STP Position Paper:

Recommended Practices for Sampling, Processing and Analysis of the Peripheral Nervous System (Nerves, Somatic and Autonomic Ganglia) during Nonclinical Toxicity Studies[1]

STP Working Group on Peripheral Nervous System Sampling:

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Abstract (232 words)

These Society of Toxicologic Pathology "best" practice recommendations should ensure consistent sampling, processing, and evaluation of the peripheral nervous system (PNS). For toxicity studies where neurotoxicity is not anticipated (Situation 1), PNS evaluation may be limited to one sensorimotor spinal nerve. If somatic PNS neurotoxicity is possible (Situation 2), analysis minimally should include three spinal nerves, cranial nerve V, and their sensory ganglia. If autonomic PNS neuropathy is suspected (Situation 3), parasympathetic and sympathetic ganglia with associated autonomic nerves should be assessed. For dedicated neurotoxicity studies where neurotoxic activity is likely (Situation 4), PNS sampling follows the strategy for Situations 2 and/or 3, as dictated by in-life data or other information for the compound/target. For all situations, bilateral sampling with unilateral processing is recommended. For Situations 1, 2, and 3, PNS is processed conventionally (immersion in formalin, paraffin embedding, H&E staining). For Situation 4 (and if feasible Situations 2 and 3), perfusion fixation with methanol-free fixative (MFF) is recommended. Where PNS neurotoxicity is possible, at least one (Situations 2 and 3) or two (Situation 4) nerve cross sections should be post-fixed with glutaraldehyde and osmium before hard plastic resin embedding; soft plastic embedding is not suitable. Special methods (axonal and myelin stains, etc.) may be used to further characterize PNS findings. Initial PNS analysis should be informed, not masked ("blinded"). Institutions should explain the basis for their sampling, processing, and evaluation strategy.

Key Words: PNS, peripheral nervous system, neuropathology, neurotoxicity, recommended practices, nerve, ganglia, autonomic

Abbreviations

CNS	central nervous system
DRG	dorsal root ganglion
EPA	(U.S.) Environmental Protection Agency
FDA	(U.S.) Food and Drug Administration
GFAP	glial fibrillary acidic protein
GLP	Good Laboratory Practices
GMA	glycol methacrylate
H&E	hematoxylin and eosin
Iba1	ionized calcium-binding adaptor molecule 1
IENFD	intra-epidermal nerve fiber density
IHC	immunohistochemistry
MFF	methanol-free formaldehyde (or fixative)
MGG	medical-grade glutaraldehyde
MIE	molecular-initiating event
MMA	methyl methacrylate
MOA	mode of action
NBF	neutral buffered 10% formalin
NME	new molecular entity
NOAEL	no observed adverse effect level
OECD	Organisation of Economic Co-operation and Development
PNS	peripheral nervous system
PPD	paraphenylenediamine
QSAR	quantitative structure/activity relationship
RT	room temperature
SOP	standard operating procedure
STP	Society of Toxicologic Pathology
TEM	transmission electron microscopy
WOE	weight of evidence

I. Background

Neurological deficits due to toxicant-induced peripheral neuropathy are a recognized consequence of accidental occupational or environmental exposures and some therapeutic treatments. Therefore, the neuropathology component of toxicity studies is a critical means for identifying potential hazards and assessing risks posed to humans by contact with new biomolecular or chemical entities.

Different regulatory agencies offer independent guidance[1] based on their distinct mandates, variable scientific levels of concern, and diverse uses of the agents they oversee regarding the specimens and procedures to be used in evaluating the integrity of the peripheral nervous system (PNS) when seeking to register new products (Bolon *et al.*, 2011, Salvo and Butt, 2011). The guidelines vary by the kind of industry (agrochemical vs. chemical vs. pharmaceutical vs. biopharmaceutical), differences in potential exposure levels, and ages of the test subjects (e.g., developing animals (EPA, 1998b, OECD, 2007) vs. adults (EPA, 1998a, OECD, 1997)). Guidelines also differ based on the aim of the study (hazard identification vs. safety assessment). For example, regulatory guidelines for performing the neuropathology analysis of Good Laboratory Practice (GLP)-type general toxicity studies (i.e., screening or "Tier I" surveys) are fairly general since such studies assess the PNS as just one system among many organs and systems to be surveyed, while guidelines for GLP-type dedicated neurotoxicity studies (i.e., advanced or "Tier II" studies) are fairly detailed since assessment of the nervous system is the primary focus of the study (Bolon *et al.*, 2011,

[1] Guidance or guideline documents provided by regulatory agencies communicate current agency thinking on topics governed by regulations. Guidances and guidelines represent legally unenforceable interpretations that are designed to help institutions achieve compliance with legally enforceable regulations.

Salvo and Butt, 2011). However, substantial differences exist in the kinds of PNS toxicity that might be encountered (**Table 1**), and current guidelines do not address variations in approach that might be required to adequately investigate these divergent scenarios. Recent compilations reviewing published regulatory guidance in this area (Bolon *et al.*, 2011, Salvo and Butt, 2011) and/or individual regulatory guidelines should be consulted because guidance is reviewed and revised over time—as is presently occurring for the Toxic Substances Control Act (administered by the U.S. Environmental Protection Agency [EPA]) and the "Redbook" guidance on food and color additives (overseen by the U.S. Food and Drug Administration [FDA]).

When sampling the PNS, considerable care must be given to selecting the appropriate methodology (sampling scheme, fixatives, tissue orientation, embedding media, special stains, etc.) to ensure that tissue morphology is optimally preserved. Basic PNS sampling and processing methods were promulgated recently by a Working Group of the Society of Toxicologic Pathology (STP) tasked with establishing "best practice" recommendations for sampling and processing the central nervous system (CNS) for nonclinical general toxicity studies (Bolon *et al.*, 2013b). Given the CNS focus, however, coverage of the PNS in this STP document was brief, and did not specifically include recommendations encompassing different divisions of the PNS—somatic (sensorimotor) vs. autonomic (parasympathetic and sympathetic)—or effectors controlled by the PNS (e.g., glands, skeletal muscle, or viscera). Accordingly, the STP established a new Working Group on PNS sampling, processing, and analysis to provide more specific recommendations appropriate to distinct varieties of neuropathies that might be encountered during the course of GLP-type toxicity studies.

The Working Group was given a charter with multiple specific aims. The first charge was to recommend what PNS structures should be regularly sampled during GLP-type toxicity studies ("Tier I" and "Tier II") performed in common vertebrate test species. The second charge was to suggest tissue processing procedures and trimming schemes to facilitate analysis of these regions. The third charge was to define what routine stains and special neurohistology procedures, if any, should be used routinely in PNS evaluations. The fourth charge was to consider when other special morphological techniques should be undertaken to provide a more complete assessment of PNS lesions. The fifth charge was to define appropriate means for assessing whether or not PNS recovery has taken place. The sixth charge was to propose what format should be used to most efficiently document histopathologic evaluation of PNS tissues in reports destined for review by regulatory bodies. The recommendations given below with respect to particular neural structures to collect (**Table 2**) and suggested sampling and processing procedures (**Table 3**), as well as the means for documenting that they have been assessed, are based on the collective experiences and opinions of the Working Group members[2] as well as selected input from the global toxicologic pathology community[3] received during a __-day-long public comment period in the ____ quarter of 2017. Where consensus among Working Group members and/or STP

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[2]The Working Group consisted of 12 individuals with formal academic and/or industrial training in some aspect of neuroscience and between 13 to 49 years of experience acquiring and analyzing neuropathology data sets for nonclinical general ("Tier I") toxicity studies and/or dedicated neurotoxicity ("Tier II") tests while working in contract research organizations; government agencies (research laboratories or regulatory bodies); industrial firms (biotechnological, chemical, or pharmaceutical companies); universities; and/or private consulting practices.

[3]The draft recommendations devised by the Working Group received several levels of internal review by STP committees before being circulated for comment to the entire STP membership. The final draft also was sent to multiple other societies of toxicologic pathology representing nations in Asia, Europe, and Latin America to obtain international feedback on the proposal. At the time of publication, these practices have been endorsed by the STP, _____.

members was lacking on certain points, several options have been included and discussed with respect to their potential advantages and disadvantages.

II. Situation-specific Recommendations for Sampling, Processing, and Analysis of the PNS during Toxicity Studies

Basic Philosophy

The Working Group concluded that a rigid "one-size-fits-all" approach to sampling, processing, and evaluating PNS tissues is inappropriate due to the variety of situations, modes of action (MOAs), molecular-initiating events (MIE), and potential target sites that might be encountered. Instead, the Working Group is of the unanimous opinion that the appropriate and achievable objective is to delineate a strategy for evaluating key PNS structures to differentiate common classes of neurotoxic lesions, but let the experiences and needs of individual institutions drive selection of the specific battery of sampling, processing, and analytical methods undertaken to provide a suitable survey of the PNS. The rationale for such decisions should be articulated clearly in the study report. Such institutional decisions should be made using a "weight of evidence" (WOE) approach, where expanded sampling and evaluation of the PNS is considered only when evidence of PNS neurotoxicity is substantial enough to be an important factor in the final risk assessment. In general, such WOE decisions incorporate such factors as the degree of PNS neurotoxicity vs. toxicity to other target systems (i.e., how sensitive is the PNS to the test item[4] relative to other systems)

[4] Or test article

for non-target species, including humans, as well as the extent of PNS neurotoxicity that develops at relevant levels of exposure.

Scenarios for PNS Neurotoxicity

Four general situations during which PNS tissues may be sampled in the course of toxicity studies were considered (**Table 1**). Each utilizes a slightly different sampling strategy, based on the different locations in which the PNS is affected. The first three situations involve general ("Tier I" or "screening") toxicity studies, while the last scenario relates to dedicated neurotoxicity ("Tier II" or "advanced") evaluations.

Situation 1 is a general toxicity study in which (1) no potential for PNS neurotoxicity was detected in data obtained during prior studies (*in vivo*, *in vitro*, and/or *in silico*) and (2) no in-life behavioral or neurological deficits are seen in the current study. This strategy represents a rational default approach when analyzing new molecular entities (NME) for which no or few prior *in vivo* toxicity studies have been done. Situation 2 is a general toxicity study in which in-life signs of peripheral neuropathy or other data reflect damage mainly to the somatic (motor and/or sensory) nerves and/or their associated ganglia. Situation 3 represents a general toxicity study in which in-life signs of peripheral neuropathy or other data suggest injury to autonomic nerves and/or ganglia, which collectively regulate involuntary, visceral homeostatic functions. For both Situations 2 and 3, other data that might trigger an expanded PNS analysis include known or presumed MOA and quantitative structure–activity relationships (QSAR) models for the test item, its metabolites, and/or related compounds or molecules. Situation 4 is the dedicated neurotoxicity study, which usually is required for test items in which human epidemiological data, experimental findings from animal studies (*in* *vivo* and/or *in vitro*), and/or MOA or QSAR similarities to known neurotoxic agents indicates a high probability that PNS neurotoxicity may occur under likely exposure scenarios. Some agents may simultaneously impact the somatic and autonomic PNS, and thus may require increased sampling (combining Situations 2 and 3) and evaluation to fully assess both arms of the PNS.

A side-by-side comparison of PNS specimens to collect as well as baseline tissue sampling and processing recommendations for the four situations are given in **Table 2** and **Table 3**, respectively. The Working Group recommends that this information be used to define one or more institutional standard operating procedures (SOPs) that describe the collection and processing practices for PNS tissues. These documents should be detailed but sufficiently flexible so that the study director and study team may adjust the PNS practices as needed to meet the recommendations for all four situations.

Best Practice Recommendations for All Four Situations

The PNS sampling strategy should be guided by observed in-life neurological signs or other information for the compound/target. The choice of which PNS samples to collect and whether or not special histology processing and/or investigative techniques should be used for a given toxicity study should be decided by the institution using a WOE approach. For all situations, PNS structures (nerves, ganglia, and effector organs) typically should be collected bilaterally but may be processed and evaluated unilaterally. Nerves and skeletal muscle (an effector organ) should be evaluated in both cross and longitudinal orientations. All PNS specimens from the treatment groups selected for initial evaluation (e.g., high-dose and control animals) should be processed in the same time frame to avoid systematic variation in processing conditions.

Where plastic embedding is required by regulatory guidelines (EPA, 1998a), hard plastic resin is the recommended medium. Soft plastic (e.g., glycol methacrylate [GMA] or methyl methacrylate [MMA]) is not an acceptable substitute for hard plastic resin.

The recommended best practice for light microscopic evaluation is to undertake a tiered, semi-quantitative analysis with foreknowledge of the study design. A subsequent masked ("blinded" or "coded") analysis of PNS tissues with findings of concern may be conducted at the discretion of the study pathologist (or peer review pathologist), but usually is done only to aid in defining the dose-response and/or establishing a no observed adverse effect level (NOAEL).

