Mapping of serotonin-immunoreactive neurons of *Anastrepha obliqua* Macquart larvae

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ABSTRACT. Serotonin-immunoreactive neurons were identified in the central nervous system (CNS) of *Anastrepha obliqua* Macquart, 1835 wandering stage larvae. The PAP immunocytochemical method was applied to the entire CNS (whole mounts). About 90 neurons were visualized in the CNS (20 in the brain and 70 in the ventral ganglion). Both somata and axons were strongly stained. These neurons showed a segmental arrangement and bilateral symmetry. All processes presented a basic projection pattern, in which the major fibres travel contralaterally. Comparison of these neurons with serotonergic neurons described in other insects suggests order-specific traits such as cerebral clusters and presence of only one 5-HT immunoreactive neuron in the 8th abdominal neuromere as well.
KEY WORDS. *Anastrepha obliqua*, fruit fly, serotonin, immunocytochemistry

Larvae of *Anastrepha obliqua* Macquart, 1835 (Diptera, Tephritidae), as occuring in other tephritid species, live and feed on the pulp of many tropical fruits (MALAVASI *et al.* 1980), becoming them unviable to consumption and causing high economic losses for the fruit-growing industry. Developing inside the fruits, they are protected from many insectivides by the fruits' rind. So, studies about fruit flies controls have been carried out on adult population size (ALBRECHT & SHERMAN 1987; MARTINEZ & MORENO 1991; RAGA *et al.* 1993, 1994).

At the end of the larval stage, the mature larvae leave the fruits to look for pupation sites. This period is known as wandering stage (FRAENKEL & BHASKARAN 1973) and corresponds to unique developmental period in the larval life susceptible to control programs.

Physiological and immunocytochemical studies have provided strong evidence that 5-hydroxytryptamine (serotonin, 5-HT) is involved in the regulation of biological processes directly or indirectly related with wandering stage as, for example, visceral and skeletal muscle contractions (BANNER *et al.* 1987; VAN HAEFTEN *et al.* 1993) and gut emptying (DAVEY & TREHERNE 1963), and also stimulation of the corpora allata activity (RACHINSKY 1994) whose juvenile hormone synthesis has an important role in the maintenance of larval development of the insects (WIGGLESWORTH 1985; RIDDIFORD 1994).

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In view of the important role that 5-HT may play in the wandering behavior and maintenance of the juvenile stages it is undoubtedly of interest to determine the neuroanatomical organization of serotonergic system, which is expected to help in the understanding of the role of serotonergic neurons in this larval phase.

Although cyclorraphous species of Diptera have been submitted to mapping of serotonergic neurons (NASSEL & KLEMM 1983; NASSEL & CANTERA 1985; VALLÉS & WHITE 1988), until now, no information is available on the occurrence of 5-HT in the nervous system of a true fruit fly (Tephritidae). So, to provide a basis for future studies on the physiology of these neurons, the objective of the present study was to report the mapping of the 5-HT immunoreactive neurons in the CNS of *A. obliqua* wandering stage larvae.

MATERIAL AND METHODS

Experimental animals

Third instar larvae of *A. obliqua* in the wandering stage were collected from infested fruits of *Spondias lutea* (cajá-mirim) (Anacardiaceae). The larval stage and the species were identified according to FRAENKEL & BHASKARAN (1973) and TELES DA SILVA (1978), rescpectively.

