Postembryonic proliferation of neuroendocrine cells expressing adipokinetic hormone peptides in the corpora cardiaca of the locust

Sheila R. Kirschenbaum* and Michael O'Shea†

Cell Biology Laboratory, Royal Holloway and Bedford New College, University of London, Egham, Surrey TW20 0EX, England

*Author for correspondence at her present address: Arizona Research Laboratories Division of Neurobiology, 611 Gould-Simpson Building, University of Arizona, Tucson, Arizona 85721, USA
†Present address: Sussex Centre for Neuroscience, School of Biological Sciences, University of Sussex, Falmer, Brighton, Sussex BN1 9Q6, England

SUMMARY

Neuroendocrine glands that synthesize and secrete peptide hormones regulate the levels of these peptide messengers during development. In this article we describe a mechanism for regulating neuropeptide levels in the corpora cardiaca of the locust *Schistocerca gregaria*, a neuroendocrine gland structurally analogous to the vertebrate adenohypophysis. A set of five colocalized peptide hormones of the adipokinetic hormone family is synthesized in intrinsic neurosecretory cells in the corpora cardiaca. During postembryonic development there are progressive changes in the absolute and relative levels of these five peptide hormones. We show that the ability of the gland to increase peptide synthesis is due to a 100-fold increase in the number of cells which make up the gland. The gland grows by the addition of new cells derived from symmetrical division of undifferentiated precursor cells within the corpora cardiaca. We show, using double-label immunocytochemistry, that cells born in the glandular lobe mature into cells that express adipokinetic hormone peptides. The pattern of cell birth and peptide expression can account for the dramatic increase in postembryonic peptide levels.

Key words: bromodeoxyuridine, insect, birthdating, locust

INTRODUCTION

The transition from larval form to adult in the locust *Schis -tocerca gregaria* is marked by a striking change in behaviour. The adult is able to make long distance flights, having acquired wings during the metamorphic changes near the end of larval life. One necessity of long term flight is release into the haemolymph of adipokinetic hormones (AKH) whose diverse actions transform the locust into an efficient, lipid-metabolizing flying machine. Adipokinetic hormones are released from the corpora cardiaca (CC), a major insect neuroendocrine organ associated with the brain. The CC resembles, anatomically and functionally, the vertebrate pituitary. It is composed of two distinct regions, both critical in neuroendocrine regulation. The storage lobe of the CC, like the neurohypophysis, consists of nerve terminals of neurosecretory cells originating in the brain. The glandular lobes of the CC, like the adenohypophysis, consist of neurosecretory cells intrinsic to the CC. The intrinsic cells, which are morphologically like unipolar neurons with short thin processes (Krogh, 1973), synthesize, store and release into the circulating haemolymph five neuropeptides. Two of these peptides, AKH I and AKH II, are released about 15 minutes after flight commences (Orchard and Lange, 1983) and are important in regulating lipid metabolism (Goldsworthy, 1983), protein synthesis (Carlisle and Loughton, 1979, 1986) and flight muscle metabolism (Robinson and Goldsworthy, 1977) amongst other physiological functions (see Orchard, 1987, for review). Three other peptides, adipokinetic hormone precursor-related peptides (APRPs) are released at the same time (Hekimi and O'Shea, 1989b) but their functions are not known. All five peptides are derived from three dimeric precursors (Hekimi et al., 1991) assembled from two monomeric subunits that are encoded by two small mRNAs (Schulz-Aellen et al., 1989; Noyes and Schaffer 1990; Hekimi et al., 1991). The biosynthesis of the five peptides involves rapid, random dimerization of the two monomer proteins to form one heterodimeric and two different homodimeric protein precursors which are enzymatically cleaved to form five peptide products. Immunocytochemical studies (Hekimi et al., 1989) demonstrate colocalization of the precursors to AKH I and AKH II in the glandular lobe cells. These data and the biosynthetic model suggest that all AKH peptides must be made by all glandular lobe cells.

