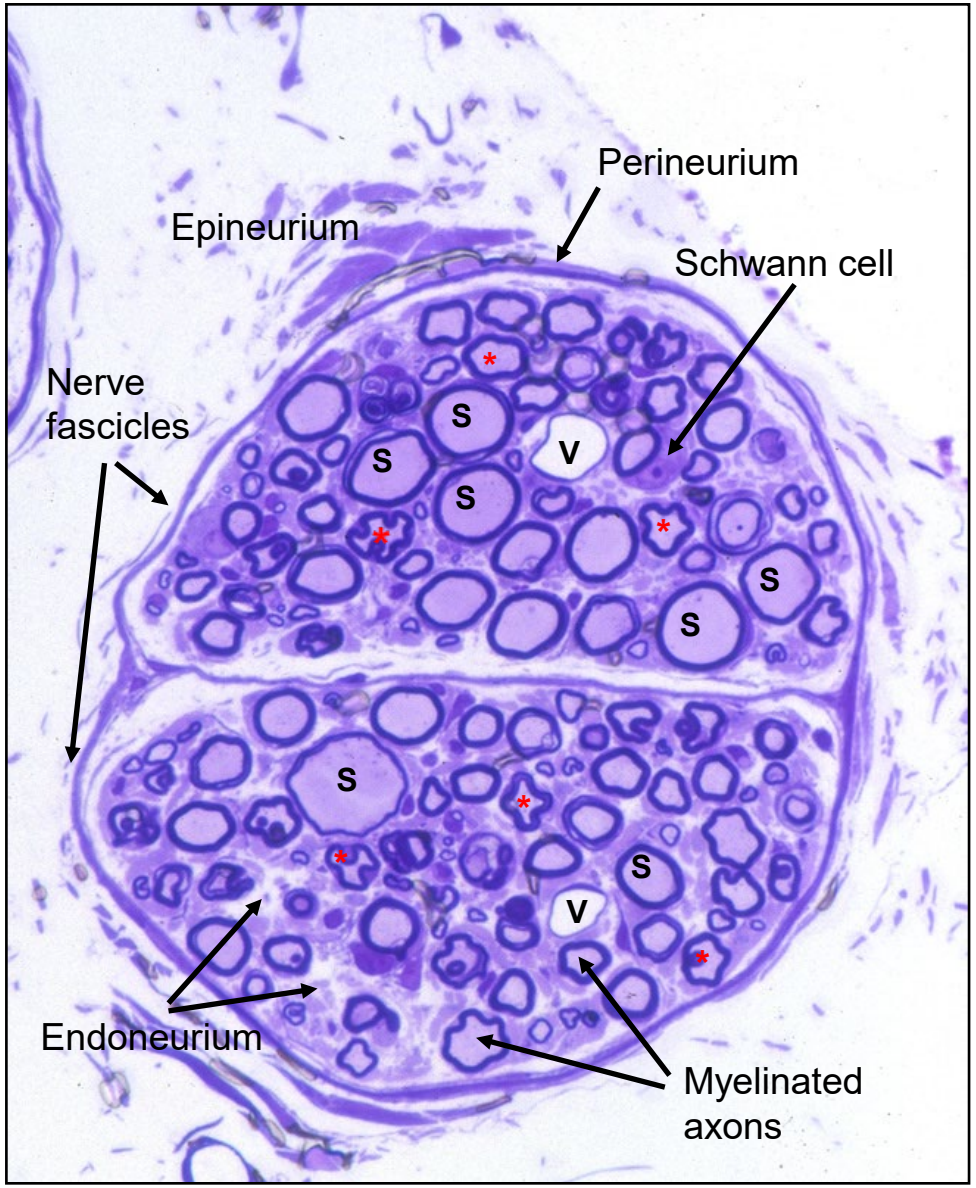
Electron Microscopy



Section of nerve stained with lead citrate and uranyl acetate. Myelin sheaths are dark black (red arrows).