Best Practice Recommendations for Situation 1

For general toxicity studies with no specific concern for PNS neurotoxicity (Situation 1), the majority of the Working Group concurs that one large, mixed (i.e., sensorimotor) somatic nerve, such as the sciatic nerve (or tibial nerve if the sciatic trunk has been traumatized), is a suitable baseline PNS survey. Additional peripheral nerves and dorsal root ganglia (DRG), either *in situ* in vertebral column segments (rodents only) or isolated, should be collected at necropsy but need not be assessed unless nerve or spinal cord lesions require additional characterization. Standard processing—immersion fixation in conventional (i.e., methanol-containing) neutral buffered 10% formalin (NBF), paraffin embedding, and hematoxylin and eosin (H&E) staining—usually is acceptable.[5]

Best Practice Recommendations for Situation 2

For general toxicity studies where somatic PNS neurotoxicity is a concern under likely exposure scenarios (Situation 2), three spinal nerves—typically the sciatic nerve and two or more of the following nerves (most of which are distal branches of the sciatic nerve): tibial, fibular (i.e., common peroneal), plantar, saphenous, sural, or (in rodents) caudal nerves—as well as cranial nerve V (trigeminal nerve) should be evaluated. The sciatic, tibial and fibular nerves in all species, and the sural and caudal nerves in rodents are mixed sensorimotor structures; the saphenous, plantar, and (in nonhuman primate) sural nerves are sensory-only branches. Nerve selection generally should be based on in-life findings. At least four DRG (two each associated with the species-specific locations of the cervical and lumbar intumescences [**Table 4**], collected *in situ* or isolated); the associated dorsal and ventral spinal nerve roots; and the trigeminal (Gasserian [cranial nerve V]) ganglion should be evaluated. Conventional processing conditions (immersion fixation in formalin, paraffin embedding, H&E staining) are suitable for PNS tissues, with three exceptions. First, methanol-free formaldehyde (MFF[6]) or medical-grade glutaraldehyde (MGG, typically 2.5%) rather than NBF ideally should be employed to minimize processing artifacts. The Working

[5] This recommendation represents the majority view of Working Group members, with the understanding that special *post hoc* processing (i.e., glutaraldehyde and osmium post-fixation, hard plastic embedding) of at least one nerve cross section, as described for Situations 2, 3, and 4 where PNS neurotoxicity is possible, may be helpful in further characterizing the PNS findings for Situation 1, especially the nature of changes observed in myelin.

[6] Methanol-free 4% formaldehyde is made from paraformaldehyde pellets or powder and thus often is referred to in the scientific literature as “4% paraformaldehyde” (PFA) (Kiernan, 2000). MFF may be purchased commercially or prepared in the laboratory shortly before use.

Group recognizes that this first adjustment may not be feasible on short notice, especially if the in-life PNS-related signs develop late in the course of a large study. Second, if nerve lesions are seen in H&E-stained sections, acquisition of serial sections for at least one mixed nerve should be considered for special neurohistological staining to highlight axonal morphology (silver stain) and explore myelin integrity (myelin stain). Third, at least one nerve cross section (usually a mixed-function distal trunk like the tibial or fibular nerve, or a mainly sensory branch like the sural or caudal nerve) should be post-fixed by immersion in MGG followed by osmium (to stabilize myelin during the processing steps with lipid-solubilizing organic solvents), processed into hard plastic resin, and then stained with toluidine blue for light microscopic evaluation. The last two adjustments should be feasible regardless of whether MMF or NBF is utilized.

Best Practice Recommendations for Situation 3

For general toxicity studies where autonomic PNS neurotoxicity is a concern at relevant levels of exposure (Situation 3), elements of the parasympathetic, sympathetic, and enteric[7] PNS should be evaluated, including nerves (vagus and sympathetic chain) and multiple autonomic ganglia. Common ganglia to assess include one post-ganglionic parasympathetic site (i.e., those in the walls of protocol-specified hollow organs [commonly the heart and urinary bladder], but ideally at sites related to in-life findings); at least two sympathetic sites (e.g., cranial cervical, cervicothoracic, cranial mesenteric, and/or sympathetic chain ganglia); and several enteric sites (i.e., submucosal [Meissner's] and myenteric [Auerbach's] ganglia).

[7] Enteric ganglia, which serve parasympathetic-like functions, form a neural net with independent reflex activity and thus are considered by some investigators to be distinct from the autonomic nervous system (Furness, 2006).

In addition to autonomic PNS nerves and ganglia, somatic PNS nerves and ganglia should be collected as described in Situation 2. Conventional processing (immersion fixation in NBF or ideally MFF, paraffin embedding, H&E staining) is suitable for most autonomic PNS samples. Post-fixation with MGG and osmium followed by hard plastic embedding may be useful despite the lower myelination of most autonomic nerves.

Best Practice Recommendations for Situation 4

For dedicated neurotoxicity studies where PNS neurotoxicity is likely or certain (Situation 4), expanded sampling includes at least three spinal nerves (sciatic, tibial, and fibular, saphenous, sural, plantar, or caudal); trigeminal (cranial n. V) nerve; DRG and their associated spinal nerve roots; and a trigeminal ganglion. At least six DRG should be examined (two or more DRG for each of the cervical, thoracic, and lumbar spinal cord divisions). In general, DRG should be removed from the vertebral column rather than processed and evaluated *in situ* to avoid soft tissue degradation associated with skeletal decalcification, but in rodents *in situ* analysis following vertebral column decalcification is acceptable. Fixation is undertaken by whole-body perfusion fixation with a methanol-free fixative (typically MFF or mixtures of MFF and MGG). Paraffin embedding is suitable for most nerves and ganglia, although at least two distal nerve cross sections (typically the tibial nerve and a more distal branch) should be post-fixed in MGG and osmium and then embedded in hard plastic resin. Paraffin-embedded nerves should be stained with H&E and, if warranted, axonal and myelin stains, while plastic-embedded nerves are stained with toluidine blue. Ganglia usually are stained only with H&E, although silver and myelin stains may be beneficial. Other special methods (see below) may be considered at the discretion of the institution to better characterize any neurotoxic lesions.

The Working Group recommendations for PNS sampling in Situations 1, 2, 3, and 4 are designed to be applicable to cases where test items have been delivered systemically (i.e., where all PNS tissues are liable to some degree of test item exposure), and thus may need to be modified for selected scenarios and/or unusual test items. Decreased PNS evaluation may be warranted if the pattern and severity of PNS lesions for the doses and/or the dosing regimen used in a study have been well defined in one or more previous studies, although the Working Group recommends that all PNS tissues described in Situation 4 be collected and archived as wet tissue. Additional PNS samples (e.g., forelimb nerves) may have to be evaluated if clinical signs suggest that PNS damage has occurred at these sites. Local delivery of a minimally diffusible test item[8] generally warrants increased collection and prioritized analysis of nerves near the administration site, while more distal PNS elements may be collected but retained as wet tissue. Such modifications in sampling and evaluation may be made at the discretion of the institution. The rationale for such adjustments should be given in the study report.

III. Rationale for Recommended PNS Sampling, Processing, and Analysis Practices

Regulatory guidelines are fairly generic with respect to prescribing the PNS sampling strategy (Bolon *et al.*, 2011, Salvo and Butt, 2011), so common sense is an essential attribute when selecting the PNS tissues to collect and evaluate. Selection of PNS sites to sample depends on the situation (**Table 2**). Reasonable flexibility is possible in the choice of PNS

[8] An example of this situation is onabotulinumtoxinA (BOTOX®), which disrupts the function of motor nerve endings at the nerve/skeletal muscle interface at the site of injection, but not the structure of PNS axons and ganglia elsewhere in the body.

tissues, depending on institutional preference. A "weight of evidence" (WOE) approach should be employed in deciding whether or not expanded PNS evaluation will provide data relevant to the risk assessment. Situations in which PNS toxicity is judged to represent a modest hazard relative to more substantial test item-related findings that are observed in more sensitive systems and/or in which PNS toxicity at high dose will not be used to define the dose response and NOAEL may preclude the need for a substantial expansion, or permit only a modest expansion, in PNS sampling and examination.

A. Situation-specific PNS Sampling Strategies

Basic Considerations

For screening in the absence of PNS neurotoxicity (Situation 1), evaluation of one large mixed (sensorimotor) nerve is a suitable survey for PNS involvement. If PNS neurotoxicity is a concern (Situations 2, 3, and 4), PNS evaluation is expanded to include additional nerves and ganglia, with the choice depending on the nature of the in-life signs. Therefore, study protocols and institutional SOPs should facilitate collection of any PNS tissues that might be needed to explain the constellation of PNS-related clinical signs seen during the in-life portion of the study.

Collection of PNS samples (nerves, ganglia, and effector organs) for all four situations usually should be done bilaterally unless such an approach would impact another endpoint (e.g., collection of unfixed tissue for biochemical or molecular analysis). The rationale for this recommendation is that bilateral sampling can be done quickly by skilled technicians, and the retention of such specimens may permit additional characterization of unexpected findings without having to repeat the entire study; again, the choice of bilateral vs. unilateral

PNS collection should remain with the institution. Sample acquisition should be undertaken in a fashion that minimizes structural artifacts produced by manipulation, compression, and traction of incompletely fixed PNS tissue. The keys to curtailing artifacts are to limit handling (pressure and stretching applied to neural tissues during sampling), to promptly place tissues into properly prepared fixative and buffer solutions, and to maintain tissues at an appropriate temperature (generally room temperature [RT] for GLP-type toxicity studies) until additional processing may be undertaken.

In general, PNS samples should be individually identified. Sample identity may be assured by either placing each specimen in its own tissue cassette, applying it to a labeled index card (to which it will adhere due to the inherent stickiness of epineurial connective tissue), or stapling it (through one end, not the middle) to an acetate strip prior to fixation to maintain it in an extended (but not "stretched") orientation (Jortner, 2000). Stapling is the least desirable method due to the likelihood for "crushing" the tissue. The orientation of the proximal and distal ends of nerves can be identified by labeling one end.

<u>Situation 1</u>

In general toxicity studies where no neurotoxic potential is expected (Situation 1), the minimal list of PNS tissues to be evaluated in all species is a readily accessible, large, spinal-origin somatic nerve and the autonomic ganglia within the walls of major viscera. This PNS sampling strategy is identical to that proposed in the STP best practices document for CNS sampling in nonclinical general toxicity studies (Bolon *et al.*, 2013b) and reflects the current practice for general toxicity studies.

Nerves. The usual PNS sample for Situation 1 is sciatic nerve. The rationale for selecting this nerve is that it contains both sensory and motor nerve fibers, which permits analysis of major peripheral sensorimotor structures in a single sample. The sciatic nerve is exposed by reflecting and/or removing the overlying skeletal muscle (**Figure 1**). Sciatic nerve samples commonly are acquired at a distal location (i.e., just proximal to where the tibial and fibular nerves branch, which occurs near the femorotibial joint). Sciatic nerve collection more proximally, typically mid-way between the vertebral column and knee, is a frequent alternative. Proximally collected sciatic nerve is populated by bigger Schwann cells covering longer axonal lengths, and these large cells appear to be more sensitive to neurotoxic agents than are distal Schwann cells (Friede and Bischhausen, 1982, Krinke, 2011). Therefore, damage to proximal Schwann cells may make myelin disruption easier to detect since damage to the larger cells tends to leave longer expanses of denuded axons. The choice of sciatic nerve site to be sampled (proximal vs. distal) is left to the discretion of the institution.

A sciatic nerve branch, typically the tibial nerve (another trunk carrying both sensory and motor nerve fibers), may be evaluated instead of the sciatic nerve if likely artifactual changes might confound sciatic nerve analysis. A common scenario in which this substitution may be warranted is in nonhuman primates that have received intramuscular injections of ketamine in the region where sciatic nerve is routinely collected. Chemical and mechanical trauma associated with such injections has been shown to damage the nearby sciatic nerve trunk (Carrier and Donnelly, 2014).

While sciatic nerve (or tibial nerve) commonly is the only PNS structure evaluated for Situation 1, additional spinal-origin somatic nerves may be collected at necropsy. Retaining other nerves in the archived wet tissues may prevent the need to repeat studies in the event that changes observed in the sciatic nerve necessitate evaluation of other portions of the PNS. A simple means for accomplishing this task in rodents is to retain an entire hind limb (after removing the skin) and the proximal tail. In non-rodent species, the distal nerve trunks should be removed at necropsy. Other nerves to consider for collection are listed below (under Situations 2-4, and in **Table 2**). The choice of which additional nerves to harvest, or whether more PNS tissue should be sampled at all, should remain the decision of the institution.

Ganglia. A majority of Working Group members, with some dissent, recommend that DRG need not be evaluated routinely for Situation 1. The Working Group does endorse collection and archiving of at least one DRG location associated with the origin of the sciatic nerve against the possibility that an explanation might need to be sought for lesions observed in the nerve. The rationale for this recommendation is that DRG, as well as the nerves they serve, lack effective neurovascular barriers (Olsson, 1990, Abram *et al.*, 2006, Sapunar *et al.*, 2012) and thus may be exposed to test items that are excluded from the CNS by the blood-brain barrier. Usually, the chosen DRG is associated with the spinal cord segments from which the sampled spinal nerve arises (i.e., the lumbar intumescence for the sciatic nerve and its branches) (**Table 4**). A fast and simple means for retaining the DRG (and their associated spinal nerve roots) in the wet tissues is to harvest an extended portion (rodents) or region-specific segments (all species) of the vertebral column (after removing the musculature and skin). The DRG may be processed and evaluated as isolated ganglia (all species) or *in situ* in decalcified vertebral column sections (rodents only). Autonomic PNS ganglia to be assessed in Situation 1 are limited to the enteric and parasympathetic ganglia already present within protocol-specified hollow viscera (e.g., heart, intestines, urinary bladder). Specific sampling of additional autonomic ganglia is not needed.

Effector Organs. In Situation 1, skeletal muscle typically is examined as a protocol-specified tissue. Reductions in myofiber diameter may serve as indirect evidence of PNS damage due to nerve fiber (i.e., motor axon) degeneration if direct evidence of myopathic injury is not seen. Although tongue is a common choice for histologic evaluation of skeletal muscle (as a means of assessing many myofibers in several orientations in a single section), other skeletal muscle groups can be collected along with their innervating nerves. Muscles commonly selected for sampling are composed mainly of type I ("slow twitch," fatigue-resistant) fibers (e.g., diaphragm and soleus) and/or type II ("fast twitch," glycolytic) fibers (e.g., biceps femoris, quadriceps femoris, and gastrocnemius) (Schiaffino and Reggiani, 2011). Some investigators substitute biceps brachii (if the forelimb appears to be affected).

The Working Group recommends the gastrocnemius as the default sample since it has a mixed (but mainly type II fiber) composition (Armstrong and Phelps, 1984); is a common site of neurogenic atrophy in both humans (Spencer and Schaumburg, 1977) and animals with peripheral neuropathy; and the size of the muscle can be assessed qualitatively during life by palpation. The biceps femoris is a suitable alternative sample as it also is a common location for detecting neurogenic atrophy. The exact choice of muscles should be left to the discretion of the institution.

