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The Gonadotropin-Releasing Hormone-Immunoreactive Neuronal System of the Freshwater Snails, *Helisoma trivolvis* and *Lymnaea stagnalis*: Possible Involvement in Reproduction

by

Kevin Gerald Young ©

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The Gonadotropin-Releasing Hormone-Immunoreactive Neuronal System of the Freshwater Snails, *Helisoma trivolvis* and *Lymnaea stagnalis*: Possible Involvement in Reproduction submitted by Kevin Gerald Young in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Cell Biology.

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Abstract

Gonadotropin-releasing hormone (GnRH) belongs to a family of peptides primarily involved in vertebrate reproductive control. These peptides function as hormones, neurotransmitters, neuromodulators, and immunomodulators. In this study I have mapped-out the distribution of a GnRH-like peptide in the hermaphroditic snails *Helisoma trivolvis* and *Lymnaea stagnalis*. As well, I have attempted to examine the possible reproductive role of GnRH in *H. trivolvis* by examining electrophysiological responses to synthetic mGnRH, and egg-laying activity following injections of synthetic mGnRH. The GnRH-like peptide was observed to be distributed throughout the CNS, including areas known to be involved in reproductive control, and in peripherally-extending fibres that innervated the male reproductive tract. No reliable effects of mGnRH on female reproductive activities were observed. A role in male reproductive control is suggested by the distribution of the endogenous peptide in the CNS and penial complex in the snails examined.

**If only I had no legs, no eyes,
No will to break or compromise,
No thoughts of mind to run astray,
Just crawl along and eat algae.**

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An unfortunate discouragement from the Department of Biological Sciences, and Dr. Bill Samuel in specific, in terms of funding, has made my life as a graduate student much less enjoyable than what it could have been. Hopefully funding in future years will be given out to allow every graduate student within the department to survive at a level not quite so far below the poverty level. Any work as a student is made much more difficult when worrying about survival becomes a daily concern.

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Chapter 1 - Introduction

Gonadotropin-releasing hormone (GnRH) belongs to a family of structurally-related peptides involved in vertebrate reproductive control (King and Millar, 1992; Sherwood et al., 1993; Muske, 1993; King and Millar, 1995). In the present study, I have examined the hypothesis that a related neuronal peptide serves also to control reproduction in an invertebrate group, the pond snails (Mollusca; Gastropoda; Pulmonata; Basommatophora).

Neuropeptidergic and Neuroendocrine Cells

Neuroendocrine cells were first identified in the hypothalamus of the teleost fish, *Phoxinus laevis* (Scharrer, 1928; reviewed in Scharrer, 1987, 1990). They were recognized by their large content of neurosecretory granules and found to be similar to endocrine cells in their ability to release proteinaceous materials. Since then, neuroendocrine cells have been shown to be important throughout the vertebrates and invertebrates in the control of many physiological functions, often acting to release hormones or neuromodulators

(Sharrer, 1990). As well, many neurons that release proteinaceous materials have been found to act as classical neurons in that they can release their peptide transmitters at synaptic sites. A good example of this is in the bullfrog sympathetic ganglion, where individual neurons release both acetylcholine and GnRH as a neurotransmitter and neuromodulator, respectively (Adams et al., 1986; Jones et al., 1987).

In snails, many types of neuropeptidergic cells have been identified in the central nervous system (CNS). Classically, techniques such as the alcian blue-alcian yellow (Boer et al., 1977; Wijdenes et al., 1980) and paraldehyde fuchsin (Simpson, 1969) staining techniques have been used to identify possible neuroendocrine cells. More recently, immunohistochemistry has been used to identify peptide-containing neurons that may serve as either neuroendocrine cells or as classical neurons (e.g. Schot et al., 1981, van Minnen et al., 1992). Neuropeptidergic cells exist as single neurons or clusters, and can be found throughout the ganglia of the CNS (Dorsett, 1986). Those that act as neuroendocrine cells can release their secretory substances into neurohemal areas in the sheath surrounding the CNS or into intercellular spaces within the ganglia through non-synaptic release sites (Dorsett, 1986).

Not only are neuropeptidergic cells wide-spread throughout the animal kingdom, diverse animal groups also share similar neurosecretory materials. For example, FMRFamide has been shown to be a common neuropeptide in many invertebrate phyla (Gerearts et al., 1988a, Greenberg and Price, 1992), and can act as a neurotransmitter, neuromodulator, or neurohormone (Saleuddin et al, 1992). Related peptides have also been demonstrated in the vertebrates (e.g. bovine YGGFMRF, Geraerts et al., 1988a). As well, the vertebrate hypothalamic peptides vasopressin and oxytocin exist within a family also containing the molluscan peptide conopressin (van Kesteren et al., 1995a).

As with many neuropeptides, neuropeptide receptors have also been shown to exist in structurally related forms throughout many phyla. Receptors for the molluscan conopressin peptide were used as outgroup representatives to demonstrate the likely characteristics of the ancestral receptors for this peptide family (van Kesteren et al., 1996). It has been suggested that neuropeptidergic systems, in general, have common ancestral roots dating back as far as the emergence of the first metazoans (Scharrer, 1987, 1990; Gorbmann, 1997).

Evolution of the GnRH Family of Peptides and their Receptors

There are at least twelve forms of GnRH within the phylum Chordata (Table I; King and Millar, 1995; Zohar et al., 1995; Powell et al., 1996; Jimenez-Linan et al., 1997). These peptides are all ten amino acids long, and are related through highly conserved C- and N-termini. All exist as monomers except for the tunicate II form, which forms a dimer via disulfide bonds at the cysteine residue (underlined in Table I; Powell et al., 1996).

Receptor sequences and ligand activation have been studied in most of the vertebrate classes (King and Millar, 1995; Sealfon et al., 1997, Lin et al., 1998). Most of the GnRH forms tested can stimulate gonadotropin release in all of the vertebrate classes, though specificity for the receptors differs (King and Millar, 1995). Notably, the mammalian pituitary receptor is only strongly activated by mammalian GnRH (mGnRH), and the Lamprey I GnRH only strongly activates the agnathan receptors. Cloning of the receptors from

Table I. The Diversity of GnRH Peptides in Chordates*

GnRH Type	Primary Structure
mammalian GnRH	**pGlu His Trp Ser** Tyr **Gly** Leu Arg **Pro Gly NH2**
guinea pig GnRH	**pGlu** Tyr **Trp Ser** Tyr **Gly** Val Arg **Pro Gly NH2**
chicken GnRH I	**pGlu His Trp Ser** Tyr **Gly** Leu Gln **Pro Gly NH2**
chicken GnRH II	**pGlu His Trp Ser** His **Gly** Trp Tyr **Pro Gly NH2**
salmon GnRH	**pGlu His Trp Ser** Tyr **Gly** Trp Leu **Pro Gly NH2**
catfish GnRH	**pGlu His Trp Ser** His **Gly** Leu Asn **Pro Gly NH2**
dogfish GnRH	**pGlu His Trp Ser** His **Gly** Trp Leu **Pro Gly NH2**
seabream GnRH	**pGlu His Trp Ser** Tyr **Gly** Leu Ser **Pro Gly NH2**
lamprey GnRH I	**pGlu His** Tyr **Ser** Leu Glu Trp Lys **Pro Gly NH2**
lamprey GnRH III	**pGlu His Trp Ser** His Asp Trp Lys **Pro Gly NH2**
tunicate I	**pGlu His Trp Ser** Asp Tyr Phe Lys **Pro Gly NH2**
tunicate II	**pGlu His Trp Ser** Leu <u>Cys</u> His Ala **Pro Gly NH2**

*Amino acids which are common to all or most of the known GnRH forms are shown in bold font.

several classes has shown some key structural differences, such as the absence of an intracellular C-terminal tail in the mammalian receptors, but not in those of other classes (Lin et al., 1998). The cloned GnRH receptors are all related by high sequence homology, in general, and all likely act as G protein-coupled receptors (King and Millar, 1995; Lin et al., 1998; Ulloa-Aguirre et al., 1998).

Within each chordate class, multiple GnRH forms exist to regulate the release of gonadotropins from the pituitary and to serve other functions such as neurotransmission/modulation within the CNS and peripheral nervous system (Jones et al., 1987; Wong et al., 1990; Oka, 1992; Oka, 1996), and immunomodulation in lymphocytes and immune system organs (Wilson et al., 1995; Marchetti et al., 1998). These peptides can also have direct effects within the gonads, as shown by the presence of gonadal receptors (Clayton et al., 1979; Pati and Habibi, 1993). In the lower chordates, it has been suggested that GnRH may serve primarily to influence the gonads via direct innervation (Powell et al., 1996).

Evidence for the existence of GnRH in invertebrates has been growing over the past decade. Most of the evidence comes from immunocytochemical staining in animals of various phyla. Positive staining for GnRH has been demonstrated in the CNS of the nematode worm, *Ascaris suum* (Brownlee et al., 1993), neurons of the coelenterate, *Renilla koellikeri* (Anctil, 1997), and in various arthropods (listed in Gorbman, 1997). In addition, a peptide with regions of 80% sequence homology to mGnRH has been isolated from yeast (Loumaye et al., 1982).

Both positive staining for GnRH and the ability of mGnRH to affect neuronal activity have been demonstrated in the pond snail *Helisoma trivolvis* (Goldberg et al., 1993). This study further demonstrated that an endogenous GnRH-immunoreactive (GnRH-IR) peptide in the CNS co-eluted with mGnRH when run on an HPLC column and that *H. trivolvis* CNS extracts could induce the release of gonadotropin from cultured goldfish gonadotrope cells. Fish gonadotrope GnRH receptors are known to bind and be activated by most of the known types of GnRH (King and Miller, 1995). Taken together, these results strongly suggested that a mGnRH-like peptide exists in the CNS of *H. trivolvis.*

Using Molluscs to Study Neuropeptide Actions

Molluscs have been used for several decades to facilitate studies into the cellular and molecular foundations of the nervous system. The study of neural circuit organization has been greatly facilitated by the relatively simple circuitry of several molluscan systems. To the neuroethologist, molluscs have provided less complicated behaviours with relatively less complicated neural foundations.

The best studied of all types of behaviours, in both molluscs and vertebrates, are those involving either repetitive patterns or reflexive acts. Repetitive behaviours such as respiration in the snail, *Lymnaea stagnalis* (Syed et al., 1990), swimming in the sea slug, *Tritonia diomedia* (Getting and Dekin, 1985), or walking in several vertebrate and invertebrate systems (listed in Pearson, 1993) have all shown common elements in their control. All of these types of behaviours are controlled by central pattern generators which, with a repetitive output, generate the rhythmic nature of the muscle contractions underlying the behaviour.

Reproduction in snails has served as a model for more complex types of behaviours in which a linear series of stereotypical events occurs to generate behaviours such as copulation and egg-laying (e.g. Chase, 1986; Geraerts et al., 1988b). Egg-laying behaviour has been shown to constitute a fixed action pattern, with a series of movements and resting periods necessarily being carried through until completion, once initiated (Ferguson et al., 1993). Internally, the eggs are passed down the female reproductive tract and packaged into an egg mass in coordination with these overt movements (Geraerts et al., 1988b). In the snails, these events can be studied at different levels of complexity, from the molecular to the whole animal.

Reproductive behaviours have been studied in detail in three molluscan systems, *Aplysia californica*, *Lymnaea stagnalis*, and *Helix aspersa*. In *A. californica* and *L. stagnalis*, a neuropeptide hormone which induces ovulation, and sets into progress the events that lead to extrusion of the egg-masses, has been cloned (reviewed in Geraerts et al., 1988b). These hormones are named egg-laying hormone in *A. californica*, and caudodorsal cell hormone in *L. stagnalis*. The two are highly related in sequence and gene structure. As well, a group of peptides has been identified which are encoded on the same gene as each of the ovulogenic peptides (Geraerts and Hogenes, 1985; Geraerts et al., 1988b; Hermann, 1994; Wayne, 1995). These peptides help to coordinate the motor events involved in the egg-laying process (Hermann, 1994; Wayne, 1995).

Neuropeptides have also been implicated in controlling male reproductive behaviours, in that the male reproductive tract of snails is innervated by many peptidergic neurons (Li et al., 1992; Li et al., 1994; Li et al., 1995; Li and Chase, 1995). Several peptides have been shown to control contractions of the vas deferens and penial complex (penis complex), as well as eversion of the penis. For instance, myomodulin causes relaxation of the penis retractor muscle (Li et al., 1994), and conopressin stimulates contractions of the vas deferens (van Kesteren et al., 1995a). Eversion of the preputium is caused by APGWamide (de Boer et al., 1997a). Several other peptides have been identified in the penial nerve and penial complex through use of mass spectrometry (Li et al., 1995; van Kesteren et al., 1995b), though the identity of all has not yet been determined.

Thus, reproduction in snails serves as an excellent model system for investigating the involvement of neuropeptides in the generation of complex behaviours, and for the examination of neuropeptide actions at the cellular and molecular levels.

The Gross Morphology of the CNS and Reproductive Tract of Pond Snails

Both the male and female reproductive tracts of snails are under direct control from the CNS (Hermann, 1994; Geraerts and Joose, 1984), as well as being hormonally regulated (Geraerts et al., 1988b). Neuropeptides affecting reproductive organ functions can, then, be delivered to the reproductive tissues via direct innervation or the open vascular system, as well as being produced within the reproductive organs.

The following sections provide background on the anatomy of the nervous system and the male and female reproductive tracts (Figs. 1.1 - 1.3).

The Nervous System

Pond snails have a nervous system consisting of a diffuse network of individual neurons in the peripheral tissues, which forms the peripheral nervous system, and a collection of ganglia in the head region, which forms the CNS. In *H. trivolvis* and *L. stagnalis*, the CNS contains five paired ganglia and one unpaired ganglion (Figs. 1.1 and 1.2). In addition, one pair of endocrine organs, the dorsal bodies, are associated with the cerebral ganglia. These are the only endocrine organs that are known to exist in snails (Saleuddin et al., 1994).

The ganglia of the CNS contain several hundred neurons each, including many neurosecretory cells and classical neurons (Dorsett, 1986; Goldberg and Cavers, 1993). The neuronal cells bodies are located in the outer layer of the ganglia, the rind, with the inner neuropil area being composed of extensive networks of neurites (Fig. 1.1A, inset). Nerve tracts extend out of the neuropil forming inter-ganglionic connectives or peripheral nerves.

The paired cerebral ganglia have been most implicated in the control of reproduction in the freshwater snails (Geraerts, 1976; Geraerts et al., 1988b; de Lange et al., 1998a). These contain several types of neurons and neurosecretory cells known to be involved in controlling both male and female reproductive processes and behaviours. Associated within the cerebral ganglia are several

Figure 1.1. The central nervous system of *Helisoma trivolvis*. A) The circumesophageal ganglia isolated in a dissection dish. The pedal commissure has been transected and the right and left pedal ganglia (RP and LP) spread laterally in order to lay the CNS flat in the dish. Other ganglia shown include the left and right cerebral (LC and RC), pleural (LPl and RPl), and parietal (LPa and RPa) ganglia and the unpaired visceral (V) ganglion. Also indicated is the cerebral extension (CE) of the left cerebral ganglion and the dorsal bodies (DB). Arrows point to individual somata (S) and areas of the sheath (Sh) surrounding the ganglia. *Inset:* A hematoxylin and eosin-stained section through one of the ganglia showing somata, neuropil (N), and an area of sheath. Bar = 50 µm. B) A schematic of the CNS showing the same ganglia as in A, as well as the left and right buccal ganglia (LB and RB). Also indicated in the schematic are the dorsal bodies, caudodorsal cells (CDCs), lateral lobes (LL), cerebral commisure (CC), and the cerebral extension. The labelled nerves are the penial nerve (penis nerve; PN), and the intestinal nerve (IN). The former innervates the male reproductive tract, and the latter innervates the female reproductive tract.

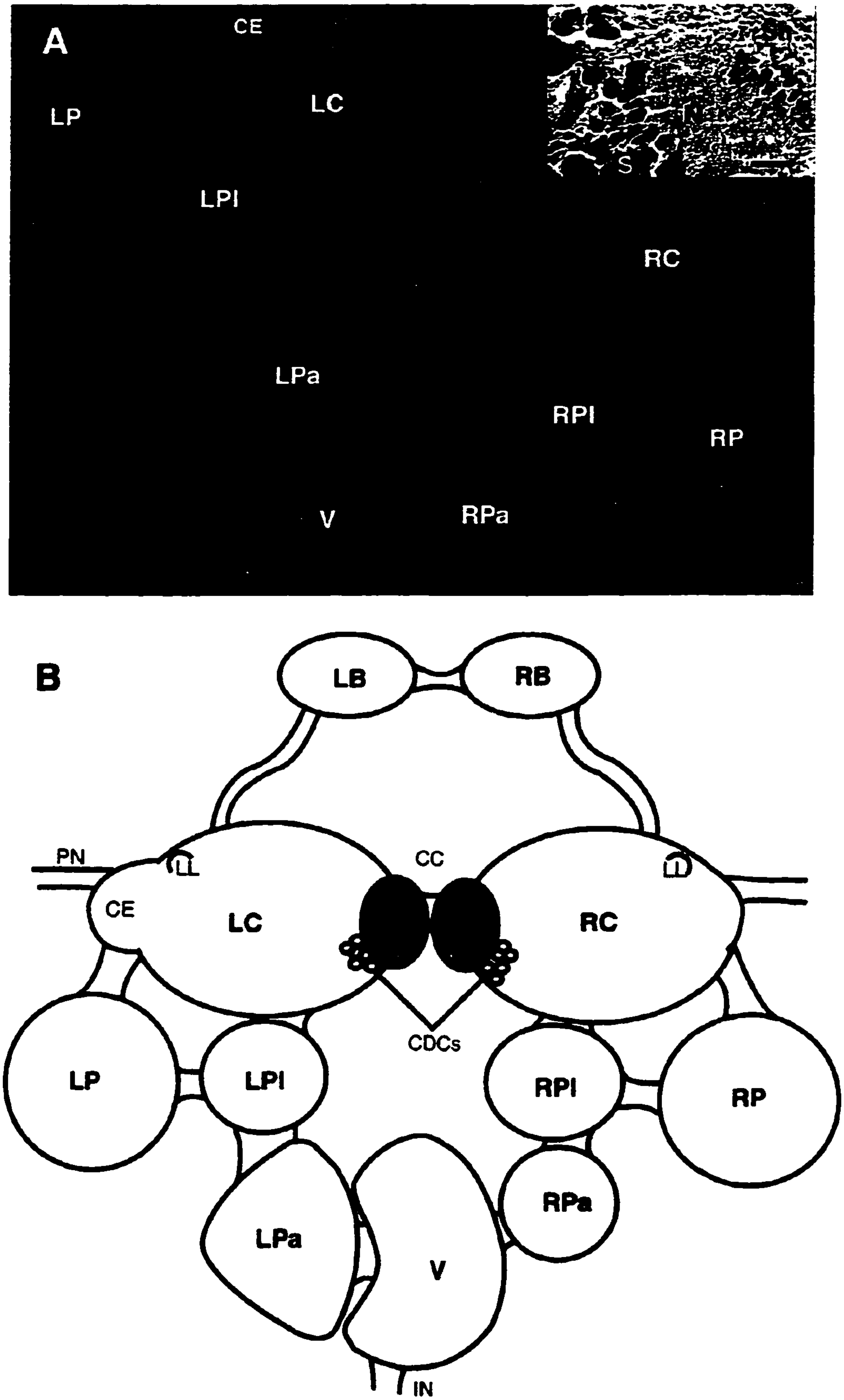
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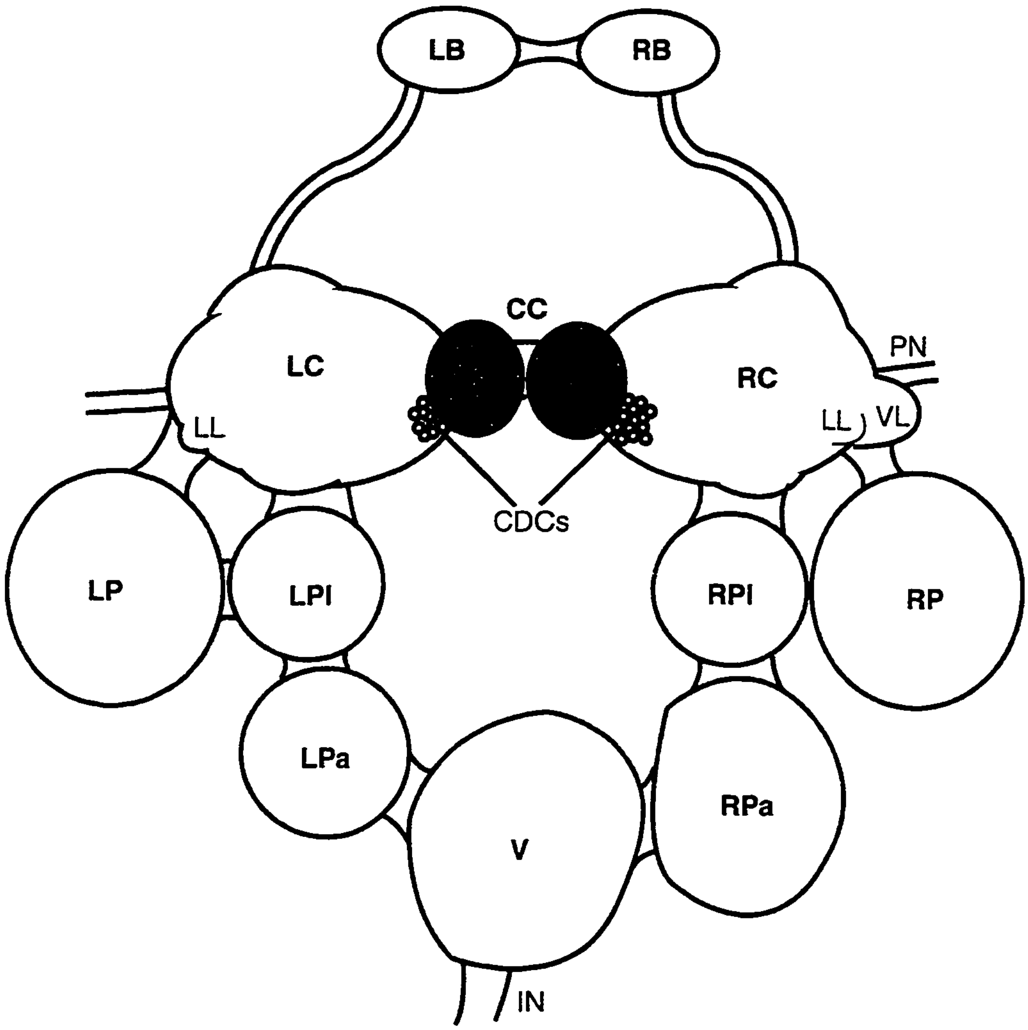
Figure 1.2. The central nervous system of *Lymnaea stagnalis*. A schematic of the circumesophageal ganglia of *L. stagnalis* indicating the left and right buccal (LB and RB), cerebral (LC and RC), pedal (LP and RP), pleural (LPl and RPl), and parietal (LPa and RPa) ganglia and the unpaired visceral (V) ganglion. Also indicated are the dorsal bodies (DB), caudodorsal cells (CDCs), lateral lobes (LL), cerebral commisure (CC), and the ventral lobe (VL) of the right cerebral ganglion. The labelled nerves are the penial nerve (PN) and the intestinal nerve (IN). Note the reversed asymmetry of the PN and the LPa and RPa as compared to *H. trivolvis* (Fig. 1.1).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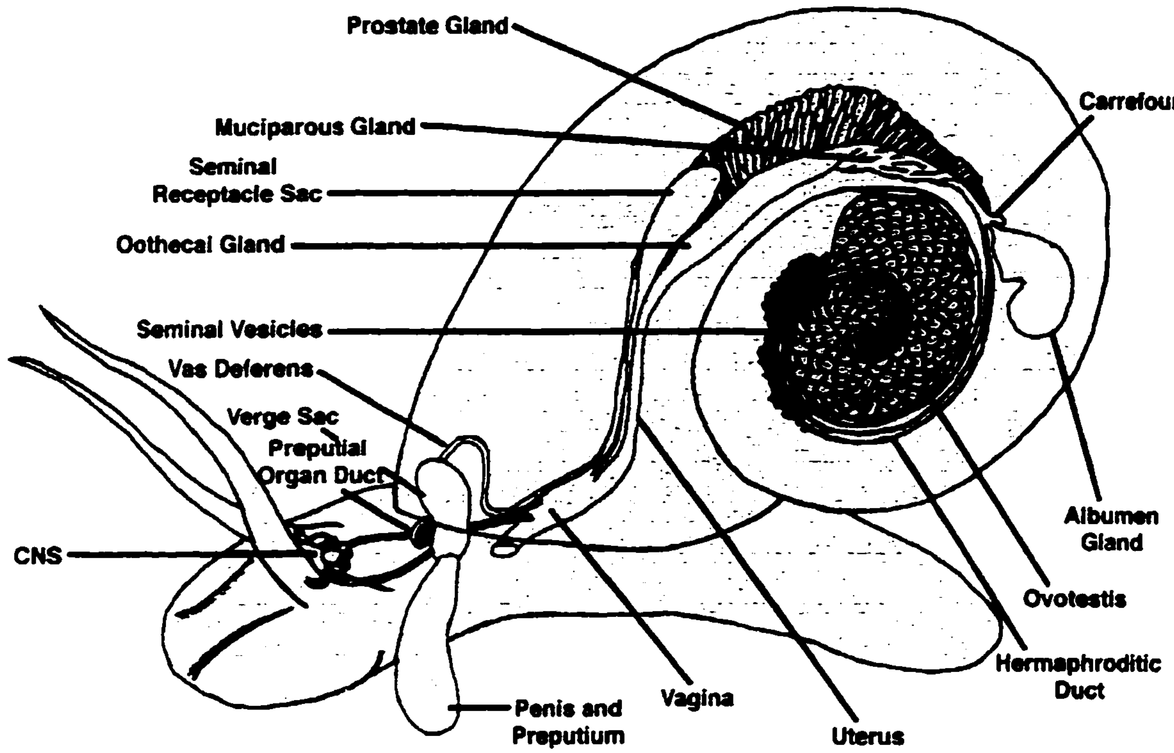
identified structures: the cerebral extensions (*Helisoma*), the ventral lobes (*Lymnaea*), the lateral lobes, and the caudodorsal cells. The significance of each of these areas in reproductive control will be referred to later.