Throughout postembryonic development the absolute amounts of the AKHs and APRPs in the CC change dramatically. High AKH levels measured in the adult CC contrast with those in larval stages. Siegert and Mordue (1986), using a sensitive radioimmunoassay to measure peptide levels, demonstrated a five-fold increase in levels of AKH I between fifth larval instar and adult CC. Hekimi et al. (1991) demonstrated that over the course of larval life the increase in AKH I is greater than the increase in AKH II so...
that in the first larval stage the AKH I to AKH II ratio is about 1:1 while in the adult the ratio approaches 5:1. Thus, in addition to the increase in absolute amounts, the ratios of these two peptides also change suggesting that different mechanisms may operate to regulate the postembryonic development of peptide expression.

We are interested in understanding the mechanisms involved in preparing the locust for adult behaviour. For example, how does the CC change through development to attain the high levels of stored AKH peptides? One model assumes that all CC cells are present at the end of embryogenesis and the increase in peptide expression is due to a continuous increase in the levels of peptide synthesis in these cells throughout development. An alternate model proposes that throughout postembryonic development new cells are added to the glandular lobes and increases in peptide levels would be due to peptide expression in these new cells. In addition, factors regulating peptide expression in established cells could regulate peptide levels in the CC.

The postembryonic growth and development of the insect nervous system has been studied extensively, particularly in holometabolous insects which undergo complete metamorphosis. In these animals although some larval neurons persist and are used again to fulfil adult requirements (Levine and Truman, 1985; Kent and Levine, 1988), the majority of the adult central nervous system is generated during postembryonic development. In the moth, Manduca sexta, for example, 60-70% of the CNS neurons arise during larval development although their differentiation is arrested until metamorphosis (Booker and Truman, 1987a). By contrast, in hemimetabolous insects like the locust, most of the adult nervous system is produced during embryogenesis. Before hatching or very early in postembryonic development, the neuroblasts in the segmental ganglia of the ventral nervous system degenerate (Edwards, 1969; Sbrenna, 1971; Bate, 1976). Cytological evaluation of postembryonic growth in the ventral nerve cord of Schistocerca gregaria confirms that the number of neurons remains constant throughout larval and adult life (Sbrenna, 1971). Postembryonic proliferation of neurons in hemimetabolous insects has been noted thus far only in the corpora pedunculata of the brain (Afify, 1960; Edwards, 1969) and in the optic lobes (Anderson, 1978).

We report here on the postembryonic development of the glandular lobes of the CC in Schistocerca gregaria. Histological studies reveal that the number of cells in the gland increases during postembryonic development. Using birthdating techniques we describe the temporal and spatial patterns of cell proliferation. New cells are born throughout larval life and into early adult life. We also examine the origin of the new cells and find that new cells arise from undifferentiated cells within the glandular lobes. New cells mature into neuroendocrine cells which have the ability to synthesize AKH peptides.

MATERIALS AND METHODS

Animals

Schistocerca gregaria were reared in our laboratory culture at 30°C in constant light. Under these conditions the larval period lasts about 4 weeks. There are five larval stages before the moult to adult and each stage lasts a characteristic number of days as follows: the first larval stage lasts 5-6 days, the second and third stages 3-4 days, the fourth 4-5 days and the fifth 7 days. Both male and female locusts were used for all experiments and no significant differences between sexes were noted in these studies.

Light and electron microscopy

Tissues for semi-thin sectioning and electron microscopy were fixed according to the method of Swales and Lane (1985) with few modifications. Briefly, CC were fixed in 3% glutaraldehyde in phosphate buffer (pH 7.4) with 6% sucrose for 1-2 hours. After postfixation in buffered osmium tetroxide, tissues were rapidly dehydrated in an ethanol series and embedded in TAAB resin. Semi-thin sections of 0.5 µm were cut, mounted on glass slides, stained with 1% toluidine blue in 1% borax and viewed with bright-field microscopy. For electron microscopy, ultra-thin sections were cut and mounted on copper grids. The sections were stained with saturated alcoholic uranyl acetate followed by Reynolds lead stain and viewed on a Zeiss EM 109 electron microscope.

