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MOLECULAR BIOLOGY OF HORMONES INVOLVED IN THE REGULATION OF REPRODUCTION AND GROWTH IN MOLLUSCS

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W. P. M. Geraerts, A. B. Smit, K.-W. Li, P. L. Hordijk and J. Joosse (1991) Molecular biology of hormones involved in the regulation of reproduction and growth in molluscs. *Bull. Inst. Zool., Academia Sinica, Monograph* **16**: 387-440. Molluscs form a large phylum, and have invaded many different habitats. A major factor that has contributed to their success is the development of specific patterns of reproduction and growth. In studies on the neuroendocrine regulation of reproduction and growth, species that function as models in fundamental neurobiological research are used (*e.g.*, the gastropods, *Lymnaea stagnalis* and *Aplysia californica*); however, these animal are of no importance for aquaculture. Nevertheless, it is expected that hormones that are identified in these animals, also occur in molluscan species of economical importance. Molluscs show a great variety of reproductive strategies. Most cephalopods display "suicide" reproduction and all reproductive activities are controlled by only one endocrine centre, the optic glands. Gastropods are hermaphrodites with complex neuroendocrine control systems, which are studied using an interdisciplinary approach that includes the molecular biological methodology.

In *Lymnaea*, the neuroendocrine caudodorsal cells (CDCs) initiate and coordinate the stereotyped egg-laying behaviours. It has been found that a family of two related though distinct genes encoding prohormones, from which overlapping sets of multiple bioactive peptides can be derived, is expressed in the CDCs. In addition, the CDCH genes are expressed in a tissue-specific fashion in various organs that are involved in distinct aspects of male and female reprodution. The APGWamide precursor is expressed in neurons in the lobus anterior that form part of an extensive network controlling male mating behaviour. Currently, additional peptidergic cell types involved in the control of copulatory behaviour are being analyzed. Growth and associated processes are regulated by the

neuroendocrine light green cells (LGCs), which express a family of four related though distinct molluscan insulin-related genes, which are regulated in a stimulus-dependent fashion. In addition, the LGCs express a gene encoding the peptide, schistosomin, which upon release excites the LGCs (autotransmission), thus stimulating growth, and inhibits reproductive activities at the same time. Schistosomin, therefore, links growth and reproductive activities, and it is suggested that schistosomin attunes these processes to each other.

Key words: Molecular biology, Hormones, Regulation, Reproduction, Growth, Mollusca.

The Mollusca are a very large phylum. With a approximately 110,000 living species there are twice as many species of molluscs as there are of vertebrates, and only the arthropods are a more successful group. Molluscs have occupied many different types of habitat in marine, freshwater and terrestrial environments. A major factor that has contributed to their success is the development of specific patterns of reproduction and growth and studies of molluscan modes of reproduction and growth, therefore, are informative, both physiologically and evolutionary. In addition, detailed knowledge of the physiological regulation of reproduction and growth will be an absolute requirement for a successful accomplishment of molluscan aquaculture.

Although (neuro)endocrine centres that control reproduction and growth have been studied in a variety of molluscs, relatively few investigations have focused on species that are of importance in aquaculture (see Table 1). In most experiments, species belonging to the cephalopods and gastropods serve as experimental molluscan models in fundamental

Table 1
Molluscs that are important in aquaculture, and molluscs that serve as experimental mode systems in fundamental neuroendocrine research

	Molluscs important in aquaculture	Molluscan models used in fundamental research
Cephalopods	squids, octopods	(*Sepia*)
Gastropods	*Helix*, abalone	*Lymnaea stagnalis* *Aplysia californica* (*Helix*)
Bivalves	mussels, oysters	—

research on the neuroendocrine regulation of reproduction and growth. The main reason is that reproduction and growth of species belonging to these two groups show characteristics that have attracted the attention of many physiologists and endocrinologists. For instance, all cephalopods are gonochorists, and the class comprises both the primitive *Nautilus* and the highly advanced squids and octopods, which display complicated behavioural patterns and compete successfully with vertebrates (such as fish) occupying the same habitats. Despite this, the hormonal regulation of cephalopod reproduction is surprisingly simple and involves only one endocrine gland. By contrast, the hermaphroditic gastropods enjoy a hormonal regulatory system almost as complex as that found in vertebrates. As we shall see, in gastropods the (neuro)endocrine control systems of reproduction and growth are intimately linked.

In addition, the CNS of some gastropod species, *i.e.*, *Lymnaea stagnalis* and *Aplysia californica*, are favoured neurobiological model systems, which are intensively studied; consequently, much is known of the endocrinological, anatomical, morphological, electrophysiological as well as molecular biological aspects of the functioning of peptidergic (neuroendocrine) systems. By contrast, far less is known of these systems in the cephalopods. Bivalves, such as mussels and oysters, are very difficult to handle experimentally, and almost nothing is known of the neuroendocrine regulation of reproduction and growth. In this review, *L. stagnalis* will be covered extensively, as much is known of the regulation of both reproduction and growth in this species. The animal may, therefore, serve as a model animal for research on molluscan species of economical importance. Attention will also be given to the cephalopods. For more detailed information, the reader should consult the extensive reviews by Arnold (1984), Tompa (1984), Hadfield and Switzer-Dunlap (1984), Joosse (1988), and Geraerts *et al.*, (1988a, 1988b, 1991).

CONTROL OF REPRODUCTION IN CEPHALOPODS AND GASTROPODS

Cephalopods: terminal reproduction controlled by a simple endocrine system

In cephalopods, males and females have single gonads with male resp.

female cells in all stages of development. The gonad is connected *via* an efferent duct with the accessory sex organs (Wells, 1978; Arnolds, 1984). A complicated sexual behaviour proceedes and accompanies mating in most species. Copulation involves the use of a modified arm, the hectocotylus, with which the spermatophores are passed to the female. Eggs are presumably fertilized in the oviduct as they pass through the spermathecae. After oviposition they are attached to a substrate. Some cephalopods, especially octopods, have been observed to display some kind of maternal care for the developing embryos and the recently hatched offsping.

Only one hormone seems to be involved in the control of nearly all of the reproductive activities

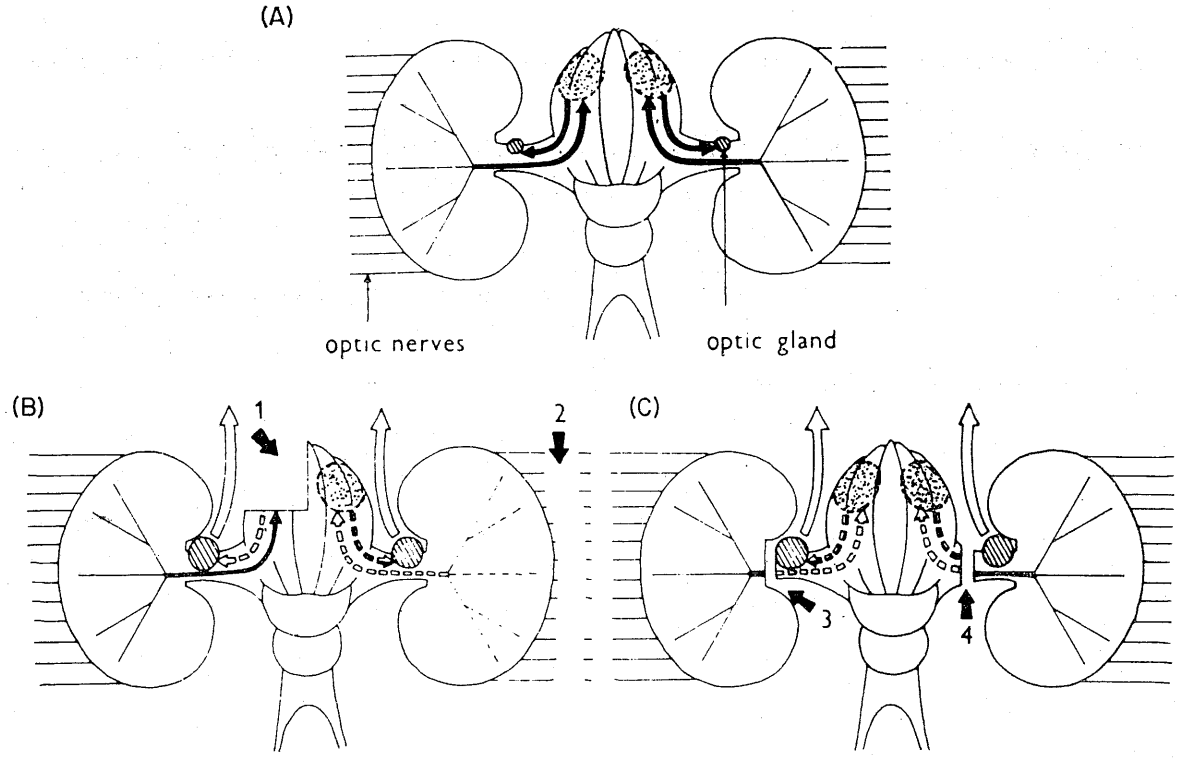


Fig. 1. The mechanism of hormonal control of gonad maturation in *Octopus*. A. Situation in an immature, unoperated *Octopus*, where secretion by the optic glands is held in check by an inhibitory nerve supply. B. Two operations that cause the optic glands to secrete a product causing the gonad to enlarge, being (1) removal of the source of the inhibitory nerve supply, and (2) optic nerves section. C. Further operations having the same effect upon the gonads, thus eliminating the possibility that there is also an excitatory innervation, being (3) optic lobe removal and (4) optic tract section. From Wells (1978).

(Richard, 1970a, 1970b; O'Dor and Wells, 1975; Wells, 1978; Wells and Wells, 1977; Joosse and Geraerts, 1983). The hormone is non-sex-specific and non-species-specific and is produced by the optic glands, endocrine organs of nervous origin that are located upon the optic tracts, which connect the brain with the large optic lobes (Fig. 1). The production and release of the hormone is under inhibitory nervous control. It stimulates the development and functioning of the gonads, including cell divisions in the germinal epithelium, production of proteinaceous yolk, and spermatogenesis and spermatophore production. It also stimulates the oviducal duct and accessory glands, and induces mobilization of muscle proteins, which are used for gamete production. The "brooding" behaviour of females is dependent on the hormone, but the mating behaviour is not.

Most cephalopods live only about one year, and nearly all are semelparous, *i.e.*, the animals reproduce only once and then die (Calow, 1983). Cephalopod reproduction represents a rather extreme case of the semelparous situation in that reproduction in these animals is an "all or none" process. As soon as the optic glands are activated, a fixed series of reproductive events (maturation, mating, egg laying) is initiated, which is inevitably followed by death. Changes in environmental conditions apparently have no or very little effect on these processes. Furthermore, no (neuro)endocrine structures affecting the activity of the optic glands have been found. This implies that releasing factors or gonadal feedback mechanisms are nonexistent in cephalopods. The reproductive period ends rather dramatically. After mating and egg laying the animals, males and females alike, cease feeding and eventually die of exhaustion. The phenomenon is called terminal or "suicide" reproduction.

Environmental cues that are able to raise the nervous inhibition of the optic glands and hence activate the glands have been investigated in detail in *Sepia officinalis* and *Octopus vulgaris*. From the experimental data a series of events that make up a reproductive cycle can be inferred, which is summarized in Fig. 2. Apparently, in cephalopods the development of terminal reproduction probably has eliminated the need for additional (neuro)endocrine or nervous control mechanisms. (These is at least one exception to this rule: the primitive *Nautilus*, which does

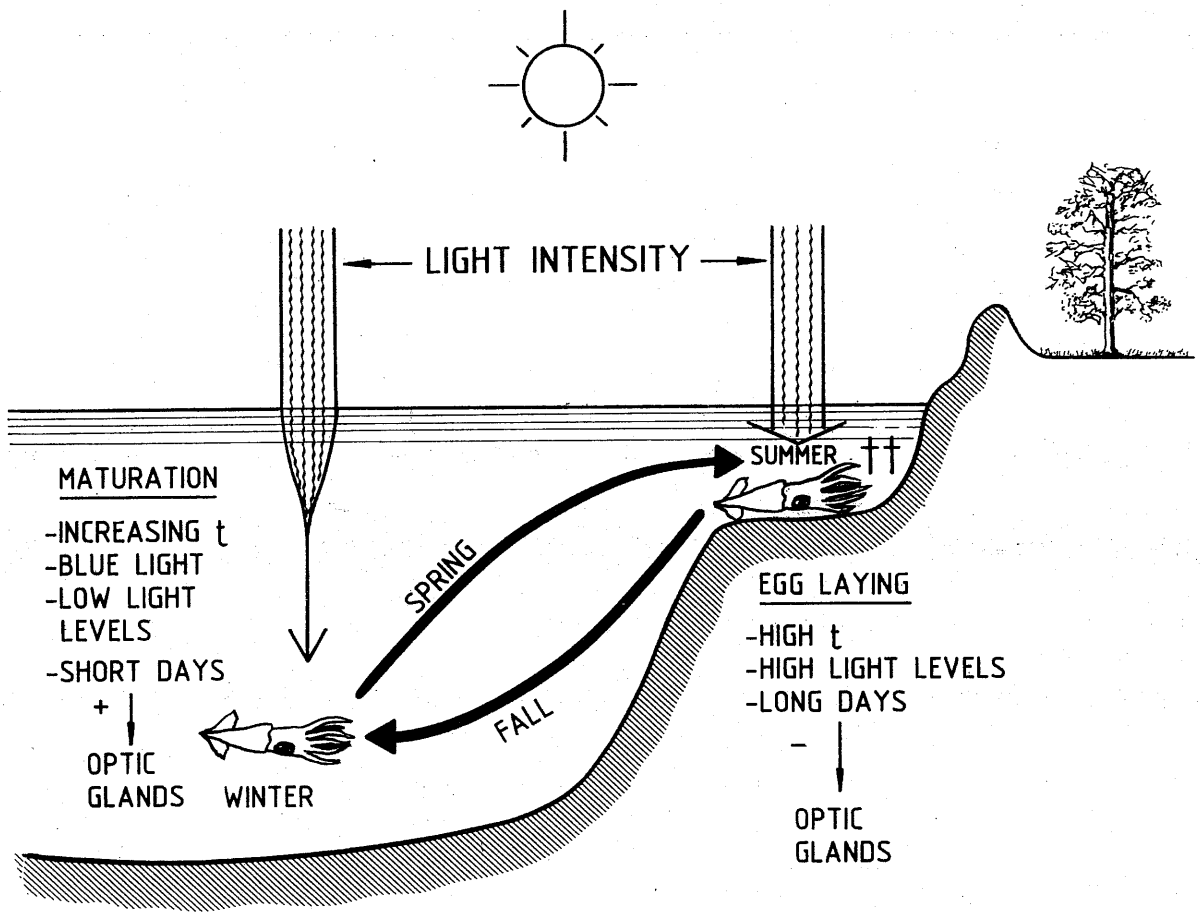


Fig. 2. Environmental regulation of the reproductive cycle in *Sepia officinalis*. During the late fall shoals of recently hatched *S. officinalis* migrate from the shore to the deep waters of the open sea, where during the winter sexual maturation is initiated. Light and water temperature are important in the control of onset of sexual maturation. First, the temperature must reach a certain minimal value. Meanwhile the animals attain the size needed for gonadal development. Optic gland activation and subsequent sexual maturation is then triggered by the short days (12 h light per day) of winter, in combination with the blue light of low intensity that prevails in deep waters. In the spring, at longer days, the animals move inshore to the warmer shallow waters with high light intensity. These conditions, in particular the long days of summer, stimulate egg laying in mature animals. After reproduction the animals die.

not know terminal reproduction. Interestingly enough, *Nautilus* does not have an optic gland).