The Reproductive Tract

All pond snails are hermaphroditic, with individuals having separate male and female tracts, and a shared ovotestis for gamete production (Fig. 1.3; Abdel-Malek, 1954). The sperm and oocytes generated in the ovotestis are passed through the hermaphroditic duct to either the male or female tracts. These gametes are kept separate by divisions within the ovotestis, thereby preventing self-fertilization in this structure (Geraerts and Joose, 1984). Self-fertilization can occur, but is thought to take place just outside of the ovotestis in the hermaphroditic duct (Geraerts and Joose, 1984).

Other structures associated with the hermaphrodite portion of the reproductive tract are the seminal vesicles. These are located at the end of the hermaphroditic duct that joins with the ovotestis

Figure 1.3. The reproductive tract of *Helisoma trivolvis.* As with all freshwater snails, this species is hermaphroditic. Shown in this schematic are the female (red) and male reproductive tracts, and the hermaphroditic portion of the system. The male organs are the penis, preputium, verge sac, vas deferens, and prostate gland. The female organs are the vagina, uterus, seminal receptacle sac, oothecal gland, muciparous gland, carrefour, and albumen gland. The ovotestis produce both sperm and eggs, which are carried through the hermaphroditic duct to either the male or female tract. At the end of this duct, joining onto the ovotestis, are the seminal vesicles. Both the male and female tracts receive innervation from the CNS.



Prostate Gland
Muciparous Gland
Seminal
Receptacle Sac
Oothecal Gland
Seminal Vesicles
Vas Deferens
Verge Sac
Preputial
Organ Duct
CNS
Penis and
Preputium
Vagina
Uterus
Hermaphroditic
Duct
Ovotestis
Albumen
Gland
Carrefour

(Fig. 1.3). The function of these is to store the individuals' own sperm (Abdel-Malek, 1954).

The female tract has three different glands associated with it, each serving to produce elements of the egg mass that will surround the individual embryos (Abdel-Malek, 1954; Plesch et al., 1971; Geraerts and Joose, 1984). These are the albumen, oothecal, and muciparous glands (Fig. 1.3). As well, the seminal receptacle sac is associated with this tract, connecting to the vagina (Fig. 1.3). Much of the sperm from a partner is transferred into this sac, and ultimately either degraded or transferred into the female tract for fertilization of the ova (Geraerts and Joose, 1984).

The male tract has only one gland associated with it, this being the prostate gland (Figs. 1.3). This gland surrounds the sperm with fluid for transfer to the female-behaving partner (Abdel-Malek, 1954; Plesch et al., 1971). As well, the prostate gland is thought to be involved in controlling the sex drive of the snail through a feedback signal to the CNS (de Boer et al., 1997b). Following transfer past the prostate gland, the sperm are carried through the vas deferens, and into the penial complex, which consists of the verge sac, preputium, preputial organ duct, and penis (Figs. 1.3).

Multiple functions of the penial complex are controlled via innervation into this structure (Li et al., 1992; de Boer et al., 1996). These functions include eversion, probing, and intromission (de Boer et al., 1996; de Lange et al., 1998b). The contractility of the vas deferens and the different muscles of the penial complex are controlled mainly by peptidergic neurons projecting into the penial nerve (Li et al., 1992; van Kesteren et al., 1995b; de Boer et al., 1996; de Lange et al., 1998a; de Lange et al., 1998b).

Objectives and Methodological Approaches

In this study, I set out to examine the distribution and possible reproductive functions of a GnRH-like peptide in the mollusc, *H. trivolvis.* To do this, I have used immunocytochemistry and neuronal tracing techniques to examine the peptide distribution, and injections of synthetic hormone to examine its effect on egg-laying. As well, I have examined two different cell populations in the CNS to determine if they may act as targets for the endogenous GnRH-like peptide.

The two populations of neurons which I examined in the CNS included one involved in female reproductive control and one involved in male reproductive control. The caudodorsal cells are neurons known to be the key regulators of ovulation, reproductive tract synthesis, and egg-laying behaviour in pond snails (Gerearts et al., 1988b; Hermann, 1994). It has previously been shown that homologous cells in opisthobranch molluscs, the bag cells, may have action potential activity affected by GnRH (Tsai et al., 1996). I therefore examined the effects of mGnRH on the electrical activity of these neuroendocrine cells in *H. trivolvis.*

In the left cerebral extension of the cerebral ganglia of *H. trivolvis*, neurons were found to innervate the male reproductive tract. As this structure is innervated by many GnRH-immunoreactive (GnRH-IR) fibres, I examined these neurons *in vitro* using a receptor staining technique (see appendix). This was done in order to initiate the establishment of an *in vitro* assay system for GnRH in snails.

A comparative study on the distribution of the GnRH-like peptide in the closely related species, *Lymnaea stagnalis*, was also performed in order to aid in predicting the main functions for the endogenous peptide. *L. stagnalis* has served as an excellent model for the study of reproductive behaviours in the past, and many aspects of reproductive control are well understood in this species (Geraerts et al., 1988b). The locations of the GnRH-like peptide, then, could be related to the known structures involved in controlling reproduction in the snails.

In summary, using information from whole animal and cellular responses to synthetic mGnRH, I have tried to link the physiological functioning of GnRH in pond snails to information obtained through histological analysis.

Chapter 2 - Methods and Materials

Animals and Dissections

Helisoma trivolvis and *Lymnaea stagnalis* were maintained as inbred colonies in 45 L flow-through aquaria. They were fed trout chow (NU-WAY - United Feeds) and lettuce *ad libitum*, and kept on a 12 hours light:12 hours dark cycle at 25°C.

Adult *H. trivolvis* at postembryonic stages 10 - 15 (10 - 15 mm shell height; staged as per Goldberg and Kater, 1989) and *L. stagnalis* at 25 - 30 mm shell height were used for most protocols. Following removal of the shell, the snails were anaesthetized in artificial pond water (APW; 0.025% Instant Ocean; Aquarium Systems) containing 25% Listerine (Warner-Lambert Co.). The animals were dissected in either *Helisoma* saline (51.3 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$,, and 5 mM HEPES; pH 7.35) or *Lymnaea* physiological saline (30 mM NaCl, 1.5 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, 0.25 mM Na_2HPO_4, 18 mM $NaHCO_3$; pH 8.2).

Histological Techniques

Haematoxylin and Eosin Staining

In order to clearly identify different tissues and cell types, a standard protocol for Harris' haematoxylin and acidified eosin staining (Drury and Wallington, 1967) was used on tissues from the reproductive tract and the CNS. The paraformaldehyde-fixed tissues were embedded in paraffin wax and sectioned at 10 μm prior to staining. These results were then compared to the results from a previous study on the morphology of the reproductive tract of *Helisoma trivolvis* (Abdel-Malek, 1954), and served to aid in identifying structures examined during immunocytochemical staining for GnRH.

Immunohistology

Immunohistochemical localization was performed in tissue sections and wholemount preparations using antibodies for mGnRH and αcaudodorsal cell peptide (αCDCP). All tissues were fixed overnight at 4°C in 4% paraformaldehyde prior to sectioning or processing as wholemounts.

Staining in Tissue Sections

Prior to sectioning, the tissues were washed in phosphate-buffered saline (PBS: 0.056 M $Na_2HPO_4 \cdot 7H_20$, 0.154 M NaCl; pH 7.2) twice, for 1 hour each, then once for at least 2 hours in PBS containing 12% sucrose. Tissues were then frozen in OCT compound (Tissue Tek, Miles Inc.) using dry ice. Sections were cut on a cryostat at a 10 µm thickness, and mounted onto chrome alum-coated microscope slides. A minimum of three preparations were sectioned for each type of tissue, unless otherwise stated. In the case of some tissues, such as the cerebral ganglia, well over a dozen preparations were sectioned.

Sections were washed three times in PBS for 20 minutes each, then in 30% horse serum (Gibco) with 0.4% Triton X-100 (Sigma) in PBS for 30 minutes. They were then incubated overnight at 4°C in a primary antibody solution. Incubations in primary antibodies were performed with two different polyclonal mGnRH antibodies (Sigma and Incstar) and a polyclonal antibody raised against αCDCP (a gift from Jan van Minnen, Vrije Universiteit, Amsterdam). The Sigma and Incstar mGnRH antibodies were diluted 1:50 and 1:500, respectively, in PBS containing 0.4% Triton X-100 and 3.3 mg/mL of bovine serum albumin (BSA; Sigma; used with the Sigma antibody) or 3 mg/mL keyhole limpet hemocyanin (Sigma; used with the Incstar antibody). The αCDCP antibody was diluted 1:1000 in PBS containing 0.4% Triton X-100 and 3.3 mg/mL BSA.

Following incubations in the primary antibodies, the sections were washed twice in PBS for 15 minutes each, then incubated for 1 hour at room temperature in a goat anti-rabbit IgG secondary antibody conjugated to either flourescein isothiocyanate (FITC; Sigma) or tetramethylrhodamine isothiocyanate (TRITC; Sigma). The secondary antibodies were diluted 1:50 in PBS containing 3.3 mg/mL BSA and 0.4% Triton X-100. The sections were then washed three times for 15 minutes in 0.4% Triton X-100 in PBS, followed by three similar washes in PBS alone. All washing was performed with gentle agitation. The sections were mounted in glycerol containing DABCO (Slowfade Light; Molecular Probes), and coverslipped.

The specificity of the mGnRH staining was determined by deletion of the primary antibodies, preabsorption of the primary antibodies, and an examination of the cross-reactivity of these antibodies to salmon GnRH (sGnRH; Peninsula) and chicken GnRH-II (cGnRH-II; Peninsula). For preabsorption of the primary antibodies, 500 µM mGnRH (Peninsula) was incubated with antibody serum for 24 hours, with the normal serum control being treated the same. No staining was noted with the preabsorbed serum (Fig. 3.1). Using an immunoblot detection with avidin-biotin and a horseradish peroxidase label (described in Loi et al., 1997), no cross-reactivity was noted with either 1 mM synthetic chicken GnRH-II or salmon GnRH. Both antibodies strongly reacted to 1 mM synthetic mGnRH.

The specificity of the αCDCP antibody has previously been tested using both preabsorption with the antigen (Griffond et al., 1992) and a demonstration of antigen-specific detection in an immunoblot (van Minnen et al., 1992). As well, this antibody labelled cells in the expected locations of the cerebral ganglia in *H. trivolvis* (Fig. 3.13).

Staining in Wholemounts

Isolated circumesophageal ganglia from two snails were prepared for wholemount staining with the Incstar mGnRH antibody for comparison to results obtained in tissue sections. This proved useful in determining both the three-dimensional locations of some of the immunoreactive neurons, and the reasons for differences found between previously published staining results (Goldberg et al., 1993) and my current results.

The wholemount stains were prepared similarly to the tissue section stains, except that washing and incubation times were increased (modified from Goldberg and Kater, 1989). As well, each CNS was incubated in 0.5% pronase prior to fixation, and 0.06% trypsin post-fixation. Washing times were typically 1 hour long at most stages. Incubation in the primary antibody (diluted 1:250) was carried-out at 4°C for 72 hours. Incubation in the secondary antibody (conjugated to FITC) was performed as with the sectioned tissue. Following staining, the preparations were dehydrated in an ascending series of ethanol and mounted on microscope slides in methyl salicylate (Sigma).

Various stages of embryos (E25 - E70; representing 25% - 70% of embryonic development; McKenney and Goldberg, 1989) were stained by a similar protocol (Goldberg and Kater, 1989) with the exception that no enzyme treatment of the preparations was used.

The preparations were otherwise incubated in identical solutions for equivalent lengths of time, and mounted similarly to the circumesophageal ganglia in methyl salicylate.

Anterograde and Retrograde Neuronal Filling

Neurons were traced in isolated CNS preparations using Lucifer yellow (dilithium salt; Sigma) for both iontophoretic injections of single neurons and backfilling of nerves. Iontophoretic injections were performed using a standard sharp-electrode recording set-up (described in Goldberg et al., 1993). A Getting M5A preamplifier was used for voltage recordings, and virtual ground circuits (WPI) were used to measure the injected current. Data were recorded on a Gould DSO 1604 digital oscilloscope and a Gould 2400 chart recorder. Current pulses were applied using a Master-8 A.M.P.I. stimulator.

The CNS was removed and pinned-out in silicone rubber-coated (General Electric, RTV627) petri dishes containing *Helisoma* saline. Prior to injection, the ganglia were bathed in 0.5% pronase (Sigma) for 8 minutes to partially digest the sheath. The microelectrodes, filled with 3% Lucifer yellow, were impaled into single neurons for current injection. Then, a tonic hyperpolarizing pulse of 0.5 - 1.5 nA was given for 10 - 15 minutes to inject the dye into the cells. After iontophoresis, the preparations were kept in the dark for at least 30 minutes while the dye spread into the neurites, following which, they were fixed in 4% paraformaldehyde overnight at 4°C. After washing the fixative out in repeated PBS washes, the CNS was processed either as wholemounts by dehydrating the preparations and mounting them on microscope slides in methyl salicylate, or sectioned and stained for mGnRH (as above).

For nerve backfilling, the isolated CNS preparations were pinned-out as with the iontophoretic injections. The cut end of the target nerve was surrounded by a well of petroleum jelly, which was filled with 10% Lucifer yellow. The preparations were incubated at 4°C overnight, and then fixed in 4% paraformaldehyde at 4°C overnight. Following fixation, the brains were sectioned and stained as described above, or processed as wholemounts.

All viewing and photography of stained tissue sections and wholemounts preparations was performed using a Nikon Diaphot TMD inverted photomicroscope equipped with epiflourescence illumination. Sections that were stained for GnRH using a TRITC-conjugated secondary antibody and labelled with Lucifer yellow backfills or injections could be viewed using a flourescein filter (excitation wavelengths from 450 - 490 nm; emission wavelengths from 510 - 520 nm) for the Lucifer yellow, and a rhodamine filter (excitation wavelengths from 510 - 560 nm; emission wavelengths from 580 - 590 nm) for the GnRH. A laser-scanning confocal microscope (multiprobe 200; Molecular Dynamics) interfaced with a computer workstation (Indigo; Silicon Graphics) was used to generate confocal reconstructions in some cases.

Electrical Recording from Caudodorsal Cells

The CDCs were tested for responsiveness to synthetic mGnRH using intracellular recording. A recording set-up similar to that used for the iontophoretic injections (described above) was used, with the exception that the microelectrodes were filled with 2.5 M KCl. The cells were first identified by iontophoretic injections of Lucifer yellow and by immunostaining in several preparations. Only a brief description of these cells has been previously given for *Helisoma* (Mukai and Saleuddin, 1988; Khan et al., 1989), making this further characterization of the CDCs in *Helisoma trivovlis* necessary. In the live preparation, the cells could be identified by their greyish colour and locations adjacent to the cerebral commisure on the ventral surface (Fig. 1.1). This allowed for repeated identification and recording from the *H. trivolvis* CDCs.

In testing for mGnRH responsiveness, synthetic mGnRH (1 - 10 μM) was superfused over the pinned-out CNS. A constant superfusion of *Helisoma* saline was used prior to, and after using the mGnRH. The mGnRH was typically applied for 1 - 10 minutes. When measuring input resistance (R_{input}), constant hyperpolarizing pulses were given prior to, during, and after application of the mGnRH to observe if changes occurred in the voltage response. In some other recordings testing only for changes in resting membrane level and/or spontaneous activity, no pulses were given.

Egg-Laying Response to mGnRH Injections

To assess *in vivo* whether mGnRH could affect reproduction, snails were monitored for their egg-laying ability following injections of mGnRH. The snails were anaesthetized in mentholsaturated artificial pond water (APW; 0.025% Instant Ocean; Aquarium Systems) prior to being injected. An AGLA micrometer syringe was used for injections through the base of the foot. The injections were done in three groups for each trial: 1) 5 µL of 100 µM mGnRH; 2) 5 µL control injections of *Helisoma* saline; 3) one of either undiluted mGnRH antibody (Sigma), 1 mM GnRH analogue ([D-pGlu^1, D-Phe^2, D-Trp^{3,6}]-mGnRH; Peninsula), or 100 µM mGnRH + 1 mM GnRH analogue (see Figs. 3.15 and 3.16). The 100 µM mGnRH concentration had previously been found to be the most effective in eliciting a putative increase in egg-laying (Zalitach, Chang, and Goldberg, unpublished results). Snails were left to recover overnight in 100 mL beakers filled with APW, and kept individually. During the next 10 days, snails were fed lettuce daily and about half of the APW was changed daily. A 12:12 (hours) light:dark cycle was used, and water temperature was kept at room temperature.