BrdU Labelling

Synthesis of DNA was assessed using the incorporation of the substituted nucleoside 5-bromodeoxyuridine (BrdU) as a marker. Immunocytochemical techniques using a monoclonal antibody to BrdU (Gratzner, 1982) reveal sites of synthesis. BrdU (Sigma), dissolved in insect saline at a concentration of 10 mM, was injected between abdominal sternites into the haemolymph space. Vegetable dye was added to the solution to confirm its distribution into the head region. Young animals (hatching through 3rd instar) were injected using glass micropipettes and older animals (4th instar through adult) were injected using 30 gauge needles. The following approximate volumes were injected: first instar, 2 µl; second instar, 5 µl; third instar, 25 µl; fourth instar, 50 µl; fifth instar, 150 µl; adult, 200 µl. The saline composition was: 140 mM NaCl, 5 mM KCl, 5 mM CaCl2, 1 mM MgCl2, 4 mM NaHCO3, 5 mM N-Tris-OH-methyl-2-aminoethanesulfonic acid, 100 mM sucrose, 5 mM trehalose, pH 7.3.

Immunocytochemistry

For detection of BrdU, CC were dissected under saline and fixed in Carnoy’s fixative for 30 minutes. They were dehydrated through a series of graded ethanol, cleared in xylene and embedded in paraffin. Serial sections (7-8 µm) were cut and mounted on gelatin-coated slides. Slides were rehydrated through an ethanol series and incubated in 2N HCl in phosphate-buffered saline (PBS) for 15 minutes. After a pretreatment with 2% non-immune horse serum or 1% bovine serum albumin (1 hour) a monoclonal antibody against BrdU (Becton-Dickinson) diluted 1:200 in the pretreatment solution was applied to the sections (1-16 hours). After several rinses in PBS, biotinylated horse anti-mouse antiserum (1:200 in PBS; Vector Labs) was applied for 1 hour, followed by avidin-biotin peroxidase complex (ABC) for 1 hour. Prepared according to the suppliers instructions (Vector Labs). The peroxidase label was revealed using 3,3’-diaminobenzidine (0.05%, Sigma) with NiCl2 intensification (0.03%). Slides were then rinsed in PBS, counterstained with Mallory-Heidenhain stain, dehydrated and mounted in DPX. Glands prepared for double labelling were fixed in a modified Bouin’s fixative (75% picric acid, 10% formaldehyde, 6% sucrose) to preserve peptide antigenicity for 2-16 hours at 4°C and then processed in an identical manner. For double labelling, sections were incubated in both primary antisera simultaneously. A polyclonal antisera to AKH II was used at a dilution of 1:2000 and has been previously described (Hekimi and O’Shea, 1989a). Following a 16 hour incubation with antibodies to BrdU and AKH, sections were rinsed and incubated for 6 hours in a mixture of two secondary antisera linked to fluorophores:
rhodamine-conjugated goat anti-mouse antiserum and fluorescein-conjugated donkey anti-rabbit antiserum both diluted 1:50 in PBS (Jackson Immunoresearch Labs Inc). Sections were rinsed in PBS, mounted in 0.1% para-phenylene diamine in 10% PBS:90% glycerol and viewed through a Zeiss microscope fitted with epi-fluorescent illumination.

For counting BrdU-labelled cells, serial sections from CC were viewed and all appearances of reaction product were counted. Counts were adjusted using the method of Abercrombie (1946) to obtain the actual numbers of labelled cells.