<u>Situation 2</u>

In general toxicity studies where in-life clinical signs or other data (e.g., MOA and QSAR similar to known PNS toxicants) suggest the potential for somatic (sensorimotor) PNS effects (Situation 2), the number of PNS specimens subjected to light microscopic analysis should be expanded. Specific neurological evidence warranting additional sampling of the somatic PNS includes local or generalized signs of paresis, paralysis, proprioceptive defects, or muscle atrophy (**Table 1**). Non-specific clinical observations related to possible somatic nervous system dysfunction (e.g., abnormal movement, circling, difficulty walking, lameness of unknown origin, and generalized skeletal muscle weakness) also may trigger collection of additional PNS samples, at the discretion of the institution.

Nerves. Multiple mixed (sensory and motor) spinal nerves are sampled bilaterally during the initial tissue analysis (**Figure 1**) (Spencer and Schaumburg, 1977). In addition to the sciatic nerve, the choice of other nerves to collect may be dictated by the spectrum of neurological signs observed in-life or may conform to a pre-defined battery specified in an institutional SOP. Typically, distal nerve branches are preferred for evaluation since they usually contain a high proportion of sensory axons, and clinical cases of peripheral neuropathy often present as altered sensation (Martyn and Hughes, 1997, Azhary *et al.*, 2010). Furthermore, hind limb nerves rather than forelimb nerves usually are sampled in toxicity testing because the longer nerve fibers that serve the hind limb usually are affected first during neuropathies (Krinke, 2011). That said, forelimb nerve branches also should be harvested if the in-life neurological signs suggest that forelimb function has been affected. Evaluation of nerves near the administration site may be prioritized in instances where a locally delivered test item has limited systemic bioavailability.

At least three spinal-origin nerves (usually sciatic nerve and two of its branches) are evaluated, but the decision regarding which nerves to assess should be left to the discretion of the institution. The tibial (all species), fibular (all species), and/or sural (rodents (Peyronnard *et al.*, 1986)) nerves are common choices as they are mixed sensorimotor tributaries of the sciatic nerve. In rodents, the caudal nerve (a mixed nerve that extends the entire length of the tail) also may be considered for evaluation as electrophysiological testing (e.g., nerve conduction velocity) combined with light microscopic examination of this nerve affords an opportunity to correlate structural and functional findings related to PNS neurotoxicity (Schaumburg *et al.*, 2010). Some Working Group members have found that aldehyde fixation of the proximal to middle tail (via intravascular perfusion or immersion) allows for later harvest and analysis of caudal nerve. In general, nerves are evaluated unilaterally (in which case nerves that are to be examined for a given animal typically are harvested from the same side), but bilateral evaluation may be considered at the discretion of the institution or if necessitated when iatrogenic nerve damage is likely due to in-life trauma (e.g., intramuscular injection sites).

Collection of dedicated sensory-only or motor-only nerves is not necessary for safety assessment since the approach to microscopic evaluation is similar for both mixed and single-modality nerves. If observed clinical signs are indicative of a sensory neuropathy (which is the most common presentation of peripheral polyneuropathy in humans and animals), the Working Group recommends that at least one PNS specimen be a sensory-predominant (often termed “sensory-only”) nerve. Readily accessible sites include the plantar (usually the lateral branch in dogs (Ghoshal, 1975a) but the medial branch in rodents (Sant'Anna *et al.*, 2016) and pig (Ghoshal, 1975b)); saphenous (dogs (Braund *et al.*, 1980) and rodents (LaMotte *et al.*, 1991)); sural (rodents and primates [including humans] (Butt *et al.*, 2014)); or caudal (rodent (Schaumburg *et al.*, 2010)) nerves. The only motor-specific nerves in all species are the ventral spinal nerve roots, which may be assessed individually or in sections that also include the sensory-only dorsal spinal nerve root and its associated DRG. For this purpose, serial DRG sections may be necessary to ensure that the desired nerve root is examined as their morphologic features are identical. The choice regarding whether or not to sample sensory-only and/or motor-only nerves should be left to the institution.

Cranial nerve V (trigeminal nerve) often is considered for evaluation since this mixed somatic nerve may be readily collected once the brain has been removed. In addition, several trigeminal nerve branches also may be evaluated *in situ* if present within standard nasal sections taken for inhalation toxicity studies (usually done only for rodents). Other cranial nerves typically are analyzed only if in-life neurological signs suggest that their function has been compromised (reviewed in (Bolon and O'Brien, 2011). The optic nerve (or cranial nerve II), while routinely included in the list of protocol-specified tissues for GLP-type general toxicity studies, develops as an evagination arising from the forebrain and is myelinated by oligodendrocytes and not Schwann cells (Butt *et al.*, 2004, Garman, 2011b), and so is not a part of the PNS.[9]

Ganglia. If evidence of a somatic peripheral neuropathy is observed, at least two DRG should be evaluated for both the cervical and lumbar divisions of the spinal cord (i.e., at least four total DRG). The best practice is to remove DRG from the vertebral column (**Figure 2**) to preclude the induction of handling artifacts associated with vertebral decalcification needed for *in situ* examination. However, an acceptable practice in rodents is to assess DRG *in situ* to avoid trauma produced during their removal. Because soft tissue gathered when

[9] Best practices for sampling optic nerve have been published previously Bolon, B., Garman, R.H., Pardo, I.D., Jensen, K., Sills, R.C., Roulois, A., Radovsky, A., Bradley, A., Andrews-Jones, L., Butt, M. and Gumprecht, L. (2013b). STP position paper: Recommended practices for sampling and processing the nervous system (brain, spinal cord, nerve, and eye) during nonclinical general toxicity studies. *Toxicol Pathol,* **41,** 1028-1048.

seeking DRGs sometimes represents connective tissue or fat, more than two DRG should be harvested to ensure that at least two DRG from each specified spinal cord level actually are available for histologic evaluation. Even more ganglia may need to be collected and examined when the test item is delivered directly nearby (e.g., epidural or intrathecal injection) or when clinical signs suggest that nerves arising from a particular spinal cord segment or segments have been affected. The DRG typically are chosen from those associated with the origins of the brachial plexus (i.e., origin of the brachial nerve) and lumbosacral plexus (i.e., origin of the sciatic nerve) because axons emanating from these ganglia are some of the longest (and thus among the most susceptible) in the body. The locations of DRG serving the brachial and sciatic nerves vary by species and sometimes strain (**Table 4**).

In addition to DRG, the trigeminal ganglion (i.e., the sensory ganglion of cranial nerve V) should be collected for evaluation. Ganglia of the autonomic PNS are assessed when seen *in situ* within routinely sampled organs (e.g., intramural parasympathetic and enteric ganglia in the heart, intestines, and urinary bladder). Similar to Situation 1, additional autonomic ganglia need not be sampled for this scenario.

<u>Effector organs</u>. Skeletal muscle from sites other than the tongue should be examined from two or more distinct muscles. The specific sampling location(s) may be left to institutional preference and the parameters of the study design (e.g., muscle near sites of locally delivered test items also should be sampled). The Working Group recommends that gastrocnemius serve as the default choice for one of the two specimens.

Muscle weights acquired at necropsy may provide an indirect but quantitative means of discriminating peripheral neuropathic effects. Weights typically are acquired from isolated biceps brachii, biceps femoris, gastrocnemius, and/or quadriceps femoris, which can be easily identified and collected in a consistent fashion (Greene, 1935, Vleggeert-Lankamp, 2007, Magette, 2012). The Working Group recommends the gastrocnemius for weighing since peripheral neuropathies usually occur first in longer axons (which in the hind limb are most distant from their supporting neurons (Krinke, 2011)). Care is required in interpreting the relevance of muscle weights if they have been gathered from samples taken near the site of local test item administration. Where present within muscle sections, muscle spindles (i.e., sensory end-organs) and intra-muscular nerves should be assessed, leaving the choice to the institution regarding how to record test item-related findings observed in these structures.

Situation 3

In general toxicity studies where autonomic PNS neurotoxicity is a concern (Situation 3), expanded sampling of autonomic PNS structures is necessary. Evidence warranting more extensive autonomic PNS sampling includes signs of visceral dysfunction including abnormalities in gastrointestinal motility, heart rhythms, micturition (urinary retention or incontinence), ocular responsiveness (mydriasis and miosis), salivation, or vascular tone (Mathias, 2003) (**Table 1**). A WOE approach is especially important in deciding whether or not to engage in expanded sampling and analysis of the autonomic PNS. In general, isolated signs of visceral distress (e.g., affecting one or two autonomic functions) usually reflect signs of toxicity to extra-neural organs rather than to the autonomic PNS, and thus would not serve as an automatic trigger for increased autonomic PNS sampling. Instead, expanded autonomic PNS collection would be undertaken if a generalized autonomic dysregulation was suggested by multiple anomalous signs originating in the autonomic CNS or PNS.

When autonomic PNS neurotoxicity is suspected, care should be taken to properly define the extent to which the histopathologic evaluation of the nervous system should be increased. Sometimes multiple autonomic divisions (i.e., enteric, parasympathetic, sympathetic) may be affected at once, which would warrant more sampling of all these divisions. In addition, autonomic neuropathies also may be accompanied by somatic neuropathies, in which case expanded sampling of the somatic PNS (as defined for Situation 2 above) also is required. As noted above, the final PNS sampling strategy should be driven by the constellation of PNS-related in-life neurological signs.

Nerve. Though the number of autonomic nerves conducive for sampling may be limited, multiple autonomic (**Figure 3**) nerves should be assessed during the initial tissue analysis for Situation 3. Autonomic PNS sampling may include parasympathetic (e.g., cranial nerve X [vagus]) and/or sympathetic (e.g., sympathetic chain branches) structures. Somatic nerve sampling often mirrors that described above for Situation 2 (**Figure 1**).

Ganglia. Intramural autonomic (parasympathetic) ganglia in protocol-specified hollow organs (e.g., gastrointestinal tract, heart, urinary bladder) should be evaluated. Ganglionic sampling should be based on in-life findings (i.e., visceral dysfunction), but enteric ganglia should be included for evaluation whenever autonomic neuropathy is suspected as they are readily identified in intestinal sections. If enteric ganglia are missing from routine sections, then preparation of additional tissue sections of protocol-specified viscera may be considered.

In addition, several sympathetic ganglia should be obtained. Frequently sampled sites include the cranial (superior) cervical ganglion, cervicothoracic ganglion, cranial (superior) mesenteric ganglion, and the celiac/cranial mesenteric ganglion. The caudal vagal (nodose) ganglion—which is a sensory [visceral afferent] portion of cranial nerve X—is easily confused with the cranial cervical ganglion since both are located in proximity to the bifurcation of the carotid artery (**Figure 3**). Somatic sensory PNS ganglia, such as multiple DRG (cervical and lumbar) and trigeminal (cranial nerve V) ganglia, also should be considered for sampling.

<u>Effector organs</u>. In most toxicity studies, the list of protocol-specified tissues will include multiple effector organs that are innervated by the autonomic PNS (e.g., glands, heart, hollow organs with abundant smooth muscle like the digestive tract and urinary bladder).

Lesions of the autonomic PNS have been linked on occasion to structural changes in some effector organs. For example, systemic administration of ganglioplegic drugs (i.e., “ganglionic blockers,” which inhibit transmission between pre-ganglionic and post-ganglionic autonomic neurons in both the parasympathetic and sympathetic systems) can induce sperm granulomas in the epididymis of rats (Bhathal *et al.*, 1974). However, sperm granulomas are a common incidental background finding in this species, so their presence should not be interpreted as confirmation that a test item produces autonomic dysfunction in the absence of additional evidence to support this conclusion.

<u>Central (CNS) autonomic centers</u>. Preganglionic neurons for autonomic nerves reside in various brain nuclei (parasympathetic role) and the lateral (intermediate) column of the thoracic ± rostral lumbar spinal cord (sympathetic role). The hypothalamus serves many significant autonomic tasks. The most important autonomic structure in this region is the paraventricular nucleus (PVN) of the hypothalamus, which contains neuroendocrine cells that innervate the median eminence and pituitary gland (Ulrich-Lai and Herman, 2009). In

rodent brains trimmed according to current STP "best practices" for CNS sampling (Bolon *et al.*, 2013b), the PVN should be present in Level 3. Cranial nerves III, VII, IX, and X carry both somatic motor and parasympathetic nerve fibers; the parasympathetic components innervate involuntary functions of multiple muscles and glands. Locations of these brainstem parasympathetic nuclei reside outside the seven levels recommended for assessment under current STP "best practices" for CNS sampling (Bolon *et al.*, 2013b), and instead will need to be localized using a species-specific neuroanatomy atlas (Paxinos *et al.*, 2000, Paxinos and Franklin, 2001, Paxinos and Watson, 2007, Palazzi, 2011) if in-life signs warrant their assessment. The lateral column of the sacral spinal cord also contains preganglionic autonomic neurons. Dogma for the past century has classed these sacral neurons as parasympathetic, but recent functional and molecular data indicates that these neurons may actually regulate sympathetic functions in pelvic viscera (Espinosa-Medina *et al.*, 2016). These CNS sites may be considered for sampling and evaluation if the potential for an autonomic neuropathy is present, at the discretion of the institution.

<u>Situation 4</u>

In dedicated neurotoxicity studies where a CNS or PNS liability is likely (Situation 4), expanded sampling is required to more fully characterize neurotoxic hazards. Because the nervous system is the main focus of the study, more extensive sampling of the PNS (and CNS) is expected by regulatory agencies. This approach is applicable to both adult (Rao *et al.*, 2011, Pardo *et al.*, 2012, Bolon *et al.*, 2013b) and developmental (Bolon *et al.*, 2006, Garman *et al.*, 2016) neurotoxicity studies in mammals, and to organophosphate-induced delayed neurotoxicity in hens (Krinke *et al.*, 1979, Krinke *et al.*, 1997).