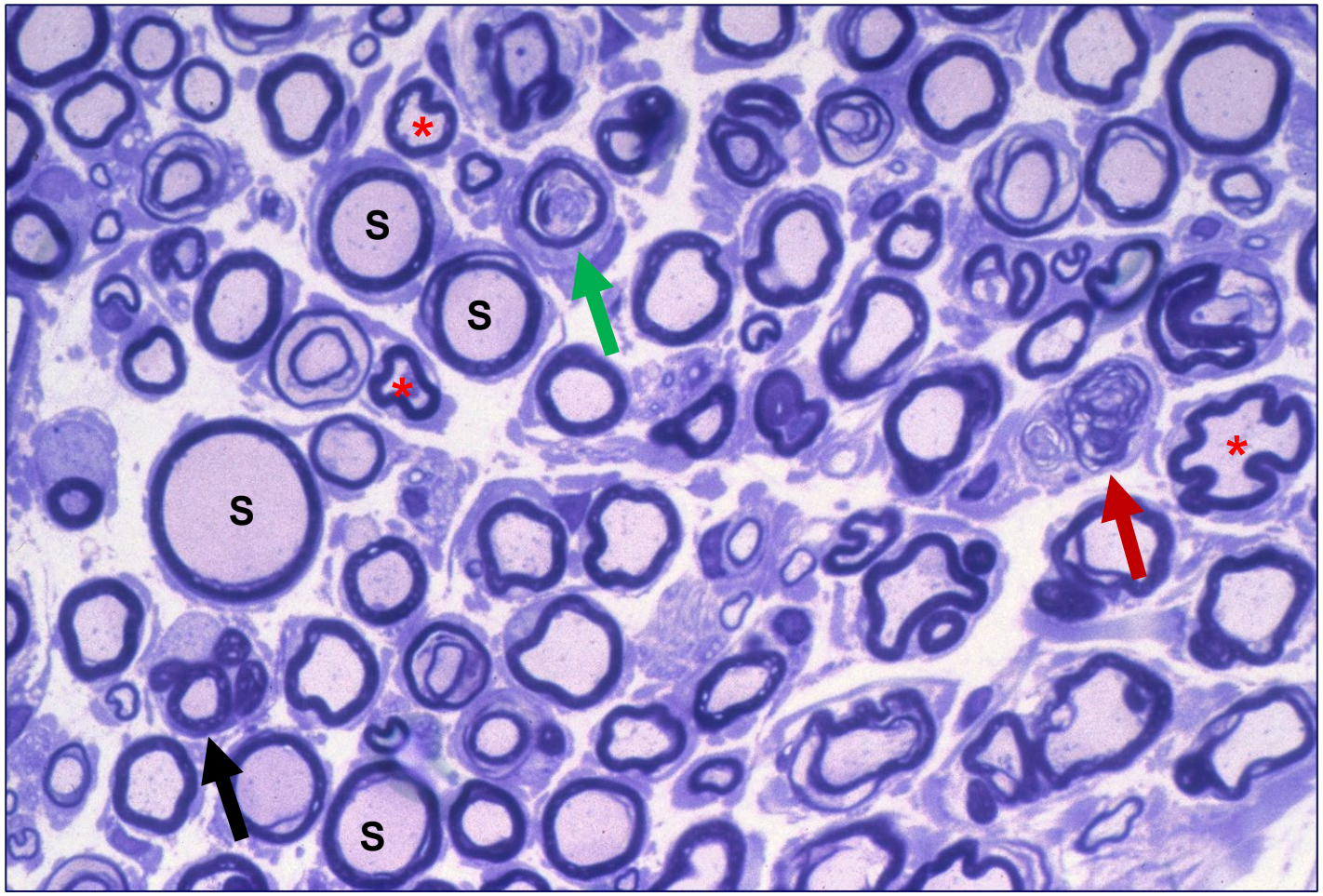
Axonal Swelling: Light Microscopy

- Cross section of the muscular branch of the posterior tibial nerve from a rat exposed to 800 ppm CS_2 for 13 weeks (toluidine blue stain)
- Many fibers display giant axonal swelling (S)
- Note the smooth contour of the myelin sheath in the swollen axons (S) compared to the irregular contour of the normal axons (*)
- V= vessel