**Mitotic figures**

To observe cells in metaphase of mitosis, locusts were injected with 0.2% colchicine in saline 16 hours before being killed. Corpora cardiaca were fixed in modified Bouin’s, sectioned and stained with 0.5 µg/ml diamidino-phenylindole (DAPI). When DAPI staining was combined with peptide immunocytochemistry, DAPI was used as a counterstain after immunocytochemical procedures were completed.

**BrdU clearance**

The disappearance of BrdU from the haemolymph was assessed by quantitative high pressure liquid chromatography (HPLC) of BrdU according to the technique of deFazio et al. (1987) with modifications. After injection of BrdU solution without vegetable dye, animals were bled by cutting a metathoracic limb. A 100 µl haemolymph aliquot was added to 50 µl of 140 mM NaCl with 5 mM EDTA to prevent clotting, and then 700 µl saturated ammonium sulphate solution was added for a final concentration of 37.8%. After centrifugation to remove precipitated proteins, the supernatant solution was subjected to reverse phase HPLC using a C18 column (Aquapore RP300; 7µm; Pierce Chemical Company). The column was eluted at a flow rate of 2 ml/minute with 10 mM potassium dihydrogen phosphate (pH 4.9) followed by 8% methanol in the same buffer to elute BrdU. A dual detection system consisting of an absorbance detector (Waters) set at 214 nm and a fluorescence detector (Kratos) set at 276 nm was used to detect BrdU. With fluorescence detection BrdU exhibits a unique negative deflection allowing unambiguous identification.

**Measurement of peptide levels**

Peptide titres in larval and adult animals were measured after quantitative HPLC separations. Corpora cardiaca from staged locusts were extracted in 20% acetonitrile in 0.1% trifluoroacetic acid (TFA) by repeated cycles of freezing and thawing. After centrifugation the supernatant was frozen until HPLC analysis. Reverse-phase separation of AKH I was achieved using a C18 column (SynChropak RP-P, SynChrom, Inc.). The column was washed at a flow rate of 2 ml/minute with 10 mM potassium dihydrogen phosphate (pH 4.9) followed by 8% methanol in the same buffer to elute BrdU. A dual detection system consisting of an absorbance detector (Waters) set at 214 nm and a fluorescence detector (Kratos) set at 276 nm was used to detect BrdU. With fluorescence detection BrdU exhibits a unique negative deflection allowing unambiguous identification.

**Results**

**Gross aspects of glandular lobes**

Throughout development the glandular lobes of the CC grow in size. In all stages the CC can be recognized as a pair of lobes that lie parallel to each other and are fused at the base where they are continuous with the storage lobe. In a first instar locust, just after hatching, the small nearly translucent glandular CC is approximately 100 µm long by 45 µm wide by 60 µm in depth although it morphologically resembles the adult form which measures approximately 350 µm long by 150 µm wide by 90 µm in depth. Histological analysis reveals that the number of cells in the glandular lobe increases from approximately 50 cells in a newly hatched individual by about two orders of magnitude to approximately 6000 in an adult CC.

Glandular lobe tissue is composed of irregular clusters of cells with each cluster containing a small number of large diameter cells surrounded by a rind of smaller cells. The size of the cells varies, due almost entirely to increases in the volume of cytoplasm. In a first larval instar (Fig. 1A), the glandular tissue has five to six clusters, each containing two to three large cells and two to three small cells. By contrast, after the last larval stage, the number of clusters has increased (Fig. 1B); about 12-15 are seen in a typical cross-section through an adult CC. A single cluster in the adult CC (Fig. 1C) has more large and smaller diameter cells than a first larval instar while the organization within the cluster is similar.

