University of Alberta

Presence of Active GnRH Receptor(s) in the Pond Snail, *Helisoma trivolvis*

by

Wanpeng Sun

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Physiology and Cell Biology

Department of Biological Sciences

Edmonton, Alberta

Fall, 2006

Your file Votre référence
ISBN: 978-0-494-22380-2
Our file Notre référence
ISBN: 978-0-494-22380-2

ABSTRACT

Gonadotropin-releasing hormone (GnRH)-like materials are present in *Helisoma trivolvis*. In this thesis, Western blot and immunofluorescence experiments using two anti-GnRH receptor antibodies demonstrated the presence of GnRH receptor-like materials, especially in the penial complex and neurons from the cerebral extension (CE) and buccal (B) ganglia of *Helisoma*. As a first step in examining the expression of GnRH receptors and possible GnRH-induced activities in cultured CE neurons, techniques for successful culturing of unidentified CE neurons were developed, followed by a general characterization. Approximately 85% of CE neurons and all B5 neurons stained positively for GnRH receptors. Application of GnRH altered input resistance and induced-firing in 96% of CE neurons; both increases and decreases in these activities were observed. In neuron B5, GnRH consistently decreased input resistance, an effect blocked by a GnRH antagonist, whereas GnRH had no effect on induced-firing. These results strongly suggest that GnRH receptors are present and functional in *Helisoma*.

ACKNOWLEGEMENTS

I wish to thank all professors, postdoctoral fellows, and graduate students in our research interest group. First of all, I would thank my supervisors, Dr. John P. Chang and Dr. Jeffrey I. Goldberg, for their excellent supervision and support. Also, I would thank Dr. Declan Ali, Dr. Greg Goss, Yi Yu, Grant Sawisky, Shandra Doran, Shawn Parries, Kee-chan Ahn, Christopher Coutts, Kessen Aroonassala Patten, Fumi Katoh, Martin Tresguerres, Scott Parks, Elizabeth Orr, Sampson Law, Bin Li, Tara Klassen, and Rheanna Sand, for their helpful discussion and technical support.

I am also grateful to Drs. X-Z Chen and Y Wu for their support in Western blot analysis. This research is supported by NSERCs to Dr. J.P. Chang and Dr. J.I. Goldberg.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

AKH: adipokinetic hormone

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate

ANOVA: analysis of variance

B: buccal (ganglion)

BC: buccal commissure

BKca: calcium-activated big conductance potassium channel

BSA: bovine serum albumin

C: cerebral ganglion

CaM: calmodulin

CAMK: CAM-dependent kinase

CBC: cerebrobuccal connective

CC: cerebral commissure

CDC: caudodorsal cells

CE: cerebral extension

CM: conditioned medium

CNS: central nervous system

CPG: central pattern generator

DAG: diacylglycerol

DIC: differential interference contrast microscopy

DM: defined medium

ET: esophageal trunk

FITC: fluorescein isothiocyanate

GALP: galanin-like peptide

GAP: GnRH associated peptide

GTH: gonadotropin

GnRH: gonadotropin-releasing hormone

cGnRH: chicken GnRH

cfGnRH: catfish GnRH

dfGnRH: dogfish GnRH

gpGnRH: guinea pig GnRH

hGnRH: herring GnRH

lGnRH: lamprey GnRH

mGnRH: mammalian GnRH

mdGnRH: medaka GnRH

octGnRH: octopus GnRH

rGnRH: *Rana* GnRH

sGnRH: salmon GnRH

sbGnRH: seabream GnRH

tGnRH: tunicate GnRH

wfGnRH: whitefish GnRH

GPCR: G-protein coupled receptor

GP: goldfish pituitary cells

HB: *Helisoma* brain

HP: *Helisoma* penial complex

HPLC: high-performance liquid chromatography

HS: *Helisoma* saline

I: injected current

IP_3: inositol 1,4,5-trisphosphate

LB: left buccal ganglion

LC: left cerebral ganglion

LH: luteinizing hormone

LL: lateral lobe

LP: left pedal ganglion

LPa: left parietal ganglion

LPl: left pleural ganglion

mAB: monoclonal anti-GnRH receptor antibody

MAPK: mitogen-activated protein kinase

MP: membrane potential

N: nerve trunk

NGF: nerve growth factor

NMDA: N-methyl-D-aspartate

NMS: normal mouse serum

NRS: normal rabbit serum

pAB: polyclonal anti-GnRH receptor antibody

PBS: phosphate buffered saline

PBST: PBS containing Tween

PIP_2: phosphatidylinositol 4,5-bisphosphate

PLC: phospholipase C

PLSD: post hoc least significant difference

PKC: protein kinase C

PN: penial nerve

PRL: prolactin

RB: right buccal ganglion

RC: right cerebral ganglion

R_{in}: input resistance

RP: right pedal ganglion

RPa: right parietal ganglion

RPl: right pleural ganglion

SDS: sodium dodecyl sulfate

SEM: standard error of mean

SKca: calcium-activated small conductance potassium channel

TEMED: tetramethyl ethylenediamine

TRITC: tetramethylrhodamine isothiocyanate

V: visceral ganglion

CHAPTER 1 – INTRODUCTION

Many well-known mammalian neurosecretory molecules are also present in invertebrates, including groups as ancient as cnidarians and flatworms (Martin and Spencer, 1983; Morita et al., 1987). This leads to the speculation that genes of some neurosecretory molecules that serve essential functions in higher vertebrates had already evolved before the diversification of metazoans over half a billion years ago. For example, one of the crucial neuropeptides controlling vertebrate reproduction, gonadotropin-releasing hormone (GnRH), has been found in many invertebrate phyla, including the ancient phylum Cnidaria (Anctil, 2000; Twan et al., 2006). This supports the hypothesis proposed by Goldberg et al. (1993) that GnRH evolved in early eukaryote evolution. Following the first demonstration of the presence of a bioactive GnRH-like factor in mollusks (Goldberg et al., 1993), a number of recent studies have focused on the possible identity and functions of molluscan GnRH-like factors. The present study examines the hypothesis that GnRH is functional in the adult pond snail *Helisoma trivolvis*. In the following sections, the evolution, structure and function of vertebrate and invertebrate GnRH peptides will be briefly reviewed. Also, progress in molluscan GnRH studies will be described. In addition, information on

the nervous and reproductive systems of *Helisoma*, especially pertaining to the possible actions of GnRH, will be discussed.

GnRH

GnRH is an ancient molecule that has been reasonably well conserved throughout metazoan evolution. GnRH isoforms with conserved amino acid sequences have been discovered in all vertebrate classes, and two invertebrate groups, urochordates (tunicates) and cephalopods (octopuses), respectively. The presence of GnRH-like factors has also been demonstrated in cnidarians (Anctil, 2000; Twan et al., 2006), nematodes (Brownlee et al., 1993), gastropods (Goldberg et al., 1993; Young et al., 1999; Zhang et al., 2000; Tsai et al., 2003), bivalves (Nakamura et al., 2005; Nakao et al., 2005), crustaceans (Fann et al., 1990), and cephalochordates (Fang et al., 1991), although amino acid sequences of these GnRH-like factors have not been determined yet.

GnRH structure and nomenclature

To date, 24 isoforms of GnRH have been identified, with 10 from invertebrates and 14 from vertebrates. There are two or more types of GnRH in each vertebrate class (Klausen et al., 2002; Millar et al., 2004). Basically, GnRH

isoforms are decapeptides with a relatively conserved N-terminus (pyr-Glu-His-Trp-Ser) and a highly conserved C-terminus (Pro-Gly-NH_2) (Figure 1.1). The N-terminus and C-terminus are important for receptor binding. There are three known atypical N-terminus configurations among the known GnRH isoforms. The octopus (oct)GnRH has twelve amino acids and a highly modified N-terminal region (pyr-Glu-Asn-Tyr-His-Phe-Ser). Interestingly, insertion of the two residues, Asn and Tyr, does not block the receptor-binding function (Iwakoshi et al., 2002). In the guinea pig GnRH, the N-terminus is pyr-Glu-Tyr-Trp-Ser (Jimenez-Linan et al., 1997), whereas in lamprey (l)GnRH-I, the N-terminus is pyr-Glu-His-Tyr-Ser (Sherwood et al., 1986).