Axonal Degeneration and Regeneration: Light Microscopy

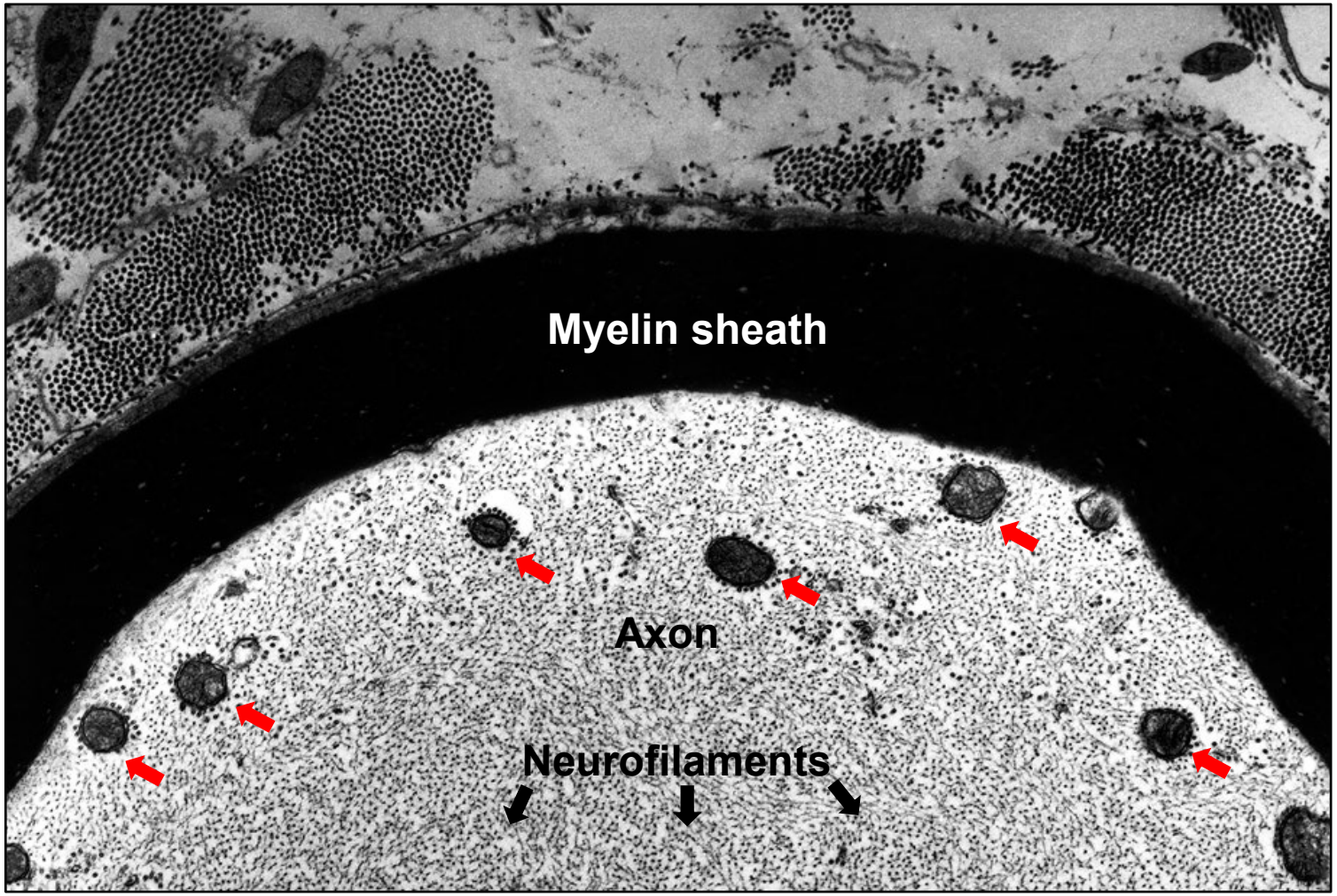
- Toluidine blue-stained section of the muscular branch of the posterior tibial nerve from a rat exposed to 800 ppm CS_2 for 13 weeks
- Green arrow indicates an axon in the early stages of axonal degeneration showing myelin debris in the axonoplasm with the myelin sheath still intact
- Red arrow shows an axon in the later stages of axonal degeneration demonstrating the progression of myelin breakdown to form a myelin ovoid
- Black arrow shows a regenerating axon with a cluster of nerve fibers
- S= Swollen axons, * = Normal axons



Axonal Swelling: Electron Microscopy

- Ultrastructural examination of the muscular branch of the posterior tibial nerve shows accumulation of 10nm neurofilaments (black stippling in axon) within swollen myelinated axons
- Mitochondria (red arrows) appear to be displaced to the periphery of the swollen axon by the neurofilaments

Cross section of a swollen myelinated nerve fiber



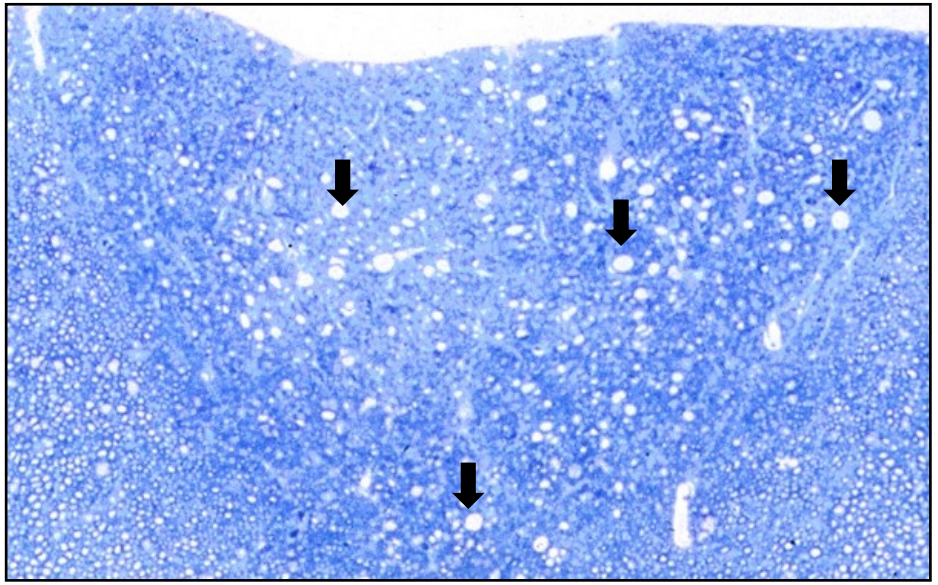
Incidence of Injury in the Muscular Branch of the Posterior Tibial Nerve