Gastropods: hermaphroditism controlled by a complex neuroendocrine system

The organization of the genital system is similar among the stylommatophorans (terrestrial pulmonates, such as the economically important *Helix*, opisthobranchs (*e.g.*, *A. californica*) and basommatophorans (freshwater pulmonates, *e.g.*, *L. stagnalis*). The gastropod pattern, as exemplified by the basommatophoran system, is shown in Fig. 3. In most hermaphrodite gastropods, both male and female cells along with follicle cells and Sertoli cells, are produced in each of the numerous acini of the ovotestis. At ovulation numerous oocytes are transported to the carrefour region where fertilization occurs. Then the egg cells are surrounded one by one by perivitellin fluid of the albumen gland. The fluid is rich in protein and

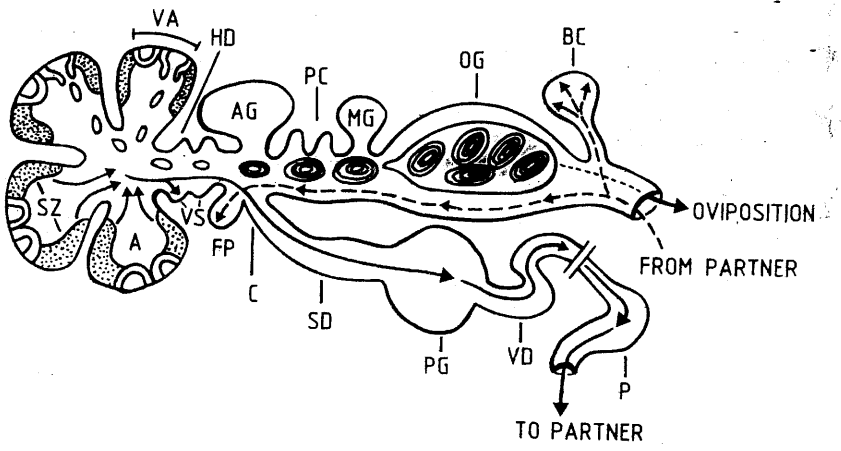


Fig. 3. Diagrammatic representation of the functional anatomy of the gastopod reproductive system. Organization based on the basommatophoran, *Lymnaea stagnalis*. Indicated are ovulation, egg formation and egg mass formation →=passage of autosperm. Sperm is stored in the vesiculae seminales (VS) and transported to the functionally female copulation partner, - - →=passage of foreign sperm. The greater part of it is hydrolyzed in the bursa copulatrix (BC), and a small part is transported to the fertilization pocket (FP) where it is stored until ovulated oocytes appear for fertilization. A=Acinus of the ovotestis; AG=albumen gland; BC=bursa copulatrix; C=carrefour; FP=fertilization pocket; HD=hermaphrodite duct; MG=muciparous gland; OG=oothecal gland; P=penis; PC=pars contorta; PG=prostate gland; SD=sperm duct; SZ=spermatogenic zone; VA=vitellogenic area; VD=vas deferens; VS=vesiculae seminales.

galactogen, and serves as nutrition during embryonic development. The eggs are packaged into an egg mass (or string), and during oviposition eggs are fixed to a substrate (or, in the case of terestrial pulmonates, in a nest that is excavated in soft and moist soil). Many gastropods display an intricate pattern of behaviours accompanying egg laying or mating. Copulation is either reciprocal or unilateral. Autosperm is transported through the male duct and provided with secretions of the male glands. Foreign sperm received during copulation is transported through the female duct to the fertilization pocket, where it is stored for fertilization of oocytes.

Separate male and female (neuro)endocrine control systems have been shown to regulate hermaphroditism in the pulmonates (Fig. 4) (Joosse, 1979; Joosse and Geraerts, 1983; Joosse, 1988). These are located in a simply organized CNS consisting

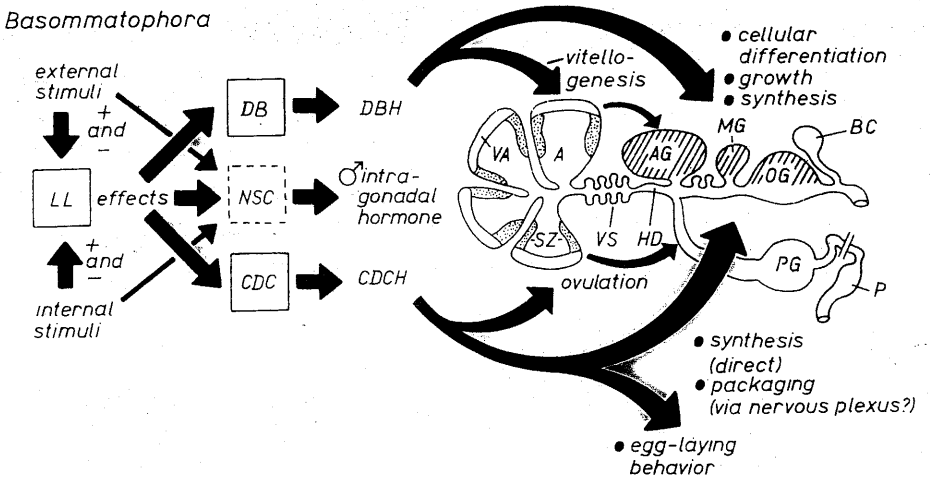


Fig. 4. Schematic representations summarizing the present data on the endocrine control of reproduction in gastropods. Based on *Lymnaea stagnalis*. All actions of hormone concern the control of female activity, except for the stimulation of male maturation by (a) center(s) in the lateral lobes (LL), which is probably effected *via* neurosecretory cells (NSC) in the central nervous system, and a male intragonadal hormone. Gonadal hormones may be involved in the control of the male and female reproductive tracts. For details, see text. A=acinus; AG=albumen gland; BC=bursa copulatrix; CDC=caudodorsal cells; CDCH=caudorsal cell hormone; CG=cerebral ganglia; DB=dorsal bodies; DBH=dorsal body hormone; HD=hermaphrodite duct; LL=lateral lobes; MG=muciparous gland; NSC=neurosecretory cells; OG=oothecal gland; P=penis; PG=prostate gland; SD=sperm duct; SZ=spermatogenic zones; VA=vitellogenetic areas; VS=vesiculae seminales.

of a ring of relatively few ganglia around the oesophagus. The control systems include neuroendocrine as well as gonadal factors. A masculinizing factor (stimulating spermatogonial mitosis, and growth and differentiation of the male part of the reproductive tract) probably is present, but has not yet been structurally identified. It is equally possible that male gonadal hormones are produced, but also in this case no structural data are available. In the basommatophorans, the lateral lobes, small ganglinonic appendices of the cerebral ganglia (Fig. 5), have a stimulating effect on spermatogenesis, probably *via* neurosecretory cells in juvenile snails, but spermatogenesis is not blocked, only delayed, after their removal (Geraerts, 1976a).

The various components of an extensive neuronal network underlying copulation as a male in *Lymnaea* have recently been identified (van Duivenboden, 1984; van Duivenboden and Ter Maat, 1988). The network consists of various types of peptidergic neuron and a cluster of serotonergic cells that all send axons to the penial complex *via* the penial nerve, the sole nerve to innervate

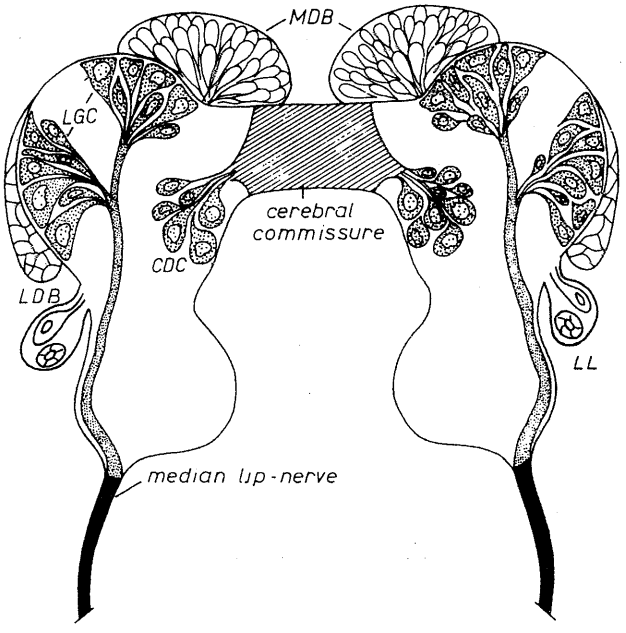


Fig. 5. Diagrammatic transverse section through the cerebral ganglia of *Lymnaea stagnalis* showing the location of the various neuroendocrine and endocrine centers. CDC=caudodorsal cells; LGC=light green cells; LL=lateral lobes; MDB and LDB=mediodorsal and laterodorsal bodies. The periphery of the cerebral commissure is the neurohemal area of the CDC; the peripheries of the median lip nerves, that of the LGC.

the complex and male accessory sex organs. A neuroethological and electrophysiological analysis along with a molecular biological and peptide-chemical analysis of the network has recently been initiated. The bioactive peptides produced by one of the constituent neuron clusters of the network, the lobus anterior of the right cerebral ganglion, have been structurally identified and the prohormone organization has been resolved (see Fig. 6). The prohormone contains 10 copies of the tetrapeptide, Ala-Pro-Gly-Trp-amide (APGWamide), and one copy of the 39 amino acid long C-terminally located anterior lobe peptide (CALP) (A.B. Smit, unpublished results; K.W. Li, unpublished results). APGWamide could also be recovered from the penial complex. The peptide, when applied *in vitro*, inhibits the contractions of the penis retractor muscles evoked by serotonin, suggesting regulation of the penial complex during copulation by an intricate interplay of peptides and classical neurotransmitters. The identification of additional peptides regulating male copulatory behaviour is currently being persued, and their role in male mating will be studied.

Female cells arise by autodifferentiation, but the further maturation of the oocytes is controlled by the dorsal body hormone (DBH) from the dorsal bodies (DBs), non-nervous endocrine organs that are located upon the cerebral ganglia (Fig. 5) (Geraerts and Joosse, 1975). DBH controls, in addition, the development of the female accessory sex organs (Geraerts and Algera,

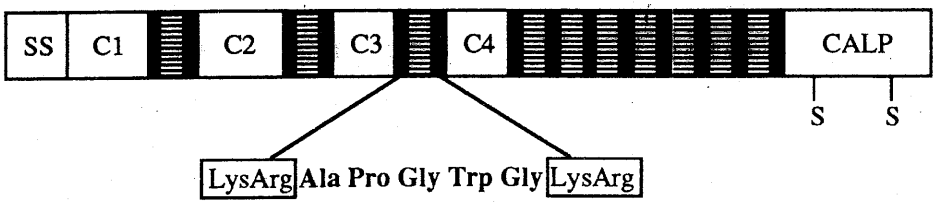


Fig. 6. Organization of the APGW amide preprohormone from the anterior lobe of the right cerebral ganglion in *Lymnaea stagnalis*. 10 copies of a peptide consisting of 5 amino acids, Ala-Pro-Gly-Trp-Gly are present on the preprohormone as encoded by a recently isolated cDNA (A.B. Smit, unpublished results). Peptide chemistry has shown that the peptide is modified to yield the C-terminally amidated APGW amide (Li *et al.*, in press). Indicated are: SS, signal sequence; C, etc. connecting peptide sequence; CALP, C-terminal anterior lobe peptide; -S, cysteine residue; black vertical bars, Lys-Arg proteolytic processing sites.

1976), possibly in conjunction with (a) gonadal (steroid) hormone(s). The chemical nature of DBH is still unknown. Recent molecular biological studies in our laboratory have shown that the DBs specifically and abundantly express a gene encoding a cytochrome P450 (Y. Teunissen, unpublished results). As this enzyme may be involved in the production of steroids, we have tested the possibility that the DBs synthesize insect or vertebrate steroids; however, the experimental evidence including attempts to estabilish precursor-product relationships was clearly negative. We are currently pursuing the possibility that the DBs synthesize juvenile hormone-like compounds or prostaglandins (juvenile hormone and related compounds are involved in the control of ecdysis and vitellogenesis in arthropods). Much more is known concerning the peptidergic control of egg laying in gastropods, and in the next section the peptidergic command system for egg laying in *Lymnaea* together with the genes involved will be reviewed.

The caudodorsal cell (CDC) system controls egg-laying behaviour in *Lymnaea*

The first indications that the caudodorsal cells (CDCs) in the cerebral ganglia of *Lymnaea* are involved in egg laying came from classical endocrinological extirpation/injection experiments (Geraerts and Bohlken, 1976). Subsequently, the CDC system was studied using a multidisciplinary approach (for details see Geraerts *et al.*, 1988a). The somata of the CDCs are located in the caudodorsal part of each cerebral ganglion (Fig. 5). Each cluster contains about 50 cells and the individual CDC somata may reach a maximal size of 100 μm. The axons emerge excentrically from the somata and first run to the anterior part of the cerebral ganglia, then form a loop in the "loop area", where they run together and contact each other electrotonically (Fig. 7). After leaving the loop area the axons run toward the ipsilateral part of the intercerebral commissure (COM) and form many thin branches that contain the neurosecretory granules and that end blindly in the neurohaemal area. The ventral CDCs form an additional branch, which runs straight to and through the COM, passes through the contralateral loop area, and then returns to the contralateral half of the neurohaemal area. In this way the ventral CDCs connect electrotonically both CDC clusters *via* the crossing

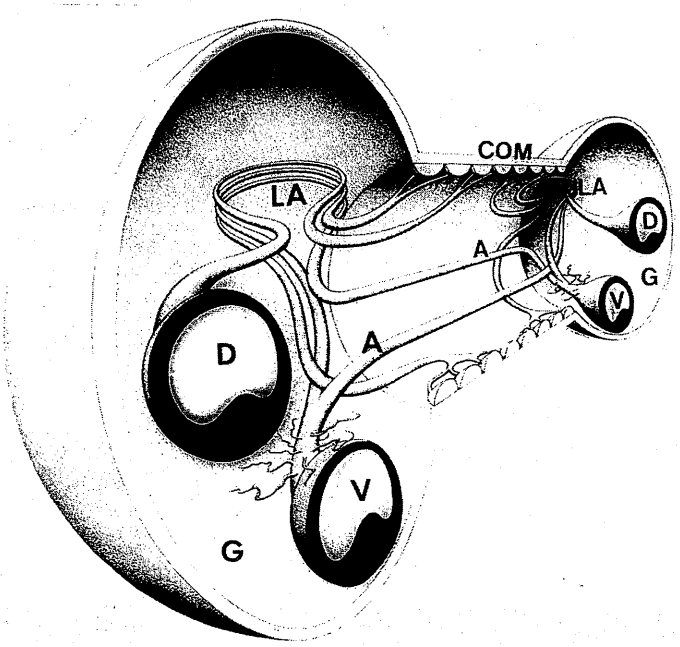


Fig. 7. Schematic representation of the cerebral ganglia (G) of *Lymnaea* with dorsal (D) and ventral (V) CDCs. Each has an axon branch that runs through the ipsilateral loop area (LA). Ventral CDCs have an additional branch (crossing axon, A) running *via* the COM through the contralateral loop area. All axon branches form neurohaemal terminals in the periphery of the COM.

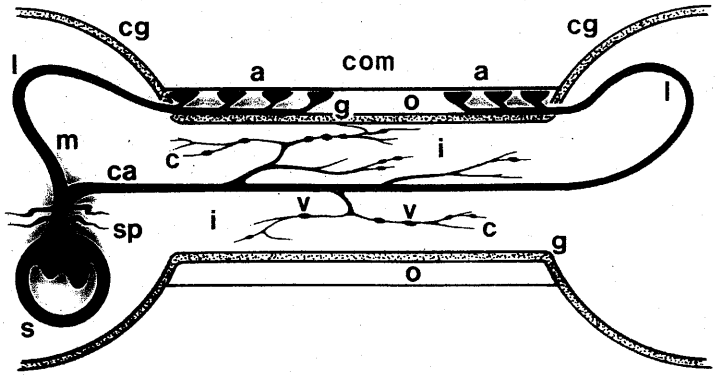


Fig. 8. Outer and inner compartments of the COM of *Lymnaea stagnalis*. The compartments are separated by a glial sheath (g). Shown is the anatomy of one ventral CDC. The main axon (m) of the ventral CDC runs through the loop area (l) to the neurohaemal area in the outer compartment (o), where the axon terminals (a) end blindly. A branch of the main axon, the crosing axon (ca), runs through the inner compartment (i) of the COM, passes through the contralateral loop area, and then runs to the outer compartment. In the inner compartment the crossing axon gives rise to the collaterals (c). cg, cerebral ganglion; s, soma; sp, spines; v, varicosity. From Schmidt and Roubos (1987).

axons and the loops. The proximal, unbifurcated part of the axon is studded with side-branches, which are involved in the reception of synaptic input. Thus, the function of the subset of specialized ventral CDCs is to receive the sensory egg-laying inducing stimuli and to relay these to the other CDCs. Furthermore, the crossing axons give rise to collaterals, which ramify and form an extensive diffuse network, the collateral system, throughout the inner compartment (Fig. 8) (Schmidt and Roubos, 1987). It seems likely that the collaterals enable the CDCs to communicate with targets within the CNS in a nonsynaptic ("paracrine" or "hormone-like") fashion (see below).