**Origin of new cells**

Additional cells in the glandular lobes could result from postembryonic proliferation of cells within the gland or migration of cells into the gland or both. To evaluate proliferation in the CC a labelling technique to identify cells undergoing DNA replication was used. Bromodeoxyuridine (BrdU), a substituted nucleoside is incorporated into DNA in place of thymidine during replication and its use in examining neurogenesis in insects has been widely reported (e.g. Truman and Bate, 1988; Shepherd and Bate, 1990; Witten and Truman, 1991). Locusts at all stages of development were injected with BrdU and 16 hours later the CC were processed for immunocytochemical staining with an antibody that recognizes BrdU (Gratzner, 1982). After a 16 hour pulse of BrdU the glandular lobes of the CC contained labelled cells in contrast to the storage lobe where there were no labelled cells (Fig. 2). Bromodeoxyuridine incorporation into neurosecretory cells cannot be taken as definitive evidence of cell division. This is because insect cells, particularly those that synthesize secretory products, are often polytene (Ashburner, 1970), in which DNA replication occurs without cell division. Three observations eliminate this possibility. First, labelled cells occur predominantly in pairs (Fig. 2). Second, CC from animals injected with colchicine, which arrests mitosis in metaphase, exhibit numerous mitotic figures (data not shown). The presence of mitotic cells in the absence of BrdU treatment also provides evidence that BrdU is not a mitogen in this system (e.g. Weghorst et al., 1991). Third, in some preparations exposed to BrdU in the absence of mitotic inhibitors, cells in other phases of mitosis are observed. Images of cells in telophase (Fig. 3) are rare, probably because the cells spend a relatively short amount of time in mitosis. Together these results clearly demonstrate that BrdU incorporation is a valid marker for identifying cell birth in the CC.

The observation that BrdU-labelled cells often occur in pairs suggests that the new cells arise within the CC. The possibility remains, however, that cells born outside the gland could migrate in, thus accounting for the increased numbers of cells. Cell migration is an important mechanism...
Fig. 1. Semi-thin transverse sections through a lobe of the corpora cardiaca at different stages of development illustrating the increase in size of the glandular lobes. (A) 1st instar. In this early stage, five to six clusters are seen in a section through the immature lobe. Each cluster contains a small number of cells; the nuclei of one cluster are labelled (n). (B) Early adult (day 2). In a mature glandular lobe the number of clusters and the size of each has increased. (C) Increased magnification of the cluster outlined in B. The large volume of cytoplasm of the two cells at the centre of the cluster is an indication of the late stage of development. Bars, 15 µm.
Proliferation of neuroendocrine cells in embryogenesis for establishing the architecture of the insect nervous system (Heathcote, 1981; Goodman et al., 1984; Copenhaver and Taghert, 1989) so that neurons born at one site migrate along specific pathways to achieve their mature distribution. To determine where new cells are born, BrdU incorporation was examined in CC in short-term (24 hours) organ culture. If all new cells are migrating in from proliferating tissues outside the CC we would expect to find no labelled cells when the CC are exposed to BrdU in vitro. In such experiments, pairs of labelled cells are distributed throughout the CC and no labelling is observed in the storage lobe or along trunks of nerves leading to the CC. Small parts of the fat body adhere to the CC when it is dissected and, although replicating fat cells often incorporate BrdU, their distinctive morphology prevents them from being mistakenly identified as contributors to the increase in glandular lobe cells. Thus new cells arise by proliferation from cells within the glandular lobe.

The immediate precursors to new pairs of glandular cells are not specifically identifiable. The tissue appears, in serial sections stained with haematoxylin and eosin, to be composed of a population of variable sized cells. The cells have no morphological distinctions that might identify some as neuroblasts (Bate, 1976). It is impossible to determine by appearance alone which cells are newly divided or predict which might divide.