All vertebrate and invertebrate GnRH genes that have been investigated to date contain the sequences for a signal peptide, a GnRH peptide, and a GnRH associated peptide (GAP); the latter may also serve as a hormone to stimulate gonadotropin (GTH) release and inhibit prolactin (PRL) release (Yu et al., 1989). In vertebrates, a single species has two or more GnRH isoforms (Mohamed and Khan, 2006), and each GnRH isoform is encoded by a single gene containing three introns and four exons (Figure 1.2; Fernald and White, 1999). Invertebrate genes encoding GnRH have been investigated in tunicates: *Ciona intestinalis* has two GnRH genes, and each gene encodes three GnRH isoforms (Adams et al.,

2003). One of the two genes has no introns, while the other one has two introns. It is noteworthy that seven of nine tunicate (t) GnRH isoforms (tGnRH 3-9) have not been isolated from tunicates and are only known from genomic analysis.

Traditionally, researchers name a GnRH peptide according to the name of the animal from which the particular peptide was initially identified. The name can be either a common one for a certain kind of animal, such as salmon (s)GnRH, or a name for a systematic class, such as mammalian (m)GnRH. However, this traditional nomenclature does not indicate the phylogenic distribution of various GnRH peptides. For example, chicken (c)GnRH-II was named after the species from which it was initially isolated (Miyamoto et al., 1984), yet it has been found in almost all vertebrate classes (King and Millar, 1995). Therefore, a new nomenclature system has been proposed to directly relate the name of GnRH peptides to their brain distributions, functions, and origins.

Fernald and White (1999) proposed that vertebrate GnRH peptides could be grouped into three types, GnRH type 1, GnRH type 2, and GnRH type 3, based mainly on their gene structures and spatial distribution in brains. According to this scheme, GnRH type 1, GnRH type 2, and GnRH type 3 refer, respectively, to the hypophysiotropic GnRH peptides, the GnRH peptides that are located only in the midbrain, and sGnRH, which has been found only in the telencephalon of bony

fish. This nomenclature does not conflict with the findings concerning the embryonic origin of GnRH neurons since GnRH type 1 and GnRH type 3 neurons originate from the olfactory placode and GnRH type 2 neurons originate within the midbrain (White and Fernald, 1998; Fernald and White, 1999). However, this nomenclature is problematic and does not account for several known properties of GnRH. It is noteworthy that the classical GnRH type 2, cGnRH-II, has been discovered not only in the midbrain but also in other regions of the brain, such as the forebrain (Licht et al., 1994; Muske and Moore, 1994). Moreover, GnRH type 1 and GnRH type 2 are also expressed in non-neuronal tissues, including kidney, liver, heart, bone, muscle, prostate, and gonad (Ohno et al., 1993; Kakar and Jennes, 1995; White et al., 1998). Also, GnRH type 3 is found in the goldfish ovary (Pati and Habibi, 1998). Furthermore, GnRH type 2 and GnRH type 3 could also play a hypophysiotropic role since they induce GTH release *in vivo* in sheep and goldfish, respectively (Peter et al., 1990; Millar et al., 2001), and GnRH type 2 (cGnRH-II) fibres also project to the anterior pituitary in goldfish. In addition, this scheme ignores the reports that a sGnRH-like molecule has also been found in the hypothalamus of the frog, *Rana esculenta* (Cariello et al., 1989; Di Matteo et al., 1996).

Despite flaws in the new GnRH nomenclature scheme, this protocol may still

be useful for vertebrates because of its largely accurate representation of the phylogenetic lineages of vertebrate GnRH isoforms. Whether and how the invertebrate GnRH peptides fit into this nomenclature scheme are questions that are largely unresolved because of the limited information on invertebrate GnRH peptides. Invertebrate GnRH isoforms have only been identified in four species representing only two groups, tunicates (*Chelyosoma productum*, Powell et al., 1996; *Ciona savignyi* and *Ciona intestinalis*, Adams et al., 2003) and octopuses (*Octopus vulgaris*, Iwakoshi et al., 2002). In addition, a hypothalamus-pituitary axis is lacking in invertebrates and the role and origin of invertebrate GnRH peptides are not as well studied relative to the situation in vertebrates. Thus for the present time, the traditional nomenclature may be more convenient (or useful) in the naming of invertebrate GnRH peptides.

GnRH functions

Reproduction

GnRH has been proposed to have conserved reproductive functions throughout evolution (Goldberg et al., 1993). In vertebrates, GnRH is the master molecule regulating reproduction because of its ability to stimulate pituitary GTH release. GTH in turn regulates gametogenesis and steroidogenesis in gonadal

tissues. GnRH may also regulate reproduction by paracrine or autocrine influences directly in the gonads. For example, GnRH can inhibit the GTH-stimulated expression of gonadal GTH receptors (Piquette et al., 1991; Tilly et al., 1992). As well, GnRH can also directly act at the level of the ovary to promote meiosis of oocytes (Pati and Habibi, 2000). Intracerebroventricular injection of GnRH also promotes mating-related behaviors, suggesting that GnRH neurons in the brain also participate directly in the regulation of reproduction (Maney et al., 1997).

In fish, GnRH can act through an alternative hypophysiotropic pathway to manipulate many biological functions. GnRH binding sites have been located on fish somatotropes (Cook et al., 1991; Stefano et al., 1999), and GnRH increases the release of growth hormone (GH) and its mRNA expression in many fish models (Habibi et al., 1992; Lin et al., 1993; Melamed et al., 1996; Klausen et al., 2001). In turn, GH stimulates the protein syntheses in a variety of biological responses, and enhances GTH-induced gonadal steroidogenesis (Van der Kraak et al., 1990). Thus, by regulating GH release, GnRH indirectly regulates many reproductive, as well as non-reproductive, functions.

In invertebrates, research has similarly indicated a role for GnRH in reproduction. In tunicates, GnRH neurons project towards the gonads (Terakado,

2001) and injection of tGnRH 2-9 enhances spawning (Adams et al., 2003). In scallops, mGnRH stimulates spermatogonial mitosis and vitellogenesis; conversely, treatment with a GnRH antagonist and/or an antibody against mGnRH attenuates the ability of pedal and cerebral ganglia extracts to stimulate these processes (Nakao et al., 2005; Osada et al., 2005). In coral, a GnRH agonist stimulates steroidogenesis (Twan et al., 2006). Furthermore, addition of vertebrate and/or invertebrate GnRH isoforms to the bathing medium stimulates the release of gametes in a hemichordate (*Saccoglossus*; Gorbman et al., 2003), a chiton (*Mopalia*; Gorbman et al., 2003) and a gastropod (*Helisoma*; Young et al., 1997). Taken together, these results suggest that GnRH acts as a neural, neuroendocrine and pheromonal regulator of reproduction in invertebrates.

Other functions of GnRH

In addition to its reproductive actions, GnRH is also a well-known immunomodulator in mammals. It stimulates the expression of interleukin-2 receptors in lymphocytes (Batticane et al., 1991) and affects the cell-mediated immune response in lymphoid tissues (Marchetti et al., 1990; Mann et al., 1999). GnRH likely suppresses immune function *in vivo* by decreasing the number of B cells, $CD4^+$ T cells and $CD25^+$ T cells (Rao et al., 1993; Ho et al., 1995), although

a recent study shows that GnRH increases B cell proliferation in cell culture conditions (Tanriverdi et al., 2004). Moreover, GnRH mRNA and its receptor mRNA have been found in lymphocytes, suggesting that locally produced GnRH regulates the immune system through paracrine and autocrine actions (Chen et al., 1999).

