# Neuroanatomical Techniques

## Insect Nervous System

Edited by
N. J. Strausfeld and Thomas A. Miller

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# Preface

Most neurobiological research is performed on vertebrates, and it is only natural that most texts describing neuroanatomical methods refer almost exclusively to this Phylum. Nevertheless, in recent years insects have been studied intensively and are becoming even more popular in some areas of research. They have advantages over vertebrates with respect to studying genetics of neuronal development and with respect to studying many aspects of integration by uniquely identifiable nerve cells.

Insect central nervous system is characterized by its compactness and the rather large number of nerve cells in a structure so small. But despite their size, parts of the insect CNS bear structural comparisons with parts of vertebrate CNS. This applies particularly to the organization of the thoracic ganglia (and spinal cord), to the insect and vertebrate visual systems and, possibly, to parts of the olfactory neuropils. The neurons that make up these areas in insects are often large enough to be impaled by microelectrodes and can be injected with dyes. Added to advantages of using a small CNS, into which the sensory periphery is precisely mapped, are the many aspects of insect behaviour whose components can be quantitized and which may find both structural and functional correlates within clearly defined regions of neuropil. Together, these various features make the insect CNS a rewarding object for study.

This volume is the first of two that describe both classic and recent methods for neuroanatomical research on insect CNS. Most techniques

are derived from methods first used on vertebrates; but there are some notable exceptions, such as methylene blue methods, special stains for synapses, and the fluorescent dye and cobalt marking techniques. The methods described in this volume are mostly biased towards correlating structure with function. Others, however, are purely descriptive. But without these even the best and most fluorescing neuron would be lost for a context in which to fit.

The editors would like to thank all the contributors for their labours, and also for their patience during the preparation of this volume which was initiated in 1976. And, lastly, we invite them all to join with us in dedicating this book to six colleagues who have played a major role in neurobiological research because they have introduced important and powerful techniques. They are, A. O. W. Stretton and E. A. Kravitz; R. M. Pitman, C. D. Tweedle and M. J. Cohen; and W. Stewart: The methods are familiar to all of us.

N. J. Strausfeld
Thomas A. Miller
September 1980

*Note to the reader:* A companion volume to this book is presently being written by an expert group of authors and edited by N.J. Strausfeld. Topics to be treated include: Electronmicroscopy (EM) of Golgi and cobalt-silver stained cells; EM resolution of transsynaptic cobalt and horse radish peroxidase; marking cells with Cytochrome C; double marking techniques for EM; High voltage EM; Interpretation of EM of freeze fracture replicas of neuropil; combined reduced silver and cobalt methods; methylene blue methods; Lucifer yellow histology; localization of functional activity by radioactive deoxyglucose; radioactive amino-acid mapping of sensory pathways; biochemistry and immunological characterization of proctolin containing neurons; histochemical distinction between octopamine, dopamine, noradrenalin and 5HT; immunocytochemical methods for identifying peptides in insect CNS; methods for studying the developing nervous system; uses for computer-graphics in structural analysis.

# Call to Authors

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Chapter 1

# The Methylene Blue Technique: Classic and Recent Applications to the Insect Nervous System

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## Historical Review

At the end of the last century Ehrlich (1886) discovered that injection of a methylene blue solution into an animal's circulatory system could stain nerve cells and their processes blue. Thereafter histologists began to use methylene blue in preference to any other method of staining, particularly for the peripheral nervous system of insects (Monti, 1893, 1894; Holmgren, 1895, 1896; Rath, 1896; Duboscq, 1898).

Their findings were, however, contradictory since these authors were unable to undertake a detailed examination of their preparations: The dye proved to be unstable and disappeared soon after it was applied.

Dogiel (1902) was the first to develop the technique of fixing and dissecting preparations, which he stabilized in a solution of ammonium molybdate. This method enabled retention of the blue coloration, and the preparations have not lost their quality for three-quarters of a century. Even today these original preparations are used for research at the University of Leningrad's cytology and histology department.

Of all its users, Zawarzin obtained superior results, applying the method to insect cerebral nervous systems. His studies, which were begun in the first decade of this century in Dogiel's laboratory, have made

him especially well known among neurohistologists. In 1911 his first work of the series "Histological Studies on Insects" was published under the title "The structure and innervation of the heart of the larval *Aeschna*." This was followed by "The sensory nervous system of the larval *Aeschna*" in 1912 (1912a), "The sensory nervous system of the larval *Melolontha vulgaris*" in the same year (1912b), "The optic ganglia of the larval *Aeschna*" in 1913, and, in 1924, "The histological composition of the unpaired abdominal nerve in insects" (1924a) and "The abdominal brain of insects (1924b)."

