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Immunohistochemical localization of histamine neuropeptide in the brain neurosecretory cells of tasar silkworm, *Antheraea mylitta* (DRURY)

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Abstract
Histamine is a putative neurotransmitter in the mammalian central nervous system where it plays a modulatory function. Recently, there has also been growing interest in histamine as a putative neurotransmitter in invertebrates and it has now been implicated as a neurotransmitter in molluscs. In the present study, the distribution and localization of histamine immunoreactive cells were investigated by using polyclonal antibody against the histamine during the larval development of tropical tasar silkworm, *Antheraea mylitta*. The result confirmed the presence of histamine immunoreactive cells in the brain of larvae. During the development of first to fifth larval instars, a pair of four groups of neurosecretory cells (MNC, LNC, PNC and VNC) were observed in the brain of larvae. In the present study it has been found that only the MNC group shows positive reactivity with anti-histamine antibody, during immunohistochemical reaction.

Keywords: Histamine, *A. mylitta*, brain, neurosecretory cells, medial neurosecretory cells (MNC)

Introduction
The insect central nervous system (CNS) includes the cerebral ganglion (brain), ventral ganglia and associated neurons. The CNS is critical in coordinating sensory information and motor activity in the insect (Chapman, 1998; Tembhare and Barsagade, 2000) [5, 39]. Many important researches in neuropeptide biology have been made in the invertebrates as experimental model animals. Invertebrates offer unique opportunities to study neuropeptides at the single cell level due to the presence of large neurons embedded in the brain and nerve ganglion as simple circuits that are easy to study for analysis of neuropeptide (O'Shea and Schaffer, 1985; Scharrer, 1987; Scheller and Kirk, 1987; Barsagade *et al*., 2019, 2021) [31, 36, 37, 2].

In insects, neuropeptides have been most extensively studied with respect to their roles as circulating hormones (Ewer and Reynolds, 2002; Dulcis, D. *et al*., (2005) [12, 9]. Although roles of neuropeptides in the insect central nervous system (CNS) are less understood, it is commonly considered that they act as neuromodulators or co-transmitters rather than as neurotransmitters (Homberg, 2002; Nässel, 2002) [19, 27].

In invertebrates the histamine acts as a neurotransmitter (Gengs *et al*., 2002, Stuart *et al*., 2007) [13, 38]. A role of histamine in neurotransmission was first suggested on the basis of its presence in identified neurons of the slug *Aplysia* (Ono and McCaman, 1980; Weinreich *et al*., 1977) [30, 40], in the compound eye and optic lobe of some insects (Maxwell *et al*., 1978) [22] and in the stomatogastric ganglion of the spiny lobster (Claiborne and Selverston, 1984) [8].

Further evidence included demonstrations of histamine synthesis, metabolism and binding sites in the nervous systems of molluscs and insects (Carpenter and Gaubatz, 1975; Elias and Evans, 1983, 1984; Gruol and Weinreich, 1979; Maxwell *et al*., 1978; Weinreich and Yu, 1977) [6, 10, 14, 22, 40]. Extensive work has been performed on the role of an identified histamine containing neuron in the cerebral ganglion of Aplysia (Chiel *et al*., 1990) [7]. This neuron uses histamine as its transmitter at several output synapses and evokes a variety of responses in its follower neurons fast or slow excitation, fast or slow inhibition, or very slow excitation (McCaman and Weinreich, 1985) [23].

However no detailed investigations have been made to demonstrate the localization of histamine neuropeptides immunohistochemically in the brain of insects. Therefore the present study provides data regarding the distribution of histamine immunoreactive cells in the whole brain of the tasar silkworm, *A. mylitta*, which can serve as a basis to better understand the physiological functions of histamine in lepidopteran species.