In addition to the above mentioned immune functions, GnRH is known to play neuromodulatory roles in vertebrates. GnRH regulates the pacemaker activity of some GnRH neurons in bony fish (Abe and Oka, 2000) and inhibits the M-current of frog sympathetic neurons (Jones, 1987). GnRH also affects visual processing by acting on amacrine and ganglion cells of the retina (Grens et al., 2005). In invertebrates, GnRH may also function as a neuromodulator. GnRH neurons has been located in the tunicate cerebral ganglion (Tsutsui et al., 1998), and GnRH affects the firing pattern of anti-GnRH immunoreactive mature neurons and the neurite outgrowth of embryonic neurons, in the snail *Helisoma trivolvis* (Goldberg et al., 1993). GnRH also alters the firing pattern of neuroendocrine bag cells in *Aplysia californica*, a sea hare (Zhang et al., 2000).

GnRH may act as a neurotransmitter or neuromodulator to regulate feeding in vertebrates. In mammals, brain regions for feeding regulation contain GnRH receptors and axon projections of GnRH neurons (Kauffman and Rissman, 2004;

Kauffman et al., 2005). In addition, intracerebral ventricular administration of cGnRH-II can decrease food intake while increasing sexual behavior (Kauffman and Rissman, 2004; Kauffman et al., 2005). Furthermore, there is evidence that neuropeptides known to regulate feeing and satiation also interacts with the GnRH neuronal system. For example, neuropeptide Y receptors are expressed in GnRH neurons (Yang et al., 2005) while central administration of neuropeptide Y inhibits mating behavior and stimulates feeding (Corp et al., 2001). Similarly, many anti-GnRH and anti-galanin immunoreactive nerve fibers are located closed to each other in brain while centrally administrated galanin-like peptide (GALP) stimulates feeding and increases plasma luteinizing hormone (LH) and testosterone levels (Kageyama et al., 2005). It is possible that GnRH neurons are part of an integrated system that regulates feeding and reproduction.

GnRH receptors and postreceptor signaling

GnRH receptors belong to the G-protein coupled receptor (GPCR) family. Like typical GPCRs, GnRH receptors have seven transmembrane domains, three extracellular loops and three intracellular loops (Figure 1.3). The extracellular domains (N-terminus and extracellular loops) are responsible for ligand binding and the intracellular domains are important in G-protein signaling. When GnRH

binds to its receptor and activates the coupled G-protein, the major signal activated is phospholipase C (PLC) and its signaling cascade (Figure 1.4). PLC, activated by G-protein, hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 causes release of calcium from intracellular IP_3-sensitive calcium stores. Both calcium and DAG can activate protein kinase C (PKC). Activated PKC and increased intracellular calcium concentration trigger other biochemical changes, such as activation of calcium and calmodulin (CaM)-dependent kinases, leading to alterations in cellular activity. Moreover, hydrolysis of PIP_2 lowers the concentration of this important membrane regulator of cation channels (Yue et al., 2002; Loussouarn et al., 2003; Rohacs et al., 2005). Thus, GnRH-induced alterations in channel activity affect many cell functions, including exocytosis (Holz et al., 2000). In addition to the PLC pathway, GnRH receptor signaling involves activation of the mitogen-activated protein kinase (MAPK) pathway (Sim et al., 1995). Activation of MAPK has been linked to the stimulation of GTH gene expression in vertebrates (Klausen et al., 2002); however, the mechanism underlying MAPK-regulated GTH gene expression is not well understood (Harris et al., 2002).

Based on similarities in amino acid sequences, vertebrate GnRH receptors can be divided into three types: type I, type II and type III (Millar et al., 2004). The

presence of a functional type I GnRH receptor appears to be conserved throughout evolution. Unlike nonmammalian type I receptor, mammalian type I receptors do not contain the C-terminal tail (Figure 1.3 B) that has been implicated in rapid receptor desensitization (Davidson et al., 1994). The type II receptor is functional in some species and silent in other species (e.g. in human). Type III receptors are only found in the lower vertebrates, fishes and frogs. In addition, other forms of GnRH receptors are thought to be present in the gonads (Klausen et al., 2002).

In invertebrates, a GnRH receptor homolog has first been cloned in the fruit fly (Hauser et al., 1998), although its ligand is adipokinetic hormone (AKH; Staubli et al., 2002). On the other hand, invertebrate forms of GnRH, including octGnRH and tGnRH 3-9 peptides, can stimulate GTH release from cultured pituitary cells (Iwakoshi et al., 2002; Adams et al., 2003), as do GnRH-like factors in snails and corals (Goldberg et al., 1993; Twan et al., 2006). Since invertebrate GnRH isoforms are recognized by at least some vertebrate GnRH receptors, it appears likely that invertebrate GnRH receptors would display a high degree of structural similarity to vertebrate GnRH receptors. Two functional GnRH receptors have been consistently revealed from the tunicate, *Ciona intestinalis*, by two separate research groups (Kusakabe et al., 2003; Tello et al., 2006). Each of them has at

least 30% amino acid identity with human GnRH receptor. Interestingly, one of them can bind both mGnRH and three tGnRH isoforms tested (Barran et al., 2005). Recently, two protostomian GnRH receptors have been revealed. An oyster GnRH receptor was cloned, but neither bioassay nor ligand-receptor binding assay was performed (Rodet et al., 2005). In addition, Kanda et al. (2006) cloned a GnRH receptor from *Octopus vulgaris* (octGnRH receptor). The octGnRH receptor shares around 30% amino acid similarity with other known GnRH receptors. Also, this receptor is expressed in *Octopus* brain and many peripheral tissues, such as gonad, oviduct, heart, stomach, and radular retractor muscle. Furthermore, octGnRH can induce inward currents in frog oocytes expressing octGnRH receptor. The current was thought to be induced by the IP_3/Ca^{2+} pathway.

GnRH studies in mollusks

Demonstration of the presence of a bioactive GnRH-like factor in *Helisoma trivolvis* by Goldberg et al. (1993) marked a milestone in GnRH studies in mollusks. Prior to this study, only three reports were published on the effects of GnRH on molluscan neurons (Barker et al., 1975; Seaman et al., 1980; Steiner and Felix, 1989). The earliest study found no effect of GnRH application on

neuron 11 of the land snail *Otala* and neuron R15 of the sea hare *Aplysia* (Barker et al., 1975). On the other hand, the two later studies suggested that GnRH may affect the activity of molluscan neurons. GnRH increased the firing rate in 4 of 5 white-cell neurons in *Aplysia* (Seaman et al., 1980), and microiontophoretic application of GnRH had only moderate effects on action potential activity in giant dopaminergic neurons of the fresh water snail *Planorbis* (Steiner and Felix, 1989). However, neither vehicle controls nor statistical analyses were performed in the study by Steiner and Felix (1989).

Goldberg et al. (1993) clearly demonstrated the presence of a bioactive GnRH-like factor in mollusks. In this study, GnRH immunoreactivity was located in the brain of the fresh water snail *Helisoma*. The presence of GnRH immunoreactive substances in the *Helisoma* brain was shown by high-performance liquid chromatography (HPLC). Two GnRH immunoreactive peaks were identified, with one peak migrating at the same rate as mGnRH. Application of mGnRH onto cultured embryonic neurons inhibited neurite outgrowth in over 50% of the neurites tested. GnRH also induced electrophysiological responses in over half of the adult *Helisoma* neurons that were surveyed. Furthermore, extracts of adult *Helisoma* brains induced the release of GTH from fish pituitary cells.

Following the seminal study of Goldberg et al. (1993), the amount of information on GnRH in mollusks has greatly increased. Evidence for the presence of a bioactive GnRH system has since been provided in four classes of mollusks, Gastropoda, Bivalvia, Polyplacophora and Cephalopoda. In *Aplysia*, anti-GnRH immunoreactivity was found in the ovotestis, hemocytes, hemolymph, central nervous system (CNS) and its surrounding sheath, and the osphradium, a chemosensory organ (Zhang et al., 2000; Tsai et al., 2003). Although brain extracts of *Aplysia* failed to stimulate LH release from mouse pituitary glands, cGnRH-II decreased the action potential afterdischarge in *Aplysia* bag cells, a characteristic firing pattern that triggers hormone release in these cells (Zhang et al., 2000; Tsai et al., 2003).

