Cellular and Molecular Mechanisms involved in the Postembryonic Development in the Central Nervous System of Holometabolous Lepidopteran insect, *Bombyx mori*

A Thesis submitted to the University of Hyderabad for the award of a Ph.D. degree in Animal Sciences

By

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June 2010

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(A Central University Established in 1974 By An Act of Parliament)

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DECLARATION

I, **Kapil M. Shah**, hereby declare that this thesis entitled ***"Cellular and Molecular Mechanisms involved in the Postembryonic Development in the Central Nervous System of Holometabolous Lepidopteran insect, Bombyx mori"*** submitted by me under the guidance and supervision of **Dr. Anita Jagota**, is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled ***"Cellular and Molecular Mechanisms involved in the Postembryonic Development in the Central Nervous System of Holometabolous Lepidopteran insect, Bombyx mori"*** is a record of bonafide work done by Mr. ***Kapil M. Shah,*** a research scholar for Ph.D. Programme in Animal Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision.

The thesis has not been submitted previously in part or in full to this or any other University or Institution for the reward of any degree or diploma.

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ACKNOWLEDGEMENTS

I am deeply indepted to my supervisor, ***Dr. Anita Jagota*** for allowing me to work on wonderful aspects of insect metamorphosis under her constant guidance and support throughout my work.

I acknowledge my doctoral committee members, ***Prof. Aparna Dutta Gupta*** and ***Prof. P. Prakash Babu*** for their valuable suggestions and assessment throughout my research work.

I thank ***Prof. Manjula Sritharan*** Head, Department of Animal Sciences and ***Prof. Aparna Dutta Gupta*** and ***Prof. S. Dayananda*** former Heads, Dept. of Animal Sciences for allowing me to use all the facilities in the Department.

I thank ***Prof. M. Ramanadham***, Dean, School of Life Sciences and former Dean ***Prof. A. S. Raghavendra*** for providing necessary facilities to carry out the research work.

I am thankful to ***Prof. P. Uma Maheswara Reddy, Dr. M. Raghunath*** *and* ***Prof. S. Dayananda*** for their critical assessment of my work, encouragement and suggestions.

I thank ***Prof. William J. Schwartz*** for critical reading of manuscripts and valuable suggestions.

I am grateful to ***Mr. S. Chidananda*** from Sericulture Board for his help in arranging different stages of *Bombyx mori*, at any point of year.

I acknowledge ***Dr. G. Padmaja*** for permitting us to use their mulberry plantation whenever required to feed our insects.

I thank all my lab mates, ***Dr. Kalyani D***, ***Dr. Jay Prakash Pandey***, ***Dr. M. Pavan Kumar***, ***Mr. M. Yallamanda Reddy***, ***Mr. Anumodh P. Mammen***, ***Mr. Dileep Kumar Reddy***, ***Ms. M. Ushodaya, Ms. B. Lakshmi*** and ***Mr. Vinod*** for their support and cooperation.

I acknowledge the help and cooperation of ***Mr. Sudhansu Sekhar Chaudhary*** and ***Mr. Gopal Krishna Pradhan.***

I thank our lab attendees ***Mr. Mallesh*** and ***Mr. Venkat*** for their help in maintaining the insect cultures.

I thank all Department staff ***Mr. Ankinedu***, ***Mrs Jyoti***, ***Mr. Jagan***, ***Mr. Gopi***, ***Mr. Pandu***, ***Mr. Khan, Mr. Babaiah*** and ***Mr. Babu Rao***.

I would like to extend my thanks to all the staff of School of Life Sciences and the University Administration for their help and cooperation.

I am thankful to CSIR for fellowship during my research work.

Lab funding from CSIR, DST, DST-Nano, ICMR, UGC, ILS, UPE, CREA and CWS is greatly acknowledged.

School of Life Sciences funding from UGC-SAP, DBT-CREB, UGC-COSIST, DST-FIST and UGC-SAP-CAS-I and II is also acknowledged.

I thank all the scholars in School of Life Sciences for their cooperation, motivation and most of all, providing a competitive atmosphere throughout my research work.

No words can acknowledge the love of my parents, sisters and friends.

Kapil M. Shah

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Introduction

The nervous system of an organism is responsible for generation of a wide variety of behaviors across different phyla by controlling a number of neuronal networks. The first nervous system appeared as a diffused network of neurons in coelenterates (Grimmelikhuijzen, 1989). The complexity of the nervous system evolved across various phyla. Cephalization of nervous system first appeared in flatworms consisting of brain and nerve cord. Later, the nervous system evolved to its most complex form as that of the higher vertebrates such as mammals with which the planarian nervous system shares many common features as morphology, electrophysiology and neurotransmitter substances (Sarnat and Netsky, 2002). The major difference between the nerve cord organization in invertebrates and vertebrates is that in the former, the nerve cord is ventral in position, while in the latter it is dorsal because of inverted body plan (Niibler-Jung and Arendt, 1994). Human beings have the most complex and highly evolved brains with greatest cognitive ability. Interestingly, chimpanzees have been recently reported to possess extraordinary working memory for numerical recollection than even the human adults (Inoue and Matsuzawa, 2007).

Understanding complexities of behavior and cognition in the brain of higher vertebrates is hampered by the fact that they contain billions of neurons and a serial reconstruction of brain through sectioning is a difficult task. However, insect nervous system offers a very simple model as it possesses fewer number of easily accessible identifiable neurons, which are larger than the vertebrate counterparts (Menzel and Giurfa, 2006). All these features make insects a good model to understand morphological, biochemical, molecular and behavioral changes during development of the nervous system. This knowledge can be later exploited to understand the mechanisms involved in development of much complex vertebrate nervous system and diseases related to human brain. Many insect models have been developed for age-related disorders of the brain such as Parkinson's disease, Alzheimer's disease etc. (Jeibmann and Paulus, 2009).

Recent lines of evidence indicate a monophyletic origin of the nervous system from a common ancestor, *Urbilateria* (Arendt *et al*., 2008). This further strengthens the importance of use of insect model for understanding neural basis of behavior and neural

development. Moreover, insects have short life-span, they are relatively easy to maintain in laboratory conditions and many samples can be studied simultaneously.

Phylum Arthropoda has been reported to contain largest number of documented species which inhabit each and every niche of earth. Every year thousands of new species of insects and other invertebrates are being discovered. According to some authors, around 75% more of earth's insects are yet to be discovered (Grimaldi and Engel, 2005). Many reasons attribute to the phenomenal success of the arthropods, including adaptability, excellent defense responses, ability to maintain large populations of individuals due to their enormous fecundity (rate of reproduction), and most importantly, their ability to metamorphose into different forms of individuals which gives them a huge chance to maximally utilize the available resources for growth and reproduction.

Arthropods, especially insects, play crucial role in the nature. With their ability to fly, they aid in pollination of fruit bearing plants. They are exploited for honey, silk, dye and royal jelly. They act as scavengers, and in turn are an important element of the food web. Apart from the many other uses to nature and man, they are generally regarded as pests, which attack our food crop. Because of their ability to harbor disease causing microbes, they spread many diseases. Most of the hematophagous diseases transmitting insects undergo metamorphosis viz., the kissing bug *Triatoma infestans* the vector of Chagas disease, the yellow fever mosquito *Aedes aegypti* that spreads dengue fever, the mosquito *Anopheles gambiense* spreads malaria, the mosquito *Culex pipiens* which is a vector transmitting filariasis etc. A knowledge of the different stages of the life cycle of an insect helps in eradication of such disease causing insects (Eisen and Eisen, 2008; Greenwood *et al*., 2008). On the other hand the same knowledge can also help in increasing the yield of useful material such as honey (Jordan *et al*., 2008) and silk (Nagaraju, 2002). In addition, it is very important to understand the metamorphosis for novel treatments for various diseases as well as methodologies for developing novel applications for benefit to mankind.

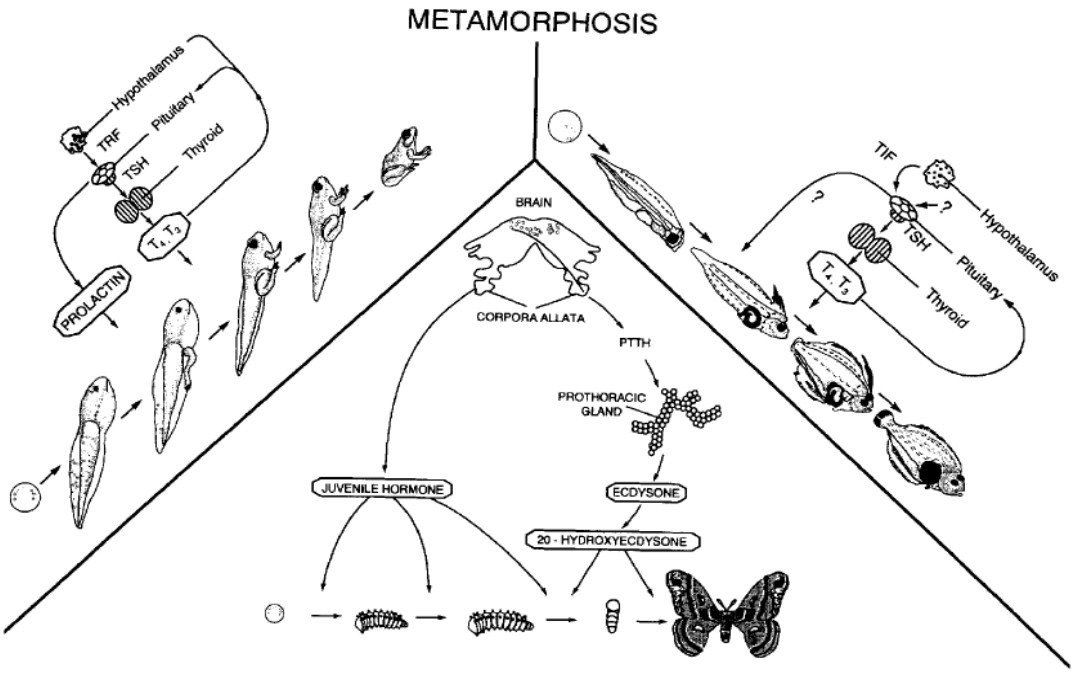
I. Metamorphosis

Metamorphosis can be defined as a marked and abrupt change in the form or structure during postembryonic development (PED). The phenomenon is common to

insects, molluscs, echinoderms, ascidians, fishes, amphibians etc. It allows organisms to fully utilize its resources while its habit and habitat show a continuous change. In the case of some molluscs, the process helps larva in evading predators (Leise and Hadfield, 2000), while in echinoderms, molluscs, and ribbon worms it gives a signal for settlement (Bishop *et al*., 2006). It is of tremendous importance to amphibians in transition from water to air breathing organism during conditions of stress, like oxygen availability, ambient temperature, predation risk and mode of feeding (Burggren and Infantino, 1994).

While metamorphosis mostly involves generation of advanced characters, in case of the ascidians, the larva demonstrates retrogressive metamorphosis where it loses many of the chordate characteristics to become a degenerate sessile adult (Woods *et al*., 2004).

Metamorphosis results in segregation of growth phase and reproductive phase during PED, and involves major processes such as generation of new cells and tissues or remodeling of tissues for novel adult-specific functions as well as elimination of entire organs or tissues that are not required for adult life. Thus metamorphosis results in developmental changes in behaviors in different animal phyla, and since the central nervous system (CNS) of all animals is responsible for generation of behavior, characteristic changes in CNS morphology and physiology are eminent. These changes include neurogenesis, programmed cell death (PCD) and remodeling of the nervous system. Metamorphic changes occur as anticipation towards new demands, and their initiation and completion are controlled by developmental hormones which are varied throughout evolution as noticed in some amphibians, fish and insects (Fig. 1). In both fishes as well as amphibians the hypothalamus produces a thyrotropin releasing factor which causes the thyrotropin cells of the pituitary to release a thyroid-stimulating hormone that activates the thyroid gland to secrete tyrosine-based thyroid hormones. The thyroid hormones which include thyroxine and 3,5,3`-triiodothyronine are responsible for initiation of metamorphosis in the fishes and amphibians (for review: Atkinson, 1994; Tata, 2006).



(Atkinson, 1994)

Figure 1. Schematic illustration of similarities in hormonal control of metamorphosis in amphibians, fishes and insects. Vertebrate metamorphosis is regulated by the action of thyroid hormones. Insect metamorphosis is regulated by ecdysteroids and juvenile hormones.

(i). Postembryonic Neurogenesis and PCD in Vertebrates

Neurogenesis during PED is widespread in invertebrates and lower vertebrate phyla including fishes, reptiles and birds, but in mammals it occurs only in certain restrictive areas of brain (Gage, 2000). Adult neurogenesis in mammals is detected in areas such as the forebrain subventricular/olfactory bulb (Bath and Lee, 2010) and the hippocampal dentate gyrus which plays a role in memory formation (Smith *et al*., 2010). Recently Ponti *et al*., (2008) demonstrated neurogenesis in cerebellum, which controls balance, stable posture and motor coordination, during early postnatal period as well as in adult rabbits.

The number of neurons and their region-specific abundance in different parts of brain determines neural function and behavior as explained by Williams and Herrup (1988). In general, mammals with larger brains such as cetaceans and primates are more clever than those with little brains, such as insectivores and marsupials. However, it is controversial to correlate neuron number to behavioral complexities. Brains of normally

intelligent humans may weigh only half of the average brain weight of 1350 gm, but still there is no evidence of lack of or a decreased cognitive abilities or intelligence (Cobb, 1965). Such correlations were however authenticated with some experimental evidence of a relation between neuron number and intelligence. A reduction in neuron number markedly lowers performance. For example, the brain mass in triploid and tetraploid newts is the same as that of the diploid controls, but they have larger neurons and only half of the normal number (Vernon and Butsch, 1957). The locomotion in polyploid newts is indistinguishable from that of the normal newts; however, they take 2-3 times more trials to learn a maze than do the normal newts. On the other hand increase in neuronal number results in improved performance. For instance, the singing abilities of the canaries correlate well with the seasonal oscillations in neuronal numbers in the song nuclei (Goldman and Nottebohm, 1983). Few workers have reported increase in neuronal numbers in immature frogs and rats upon growth hormone administration (Zamenhof *et al*., 1966; for review: Williams and Herrup, 1988).

Neurogenesis and cell death shape the nervous system in a number of phyla and are regulated by hormones such as steroids in lower vertebrates such as fishes and amphibians, and in higher vertebrates such as mammals. Thyroid hormone controls remodeling of the nervous system during amphibian metamorphosis (Marsh-Armstrong *et al*., 2004) by removing larval-specific neural circuits (Coen *et al*., 2001), by regulating proliferation of neural progenitors in the developing retina (Marsh-Armstrong *et al*., 1999) and by generation of spinal cord limb motoneurons in tadpoles (Schlosser *et al*., 2002). Thyroid hormone regulates apoptosis in a number of larval specific tissues in amphibia such as tail (Rowe *et al*., 2005), skin (Schreiber and Brown, 2003) etc.

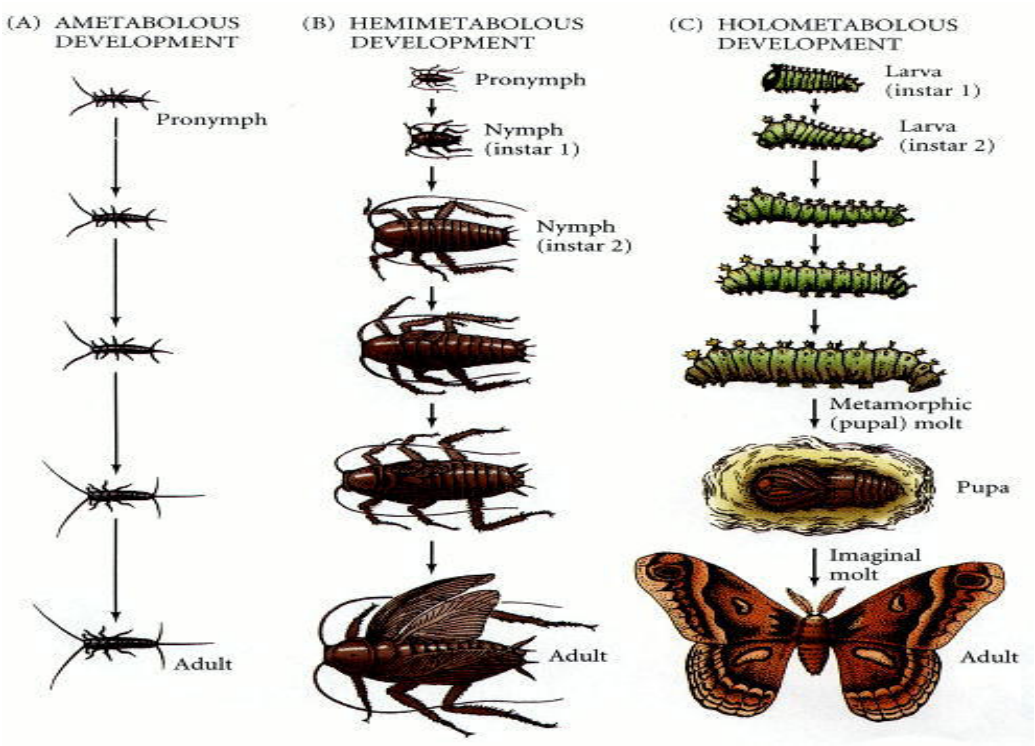
In mammals, adult neurogenesis is regulated by steroid hormones (e.g., adrenal steroids, estrogen, and testosterone) and peptide hormones (prolactin). High estrogen levels increase cell proliferation in hippocampal dentate gyrus (Tanapat *et al*., 1999), while prolactin has been shown to have a similar effect in the subventricular zone (Shingo *et al*., 2003). On the other hand, the adrenal steroids have an inhibitory effect on neurogenesis in the hippocampal dentate gyrus i.e., the removal of adrenal steroids by adrenalectomy results in robust neurogenesis in the dentate gyrus (Krugers *et al*., 2007).

Steroidal influence on neuronal death has been reported in vertebrate development (Sengelaub and Arnold, 1989). The gonadal steroids induce caspase-3 dependent cell death especially during sexual differentiation of the brain: cell death markers are elevated under influence of testosterone (Tsukahara *et al*., 2006) in males and estrogens in females in certain areas of brain (Waters and Simerly, 2009). Marked changes in neuronal arbors in vertebrates during development and in the adulthood are also induced by steroid hormones such as the thyroid hormone (Gould *et al*., 1990), gonadal hormones (Goldstein *et al*., 1990) etc.

(ii). Insect Metamorphosis

Insect metamorphosis is of three general types: ametabolous, hemimetabolous and holometabolous (Fig. 2) (for review: Gilbert, 2006). Ametabolous insects do not undergo metamorphosis and include insects belonging to Thysanura such as the silverfish (*Lepisma saccharina*) that undergoes little or no changes during young to adult transformation except in size and development of sexual organs. In hemimetabolous insects such as the Blatteria which include cockroaches (e.g., *Periplaneta americana*) the nymphs resemble adults but are smaller and lack wings and sexual organs. Holometabolous insects such as the Lepidoptera which include butterflies and moths undergo complete metamorphosis and here, there is not even a remote resemblance between the juvenile larvae and the adults.

Insects that undergo complete metamorphosis face significant behavioral and developmental challenges. During metamorphosis typical larval behaviors like crawling, feeding, defensive thrashing and ecdysis are replaced by adult behaviors like walking, flight, mating and egg laying. These behaviors understandably require different intrinsic properties and synaptic interactions of neurons, changes in overall musculature as well as sensory inputs. While there is little change in the central nervous system (CNS) of metamorphosing insect in ametabolous or hemimetabolous insect, extensive changes occur in CNS during holometabolous metamorphosis. This could be a prerequisite to the behavioral transformations observed in the insects during metamorphosis, which is achieved by remodeling of neural and motor systems (Consoulas *et al*., 2000).



(Gilbert, 2006)

Figure 2. Different types of Insect Metamorphosis include ametabolous (e.g. silverfish), hemimetabolous (e.g. cockroaches) and holometabolous (e.g. moths).

(iii). Postembryonic Developmental Changes in Holometabolous Insects

Holometabolous metamorphosis involves complete remodeling of the larval insect during the resting pupa to generate *de novo* the adult insect with higher specialized functions. Larval tissues with obsolete functions in the adult are eliminated by PCD during metamorphosis. These include: the larval specific intersegmental muscles (ISMs) in the Pernyi silkmoth *Antheraea pernyi* (Lockshin and Williams, 1965), the tobacco hawkmoth *Manduca sexta* (Myer *et al*., 2009); motoneurons innervating the ISMs in *M. sexta* (Streichert *et al*., 1997); the silk glands of the silkworm *Bombyx mori* (Manaboon *et al*., 2009), the salivary glands of the fruit fly, *Drosophila melanogaster* (Ianella *et al*., 2008), the labial glands of *M. sexta* (Halaby *et al*., 2003) etc.

Some larval tissues are remodeled into adult tissues. The larval midgut undergo PCD to be replaced by the adult midgut as observed in *D. melanogaster* (Denton *et al*., 2010), the crucifer root maggot *Delia radicum* (Hegedus *et al*., 2002), the greater wax moth, *Galleria mellonella* (Uwo *et al*., 2002), *B. mori* (Kaji *et al*., 2009), *A. aegypti*

(Parthasarathy and Palli, 2007a), the tobacco budworm *Heliothis virescens* (Parthasarathy and Palli, 2007b) etc. The fat body remodeling in insects involves dissociation of the larval fat body into individual cells e.g. in the flesh fly, *Sarcophaga peregrine* (Kurata *et al*., 1991), *B. mori* (Gui *et al*., 2006), *D. melanogaster* (Liu *et al*., 2009) etc.

Tissue required for adult function are generated *de novo* from imaginal discs (Cranna and Quinn, 2009) include adult head structures such as eyes and antennae, appendages such as wings (Baker, 2007) and legs (McClure and Schubiger, 2008), genitalia (Chen *et al*., 2005) etc.

(iv). Postembryonic Developmental Changes in Central Nervous System of Insects (Neurometamorphosis)

In general it is thought that more complex the behavior more is the number of neurons involved in the behavioral circuitry (Ward *et al*., 1998). During postembryonic development of insects, the simpler larval/nymph behaviors are replaced by complex behaviors in the adult (Consoulas *et al*., 2000). In hemimetabolous insects, such behavioral transformations are incorporated in the CNS during a relatively active and mobile last nymphal instar. Major transformations are incorporated in the CNS of holometabolous insects during the resting pupal stage.

The adult insect CNS show regional differences in the neuronal numbers found within the segmental ganglia (Booker *et al*., 1996). These regional differences in neuronal numbers are achieved by different ways in insects. In hemimetabolous insects, such as grasshoppers and locusts, segmental ganglia are developed by the end of embryogenesis and no neurogenesis occurs during PED (Shepherd and Bate, 1990). For example the newly hatched grasshopper has adult complement of neurons which show basic morphology characteristic of that of the adult. The neural circuits for adult specific behaviors such as flight (Stevenson and Kutsch, 1988) or oviposition (Thompson, 1993) are readily recognizable in nymphs at the time of hatching. For this reason the CNS of nymph is considered a miniature version of the adult CNS as the latter is virtually established during embryogenesis. However, the CNS of hemimetabolous insects is far from being static, and new neurons are always being added during larval life to accommodate growth and addition of new sensory elements (Murphey and Chiba, 1990)

which show quantitative as well as qualitative growth as in case of receptive structure such as antennae, compound eye, cerci etc. A qualitative change is especially observed during its final molt to the adult during which the insects add receptors required for novel adult behaviors. This includes the sex-pheromone detecting-antennal sensilla in cockroaches which differentiate only during the adult molt (Schafer and Sanchez, 1973; for review: Truman, 1996). Postembryonic neurogenesis is however prominent in certain areas of the brain (Cayre *et al*., 2002).

In the holometabolous insects such as flies and moths, there is a small difference in the number of neurons found in thoracic and abdominal ganglia by the end of embryogenesis (Truman and Bate, 1988). The embryo produces only a fraction of the neurons that make up the adult CNS. Postembryonic neurogenesis plays a major role in generating the regional differences observed in segmental ganglia of adult holometabolous insects. Postembryonic neurogenesis has been reported in larvae of many species of Holometabola, including *M. sexta* (Booker *et al*., 1996), *D. melanogaster* (Ito and Hotta, 1992), the honey bee, *Apis mellifera* (Ganeshina *et al*., 2000) etc. Though neurogenesis is rare in adult Holometabola, it has been reported in the turnip moth *Agrotis ipsilon* (Dufour and Gadenne, 2006), the rove beetle *Aleochara curtula* (Bieber and Fuldner, 1979) and few Coleopterans (Cayre *et al*., 1996). Recently, adult neurogenesis was reported to occur in *D. melanogaster* (Ben Rokia-Mille *et al*., 2008; von Trotha *et al*., 2009).

Differences in early life histories and behavioral repertoires are responsible for the variations in neurogenesis patterns between hemimetabolous (Farris and Strausfeld, 2001) and holometabolous insects (Farris *et al*., 1999) during PED. In hemimetabolous insects, higher functional regions such as mushroom bodies (MBs) are well developed in the nymph since immediately after hatching the nymph must search for food and shelter (Farris and Sinakevitch, 2003). However, in holometabolous insects MBs are less developed in the larvae that typically hatch on or inside their source of food and shelter and hence they have relatively less role in non-feeding or defensive behaviors (Bernays, 2001; Wennström *et al*., 2010). Such simple behaviors do not require MBs and correspondingly sensory centers such as eyes and antennal lobes are rather rudimentary in newly hatched larvae. The *Drosophila* larvae and adult possesses different ecological

niches, with the former maintaining a constant contact with food till pupation (Wu *et al*., 2003), while the latter are flying insects that use the sense of smell for finding suitable food sources, mating and egg laying (Zhu *et al*., 2003). Like wise, there are fewer larval odorant receptor neurons (ORNs) in *Drosophila* as compared to the adult ORNs depicting the stage-specific changes in the population of a particular set of neurons for specialized functions during PED (Couto *et al*., 2005; Fishilevich *et al*., 2005). Such a developmental delay is extreme in the social insects such as the Hymenoptera in which the larvae are completely cared for by adult workers and hence in their earlier instars have poorly developed brains (Farris *et al*., 1999). Further age related behavioral changes in honeybee workers include morphogenetic changes in the MBs (Withers *et al*., 1993) which occurs in the absence of neurogenesis (Fahrbach *et al*., 1995).

Some reports indicate that the CNS architecture is remodeled during metamorphosis (primarily through ganglionic fusion) in the holometabolous insects of different orders such as the Hymenoptera: stingless bee, *Melipona quadrifasciata* (Pinto *et al*., 2003), the common Indian wasp *Polistes hebraeus* Fabr. (Singh, 1974a); Diptera: *D. melanogaster* (Truman, 1990), *S. peregrine* (Fujii *et al*., 1999), Coleoptera: the black ladybird, *Chilocorus nigritus* (Singh, 1974b), the mealworm beetle, *Tenebrio molitor* (Breidbach, 1990), the white pine weevil, *Pissodes strobi* (Whitney and Godwin, 1979), Lepidoptera: *M. sexta* (Amos *et al*., 1996), and the cabbage butterfly, *Pieris brassicae* (Heywood, 1965). However, there are some rare instances where the CNS remodeling accompanies ganglionic separation as observed in the Dipteran insect, the hoverfly *Syrphus ribesii* (for review: Niven *et al*., 2008).

In *M. sexta*, the larval thoracic legs are replaced by new adult legs; however, the leg motor neurons do not die after loss of target muscles, but persist to innervate the new adult leg muscles. The accumulation and proliferation of adult leg muscle precursors in *M. sexta* are dependent on innervations from the CNS (Consoulas and Levine, 1997).

The larval neurons that persist through metamorphosis into adult undergo changes in dendritic morphology (Consoulas *et al*., 2000) and biophysical properties (Börner *et al*., 2006; Meseke *et al*., 2009). Robust reorganizations of synaptic connections in Kenyon cells of the MBs have been correlated with behavioral plasticity in holometabolous insects indicating that they have different targets and perform different

functions in the larval and adult behaviors (Armstrong *et al*., 1998; Lee *et al*., 1999). However, in hemimetabolous insects such reorganizations in Kenyon cells in MBs are absent probably because the juvenile and adults behaviors are not as vastly different as observed in the holometabolous insects (Strausfeld and Li, 1999).

Many factors regulate vertebrate as well as invertebrate neurogenesis including the hormonal influences during the molt cycle (Harrison *et al*., 2001) or adulthood (Rasika *et al*., 1994), neurotransmitter serotonin levels (Benton *et al*., 2008), the day-night cycle (Goergen *et al*., 2002; Tamai *et al*., 2008), seasonality (Hansen and Schmidt, 2004; Sherry and Hoshooley, 2010), physical activity (Lafenêtre *et al*., 2010) and environmental living conditions (Scotto-Lomassese *et al*., 2002; Schloesser *et al*., 2010). Cell proliferation in insects is negatively regulated by molecules such as the nitric oxide (NO) which was demonstrated to inhibit cell proliferation during eye development in *D. melanogaster* (Kuzin *et al*., 2000) and *M. sexta* (Champlin and Truman, 2000).

(v). Genetic regulation of Neurogenesis in Insects

It has been long known that neurogenesis as well as distribution of neuronal precursors (neuroblasts) during embryonic and postembryonic development is regulated by *Notch* gene expression (Fehon *et al*., 1991). Notch signaling promotes formation of neuroblasts in the brain while the same signaling event inhibits neuroblast formation in the ventral nerve cord (Wheeler *et al*., 2008). Recently Monastirioti *et al*., (2010) reported that Notch signaling involves the target protein 'Hey' during embryonic and larval neurogenesis. The projection neurons and the interneurons in the thoracic ganglia of *D. melanogaster* have different Notch signaling requirements during generation of secondary neurons (Truman *et al*., 2010).

Neurogenesis is also regulated by the MAGE (melanogen antigen) family of proteins, and recently one of the MAGE members, 'necdin' has been shown to be highly expressed during early embryonic neurogenesis in *D. melanogaster*, while its levels were undetectable in postmitotic neurons during late embryonic period. However, MAGE expression was observed throughout the postembryonic neurogenesis in the neuroblasts and their progeny (ganglionic mother cells and postmitotic neurons) during larval and pupal stages. High levels of MAGE expression were also reported in postmitotic neurons

during adulthood in MBs and retinal photoreceptors (Nishimura *et al*., 2008). Baboon/dSmad2 TGF-β signaling has been shown to be essential for high expression levels of EcR-B1 in larval neurons that are to be remodeled during early metamorphosis to perform novel adult functions (Zheng *et al*., 2003). Recently the same signaling pathway was reported to be involved in proper morphogenesis of adult-specific neurons during the late larval stages (Zheng *et al*., 2006).

II. Programmed Cell Death

The term "programmed cell death" (PCD) was coined by Lockshin and Williams (1965) while studying steroid regulated degeneration of ISMs during metamorphosis. In all higher organisms, PCD plays an essential role in maintaining homeostasis by eliminating obsolete cells and tissues, control of cell number, and by removal of damaged or abnormal cells, or cells no longer required for further functions (Jacobson *et al*., 1997). Developmental PCD is defined as the spatially or temporally reproducible, tissue and species-specific loss of cells, which serves distinct developmental functions (Buss *et al*., 2006). PCD is an evolutionarily conserved process observed in all the kingdom of life, including bacteria, prokaryotes as well as eukaryotes (Ameisen, 2004). In multicellular organisms PCD is thought to be a defense mechanism that is required for elimination of damaged or abnormal cells and healthy survival of the whole organism (Brodersen *et al*., 2002). PCD is highly regulated by pro- and anti-apoptotic genes, and their aberrant expression can manifest into a number of disease and pathological conditions (Rudin and Thompson, 1997).

(i). Mechanisms of Programmed Cell Death

PCD is characterized by biochemical and morphological characteristic features that are distinct from those of necrosis (Kerr *et al*., 1972; Wyllie, 1980; Vaux and Korsmeyer, 1999) and is controlled by genetic regulatory pathways conserved throughout the evolution (Abrams, 1999). PCD can be classified into three categories: type 1, or apoptosis; type 2, or autophagic cell death (also called as "autophagocytosis"); and type 3, or non-lysosomal PCD (Schweichel and Merker, 1973; Clarke, 1990). These different types of PCD are distinguished based on electron microscopy. Apoptotic features (Type 1

PCD) include various processes such as chromatin condensation, nuclear shrinkage, cell cross linkage, increase in electron density of cytoplasmic contents, increase in number of free ribosomes and formation of apoptotic bodies that are phagocytosed by other cells (Kerr *et al*., 1972). The apoptotic bodies do not increase in number and the materials from the dying cells are digested by lysosomes of the other cells (heterophagy). However, during autophagic cell death (Type 2 PCD), cellular contents are sequestrated into autophagic bodies and are destroyed by the cell's own lysosomes. Thus autophagic PCD is considered as the true form of cell suicide (Levine and Yuan, 2005). The autophagic bodies increase in size as well as in numbers following increase in electron density of cytoplasmic contents but chromatin condensation is either absent or occurs relatively later. The nonlysosomal cell death (Type 3 PCD) in EM is characterized by pronounced dilation of organelles and empty spaces while the degeneration proceeds without any detectable involvement of lysosomes.

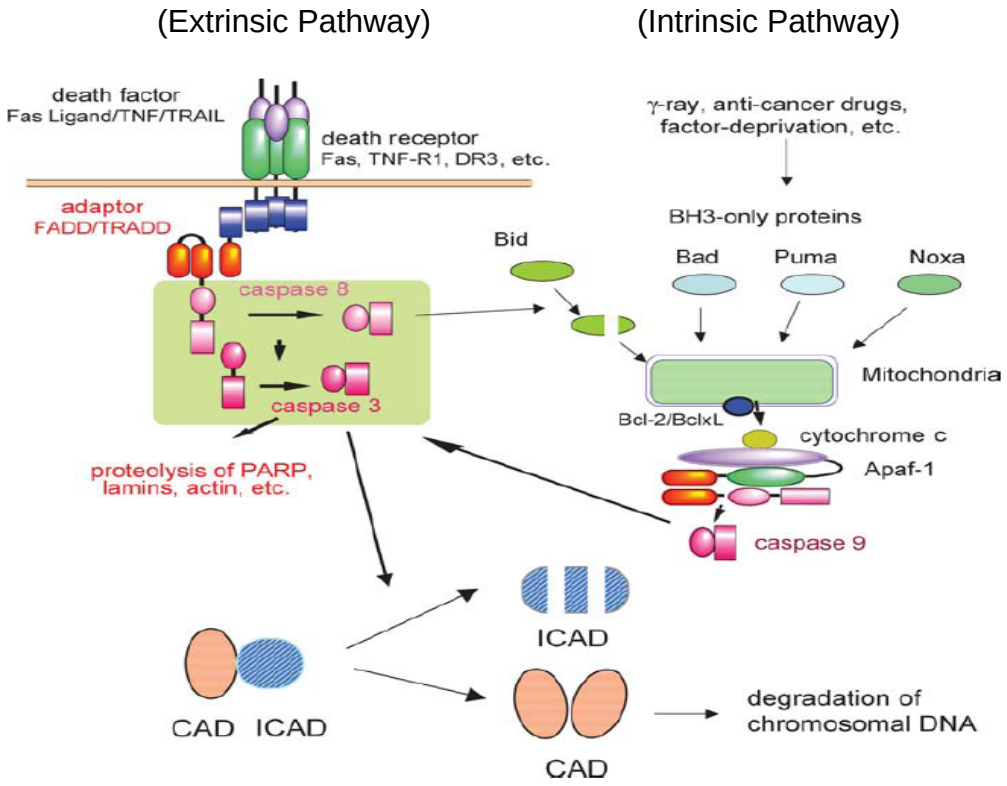
Apoptotic cell death is considerably distinct from necrotic cell death. Necrosis leads to loss of membrane integrity, swelling of cells, organelle damage, disruption of lysosomes and uncontrolled cell disruption and lysis releasing cellular components into exterior, eventually leading to tissue inflammation, in contrast with apoptosis which does not lead to any inflammation (Krysko *et al*., 2008).

a. Major Pathways involved in PCD

Pathways leading to apoptosis have been nicely reviewed by Nagata (2005). PCD is triggered by intrinsic and extrinsic stimuli (Fig. 3) and require caspases, a family of cysteine proteases. Caspases that are involved in cellular apoptosis include caspases 3 and 6-10 which are further divided into initiator caspases (caspase 8 and 9) and executor caspases (caspase 3, 6 and 7) (Fuentes-Prior and Salvesen, 2004).

The intrinsic pathway can be triggered by anticancer drugs, γ-irradiation, antioxidants and deprivation of growth factors or serum, and can be blocked by the oncogene Bcl-2. The Bcl-2 homolog (BH) 3-only proteins are transcriptionally activated by p53 or released from their adaptor by dephosphorylation (Bad). These BH3-only proteins stimulated mitochondrial release of cytochrome c that can activate the initiator

caspase 9 (Tichý *et al*., 2008) along with an adaptor called Apaf-1 (apoptotic protein activating factor 1) (Fig. 3).



(Nagata, 2005)

Figure 3: Apoptotic signaling pathways leading to DNA degradation. Intrinsic pathway activates caspase 3 through caspase 9, while the extrinsic pathway activates caspase 3 through caspase 8. Once activated, caspase 3 cleaves a number of cellular proteins and also results in DNA fragmentation by dissociating CAD from its inhibitor ICAD.

The extrinsic pathway is triggered by death factors such as Fas ligand, tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL). Following extrinsic activation a death-inducing signaling complex (DISC) consisting of a receptor, adaptor and procaspase 8 is formed which results in autocatalysis of procaspase 8 to the active caspase 8 (Pennarun *et al*., 2010). Both caspase 8 and caspase 9 directly activate the executor caspase 3 (Fig. 3), which is known to cleave more than 280 nuclear and cytoplasmic proteins that are essential cell components such as kinases, transcription factors, DNA polymerases, RNA polymerases and cytoskeletal proteins (for review: Fischer *et al*., 2003). Cleavage of these essential molecules is responsible for the morphological and biochemical changes that characterize apoptosis.

b. DNA Degradation during PCD

DNA fragmentation has been considered to be a classical hallmark feature of apoptotic cell death (Wyllie, 1980). Nagata (2005) reviewed the importance of DNA degradation in various developmental processes and for maintaining homeostasis in animals. If DNA is degraded, the cells do not survive, except in few cases such as red blood cells (erythrocytes) and lens fiber cells, in which DNA is removed or degraded in their final stage of differentiation, but perform their functions normally. Specific nucleases have been reported to degrade DNA in erythroid precursor cells and lens fiber cells and deficiencies in the respective DNases result in conditions such as anemia (Nagata, 2007) and cataract (Nagai *et al*., 2006).

DNA degradation occurs in two stages in apoptosis. In early stages, DNA is cleaved into large fragments (50-100 kb), which are subsequently cleaved in the later stages of apoptosis into nucleosomal units (180 bp), that are evident as DNA ladder formation on agarose gel electrophoresis (Oberhammer *et al*., 1993; Lagarkova *et al*., 1995). DNA fragmentation forms the basis for TUNEL technique that is widely used to detect apoptotic cells (Gavrieli *et al*., 1992).

Among the many enzymes and factors responsible for DNA degradation, the important ones include DNase 1, DNase 2, DNase γ, caspase activated DNase (CAD) and endonuclease G. CAD has been reported to be responsible for apoptotic DNA degradation, since in CAD-deficient mouse (Kawane *et al*., 2003) or *D. melanogaster* cells (Mukae *et al*., 2002) no DNA fragmentation is observed. CAD is active at neutral pH and generates double stranded-DNA breaks at the 3'-end exposing the hydroxyl group (-OH) that serves as a good substrate for terminal deoxyribonucleotidyl transferase (TdT). CAD cleaves DNA only at the linker region between nucleosomes, thus generates oligonucleosomal ladder on agarose gels (Widlak *et al*., 2000).

CAD possesses high specific activity that is potentially harmful to cells. Hence, CAD is synthesized along with its inhibitor (ICAD) which is required for its proper functional folding (Sakahira and Nagata, 2002). When cells receive the stimuli for undergoing apoptosis, caspase 3 cleaves ICAD which then loses its affinity for CAD. The free CAD which carries a nuclear localization signal migrates into nucleus and cleaves

chromosomal DNA (Murgia *et al*., 1992). Abundant expression of CAD has been reported in lymphocytes that show prominent DNA fragmentation during apoptosis. However, apoptotic DNA fragmentation is not very apparent in nerve cells, hepatocytes or embryonal fibroblasts in which CAD expression is low (Nagase *et al*., 2003).

DNA fragmentation is not a compulsory event and is considered to be dispensable for PCD. For instance, even enucleated cells undergo apoptotic cell death displaying typical morphological changes characteristic of apoptotic cell death (Föller *et al*., 2009). Further, it has been reported that mice deficient in CAD or ICAD develop normally and show no abnormal morphological phenotypes (McIlroy *et al*., 2000). However, recently mice deficient in CAD have been reported to be vulnerable to different types of cancers (Widlak and Garrard, 2009).

c. Deoxyribonuclease 1 (DNase 1) in Apoptotic DNA fragmentation

DNase 1, a Ca^{2+}/Mg^{2+} dependent endonuclease, mediates internucleosomal DNA degradation in human cells undergoing apoptosis (Falcone *et al*., 1999; Oliveri *et al*., 2001). Treatment of cells with anti-DNase 1 antibody was shown to completely inhibit enzymatic activity. Inhibition of DNase 1 results in resistance to apoptotic stimuli and inhibition of the morphological features of apoptosis and DNA fragmentation (Madaio *et al*., 1996). Over expression of DNase 1 induce typical morphological and biochemical changes observed during apoptosis and tissue distribution confirmed a preferential localization and concentration of the enzyme in cells undergoing apoptotic cell death (Oliveri *et al*., 2001).