Each day, egg masses were collected, and the number of embryos within each was counted. In some experiments, the egg-mass production was measured for 10 days prior to injections, while the animals were maintained in the beakers. The animals were then divided into the different treatment groups so as to have animals of equal egg-laying ability in each of the groups.

Results of the egg-laying were compared between mGnRH-injected and the control groups using a Kruskal-Wallis test, or a Mann-Whitney U test for comparing two groups alone ($p \leq 0.05$ for significance).

Chapter 3 - Results

Distribution and Ontogeny of GnRH-Immunoreactivity in *Helisoma trivolvis*

Distribution

Immunohistochemical staining for GnRH in adults was performed with CNS and reproductive tract tissue, and in one case representative sections were taken from an entire animal following removal of its shell. Specific staining was only identified in neuronal tissues (Fig. 3.1). This included cell bodies and neurites in all of the ganglia (Figs. 3.2 - 3.4), and within some of the peripherally-extending nerves (Figs. 3.2, 3.4, and 3.5). As well, GnRH-immunoreactive neurites were identified in sections taken from the penial complex (Fig. 3.5).

In the CNS, GnRH-IR somata generally occurred in either small clusters or individually. The soma sizes in the adults ranged from around 20 µm to around 45 µm in diameter, with the larger somata being in the more posterior visceral and parietal ganglia, and the smallest ones in the cerebrals. The ganglia with the largest number

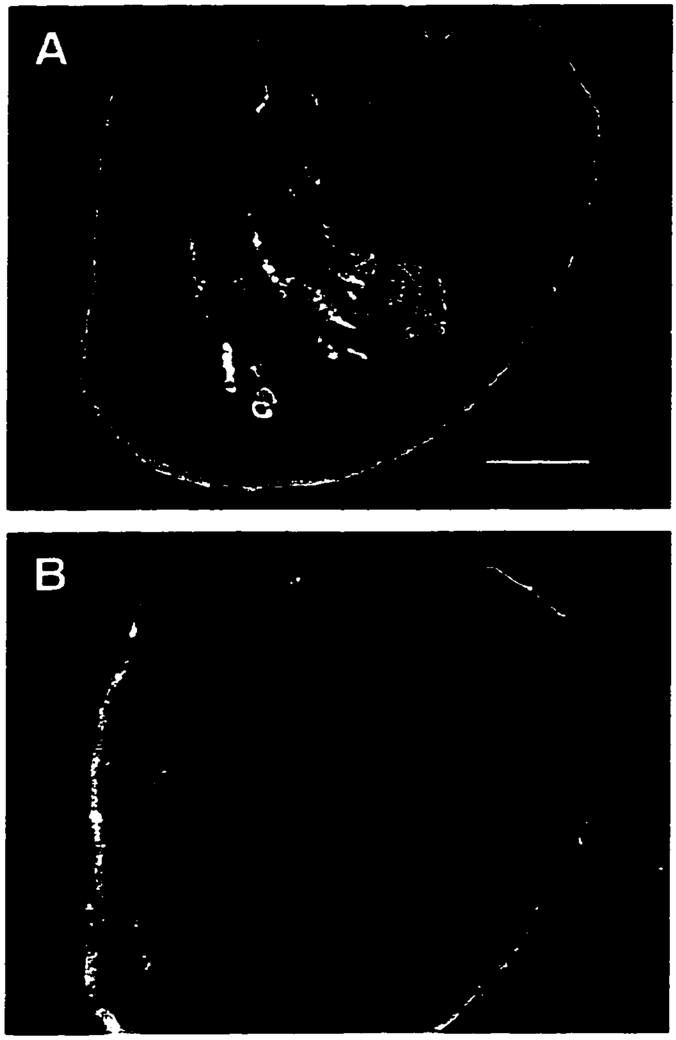


Fig. 3.1. Specificity of mGnRH antibody staining. A) A cerebral ganglion section stained with mGnRH antiserum (Incstar). B) An adjacent section stained with preabsorbed serum. Preabsorption with synthetic mGnRH eliminated all staining. The diffuse fluorescence around the edge of the tissue sections is an artifact of the staining process. Scale bar = 100 µm in A.

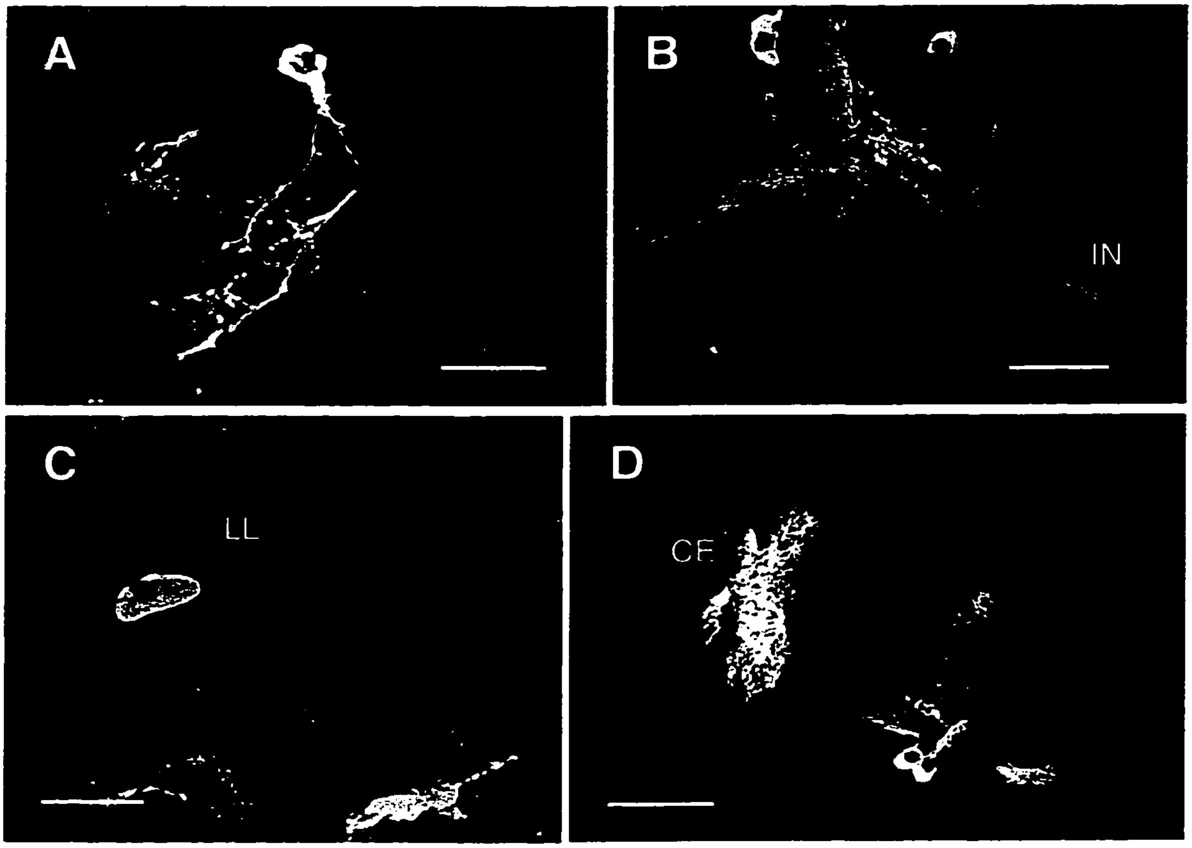


Fig. 3.2. Localization of GnRH-immunoreactivity in the CNS of *Helisoma trivolvis*. A) A cerebral neuron showing clear staining throughout the perikaryon and neurites. B) Immunoreactive somata and neurites in the visceral ganglion and intestinal nerve (IN). C) A single neuron stained within a lateral lobe (LL). D) Immunoreactive somata adjacent to, and extensive fibre-like staining within the left cerebral extension (CE). Bar = 50 µm in A and C; 100 µm in B and D.

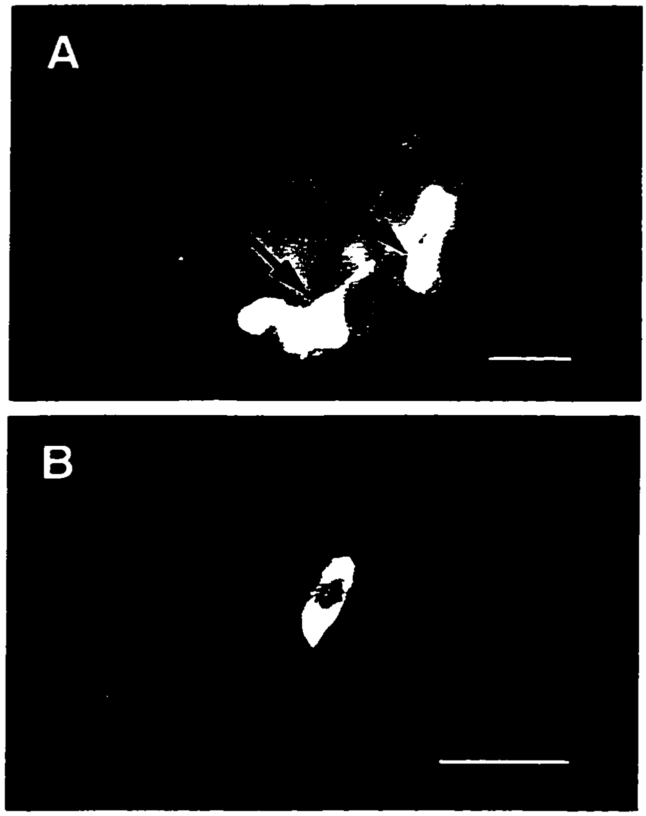


Figure 3.3. Staining for GnRH in wholemounts of the visceral (A; arrows) and cerebral (B) ganglia. Fewer immunoreactive neurons were observed in wholemounts than in sectioned tissue, however those that were observed were consistent in locations with those seen in sections. Bar = 50 µm in A and B.

Figure 3.4. Distribution of GnRH-immunoreactivity in the CNS of *Helisoma trivolvis*. Labelled cell bodies and neurites were distributed throughout all of the ganglia of the CNS, and immunoreactive neurites occurred in three nerves, including the penial nerve (PN) and the intestinal nerve (IN). In clusters with greater than three cells showing, the actual number may be underrepresented for the purpose of clarity. Paired dashed lines represent the area taken up by GnRH-IR neurite pathways. In the cerebral ganglia, the thicker dashed lines indicate ventral projections, thinner ones being dorsal. The unfilled somata represent the cluster targeted for iontophoretic injections (see Fig. 3.10).

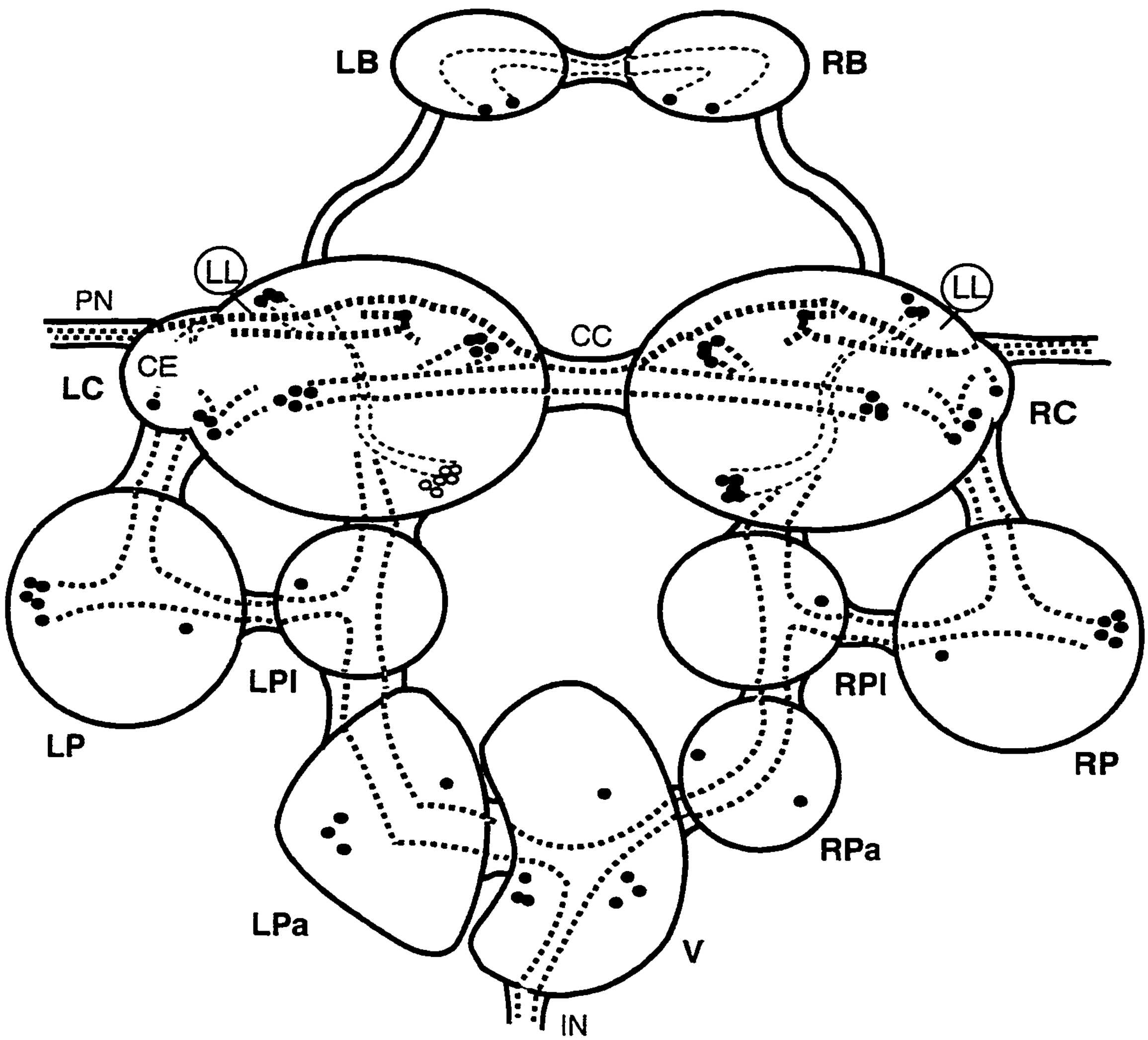

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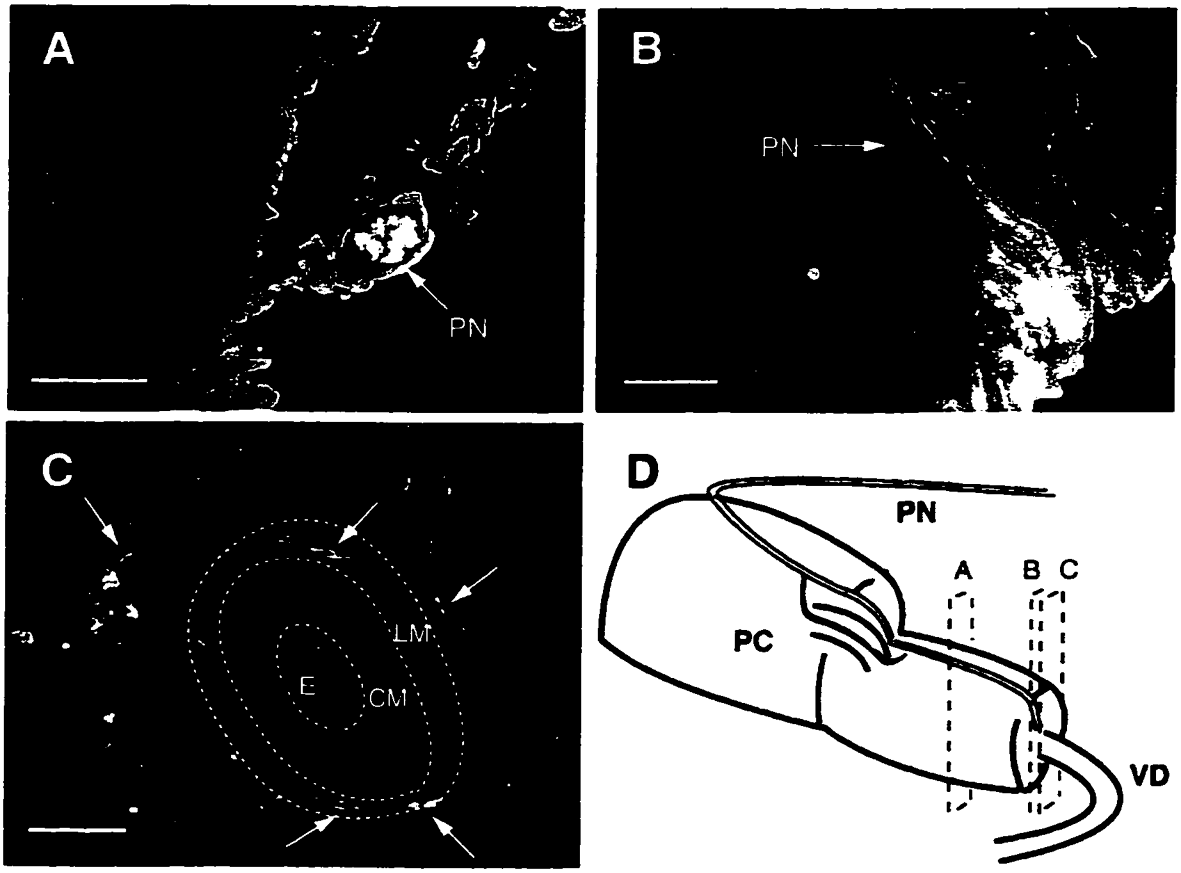
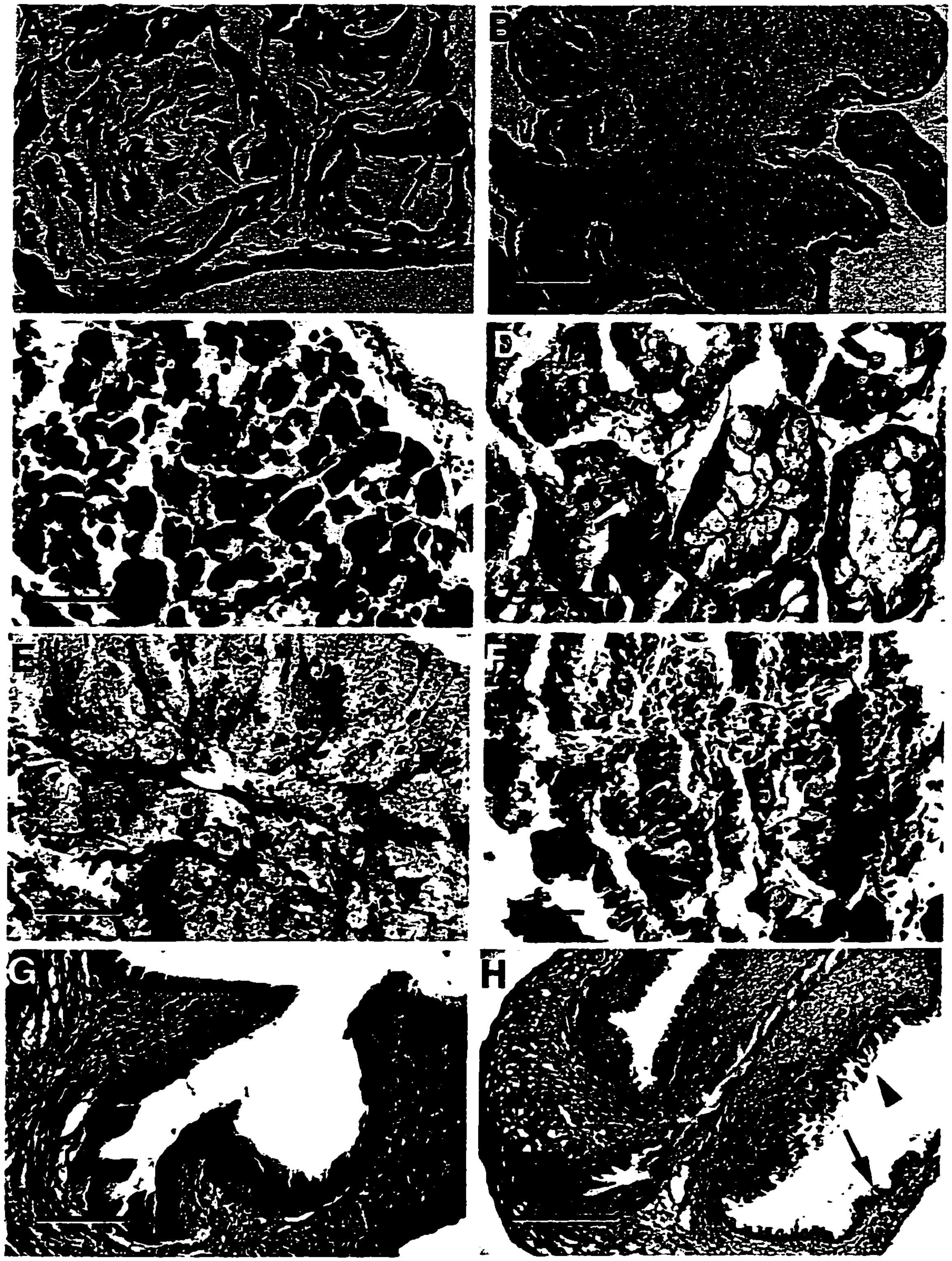
Figure 3.5. Innervation of the penial complex by GnRH-IR fibres. A) A cross-section of the penial nerve (PN) as it runs adjacent to the penial complex. Much of the nerve is labelled by the mGnRH antibody. B) The penial nerve as it penetrates into the muscle tissue near the vas deferens. C) Immunoreactive fibres (arrows) surrounding and within the outer muscle layer of the vas deferens. The epithelial layer (E), circular muscle (CM), and longitudinal muscle (LM) of the vas deferens are outlined by dotted lines. D) A schematic indicating the relative positions of the sections used in A, B, and C. Bar = 50 µm in A, B, and C.