The organization and expression of the ovulation hormone (CDCH) gene family

The ovulation hormone (CDCH) was the first peptide to be structurally characterized from the CDC system (Ebberink *et al.*, 1985). With the help of oligonucleotides derived from the CDCH primary sequence a small family of CDCH genes encoding two related prohormones, the CDCH I and II prohormones, was identified using both a cDNA library of the *Lymnaea* CNS and a genomic library of *Lymnaea* (Vreugdenhil *et al.*, 1988; see also Geraerts *et al.*, 1991). The CDCH genes contain two exons, of which exon I contains the 5′ non-coding part and exon II the complete coding region (Fig. 9). The most

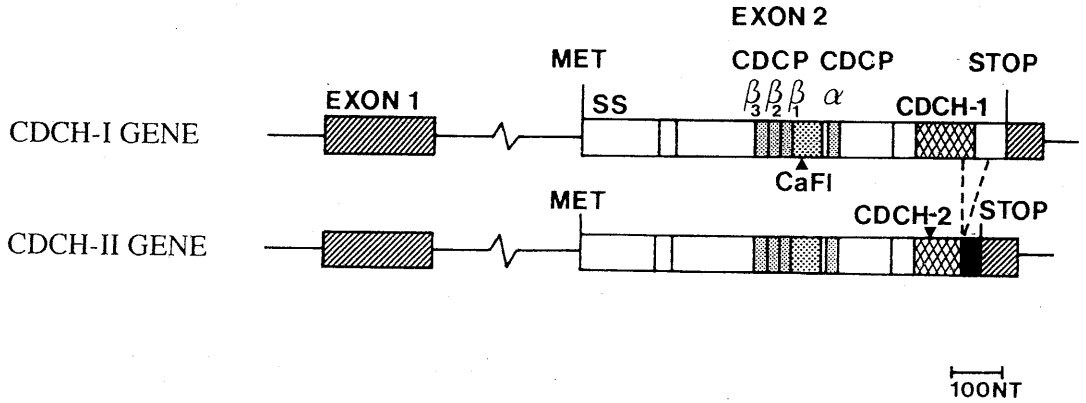


Fig. 9. Organization of the CDCH gene family. Indicated are exons, introns, the translation initiating codon methionine (MET), the stop codon (STOP) and the various possible proteolytic cleavage sites (vertical bars). SS, signal sequence; CDCP, caudodorsal cell peptide; CDCH, caudodorsal cell hormone; CaFl, calfluxin; NT, nucleotides.

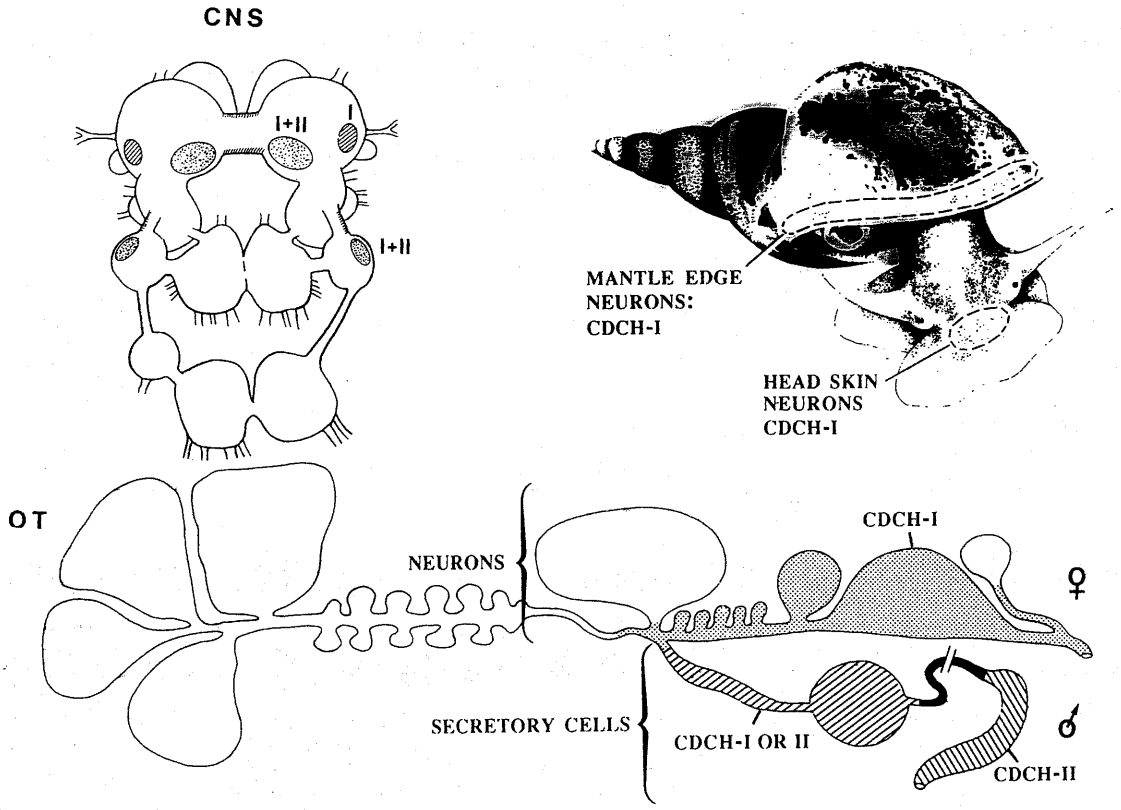


Fig. 10. Summary of the central and peripheral expression of the CDCH I and II genes (indicated as I and II) in *Lymnaea stagnalis*. The CDCH genes are expressed in the CDCs and other neurons of the CNS, the skin of the head region, the mantle edge and the female part of the reproductive duct (stipled), except for the albumen gland. In the male glands (cross-hatched), the genes are expressed in exocrine cells. CNS, central nervous system; OT, ovotestis. Courtesy of Dr. Joosse.

striking difference between both CDCH prohormones is a 17 bp deletion near the C-terminal region of CDCH including its proteolytic processing site. This gives rise to a truncated form of CDCH, which is flanked C-terminally by a completely different peptide. The truncated CDCH and the C-terminal peptide together have been termed CDCH II. In addition, several amino acid substitutions occur in other peptide domains. As a result, each type of CDCH gene encodes a different though overlapping set of peptides.

The CDCH gene family is expressed, in a tissue-specific way, both in the CNS in cell clusters or dispersed throughout the ganglia, and in neurons or secretory cells in the skin, the mantle edge, and the male and female accessory sex organs

(Figs. 10-12) (van Minnen *et al.*, 1988, 1989). Both CDCH genes are expressed in the CDC system. In addition, the female glands the CDCH I gene is expressed in neurons that control the activities of muscles and glandular cells involved in egg mass production. In the male glands, the

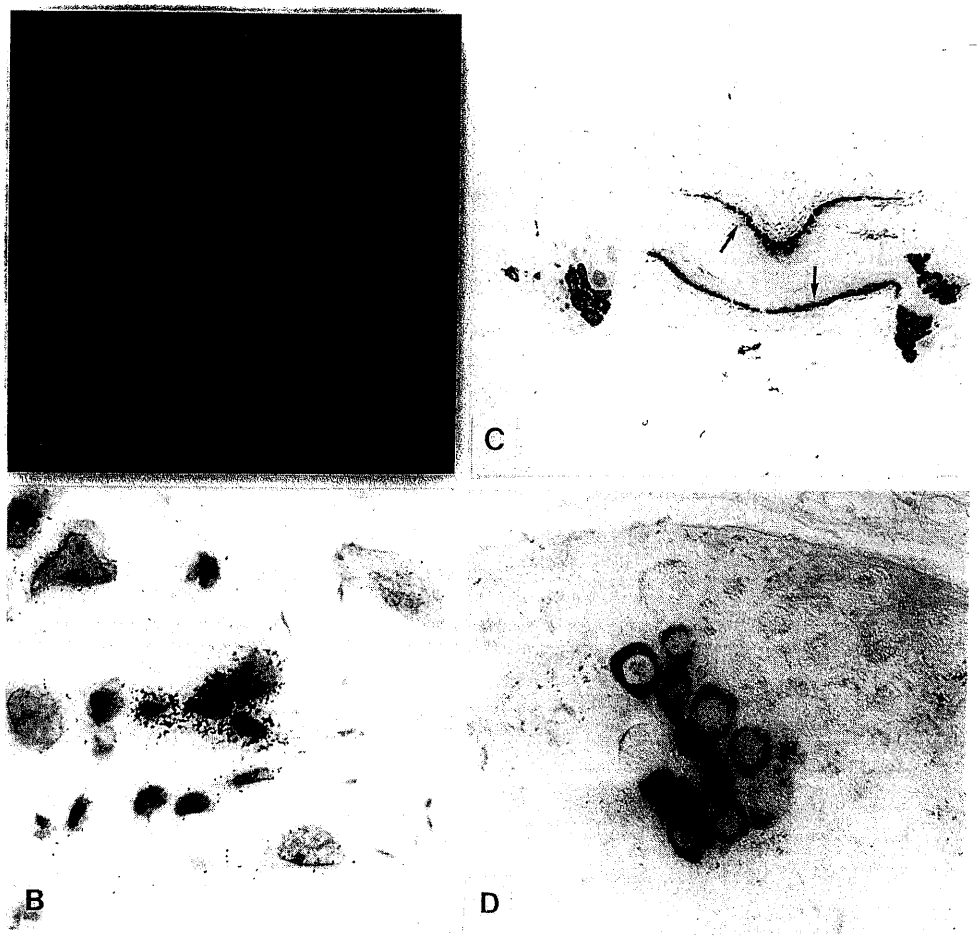
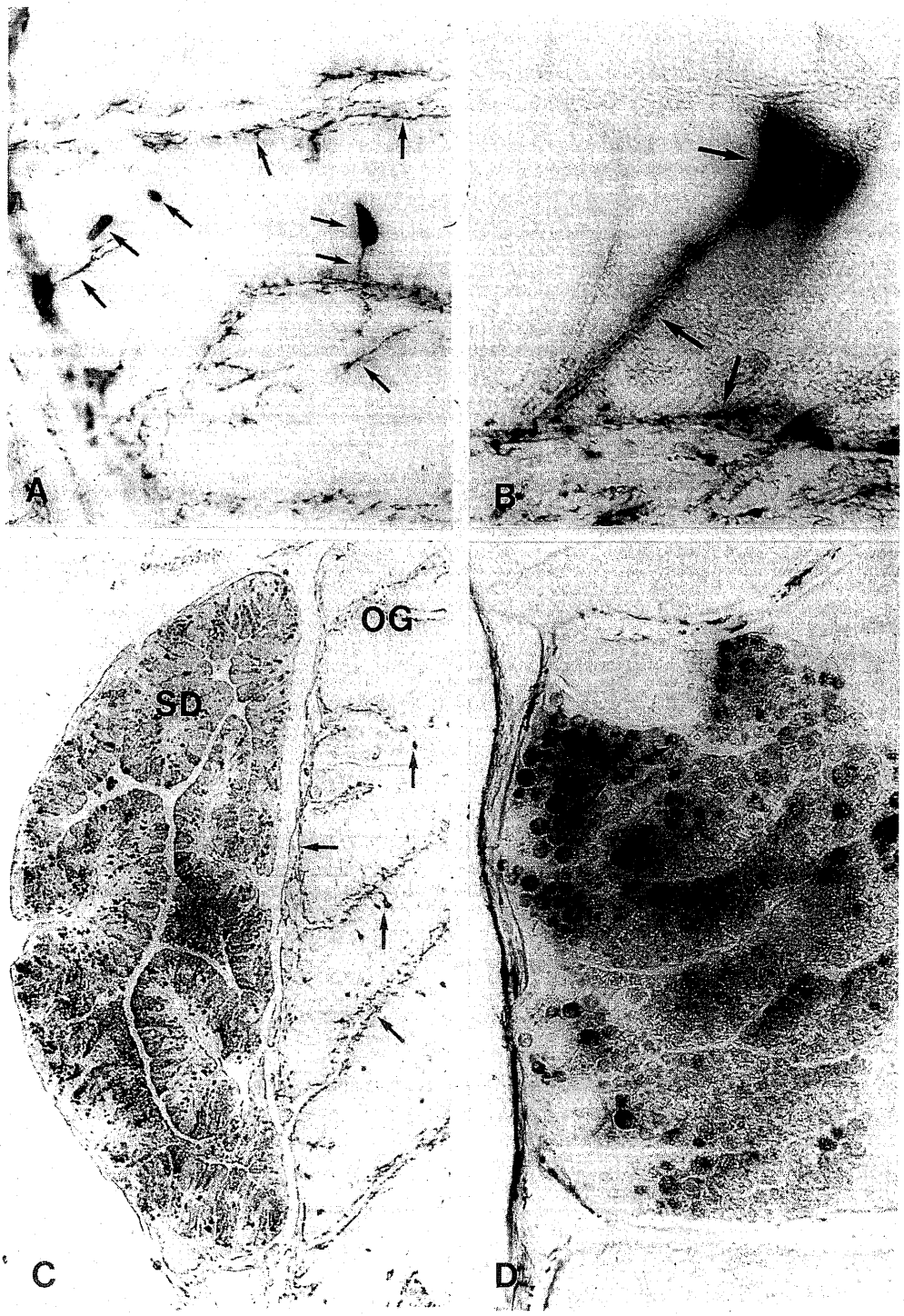


Fig. 11. *In situ* hybridization and immunocytochemical localization of CDCH and CDCH-like peptide expressing neurons in the CNS of *Lymnaea stagnalis*. A, B: *In situ* hybridization. Bouin-fixed and frozen sections of the CNS were exposed to a CDCH-specific cDNA probe labelled with ^{35}S. The probe hybridized with CDCH mRNA, whose location was revealed by autoradiography. A: Dark-field autoradiographs. Arrows show hybridization in the clusters of CDCs in the cerebral ganglia. ×40. B: The black dots show hybridization in cells other than the CDCs in the cerebral ganglia. ×400. C, D: Immunocytochemistry using a polyclonal antibody to synthetic CDCH. C: AntiCDCH-positive perikarya of the cerebral ganglia and their axon terminals in the COM (arrows). ×40. D. AntiCDCH-positive perikarya of a group of cells other than the CDCs in the left cerebral ganglion. These cells express only the CDCH I gene (*cf.*, Fig. 10). ×500. Courtesy Dr. van Minnen.


A
B
SD
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D

CDCH I and II genes are expressed in glandular cells. The materials of these cells are secreted and passed on to the partner during copulation. They possibly regulate reproductive behaviour of the partner acting as the female copulant. It is furthermore suggested that sensory and other types of neuron that express the CDCH genes in the skin, female tract and CNS are involved in transmitting egg-laying-inducing stimuli to the CDC clusters in the cerebral ganglia.

Structure and processing of the CDCH prohormones: An evolutionary considerations

The structural organization of both CDCH precursors are quite similar, with conservation of all but one of the potential proteolytic cleavage sites (see above at Fig. 9). In Fig. 13, the CDCH-I precursor of *L. stagnalis* is shown together with the egg-laying hormone (ELH) precursors of *A. californica* and the ELH precursor of a second *Aplysia* species, *A. parvula* (Scheller *et al.*, 1982; Nambu and Scheller, 1986). ELH precursors are expressed in the bag cells, neuroendocrine cells with a similar function as the CDCs. Comparison of the precursors and peptides is of interest with regard to the evolutionary history of these species. The structural organizations of the ELH precursors of both *Aplysia* species are quite similar, with conservation of all but one of the potential proteolytical cleavage sites. The overall amino acid homology between the ELH precursors is 66%. The regions containing ELH, α-bag cell peptide (α-BCP), and the β-BCPs are even more highly conserved, indicating that these peptides are of importance in egg laying. Three regions of the CDCH precursor identified so far are of interest: CDCH has a high degree of homology with ELH, α-CDCP with α-BCP, and a region containing three peptides,

Fig. 12. CDCH-immunoreactive cells and processes in the accessory sex organs of *Lymnaea stagnalis*. A: Oothecal gland. Arrows show antiCDCH-positive perikarya of neurons as well as positive varicosities of axons apparently contacting secretory cells. ×200. B: AntiCDCH-positive perikaryon and a ramifying process (arrows) of a neuron in the oothecal gland. ×500. C: Cross-section of the spermduct (SD) and the oothecal gland (OG). The secretory cells of the spermduct are filled with antiCDCH-positive material. In the oothecal gland, antiCDCH-positive neurons and varicosities of axons are present (arrows). ×40. D: Cross-section of the prostate gland with secretory cells containing antiCDCH-positive secretory granules (SG). ×500. Courtesy Dr. van Minnen.