DNase 1 is a cytoskeletal protein with its activity regulated by actin (Lazarides and Lindberg, 1974). However, DNase 1 plays major role in the formation and function of actin filaments rather than the degradation of DNA (Moraczewska *et al*., 2004). DNase 1 expression is low but is distributed throughout the cytoplasm. It is especially active during the execution phase of apoptosis. DNase 1 expression increases prior to apoptotic induction and higher enzyme concentrations were observed in the perinuclear space, particularly in the cells displaying rapid turnover rate (Polzar *et al*., 1994).

d. Role of Caspases in PCD

As discussed earlier cleavage of cellular proteins by executor caspase such as caspase 3 (Fig. 3) has been linked to some of the discrete morphological changes characteristic of apoptosis that have been extensively reviewed by Fischer *et al*., (2003). Caspase 3 cleaves caspase activated DNase (CAD) from its inhibitor (ICAD) and results in apoptotic DNA fragmentation. Chromatin and nuclear condensation are a result of cleavage of DNA helicases: acinus (Sahara *et al*., 1999) and helicard (Kovacsovics *et al*., 2002). Membrane blebbing, another classical feature of apoptosis is a result of cleavage of substrates such as gelsolin (Koya *et al*., 2000) and kinases such as the Rho kinase which affect the integrity of F-actin and myosin (Orlando *et al*., 2006). Cleavage of proteins involved in maintenance of the cytoskeletal architecture such as the intermediate filaments directly contributes to apoptotic changes in cell shape (Gilbert *et al*., 2008). Caspases cleave proteins involved in cortical actin network (such as fodrin) and those of focal adhesion complex such as focal adhesion kinase or paxillin resulting in cell shrinkage and cell detachment (for review: Fischer *et al*., 2003).

Apoptosis results in disruption of the endoplasmic reticulum (Heath-Engel *et al*., 2008), Golgi complex (Mukherjee *et al*., 2007), and impairs vesicular transport processes (Machamer, 2003). Caspases initiate destruction of nucleus as a result of cleavage of proteins involved in nuclear matrix (Gerner *et al*., 2002). Nuclear transport is also impaired as a result of cleavage of proteins involved in nuclear lamina and several components of nuclear pores (Kramer *et al*., 2008). Inhibition of DNA repair by cleavage of proteins such as Poly (ADP-ribose) polymerase 1 (PARP-1) promote apoptosis and offers great potential for treatment of cancers (Peralta-Leal *et al*., 2008; Chevanne *et al*., 2010). Caspases target proteins involved in DNA synthesis and replication, such as DNA polymerase Pol ε (Liu and Linn, 2000), or replication factor RFC140 (Rhéaume *et al*., 1997). Cleavage of RNA helicase A (Myohanen and Baylin, 2001), multiple splicing factors (Degen *et al*., 2000) as well as heterogeneous nuclear ribonucleoproteins (Brockstedt *et al*., 1998), leads to general shut-down of RNA synthesis. Inactivation of translation initiation factors, including eukaryotic initiation factor (eIF) 2 alpha (eIF2α) (Marissen and Lloyd, 1998), eIF3 (Bushell *et al*., 2000) result in inhibition of protein synthesis.

Apoptosis is regulated by anti-apoptotic proteins which are involved in cell-protective mechanisms. Caspases cleave these apoptosis inhibitors and activate cell destructive mechanisms in which the cleavage of apoptosis inhibitors such as Bcl-2 proteins leads to their loss of antiapoptotic function and converts them to proapoptotic proteins. Death-receptor mediated apoptosis (extrinsic pathway) results in cleavage of Bcl-2 member Bid that induces the release of cytochrome c from mitochondria and this in turn activates the effector caspases (Desagher *et al*., 2001).

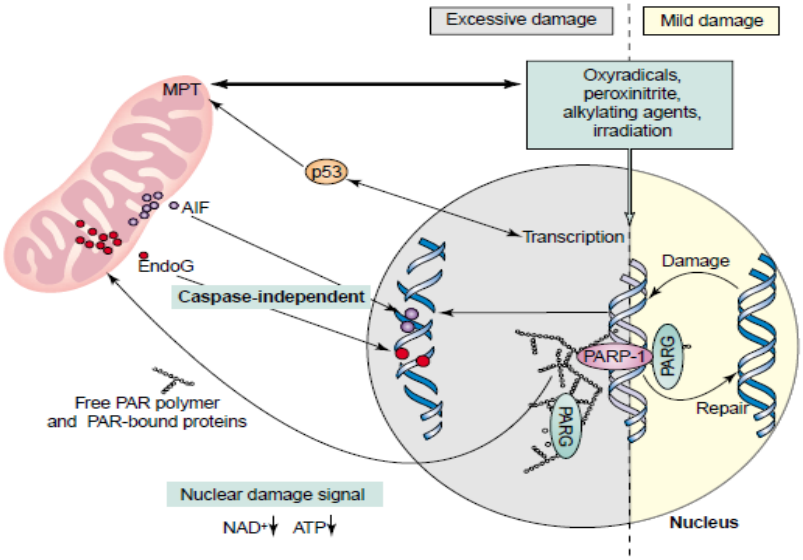
e. Role of Poly (ADP-ribose) polymerase 1 (PARP-1) in PCD

PARP-1 is an abundant nuclear protein which in response to DNA damage increases in activity upto 500 fold. It acts as a nick sensor and recognizes both single stranded as well as double stranded breaks in DNA. Activation of PARP regulates cellular DNA repair, transcription and replication of DNA, cytoskeletal organization, protein degradation etc., through (ADP-ribosyl)ation of PARP-1 substrates (Bouchard *et al*., 2003). On the other hand excessive activation of PARP-1 as a result of extensive DNA damage leads to generation of large branched-chained PAR polymers which leads to activation of cell death program (Yu *et al*., 2003). Hence because of rapid activation of PARP-1, the intensity of PARP-1 activation is a key factor that regulates whether cells will survive or will undergo PCD following DNA damage.

The primary structure of PARP-1 enzyme is highly conserved between species. It is a 116 kDa protein that contains three functional domains: 42 kDa DNA-binding domain (N-terminal), a central auto modification domain (16 kDa), and 55 kDa catalytic domain (C-terminal). The DNA binding domain utilizes two zinc finger motifs that recognize either single or double stranded breaks in DNA. Over activation of PARP-1 and (ADP-ribosyl)ation leads to massive nicotinamide adenine dinucleotide (NAD+) utilization and rapid loss of cellular NAD+ and adenosine-5'-triphosphate (ATP) affecting cellular energy metabolism and ultimately leads to cell death (for review: Bouchard *et al*., 2003).

PARP-1 transfers poly (ADP-ribose) (PAR) to itself and to various cytoplasmic as well as nuclear proteins such as histones, DNA polymerases, topoisomerases, DNA ligases, high mobility group proteins and transcription factors. This modifies the charge

distribution of acceptor proteins and increases the hindrance of proteins by the addition of a bulky and complex PAR structure. The physiological function of acceptor proteins are adversely affected and thus might lead to cell death. Many proteins related to apoptosis interact with PARP, including p53, p21 and caspases (Pleschke *et al*., 2000) indicating that PAR polymer might activate cell death effectors. PARP-1 mediated cell death pathway appears to involve apoptosis inducing factor (AIF). PARP-1 is activated by DNA alkylating agents, H_2O_2 and N-methyl-D-aspartic acid (NMDA) which induce the translocation of AIF from mitochondria to nucleus, nuclear condensation, dissipation of mitochondrial membrane potential and cell death (Fig. 4). This indicates that AIF acts downstream to PARP-1 to mediate cell death (Yu *et al*., 2003; for review: Hong *et al*., 2004).



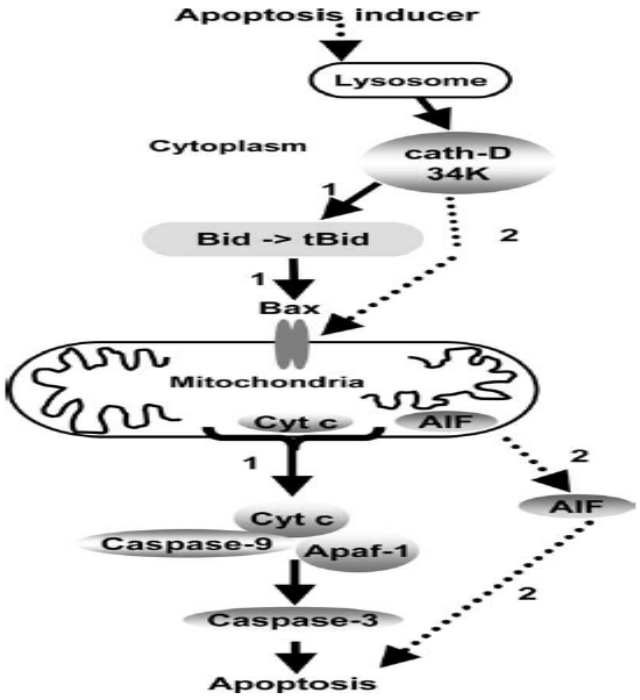
(Hong *et al*., 2004)

Figure 4. Caspase-independent cell death in poly (ADP-ribose) polymerase 1 (PARP-1)-mediated cell death. Poly (ADP) ribosylation of cellular proteins increases their bulk and causes hindrance of proteins, and may activate effector proteins involved in cell death.

Interestingly, during the execution phase of apoptosis PARP-1 is inactivated and is cleaved by the caspases 3 and 7, into a 25 kDa N-terminal fragment and a 85 kDa C-terminal fragment. This protects cells from ATP depletion and necrotic cell death. In fact, by preventing DNA repair, PARP-1 cleavage commits cells to PCD by apoptotic pathway (Soldani and Scovassi, 2002).

f. Role of Lysosomal protein Cathepsin D in PCD

Cathepsin D is an aspartic proteinase distributed ubiquitously in the lysosomes and has numerous functions within lysosomal compartments, especially in proteolysis of endocytosed and autophagocytosed proteins at low pH leading to protein turnover and antigen presentation (Moss *et al*., 2005). It is also involved in cell growth and tissue homeostasis (Koike *et al*., 2003). Cathepsins also play extra-lysosomal functions including degradation of extracellular matrix and execution of PCD (Fig. 5) when released into cytosol (Fehrenbacher and Jäättelä 2005). Cathepsin D is synthesized on the rough endoplasmic reticulum as a 52 kDa immature pre-pro-enzyme that undergoes a series of proteolytic cleavages during biosynthesis to first give a 48 kDa intermediate and finally a 34 kDa and a 14 kDa mature cathepsin D (Laurent-Matha *et al*., 2006).



(Liaudet-Coopman *et al*., 2006)

Figure 5: Role of Cathepsin D in apoptotic induction. Release of cathepsin D into cytosol results in destabilization of mitochondrial membrane releasing cytochrome c, which associates with Apaf-1 and caspase 9 to activate caspase 3 and leads to apoptosis.

Kågedal *et al*., (2001) reported the participation of cathepsin D in apoptosis. Upon oxidative stress by reactive oxygen species such as H_2O_2, there is damage of lysosomal

membrane and subsequent leakage of cathepsin D into cytosol. Cathepsin D destabilizes the mitochondrial membrane releasing cytochrome c which activates caspase 9 after binding to Apaf-1 (Heinrich *et al*., 2004). This complex in turn activates the executor caspase 3 and brings about apoptosis (for review: Liaudet-Coopman *et al*., 2006).

(ii). PCD during Insect Metamorphosis

It is known that pulses of 20-hydroxyecdysone (20E) control insect metamorphosis. 20E pulses at the end of larval development triggers puparium formation and signals for the onset of metamorphosis. 20E pulse after pupariation signals stage-specific destruction of obsolete larval tissues by PCD and development of adult specific tissues and structures, thus resulting in the transformation of a larva to adult (for review: Truman and Riddiford, 2002). In insects, PCD in the CNS is essential for removal of larval specific neurons that do not have any functional role in the adult nervous system. It is also responsible for generation of segmental specialization and sexual dimorphism within the CNS and for adjusting the final neuronal numbers. PCD results in matching cell numbers between CNS and periphery (Hollyday and Hamburger, 1976), dispose off cells that function transiently during development (Bate *et al*., 1981) and remove cells that arose in an inappropriate manner (Horvitz *et al*., 1982).

PCD in the insect CNS removes larval neurons located in the abdominal ganglia reflecting the reduced importance of abdomen for locomotion as the insect transforms from a vermiform larva into an adult with well developed thoracic legs and wings. This neuronal death occurs in 2 waves. The first one occurs at larval-pupal transformation. Pupal ecdysis in *M. sexta* is followed by loss of most of larval musculature. While some of the larval muscles are replaced by adult muscles, many others are not. In insects, the fates of some of the larval motoneurons are correlated with those of their muscle targets. Hence, motoneurons associated with muscles that are replaced persist to the adult stage, whereas, motoneurons of muscles that are eliminated degenerate after larval-pupal transition. The best studied example of the latter is the principal planta retractor (PPR) motoneuron which innervates the principal retractor muscles of the proleg. However the accessory planta retractor (APR) motoneurons also undergo dendritic regression but not

PCD. The second major period of larval neuron degeneration occurs at adult emergence and it is associated with loss of ecdysial muscles (for review: Tissot and Stocker, 2000).

Cell death in terminal abdominal ganglion has been associated with generation of sexual dimorphism in the adult CNS in *M. sexta* (Thorn and Truman, 1994). The motoneurons innervating genitalia and reproductive tract differentiate during metamorphosis and are amongst the last neurons to develop. Some examples of anatomical correlates of sexual dimorphism in insects in the brains of the two sexes that arises because of cell death include the macroglomerular complex in the antennal lobes of males which responds to female pheromones in moths (Matsumoto and Hildebrand, 1981) and cockroaches (Prillinger, 1981).

a. DNA degradation during PCD in Insects

Cellular morphological characteristics differ in apoptosis in insects and the mammals. PCD in ISMs (Beaulation and Lockshin, 1977) and labial glands (Zakeri *et al*., 1993) of insect *M. sexta* have been shown to involve early cytoplasmic destruction while nuclear condensation occurs to a lesser extent as compared to during mammalian apoptosis. Further, in insects all cells fated to die, do so in synchrony while retaining their normal nuclear morphology, indicating that DNA cleavage doesn't initiate cell death. In contrast PCD in the mouse limbs is asynchronous but gives a prominent DNA ladder formation indicating the role of DNA degradation in apoptosis. A major reason for absence of DNA fragmentation in insects has been attributed to their prominent heterochromatin which is less readily attacked by endonucleases and an overall decrease in protein synthesis as metamorphosis begins (Zakeri *et al*., 1993). DNA fragmentation is however prominently observed in the fat body and gut (Gui *et al*., 2006), and also in anterior silk glands (Kaneko *et al*., 2006) in *B. mori* pupae.

b. Role of Deoxyribonucleases in PED in Insects

Abrupt changes in deoxyribonuclease levels were reported during histolysis of larval-specific tissue at the onset of metamorphosis in some insects such as *D. hydei* (Boyd and Boyd, 1970) and the midge *Chironomus thummi* (Laufer and Nakase, 1965). DNases active at high pH display tissue-specific distribution, while the acid DNases have been reported in a variety of tissues at the onset of metamorphosis in *D. hydei* (Boyd and

Boyd, 1970). Detwiler and MacIntyre (1978) identified the genetic locus for acid active DNase 1 in *D. melanogaster* and reported the enzyme activity to be higher in 3rd instar larva and throughout pupation, but decreases in the adults. Some authors related this to reutilization of larval DNA in the formation of adult tissues (Agrell, 1964). The relative DNase activity at neutral pH has been shown to increase in extracts of different developmental stages of *D. melanogaster* in the following order: eggs, 2 day old pupae, l day old pupae, adults, and 3, 5, and 7 day old larvae. Low DNase activity in the pupa was suggested to indicate the down regulation of enzyme activity during the period of extensive histogenesis (Muhammed *et al*., 1967).

Yokoyama *et al*., (2000) identified a DNase in *D. melanogaster* Schneider line (S2) cells that can be activated by mammalian caspases and reported it to be a *Drosophila* counterpart of CAD (dCAD). However, the same group also showed that dCAD has DNase activiy but no DNA fragmentation activity as dCAD cannot enter nucleus on its own unlike mouse CAD (mCAD). Further, inhibition of dCAD with *Drosophila* inhibitor of caspase activated DNase (dICAD) was demonstrated by Mukae *et al*., (2000) who showed that dCAD and dICAD form complexes in the proliferating cells. Interestingly several protein binding sites for the ecdysone responsive elements such as the E74 protein (Urness and Thummel, 1990) or the BR-C (von Kalm *et al*., 1994) are found in the promoter region of *dICAD* gene.

c. Role of Caspases in PCD during Insect Metamorphosis

Caspases are involved in PCD in holometabolous insects, and well studied in *D. melanogaster* (Kumar and Doumanis, 2000). The caspases, *Drosophila* Nedd2-like caspase (DRONC) (Dorstyn *et al*., 1999a), Death related ced-3/Nedd2-like protein (DREDD) (Chen *et al*., 1998), *Drosophila* caspase-1 (DCP-1) (McCall and Stellar, 1998), death executioner caspase related to Apopain/Yama (DECAY) (Dorstyn *et al*., 1999b) and *Drosophila* Interleukin-1β converting enzyme (DRICE) (Fraser and Evan, 1997) have been shown to play a role in the execution of cell death in *Drosophila*. DRONC was reported to play similar role as that of the initiator caspase 9 in mammal and CED-3 in *Caenorhabditis elegans* (Dorstyn *et al*., 1999a) as it is the only *Drosophila* caspase with a caspase-recruitment domain. Its essential role for developmental PCD and its requirement

for cell death in the fly eye mediated by Reaper, Hid and Grim, has been demonstrated by genetic and gene ablation studies (Hawkins *et al*., 2000). DRICE has been reported to function as an effector caspase which is activated by DRONC, the initiator caspase (Hawkins *et al*., 2000). Massive up regulation of *dronc* transcript by the hormone ecdysone has been reported to mediate PCD of larval tissues including the larval salivary glands and midgut during larval/pupal transformation (Baehrecke, 2000). DECAY, has been shown to be related to mammalian caspase-3 and caspase-7, and has the substrate specificity similar to that of caspase-3 (Dorstyn *et al*., 1999b). Transcripts of *decay* have been shown to be elevated in the larval salivary glands and midgut, and probably could be regulating their programmed removal (Dorstyn *et al*., 1999b). The *Drosophila* caspase DCP-1 (Song *et al*., 1997) and DRICE (Fraser and Evan, 1997) have been shown to cleave human PARP and *D. melanogaster* lamin A, respectively.

Cytochrome c release from mitochondria is known to be mediating caspase activation in mammalian cells (Green and Reed, 1998). However, some reports have shown that PCD in *D. melanogaster* doesn't involve release of cytochrome c from mitochondria. Though cytochrome c is released, it remains near the mitochondrial membrane and the apoptosome complex may form in the vicinity of mitochondria which could play a role in apoptosis in *D. melanogaster* (Dorstyn *et al*., 2002).

Neuronal PCD also involves caspases in insects, as has been shown by immunolabeling of neurons dying posteclosion in the fly brain (Kato *et al*., 2009). Kim *et al*., (2007) have implicated role of caspases in neuronal PCD in thoracic ganglia of *B. mori* and have demonstrated inhibition of cellular apoptosis by injection of inhibitors of caspase-8 and caspase-3. Similar results were reported in PCD in the brain of *B. mori* during PED (Kim *et al*., 2009). DRONC was demonstrated to control neuroblast number in *D. melanogaster* CNS by inducing apoptosis (Waldhuber *et al*., 2005). *B. mori* interleukin-1β-converting enzyme (BmIce) from *B. mori* was also characterized as a caspase and demonstrated to play a role in apoptosis (Duan *et al*., 2005).

d. Role of PARP-1 in PED in Insects

PARP-1 plays vital role in *D. melanogaster* development as its enzymatic activity facilitates transient loosening of chromatin and regulates transcriptional activation of

many genes that are dependent on heat shock, NF-kB and ecdysteroids (Tulin and Spradling, 2003). PARP cleavage was demonstrated in the apoptotic pathway in Schneider line cells of *D. melanogaster* (Poltronieri *et al*., 1997).

Poly (ADP-ribosylation) reactions have been described in some of the holometabolous insects including the Mediterranean fruit fly *Ceratitis capitata* (Cavalloro *et al*., 1988), *D. melanogaster* (Ji and Tulin, 2009) and *S. peregrine* (Masutani *et al*., 1994). PARP activity has also been reported in hemimetabolous insects such as *P. americana* (Denegri *et al*., 2000).

e. Role of Cathepsin D in Insect Metamorphosis

Gui *et al*., (2006) and Lee *et al*., (2009) showed that *B. mori* cathepsin D (BmCatD) is an ecdysone-induced protease which is expressed in the larval fat body of the final instar and in the larval gut of pupal stage. While over expression of cathepsin D led to PCD, RNA intereference (RNAi)-mediated BmCatD knock-down inhibited PCD (studied by DNA fragmentation) in the above mentioned stages. BmCatD RNAi also resulted in inhibition of larval-pupal transformation which clearly indicates that BmCatD is a metamorphosis specific lysosomal proteinase. A similar role of cathepsin D was reported in amphibian metamorphosis (Seshimo *et al*., 1977). Ecdysone has been reported to be a negative translational regulator of a cathepsin D like lysosomal aspartic protease (LAP) mRNA in the mosquito *A. aegypti*, and the rise in the mRNA coincides with a decline in ecdysone levels (Dittmer and Raikhel, 1997).

Cathepsin D deficiency in humans has been implicated in several neurological disorders. Cathepsin D deficiency usually occurs as a result of single nucleotide mutation that results in conversion of the active site aspartic acid to asparagine and renders the protein structurally stable but functionally inactive (Tyynelä *et al*., 2000). Cathepsin D deficiency and its enzymatic inactivation result in an early-onset, progressive and fatal neurodegenerative disorders, classified as 'neuronal ceroid lipofuscinoses' (NCL) in mammals such as the american bulldogs (Awano *et al*., 2006), mice (Koike *et al*., 2000; Partanen *et al*., 2008) and humans (Siintola *et al*., 2006). The loss of Cathepsin D leads to accumulation of ceroid lipofuscin-containing lysosomes (Koike *et al*., 2000) which leads to disruption of lysosome function, inhibits recycling of autophagosomes and

accumulation of autophagic vacuoles that induce neuronal apoptosis (Shacka and Roth, 2005). Cathepsin D deficiency in *D. melanogaster* has been shown to be involved in synucleopathy, whereby there is a reduced degradation of α-synuclein (Cullen *et al*., 2009). Interestingly the aggregation of insoluble α-synuclein is a hallmark feature of many neurodegenerative diseases such as sporadic Parkinson's disease (Klein and Schlossmacher, 2007), dementia with Lewy bodies (Spillantini *et al*., 1997) etc.

(iii). Genetic Regulation of Programmed Cell Death in Insects

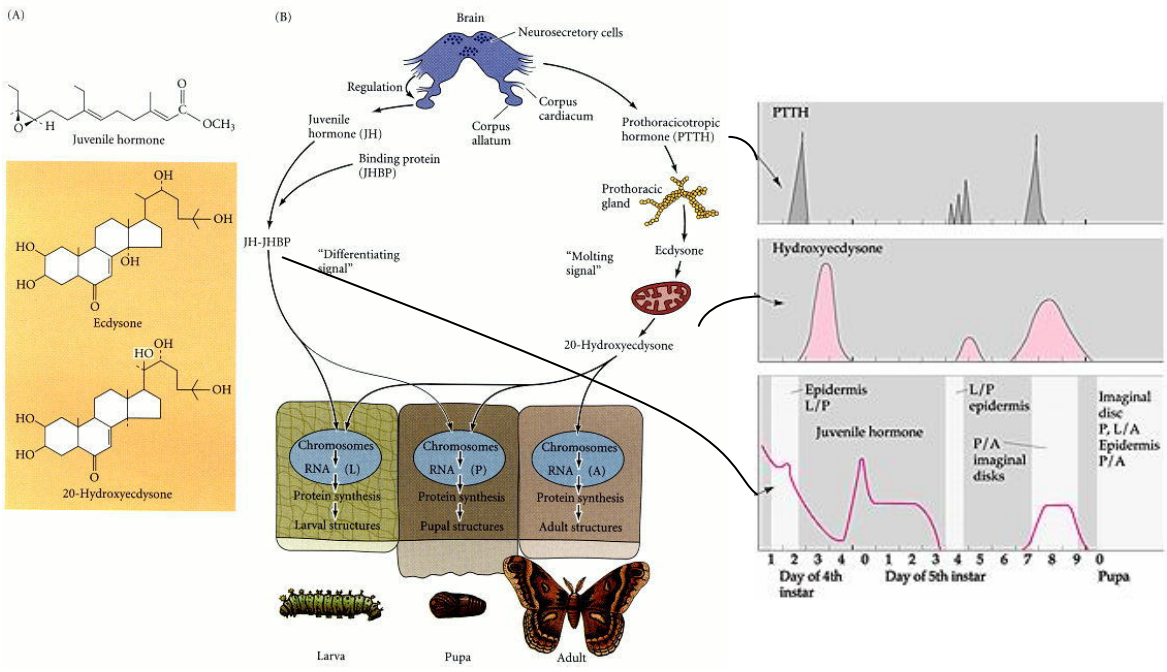
White *et al*., (1994) demonstrated effective blockage of PCD upon deletion of the locus *Df(3L)H99* which contains the cell death genes *rpr*, *hid* and *grim*. Ectopic expression of these genes was reported to induce cell death in a caspase dependent manner in *Drosophila* (Grether *et al*., 1995; Chen *et al*., 1996; White *et al*., 1996). PCD in insects is modulated by inhibitors of apoptosis protein (IAP) homologs (Clem and Miller, 1994). The ectopic expression of *Drosophila* IAP (*diap1*) blocks *rpr, hid* and *grim* driven cell death, while *diap1* null mutants die in embryogenesis with extensive cell death (Wang *et al*., 1999; Goyal *et al*., 2000).

Inhibition of PCD by mutations in the early pupal stages results in pathological manifestations in the later stages of insect development and neurodegeneration (Usui-Aoki *et al*., 2002). The spin mutation results in the long-abdominal-ganglion phenotype as a result of failure of PCD, a feature similar to that of *H99* deletion mutants and both phenotypes demonstrated intense autofluorescence associated with extensive degeneration of neurons (Draizen *et al*., 1999; Usui-Aoki *et al*., 2002).

Segment-specific specialization of CNS is under the control of the Hox genes such as *Ultrabithorax*, *Antennapedia* and *AbdominalA* which act in antagonistic manner regulating segment-specific PCD of differentiated neurons in embryonic as well as postembryonic periods (Rogulja-Ortmann and Technau, 2008). Sexual differentiation in neuronal population involves regulation of sex-specific neuronal PCD and cell survival by the genes *fruitless* and *doublesex* (Kimura *et al*., 2008; Sanders and Arbeitman, 2008). The clearance of neurons undergoing apoptosis, and also the axonal pruning events occurring during *Drosophila* metamorphosis are all regulated by the apoptotic cell engulfment genes *draper* and *ced-6* (Awasaki *et al*., 2006).

III. Hormonal Control of Insect Metamorphosis

Two major classes of hormones control molting and metamorphosis in insects, viz., the ecdysteroid hormones such as ecdysone, and the sesquiterpenoid hormones such as the juvenile hormone (JH) (Fig. 1 and 6) (Truman and Riddiford, 2002). Ecdysteroids are the major steroid hormones secreted by the prothoracic glands (PGs) and regulate molting and metamorphosis in insects. The prothoracicotropic hormone (PTTH) synthesized and released by neurosecretory cells in the brain stimulate the PGs to synthesize and release ecdysone which is converted into the insect molting hormone, 20E. Initiation of metamorphosis by ecdysteroids is markedly influenced by the levels of JH. High ecdysone pulses in presence of high JH result in larval-larval transformation, in presence of moderate JH levels result in larval-pupal, and in absence of JH lead to pupal-adult transformation (for review: Gilbert, 2006).



(Gilbert, 2006)

Figure 6. Hormonal Regulation of Insect Metamorphosis. Ecdysone triggers molting and metamorphosis, while the JH act as a "status quo" hormone determining whether the molt will be to larval/pupa/adult.

Ecdysteroid synthesis by PGs is also regulated by FMRFamide (Phe-Met-Arg-Phe-NH2)-related peptides (FaRP) expressed in the neurosecretory cells of thoracic

ganglia which directly innervate the PGs to supply the FaRPs to the gland surface (Yamanaka *et al*., 2006).

(i). Ecdysteroid Hormones in Insect Metamorphosis

The principal ecdysteroids in insects that initiate post-embryonic development and metamorphosis are ecdysone (α-ecdysone) and 20E (β-ecdysone). PG synthesizes and release ecdysone into the hemolymph, then it is metabolized by other tissues to 20E which is considered to be the "true" molting hormone. In fact 20E is biologically more active than ecdysone. Ecdysone is converted to 20E by ecdysone 20-monooxygenase which is a steroid mixed-function oxidase, and the reaction is catalyzed by cytochrome P450 which is distributed in various tissues (fat body, Malpighian tubules, midgut etc). The enzyme may be localized in mitochondria in *M. sexta* or microsomes in *L. migratoria* (for review: Gilbert *et al*., 1980).

a. Ecdysteroid Receptors

Ecdysteroids play a central role in the onset of molting and metamorphosis via stage- and tissue-specific expression of different types of ecdysteroids receptors (EcRs) (Tan and Palli, 2008). 20E plays dual role in metamorphosis regulating both neurogenesis and PCD (reviewed by: Tissot and Stocker, 2000). The CNS responses to ecdysteroids are different, and are dependent on the type of EcR expressed. EcR-A is involved in PCD (Robinow *et al*., 1993) and EcR-B in proliferation or remodeling (Truman *et al*., 1994). The effect of ecdysteroids on metamorphic events is modulated by JH (Lobbia *et al*., 2007) and its analogues (JHA) (Parthasarathy and Palli, 2007b), where expression of EcR-B1, E75-B and HR3 are modulated (Siaussat *et al*., 2004).

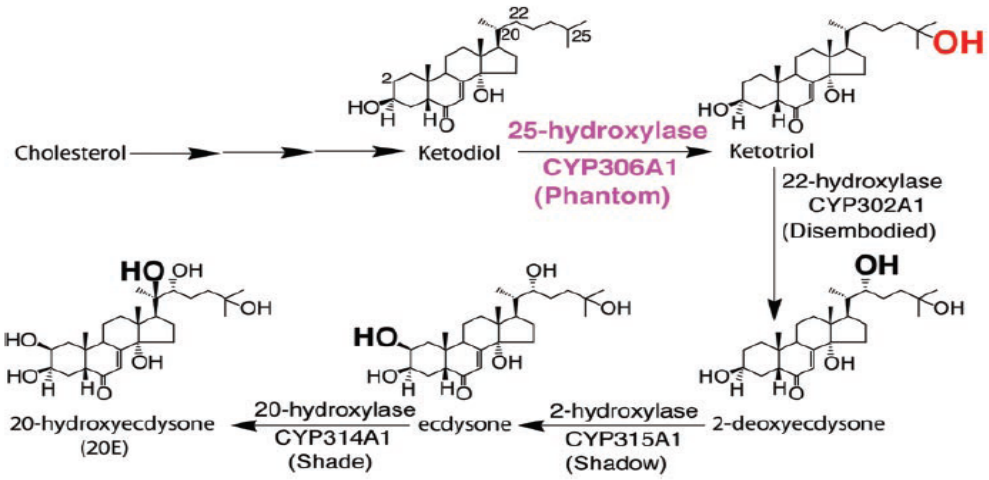
b. Chemical Structure of Ecdysteroids

20E was first crystallized and termed “ecdysone” by Butenandt and Karlson in 1954, while its steroidal nature was confirmed by Karlson and Hoffmeister in 1963 who showed that cholesterol is the precursor of the ecdysone (Karlson, 1996). Ecdysteroids are polyhydroxylated ketosteroids with a common tetracyclic ring structure and ubiquitously found in arthropods (Goodwin *et al*., 1978). Ecdysone (2B,3B,14a,-22R,25-pentahydroxy-5B-cholest-7-en-6-one) was the first of the ecdysteroids identified and is

the parent molecule from which other ecdysteroids are derived. So far, seven ecdysteroids have been identified in various stages of the insect life cycle. The ecdysteroids have a 27-carbon (C-27) skeleton structure with a single exception of makisterone A, a C28 steroid synthesized in the honey bees (Feldlaufer *et al*., 1986), varying only in the number and/or stereochemistry of the hydroxyl groups present (Morgan and Poole, 1977).

c. Ecdysteroid Biosynthesis

The pathway for biosynthesis of 20E (Fig. 7) (Niwa *et al*., 2004) has been well characterized. Phytophagous insects such as *B. mori* ingest phytosterols from their plant diet, primarily β-sitosterol, the major sterol in mulberry leaves (Nagata *et al*., 2006). The phytosterols are first dealkylated to cholesterol, the immediate sterol precursor of ecdysteroids. Cholesterol is first converted into 7dC (7-dehydrocholesterol) in the PG cells, by the action of the 7,8-dehydrogenase enzyme. Members of the Halloween gene family, including *disembodied* (*dib*), *shadow* (*sad*) and *shade* (*shd*), encode cytochrome P450 enzymes and catalyze several steps of the ecdysteroid biosynthetic pathway (Rewitz *et al*., 2006).



(Niwa *et al*, 2004)

Figure 7: Biosynthetic Pathway of Synthesis of 20-hydroxyecdysone (20E). The cytochrome P450 enzymes modify the cholesterol to give the ecdysteroid, ecdysone, which is then hydroxylated at the 20th carbon position to give 20E in the peripheral tissues such as epidermis, midgut, Malphighian tubules and fat body.

Mutations disrupting the P450 steroid hydroxylases result in drastic morphogenetic abnormalities such as failure of head involution and cuticle formation,

leading to embryonic death in *D. melanogaster* and *B. mori* (Niwa *et al*., 2004; Warren *et al*., 2004). The embryonic lethal phenotypes of the *D. melanogaster* Halloween family of mutants have been reported to be caused by a low ecdysteroids titer (Chávez *et al*., 2000). Mutations disrupting 20E signaling result in a wide spectrum of defects during metamorphosis (D'Avino and Thummel, 2000), molting (Neubeuser *et al*., 2005) and cause sterility in adults (Wismar *et al*., 2000).

d. Morphogenetic Effects of Ecdysteroids

Exogenous application of ecdysteroids has various morphogenetic effects on many tissues, and the same tissue may respond differently due to developmental differences in hormone receptors, metabolism or other intrinsic properties (Riddiford, 1985). For example, under the influence of 20E the corpus allatum (CA) in the cockroach may reduce JH synthesis (Stay *et al*., 1980) or regress in cell size (Chiang *et al*., 1991). Chiang *et al*., (1997) reported that 20E stimulates DNA synthesis, but not cell proliferation in the CA of *B. mori.* Mitogenic action of 20E on epidermis has been demonstrated in many insects (Kato and Riddiford, 1987).

20E has different effects on neurogenesis in different insects: it reduces neurogenesis in the MBs of the house cricket, *Acheta domesticus* (Cayre *et al*., 2000), but it has a mitogenic effect (increased neurogenesis) on the MB neuroblasts of the adult cockroach *Diploptera punctata* (Gu *et al*., 1999). Moderate levels of 20E stimulate proliferation in resting neuroblasts in differentiating optic lobes (Champlin and Truman, 1998a) and in glial cells of *M. sexta* pupae (Kirschenbaum *et al*., 1995). 20E was shown to inhibit mitotic activity of the neural precursors in the developing MB in the pupal stages of *A. mellifera* (Malun *et al*., 2003).

e. Ecdysteroid regulation of Insect Metamorphosis

Periodic surges in ecdysone regulate diverse biological responses during insect life history (Riddiford *et al*., 2003; Chittaranjan *et al*., 2009; Cranna and Quinn, 2009). In fact development and metamorphosis of insects are impossible without ecdysone as evidenced by the mutations affecting the synthesis of ecdysteroids (Sliter and Gilbert, 1992) or loss of tissue sensitivity to 20E (Fristrom *et al*., 1981; for review: Mitrofanov, 2007).

Ecdysone induces three sets of puffs (zones of gene activity) in chromosomes: intermolt, early and late. The genes expressed during intermolt puffs are the first to be induced, and these control the synthesis of glue proteins which are used by larvae for sticking to a hard substrate and complete metamorphosis. The early puffs include 2B5, 74EF and 75B, which activate more than 100 late genes. One of the products of genes of early prepupal puffs, *βFTZ-F1* is involved in response of the prepupal genes to ecdysone and is involved in hormonal signal transmission (Lavorgna *et al*., 1993). At this stage many tissues change their developmental programs, including salivary gland histolysis, imaginal disc differentiation and proliferation (Cranna and Quinn, 2009).

Ecdysone induced *E74* and *BR-C* products encode family of transcription factors that regulate the genes of primary and secondary response to ecdysone. They are involved in puparium formation, pupation and induction of early genes (Fletcher and Thummel, 1995; Zhimulev *et al*., 1995). On the other hand, the nature of steroid-inducible biological response is determined by the gene *E93* (Lee *et al*., 2000). *D. melanogaster Ets* gene product play important role developmental processes such as metamorphosis, oogenesis, neurogenesis, myogenesis, and eye development; malfunction of *Ets* gene activities has been associated with oncogenic processes (Hsu and Schulz, 2000).

(ii). Juvenile Hormones in Insect Metamorphosis

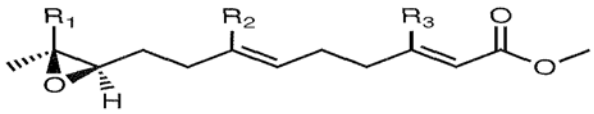
a. Physiological Actions of JH

The importance of JH in regulating insect development has been extensively reviewed by Minakuchi and Riddiford, (2006). JH was first discovered by Wigglesworth in 1934 as a humoral factor in the blood-sucking insect, *Rhodnius prolixus* that prevents metamorphosis of the final instar nymph into the adult. JH is synthesized in the corpora allata (CA) in all the insects. JH action during larval development allows proper larval molting and prevents premature metamorphosis as elimination of JH during earlier stage by removal of CA (allatectomy) results in precocious metamorphosis of the larvae (Fujita *et al*., 2008). JH was shown to suppress morphogenesis of imaginal discs and growth as starved allatectomized *M. sexta* larvae showed formation and growth of imaginal discs (Truman *et al*., 2006). At the end of the larval developmental period circulating JH is degraded while elevated ecdysteroid levels trigger metamorphosis. In Hemipteran and

Lepidopteran larvae, withdrawal of JH resulted in 20E induction of precocious metamorphosis, whereas application of JH during the last instar resulted in a supernumerary instar or larval-pupal transformation. It was earlier reported that higher JH titers are required to maintain proliferation of neuroblasts in the brain of adult cricket *A. domesticus* (Cayre *et al*., 1994) and the late feeding phase (last instar larva) of honey bee *A. mellifera* (Vitt and Hartfelder, 1998), where low mitotic activity correlates with low JH titer (Rachinsky *et al*., 1990). JH also regulates many physiological aspects such as development, reproduction, diapause and polyphenism (reviewed by Shinoda and Itoyama, 2003).

b. Chemical Structure of JH

JH was chemically identified as a sesquiterpenoid compound (a terpene consisting of three isoprene (5-C) units) and led to identification of JH 1 from the cecropia moth *Hyalophora cecropia*. However, many other JH structures have been identified. JH III has been reported in many insect orders, while JH 0, JH I, JH II and 4-methyl-JH I are present exclusively in Lepidoptera. JH 0 to III differ from each other in the ethyl or methyl group substitutions at the positions R1-3 (Fig. 8). Higher Diptera, such as *Drosophila* produce JH III bisepoxide (for review: Minakuchi and Riddiford, 2006).