In the fresh water snails *Helisoma* and *Lymnaea*, anti-GnRH immunoreactivity has been detected in the brain, penial nerve and penial complex of the male reproductive tract (Young et al., 1999). Using a commercially available anti-mGnRH antibody on sectioned tissues, this study expanded the location of anti-GnRH immunoreactivity in the *Helisoma* brain originally described by Goldberg et al. (1993). Furthermore, research on *Helisoma* showed that exposure of snails to mGnRH increased egg laying (Young et al., 1997), and that GnRH altered the firing activity of buccal neuron B19 (Ramakrishnan and Murphy, 2003).

GnRH expression and activity have also been demonstrated in other molluscan classes.

In two marine bivalves, *Crassostrea* and *Mytilus,* several GnRH isoforms (mGnRH, sGnRH, cGnRH-I, cGnRH-II, and lGnRH-I) stimulated gonadal DNA synthesis (Pazos and Mathieu, 1999). In the scallop *Patinopecten*, anti-GnRH immunoreactivity and GnRH bioactivity have been found in the cerebral and pedal ganglia (Nakamura et al., 2005) and mGnRH promoted spermatogenesis and vitellogenesis (Nakao et al., 2005; Osada et al., 2005). Moreover, exposure to lGnRH-I and tGnRH-2, but not lGnRH-III, tGnRH-1 and cGnRH-II, stimulated spawning in the chiton *Mopalia* (Gorbman et al., 2003). In cephalopods, octGnRH has been purified from *Octopus* (Iwakoshi et al., 2002), and anti-GnRH immunoreactivity has been located in the CNS and many peripheral organs, such as the optic gland, heart, and reproductive ducts (Di Cosmo and Di Cristo, 1998; Di Cristo et al., 2002; Iwakoshi-Ukena et al., 2004). Furthermore, octGnRH was found to modulate contractions of the heart and oviduct (Iwakoshi-Ukena et al., 2004). In addition, as discussed previously, GnRH receptors have recently been cloned from oyster (Rodet et al., 2005) and octopus (Kanda et al., 2006).

Background of experimental animal

The experimental animal, *Helisoma*, has a simple nervous system that has been exploited as an experimental model for neuroscience research. First of all, the number of neurons in *Helisoma* is few compared with that in mammals. The brain of pond snails contains only a few thousand neurons, while the human cortex contains over 10 billion (Pakkenberg and Gundersen, 1997). Moreover, the organization of the nervous systems in *Hellsoma* is also quite simple. The snail brain consists of a circumoesophageal ganglia complex and a pair of buccal ganglia (Figure 1.5). The circumoesophageal ganglia consist of paired cerebral ganglia, pedal ganglia, parietal ganglia, pleural ganglia and a single visceral ganglion. It is noteworthy that anti-GnRH immunoreactive neurons have been located in all eleven ganglia (Young et al., 1999). Not all pairs of ganglia are symmetrical. The left cerebral ganglion has a lateral extension, named the cerebral extension (CE), and the left parietal ganglion is much bigger than the right parietal ganglion. There are also identifiable giant neurons (with soma of greater than 40 μm in diameter) in the *Helisoma* brain. These two properties, the simple organization of the nervous system and existence of large identifiable neurons, facilitate the ease and reproducibility of electrophysiological manipulations. Furthermore, some of these identifiable neurons have known

functions and can be isolated and maintained in culture for extended periods of time. These characteristics make them prime candidates for both *in situ* and *in vitro* manipulations in studies on neural control of select physiological functions.

Historically, the successful establishment of an embryonic neuronal culture system (Goldberg et al., 1988) rendered *Helisoma* as one of the first animal models with parallel adult and embryonic neuronal culture systems. This seminal research provided a new opportunity for comparing the mechanisms underlying regeneration and development of neurites. Later studies on the effects of serotonin on neurite outgrowth suggested that the regulatory mechanisms are not completely conserved between neurite regeneration in adult neurons and neurite development in embryonic neurons (Goldberg et al., 1991). The expanding structure at the tip of a neurite is called a growth cone, which navigates the outgrowth direction and extends the leading edge of the neurite during elongation (Nicholls et al., 1992). Active growth cones contain a membranous veil, the lamellipodium, and many fingerlike protrusions named filopodia. Research from both adult and embryonic culture systems of *Helisoma* neurons has shown that the growth cone is a unit, relatively independent from the soma, to sense environmental stimuli (Kater and Mattson, 1988; Goldberg et al., 1991).

Helisoma are hermaphrodites, and thus possess both male and female

reproduction systems (Lutz, 1985). They have shared ovotestis and a hermaphroditic duct to generate and carry gametes, respectively. In the junction area between the ovotestis and hermaphroditic duct, there are seminal vesicles that store sperms. Downstream of the hermaphroditic duct, the female reproductive tract consists of the oviduct, bursa copulatrix, vagina and associated glands, while the male reproductive tract consists of the vas deferens, penial complex and prostate gland. The male reproductive tract is innervated by the penial nerve, which extends out mainly from the CE and is covered by connective tissues. Anti-GnRH immunoreactivity has been found in the ovotestis, female reproductive glands, prostate gland and penial nerve (Young et al., 1997; Young et al., 1999). Prominent anti-GnRH immunoreactivity is located in the penial complex, especially in nerve fibers in the area where vas deferens enters the penial complex (Young et al., 1999). Furthermore, prominent anti-GnRH immunoreactivity also occurs in a dense array of neurites within the neuropil of the CE, and anti-FMRFamide immunoreactivity is also found in this structure (Saleuddin et al., 1992; Young et al., 1999). These observations lead to the speculation that neuropeptides act on CE neurons that subsequently innervate the male reproductive tract through the penial nerve. In addition, neurons outside the CE area project neurites that pass through the CE area and enter the penial

nerve. These neurons may also release neuropeptides in the male reproductive tract of pond snails. Evidence for the possible participation of multiple neuropeptides in the regulation of pond snail reproductive tract activities has come from experiments on *in vitro* preparations of the vas deferens, the preputium of the penial complex, and the penis retractor muscle of the pond snail *Lymnaea* (van Golen et al., 1995a; van Golen et al., 1995b; van Golen et al., 1996; de Boer et al., 1997; de Lange et al., 1997). APGWamide causes relaxation of the preputium and the anterior vas deferens (van Golen et al., 1995b; de Boer et al., 1997), while conopressin causes contraction of the vas deferens (van Golen et al., 1995b). FMRFamide and FLRFamide cause contraction of the penial retractor muscle, whereas SDPFLRFamide and GDPFLRFamide can cause relaxation of the penial retractor muscle (van Golen et al., 1995a). To date, our knowledge about the reproductive control of pond snails is mostly based on studies on *Lymnaea*, whereas less research has been done to explore the neuroendocrine control of *Helisoma* reproduction.

Neural control of feeding behavior has been studied in *Heiisoma*. Snails find their food using tentacles which detect environmental information, move toward the food, and then eat the food (Elliott and Susswein, 2002). The eating process of *Helisoma* can be divided into three rhythmically repetitive stages, protraction,

retraction, and hyper-retraction (swallowing) (Murphy, 2001). Unique patterns for each stage of activity have been detected in buccal motor neurons, such as neuron B5 and neuron B19 (Quinlan and Murphy, 1991; Quinlan et al., 1995). These motor neuron activities were controlled by the central pattern generator (CPG), which is made up of identified interneurons (Quinlan and Murphy, 1996). The CPG underlying feeding consists of three subunits, S1, S2, and S3 (Quinlan et al., 1995; Murphy, 2001). Each unit controls their corresponding motor neurons, and subsequently controls the three stages of consummatory process. Also, many neurotransmitters may play a role in *Helisoma* feeding by altering the activities of neurons controlling feeding. Dopamine induced fictive feeding behavior and corresponding patterns of neuronal activity, which could be blocked by a dopamine antagonist (Quinlan et al., 1997). Serotonin failed to induce the classic feeding patterns, although it did increase the intensity of burst firing in neuron B5 and B19 *in situ* (Quinlan et al., 1997). FMRFamide and GnRH reduced neuron B19 firing *in situ* (Murphy, 1990; Ramakrishnan and Murphy, 2003). GnRH also increased neuron B5 firing *in situ* (Garofalo et al., unpublished).

