PART -1

STUDIES ON PROTEIN SYNTHESIS AND THEIR HORMONAL REGULATION IN THE CENTRAL NERVOUS SYSTEM OF BOMBYXMORI DURING POSTEMBRYONIC DEVELOPMENT
CHAPTER 1

INTRODUCTION
Insects showing complete metamorphosis (holometabolous) undergo some of the most complex transformations seen in the animal kingdom. After a period of extensive growth during the larval stage, the animal undergoes a dramatic reorganization at pupal stage during which many of larval tissues are partially and/or completely destroyed. Adult specific structures are formed either from reorganization of larval cells/tissues or groups of precursor cells of embryonic origin retained through the larval life. These profound changes in the morphology and behaviour during metamorphosis are accompanied by equally dramatic changes in the central nervous system (CNS) of the insect. These include neurogenesis, programmed cell death and reorganization of the larval neurons to perform new functions in the adult insect (Truman and Levine, 1983; Truman, 1996). Unlike in the embryo, these processes are carried out in an animal that is capable of showing specific behavioural response to the surroundings during the entire transition. Consequently provisions must be made for restructuring parts of the CNS, while other regions remain functional. Studies on the postembryonic development of CNS of insects attracted considerable attention. These studies revealed extensive morphological, biochemical, electrophysiological and molecular changes during metamorphosis.

Earliest work on the postembryonic developmental changes in the CNS of holometabolous insects was that of Lyonet (1762) who dissected and described the differences in the organization of nervous system of larval and adult forms of the insect, *Cossus*. This work laid foundation for knowledge on the extent of reorganization and remodelling of the nervous system during larval-pupal-adult transformation of holometabolous insects. Later, Weismann (1864) described histological changes during the development of nervous system in the house fly, *Musca domestica*, a holometabolous insect. Work done since then has established that development of nervous system in holometabolous insects occurs in two distinct phases, viz. embryonic and postembryonic development.

During embryogenesis, a set of neuroblasts delaminate from the ectodermal layer and serve as pre-runners of future nervous system. These neuroblasts become arranged in a repeating pattern to form sequential ganglia (Poulson, 1956). A key
observation made by Bate (1976a, b) was that each neuroblast has a fixed position and neuroblasts are arranged in a stereotyped array. Each neuroblast produces a lineage of neurons by a repeated series of unequal divisions (Schrader, 1938). The smaller product of each division, ganglion mother cell (GMC), subsequently undergoes an equal division, thereby producing two daughter cells which then differentiate into neurons. For at least the early neurons produced in each lineage, the cellular phenotype of a given cell is determined by its parent neuroblast and the order of birth by its GMC (Taghert and Goodman, 1984). The mechanism by which the two daughter cells of a given GMC may assume different fates apparently involves interactions between the two cells after their birth (Kuwada and Goodman, 1985). In addition to GMC's, each segment also has a smaller number of median precursor cells, which undergo a single symmetrical division to produce two daughter neurons (Bate and Grunewald, 1981, Thomas et al., 1984). They along with other segmental neurons, extend axons and act as central pioneer neurons to establish a characteristic pattern of longitudinal tracts and transverse commisures to form the segmental architecture of neuropil. These initial pathways are used for guiding axons of neurons that are formed later (Bastiani et al., 1985). This phase of the embryonic development of nervous system is common for both hemi- and holometabolous insects (Truman, 1996).

Subsequent postembryonic development of CNS of hemi- and holometabolous insects differ considerably. In the former group of insects, the adult nervous system is established by the end of embryogenesis (Bate, 1976a; Doe and Goodman, 1985). Many of the central neurons of the nymphs can be readily recognized because their pattern of central branching is very similar to that seen in the adult (Shankland and Goodman, 1982; Raper et al., 1983). Likewise, neuronal numbers remain quite stable after hatching (Gymer and Edwards, 1967; Sbrenna, 1971). Two notable exceptions occur in the brain: (i) the optic lobes continue to add visual interneurons to accommodate the new ommatidia that are added to the compound eye at each larval moult (Anderson, 1978) (ii) also the mushroom bodies, areas of the brain associated with learning and memory (Davis, 1993), continue to add neurons throughout larval life and even during the adult stage (Cayre et al., 1994). Nevertheless, the essence of
the adult nervous system in terms of the number of neurons and their main pattern of connections, is clearly evident in hemimetabolous nymph by the end of embryogenesis.