These observations suggested that all cells in the glandular lobes might be equally capable of proliferation. Thus, it seemed possible that differentiated cells expressing AKH peptides were dividing. To determine whether dividing cells contain peptide, locusts were injected with colchicine and 16 hours later the CC were examined with immunocytochemical staining for peptide, combined with DAPI staining of chromosomes. In cells in which we observed mitotic chromosomes there was no peptide staining. It was possible, however, that the dividing cells contained peptide but the peptide level was too low to detect by immunocytochemical means. Colchicine treated CC were therefore examined with electron microscopic techniques to determine the peptide content of mitotic cells. Glandular lobe cells expressing AKH peptides, like other peptidergic cells, are packed with dense cored granules (Rademakers, 1977; Schooneveld et al., 1983; Diederen et al., 1987) so we can use these ultrastructural features as markers for peptide-expressing cells. We specifically searched for mitotic cells in regions of peptide expression and looked for the presence of dense cored granules in mitotic cells. Cells in metaphase of mitosis are devoid of dense cored granules (Fig. 4). A small area of clear cytoplasm is sometimes seen adjacent to the nuclear material that fills the cell after the breakdown of the nuclear envelope. We cannot rule out the possibility that such cells deplete their peptide stores by release or enzymatic degradation, but during the process of division cells appear to contain no peptide.

Temporal patterns of cell birth and peptide expression

The temporal pattern of growth in the CC was assessed by examining cellular proliferation during all stages of postembryonic development. Animals undergoing synchronous development were exposed to BrdU and killed 16 hours later. Incorporation of BrdU into the CC is stage specific (Fig. 5). In every larval instar cells are labelled but the number of cells labelled is dependent on the instar. From the first instar through the fourth instar there is a dramatic increase in the number of cells labelled during the 16 hour period. In the fourth instar, when about 660 cells (±198) are labelled, we estimate that about 20% of the cells are new. During the fifth instar there is a sharp decline in the number of labelled cells (212±43) continuing into the early part of
the adult stage (77±19). About 14 days after adult metamorphosis the animals become sexually mature and at this time there is no BrdU incorporation in the CC.

Changes during postembryonic development in the levels of one of the peptide hormones synthesized in the glandular cells were evaluated following chromatographic separation of CC extracts. Adipokinetic hormone I levels change by more than 100-fold throughout development (Fig. 5), most of the change occurring in the later stages. Thus, the levels of AKH I change 10-fold during the first four larval stages and a further 10-fold increase between the fourth instar and adult life. The most rapid increase in peptide levels, therefore, takes place when the locust is undergoing partial metamorphosis and the acquisition of wings.

Spatial pattern of cell birth

The histological distribution of new cells appears to be random but, in fact, occurs at specific sites in the architecture of the CC. The pattern of cell birth can be defined by the cellular organization of the glandular lobes. The lobes consist of clusters of heterogeneously sized cells with larger cells at the core surrounded by a rind of smaller cells. All clusters in the glandular lobes gain new cells but the new cells always form on the peripheries of the clusters (Fig. 6A). Thus the smallest cells in the outer edge of the cluster are newly generated cells. This pattern of cell birth is observed during all stages of larval growth.

Differentiation of new cells

Since glandular lobe cells are those that synthesize AKH, immunocytochemical techniques were used to demonstrate the peptide content of new cells to confirm their identity. Glandular lobe cells were examined with immunocytochemical techniques using both anti-BrdU and anti-AKH antisera 16 hours after incorporation of BrdU. No instances of double labelling occurred in new cells, indicating that shortly after incorporation (a maximum of 16 hours), new cells do not contain peptide (Fig. 6A). In contrast, CC from animals that had survived for two weeks after exposure to BrdU were labelled with both AKH and BrdU antisera (Fig. 6B). The double-labelled cells were born shortly after the animals were exposed to BrdU, since BrdU is rapidly cleared from the haemolymph (Fig. 7). Thus, new cells do become peptide-producing glandular lobe cells. These instances of double-labelled cells are, however, surprisingly rare. For example, when we examined an animal injected two weeks previously during the fourth larval instar, we had expected to see several hundred BrdU labelled cells with a percentage of this labelled population also labelled for peptide. The number of cells labelled with BrdU, however, decreased with increasing time between exposure and examination (data not shown). In young adults examined 7 days after BrdU injection, the number of cells labelled was 60% less than when examined one day after injection. The decrease appears to reflect loss of BrdU from the cells rather
than loss of cells since the total number of cells in the gland increases continuously through development.