With respect to the study of *in toto* preparations, stained *in vivo* with methylene blue, Zawarzin comprehensively described features of the peripheral nervous system, including the sensory innervation of lip fibrils, the mandibuli, jaws, labium, abdomen, chordotonal organs, and chemosensory apparatus. These studies made the simple distinction between two major classes of sensory cells. The first kind is a bipolar element that lacks peripheral ramifications and simply invests or inserts into the base of the sensory apparatus, whereas the second kind gives rise to apical processes and forms tree-like terminal ramifications. The latter are almost always located at articulations, where they innervate the hypoderma of the membrane, that is, the softest and most delicate part of the chitinous skeleton.

The usefulness of the methylene blue technique for staining insect nervous system was, however, best demonstrated by Zawarzin's studies on the abdominal chain of the larval *Aeschna*, which he began in 1914 and worked on for the following eight years.

The characteristic feature of the methylene blue method is that in each preparation only single elements of the whole ganglionic mass can be identified. In order to collect his results Zawarzin systematically recorded every element in all the ganglia that had taken up the stain. He claimed that usually no novel elements were revealed after the 400th preparation. In all, Zawarzin prepared and examined ventral cords of about 750 animals and ten times that many ganglia.

He found that neuronal elements that belong to the same ganglion are characterized by their astonishingly constant forms and numbers, and because of this, specific cell types can be easily recognized, even in preparations that are only partially stained. One essential feature of a neuron is the position of its cell body and processes. Zawarzin carefully described the position of each single element in the horizontal and dorsoventral planes and so revealed the precise topography of neurons, from which he was then able to speculate about their possible functional significance. He showed, for example, that there was a dorsoventral differentiation within the ganglia. This comprised special loci for inter- and intraganglionic conduction pathways that could be distinguished from sensory and motor regions and the central bulk of neuropil.

Zawarzin described the neuronal composition of the ganglia more comprehensively and more precisely than any other contemporary author, and it is claimed that for some ganglia he identified more than 75% of the constituent neurons. Although his extensive and complex investigations recognized cardinal features of each thoracic and abdominal ganglion, his publications describe only two of these in detail, namely, the second (thoracic) and fourth (1st abdominal) ganglia. It was presumably his intention at some stage to describe the others but he was unable to complete this study. This work has been continued in Zawarzin's tradition by Tsvileneva, who has since published several extensive works on this and other arthropod nervous systems (see, for example, Tsvileneva, 1950, 1951, 1970).

Zawarzin's studies on the insect visual system precede descriptions of the ventral nerve cord. At that time little was known about the interrelationships between various elements of the visual neuropil, and terminology was not even defined. Zawarzin referred to the three neuropil masses that subserve the retina as the first, second, and third optic "ganglia."[1] By using both reduced silver techniques and methylene blue preparations, he was able to reveal both the general structures of these neuropils and the course of many single fibers between them.

Zawarzin was also the first to make detailed comparison between the fiber interrelationships in the visual systems of insects, vertebrates, and cephalopods and to draw specific analogies between them. He was struck by the similarity of organization between the visual neuropils of these three groups of animals, and although some similar comparisons had attracted the attention of other authors, such as Radl (1912), their studies did not take into account specific neuronal arrangements and were derived from gross preparations alone.

Zawarzin's analysis, however, demonstrated that throughout the visual system cell processes are organized as stratifications in which synaptic specializations ought to be located. He proposed that these layered structures should be termed "screening centers" and that they were invariably located at the same geometric position in the neuropil: Similar elements were arranged across each layer.

He suggested that the structure of the retina, as well as that of the cerebral cortex, midbrain, and cerebellum of all higher vertebrates, contains layered arrangements that correspond to those found in the insect visual system. Zawarzin then proposed that many centers, including the cortex, may find reference to a model system of development represented by the stratified vertebrate retina.

[1] The first, second, and third optic ganglia are, respectively, the lamina, medulla, and lobula, by modern terminology. Zawarzin used the term "ganglion" for the synaptic part of the nervous system, not considering whether this part is anatomically similar to the segmental ganglia. [Eds.].