Immunocytochemistry

Twenty larvae were used. The animals were immobilized and sacrificed by freezing in liquid nitrogen. The nervous system was dissected in phosphate buffer saline (0.1M pH 7.4; 0.9% NaCl) (PBS) and fixed for 2-3h with an ice-cold solution of 4% paraformaldehyde/8% saturated picric acid in phosphate buffer (0.1 M pH 7.4). After prolonged washing (72h) in PBS + 5% sucrose, the preparations were submitted to immunocytochemistry. The immunoreaction was carried out on whole mounts by the indirect peroxidase-antiperoxidase (PAP) technique (BOURNE 1984). To reduce nonspecific background staining, the specimens were incubated for one hour in 0.1M Tris-HCl, pH 7.4, containing 10% normal guinea-pig serum (Dako-patts). Rabbit-antiserotonin antiserum (kindly donated by Dr. Jean Lauder; University of North Carolina, Chapell Hill) was diluted 1:500 in PBS + 0.25% Triton X-100 (Sigma) (PBS-T) and specimens were incubated for 72h at 4°C. After washing in PBS-T, the specimens were incubated with unlabeled guinea-pig antirabbit antiserum (Dakopatts) and rabbit PAP-complex (Dakopatts) diluted 1:50 and 1:100 in PBS-T for 12h at 4°C. The peroxidase reaction was visualized in 0.025% 3,3' diaminobenzidine tetrahydrochloride (DAB, Sigma) with 0.01% H_2O_2 in PBS. After staining, some preparations were dehydrated in alcohol, cleared in xylene, and mounted in Entellan, and others were embedded in paraplast for serial sectioning (10 μm).

The absence of endogenous peroxidase was confirmed by incubating the entire nervous system in the absence of the primary antiserum. The specificity of the immunoreaction was tested with antiserum preincubated with antiserum (STEINBUSCH et al. 1983).

RESULTS

Serotonergic neurons were immunocytochemically identified in the central nervous system of *A. obliqua* third instar larvae.

Staining of whole mounts revealed immunoreactive cell bodies, axons and fine dendrites.

A total of 90 neurons were located (20 in the brain and 70 in the ventral ganglion), whose cell body and axons were strongly stained.

These neurons are uniform in size and show a bilateral symmetrical arrangement.

The larval brain

The proto-, deuto- and tritocerebrum are not clearly distinguishable in the larval's central nervous system. Thus, the protocerebral cell bodies were considered to be the most dorsal somata in the cerebral hemisphere and the tritocerebral cell bodies were considered to be the most ventral.

The cerebral 5-HT immunoreactive neurons were grouped into four distinctive clusters, as follows: cluster 1, is formed by one neuron localized in the dorso-anterior cortex (protocerebral region). The axons of this cluster project ventro-posteriorly within the ipsilateral brain hemisphere toward the intercerebralis commissure (Figs 1a,b, 2a); cluster 2, consists of four neurons situated in the dorso-medial cortex (protocerebrum), after cluster 1. Their axons travel ventrally to the contralateral hemisphere forming an immunoreactive tract and arborizing in the dorsal contralateral neuropil lamina (Figs 1a,b, 2a); cluster 3, is formed by two neurons in the dorso-posterior cortex (protocerebrum). The axons run anteroventrally to the ipsilateral neuropil and then laterally to the intercerebralis commissure, where they also form another tract (Figs 1a, 2a); cluster 4, is formed by three neurons lying in the ventro-posterior cortex (tritocerebrum). Their axons travel ventro-anteriorly to the contralateral side forming a tract (Figs 1b, 2a).

The axons of clusters 2 and 3 form a large-field of fine immunoreactive arborization in the dorsal contralateral midbrain neuropile, while the axons belonging to cluster 4 arborize in the ventral neuropile.

Ventral ganglion

The ventral ganglion is formed by the fusion of the suboesophageal, thoracic and abdominal neuromeres (Figs 1c, 2b).

About 26 neurons were identified in the suboesophageal neuromeres. These neurons are distributed unevenly among the three neuromeres that form this ganglion, *e.g.*, mandibular, maxillary and labial neuromeres. The number and arrangement of the 5-HT immunoreactive neurons in the mandibular neuromere differ from those observed in the maxillary and labial neuromere. The mandibular neuromere presents a cluster formed by three somata located laterally in each hemiganglion, and whose axons ascend contralaterally into the tritocerebrum. The maxillary and labial neuromeres have five somata per hemiganglion, which were grouped into two clusters: cluster 1, consisting of three neurons located in the lateral region and corresponding to the maxillary neuromere cluster; and cluster 2, formed by two neurons situated ventrally in the hemiganglion. The axonal projections of this second cluster were not clearly observed.