Material and Methods

The larvae of tasar silkworm were collected from the tasar rearing field of Central Tasar Research and Training Institute, Bhandara (MS) and kept in insect rearing cages at laboratory, where larvae fed on fresh leaves of *Terminalia tomentosa* to acclimatize. The larvae were anesthetized with chloroform soaked in cotton pad. The brain were dissected out in PBS and fixed in cold Bouin's fixative for 24 hr. Thereafter, the material was given the changes of 10%, 20% and 30% cold sucrose solution for 24 hr. The frozen sections of material of 10µm thickness were cut on the Cryostat (Leica-CM 1520) at -20 ºC. The sections were fixed to Poly-L-Lysin coated slides. The slides were preserved in 4 ºC in freezer till they were proceeding for immunohistochemical staining.

The Streptavidin-biotin-peroxidase method was used during the present study. The sections were washed in PBS (pH- 7.4) for 15 min. and treated with 1% bovine serum albumin (BSA) in PBS containing 0.3% Triton X-100. The polyclonal antibody of Histamine (IMMUNOSTAR, CAT NO. 22939, 1:1000 dilution) were diluted in PBS in concentration containing 0.3% Triton X-100 and 1% BSA. The sections were then incubated for 2hr at 25 ºC. Sections were washed in PBS for 10min. and incubated with biotinylated secondary antibody for 1hr. followed by Streptavidine-Peroxidase conjugate (Sigma 3-Amino-9-Ethyl Carbazole AEC 101) was used as chromogen to visualize the reddish brown reaction.

Results

The histamine immunoreactivity against were tested on larval brain of tasar silkworm *A. mylitta.* The brain of larvae were dissected out in phosphate buffer saline (PBS) and preserved. After the staining procedure the sections were observed under light microscope. The positive reactive neurosecretory cells were darkly stained and background was light as compared to control cells.

Sections of first to fifth instar larval brain were used to localize histamine neuropeptide. The section of brain in fifth instar larva of *A. mylitta* omitted with antibody (Negative control) and for positive control we preferred vertebrate brain section of fish *C. gariepinus* showing immunoreactivity. (Fig. A &B).

In the first instar, four groups of neurosecretory cells (MNC, LNC, PNC and VNC) were observed during histological study. In the present observation only four cells of the MNC group shows positive reactivity with anti-histamine antibody. From the MNC group 2 A and 2C cells were showing positive reaction with anti-histamine antibody (Fig. C). In the second instar MNC group shows positive reactivity with anti-histamine antibody. From the MNC group total four cells, 2A and 2C cells were histamine positive in each half of the brain (Fig. D). The histamine immunoreactive cells were found in third instar larva. In each half of the brain 3A and 2C cells of MNC group were found positive with anti-histamine antibody, whereas the LNC, VNC and PNC cells were not showing any positive reaction (Fig. E). In the fourth instar larva number and cell size of NSC increases gradually as compare to first, second and third instar larva. In the MNC group total 5 cells are histamine positive. 3A and 2C cells in each half of the brain were positive for anti-histamine antibody, while other groups were not showing any reaction (Fig. F). In the fifth instar larva the number of NSC increases. Total 5 cells are positive for anti-histamine antibody. From that in MNC group 3A and 2C cells were positive for anti-histamine antibody (Fig. G).

From the results it has been found that the histamine neurosecretory cells were present in the brain of *A. mylitta* and the number of these cells were increases from first to fifth instar larvae.

Table 1: Immunocytochemical localization of Histamine reactivity in the brain neurosecretory cells

	MNC			LNC		VNC		PNC	
	A	B	C	B	C	B	C	B	C
I	+	-	+	-	-	-	-	-	-
II	+	-	+	-	-	-	-	-	-
III	+	-	+	-	-	-	-	-	-
IV	+	-	+	-	-	-	-	-	-
V	+	-	+	-	-	-	-	-	-

Table 2: Number of Histamine positive neurosecretory cells present in the brain of *A. mylitta*

	MNC	LNC	VNC	PNC
I	3	-	-	-
II	4	-	-	-
III	5	-	-	-
IV	5	-	-	-
V	5	-	-	-

Table 3: Number of Histamine reactive neurosecretory cells in the brain of *A. mylitta*