A
PN
B
PN
C
LM
E
CM
D
PN
A
B C
PC
VD

of GnRH-IR neurons were the cerebrals (Fig. 3.4). Immunoreactive neurites ran through the neuropil area of every ganglion, and were seen to extend peripherally from the left cerebral ganglion through the penial nerve, and from the visceral ganglion through the intestinal nerve. As well, a few immunoreactive fibres were observed in the nerve opposite to the penial nerve, extending from the right cerebral ganglion into the body wall. Of these peripheral nerves, labelled neurites were most prominent in the penial nerve. In the penial complex, the GnRH-IR neuronal fibres were found in the muscle of the preputium, and innervating the vas deferens at its point of entry into the penial complex (Fig. 3.5). Most of the GnRH-IR fibres appeared to terminate in the outer muscle layer of the vas deferens.

Other tissues examined for GnRH-immunoreactivity included the ovotestis, seminal vesicles, hermaphroditic duct, seminal receptacle sac, and all of the glands and muscle tissue of the female and male reproductive tracts (Fig. 3.6). As well, salivary gland, buccal mass, hepatopancreas, pedal muscle, integument, mantle, and tentacles were all easily identified in the sectioned whole animal. No specific staining was observed in any of these, as judged by

Figure 3.6 Tissues of the reproductive tract of *Helisoma trivolvis*. Shown are haematoxylin and eosin-stained sections from various reproductive organs. A) The ovotestis, showing sperm and oocytes being produced in acinar structures. The large arrowhead indicates the heads of some mature sperm; the small arrowhead indicates their tails; arrow indicates a mature ovum. B) The seminal vesicles, containing a dense amount of stored sperm. C) The albumen gland, showing secretory cells which produce the perivitelline fluid during egg mass production. D) The prostate gland, showing secretory cells which produce the seminal fluid during the transfer of sperm. E and F) Sections through the oothecal (E) and muciparous (F) glands. These contain secretory cells involved in producing materials for the egg mass structure as it is passed through the female reproductive tract. G) The vagina, lined with ciliated epithelial cells used for the movement of gametes and egg masses. H) The penis (arrowhead) and preputium (arrow). The preputium is a muscular sheath which encloses the penis, and is everted with it during copulation. Aside from neurites in the penial complex, no GnRH-immunoreactivity was noted in these tissues. Bar = 50 μm for A, C, D, E, F; 100 μm for B and G; 600 μm for H.


A
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comparison to control staining with preabsorbed antibody. Simultaneously stained CNS preparations consistently contained staining.

When comparisons of the staining in the cerebral ganglia were made between juvenile *H. trivolvis* (postembryonic stage 3 (P3); = 3 mm shell height), and age-matched adult virgins, newly-mated, and randomly mating adults, no obvious differences in soma numbers, or staining intensities were noted. The cross-sectional area of the juvenile and virgin GnRH-IR somata was smaller on average, though this was not compared in detail.

Embryonic Expression

Expression of GnRH immunoreactivity in *H. trivolvis* embryos was observed to develop by at least embryonic stage E70 (Fig. 3.7). No immunoreactivity was noted in younger embryos (stages E25 - E50). In wholemount preparations, staining was observed in the head region, just below the tentacles. In tissue sections, most of the GnRH-IR cells were localized within at least two different clusters of 5 - 10 cells each. Cerebral ganglia from the CNS are known to begin development as early as stage E20 within the area where

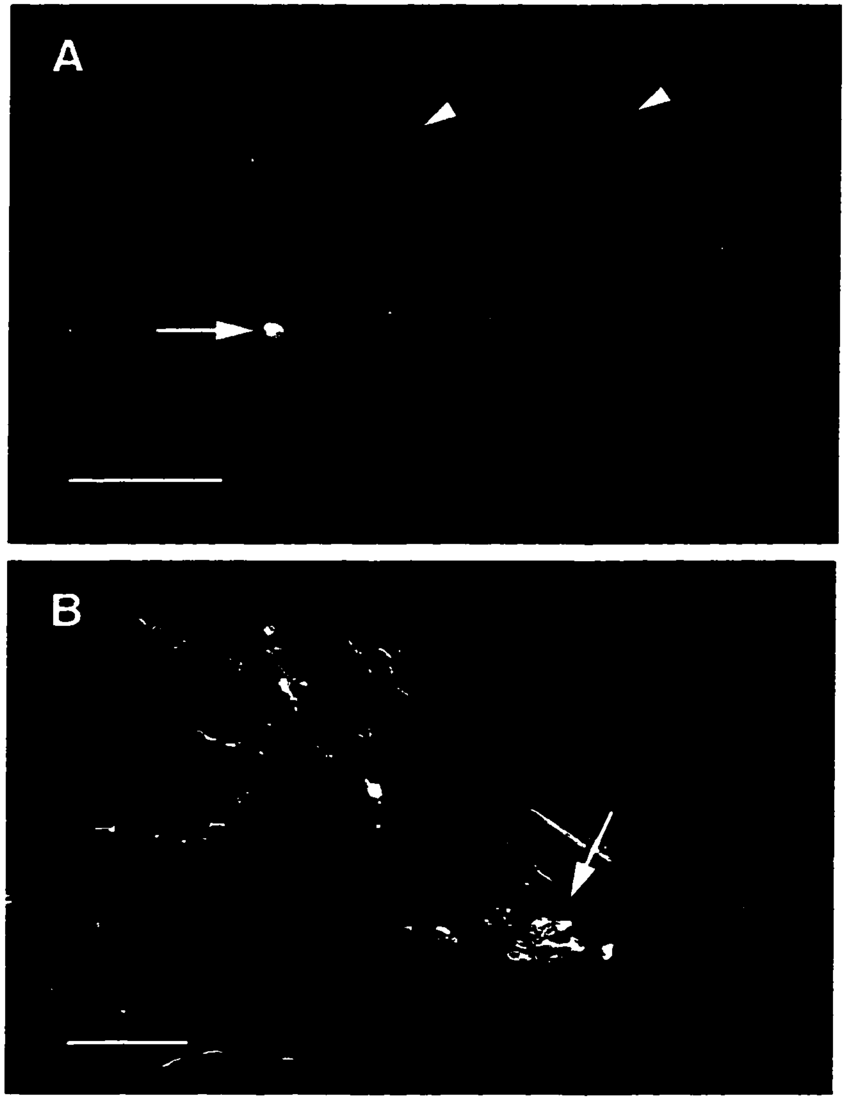


Figure 3.7. Embryonic expression of GnRH-immunoreactivity. A) A wholemount preparation of an E70 embryo showing immunoreactive cells in the head region (arrow). The tentacles are indicated with arrowheads. B) In section, clusters of immunoreactive neurons were present. The exact position of these was not determined.

these cells are located (Goldberg, 1995).

As noted above, GnRH-IR neurons in the cerebral ganglia of juvenile (P3) *H. trivolvis* are already distributed in a pattern similar to that of sexually mature adults. Thus, it appears that the pattern of GnRH-IR expression is established early in the development of the snail.

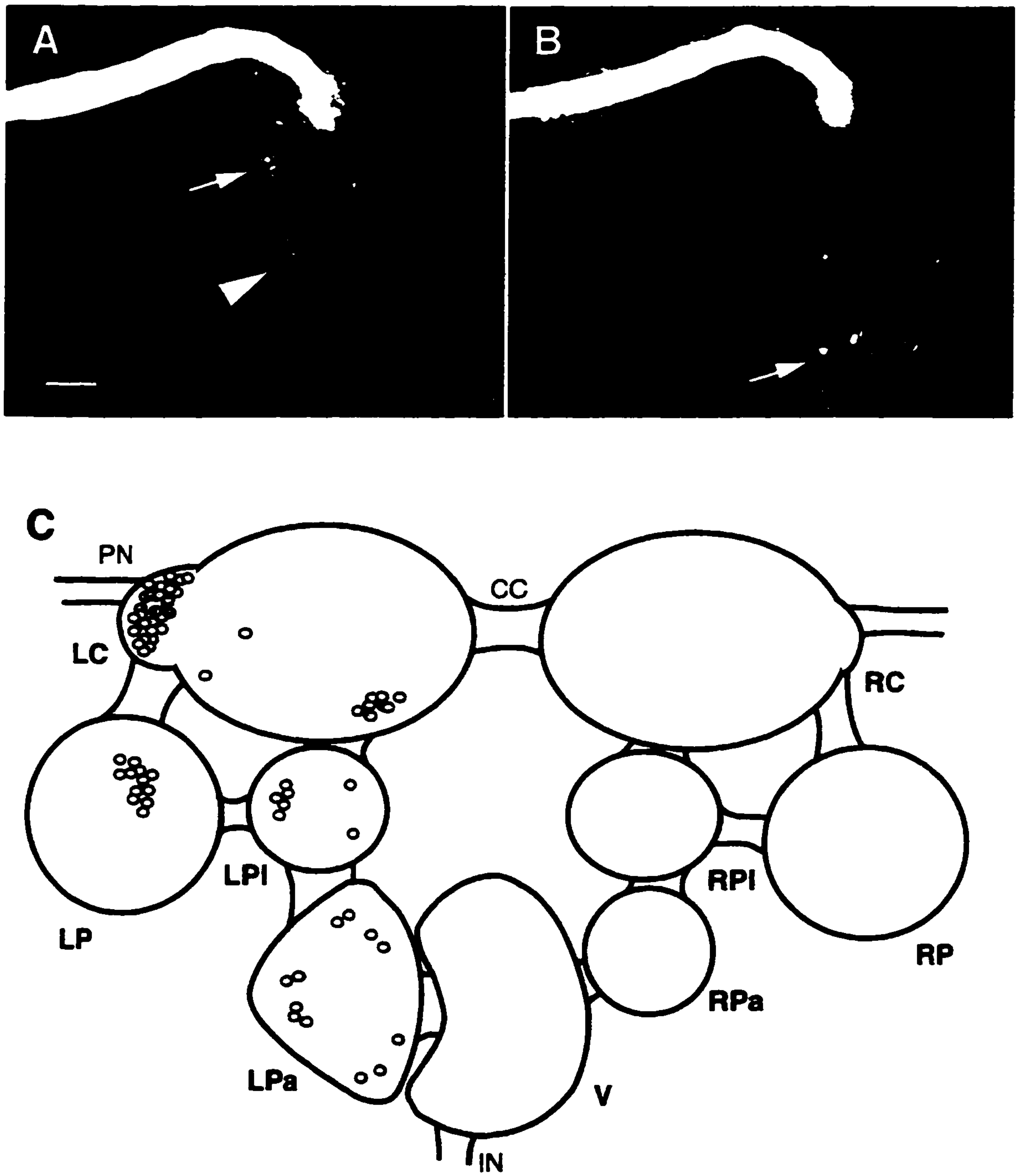
Tracing of GnRH-IR Neurons

In order to help define the projection patterns of the GnRH-IR neurons, a tracing of neurons which sent axons into the penial nerve was performed. As it was impossible to trace the complete pathways of any individual GnRH-IR neurons using immunostaining alone, an attempt to trace at least some of them through both retrograde and iontophoretic loading of the fluorescent dye, Lucifer yellow, was made. Using retrograde filling from the penial nerve, many somata were stained, though the projection of individual neurites was still difficult to follow in entirety. Iontophoretic injections, performed on neurons from the GnRH-IR cluster indicated in Figure 3.4, allowed for a more complete tracing of a few GnRH-IR cells.

The neurons that were Lucifer yellow-positive in penial nerve backfills were found in the left cerebral, left pleural, left pedal, and left parietal ganglia (Fig. 3.8). Some Lucifer yellow-positive cell bodies in the left cerebral, pleural, and parietal ganglia, were observed to be double-labelled when stained for GnRH (Fig. 3.9). As well, much of the neuropil area in the cerebral extension of the left cerebral ganglion, which contained most of the backfilled neurons, densely stained for GnRH (Fig. 3.9).

For the tracing of an individual GnRH-IR neuron, a cluster of small neurons occurring in the posterior, medial area of the left cerebral ganglia was examined. These neurons were double-labelled in the experiments described above (Fig. 3.9). While the easily identifiable position due to nearby landmarks made these the most suited to electrode penetration, neither these, nor any of the other GnRH-IR neurons were easily visible within the live CNS preparation. This is because they are generally located below the surface of the ganglion, with other cells completely or partially covering them. In three separate preparations, a single neuron from this cluster

Figure 3.8. Neurons backfilled with Lucifer yellow through the penial nerve (PN). A) A wholemount preparation focussed on Lucifer yellow-filled neurons in the cerebral extension (arrow) and the left pedal ganglion (arrowhead). B) The same preparation, focussed on neurons in the left parietal ganglion (arrow). C) A schematic of the locations of the backfilled neuronal cell bodies. This pattern of backfilling is similar to that established for *L. stagnalis* (de Boer et al., 1996; de Lange et al., 1998a). Bar = 100 μm in A.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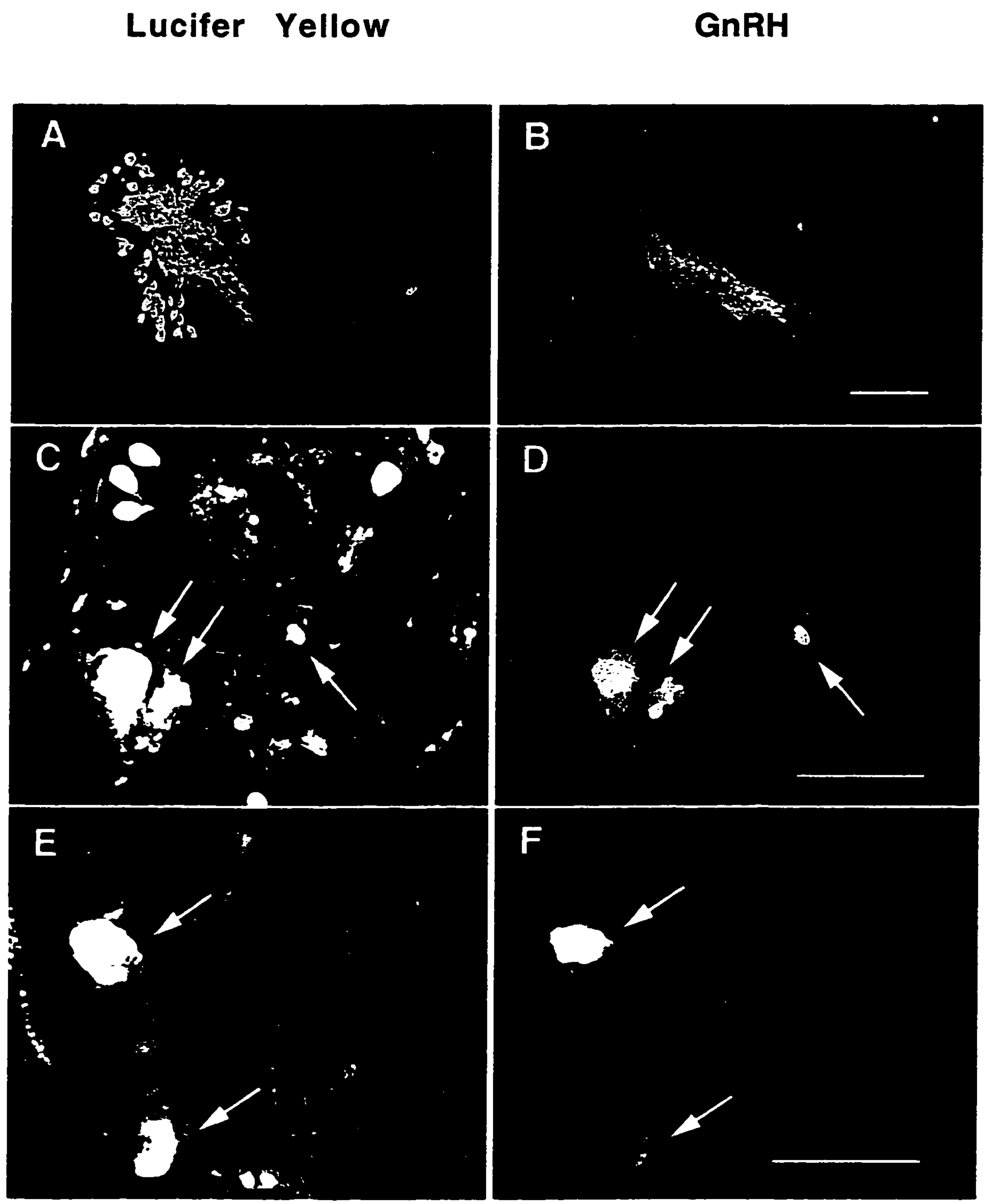
IN

Fig. 3.9. Sections double-labelled for penial nerve Lucifer yellow backfilling and GnRH-immunoreactivity. The left panels (A, C, E) show Lucifer yellow fluorescence and the right panels (B, D, F) show GnRH-immunoreactivity in the same corresponding sections. A) The largest cluster of backfilled neurons occurred in the left cerebral extension, through which the penial nerve exits the left cerebral ganglion (Fig. 3.8). B) This lobe consistently contained a dense amount of GnRH staining in the neuropil area, in what appeared to be a network of fibres (see also Fig. 2). C and D) Backfilled neurons in the left pleural (top) and parietal (bottom) ganglia, several of which were GnRH-IR (arrows in C and D). E and F) Backfilled neurons in the left parietal ganglion; the two neurons indicated are double-labelled for GnRH and Lucifer yellow (arrows in E and F). Bar = 100 μm in B, D, and F.


Lucifer Yellow
GnRH
A
B
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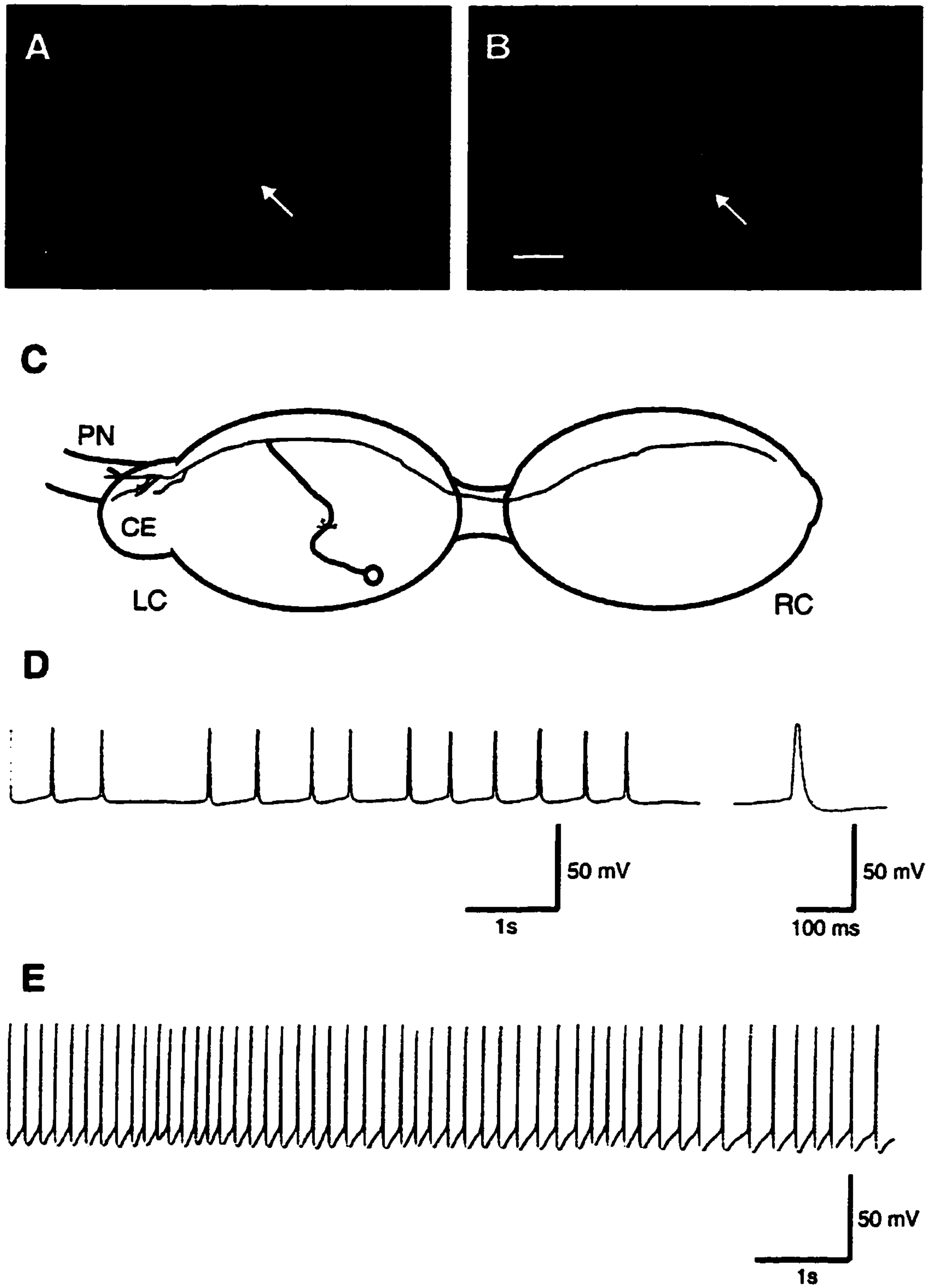
was filled by iontophoretic injections of Lucifer yellow dye, with two being used for positive identification with GnRH staining in tissue sections and the third being viewed in wholemount. The primary neurites of these projected anteriorly through the cerebral ganglia, then laterally, exiting through the penial nerve on the left side, and having fibres end in the anterior part of the cerebral extensions on both sides (Fig. 3.10). This coincides well with the pattern seen from the antibody stains, alone.