Nerve. Multiple (three or more) spinal-origin nerves and cranial nerve V are sampled, as defined in Situation 2 above. The precise choice of spinal-origin nerves is left to the discretion of the institution, although more distal locations and predominantly sensory nerves should be emphasized due to their early involvement in toxicant-induced peripheral neuropathies. Where nerve conduction velocity is tested (e.g., in dogs, the fibular nerve for motor fibers and the sural nerve for sensory fibers; in rats, the caudal nerve), the same nerves for the ipsilateral and/or contralateral limb should be considered for microscopic examination to permit structure-to-function correlations. Autonomic nerves typically are not collected unless in-life neurological signs suggest that lesions may exist in the autonomic PNS, in which case additional autonomic nerves as defined in Situation 3 should be collected as well.

Ganglia. Multiple DRG (more than the four collected in Situation 2) should be examined. At least two should be harvested and assessed bilaterally for each spinal cord division (cervical, thoracic, and lumbar); some institutions collect a dozen or more, especially in studies that involve direct epidural or intrathecal delivery or in which in-life neurological signs show that the sensory PNS represents a sensitive target organ. In studies where the PNS findings seen at relevant exposure levels are likely to contribute to the risk assessment, the Working Group members concur that it is impossible to assess too many DRG since neurotoxic changes in these structures do not develop in a uniform manner in these organs. The Working Group recommends removal of the DRG from the vertebral column as the best practice (to avoid decalcification-related tissue artifacts). In rodents, DRG may be evaluated *in situ* following vertebral decalcification.

Sites for collecting cervical and lumbar DRG are the same ones recommended above for Situation 2 (**Table 4**). The thoracic DRG typically are collected from the middle of that division. For DRG investigations, it is important to remember that while all DRG are located immediately adjacent to the vertebra of the same designation (i.e., DRG L_5 is immediately caudal to vertebra L_5), the spinal cord segment associated with a DRG frequently is present cranial to the vertebra bearing the same designation (i.e., spinal cord segment L_5 is located in vertebra $L_{1\text{-}2}$ in rodents (Bolon *et al.*, 2013b)).

As with Situation 2, the trigeminal ganglion (for cranial nerve V) and autonomic (parasympathetic) and enteric ganglia as available in other protocol-specified organs should be examined. If neurological signs suggest that autonomic dysfunction may be present, sampling of autonomic ganglia may be expanded to include the specimens listed for Situation 3.

<u>Effector organs</u>. If the known potential for neurotoxicity suggests that neural lesions are localized to somatic nerves and/or ganglia, skeletal muscle should be examined for at least two distinct sites, as defined above for Situation 2. Organ weights may be obtained after whole-body perfusion fixation for one or more isolated muscle bellies, at the discretion of the institution, and the isolated muscles may be employed thereafter for histopathologic analysis.

B. Situation-specific Fixation Options for PNS

<u>Situation 1</u>. For general toxicity studies in which PNS neurotoxicity is not known, suspected, or observed during life, the PNS is fixed using the same regimen applied to the non-neural tissues: immersion in NBF, commercial formulations of which contain 3.7 to 4% formaldehyde and approximately 1% (v/v) methanol (included as a stabilizer to extend the shelf-life by slowing polymerization of formaldehyde monomers into paraformaldehyde polymers (Kiernan, 2000, Kiernan, 2008)). Methanol is a solvent and therefore may induce

morphologic artifacts in PNS, especially vacuoles and splitting of myelin sheaths (Garman, 2011a). Nonetheless, due to cost and ready availability, NBF is still the preferred PNS fixative for general toxicity studies without a pre-defined need for a special assessment of the nervous system.

Immersion fixation in NBF is conducted at RT for at least 24 hours. The ratio of fixative solution to tissue should be at least 10 volumes of fluid to one volume of tissue. The quality of PNS preservation using methanol-containing NBF is acceptable provided that tissues are harvested quickly and not handled excessively (to avoid crush and stretch artifacts). If desired, MFF may be utilized for selected specimens at the discretion of the institution to preserve methanol-sensitive antigens for later immunohistochemical (IHC) detection, but this practice is not undertaken for entire studies for Situation 1.

Situations 2, 3. For general toxicity studies in which a concern for somatic (Situation 2) or autonomic (Situation 3) PNS neurotoxicity is projected by in-life neurological signs, PNS fixation typically is identical to that employed in Situation 1: immersion in NBF (3.7% formaldehyde with 1% methanol). Where feasible (e.g., where in-life neurological signs develop early enough in the course of a study to allow bulk acquisition of specialty reagents), a preferred choice for immersion fixation is MFF (e.g., methanol-free 4% formaldehyde) as the absence of methanol improves myelin integrity.

Some institutions may prefer to employ whole-body perfusion fixation if PNS neurotoxicity is suggested by in-life neurological signs (**Table 3**) and providing that additional study endpoints do not preclude this manner of fixation. Perfusion fixation may alter certain parameters commonly included in the data sets of GLP-type toxicity studies, particularly organ weights and the microscopic integrity of highly vascular organs (e.g., lung, spleen). Except for the lungs and possibly the spleen and heart, comparison of organ weights among groups should be possible for perfusion-fixed tissues from animals in the same study, if the laboratory has an established track record of successfully performing the perfusion procedure. Comparison of organ weights from perfusion-fixed animals with historical control data from immersion-fixed animals is not recommended. Technical details for whole-body perfusion fixation are given below under Situation 4.

Situation 4. For dedicated neurotoxicity studies in which an impact on the nervous system (PNS or CNS or both) is likely or certain (Situation 4), whole-body perfusion using MFF or another methanol-free fixative (e.g., 2.5% MGG) is recommended. Because perfusion fixation can impact the ability to assess other protocol-specified organs, collection of PNS (and CNS) samples commonly is done on a satellite group specifically slated for neuropathology evaluation.

For intravascular perfusion, fixative is introduced into either the left cardiac ventricle or aorta of a deeply anesthetized animal through a blunt metal needle or plastic cannula at a pressure of 120 to 150 mm Hg (approximately equal to vertebrate systolic blood pressure) by perfusion pump or a gravity drip system (Fix and Garman, 2000). Species-appropriate needle sizes are 21-25 gauge in mice and young rats, 19 to 21 gauge in adult rats, and 14 to 18 gauge (or even greater) in non-rodents (Hancock *et al.*, 2005, Bolon and Butt, 2014). A pre-flush of physiological saline may be given to prevent thrombi from forming in small blood vessels as the fixative contacts blood cells and plasma proteins. Inclusion of a vasodilator (e.g., sodium nitrite, 1 mg/ml) and/or anti-coagulant (e.g., sodium heparin, 1000 IU/L of solution) in the pre-flush maximizes vessel patency. The choice of using a pre-flush (with or without anti-coagulants and vasodilators) should be left to the institution's discretion. The volumes of pre-flush and fixative to infuse usually are determined by the need to adequately preserve the brain and spinal cord, and vary by the species. Each laboratory should develop their own protocols for intravascular perfusion especially concerning the duration, volume, and rate of perfusion. Fifty to 100 mL in adult mice, 500 to 1000 mL in adult rats, and 3 to 5 L (or more) in non-rodents are suggested as starting points for the amount of fixative solution to instill; the amount of pre-flush typically is between 30% to 50% of these volumes. Both pre-flush and fixative solutions may be perfused at either RT or 4°, but RT solutions may produce fewer artifacts (Hancock *et al.*, 2005, Bolon and Butt, 2014).

The consensus recommendation of the Working Group is that MFF is a perfusion fixative of choice for preserving PNS (and CNS) tissues for routine light microscopic analysis. If transmission electron microscopy (TEM) also is to be undertaken, inclusion of MGG is recommended as another component of the perfusate to better preserve cytoarchitectural details and reduce artifactual changes in myelin. These two aldehydes may be applied sequentially (usually using MFF to begin) or in combination. Two common mixtures are modified Karnovsky's solution (2% MFF and 2.5% MGG) and McDowell/Trump solution (4% MFF and medical-grade 1.0% MGG); in the Working Group's experience, the most common choice is modified Karnovsky's solution. Fixatives for TEM often are made in 0.1 M cacodylate or phosphate buffer (pH 7.4). Cacodylate solutions have a longer shelf-life but contain arsenic and thus require extra care during use and disposal. For combination fixatives, intact ganglia or nerves are post-fixed by immersion in fresh fixative at 4°C for 2 to 24 hours, after which tissue is transferred to fresh, ice-cold buffer. The reason for reduced fixation length with glutaraldehyde is that this agent renders tissues hard and brittle through its ability to more effectively cross-link molecules (Kiernan, 2000). Extended storage in glutaraldehyde-containing fixatives results in excessive tissue hardening that may lead to fragmentation of the samples during sectioning.

Post-fixation. For settings in which PNS neurotoxicity is suspected (Situations 2 and 3) or likely (Situation 4), or where regulatory guidelines require plastic embedding of nerve (EPA, 1998a), selected nerve samples require additional fixation to stabilize myelin lipids. For this purpose, one (Situations 2 and 3) or at least two (Situation 4) nerves—usually spinal-origin somatic trunks rather than autonomic branches—are post-fixed in glutaraldehyde and then osmium tetroxide[10] (Bolon *et al.*, 2008, Raimondo *et al.*, 2009). Osmium must be used with glutaraldehyde to best maintain cellular structures (Penttila *et al.*, 1974).

Isolated PNS samples (typically nerve cross sections) first are immersed in MGG for at least two hours (Dyck, 2005, Bilbao and Schmidt, 2015). A common composition is 2.5% MGG in 0.025 M cacodylate buffer, pH 7.4, at an osmolarity of 300-330 mOsm. Fixation may be done at RT or 4°C, after (usually overnight to 24 hours) which fixed tissue may be stored in buffer. Post-fixation in MGG is utilized for tissues fixed in NBF or MFF but is not needed for samples in which MGG was part of the perfusate. Subsequently, samples are immersed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4 at RT for 1 to 4 hours, after which tissues are shifted to buffer. Osmium penetrates poorly (approximately 1 mm total (Dykstra, 1992)), so prior to osmication PNS samples must be cleaned of surrounding adipose and connective tissue—without injuring the neural elements. Large samples (e.g., sciatic nerves of non-rodents) may need to be trimmed into thin slices to facilitate osmium permeation into the nerve center.

[10] Fixation in osmium is typically termed “osmication” (though sometimes is rendered as “osmification”).

C. Strategies for Trimming PNS Samples

Great care should be exercised when handling nerves and ganglia (even when fixed) as even subtle manipulation may cause artifactual changes. Tissue trimming of the PNS includes one or more nerve trunks and skeletal muscle (an effector organ) in all four Situations as well as DRG (including spinal nerve roots) and/or autonomic ganglia for Situations 2, 3, and 4. Nerves and skeletal muscle should be trimmed to permit analysis of fibers in both cross (transverse) and longitudinal orientations. Particular attention should be given to evaluating skeletal muscle in cross sections because the morphological features of myofibers affected by PNS lesions (e.g., “fiber group atrophy” from denervation) are assessed most readily in this orientation. Myofibers in the diaphragm and tongue are arranged in crisscrossing patterns that preclude most fibers from being viewed in truly longitudinal and cross orientations, thereby adding to the challenge of detecting “fiber group atrophy.”

A properly prepared nerve cross section (**Figure 4**) allows for an assessment of the density and numbers of myelinated axons, and to a lesser extent those of unmyelinated axons (Raimondo *et al.*, 2009). The cross-section also allows for an evaluation of myelin integrity (including discrimination between demyelination and remyelination), and may reveal Schwann cell changes not readily seen on a longitudinal section. The longitudinal section provides a means for demonstrating axonal or myelin damage spanning several internodes (**Figure 4**) and may, due to the length of nerve examined, allow for a better assessment of associated changes such as inflammatory reactions. Longitudinal nerve samples should be approximately 1 cm long if feasible (Bolon *et al.*, 2013b) to ensure that sufficient numbers of nerve fibers will be visible over extended lengths. Spinal nerve roots may be isolated if necessary for evaluation (after embedding in plastic resin, but generally are embedded along with their associated DRG, typically in longitudinal orientation (**Figure 5**).

For Situation 3, isolated sympathetic ganglia should be processed in a fashion similar to other ganglia.

D. Situation-specific PNS Embedding Strategies

Embedding of PNS tissues is a critical factor in determining the data quality derived from evaluation of PNS tissues. Paraffin allows detection of primary degenerative and infiltrative processes and therefore is a suitable embedding medium for PNS samples in general toxicity studies where PNS neurotoxicity is not a concern (Situation 1). Paraffin also is used for most specimens in general toxicity studies where PNS neurotoxicity is a concern (Situations 2 and 3) as well as in dedicated neurotoxicity studies where neurotoxicity (CNS and/or PNS) is likely or certain (Situation 4) due to its low cost and ready availability. One neurotoxicity testing guideline states that "[p]lastic embedding is required for tissue samples from the peripheral nervous system" (EPA, 1998a). The intent of this recommendation is to improve discrimination of fine cellular detail in myelinated and unmyelinated fibers. Use of plastic embedding media permits acquisition of thinner sections, thus providing improved resolution of cellular features.

Plastic embedding is expensive and labor-intensive. In studies where it is deemed that plastic embedding will be to costly for use with all PNS samples, the Working Group advises the following adaptation of regulatory guidance requiring plastic embedding for the PNS.

The Working Group recommends plastic embedding for at least one (Situations 2 and 3) or two (Situations 4) nerve cross sections (**Figure 4**), which are scenarios in which a concern exists that a test item may elicit PNS neurotoxicity. Indeed, for Situation 3, nerve fibers (and especially the myelin sheaths) of autonomic nerves often are so small that plastic sections of osmicated nerves may be essential for light microscopic assessment. In such cases, PNS specimens slated for plastic embedding have been post-fixed in glutaraldehyde and osmium. Cross sections of these nerve samples permit ready evaluation of the features and diameters for both axons and complete nerve fibers (i.e., axons plus myelin). Plastic embedding of longitudinal nerve sections is used less often as osmium deposition in myelin may obscure features in superimposed PNS nerve fibers due to overlap of the metal-impregnated myelin sheaths; however, plastic-embedded longitudinal nerve sections may be useful for evaluating nodes of Ranvier. Several Working Group members suggest that laboratories and sponsoring institutions be encouraged to consider adjusting their PNS processing procedures for Situation 1 to incorporate routine preparation of osmicated, plastic-embedded nerve cross sections as a means of attaining ideal morphologic preservation for PNS samples. However, the majority of the Working Group accepts that this proposed modification, while technically correct, may not be practical for the many general toxicity studies where no concern exists that the test item has induced PNS neurotoxicity.