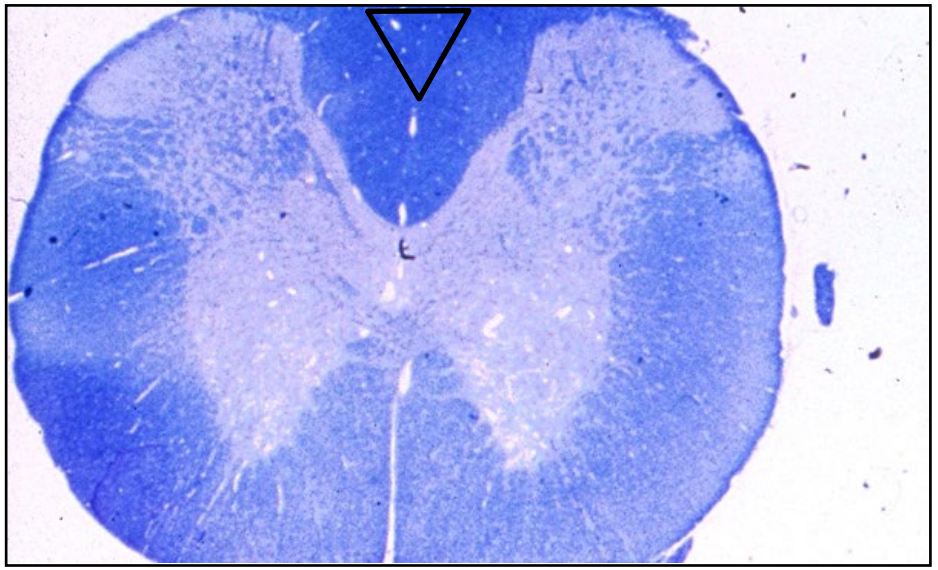
Exposure Time		8 weeks		13 weeks	
Sex		M	F	M	F
No.		4	4	8	8
Axonal Swelling	800[a]	1	3†	7**	8**
Degeneration	800[a]	0	0	3†	4*
Regeneration	800[a]	0	0	4*	6*

[a]Exposure concentration expressed in ppm
*p≤0.05 vs controls (Fisher's exact test)
**≤0.001 vs controls (Fisher's exact test)
†p≤0.05 vs controls (Exact permutation trend test)

Axonal Swelling: Light Microscopy

- Bottom panel is the cervical spinal cord of a control rat showing the location of the fasciculus gracilis nerve tracts (triangle)
- Top panel shows diffuse axonal swelling (arrows) in the fasciculus gracilis nerve tracts in a rat exposed to 800 ppm CS_2 for 13 weeks
- Mild, multifocal axonal swelling was first detected at 8 weeks and progressed to moderate, diffuse swelling at 13 weeks
- Similar changes were also seen in the lumbar spinal cord





Incidence of Axonal Swelling in Cervical Spinal Cord

Exposure Time		8 weeks		13 weeks	
Sex		M	F	M	F
No.		4	4	8	8
	800[a]	4*	4*	8**	8**
	500	4*	2	8**	8**
	50	0	0	0	0
	0	0	0	0	0

[a]Exposure concentration expressed in ppm
*p≤0.05 vs controls (Fisher's exact test)
**p≤0.001 vs controls (Fisher's exact test)

Molecular Study

Valentine WM, Amarnath V, Graham DG, Morgan DL, Sills RC. CS2-mediated cross-linking of erythrocyte spectrin and neurofilament protein: dose response and temporal relationship to the formation of axonal swellings. Toxicol Appl Pharmacol. 1997 Jan;142(1):95-105.