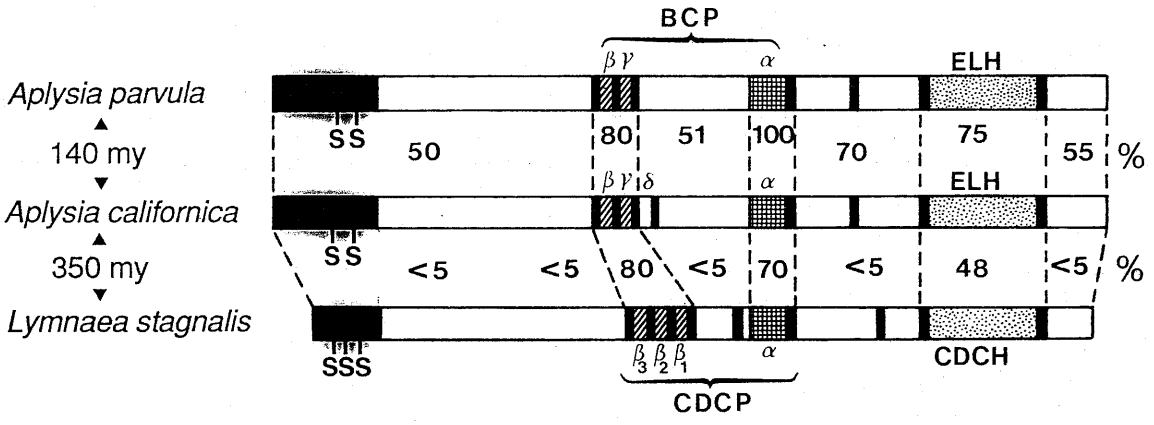

Fig. 13. Comparison of the CDCH and egg-laying hormone (ELH) precursors from the CDCs of *Lymnaea stagnalis* and the bag cells of *Aplysia californica* and *Aplysia parvula*. Percentage homologies between regions are indicated. Further indicated are the positions of the identified peptides, including CDCH and ELH, α-bag cell petpide (α-BCP) and α-CDCP, β-, γ-BCP and β_1-3-CDCP. Cleavage sites are shown as vertical black bars. Signal peptides are shown as black areas. -S, position of cysteine residue.

the β-CDCPs, have a remarkable sequence homology with β- and γ-BCP, and with α-CDCP and α-BCP. In comparison with ELH precursors, one extra pentapeptide (β2-CDCP) has been generated in the CDCH precursor. Compared with *A. parvula* and *L. stagnalis* precursors, the δ-BCP region in the *A. californica* precursor is completely different. The region between the β-CDCPs and α-CDCP is much shorter than the counterpart on the ELH precursor. This difference suggests the

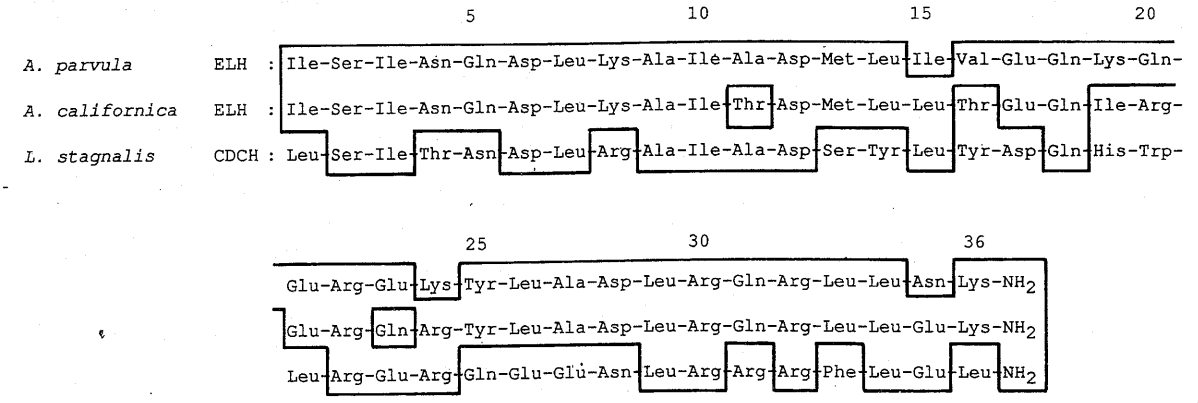

Fig. 14. Amino acid sequence homologies among CDCH of *Lymnaea stagnalis* and the ELHs of *Aplysia californica* and *A. parvula*. Identical amino acids are enclosed within boxes.

occurrence of one or more deletions in the *Lymnaea* gene during the course of evolution. Another difference between the CDCH and ELH precursors is that, apart from the homologies described above, the CDCH and ELH precursors are not homologous.

CDCH and the ELHs each consists of 36 amino acids (Fig. 14). They have a basic character. The ELHs of both *Aplysia* species exhibit 78% homology, with nearly all of the amino acid alterations in the middle third of the molecule. CDCH shows 44% homology with ELH of *A. californica*, with amino acid alterations predominantly in the middle part of the peptide. α-BCPs share 100% homology, and α-CDCP has 5 uninterrupted amino acids in common with the α-BCPs (Fig. 15), suggesting that the α-CDCP exhibits similar functions as α-BCP, including the autotransmitter function (see below). β_1-CDCP is completely homologous with the *Aplysia* β-BCPs (Fig. 15), and further exhibits strong homology with β_2- and β_3-CDCP, and with the γ-BCPs. *A. californica* and *A. parvula* diverged from each other some 140 million years ago, while pulmonate

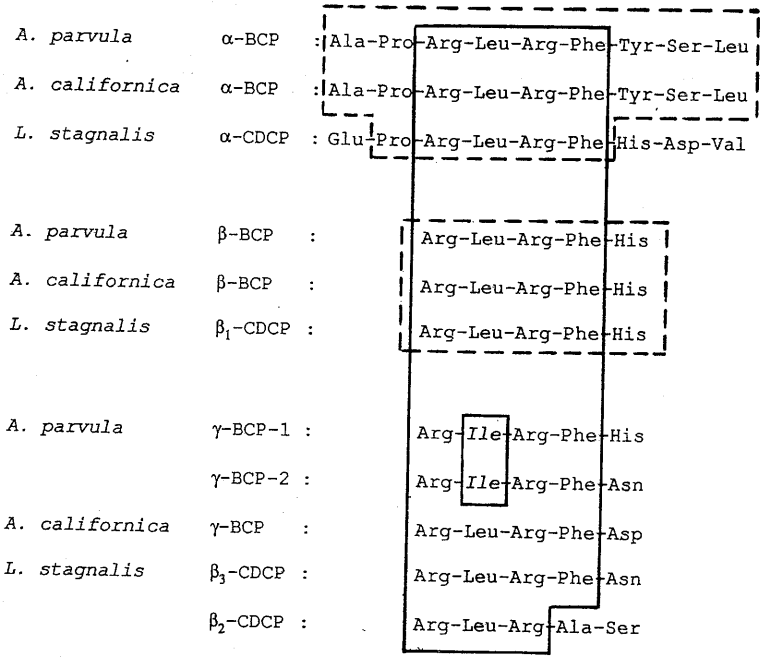

Fig. 15. Amino acid sequence homologies among α-CDCP, α-BCPs and the pentapeptides of CDCH and ELH precursors. Identical amino acids are enclosed within boxes.

and opisthobranch gastropod subclasses diverged from each other about 350 million years ago. Also, the Lymnaeidae are evolved from marine-based molluscs through land-based intermediates, and one only distantly related to the aplysiids. Regions on the precursors that exhibit strong homology are peptides that control egg laying. Apparently, these peptides have been under intense selective pressure. It is tempting to speculate that the diverged peptides regulate aspects of egg laying that are species-specific.

The precursors of neuropeptides undergo a variety of posttranslational modifications resulting in biologically active peptides that are released by exocytosis. The hydrophobic signal sequence is removed as the nascent

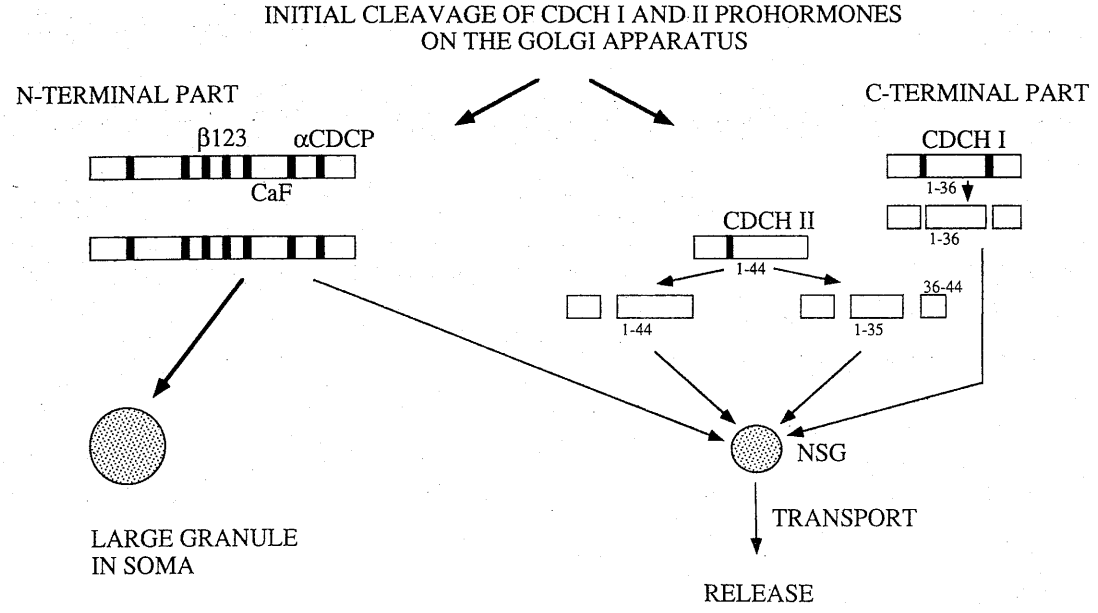

Fig. 16. Proteolytic processing of the CDCH-I and II precursors in the CDC system of *Lymnaea stagnalis*. The N-terminally located signal sequence is not shown. Small vertical black bars represent the endoproteolytical cleavage sites. The first cleavage in the Golgi apparatus occurs at the tetrabasic sequence, separating both precursors into N- and C-terminal fragments, which are further processed as shown. 90% of the total of N-terminal fragments are packaged into large granules that stay in the soma, and 10% is packaged into neurosecretory granules (NSG) together with the C-terminal fragments. There is also a population of NSG that contains solely C-terminal fragments. The NSG are transported to axon terminals both in the inner and outer compartments of the CDC system where the peptides derived from the fragments are released by exocytosis.

precursor enters the lumen of the endoplasmic reticulum. The prohormone is then transported to the Golgi apparatus where glycosylation, sulphation, and packaging into secretory granules takes places. The bioactive domains on prohormones most frequently are flanked by basic amino acid residues, which serve as proteolytic processing sites. Processing of the prohormones involves a number of enzymes, such as endopeptidases, exopeptidases, and nonproteolytic enzymes, which modify the N- or C-terminus of the peptide.

The processing of the CDCH prohormones in the CDCs shows unique features. Both prohormones are cleaved at least nine sites, which results in 10 peptide products, some of which are further processed or modified by amidation or proteolytic trimming of the N- or C-terminal ends (Fig. 16) (K. W. Li, unpublished results). The first endoproteolytic cleavage occurs at a site consisting of four consecutive basic residues.

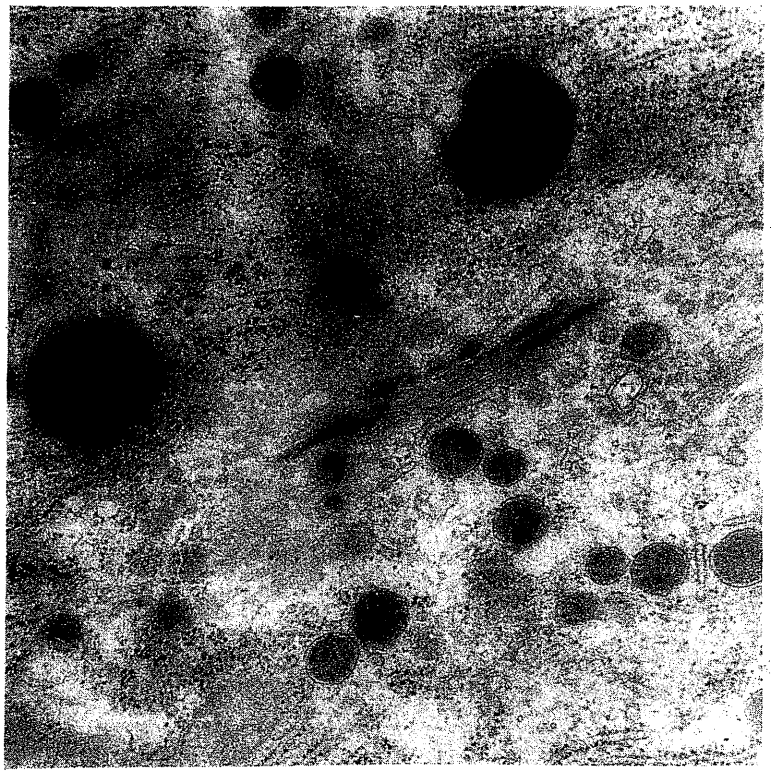

Fig. 17. Cytoplasm of CDC double immuno-stained with anti-CDCH-I (small dots; 5 nm) and anti-α-CDCP (large dots: 15 nm) $\times$50,000. The larger granules contain mainly α-CDCP whereas the smaller granules (neurosecretory granules) contain mainly CDCH-I. Note also Golgi zones filled with electron dense material. Courtesy Dr. van Heumen.

The C-terminal region of the precursor is then rapidly further processed to yield three peptides including CDCH. The N-terminal intermediate is processed more slowly, yielding three types of physiologically active peptide, the α-, β-, and β_{1-3} CDCPs, together with a number of other peptides. The peptides C-terminal to the first cleavage, including CDCH, are present at much higher levels, 10-fold higher than the N-terminal cleavage products. There is quite some evidence suggesting that the first cleavage of the CDCH prohormones occurs in the Golgi apparatus. Next, *via* an unknown mechanism, sorting takes place and vesicles bud off the trans Golgi, containing either the C-terminal fragment, the N-terminal fragment, or both. Vesicles (the neurosecretory granules) containing the C-terminal peptides are transported to the CDC axon terminals in the neurohaemal area and the inner compartment in much larger quantities than vesicles (the large granules) containing the N-terminal peptides (Fig. 17). These large vesicles stay in the somata and are not seen in the axon terminals. Eventually they fuse with lysosomes. The differential vesiculation and transport is significant in the context of the physiological functions of the peptides. CDCH functions largely as a circulatory hormone, whereas α-CDCP together with CDCH (see below) are thought to have autocrine actions on the CDCs and, thus, are not released into the circulatory system in large quantities.

The fact that the peptides derived from the CDCH-I and II precursors are packaged in distinct vesicle classes that have unique subcellular localizations, and, furthermore, that the peptides are not recovered in stoichiometrics defined by the prohormones suggests that cellular trafficking, not precursor structure, defines the levels and sites of release of the various bioactive peptides. In addition, quantitative data on the peptides derived from each of the CDCH prohormones learns that under standard laboratory conditions the ratio of expression of the CDCH-I and II genes is not equal, but is ~10 to 1 (K.W. Li, unpublished results), suggesting the possibility of an independent regulation of the CDCH-I and II genes. In principle, regulation of expression level of the CDCH-I and II genes, in combination with the separation of packaging, transport and release

enormously expands the information-handling capacity of the CDCs. Under changing physiological conditions various vesicle classes containing different combinations of bioactive peptides may be generated that are able to induce distinct though related (and overlapping) physiological processes including behaviours.

Discharges, exocytosis, and peptide release of the CDC system

Electrophysiologically, the CDCs are characterized by 3 different states of excitability, the active, inhibited and resting state (Kits, 1980). Their occurrence is closely related to the egg-laying cycle (Fig. 18). The central event in egg laying is a long-lasting period of electrical activity (discharge) of the cells (Fig. 19). *In vivo* recordings using chronically implanted cuff- and fine wire electrodes on the CDCs have revealed that discharges always precede spontaneous egg laying (*e.g.*, Ter Maat *et al.*, 1986). The CDC discharges last about 60 min and during the discharge all cells of the network are simultaneously active. Synchrony between the cells of the network is achieved through electrotonic connections (de Vlieger *et al.*, 1980). In the laboratory, discharges can be triggered by conditions that, in the wild, are favourable for survival of offspring, such as clean water, optimal temperature and high oxygen content (Ter Maat *et al.*, 1983a, 1983b). First and second messengers, as well as autotransmission play a role in generating discharges. First messenger regulation of the CDCs involves a biphasic response to acetylcholine, which is mediated through nicotinic and muscarinic acetylcholine receptors. *In vitro* studies have shown that there is an initial transient depolarization and a late hyperpolarization (Ter Maar *et al.*, 1983b). The initial response may play a role in the initiation of the CDC discharge. The hyperpolarizing response considerably decreases the excitability of the CDCs and thus prevents CDC discharge activity and egg laying. It probably plays a role in interrupting egg mass production during escape behaviour of the animal, *e.g.*, when it is attacked by predators.