Motor neuron B5 innervates the esophagus and gut of *Helisoma* and fires regularly *in situ* (Murphy and Kater, 1980; Murphy et al., 1985; Quinlan and Murphy, 1996). However, neurons that stimulate the firing of B5 have not been

identified, although the firing of B5 is inhibited by some interneurons, such as N3a (Quinlan and Murphy, 1996). Since anti-GnRH immunoreactive neurons are present in the buccal ganglia (Young et al., 1999), it is possible that some interneurons release GnRH to regulate B5 activity. In contrast, 5-HT has been excluded from playing a stimulatory role since 5-HT actually reduces firing of neuron B5 in culture (Achee and Zoran, 1997). To test the potential role of GnRH, direct effects of GnRH can be investigated in single cell cultures of neuron B5.

Hypotheses and objectives

Given that there is a bioactive GnRH-like factor in *Helisoma* adults, the main hypothesis of this thesis is that GnRH has physiological actions in this species. In order to test this hypothesis, I constructed a series of three questions based on the fact that GnRH requires the presence of a GnRH receptor to be functional. The three questions addressed are:

1) Is a GnRH receptor-like molecule present?

2) If it is present, where would these potential GnRH receptors be found?

3) Do potential target cells containing these GnRH receptor-like molecules respond to exogenously applied GnRH?

Briefly, since anti-GnRH immunoreactivity has been detected in the *Helisoma*

brain and penial complex (Goldberg et al., 1993; Young et al., 1999), I hypothesized that GnRH target cells, and consequently GnRH receptors, are likely present in these tissues. To test this hypothesis, Western blot analysis of tissue protein extracts was carried out using two commercially available antibodies against the human GnRH receptor type I: a polyclonal antibody raised against its third intracellular loop and a monoclonal antibody raised against its extracellular N-terminus. Immunohistochemistry on wholemounts of *Helisoma* brains, especially CEs, and penial complexes were also carried out in an attempt to visualize the distribution of GnRH receptor in these tissues. Since previous studies showed that intensive anti-mGnRH immunoreactivity is located in the neuropil area of the CE (Young et al., 1999) and that mGnRH can affect the firing of buccal neuron B5 in ganglionic preparations (Garofalo et al., unpublished), I further hypothesized that CE and B5 cells are GnRH targets. Thus, immunocytochemistry was also carried out in cultures of mixed populations of dispersed CE cells and cultures of individual B5 neurons. In addition, the effects of exogenous mGnRH on the electrophysiological activities of cultured CE neurons and B5 neurons were studied. To facilitate these studies on cultured neurons, a system for culturing CE neurons was developed *de novo* and characterized; concurrently, existing protocols for culturing neuron B19 (Price and

Goldberg, 1993) were modified for studies on neuron B5.

Figure 1.1 Amino acid sequences of all known GnRH isoforms. (A) Green color indicates the relatively conserved N-terminus (pyr-Glu-His-Trp-Ser) and highly conserved C-terminus (Pro-Gly-NH_2). cGnRH: chicken GnRH; cfGnRH: catfish GnRH; dfGnRH: dogfish GnRH; gpGnRH: guinea pig GnRH; hGnRH: herring GnRH; lGnRH: lamprey GnRH; mGnRH: mammalian GnRH; mdGnRH: medaka GnRH; rGnRH: *Rana* GnRH; sGnRH: salmon GnRH; sbGnRH: seabream GnRH; tGnRH: tunicate GnRH; and wfGnRH: whitefish GnRH. (B) Octopus GnRH (octGnRH) has twelve amino acids and a modified N-terminal region (pyr-Glu-Asn-Tyr-His-Phe-Ser), with the insertion of two extra residues, Asn and Tyr. tGnRHs and octGnRH are invertebrate GnRH; all the others are vertebrate GnRH (adopted from Millar et al., 2004).

A

	N'									C'
cGnRH-I	pyr-Glu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly-NH_2
cGnRH-II	pyr-Glu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH_2
cfGnRH	pyr-Glu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly-NH_2
dfGnRH	pyr-Glu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly-NH_2
gpGnRH	pyr-Glu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	Gly-NH_2
hGnRH	pyr-Glu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly-NH_2
lGnRH-I	pyr-Glu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly-NH_2
lGnRH-III	pyr-Glu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly-NH_2
mGnRH	pyr-Glu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2
mdGnRH	pyr-Glu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	Gly-NH_2
rGnRH	pyr-Glu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	Gly-NH_2
sGnRH	pyr-Glu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly-NH_2
sbGnRH	pyr-Glu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly-NH_2
tGnRH 1	pyr-Glu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly-NH_2
tGnRH 2	pyr-Glu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly-NH_2
tGnRH 3	pyr-Glu	His	Trp	Ser	Tyr	Ala	Leu	Ser	Pro	Gly-NH_2
tGnRH 4	pyr-Glu	His	Trp	Ser	Leu	Ala	Leu	Ser	Pro	Gly-NH_2
tGnRH 5	pyr-Glu	His	Trp	Ser	Asn	Gln	Leu	Thr	Pro	Gly-NH_2
tGnRH 6	pyr-Glu	His	Trp	Ser	Tyr	Glu	Phe	Met	Pro	Gly-NH_2
tGnRH 7	pyr-Glu	His	Trp	Ser	Tyr	Glu	Tyr	Met	Pro	Gly-NH_2
tGnRH 8	pyr-Glu	His	Trp	Ser	Lys	Gly	Tyr	Ser	Pro	Gly-NH_2
tGnRH 9	pyr-Glu	His	Trp	Ser	Asn	Lys	Leu	Ala	Pro	Gly-NH_2
wfGnRH	pyr-Glu	His	Trp	Ser	Tyr	Gly	Met	Asn	Pro	Gly-NH_2