Plastic embedding for nerve samples usually employs one of two variants: “hard plastic” (hydrophobic) resins such as araldite, epon, or Spurr’s, or combinations thereof (e.g., epon-araldite); or “soft plastic” (hydrophilic) materials like glycol methacrylate (GMA) and methyl methacrylate (MMA). Section thicknesses that are reproducibly attainable for PNS using hard plastic (<1 µm, **Figure 4**) and soft plastic (2 µm, **Figure 6**) are considerably reduced relative to that which is readily achievable for paraffin (4-6 µm, **Figure 4** and **Figure 6**). Soft plastics are more expensive than paraffin but are less costly and easier to process and section than are hard plastics. However, hard plastics can be used with osmicated PNS samples while soft plastics are not compatible with osmium; thus, myelin lamellae are only imperfectly conserved in soft plastic sections, which negates the original reason why plastic embedding of PNS tissues was required (EPA, 1998a). The Working Group is of the unanimous opinion that soft plastic embedding media offer little improvement in cytological resolution over paraffin embedding for non-osmicated nerve samples (**Figure 6**), and that soft plastic offers substantially inferior tissue preservation relative to hard plastic combined with osmication (**Figure 4**). Accordingly, the Working Group recommends hard plastic resin (of osmicated samples) as the best practice for plastic embedding of PNS, and further advises that the use of soft plastic is not a suitable alternative for PNS embedding. Methodological details for hard plastic embedding are found in the manufacturer's instructions available with commercially available kits.

Osmium-impregnated nerves may be embedded in paraffin (Bolon *et al.*, 2013a). The preservation and visualization of myelin is enhanced in osmicated, paraffin-embedded nerve sections in comparison to non-osmicated, paraffin-embedded nerve sections but remains inferior to osmicated, hard plastic resin-embedded sections. Therefore, the Working Group recommends that paraffin embedding of osmicated tissues be avoided as a substitute for hard plastic resin embedding.

For dedicated neurotoxicity studies (Situation 4), the Working Group recommends that nerves and DRG should be embedded in individual blocks (with or without other tissues) so that lesions may be tracked to identifiable PNS sites. Alternatively, some institutions place many DRG from all spinal cord divisions in one cassette (**Figure 5**), or group DRG from specific spinal cord segments into separate cassettes. The Working Group recommends that sampling more DRG is preferable, even if exact locations of individual DRG are not tracked.

For both paraffin- and plastic-embedded specimens, the Working Group recommends that all PNS tissues from the treatment groups selected for initial evaluation (e.g., high-dose and control animals) should be processed in the same time frame to avoid any systematic variation in such technical factors as the lengths of time spent in fixative or dehydrating solutions. If a quantitative endpoint has been built into the study design, tissues from all study groups for which the endpoint might need to be collected should be processed into blocks at the beginning of the study, even if sectioning of the blocks for intermediate doses groups will be delayed; this strategy will greatly reduce the likelihood that variations in processing will impact the quantitative data. A key means of standardizing the effects of fixation and processing across treatment groups is to include cassettes from animals in the different cohorts within each processing "run" so that handling-related artifacts are balanced by mixing samples from all dose groups.

E. Situation-specific PNS Staining Strategies

Staining of PNS tissues employs hematoxylin and eosin (H&E) for paraffin sections and toluidine blue for hard plastic resin sections (**Table 3**). The Working Group recommends these two stains as a suitable initial screen for PNS specimens in toxicity studies. When preparing PNS tissues, an important consideration is that delayed processing of some treatment groups (as opposed to immediate processing of all groups into blocks) may result in altered tinctorial intensity in H&E-stained nerve sections, which might confound any quantitative or *post hoc* coded histopathologic evaluations. For studies in which PNS neurotoxicity represents a possible concern (Situations 2, 3, and 4), other neurohistological methods may be undertaken in non-osmicated, paraffin-embedded nerves at the discretion of the institution to further characterize any PNS findings discerned during the initial analysis. Stain quality for such special procedures varies depending on many factors, including section thickness, technician experience, and regularity with which the procedure is performed.

When needed, the Working Group recommends stains for axons and myelin as the most useful special methods for further characterizing PNS findings related to test item exposure. Silver stains (e.g., Bielschowsky's [**Figure 7**], Bodian's, or Holmes) are helpful to highlight neurofilament-rich structures, including axons and cytoplasmic organelles in neurons, and for demonstrating damaged axons in axonopathy (e.g., fragmentation) or neuroaxonal dystrophy (e.g., axonal spheroids). Myelin-staining methods used in PNS tissues include Luxol fast blue (LFB; **Figure 7**) and Sudan black, which are especially beneficial for intact myelin, and the Marchi stain, which often is used to reveal demyelination (Strich, 1968). The reasons for recommending these procedures are that axons and myelin are the two key components of PNS structures, and thus many laboratories routinely perform these stains.

Special neurohistological methods used to showcase neurotoxic damage in the CNS typically are not utilized when evaluating PNS neurotoxicity. Routine IHC methods to demonstrate glial fibrillary acidic protein (GFAP, upregulated in reactive astrocytes and in some satellite glial cells) and ionized calcium-binding adapter molecule 1 (Iba1, expressed by microglia and macrophages) may be used in DRG to detect satellite glial cells and activated macrophages, respectively (Patro *et al.*, 2010, Ton *et al.*, 2013), but such techniques typically are deployed in the research setting rather than in toxicity testing. Glutamine synthetase (a preferred marker for satellite glial cells (Miller *et al.*, 2002, Schaeffer *et al.*, 2010)) and CD68 (a macrophage marker (Jimenez-Andrade *et al.*, 2006)) may be used with or in place of anti-GFAP and anti-Iba1 procedures; leukocyte biomarkers (e.g., anti-CD3 for T-lymphocytes) may be helpful in differentiating inflammation from increased satellite cell numbers. Fluoro-Jade, a fluorescent stain used to detect necrotic neurons in the CNS (Schmued and Hopkins, 2000, Schmued *et al.*, 2005), may be attempted in the PNS to detect degenerating neurons in ganglia (Marmiroli *et al.*, 2009). In the experience of several Working Group members, Fluoro-Jade does not specifically highlight necrotic ganglionic neurons, presumably because dead neurons in DRG do not express the as yet unidentified marker labeled by Fluoro-Jade stains in dead CNS neurons. Accordingly, the Working Group does not recommend the routine use of these CNS-oriented special methods for evaluating PNS lesions.

While the use of soft plastic is not recommended for PNS tissues, archival samples embedded in this medium may be stained routinely with H&E. Other procedures that may be undertaken in soft plastic-embedded PNS tissues include silver stains for axons and histochemical stains (e.g., Sudan black; (Cerri and Sasso-Cerri, 2003)) or IHC methods (e.g., myelin basic protein (Mueller *et al.*, 2000)) to reveal myelin lipids. The experience of Working Group members is that special techniques are applied to soft plastic-embedded PNS specimens mainly in the research setting.

Hard plastic-embedded nerve cross sections usually are osmicated during processing and then stained with toluidine blue (**Figure 1**). The concentration of toluidine blue used for this purpose varies among laboratories but typically is set at 1% (1:100) (Hancock *et al.*, 2005). Paraphenylenediamine (PPD) also may be employed to highlight lipid-rich cell membranes in osmicated, hard plastic resin-embedded sections (Shirai *et al.*, 2016), including myelin sheaths (Sadun *et al.*, 1983).

Special histochemical procedures may be applied to differentiate various myofiber types in skeletal muscles (Armstrong and Phelps, 1984, Kremzier, 1984, Staron *et al.*, 1999). These methods generally are not used to evaluate muscle samples in situations where PNS neurotoxicity is a concern as muscle lesions due to PNS damage (i.e., fiber group atrophy) may be seen easily by H&E.

F. Special Procedures for Evaluating PNS Neurotoxicity

If warranted, additional techniques may be undertaken to better characterize PNS lesions. Examples include TEM (Peters *et al.*, 1991), morphometry (Diemer, 1982, Kristiansen and Nyengaard, 2012, Butt *et al.*, 2014), stereology (Hyman *et al.*, 1998, Butt *et al.*, 2014), teased fiber preparations (Krinke *et al.*, 2000), and quantification of intra-epidermal nerve fiber ending density (IENFD; (Lauria *et al.*, 2005a, Lauria *et al.*, 2005b, Myers and Peltier, 2013, Mangus *et al.*, 2016)), motor end plates (Francis *et al.*, 2011), and muscle spindles (Krinke *et al.*, 1978, Muller *et al.*, 2008). A detailed consideration of such special procedures is beyond the scope of this paper. Decisions regarding whether or not to deploy these methods should be guided by data showing the neurotoxic potential of a test item to the PNS, usually the presence of in-life neurological signs or prior knowledge that the test item or a related molecule produces morphological effects in the PNS. Another important reason for quantifying IENFD, motor end plates, and/or muscle spindles may be to show an absence of neuropathy, to provide evidence that nerve signaling is intact. The choice regarding whether or not to use these special procedures should be left to the discretion of the institution.

IV. Strategy for PNS Neuropathology Analysis

The approach used for initially evaluating the PNS during nonclinical toxicity studies in all four situations is a tiered, semi-quantitative light microscopic examination (i.e., assigning lesion grades) equivalent in concept to that for any other organ or tissue. Criteria used to set histopathologic grades for unusual findings should be defined using text descriptions and/or visual illustrations of concrete features, or should be established by citing published, well-established grading schemes. The analysis should identify the existence of morphological changes in PNS tissues and characterize the lesion pattern so that the cell populations (e.g., neurons and/or Schwann cells) and structures (e.g., cell body vs. axon vs. myelin) targeted by the test item can be determined.

The initial microscopic evaluation of PNS tissues from nonclinical toxicity studies for all four situations generally should be conducted in an informed ("unblinded" or "unmasked") fashion. In other words, the study pathologist should receive *in advance* full knowledge of the dose level and group assignment for each animal as well as other data (macroscopic findings, organ weights, clinical observations and outcomes of behavioral testing), all of which might help in interpreting the microscopic pathology data. This recommendation represents a consensus opinion among experienced toxicologic pathologists (Gosselin *et al.*, 2011), including members of the Working Group, and conforms to STP recommended "best practices" for histopathologic evaluation of tissues from toxicity studies (Crissman *et al.*, 2004). The initial uncoded assessment may be limited to the control and high-dose groups or may include all dose groups at the discretion of the institution. The rationale for this recommendation is that informed examination greatly enhances the quality of the pathology data set by (1) permitting development of more objective criteria for grading changes, (2) increasing the likelihood of detecting subtle PNS findings, and (3) enhancing the speed with which the analysis may be undertaken (which reduces diagnostic drift). This logic is no different from that used when designing the assessment for any other organ or system.

Once a PNS finding has been identified, a masked ("blinded" or "coded") *post hoc* assessment of specific changes may be performed at the discretion of the study pathologist (or peer review pathologist (Morton *et al.*, 2010)). Such blinded evaluations should be limited, performed only as needed to clarify the incidence of subtle findings, tighten severity grade assignments, discern treatment-associated exacerbation of background lesions, and/or establish a dose-effect relationship (including definition of a no observed adverse effect level [NOAEL]). The choice of which dose groups and findings to include in a masked evaluation is not defined by existing regulatory guidelines but rather is chosen by the pathologist; for example, the "blinded" assessment may be limited to the control and low-dose animals and ignore any other dose groups if clear neuropathologic changes are evident in the mid-dose and high-dose animals. This same strategy—informed initial analysis followed if necessary by a supplemental masked evaluation—also should be the usual practice for neuropathology peer reviews oriented toward PNS lesions.

IV. Neuropathology Documentation

With respect to communication of PNS neuropathology data, the final report for a toxicity study should contain all the parts of a conventional pathology report (e.g., a narrative together with individual animal and summary data tables) while providing detailed descriptions of the particular neuropathology techniques (e.g., fixative solutions and methods, embedding and staining procedures) used for the study, and ideally the reasons why they were chosen by the institution. Regulatory scientists have repeatedly expressed a preference that PNS lesions in the individual animal data tables be referenced to specific anatomical sites (e.g., “sciatic nerve” or “dorsal root ganglion”) rather than more generic terms (e.g., “nerve” or “peripheral nerve” or “ganglion”), and that the key PNS structures that were sampled are explicitly stated in the report. When assessing autonomic ganglia, institutions retain the discretion regarding whether or not findings in non-protocol-specified neural structures (e.g., intramural autonomic ganglia in protocol-specified organs, muscle spindles) are to be recorded as a separate tissue (i.e., “enteric ganglia”) or as a notation under the tissue in which they reside (i.e., “heart”, “jejunum”, or “urinary bladder”). This choice may differ for Situation 1 (general toxicity study with no in-life PNS signs) vs. Situations 2, 3, or 4 (studies for which PNS neurotoxicity is a concern). The Working Group concurs that the use of specific terms for protocol-specified PNS sites represents the optimal practice for reporting PNS lesions. If full methodological details are not included specifically in the final report, they should be made available in an institutional reference document (e.g., SOP) detailing the PNS sampling and trimming scheme.