JH 0 (R_1=R_2=R_3=C_2H_5)
JH I (R_1=R_2=C_2H_5, R_3=CH_3)
JH II (R_1=C_2H_5, R_2=R_3=CH_3)
JH III (R_1=R_2=R_3=CH_3)

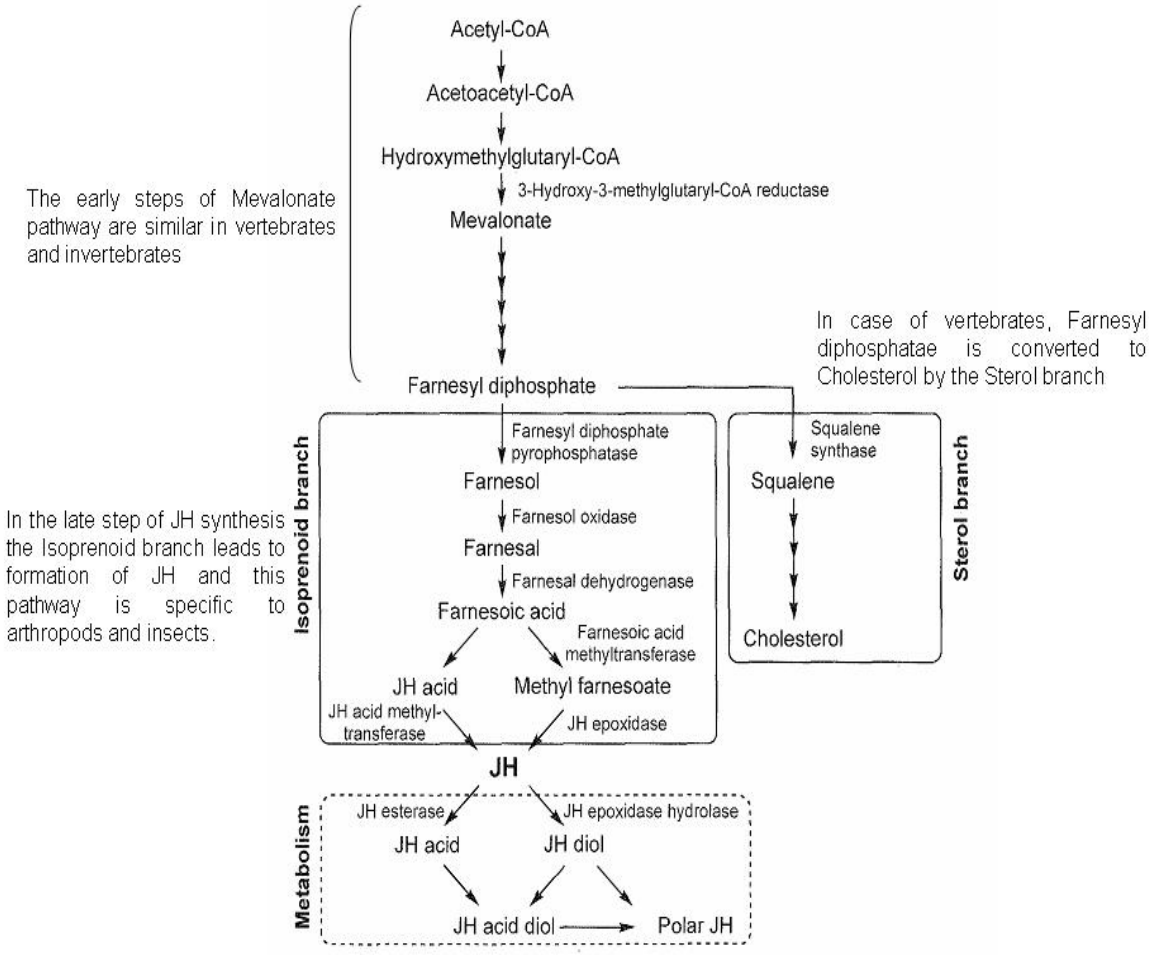
(Minakuchi and Riddiford, 2006)

Figure 8: The Chemical structure of JH. The basic backbone structure of JH is modified at different positions (R1, R2, R3) in a species specific manner to give the distinct JHs.

c. JH Biosynthetic Pathway

JH is synthesized in insects by the mevalonate pathway via isoprenoid branch which is specific to arthropods and insects. JH biosynthesis has been conventionally divided into a two-step process: the early step and the late step. In the early step, acetyl-CoA is converted to farnesyl diphosphate via the classical mevalonate pathway, which is

common to vertebrates and invertebrates. The genes encoding the enzymes involved in conversion of acetyl-CoA to farnesyl diphosphate have been identified in *Drosophila* and *Anopheles*. The late step involves hydrolysis of farnesyl diphosphate to farnesol, followed by successive oxidation to farnesol and farnesoic acid. The farnesoic acid is converted to active JH by the action of JH acid methyltransferase (JHAMT) and a cytochrome P450 monooxygenase (CYP15A1) (Fig. 9). The insect specific late-step enzymes are excellent targets for selective insect growth regulators (for review: Minakuchi and Riddiford, 2006).



(Minakuchi and Riddiford, 2006)

Figure 9: Biosynthesis and Metabolic Pathways of JH is specific to arthropod and insects, and thus forms the target for selective insect growth regulators.

Mevalonate synthesized from acetyl-CoA undergoes three phosphorylations followed by a decarboxylation reaction to generate isopentenyl diphosphate (IPP) which

covalently links with dimethyl allyl diphosphate to form 15-carbon farnesyl diphosphate (FPP). Removal of pyrophosphate from FPP generates farnesol, which is oxidized to give farnesal and later farnesoic acid. Farnesoic acid is methylated by S-adenosyl-Met-dependent methyl transferase, followed by epoxidation between C-10 and C-11 to finally produce JH III (Fig. 9). Biosynthesis of active JH is regulated by JHAMT, which is considered as a key regulatory enzyme for insect metamorphosis (Niwa *et al*., 2008). The ethyl branches of JH 0, JH I and JH II (Fig. 8) synthesized exclusively in the Lepidopterans, are derived from iso-leucine and valine; they are first metabolized to propionate and incorporated in the early steps of biosynthesis (Brindle *et al*., 1992). Further, epoxidation of farnesoic acid to JH acid occurs before final methylation (Cusson *et al*., 1991). In Lepidoptera, JH 0 and methyl JH I are exclusively produced in embryos (Bergot *et al*., 1980), JH I and JH II are produced in larvae (Baker *et al*., 1987) and JH II and JH III are synthesized in adult (Unni *et al*., 1991).

In the Lepidopteran insects, the CA stop JH synthesis in the last larval instar, while the JH rapidly disappears from the hemolymph. A small surge in ecdysteroid titers at the end of the feeding stage triggers wandering behavior and pupal commitment. The physiological inactivation of CA during metamorphosis occurs as a result of loss of JHAMT activity in the CA as observed in *M. sexta* (Sparagana *et al*., 1984) and *B. mori* (Niimi and Sakurai, 1997). The transcriptional inactivation of the *JHAMT* gene has been reported to be a crucial step for termination of JH biosynthesis in CA before metamorphosis (Shinoda and Itoyama, 2003).

JH released from CA binds to juvenile hormone binding protein (JHBP), which functions to transport JH in hemolymph, protect JH from degradation from the enzymes in hemolymph and facilitate JH recognition and uptake by the target cells (Touhara *et al*., 1995; for review: Minakuchi and Riddiford, 2006).

d. JH Degradation Pathway

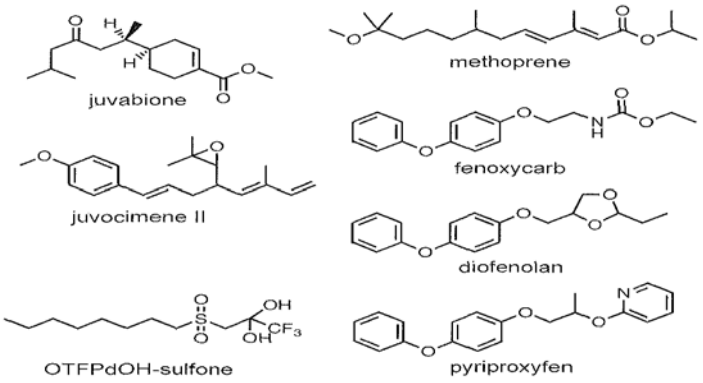
JH can be degraded by two major metabolic pathways: hydrolysis by juvenile hormone esterase (JHE) and hydration by juvenile hormone epoxide hydroxylases (JHEH) (for review: Minakuchi and Riddiford, 2006). Hydration of JH by JHEH has been reported in some Lepidopteran insects such as *B. mori* (Zhang *et al*., 2005), *M. sexta*

(Severson *et al*., 2002) etc. The other pathway involves hydrolysis of the methyl ester moiety by JHE. JHE has been well characterized in many insect orders including Lepidoptera (e.g. Spruce budworm *Choristoneura fumiferana* (Feng *et al*., 1999), *H. virescens* (Hanzlik *et al*., 1989), *M. sexta* (Hinton and Hammock, 2001), *B. mori* (Hirai *et al*., 2002)), Coleoptera (e.g. *T. molitor* (Hinton and Hammock, 2003)) and Diptera (e.g. *D. melanogaster* (Kethidi *et al*., 2005)).

(iii). Juvenile Hormone Analogues (JHA)

a. Discovery of JH Analogues

Earliest JH active compound discovered was "juvabione" in the paper products derived from the wood of balsam fir, which was found to affect the metamorphosis of the Hemipteran bug, *Pyrrocoris apterus* (Bowers *et al*., 1966; Slama and Williams, 1966). However, the compound was less effective against the Coleopteran mealworm beetle *T. molitor* (Bowers *et al*., 1966) and had no activity against the Lepidopterans (Slama and Williams, 1966). Juvocimenes isolated from the sweet basil *Ocimum basilicum*, possessed JH activity against the Hemipteran, the milkweed bug *Oncopeltus fasciatus* (Bowers and Nishida, 1980).



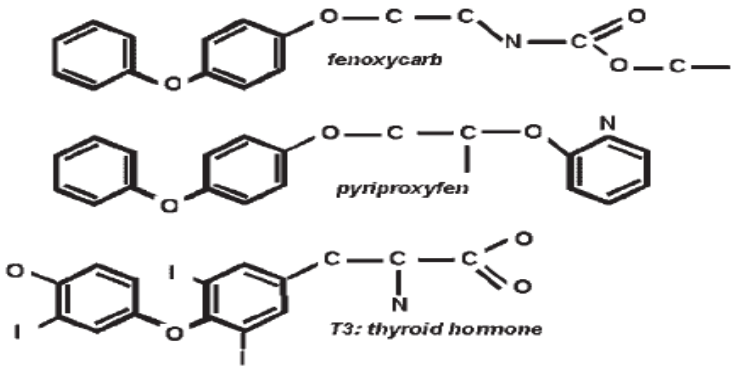
(Minakuchi and Riddiford, 2006)

Figure 10: Various Insect Growth Regulators (IGRs) are chemically synthesized and commercially available.

These discoveries led to development of a number of potent JH mimics (analogs) that disrupt insect endocrine system and metamorphosis and are now being used as pesticides. Several JH analogs (JHA) are chemically synthesized and commercially available in the market as Insect Growth Regulators (IGRs) (Fig. 10). These include

methoprene, fenoxycarb, diofenolan and pyriproxyfen (for review: Minakuchi and Riddiford, 2006).

JH activity can be increased by incorporation of aromatic rings at various locations within the carbon chain of JH molecule. Interestingly the structure of the vertebrate thyroid hormone triiodothyroxin (T3) closely resembles that of certain JH mimics (Fig. 11). T3 induces JH-mediated volume reduction in the follicle cells of *Locusta migratoria* and *R. prolixus* (Wheeler and Nijhout, 2003).



(Wheeler and Nijhout, 2003)

Figure 11: Aromatic rings connected to the JH molecule increase the potency of JH mimics.

b. Effects of JHAs

JHAs have species specific activity. For example, methoprene is very effective against Dipteran insects, but to a lesser extent against Lepidopteran insects. However, JHAs are not very effective against major crop pests since they lack the speed with which the pyrethroid or organophosphate insecticides kill, and hence are seldom considered when rapid depletion of insect population is desired. Methoprene is particularly widely used against mosquitoes and thus used in controlling the vector population which is responsible for many human diseases. Methoprene induces various effects in insects such as toxicity and morphogenetic defects, failure of hatching of eggs, sterility in adults and mortality during pupal development. Methoprene mimics JH III and their application to *Drosophila*, especially the white puparia, has been shown to result in metamorphic disruption followed by morphogenetic effects such as disruption of abdominal bristles and abnormal rotation of male genitalia as well as toxicity leading to death during the

pharate adult stage (for review: Wilson, 2004). Methoprene induces permanent larvae in *B. mori* (Sakurai and Imokawa, 1988).

Methoprene-tolerant (Met) gene is essential for the manifestation of the toxic and morphogenetic effects observed in *Drosophila* upon application of JH/JHA. Met mutants are resistant to the above mentioned effects of methoprene and since Met is capable of binding with specificity and nanomolar affinity, it is a potential candidate as a JH receptor (Konopova and Jindra, 2007).

c. Fenoxycarb: A potent JHA

Fenoxycarb (FX) is a non-neurotoxic carbamate JHA which exhibits potent JH activity in a wide variety of insect species (Grenier and Grenier, 1993; Monconduit and Mauchamp, 1998). It was the first JHA introduced to control agricultural pests. The advantages of using FX in pest managements include very low toxicity to mammals via ingestion but it is considered to be moderately to highly toxic to fishes. It is practically non-toxic to honey bees which are well known for their economic importance (reviewed by Sullivan, 2000). Contamination of mulberry plantation and subsequently the diet of *B. mori* with FX results in deleterious effects on sericulture such as induction of "non-spinning" syndrome. The silk spinning is triggered by the 20E peak in the late stages of last instar larvae. FX treatment inhibits the secretory activity of the PGs, and subsequently the absence of 20E peak results in failure of spinning and pupation (Monconduit and Mauchamp, 1998). *B. mori* is extremely sensitive to FX and treatment of insects at the beginning of the last instar with even picogram quantities leads to prolongation of larval duration (dauer larva) (Dedos and Fugo, 1996) and inhibits normal pupation (Monconduit and Mauchamp, 1998). This prolongation of the last larval instar has been shown to occur by inhibition of prothoracicotrophic hormone (PTTH) by the brain-corpora cardiaca-corpora allata (Br-CC-CA) complex (Dedos and Fugo, 1999b) and also by affecting the steroidogenic competence of PGs as observed in *M. sexta* (Watson and Bollenbacher, 1988; Smith, 1995). Similarly, FX treatment of last instar larvae at around 5^{th} day did not induce any morphological abnormalities in the pupa stage, but induces dauer (permanent) pupa (Dedos *et al*., 2002), apparently because of non-

degenerating PGs in pupa which do not respond to PTTH and continue secreting low amounts of ecdysteroids which maintain high titer throughout pupa.

FX treatment during the middle of the last instar induces an extra larval molt (Dedos and Fugo, 1996; Kamimura and Kiuchi, 1998) and the resulting 6^{th} instar larvae possess some pupal characteristics such as evaginated pupal antennae and wings, and eventually die. However FX treatment at a younger instar larva induces a perfect extra-larval molt to the 6^{th} instars which pupate and emerge as normal adults (Kamimura and Kiuchi, 2002). Treatment with higher doses of FX during pupal ecdysis reportedly results in disturbance in adult eclosion (Dedos and Fugo, 1999a). It has been suggested that FX treatment during pupal ecdysis prevents normal formation of rectum leading to the failure of rectum to eliminate the ecdysteroids from the hemolymph, a critical process which is a prerequisite for the completion of imaginal differentiation and for response to the eclosion hormone (Schwartz and Truman, 1983). Malformation of rectum prevents the decline of ecdysteroids titer and this result in eclosion failure, by preventing the contraction of abdominal muscles that initiate adult eclosion (Dedos and Fugo, 1999a).

d. JH Regulation of Insect Metamorphosis

JH and JHAs are reported to suppress chromosome puffs induced by ecdysone (Richards, 1978). Mutations of the gene *apterous (ap)* lead to JH deficiency resulting in abnormal histolysis of the larval fat bodies, inhibition of vitellogenesis, sterility and irregular nuptial behavior (Shtorch *et al*., 1995). JH has been shown to regulate the expression of the early gene product Broad Complex (BR-C) by acting downstream of the JH receptor, Met (Konopova and Jindra, 2008). BR-C shows developmental changes in temporal distribution of its different isoforms and plays key role in BR-C dependent CNS morphogenesis (Consoulas *et al*., 2005; Spokony and Restifo, 2009).

IV. Role of Neurotransmitter Serotonin in Neurometamorphosis

(i). Serotonin and Brain Development

The biogenic amine serotonin (5-Hydroxytryptamine, 5-HT) plays important role in mammalian brain development before even it assumes the role of a neurotransmitter

(Whitaker-Azmitia, 2001; Sodhi and Sanders-Bush, 2004). The serotonergic system is the earliest appearing afferent system which is detected as early as 5th gestational week in mammalian embryo (Sundström *et al*., 1993; for review: Zafeiriou *et al*., 2009) and has been implicated in playing important role in brain development even in postnatal development (Jagota and Kalyani, 2008, 2010). It reportedly influences morphogenetic processes such as neurogenesis (Banasr *et al*., 2004; Wang *et al*., 2010), neuronal PCD (Zilkha-Falb *et al*., 1997), differentiation (Menegola *et al*., 2004), synaptogenesis (Faber and Haring, 1999), dendritic development (Jones *et al*., 2009) etc.

a. Serotonin Distribution and Metabolism

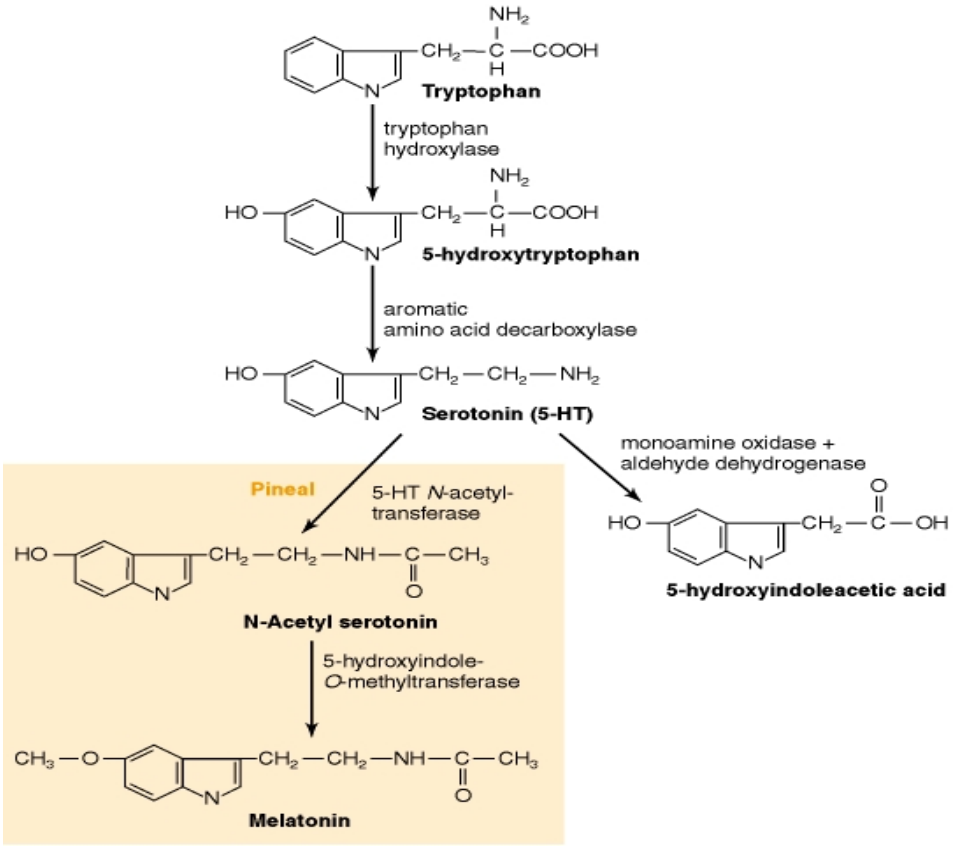
5-HT is primarily found in gastrointestinal tract (GI), platelets and CNS. Almost 80% of serotonin is located in enterochromaffin cells of gut while the remainder is synthesized in serotonergic neurons in the CNS (Zhu *et al*., 2001). Majority of 5-HT containing neurons are restricted to midbrain regions including the median and dorsal raphe nuclei, but the serotonergic system innervates the entire brain (Sodhi and Sanders-Bush, 2004).

Mechanism of synthesis of 5-HT from the essential amino acid (L-tryptophan) (Fig. 12) is evolutionarily conserved throughout the animal kingdom (Andrés *et al*., 2007). Tryptophan is hydroxylated to 5-hydroxytryptophan by the rate limiting enzyme tryptophan hydroxylase (TPH), and subsequently decarboxylated to 5-HT by tryptophan decarboxylase. 5-HT is mainly catabolized in liver by monoamine oxidase A (MAOA) to the metabolite 5-hydroxyindoleacetic acid (5-HIAA) and is later excreted by the kidneys in urine (for review: Siegel *et al*., 1998).

TPH is present in higher vertebrates in two isoforms depending upon the site of synthesis. TPH1 is primarily synthesized in the pineal gland and non-neural tissues such as the enterochromaffin cells of GI tract (Côté *et al*., 2003; Patel *et al*., 2004). TPH2 is distributed exclusively in neuronal cells, such as the dorsal raphe and myenteric plexus (Walther *et al*., 2003; Patel *et al*., 2004).

Serotonin is also acetylated to N-acetyl serotonin by the action of N-acetyl transferase, followed by methylation by 5-hydroxyindole-O-methyltransferase (HIOMT) to produce melatonin (Fig. 12) (Siegel *et al*., 1998). The circulating levels of melatonin

vary in daily cycle and regulate circadian rhythms of several biological functions (Altun and Ugur-Altun, 2007).



(Siegel *et al*., 1998)

Figure 12: Serotonin Metabolism Pathway. 5-HT is synthesized from Tryptophan by successive hydroxylation and decarboxylation reactions. 5-HT is metabolized to Melatonin by the action of HIOMT. 5-HT is excreted in urine as the major metabolite 5-HIAA after oxidative deamination by the action of monoamine oxidase (MAO).

b. Serotonin Receptors

Serotonin mediates its actions through its receptors, which are of 7 types and 14 subtypes, all of which are involved in development (Hannon and Hoyer, 2008). Each receptor has a unique function in the CNS. 5-HT_{1A} receptor activation influences the length and number of dendrites and dendritic spines in hippocampus (Sikich *et al*., 1990); it also regulates neurogenesis (Huang and Herbert, 2005; Grabiec *et al*., 2009). 5-HT_{2A} receptor activation is involved in neuronal death (Capela *et al*., 2007) as well as in neuronal differentiation and dendritic maturation (Dooley *et al*., 1997; Vaidya *et al*., 1997). 5-HT_{2C} receptor activation is involved in long-term potentiation in the

hippocampus (Tecott *et al*., 1998). All 5-HT receptors are G-protein coupled receptors except 5-HT3 (Hannon and Hoyer, 2008).

c. Serotonin Functions

Since serotonin shows widespread distribution throughout body, it regulates many vital functions such as memory, mood, sleep-wake cycle, hormonal secretion from hypothalamus, thermoregulation, food intake, reproduction and rhythmic activities of cardiac and respiratory organs (for review: Lanoir *et al*., 2006). Any alterations in serotonergic system result in abnormal brain development leading to neurological disorders such as autism (Zafeiriou *et al*., 2009), schizophrenia (Veltman *et al*., 2010), depression and anxiety (Baldwin and Rudge, 1995; Lacasse and Leo, 2005), Alzheimers disease (Muck-Seler *et al*., 2009), Parkinson's disease (Fox *et al*., 2009) etc. In vertebrates, serotonin serves as an important modulatory signal in neural development (Gaspar *et al*., 2003). Excessive blood serotonin levels result in "serotonin syndrome" a potentially life threatening syndrome that causes aberrations in cognitive functions, neuromuscular functions and many functions related to autonomic nervous system (Ables and Nagubilli, 2010).

d. Serotonin as a Morphogen

Morphoregulatory actions of 5-HT are well known in vertebrates and molluscan systems (Lipton and Kater, 1989; Lauder, 1993). Higher 5-HT levels have been implicated in the developmental neuropathologies in the brain regions of autistic individuals such as the hippocampus, amygdala and cerebellum which occur as a result of decreased neurite branching (Raymond *et al*., 1996). This suggests that 5-HT probably functions to maintain proper neuronal architecture and its withdrawal causes excessive branching. Moreover 5-HT has been demonstrated to be involved in induction of metamorphosis in other invertebrates, including the marine snail *Ilyanassa obsoleta* (Gharbia *et al*., 2009), the Japanese short-neck clam *Ruditapes philippinarum* (Urrutia *et al*., 2004), the Hydrozoan *Phialidium gregarium* (McCauley, 1997) etc. Morphogenetic effects of 5-HT on metamorphosis, such as PCD has been reported in the apical ganglion of the mollusc *I. obsoleta* (Gifondorwa and Leise, 2006). 5-HT also regulates adult

neurogenesis by multiple pathways in a concentration dependent manner in the crustacean brain (Benton *et al*., 2008).

(ii). Role of Neurotransmitters in regulating Insect Metamorphosis

Animals undergoing metamorphosis undergo profound changes in behavior during PED that adapts them to changing needs and conditions at different stages in their life histories. Such behavior plasticity is affected by neuromodulation and changes in neuronal networks as well as CNS architecture, while many others have also reported intrinsic changes in the amounts as well as the major types of neurotransmitters functioning within these networks (Witten and Truman, 1991). The behavioral changes affected include the onset of sexual maturity or reproductive status (Fabre-Nys *et al*., 1997), the development and metamorphosis of insects (Mercer and Hildebrand, 2002) and amphibians (Sillar *et al*., 1998), caste differentiation (Sasaki and Nagao, 2002) and division of labour between workers in social insects (Wagener-Hulme *et al*., 1999) and phase change including gregarization and solitarization in the locusts (Rogers *et al*., 2004).

Biogenic amines such as serotonin, octopamine and dopamine play important roles in the insect CNS as neurotransmitters, neuromodulators and neurohormones, influencing the expression of different sets of behaviors during PED in holometabolous insects (Wagener-Hulme *et al*., 1999).

a. Serotonin Functions

Serotonin affects diverse arthropod behaviors such as aggression in *D. melanogaster* (Johnson *et al*., 2009), *G. bimaculatus* (Stevenson *et al*., 2000); motor pattern generation in *M. sexta* (Claassen and Kammer, 1986); learning in *D. melanogaster* (Sitaraman *et al*., 2008), *A. mellifera* (Menzel *et al*., 1999); and circadian rhythms in *D. melanogaster* (Yuan *et al*., 2005), *G. bimaculatus* (Saifullah and Tomoika, 2002), *P. americana* (Jagota and Habibulla, 1992a), the American lobster, *Homarus americanus* (Wildt *et al*., 2004) etc. Serotonin modulates olfactory sensitivity in a number of insects in the peripheral nervous system in *M. sexta* (Dolzer *et al*., 2001), the cabbage moth *Mamestra brassicae* (Grosmaitre *et al*., 2001) and the central nervous

system in *D. melanogaster* (Dacks *et al*., 2009), *B. mori* (Iwano and Kanzaki, 2005), *M. sexta* (Dacks *et al*., 2008) etc.

b. Morphogenetic Role of Serotonin in Insects

Serotonin appears in the neurons of *Drosophila* (Lundell and Hirsh, 1994) and its presence in outgrowing neurites suggests its involvement in the neuronal circuits as a neurotransmitter as well as in developmental functions. Serotonin may also regulate neural outgrowth during insect development. Evidences supporting the role of serotonin in morphogenesis of insect CNS include development of a *Ddc* mutant *Drosophila* (Budnik *et al*., 1989), in which the branching pattern of peripheral serotonin containing fibers innervating the gut show a 2-fold increase in the extent of branching as compared to the wildtype flies. Growth enhancement of antennal lobe neurons of *M. sexta* under *in vitro* conditions (Mercer *et al*., 1996) also suggests its role as a morphogen (reviewed by Buznikov *et al*., 1999).

Serotonin is known to autoregulate the differentiation of serotonergic neurons in invertebrates (Baker *et al*., 1993) and vertebrates (Whitaker-Azmitia *et al*., 1995). Serotonin regulates major developmental processes such as neurogenesis (Sandeman *et al*., 2009) which is proved to be under circadian control (Goergen *et al*., 2002). Thus serotonin plays an important role in proper development of the nervous system in the animal kingdom.

c. Other neurotransmitters playing important role in Insect Metamorphosis

i. Dopamine Functions

In insects, Dopamine has been shown to affect lifespan and fertility, tanning, locomotion, learning and memory, and sexual behavior (reviewed by Wicker-Thomas and Hamann, 2008). Dopamine significantly affects metamorphosis as it has been shown to regulate metabolism of JH and 20E. Dopamine regulates JH metabolism in *M. sexta* (Granger *et al*., 1996), *D. melanogaster* (Gruntenko *et al*., 2005), the German cockroach *Blattella germina* (Pastor *et al*., 1991), the cricket *Gryllus bimaculatus* (Woodring and Hoffman, 1994) etc. Dopamine also regulated metabolism of 20E in *D. virilis* (Rauschenbach *et al*., 2006).

ii. Octopamine Functions

Octopamine is major insect and invertebrate specific biogenic amine involved in regulation of *Drosophila* reproductive function by controlling the process of ovulation and oviposition (Gruntenko *et al*., 2007). Defects in Octopamine synthesis leads to sterility in *Drosophila* females because of inability in egg laying (Monastirioti *et al*., 1996). Octopamine levels in hemolymph trigger flight or fight responses in *G. bimaculatus* (Adamo *et al*., 1995) and the locust *Schistocerca gregaria* (Rogers *et al*., 2004).

Octopamine was shown to regulate JH release in different ways in different insect species. Octopamine inhibited JH release in *D. punctata* (Thompson *et al*., 1990) and *G. bimaculatus* (Woodring and Hoffman, 1994), whereas it enhanced JH release in *L. migratoria* (Lafon-Cazal and Baehr, 1988) and *A. mellifera* (Kaatz *et al*., 1994). Hirashima *et al*., (1999) pointed out that the octopamine peak on day-2 or day-3 of *B. mori* might cause ecdysteroid release that could be triggering developmental programs that elicits wandering behavior, cessation of feeding and initiation of spinning that occurs in the late 5^{th} instar. Steroid regulation of octopamine expression during metamorphosis was also reported in *M. sexta* (Lehman *et al*., 2000).

Objectives

Scope and Objectives of the present Work:

Based on this literature background the following objectives were planned

1. Neurogenesis studies in the CNS during PED
2. Cell death studies in the CNS during PED
3. Effect of developmental hormones (JH and 20E) on neurogenesis and cell death during PED
4. Role of serotonin in CNS remodeling during PED

We have used *B. mori* as a model insect to study these objectives as it has distinct stages during its development such as egg, larvae, pupa and adult. It is easy to maintain in laboratory conditions with short life-cycle. Present work may prove a step towards understanding the complexities in the development of the nervous system and may prove useful in understanding the neuronal developmental disorders and pest management studies.

Chapter 1

Neurogenesis studies in the CNS during PED

Contents

I. Introduction

The basic architecture of CNS is same in the embryo and nymph of hemimetabolous insects. The CNS of the nymph is considered a replica of that of the adult. In holometabolous insects, the embryonic CNS closely resembles the larval CNS, but there is remote resemblance of the larval CNS to that of the adult. This explains the behavioral differences observed more conspicuously in the juvenile and adult of the holometabolous insects, while it is not so apparent in the hemimetabolous group (Farris and Sinakevitch, 2003). Novel adult behaviors appear to be incorporated in the insect CNS during the dormant and immobile pupal phase (Consoulas *et al*., 2000). During these developmental transformations, there occurs tremendous variability in neuronal population accompanied with developmental changes in the CNS.

(i). Reorganization of the CNS in Holometabolous Insects

Neurometamorphosis in holometabolous insects involves extensive reorganization of the larval CNS to accommodate novel adult behaviors. The multiple segmental ganglia in the larvae undergo fusion during PED to form compound ganglia characteristic of the adult. Ganglionic fusion (GF) in the insect nervous system of endopterygote insects have been described in many old scientific reports as reviewed by Singh and Srivastava, (1973) such as in the sphinx moth *Sphinx ligustri* by Newport in 1834, *P. brassicae* and the small tortoise shell butterfly *Vanessa urticae* by Brandt in 1879, the grain weevil, *Calandra oryzae* by Murray and Tiegs in 1935 etc. Ashhurst and Richards (1964) studied the nature of connective tissue sheath and the fusion of ganglia in the nerve cord of *G. mellonella* and described the formation of thoracicoabdominal ganglion by fusion of the two anterior abdominal ganglia with the meso- (2^{nd} thoracic) and metathoracic (3^{rd} thoracic) ganglia. Similar studies were reported by many authors in the nervous systems of holometabolous insects including the Lepidopteran insects such as, *G. mellonella* (Pipa, 1969), *M. sexta* (Amos *et al*., 1996), *P. brassicae* (Tsujimura, 1983), *A. segetum* (Cantera *et al*., 1995), the castor silk moth *Philosamia ricini* (Singh and Srivastava, 1973) etc; the Dipteran insects such as *D. melanogaster* (Olofsson and Page, 2004); the Hymenopteran insects such as *M. quadrifasciata* (Pinto *et al*., 2003), *P. hebraeus* (Singh, 1974a) etc; the Coleopteran insects such as, *C. nigritus* (Singh, 1974b), *T. molitor*

(Breidbach, 1990), *P. strobi* (Whitney and Godwin 1979), the European chafer *Amphimallon majalis* (Menees, 1961) etc.

Ganglionic fusion results in changes that may facilitate communication between neural networks that generate adult behavior. Individual thoracic ganglia that contain neural circuitry for flight fuse with few anterior abdominal ganglia (Amos and Mesce, 1994) in order to reduce neuronal conduction and integration times, facilitating walking and flight mechanisms in the adult. Ganglionic consolidation ($T_2T_3Ab_1Ab_2$) in the thorax region of *M. sexta*, comprising of the pterothoracic ganglion which is formed by the fusion of thoracic ganglia (2-3) and abdominal ganglia (1-2) has been shown to facilitate flight by permitting the formation of novel synaptic contacts in the central neuropil of the fused ganglion and by reducing interganglionic conduction times (Duch and Pfluger, 1999). Shortening of interganglionic connectives results in extensive looping of axons running in these connectives, and such loops can be observed in the adults and represent the persistent neurons. During ganglionic migration and fusion axons are coiled within shortened interganglionic connectives but are elongated in nerve roots, whereas the clusters of neuronal somata lose their ganglionic sheaths to migrate toward adjacent ganglia (Pipa and Woolever, 1964). These processes culminate in the fusion of adjacent ganglia (Amos and Mesce, 1994). The individual neurons in each ganglion undergo positional rearrangements relative to each other and migrate either anteriorly or posteriorly to fuse with other segmental ganglia (Amos *et al*., 1996).

Role of glial cells in the process of ganglionic fusion has been reported in *G. mellonella* (Tung and Pipa, 1972), *M. sexta* (Cantera *et al*., 1995). During neurometamorphosis, the glial cell population increases in the shortening connectives. The glias wrap around the neurons of the fusing ganglia and "tow" them moving closer to their destined future segmental ganglion (Oland and Tolbert, 2003). With lack of glial-specific markers in insects (Loesel *et al*., 2006) it has been difficult to understand neuron-glial interactions during nerve cord reorganization. However, focus on insect cell adhesion molecules in the context of neuronal migration during insect embryogenesis (Wright and Copenhaver, 2000) and recent work by Himes *et al*., (2008), have led to understanding of the role of hormone-mediated expression of Fasciclin II in GF and migration during the CNS reorganization in *M. sexta*. Apart from glial cells, even

hemocytes have been suggested to play an important role in ganglionic fusion events as they provide phagocytic functions and help in breakdown of neural lamella as reported for *P. ricini* (Singh and Srivastava, 1973) and *G. mellonella* (Ashhurst and Richards, 1964).

During ganglionic fusion, several events coincide (Amos and Mesce, 1994) including the death of some larval neurons (Weeks, 1987), remodeling of persistent neurons (Levine, 1989), differentiation of postembryonically generated neurons (Booker and Truman, 1987; Witten and Truman, 1991) and associated changes in neurotransmitter expression (Tublitz *et al.,* 1991; Witten and Truman, 1991).

(ii). Postembryonic Neurogenesis

Insects possess species or family-specific innate behaviors (Hofmann and Schildberger, 2001) and it is easy to relate a particulate set of neurons to a particular behavior. The embryonic development of CNS is very similar in hemi- and holometabolous insects (Truman, 1996). A set of neuroblasts delaminate from the ectodermal layer during embryogenesis serving as precursor cells for future nervous system (Campos-Ortega, 1993). These neuroblasts form sequential repeated arrays to form segmental ganglia each neuroblast having a fixed position in a repetitive arrangement (Bate, 1976). The neuroblasts divide unequally to produce a smaller ganglionic mother cell which in turn divides equally to give two daughter cells that differentiate into neurons (Campos-Ortega, 1993).

In hemimetabolous insects such as grasshoppers and locusts the adult CNS is already established by the end of embryogenesis (Doe and Goodman, 1985; Shepherd and Bate, 1990). The pattern of neuronal branching in the CNS is very similar in the nymphs and adult (Raper *et al*., 1983). The newly hatched insect has all the neurons that it needs for life and neuronal numbers remain fairly stable as observed in *A. domesticus* (Gymer and Edwards, 1967) and *S. gregaria* (Sbrenna, 1971). The larval CNS show basic morphology of neurons characteristic of that of adult. The neural circuits for adult specific behaviors such as flight (Stevenson and Kutsch, 1988) or oviposition (Thompson, 1993) are readily recognizable in nymphs at the time of hatching. However, the CNS of hemimetabolous insects is far from being static, and new neurons are always

being added during larval life to accommodate growth and addition of new sensory elements (Murphey and Chiba, 1990) which show quantitative as well as qualitative growth as in case of receptive structure such as antennae, compound eye, cerci etc. During the final molt to the adult, receptors are added to the CNS for novel adult behavior; for example, the sex-pheromone detecting-antennal sensilla in cockroaches which differentiate only during the adult molt (Schafer and Sanchez, 1973; for review: Truman, 1996). Visual interneurons are continually added to the optic lobes during development of compound eyes for accommodation of new ommatidia during each molt (Stark and Mote, 1981). Since the behavior is similar in immature nymphs and adults in the hemimetabolous insects, the MBs in the newly hatched nymph are replica of the adult MB, but continuous neurogenesis occurs in the MBs throughout the larval and the adult life as reported in *P. americana* (Farris and Strausfeld, 2001) and *A. domesticus* (Malaterre *et al*., 2002).

The embryos of holometabolous insects produce only a fraction of the neurons that make up the adult nervous system (Shepherd, 1994) and neurogenesis is extended into postembryonic period in both the ventral nerve cord (Taylor and Truman, 1992) and brain (Fahrbach, 2006), which explains the different complement of neurons sufficient for a particular set of behaviors during the larval and adult period. The brain of holometabolous insects is larger in the adult as compared with that of the larvae and this increase in size reflects postembryonic neurogenesis in the mushroom bodies (areas of learning), the antennal and the optic lobes e.g., *D. melanogaster* (Ito and Hotta, 1992), *A. mellifera* (Vitt and Hartfelder, 1998), *M. sexta* (Booker *et al*., 1996), the monarch butterfly, *Danaus plexippus* (Nordlander and Edwards, 1970), the carpenter ant *Camponotus japonicus* (Ishii *et al*., 2005) etc.

In the holometabolous insects such as flies and moths, there is a small difference in the number of neurons found in thoracic and abdominal ganglion by the end of embryogenesis. Postembryonic neurogenesis plays a major role in generating regional differences observed in segmental ganglia of adult as reported in *M. sexta* (Booker and Truman, 1987) and *D. melanogaster* (Truman and Bate, 1988). The neurons that make up the adult moth CNS are derived from 2 sources: larval neurons, some of which are remodeled to serve new functions in the adult (Levine and Truman, 1985) and adult

specific neurons which are added to the CNS during larval stages (Truman and Bate, 1988). Soon after their birth, the adult specific neurons extend a small axon-like projection into the neuropil, and then arrest their development. Their development is resumed at the onset of metamorphosis (Truman and Bate, 1988).

Studies involving developmental changes in neuronal population have been seldom carried out as the process is tedious and time consuming. In the present study, using the holometabolous Lepidopteran insect *B. mori*, gross regional changes in neuronal population in the entire CNS were studied in different stages during postembryonic development using wholemount toluidine blue staining (Altman and Bell, 1973). This staining method has several advantages in wholemount applications, in insect CNS especially because of its small size, over methods involving sectioning of more complex biological material where serial reconstruction is extremely difficult. Thus the use of the wholemount method using toluidine blue is a prerequisite for understanding the changes in gross neuronal numbers and morphology before an attempt towards underpinning the more complex changes in the nervous system and behavior.

Stage- and region-specific changes in new-born neurons were studied by *in vitro* bromodeoxyuridine (BrdU) labeling, which is suitable marker for proliferating cells. Cells synthesize DNA (i.e., undergo DNA replication) before cell division. BrdU which is a thymidine analogue gets incorporated into the DNA of dividing cells during DNA synthesis and can be later detected with specific anti-BrdU antibodies (Gratzner, 1982).

BrdU is not harmful to cell differentiation. There is no difference in distribution of neurons labeled *in vitro* with either tritiated Thymidine and BrdU in rat, mouse (Miller and Nowakowski, 1988) and chick (Bannigan, 1987). Differentiation of cells upon BrdU treatment occurs normally in Hydra (Plickert and Kroiher, 1988), *Drosophila* peripheral neurons (Bodmer *et al*., 1989) and locust CNS (Shepherd and Bate, 1990). There are many reports which use BrdU as the marker for neurogenesis in insects in both *in vitro* as well as *in vivo* conditions (e.g., *D. melanogaster* (Truman and Bate, 1988), *M. sexta* (Booker *et al*., 1996), *A. domesticus* (Cayre *et al*., 2005), *A. mellifera* (Fahrbach *et al*., 1995; Ganeshina *et al*., 2000). However, toxic effects of BrdU have been reported in some cases such as: it affects gene expression (Masterson and O'Dea, 2007), PCD of the limb buds in mice (Nakamura *et al*., 2000) and chick (Toné *et al*., 1988), differentiation

of cancer cell lines (Sugimoto *et al*., 1988) and frequency of recombination during sister chromatid exchange in cultured mammalian cells (Rodríguez-Reyes and Morales-Ramírez, 2003).

With this literature background, neurogenesis in the CNS of *B. mori* during PED was studied as following:

1. Gross morphological changes were studied by neuronal counts during PED
2. Birth-dating of neurons during different developmental stages during PED

II. Materials and Methods

(i). General Methodologies for Maintaining B. mori

Different larval stages were obtained from local sericulture board and housed in a facility maintained at LD 12:12 photoperiod with temperature of 26 ± 1 °C and relative humidity 70 ± 5 %. Various developmental stages studied include 4^{th} instar (4I), 5^{th} instar (day 1-3 early (5EI), day 4-6 mid (5MI), day 7-9 late (5LI)), pupa (day 1-3 early (EP), day 4-6 mid (MP), day 7-9 late (LP)) and 1 day old adults (A). The larvae were fed with fresh mulberry leaves provided *ad libitum*.