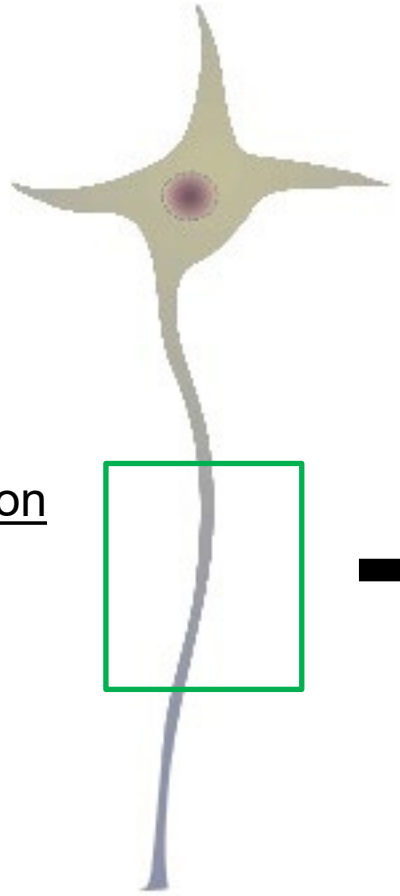
Rationale for Molecular Studies

- Chronic studies in rats have shown that exposures to CS_2 result in axonopathy
- Structural changes in axons include pre-nodal axonal swellings containing increased numbers of neurofilaments with a complex arrangement
- At the ultrastructural level, axonal changes include an increased number of 10 nm neurofilaments that displace organelles to the periphery of the axons
- Studies have shown that CS_2 is able to covalently cross-link proteins including hemoglobin and erythrocyte spectrin in a dose-dependent manner
- This ability of CS_2 to crosslink proteins including neurofilaments, represents a potential molecular mechanism for CS_2 -induced axonopathy

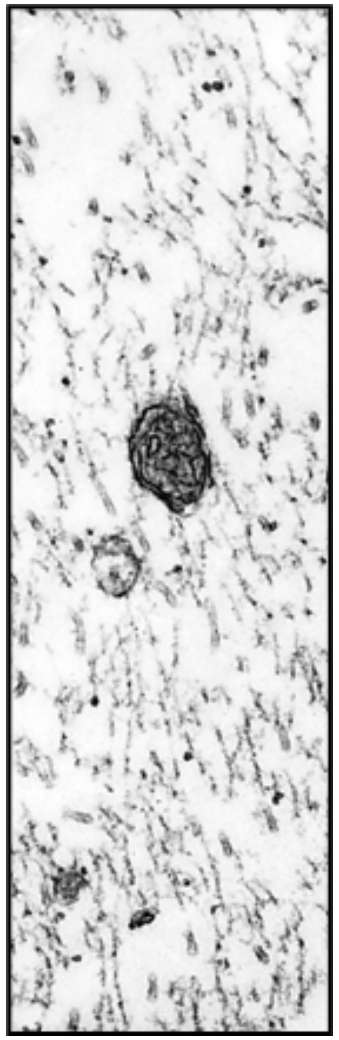
Ultrastructure of Axonal Swelling

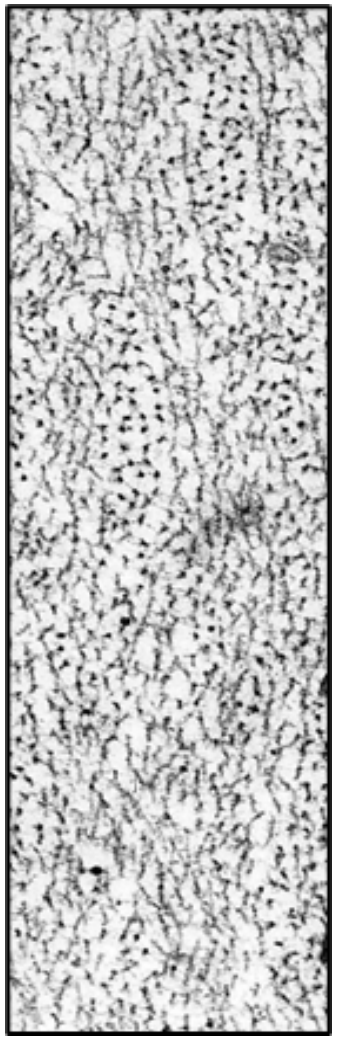
(Lumbar Spinal Cord)

Air Control



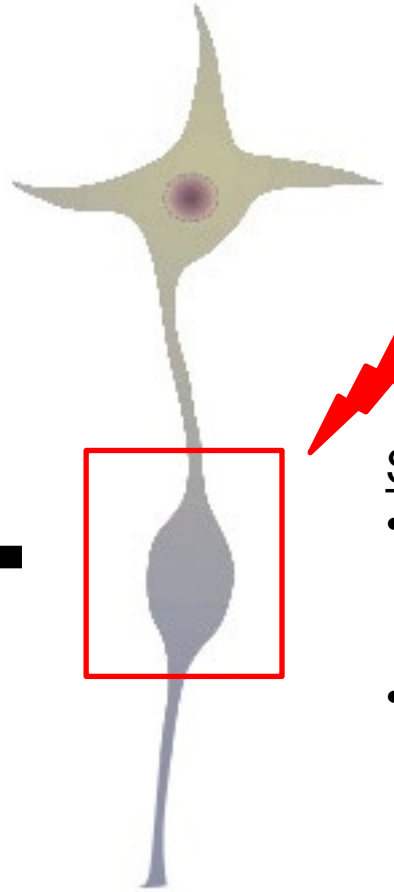
Normal axon





Carbon Disulfide

(13 weeks, 800 ppm)