V. Discussion

The Working Group unanimously holds that these “best practice” recommendations for PNS sampling, processing, and evaluation are detailed enough to provide for a systematic analysis of the PNS in GLP-type toxicity studies for four distinct neurotoxicity scenarios and yet still sufficiently flexible to allow their implementation via relatively modest revisions of existing institutional practices. The experiences of Working Group members suggest that

PNS sampling at many institutions already approaches or conforms to the recommendations set forth here, especially for Situations 1, 2, and 4—with the likely exception of the preferred plastic embedding medium (as discussed below). Therefore, adoption of these best practice recommendations should not represent a major departure from current practice for these three scenarios. The sampling recommendations where autonomic PNS neurotoxicity is a concern (Situation 3) likely will require adjustments to existing institutional practices; given the extensive autonomic control of many physiologic processes, a discussion of what spectrum of clinical signs might suggest a general effect on the autonomic nervous system warranting increased autonomic PNS sampling also will be in order. Common sense will need to be utilized during implementation of these recommendations as certain common clinical observations (e.g., emesis in dogs and nonhuman primates) occurring in isolation seldom will indicate the existence of autonomic PNS neurotoxicity, and should not automatically be investigated as such. In short, the decision regarding which PNS tissues to sample and evaluate should be made using a "weight of evidence" approach where expanded PNS sampling and evaluation is done only in Situations where the PNS represents an important target system that is likely to be an important factor in the risk assessment.

The principal adjustment that may be needed at many institutions to conform to the Working Group's "best practice" recommendations is to modify the plastic embedding protocol for PNS tissues. Current practice where PNS neurotoxicity is a concern (i.e., Situations 2, 3, and 4) often employs soft plastic media (e.g., GMA or MMA) for routinely fixed (i.e., NBF only) tissue. However, the Working Group unanimously agrees that optimal PNS preservation (especially of myelin) requires initial fixation in MFF, post-fixation in glutaraldehyde followed by osmium, and embedding in hard plastic resin. The Working

Group recognizes that many test facilities and contract histology laboratories may not be equipped at present with the specialized microtomy and hazardous waste reclamation equipment and procedures required to prepare hard plastic blocks and sections. Nonetheless, the Working Group unanimously judges that the data quality obtained using hard plastic-embedded cross sections of osmicated nerves offers the most effective means for meeting the intent of regulatory guidelines that require plastic embedding (EPA, 1998a). Indeed, the Working Group consensus is that time spent evaluating one optimally processed nerve sample—a cross section post-fixed in both glutaraldehyde and osmium tetroxide, embedded in hard plastic resin, and then cut at 1 µm—will be of greater value in understanding the nature and mechanism of toxicant-induced lesions in the PNS than will be the examination of multiple sections made using routine methods (i.e., NBF-fixed, non-osmicated, paraffin-embedded, 5 µm thick) or currently accepted specialty techniques (i.e., NBF-fixed, non-osmicated, soft plastic-embedded, 2 µm thick). A majority of the Working Group agrees that hard plastic embedding of nerve for general toxicity studies where PNS neurotoxicity is not expected (Situation 1) is not feasible as a routine practice.

VI. Concluding Remarks

Current approaches to investigating PNS neurotoxicity during GLP-type toxicity studies vary to some degree across institutions, and appear to be distinguished more by application of a few time-tried methods rather than a reasoned exploration of the PNS as a potential target site for toxicity. The procedures for PNS collection, processing, analytical, and reporting practices should depend on the aims of the study, and thus to a fair degree on institutional preference. However, a substantial improvement in the risk assessment for PNS neurotoxicity may be gained by improving the consistency of PNS sampling, processing, and evaluation. The STP believes that adoption of these “best practice” recommendations will provide a systematic yet malleable strategy for increasing the consistency, and thus the quality, of PNS sampling, processing, and analysis among institutions and across geographic regions over time.

Continuing advances in diverse fields like computational biology and non-invasive imaging (structural and functional) are transforming the modern practice of toxicologic neuropathology and human risk assessment. The STP believes that these “best practice” recommendations for PNS collection, processing, and evaluation may serve as a logical morphological “gold standard” against which emerging technologies and experimental neurotoxicity models may be measured.

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VIII. References

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VII. Tables

Table 1: Different GLP-type Nonclinical Toxicity Studies in Which PNS Neurotoxicity is Evaluated

	"Tier I" Studies			"Tier II" Studies
	General Toxicity Studies			Dedicated Neurotoxicity Studies
Parameter	Situation 1	Situation 2	Situation 3	Situation 4
PNS Neurotoxic Presentation	None	Somatic (suspected)	Autonomic (suspected)	Somatic and/or Autonomic (expected)
Historical Evidence of Neurotoxicity				
• Known potential for neurotoxicity (CNS or PNS)	X	X	X	✓
• Suspected potential for neurotoxicity based on the putative mode of action (MOA) and/or quantitative structure-activity relationships (QSAR) modeling	X	X	X	±
In-life Evidence of PNS Neurotoxicity				
• None	✓	N/A	N/A	N/A
• Signs suggest **somatic (sensorimotor) PNS neurotoxicity**—abnormal movement, circling, difficulty walking, lameness of unknown origin, generalized muscle weakness)	N/A	✓	±	✓
• Signs suggest **autonomic PNS neurotoxicity**—anomalies in gastrointestinal motility, heart rhythms, micturition (urinary retention or incontinence), ocular responsiveness (mydriasis and miosis), salivation, or vascular tone, and formation of sperm granulomas	N/A	±	✓	±

Abbreviations: CNS = central nervous system, N/A = not applicable, PNS = peripheral nervous system

Symbols: ✓ = present, X = not present, ± = may be present

Table 2: Recommended Sampling for Peripheral Nervous System (PNS) Tissues During Nonclinical Toxicity Studies

		General Toxicity Studies			Dedicated Neurotoxicity Studies
Parameter	Parameter Options	Situation 1	Situation 2	Situation 3	Situation 4
PNS Neurotoxic Presentation		None	Somatic (suspected)	Autonomic (suspected)	Somatic and/or Autonomic (expected)
Sampling (nerves and ganglia)	Bilateral (all species)	✓	✓	✓	✓
Somatic PNS					
Mixed nerves	Sciatic	✓	✓	✓	✓
	Tibial	Alternative (instead of sciatic)	✓	✓	✓
	Fibular (common peroneal)	X	✓ [d]	✓	✓ [d]
	Sural (rodent)	X	✓ [d]	✓	✓ [d]
	Caudal (rodent)	X	✓ [d]	✓	✓ [d]
Motor-only nerve	Ventral spinal nerve root	X	X	X	✓ [d]
	Muscle spindles (in large skeletal muscles)	X	X	X	Assess if present
Sensory-only nerve	Saphenous (canine, rodent)	X	✓ [d]	✓	✓ [d]
	Sural (nonhuman primate)	X	✓ [d]	✓	✓ [d]
	Plantar (canine, rodent)	X	✓ [d]	X	✓ [d]
	Dorsal spinal nerve root	X	X	X	✓ [d]
Cranial nerve	V (trigeminal) [a]	X	✓	✓	✓
Ganglia	Dorsal root ganglia (DRG) – cervical (C) and lumbar (L) ± thoracic (T) regions [a]	X	2 or more (for both C and L)	2 or more (for both C and L)	2 or more (for all regions: C, T, L)
	Trigeminal (cranial nerve V) [a]	X	✓	✓	✓
Other	Vertebral column [b]	✓	✓	✓	✓
	Hind limb (intact) [c]	✓	✓	✓	✓
Autonomic PNS					
Autonomic Nerve	Cranial nerve X (vagus = parasympathetic)	X	X	✓	Assess if present
	Sympathetic chain (sympathetic)	X	X	✓	Assess if present
	Nerve trunks attached to autonomic ganglia	X	X	X	✓
Autonomic Ganglia	Enteric ganglia	As available *in situ* in protocol-specified organs (e.g., intestines)	As available *in situ* in protocol-specified organs (e.g., intestines)	As available *in situ* in protocol-specified organs (e.g., intestines)	As available *in situ* in protocol-specified organs (e.g., intestines)
	Parasympathetic ganglia	As available *in situ* of protocol-specified organs (e.g., urinary bladder)	As available *in situ* in protocol-specified organs (e.g., urinary bladder)	Examine at least 2 specific ganglia (e.g., in walls of heart or urinary bladder)	As available *in situ* in protocol-specified organs – use scheme for Situation 3 if in-life autonomic signs are observed

	Sympathetic ganglia	X	X	Sample at least 2 distinct sites (e.g., cervicothoracic, cranial cervical, cranial mesenteric, or sympathetic chain)	As available *in situ* in protocol-specified organs – use scheme for Situation 3 if in-life autonomic signs are observed
Autonomic CNS centers	Hypothalamus: paraventricular nucleus (PVN) – present in routine brain sections	✓	✓	✓	✓
	Brain nuclei (parasympathetic) for cranial nerves III, VII, IX, and X	X	X	Sample (using neuroanatomic atlas) as needed based on neurological signs	Use scheme for Situation 3 if in-life autonomic signs are observed
	Spinal cord, lateral/intermediate column in thoracic division (sympathetic)	X	X	✓	✓

Effector Organs					
Skeletal Muscle	One or more (biceps femoris, a specific head of the quadriceps femoris, gastrocnemius, soleus, and/or diaphragm)	One site (gastrocnemius recommended)	Two or more sites (gastrocnemius recommended)	One site (gastrocnemius recommended)	Two or more sites (gastrocnemius recommended)
	Muscle weights	X	As needed [c]	X	As needed [c]

Abbreviations: C = cervical, DRGs = dorsal root ganglia, L = lumbar, T = thoracic

Symbols: ✓ = collected, X = not collected

[a] May be prepared *in situ* in rodents followed by decalcification of vertebral cross sections or skull.

[b] Vertebral column (intact in rodents, cervical and lumbar segments in non-rodents) should be kept in case DRGs and spinal nerve roots are needed.

[c] A hind limb (intact in rodents, intact or distal in non-rodents) should be kept in case additional somatic nerves are needed; overlying muscle should be reflected to expose nerves

[d] Denotes examples of distal nerve branches that may be evaluated (along with sciatic and tibial nerves) as the minimal set needed for systematic evaluation of neurotoxicity affecting the somatic PNS; in general, a sensory-only nerve (i.e., more distal branch) will be the preferred choice. Situations 2, 3, and 4 suggest sampling at least three spinal nerve locations (inclusive of sciatic and/or tibial nerves), at least one of which should be sensory-only if a sensory neuropathy is suspected.

Table 3: Recommended Baseline Processing Strategies for Peripheral Nervous System (PNS) During Nonclinical Toxicity Studies

		General Toxicity Studies			Dedicated Neurotoxicity Studies
Parameter	Parameter Options	Situation 1	Situation 2	Situation 3	Situation 4
PNS Neurotoxic Presentation		None	Somatic (suspected)	Autonomic (suspected)	Somatic and/or Autonomic (expected)
Sampling (nerves and ganglia)	Bilateral (all species)	✓	✓	✓	✓
Processing (nerves and ganglia)	Unilateral (all species)	✓	✓	✓	✓
Trimming Orientation – Nerve	Longitudinal and cross (transverse)	✓	✓	✓	✓
Fixation Method		Immersion	Immersion	Immersion	Perfusion
Fixative	Neutral buffered 10% formalin (standard)	✓	✓	✓	X
	4% formaldehyde (methanol-free) [a]	X	Nerves and ganglia	Nerves and ganglia	✓
	TEM fixative (with glutaraldehyde at 1% or greater concentration) [b]	X	As needed [d] (for nerves)	As needed [d] (for nerves)	As needed [d] (for nerves)
Post-fixative (by immersion)	Glutaraldehyde (at concentration of 1% or greater) [b]	X	Used as needed for TEM and/or prior to osmium post-fixation	Used as needed for TEM and/or prior to osmium post-fixation	Used as needed for TEM and/or prior to osmium post-fixation
	Osmium tetroxide (at 1%) [b]	X	At least 1 somatic N (C section for hard plastic embedding)	At least 1 somatic N and 1 autonomic N (C section for hard plastic embedding)	At least 2 somatic N (C sections for hard plastic embedding)
Embedding Medium	Paraffin	Ganglia Somatic N (C/L)	Ganglia Somatic N (C/L) Cranial N	Ganglia Somatic N (C/L) Cranial N	Ganglia Somatic N (C/L) Cranial N
	Hard plastic resin [c]	X	1 Somatic N (C) – after osmium post-fixation	1 Somatic N (C) and if possible 1 autonomic N (C) – after osmium post-fixation	At least 2 Somatic N (C) – after osmium post-fixation
Staining (paraffin sections)	Hematoxylin and eosin (H&E)	✓	✓	✓	✓
	Silver stains (for axons – Bielschowsky's, Bodian's. or Holmes)	X	As needed [d]	As needed [d]	As needed [d]
	Myelin stains (e.g., Luxol fast blue, Marchi)	X	As needed [d]	As needed [d]	As needed [d]
	Cell type-specific biomarkers (e.g.,	X	As needed [d]	As needed [d]	As needed [d]

	intermediate filaments, neurotransmitters)				
Staining (hard plastic sections)	Toluidine blue	X	✓	✓	✓

Abbreviations: C = cross (transverse) orientation, L = longitudinal orientation, N = nerve, TEM = transmission electron microscopy

Symbols: ✓ = utilized, X = not utilized

[a] Methanol-free 4% formaldehyde is prepared from paraformaldehyde powder to avoid the presence of methanol (a stabilizing agent that can induce myelin vacuolation as an artifact)

[b] Post-fixation in glutaraldehyde (e.g., modified Karnovsky's solution: methanol-free 2% formaldehyde [from paraformaldehyde powder] combined with medical-grade 2.5% glutaraldehyde) followed by osmium are required for optimal myelin preservation

[c] Soft plastic (e.g., glycol methacrylate [GMA]) is ***not an acceptable substitute*** for hard plastic resin (e.g., araldite, epon, or Spurr's)

[d] "As needed" decisions remain at the discretion of the institution

Table 4: Spinal Cord Origins of the Principal Forelimb (Brachial) and Hind Limb (Sciatic) Nerves for Common Vertebrate Species

Species	**Nerve**	**Main Source Segments of Spinal Cord**	**Reference**
Chicken	Brachial	C12 – C15	(Jungherr, 1969)
	Sciatic	Syn3 – Syn8	(Jungherr, 1969)
Mouse	Brachial	C4 – T2	(Kaufman and Bard, 1999)
	Sciatic	L3 – L4 **	(Rigaud *et al.*, 2008)
Rat	Brachial	C4 – T1 (± T2)	(Greene, 1935)
	Sciatic	L4 – L5 **	(Rigaud *et al.*, 2008)
Dog	Brachial	C6 – T2 (± C5)	(Ghoshal, 1975a, Sharp *et al.*, 1990)
	Sciatic	L4 – S2	(Ghoshal, 1975a, Bailey *et al.*, 1988)
Pig	Brachial	C5 – T1	(Ghoshal, 1975b)
	Sciatic	L5 – S2 (± L4)	(Ghoshal, 1975b)
Primate	Brachial	C5 – T1	(Turnquist and Minugh-Purvis, 2012)
	Sciatic	L1 – S2	(Turnquist and Minugh-Purvis, 2012)

Abbreviations: C = cervical, L = lumbar, S = sacral, Syn = synsacral (representing the fused lumbar and sacral vertebral segments)

** Denotes that the origin varies with the strain

VII. Figures

Fig 1. Locations for harvesting somatic nerves from the rodent hind limb. Nerves: a = sciatic; b = tibial; c = common peroneal (fibular); d = lateral sural; e = plantar. Muscles: 1 = gluteus medius; 2 = biceps femoris; 3 = semitendinosus; 4 = quadriceps femoris; 5 = gastrocnemius lateralis; 6 = rectus femoris; 7 = gastrocnemius medialis; 8 = tibialis cranialis. Bones: P = patella; T = tibia. (Schematic diaphragm adapted from (Popesko *et al.*, 2003) by permission of the Publisher).