(ii). Tissue Preparation

The insects were narcotized on ice for 15 minutes and the CNS was dissected out in insect Ringer's solution (215 mM NaCl, 5 mM KCl, 1 mM $CaCl_2.6H_2O$, 2.7 mM $NaHCO_3$, 0.1 mM NaH_2PO_4) (Miller, 1979).

(iii). Wholemount Toluidine Blue Staining

The method of toluidine blue wholemount CNS staining was adapted from that of Altman and Bell (1973). Freshly dissected CNS (consisting of supraesophageal ganglion (Brain), subesophageal ganglion (SEG), three thoracic ganglia (T_1 to T_3) and seven abdominal ganglia (Ab_1 to Ab_7)) were immersed in 1% Toluidine blue (Sigma) in Borax solution (6 g borax ($Na_2B_4O_7$), 1 g boric acid (H_3BO_3) in 100 ml distilled H_2O, pH 7.6) for 15-20 min at room temperature. The CNS was then fixed with Bodian's No. 2 fixative (5 ml formalin-5 ml glacial acetic acid-90 ml 80% ethanol) which acts as a differentiator as well as a fixative. The CNS was dehydrated in absolute alcohol for 1-2 min, cleared

with methyl benzoate and mounted on clean, grease-free microscopic glass slide with dibutyl phthalate in xylene (DPX) mountant.

(iv). DNA Isolation

DNA was isolated from CNS tissues pooled to 10 mg weight in the respective stages (from 20 insects in 4I, 10 in 5EI, 5 each in 5MI, 5LI, EP, MP, LP and A) using Isopropanol method (Laird *et al*., 1991) which does not involved phenol-chloroform extraction procedures. The tissue was homogenized in SNET buffer (containing 1% sodium dodecyl sulfate (SDS), 200 mM NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 50 mM trisaminomethane hydrochloride (Tris-HCl) pH 9.0, 0.5 mg/ml Proteinase K, and 100 μg/ml RNase A) and incubated at 55 °C overnight. The clear lysate was centrifuged at 13,400 revolutions per minute (RPM) for 10 min to remove undigested tissue debris and the supernatant was transferred to a fresh microfuge tube. Supernatant was mixed with equal volume of isopropanol and gently invert till DNA precipitates. Isopropanol was allowed to evaporate and DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA yield was assessed by measuring absorbance at 260 nm using the NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific). (Hansen *et al*., 2007). The ratio of the absorbance at 260 and 280nm ($A_{260/280}$) was around 1.8 indicating that the DNA isolated was of high purity.

(v). Wholemount BrdU labeling

Freshly dissected CNS were labeled *in vitro* in 5mg/ml BrdU Ringer's solution for 5 h at room temperature (Vitt and Hartfelder, 1998), washed in Ringer's three time, 10 min each, and then fixed in 10% formalin in phosphate buffered saline (PBS) (136 mM NaCl, 2.7 mM KCl, 0.5 mM $CaCl_2.6H_2O$, 0.5 mM $MgCl_2.6H_2O$, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) (Ganeshina *et al*., 2000). The BrdU immunohistochemistry for labeling new born neurons in the CNS was adapted from Gu *et al.,* (1999). The fixed labeled tissues were washed in PBS three times, 10 min each and permeabilized for 1 h at room temperature in PBS containing 0.5% Triton-X100 (PBS-Tx). The DNA was denatured by incubating the tissues in 2N HCl in PBS-Tx for 30 min at room temperature. This was followed by three washes, 10 min each in PBS-Tx to neutralize the acid. The tissues were incubated for 5 h at room temperature with 1:20 anti-BrdU

antibody (Boehringer Mannheim) coupled with alkaline phosphatase (ALP) in PBS-Tx containing 1% BSA as a blocking agent to prevent non-specific binding. The enzyme tagged antibody was detected by incubating the tissue in nitro blue tetrazolium chloride / 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) ready to use substrate solution (Bangalore Genei) while the color development was monitored in a dissection microscope. BCIP is hydrolyzed by alkaline phosphatase to form an intermediate that undergoes dimerization to produce an indigo dye. The NBT is reduced to the NBT-formazan by the two reducing equivalents generated by the dimerization. Together they yield an intense, insoluble black-purple precipitate when reacted with alkaline phosphatase. The tissue was later dehydrated in absolute alcohol for 1-2 min, followed by clearing in methyl benzoate for 1-2 min, and finally mounted on a glass slide with DPX mountant.

(vi). Method of Neuronal Counting

Following preparation of toluidine blue stained wholemount slides, or BrdU immunodetection, images were captured from whole mount preparations using Olympus BX-41 microscope fitted with DP-12 camera. For each ganglion images were captured at various optical levels, neuronal maps of labeled cells at each level were prepared and then these were overlapped to prepare the neuronal maps of the ganglion (Jagota and Habibulla, 1992b). Cell counting was also verified using Image Pro-Plus software (Media Cybernetics) (DeCoster, 2007).

(vii). Statistical Analysis

Data were analyzed by one way ANOVA followed by posthoc Duncan's test and by Student's t-test using *Sigma Stat*™ (Jandel Scientific). Data were expressed as mean ± SE (n = 4-6) ($p \leq 0.05$).

III. Results

(i). CNS reorganization during PED

B. mori CNS is composed of Brain, SEG, three thoracic (T_1 to T_3) and seven abdominal ganglia (Ab_1 to Ab_7), where the last abdominal ganglion being a paired

ganglion. During development in the larval stages, the length of the CNS increases due to increase in the length of the interganglionic connectives (IGCs). However, during the larval-pupal transformation, there is a significant reduction in the length of all the IGCs and hence a decrease in the length of the CNS. These further decreases during developmental progression from EP to MP as a result of ganglion fusion of T_3, Ab_1 and Ab_2 (GF_1:T_3-Ab_1; GF_2: Ab_1-Ab_2). The last abdominal ganglion Ab_7 fuses with the Ab_6 to form the compound ganglion 2 (GF_3) during development from EP to MP. Later T_2 also fuses with T_3-Ab_1-Ab_2 during development from MP to LP to form the pterothoracic ganglion (compound ganglion 1) (GF_4: T_2-$T_3Ab_1Ab_2$). Thus, the adult CNS consists of Brain, SEG, T_1, $T_2T_3Ab_1Ab_2$ (GF_4), Ab_3, Ab_4, Ab_5 and $Ab_{6\text{-}7}$ (GF_3) (Fig. 13).

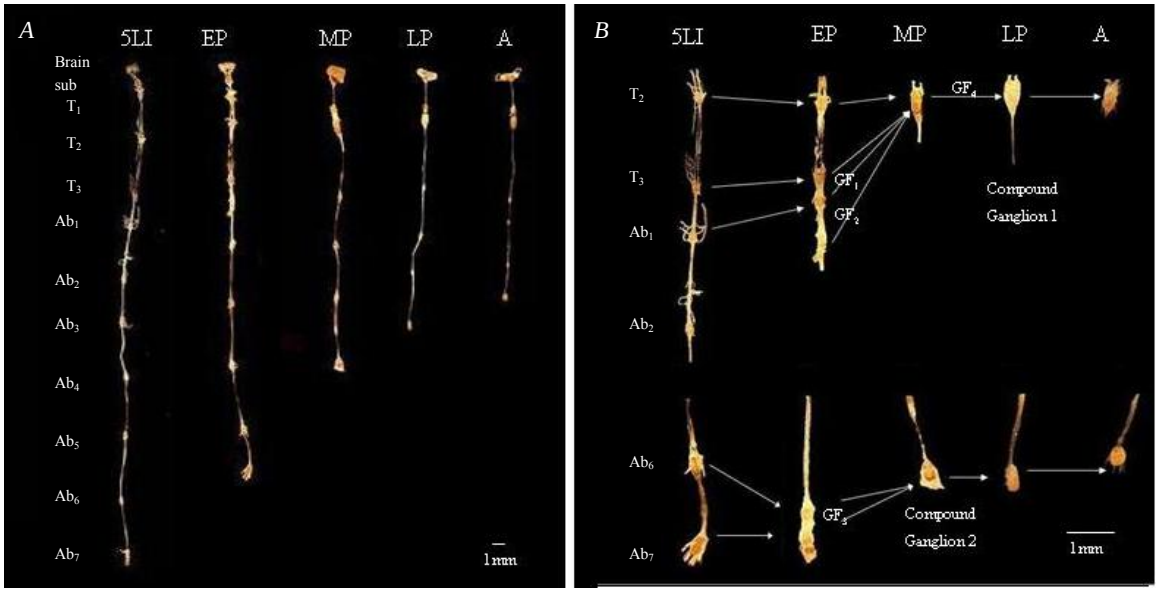


Figure 13: CNS reorganization during Postembryonic Development. A. Dorsal view of CNS (with Brain and chain of segmental ganglia [Subesophageal (SEG), 3 thoracic (T_1, T_2 and T_3) and 7 abdominal (Ab_1, Ab_2, Ab_3, Ab_4, Ab_5, Ab_6 and Ab_7)] of *B. mori* in various stages [5th late instar (5LI), early (EP), mid (MP) and late (LP) pupa and adult (A)]. B. Ganglionic Fusion - GF1, GF2, GF3, and GF4 and formation of compound ganglia 1 and 2.

(ii). Gross Neuronal counting in the CNS during PED

The neuronal profiles of the entire CNS (including brain, three thoracic and eight abdominal ganglia) were studied. Neuronal maps were prepared by taking images at multiple optical images of the wholemount toluidine blue stained ganglia (Fig. 14). There is a considerable increase in neuronal numbers in the entire CNS in different stages during PED including 4I, 5EI, 5MI, 5LI, EP, MP, LP and A (Table 1; Fig. 15).

With the present study we can report the change in the neuronal numbers in each ganglion in the CNS of *B. mori* during various developmental stages. We report here that the changes in the brain neuroanatomy were robust. The neuronal counts in brain increase significantly during development from 4I (400 ± 6) to 5EI (586 ± 10) ($p_a \leq 0.05$) (where a refers to comparison with 4I). However, there was no significant change in neuronal counts in postembryonic developmental stage 5th instar larva (5EI, 5MI (692 ± 15) and 5LI (721 ± 11)). However there is consistent increase in neuronal numbers during various pupal stages (EP (1132 ± 40) ($p_d \leq 0.05$), MP (1428 ± 28) ($p_e \leq 0.05$) and LP (2170 ± 71) ($p_f \leq 0.05$) and adult (2775 ± 94) ($p_g \leq 0.05$) (Fig. 15) (where d, e, f and g refer to comparisons with 5LI, EP, MP and LP respectively).

In the SEG, the neuronal numbers do not vary between 4I (190 ± 6) and 5EI (201 ± 8), but increase significantly in 5MI (259 ± 6) ($p_b \leq 0.05$) and 5LI (337 ± 8) ($p_c \leq 0.05$) (where b and c refer to comparison with 5EI and 5MI respectively). However, there is no significant increase in neuronal counts during larval-pupal transformation from 5LI to EP (350 ± 15). The neuronal numbers increase continuously in MP (450 ± 13) ($p_e \leq 0.05$) and LP (652 ± 25) ($p_f \leq 0.05$), while it remains same in the adult (670 ± 24) as in LP (Fig. 15).

Neuronal numbers in T_1 increase from 4I (264 ± 4) to 5EI (295 ± 6) ($p_a \leq 0.05$), and decreases in 5MI (250 ± 5) ($p_b \leq 0.05$). It doesn't vary significantly during development to 5LI (275 ± 3) and EP (290 ± 13), but a robust increase is observed in the MP (450 ± 11) ($p_e \leq 0.05$). There is no significance difference in the neuronal counts between MP and LP (463 ± 14), but the numbers increase significantly in adult (613 ± 10) ($p_g \leq 0.05$) (Fig. 15).

Since T_2, T_3, Ab_1 and Ab_2 ganglia fuse during the pupal development to form a compound ganglion $T_2T_3Ab_1Ab_2$ we have compared the neuronal numbers in this region by adding up the number of neurons counted for each of unfused ganglia during the larval period. The numbers increase robustly from 4I (509 ± 16) to 5EI (700 ± 15) ($p_a \leq 0.05$), but do not vary significantly throughout the 5th instar. The numbers increase significantly from 5LI (801 ± 14) to EP (948 ± 34) ($p_d \leq 0.05$), but they are not significantly different throughout the pupal development. The neuronal counts then increase significantly during development from LP (947 ± 32) to A (1121 ± 17) ($p_g \leq 0.05$) (Fig. 15).

Neuronal numbers in Ab_3 do not vary significantly between 4I (114 ± 4) to 5EI (119 ± 3), but a robust increase is observed in 5MI (160 ± 2) ($p_b \leq 0.05$). There is no significant difference in the neuronal numbers between 5MI and 5LI (169 ± 3), but a robust and a continuous increase is observed in EP (260 ± 17) ($p_d \leq 0.05$) and MP (413 ± 16) ($p_e \leq 0.05$).

While there is no significance difference in neuronal numbers between MP and LP (450 ± 17), a significant reduction is observed in the adult (300 ± 9) ($p_g \leq 0.05$) (Fig. 15).

In Ab_4, the neuronal numbers increase significantly from 4I (98 ± 4) to 5EI (135 ± 5) ($p_a \leq 0.05$). There is no significant change in neuronal numbers between 5EI and 5MI (144 ± 4), but a continuous increase is observed during development to 5LI (187 ± 4) ($p_c \leq 0.05$), EP (252 ± 8) ($p_d \leq 0.05$), MP (343 ± 9) ($p_e \leq 0.05$) and LP (475 ± 13) ($p_f \leq 0.05$), while a decrease is observed in A (400 ± 11) as compared to LP ($p_g \leq 0.05$) (Fig. 15).

In the Ab_5, there is a significant increase in neuronal numbers between 4I (101 ± 4) and 5EI (137 ± 1) ($p_a \leq 0.05$), but the numbers do not vary significantly throughout the 5th instar larvae. A continuous increase in the neuronal numbers is observed during development from 5LI (152 ± 3) to EP (236 ± 13) ($p_d \leq 0.05$) and MP (337 ± 13) ($p_e \leq 0.05$), which reduces in LP (307 ± 13) ($p_f \leq 0.05$) and then increases in A (363 ± 12) ($p_g \leq 0.05$) (Fig. 15).

During development from EP to MP, Ab_6 fuses with Ab_7 to form the terminal abdominal ganglion. Hence in order to compare the neuronal counts we added the number of neurons counted for individual unfused Ab_6 and Ab_7 during the larval development. The neuronal numbers increase continuously during development from 4I (361 ± 9), 5EI (472 ± 17) ($p_a \leq 0.05$), 5MI (511 ± 9) ($p_b \leq 0.05$), 5LI (572 ± 11) ($p_c \leq 0.05$) and EP (866 ± 14) ($p_d \leq 0.05$), but decrease in the MP (758 ± 6) ($p_e \leq 0.05$) followed by an increase in the LP (930 ± 7) ($p_f \leq 0.05$) and then again decrease from LP to A (875 ± 7) ($p_g \leq 0.05$) (Fig. 15).

The total neuronal population in the entire CNS increases significantly from 2047 ± 39 neurons in the 4I to 2617 ± 25 neurons in 5EI ($p_a \leq 0.05$). There is no significant increase in neuronal numbers observed during development from 5EI to 5MI (2915 ± 74), and 5MI to 5LI (3219 ± 43). However, a continuous increase is observed in subsequent development from 5LI to EP (4277 ± 101) ($p_d \leq 0.05$), MP (5139 ± 243) ($p_e \leq 0.05$), LP (6396 ± 112) ($p_f \leq 0.05$) and A (7108 ± 320) ($p_g \leq 0.05$) (Fig. 15).

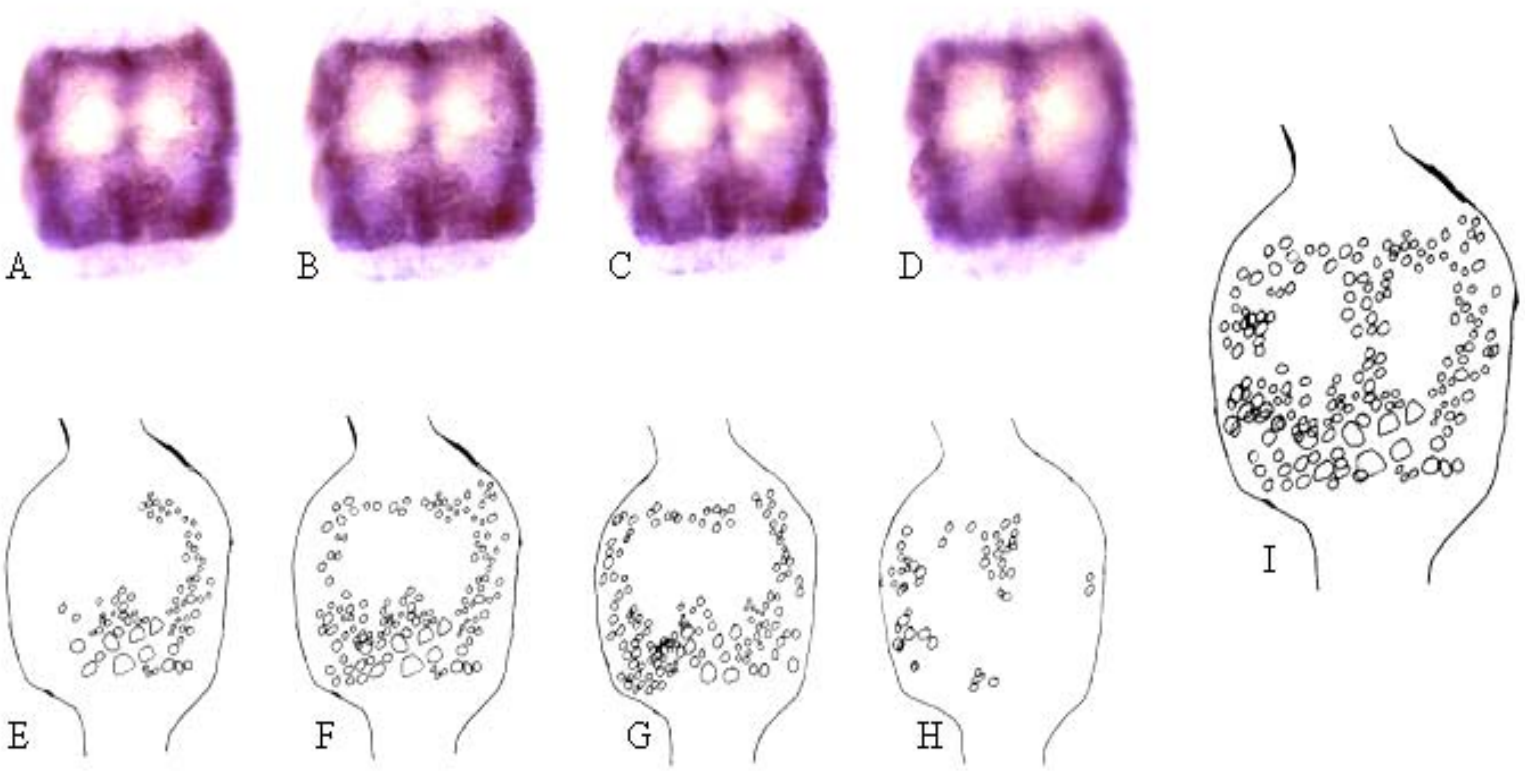


Figure 14: Representative toluidine blue stained wholemount ganglia. Neuronal counts done from the neuronal maps prepared by taking pictures of wholemount toluidine blue ganglia at multiple optical levels. A, B, C and D: Photographs taken at 100X magnification using Olympus BX-41 with DP12 camera. E, F, G and H: neuronal maps traced from the respective optical image and overlapped to give a final neuronal map (I) from which counting was done. Neuronal counts were later verified using Image Pro Plus software (Media Cybernetics).

Table 1: Developmental changes in neuronal numbers in the CNS of *B. mori*

Ganglion	4I	5EI	5MI	5LI	EP	MP	LP	A
Brain	$400 \pm 6^{b\text{-}h}$	$568 \pm 10^{a,d\text{-}h}$	$692 \pm 15^{a,e\text{-}h}$	$720 \pm 12^{a,b,e\text{-}h}$	$1132 \pm 40^{a\text{-}d,f\text{-}h}$	$1428 \pm 28^{a\text{-}e,g,h}$	$2170 \pm 71^{a\text{-}f,h}$	$2775 \pm 94^{a\text{-}g}$
SEG	$190 \pm 6^{c\text{-}h}$	$201 \pm 8^{c\text{-}h}$	$259 \pm 6^{a,b,d\text{-}h}$	$337 \pm 8^{a\text{-}c,f\text{-}h}$	$350 \pm 15^{a\text{-}c,f\text{-}h}$	$450 \pm 13^{a\text{-}e,g,h}$	$652 \pm 25^{a\text{-}f}$	$670 \pm 24^{a\text{-}f}$
T_1	$264 \pm 4^{b,f\text{-}h}$	$295 \pm 6^{a,c,f\text{-}h}$	$250 \pm 5^{b,e,f\text{-}h}$	$275 \pm 3^{f\text{-}h}$	$290 \pm 13^{c,f\text{-}h}$	$450 \pm 11^{a\text{-}e,h}$	$463 \pm 14^{a\text{-}e,h}$	$613\pm 10^{a\text{-}g}$
T_2	$149 \pm 6^{c\text{-}f}$	$175 \pm 2^{c\text{-}f}$	$228 \pm 6^{a,e\text{-}f}$	$229 \pm 4^{a,b,e}$	$331 \pm 15^{a\text{-}d}$	$320 \pm 15^{a\text{-}d}$	$947 \pm 32^{a\text{-}d,h}$ GF1	1121 ± 17
T_3	$133 \pm 3^{b\text{-}e}$	198 ± 3^{a}	187 ± 6^{a}	210 ± 4^{a}	199 ± 6^{a}	677 ± 18 GF1		
Ab_1	$110 \pm 5^{b\text{-}e}$	171 ± 6^{a}	166 ± 3^{a}	180 ± 3^{a}	169 ± 8^{a}			
Ab_2	$118 \pm 3^{b\text{-}e}$	$155 \pm 3^{a,d,e}$	$167 \pm 2^{a,e}$	$180 \pm 2^{a,b,e}$	$248 \pm 9^{a\text{-}d}$			
Ab_3	$115 \pm 4^{c\text{-}h}$	$120 \pm 3^{c\text{-}h}$	$160 \pm 2^{a,b,e\text{-}h}$	$169 \pm 3^{a,b,e\text{-}h}$	$260 \pm 17^{a\text{-}d,f\text{-}h}$	$413 \pm 16^{a\text{-}e,g,h}$	$450 \pm 17^{a\text{-}f,h}$	$301\pm 9^{a\text{-}e,g}$
Ab_4	$98 \pm 4^{b\text{-}h}$	$135 \pm 5^{a,d\text{-}h}$	$144 \pm 4^{a,d\text{-}h}$	$187 \pm 4^{a\text{-}c,e\text{-}h}$	$252 \pm 8^{a\text{-}d,f\text{-}h}$	$343 \pm 9^{a\text{-}e,g,h}$	$476 \pm 13^{a\text{-}f,h}$	$400 \pm 11^{a\text{-}g}$
Ab_5	$100 \pm 4^{b\text{-}h}$	$137 \pm 1^{a,e\text{-}h}$	$151 \pm 3^{a,e\text{-}h}$	$162 \pm 3^{a,e\text{-}h}$	$237 \pm 13^{a\text{-}d,f\text{-}h}$	$337 \pm 13^{a\text{-}e,g}$	$308 \pm 13^{a\text{-}f,g}$	$363\pm 12^{a\text{-}e,g}$
Ab_6	$110 \pm 5^{b\text{-}e}$	$144 \pm 2^{a,c\text{-}e}$	$164 \pm 4^{a,b,d,e}$	$197 \pm 5^{a\text{-}c,e}$	$344 \pm 8^{a\text{-}d}$	758 ± 15 GF2	930 ± 17	875 ± 18
Ab_7	$251 \pm 5^{b\text{-}e}$	$328 \pm 15^{a,d,e}$	$346 \pm 5^{a,d,e}$	$375 \pm 8^{a\text{-}c,e}$	$522 \pm 7^{a\text{-}d}$			
Total CNS	$2047 \pm 39^{b\text{-}h}$	$2617 \pm 25^{a,d\text{-}h}$	$2915 \pm 74^{a,e\text{-}h}$	$3219 \pm 43^{a,b,e\text{-}h}$	$4277 \pm 101^{a\text{-}d,f\text{-}h}$	$5139 \pm 243^{a\text{-}e,g,h}$	$6396 \pm 112^{a\text{-}f,h}$	$7108 \pm 320^{a\text{-}g}$

The neuronal number increases in the entire CNS continuously from 4I to A. There is a 3.5 fold increase in total neuronal population in A as compared to 4I. Each value is expressed as Mean ± SE (n = 6). $p_a \leq 0.05$, $p_b \leq 0.05$, $p_c \leq 0.05$, $p_d \leq 0.05$, $p_e \leq 0.05$, $p_f \leq 0.05$, $p_g \leq 0.05$ and $p_h \leq 0.05$, where a, b, c, d, e, f, g and h are comparisons with 4I, 5EI, 5MI, 5LI, EP, MP, LP and A respectively.

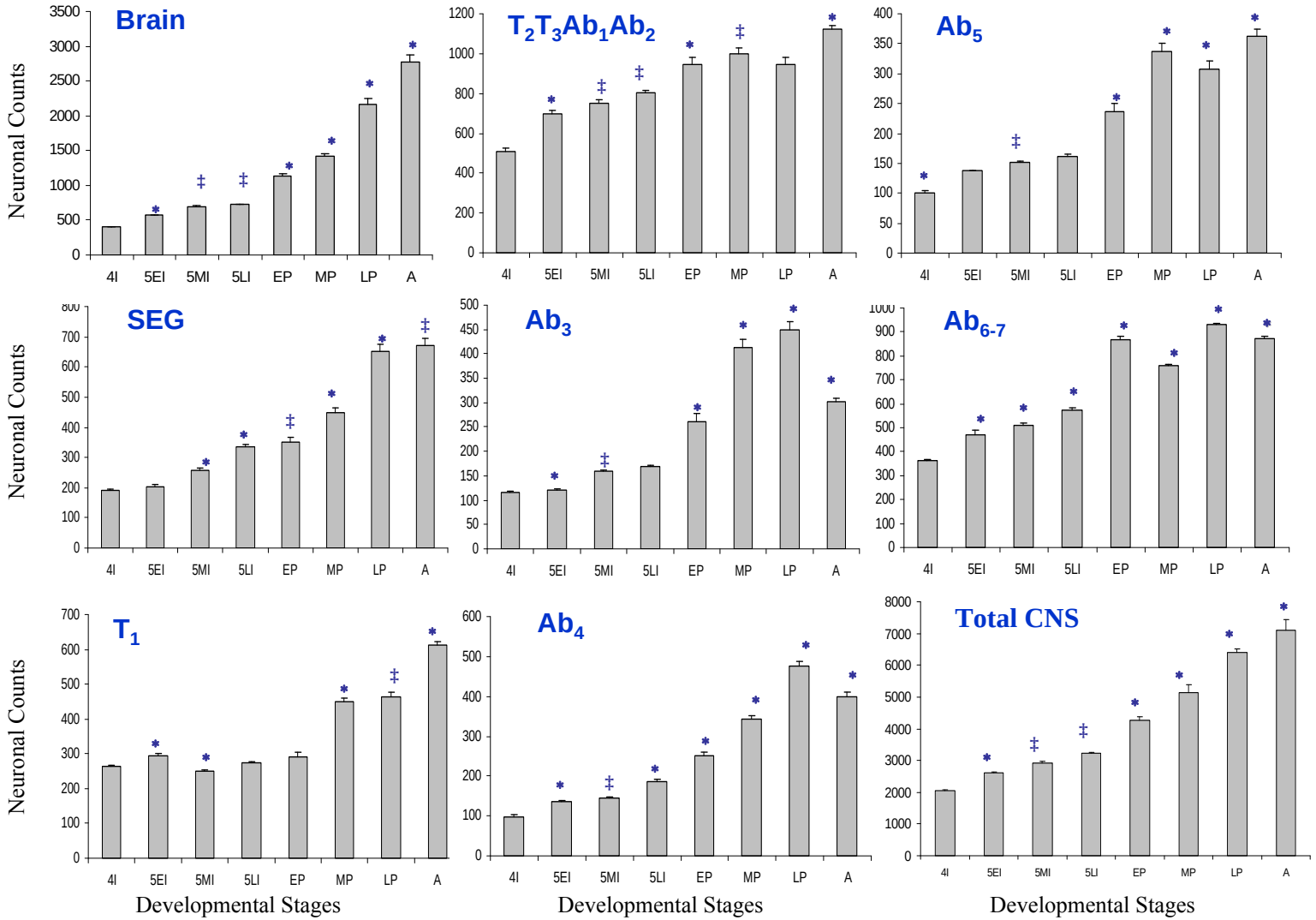


Figure 15: Stage-specific and region-specific changes in neuronal population in the CNS of *B. mori* during PED. Brain showed a maximum 7 fold increase in neuronal population during development from 4I to A. Other ganglia showed only a 2-4 fold increase. Neuronal numbers increased continuously until A in almost all the ganglia except in Ab_3 , Ab_4 and Ab_{6-7} where a decrease was observed during development from LP to A. Total neuronal population showed a 3.5 fold increase in A as compared to 4I. Each value is Mean ± SE (n = 6). * is significant difference with previous stage. ‡ is significant difference with last but one stage ($p \leq 0.05$).

(iii). Changes in DNA levels during PED

DNA levels did not vary significantly between 4I (0.9 ± 0.04), 5EI (1.12 ± 0.15), 5MI (1.1 ± 0.06) and 5LI (0.71 ± 0.07) µg/CNS. However after larval-pupal transformation more than 2 fold increase in DNA content was observed during development from 5LI (0.71 ± 0.07) to EP (1.96 ± 0.23) µg/CNS. DNA levels did not increase significantly between EP (1.96 ± 0.23) to MP (2.2 ± 0.15) µg/CNS, but a significant increase was observed between MP (2.2 ± 0.15) to LP (2.97 ± 0.21) and LP to A (3.63 ± 0.13) µg/CNS. The adult CNS had 3.5 fold higher DNA content as compared to that of the 4I larvae (Table 2; Fig. 16).

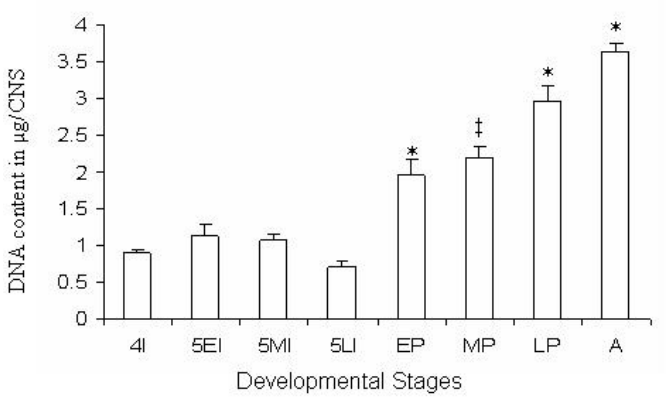


Figure 16: Changes in DNA content in the CNS of *B. mori* during PED. * indicates significant difference with previous stage. ‡ is significant difference with last but one stage ($p \leq 0.05$).

Table 2: Postembryonic developmental changes in DNA content in the CNS

Developmental stages	DNA content µg/CNS
4I	$0.9 \pm 0.04^{\text{e-h}}$
5EI	$1.12 \pm 0.15^{\text{e-h}}$
5MI	$1.08 \pm 0.06^{\text{e- h}}$
5LI	$0.71 \pm 0.07^{\text{e-h}}$
EP	$1.96 \pm 0.23^{\text{a-d, g, h}}$
MP	$2.2 \pm 0.15^{\text{a-d, g, h}}$
LP	$2.97 \pm 0.21^{\text{a-f, h}}$
A	$3.63 \pm 0.13^{\text{a-g}}$

Each value is Mean ± SE, (n = 6). $p_a \leq 0.05$, $p_b \leq 0.05$, $p_c \leq 0.05$, $p_d \leq 0.05$, $p_e \leq 0.05$, $p_f \leq 0.05$, $p_g \leq 0.05$ and $p_h \leq 0.05$, where a, b, c, d, e, f, g and h are the same as in Table 1.

(iv). Neurogenesis in the CNS during PED

A continuous increase in neuronal counts was observed with toluidine blue staining in all the ganglia of the CNS during PED. In order to confirm neurogenesis, we studied stage-specific and region-specific changes in new-born neurons by *in vitro* BrdU labeling of CNS of *B. mori* (Fig. 17) during different developmental stages including 4I, 5EI, 5MI, 5LI, EP, MP, LP and A (Table 3; Fig. 18). Except in the adult CNS neurogenesis was observed in all other stages.

Neurogenesis in brain increased significantly during development from 83 ± 8 in 4I to 171 ± 9 BrdU positive cells in 5EI ($p_a \leq 0.05$). However, the increase in the number of BrdU positive cells between 5EI to 5MI (218 ± 21) were not significant, as also between 5MI to 5LI (270 ± 8). BrdU cell counts increased robustly from 5LI to EP (656 ± 45) ($p_d \leq 0.05$) followed by a robust decrease in the MP (272 ± 22) ($p_e \leq 0.05$), and it increased significantly in the LP (352 ± 13) ($p_f \leq 0.05$), though the increase was not that robust (Fig. 18).

In the SEG, neurogenesis decreased from 4I (44 ± 1) to 5EI (23 ± 2) neurons ($p_a \leq 0.05$), followed by a significant increase in 5MI (58 ± 4) ($p_b \leq 0.05$) and then decreased in 5LI (28 ± 1) neurons ($p_c \leq 0.05$). Neurogenesis increased greatly in EP (164 ± 7) neurons ($p_d \leq 0.05$) as compared to 5LI. A continuous decrease in neurogenesis was observed during development from EP to MP (116 ± 2) ($p_e \leq 0.05$) and LP (92 ± 3) neurons ($p_f \leq 0.05$) (Fig. 18).

Neurogenesis levels in T_1 did not vary significantly in 4I (46 ± 4), 5EI (33 ± 2) and 5MI (48 ± 2) neurons, while it was not at all detected in 5LI. Robust neurogenesis occurred in T_1 in EP (248 ± 25) ($p_d \leq 0.05$), and the levels were similar in MP (221 ± 8) neurons. However, in comparison with MP, neurogenesis decreased significantly in LP (134 ± 9) neurons ($p_f \leq 0.05$) (Fig. 18).

In $T_2T_3Ab_1Ab_2$ region, a robust decrease in the number of BrdU positive cells was observed during development from 4I (285 ± 16) to 5EI (131 ± 10) neurons ($p_a \leq 0.05$). Neurogenesis levels did not differ significantly between 5EI and 5MI (191 ± 14), but increased significantly in 5LI (288 ± 30) ($p_c \leq 0.05$) and EP (863 ± 42) neurons ($p_d \leq 0.05$), followed by a continuous decrease in MP (562 ± 42) ($p_e \leq 0.05$) and LP (277 ± 10) neurons ($p_f \leq 0.05$) (Fig. 18).

In Ab_3 neurogenesis decreased to undetectable levels during development from 4I (77 ± 3) neurons to 5EI ($p_a \leq 0.05$), and then increased in 5MI with (37 ± 2) neurons ($p_b \leq 0.05$). Neurogenesis in Ab_3 does not vary significantly between 5MI and 5LI (43 ± 2) neurons, but increases significantly in EP (226 ± 18). A significant decrease in neurogenesis during pupal progression from EP to MP (168 ± 14) and LP (60 ± 2) cells is observed in Ab_3 ($p_e \leq 0.05$) (Fig. 18).

In Ab_4, neurogenesis decreased to undetectable levels during development from 4I (77 ± 5) neurons to 5EI ($p_a \leq 0.05$), and then increased in 5MI (65 ± 4) neurons ($p_b \leq 0.05$). Neurogenesis in Ab_4 doesn't vary significantly between 5MI and 5LI (69 ± 4), but increases robustly during 5LI to EP (179 ± 3) neurons ($p_d \leq 0.05$). Neurogenesis doesn't vary significantly between EP to MP in Ab_4 (157 ± 24) neurons but decreases significantly in LP (65 ± 1) neurons ($p_f \leq 0.05$) (Fig. 18).

In Ab_5, neurogenesis decreases from 4I (89 ± 1) to 5EI (53 ± 3) neurons ($p_a \leq 0.05$), but doesn't vary significantly throughout the 5^{th} instar (5MI 53 ± 4 and 5LI 50 ± 2) neurons. Neurogenesis increases robustly in EP (137 ± 6) neurons ($p_d \leq 0.05$) and the levels remain high in MP (146 ± 17), thereafter decrease significantly in LP (64 ± 9) neurons ($p_f \leq 0.05$) (Fig. 18).

In $Ab_{6\text{-}7}$ region, neurogenesis reduces to undetectable levels during development from 4I (238 ± 16) neurons to 5EI ($p_a \leq 0.05$), and then increases in 5MI (131 ± 13) ($p_b \leq 0.05$). There is no significant difference in the neurogenesis in 5MI and 5LI (116 ± 7), but a robust increase is observed in EP (516 ± 7) neurons ($p_d \leq 0.05$), followed by a continuous decrease in MP (267 ± 16) ($p_e \leq 0.05$) and LP (164 ± 15) neurons ($p_f \leq 0.05$) (Fig. 18).

Neurogenesis in the entire CNS decreases from 4I (941 ± 24) to 5EI (414 ± 22) neurons ($p_a \leq 0.05$), increases in 5MI (802 ± 31) ($p_b \leq 0.05$) and remains at similar levels till 5LI (861 ± 16) neurons. A robust increase in neurogenesis was observed in EP (2992 ± 60) ($p_d \leq 0.05$), followed by a continuous decrease in MP (1916 ± 41) ($p_e \leq 0.05$) and LP (1204 ± 20) neurons ($p_f \leq 0.05$) (Fig. 18).

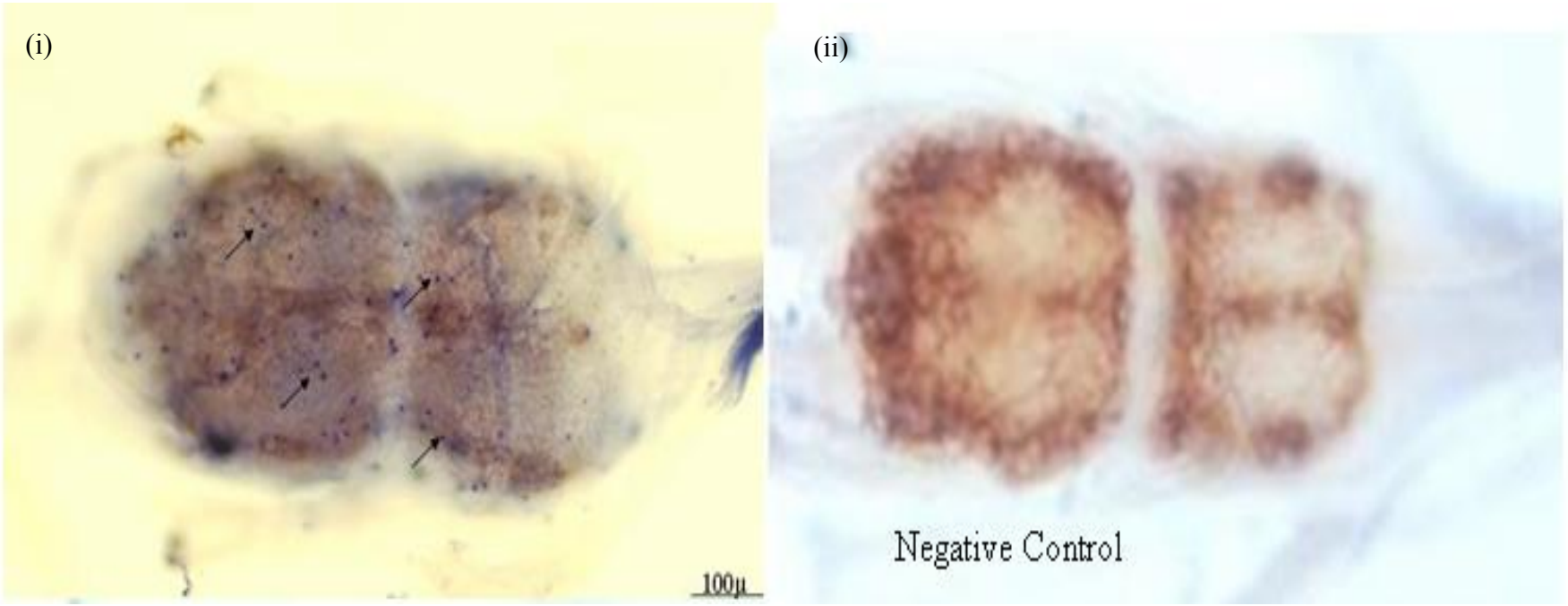


Figure 17: (i) Neurogenesis in the wholemount of Ab_7 of late 5th instar stage during PED in *B. mori*. Arrows: BrdU labeled neurons showing purple blue precipitate. (ii) Negative control: no primary antibody used. Photographs taken at 100X magnification using Olympus BX-41 with DP12 camera.