Electrically, the GnRH-IR neurons that were traced fired broader spontaneous action potentials than the surrounding neurons. As well, the frequency of firing of the three identified neurons from the GnRH-IR cluster was typically slower than the surrounding neurons, being around 2 - 4 Hz, with the surrounding ones firing around or over 4 Hz ($n > 10$). Similar electrical profiles were obtained from several additional recordings in the GnRH-IR cluster location (AP frequency = 2.6 ± 0.57 Hz, $n = 8$). Neurons that were observed to have spontaneous activity differing from the GnRH-IR cluster all had clearly different neurite projections compared to those within the GnRH-IR cluster (Fig. 3.11).

Figure 3.10. Iontophoretic tracing of a single GnRH-IR neuron. A) A cluster of GnRH-IR neurons which occurred in the mediodorsal area of the left cerebral ganglia (see Fig. 3.4). The exact position of these neurons could be identified in sectioned tissue based on nearby landmarks. Though they are positioned underneath a layer of more visible neurons, it was possible to place an electrode into these cells on several attempts. B) The Lucifer yellow fill of the single GnRH-IR neuron which was traced in the preparation (arrow in A and B). C) A schematic based on two tracings performed in serial sections on GnRH-IR neurons, and one neuron from this cluster which was viewed in wholemount (see Fig. 3.11). D) The intracellular recording from the neuron shown in A and B. An expanded trace is shown to the right. The cell fired relatively broad (30 ms at half the AP amplitude), spontaneous action potentials. E) A typical recording from neurons adjacent to the GnRH-IR cell cluster. The spontaneous activity was clearly different, with higher frequency, shorter duration action potential firing. Bar = 25 μm in B.


A
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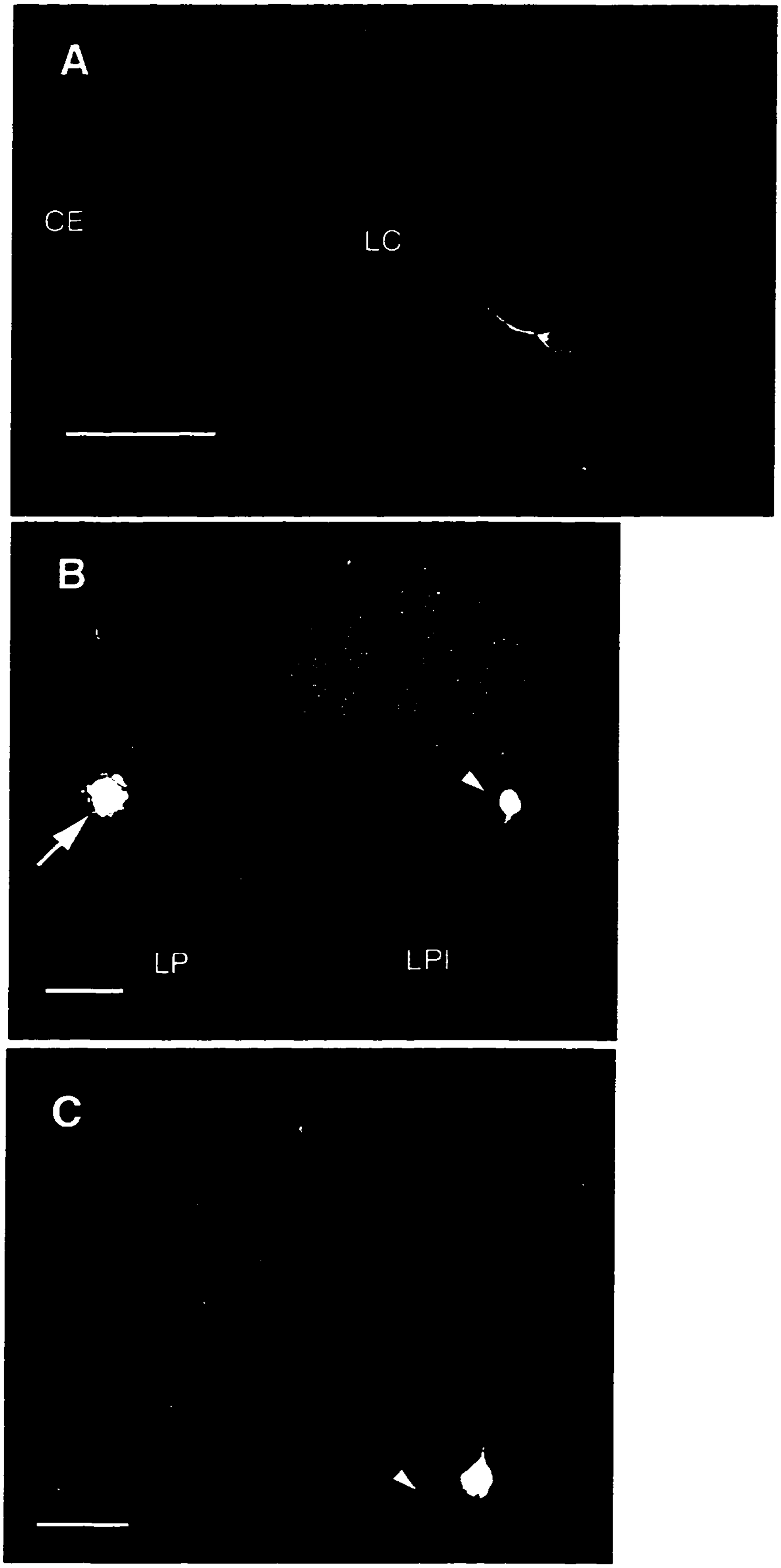
RC
D
50 mV
1s
50 mV
100 ms
E
50 mV
1s

Figure 3.11. Lucifer yellow fills of cells adjacent to the mediodorsal cerebral GnRH-IR neuron cluster. A) A confocal reconstruction of a Lucifer yellow-filled cell body and primary neurite of a neuron from the cluster of GnRH-IR neurons located in the dorsal, medial area of the left cerebral ganglion. The smaller lateral neurites and secondary neurites in the neuropil were not visible at this magnification. B) Immediately adjacent to the GnRH-IR cluster (indicated by the arrowhead), this neuron projected through the cerebral-pleural connective, into the pleural-pedal connective, then back into the left cerebral ganglion through the pedal-cerebral connective. The neuron extended secondary neurite branches into the pedal ganglion, and continued into the cerebral extension where it dye-coupled with a large cell (arrow). C) Further to the right are the mediodorsal cells (GnRH-IR cell cluster location is indicated with the arrowhead). The mediodorsal cells project into the median lip nerve, where they release molluscan insulin-like peptide into a neurohemal area in the sheath of that nerve (Khan and Saleuddin, 1992). Bar = 100 µm in A, B, and C.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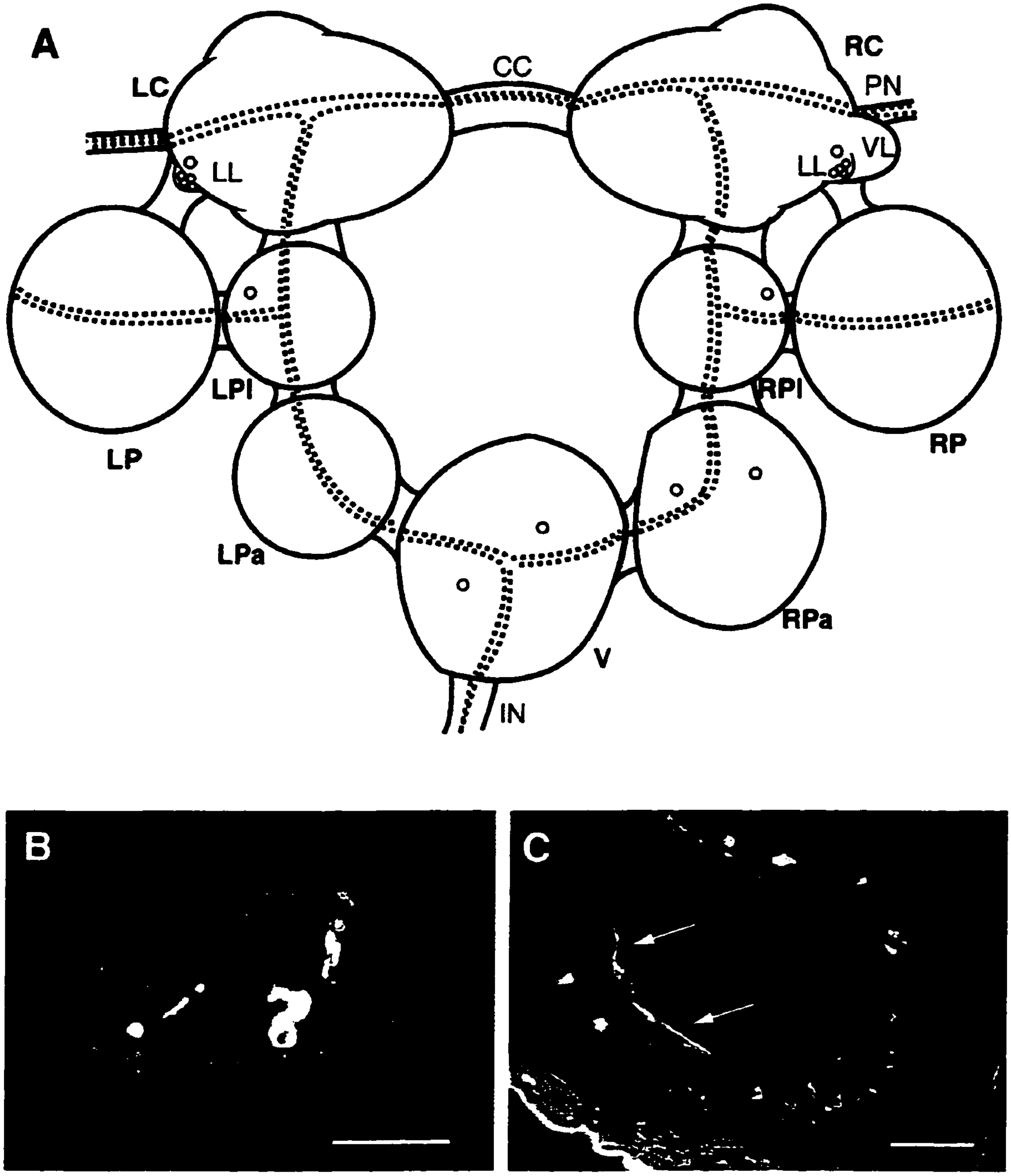
Distribution of GnRH-immunoreactivity in *Lymnaea stagnalis*

I sectioned and stained the CNS and penial complexes from five *Lymnaea stagnalis* snails for comparison to the results obtained from *H. trivolvis*. In *L. stagnalis*, the distribution of immunoreactivity was far less widespread, with only 14 - 16 neurons in the entire CNS staining for GnRH (Fig. 3.12). However, the neurons which were immunoreactive were found in locations consistent with those of the *H. trivolvis* CNS. As well, in *L. stagnalis*, immunoreactive fibres were seen in the corresponding nerves to those containing them in *H. trivolvis*. With the number of immunoreactive neurons being fewer in *L. stagnalis*, the amount of fibre staining in these nerves, and in the neuropil regions of each ganglion, was also much less. Staining in the buccal ganglia was completely absent. The regions with the greatest number of GnRH-IR cell bodies in *L. stagnalis* were the lateral lobe regions (Fig 3.12).

Within the penial complex, only a few GnRH-IR fibres were observed. However, these were located in positions consistent with what was observed for *H. trivolvis*. A few fibres projected into the preputial muscle, with the remaining ones innervating the muscle of

Figure 3.12. The distribution of GnRH-immunoreactivity in *Lymnaea stagnalis*. A) A schematic of the distribution within the CNS. Much fewer cell bodies stained as compared to the CNS of *H. trivolvis*. The narrower representation of the fibre tracts (as compared to Fig. 3.4) indicates the lower number of GnRH-IR fibres in *L. stagnalis*, occupying narrower bands. B) The left lateral lobe of *L. stagnalis*, showing GnRH staining in cell bodies and fibres. Both left and right lobes stained similarly. C) Immunoreactive fibres (arrows) in the outer muscle layer of the *L. stagnalis* vas deferens. The stained soma-like structures outside of the vas deferens were non-specifically labelled, as judged by their appearance in preabsorbed antiserum control stains. Bar = 50 μm in B and C.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the vas deferens (Fig. 3.12).

Possible Neuronal Targets for GnRH in the Central Nervous System of *Helisoma trivolvis*

To examine further the role that the endogenous GnRH-like peptide may have on reproductive control, I tested the responsiveness of the caudodorsal cells, the key regulators of snail egg-laying, to mGnRH. As the CDCs were identifiable in isolated CNS preparations (Fig. 3.13), I chose to use intracellular electrical recordings to investigate whether mGnRH could affect the electrical activity of these neurons.

The CDCs recorded from were normally silent, but fired spontaneous action potentials in a few (approximately 20%) of the CNS preparations (Fig. 3.13). This is consistent with results from *L. stagnalis* (Kits, 1980; Vlieger et al, 1980), with the exception that *H. trivolvis* CDCs did not produce afterdischarge firing following repeated depolarizing stimuli.

In each cerebral ganglion, a cluster of GnRH-IR neurons is located close to the CDCs, and GnRH-IR fibres run through the

Figure 3.13. Identification of the caudodorsal cells in the cerebral ganglia. A) A confocal reconstruction of a caudodorsal cell filled with Lucifer yellow. The axon bifurcates, and sends two branches into the cerebral commissure (CC) which end in the neurohemal area of the commissure. DB = dorsal bodies, LC = left cerebral ganglion, RC = right cerebral ganglion. B) αCDCP-immunoreactivity in the cerebral ganglia. A cluster of stained cells (broad arrows) was observed on either side of the cerebral commissure. A few αCDCP-IR neurons could also be identified lateral to the main cluster (small arrow). The neurohemal area in the commissure stained intensely. C) CDC neurons were typically silent, and showed rapid accommodation with a depolarizing stimulus, firing only one, or a few action potentials (APs). D) In some preparations, the CDCs fired spontaneous APs. Bar = 100 μm in A and B.

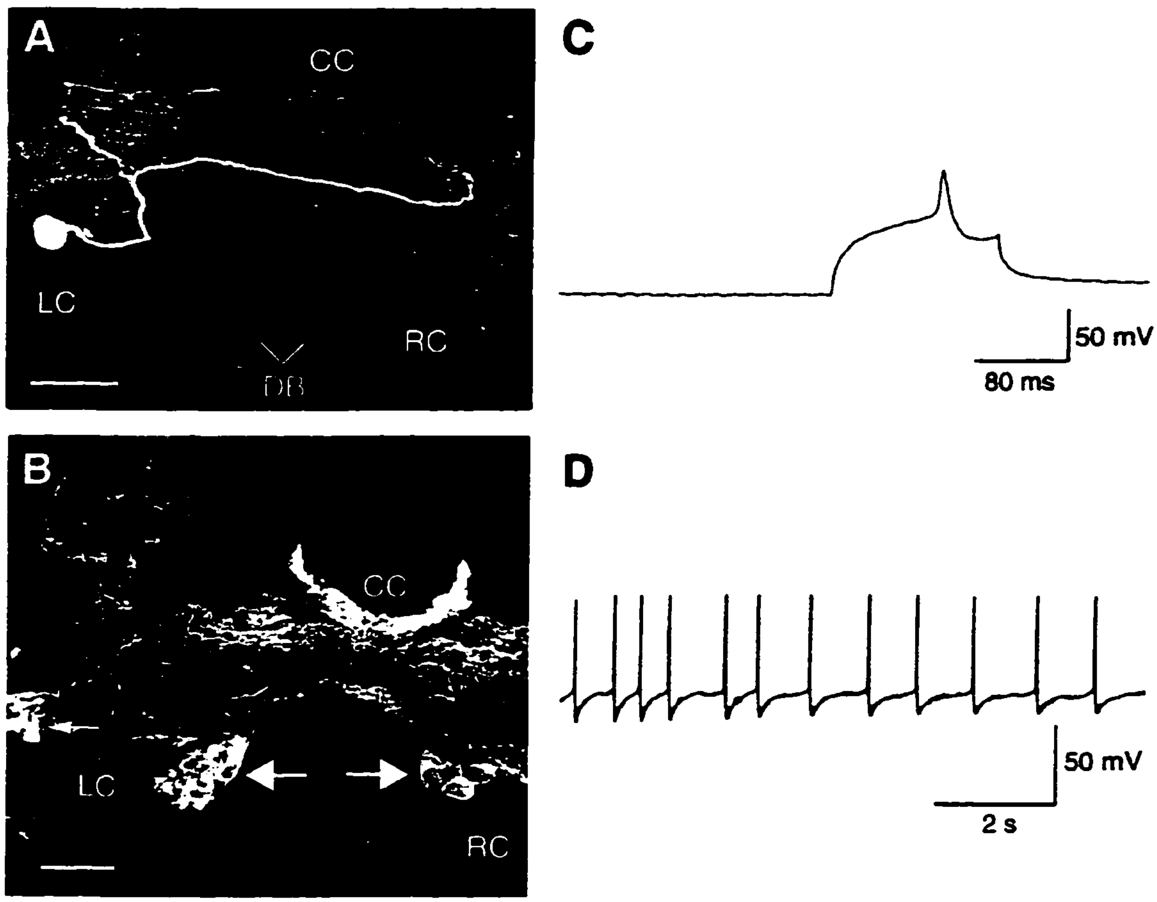
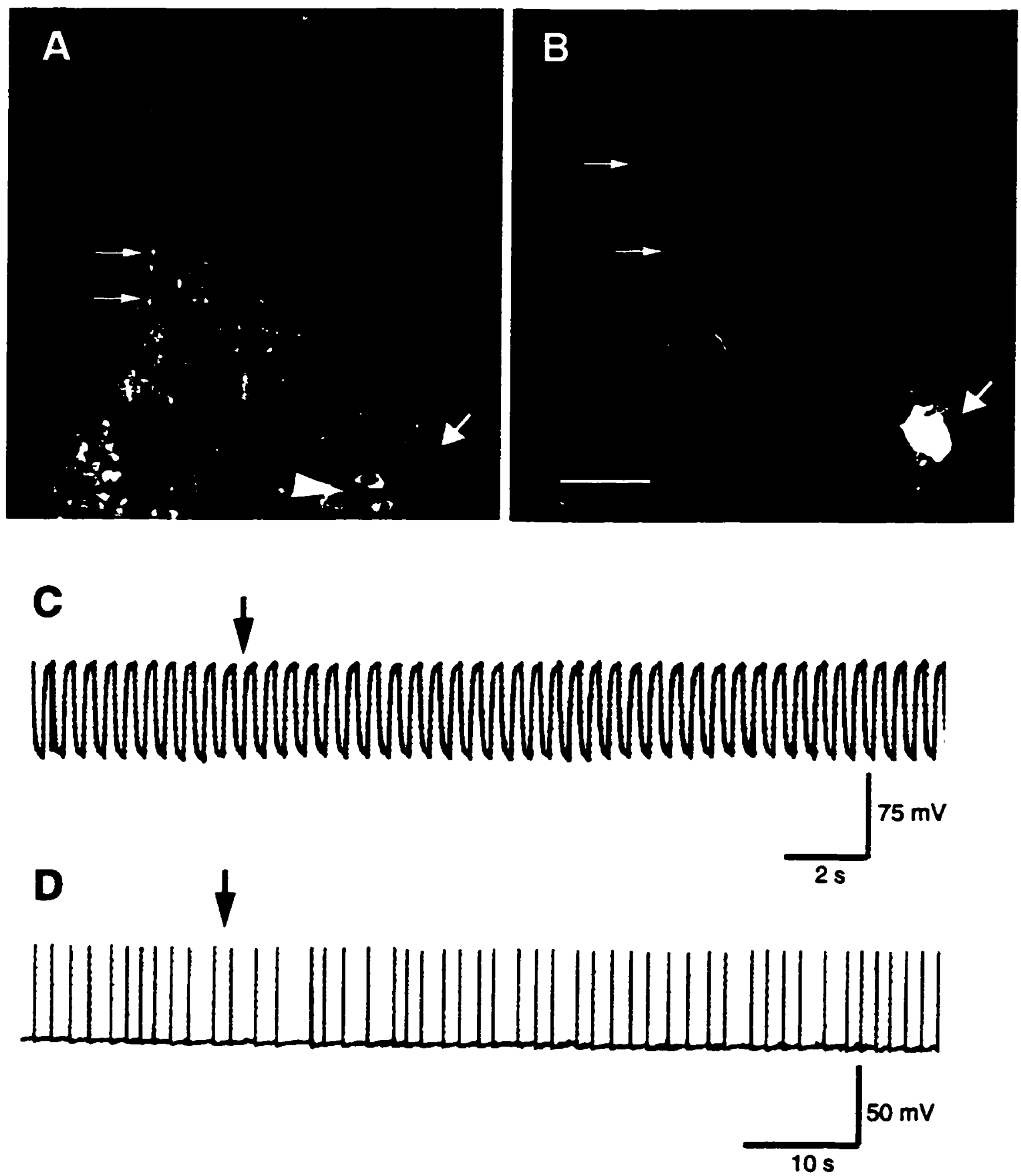

A
CC
LC
RC
DB
B
CC
LC
RC
C
50 mV
80 ms
D
50 mV
2 s

Figure 3.14. Effect of mGnRH on caudodorsal cell electrical activity. A) Two GnRH-IR neurons (arrowhead) are shown next to a CDC neuron (position indicated by the broad arrow). The Lucifer yellow filled CDC neuron is shown in the same section in B. GnRH-IR neurites could also be observed in the same fibre tract as the CDC neurites (thin arrows in A and B). C) The input resistance of CDC neurons did not change in response to mGnRH (arrow; 10 μM; n = 2). In order to measure R_{input}, hyperpolarizing pulses of 0.5 nA were continuously applied, and the resulting voltage deflections were measured. The mGnRH was applied for 1 minute prior to a washout with saline in the trial shown. D) In spontaneously active CDCs, action potential activity was not altered by mGnRH (arrow; 10 μM; n = 2). The mGnRH was applied for 8 minutes prior to a washout with saline in the trial shown. Bar = 100 μm in B.