The way in which a discharge is triggered can be viewed as a two-stage process. An external stimulus is relayed to the system and excites (a subset) of the peptidergic network sufficiently to trigger spikes and hence the release of autotransmitters. The next stage is one of amplifica-

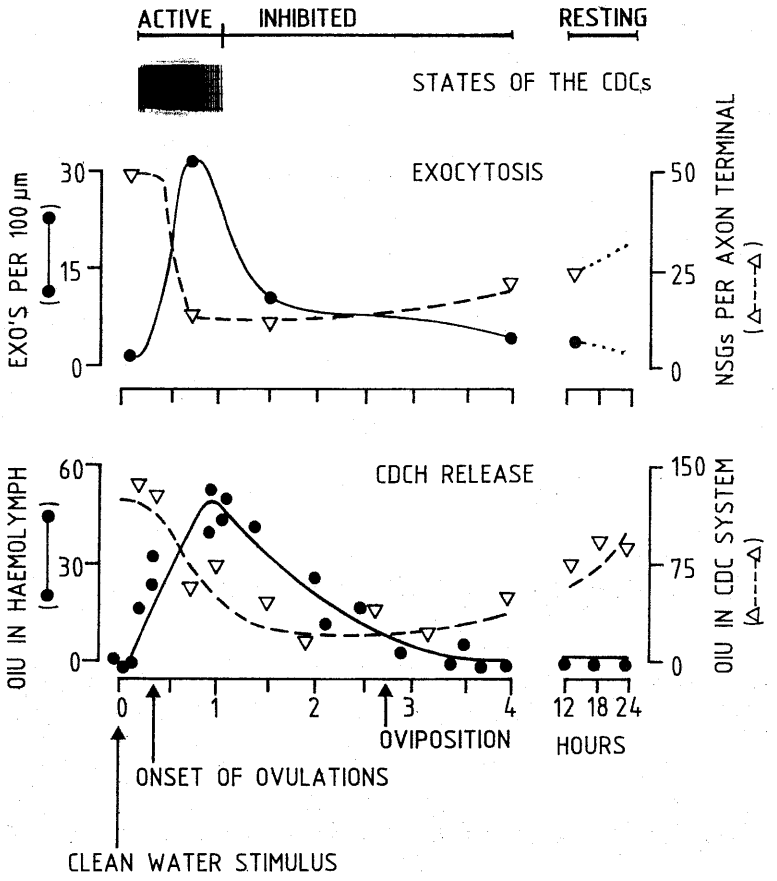

Fig. 18. Discharge, exocytosis, CDCH release, and egg laying in *Lymnaea stagnalis*. Animals were induced to lay egg masses by application of the clean water stimulus. Top, the physiological cycle of the CDCs (active, inhibited, and resting state); Middle part, morphometry of CDC axon terminals. Storage of neurosecretory granules, expressed as numbers of granules (NSG) per axon terminal profile; release of granule contents expressed as numbers of exocytoses (EXO's) per outline of the COM (100 μm). Bottom, CDCH titers in the blood and CDCH contents of the CDC system, expressed in ovulation inducing units (OIU). (1 OIU is the threshold dose for the induction of ovulation.)

tion. As a result of excitatory action of the autotransmitters, all cells constituting the network are depolarized until maximum excitation is achieved (Brussard *et al.*, 1990). In this respect it is interesting to note that the hormonal output of the CDC system during the discharge is as high as 50 times the threshold dose of CDCH for the triggering of egg laying in *Lymnaea* (Fig. 18) (Geraerts *et al.*, 1984). Thus, this positive feedback mechanism ensures the maximum output of the system

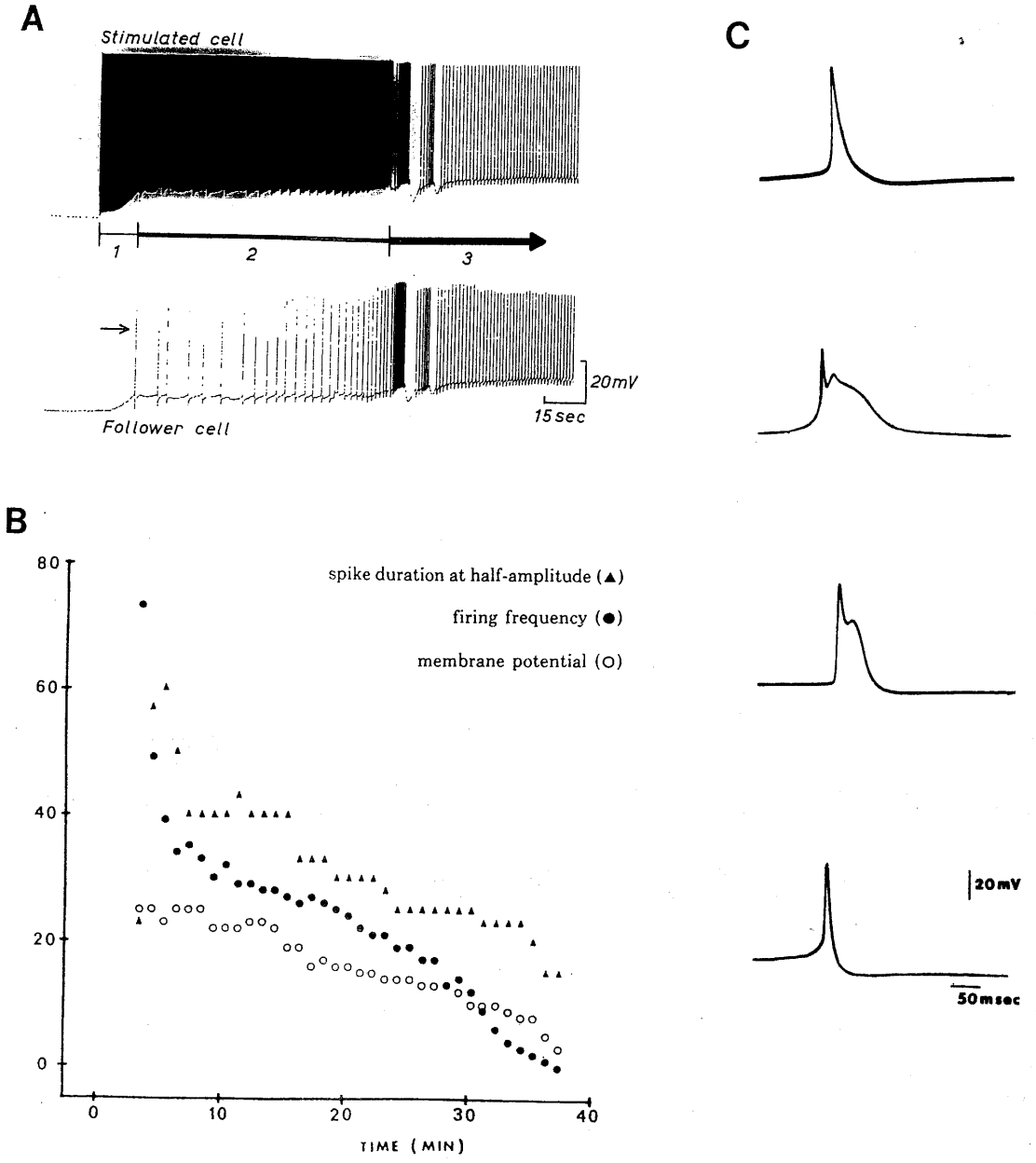

Fig. 19. Characteristics of the CDC discharge in *Lymnaea stagnalis*. A) Activation of all CDCs by stimulation of one cell of the system. After an initial depolarization (1), other CDCs are recruited (note the spikes in the follower cell), and additional spikes are fired with increasing frequency (2) until stimulation can be stopped and the afterdischarge develops (3). B) Time courses of spike width, firing frequency, and membrane potential. C) Shapes of spikes in resting and active states. From top to bottom: action potentials in resting state, after 1 and after 5 min following the onset of the afterdischarge and near the end of the afterdischarge. Note the considerable broadening during the active state which is due to an additional slow Ca/Na-component (Courtesy Dr. Kiti).

once triggered.

Autotransmitters are released by the CDCs upon depolarisation of the cells (Ter Maat *et al.*, 1987; Brussa ard *et al.*, 1990). It has been demonstrated that a specific combination of four peptides is necessary and sufficient to elicit the CDC discharge (Brussaard *et al.*, 1990). These peptides, CDCH, α-CDCP(1-11), α-CDCP(3-11) and α-CDCP(3-10), are encoded by the CDCH I gene and, with the exception of CDCH, by the CDCH II gene. α-CDCP(3-11) and α-CDCP(3-10) are derived from α-CDCP(1-11). The CDCs offer the first example of an excitatory feedback mechanism employing peptides to build up their characteristic firing pattern. Similar mechanisms might apply to functionally related systems, such as the oxytocin and vasopressin cells in vertebrates. The biochemical mechanisms underlying CDC discharges involve the phosphorylation of proteins, both enzymes and ionic channels. Several second messengers, such as cAMP, calcium, phorbol esters, and pH, appear to be involved and the reader is referred to the review of Geraerts *et al.* (1988a) for or a more detailed discussion of this topic.

There is a close relationship between electrical activity, exocytosis, and release of peptides by the CDCs (Ter Maat *et al.*, 1983b; Geraerts *et al.*, 1984; Roubos, 1984). This is particularly clear from experiments with *L. stagnalis*, in which the animals were induced to lay eggs with the help of the clean water stimulus. The onset of the CDC discharge precedes the appearance of CDCH in the blood and, furthermore, ovulations do not begin until the homone has appeared in the blood (Fig. 18). Release of CDCH takes place almost exclusively during the CDC discharge and is accompanied by a decrease of the CDCH contents of the CDC system. These phenomena are closely paralleled by an enormous increase of exocytosis profiles in CDC terminals of the neurohaemal area in the COM during the CDC discharge. *In vitro* experiments have shown that during a discharge of an isolated CDC system prelabelled with radioactive amino acids, various different peptides are released that are encoded by the egg-laying peptide genes (Geraerts and Hogenes, 1985).

Role of CDC peptides in the control of egg laying in *Lymnaea*

The onset of the discharge marks the start of egg-laying behaviour. The egg-laying behaviour comprises both internal (covert) and externally

observable (overt) behaviours. The covert behaviours relate to egg-mass formation and the overt behaviours consist of a number of action patterns that serve to prepare the substrate for egg-mass deposition. The covert and overt behaviours will be briefly described.

In *Lymnaea*, 100-200 oocytes are released within 5-10 min after the start of the CDC discharge and the subsequent appearance of the ovulation hormone in the blood. The oocytes are fertilized and packaged one by one into eggs and subsequently into an egg mass, which appears on the substrate about 2 h following the onset of the discharge. The overt egg-laying behaviour is illustrated in Fig. 20. Its onset is characterized by a cessation of locomotion and a period during which the animal retains a fixed posture with the shell drawn over the anterior head-foot and tentacles drooping (the "resting" phase). After about 60 min the animal starts crawling about, very slowly, along a tortuous path such that it virtually remains in the same place. At this point the animal cleans the surface of the substrate with rasping movements of the buccal mass, exactly where the egg mass is to be deposited. This "turning" phase is followed by the actual deposition of the egg

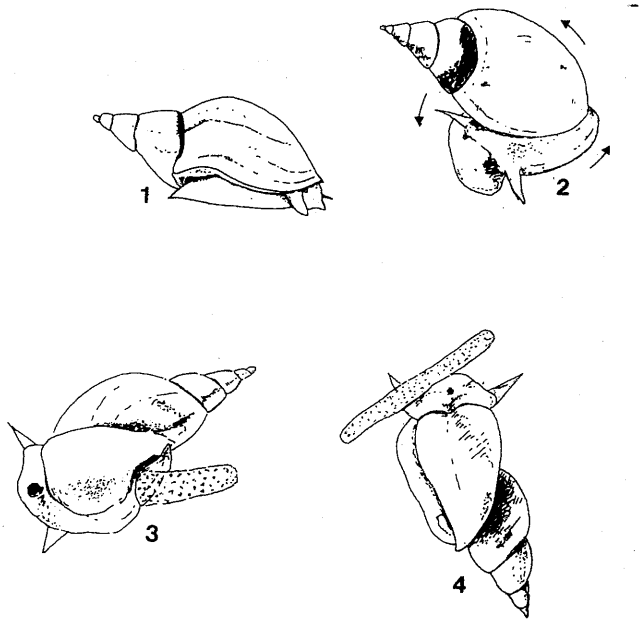

Fig. 20. Overt egg-laying behaviour of *Lymnaea stagnalis*. Postures during the four phases of egg-laying behaviour: 1, resting; 2, turning; 3, deposition of the egg mass; 4, inspection.

mass and by "inspection" of the egg mass. Thus, overt egg-laying behaviour in *Lymnaea* is a rather complex sequence of behavioural acts that involve a variety of command and motor systems. The effects of CDC peptides on these systems and on the reproductive tract have been studied in quite some detail, and our present knowledge of the organization of egg-laying in *Lymnaea* is summarized in Fig. 21.

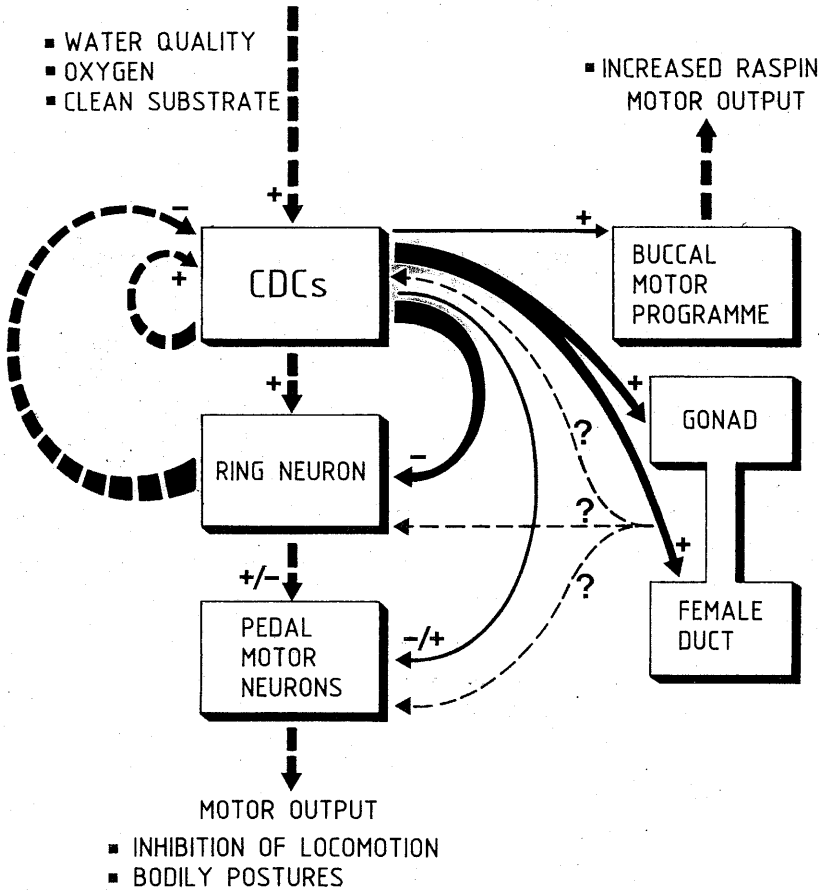

Fig. 21. Scheme summarizing the organization of egg laying in *Lymnaea*. External stimuli trigger the CDC discharge. Autoexcitation leads to maximal output of the CDC system and hence to ovulation. There is an immediate excitation of the Ring Neuron, which in turn affects the firing of pedal motor neurons. In addition, there are hormonal effects on the Ring Neuron and on motor neurons in both pedal and buccal ganglia. The activities of the motor neurons can explain the elevated rate of rasping and the shell forward position, which are characteristic of egg laying. Heavily dashed arrow, neuronal pathway within the brain; solid arrow, blood borne; lightly dashed arrow, presumed neuronal or hormonal pathway.

The unpaired Ring Neuron of the right cerebral ganglion of *Lymnaea* deserves special attention, because it plays a crucial role in egg-laying behaviour. The Ring Neuron provides a direct pathway from the CDCs to the musculature involved in the early phase of egg laying.

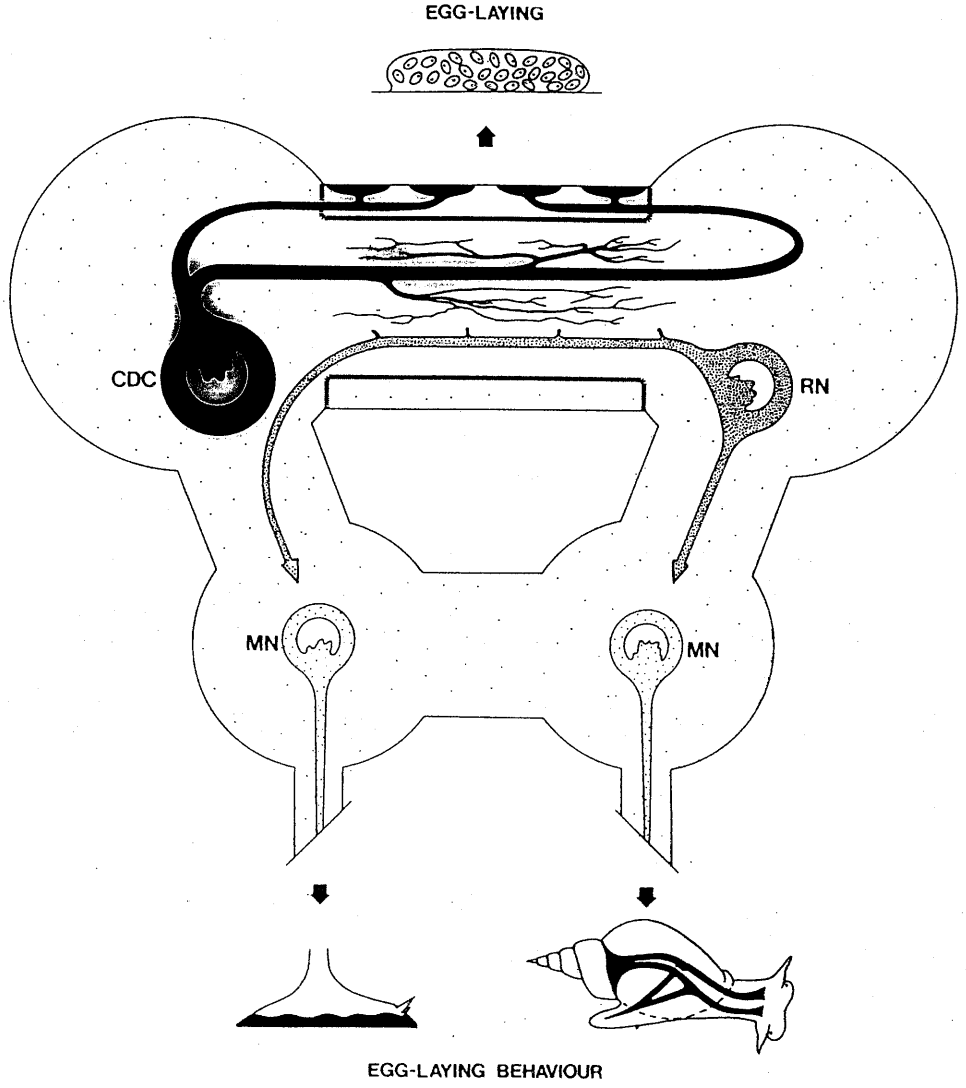

Fig. 22. Tentative scheme of the role of neurohormonal and nonsynaptic release of peptides from the CDC neurohaemal and collateral systems in the control of egg-laying behaviour in *Lymnaea stagnalis*. The peptides are released in the circulatory system to act on peripheral and central targets. In addition, the peptides are released in intercellular spaces in the inner compartment of the COM and influence the activities of the Ring Neuron. The Ring Neuron in turn modulates the activities of motor neurons in the pedal ganglia controlling muscle systems involved in the control of movement and of shell position.