Table 3: Neurogenesis in CNS of *B. mori* during PED

Ganglion	4I	5EI	5MI	5LI	EP	MP	LP	A
Brain	83 ± 8 b-h	171 ± 9 a, d-h	218 ± 21 a, e, g, h	270 ± 8 a,b,e,g,h	656 ± 45 a-d, f-h	272 ± 22 a,b,e,g,h	352 ± 13 a-f, h	0
SEG	44 ± 1 b-h	23 ± 2 a,c,e-h	58 ± 4 a,b,d-h	28 ± 2 a,c,e-h	164 ± 7 a-d, f-h	116 ± 2 a-e, g, h	92 ± 3 a-f,h	0
T_1	46 ± 4 d-h	33 ± 2 d-h	48 ± 2 d-h	0	248 ± 25 a-d, g,h	221 ± 8 a-d, g,h	134 ± 9 a-f,h	0
T_2	54 ± 4 b,e-h	0	24 ± 3 e-h	53 ± 5 b,e-h	225 ± 16 a-d, f-h	197 ± 17 a-d,g,h	277 ± 10 b,c,e,f,h	0
T_3	69 ± 2 c-h	58 ± 6 c-h	37 ± 2 a,b,d-h	96 ± 8 a-c,e-h	185 ± 6 a-d, f-h	370 ± 26 a-e, g,h		
Ab_1	93 ± 3 b,c,e-h	0	66 ± 1 a,b,e-h	81 ± 11 b,e-h	215 ± 7 a-d, f-h			
Ab_2	70 ± 6 e-h	73 ± 3 e-h	64 ± 8 e-h	58 ± 6 e-h	237 ± 12 a-d, f-h			
Ab_3	77 ± 3 b-f,h	0	37 ± 2 a,b,e,f,h	43 ± 2 a,b,e,f,h	226 ± 18 a-d,f-h	168 ± 14 a-e,g,h	60 ± 2 b,e,f,h	0
Ab_4	77 ± 5 b,e,f,h	0	65 ± 4 b,e,f,h	69 ± 4 b,e,f,h	179 ± 3 a-d,g,h	157 ± 24 a-d,g,h	65 ± 1 b,e,f,h	0
Ab_5	89 ± 11 b-h	53 ± 3 a,e,f,h	53 ± 4 a,e,f,h	50 ± 2 a,e,f,h	137 ± 6 a-d,g,h	146 ± 17 a-d,g,h	64 ± 9 a,e,f,h	0
Ab_6	103 ± 5 b-h	0	52 ± 7 a,b,d-h	0	173 ± 1 a-d, f-h	267 ± 16 a-e,g,h	164 ± 15 a-f,h	0
Ab_7	135 ± 10 b,c,e-h	0	75 ± 6 a,b,d-h	116 ± 7 b,c,e-h	343 ± 8 a-d, f-h			
Total CNS	941 ± 24 b-h	414 ± 22 a, c-h	802 ± 31 a, b, e-h	861 ± 16 b, e-h	2992 ± 60 a-d, f-h	1916 ± 41 a-e, g, h	1204 ± 20 a-f, h	0

Neurogenesis was observed in CNS of every stage, except the 1-day old adult. Neurogenesis was maximum in EP and minimum in 5EI. Each value is mean of BrdU positive cell counts ± SE (n = 4). $p_a \leq 0.05$, $p_b \leq 0.05$, $p_c \leq 0.05$, $p_d \leq 0.05$, $p_e \leq 0.05$, $p_f \leq 0.05$, $p_g \leq 0.05$ and $p_h \leq 0.05$, where a, b, c, d, e, f, g and h are the same as in Table 1

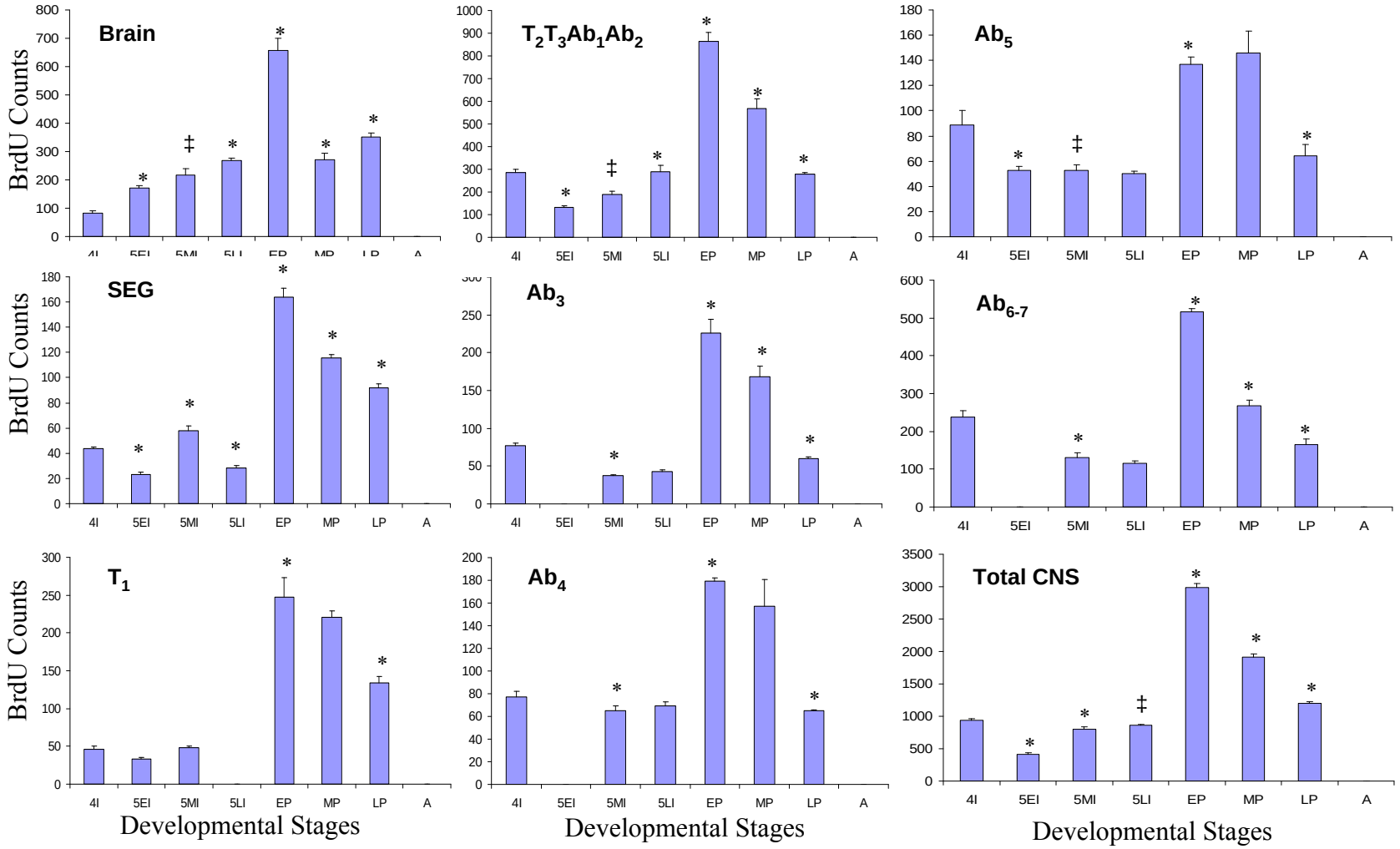


Figure 18: Stage-specific and region specific changes in neurogenesis in the CNS of *B. mori* during PED. Maximum neurogenesis was observed in the entire CNS during EP. Neurogenesis decreased from EP to LP, except in brain, where it initially decreased in MP, and then increased in LP. BrdU positive cells could not be detected in the CNS of the adult; in Ab_3, Ab_4, Ab_6 and Ab_7 of 5EI, and in T_1 and Ab_2 of 5LI larvae. Each value is Mean ± SE (n = 4). * indicates significant difference with the previous stage ($p \leq 0.05$).

IV. Discussion

During transformation from EP to LP there is a significant shortening of IGCs leading to fusion of ganglia. The CNS in thoracic segments as well as abdominal segments undergo extensive transformation during PED as indicated by GFs between T_2-T_3, T_3-Ab_1, Ab_1-Ab_2 and Ab_6-Ab_7 by resorption of interganglionic connectives between the respective ganglia. The selective shortening of these connectives could to be due to differential expression of ecdysone receptors while higher ecdysone levels in hemolymph in this period have been reported in *M. quadrifasciata* (Pinto *et al*., 2003), *M. sexta* (Amos *et al*., 1996) etc. Ecdysteroids have been reported to regulate GF in *M. sexta* by directing stage-specific expression patterns of Fasciclin II, a cell adhesion molecule (Himes *et al*., 2008).

Though GF is a phenomenon specific to the arthropod CNS, biological significance of these fusions have not been yet determined. However, GFs have been related to altered behavior as fusion parallels behavioral plasticity observed in present insect model. The fused ganglia T_2-T_3-Ab_1-Ab_2 can be related to altered locomotor and flight behavior in *B. mori* (Tsujimura, 1988) and Ab_6-Ab_7 to innervations by copulation and ovipositor apparatus as suggested in *P. rapa* (Sugawara, 1981) and *M. sexta* (Giebultowicz and Truman, 1984).

The gross number of neurons in the CNS, counted using toluidine blue staining showed increase in neuronal population in subsequent stages during PED of *B. mori*. Development of 4I to 5LI showed a significant 1.6 fold increase ($p_a \leq 0.05$) in neuronal counts in the CNS, while the number increased 3.5 fold in adult CNS ($p_a \leq 0.05$) as compared with that of 4I.

In the brain, the neuronal counts significantly increased from 4I to 5EI ($p_a \leq 0.05$), but the neuronal profiles were similar throughout the last larval instar. There were significant changes in neuronal counts in the subsequent EP, MP, LP and A stages. There is a 1.8 fold increase in neuronal numbers in the brain from 4I to 5LI ($p_d \leq 0.05$), a 7-fold increase 4I to A ($p_h \leq 0.05$), while the difference was 4-fold between 5LI and A ($p_h \leq 0.05$). The BrdU counts in brain are not significantly different in 5MI, 5LI and MP, and between 5EI and 5MI, though were significantly different from other stages. Maximum neurogenesis was observed in EP and minimum in the 4I with an 8 fold difference in

BrdU counts between the two stages. Substantial numbers of adult-specific neurons are added to the brain of *B. mori* during PED. Using toluidine blue staining technique the neuronal numbers in adult brain of *B. mori* obtained is 2775 ± 94 neurons using both manual as well as by Image Pro-Plus software. Earlier workers estimated higher neuron numbers in the brain of the cockroach, *P. americana* (12,00,000 neurons) and *D. melanogaster* (2,00,000 neurons) (Strausfeld, 1976). Using specific and sensitive technique such as BrdU labeling in the present study further strengthens the total counts as the numbers were always in the range of neurons counted by toluidine blue staining.

Region-specific differences in neuronal numbers in the CNS of holometabolous insects are a result of postembryonic neurogenesis. We observed that gross neuronal numbers obtained with toluidine blue staining in the $T_2T_3Ab_1Ab_2$ region do not vary during 5^{th} instar, as well as during the pupal development. However, BrdU labeling in this region decreases to half during development from 4I to 5EI, but increased 2-fold between 5EI and 5LI. Extensive neurogenesis in EP lead to a 3-fold increase in BrdU counts as compared to 5LI, while there was almost 30% decrease in BrdU counts in MP, which further reduced to half in LP as compared with MP. We found that gross neuronal numbers in $T_2T_3Ab_1Ab_2$ in adult were 3-fold higher as compared with 4I. In the other abdominal ganglia, there was a 2-4 fold increase in gross neuronal counts in the adult CNS as compared with that of 4I, while stage-specific and region specific differences in neurogenesis were observed in different developmental stages. In *M. sexta*, neuroblasts generate around 3000 cells in each of the thoracic ganglia, while around 40-80 cells are added to the abdominal ganglia during PED (Booker and Truman, 1987). A similar range of region-specific changes in neuronal populations were observed in *D. melanogaster* (Truman and Bate, 1988).

In the present study no neurogenesis was observed in the CNS of newly emerged one-day old adult *B. mori*. Adult neurogenesis has been reported in some insect species of Orthoptera and Coleoptera (Cayre *et al*., 1996), although neurogenesis was thought to be an extremely rare event in adult holometabolous insects earlier (Edwards, 1969; Technau, 1984). However persistent neurogenesis is now reported to occur in the MBs of *A. ipsilon* (Dufour and Gadanne, 2006) as well as *D. melanogaster* (Ben Rokia-Mille *et al*., 2008; von Trotha *et al*., 2009).

We report that there are no significant changes in DNA content in the CNS of the larval stages, but the DNA levels increase continuously from larval-pupal transformation till the adult eclosion. This indicates that continuous neurogenesis occurs in the CNS in order to accommodate new neurons required for higher functions in the adult. Metamorphosis in holometabolous insects involves stage-specific distribution of DNA among tissues in a closed system which undergoes extensive histolysis and reconstruction. Chinzei (1975) reported disappearance of DNA as well as RNA from the silk glands of *B. mori*, and a simultaneous increase in their levels in the fat body during pharate pupa. However during development of pharate adult, while the fat body lost both the nucleic acids, the ovary, the integument, and some internal imaginal organs gained them (Chinzei and Tojo, 1972). Lang *et al*., (1965) reported a steady decrease in DNA content during development from pharate pupa to the adult in *A. aegypti*. Total DNA content per insect in the blowfly *Lucilia cuprina* decreased in the pharate pupa and then increased continuously till adult emergence (Lennie *et al*., 1967).

We report here that neurogenesis takes place throughout the larval stages, and that in pupal stages it is extensive in EP though continues till LP. Neurogenesis appears to be absent in 1-day old adults in our study.

Chapter 2

Cell death studies in the CNS during PED

Contents

I. Introduction

Programmed cell death (PCD) plays an important adaptive role during the development of the nervous system (for review: Buss *et al*., 2006). Biological functions of PCD were divided into three main types by Glücksmann (1951): morphogenetic process (e.g., formation of digits by interdigital PCD); histogenetic (PCD that accompanies formation of tissues, including the nervous system); and phylogenetic (loss of vestigial organs such as tail and the pronephros and mesonephros in mammals, or the loss of larval-specific structures during vertebrate and invertebrate metamorphosis). Many evolutionary adaptations are mediated by developmental PCD, including interdigital cell death (Mori *et al*., 1995), deletion of self-reactive immune cells (negative selection) preventing autoimmunity (Gronski and Weinem, 2006), loss of Müllerian duct in male embryos (Klattig and Englert, 2007) and loss of Wolffian duct in female embryos leading to sexual differentiation (Jirsová and Vernerová, 1993), loss of larval structures during metamorphosis in insects and amphibians (Truman, 1996) etc.

The nervous system is one tissue which is not subject to homeostasis i.e., animals die with the nerve cells they are born with (Peters and Rosene, 2003). PCD is essential for segment-specific specialization of the nervous system and regulation of neuronal population controlling the movements and functions of the respective body segments (Jeffs *et al*., 1992). It is responsible for generation of sexual dimorphism in neuronal population in vertebrates (Morris *et al*., 2004). Farah (2006) has reviewed neurogenesis and cell death during formation of vertebrate retina. Differences have been observed in relation to occurrence of PCD in some neuronal population in different classes of animals. For example, PCD is observed in the spinal interneurons and photoreceptors in retina of mammals, but not in birds (Cook *et al*., 1998; Lowrie and Lawson, 2000); on the other hand, PCD is observed in sympathetic preganglionic neurons in birds, but not in mammals (Wetts and Vaughn, 1998).

PCD is primarily involved in regulating the population size of neuronal precursors, which in turn affects the size and morphology of the functional neuronal structure. Significant PCD reduces proliferative cells in the germinal regions of retina (Dünker and Krieglstein, 2003) and spinal cord (Frade and Barde, 1999), cerebellum (Jankowski *et al*., 2009), cortex (Sanno *et al*., 2010) etc. Perturbations in PCD of

progenitor cells results in abnormalities in size and structure of brain such as increased forebrain size (Putz *et al*., 2005) or in severe cases causes exencephaly where the brain is located outside the skull (Depaepe *et al*., 2005).

Developmental PCD also plays important role during 'error-correction' function i.e., selective removal of neurons that migrate to ectopic positions, those with abnormal axonal path finding, or removal of neurons that have innervated inappropriate targets. In short the 'error correction' functions by PCD results in establishment and refinement of proper neuronal circuitry as observed in developing mammalian visual system and in frog brain. In general neurons are generated in excessive numbers so that they compete with other cells to form proper innervations of their targets and adjust their numbers accordingly by PCD (Pettmann and Henderson, 1998). This results in 'Systems matching', i.e., optimization of connections between neurons and their afferent inputs and efferent targets (for review: Buss *et al*., 2006).

(i). PCD in Holometabolous Insects

Development of larval forms of holometabolous insects into adult forms require remodeling of neural circuits underlying feeding and simple locomotor behavior (crawling on prolegs) and converting them into circuits controlling advanced adult specific behaviors such as walking, flight, mating and egg laying. Novel sensory inputs and neuromuscular interactions have to be accommodated in a CNS which is already functional. The nervous system of an adult holometabolous insect doesn't develop *de novo* during metamorphosis, but is developed around an existing scaffold of larval neural elements including sensory neurons, motoneurons and interneurons (Consoulas *et al*., 2000). The major events occurring in the CNS of a typical holometabolous insect during neurometamorphosis include neurogenesis, cell death and remodeling of persistent larval neurons to perform new adult-specific functions (reviewed by: Tissot and Stocker, 2000).

Segment-specific specialization of insect nervous system also requires PCD. Typically, the embryonically derived larval sensory organs and their innervating neurons degenerate (Ghysen *et al*., 1986) and are replaced by adult-specific sensory neurons and organs (Jan *et al*., 1985), while some larval sensory neurons persist (Shepherd and Smith, 1996). Most of the motor neurons innervating the leg survive through metamorphosis

(Kent and Levine, 1993). However, after the loss of larval musculature the motor axons remain in the periphery and innervate the new adult leg muscles and the related sensory neurons (Consoulas *et al*., 1996). PCD is also involved in proper formation of optic lobes in insects (Monsma and Booker, 1996). Sex-specific cell death has been reported in the CNS (Thorn and Truman, 1994) and is genetically regulated by *fruitless* and *doublesex* in *M. sexta* (Kimura *et al*., 2008) and *D. melanogaster* (Sanders and Arbeitman, 2008).

Within CNS PCD removes larval-specific neurons that are no longer required to function in adults in two major phases of cell removal. The first phase of neuronal PCD occurs 12-18 h immediately after puparium formation, while the second phase of neuronal PCD occurs at the time of adult emergence. This is the major route for simplification of abdominal musculature in the adults where thorax takes the major role of locomotor behaviors (for review: Tissot and Stocker, 2000).

The survival of neurons is target-dependent. The thoracic leg motoneurons of *M. sexta* survive and are extensively remodified to accommodate the changing behavioral roles of muscles of true larval legs which are replaced by new muscles in the adult legs (Kent *et al*., 1995). However, larval behaviors that are facilitated by the accessory planta receptors (APRs) innervating prolegs have to be dismantled during metamorphosis ultimately leading to the loss of proleg musculature, and hence regression of specific larval dendrites and PCD (Streichert *et al*., 1997).

At the end of metamorphosis, the holometabolous insects demonstrate a set of highly specialized behaviors that are used during emergence from the puparium and expansion of new wings. These behaviors are not used later in the adult life, and the dedicated motoneurons become obsolete and rapidly degenerate (Truman *et al*., 1994). Even the abdominal muscles in *M. sexta* that innervate the intersegmental muscles are removed by PCD following adult emergence (Truman and Schwartz, 1984).

(ii). Mechanisms of Cell Death in Insects

PCD during insect development involves both apoptosis (Draizen *et al*., 1999) as well as autophagy (Baehrecke, 2000). The autophagic mode of cell death is usually seen when entire tissues such as the midgut (Denton *et al*., 2010) and salivary glands (Berry and Baehrecke, 2008) are committed to destruction especially during onset of

metamorphosis. Incidentally Lee and Baehrecke (2001) were able to demonstrate that autophagic cell death involves caspase activation, while some authors reported otherwise (Denton *et al*., 2010). However, Kinch *et al*., (2003) demonstrated that PCD of motoneurons innervating the accessory planta receptor 6 is caspase-dependent and autophagic in nature, but not apoptotic.

Cell death in the CNS has been traditionally studied using TUNEL assay in insects such as *A. mellifera* (Roat and da Cruz Landim, 2010), *D. melanogaster* (Peabody *et al*., 2008), *M. quadrifasciata* (Pinto *et al*., 2003); by modified toluidine blue staining in *M. sexta* (Fahrbach *et al*., 1994), *D. melanogaster* (Kimura and Truman, 1990); or by routine light microscopic observations following hematoxylin-eosin staining of sectioned materials as in optic lobes (Monsma and Booker, 1996) or the ventral nerve cord in *M. sexta* (Truman and Schwartz, 1984). TUNEL has been the hallmark assay for demonstration of DNA fragmentation *in situ* in a number of metamorphosing tissues, including gut (Parthasarathy and Palli, 2007a), fat body, salivary glands (Lee and Baehrecke, 2001), prothoracic gland (Dai and Gilbert, 1999), larval abdominal muscles (Bayline *et al*., 1998) and ovaries (Capella and Hartfelder, 1998).

Deoxyribunuclease 1 is involved in fragmentation of nuclear material and has been implicated in apoptosis (Falcone *et al*., 1999). There are earlier reports of developmental changes in deoxyribonucleases active at both neutral (Muhammed *et al*., 1967) as well as acidic pH (Detwiler and MacIntyre, 1978) in the holometabolous insect *D. melanogaster*.

PCD in insect neurons has also been shown to involve caspases (Kato *et al*., 2009; Kim *et al*., 2009). DRONC controls neuroblast numbers in *D. melanogaster* CNS by inducing apoptosis (Waldhuber *et al*., 2005). Characterization of BmIce from *B. mori* revealed that it shares significant sequence similarity with DRICE of *D. melanogaster* and CED3 of *C. elegans*, and hence could play a role in apoptosis (Duan *et al*., 2005). Arya and Lakhotia (2008) reported the role of the heat shock protein Hsp60D which interacts with *Drosophila* inhibitor of apoptosis 1 (DIAP1) and induces caspase-mediated apoptosis. Absence of Hsp60D resulted in inability of dissociation of DIAP1 with the initiator and executioner caspases in *D. melanogaster*.

There are very few reports linking PARP 1 to PCD in insects. PARP cleavage by caspases results in activation of cell death machinery in S2 cells of *D. melanogaster* (Poltronieri *et al*., 1997). However, some authors have reported that PARP-1 enzymatic activity facilitates transient loosening of chromatin and regulates transcriptional activation of many genes during *D. melanogaster* development that are dependent on heat shock, NF-kB and ecdysteroids (Tulin and Spradling, 2003). PARP mRNA levels during PED in *D. melanogaster* was detected in the pupae and the adult flies, but not in the larvae (Hanai *et al*., 1998). Though Poly (ADP-ribosylation) has been described in some of the holometabolous insects including *C. capitata* (Cavalloro *et al*., 1988), *D. melanogaster* (Ji and Tulin, 2009) and *S. peregrine* (Masutani *et al*., 1994), its role in PCD in insects is not clear.

Some workers have recently reported Cathepsin D as an ecdysteroids-inducible protein which plays an important role in PCD in fat body and gut in *B. mori* (Gui *et al*., 2006; Lee *et al*., 2009). Overexpression of cathepsin D was demonstrated to induce PCD, but RNA interference (RNAi)-mediated BmCatD knock-down was shown to inhibit PCD of the larval fat body and larval gut.

Based on the above literature background we studied PCD in the CNS of *B. mori* by various methods. In this chapter we studied:

1. DNA Fragmentation studies in the CNS during PED
2. Role of Deoxyribonuclease 1 in the CNS during PED
3. Cell death detection using wholemount TUNEL assay
4. Studies involving changes in the levels of various cell death marker proteins including – caspase 3, PARP-1 and Cathepsin D

II. Materials and Methods

(i). DNA Fragmentation Assay

DNA isolation procedure was as described in Chapter 1 (Page No.52).

A 1.5 % agarose mini gel was prepared in Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA), and mixed with 0.5 μg/ml Ethidium Bromide. After polymerization the gel was transferred to electrophoresis chamber (Broviga, India) and

submerged in TAE buffer completely avoiding any air bubble formation. 1 μg DNA from the DNA isolated from distinct developmental stages was mixed with 0.2 volumes of 6X gel loading buffer (0.25% Bromophenol blue, 0.25% Xylene cyanol and 40% w/v Sucrose in distilled. H_2O) and loaded into the respective wells along with a 100 base pair (bp) DNA ladder marker (Bangalore Genei). Agarose electrophoresis was performed for 30-60 min with 5-20 V/cm voltage. Various samples (1 μg DNA each) from different developmental stages (3I, 4I, 5EI, 5MI, 5LI, EP, MP, LP and A) were electrophoresed to detect DNA fragmentation (Jagota *et al*., 1997).

(ii). Deoxyribonuclease 1 (DNase 1) Assay

DNase 1 activity was studied in CNS in different developmental stages using Native PAGE Zymography (Napirei *et al*., 2004). Native PAGE Zymography (in-gel activity staining) is a two-stage technique involving protein separation by electrophoresis performed under native conditions followed by in-gel assay of enzymatic activities. During native PAGE polyacrylamide gels were prepared in the absence of sodium dodecyl sulphate (SDS) and samples were diluted in gel loading buffer without SDS and β-mercaptoethanol (BME) or dithiothreitol (DTT). SDS denatures proteins by linearization. BME or DTT denatures proteins by disrupting the disulfide bonds within the polypeptide chain. Hence, they were avoided in the Native PAGE. CNS was pooled from various stages (from 20 insects in 4I, 10 in 5EI, 5 in 5MI, 5LI, EP, MP, LP and A) to get 10mg tissue sample as described earlier (Chapter 1, Page No. 52) and 5% homogenate was prepared in 50 mM Tris-HCl (pH 7.5) in the presence of 1 mM phenylmethanesulfonylfluoride (PMSF) to prevent protease activity. After centrifugation of homogenates at 4 °C at 10,000 RPM, the supernatants were transferred to fresh tubes and the protein content was estimated by Bradford method (1976) which is based on the binding of the dye Coommassie blue G250 to protein which gives maximum absorbance at 595 nm. The standard graph was prepared using bovine serum albumin (BSA) with concentrations ranging from 1 μg to 10 μg protein.

Preparation of Native PAGE Gels

The gel plates were sealed with 2% agar-agar and were checked for any leakage, and then a 12 % native resolving gel (4 ml of 30% Acrylamide-bisacrylamide mix, 200 µl of 100 µg/ml salmon testis DNA, 2.5 ml of 1.5 M Tris (hydroxymethyl)-aminomethane or Tris base pH 8.8, 100 µl of 10% ammonium persulphate (APS), 5 µl of tetramethylethylenediamine (TEMED), 3.3 mL H_2O) was prepared upto 2/3rd of the gel plate. A 5 % stacking gel (0.83 ml of 30% Acrylamide-bisacrylamide mix, 100 µL of 100 µg/ml salmon testis DNA, 0.63 ml of 1 M Tris pH 6.8, 50 µl of 10% APS, 5 µl of TEMED, 3.4 ml H_2O) was prepared over the resolving gel with the appropriate comb set. After polymerization, the plates were fixed to a vertical slab gel electrophoresis unit. The electrophoresis buffer (25 mM Tris base, 250 mM Glycine) was poured in both the electrode tanks. The whole apparatus was cooled to 4 °C by keeping it in a cooling cell for at least 1 h.

Samples were prepared in 4X gel loading buffer (200 mM Tris-HCl, 0.4% bromophenol blue, 40 % v/v glycerol on ice. For negative control the gel loading buffer was added as such (Blank). For positive control, protein homogenate was prepared from the Gut of the 5th instar larvae of *B. mori* as DNases have been reported as an integral part of larval digestive system in insects (Schernthaner *et al*., 2002). 50 µg protein sample was mixed with gel loading buffer to get 1X concentration and then loaded into the wells with gel loading tips. The voltage of the electrophoresis power pack (Broviga, India) was set to 80 V and electrophoresis was run till the sample reaches the end of stacking gel and then, electrophoresis was continued at 160 V for 2-3 h till the bromophenol blue indicator migrated out of the resolving gel.

After electrophoresis, the gel was washed with distilled H_2O, and transferred to 5 volumes of DNase 1 reactivation buffer (20 mM Tris-HCl, 5 mM $MgCl_2.6H_2O$, 5 mM $CaCl_2.6H_2O$, 10 µg/ml). It was then incubated at 37 °C and observed for DNase 1 activity every 2 h in a UV transilluminator. This assay is based on the principle that DNase 1 digests the native Salmon testis DNA in the PAGE gel which cannot be bound by ethidium bromide. Ethidium bromide binds to the native DNA and gives a fluorescent

background with dark bands representing degraded DNA. The gel was later photographed under UV light using Bio-Rad Gel documentation system.

(iii). Whole-mount TUNEL Assay

TUNEL labeling was performed with the *in situ* Cell Death Detection kit, with alkaline phosphatase label (Roche Molecular Diagnostics) according to the method of Draizen *et al*., (1999), with some modifications. The CNS was rapidly dissected in insect Ringer's solution, and immediately fixed in 10% formalin in PBS overnight. The fixed tissue was washed with PBS for 30 min, and then subjected to permeabilization by proteinase K treatment for 30 min at 37 °C. It was washed first in PBS and subsequently in PBSTx for 30 min each. Later the tissue was subjected to TUNEL reaction mixture according to kit instructions (Roche Molecular Diagnostics) for a couple of hours at 37 °C. Terminal deoxyribonucleotidyl transferase (TdT) catalyzes the addition of Digoxygenin (DIG) tagged-dUTP molecules to the 3`-hydroxyl termini of DNA molecule in the presence of Co^{2+}. It was then washed in PBST, and incubated with Convertor-AP (Roche Molecular Diagnostics) provided in the kit for a couple of hours. The Convertor-AP solution contained the anti-DIG antibody which in turn was tagged with an alkaline phosphatase label. The tissue was then washed in PBS and treated with NBT-BCIP till color development. The tissue was later dehydrated and mounted in DPX-mountant and photographed with DP-12 camera. For negative control sample, the Convertor-AP solution from the kit was omitted in the reaction mixture, and later the tissue was processed in the same way as mentioned. TUNEL positive cells were counted in the same way as described in Chapter 1 (Page No. 53).

(iv). Western Blot of Cell death marker proteins (Caspase 3, PARP 1 and Cathepsin D)

SDS-PAGE electrophoresis was performed by the method of Sambrook and Russell (2001). The tissues were pooled to 10mg weight as discussed earlier in Chapter 1 (Page No. 52). The samples were homogenized in lysis buffer (50 mM Tris-HCl buffer (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM PMSF) and the lysates were collected after centrifugation at 10,000 RPM for 10 min at 4 °C (Crljen *et al*., 2004). Lysates were

mixed with the SDS gel loading buffer (4% SDS, 10% BME, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 6.8). 20 µg protein was loaded into the wells of 10% SDS-PAGE (3.3ml of 30% Acrylamide-bisacrylamide mix, 2.5 ml of 1.5 M Tris pH 8.8, 100 µl of 10% SDS, 100 µl of 10% APS, 5 µl of TEMED, 4 mL H_2O) with vertical slab gel electrophoresis under Tris-glycine buffer (25 mM Tris base, 190 mM Glycine, 0.1 % SDS, pH 8.3) at room temperature. After the dye-front had reached 0.5 cm above the bottom of resolving gel, electrophoresis was stopped, and the gel was washed in cold polyvinylidene fluoride (PVDF) transfer buffer (25 mM Tris base, 190 mM Glycine, 5% methanol) to remove excess SDS.

The proteins from the SDS-PAGE gel were transferred to 0.2 µ pore size PVDF membrane (FluoroTrans®; PALL Life Sciences) as PVDF membrane possesses many advantages over nitrocellulose membranes including better protein retention, greater physical strength and chemical compatibility (Pluskal *et al*., 1986) and are highly hydrophobic in nature and require brief pretreatment with methanol, which stabilizes the gel and also removes complexed SDS from protein molecules (Mozdzanowski and Speicher, 1992). The membranes were then soaked in cold transfer buffer. For assembly of the transfer cassette, the SDS-PAGE gel and the PVDF membrane was sandwiched between Whatman 3mm filter papers in the following order: the cathode carbon plate with black electrode, Scotch Brite pads, 2 thick filter papers, 2 thin filter papers, the gel, the PVDF membrane, 2 thin filter papers, 2 thick filter papers, Scotch Brite pads and finally the anode plate with red electrode on the top. The filter papers and the Scotch Brite pads were soaked in transfer buffer before the assembly and care was taken to avoid formation of any air bubbles. The proteins were transferred in western blot tank (Broviga, India) using the wet transfer method at 30V overnight (Meiring *et al*., 2005) at 4 °C.

The transfer of proteins was verified by Ponceau S staining. The PVDF membranes were washed with distilled H_2O to remove excess Glycine from the transfer. The membrane was flooded with Ponceau S staining (0.2% w/v Ponceau S, 3% acetic acid) for 10-15 minutes, and then transferred to distilled H_2O for visualization of efficient protein transfer. The PVDF membrane was blocked overnight with 5% non-fat milk (Nestle Carnation) in Tris buffered saline with Tween 20 (TBST) (20 mM Tris base, 150 mM NaCl, pH 7.6, 0.4% Tween 20). The transferred proteins were later probed using

monoclonal mouse anti-human PARP 1 (Santa Cruz sc-8007), polyclonal rabbit anti-human caspase 3 (sc-7148) and polyclonal goat anti-human cathepsin D (sc-6486) at 1:1000 dilutions in TBST. For equal protein loading, the membranes were probed with polyclonal rabbit anti-human β-tubulin (H-235) at 1:1000 dilutions in TBST. After washing off the excessive unbound primary antibody, the blots were later probed using the respective secondary antibodies diluted to 1:2000 in TBST: goat anti-mouse-alkaline phosphatase (ALP), goat anti-rabbit-horse radish peroxidase (HRP) and rabbit anti-goat HRP. The membrane for PARP 1 detection was probed with alkaline phosphatase coupled secondary antibody and the purple-blue color reaction was developed by incubating the membrane in NBT/BCIP solution for 5-10 min. In order to detect HRP, the membranes were incubated with 3,3´-diaminodbenzidine (DAB) substrate solution (0.05% DAB**,** 0.015% H_2O_2, in 50 mM phosphate buffer (50 mM Na_2HPO_4, 50 mM NaH_2PO_4)) till reaction is observed as dark brown precipitate.

(v). Coomassie Blue Staining

Equal loading of proteins was verified after electrophoresis by staining of the polyacryamide gel with Coomassie blue staining (Muro *et al*., 2002). PAGE gels were stained with modified Coomassie blue method of Wang *et al*., (2007). After electrophoresis, the gel was fixed in 20 volumes of fixing solution (10% acetic acid, 10% methanol, 40% Ethanol) for 1 h. The fixing solution was discarded and the gel was later kept in sensitization solution (1% acetic acid, 10% $(NH_4)_2SO_4$) for 2 h. The sensitization solution was discarded and the gel was kept in staining solution (5% acetic acid, 45% ethanol, 0.125% Coomassie Brilliant Blue R-250) for 4h to overnight on a rocker. After discarding the staining solution, the gel was transferred to destaining solution I (5% acetic acid, 40% ethanol) for 1 h, and later to destaining solution II (3% acetic acid, 30% ethanol). The gel was then preserved in 5% acetic acid solution.

(vi). Statistical Analysis

The data was analyzed using SigmaStat software (Jandel Scientific). One way ANOVA was followed by Duncan's post-hoc test for multiple comparisons of neuronal numbers i.e., number of TUNEL positive cells, in various stages during development.

III. Results

(i). DNA Fragmentation Assay

Agarose gel electrophoresis of genomic DNA isolated from various developmental stages including 4I, 5EI, 5MI, 5LI, EP, MP, LP and A, did not reveal any DNA fragmentation (Fig. 19).

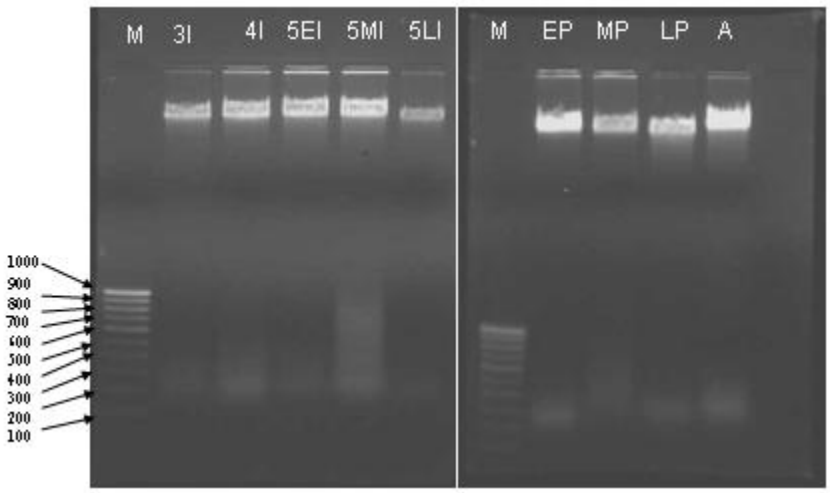
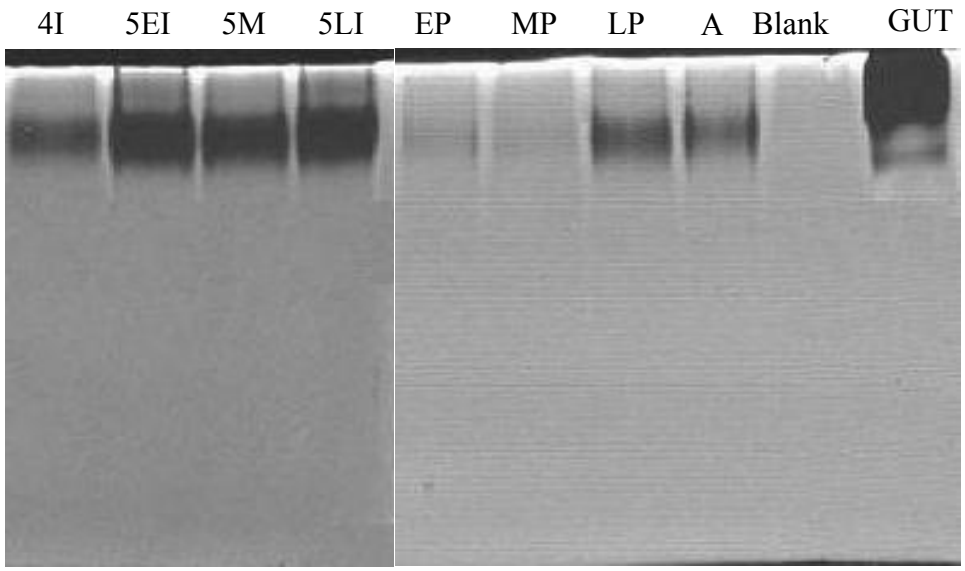


Figure 19: DNA Fragmentation assay of the genomic DNA isolated from the CNS in different stages during PED. M: Molecular weight marker ranging from 100 bp to 1000 bp. 3I: 3rd instar; 4I: 4th instar; 5EI: 5th early (day 1-3); 5MI: 5th mid (day 4-6); 5LI: 5th late (day 7-9); EP: early pupa (day1-3); MP: mid pupa (day 4-6); LP: late pupa (day 7-9); A: 1 day old adults.

(ii). Deoxyribonuclease 1 Assay (Native PAGE Zymography) in CNS in various stages during postembryonic development

DNase 1 activity is detected as dark bands against fluorescent native DNA (Fig. 20 (i)). Densitometric analysis revealed that DNase 1 levels were higher in the larval stages from 4I to 5LI though slightly lower in 4I. In EP and MP the DNase 1 appeared very faint, but again there was increase in LP and A (Fig. 20 (ii)). The gut homogenate from *B. mori* larva was taken as a positive control of DNase 1 activity. Densitometric analysis of the DNase 1 activity was performed using Scion Image software.

i.



ii.

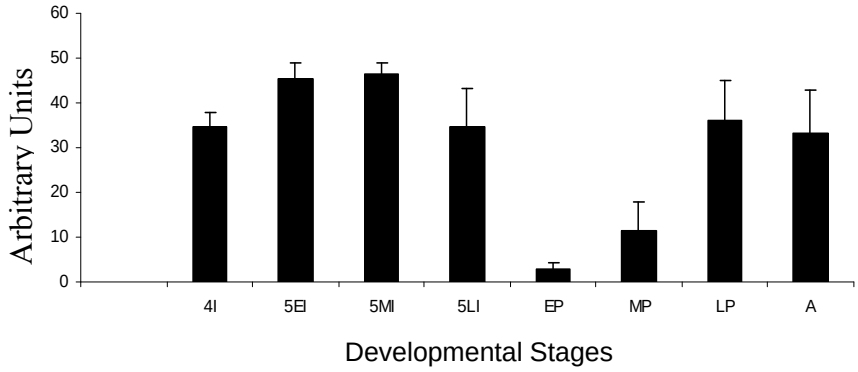


Figure 20 (i). Native PAGE Zymogram of Deoxyribonuclease 1 activity in the CNS of *B. mori* during PED in various stages such as 4I, 5EI, 5MI, 5LI, EP, MP, LP and A. Gut homogenate was used as a positive control, while gel loading buffer was added in the Blank as negative control. (ii). Densitometric analysis of the DNase 1 activity (n = 3). The enzymatic activity is higher in larval stages, decreases robustly in EP and MP, and MP onwards remained high in LP and A. Each densitometry value is Mean ± SE (n = 3).

(iii). Cell death in the CNS in various stages during PED

Stage-specific and region-specific changes in the number of apoptotic cells (TUNEL positive cells) were observed in the CNS during PED. Cell death was detected by TUNEL assay in the pupa and the adult as dark bluish-purple reaction products (Fig. 21). However, no TUNEL positive cells were detectable in the 4^{th} or the 5^{th} instar larvae. (Table 4; Fig. 22).