B

Octopus GnRH

N'										C'
pry-Glu	Asn	Tyr	His	Phe	Ser	Asn	Gly	Trp	His	Pro	Gly-NH_2

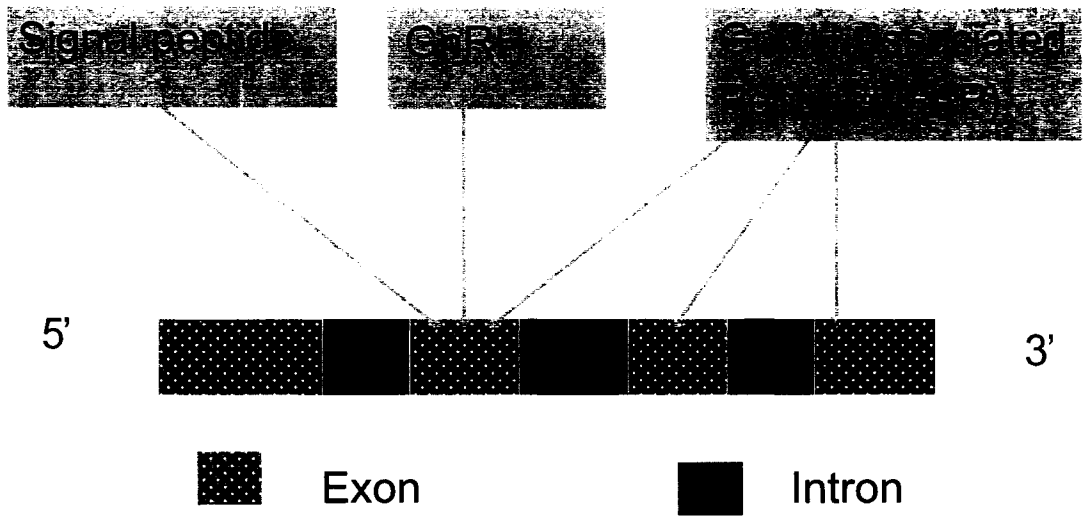
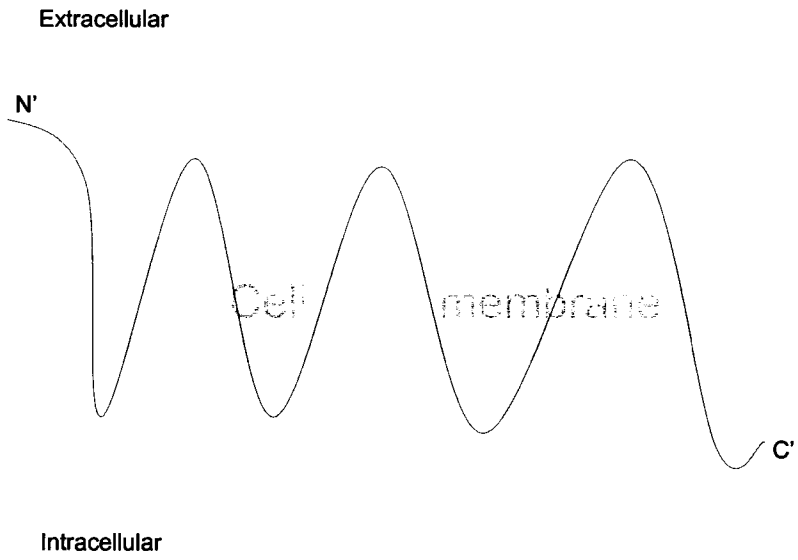


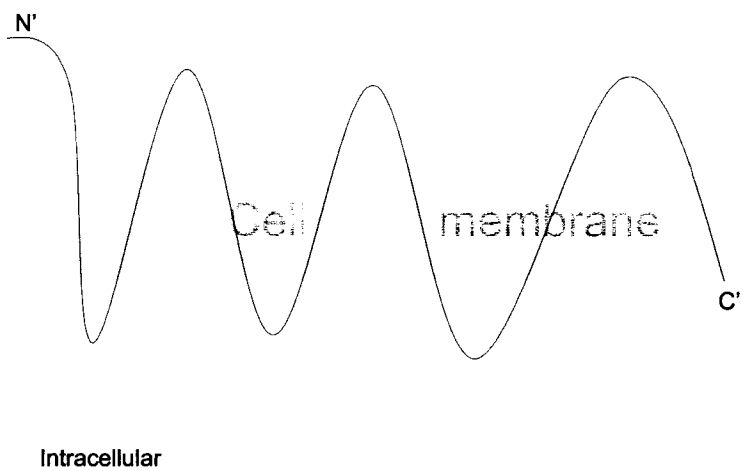
Figure 1.2 Typical vertebrate GnRH gene structure containing three introns and four exons. The second exon contains gene sequences encoding a signal peptide, GnRH and part of GAP. GAP is also encoded by the gene sequences in the third exon and part of the fourth exon (adopted from Fernald and White, 1999).

Figure 1.3 Schematic structure of GnRH receptor. GnRH receptor contains seven transmembrane domains, three extracellular loops and three intracellular loops. (A) Typical structure of GnRH receptor. (B) Mammalian type I GnRH receptor does not have the C-terminal tail.

A



B



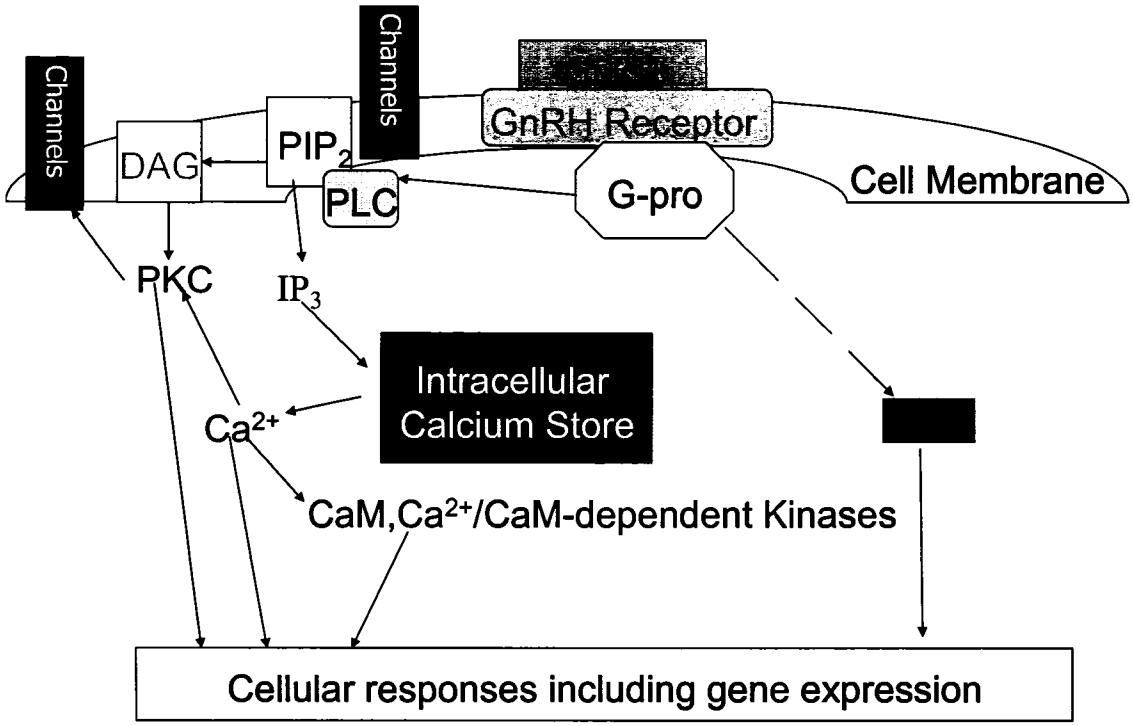


Figure 1.4 Schematic of the major signal transduction pathways of GnRH. Broken line: the mechanism for the activation of MAPK pathway is still under investigation; GnRH could activate this pathway through different mechanisms (Naor et al., 2000; Harris et al., 2002; Kraus et al., 2004).