This neuron projects towards the pedal ganglia where it shows extensive branching and traverses the pedal commissure, thus completing a ring (Fig. 22) (Jansen and Bos, 1984). Some of the motor neurons in the pedal ganglia that innervate the columellar muscle, which causes the movements of the shell, receive excitatory input and others inhibitory input from the Ring Neuron (Jansen and Ter Maat, 1985). As it has recently been shown that the Ring Neuron expresses the APGWamide gene (J. van Minnen, unpublished results), it is the bioactive peptides that are derived from the APGWamide prohormone (APGWamide and CALP) that may be active on the motor neurons. The CDC discharge increases the firing rate of the Ring Neuron. This effect has a latency of seconds, and it may last as long as the CDC discharge (Jansen and Bos, 1984). It seems very likely that these responses are the result of local actions of CDC peptides, probably mediated *via* non-synaptic (paracrine) release of material from the collateral system in the inner compartment of the COM (see above). As the excitation of the Ring Neuron lasts as long as the CDC discharge, it is sufficient to explain the first part of the egg-laying behaviour (the "resting" phase). Thus, *Lymnaea* provides a neural analogue for parts of the egg-laying behaviour, in that within the brain a pathway is present that relays the activity of the CDCs through the Ring Neuron to motor neurons innervating the columellar muscle and probably also the muscle systems involved in locomotion.

GENES ENCODING INSULIN-RELATED PEPTIDES INVOLVED IN THE CONTROL OF GROWTH AND ASSOCIATED PROCESSES IN *LYMNAEA*

Animal growth is characterized by an increase in dry weight of the organic material (mainly protein) of the body. Body growth and body structure are highly interrelated and the organs, in particular the skeleton, grow in proportion to the body as a whole. In vertebrates, this is achieved by an intricate interaction of many hormones, *e.g.*, growth hormone, insulin, insulin-like growth factors (IGFs I and II), prolactin, thyroid hormones, and androgens. In the next section we will review our knowledge of the neuroendocrine regulation of growth in *Lymnaea*. We focus on the genes that code for the insulin-related peptides that are synthesized by the LGCs, as well as

on a possible role for a vertebrate-like growth hormone. In the next section, we discuss the dual role in the regulation of both growth and reproduction of another LGC peptide, called schistosomin.

LGCs and the control of body growth

In molluscs, neuroendocrine centres are involved in the regulation of body growth (see reviews of Joosse, 1988; Geraerts *et al.*, 1988b). This has been studied in detail in *L. stagnalis*, where the LGCs regulate the growth of the soft body parts and the shell. The LGCs are giant neurons (diameter ~100 μm), which are located in the cerebral ganglia in two paired groups with together about 150 cells (Figs. 5 and 23). They use the periphery of the long median lip nerves as their neurohaemal area.

The experimental proof that the LGCs are involved in the control of growth of *Lymnaea* comes from classical endocrinological extirpation and implantation experiments. Cauterization of the LGCs of rapidly growing juvenile snails results in a markedly retarded body growth, which can be restored by implantation of cerebral ganglia containing the LGCs (Geraerts, 1976b). The LGCs affect also various aspects of the metabolism related to growth (reviewed in Joosse, 1988; Geraerts

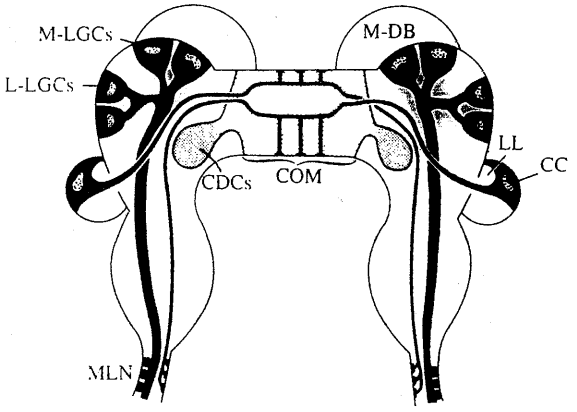

Fig. 23. Location and anatomical organization of the LGC system in the cerebral ganglia of *Lymnaea stagnalis*, based on Lucifer Yellow fillings (Benjamin *et al.*, 1976) and light and electron microscopy (van Minnen *et al.*, 1980). Notice the intricate axonal topology of the canopy cells (CC) in the lateral lobes (LL), which strongly suggests that the CC control the activities of the other LGCs. M-LGC and L-LGC, medio- and laterodorsal groups of LGCs, resp.; MLN, median lip nerve; CDC, caudodorsal cells; COM, cerebral commissure; M-DB and L-DB, medio- and laterodorsal bodies, resp.

et al., 1988b). Thus, factors released from the LGCs keep glycogen stores low (Geraerts, in press), and stimulate the activity of ornithine decarboxylase, an enzyme which shows high levels of activity in growing animal tissues. The LGCs further strimulate various processes of shell growth: formation of the periostracum, the proteinaceous component of the shell, as well as calcium and bicarbonate incorporation into the shell edge, and the maintenance of high concentrations of a calcium-binding protein, important for cellular calcium transport in the mantle edge.

In *Lymnaea*, a second centre involved in growth control is located in the lateral lobes (Figs. 5 and 23). Cauterization of the lobes results in giant growth, whereas reimplantation of cerebral ganglia with lateral lobes restores normal growth (Geraerts, 1976a). These effects of the lateral lobes are mediated *via* changes of the activities of the LGCs (Geraerts, 1976b). An interesting observation is that in each lobe an ectopic LGC, termed the canopy cell, is located. The axons of both canopy cells show an intricate branching pattern and run very close to axons coming from the clusters of LGCs in the cerebral ganglia (Fig. 23) (Benjamin *et al.*, 1976; van Minnen *et al.*, 1980). This suggests that the canopy cells are specialized LGCs that transmit regulatory stimuli to the LGC clusters in the cerebral ganglia.

The peptide messengers produced by the LGCs have been identified using the methodologies of both molecular biology and peptide chemistry. The LGCs produce at least four insulin-related peptides, called molluscan insulin-related peptides (MIPs), each of which is thought to control a different aspect of growth and associated processes in *Lymnaea* (Smit *et al.*, 1988; A.B. Smit, unpublished results). The MIPs possess the basic three-dimensional configuration, with disulphide bridges, a hydrophobic core, α-helices, and sharp turns of the peptide chains, typical of other members of the insulin superfamily, *i.e.*, the insulins, IGFs, and relaxins of the vertebrates (Froesch *et al.*, 1985; Steiner *et al.*, 1985; Blundell and Wood, 1975; Blundell and Humbel, 1980), and the bombyxins of the insects (Kawakami *et al.*, 1989). The organization, expression, and the evolutionary aspects of the MIPs and the gene family encoding them are reviewed in the sections that follow.

The organization of the MIP gene family

Using a plus/minus screening strategy, four LGC-specific cDNA clones encoding the precursors of MIPs I, II, III and V were identified. The corresponding MIP I, II, III and V genes were isolated with the help of radioactive probes made from the MIP cDNAs and from synthetic gene-specific oligonucleotide sequences derived from the cDNAs. Two more genes, the MIP IV and VI genes, were identified in the course of the genomic analysis (Smit *et al.*, 1988; A. B. Smit, unpublished results). Highly interesting features of the organization of the MIP genes were revealed when the MIP genes were compared with the vertebrate insulin genes, *e.g.*, the human insulin gene (Fig. 24). All MIP genes show the overall structure typical of the insulin genes, with three exons interspaced by two introns. The exons code for similar domains of the precursors in both the MIP genes and insulin genes. The positions of the introns are also conserved, one within the sequence coding for the mRNA leader region and the other interrupting the C peptide coding region. The six MIP genes are present in the genome as three couples of closely linked genes, which are transcribed in the same direction. They represent all homologous members of the MIP gene family in the *Lymnaea* genome, as indicated by genomic Southern blotting and restriction analysis of separate genomic clones. The family may, however, include additional members with undetectably low homology to the

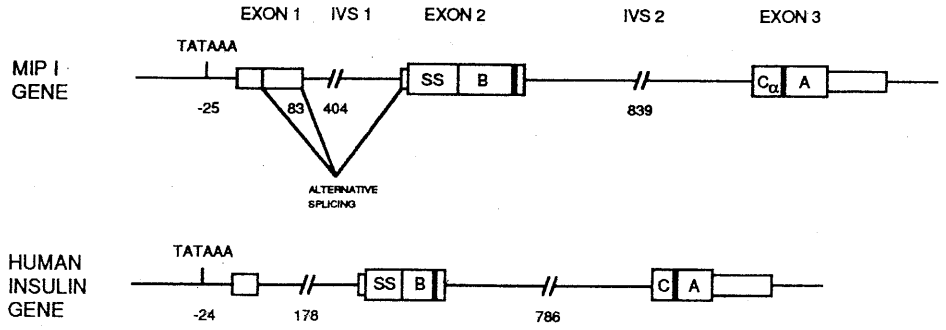

Fig. 24. Schematic representation of the MIP I gene and the human insulin gene. The MIP genes II–VI have a similar organization. Indicated are the exons and the intervening sequences (IVSs). SS, signal sequence; B, B chain; A, A chain; Cα, Cα peptide; C, C peptide. Goldberg-Hogness box, TATAAA. Numbers refer to nucleotide length. The alternative splicing in exon 1 of the MIP I gene is also indicated.

identified MIP genes. The genomic organization of the MIP gene family differs markedly from that of the bombyxin gene family (Kawakami *et al.*, 1989). Bombyxin genes do not contain introns and are arranged as transcription units of paired genes with opposite orientation.

A TATA box is present at the expected position (approx. ~25) in the 5′-region of the MIP I, II, III, and V genes. By contrast, in both the MIP IV and VI genes, a putative TATA box is located further upstream (at position-178). Two transcripts, which differ in their leader sequence by 83 nucleotides, can be generated from the MIP I gene by alternative splicing. The stretches of the 5′-flanking regions near the transcription initiation sites of the MIP I and II genes are quite similar, but sequence divergence becomes gradually stronger further upstream. The 5′-flanking regions of the MIP II and V genes are also very similar, except for a few interspersed stretches of pronounced sequence divergence. By contrast, the 5′-flanking region of the MIP III gene has no sequence resemblance with those of other MIP genes. The upstream regions, the signal peptide, and the first amino acids of the B chain in the MIP IV and VI genes are very similar to each other. Also, the upstream regions do not resemble those of other MIP genes. The MIP VI gene probably is a pseudogene, because an insertion of 7 nucleotides in exon 2 causes a shift of the reading frame resulting in a stop codon at the end of exon 2.

Biosynthesis, structure and central expression of the MIPs and their precursors

The MIP precursors encoded by the completely characterized MIP I, II, III and V genes represent pre-proinsulin-related proteins containing a signal sequence. A and B chains and a connecting C peptide, as well as dibasic amino acid processing sites for the generation of MIPs and C peptides (Fig. 25). Pulse-label and pulse-chase analysis of newly synthesized proteins and peptides in the LGC system *in vitro* showed that putative MIP precursors were present in the LGC somata after a 20 min pulse with radioactive cysteine. The synthesis and subsequent conversion of the proMIPs to end products probably is confined to the LGC somata (and perhaps the proximal parts of the LGC axons that were not studied in these experiments). The MIPs and C peptides were

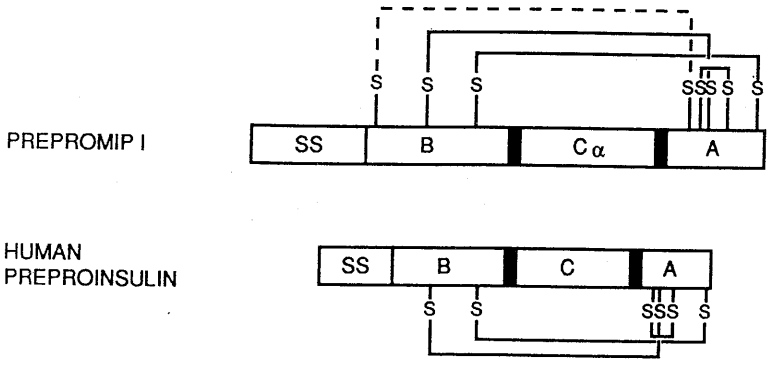

Fig. 25. Schematic representation of the precursors of MIP I and human insulin. The precursors are aligned on the positions of the cysteine residues involved in the inter chain disulphide bridges in the B chain domains. SS, signal sequence; B, B chain; A, A chain; Cα, Cα peptide; C, C peptide; S, position of cysteine residue. The extra putative disulphide bridge in MIP is indicated by a dotted line, others by solid lines. Vertical black bars indicate proteolytic cleavage sites.

transported to the LGC axon terminals in the median lip nerves, where they appeared between 1.5 h and 6 h following the pulse period. Sequencing of the material purified from the median lip nerves showed that various MIPs were present as encoded on the genes.

Close inspection of the cDNA and peptide data revealed a number of differences between the various MIPs. The A and B chains of MIPs I, II and V appeared to be terminally blocked (pyroglutamate), and the B chain N-terminus of MIP III is two amino acids longer than predicted by the gene work. Moreover, the two C-terminal amino acids of all B chains are post-translationally removed (K. W. Li, unpublished results). In conclusion, these data indicate that preproMIPs are processed to form mature 2-chain MIP molecules that together with the various C peptides are stored in the LGC axon terminals. Comparison of MIPs I, II, III and V revealed that they are homologous with only about 45-75% of the amino-acid residues being identical among them throughout the A and B chains (Fig. 26). However, all MIPs share the amino acids that are important in adopting the basic insulin configuration. This is apparent when MIPs are compared with other members of the insulin superfamily. In the A and B chains of the MIPs, cysteines are present at positions typical for the insulin superfamily, suggesting that the three characteristic disulphide bridges in the MIP molecules have

A Chains

		-4	-3	-2	-1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
Insulin(-related)																											
MIP I	Lymnaea	Q	G	T	T	N	I	V	C	E	C	C	M	K	P	C	T	L	S	E	L	R	Q	Y	C	P	
MIP II	Lymnaea	Q	R	T	T	N	L	V	C	E	C	C	F	N	Y	C	T	P	D	V	V	R	K	Y	C	Y	
MIP III	Lymnaea	E	S	R	P	S	I	V	C	E	C	C	F	N	Q	C	T	V	Q	E	L	L	A	Y	C	-	
MIP V	Lymnaea	Q	R	T	T	N	L	V	C	E	C	C	Y	N	V	C	T	V	D	V	F	Y	E	Y	C	Y	
bombyxin II	Bombyx	-	-	-	-	G	I	V	D	E	C	C	L	R	P	C	S	V	D	V	L	L	S	Y	C	-	
Insulin (-related)	Sponge	-	-	-	-	-	I	V	Q	Q	C	T	S	G	I	C	S	L	Y	Q	-	E	N	Y	C	N	
IGF I	Human	-	-	-	-	G	I	V	D	E	C	C	F	R	S	C	D	L	R	R	L	E	M	Y	C	A	...
IGF II	Human	-	-	-	-	G	I	V	E	E	C	C	F	R	S	C	D	L	A	L	L	E	T	Y	C	A	...
Relaxin	Human	R	P	Y	V	A	L	F	E	K	C	C	L	I	G	C	T	K	R	S	L	A	K	Y	C	-	
Relaxin	whale	-	-	R	M	T	L	S	E	K	C	C	Q	V	G	C	I	R	K	D	I	A	R	L	C	-	
Insulin	Hagfish	-	-	-	-	G	I	V	E	Q	C	C	H	K	R	C	S	I	Y	N	L	Q	N	Y	C	N	
Insulin	Guinea pig	-	-	-	-	G	I	V	D	Q	C	C	T	G	T	C	T	R	H	Q	L	Q	S	Y	C	N	
Insulin	Rat I	-	-	-	-	G	I	V	D	Q	C	C	T	S	I	C	S	L	Y	Q	L	E	N	Y	C	N	
Insulin	Human	-	-	-	-	G	I	V	E	Q	C	C	T	S	I	C	S	L	Y	Q	L	E	N	Y	C	N	

