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Localization of neuropeptide γ- amino butyric acid (GABA) immunoreactivity in the sub oesophageal ganglion (SOG) of *Antheraea mylitta*

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Abstract
The GABA is considered as an inhibitory neurotransmitter in the CNS across all vertebrates and in invertebrates also. The studies on the developing brain and primary brain culture provided the evidence for varieties of function of GABA in the synapse formation. In the present study, the distribution and localization of GABA immunoreactive cells were investigated by using polyclonal antibody against the GABA during the larval development of *Antheraea mylitta*. The results of the present study showed the presence of GABA immunoreactive cells in MLNC of mandibular group in SOG ganglion. Two GABA immunoreactive cells were localize from first instar to third instar whereas the three and four GABA immunoreactive cells were localize in the fourth and fifth larval instar of *A. mylitta* respectively. The numbers of GABA immunoreactive cells were increased from first to fifth larval instar.

Keywords: γ- amino-butyric acid (GABA), subesophageal ganglion (SOG), *A. mylitta*

1. Introduction
During the specific developmental stages of lepidopteran insect and also in response to various environmental conditions the suboesophageal ganglion (SOG) produces various neurohormones which plays an important role in development and growth of insects [1, 2]. The suboesophageal ganglion consist of three fused segmental ganglia that innervate the structures surrounding the insect mouth [3]. Recently, it has been found that SOG region of Drosophila contains the circuitry network that regulate feeding behaviour and play an important role in many other physiological aspects of sensory processing and locomotor control [4].
γ-Amino Butyric Acid (GABA) is a well-known primary inhibitory neurotransmitter in the central nervous system of across the vertebrates and also in invertebrates [5, 6, 7, 8, 9]. It is found that in fruit fly Drosophila melanogaster GABA produced in the large number of neurons throughout the central nervous system [10, 11, 12, 13, 14]. Only central interneurons of Drosophila melanogaster use GABA as a neurotransmitter [13, 14, 15]. In contrast to this, in some other insect such as locust and cockroaches also possesses GABAnergic motor neuron called as common inhibitory neurons and GABA receptors on muscle fibre [16, 17, 18, 19].
The distribution and localization of GABA in central complex of insect is found to be highly conserved among the different insect species. Various studies have been demonstrated the GABA immunostaining in the central complex of the honey bee, the sphinx moth, the fruit fly, the cockroach and beetle [20, 21, 22, 23].
Considering the conserve nature of GABA in the central complex of insects the present study was undertaken with the aim to study the localization and distribution of GABA immunoreactive cells in the SOG of tropical tasar silkworm *Antheraea mylitta*.

2. Materials 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 Subesophageal ganglion 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 10µm thickness were cut on the Cryostat (Leica- CM 1520) at -20 ^{0}C. The sections were

affixed to Poly- L- Lysin coated slides. The slides were preserved in 4 ^{0}C in freezer till they were proceeding for immunocytochemical 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 antibodies against the µ-amino- butyric acid (GABA) GABA antibody (SIGMA, CAT NO. A-2052, 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^{0}C. 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.

3. Results

In *A. mylitta* suboesophageal ganglion consists of various neurosecretory cells. The neuroendocrine cells named according to their location as a Mandibular neurosecretory cells (MdNC) located in anterior region, Maxillary neurosecretory cells (MxNC) in medial region and Labial neurosecretory cells (LbNC) in posterior region. Lateral region of subesophageal ganglion also consists of some neurosecretory cells in anterior, medial and posterior regions and denoted as anterior lateral neurosecretory cells (ALNC), Medial lateral neurosecretory cells (MLNC) and Posterior lateral neurosecretory cells (PLNC) respectively (fig. B).

By analyzing the GABA immunoreactivity in SOG of A. mylitta we recognize that the immunoreactivity of GABA increases from first instar to fifth instar as the development proceeds (Table no.1). The number of immunoractive cells were found to increase as the development of the larval instar take place (Fig. 1B). In all the larval instars, mandibular cell of SOG showed positive immunoreactivity as the development proceeds from first instar to last instar. In accordance with results of the immunohistochemistry, the mandibular group of first instar larva showed positive neurosecretory cells in the subesophageal ganglion, where two cells showed the positive immunoreactivity of GABA. (Fig. C). In the second instar larva the mandibular group showed positive reactivity with anti-GABA-antibody. From this group two mandibular cells were positive with GABA antibody and in third instar two cells were showed positive reaction (fig. D, E). Similarly in the fourth and fifth instar larvae three and four mandibular cells were positive to the GABA immunoreavtivity respectively (fig. F, G). From the first to fifth instar larvae the number of immoreactive positive cell was increased (Table. 1, and fig. 1).