In the thoracic neuromeres, 14 neurons were stained. They are arranged as follows: three pairs of serotonergic somata in the prothoracic neuromere, and two
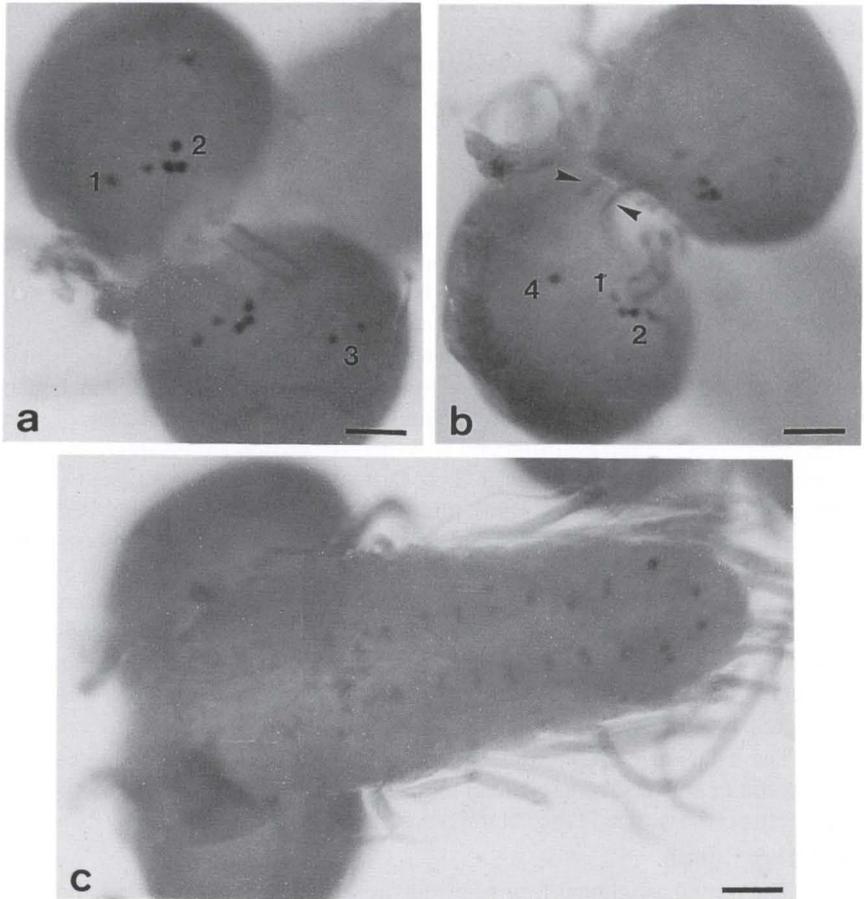
Fig. 1. Serotonin immunoreactive neurons of wandering third instar larvae of *Anastrepha obliqua*. Photomicrographs of whole mount preparations of cerebral ganglion in dorsal (a) and frontal (b) view, and suboesophageal ganglia in a ventral view (c). Arrows in the Figure 1b indicate the commissural tracts. Scale bar: in (a) and (b) = 180 μm, in (c) = 190 μm, (1-4) cerebral clusters.

pairs in the meso- and metathoracic neuromeres. The somata are located laterally in each hemineuromere and the axons run contralaterally and then appear to project in an ascending and descendeing fashion.