In brain, the number of dead cells significantly increased 1.4 folds from EP (321 ± 25) to MP (446 ± 12) ($p_e \leq 0.05$), decreased more than 60% in LP (177 ± 11) ($p_f \leq 0.05$), and then again showed a 2-fold increase following adult eclosion (381 ± 11) ($p_g \leq 0.05$) (Table 4; Fig. 22).

Cell death in SEG did not show any change between EP (200 ± 5) and MP (201 ± 22), but showed a 50% reduction in LP (90 ± 6) ($p_f \leq 0.05$) followed by robust 3-fold increase in adult (259 ± 12) ($p_g \leq 0.05$) (Table 4; Fig. 22).

In T_1 region, the number of TUNEL positive cells increased significantly by 1.3 folds during development from EP (158 ± 5) to MP (201 ± 4) ($p_e \leq 0.05$). The counts later decreased to more than 50%, in LP (89 ± 7) ($p_f \leq 0.05$) followed by a robust 3-fold increase in A (285 ± 19) ($p_g \leq 0.05$) (Table 4; Fig. 22).

In $T_2T_3Ab_1Ab_2$ region cell death increases 1.5 folds during development from EP (328 ± 25) to MP (479 ± 16) ($p_e \leq 0.05$), decreases by 40% in the LP (276 ± 13) ($p_f \leq 0.05$) followed by a 2 fold increase in the A (591 ± 16) ($p_g \leq 0.05$) (Table 4; Fig. 22).

Cell death in Ab_3 shows a significant increase during development from EP (100 ± 15) to MP (136 ± 8) ($p_e \leq 0.05$), followed by a 40% decrease in LP (78 ± 5) ($p_f \leq 0.05$), and another similar decrease in A (48 ± 5) ($p_g \leq 0.05$) (Table 4; Fig. 22).

In Ab_4 cell death does not vary significantly during development from EP (122 ± 8) to MP (113 ± 18), but decreases by 25% in LP (84 ± 8) ($p_f \leq 0.05$) followed by 50% reduction in A (41 ± 4) ($p_g \leq 0.05$) (Table 4; Fig. 22).

Interestingly in the Ab_5 there is no significant variation in cell death throughout the pupa (EP (68 ± 12), MP (70 ± 3) and LP (73 ± 5) and following adult emergence (68 ± 6) (Table 4; Fig. 22).

In $Ab_{6\text{-}7}$, cell death significantly decrease by 50% during development from EP (333 ± 17) to MP (149 ±4) ($p_e \leq 0.05$), and then shows a small but significantly increases in LP (192 ± 7) ($p_f \leq 0.05$) and adult (218 ±3) ($p_g \leq 0.05$) (Table 4; Fig. 22).

Gross cell death doesn't vary significantly between EP (1627 ± 110), MP (1788 ± 83) and A (1892 ± 75), but is lowest in LP (1058 ± 75). However, cell death decreases from MP to LP ($p_f \leq 0.05$) and then significantly in A ($p_g \leq 0.05$) (Table 4; Fig. 22).

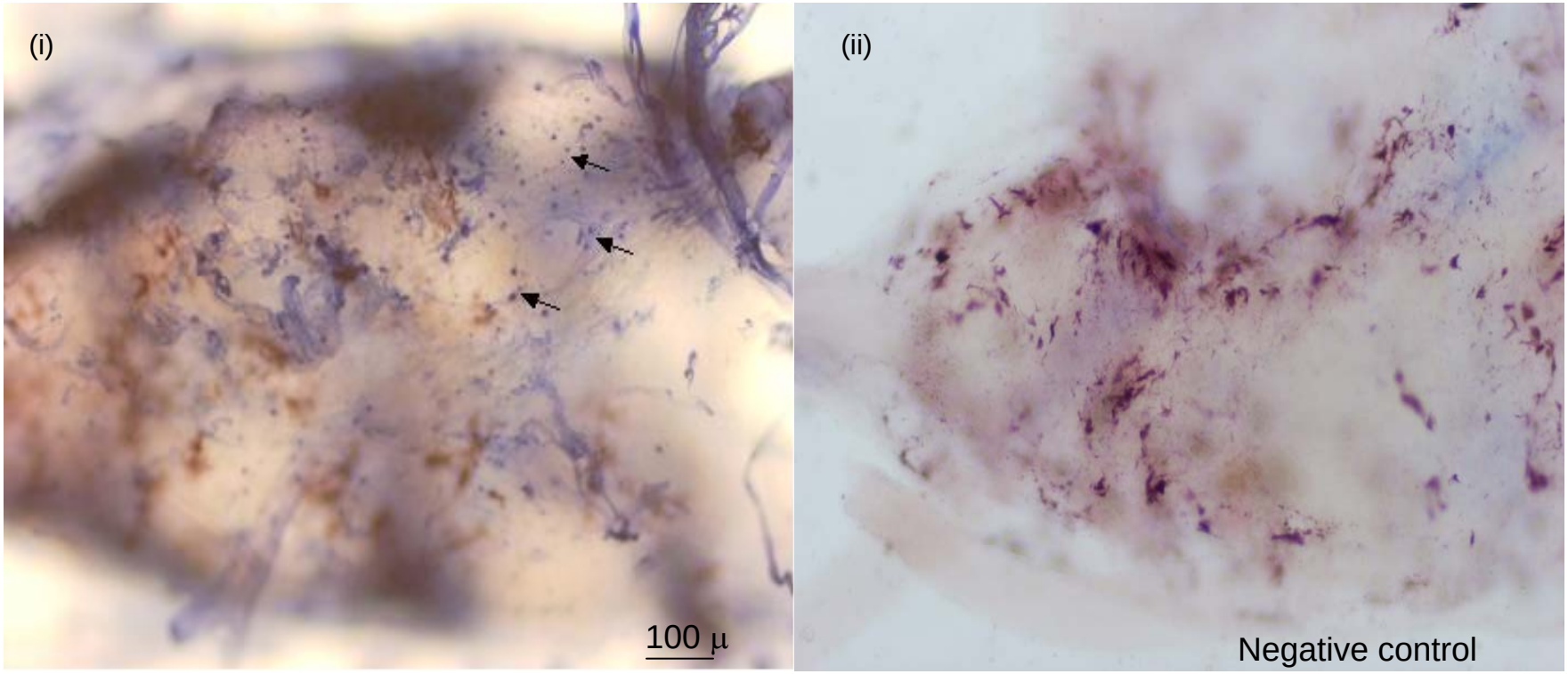


Figure 21: TUNEL Assay showing cell death in the CNS of *B. mori*. (i) Representative image of wholemount TUNEL Assay of compound ganglion 1 ($T_2T_3Ab_1Ab_2$) in adult. Arrows: TUNEL positive cells showing purple blue precipitate. (ii) Negative control in which converter AP antibody was omitted from the reaction mixture.

Table 4: Cell death in the CNS of *B. mori* during PED

Ganglion	EP	MP	LP	A
Brain	321 ± 25^{f-h}	446 ± 12^{e,g,h}	177 ± 11^{e,f,h}	381 ± 11^{e-g}
SEG	200 ± 5 ^{g,h}	201 ± 22 ^{g,h}	90 ± 6 ^{e,f,h}	259 ± 12 $^{e-g}$
T_1	158 ± 5 $^{f-h}$	201 ± 4 ^{e,g,h}	89 ± 7 ^{e,f,h}	285 ± 19 $^{e-g}$
T_2	39 ± 1 ^{f}	235 ± 9 ^{e}	276 ± 13 ^{e,f}	591 ± 15 $^{e-g}$
T_3	69 ± 10	236 ± 4 ^{e}		
Ab_1	155 ± 5			
Ab_2	60 ± 8			
Ab_3	100 ± 15 ^{f,h}	136 ± 8 ^{e,g,h}	78 ± 5 ^{f,h}	48 ± 5 $^{e-g}$
Ab_4	122 ± 8 ^{g,h}	113 ± 18 ^{g,h}	84 ± 8 ^{e,h}	41 ± 4 $^{e-g}$
Ab_5	68 ± 12	70 ± 3	73 ± 5	68 ± 6
Ab_6	131 ± 6	149 ± 4^{e,g,h}	192 ± 7 ^{e,f,h}	218 ± 3 $^{e-g}$
Ab_7	203 ± 10			
Total CNS	1627 ± 110^{g}	1788 ± 83^{g}	1058 ± 62^{e,f,h}	1892 ± 75^{g}

Cell death in the CNS of EP, MP and A did not vary significantly, but was observed to be lowest in LP. Cell death could not be detected in the CNS of 4I, 5EI, 5MI and 5LI. Each value is mean of TUNEL positive cell counts ± SE (n = 4), $p_e \leq 0.05$, $p_f \leq 0.05$, $p_g \leq 0.05$, $p_h \leq 0.05$, where e, f, g and h are same as in Table 1.

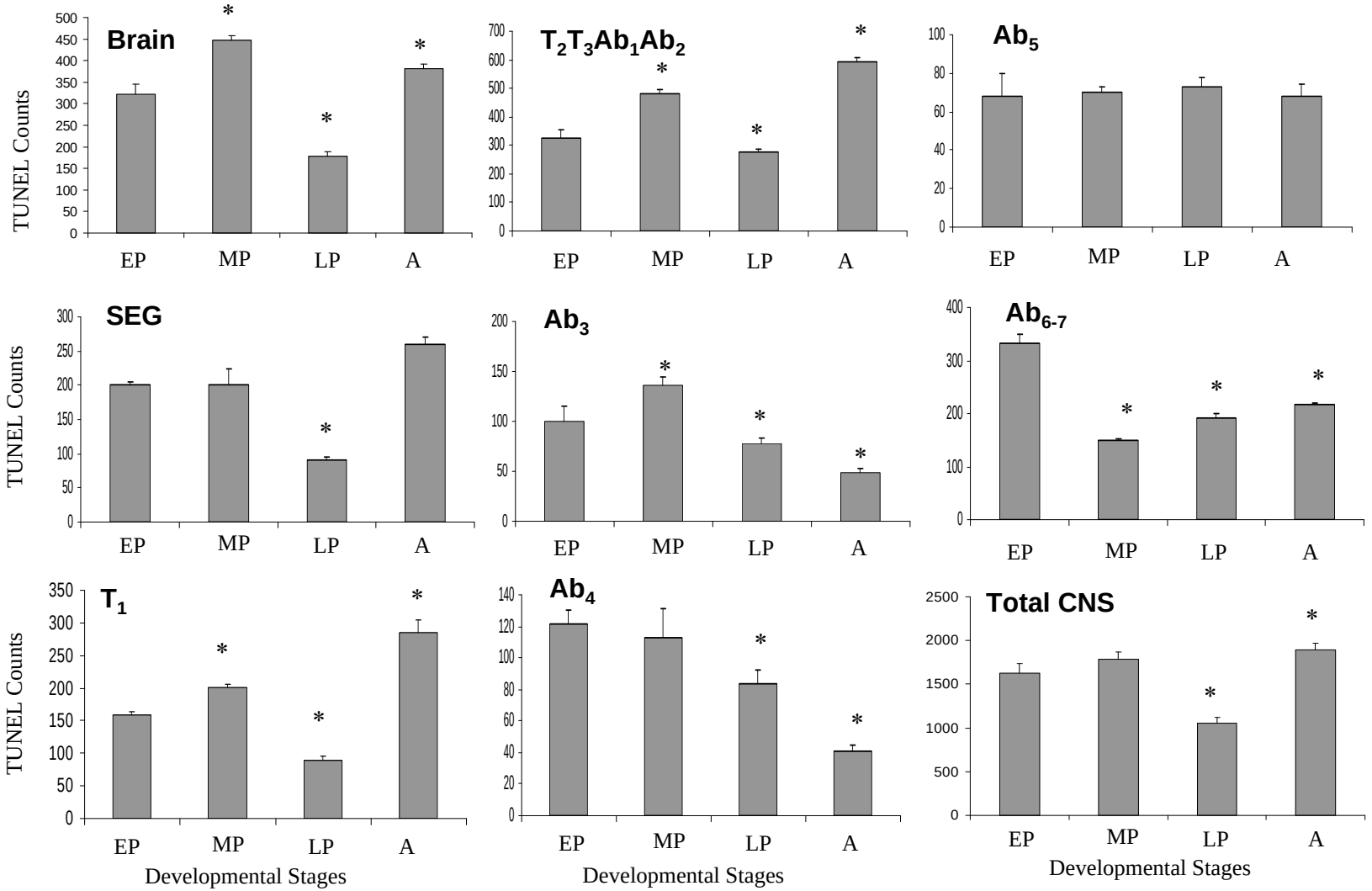


Figure 22: Stage-specific and region-specific changes in cell death (TUNEL positive cell counts) in the CNS of *B. mori* during PED. Cell death in Brain, SEG, T_1 and $T_2T_3Ab_1Ab_2$ region decreases significantly between MP to LP followed by a significant increase in A. In Ab_3 and Ab_4, cell death shows a gradual reduction during development from MP to LP and A. In Ab_5, there is no significant changes in cell death, while in Ab_{6-7} region, cell death significantly reduces between EP to MP, followed by a small but significant increase till A. Cell death in the entire CNS does not change significantly between EP and MP, but decreases significantly in LP and again shows a significant rise in adult CNS. Each value is expressed as Mean ± SE (n = 4). * indicates significant difference between successive developmental stages ($p \leq 0.05$).

(iv). Western Blot Analysis of Cell Death marker proteins

Prominent caspase 3-like immunoreactive bands were observed at 48 kDa and 26 kDa. These bands were considered for densitometric analysis though heavy molecular weight bands were also observed. Caspase 3 cleavage was complete in EP and MP as only 26 kDa immunoreactive band was observed, while in LP and A, it was incomplete as both 26 kDa as well as 48 kDa bands were detected (Fig. 23 (iii)).

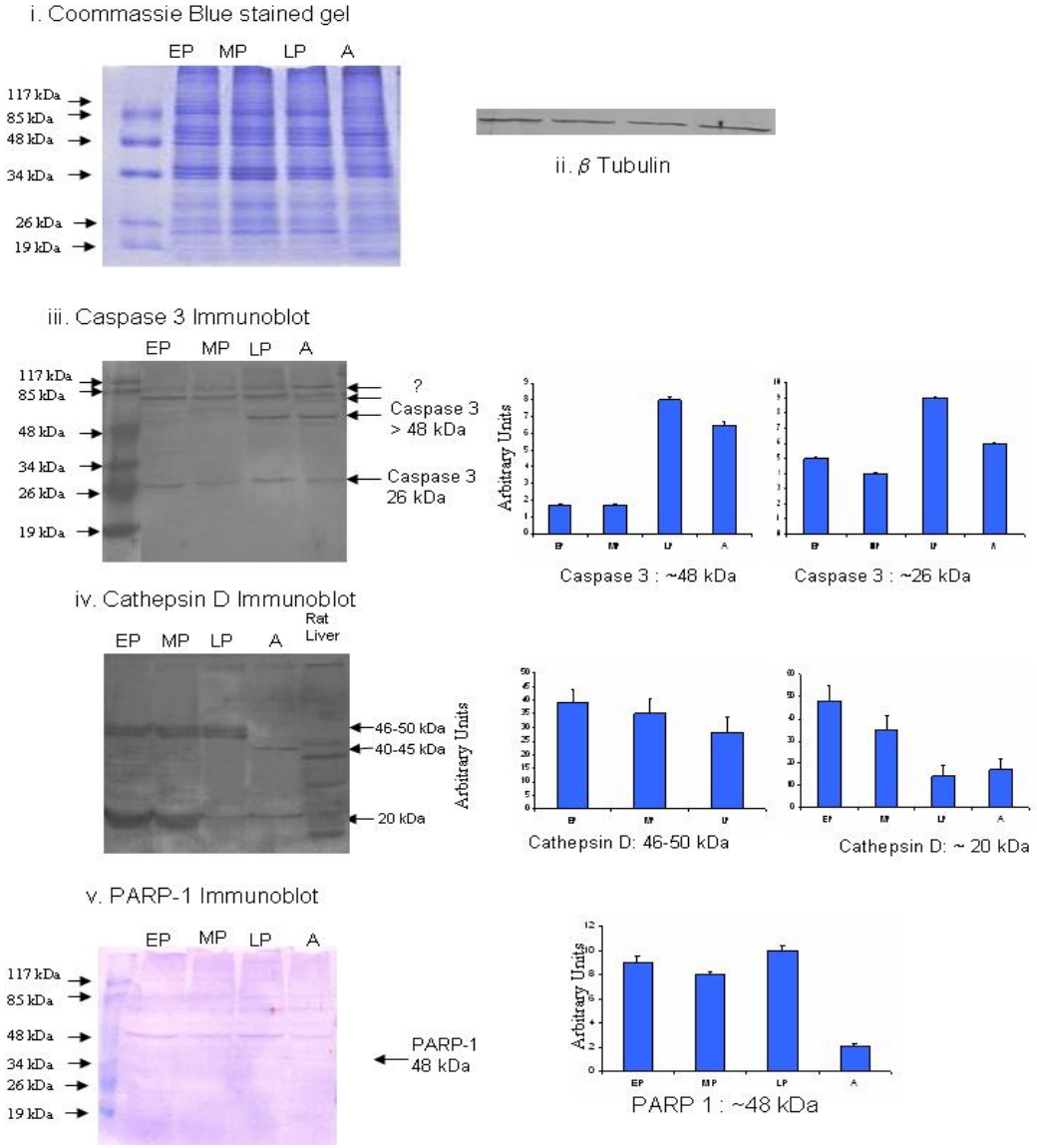


Figure 23: Western blot analysis of cell death proteins. Coomassie Blue staining (i) and β-tubulin (ii) showing equal protein loading. Cell death proteins Caspase 3 (iii), PARP-1 (iv) and Cathepsin D (v) were studied. Caspase 3 is completely cleaved in EP and MP as compared to LP and A. Cathepsin D shows prominent 46-50 kDa immunoreactive bands during pupal development, while the 20 kDa band are higher during EP and MP, but gradually decrease in LP and A. However, in adults, additional 40-45 kDa Cathepsin D

like immunoreactivity is detected. PARP 1 shows prominent immunoreactivity in Pupal stages, but least in 1-day old adult.

In case of PARP-1, a prominent 48 kDa immunoreactive band was observed which was higher throughout the pupa but decreased in the adult (Fig. 23 (iv)).

Two close prominent cathepsin D-like immunoreactive bands in the range of 46-50 kDa and another lower molecular weight band near 20 kDa range were observed throughout the pupal stages (Fig. 23 (v)). However, in the adult a single prominent cathepsin D-like immunoreactive band was observed near 40-45 kDa range, while the lower molecular weight band at 20 kDa region was not as prominent as EP and MP.

Equal loading of proteins was confirmed by Coomassie Blue staining (Fig. 23 (i)) and by using β-tubulin as loading control (Fig. 23 (ii)).

IV. Discussions

Stage- and region-specific changes in cell death were observed in the CNS of *B. mori*. We report here PCD in the CNS is prominent during pupal development and continues till adult emergence. TUNEL assay could not detect PCD in any of the larval stages. However, little amount of PCD has been reported in brain and thoracic ganglia of *B. mori* last instar larvae (Kim *et al*., 2007; Kato *et al*., 2009). Further, PCD is reported to be restricted to the early pupation and upon adult emergence in the CNS of *D. melanogaster* (for review: Truman, 1996). However we report here that cell death occurs throughout pupal development and continued in the adult CNS.

In present study we report that in Ab_4 of *B. mori* cell death was higher in the pupal stages as compared to the adults. Similar results were reported in case of *M. sexta*, where the 4^{th} abdominal ganglion (Ab_4) reported to contain around 847 ± 23 neurons just before adult eclosion, but the counts decreased to 508 ± 16 neurons within 3 days post-emergence (Truman, 1983).

The number of cells undergoing PCD in brain region increases from EP (321 ± 25) to MP (446 ± 12), decreases in LP (177 ± 11) and then increases in the adult (381 ± 11). On the other hand in Ab_{6-7} region, cell death is highest in the EP (334 ± 17), before the ganglia fuse in the MP (149 ± 4) in which the cell death decreases robustly, but continues to increase through LP (192 ± 7) and A (218 ± 3).

We could not detect PCD by DNA fragmentation in the CNS of *B. mori* in any of the developmental stages. DNA fragmentation has been readily demonstrated in the fat body and gut (Gui *et al*., 2006) and also in anterior silk glands (Kaneko *et al*., 2006). However, in other cases as in ISMs (Beaulation and Lockshin, 1977) and labial glands (Zakeri *et al*., 1993) of *M. sexta*, or the silk glands of *B. mori* (Akai, 1984), there is readily detectable early cytoplasmic destruction, but nuclear destruction and DNA fragmentation occurs to a much lesser extent that cannot be detected as compared to mammalian apoptosis. Absence of DNA fragmentation in insects has been attributed to their prominent heterochromatin which is less readily attacked by endonucleases and overall decrease in protein synthesis as metamorphosis begins. Oligonucleosomal DNA ladder formation in the CNS of Lepidopteran insects has not been demonstrated so far. Though DNA ladder formation has been considered as a hallmark event of apoptosis, it has been reported that it is not the only essential event (Zakeri *et al*., 1993; Jagota *et al*., 1997; Nagase *et al*., 2003).

DNase 1 levels were found to be higher in the larval stages as compared to pupa and adult, and were found to be in agreement with the results obtained in *D. melanogaster* (Muhammed *et al*., 1967). DNase 1 could be involved with tissue histolysis during larval stages, and decreases in activity during histogenetic processes during pupal development. We report here for the first time changes in DNase 1 activity in the CNS of *B. mori* during PED.

Western blot studies revealed that a correlation existed between cell death levels and the levels of caspase-3, PARP-1 and cathepsin D in the CNS of *B. mori*. PCD was high in EP and MP which correlated with complete caspase 3 cleavage, high PARP 1 levels and cleavage of cathepsin D to its subunits. In LP, where PCD is lowest, caspase 3 and cathepsin D cleavage were not so prominent, but PARP 1 levels were higher. In adult CNS, PCD again increases and could be involving a different mechanism altogether as the caspase 3 cleavage is not prominent, PARP 1 levels decrease, while a novel cathepsin D immunoreactive band is observed as compared to those observed in the pupal developmental period. In *B. mori* BmIce has been reported to be involved in apoptosis (Duan *et al*., 2005). Specific inhibitors of caspase 3 and caspase 8 have been shown to inhibit neuronal cell death (Kim *et al*., 2009). In fact caspases play very important role in

developmental cell death in all metazoans including nematodes, *Drosophila* and mouse (Shearwin-Whyatt and Kumar, 1999).

In *B. mori* we found that PARP-1 levels were higher in the CNS of pupa and decreased significantly in the adult. Probably PARP-1 could be playing important role in modulating ecdysteroids triggered changes in gene expression during the pupal stage (Tulin and Spradling, 2003). The role of PARP-1 in apoptosis (Poltronieri *et al*., 1997; Yu *et al*., 2003), in chromatin loosening and transcriptional activation (Tulin and Spradling, 2003) has been well documented.

We report that cathepsin D plays important role during PCD in the CNS of *B. mori.* This is in agreement with recent reports where cathepsin D was demonstrated to play significant role in PCD in the gut and larval fat body in *B. mori* (Gui *et al*., 2006; Lee *et al*., 2009).

Chapter 3
Effect of Developmental Hormones (JH and 20E) on Neurogenesis and Cell Death during PED

Contents

I. Introduction

Holometabolous insects molt periodically during the larval growth phase, undergo complete metamorphosis during the resting pupa and transform into the reproductive adult phase. The entire life cycle is regulated by the steroid hormones (ecdysteroids) and the sesquiterpenoid hormones (JH) (for review: Truman and Riddiford, 2002).

(i). Role of Ecdysteroid hormones during Neurometamorphosis

The ecdysteroids control the onset of molting and metamorphosis (Truman, 2005) by influencing stage-specific and tissue-specific expression of ecdysteroid receptor (EcR) isoforms in many insects such as *M. sexta* (Jindra *et al*., 1996), *A. aegypti* (Parthasarathy and Palli., 2007a), *D. melanogaster* (Talbot *et al*., 1993), the red flour beetle *Tribolium castaneum* (Tan and Palli, 2008) etc. Ecdysteroids regulate both neurogenesis (Champlin and Truman, 2000), cell death (Weeks, 2003), remodeling of persistent larval neurons (Hewes, 2008), and complete reorganization of the CNS (Amos *et al*., 1996; Himes *et al*., 2008) such that the larval CNS differs significantly from that of its adult counterpart. All these morphogenetic changes are regulated by the type of EcR expressed by a particular cell during its time of development and may involve either EcR-A in cells undergoing programmed cell death (Robinow *et al*., 1993) or EcR-B by proliferating cells or those that undergo extensive remodeling (Truman *et al*., 1994; for review: Tissot and Stocker, 2000).

Sequential pulses of 20E regulate developmental changes in CNS architecture. The decline in prepupal peak and the subsequent preadult rise in ecdysteroids are necessary for ganglionic migration and fusion to proceed normally (Amos *et al*., 1996). Initiation of ganglionic fusion under influence of ecdysteroids has been reported in case of *G. mellonella* (Robertson, 1974) and *M. sexta* (Amos *et al*., 1996). In the absence of 20E, as a result of ligation, the ganglia remain in their larval locations and immunoreactivity to transmembrane Fasciclin II (TMFasII) (cell adhesion molecule) is reduced (Himes *et al*., 2008). Such ligated abdomens show increased TMFasII immunoreactivity upon administration of 20E. The decrease in TMFasII expression could also be noted upon treatment of nerve cords with RNA or protein synthesis inhibitors (Himes *et al*., 2008) which indicates that steroid-regulated expression of TMFasII is

required for ganglionic migration and fusion. The neuroglial cells have been reported to play prominent role mediating ganglionic fusion under ecdysteroid influence (Oland and Tolbert, 2003). The selective shortening of the interganglionic connective that leads to ganglionic fusion could be due to differential expression of EcR and high ecdysone levels in hemolymph during early pupal period as shown in *M. quadrifasciata* (Pinto *et al*., 2003).

Ecdysteroids exert stage-specific effects on neuronal cell death and survival. While the prepupal peak of ecdysteroids triggers neuronal cell death (Weeks *et al*., 1992), a decline in ecdysteroids levels causes cell death post adult eclosion (Draizen *et al*., 1999). Low levels of 20E are also known to induce proliferation during development of eye in *M. sexta* (Champlin and Truman, 1998a). Like wise, role of ecdysteroids on neuronal remodeling is also well documented. The prepupal ecdysteroids peak triggers dendritic regression because of death of larval specific musculature (Weeks *et al*., 1992), while the preadult rise in ecdysteroids triggers dendritic regrowth with formation of adult musculature (Weeks and Ernt-Utzschneider, 1989).

Development of adult moth after pupation is triggered by the ecdysteroids levels which continuously increase and peak in mid pupal stage, and later decline to basal levels by adult emergence (Bollenbacher *et al*., 1981). The ecdysteroids exert their action directly on the CNS: cultured ganglia under the influence of physiological levels of 20E survived neuronal death, while its withdrawal leads to cell degeneration (Bennett and Truman, 1985). The steroid triggered neuronal cell death can be blocked by injecting the protein synthesis inhibitor cycloheximide which was shown to inhibit cell death during prepupal ecdysteroid peak in *M. sexta* (Weeks *et al*., 1993). Cycloheximide treatment also prevents the metamorphic degeneration of the ISMs (Schwartz *et al*., 1990) and post-eclosion cell death in motoneurons innervating the ISMs in *M. sexta* (Fahrbach and Truman, 1988). Himes *et al*., (2008) reported that treatment of the CNS with cycloheximide and actinomycin D (RNA synthesis inhibitor) can block cell death and consequently events leading to fusion of multiple ganglia to form compound ganglia which is again ecdysteroid regulated (Amos *et al*., 1996). Ecdysteroids also exert its mitogenic potential on *T. mollitor* accessory glands (Szopa *et al*., 1985) and epidermis in

M. sexta (Kato and Riddiford, 1987), corpora allata in *D. punctata* and *B. mori* (Chiang *et al*., 1997).

(ii). Effect of JH Analogue (Fenoxycarb) on Metamorphosis

JH synthesized in the corpora allata of all insect species, allows larval molting in presence of ecdysteroids and also prevents the switching on of gene expression necessary for metamorphosis (Truman and Riddiford, 2002). JH regulates many physiological aspects in addition to molting and metamorphosis such as reproduction (Tibbetts and Izzo, 2009), diapause (Shiga *et al*., 2003) and polyphenism (Scharf *et al*., 2007). JH has been shown to stimulate persistent neurogenesis in the adult insect brain (Cayre *et al*., 1997). It appears to indirectly regulate cell death where it modulates ecdysteroid action on metamorphosis (Wilson, 2004). JH prevents cell death in PGs of *M. sexta* (Dai and Gilbert, 1998). JH was shown to reduce the expression of ecdysteroid inducible Broad Complex protein (Reza *et al*., 2004), which in turn is responsible for regulating expression of the caspase DRONC during PCD in *D. melanogaster* (Cakouros *et al*., 2002). Very recently JH has also been shown to down regulate 20E-induced cell death by restricting mRNA levels of *dronc* and *drice*, thus preventing caspase-dependent PCD in the fat body of *D. melanogaster* (Liu *et al*., 2009).

Treatment of insects with JH or JH analogues (JHA) are known to induce morphological changes during larval-pupal (Nakamura *et al*., 2007) and pupal-adult differentiation (Rose, 2004). Fenoxycarb (FX) is a non-neurotoxic carbamate compound which acts as an effective JH mimic in a number of insects (Grenier and Grenier, 1993). It regulates insect growth in Lepidopterans by prolonging duration of larval instar, inducing permanent (dauer) larvae (Dedos and Fugo, 1999b), dauer pupae (Dedos *et al*., 2002) and also induces a supernumerary molt (Kamimura and Kiuchi, 2002). FX induces non-viable supernumerary stages in a number of insect pests (Dhadialla *et al*., 1998).

FX application at 129 to 132 h of last instar larvae of *B. mori* doesn't induce any morphological abnormalities in the pupal stages, but these become dauer pupae due to persistently higher ecdysteroid titer (Dedos *et al*., 2002). Interestingly the induction of dauer larvae upon application of FX to early last instar larvae is because of persistently low ecdysteroid titer (Monconduit and Mauchamp, 1998). However, FX treatments done

at younger larval instars (such as 3rd or 4th instar) induce an extra molt to perfect 6th instar larvae which pupate and emerge normally (Kamimura and Kiuchi, 2002).

FX injections at the pupal ecdysis in *B. mori* result in higher ecdysteroid production by PGs and elevated PTTH-secretion by the neurosecretory cells of brain. As a result the hemolymph of FX treated pupae contains higher ecdysteroid titers, which results in developmental defects in rectum formation in pharate adults and hence disturbs adult eclosion (Dedos and Fugo, 1999a). FX treatment induces abnormal rectum formation in the cat flea, *Ctenophalides felis* (Marchiondo *et al*., 1990). However, recently, it was shown that JH also antagonizes 20E i.e., JH inhibits 20E induced PCD by reducing the expression of the caspases Dronc and Drice in *D. melanogaster* (Liu *et al*., 2009).

In the previous two chapters, we studied stage-specific and region-specific changes in neurogenesis and cell death in the entire CNS during PED. In this chapter we studied:

i. Effect of 20E on neurogenesis and cell death in distinct developmental stages during PED.
ii. Effect of JHA FX on neurogenesis and cell death in distinct developmental stages during PED.

II. Materials and Methods

(i). General Methodologies for Maintaining B. mori

As discussed in Chapter 1 (Page No. 51). For hormone treatment, 4I, 5EI, 5LI and EP were used.

(ii). Fenoxycarb Treatment

FX (ethyl 2-(4-phenoxyphenoxy) ethylcarbamate, $C_{17}H_{19}NO_4$, PESTANAL®, analytical standard grade) was commercially purchased from Fluka (Sigma-Aldrich). Stock solutions (200 mg/ml) of FX were made in acetone, as it is relatively less soluble in water. However, prior to application working dilutions consisting of 1 µg/ 5 µl of acetone was prepared from stock solution. 5 µl of this diluted hormone was topically applied to

each insect along dorsal midline with the help of a micropipette (Dedos and Fugo, 1999b). For vehicle control, 5 µl of acetone was topically applied in the same manner.

(iii). Ecdysone Treatment

20E (Sigma) was dissolved in 10% ethanol in Insect Ringer's saline (vehicle), and topically applied onto fresh mulberry leaves (50 µg/leaf) of approximately 100 cm^2 area (Chiang *et al*., 1997). EP were injected with 50 µg 20E in 20 µl vehicle (Kamimura *et al*., 2007). Control larvae were fed on leaves treated topically with the vehicle (10% ethanol in Insect Ringer's saline), control pupae were injected with 20 µl of vehicle alone.

(iv). Neurogenesis and Cell Death

The CNS from treated insects was removed for studying neurogenesis or cell death 24 h after treatment with either hormone. Neurogenesis was studied using *in vitro* wholemount BrdU labeling, as described in the methodologies in Chapter 1 (Page No. 52). Cell death was studied using wholemount TUNEL labeling method as described in Chapter 2 (Page No. 75).

(v). Statistical analysis

Effect of FX and 20E on neurogenesis and cell death in different ganglia of the CNS in different developmental stages was studied by performing Students t-test. All statistical tests were performed with SigmaStat™ (Jandel Scientific). Data were expressed as Mean ± SE (n = 4) ($p \leq 0.05$).

III. Results

(i). Effect of developmental hormones on Neurogenesis

(a) Effect of FX on Neurogenesis

FX treatment resulted in stage-specific and region-specific changes in neurogenesis in the CNS of distinct stages during PED. FX treatment significantly reduced BrdU labeled cells in different regions of the CNS of 4I larvae ($p \leq 0.05$), except in brain (vehicle 246 ± 14 to hormone 198 ± 26) where the decrease was not significant

(Table 5; Fig. 24). FX treatment resulted in significant decrease in neurogenesis by as much as 50% decrease in number of BrdU positive cells in SEG (46 ± 7 to 21 ± 3), 45% in T_1 (64 ± 6 to 36 ± 6), T_2 (105 ± 5 to 47 ± 2), Ab_2 (105 ± 12 to 58 ± 10) and Ab_3 (67 ± 3 to 36 ± 2), 40% in T_3 (105 ± 7 to 65 ± 6) and Ab_7 (122 ± 6 to 73 ± 6), 65% in Ab_1 (115 ± 16 to 40 ± 9), 75% in Ab_4 (72 ± 7 to 17 ± 1), 60% in Ab_5 (87 ± 4 to 34 ± 1) and 25% in Ab_6 (88 ± 2 to 65 ± 5) ($p \leq 0.05$) (Table 5; Fig. 24).

In 5EI, FX treatment resulted in 1.5 fold increase in neurogenesis in brain (196 ± 14 to 293 ± 12), almost a 2-fold increase in SEG (41 ± 3 to 95 ± 2), T_1 (106 ± 12 to 189 ± 8) and Ab_4 (48 ± 3 to 93 ± 3), but a 60% decrease in neurogenesis was observed in T_3 as compared to the vehicle treatment (93 ± 3 to 36 ± 4) ($p \leq 0.05$). There was no change in neurogenesis counts in Ab_2 (58 ± 2 to 61 ± 3), Ab_3 (43 ± 3 to 46 ± 6) and Ab_5 (Fig. 24). Interestingly, no neurogenesis was observed upon vehicle treatment in T_2 and Ab_1 but FX resulted in neurogenesis in T_2 (81 ± 7) and Ab_1 (62 ± 3) ($p \leq 0.05$). However, FX treatment did not induce any neurogenesis in Ab_6 and Ab_7 (Table 5; Fig. 24).

In 5LI, FX treatment decreased neurogenesis by as much as 45% in SEG (80 ± 5 to 45 ± 7), Ab_2 (33 ± 1 to 18 ± 1) and Ab_3 (44 ± 4 to 25 ±2), 50% in T_1 (101 ± 13 to 53 ± 7), 65% in T_2 (61 ± 3 to 22 ± 2) and 55% in Ab_4 (55 ± 5 to 24 ± 5). FX treatment resulted in significantly increased neurogenesis by 1.25 fold in brain (369 ± 9 to 463 ± 13) and Ab_6 (43 ± 3 to 53 ± 1), and 1.4 fold in Ab_7 (78 ± 6 to 110 ± 2) ($p \leq 0.05$); no change was observed in BrdU counts in T_3 (60 ± 11 to 42 ± 5), Ab_1 (33 ± 4 to 26 ± 5) and Ab_5 (44 ± 5 to 42 ± 6) (Table 5; Fig. 24).

In EP, FX treatment resulted in significant decrease in neurogenesis by as much as 30% in brain (597 ± 24 to 408 ± 14), while neurogenesis showed a robust 4-fold increase in T_2 (43 ± 4 to 164 ± 9), 3 fold in T_3 (71 ± 7 to 224 ± 13), 1.8 fold in Ab_1 (120 ± 15 to 224 ± 9), 1.5 fold in Ab_2 (142 ± 7 to 214 ± 10) and 1.3 fold in Ab_5 (139 ± 10 to 184 ± 12) ($p \leq 0.05$). FX treatment did not have any affect on neurogenesis counts in SEG (196 ± 13 to 161 ± 16), T_1 (144 ± 6 to 172 ± 10), Ab_3 (148 ± 18 to 173 ± 15), Ab_4 (127 ± 12 to 156 ± 14), Ab_6 (202 ± 19 to 236 ± 9) and Ab_7 (248 ± 16 to 263 ± 8) (Table 5; Fig. 24).

(b) Effect of 20E on Neurogenesis

Neurogenesis was significantly increased upon treatment with 20E in CNS of 4I by 1.2 fold in SEG (176 ± 5 to 214 ± 13) and T_3 (144 ± 2 to 167 ± 7), 1.4 fold in Ab_1 (99 ± 5 to 138 ± 11) and 2 fold in Ab_2 (75 ± 2 to 142 ± 7), but decreased by 26% in T_1 (139 ± 10 to 103 ± 5), 40% in T_2 (147 ± 2 to 88 ± 6) and Ab_4 (65 ± 2 to 39 ± 4), and 50% in Ab_7 (189 ± 6 to 96 ± 4) ($p \leq 0.05$), while no changes were observed in brain (356 ± 13 to 321 ± 31), Ab_3 (75 ± 7 to 97 ± 15), Ab_5 (84 ± 3 to 71 ± 8) and Ab_6 (53 ± 4 to 51 ± 4) (Table 6; Fig. 25).

In 5EI, significant increase in BrdU counts were observed by as much as 1.5 fold in brain (167 ± 3 to 252 ± 17), 3.5 fold in SEG (38 ± 5 to 134 ± 6), 2.7 fold in T_1 (73 ± 5 to 199 ± 15), 2 fold in T_3 (34 ± 3 to 70 ± 7), Ab_1 (40 ± 5 to 88 ± 4) and Ab_2 (60 ± 3 to 135 ± 3), and 2.4 fold in Ab_3 (43 ± 4 to 109 ± 4) ($p \leq 0.05$), while no changes in BrdU counts were observed in Ab_5 (41 ± 6 to 49 ± 5) (Table 6; Fig. 25). However, 20E treatment resulted in neurogenesis in T_2 (90 ± 2), Ab_4 (71 ± 5), Ab_6 (58 ± 3) and Ab_7 (100 ± 12) (Fig. 25) ($p \leq 0.05$) where as neurogenesis was absent in control insects.

In 5LI, 20E treatment increased neurogenesis counts in the entire CNS: 1.6 fold in brain (344 ± 15 to 606 ± 40) and Ab_5 (81 ± 6 to 135 ± 7), 2 fold in SEG (63 ± 6 to 122 ± 15), Ab_1 (108 ± 12 to 198 ± 9), Ab_3 (78 ±3 to 158 ± 4) and Ab_7 (166 ± 8 to 349 ± 4), 2.6 fold in T_1 (53 ± 5 to 137 ± 6) and T_2 (50 ± 5 to 132 ± 9), 3.2 fold in T_3 (62 ± 8 to 201 ± 6), 2.5 fold in Ab_2 (72 ± 11 to 178 ± 3) and Ab_4 (78 ± 4 to 191 ± 7), and 4 fold in Ab_6 (24 ± 2 to 104 ± 9) (Table 6; Fig. 25) ($p \leq 0.05$).

In EP upon 20E treatment neurogenesis increased by 1.3 fold in brain (712 ± 29 to 946 ± 17), but decreased by more than 60% in SEG (245 ± 4 to 93 ± 3), 55% in T_1 (291 ± 8 to 129 ± 4), 35% in T_2 (247 ± 3 to 162 ± 4) and Ab_6 (267 ± 6 to 177 ± 3), 40% in Ab_3 (260 ± 6 to 160 ± 5), 30 % in Ab_4 (228 ± 6 to 159 ± 3) ($p \leq 0.05$). The neurogenesis was not affected significantly by 20E treatment in T_3 (158 ± 4 to 154 ± 4), Ab_1 (112 ± 8 to 108 ± 5), Ab_2 (99 ± 3 to 100 ± 6), Ab_5 (216 ± 3 to 208 ± 4) and Ab_7 (400 ± 10 to 418 ± 7) (Table 6; Fig. 25).

(ii). Effect of developmental hormones on PCD

In the present study, both FX and 20E caused region specific changes in apoptotic cell counts (TUNEL) in EP only. However, cell death was not detectable in 4I, 5EI and 5LI upon treatment with either hormones or their vehicle controls.