Fig 2. Diagrams show how to approach and localize dorsal root ganglia (DRG) associated with the origin of the sciatic nerve. Left panel: Note that caudal segments of lumbar spinal cord (L4 to L7) are displaced cranially relative to the DRG and vertebrae of the same number. Regional anatomy is based on the dog vertebral column. Right panel: DRG are best approached via removing the vertebral arches (at the location of the dotted lines at 2 o'clock and 10 o'clock) using bone-cutting rongeurs. Images crafted by Mr. Tim Vojt.

Fig 3. Two cervical ganglia, the cranial (superior) cervical ganglion (C, a part of the sympathetic division) and the caudal vagal (nodose) ganglion (X, a visceral afferent [i.e., sensory] element), may be isolated adjacent to the trachea in the vicinity of the bifurcation of the carotid artery. Samples: left column = adult rat (provided courtesy of Dr. Magalie Boucher, Pfizer, Inc.); right = adult Beagle dog showing collection of both the cranial cervical ganglion (C) and caudal vagal ganglion (X) in the same histologic section. Processing (right image): immersion fixation in neutral buffered 10% formalin, paraffin embedding, sectioning at 4 μm, H&E staining.

Fig 4. Nerves should be available for histopathologic analysis in both cross (top row) and longitudinal (bottom row) orientations. The cross (transverse) view allows comparison of the numbers and densities of myelinated nerve fibers (large-caliber, pale blue axons bounded by thick, dark myelin sheaths) and possibly unmyelinated fibers (small-caliber axons with minimal myelin [often found in small clusters]), although such fine discrimination is only possible in specially prepared nerves exhibiting high contrast between axons (pale) and myelin sheaths (dark) (upper left panel) and not in routinely processed sections (upper right panel) where contrast is modest and extensive clear space exists between as a very common processing artifact. The longitudinal plane permits axonal and myelin integrity to be assessed over extended distances. Samples: sciatic nerve from normal (i.e., control) adult rat. Processing: left column = whole-body perfusion fixation with 4% glutaraldehyde, post-fixation in 1% osmium tetroxide, hard plastic resin (epon) embedding, sectioning at 1 μm, toluidine blue staining; right column = immersion fixation in neutral buffered 10% formalin, no glutaraldehyde or osmium post-fixation, paraffin embedding, sectioning at 4 μm, H&E staining. [Images of hard plastic-embedded nerves (left column) were provided courtesy of Dr. William Valentine, by permission.]

Fig 5. Placement of multiple ganglia in a single cassette ensures that sufficient tissue is available for histopathologic evaluation. The grouping may be ganglia from all spinal cord divisions (as shown here) or alternatively grouping as a single spinal cord division (e.g., cervical, thoracic, or lumbar). Sample: dorsal root ganglia and spinal nerve roots (arrows) from adult control rat. Processing: immersion fixation in neutral buffered 10% formalin, paraffin embedding, sectioning at 4 µm, H&E staining.

Fig 6. Soft plastic (left panel) is not a suitable medium to comply with regulatory guidelines that mandate plastic embedding of nerves, as soft plastic provides no significant improvement in resolution relative to conventional paraffin embedding (right panel). Samples: sciatic nerve from normal (i.e., control) adult rat. Processing: left column = whole-body perfusion fixation in neutral buffered 10% formalin, no osmication, soft plastic (glycol methacrylate) embedding, sectioning at 2 µm, H&E staining; right column = immersion fixation in neutral buffered 10% formalin, no osmication, paraffin embedding, sectioning at 4 µm, H&E staining.

Fig 7. Special methods used to highlight nerve fibers include silver and myelin stains. Upper panel: Bielschowsky's silver stain demonstrates axons and neuronal cytoplasm as dark profiles against a pale background. Arrows indicate swollen axons. Lower panel: Luxol fast blue stain reveals myelin as intact dark blue sheaths. A single degenerating axon is revealed at the bottom of the image as a series of vacuoles containing fragmented debris. The myriad tiny, clear vacuoles in the myelin sheaths represent a processing artifact. Processing: immersion fixation in neutral buffered 10% formalin, paraffin embedding, sectioning at 4 µm.

Figure 1

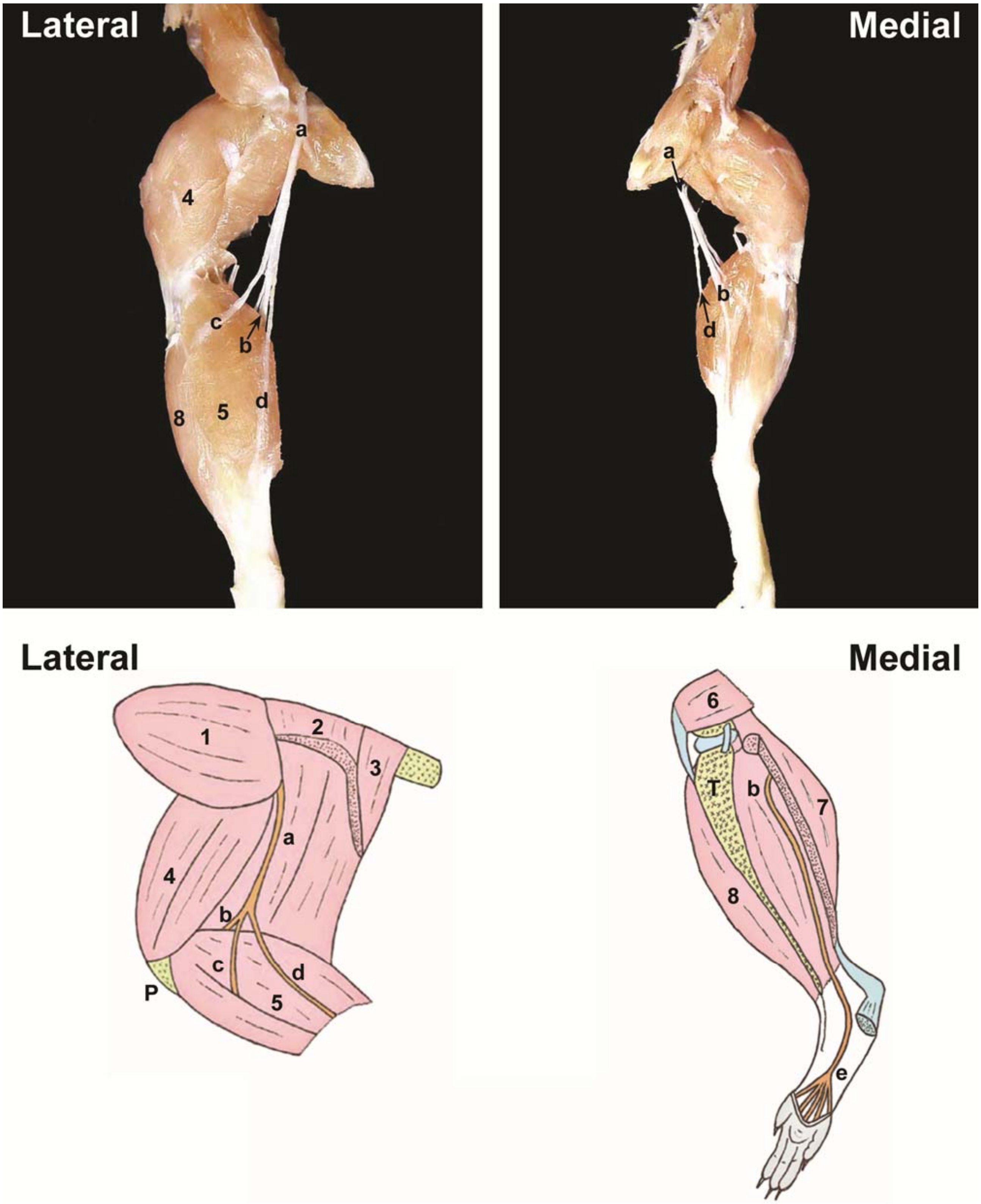

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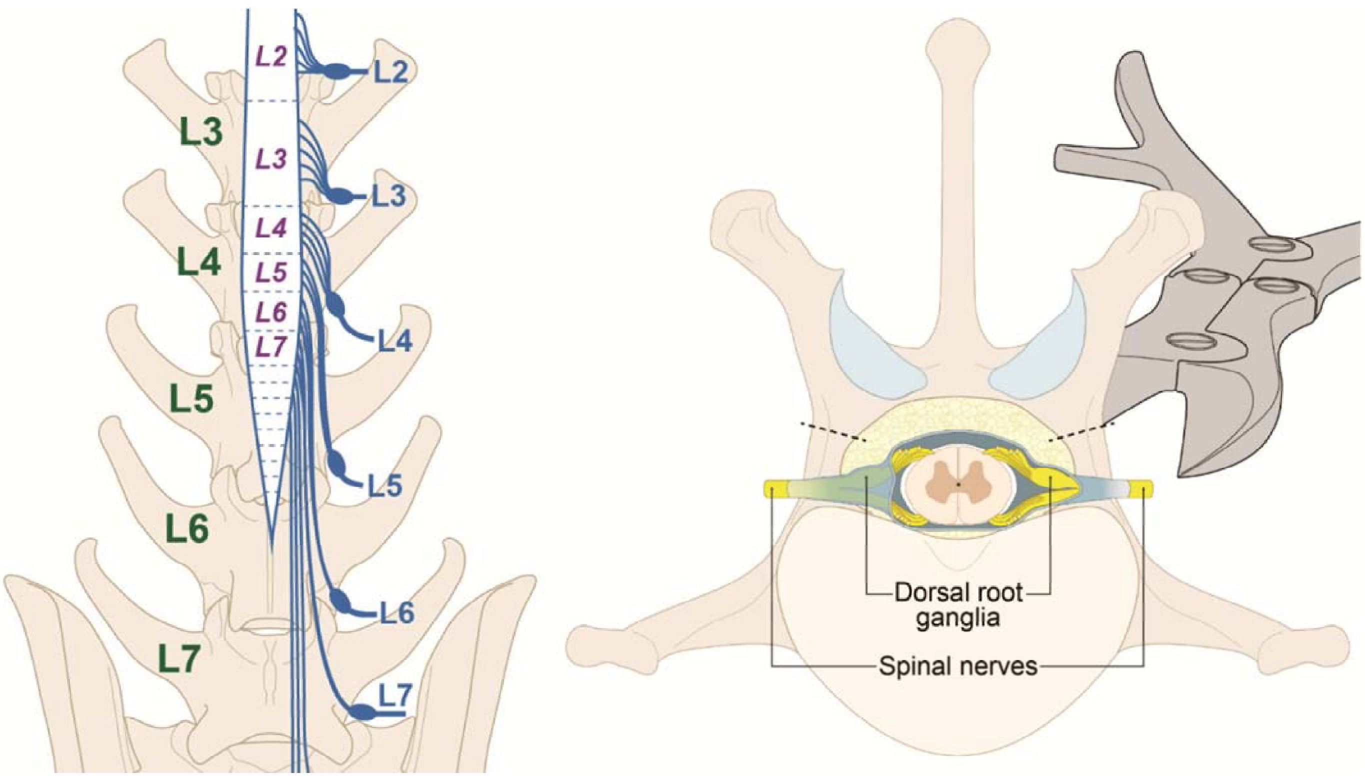
Figure 2



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Dorsal root ganglia
Spinal nerves