A
B
C
75 mV
2 s
D
50 mV
10 s

cerebral commisure adjacent to the CDC axons (Fig. 3.14). This indicated the possibility that GnRH neurons can interact with the CDC neurons. To test whether mGnRH could alter the normal electrical properties of the CDCs, I measured its effects on input resistance, membrane potential, and spontaneous activity. Synthetic mGnRH (1 - 10 μM) had no effect on these electrical parameters in the CDCs of *H. trivolvis* (Fig. 3.14).

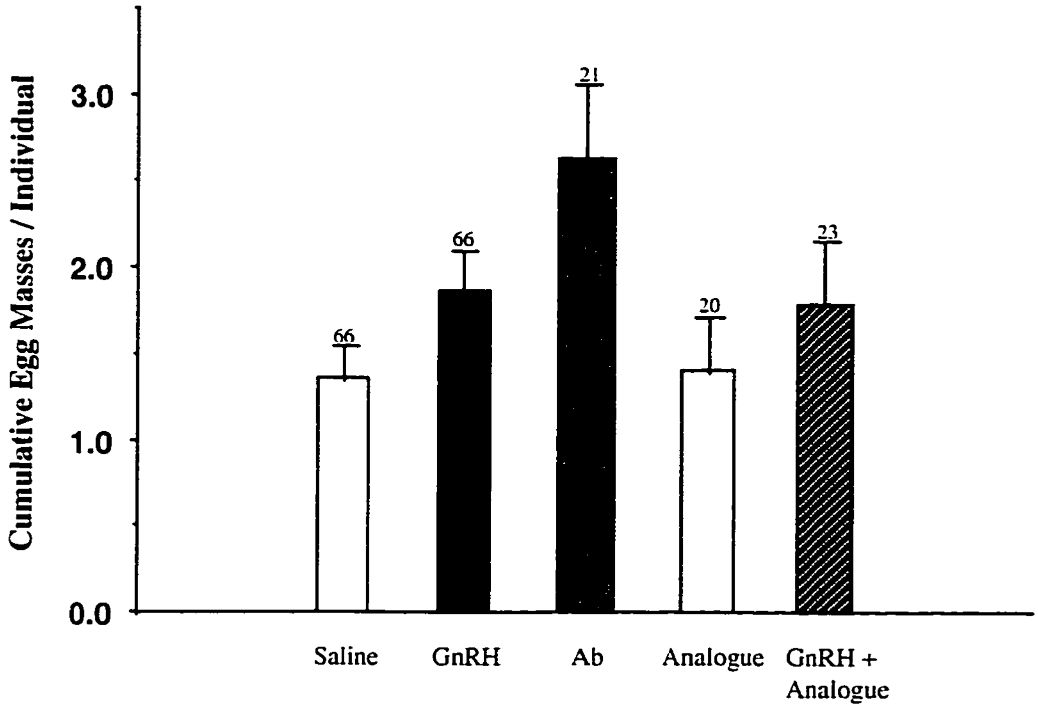
<u>Effects of mGnRH Injections on Egg-laying</u>

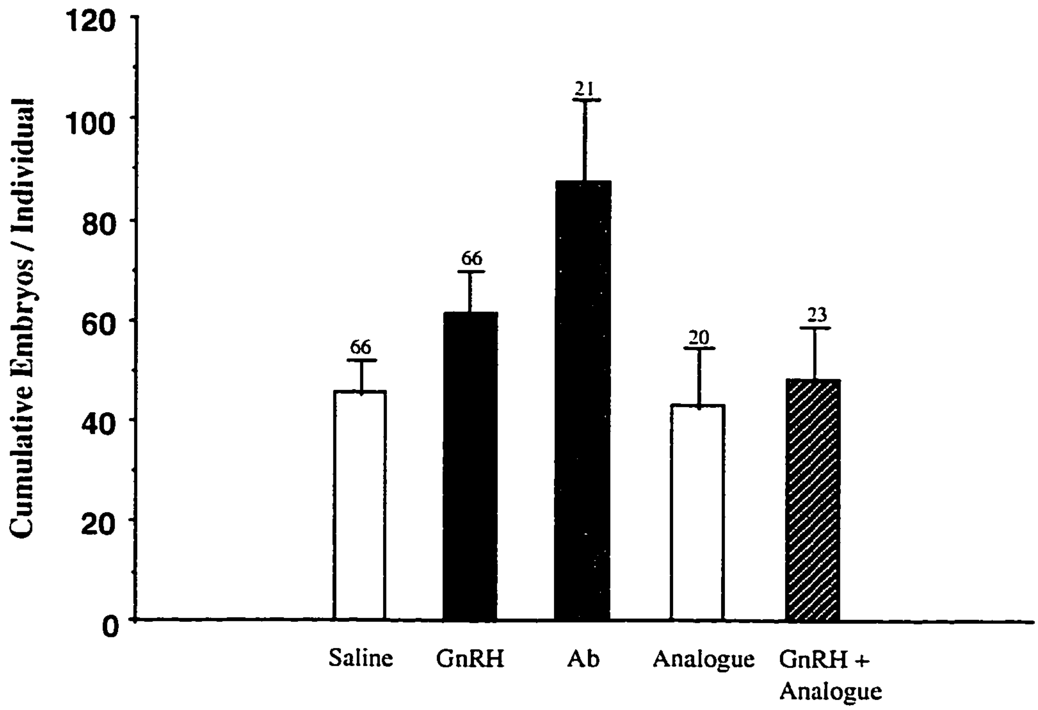
Preliminary data suggested that injections of mGnRH into the hemocoel of *H. trivolvis* could cause a small increase in egg-laying over a several-days period (Zalitach, Chang, and Goldberg, unpublished observations). These results further supported that mGnRH has biological activity in snails, and indicated that it may affect reproductive tissues or the regulation of reproduction by the nervous system. I tried to confirm these results using injections of mGnRH, mGnRH antibody serum, [D-pGlu^{1}, D-Phe^{2}, D-Trp^{3,6}]-mGnRH (a GnRH analogue known to act as an antagonist in mammalian systems; Rivier and Vale, 1978), and co-injections of mGnRH and the analogue.

Though some trials indicated that mGnRH did cause a small increase in egg-laying, this did not hold true throughout repeated trials. The mGnRH-injected snails did not lay significantly more egg-masses over a 10 day period than did saline-injected snails (Figs. 3.15 - 3.17). In one set of trials, the antibody-injected group did lay significantly more egg masses than the saline-injected snails (Fig. 3.15). However, this did not hold true in the second set of trials (Fig. 3.17). In the second set of trials, the analogue-injected group laid more egg-masses than the all of the other groups (Fig. 3.17), however this was not true for the first set of trials (Fig. 3.15). As well, co-injections of the analogue and mGnRH did not lead to any increases in egg-laying (Fig. 3.15 - 3.17).

In order to see if injections of mGnRH could induce egg-laying in non-egg-laying adults, I injected mGnRH into virgin adult snails. Virgin adults are capable of egg-laying, but do so with very low frequency, or not at all (Saleuddin et al., 1980; Paraense and Corrêa, 1988). It has been shown that injections of extracts from the CNS can induce egg-laying in virgin adults after a period of five to six days post-injection (Saleuddin et al., 1980). The factor(s) responsible for this have not been identified.

Figure 3.15. The effect of mGnRH on egg-laying. A) The cumulative number of egg-masses laid per individual over a ten day period. There is no statistical difference among the groups using a Kruskal-Wallis test, however there is a difference between the antibody (Ab)-injected and saline-injected groups using a Mann-Whitney U comparison ($p = 0.0058$). Numbers of individuals used in each group are shown above the bars. The data represents numbers from six trials for the GnRH and saline groups, and two trials each for the antibody, analogue, and mGnRH + analogue groups. The data is shown grouped from all trials. B) The cumulative numbers of embryos, from the trials shown in A, laid per individual over a ten day period. No significant differences existed among the treatment groups. Numbers at the top of each column represent the total number of individuals in the group.


Cumulative Egg Masses / Individual
3.0
2.0
1.0
0.0
66
66
21
20
23
Saline
GnRH
Ab
Analogue
GnRH + Analogue


Cumulative Embryos / Individual
120
100
80
60
40
20
0
66
66
21
20
23
Saline
GnRH
Ab
Analogue
GnRH + Analogue

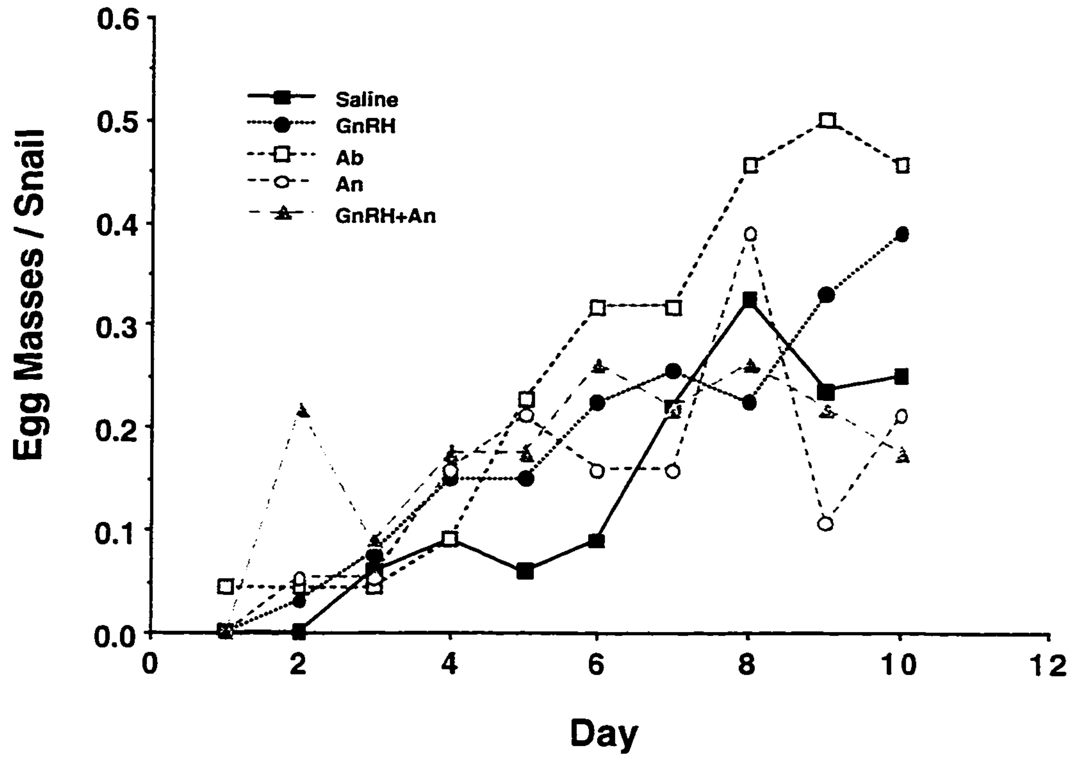
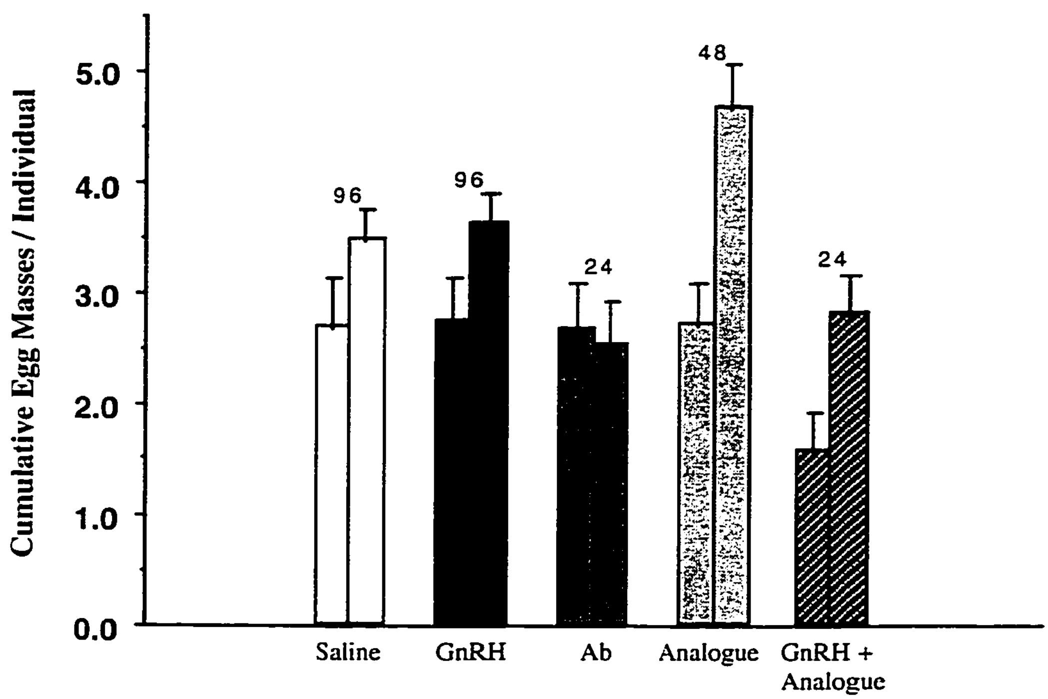


Fig. 3.16. Egg-laying over the ten day measurement period following injections. Error bars are not shown for the purpose of clarity (numbers = mean; SE ≤ 0.127 for the data points). The pattern of laying was highly variable for each treatment group on most days. There was a general trend of increased egg-laying over the ten day period, likely due to the freshwater stimulus; this trend was similar in all of the treatment groups. The data was taken from the same trials shown in Fig. 3.15.

Figure 3.17. The effect of mGnRH on egg-laying with treatment groups assigned based on preinjection laying. Measurements of egg-laying were made for 10 days pre-injection, and individuals were placed into each group so as to ensure that the groups contained snails of equal egg-laying ability. The number of egg-masses within each group was, therefore, similar within the preinjection periods in each trial. (The two trials involving the GnRH + analogue control show lower numbers preinjection as the overall egg-laying in these trials was lower. However, these preinjection numbers are not statistically different.) The data represent individual numbers from eight trials for the mGnRH and saline groups, four for the analogue group, and two for the antibody (Ab) and mGnRH + analogue groups. The cumulative numbers of egg masses laid during the pre-injection period are presented on the left of each of the column pairs. The analogue injected group laid significantly more egg-masses than the other groups, post-injection (Kruskal-Wallis; $p = 0.013$). Numbers on the top of the column pairs represent the total number of individuals in the group.


Cumulative Egg Masses / Individual
5.0
4.0
3.0
2.0
1.0
0.0
96
96
24
48
24
Saline
GnRH
Ab
Analogue
GnRH +
Analogue

In trials involving virgin snails, neither the mGnRH-injected nor the saline injected group laid any viable embryos. A few empty egg-masses were laid from snails in each group, but not enough egg-laying occurred to continue the analysis.

Chapter 4 - Discussion

In this study we have been able to correlate GnRH-immunoreactivity in two species of pond snails to CNS and peripheral areas that are involved in reproduction. This fits with an earlier hypothesis (Goldberg et al., 1993) that an endogenous GnRH-like peptide may play a role in reproductive control in snails. Though the physiological testing that was performed showed no consistent indication of GnRH affecting reproductive processes, the anatomical findings would suggest that further physiological testing should focus on the male reproductive tract and neurons innervating this tract.

Distribution in the CNS

The pattern of GnRH-immunoreactivity in the *Helisoma trivolvis* CNS found in this study varies from that detailed in Goldberg et al. (1993). This previous study used polyclonal antibodies raised against sGnRH, cGnRH-II, and mGnRH, and staining was assessed exclusively in wholemounts. The differences in staining may be accounted for by the presence of different forms of GnRH in the CNS, and by the differences in antibody penetration in wholemounts versus sections. While this previous work showed that most of the immunoreactive neurons were present in the more posterior ganglia, some staining in the cerebral ganglia had been observed when using the mGnRH antibody, but not the sGnRH or cGnRH-II antibodies. Even more GnRH-IR cerebral neurons were observed in both wholemounts and tissue sections during the current study, likely due to the use of antibodies which bound more strongly with the antigen.

As well, Goldberg et al. (1993) found that while mGnRH co-migrated on an HPLC column with the major GnRH radioimmunoassayed chromatograph peak from a snail CNS homogenate, there was a second peak from the homogenate that was detectable in the assay, suggesting the presence of two GnRH-like peptides. Indeed, multiple forms of GnRH in a single species seems to be more the norm rather than the exception (King and Millar, 1995).

That the relatively few GnRH-IR neurons in *L. stagnalis* were found in locations similar to those in *H. trivolvis* gives some indication as to the function of the endogenous peptide. Staining in both species was found in and around the lateral lobe regions. As neurons from these lobes are known to be involved in the stimulation of egg-laying (Geraerts, 1976; Roubos et al., 1980; Geraerts and Joose, 1984; Saleuddin et al., 1994; Saleuddin and Ashton, 1996), it seems likely that the GnRH-like peptide serves to influence CNS neurons involved in reproductive control. The lateral lobes are thought to stimulate reproduction by inactivating growth hormone-producing neurons, which in turn stimulates the caudodorsal cells to release peptides involved in inducing ovulation and egg-laying (Geraerts et al., 1976; Saleuddin et al., 1994), and by stimulating the endocrine dorsal bodies to release a vitellogenic hormone (Roubos et al., 1980; Saleuddin et al., 1994; Saleuddin and Ashton, 1996). Lateral lobe neurons also act on other centers within the cerebral ganglia, as they send projections throughout these ganglia (Saleuddin and Ashton, 1996).

Furthermore, an involvement of the GnRH-IR neurons in the regulation of reproductive neurons and neuroendocrine cells is indicated by their presence in several other areas throughout the cerebral ganglia. The cerebral ganglia contain most of the key cells known to be important in regulating both male and female aspects of reproduction in freshwater snails (Geraerts and Joose, 1984; Geraerts et al, 1988b; Saleuddin et al., 1994).

In the cerebral ganglia, the basic pattern of staining was similar in juveniles and adults of different reproductive status, and the GnRH-IR expression was observed as early as E70 embryos. As well, embryonic neurons can have neurite outgrowth affected by mGnRH in culture (Goldberg et al., 1993). While this may indicate that the GnRH-like peptide is not produced only for the initiation of specific reproductive events, it does not at all preclude its involvement in reproduction. GnRH is both expressed and released in juvenile vertebrate animals (Becu-Villalobos and Libertun, 1995; Parhar et al., 1995; Silverin and Sharp, 1996). As well, the key ovulogenic hormone in snails, caudodorsal cell hormone, is expressed in juveniles (de Lange et al., 1994). This hormone was proposed to play a role in the maturation of the reproductive tract in the juvenile snails.

Innervation of the Reproductive Tract

In *Lymnaea stagnalis*, the penial complex is known to be innervated by multiple peptides (Li et al., 1992; van Kesteren et al., 1995a; de Lange et al., 1998b). Neurons innervating the penial complex originate in the right cerebral, pleural, pedal, and parietal ganglia (de Boer et al., 1996; de Lange et al., 1998a). The mainly peptidergic neurons involved serve to regulate the complex control of penial eversion, probing, and intromission (de Lange et al., 1998b).

This study adds a GnRH-like peptide to the list of peptides that may be involved in the control of the penial complex in pond snails. While in *L. stagnalis* only a few GnRH-IR fibres were found in the penial nerve and complex, the *H. trivolvis* nerve and complex were much more heavily labelled, suggesting a greater role for the GnRH-like peptide in this species. Alternatively, the reduced staining in *L. stagnalis* may reflect the existence of another GnRH isoform that is less immunoreactive to the antibody used, rather than a difference in functional importance.