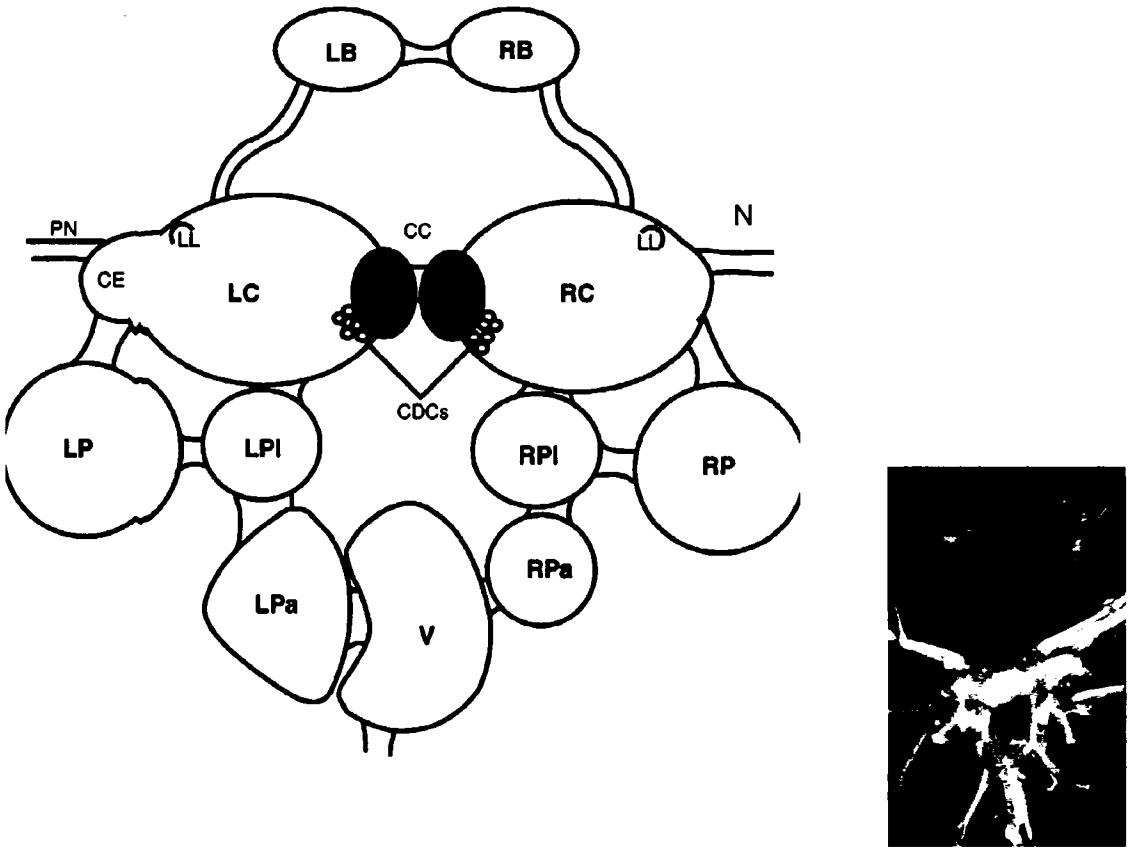


Figure 1.5 A schematic of *Helisoma* brain. Inset: a picture of the *Helisoma* brain pinned in dish with pedal commissure cut to spread all ganglia in one plate. CC: cerebral commissure; CDCs: caudodorsal cells; CE: cerebral extension; LB: left buccal ganglion; LC: left cerebral ganglion; LL: lateral lobe; LP: left pedal ganglion; LPa: left parietal ganglion; LPl: left pleural ganglion; N: nerve trunk; PN: penial nerve; RB: right buccal ganglion; RC: right cerebral ganglion; RP: right pedal ganglion; RPa: right parietal ganglion; RPl: right pleural ganglion; V: visceral ganglion (adopted from Young, 1998; Ahn and Goldberg, 2003).

CHAPTER 2 - MATERIALS AND METHODS

Experimental animal

Adult pond snails, *Helisoma trivolvis*, with vertical shell diameter between 8mm and 15mm were used as experimental animals. They were reared at around 25 °C in de-chlorinated city of Edmonton water in flow-through aquaria under a 12 hr light/12 hr dark photoperiod. Each aquarium contained around 30 adult snails and an oyster shell substratum. A piece of lettuce and around 30 pieces of trout chow (United Feeds) were added to each aquarium every week. All experiments were carried out at room temperature except where indicated otherwise.

Deshelling

Snails were anaesthetized with 25% listerine in artificial pond water (0.025% Instant Ocean; Aquarium Systems) for 15 minutes. From the aperture, snail shells were cut along the dorsal midline. Then shells were gently split into two parts. The columellar muscle attaching each snail to its shell was transected using stainless steel scissors (Fisher) to release snails from shells. Following this, snails were soaked in 25% listerine in artificial pond water for 15 minutes, washed with artificial pond water twice for 1 minute each, and washed with *Helisoma* saline

(HS: 51.3 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 5 mM HEPES; pH 7.45) for 1 minute.

Dissection

Deshelled snails were pinned onto silicone rubber (General Electric)-coated dissecting dishes filled with antibiotic *Helisoma* saline (HS containing 150 mg/ml gentamicin, Sigma). The mantle was cut along the midline to expose the penial complex and brain. Either one or both these structures were removed, depending upon the experimental needs.

Protein extraction and Western blot

Brains or penial complexes from 10 snails were washed with ice-cold phosphate buffered saline (PBS: 0.056 M $Na_2HPO_4 \cdot 7H_2O$, 0.154 M NaCl; pH 7.2) 3 times at 10 minutes each and mechanically homogenized in a microhomogenizer (Radnoti) containing ice-cold 100 µl homogenization buffer (1% Triton-X 100, 250 mM sucrose, 1 mM ethylenediaminetetraacetic acid, 30 mM Tris, 2 mM phenylmethylsulphonylfluoride, 1mM benzamidine, and 0.15 mM aproteinin; pH 7.4). The homogenate was centrifuged at 5000 x g for 5 minutes at 4 ℃. The supernatant was mixed with an equal volume of 2X Laemmli loading

buffer (4% sodium dodecyl sulfate (SDS), 20% glycerol, 125 mM Tris-Cl pH 6.8, 200 mM dithiothreitol, and 1% bromophenol blue), heated in a waterbath at 55 °C for 15 minutes, and then separated by gel electrophoresis for 20 minutes at 100 mV and 1 hour at 200 mV. The gel consisted of a thin upper layer of stacking gel (0.1% SDS, 120 mM Tris-Cl pH 6.8, 4% acrylamide/bis, 0.05% ammonium persulfate, and 0.1% tetramethyl ethylenediamine (TEMED)) and a thick bottom layer of separating gel (0.1% SDS, 375 mM Tris-Cl pH 8.8, 10% acrylamide/bis, 0.05% ammonium persulfate, and 0.05% TEMED). After electrophoresis, proteins in the gel were transferred onto a nitrocellulose membrane which was then stained by ponceau red to monitor protein transfer. Further processing of the membrane was performed with shaking. The membrane was treated with 3% non-fat milk in PBST (PBS containing 0.5% Tween 20) for one hour to minimize nonspecific binding and then incubated with primary antibodies at 1:300 dilution in 3% non-fat milk in PBST for 18 hours at 4°C. The primary antibody was either rabbit polyclonal antibody (Sigma) raised against the 3rd intracellular loop of human GnRH receptor type I or mouse monoclonal antibody (Lab Vision, California) raised against the N-terminus of human GnRH receptor type I. The membrane was washed with 3% non-fat milk in PBST for 3 times at 8 minutes each and then incubated with horseradish peroxidase-conjugated secondary

antibodies (1:1000 dilution in 3% non-fat milk in PBST) for one hour. The secondary antibody was either goat anti-rabbit IgG antibody or goat anti-mouse IgG antibody (Sigma), depending on which primary antibody was used. The membrane was washed with 3% non-fat milk in PBST 3 times for 10 minutes each, followed by a single wash with PBST for 1 minute. Finally, under non-shaking conditions, bands were detected using the ECL Western Blotting Detection kit (Amersham Biosciences) according to instructions provided by the manufacturer. Positive controls were performed using goldfish pituitary cells (kindly provided by Dr. Chang's lab), whereas negative controls were performed by replacement of primary antibodies with normal rabbit or mouse sera.

Cell culture

Mass culture of CE neurons

Culturing method

Brains were dissected from deshelled *Helisoma* under sterile conditions and placed in defined medium (DM: 50% Liebovitz (Life Technologies), 40 mM NaCl, 1.7 mM KCL, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5 mM HEPES, 50 mg/ml gentamicin, and 0.015% glutamine; pH 7.45) for 5 minutes. They were then treated with 0.2% trypsin (Sigma) in DM for 30 minutes, 0.1% trypsin inhibitor (Sigma) in DM for 20

minutes, and washed in DM 3 times for 10 minutes each. The brains were pinned ventral side up in a silicone rubber-coated dish containing DM (Figure 2.1). After the cerebral commissures were crushed by forceps, a slit was made through the sheath covering the CE area using an electrically sharpened tungsten microknife. Cells were gently sucked into a fire-polished, Sigmacote-coated micropipette and plated onto poly-L-lysine-coated (1 μg ml^{-1}; Sigma) 35mm culture dishes (Falcon 3001) containing 2 ml of conditioned medium (CM: made by incubating 2 snail brains per ml DM for 96 hours). Cell cultures were kept in the dark for 12 hours prior to use.