B Chains

		-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Insulin(-related)																																										
MIP I	Lymnaea	Q	F	S	A	C	N	I	N	D	R	P	H	R	R	G	V	C	G	S	A	L	A	D	L	V	D	F	A	-	C	S	S	S	N	Q	P	A	M	V	-	-	-
MIP II	Lymnaea	Q	-	S	S	C	S	L	S	S	R	P	H	P	R	G	I	C	G	S	N	L	A	G	F	R	A	F	I	-	C	S	N	Q	N	S	P	S	M	V	-	-	-
MIP III	Lymnaea	Q	-	H	T	C	S	I	L	S	R	P	H	P	R	G	L	C	G	S	T	L	A	N	M	V	Q	W	L	-	C	S	T	Y	T	T	S	S	K	V	-	-	-
MIP V	Lymnaea	Q	F	S	A	C	S	F	S	S	R	P	H	P	R	G	I	C	G	S	D	L	A	D	L	R	A	F	I	-	C	S	R	R	N	Q	P	A	M	V	-	-	-
Bombyxin II	Bombyx	-	-	-	-	-	-	-	E	N	P	N	A	V	H	T	T	C	G	R	H	L	A	R	T	L	A	D	L	-	C	W	E	A	G	V	D	-	-	-	-	-	-
Insulin (-related)	Sponge	-	-	-	-	-	-	-	-	-	-	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	I	L	V	C	G	E	R	G	F	F	Y	T	P	M	S	-
IGF I	Human	-	-	-	-	-	-	-	-	-	-	-	G	P	E	T	L	C	G	A	E	L	V	D	A	L	Q	F	V	-	C	G	D	R	G	F	Y	F	N	K	P	T	G
IGF II	Human	-	-	-	-	-	-	-	-	A	Y	R	P	S	E	T	L	C	G	G	E	L	V	D	T	L	Q	F	V	-	C	G	D	R	G	F	Y	F	S	R	P	A	S
Relaxin	Human	-	-	-	-	-	-	-	K	W	K	D	D	V	I	K	L	C	G	R	E	L	V	R	A	Q	I	A	I	-	C	G	M	S	T	W	S	K	R	S	L	-	-
Relaxin	Whale	-	-	-	-	-	-	-	-	S	T	N	D	L	I	K	A	C	G	R	E	L	V	R	L	W	V	E	I	-	C	G	S	V	S	W	G	R	T	A	L	-	-
Insulin	Hagfish	-	-	-	-	-	-	-	-	-	-	R	T	T	G	H	L	C	G	K	D	L	V	N	A	L	T	I	A	-	C	G	V	R	G	F	F	Y	D	P	T	K	M
Insulin	Guinea pig	-	-	-	-	-	-	-	-	-	-	F	V	S	R	H	L	C	G	S	N	L	V	E	T	L	T	S	V	-	C	Q	D	D	G	F	F	Y	I	P	K	D	-
Insulin	Rat I	-	-	-	-	-	-	-	-	-	-	F	V	K	N	H	L	C	G	P	H	L	V	E	A	L	Y	L	V	-	C	G	E	R	G	F	F	Y	T	P	K	S	-
Insulin	Human	-	-	-	-	-	-	-	-	-	-	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	-	C	G	E	R	G	F	F	Y	T	P	K	T	-

been conserved. In addition, the important hydrophobic core residues of the globular insulin structure are either conserved as identical residues, or are replaced by residues with an equally hydrophobic character. The only exception is arginine at B15 in MIPs II and V. Also, α-helices, which are present in the A and B chains of vertebrate insulins, can be predicted for the MIPs.

Although the MIPs are 2-chain peptides that will adopt the insulin core structure, they cannot be considered genuine insulin molecules. MIPs exhibit strong divergence of surface residues that are important in many functions of the vertebrate insulin molecules (Blundell and Wood, 1975), such as receptor binding, solubility of the molecule, processing of the precursor, and monomer and dimer interactions, as well as hexamer formation. A striking example is the almost complete divergence of the amino-acid residues involved in receptor binding, including residues at A1-A5, A8, and B23-26. Thus, it is unlikely that the MIP receptor is of the vertebrate type. Interestingly, these putative receptor binding residues are divergent among MIPs, which suggests either that each MIP binds a different receptor, or alternatively, that various MIPs bind the same receptor with different affinities. Thus, each MIP may fulfil a different function in the control of growth and associated processes in *Lymnaea*. In addition to these differences, MIPs possess features that classify them into a distinct group of the insulin superfamily. Both the A and B chains are N-terminally extended (Fig. 26), and the B chain extension contains an extra cysteine at B-6 that may form a third interchain disulphide bridge with an extra cysteine at A4. Due to these properties, the MIPs are the most complexely folded molecules of the insulin superfamily.

Expression of the MIP I, II, III and V genes in adult animals is entirely restricted to the LGCs, as could be assessed by various methods. Northern blotting experiments showed that MIP I, II, III and V transcripts are only present in the cerebral ganglia of the CNS, while no MIP IV and VI transcripts could be identified. Because the MIP VI gene probably is a pseudogene, this is not surprising. For the same reason the MIP IV transcript may not have been found. Alternatively, however, MIP IV gene transcription may take place under hitherto unknown conditions. The MIP I, II and

V specific transcripts each had a length of 650-700 nucleotides. Two MIP III specific transcripts of 800 and 1,150 nucleotides were found that are probably generated by the use of two distinct termination signals in the 3′-region of the MIP III gene.

Hybridization histochemistry showed that the MIP I, II, III and V genes are expressed in the LGCs and in the canopy cells of the lateral lobes. No other cell type of the central nervous system showed a positive signal. Unexpectedly, MIP transcripts were also detected in LGC axons. The significance of the phenomenon is not well understood and further experiments addressing this question are in progress. The immunohistochemistry was in agreement with the *in situ* hybridization studies and showed immunostaining of only LGCs and canopy cells. Furthermore, it revealed a complex axonal topology of the LGCs and canopy cells that confirms the dye injections and the ultrastructural studies in all details (*cf.*, Fig. 23).

To determine the intracellular localisation of the (pro)MIPs more precisely, immunogold electron microscopy was performed. Immunogold labelling in both the LGCs and canopy cells was observed above neurosecretory granules budding off from the Golgi apparatus, above mature granules in both the cell bodies and axon terminals, and over

Fig. 26. Amino acid sequence comparison of MIPs I, II, III and V to those of other insulin(-related) peptides. The sequence similarity in the A and B chains of the MIPs ranges from 45%-75%. The sequence similarity of the MIPs with bombyxin II, sponge insulin-related peptide (Robitzki *et al.*, 1989), and vertebrate insulins, relaxins, and IGFs is 20%-40%. The sequence similarity among insulins and related peptides from vertebrates is 55%-95%. In the MIP A and B chains cysteines are present at positions A6, A7, A11, A20, B7 and B19, an arrangement which is typical for members of the insulin superfamily. Two additional cysteines are present at positions A4 and B6. Like the insulins, MIPs have a glycine at B8, but lack glycines at B20 and B23, which are present in most insulins and introduce a sharp turn in the B chain. Most residues of the hydrophobic core of insulin are conserved as hydrophobic in the MIPs, namely A2, A11, A16, A20, B11, B15 and B19, except for Arg at B15 in MIPs II and V. Other hydrophobic residues are identical or conserved: A3, A19, B6, B12 and B18. Residues A1-A5, A8, and B23-B26, which interact with the insulin receptor, are diverged among MIPs. Number 1 designates the first residues of the A and B chains of human insulin.

exocytosis profiles in LGC axon terminals. This suggests that pro-MIPs are packaged into granules in the Golgi apparatus and transported to the axon endings, where the peptides derived from the precursors are released. There is endocrinological evidence that the lateral lobes are involved in the control of the synthesis and release activities of the LGCs and of female gonadotropic centres in the cerebral ganglia (Geraerts, 1976a). Therefore, the canopy cells probably are specialized LGCs that transmit regulatory stimuli to both the LGC clusters and the female gonadotropic centres (*cf.*, Fig. 23) of the cerebral ganglia.

Since each MIP may have a different function, we reasoned that physiological conditions with different effects on growth and associated processes in *L. stagnalis* might induce a differential pattern of expression of the MIP genes in the LGCs. We first investigated the effects of starvation on MIP transcript levels. During starvation growth is arrested and glycogen stores are considerably depleted. Northern blot analysis of MIP II and III transcripts in the LGCs showed that during starvation the MIP II transcript had disappeared completely, while the level of the MIP III transcript was severely reduced. Also the length of the MIP III transcript was reduced in starved animals, indicating changes in mRNA stability and/or translation efficiency. In a second series of experiments, the effects of extirpation of the lateral lobes, a treatment which causes giant growth and a depletion of glycogen stores, and the effects of a carbohydrate-rich diet, which results in an arrest of growth and enormously increased glycogen stores, were studied. The results to date suggest indeed a stimulus-dependent differential pattern of expression of the MIP genes in the LGCs.

Electrical activity and release of MIPs

Data concerning the electrophysiological characteristics of the LGC system are restricted to *in vitro* experiments on LGCs in the isolated CNS or on dissociated LGCs in primary culture. These *in vitro* studies have shown that the LGC system can act as a site of integration of diverse information. Among the first messengers involved in the regulation of LGC activities are several peptides and amino acids, as well as dopamine and glucose,

Only dopamine and glucose responses are studied in some detail. The LGCs of *Lymnaea* display a complicated response to dopamine (de Vlieger *et al.*, 1987). Initially, the LGCs are rendered inexcitable. This is caused by a hyperpolarization, due to the opening of K-channels, and by a concomittant reduction of the voltage dependent Ca-current. The effect on the K-channels is mediated *via* a D-2 like receptor. This initial phase of the response lasts from one to a few min and is followed by a slow rise in excitability, during which the cells gradually depolarize. The effect is mediated through a D-1 like receptor. Because no effects of dopamine application on intracellular cAMP levels of the LGCs were found, it is unlikely that the responses are mediated through inhibition or stimulation of adenylate cyclase activity, as is the case in the classical vertebrate D-2 and D-1 receptors. However, an increase in the intracellular levels of cAMP using non-hydrolysable analogues, such as 8-CPT-cAMP, prevented the inhibitory response and mimicked the stimulatory one. Therefore, the role of cAMP in the regulation of the responses to dopamine remains as yet unresolved.

An intresting finding is that physiological concentrations of glucose (0.1-10 mM) applied *in vitro* can induce long-lasting discharges of action potentials in the LGCs (Fig. 27). Thus, blood glucose may play an important role as an excitatory first messenger. This creates an interesting parallel with the regulation of the electrical activities of the β-cells in the pancreas of

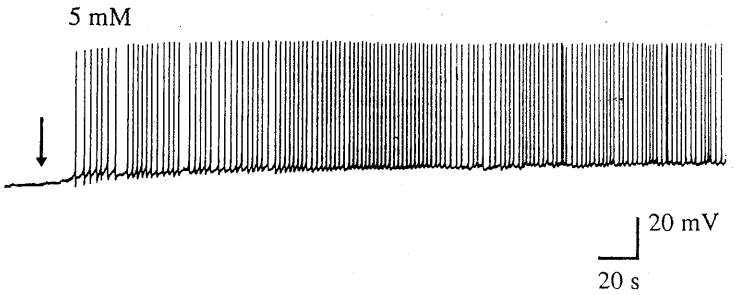

Fig. 27. Excitatory effects of glucose on LGCs. D(+)-glucose directly evokes long-lasting spiking activity in the LGCs of cerebral ganglia *in vitro*. D-glucose was applied at the arrow. The threshold is 5 mM glucose. The effect is not seen with L-glucose or with other monosaccharides such as galactose, etc. Courtesy Dr. Kits.

vertebrates (Petersen *et al.*, 1986). There is strong evidence that the regulation of LGC excitability involves cAMP acting on Ca-channels (Kits and Lodder, 1988). When the level of cAMP is experimentally increased, inexcitable cells turn into excitable ones and cells that already displayed enhanced excitability initiate a long-lasting spiking activity. Application of 8-CPT-cAMP, IBMX and forskolin cause enhancement of the Ca-current, which can be mimicked by intracellular application of the catalytic subunit of cAMP-dependent kinase. This suggests that the effect is brought about by phosphorylation of Ca-channels or associated proteins. Therefore, it is thought that an increase in the intracellular levels of cAMP and calcium is necessary for release of MIPs from the LGCs. This is entirely corroborated by the results of *in vitro* experiments employing LGC systems, prelabelled with radioactive cysteine and treated with cAMP (K. W. Li, unpublished results). Application of cAMP clearly stimulates release of MIPs above background levels. Other compounds that induce spiking activity in the LGCs, such as glucose and 4-aminopyridine, are also capable of stimulating the release of MIPs.

Evolutionary perspective

The insulin superfamily of regulatory peptides signals essential steps in growth, development, reproduction, and metabolism in both vertebrates and invertebrates. The members belonging to the insulin superfamily are quite divergent, structurally and functionally. In vertebrates, the 2-chain insulins are released by the pancreas and function as metabolic hormones, whereas the single-chain IGFs and the 2-chain relaxins are produced by different tissues, bind different receptors, and serve other functions. IGFs are important growth regulators, and relaxins have a role in reproduction (Froesch *et al.*, 1985; Steiner *et al.*, 1985; Girbau *et al.*, 1987). In molluscs MIPs are involved in the control of growth and associated metabolic processes (Joosse, 1988; Geraerts *et al.*, 1988b). The function of sponge insulin-related peptide is still unknown (Robitzki *et al.*, 1989). The members of the insulin superfamily can be subdivided into distinct groups of related peptides, and their phylogenetic relationships can be tentatively proposed as shown in Fig. 28. The functional and structural diversity of the members of the insulin superfamily raises

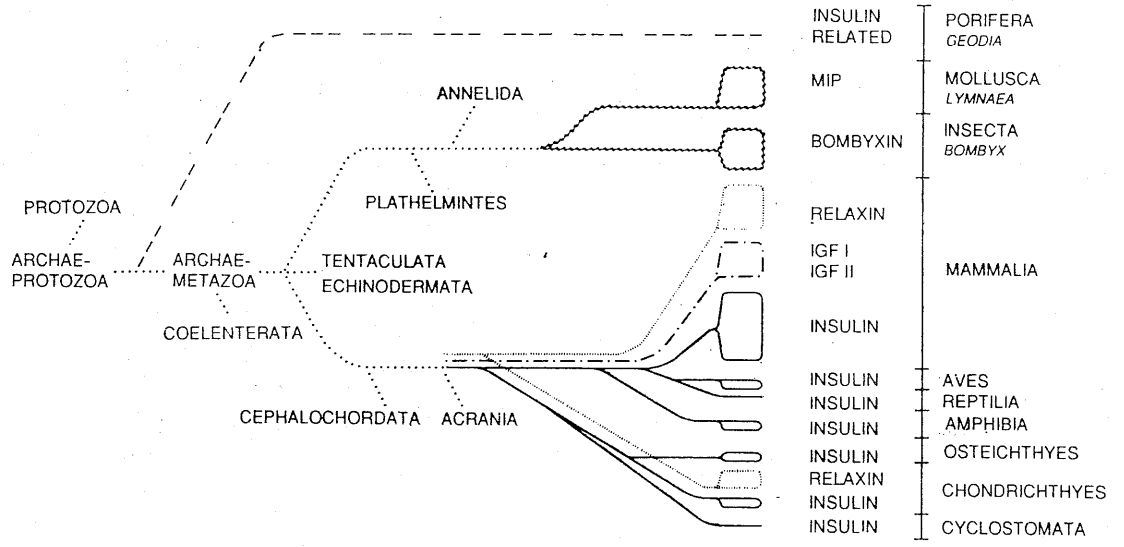

Fig. 28. Tentative scheme of the phylogenetic relationships of the animal phyla and the known members of the insulin superfamily. Dotted lines, hypothetic phylogeny. Dashed line, insulin-related peptide of sponges. Wavy lines, insulin-related peptides of insects and molluscs. Cross-hatched lines, relaxins of vertebrates. Dashed/dotted lines, IGFs of vertebrates. Solid lines, insulins of vertebrates.

many important questions and urges the development of new concepts concerning the evolution, phylogenetic distribution, and function of the peptides belonging to the insulin superfamily. Some of these issues will be discussed below.