DISCUSSION

During postembryonic development the glandular lobes of the corpora cardiaca in *Schistocerca gregaria* undergo a dramatic change in size. At hatching the CC is composed of fewer than 100 cells. Cells are added to the glandular lobes throughout the period of larval development, which lasts about 4 weeks. The largest numbers of cells are added

Fig. 5. Summary of stage-specific patterns of cell birth and peptide levels. Top: changes in expression of AKH I throughout larval development. Each point represents the average amount of peptide hormone in a single corpora cardiaca at different stages. Bottom: each bar represents the average number of new cells labelled with BrdU in 16 hours in each stage (n=6). The scale of the x-axis reflects the number of days in each larval stage and the position of the bars indicates the time within each stage during which BrdU incorporation was assessed.

Fig. 6. Sections through corpora cardiaca that have been immunocytochemically labelled with antisera to BrdU (orange) and AKH II (green). (A) After 16 hours exposure, many cells have incorporated BrdU. Shortly after incorporation, BrdU-labelled cells are found only on the periphery of the clusters of peptide-containing cells. Cells labelled for BrdU do not label for peptide. (B) Two weeks after exposure to BrdU, labelled cells also label for peptide and are found at the centre of clusters. Bars, 10 µm.
during the third and fourth larval instars with a continuing increment at a lower rate during the fifth instar and early adult life. At the onset of sexual maturity, the adult glandular lobes resemble the larval form but the number of cells has increased by about 100-fold.

Developmental changes in peptide synthesis

A previous study (Hekimi et al., 1991) demonstrated that the levels of peptides synthesized in the glandular lobes change during development. Levels of both AKH I and AKH II increase throughout the postembryonic period. That shifts in peptide levels must be due, at least partially, to the expression of peptide in new cells is confirmed by double-label immunocytochemistry, which showed that new cells do eventually express peptide. The time course of cell proliferation and the changes in peptide levels differ, with the largest increase in cell birth occurring during the third and fourth larval instars and the largest increase in peptide levels occurring mainly between the fourth instar and adult maturation. This time difference is approximately 5 days and most likely reflects the time required for differentiation of the new cells and accumulation of sufficient peptide for measurement by HPLC techniques. It is impossible, however, to relate the increase in absolute levels of peptide to the prior birth of new cells because peptide levels in individual cells cannot be accurately measured. In addition, factors may be acting on all cells in the gland, both new and pre-existing, to influence the synthesis and expression of peptide. Such regulatory mechanisms, along with the birth of new cells, may be the means by which the ratios of AKH I to AKH II change during development.

Further support for this model of peptide regulation may come from determining the time delay between peptide expression and cell birth. This was not possible because the BrdU label disappears from new cells. Since DNA replication is semi-conservative, the loss of label is surprising. Even if after the first division in the presence of BrdU a cell were to divide several times (in the absence of BrdU) the original label would be present in two strands of DNA, which are no longer paired. A high level of sister chromatid exchange during repeated divisions could dilute the label to a point at which it might be undetectable by immunocytochemical methods. The loss could also be due to DNA repair mechanisms, which excise and replace the foreign nucleoside.