Backfilling from the penial nerve indicated that the groups of neurons that project axons to the penial nerve in *H. trivolvis* (Fig. 3.8

and 3.9) are very similar in location to those in *L. stagnalis* (de Boer et al., 1996; de Lange et al., 1998a). While the Lucifer yellow used in this study can fill neurons beyond those which send axons to a nerve, through the process of dye-coupling, the similarity in the pattern of backfilling through the penial nerve to that established for *L. stagnalis* would suggest that little dye-coupling took place. Previous work in *L. stagnalis* has used both cobalt and nickel-lysine for backfilling (de Boer et al., 1996; de Lange et al., 1998a).

The lobe which possesses most of the backfilled neurons in *H. trivolvis* is the left cerebral extension, through which the penial nerve exits the CNS (Fig. 3.8). This lobe consistently contained heavy GnRH-labelling in what appeared to be a dense network of neurites within the neuropil. While the origin of the neurites is not known, the posterior-medial cluster of GnRH-IR neurons, which were iontophoretically injected with Lucifer yellow, appear to contribute to this network. It is possible that GnRH-containing axons serve to regulate at least some of the neurons within this lobe, as some of these neurons may express GnRH receptors (see appendix).

With respect to the female reproductive tract, though we did not observe GnRH-IR fibres in any of the female reproductive organs, staining in the intestinal nerve would suggest that the GnRH-like peptide may innervate at least one of these as well. This nerve can be seen to run adjacent to the female tract in *H. trivolvis* (personal observation), and is known to innervate the ovotestis in other pond snails (Geraerts and Joose, 1984; Hermann, 1994). In *L. stagnalis*, the intestinal nerve relays feedback signals from the reproductive tract to the CNS during egg-laying (Ferguson et al., 1993; Hermann, 1994).

Targets and Possible Functions for GnRH in Freshwater Snails

In the initial characterization of the presence and actions of GnRH in *H. trivolvis*, it was demonstrated that mGnRH could affect the electrical activity of visceral and left parietal ganglion neurons (Goldberg et al., 1993). In this study, I focussed on neurons which are known to be key regulators of reproductive functioning, the caudodorsal cells. That these cells may be responsive to GnRH was supported by a previous report that the homologous cells in *A. californica*, the bag cells, have their electrical activity altered by the application of cGnRH-II (Tsai et al., 1996). However, the results found in this report showed only a small effect.

In *H. trivolvis*, there were no observed effects of mGnRH on the electrical activity of the CDCs. As the number of successful recordings was limited in this study, and not all electrical properties were tested, concluding that GnRH does not affect the functioning of the CDCs may be premature. If GnRH does have a modulatory effect on these neurons, it may be a subtle one, observable only with more extensive testing. For example, the putative effect of GnRH on bag cells was observed only during the afterdischarge phase of activity (Tsai et al., 1996). However, there is no strong indication that a modulatory effect does exist.

As well, no strong evidence was found that the main process affected by CDCs, egg-laying, was affected by the injections of mGnRH (Figs. 3.15 - 3.17). However, if a small effect on egg-laying does exist, as indicated in some trials, it would be lost in the variability of the data. Individual egg-laying ability varies greatly between snails, thus it was difficult to discern changes in the patterns of laying following the injections. No discrete changes in laying on any one day, or group of days, were noted following the injections (Fig. 3.16). Statistically significant differences with cumulative egg-laying did exist with antibody-injected and analogue-injected groups laying higher numbers of egg-masses in different sets of trials. However, these differences were not consistent between the two sets of data shown (Figs. 3.15 and 3.17). Also, in the set of trials where the analogue-injected group laid more egg-masses, the group co-injected with mGnRH and the analogue showed no significant difference from the remaining treatment groups (Fig. 3.17). This indicates that the observed differences may have been artifactual.

Even if the GnRH-like peptide does play a role in the egg-laying process, this type of analysis may not indicate it. Peptides already demonstrated to affect reproductive processes in snails may have no observable effects on reproductive behaviours upon injection. For instance, conopressin controls contractions of the vas deferens, however no effects on male reproductive behaviours are observed upon injection of this peptide in *L. stagnalis* (de Boer et al., 1997).

Involvement of the snail GnRH-like peptide in male, rather than female, reproductive function is more strongly supported by the anatomical data presented in the present study. This involvement is indicated by the extensive fibre-like staining in the cerebral extension in *H. trivolvis*, and innervation of the male reproductive tract by GnRH-IR neurons in *H. trivolvis* and *L. stagnalis*. As well, a subset of cerebral extension neurons may express GnRH receptors in culture (see appendix). In the left parietal ganglion, many neurons innervate the male reproductive tract (Fig. 3.8), where mGnRH has already been demonstrated to affect neuronal electrical activity (Goldberg et al., 1993).

Any further characterization of the physiological responses to GnRH in pond snails would also be greatly facilitated by the elucidation of the primary structure of the endogenous peptide(s). In the bullfrog sympathetic ganglion, initial studies were performed with the assumption that the endogenous peptide was similar to either mGnRH (Jones, 1987) or a teleost GnRH (Adams et al., 1986). More recently, it has been found that the endogenous form is cGnRH-II (King and Millar, 1995; Troskie et al., 1997). The cGnRH-II form is also about 1000 times more potent than mGnRH in its neuromodulatory effect in this system (King and Millar, 1995; Troskie et al., 1997).

The Functional Conservation of GnRH Neurons

In vertebrates, an ever-increasingly studied role for GnRH is that of neurotransmission/neuromodulation (Jones et al., 1987; Wong et al., 1990; Oka, 1992; Oka, 1996). In the CNS, GnRH neurons and fibres are found in the terminal nerve-septo-preoptic system and throughout additional extrahypothalamic areas not involved in gonadotropin release such as the limbic system and midbrain tegmentum (Muske, 1993; Millam et al., 1993; Muske et al., 1994; D'Aniello et al., 1994). These neurons have been implicated in controlling reproductive behaviours through actions within the CNS (Wirsig, 1987; Dellovade et al., 1995). In tunicates, GnRH immunostaining was found exclusively in the nervous system, in neurons which may innervate the gonads (Powell et al., 1996).

In the present study, staining of a GnRH-like peptide was limited to CNS neurons. At least a few of these neurons exhibited relatively broad, rhythmic action potential firing (Fig. 3.10) which is typical of neuromodulatory neurons (Oka, 1992; Oka, 1996). While the role(s) that GnRH plays in any animal is likely limited by the constraints of each animal's unique physiology, these data are consistent with the idea that one function of the GnRH family of peptides is to act as neuronal transmitters/modulators to control reproductive function throughout the phyla in which it is present.

As with GnRH peptides in chordate species, though, it is likely that the endogenous peptide in snails has more than one key function. The GnRH-IR somata and neurites are dispersed throughout the ganglia of the CNS, providing for opportunities to communicate with many types of neurons. No doubt further studies examining the physiological responses to GnRH in the snails will further help to define the role(s) of the endogenous peptide.

Literature Cited

Abdel-Malek, E.T. (1954) Morphological studies on the family planorbidae (Mollusca: Pulmonata). 1. Genital organs of *Helisoma trivolvis* (Say) (Subfamily Helisomatinae F.C. Baker, 1945). Trans. Amer. Microscop. Soc. *73* : 103-124.

Adams, P.R., S.W. Jones, P. Pennefather, D.A. Brown, C. Koch, and B. Lancaster (1986) Slow synaptic transmission in frog sympathetic ganglia. J. Exp. Biol. *124* : 259-285.

Anctil, M. (1997) Gonadotropin releasing hormone-like factor in a primitive metazoan: immunohistochemical evidence and bioactivity. In S. Kawashima and S. Kikuyama (Eds.): Advances in Comparative Endocrinology - Proceedings of the XIIIth International Congress of Comparative Endocrinology. Yokahama. pp. 703-707.

Becu-Villalobos, D. and C. Libertun (1995) Development of gonadotropin-releasing hormone (GnRH) neuron regulation in the female rat. Cell. Mol. Neurobiol. *15* : 165-176.

Boer, H.H., E.W. Roubos, H. van Dalen, and J.R.F.Th. Groesbeek (1977) Neurosecretion in the basommatophoran snail *Bulinus truncatus* (Gastropoda, Pulmonata). Cell Tiss. Res. *176* : 57-67.

Brownlee, D.J.A., I. Rairweather, C.F. Johnston, D. Smart, C. Shaw, and D.W. Halton (1993) Immunocytochemical demonstration of neuropeptides in the central nervous system of the roundworm, *Ascaris suum* (Nematoda: Ascaroidea). Parasitology *106* : 305-316.

Chase, R. (1986) Brain cells that command sexual behaviour in the snail *Helix aspersa*. J. Neurobiol. *17* : 669-679.

Clayton, R.N., J.P. Harwood, and K.J. Catt (1979) Gonadotropin-releasing hormone analoque binds to luteal cells and inhibits progesterone production. Nature *282* : 90-92.

D'Aniello, B., C. Pinelli, J.A. King, and R. K. Rastogi (1994) Neuroanatomical organization of GnRH neuronal systems in the lizard (*Podarcis s. sicula*) brain during development. Brain Res. *657* : 221-226.

de Boer, P.A.C.M., R.F. Jansen and A. ter Maat (1996) Copulation in the hermaphroditic snail *Lymnaea stagnalis*. Invert. Reprod. Dev. *30* : 167-176.

de Boer, P.A., A. ter Maat, A.W. Pieneman, R.P. Croll, M. Kurokawa, and R.F. Jansen (1997a) Functional role of peptidergic anterior lobe neurons in male sexual behaviour of the snail *Lymnaea stagnalis*. J. Neurophysiol. *78* : 2823 - 2833.

de Boer, P.A.C.M., R.F. Jansen, J.M. Koene, A. ter Maat (1997b) Nervous control of male sexual drive in the hermaphroditic snail *Lymnaea stagnalis*. J. Exp. Biol. *200* : 941-951.

de Lange, R.P.J., J. van Minnen, and H.H. Boer (1994) Expression and translation of the egg-laying neuropeptide hormone genes during post-embryonic development of the pond snail *Lymnaea stagnalis*. Cell Tissue Res. *275* : 369-375.

de Lange, R.P.J., P.A.C.M. de Boer, A. ter Maat, C.P. Tensen, and J. van Minnen (1998a) Transmitter identification in neurons involved in male copulation behaviour in *Lymnaea stagnalis*. J. Comp. Neurol. *395* : 440-449.

de Lange, R.P.J., J. Joosse, and J. van Minnen (1998b) Multi-messenger innervation of the male sexual system of *Lymnaea stagnalis*. J. Comp. Neurol. *390* : 564-577.

Dellovade, T.L., M.A. Ottinger, and E.F. Rissman (1995) Mating alters gonadotropin-releasing hormone cell number and content. Endocrinology *136* : 1648-1657.

Dorsett, D.A. (1986) Brains to cells: The neuroanatomy of selected gastropod species. In A.O.D. Willows (ed.): The Mollusca. Toronto: Academic Press.

Drury, R.A.B. and E.A. Wallington (1967) Carleton's Histological Technique. Toronto: Oxford University Press.

Ferguson, G.P., A.W. Pieneman, R.F. Jansen, and A. ter Maat (1993) Neuronal feedback in egg-laying behaviour of the pond snail, *Lymnaea stagnalis*. J. Exp. Biol. *178* : 251-259.

Geraerts, W.P.M. (1976) The role of the lateral lobes in the control of growth and reproduction in the hermaphrodite freshwater snail *Lymnaea stagnalis*. Gen. Comp. Endocrin. *29* : 97-108.

Geraerts, W.P.M. and J. Joosse (1984) Freshwater snails (Basommatophora). In A.S. Tompa, N.H. Verdonk, and J.A.M. van den Biggelaar (eds.): The Mollusca: Reproduction. Toronto: Academic Press. pp. 142-207.

Geraerts, W.P.M., and Th.M. Hogenes (1985) Heterogeneity of peptides released by electrically active neuroendocrine caudodorsal cells of *Lymnaea stagnalis*. Brain Res. *331* : 51-61.

Geraerts, W.P.M., E. Vreugdenhil, and R.H.M. Ebberink (1988a) Bioactive peptides in molluscs. In M.C. Thorndyke, and G.J. Goldsworthy (eds.): Neurohormones in Invertebrates. New York: Cambridge University Press. pp. 261-281.

Geraerts, W.P.M., A. Ter. Maat, and E. Vregdenhil (1988b) The peptidergic neuroendocrine control of egg-laying behaviour in *Aplysia* and *Lymnaea*. In H. Laufer and R.G.H. Downer (eds.): Endocrinology of Selected Invertebrate Types. New York: Liss. pp. 144-231.

Getting, P.A. and M.S. Dekin (1985) A model system for integration within rythmic motor systems. In A.I. Selverston (ed.): Model Neural Networks and Behavior. New York: Plenum. pp. 3-20.

Goldberg, J.I., and S.B. Kater (1989) Expression and function of the neurotransmitter serotonin during development of the *Helisoma* nervous system. Dev. Biol. *131* : 483-495.

Goldberg, J.I. and K.J. Cavers (1993) Growth of neuronal populations during postembryonic development in *Helisoma trivolvis.* Soc. Neurosci. Abstr. *19* : 533.8.

Goldberg, J.I., R. Garofalo, C.J. Price, and J.P. Chang (1993) Prescence and biological activity of a GnRH-like factor in the nervous system of *Helisoma trivolvis.* J. Comp. Neurol. *336* : 571-582.

Goldberg, J.I. (1995) Neuronal development in embryos of the mollusk, *Helisoma trivolvis*: multiple roles of serotonin. Adv. Neural Sci. *2* : 67-87.

Gorbman, A. (1997) The ancestary of vertebrate hormonal peptides. In Molecular Ancestary of Vertebrate Polypeptide Hormones and Neuropeptides. Tokyo. pp. 6-8.

Greenberg, M.J. and D.A. Price (1992) Relationships among the FMRFamide-like peptides. Prog. Br. Res. *92* : 25-37.

Griffond, B., J. van Minnen, C. Colard (1992) Distibution of αCDCP-immunoreactive neurons in the central nervous system of the snail *Helix aspersa.* Reprod. Nutr. Dev. *32* : 113-121.

Hermann, P.M. (1994) Behavioural and neural effects of multiple peptides encoded on a single gene. Ph.D. thesis. Vrije Universiteit.

Jimenez-Linan, M., B.S. Rubin, and J.C. King (1997) Examination of guinea pig luteinizing hormone-releasing hormone gene reveals a unique decapeptide and existence of two transcripts in the brain. Endocrinology *138* : 4123-4130.

Jones, S.W. (1987) Luteinizing hormone-releasing hormone as a neurotransmitter in bullfrog sympathetic ganglia. Ann. N.Y. Acad. Sci. *519* : 310-322.

Khan, H.R. and A.S.M. Saleuddin (1992) Neurosecretion of the mediodorsal cells of the central nervous system of the snail *Helisoma duryi.* Cell Tissue Res. *268* : 131-139.

Khan, H.R., M.L. Ashton, S.T. Mukai, and A.S.M. Saleuddin (1989) The effects of mating on the fine structure of neurosecretory caudodorsal cells in *Helisoma duryi* (Mollusca). Can. J. Zool. *68* : 1233-1240.

King, J.A., and R.P. Millar (1992) Evolution of gonadotropin-releasing hormones. TEM *3* : 339-345.

King, J.A., and R.P. Millar (1995) Evolutionary aspects of gonadotropin-releasing hormone and its receptors. Cell. Mol. Neurobiol. *15* : 5-23.

Kits, K.S. (1980) States of excitability in ovulation hormone producing neuroendocrine cells of *Lymnaea stagnalis* (Gastropoda) and their relation to the egg-laying cycle. J. Neurobiol. *11* : 397-410.

Li, K.W., A.B. Smit, and W.P.M. Geraerts (1992) Structural and functional characterization of neuropeptides involved in the control of male mating behaviour of *Lymnaea stagnalis*. Peptides *13* : 633-638.

Li, K.W., F.A. van Golen, J. van Minnen, P.A. van Veelen, J. van der Greef, and W.P.M. Geraerts (1994) Structural identification, neuronal synthesis, and role in male copulation of myomodulin-A of *Lymnaea*: a study involving direct peptide profiling of nervous tissue by mass spectrometry. Mol. Br. Res. *25* : 355-358.

Li, K.W., Z. el Filali, F.A. van Golen, and W.P.M. Geraerts (1995) Identification of a novel peptide, GLTPNMNSLFF-NH_2, involved in the control of vas deferens motility in *Lymnaea stagnalis*. Eur. J. Biochem. *229* : 70-72.

Li, G. and R. Chase (1995) Correlations of axon projections and peptide immunoreactivity in mesocerebral neurons of the snail *Helix aspersa*. J. Comp. Neurol. *353* : 9 - 17.

Lin, X., J.A. Janovick, S. Brothers, M. Blomenrohr, J. Bogerd, and P.M. Conn (1998) Addition of catfish gonadotropin-releasing hormone (GnRH) receptor intracellular carboxyl-terminal tail to rat GnRH receptor alters receptor expression and regulation. Mol. Endocrin. *12* : 161-171.

Loi, P.K., H.F. McGraw, and N.J. Tublitz (1997) Peptide detection in single cells using a dot immunoblot assay. Peptides *18* : 749-753.

Loumaye, E., J. Thorner, and K. Catt (1982) Yeast mating pheromone activates mammalian gonadotrophs: evolutionary conservation of a reproductive hormone. Science *218* : 1323-1325.

Marchetti, B., F. Gallo, Z. Farinella, C. Tirolo, N. Testa, C. Romeo, and M.C. Morale (1998) Luteinizing hormone-releasing hormone is a primary signaling molecule in the neuroimmune network. Ann. N.Y. Acad. Sci. *840* : 205-248.

McKenney, K. and J.I. Goldberg (1989) *Helisoma* embryogenesis: morphological, behavioural and neural development. Soc. Neurosci. Abstr. *15* : 1016.

Millam, J.R., P.L. Faris, O.M. Youngren, M.E. El Halawani, and B.K. Hartman (1993) Immunohistochemical localization of chicken gonadotropin-releasing hormones I and II (cGnRH I and II) in the turkey hen brain. J. Comp. Neurol. *333* : 68-82.

Mukai, S.T., and A.S.M. Saleuddin (1988) Mating increases the synthetic activity of the neurosecretory caudodorsal cells of *Helisoma duryi* (Mollusca: Pulmonata). Can. J. Zool. *67* : 2363-2367.

Muske, L.E. (1993) Evolution of gonadotropin-releasing hormone (GnRH) neuronal systems. Brain Behav. Evol. *42* : 215-230.

Muske, L.E., J.A. King, F.L. Moore, and R.P Millar (1994) Gonadotropin-releasing hormones in microdissected brain regions of an amphibian: concentration and anatomical distribution of immunoreactive mammalian GnRH and chicken GnRH II. Reg. Peptides *54* : 373-384.

Oka, Y. (1992) Gonadotropin-releasing hormone (GnRH) cells of the terminal nerve as a model neuromodulator system. Neurosci. Letters *142* : 119-122.

Oka, Y. (1996) Characterization of TTX-resistant persistent Na^+ current underlying pacemaker potentials of fish gonadotropin-releasing hormone (GnRH) neurons. J. Neurophys. *75* : 2397-2404.

Paraense, W.L. and L.R. Corrêa (1988) Self-fertilization in the freshwater snails *Helisoma duryi* and *Helisoma trivolvis*. Mem. Inst. Oswaldo Cruz *83* : 405-409.

Parhar, I.S., M. Iwata, D.W. Pfaff, and M. Schwanzel-Fukuda (1995) Embryonic development of gonadotropin-releasing hormone neurons in the sockeye salmon. J. Comp. Neurol. *362* : 256-270.

Pati, D., and H. Habibi (1993) Characterization of gonadotropin-releasing hormone receptors in goldfish ovary: variation during follicular development. Am. J. Physiol. *33* : R227-R234.

Pearson, K.G. (1993) Common principles of motor control in vertebrates and invertebrates. Ann. Rev. Neurosci. *16* : 265-297.

Plesch, B., M. de Jong-Brink, and H.H. Boer (1971) Histological and histochemical observations on the reproductive tract of the hermaphrodite pond snail *Lymnaea stagnalis* (L.). Netherlands J. Zool. *21* : 180-201.

Powell, J.F.F., S.M. Reska-Skinner, M.O. Prakash, W.H. Fischer, M. Park, J.E. Rivier, A.G. Craig, G.O. Mackie, and N.M. Sherwood (1996) Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. Proc. Natl. Acad. Sci. USA *93* : 10461-10464.

Rivier, J.E.a.W.W.V. (1978) [D-pGlu^1, D-Phe^2, D-Trp^{3,6}]-LRF. (1978) A potent luteinizing hormone releasing hormone factor antagonist *in vitro* and inhibitor of ovulation in the rat. Life Sci. *23* : 869-876.

Roubos, E.W., W.P.M. Geraerts, G.H. Boerrigter, and G.P.J. van Kampen (1980) Control of activities of the neurosecretory light green cells and caudodorsal cells and of the endocrine dorsal bodies by the lateral lobes in the freshwater snail *Lymnaea stagnalis* (L.). Gen. Comp. Endocrin. *40* : 446-454.

Saleuddin, A.S.M., L.E. Wilson, H.R. Khan, and G.M. Jones (1980) Effects of brain extracts on oocyte maturation in *Helisoma* (Pulmonata: Mollusca). Can. J. Zool. *58* : 1109-1124.