Figure 3

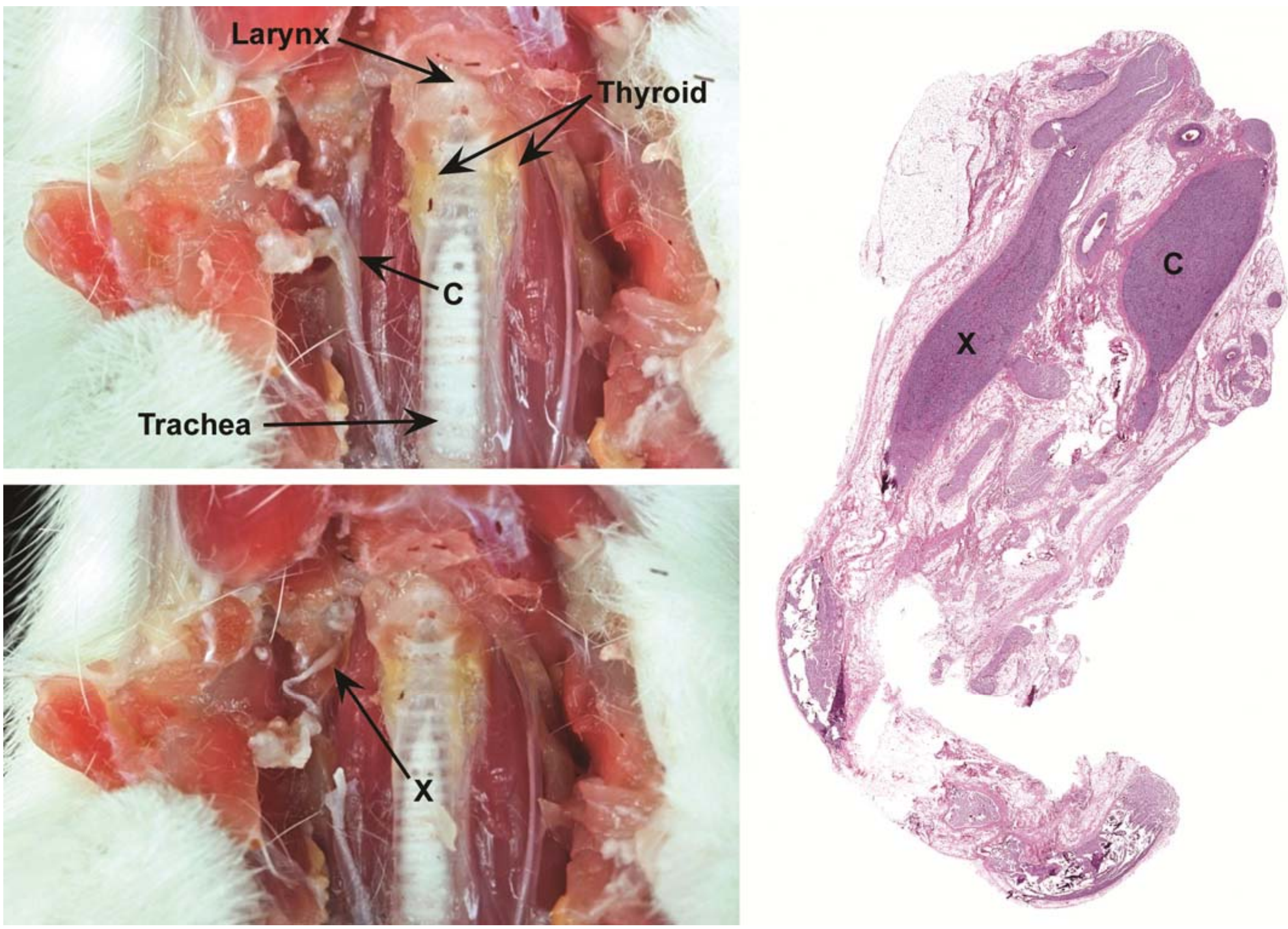

Figure 4

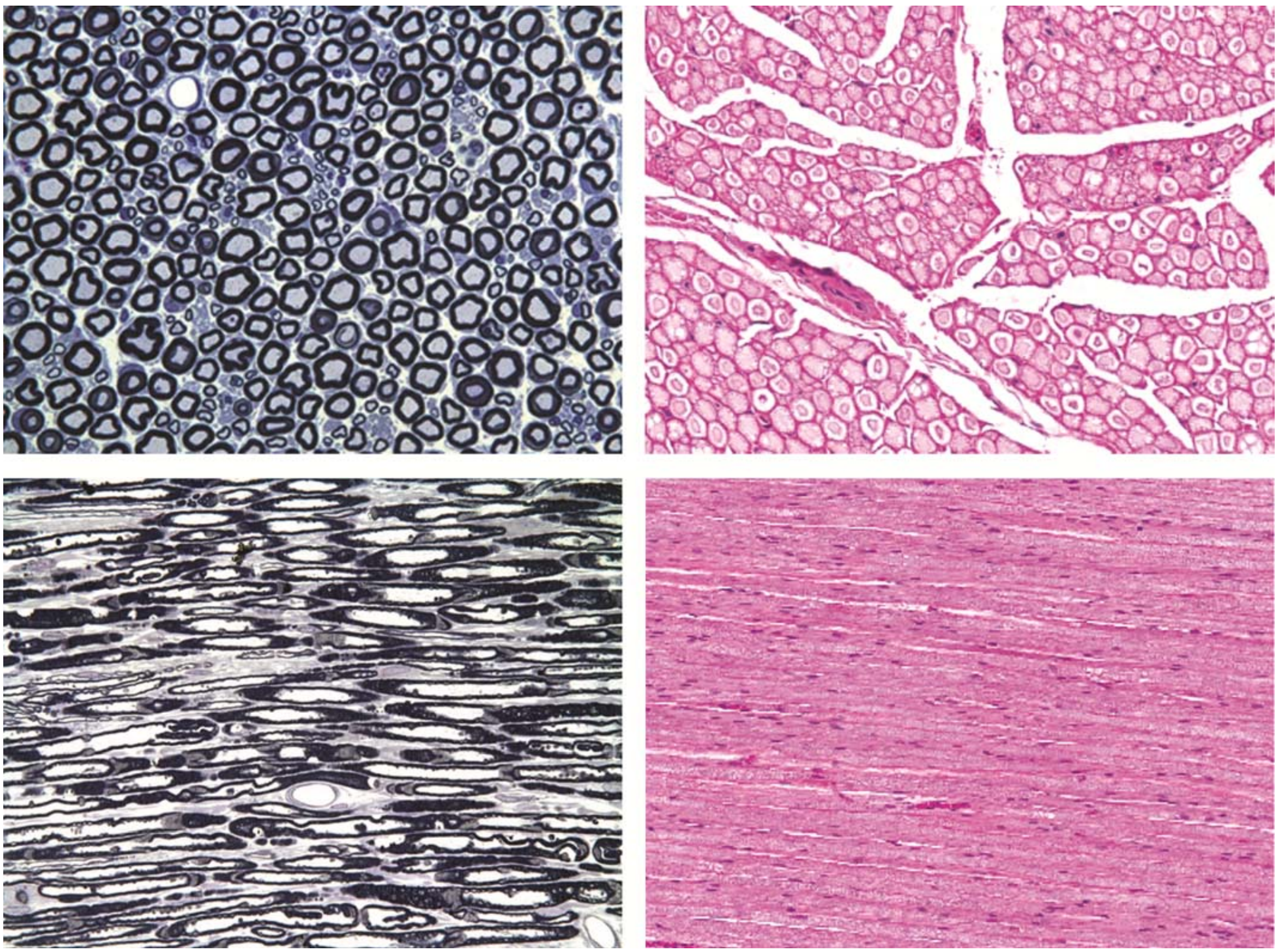

Figure 5

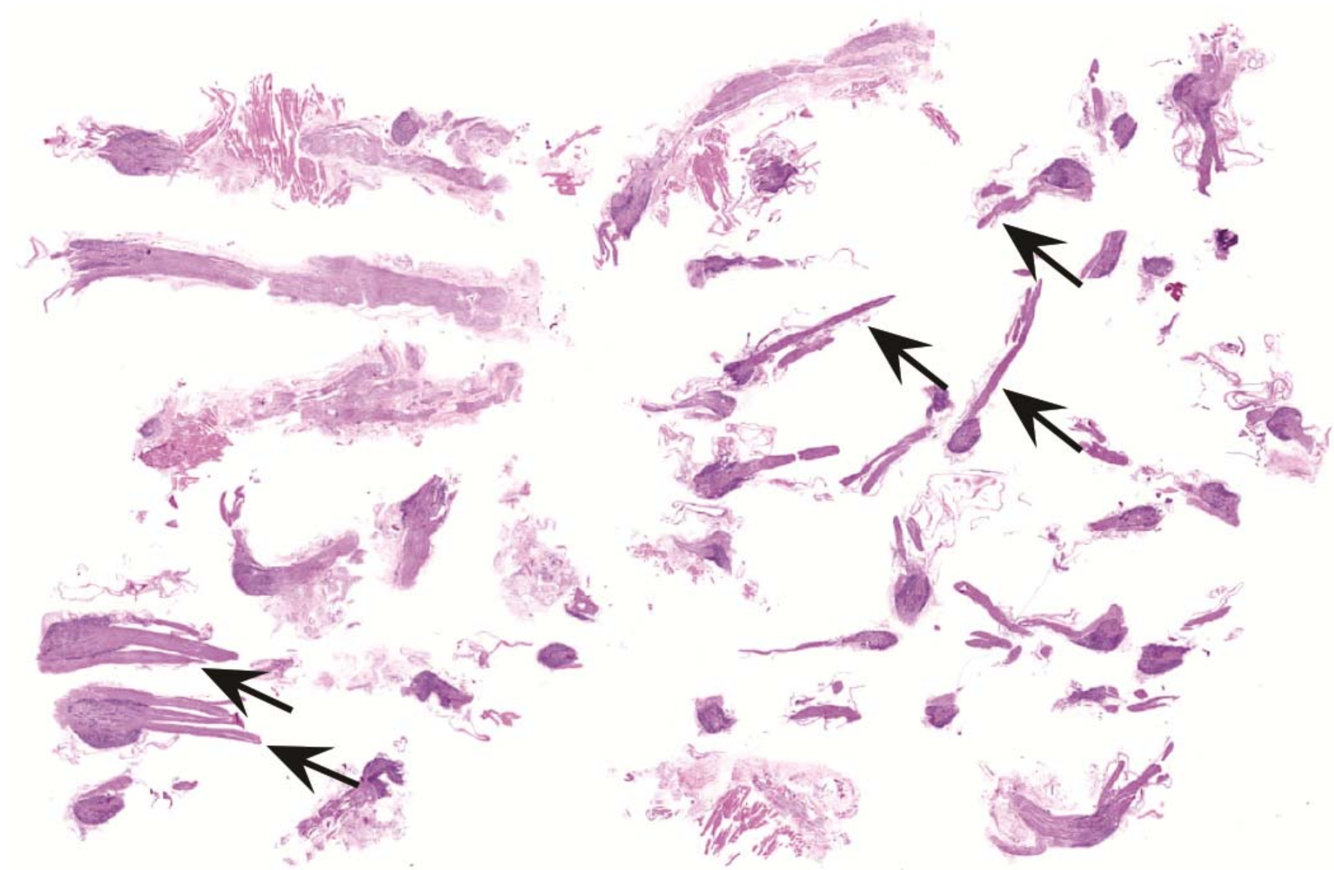

Figure 6

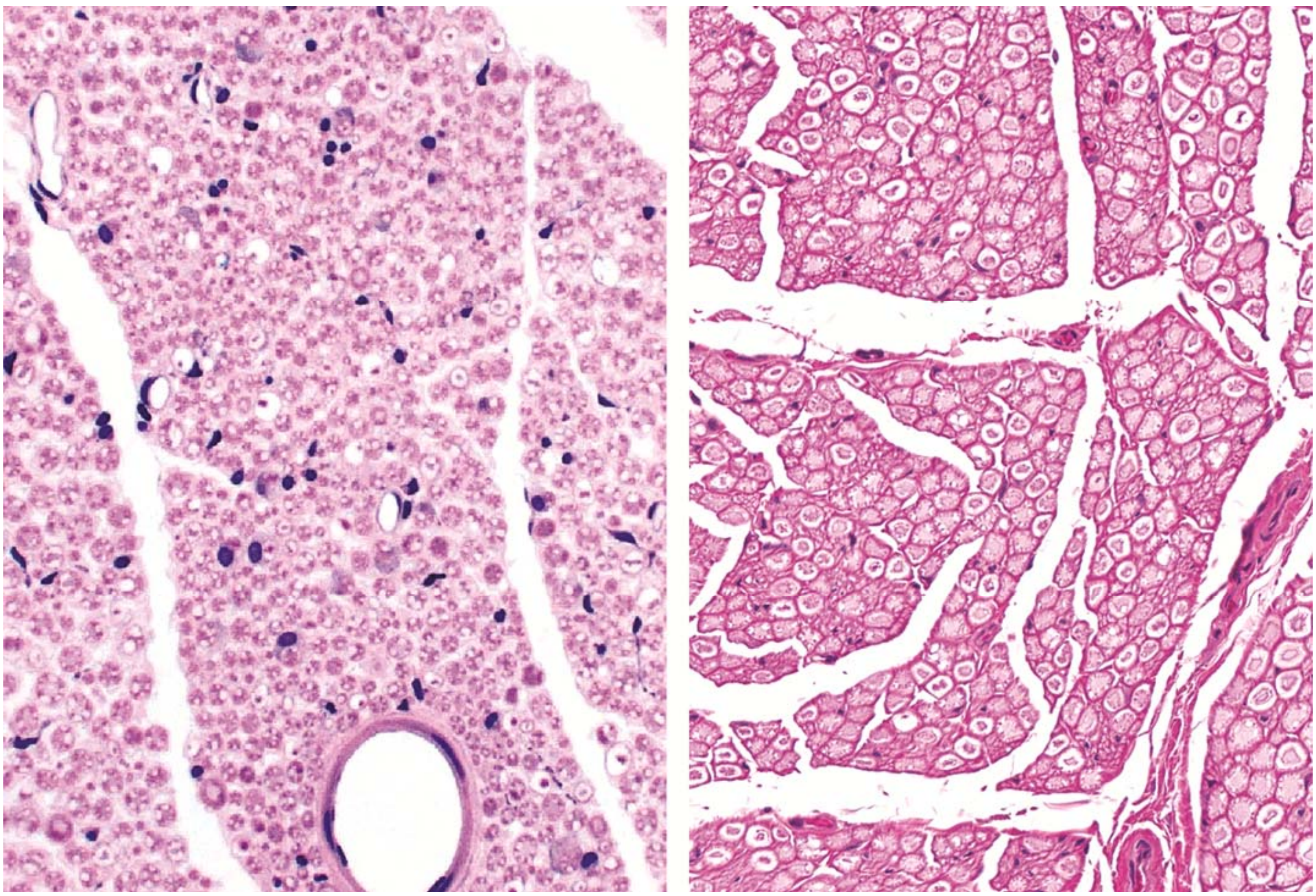

Figure 7