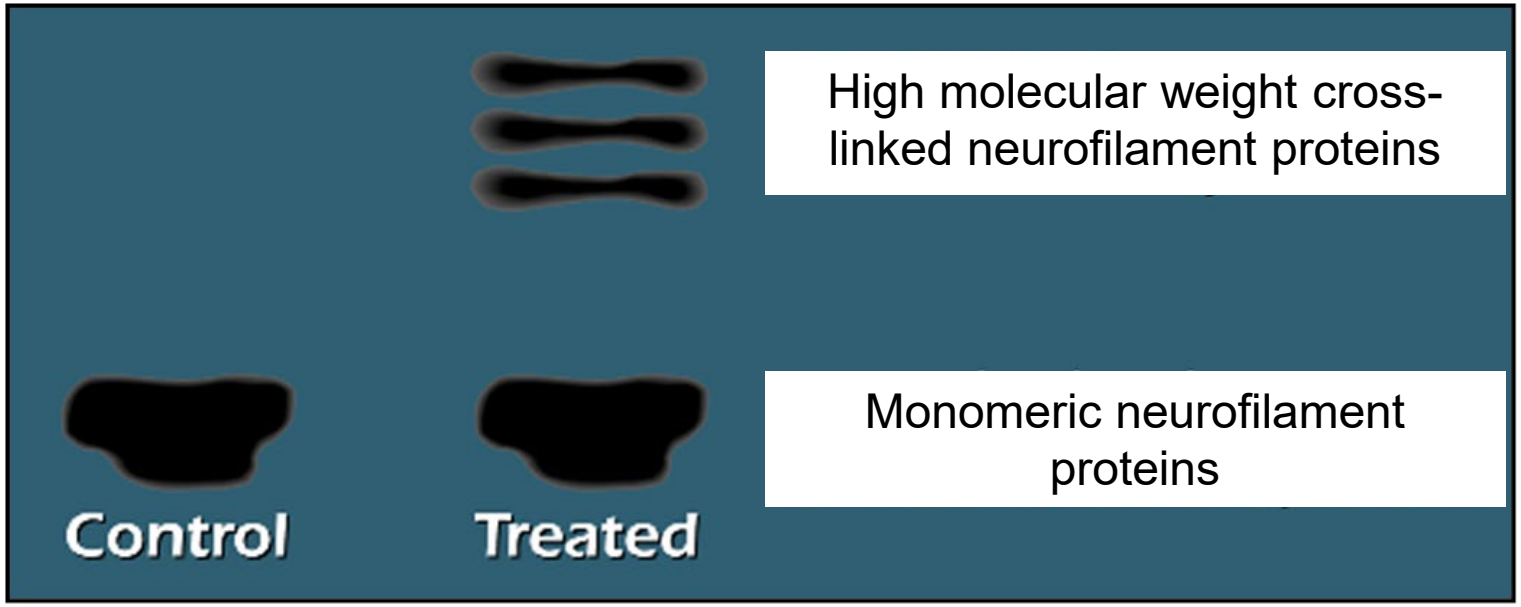
Cross-linking of neurofilament proteins by CS_2

Swollen axon

- Increased numbers of disorganized 10 nm neurofilaments
- Displacement of organelles to the periphery of the axon by the neurofilaments