Thirty neurons arranged in pairs were mapped in the neuromeres corresponding to the abdominal ganglia. All the abdominal neuromeres present two somata per hemineuromere, except the last, which contains only one. The axonal projections travel contralaterally and appear to ascend into the anterior ganglia and to descend into the posterior ganglia.
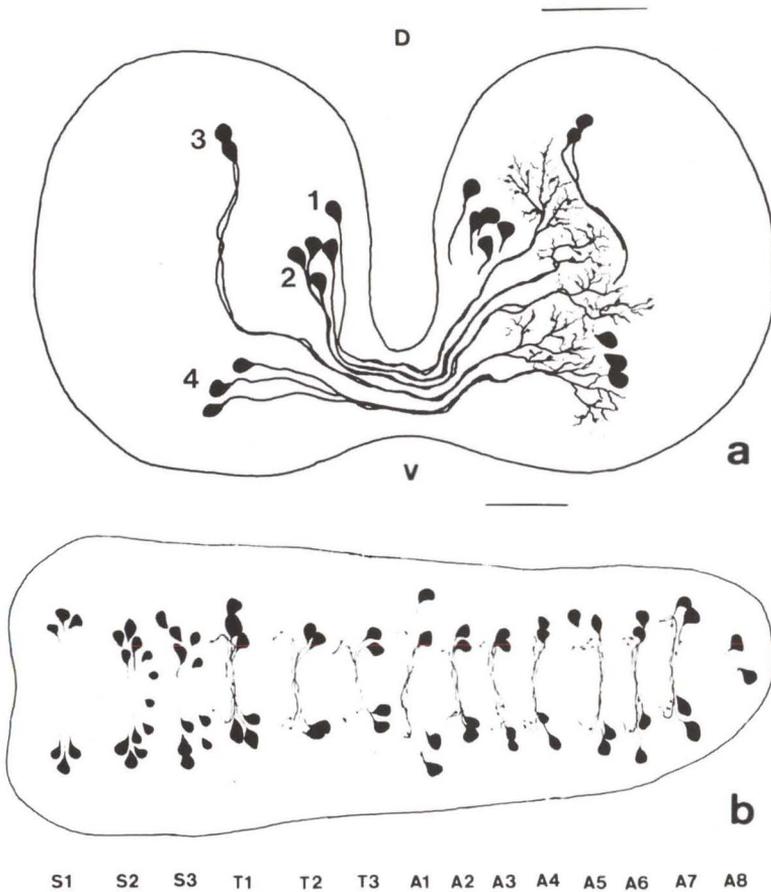
Fig. 2. Camara lucida drawing indicating the neuronal clusters of the brain (a) and of the ventral ganglion (b) of *Anastrepha obliqua*. Scale bar: in (a) and (b) = 200μm, (1-4) cerebral clusters, (D) dorsal, (V) ventral, (S1-3) suboesophageal clusters, (T1-3) thoracic clusters, (A1-8) abdominal clusters.

DISCUSSION

This paper contains a basic description of serotonin-like immunoreactive neurons in the CNS of *A. obliqua* wandering stage larvae.

Altogether 90 neurons and their processes were identified in the brain and ventral ganglion, where they showed a segmental and bilateral symmetrical arrangement. This organizational pattern of serotonergic neurons reflects the segmentation and bilateral symmetry plan of the nervous system of Arthropods, which are believed to have descended from segmented, annelid-like ancestors (BRUSCA & BRUSCA 1990).

The segmental neuroanatomical disposition of the 5-HT immunoreactive neurons in the ventral ganglion unabled us to distinguish the neuromeres of the basic

functional units or tagmas of insects: three suboesophageal neuromeres, three thoracic neuromeres and eight abdominal neuromeres. The presence of bilateral symmetric pairs of neurons in all of the neuromeres suggests a serial homology (LONGLEY & LONGLEY 1986), which has long been recognized as a result of repetition of development instructions to form several similar segments along the antero-posterior axis (BATE 1976). The immunoreactivity pattern also showed some segment-specific variations in the number of cells, whose function is not clear.

An interesting aspect of these neurons is the contralateral projections of their axons. The functional role of these axonal projections toward the opposite hemi-ganglion and to the somata appears to be the interaction of the right and left nervous system (HOMBERG & HILDEBRAND 1989), supposedly important to coordinate the movements of lateral body undulations during the wandering behavior, since the muscles are organized as segmental bands and muscle contractions appear to be regulated by 5-HT (BANNER et al. 1987; VAN HAEFTEN et al. 1993). However, the ausence of serotonin immunoreactivity in the peripheral nerves makes it difficult to consider the serotonergic neurons mapped in the ventral nervous system of *A. obliqua* wandering stage larvae as motoneurons.