Table 1: Number of GABA positive immunoreactive cells in First to Fifth instar larva of *A. mylitta*

Developmental stages	No. of GABA Positive Mandibular Cells
I Instar	2
II Instar	2
III Instar	2
IV Instar	3
V Instar	4

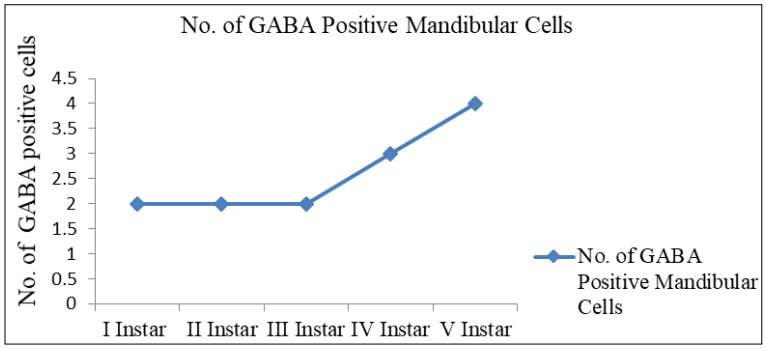

Fig 1: Graphical representation of GABA positive immunoreactive cells in First to Fifth instar larva of *A. mylitta*

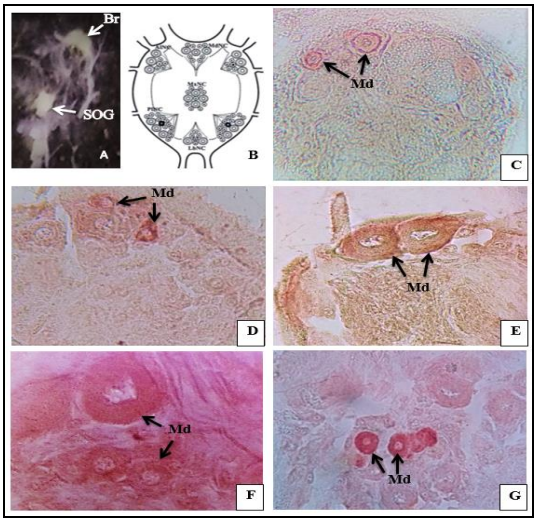

Fig 2: A In situ structure of cephalic neuroendocrine complex in larva of *A. mylitta*. B: Diagrammatic representation of group of neurosecretory cells of SOG. C-G: GABA immunoreactive mandibular neurosecretory cells in First to fifth instar larva. Abbr.: Br- Brain, SOG- Sub-oesophageal ganglion, MdNC- Mandibular neurosecretory cells, MxNC- Maxillary neurosecretory cells, LbNC- Labial neurosecretory cells.

4. Discussion

In present investigation, three groups of neurosecretory cells were found in subesophagial ganglion of the tasar silkworm *A. mylitta*, as mandibular, maxillary and labial group of neurosecretory cells. Sato *et al*, [25] explain the presence of mandibular, maxillay and labial group of neurosecretory cells in in the subesophageal ganglion of *B. mori*, and in *Agrotis ipsion* by Duportets *et al*, [26]. These neurosecretory group of cells also found in SOG of *Achaea janata* [27] and *Orgyia thyellina* [28] are similar to our observations. In insect, subesophageal ganglion is formed by fusion of mandibular, maxillary and labial neuromeres and houses central circuits which is responsible for feeding nature of insects [29, 30, 31, 32, 33].

γ- Amino butyric acid (GABA) is the major inhibitory neurotransmitter in the insect brain [34, 6]. In earlier study, GABA immunostaining was found in all neuropiles of the optic lobe of locusts [35]. In different insects GABA receptors were localize in various ganglion like in locust thoracic ganglia [36], in optic lobe neurons [37], and mushroom body Kenyon cells [38]. During the present study, the subesophageal ganglion of the tasar silkworm *A. mylitta* after reacting with polyclonal GABA- antibody, few GABA immunoreactive cells were localized in mandibular group of neurosecretory cells in sub oesophageal ganglion. Presence of GABA has been reported earlier in invertebrates the brain of the catfish and goldfish [39, 40]. While, GABA-immunoreactive neurons have also been demonstrated in the brain of mormyrid fish and the goldfish [41, 42], however in vertebrate γ-amino butyric acid (GABA) now accepted as major inhibitory neurotransmitters in the central nervous system of vertebrates. Leitch and Laurent [43], discovered that both immunoreactive and immunonegatie profiles were in contact with GABA-immunoreactive presynaptic terminals in the antennal lobe of the locust. In contrast, both reactive and negative terminals got immediate feedback from GABA-immunoreactive profiles. The neurons of the antennal lobe utilize GABA as primary neurotransmitters [44, 45]. The presence of GABA secreting neuroendocrine cells of SOG in *A. mylitta* might be stimulating brain antennal lobe as a neurotransmitter.

Earlier, Hidaka *et al*. [46], Ito *et al*. [47], Tashiro and Kuriyama [48], Gardner and Walker [49] noticed that the physiological role of GABA in the nervous system of worms as both excitatory and inhibitory effects on musculature of body wall. Localization of GABA immunoreactive cells of SOG in *A. mylitta* might be function as excitatory and inhibitory role to control the thoracic musculature. In locust *S. gregaria* inhibitory motor neurons in the thoracic ganglion whose somata contain glutamate decarboxylase and for that GABA is implicated for the inhibitory action on muscles [50, 16, 51], thus providing supporting evidences of SOG GABA positive cells may regulate the muscles of peripheral region of their surrounding tissue in *A. mylitta*.

5. Conclusion

During the present study it has been found that GABA immunoreactive cells were present in SOG as well as increase intensity of GABA immunoreactivity during post embryonic development from first instar to adult, confirm the synthesis and release of GABA neurotransmeter. The increase intensity of immunostaining in fifth instar larval SOG neurosecretory cells indicates the use of GABA in larval pupal transformation and it may regulate in musculature transformation during pupal metamorphosis. It might also play role in excitatory and inhibitory effects for transformation of nervous system from larva to pupa as central nervous system was different in pupa. Perhaps this is the first observation of GABA during metamorphosis in *A. mylitta*.

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