	MNC			LNC			VNC			PNC		
	A	B	C	A	B	C	A	B	C	A	B	C
I	2	-	1	-	-	-	-	-	-	-	-	-
II	2	-	2	-	-	-	-	-	-	-	-	-
III	3	-	2	-	-	-	-	-	-	-	-	-
IV	3	-	2	-	-	-	-	-	-	-	-	-
V	3	-	2	-	-	-	-	-	-	-	-	-

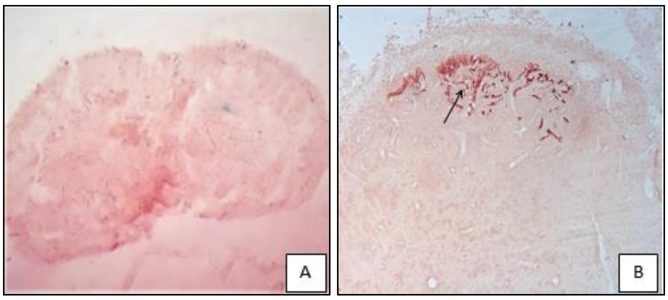

Fig A & B: Control sections. T. S. passing through brain of fifth instar larva of *A. mylitta*, omitted with histamine antibody showing histamine negative immunoreactivity (Negative control) and T. S. passing through brain of fish after applying antibody showing immunoreactivity (positive control)

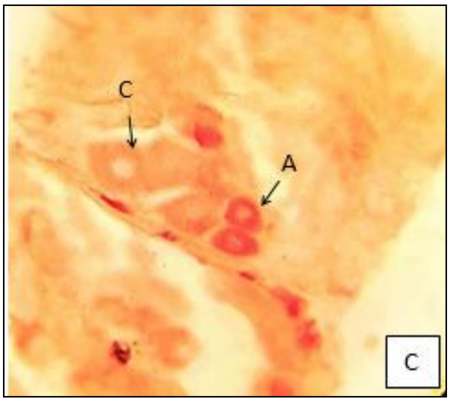

Fig C: T. S. passing through brain of first instar larvae of *A. mylitta* showing histamine positive A and C cells of MNC

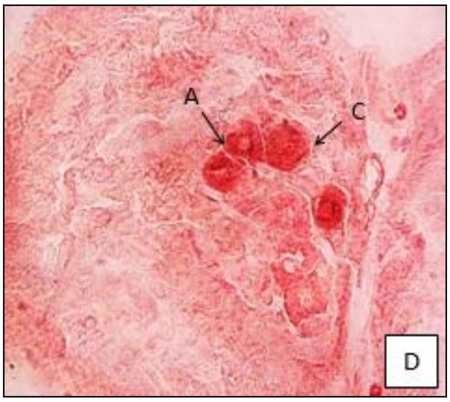

Fig D: T.S. passing through brain of second instar larvae of *A. mylitta* showing histamine positive A and C cells of MNC

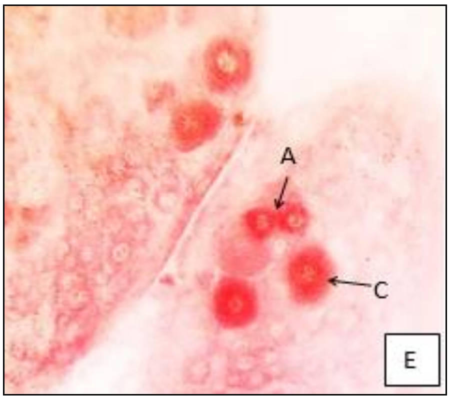

Fig E: T.S. passing through brain of third instar larvae of *A. mylitta* showing histamine positive A and C cells of MNC

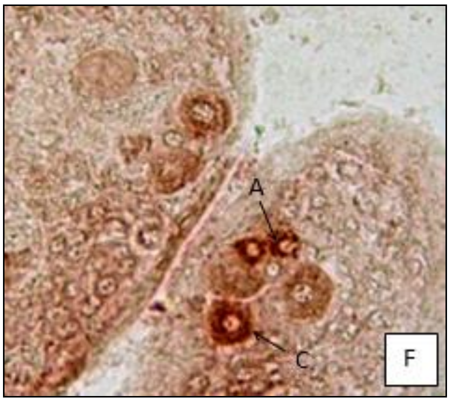

Fig F: T.S. passing through brain of fourth instar larvae of *A. mylitta* showing histamine positive A and C cells of MNC