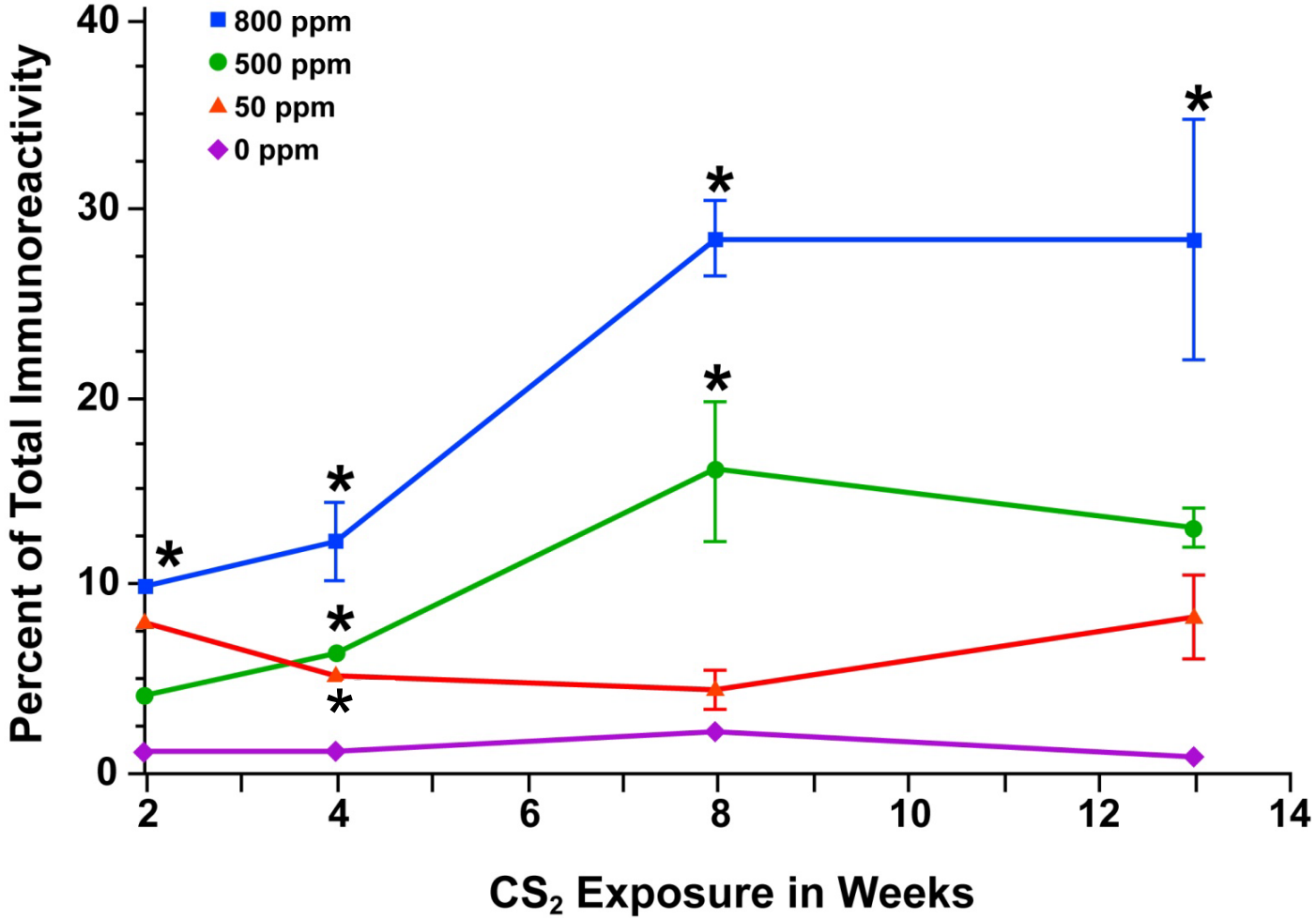
Evaluation of Neurofilament Proteins

- Neurofilament (NF) proteins were isolated from rat spinal cord preparations
- NF proteins were separated using polyacrylamide gel electrophoresis
- Western blots were probed using antisera directed against NF heavy-chain, medium-chain and light-chain subunit proteins (NFH, NFM and NFL)
- Proteins which migrate at a slower rate are of a higher molecular weight and represent cross-linked proteins



