**Supplementary file 2: Protocol for histopathological examination of brain tissues of Drosophila flies**

**PROTOCOL FOR HISTOPATHOLOGICAL EXAMINATION OF BRAIN TISSUES FOR FLIES**

Histological tests were performed on 10-day-old and 16-28-day-old male flies, and treatments were administered for 1-3 hours and 6-18 days respectively for acute and chronic histological tests, at 22-30oC using modifications of general and special histological techniques, as previously described 1–4.

***Tissue preparation and sectioning.*** Tissue preparation and sectioning were done using modifications of standard methods 1–4. Briefly, 100 *Drosophila melanogaster* flies in each group, in replicates of 10 flies per plastic fly vial (Genesee Scientific, 32-116, USA), were randomly selected, anesthetized with CO2 (Fly CO2 anesthesia setup; Genesee Scientfic, 59-114/54-104M, USA), and placed on a CO2 perfused pad for collecting. The flies were decapitated under a dissecting stereo microscope (Leica Microsystems, [Leica M60](https://www.leica-microsystems.com/products/stereo-microscopes-macroscopes/p/leica-m80/) CMO), heads placed in 10% neutral buffered formalin fixative at room temperature (22- 30oC) for 24 hours, fixed fly heads were washed with 70% ethanol after the 24 hours. The tissues were then folded in a piece of filter paper, put in a plastic tissue cassette, and processed in a paraffin automated tissue processor (Histokinette-SLEE MAINZ, MTP type) overnight. The paraffin-impregnated heads were then removed from the tissue cassettes and transferred to 100% liquid paraffin for embedding in a molten wax bath on the tissue embedding center, placed at 60°C for 15 minutes, then the paraffin was allowed to cool and harden at room temperature on a cold plate of embedding center. Excess wax was trimmed away from the tissue block using a blade and the cold block was then mounted to the Rotary microtome (SLEE MAINZ, CUT4062 model) for transverse sectioning into 6 μm-thick histological sections onto salinized microscope slides. The sections were processed further until they were made ready for manual hematoxylin and eosin (H and E), and combined Luxol fast blue (LFB) and Nissl (Klüver’s) staining techniques following standard protocols 1–4.

***H & E staining technique of Drosophila brain tissue sections.*** Deparaffinized the section by flaming a slide onto which the tissue was fixed on a burner and placed in xylene (2 changes; 2minutes each). Hydrated the tissue section by passing the slide through decreasing concentrations of alcohol baths and water (100%, 90%, 80%, 70%; 2 minutes each). Stained in hematoxylin for 4 minutes, washed in running tap water until sections “blued”, this took 4 minutes. Differentiated in 1% acid alcohol (1% HCl in 70% alcohol) for 5 minutes, washed in running tap water until the sections were again blue by dipping in an alkaline solution (ammonia water) followed by tap water wash. Stained in 1% Eosin Y for 10 minutes, washed in tap water for 4 minutes. Dehydrated in increasing concentration of alcohol (70%, 80%, 90%, 100%; 1 minute each) and cleared in xylene (2 changes; 2 minutes each). Mounted in mounting media for microscopic analysis 1,3,4.

***Combined Luxol fast blue and Nissl (Klüver's) staining technique of Drosophila brain tissue sections.*** Deparaffinized the section by flaming the slide on a burner and placing it in xylene (2 dips, 2 minutes per dip). Hydrated sections to 95% alcohol (100%, 100%, 95%; 2 minutes each).Stained in LFB overnight at 56oC to 58oC in a tightly capped container to avoid evaporation of the alcohol stain. First thing in the morning, added 10% acetic acid to the cresyl violet, filtered and preheated solution to 57oC, and maintained it at this temperature in a dry air oven for 6 minutes.Rinsed sections in 95% alcohol for 4 minutes to remove excess stain.Rinsed in distilled water for 4 minutes.Differentiated initially in lithium carbonate for 20 seconds, continued differentiating in 70% alcohol solution until grey and white matter can be distinguished. Ensured not to over-differentiate (3 minutes in 70%I, 3 minutes in 70%II, 3 minutes in 70%III).Washed sections in distilled water for four minutes.Finished differentiation by rinsing briefly for 20 seconds in lithium carbonate solution and then putting the sections through several changes of 70% alcohol solution until the greenish-blue of the white matter contrasts well with the colorless gray matter (1 minute in each of the three 70% alcohol).Rinsed thoroughly in distilled water for four minutes. Placed slides in the cresyl echt violet solution for 6 minutes. Ensured the staining solution was kept hot (57oC) during the staining.Differentiated in several changes of 95% alcohol (3 changes, 1 minute each).Dehydrated in absolute alcohol (2 changes, 1 minute each) and cleared in xylene (3 changes; 2 minutes each) and mounted in mounting media for microscopic analysis 2,3.

***Microscopic examination of brain tissues.*** Images were collected on the mounted stained tissue preparations on a light microscope (Nikon Eclipse Ci, 104C type) using 20x and 40x objectives, equipped with a digital camera (Nikon digital sight DS, Fi 1) and connected to a computer with software (NIS-Elements F3.00, SP7; Build 547) for photography. The hematoxylin and eosin slides enabled visualization of the general morphology of brain tissue (Palladino *et al.,* 2002, 2003) whereas Klüver's stained tissues aided the demonstration of axonal structure, Nissl substance, nuclei of glial cells, and individual neurons in tissue sections 1,5,6. Brain neurodegeneration was categorized and quantified based on the prominence (size) and frequency of brain vacuolations in H and E stained-sections following previous methods 7,8, none significant lesions = brains having no gross neuropathology or a single small vacuolar lesion (˂3 µm in diameter) and brains having sporadic small individual vacuolar lesions (˂3µm in diameter) in multiple sections; moderate neurodegeneration = individual vacuolar lesions (3-5 µm in diameter) that occur more frequently and appear in most sections of the brain; severe neurodegeneration = brains with numerous vacuolar lesions (˃5 µm in diameter) in individual sections 7,8. *Drosophila* does not have axons surrounded by myelin sheath or Schwann cells 9, however, the non-myelinating Schwann cells in mammals are comparable to the ensheathingglial cells within the CNS of *Drosophila* whose role is to encase the axons and neuropil of the flies 10,11, therefore, LFB in Klüver LFB stain was used in the current study to demonstrate axonal degeneration of *Drosophila* neurons instead of axonal demyelination 12. The nature of the Nissl substance was analyzed using photomicrographs from tissues stained with Klüver LFB stain using previously described methods 13,14, nerve tracts (axons) were shown by blue color and Nissl substance was shown by magenta (violet) color with the stain. A weak LFB stain (light blue patchy areas) indicated axonal degeneration of nerve tracts, while a weak cresyl echt violet stain (light violet) indicated abnormalities in Nissl substance 13,14.

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