All members of the insulin superfamily share the basic insulin globular configuration, indicating that an early genesis of this information-rich structure has been an important step in the evolution of the insulin superfamily (Steiner and Chan, 1988). Because this basic insulin motif is found in vertebrates, insects and molluscs, it must have been present in the common ancestors, the Archaemetazoa, which date back as far as 6.10^8 years ago. [Insulin-related peptide of sponges exhibits very high amino-acid sequence similarity with the vertebrate insulins (*cf.*, Fig. 26). However, the intrachain disulphide bridge in the A chain is absent. Moreover, the positions of the other cysteine residues are atypical, due to a deletion in the A chain and an insertion in the B chain. These modifications may have important consequences for the three dimensional structure. The absence of the intrachain bridge in sponge insulin-related

peptide may perhaps indicate that a simpler ancestor molecule, probably possessing only two disulphide bridges, has been present in the Archaeprotozoa, from which both the sponges and the Archaemetazoa have arisen]. In all other members of the insulin superfamily 3 disulphide bridges are present. The occurrence of a 4th disulphide bridge in the MIPs represents very likely a relatively recent development towards a more complex insulin core structure.

The studies on the MIP genes for the first time make it likely that the ancestral insulin-related peptides of the Archaemetazoa were encoded by genes that already possessed the strctural organization of the insulin gene. They furthermore suggest that these ancestral peptides were proteolytically cleaved from a prohormone in much the same way as the insulins and MIPs. Thus, the IGF genes, which have a modified intron-exon organization (Daughaday and Rotwein, 1989) and code for single-chained peptides, and the bombyxin genes, which lack introns altogether (Kawakami *et al.*, 1989), represent probably more recently evolved modifications of the ancestral insulin genes.

Interestingly, in the molluscs and insects the insulin-related peptides are produced by neuroendocrine cells in the CNS. The claim that insulin is produced by neurons in the CNS of vertebrates is controversial, however, in view of the growing evidence that pancreatic islet cells share a number of features with neuroendocrine cells (Petersen *et al.*, 1986; Alpert *et al.*, 1988), it seems possible that in the Archaemetazoa the evolution of the insulin superfamily may have been within primitive neuroendocrine cells, probably associated with the digestive system (Steiner and Chan, 1988). In *Lymnaea* and other invertebrates, there is evidence for the presence of immunoreactive insulin in the gut, however, structural data about intestinal insulins of invertebrates are as yet not available.

An intriguing aspect of the evolution of the insulin superfamily concerns the striking difference in the degree of conservation of its members. The vertebrate insulins (and the highly similar sponge insulin-related peptide) and to a lesser extent also the bombyxins are conserved, suggesting a low acceptance of mutational change. By contrast, the MIPs, like the relaxins, are widely divergent among themselves, indicating a high degree of

acceptance of mutational change. Could different mechanisms account for these uneven rates of evolution in the insulin superfamily? Blundell and Wood (1975) have previously pointed out that in insulins most amino acid replacements are deleterious, due to a critical interdependence of the various residues in the molecule and the their strict relationship to the three dimensional structure and the role of this structure in the physiology of (pro)insulin, *e.g.*, synthesis, cleavage, packaging, transport, and receptor interaction. All these aspects could act as restraints during the evolution of insulin, resulting in a highly selective fixation of random amino acid replacements. This model, however, does not hold for the extensive molecular differences of MIPs and relaxins.

The MIP genes possess structural patterns that are reminiscent of important macroscale events that have taken place during evolution. The A and B chain domains of the MIP genes code for highly different peptides, while other parts, for example those encoding C peptides, are rigorously conserved. Also, in the introns, conserved and diverged regions alternate. These phenomena can be explained by macroscale events, such as reshuffling and/or gene conversion. A convincing example is the MIP V gene, which is clearly organized as a complex mosaic pattern of nucleotide sequences derived from

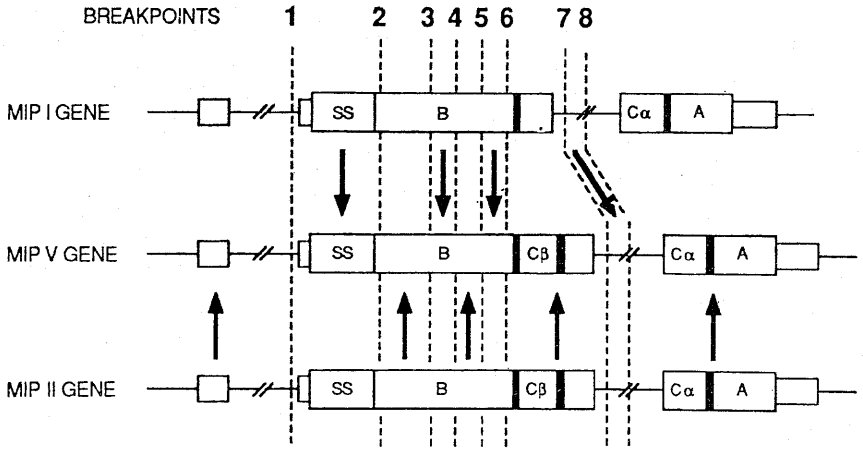

Fig. 29. Schematic representation of the hybrid MIP V gene. The MIP V gene may have evolved by an exchange of large stretches of the parental MIP I and II genes, very likely by intergenic reshuffling. Indicated are breakpoints, as indicated by the nucleotide sequence data. SS, signal sequence; A, A chain; B, B chain; Cα, Cα peptide; Cβ, Cβ peptide.

the MIP 1 and II genes. Here, reshuffling events have created MIP diversity, which is especially clear in the B chain domain of the MIP V gene (Fig. 29). The duplication of an ancestor MIP gene very likely has been an initial step towards MIP diversity. This early event released the constraint on mutational divergence, and subsequent exchanges of large parts of the MIP genes significantly enhanced the rate of successful sequence variations. Whether this molecular mechanism is at the basis of the relaxin sequence diversity remains an intriguing question. The macroscale evolutionary events as described for the MIP genes may have a wider significance, *i.e.*, in the generation of complex proteins in general.

Vertebrate growth hormone-like protein in gastropod molluscs

It has been demonstrated (Morse, 1981) that the abalone, *Haliotis rufescens*, treated with bovine growth hormone showed increased growth rates. Recently, Kawauchi and co-workers (Moriyama *et al.*, 1989), using anti-bodies against chum salmon growth hormone, showed the presence

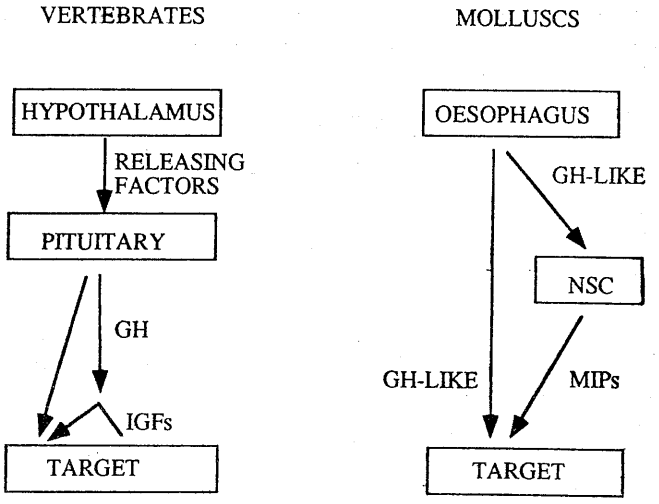

Fig. 30. Hypothetical model for the control of growth in molluscs. The left panel outlines the situation in the vertebrates. Growth hormone (GH) induces the release of insulin-like growth factors (IGFs) in the targets and, at least in some cases, acts together with IGFs on the target to control growth. The right panel outlines the hypothetical interplay between the GH-like factor and MIPs in molluscs. In analogy of the situation in the vertebates the GH-like factor from the oesphagus induces the release of MIPs from neurosecretory cells (NSC) and presumably acts together with the target to control growth.

of immunopositive cells in both renal and oesophageal tissues of the abalone, *H. discusus hamai*. Both the chum salmon growth hormone and the partially purified abalone growth hormone-like substance increased the growth rate of *H. discusus hamai*, when injected intramuscularly. And partial sequencing of the abalone protein revealed some sequence similarity with chum almon growth hormone.

These results might indicate that in addition to MIPs, a growth hormone-like protein might be involved in the regulation of growth in molluscs, at least in gastropods. In vertebrates, growth is regulated by a number of hormones, including growth hormone and IGFs, which show an intricate interplay. In analogy to the situation in vertebrates, we hypothesize that in gastropods there is also a complex interplay of hormones involving both a growth hormone-like protein and MIPs. The general outlines of the hypothetic control of molluscan growth by MIPs and a growth hormone-like hormone are shown in Fig. 30.

SCHISTOSOMIN, A LGC PEPTIDE WITH A DUAL FUNCTION: STIMULATION OF GROWTH AND INHIBITION OF REPRODUCTION

In addition to the four MIP genes, the LGC system expresses another neuropeptide gene, a gene encoding a peptide, called schistosomin. Schistosomin was discovered in experiments designed to uncover the molecular basis of the endocrine interactions between the schisostome parasite, *Trichobilharzia ocellata* and *L. stagnalis*. [Schistosome parasites cause the wide-spread tropical disease, schistosomiasis (bilharziasis).

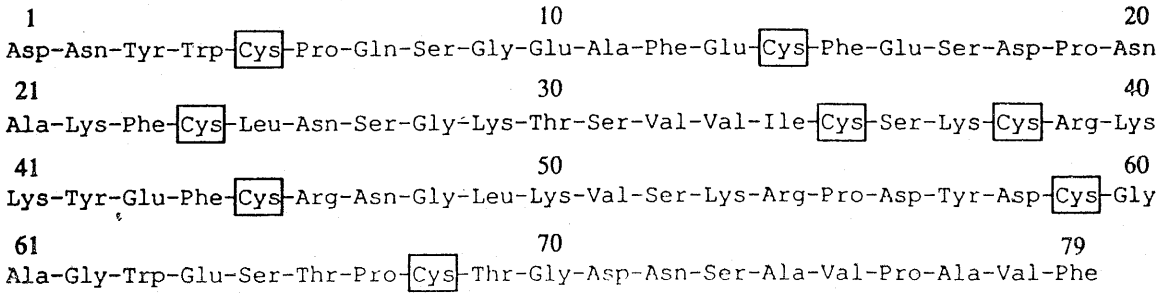

Fig. 31. Primary structure of schistosomin isolated from the central nervous system of *Lymnaea stagnalis*. Cysteine residues are boxed.

Freshwater snails are the intermediate hosts of these digenetic trematode parasites. In our laboratory, the combination of the avian schistosome, *T. ocellata* and the snail *L. stagnalis* is used to study host-parasite interactions.] The inhibition of reproductive activities and the simultaneous stimulation of somatic growth, which occur in infected snails, is caused by schistosomin, which peptide is specifically present in the haemolymph of parasitized animals. Further experiments have shown that schistosomin is stored in the LGC axons in the median lip nerves of normal, non-infected animals. Schistosomin from the CNS of normal animals has been purified and sequenced. It is a 79 amino acid complexely folded peptide, due to the presence of 8 cysteine residues, which may form four intramolecular disulphide bridges (Fig. 31) (Hordijk *et al.*, in press). Immunocytochemical studies indicate that schistosomin is synthesized by the LGCs, including the canopy cells (Fig. 23), as well as small neurons in the pedal ganglia. In infected snails, schistosomin is released under the influence of an as yet unidentified factor produced by the developing parasite. With the help of oligonucleotides derived from known peptide sequences the concentration of transcript coding for schistosomin was determined in normal and in infected snails. It appeared that the gene encoding schistosomin is quite inactive in uninfected snails, but becomes activated after infection, presumably by the action of the unidentified compound produced by the parasite.

Schistosomin inhibits the effects of injected CDCH (CDCH induces ovulation and egg laying) and calfluxin (induces calcium fluxes in the female albumen gland). Both peptides are derived from the CDCH prohormones (see above). Furthermore, in additional experiments it was shown that schistosomin is capable of inhibiting the biological effects of DBH (stimulation of vitellogenesis and of synthetic activities in the albumen gland). The mentioned inhibiting effects of schistosomin on reproduction are peripheral effects and consist, probably, of interactions with CDCH, calfluxin and DBH on the receptor level. In addition to these peripheral effects, schistosomin has clear central effects. It decreases the excitability of the CDCs, which results in the inhibition of release of the CDC peptides. At the same time, it increases the excitability of the LGCs, thus acting as an excitatory autotransmitter,

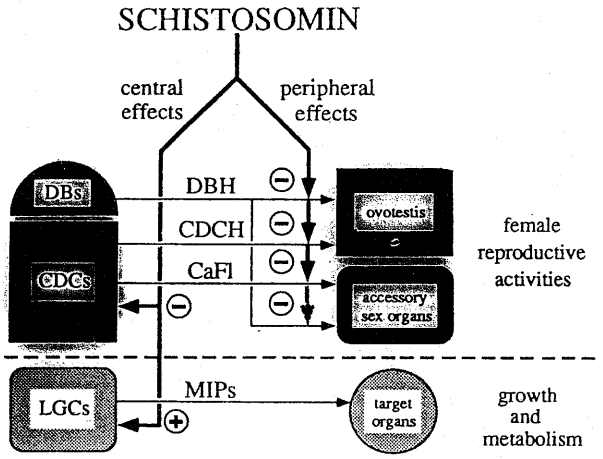

Fig. 32. Schematic representation of the central and peripheral effects of schistosomin in *Lymnaea*. The effects of purified schistosomin on female reproductive activities can be distinguished as central and peripheral effects. Centrally, schistosomin reduces the excitability of the caudodorsal cells (CDCs). In the periphery, schistosomin inhibits the activities of the dorsal body hormone (DBH) and the caudodorsal cell hormone (CDCH) on the ovotesits and of calfluxin (CaFl) on the albumen gland. Furthermore, schistosomin increases the excitability of the light green cells (LGCs), which leads to release of MIPs.

which leads to an increased release of MIPs and thus, to an increased growth rate. The various effects of schistosomin are summarized in Fig. 32. The opposing effects of schistosomin on neuronal systems that control reproduction and growth suggest that the peptide may play a major role in the coordination of the two processes. And, although this has not been shown directly, we assume that in normal, intact snails schistosomin has a physiological role in attuning both processes.

CONCLUDING REMARKS

From the studies on the molluscan peptidergic model systems several interesting conclusions can be drawn. As we have seen, the mechanism responsible for the control of such complex behaviours as egg-laying in *Lymnaea* and *Aplysia* involves the synthesis of large prohormones containing multifunctional sets of different peptides that are essential in mediating the full physiological and behavioural array associated with egg laying. These peptides are released during long-lasting bursts of electrical activity of the peptidergic systems in order to act as neuromodulators, local hormones (nonsynaptic release or

paracrine actions), or circulatory hormones on targets in the CNS and the reproductive tract. Several peptides act as autotransmitters and initiate discharge activity in all cells of the peptidergic network. In addition, the electrotonic coupling among the cells probably is a means for the synchronization of the activities of the constituent neurons. These features provide the means for the sudden release of large amounts of bioactive peptides. Other peptide-secreting systems displaying episodic release, *e.g.*, in the hypothalamus and pituitary of vertebrates, may have similar properties. The peptides derived from the egg-laying peptide precursors are packaged in distinct vesicle classes that have unique subcellular localizations. The resulting separation of synthesis, packaging, transport and release expands the information content of a prohormone by generating various vesicle classes containing different combinations of bioactive peptides.

The neuropeptide gene families controlling egg laying and growth in molluscs code for different though related (sets of) bioactive peptides. This has several interesting consequences. First, the diversity and complexity of physiological and behavioural processes that can be controlled by a gene family coding for different though related sets of neuropeptides is greatly expanded. Thus, neuropeptide gene families coding for different peptides are extremely well suited for the regulation of vital and often long-lasting life processes. Second, the expression of the members of neuropeptide gene families may be controlled in a tissue-specific way. The CDCH genes are expressed in neurons of the CNS and of the peripheral nervous system and in secretory cells of the reproductive tract. It seems likely that the peptides generated in these tissues have a role in the coordination of some (behavioural) aspect related to egg laying. Thus, tissue-specific expression of neuropeptide gene families is a means to control local processes that are part of a larger physiological or behavioural programme. Third, a large number or even all members of a neuropeptide gene family may be expressed in one type of neuron. In *Lymnaea*, several genes of the MIP gene family together with the schistosomin gene are expressed in a stimulus-dependent way in the LGCs. A neuron's ability to express alternative peptides indicates that it has available a greater number of codes for communication with its

targets; therefore, this type of differential expression and release of different neuropeptides endows the peptidergic neuron with a considerably increased adaptive function for information-handling. Fourth, in *Lymnaea* there is "cross-talk" between the peptidergic systems controlling growth and reproduction. A clear example is schistosomin, which is released by the LGC system under specific physiological conditions. Schistosomin has a dual function: it excites the LGCs and inhibits the gonadotropic centres.

Finally, as it is expected that the genes controlling growth and reproduction in *Lymnaea* are present in other molluscan species, it seems worthwhile to start a search for the genes, their products and functions in those species that are of importance for aquaculture.

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