In contrast to that of hemimetabola, the CNS of holometabolous insects undergoes profound changes during the postembryonic life. Neurogenesis, programmed cell death and reorganization and remodelling of existing neurons transforms the larval CNS into that of the adult. Neurogenesis plays an prominent role in forming parts of the adult CNS especially the brain (Nordlander and Edwards, 1969a, b; White and Kankel, 1978; Ito and Hotta, 1992) and thoracic ganglia (Booker and Truman, 1987a; Truman and Bate, 1988). The segmental ganglia of larvae possess a stereotyped array of neuroblasts, which get mitotically activated during early larval life, and undergo a pattern of division typical of insect neuroblasts (Edwards, 1970). Each neuroblast generates a lineage of up to 100 cells. However, unlike the embryonic cells, development of the progeny is arrested soon after the young one is born. With the onset of metamorphosis, these arrested cells undergo development and differentiation and mature into functional adult neurons. During postembryonic development of Manduca sexta between 2000-3000 new neurons are added to each thoracic ganglion and about 50-100 cells to each unfused abdominal ganglion (Truman, 1988). Accordingly, in Manduca about 60-70% of the adult ventral CNS is produced postembryonically (Booker and Truman, 1987a) while in Drosophila, the proportion is over 90 % (Truman and Bate, 1988).

Metamorphosis is always accompanied by the programmed cell death of a subset of larval neurons throughout the CNS. This process is more pronounced in the abdominal ganglia, which is in accord with the shift in locomotor function from the abdomen of the larva, to the thorax of the adult. As a result in Manduca, an abdominal ganglion in the adult contains only about 350 neurons as compared to 700-800 in the larva (Taylor and Truman, 1974). The larval neurons die in two waves. The first wave of death occurs about 2 days after pupal ecdysis (Weeks and Truman, 1985; Weeks and Ernst-Utzschneider, 1989). The second wave of neuronal death takes place after the emergence of the adult. Most of the neurons that die at this time
are either involved in maintaining the behavioural pattern during the pupal-adult transition or in the performance of ecdysis and associated behaviours. Because adult is a terminal stage that does not molt again, neurons and muscles dedicated to ecdysial behaviour undergo extensive programmed degeneration after adult emergence (Truman, 1983; Kimura and Truman, 1990).

An important change which occurs during the transformation of larval nervous system to that of adult is the reduction in the length of the nerve cord and in the number of ganglia, especially in the thoracic and abdominal regions. Based on the observations made on larval and adult nervous systems of Lepidoptera, Brandt (1879) identified four different patterns of reduction in the number of ganglia. In two of these groups, there are 2 cerebral ganglia, 2 thoracic and 4 abdominal ganglia - difference between these two groups is the presence of a constriction in one of the thoracic ganglia giving the false appearance of two separate ganglia instead of one. In the third group, there are 2 cerebral, 3 thoracic and 4 abdominal ganglia, while in the fourth group, there are 2 cerebral, 3 thoracic and 5 abdominal ganglia. However, larval forms of all these groups show the presence of 2 cerebral, 3 thoracic and 8 abdominal ganglia.

Reduction in the number of ganglia during metamorphosis of various holometabolans has attracted considerable attention. Newport's (1832, 1834) concept of degeneration and disappearance as the cause for reduction in ganglionic number has been ruled out by the investigations of later workers. Further, it has been suggested that fusion of ganglia leads to the reduction in their number. In rice weevil, *Calandra oryzae*, mesothoracic, metathoracic and first abdominal ganglia fuse to form the anterior ganglionic complex and it was proposed that fusion of ganglia is due to proliferation and overgrowth of the neurons in the ganglia (Murray and Tiegs, 1935). Pipa and his colleagues (1963, 1964, 1965) have done extensive investigations on ganglionic fusion in greater wax moth, *Galleria mellonella*. In this insect during metamorphosis, interganglionic connectives between first and second thoracic ganglia are reduced by 75% while third thoracic, first and second abdominal ganglia fuse to form a single ganglionic mass. Further, they have shown that the cell number in the
ganglion remains constant, thus discarding the hypothesis of Murray and Tiegs (1935). These investigators have also demonstrated that ganglionic fusion in *Galleria mellonella* is accompanied by coiling and looping of axons of interganglionic connectives (Pipa, 1963; Pipa and Woolever, 1965). This was shown to occur within the neurilemma and glial cells that are involved in the shortening of these axons. They also suggested that the glial cells may unwrap their elaborate foldings and withdraw from the interganglionic spaces during the shortening of the ventral nerve cord axons. Shortening of interganglionic connectives and fusion of ganglia was also demonstrated in another Lepidopteran insect, *Pieris brassicae* (Heywood, 1965). In this insect, the nerve cord gets shortened by about 30% of its original length during metamorphosis and that the process of shortening is entirely different from that of *Galleria mellonella* (Pipa and Woolever, 1965). In *Pieris brassicae*, no coiling and looping of axons was observed during the shortening process, however this process occurs few hours after the reduction in the length of the body wall. It was suggested that "resorption" of axonic material is responsible for the reduction in the length of interganglionic connectives. As a result, neuronal perikarya are pulled forwards from their original abdominal position to thoracic portion of the body. Shortening of neural lamella is passive and is attributed to its elasticity (Heywood, 1965). Beals and Berberet (1976) reported similar pattern of fusion of ganglia in, *Elasmopalpus lignosellus* (Lepidoptera).