Anatomical development of a neuroendocrine gland

The temporal and spatial patterns of cell birth in the glandular lobes have suggested a model for the postembryonic development of the CC. At hatching the gland is composed of a small population of cells some of which are differentiated and expressing AKH peptides. Another population distributed throughout the gland are undifferentiated and mitotically active. During development these cells undergo symmetrical division resulting in pairs of cells. What is the fate of these new cells? The number of cells expressing peptide is increasing throughout development but dividing cells do not contain peptide. It is likely therefore that after division new cells choose between two fates: they may remain mitotically active or they may stop dividing and differentiate. What factors influence the decision to differentiate are not known but both extrinsic and intrinsic mechanisms are suggested by developmental studies in invertebrate systems. For example, in Manduca, steroid hormones involved in metamorphosis control the timing of maturation of neurons generated postembryonically (Booker and Truman, 1987b). Interactions between equivalent sibling cells during embryogenesis in the grasshopper determines the axonal growth pattern (Doe and Goodman, 1985; Kuwada and Goodman, 1985). Intrinsic mechanisms related to cell lineage that count cycles of DNA replication and cell division may also play a role in determining the fate of new cells (reviewed by Williams and Herrup, 1988). This model for glandular growth assumes that cells capable of dividing are not migrating into the gland. The limitations of long term in vitro culture of the gland make it impossible to determine if all precursor cells are part of the glandular tissue at hatching, but the distribution of pairs of cells throughout the glandular lobes suggests it is unlikely that precursors are migrating into the CC.

Division and differentiation of glandular lobe cells takes place at multiple sites at the same time throughout the glandular lobes. New cells arising along the peripheries of cell clusters increase the volume of each cluster. Cells that choose to differentiate accumulate peptide in the cytoplasm and thus increase the volume of the peptide-expressing cells in the cluster. During adult life, cells mature to uniformly large cells while clusters have grown to a maximum volume. Adjacent clusters appear to merge, creating the appearance of a homogenous glandular tissue.

Towards the end of larval life cell proliferation slows despite the increase in the number of cells. In the fourth
larval instar we estimate that about 20% of the cells are new in contrast to the fifth larval instar and the adult where about 2% and less than 1%, respectively, are new. During the last instars near the end of larval life, there is a partial metamorphosis involving the rapid development of wings. Juvenile hormone is known to play an important role in insect metamorphosis and may be involved in the decline in proliferative activity. Spatial constraints in the glandular tissue may also determine the rate of proliferation. Cells in the glandular lobe are connected by symmetrical scleriform junctions associated with mitochondria (Pow and Golding, 1989). These junctions, found in all larval stages, lock the glandular tissue into a stable cellular network and mitotically active cells, enclosed by connecting cells, may be inhibited to divide.

**Mechanism of proliferation**

The mechanism of proliferation of the neuroendocrine cells in the CC contrasts with neuron proliferation in the insect CNS. Neurogenesis during embryonic and postembryonic development follows a stereotypic pattern of cell division (Doe and Goodman, 1985). Neuroblasts, derived from ectodermal cells, are large stem cells that divide asymmetrically to generate smaller ganglion mother cells and neuroblasts. Multiple rounds of neuroblast division yield ganglion mother cells each of which divide once, symmetrically, producing two postmitotic neurons that are clonally related to all the neurons derived from the neuroblast division. In the CC there are no morphologically distinctive stem cells although mitotically active cells are often smaller than the mature differentiated cells that ultimately result. Unlike the nervous system, all observed divisions are symmetrical and the two cells resulting from a division do not appear to be different. Like the products of neuroblast division, however, they may have different fates so that some cells immediately differentiate while others continue to divide and differentiate at a later stage of development.

The elaborate mechanisms for establishing the adult CC suggest a functional significance for the changes in peptide levels and ratios during larval development. The continuous addition to the CC of new cells that synthesize peptide could be a means of regulating both absolute peptide levels and peptide ratios. New cells added to the gland throughout development mature and differentiate resulting in an increase in the absolute levels of peptides. New cells may synthesize peptides in a ratio specific to the stage during which they were generated. Fischer-Lougheed et al. (1993) have recently described transcriptional and translational mechanisms that alter the neupeptide ratios during development in the CC. Thus, while all cells produce all peptides, the continuous addition of new cells synthesizing and accumulating different peptide ratios could account for the progressive shift in peptide ratios throughout development.

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