(a) Effect of FX on PCD

FX treatment significantly increased cell death in EP by about 1.5 fold in SEG (197 ± 7 to 290 ± 27), T_1 (170 ± 23 to 272 ± 10) and Ab_6 (73 ± 6 to 117 ± 16), 1.2 fold in Ab_4 (121 ± 5 to 150 ± 8) and Ab_7 (193 ± 15 to 247 ± 6), and 2 fold in Ab_5 (67 ± 15 to 126 ± 5) ($p \leq 0.05$), while the counts did not change significantly in other regions of the CNS including brain (488 ± 33 to 676 ± 77), T_2 (128 ± 11 to 107 ± 7), T_3 (132 ± 5 to 142 ± 12), Ab_1 (152 ± 17 to 96 ± 18), Ab_2 (95 ± 7 to 100 ± 14) and Ab_3 (112 ± 7 to 138 ± 15) (Table 7; Fig. 26).

(b) Effect of 20E on PCD

Treatment with 20E significantly increased cell death in EP by 1.1 fold in brain (658 ± 7 to 699 ± 12), 1.3 fold in T_2 (153 ± 5 to 203 ± 8), 1.7 fold in Ab_3 (82 ± 5 to 143 ± 3) and Ab_6 (82 ± 3 to 147 ± 4), and 2 fold in Ab_7 (203 ± 9 to 384 ± 9). 20E treatment significantly decreased cell death by 30% in Ab_4 (267 ± 8 to 183 ± 3) and Ab_5 (243 ± 3 to 173 ± 4) ($p \leq 0.05$), while in other regions including SEG (180 ± 10 to 176 ± 9), T_1 (112 ± 4 to 101 ± 6), T_3 (123 ± 3 to 131 ± 5), Ab_1 (144 ± 4 to 144 ± 6) and Ab_2 (107 ± 3 to 106 ± 5) was not affected (Table 7; Fig. 27).

Table 5: Effect of Fenoxycarb on Neurogenesis in CNS of *B. mori*

Developmental Stages	4I		5EI		5LI		EP	
	Control	FX	Control	FX	Control	FX	Control	FX
Brain	246 ± 14	198 ± 26	196 ± 14	293 ± 12*	369 ± 9	463 ± 13*	597 ± 24	408 ± 14*
SEG	46 ± 7	21 ± 3*	41 ± 3	95 ± 2*	80 ± 5	45 ± 7*	196 ± 13	161 ± 16
T_1	64 ± 6	36 ± 6*	106 ± 12	189 ± 8*	101 ± 13	53 ± 7*	144 ± 6	172 ± 10
T_2	105 ± 5	47 ±2*	0	81 ± 7*	61 ± 3	22 ± 2*	43 ± 4	164 ± 9*
T_3	105 ± 7	65 ± 6*	93 ± 3	36 ± 4*	60 ± 11	42 ± 5	71 ± 7	224 ± 13*
Ab_1	115 ± 16	40 ± 9*	0	62 ± 3*	33 ± 4	26 ± 5	120 ± 15	224 ± 9*
Ab_2	105 ± 12	58 ± 10*	58 ± 2	61 ± 3	33 ± 1	18 ± 1*	142 ± 7	214 ± 10*
Ab_3	67 ± 3	36 ± 2*	43 ± 3	46 ± 6	44 ±4	25 ± 2*	148 ± 18	173 ± 15
Ab_4	72 ± 7	17 ± 1*	48 ± 3	93 ± 3*	55 ± 5	23 ± 5*	127 ± 12	156 ± 14
Ab_5	87 ± 4	34 ±1*	54 ± 5	52 ± 3	44 ± 5	42 ± 6	139 ± 10	184 ± 12*
Ab_6	88 ± 2	65 ± 5*	0	0	43 ± 3	53 ± 1*	202 ± 19	236 ± 9
Ab_7	122 ± 6	73 ± 6*	0	0	78 ± 6	110 ± 2*	248 ± 16	263 ± 8

Stage-specific and region-specific changes in neurogenesis were observed in the CNS of *B. mori* in 4I, 5EI, 5LI and EP. Each value is Mean ± SE (n = 4). * indicates significant changes in number of BrdU positive cells upon FX treatment as compared with vehicle controls (Student's t-test; $p \leq 0.05$).

Table 6: Effect of 20E on Neurogenesis in CNS of *B. mori*

Developmental Stages	4I		5EI		5LI		EP	
	Control	20E	Control	20E	Control	20E	Control	20E
Brain	356 ± 13	321 ± 31	167 ± 3	252 ± 17*	344 ± 15	606 ± 40*	712 ± 29	946 ± 17*
SEG	176 ± 5	214 ± 13*	38 ± 5	134 ± 6*	63 ± 6	122 ± 15*	245 ± 4	93 ± 3*
T_1	139 ± 10	103 ± 5*	73 ± 5	199 ± 15*	53 ± 5	137 ± 6*	291 ± 8	129 ± 4*
T_2	147 ± 2	88 ± 6*	0	90 ± 2*	50 ± 5	132 ± 9*	247 ± 3	162 ± 4*
T_3	144 ± 2	167 ± 7*	34 ± 3	70 ± 7*	62 ± 8	201 ± 6*	158 ± 4	154 ± 4
Ab_1	99 ± 5	138 ± 11*	40 ± 5	88 ± 4*	108 ± 12	198 ± 9*	112 ± 8	108 ± 5
Ab_2	75 ± 2	142 ± 7*	60 ± 3	135 ± 3*	72 ± 11	178 ± 3*	99 ± 3	100 ± 6
Ab_3	75 ± 7	97 ± 15	43 ± 4	109 ± 4*	78 ± 3	158 ± 4*	260 ± 6	160 ± 5*
Ab_4	65 ± 2	39 ± 4*	0	71 ± 5*	78 ± 4	191 ± 7*	228 ± 6	159 ± 3*
Ab_5	84 ± 3	71 ± 8	41 ± 6	49 ± 5	81 ± 6	135 ± 7*	216 ± 3	208 ± 4
Ab_6	53 ± 4	51 ± 4	0	58 ± 3*	24 ± 2	104 ± 9*	267 ± 6	177 ± 3*
Ab_7	189 ± 6	96 ± 4*	0	100 ± 12*	166 ± 8	349 ± 4*	400 ± 10	418 ± 7

Stage-specific and region-specific changes in neurogenesis were observed in the CNS of *B. mori* in 4I, 5EI, 5LI and EP. Each value is Mean ± SE (n = 4). * indicates significant changes in number of BrdU positive cells upon 20E treatment as compared with vehicle controls (Student's t-test; $p \leq 0.05$).

Table 7: Effect of FX and 20E on PCD in the CNS of *B. mori*

Developmental Stages	EP		EP	
	Control	FX	Control	20E
Brain	488 ± 33	676 ± 77	658 ± 7	699 ± 12*
SEG	197 ± 7	290 ± 27*	180 ± 10	176 ± 9
T_1	170 ± 23	272 ± 10*	112 ± 4	101 ± 6
T_2	128 ± 11	107 ± 7	153 ± 5	203 ± 8*
T_3	132 ± 5	142 ± 12	123 ± 3	131 ± 5
Ab_1	152 ± 17	96 ± 18	144 ± 4	144 ± 6
Ab_2	95 ± 7	100 ± 14	107 ± 3	106 ± 5
Ab_3	112 ± 7	138 ± 15	82 ± 5	143 ± 3*
Ab_4	121 ± 5	150 ± 8*	267 ± 8	183 ± 2*
Ab_5	67 ± 15	126 ± 5*	243 ± 3	173 ± 4*
Ab_6	73 ± 6	117 ± 16*	82 ± 3	147 ± 4*
Ab_7	193 ± 15	247 ± 6*	203 ± 9	384 ± 9*

Region-specific changes in PCD were observed in the CNS of *B. mori*. FX and 20E treatment did not induce any cell death in the CNS of 4I, 5EI and 5LI. Each value is Mean ± SE (n = 4). * indicates significant changes in number of TUNEL positive cells upon 20E treatment as compared with vehicle control (Student's t-test; $p \leq 0.05$).

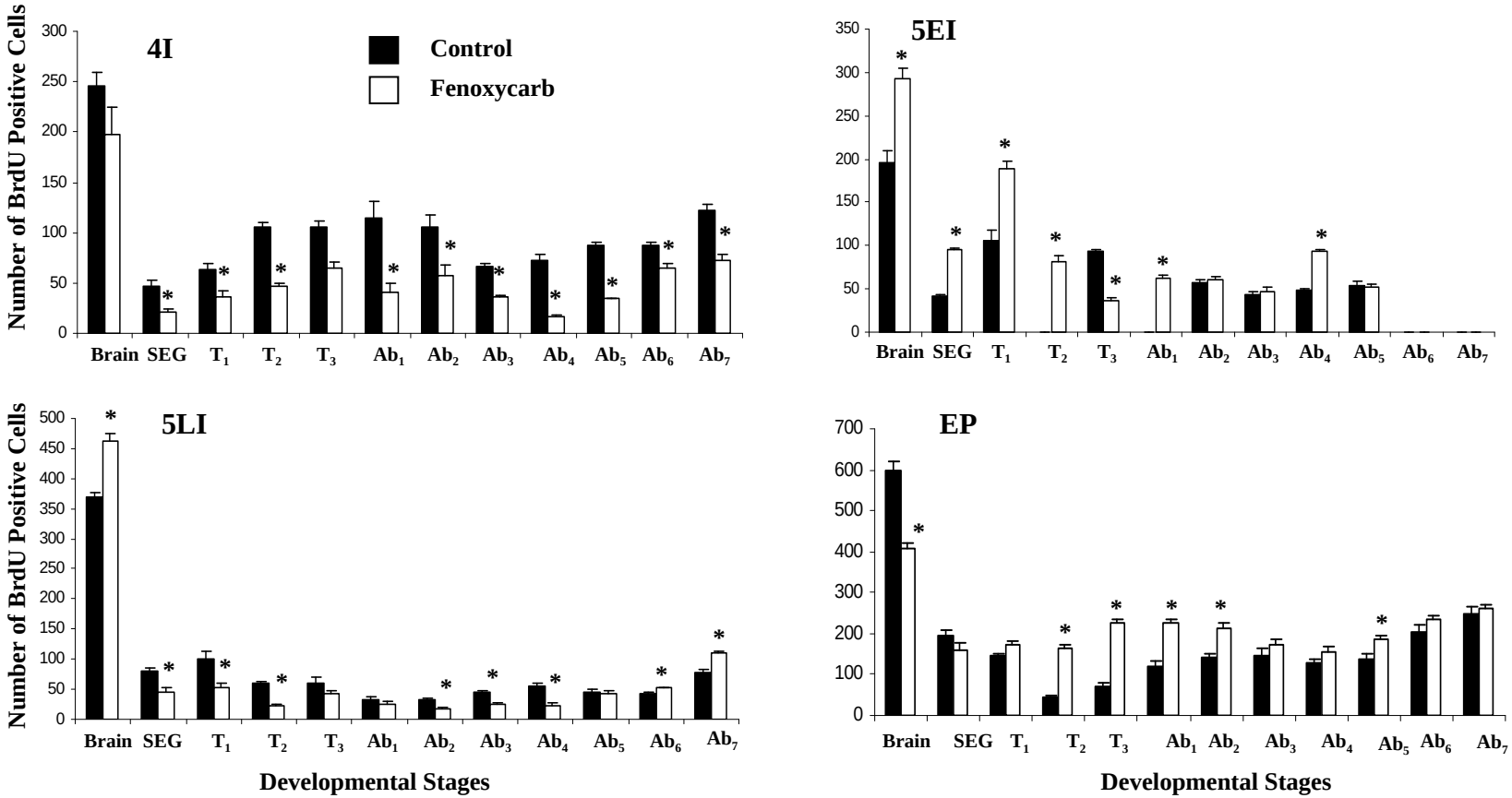


Figure 24: Stage-specific and region-specific changes in neurogenesis upon treatment with FX. FX treatment significant reduced neurogenesis in the entire CNS in 4I. In 5EI, neurogenesis increased in brain, SEG, T_1, T_2, Ab_1 and Ab_4, but reduced in T_3. In 5LI neurogenesis increased in brain, Ab_6 and Ab_7, but reduced in SEG, T_1, T_2, Ab_2, Ab_3 and Ab_4. In EP neurogenesis increased in T_2, T_3, Ab_1, Ab_2 and Ab_5, but reduced in Brain. Each value is Mean ± SE (n = 4). * indicates significant changes between hormone and vehicle treatment (Student's t- test; $p \leq 0.05$).

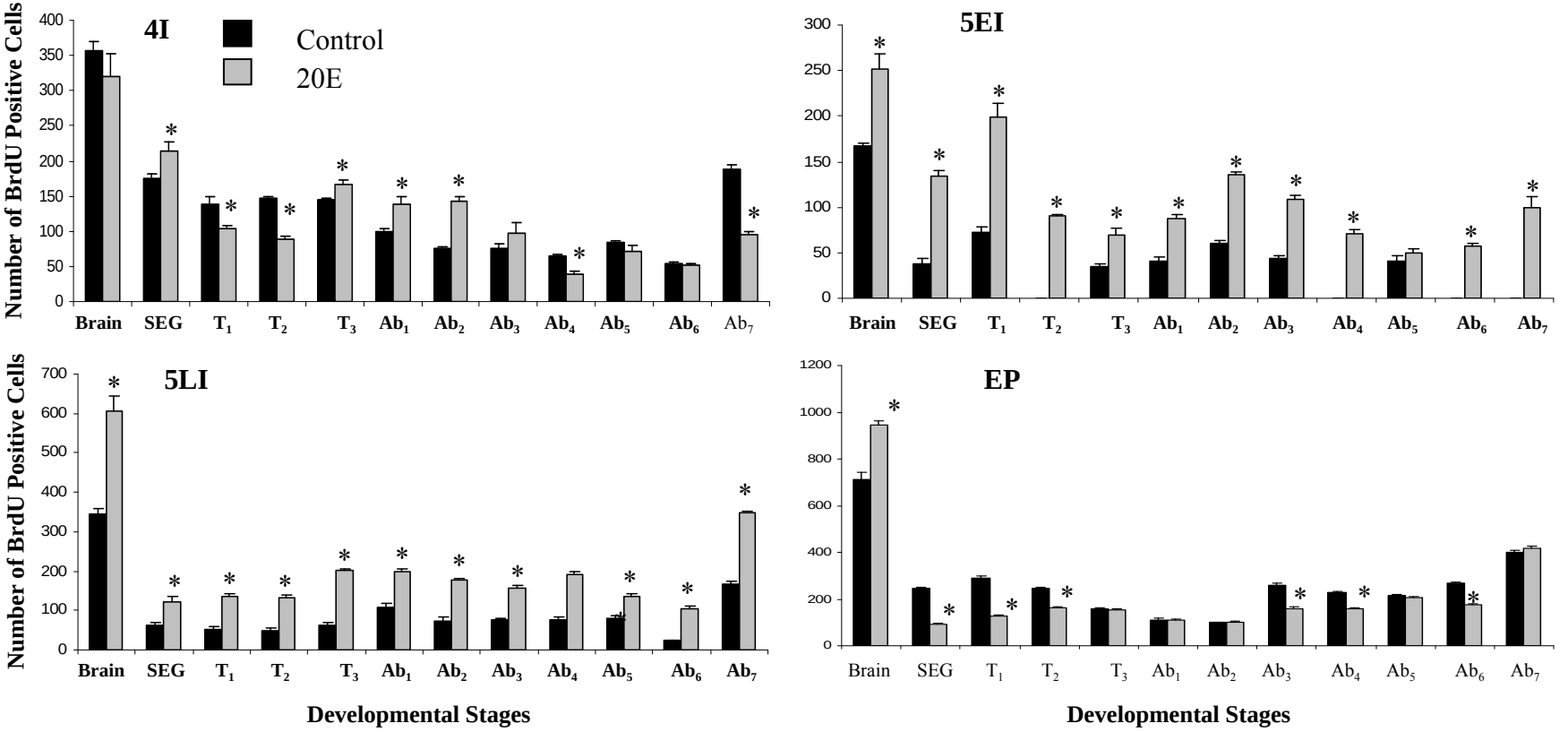


Figure 25: Stage-specific and region-specific changes in neurogenesis upon treatment with 20E. Neurogenesis increased in 4I in SEG, T_3, Ab_1 and Ab_2 but reduced in T_1, T_2, Ab_4 and Ab_7. 20E treatment significant increased neurogenesis in the entire CNS in 5EI as well as 5LI. In EP, neurogenesis increased in brain, but reduced in SEG, T_1, T_2, Ab_3, Ab_4 and Ab_6. Each value is Mean ± SE (n = 4). * indicates significant changes between hormone and vehicle treatment (Student's t-test; $p \leq 0.05$).

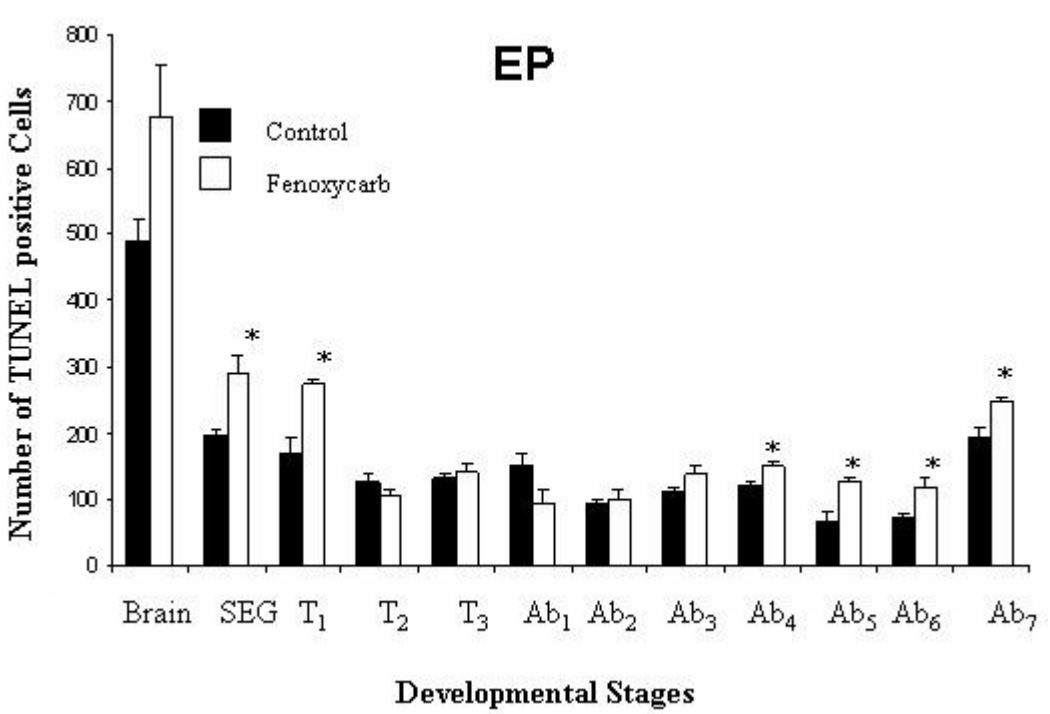


Figure 26: Region-specific changes in cell death as a result of FX treatment. Cell death increased in SEG, T_1, Ab_4, Ab_5, Ab_6 and Ab_7, but in the other ganglia the changes were not significant. No cell death was detected in the 4I, 5MI and 5LI upon treatment with vehicle or the hormone. Each value is Mean ± SE (n = 4). * indicates significant changes between hormone and vehicle treatment (Student's t- test; $p \leq 0.05$).

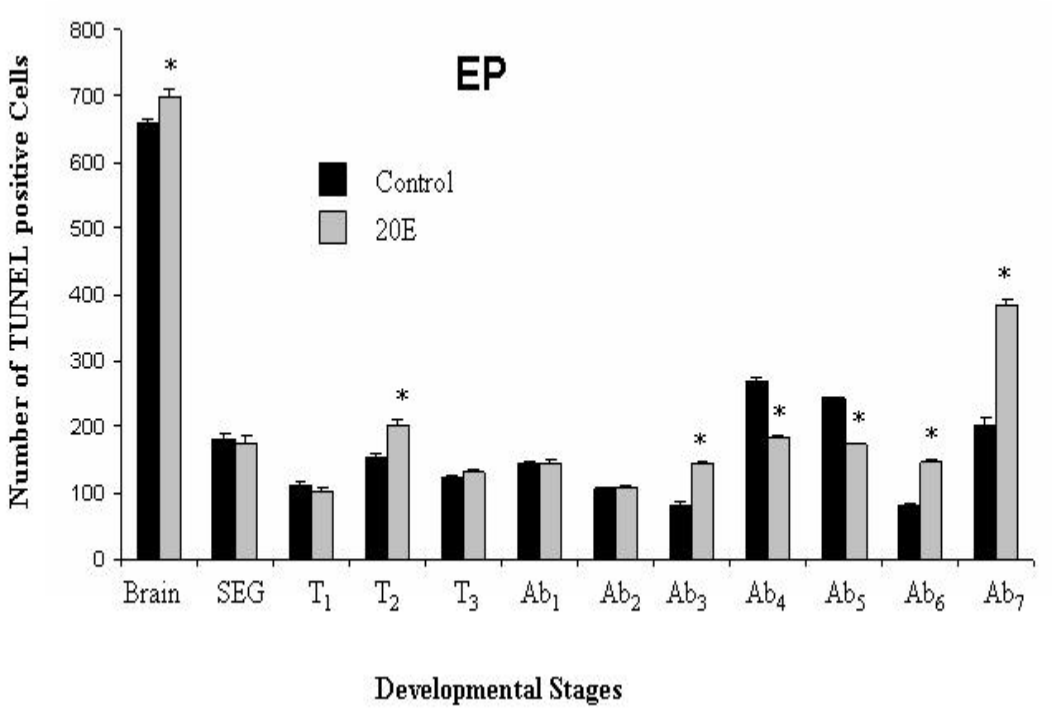


Figure 27: Region-specific changes in cell death induced by treatment with 20E. Cell death increased in brain, T_2, Ab_3, Ab_6 and Ab_7, but decreased in Ab_4 and Ab_5. No cell death was detected in the 4I, 5MI and 5LI upon treatment with vehicle or the hormone. Each value is Mean ± SE (n = 4). * indicates significant changes between hormone and vehicle treatments (Student's t- test; $p \leq 0.05$).

IV. Discussion

(i). Effect of 20E and FX on neurogenesis

Stage-specific and region-specific changes in neurogenesis were observed in the CNS of *B. mori* upon treatment with the ecdysteroid (20E) and the JH analogue (FX). In some ganglia, both hormones had similar effects on neurogenesis or cell death, while in other ganglia, the effects were hormone-specific. For example neither FX nor 20E treatment results in any significant changes in neurogenesis in the brain of 4I. In 5EI and 5LI both FX and 20E treatment resulted in significant increase in neurogenesis in brain. FX decreased neurogenesis in brain in EP, but 20E showed an opposite effect.

Neurogenesis in SEG increased in 5EI upon treatment with either of the hormones, but FX treatment resulted in a decrease in 4I and 5LI, while 20E caused an increase in the same stages. FX treatment did not produce any significant change in neurogenesis in SEG EP, while 20E treatment resulted in decreased in neurogenesis.

In the thoracic ganglia (T_1, T_2 and T_3) of 4I, treatment with either FX or 20E resulted in decrease in neurogenesis, except in T_3, in which 20E treatment caused an increase. FX and 20E treatment increased neurogenesis in T_1 in 5EI, while these resulted in neurogenesis in T_2 where it was absent in controls. In T_3 of 5EI, the hormones had contrasting effects on neurogenesis, where FX treatment reduced neurogenesis, while 20E treatment increased it. In 5LI, 20E treatment reduced BrdU counts in all thoracic ganglia, while FX reduced the counts in T_1 and T_2 only. FX did not alter neurogenesis counts in T_3 of 5LI. FX treatment increased neurogenesis in T_2 and T_3 of EP, while no changes were observed in T_1. However, 20E treatment reduced neurogenesis in T_1 and T_2 in EP, but did not alter it in T_3.

FX treatment reduced neurogenesis in all the abdominal ganglia (Ab_1 to Ab_7) of 4I. 20E similarly reduced neurogenesis in Ab_4 and Ab_7 in 4I, but increased neurogenesis in Ab_1 and Ab_2, while no changes were observed in neurogenesis in Ab_3, Ab_5 and Ab_6. FX treatment resulted in neurogenesis in Ab_1 of 5EI where it was absent in controls, while it increased in Ab_4. No changes were observed in neurogenesis upon FX treatment in Ab_2, Ab_3 and Ab_5, while neurogenesis was not detected in Ab_6 and Ab_7. 20E treatment increased neurogenesis in Ab_1, Ab_2 and Ab_3 of 5EI, while it resulted in neurogenesis in Ab_4, Ab_6 and Ab_7 where neurogenesis was absent in controls; however it did not affect

neurogenesis counts in Ab_5. In 5LI, FX reduced neurogenesis in Ab_2, Ab_3 and Ab_4, but increased it in Ab_6 and Ab_7, while it did not affect the counts in Ab_1 and Ab_5. However, 20E increased neurogenesis in all the abdominal ganglia of 5LI. FX increased neurogenesis in Ab_1, Ab_2 and Ab_5 of EP, while it did not affect neurogenesis levels in other abdominal ganglia. 20E reduced neurogenesis in Ab_3, Ab_4 and Ab_6 of EP, while it did not affect neurogenesis in other abdominal ganglia. Interestingly, FX and 20E treatment had little effect on neurogenesis on Ab_5, where FX treatment reduced neurogenesis in 4I, while decreased it in EP; however, 20E treatment increased neurogenesis in Ab_5 in 5LI, while it did not show any effect in other stages.

In the present study using *B. mori* as a model we found that 20E caused stage-specific and region-specific changes in neurogenesis in brain and all ganglia of the CNS during different developmental stages which have not been reported earlier. 20E treatment resulted in increase in neurogenesis in brain in EP which is in agreement with earlier reports that moderate levels of 20E stimulate proliferation in resting neuroblasts in differentiating optic lobes of *M. sexta* pupae (Champlin and Truman, 1998b). But 20E appears to influence neurogenesis in species-specific manner as it inhibits mitotic activity of neuronal precursors in the developing MB in the pupal stages of honeybee (Malun *et al*., 2003).

In present study, FX treatment resulted in increased proliferating potential in the entire CNS of the last instar larvae (5EI and 5LI). Our results in *B. mori* are in agreement with earlier reports that higher JH titers are required to maintain proliferation of neuroblasts in the brain of the late feeding phase (last instar larva) of honey bee (Vitt and Hartfelder, 1998), where low mitotic activity correlates with low JH titer (Rachinsky *et al*., 1990).

(ii). Effect of 20E and FX on Cell Death

Stage-specific and region-specific changes in cell death as detected by TUNEL assay were observed in the CNS of *B. mori* upon treatment with the ecdysteroid, 20E and the JH analogue.

In present study, we report that cell death in brain was increased slightly upon treatment with 20E. There was no significant change in cell death counts in T_3, Ab_1 and

Ab_2, which specifically are destined to undergo ganglionic fusion by the mid pupa (MP); however, Ab_6 and Ab_7 which do fuse in MP, show higher cell death upon treatment with 20E. It has been reported that supernumerary adult specific neuron precursors undergo PCD upon exposure with high ecdysteroids levels in prepupal and pupal stages (Truman *et al*., 1994). Exogenous application of 20E accelerates demise of nuclear material from muscles of the adult (Hegstrom and Truman, 1996) as well as muscles and motoneurons of larvae (Weeks and Truman, 1985) in the *M. sexta*. High levels of 20E have also been reported to increase apoptosis in the optic lobes of *M. sexta* pupa (Champlin and Truman, 1998a).

FX treatment significantly increased PCD in EP in SEG, T_1, Ab_4, Ab_5, Ab_6 and Ab_7. It showed an antagonistic activity to 20E in Ab_4 and Ab_5 regions only in which PCD is decreased upon 20E treatment. Recent reports have demonstrated this effect on fat body destruction during metamorphosis in *D. melanogaster* (Liu *et al*., 2009).

Chapter 4

Role of Serotonin in CNS remodeling during PED

Contents

I. Introduction

Postembryonic development (PED) in holometabolous insects involves major modifications in the CNS including neurogenesis, cell death and remodeling of existing larval neurons such that larval behaviors are replaced by the adult behavior (for review: Tissot and Stocker, 2000). Apart from developmental changes in the neuronal architecture, major biochemical changes could also involve intrinsic changes in the amount of neurotransmitter within neural networks and in some cases alteration of neurotransmitter phenotype itself (Witten and Truman, 1991).

Biogenic amines (BAs) such as serotonin, octopamine and dopamine play important roles in the insect CNS as neurotransmitters, neuromodulators and neurohormones, influencing the expression of different sets of behaviors during PED in holometabolous insects such as the bertha armyworm *Mamestra configurata* (Bodnaryk, 1980), *A. mellifera* (Wagener-Hulme *et al*., 1999), *M. sexta* (Homberg and Hildebrand, 1989), *D. melanogaster* (Monastirioti, 1999) etc.

In Lepidopteran insects highly coordinated changes occur in the biosynthesis, release and inactivation of prothoracicotrophic hormones (PTTH) (Bollenbacher and Granger, 1985), ecdysteroids and JH (Baker *et al*., 1987). These hormonal changes are necessary for larval-pupal metamorphosis. The BAs could be playing a major role in hormonal regulation of metamorphic events, as their presence has been reported in the insect brain, corpora allata (CA) and corpora cardiaca (CC) in many insect species including *S. gregaria*, *P. americana* (Evans, 1978), *L. migratoria*, (David and Lafon-Cazal, 1979), *D. punctata* (Thompson *et al*., 1990), *M. sexta* (Krueger *et al*., 1990) etc. Mesce (2002) reported that ecdysteroids also modulate the BAs: serotonin, octopamine and dopamine in *M. sexta* through feedback mechanisms.

Alterations in the type of major aminergic neurotransmitter have been reported in the brain of developing honeybee. Taylor *et al*., (1992) reported an increase in serotonin levels in honeybee brain upon adult emergence, but the levels remain relatively constant throughout the adulthood; on the other hand dopamine levels were not significantly different between the late pupa and newly emerged adults, but increased significantly during adult behavioral development. Such plasticity in neurotransmitter expression has been observed even in case of peptidergic neurons in the hawk moth *M. sexta* (Mcgraw *et*

al., 1998). The lateral neurosecretory cells in this insect primarily express cardioacceleratory peptide 2 (CAP2) during embryonic and larval stages. However, during metamorphosis they begin producing the peptide hormone, bursicon, which is responsible for cuticular sclerotization (Tublitz and Loi, 1993). Developmental changes in BAs have been reported in many insects including *L. gregaria* (Rogers *et al*., 2004), *B. mori* (Takeda *et al*., 1991; Hirashima *et al*., 1999), *M. sexta* (Geng *et al*., 1993) etc.

(i). Metabolism of Serotonin in Insects

Serotonin (5-hydroxytryptamine, 5-HT) in invertebrates is synthesized in the interneurons from the essential amino acid tryptophan (Livingstone and Tempel, 1983; Osborne and Neuhoff, 1974). Tryptophan is taken up in serotonergic neurons and converted to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase. 5-HTP is then decarboxylated into serotonin (5-hydroxytryptamine, 5-HT) by 5-HTP decarboxylase (Siegel, *et al*., 1998). Tryptophan hydroxylase occurs in two isoforms in insects (Coleman and Neckameyer, 2005). *Drosophila* tryptophan hydroxylase (DTRH) is primarily expressed in neurons, and shares significant homology with mammalian neuronal TPH2 (Walther *et al*., 2003; Coleman and Neckameyer, 2005). *Drosophila* tryptophan-phenylalanine hydroxylase (DTPH) has a dual role in hydroxylating tryptophan as well as phenyalanine (Neckameyer *et al*., 2007) plays a crucial role in peripheral functions and expression, and is homologous to TPH1 in mammalian systems.

In *D. melanogaster*, the DTPH gene itself has two alternative transcripts that are expressed differentially during PED. The DTPH mRNA type I appear throughout PED, while DTPH mRNA type II levels are scarce in first or second larval instars but are not detectable in 2 or 3-day-old pupae (Ruiz-Vázquez *et al*., 1996).

The pathway for inactivation of 5-HT is not clear, as the activity of monoamine oxidase is very low in insect nervous system (Sloley, 2004)). In insects 5-HT is metabolized by either N-acetylation as in *D. melanogaster* (Dewhurst *et al.,* 1972), *A. mellifera* (Evans and Fox, 1975), *L. migratoria* (Hayashi *et al.,* 1977) or by sulphate conjugation in the mosquito *Aedes togoi* (Khoo and Wong, 1993).

In insects 5-HT exerts its action through its receptors. In *Drosophila* four 5-HT receptors have been identified: d5-HT_{1A}, d5-HT_{1b}, d5-HT_2 and d5-HT_7 (Witz *et al*., 1990;

Saudou *et al*., 1992; Colas *et al*., 1995), all of which are G-protein coupled receptors sharing significant sequence similarities with their mammalian counterparts. The d5-HT_{1A} receptor has been implicated in regulating sleep-wake cycle, as the mutant flies have short-fragmented sleep (Yuan *et al*., 2006). On the other hand d5-HT receptor functions in circadian response to light (Yuan *et al*., 2005). 5-HT receptors have been identified in Lepidopteran insects such as *M. sexta* (Dacks *et al*., 2006), *B. mori* and *H. virescens* (von Nickisch-Rosenegk *et al*., 1996).

(ii). Serotonin Functions in Insect Physiology

Serotonin affects a number of physiological processes. 5-HT was suggested to inhibit neurosecretory activity of brain during early days of diapause induction and high 5-HT levels are required for maintenance of diapause in *P. brassicae* pupa (Puiroux *et al*., 1990). 5-HT stimulates release of JH from the corpora allata of honey bee worker larvae regulating caste production (Rachinsky, 1994). It could be involved in release of PTTH from neurosecretory cells in the brain of silkworm pupae (Aizono *et al*., 1997). 5-HT modulates circadian rhythmicity of insect behaviors (Jagota and Habibulla, 1992a; Cymborowski, 1998) mediated via inhibitory affect on brain neuroseceretory cells, which control PED (Muszyńska-Pytel and Cymborowski, 1978). 5-HT has been shown to regulate sensitivity of photoreceptors between day and night (Cuttle *et al*., 1995). It acts as a circadian modulator of olfactory sensitivity (Gatellier *et al*., 2004). An increase in brain 5-HT levels increases aggression, whereas its depletion results in decreased aggression in insects (Libersat and Pflueger, 2004).

(iii). Morphoregulatory actions of Serotonin on Insect Growth and Development

Detection of 5-HT in the neurons of *D. melanogaster* (Lundell and Hirsh, 1994) and its presence in outgrowing neurites indicates its involvement in the neuronal circuits as a neurotransmitter as well as in developmental functions. 5-HT may also regulate neural outgrowth during insect development as it was shown in *Ddc* mutant *D. melanogaster* unable to synthesize 5-HT (Budnik *et al*., 1989) that the branching pattern of peripheral 5-HT containing fibers innervating the gut show a 2-fold increase in the extent of branching as compared to the wild type. Growth enhancement of antennal lobe

neurons of *M. sexta* in vitro (Mercer *et al*., 1996) also suggests its role as a morphogen (Butkevich, *et al*., 2003). Postembryonic developmental changes in 5-HT levels are known to modulate serotonin varicosity densities in the fly CNS; exposure of serotonergic neurons to 5-HT in younger larvae increases serotonin varicosity, while in the older larvae it decreases (Sykesa and Condron, 2005).

Serotonergic neurons are important regulators of growth in insects, since disturbances in serotonergic function slow larval growth (Vallés and White, 1986). Kaplan *et al*., (2008) reported that the levels of 5-HT per body weight were increased by around 50% in nucleostemin 3 (NS3) mutants and showed that serotonergic neurons control insect growth through regulation of insulin secretion from the insulin producing cells (IPCs). Serotonin immunoreactive (5-HTi) neurons occur in relatively small numbers in the CNS of insects (Beltz and Kravitz, 1983; Elofsson, 1983). 5-HT immunoreactivity (5-HTi) patterns greatly vary in different insect species (Klemm, 1976). For instance the highly developed mushroom bodies in bees and ants possess extensive and complex 5-HTi innervation patterns as compared to the relatively simpler mushroom bodies of flies where only scarce immunoreactivity occurs in the calyx and peduncle.

In this chapter we studied the role of serotonin during neurometamorphosis:

i. Changes in the levels of serotonin (5-HT) and its major metabolite, 5-hydroxyindole acetic acid (5-HIAA) during PED
ii. The effect of Fenoxycarb, (FX) on 5-HT and 5-HIAA levels
iii. The effect of 20E on 5-HT and 5-HIAA levels

II. Materials and Methods

(i). General Methodologies for Maintaining B. mori

As discussed in Chapter 1 (Page No. 51)

(ii). Fenoxycarb Treatment

FX treatment was done as discussed in 3rd chapter (Page No. 92).

(iii). Ecdysone Treatment

20E treatment was done as discussed in 3rd chapter (Page No. 93).

(iv). Sample Preparation

CNS was pooled in each stage 4I, 5EI, 5MI, 5LI, EP, MP, LP and A, till 10 mg weight as mentioned in Chapter 1 (Page No. 52) and 5% homogenates were prepared in 0.1 N perchloric acid containing 1 mM sodium bisulfite. The tissue lysates were centrifuged at 12,800 g to remove tissue debris and filtered with 0.2 µ syringe filters before injecting into chromatographic system (Waters Alliance System) with EC detector. C-18 reversed phase silica column was used for chromatographic separation and the samples were eluted using 10% methanol containing 0.1 M citric acid, 0.1 M sodium acetate, 50 mg/L EDTA at pH 4.1. The stock solutions of 5-HT and 5-HIAA standards were prepared in 0.1 N perchloric acid at a concentration of 1 mg/ml and stored at -20 °C. The daily working standards were prepared by diluting the stock solutions in 0.1 N perchloric acid. Linear standard curves were obtained by diluting the standards in the range of 1 ng to 20 ng. The standard retention time was found to be 5.8 min for 5-HT and 9.5 min for 5-HIAA. Concentrations of 5-HT and 5-HIAA in the tissue homogenates were determined by comparing with the peak area obtained with the standards (Fig. 28) (Jagota and Reddy, 2007). All samples were collected between 9 AM to 11 AM.

(v). Statistical Analysis

Data for developmental changes in 5-HT and 5-HIAA levels were analyzed by One-way ANOVA followed by a post hoc Duncan's multiple range test**.** Effect of FX and 20E on 5-HT and 5-HIAA levels was studied by performing Students t-test. All statistical tests were performed with SigmaStat™ (Jandel Scientific). Each value was mean ± SE (n = 4) ($p_a \leq 0.05$, $p_b \leq 0.05$, $p_c \leq 0.05$, $p_d \leq 0.05$, $p_e \leq 0.05$, $p_f \leq 0.05$, $p_g \leq 0.05$ and $p_h \leq 0.05$, where a, b, c, d, e, f, g and h refer to comparisons with 4I, 5EI, 5MI, 5LI, EP, MP, LP and A respectively).

III. Results

(i). Developmental Changes in levels of 5-HT and 5-HIAA

Both 5-HT (13.7 ± 1.63) and 5-HIAA (45.6 ± 10.2) µmol/g protein levels were highest in 4I (Table 8; Fig. 29 (i)) as compared to all other developmental stages studied ($p_{b\text{-}h} \leq 0.05$). There was almost 9-fold decrease in the levels of both 5-HT (1.4 ± 0.3) and

5-HIAA (5 ± 1.2) µmol/g protein upon larval-larval transformation from 4I to 5EI (Fig. 29 (i)) ($p_a \leq 0.05$). The 5-HT levels in 5EI (1.4 ± 0.3), 5MI (1.05 ± 0.3) and 5LI (1.34 ± 0.3) µmol/g protein and 5-HIAA levels in 5EI (5 ± 1.2), 5MI (4.5 ± 2.6) and 5LI (12.5 ± 3.6) µmol/g protein did not show any significant changes (Fig. 29 (i)). During larval-pupal transformation, almost 4 fold increase in 5-HT (5.4 ± 1.3) µmol/g protein levels were observed in EP as compared to 5LI ($p_d \leq 0.05$), while the levels of 5-HIAA (12.65 ± 3) µmol/g protein were not significantly different between 5LI and EP (Table 8; Fig. 29 (i)). During pupal development from EP to MP, almost 6 fold decrease in the levels of both 5-HT (88 ± 5) and 5-HIAA (2.1 ± 0.6) µmol/g protein ($p_e \leq 0.05$) was observed, followed by a robust 10 and 14 fold increase in 5-HT (8.4 ± 0.09) and 5-HIAA (27.7 ± 0.8) µmol/g protein ($p_f \leq 0.05$) levels respectively during development from MP to LP (Table 8; Fig. 29 (i)). Following adult emergence, we found no significant changes in 5-HT levels (7 ± 1.3) µmol/g protein, while those of 5-HIAA decreased by 5-folds (5.7 ± 0.9) µmol/g protein as compared to LP ($p_g \leq 0.05$) (Table 8; Fig. 29 (i)).

5-HT/5-HIAA ratio (Jagota and Habibulla, 1992a) was calculated for each of the developmental stages (Table 8; Fig. 29 (ii)). Even though the levels of 5-HT and 5-HIAA were highest in 4I ($p_{b-h} \leq 0.05$) as compared to all other stages studied, the 5-HT/5-HIAA ratio was not significantly different between 4I (0.32 ± 0.03), 5EI (0.28 ± 0.04) and 5MI (0.49 ± 0.18). A significant decrease in the 5-HT/5-HIAA ratio was observed in 5LI (0.11 ± 0.01) as compared to 5MI ($p_c \leq 0.05$), indicating high conversion of 5-HT to 5-HIAA during 5LI (Table 8; Fig 29 (ii)). During larval pupal transformation, there was a 4 fold increase in 5-HT/5-HIAA ratio in EP (0.45 ± 0.06) as compared to 5LI ($p_d \leq 0.05$). 5-HT/5-HIAA ratio showed no significant changes throughout the pupal development, EP (0.45 ± 0.06), MP (0.5 ± 0.1) and LP (0.3 ± 0.07). 5-HT/5-HIAA ratio again increased 4-fold during transformation from LP to A (1.22 ± 0.03) ($p_g \leq 0.05$) (Table 8; Fig. 29 (ii)).