Cumulative Cross-linking of Neurofilament Proteins

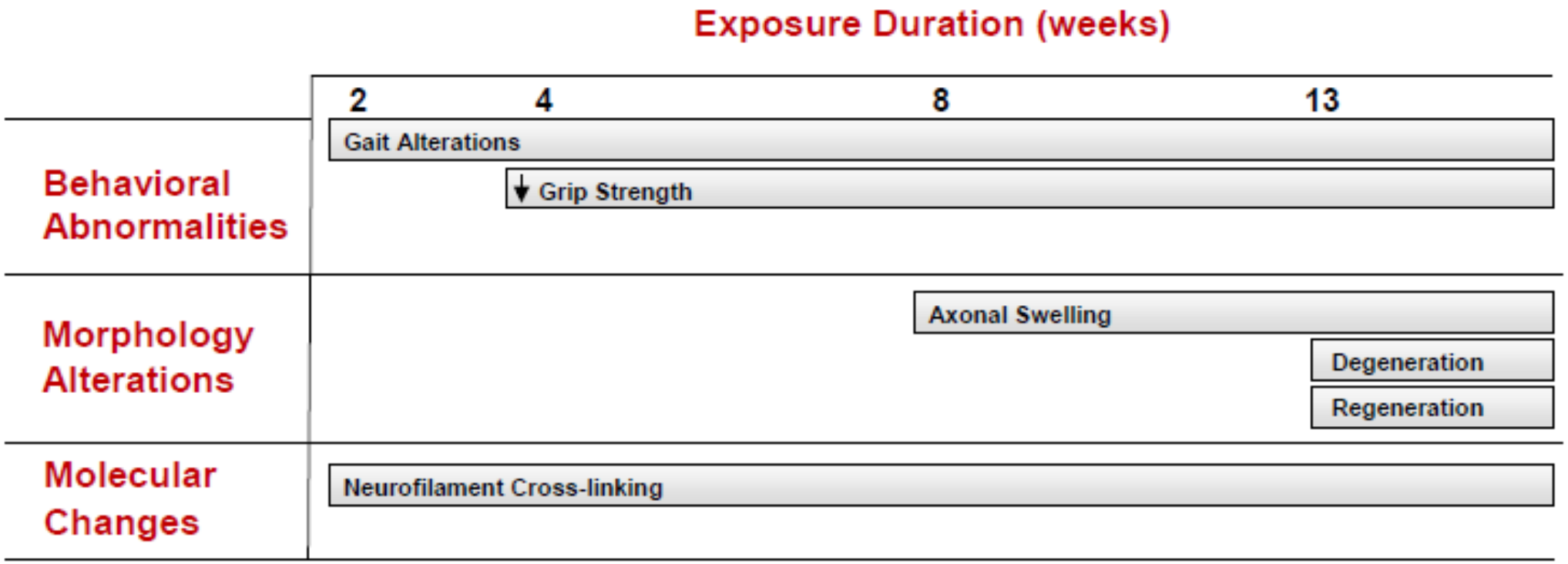
- A high molecular weight protein immunoreactive to anti-NFL and migrating slower than monomeric NFL (crossed-linked) was expressed as a percentage of total immunoreactive NFL protein
- The earliest significant increase over controls (*) was seen at 2 weeks in the 800 ppm group
- At 4 weeks, significant increases over controls were present in all treatment groups



Summary

Harry GJ, Graham DG, Valentine WM, Morgan DL, Sills RC. Carbon disulfide neurotoxicity in rats: VIII. Summary. Neurotoxicology. 1998 Feb;19(1):159-61.

Overall Summary: Carbon Disulfide Inhalation Studies



Behavioral Study: Summary

- FOB provided a profile of neuro-behavioral consequences of inhalational exposure to CS_2
- Neuromuscular deficits were the primary consequence of CS_2 exposure
- Deficits were more pronounced in the hindlimbs and were detected in rats of both sexes
- Mild gait changes occurred as early as 2 weeks
- Decreases in hindlimb grip strength occurred as early as 4 weeks
- Other deficits seen mostly at 13 weeks included mild tremors and decreased responsiveness to visual stimuli

Morphology Study: Summary

- Peripheral nervous system (800 ppm CS_2)
  - Axonal swelling was seen in the muscular branch of the posterior tibial nerve beginning at 8 weeks exposure
  - By 13 weeks, there were giant swollen axons accompanied by axonal degeneration and regeneration
- Central nervous system (500 and 800 ppm CS_2)
  - At 8 weeks, white matter changes were seen in cervical spinal cord that consisted of prominent axonal swelling in the fasciculus gracilis nerve tracts
  - By 13 weeks, axonal swelling was diffuse and was also present in the lumbar spinal cord

Molecular Study: Summary

- Neurofilament cross-linking in axons involved all 3 subunits of the protein
- The temporal relationship of NF protein cross-linking was consistent with a contributing role in the development of axonal swellings
- The dose-response characteristics for NF protein cross-linking support a direct role for covalent modification of neurofilament subunits by CS_2 in the pathogenesis of axonopathy

References

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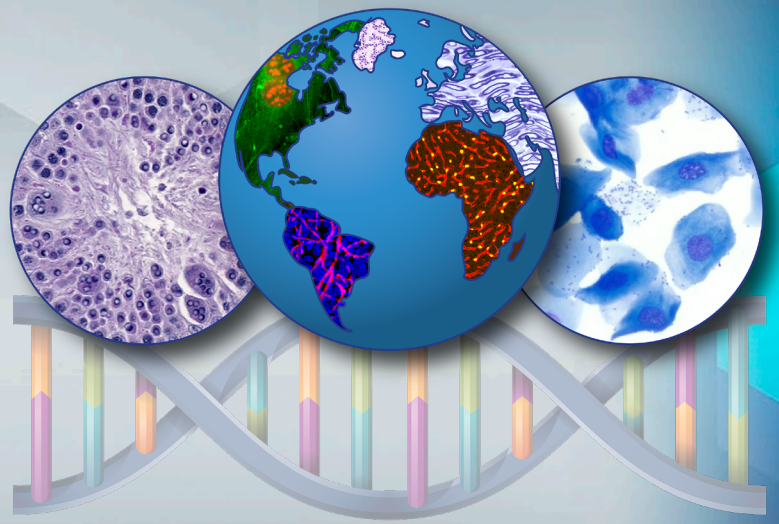

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