During metamorphosis the first and second abdominal ganglia fuse with the third thoracic ganglion to form a last compound abdominal ganglion in *Philosamia cynthia* (Tsui-Yin and Fang, 1966). In the silkworm, *Bombyx mori*, the shortening of the interganglionic connectives was found to be accompanied by the formation of two ganglionic complexes - i.e., anterior ganglionic complex (AGC) and posterior ganglionic complex (PGC). AGC is formed by the fusion of second and third thoracic ganglia and first and second abdominal ganglia, while the PGC is formed by the fusion of fourth, fifth, sixth, seventh and eighth abdominal ganglia (Sivaprasad, 1987).
Role of hormones in insect neurometamorphosis

In insects, the role of hormones in the development of embryonic nervous system is largely unexplored. Their effects during postembryonic life, by contrast, has received more attention and are better understood, especially in the holometabolous forms. These insects produce a larval stage with a simple CNS with few sensory systems. The growth of the nervous system and its synaptic field occurs mainly during the postembryonic development which consists of growth punctuated by a series of moults followed by metamorphosis, thereby it comes under the control of the endocrine cues which regulate the larval growth, moults and metamorphosis (Truman, 1988). These moults and metamorphosis are initiated and coordinated by morphogenetic hormones (Riddiford, 1994). It is generally accepted that the interplay of ecdysteroids, a group of steroid hormones, and juvenile hormones, sesquiterpenes, serves to orchestrate the progression from one developmental stage to the next, with ecdysteroids regulating the onset and timing of moult and JH regulating the quality of moult (Sehnal and Mayer, 1968; Gilbert et al., 1988, 1996; Sehnal, 1989, Riddiford, 1994, 1996)

Larval-pupal transition

The decline and disappearance of JH during the final larval instar development allows metamorphosis to occur (Gilbert et al., 1996). In the absence of JH, ecdysteroids are known to initiate the metamorphic moult resulting in the formation of the pupal stage (Bollenbacher et al., 1975; Riddiford, 1976). In Lepidopteran insects, there are two peaks of ecdysteroids during the larval-pupal transformation. The initial commitment peak is relatively small; it turns off the feeding behaviour and also commits larval tissues such as epidermis for a pupal response (Riddiford, 1978, 1985; Dominick and Truman, 1985; Gu and Chow, 1993). This is followed by the prepupal peak that actually causes the molt to the pupal stage (Bollenbacher et al., 1981; Sehnal et al., 1986, 1996).

Calvez et al., (1976) demonstrated that in the larvae of the silk worm, Bombyx mori, a difference exits in baseline haemolymph ecdysteroid levels between the fourth and fifth (final) larval instars: low but significant levels of ecdysteroids (30-40 ng/ml).
were observed during the early stages of last instar (Kiguchi and Agui, 1981). The physiological significance of this difference has recently been demonstrated (Gu and Chow, 1993, 1996, 1997). It was found that very low ecdysteroid levels during early stages of last instar larvae are a prerequisite for larvae to undergo metamorphosis. In *Bombyx*, like other Lepidopteran insects, two distinct peaks of ecdysteroids have been reported, a commitment peak at prepupal stage followed by major pupal peak at mid-pupal stage (Calvez et al., 1976).