Saleuddin, A.S.M., H.R. Khan, M.-L. Ashton, and B. Griffond (1992) Immunocytochemical localization of FMRFamide in the central nervous system and the kidney of *Helisoma duryi* (Mollusca): its possible antidiuretic role. Tiss. Cell *24* : 179-189.

Saleuddin, A.S.M., S.T. Mukai, and H.R. Khan (1994) Molluscan endocrine structures associated with the central nervous system. In K.G. Davey, R.E. Peter, and S.S. Tobe (Eds.): Perspectives in Comparative Endocrinolgy. Ottawa. pp. 257-263.

Saleuddin, A.S.M., and M.-L. Ashton (1996) Neuronal pathways of three neurosecretory cells from the lateral lobes in *Helisoma* (Mollusca): innervation of the dorsal body. Tiss. Cell *28* : 53-62.

Scharrer, B. (1987) Neurosecretion: beginnings and new directions in neuropeptide research. Ann. Rev. Neurosci. *10* : 1-17.

Scharrer, B. (1990) The neuropeptide saga. Amer. Zool. *30* : 887-895.

Scharrer, E. (1928) Die Lichtempfindlichkeit blinder Elritzen (Untersuchungen über das Zwischenhirn der Fische). Z. Vergl. Physiol. *7* : 1-38.

Schot, L.P.C., H.H. Boer, D.F. Swaab, and S. van Noorden (1981) Immunocytochemical demonstration of peptidergic neurons in the central nervous system of the pond snail *Lymnaea stagnalis* with antisera raised to biologically active peptides of vertebrates. Cell Tissue Res. *216* : 273-291.

Sealfon, S.C., H. Weinstein, and R.P. Millar (1997) Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. Endocrine Rev. *18* : 180-205.

Sherwood, N.M., D.A. Lovejoy, and I.R. Coe (1993) Origin of mammalian gonadotropin-releasing hormones. Endocrine Rev. *14* : 241-254.

Silverin, B. and P. Sharp (1996) The development of the hypothalamic-pituitary-gonadal axis in juvenile great tits. Gen. Comp. Endocrin. *103* : 150-166.

Simpson, L. (1969) Morphological studies of possible neuroendocrine structures in *Helisoma tenue* (Gastropoda: Pulmonata). Z. Zellforsch. *102* : 570-593.

Syed, N.I., A.G. Bulloch, and K. Lukowiak (1990) *In vitro* reconstruction of the respiratory central pattern generator of the mollusk *Lymnaea*. Science *250* : 282-285.

Troskie, B., J.A. King, R.P. Millar, Y.Y. Peng, J. Kim, H. Figueras, and N. Illing (1997) Chicken GnRH II-like peptides and a GnRH receptor selective for chicken GnRH II in amphibian sympathetic ganglia. Neuroendocrinology *65* : 396-402.

Tsai, P.-S., N.M. Sherwood, and N.L. Wayne (1996) Biological and immunological characterization of a gonadotropin-releasing hormone (GnRH)-like substance in a mollusk, *Aplysia californica*. Society for Neuroscience abstr. *22* : 625.11.

Ulloa-Aguirre, A., D. Stanislaus, V. Arora, J. Vaananen, S. Brothers, J.A. Janovick, P.M. Conn (1998) The third intracellular loop of the rat gonadotropin-releasing hormone receptor couples the receptor to Gs- and G(q/11)-mediated signal transduction pathways: evidence from loop fragment transfection in GGH3 cells. Endocrinology *139* : 2472-2478.

van Kesteren, R.E., A.B. Smit, R.P.J. de Lange, K.S. Kits, F.A. van Golen, R.C. van der Schors, N.D. de With, J.F. Burke, and W.P.M. Geraerts (1995a) Structural and functional evolution of the vasopressin-oxytocin superfamily: vasopressin-related conopressin is the only member present in *Lymnaea*, and is involved in the control of sexual behaviour. J. Neuroscience *15* : 5989-5998.

van Kesteren, R.E., F.A. van Golen, K.W. Li, A.B. Smit, and W.P.M Geraerts (1995b) A novel method to study diversity and functions of peptides in neuronal networks: peptides of the network underlying male copulation behaviour in the mollusc *Lymnaea stagnalis*. Neth. J. Zool. *45* : 57-63.

van Kesteren, R.E., C.P. Tensen, A.B. Smit, J. van Minnen, L.F. Kolakowski, Jr., W. Meyerhof, D. Richter, H. van Heerikhuizen, E Vreugdenhil, and W.P.M. Geraerts (1996) Co-evolution of ligand-receptor pairs in the vasopressin/oxytocin superfamily of bioactive peptides. J. Biol. Chem. *271* : 3619-3626.

van Minnen, J., H.D.F. Schallig, and M.D. Ramkema (1992) Identification of putative egg-laying hormone containing neuronal systems in gastropod molluscs. Gen. Comp. Endocrin. *86* : 96-102.

Vlieger, T.A. de, K.S. Kits, A. Ter Maat, and J.C. Lodder (1980) Morphology and electrophysiology of the ovulation hormone producing neuro-endocrine cells of the freshwater snail *Lymnaea stagnalis* (L.). J. Exp. Biol. *84* : 259-271.

Wayne, N.L. (1995) The neuroendocrine bag cells of Aplysia: a model system for neural control of hormone secretion. J. Endocrin. *147* : 1 - 4.

Wijdenes, J., J. van Minnen, and H.H. Boer (1980) A comparative study on neurosecretion demonstrated by the alcian blue-alcian yellow technique in three terrestrial pulmonates (Stylommatophora). Cell Tissue Res. *210* : 47-56.

Wilson, T.M., L.-Y. Yu-Lee, and M.R. Kelley (1995) Coordinate gene expression of luteinizing hormone-releasing hormone (LHRH) and the LHRH-receptor after prolactin stimulation in the rat Nb2 T-cell line: implications for a role in immunomodualtion and cell cycle gene expression. Mol. Endocrin. *9* : 44-53.

Wirsig, C.R. (1987) Effects of lesions of the terminal nerve on mating behaviour in the male hamster. Ann. N.Y. Acad. Sci. *519* : 241-251.

Wong, M., M.J. Eaton, and R.L. Moss (1990) Electrophysiological actions of luteinizing hormone-releasing hormone: intracellular studies in the rat hippocampal slice preparation. Synapse *5* : 65-70.

Zohar, Y., A. Elizur, N.M. Sherwood, J.F.F. Powell, J.E. Rivier, and N. Zmora (1995) Gonadotropin-releasing hormone activities of the three native forms of gonadotrpin-releasing hormone present in the brain of gilthead seabream, *Sparus aurata*. Gen. Comp. Endocrin. *97* : 289-299.

Appendix

As the distribution of GnRH-immunoreactivity in *Helisoma trivolvis* shown in this study suggested that an endogenous GnRH-like peptide may affect male reproductive function, I began to test whether mGnRH could regulate the functioning of neurons which innervate the male reproductive tract. Neurons of the cerebral extension were chosen as a possible target for such an investigation. These neurons are fairly homogenous in that they mostly contain an FMRFamide-IR peptide (Saleuddin et al., 1992; Fig. A1), and most project into the penial nerve (Fig. 3.9). The CE was also filled with fibre-like GnRH staining throughout the neuropil area (see Figs. 3.2 and 3.9). This relationship between GnRH-immunoreactivity and the CE neurons would suggest that GnRH may play a role in regulating the neuronal activity within this lobe.

As an initial attempt to test whether mGnRH could affect the functioning of cerebral extension neurons, I have examined the possible existence of GnRH receptors on dispersed CE cells in culture. Efforts to examine the possible existence of a physiological response of these neurons to mGnRH *in vitro* by calcium imaging are in the preliminary stages, and results from such experiments are not shown.

Methods

Cell Cultures

Neurons from the left cerebral extension were isolated and used for *in vitro* testing for the presence of GnRH receptors. The CE neurons were isolated under sterile conditions using the following procedure.

Animals were anaesthetized and placed into sterile antibiotic-containing saline (*Helisoma* saline + 150 µg/mL gentamicin; Sigma). The CNSs from ten snails were dissected out, washed briefly with sterile saline, then incubated in fresh sterile saline containing 0.1% trypsin for 90 minutes. They were then washed briefly in culture medium (CM: 50% Liebovitz-15 (Gibco), 40 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5.0 mM HEPES, 50 µg/mL gentamicin, 0.015% L-glutamine (Sigma); pH 7.35) and transferred to a silicone rubber-lined petri dish containing fresh CM for dissection. The CE lobes were excised from the left cerebral ganglia, and placed into fresh CM. Neurons from the CE were dissociated by repeatedly passing the lobes through a 63 μm mesh placed over the end of a 3 mL syringe. Harvested cells were plated onto poly-L-lysine-coated (hydrobromide; MW 4000 - 15000; Sigma) coverslips attached to the bottoms of 35 mm tissue culture dishes (Falcon 1008). The cultures were incubated in CM at room temperature in a dark, humidified chamber. Fresh medium was added to each dish after a day in culture.

Receptor Staining

I performed receptor staining on cultured CE neurons using biotinylated mGnRH (Peninsula) as a receptor probe. Dispersed neurons that were grown in culture for 48 hours were rinsed with fresh CM and kept at 4°C for 30 minutes. The CM was then replaced with fresh CM containing either 5 μM biotinylated mGnRH, 5 μM biotinylated mGnRH with 1 mM unlabelled mGnRH, or no additional peptide (no staining was observed with a lower concentration of 500 nM biotinylated mGnRH). They were incubated in these solutions for 4 hours at 4°C, following which they were fixed in ice-cold 4% paraformaldehyde. In a separate control, the cells were preincubated with 1 mM unlabelled mGnRH for 4 hours at 4°C, followed by the addition of biotinylated mGnRH (to a final concentration of 5 μM) for 4 hours at 4°C, prior to fixation.

Fixed cultures were washed three times 15 minutes in PBS, and then once in 30% horse serum and 0.4% Triton X-100 in PBS for 30 min. The biotinylated GnRH was detected using 10 μg/mL streptavidin-FITC (Sigma) diluted in PBS containing 0.4% Triton X-100, and incubated for 1 hour. The cells were then washed once in 0.4% Triton X-100 in PBS for 10 minutes, and three times in PBS for 10 minutes. Finally, they were mounted in Slowfade Light.

FMRFamide Immunostaining in Culture

Neurons from the cerebral extensions were processed for FMRFamide-immunoreactivity after 2 days in culture using the following protocol. Following a 2 hour fixation in 4% paraformaldehyde at 4°C, the cells were washed three times for 10 minutes each in PBS, and two times for 10 minutes in tris-buffered saline (0.15 M NaCl in 50 mM tris buffer). Blocking was performed with 10% horse serum in PBS containing 0.4% Triton X-100. The cells were then incubated overnight at 4°C in a polyclonal

FMRFamide antibody (1:200; Affiniti Research Products Ltd.) diluted in PBS containing 0.4% Triton X-100 and 3.3 mg/mL BSA. Following this, they were washed in PBS containing 0.4% Triton X-100 six times for 5 minutes each, and incubated in secondary antibody conjugated to either FITC or TRITC diluted in PBS containing 0.4% Triton X-100 and 3.3 mg/mL BSA . Finally, the cells were washed four times for 5 minutes each in PBS containing 0.4% Triton X-100, two times for 10 minutes each in PBS alone, and mounted in Slowfade Light.

FMRFamide Immunostaining in Tissue Sections

Staining for FMRFamide in cerebral ganglion sections was performed using the protocol for tissue section immunostaining outlined in the Chapter 2. Polyclonal FMRFamide antibody was used at a 1:300 dilution. The FMRFamide antibody was not tested for the specificity of its labelling, however, it selectively labelled cells that have previously been demonstrated to be FMRFamide-IR in *Helisoma duryi* using a different antibody (Saleuddin et al., 1992).

All viewing and photography was performed as outlined in Chapter 2.

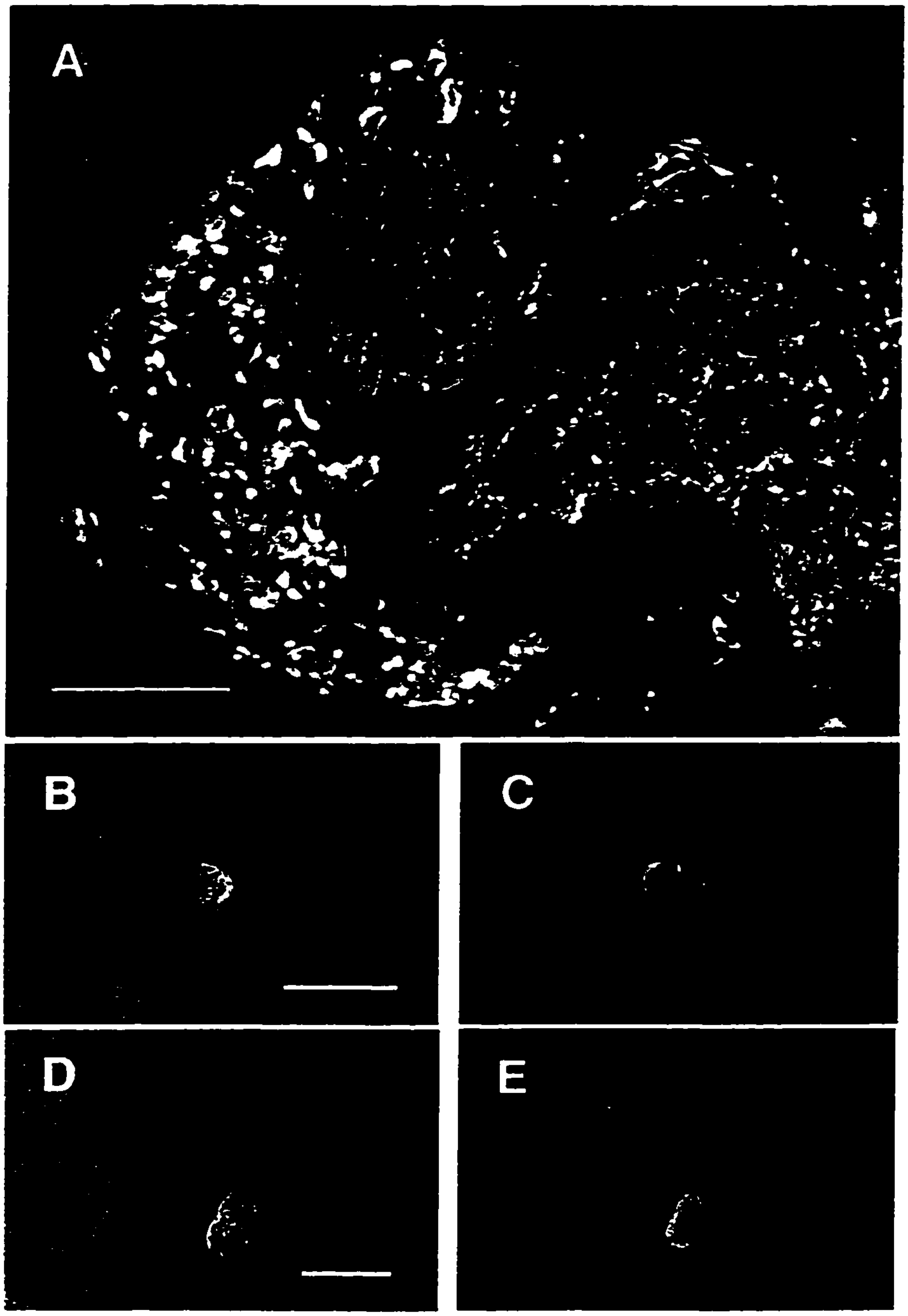
Results and Discussion

Labelling of the cerebral extension neurons for FMRFamide in *Helisoma trivolvis* showed that they stain similarly to those of *H. duryi* (Saleuddin et al., 1992; Fig. A1). Cells from the corresponding lobe in *Lymnaea stagnalis*, the ventral lobe, are known to contain authentic FMRFamide (de Boer et al., 1996). In culture, most of the neurons expressing this peptide were amongst the smaller neurons in each dish (around 20 µm), though some larger and moderate sized neurons stained as well (up to about 35 µm; Fig. A1). This provides a means for identifying these neurons in any work regarding the effect of GnRH on CE neurons.

Using a biotinylated mGnRH as a receptor probe, many small to medium sized cells (around 20 - 30 µm in diameter) in each culture dish, with little or no neurite outgrowth, were observed to be labelled (Fig. A2). The appearance of these cells corresponds to that of many of the FMRFamide-IR neurons in culture. In control staining, no FITC fluorescence was noted in cultures where biotinylated mGnRH had not been added, and only a few cells were observed to be weakly labelled in cultures which had been incubated with unlabelled mGnRH prior to addition of the labelled mGnRH. However, coincubation of a 200-fold excess amount of unlabelled mGnRH did not noticeably reduce the FITC fluorescence. This may be due to a higher affinity for the receptor of the biotinylated peptide, or may reflect the need for higher concentrations of the unlabelled form to noticeably reduce the fluorescence. Further work is thus needed to conclusively demonstrate the presence of GnRH receptors and GnRH responsiveness in the CE neurons.

Combined with immunohistological staining for GnRH in the CNS, this work provides an initial analysis which suggests that neurons involved in male reproductive control in *H. trivolvis* are targets for regulation by a GnRH-like peptide.

Figure A1. FMRFamide-immunoreactivity in the cerebral extension neurons of *Helisoma trivolvis.* A) Staining for FMRFamide in a sectioned cerebral extension. Most of the neurons within the lobe were stained. B - E) Cultured neurons, shown under Nomarski differential interference contrast optics in B and D, stained for FMRFamide (C and E, respectively). Approximately 40% of the cells in culture could be identified as FMRFamide-IR. These cells were typically like the one shown in B, with little or no neurite outgrowth, and often possessed a lammellapodial veil. Only a few cells with more extensive neurite outgrowth, as in D, were stained. Bar = 100 µm in A; 50 µm in B and D.


A
B
C
D
E

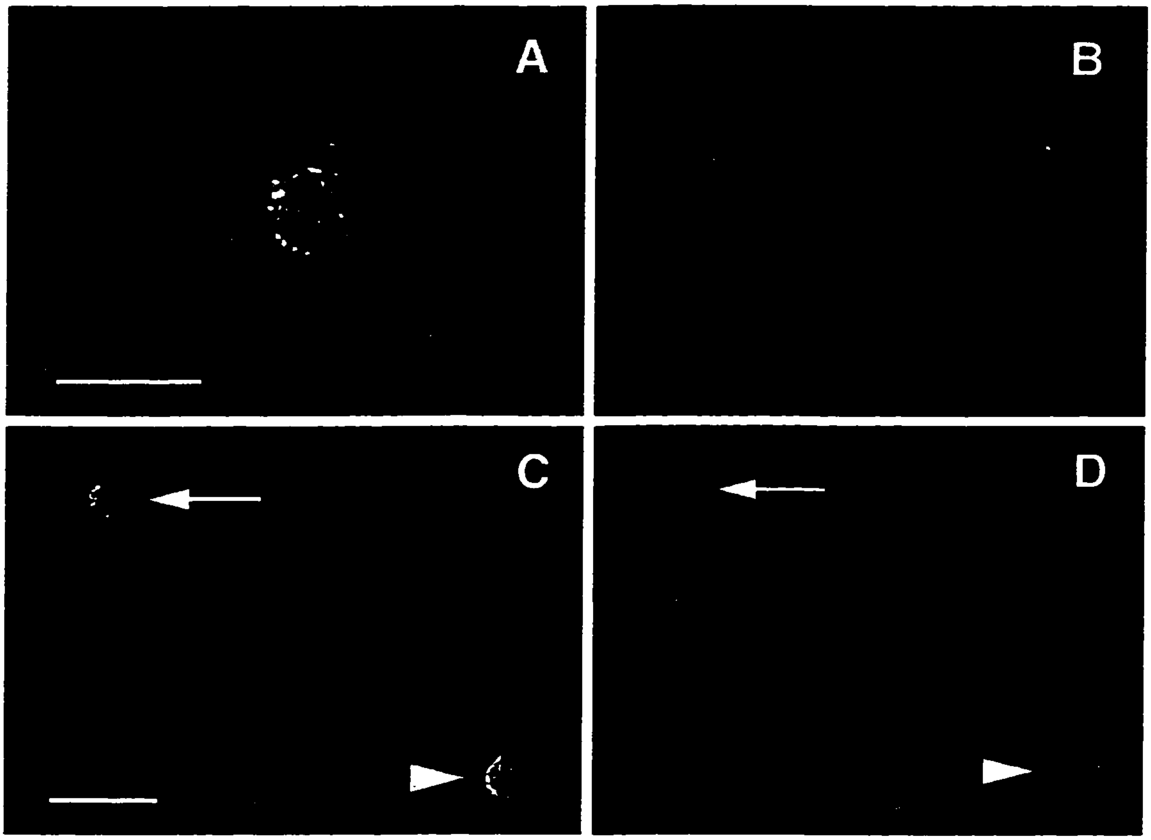


Figure A2. Using biotinylated mGnRH as a receptor probe on cerebral extension neurons. A and C) Nomarski differential interference contrast micrographs of cultured CE neurons. B and D) The respective fluorescence images of these neurons. The positions of the two cells in C (arrows) are indicated in D (arrows). Only one was stained. Only a few of the overall number of cells in each culture dish were clearly stained, with almost all of these being small to medium sized cell with little or no neurite outgrowth. Bar = 50 µm in A and C.

IMAGE EVALUATION TEST TARGET (QA-3)

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6″

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