On the physiological view, another important aspect about the serotonergic system of *A. obliqua* is the strong immunoreactivity showed by the neurons, axons and areas of the neuropile, whose presence in the wandering stage indicates that large amounts of 5-HT are sintetized and secreted. In this same developmental phase, it was observed a high synthesis of juvenile hormone in vitro by corpora allata (data not showed). So, considering that, according to RACHINSKY (1994), serotonin is a strong stimulant of the corpora allata activity, it's probably that the 5-HT really has a stimulant role on the corpora allata of the wandering stage. This point of view is reinforced by the presence of strongly stained processes in the neuropile, from which the corpora allata nerve emerge.

By means of detection of BUdR incorporation, TRUMAN & BATE (1988) have demonstrated that a large number of new neurons are produced during larval life by embryonic neuroblasts persisting into the larval stage. According to LAUDER (1993), neuronal development is regulated by serotonin. So it is possible that the immunoreactivity to serotonin found in areas of neuropile of *A. obliqua* wandering stage larvae is involved in the control of neurogenesis.

Comparison among different insect species

A comparison of the arrangement of serotonergic neurons in the larval nervous system of Diptera, *e.g.*, *A. obliqua* (present data), *Calliphora erythroce-phala* Meigen, 1830 (Calliphoridae) and *Sarcophaga bullata* Parker, 1976 (Sarco-phagidae) (NASSEL & CANTERA 1985) and *Drosophila melanogaster* Meigen, 1830 (Drosophilidae) (VALLÉS & WHITE 1988), shows some similarities in the location and axonal projection pattern. Concerning the cerebral neurons, it was observed that these similarities among the four species mentioned are also related to the clusters. However, with respect to the number of neurons per cluster, there is correspondence only between *A. obliqua* and *D. melanogaster*, a fact reflecting the higher phyloge-netical proximity between Tephritidae and Drosophilidae.

In the suboesophageal ganglia, *A. obliqua*, *Calliphora* and *Sarcophaga* all present three neuron pairs in the mandibular neuromere and five in the maxillary neuromere; in *Drosophila* only two mandibular and three maxillary neurons are identified. A total of five neurons were stained in the labial neuromere of *A. obliqua*. But, this number does not correspond to that obseved in the other three dipteran species, *i.e.*, of three neurons. These differences may be related to some species-specific role whose involvement in insect development is still unknown.

No difference was observed among these species in the total number of thoracic and abdominal somata. All of them presented three 5-HT immunoreactive neurons in the prothoracic neuromere, two in the mesothoracic, metathoracic and 1st-7th abdominal neuromeres, and one in the last (8th) abdominal neuromere. The presence of bilateral symmetrical neurons, also denominated twin neurons, in the ventral ganglion has been demonstrated in cockroach (BISHOP & O'SHEA 1983), grasshopper (TAGHERT & GOODMAN 1984), dragonfly (LONGLEY & LONGLEY 1986), beetle (BREIDBACH 1991; VAN HAEFTEN & SCHOONEVELD 1992), honeybee (BOLELI *et al.* 1995), fly (NASSEL & CANTERA 1985; VALLÉS & WHITE 1988). This similarity of cellular and process staining among a wide range of insects belonging to distinct orders, as cited by LONGLEY & LONGLEY (1986), indicates that this organizational pattern of serotonergic neurons was maintained phylogenetically. However, particularly interesting is the fact that no other insects present a single neuron pair in the 8th abdominal neuromere, except Diptera. Further studies are needed to determine whether the absence of this neuron pair in the last neuromere corresponds to a loss of activity related to some different role of the abdominal ganglia.

Another interesting fact about the serotonergic system of *A. obliqua* wandering stage larvae is its correspondence in the location of cells with the groups of cerebral paraldehyde fuchsin (PF) positive neurosecretory cells studied by BOLELI *et al.* (1994). PF-neurosecretory cells have been described as A-type cells and their significance has often been debated. The presente paper, however, indicates that in *A. obliqua* wandering stage larvae these cells contain biogenic amine as occurring in locust (VIEILLEMARINGE *et al.* 1982).

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