Fig G: T.S. passing through brain of fifth instar larvae of *A. mylitta* showing histamine positive A and C cells of MNC

Discussion

Panula *et al*., (1988, 1990) [32] and Pirvola *et al*., (1988) [33] investigated histamine immunoreactive neurons in the cockroach brain *Leucophaea maderae.* They found that in the brain of *Leucophaea maderae* the total number of distinctly histamine immunoreactive (HAIR) neuronal cell bodies is about 30. These strongly labelled cell bodies are distributed in the deutocerebram and tritocerebrum in variable number with distinct location. In the present study it has been observed that the neurosecretory cells in the brain of *A. mylitta* show positive reaction against anti-histamine antibody. The immunoreactivity was observed gradual increase from first to fifth instar larval brain. A result of the present study is showed the presence of intense histamine immunoreactive cell in MNC of the 5th instar larval brain as compared to the other.

Histamine-containing somata and fibers are widespread in arthropod brains, with the most intense labeling in the retinal photoreceptors and in the first optic ganglion, where the short visual fibers contact the monopolar neurons (Nässel, 1999; Pirvola *et al*., 1988; Stuart *et al*., 2007) [28, 33, 38]. Histamine is released from arthropod photoreceptors and gates chloride channels on postsynaptic interneurons; and mediates the light response of the postsynaptic large monopolar cells Gengs *et al*., (2002) [13] have provided unequivocal evidence that histamine is the transmitter at the photoreceptor synapse of Drosophila and likely in all arthropods (Hardie, 1989; Stuart *et al*., 2007; Zheng, 2006) [16, 38, 41]. In the compound eye of flies, output from photoreceptors that share the same visual field is pooled and transmitted via histaminergic synapses to two classes of interneurons, large monopolar and amacrine cells. Furthermore, histamine modulates insect clock neurons and is crucial for insect temperature preferences (Hong *et al*., 2006) [20].

Presence of histamine in a variety of neuron types in the brain and optic lobes as well as in the ganglia of the ventral nerve cord of several insect species, suggesting a more widespread role as a neurotransmitter or modulator (Bornhauser and Meyer, 1997; Buchner *et al*., 1993; Helle *et al*., 1995; Homberg and Hildebrand, 1991; Nässel, 1999; Nässel and Elekes, 1992; Nässel *et al*., 1988, 1990; Pirvola *et al.*, 1988; Pollack and Hofbauer, 1991) [3, 4, 17, 18, 28, 27, 25, 33, 34].

The histamine immunoreactivity were detected in mechanosensory cells and their axons in Drosophila by Buchner *et al*., (1993) [4] and Melzig *et al*., (1996) [24]. Earlier it has been shown that the histamine not only worked as the neurotransmitter of photoreceptors in compound eyes and ocelli in insects (Hardie, 1987, 1989; Sarthy, 1991) [15, 35], but it also appears to be the neurotransmitter of certain extraocular photoreceptors in the locust brain (Lundquist *et al*., 1996).

During the present study it has been observed that mostly MNC group of neurosecretory cells show histamine positive reaction in first to fifth instar larva confirm the secretion of histamine and utilization of histamine during post embryonic development. Furthermore the number of histamine positive A and C cells were variable from first to fifth instar larva indicated that different amount of histamine released time to time. It was also observed that in MNC group, B cells were not involved in histamine synthesis activity.

The presence of histamine-immunoreactivity in specific sets of neurons in the brain of different insect species suggests neurotransmitter or neuromodulatory roles of histamine in numerous central circuits (Nässel, 1999) [28].

The presence of histamine immune positive cells in the brain of tasar silkworm *A. mylitta* confirms the synthesis of histamine during development and may be worked as neuromodulators related activity as it has been confirmed in other insects.

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