(ii). Effect of FX on 5-HT and 5-HIAA levels

We found that FX treatment resulted in a robust 14 fold increase in levels 5-HT (0.063 ± 0.006 to 0.9 ± 0.16 µmol/g protein) and a significant 1.16 fold increase in levels of 5-HIAA (4.8 ± 0.25 to 5.95 ± 0.17 µmol/g protein) in 4I as compared to controls ($p \leq 0.05$) (Table 9; Fig. 30 (i)). However, in 5EI and 5LI FX treatment did not result in any

significant changes in the levels of both compounds (Table 9; Fig. 20 (i)). In 5EI, 5-HT and 5-HIAA levels in control group were 0.1 ± 0.03 and 7.2 ± 1.2 µmol/g protein respectively, while in FX treated insects, the 5-HT and 5-HIAA levels were 0.28 ± 0.1 and 11.3 ± 2.1 µmol/g protein respectively. In 5LI, 5-HT and 5-HIAA levels in control group were 0.56 ± 0.26 and 15 ± 4.4 µmol/g protein respectively, while in FX treated insects, the 5-HT and 5-HIAA levels were 0.18 ± 0.06 and 9.8 ± 3.1 µmol/g protein. In EP, FX treatment resulted in 45% decrease in 5-HT levels (0.97 ± 0.11 to 0.52 ± 0.06 µmol/g protein) ($p \leq 0.05$), while 5-HIAA levels were not affected (3.7 ± 0.4 to 2.8 ± 0.4 µmol/g protein) (Table 9; Fig. 30 (i)).

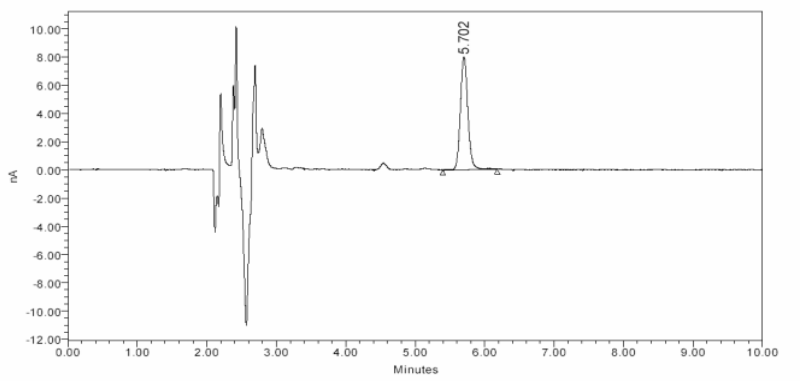
5-HT/5-HIAA ratio was robustly increased by as much as 9-folds in 4I (0.013 ± 0.0007 to 0.151 ± 0.024) upon FX treatment ($p \leq 0.05$) (Table 9; Fig. 30 (ii)). 5-HT/5-HIAA ratios were not affected in 5EI (0.014 ± 0.002 to 0.023 ± 0.002) and 5LI (0.03 ± 0.01 to 0.02 ± 0.002) upon FX treatment but decreased by 25% in EP (0.265 ± 0.024 to 0.197 to 0.024) ($p \leq 0.05$) (Table 9; Fig. 30 (ii)).

(iii). Effect of 20E on 5-HT and 5-HIAA levels

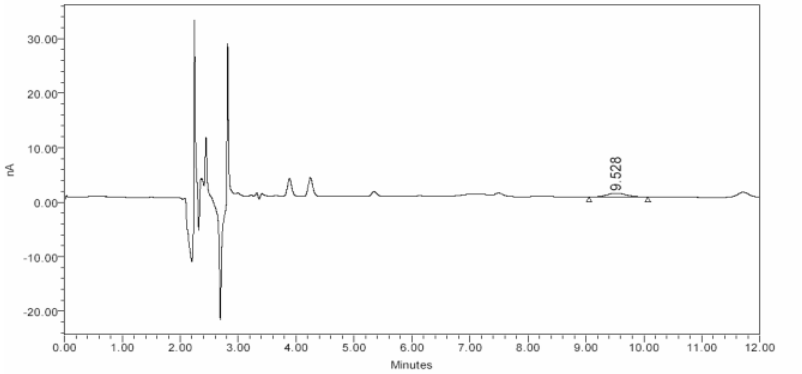
20E treatment resulted in 80% decrease in 5-HT levels (0.17 ± 0.06) µmol/g protein in 4I as compared to control treated insects (0.8 ± 0.14) µmol/g protein ($p \leq 0.05$) (Table 10; Fig. 31 (i)), while the levels were not significantly altered in 5EI (0.25 ± 0.07 to 0.3 ± 0.1), 5LI (0.15 ± 0.01 to 0.25 ± 0.1) and EP (4.2 ± 0.9 to 3.4 ± 0.5 µmol/g protein). 5-HIAA levels were significantly elevated by 6-folds as compared to control treatment in 5LI (2.5 ± 0.2 to 12.8 ± 1.4 µmol/g protein) ($p \leq 0.05$) (Table 10; Fig. 31 (i)), while in 4I (6.7 ± 0.5 to 6.9 ± 0.5), 5EI (14.4 ± 3.2 to 10 ± 1) and EP (20 ± 4.7 to 12.2 ± 0.5 µmol/g protein). 5-HIAA levels did not show any changes upon 20E treatment (Table 10; Fig. 31 (i)).

5-HT/5-HIAA ratio was also significantly decreased upon treatment with 20E in 4I as compared to ratio in control treated insects (0.114 ± 0.01 to 0.023 ± 0.007) ($p \leq 0.05$) (Table 10; Fig. 31 (ii)), while in 5EI (0.017 ± 0.001 to 0.028 ± 0.008), 5LI (0.062 ± 0.003 to 0.02 ± 0.005) and EP (0.21 ± 0.02 to 0.28 ± 0.03), there were no significant changes in 5-HT/5-HIAA ratios upon 20E treatment (Table 10; Fig. 31 (ii)).

i. 5-HT peak occurs at 5.8 minutes



ii. 5-HIAA peak occurs at 9.5 minutes



iii. 5-HT and 5-HIAA peaks in EP

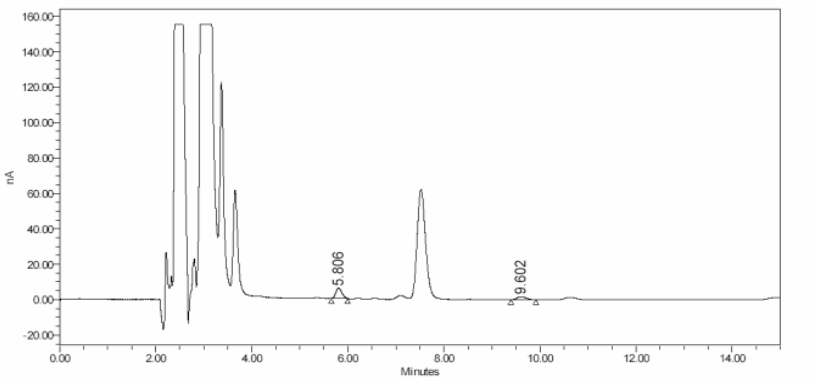


Figure 28: Standards of (i) serotonin (5-HT) and (ii) its metabolite (5-HIAA) detected in Waters Alliance HPLC-ECD at different times of elution from reverse phased columns. Figure (iii) shows both 5-HT and 5-HIAA detected in the same sample from the CNS in early pupa (EP).

Table 8: Changes 5-HT and its metabolite 5-HIAA in CNS of *B. mori* during PED.

Developmental Stages	5-HT (µmol/g Protein)	5-HIAA (µmol/g Protein)	5-HT/5-HIAA Ratio
4I	13.7±1.63 $^{b\text{-}h}$	45.6 ± 10.2 $^{b\text{-}h}$	0.32 ± 0.03 ^{h}
5EI	1.4 ± 0.3 ^{a,e,g,h}	5 ± 1.2 ^{a,g}	0.28 ± 0.04^{h}
5MI	1.05 ± 0.3 ^{a,e,g,h}	4.5 ± 2.6 ^{a,g}	0.49 ± 0.18^{d,h}
5LI	1.34 ± 0.3 ^{a,e,g,h}	12.5 ± 3.6 ^{a,g}	0.11 ± 0.01$^{d\text{-}f,\,h}$
EP	5.4 ± 1.3 $^{a\text{-}d,f,g}$	12.6 ± 3 ^{a,g}	0.45 ± 0.06^{d,h}
MP	0.9 ± 0.05 ^{a,e,g,h}	2.1 ± 0.6 ^{a,g}	0.50 ± 0.10^{d,h}
LP	8.4 ± 0.09 $^{a\text{-}f}$	27.7 ± 0.8 $^{a\text{-}f,h}$	0.30 ± 0.07^{h}
A	7 ± 1.3 $^{a\text{-}d,f}$	5.7 ± 0.9 ^{a,g}	1.22 ± 0.03$^{a\text{-}g}$

Both 5-HT and 5-HIAA levels were maximum in CNS of 4I, and minimum in MP. Upon adult emergence 5-HT levels did not vary significantly between LP and A, but the 5-HIAA levels were significantly reduced. 5-HT/5-HIAA ratio was lowest in the CNS of 5LI, but highest in 1 day old adult. Each value is Mean ± SE (n = 4). $p_a \leq 0.05$, $p_b \leq 0.05$, $p_c \leq 0.05$, $p_d \leq 0.05$, $p_e \leq 0.05$, $p_f \leq 0.05$, $p_g \leq 0.05$ and $p_h \leq 0.05$, where a, b, c, d, e, f, g and h are the same as in Table 1.

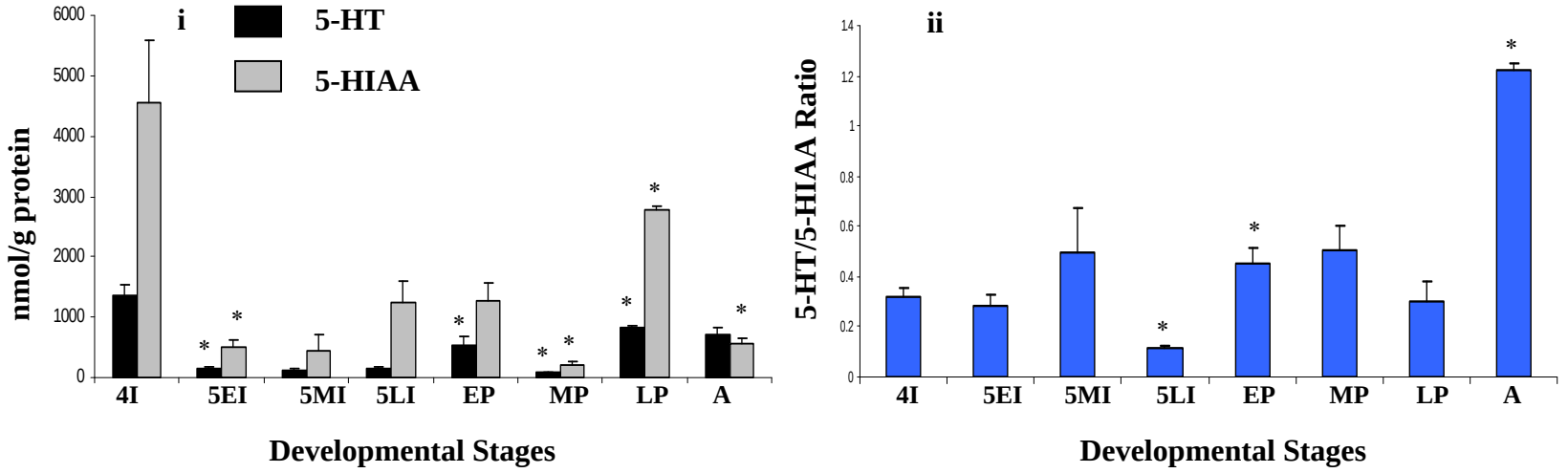


Figure 29 (i) Postembryonic developmental changes in the levels of serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA). (ii) 5-HT/5-HIAA ratios during PED. Different developmental stages were studied including 4I, 5EI, 5MI, 5LI, EP, MP, LP and A. Both 5-HT and 5-HIAA levels were maximum in CNS of 4I and minimum in MP. 5-HT levels did not vary significantly between LP and A, but the 5-HIAA levels were significantly reduced in A as compared with LP. 5-HT/5-HIAA ratio was lowest in the CNS of 5LI, but highest in 1 day old adult. Each value is Mean ± SE (n = 4). * indicates significant difference with the previous developmental stage ($p \leq 0.05$) .

Table 9: Effect of FX on 5-HT and 5-HIAA levels in the CNS of *B. mori*

Developmental Stages	5-HT (µmol/g protein)		5-HIAA (µmol/g protein)		5-HT/5-HIAA Ratio	5-HT/5-HIAA Ratio
	Control	FX	Control	FX	Control	FX
4I	0.063 ± 0.006	0.9 ± 0.16*	4.8 ± 0.25	5.95 ± 0.17*	0.013 ± 0.001	0.15 ± 0.02*
5EI	0.1 ± 0.03	0.28 ± 0.1	7.2 ± 1.2	11.3 ± 2.1	0.014 ± 0.002	0.023 ± 0.002
5LI	0.56 ± 0.26	0.18 ± 0.06	15 ± 4.4	9.8 ± 3.1	0.03 ± 0.01	0.02 ± 0.002
EP	0.97 ± 0.11	0.52 ± 0.06*	3.7 ± 0.4	2.8 ± 0.4	0.265 ± 0.02	0.197 ± 0.02*

FX treatment affected the levels of 5-HT and 5-HIAA in a stage specific manner. It increased 5-HT and 5-HIAA levels in 4I, but reduced 5-HT levels alone in EP. 5-HT/5-HIAA ratios were significantly increased in 4I, but reduced in EP. Each value is Mean ± SE (n = 4). * indicates significant difference with control treatment (Student's t-test; $p \leq 0.05$).

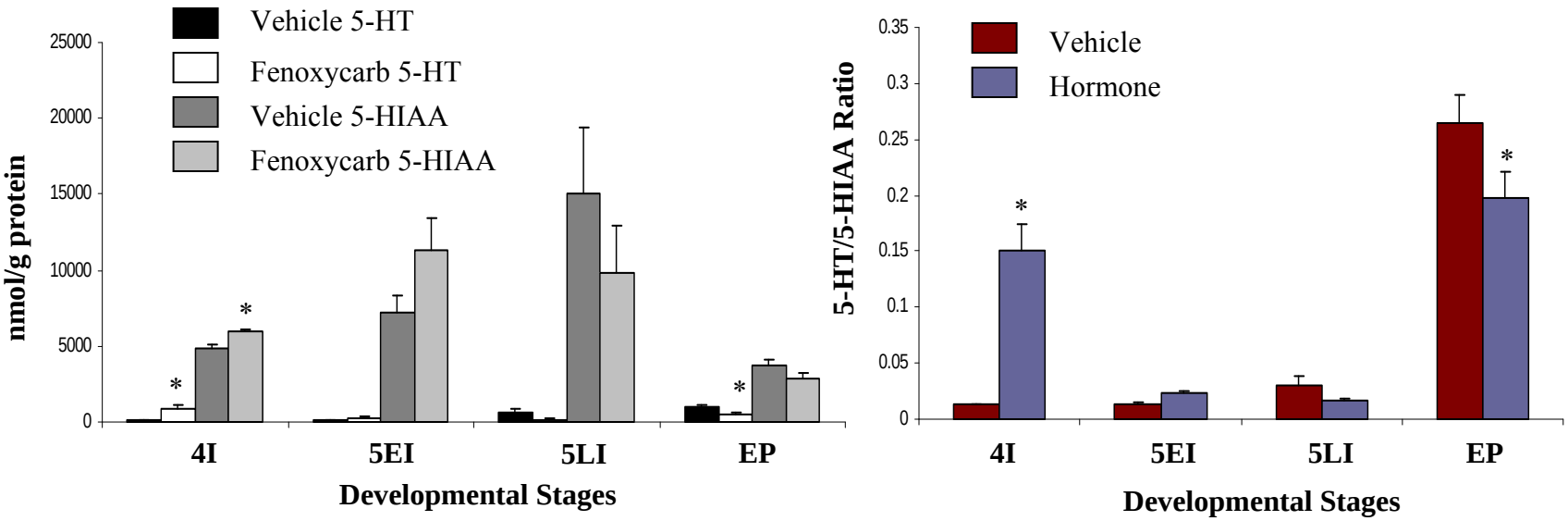


Figure 30: (i) Effect of FX on postembryonic developmental changes in the levels of serotonin (5-HT) and its metabolite (5-HIAA) in distinct developmental stages including 4I, 5EI, 5LI and EP. (ii) 5-HT/5-HIAA ratios in the CNS of FX treated insects during PED. Each value is Mean ± SE (n = 4). * indicates significant differences upon control treatment (Student's t-test; $p \leq 0.05$).

Table 10: Effect of 20E on 5-HT and 5-HIAA levels in the CNS of *B. mori*

Developmental Stages	5-HT (µmol/g protein)		5-HIAA (µmol/g protein)		5-HT/5-HIAA ratio	
	Control	20E	Control	20E	Control	20E
4I	0.8 ± 0.14	0.17 ± 0.06*	6.7 ± 0.5	6.9 ± 0.5	0.114 ± 0.01	0.023 ± 0.007*
5EI	0.25 ± 0.07	0.3 ± 0.1	14.4 ± 3.2	10 ± 1	0.017 ± 0.001	0.028 ± 0.008
5LI	0.15 ± 0.01	0.25 ± 0.1	2.5 ± 0.2	12.8 ± 1.4*	0.062 ± 0.003	0.02 ± 0.005
EP	4.2 ± 0.9	3.4 ± 0.5	20 ± 4.7	12.2 ± 0.5	0.21 ± 0.02	0.28 ± 0.034

20E treatment affected the levels of 5-HT and 5-HIAA in a stage specific manner. It decreased 5-HT in 4I, increased 5-HIAA levels alone in 5LI. 5-HT/5-HIAA ratio was significantly decreased in 4I, but the effects were not significant in 5EI, 5LI and EP. Each value is Mean ± SE (n = 4). * indicates significant difference with control treatment (Student's t-test; $p \leq 0.05$).

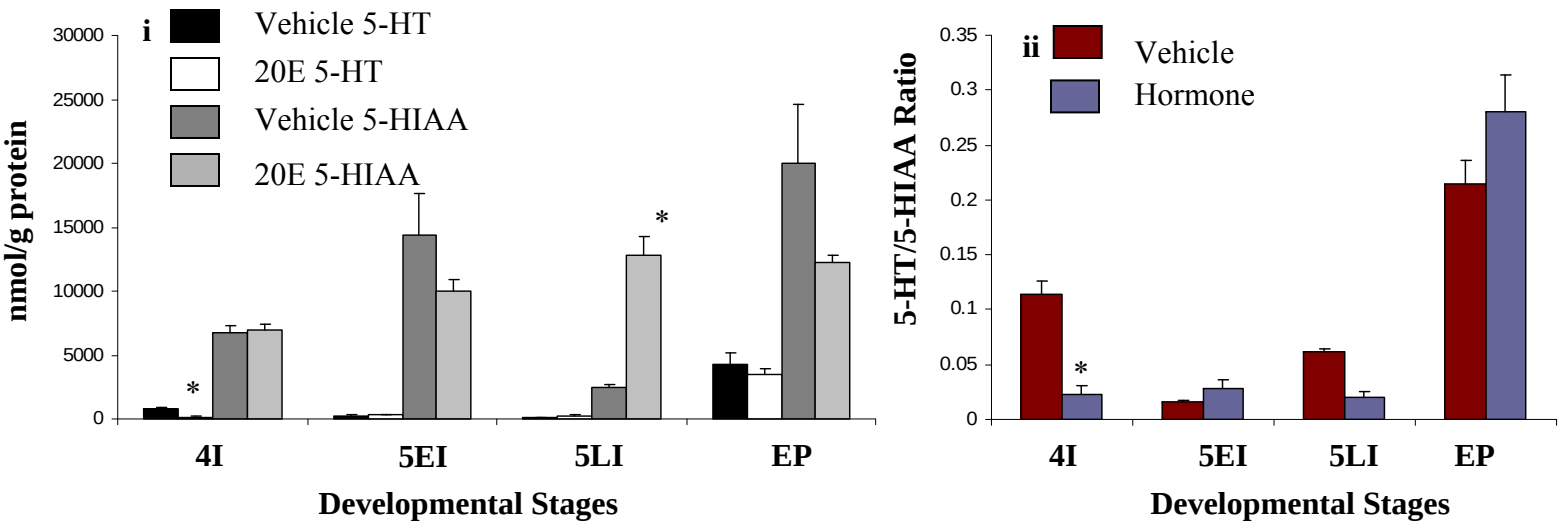


Figure 31: (i) Effect of 20E on postembryonic developmental changes in the levels of serotonin (5-HT) and its metabolite (5-HIAA) in distinct developmental stages including 4I, 5EI, 5LI and EP. (ii) 5-HT/5-HIAA ratios in the CNS of 20E treated insects during PED. Each value is Mean ± SE (n = 4). * indicates significant differences with control treatment (Student's t-test; $p \leq 0.05$).

IV. Discussion

(i). Developmental Neurotransmitter Plasticity

In present study we have detected 5-HT and its metabolite 5-HIAA from homogenates of entire CNS. We report here that both 5-HT and 5-HIAA levels were highest in 4I, but their levels were reduced robustly in the 5EI. Moreover we found that both 5-HT and 5-HIAA levels did not vary significantly throughout the progressive period during 5th instar. 5-HT levels increased robustly from 5LI to EP, while no significant changes were observed in the levels of 5-HIAA. The levels of both the metabolites decreased 6 folds from EP to MP, followed by 10-fold increase in LP. In adults, only 5-HIAA levels decreased significantly, while no changes were observed in 5-HT levels. There are very few reports on the levels of 5-HT and 5-HIAA in the complete CNS of any insect. Studies in the blow fly *Calliphora erythrocephala* have shown that 5-HT levels are lower in larvae, than in the adult (Nässel and Laxmyr, 1983). Cantera and Carlberg (1988) demonstrated that 5-HT levels in *C. erythrocephala* increase gradually in last instar larvae and peak at the end of larval development when the larva is about to transform to pupa; the levels robustly decrease in early pupa, peak during the mid pupa, and again decrease in the late pupal stage 2-days prior to adult eclosion; following this 5-HT levels increase in the adults. Region-specific changes in 5-HT were reported in CNS of *B. mori* (Takeda *et al*., 1991), in which they found that 5-HT in brain increases progressively from early 4I to late 4I, it increases progressively from 5EI to 5LI, but decreases in post-spinning stage. 5-HT in SEG was not detectable in early 4I and post-spinning stage, but increased progressively during the 5EI to 5LI. 5-HIAA was detected in only the SEG of 4I, but not in 5th instar and post-spinning stage (Takeda *et al*., 1991). Cantera and Carlberg (1988) also could not detect 5-HIAA in the CNS of larval or adult *C. erythrocephala*. In honey bee brains Taylor *et al*., (1992) reported a dramatic rise in 5-HT levels following adult emergence.

5-HT/5-HIAA ratio during PED has not been reported in CNS of holometabolous insects so far. In the present study we report that 5-HT/5-HIAA ratio in the CNS of *B. mori* does not vary significantly between 4I, 5EI, 5MI, EP, MP and LP, but the ratio is lowest in 5LI and highest in the newly emerged adults. Interestingly the ratio significantly increases during larval-pupal and pupal-adult transformation. According to some earlier reports insect CNS possesses very low monoamine oxidase (MAO) activity (Colhoun, 1967; Evans and Fox, 1975), which is responsible for conversion of 5-HT to 5-HIAA. It has been suggested

that 5-HT is metabolized by N-acetylation (Evans, 1980; Sloley, 2004). We did not study MAO activity in the CNS of *B. mori*, but with stage-specific variations in the levels of 5-HT and 5-HIAA, our study suggests that monoaminergic systems could be playing a major role in serotonin metabolism the insect CNS. Low 5-HT/5-HIAA ratio in the 5LI could be indicative of high MAO activity and hence high conversion of 5-HT to 5-HIAA. High 5-HT/5-HIAA ratio in adult CNS is indicative for low 5-HT to 5-HIAA conversion and could be due to reduced MAO activity.

(ii). Effect of developmental hormones (JH and 20E) on 5-HT and 5-HIAA levels

In present study, we report for the first time stage-specific effects of the JHA FX and 20E on the levels of 5-HT and its major metabolite, 5-HIAA in the CNS of *B. mori*. FX treatment to 4I resulted in 14-folds increase in 5-HT, while the increase in 5-HIAA, though statistically significant, was only 1.2-fold as compared to the vehicle controls. FX treatment did not induce any significant alterations in 5-HT and 5-HIAA levels in 5EI or 5LI. In EP we observed a significant reduction in 5-HT levels upon FX treatment, while the 5-HIAA levels were not affected significantly. 5-HT/5-HIAA ratio increased robustly by more than 7-folds in 4I, while in EP, it significantly reduced by 20%. Treatment of 4I larvae with 20E resulted in 80% decrease in 5-HT levels, while there were no changes in the levels of 5-HIAA. In 5EI, 5LI and EP, 20E treatment did not induce any variations in 5-HT and 5-HIAA levels, except in 5LI where 5-HIAA levels showed a robust 4.5-fold increase.

Changes in 5-HT and 5-HIAA levels upon treatment during PED with the insect developmental hormones JH and 20E have not been reported earlier. However, synthesis and degradation of other BAs have been shown to be regulated by the JH (Rauschenbach *et al*., 2004) and the ecdysteroids (Lehman, 2000; Gruntenko *et al*., 2005).

Changes in BAs upon hormone treatment appear to be stage specific. In our study the JH analog, FX, induced increase in 5-HT in CNS of 4I, while 20E treatment results in tremendous decrease. On the other hand FX treatment significantly reduced 5-HT levels in EP, whereas 20E treatment did not result in any change. Further work has to be done to study if the developmental hormones have any dose-dependent effects on the CNS neurotransmitter changes.

Conclusion

Conclusion

The economically important holometabolous Lepidopteran insect, the silkworm, (*Bombyx mori*) passes through 4 distinct stages in its entire life-cycle: egg, larva, pupa and adult. Extensive behavioral transformations are observed in *B. mori* during postembryonic development (PED). During the larval period it passes through 5 distinct larval stages (instars) during which it feeds voraciously on fresh mulberry leaves (*Morus alba*) and exhibits larval-specific behaviors such as crawling, defensive thrashing and molting. The larval period is mainly a dedicated growth phase during PED. The adult phase is mainly responsible for reproduction and egg laying and has well developed histological apparatus for walking and flight mechanisms as well as reproduction and egg laying. This distinct set of behaviors during the larval and adult period imposes many demands on the nervous system and as a result, the larval CNS is significantly different in organization as compared to adult CNS. This creates an overall need for characteristic changes in the nervous system which is remodeled during the resting pupal period, where 'neurometamorphosis' events are prominent

During neurometamorphosis many processes occur at both cellular and tissue level within the CNS. The cellular events in the CNS during metamorphosis include neurogenesis, cell death and remodeling of larval neurons to perform new functions in the adult (for review: Tissot and Stocker, 2000). At the histological levels, the CNS is extensively reorganized and some sets of ganglia undergo fusion to form larger compound ganglia for higher complex functions such as locomotion and reproduction. The larval CNS consists of brain (supraesophageal ganglion), subesophageal ganglion (SEG), three thoracic and seven abdominal ganglia. During larval-pupal transformation i.e. from 5LI to EP, there is decrease in the length of the CNS due to shortening of the interganglionic connectives. During progression from EP to MP, T_3 fuses with Ab_1 and Ab_2 in the anterior region of the CNS, while Ab_6 fuses with Ab_7 in the posterior region. During progression from MP to LP, T_2 fuses with $T_3Ab_1Ab_2$ to form compound ganglion 1. The fused Ab_{6-7} ganglion is called as the compound ganglion 2. However, no more ganglionic fusion of CNS reorganization occur in the CNS during adult Eclosion i.e., from LP to A. Finally, the adult CNS consists of brain, SEG, T_1, compound ganglion 1

($T_2T_3Ab_1Ab_2$), three individual abdominal ganglia (Ab_3, Ab_4 amd Ab_5) and the compound ganglion 2 (Ab_{6-7}).

The extensive reorganization of the CNS has been implicated in behavioral plasticity observed in holometabolous insect. This could also be a result of changes in neuronal population during PED. In *B. mori*, we found that there is a continuous increase in the numbers of neurons in different stages including 4I, 5EI, 5MI, 5LI, EP, MP, LP and A. In brain, we observed almost a 7 fold increase in neuronal counts in A as compared to 4I, while in the other ganglia of the CNS only a 2-4 fold increase was observed. This indicates a continuous addition of adult-specific neurons during the larval and the pupal stages. The total neuronal population of the CNS showed a 3.5 fold increase in adult as compared to 4I.

DNA content estimates in the CNS revealed that there was no significant difference between 4I, 5EI, 5MI and 5LI larvae. However the DNA levels continuously increase following larval-pupal transformation till adult eclosion. Interestingly DNA content was observed to be 3.5 fold higher in the adult as compared to 4I, the same fold difference coincident with that of neuronal population.

This net increase in neuronal population as well as DNA levels could be a result of neurogenesis as well as programmed neuronal death in addition to a certain population of neurons that persist during metamorphosis. These persistent neurons could be extensively remodeled losing their larval functional properties to perform novel functions in the adult CNS.

Neurogenesis studies with *in vitro* BrdU labeling method revealed that the rate of addition of neurons was not constant even though the neuronal populations showed a continuous increase throughout PED. Stage-specific and region-specific changes were observed in the number of BrdU positive cell counts. Neurogenesis was highest in the entire CNS during EP, but decreased gradually in the LP in all the ganglia except the brain. Interestingly we did not detect any BrdU positive cells in any part of the CNS of 1-day old adult *B. mori* which indicates absence of neurogenesis. We report that neurogenesis is absent in T_2, Ab_1, Ab_3, Ab_4, Ab_6 and Ab_7 ganglia in the 5EI, as well as T1 and Ab_6 of 5LI larvae. Gross neurogenesis among the larval stages appeared to be

higher in 4I as compared to 5EI, 5MI and 5LI, while during the pupal developmental period, neurogenesis was robust in EP as compared to MP and LP.

Cell death study using DNA fragmentation assay did not reveal oligonucleosomal DNA ladder formation on agarose gels. However, using Native PAGE Zymography deoxyribonuclease 1 levels were found to be higher in the larval stages as compared to the adult stages.

We studied cell death using more sensitive TUNEL assay and found that cell death was prominent throughout the pupal period as well as the adults, but was not detectable in the 4^{th} or 5^{th} instar larvae. Stage-specific and region-specific changes in the number of TUNEL positive nuclei were observed indicating that cell death doesn't occur in a uniform pattern during PED. In brain, T_1 and $T_2T_3Ab_1Ab_2$ ganglia, cell death increased during development from EP to MP, while no significant changes were observed in this period in SEG. However, in these ganglia, cell death decreased significantly from MP to LP and then increased robustly upon adult eclosion. In Ab_3 and Ab_4, the number of TUNEL positive cells decreased gradually during development from MP to LP and A. Cell death levels were constant throughout the pupa and adult in Ab_5. In Ab_{6-7}, cell death was highest in EP, decreased significantly in MP and then steadily increased till the adult. Gross cell death levels were not significantly different between EP, MP and A, but were lowest in the LP. We observed developmental changes in proteins involved in cell death: caspase 3, PARP-1 and cathepsin D. Their levels correlated with the TUNEL counts indicating their possible role in PCD in the CNS of *B. mori*.

Insect developmental hormones including ecdysteroids and juvenile hormones are known to regulate molting and metamorphosis in insect (Truman and Riddiford, 2002). In the present study, we used the JHA, Fenoxycarb and the ecdysteroid, 20-hydroxyecdysone (20E) to study their effects on neurogenesis and cell death during selected stages during PED including 4I, 5EI, 5LI and EP. Both the hormones induced stage-specific and region-specific changes on neurogenesis. Fenoxycarb treatment decreased neurogenesis in the entire CNS of 4I larvae while it induces differential changes on neurogenesis in the 5EI, 5LI and EP. 20E treatment resulted in significant increase in neurogenesis in 5EI and 5LI, while the effects were differential in 4I and EP.

Fenoxycarb treatment significantly increased PCD in EP, while 20E treatment induced differential changes. However, neither Fenoxycarb nor 20E treatment could induce any detectable PCD in the CNS of 4I, 5EI and 5LI larvae.

Serotonin is a biogenic amine which is known to play an important role as a neurotransmitter, neuromodulator and a neurohormone (Dacks *et al.*, 2006). Its role in inducing metamorphosis is well established in other invertebrates such as the molluscs. Further, its morphogenetic role in influencing embryonic as well as postnatal neurogenesis, cell death and neuronal remodeling in the CNS are well established in the higher vertebrates such as mammals. Postembryonic developmental changes in the levels of serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were studied in the CNS of *B. mori*, using reverse phase HPLC with electrochemical detection (Jagota and Reddy, 2007). 5-HT and 5-HIAA levels were highest in 4I, but significantly decreased by 10-folds during 5EI. The levels did not vary significantly between the 5EI, 5MI and 5LI, however, from 5LI to EP, 5-HT alone showed a significant 4-fold increase. During EP to MP, both 5-HT and 5-HIAA levels decreased by 6-folds, and during MP to LP showed a robust 10-fold increase in levels. During development from LP to adult, 5-HT levels did not vary significantly, but the 5-HIAA levels decreased almost 5-folds.

5-HT/5-HIAA ratios did not vary significantly between 4I, 5EI and 5MI, but decreased significantly in 5LI. The 5-HT/5-HIAA ratio increased significantly in EP, but was consistent during the pupal development. 5-HT/5-HIAA ratio significantly increased during LP to adult.

Fenoxycarb treatment significantly increased 5-HT/5-HIAA ratio in 4I, but had an opposite affect during the EP. It did not result in any significant change in the levels of 5-HT or 5-HIAA in 5EI and 5LI. However, 20E treatment significantly reduced 5-HT/5-HIAA ratio in 4I, but it did not induce any alterations upon treatment of 5EI, 5LI and EP. High serotonin levels can be related to comparable higher neurogenesis levels in the 4I, neurogenesis as well as cell death in EP, while in the adult it could be mainly responsible for PCD (Fig. 32).

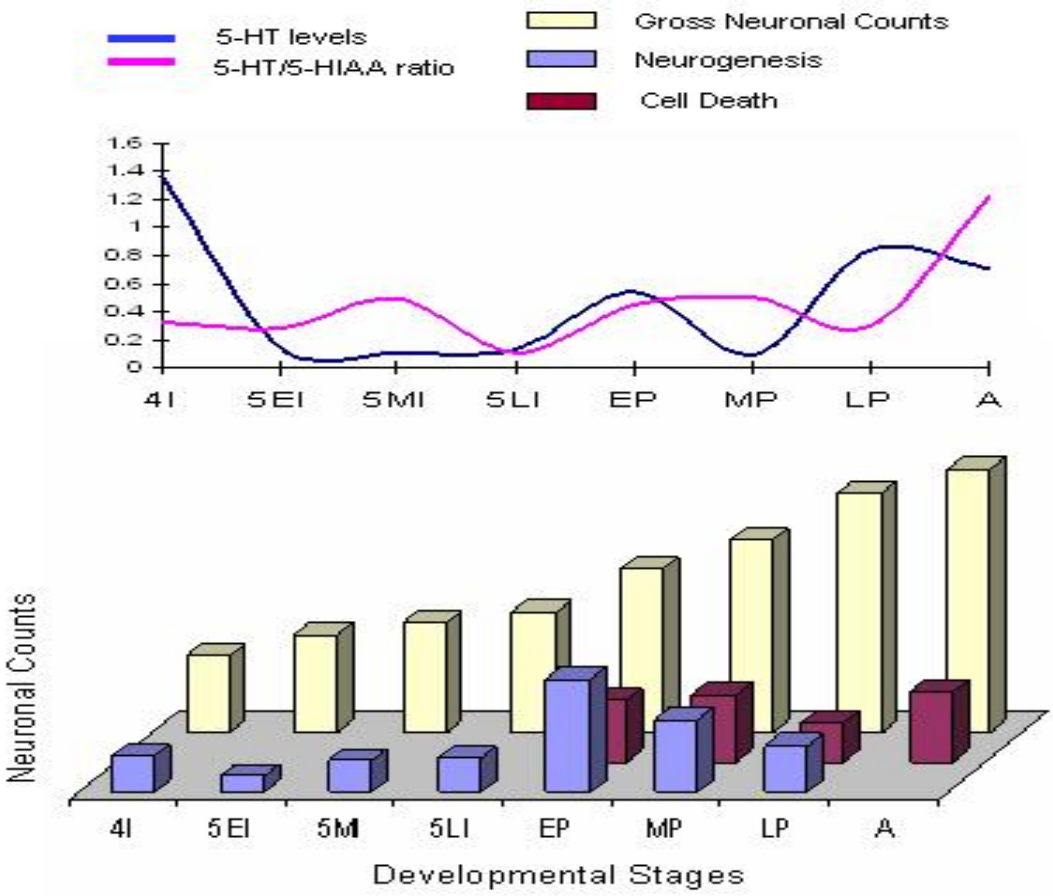


Figure 32: Serotonin levels correlate with extensive neurogenesis in 4I, neurogenesis and programmed cell death in EP, and PCD in the adult CNS.

Finally, we conclude that (i) in the larval stages neurogenesis is much higher than PCD; (ii) in EP, neurogenesis is greater than cell death; (iii) in MP and LP, neurogenesis levels are similar to that of PCD; (iv) in the adult CNS, neuronal cell death is much greater than neurogenesis. The high levels of serotonin observed in the present study during specific stages coincide with period of high ecdysteroids as well as high juvenile hormone.

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Abbreviations

°C :	degree Celsius
20E:	20-hydroxyecdysone
5-HIAA :	5-Hydroxyindole acetic acid
5-HT:	5-hydroxytryptamine
Ab_{1-7}:	Abdominal ganglia 1 to 7
ALP:	Alkaline phosphatase
APS :	Ammonium per sulphate
BCIP:	5-bromo-4-chloro-3-indolyl phosphate
BME:	β-mercaptoethanol
B. mori:	*Bombyx mori*
BrdU:	5-bromo-2-deoxyuridine
BSA:	Bovine serum albumin
Ca^{2+} :	Calcium ion
cAMP :	cyclic adenosine monophosphate
CNS :	Central nervous system
DAB:	3, 3`-Diaminobenzidine tetrahydrochloride
DNA :	Deoxyribonucleic acid
DNase 1	Deoxyribonuclease 1
DTT:	Dithiothreitol
dUTP:	2´-Deoxyuridine, 5´-Triphosphate
ECD:	Electrochemical Detector
EtBr:	Ethidium Bromide
FX:	Fenoxycarb
HIOMT :	Hydroxyindole-O-methyltransferase
HPLC:	High performance liquid chromatography
HRP:	Horse radish peroxidase
JH:	Juvenile hormone
JHA:	Juvenile hormone analogue
kDa:	Kilo Dalton
LD cycle :	Light-dark cycle
MAO :	Monoamine oxidase
mg :	milligram
mg/L:	milligram per liter
mg/ml:	milligram per milliliter
Mg^{2+}:	Magnesium ion
ml :	milliliter
mM :	millimolar
mmol:	millimoles
NAT :	*N*-acetyl transferase
NBT:	Nitro blue tetrazolium
NFM:	Non-fat milk powder
nm :	nanometer
NPZ:	Native PAGE Zymography
PAGE :	Polyacrylamide gel electrophoresis

PARP 1:	Poly (ADP-ribose) polymerase 1
PBS:	Phosphate buffered saline
PBS-Tx:	Phosphate buffered saline with Triton X-100
PCA:	Perchloric acid
PED:	Postembryonic development
PVDF:	Polyvinylidene Fluoride
RP-HPLC :	Reverse phase high pressure liquid chromatography
RPM:	Revolutions per minute
SDS :	Sodium dodecyl sulphate
SNET buffer:	SDS, NaCl, EDTA and Tris-HCl containing buffer
SEG:	Subesophageal ganglion
T_{1-3}:	Thoracic ganglia 1 to 3
TAE:	Tris-Acetate-EDTA buffer
TBS:	Tris buffered saline
TBST:	Tris buffered saline with Tween-20
TdT Buffer:	Terminal deoxynucleotidyl transferase buffer
TE Buffer:	Tris-EDTA buffer
TEMED :	*N, N*,N_1,N_1-Tetramethylethylenediamine
TPH :	Tryptophan hydroxylase
TRH :	Thyrotropin releasing hormone
Tris :	Tris-(Hydroxymethyl) aminoethane
TRM:	TUNEL Reaction mix
TUNEL:	Terminal deoxynucleotidyl transferase dUTP nick end labeling
µ:	microns
µg:	microgram
µl :	microliter
µM :	micro molar
µmol:	micromoles
p_a:	a refers to comparison with 4I
p_b:	b refers to comparison with 5EI
p_c:	c refers to comparison with 5MI
p_d:	d refers to comparison with 5LI
p_e:	e refers to comparison with EP
p_f:	f refers to comparison with MP
p_g:	g refers to comparison with LP
p_h:	h refers to comparison with A