Studies on the greater wax moth, *Galleria mellonella* have provided clear cut evidence for hormonal stimulation of interganglionic connective shortening, by implanting sections of connectives from larval stages into metamorphosing hosts. The implant shortened in concert with the connectives of the host animal (Pipa, 1967). Subsequently, injections of ecdysone proved effective in causing the shortening response (Pipa, 1969). These *in vivo* studies were then complimented with *in vitro* studies in which isolated connectives from final larval stage were cultured in the presence of 20-hydroxyecdysone (20E). Interestingly, the hormone could not initiate the process *in vitro*, but it could maintain shortening in culture if the process had already begun *in vivo*, suggesting the involvement of additional factor(s) in the initiation of nerve cord shortening (Robertson and Pipa, 1973, Robertson, 1974). Similar factors which mediate the action of 20E have been reported in these insects (Caglayan, 1990; Ashok and Dutta-Gupta, 1991)

Detailed studies have demonstrated that the prepupal peak of ecdysteroids causes dendritic regression in larval motor neurons (Runion and Pipa, 1970; Weeks and Truman, 1985). The regulation of this regression has been examined in detail for the motorneuron, PPR (principal proleg retractor) which innervates an abdominal proleg retractor muscle in *Manduca sexta*. When larval abdomens were isolated prior to the prepupal peak of ecdysteroids, PPR retained its morphology. Ecdysteroid infusion into such abdomens triggered dendritic loss followed by the death of the cell a few days later (Weeks and Truman, 1985). The other hormone regulating the fate of PPR is JH, but JH can act only during the small commitment peak. The low levels of ecdysteroids normally present during this peak are not sufficient to cause neurite loss
but regression can be experimentally induced by large doses of 20E. Treatment with **JH** prior to 20E infusion prevents this neurite loss (Weeks and Truman, 1986). In addition to inducing dendritic regression, prepupal ecdysteroid peak also causes the death of some of the larval neurons after pupal ecdysis (Streichert and Weeks, 1994).

Larval neurons are known to respond to prepupal ecdysteroid peak by dendritic regression, while the **imaginal** neurons respond to this peak by initiating maturation. In *Manduca*, resumption of maturation is first evident as an increase in the cell body size (Booker and Truman, 1987b). Studies on *Drosophila* indicate that this size increase is preceded by an **up-regulation** of the homeotic gene **ultrabithorax** (Glicksman and Truman, 1990).

**Pupal-adult transition**

A prolonged release of ecdysteroids causes the transformation of the pupa into the adult (Warren and Gilbert, 1986). During this stage there is considerable dendritic sprouting of the surviving larval neurons (Prugh et al., 1992). In the **motorneuron** MN-1 of *Manduca*, this outgrowth includes an extensive new arbour ipsilateral to the cell body. Neurite extension starts on day 3, after pupal ec dysis and continues through the next 8-10 days (Truman and Reiss, 1988). The adult-specific growth of MN-1 is also sensitive to JH as treatment with JH mimics up to 2-3 days after pupal edysis block adult outgrowth (Truman and Reiss, 1988; Prugh et al., 1992).

The final **metamorphic** change in CNS is the wave of neuronal death that occurs after adult emergence. In both *Manduca* and *Drosophila* the withdrawal of ecdysteroids at the end of metamorphosis is essential for these cells to die. Treatment with 20E either *in vivo* or to cultured ganglia delays or prevents the death of these neurons (Truman and Schwartz, 1984; Bennett and Truman, 1985; Robinow et al., 1993). Recent studies have found a strong correlation between ecdysteroid receptor expression in the CNS and the nature of the neurons response to ecdysteroids, but there is not yet any direct evidence to indicate that the relationship is casual (Rabinow et al., 1993; Truman et al., 1994).
Scope and objectives of the present study

Among the most remarkable events of CNS development and differentiation are the process of extension and the assumption of highly characteristic cellular morphology. Though there are considerable number of studies on various aspects of insect neurometamorphosis, there are no reports on the role of cytoskeletal elements in the ganglionic fusion and nerve cord shortening process during the metamorphosis of holometabolous insects. Cellular movements such as extension of neurites, coiling or looping of axons, resorption of axonic material involve the active participation of the cytoskeletal components (Hollenback, 1989; Oblinger et al., 1989; Mizobuchi et al., 1990; Warn et al., 1993; Barklow and Hartwig, 1995, Haendel et al., 1996; Caroni, 1997). The present study focuses on the changes in the synthesis and content of the two major cytoskeletal proteins - actin and tubulin in the nervous system of the silk worm, *Bombyx man* during the postembryonic development. In the present study, hormonal involvement in the regulation of expression of these proteins was also examined. Phosphorylation was shown to play a central role in dynamic remodelling of cytoskeletal architecture (Vallano et al., 1986, Nixon and Sihag, 1991; de Freitas et al., 1995). Wandosell et al., (1987) demonstrated the phosphorylation of both the subunits of tubulin (a and β). Further, phosphorylation of tubulin has been shown to prevent its incorporation into microtubules. (Yamomoto et al., 1985; Wandosell et al., 1987). Thus, an attempt was also made to find out whether these cytoskeletal proteins undergo phosphorylation during nervous system development and reorganization.