Morphological characterization

Cultured CE neurons were observed daily for up to 6 days. Cells were observed under phase-contrast optics with an inverted microscope (Zeiss). Images were captured by a digital video camera (Q Imaging) mounted on the microscope coupled to a Pentium-4 computer containing Northern Eclipse acquisition and processing software (Empix Imaging).

To determine the optimal conditions for survival of CE neurons, several preparations of day 1 CE neurons cultured in either DM or CM were stained by 0.01% tripan blue for 10 minutes, and then examined under light microscopy.

After the preliminary characterization experiments, CM was used as the culture medium for all cultures.

Single cell culture of neuron B5

The culturing method for neuron B5 was similar to that described above for the CE neuron mass culture except: 1) only the cerebral and buccal ganglia were removed from snails during dissection; 2) the duration for trypsin and trypsin inhibitor treatments were 10 minutes and 5 minutes, respectively; 3) the buccal commissure, esophageal trunks (ETs), and the cerebrobuccal connectives were crushed; 4) the slit was made adjacent to neuron B5; 5) one B5 cell was plated per culture dish; and 6) the pinning was done as shown in Figure 2.2. Ganglia were pinned with sufficient tension such that the soma of neuron B5 would easily pop out when the slit in the sheath was made.

Anti-GnRH receptor immunofluorescence

Wholemount preparations

Immediately following dissection, brains or penial complexes dissociated from the *Helisoma* bodies were washed with ice-cold PBS 3 times, 10 minutes each, and then pinned in silicone rubber-coated dishes. The tissues were fixed

for 18 hours in Zamboni's 2X fixative (4% paraformaldehyde in 7.5 % picric acid in PBS; pH 7.3) at 4 °C. Shaking was applied after fixation until the mounting step. To help the antibody to penetrate the sheath covering the brain, two extra enzyme treatments were applied in brain preparations: the sample was treated with 0.5% pronase (Sigma) in HS for 30 minutes right before fixation and 0.2 % Trypsin in HS for 1 hour right after fixation. Subsequently, samples were washed 4 times, 20 minutes each, with PBS and then washed in 10% horse serum (Gibco) in 4% Triton X-100 in PBS for 45 minutes. Tissues were then incubated in a polyclonal anti-GnRH receptor antibody diluted 1:300 in PBS containing 1% horse serum, 4% Triton X-100 and 3.3 mg/ml bovine serum albumin (BSA; Sigma). After a seventy-two-hour incubation in primary antibody at 4 °C, samples were washed with 4% Triton X-100 in PBS 6 times, 20 minutes each, and then treated with 2% horse serum in 4% Triton X-100 in PBS for 20 minutes. All subsequent steps were carried out in darkness. The secondary antibody was applied at a 1:50 dilution in PBS containing 1% horse serum, 4% Triton X-100 and 3.3 mg/ml BSA. The secondary antibody was an anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC; Sigma). Following a two-hour incubation in the secondary antibody, samples were washed in 4% Triton X-100 in PBS for 10 minutes, followed by 0.4% Triton X-100 in 0.01 M PBS 5 times, 15 minutes each, and then

PBS 3 times, 20 minutes each. The samples were dehydrated through an ascending series of ethanol and then mounted in methyl salicylate (Sigma) for fluorescence microscopy. Negative controls were performed by deletion of the primary antibody.

Cultured neurons

Neuron B5 or CE neurons cultured overnight in glass-bottom culture dishes were fixed for 10 minutes in Zamboni's 2X fixative. After fixation, cells were washed 3 times, 10 minutes each, with PBS to remove all traces of the fixative. To minimize nonspecific binding, cells were washed in PBS containing 10% horse serum and 0.4% Triton X-100 for 30 minutes. Cells were incubated for 18 hours at 4 °C in primary antibody diluted 1:300 in PBS containing 1% horse serum, 0.4% Triton X-100 and 3.3 mg/ml of BSA. Cells were then washed 3 times, 10 minutes each, with 0.4% Triton X-100 in PBS. All subsequent steps were carried out in darkness. The secondary antibody was applied at a 1:50 dilution in PBS containing 1% horse serum, 0.4% Triton X-100 and 3.3 mg/ml of BSA. The secondary antibody was either the goat anti-rabbit IgG conjugated to FITC or a goat anti-mouse IgG conjugated to tetramethylrhodamine isothiocyanate (TRITC; Sigma), depending on which primary antibody was used. Following one-hour

incubation in the secondary antibody, cells were washed in 0.4% Triton X-100 in PBS for 3 times, 10 minutes each, and then PBS 3 times, 10 minutes each. The samples were mounted in 25% glycerol (Sigma) in PBS for fluorescence microscopy. Negative controls were performed by replacement of the primary antibody with normal rabbit or mouse sera.

Electrophysiological recording and pharmacological study

Sharp-electrode intracellular recordings were performed on cells in culture dishes containing 2 ml of CM. Sharp electrodes, with tip resistances of 20 to 60 MΩ, were made from borosilicate glass micropipette (World Precision Instruments) pulled by a micropipette puller (Sutter Instrument). Each electrode was filled with 3 M KCl and connected to a Getting, Model 5A amplifier. Cell penetration was achieved under observation through phase-contrast optics (Nikon TMD). Current injections were achieved through a Master-8 stimulator (A.M.P.I.), and measured with a virtual ground circuit (WPI). A bridge-circuit was used to cancel the stimulus-induced voltage error. Data acquisition was achieved either using a digitizer and pCLAMP software (Axon Instruments) or using a chart recorder (Cole-parmer Instrument).

Recordings were performed in the first day after cell-plating. During

recordings, two sequential applications of vehicle or vehicle followed by drug stock solution were conducted. The vehicle was 2 µl HS. The drug stock solution was 2 µl mGnRH (Sigma or Bachem), mGnRH receptor antagonist ([D-pGlu^{1}, D-Phe^{2}, D-Trp^{3,6}] GnRH; Sigma), or the mixture of mGnRH and antagonist. The drug stock solution was of 1000X fold final concentration.

Statistics

Appropriate statistical methods were utilized according to different situations. Chi-squared test was applied to compare the distribution of morphological subpopulations of CE neurons between DM and CM and to compare proportions of different kinds of cells between DM and CM. *t*-test was performed to compare the length of neurites between DM and CM. Analysis of Variance (ANOVA; factorial analysis) was performed to compare the length of neurites between morphological subpopulations of CE neurons. Fisher's exact test was performed compare input resistance between morphological subpopulations of CE neurons. In neuron B5 electrophysiological studies, data was analyzed by ANOVA, repeated measures, following by Fisher's post hoc least significant difference (PLSD) test. For all statistical methods mentioned above, differences were considered significant when $P<0.05$.

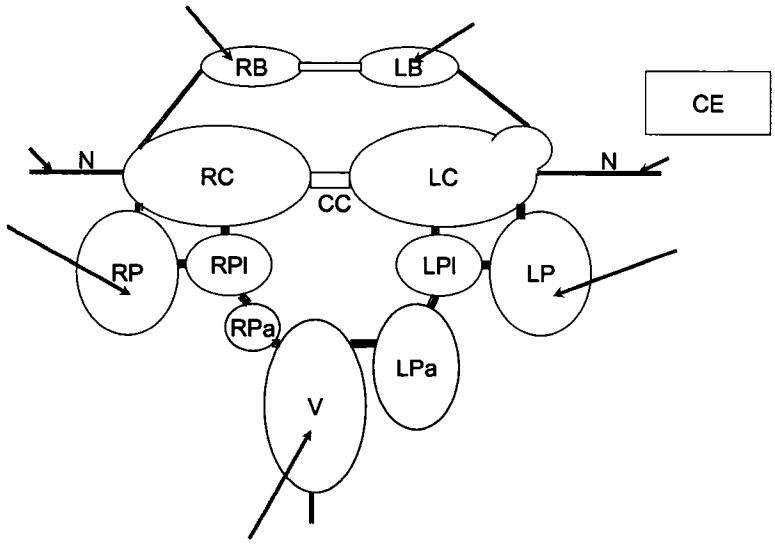
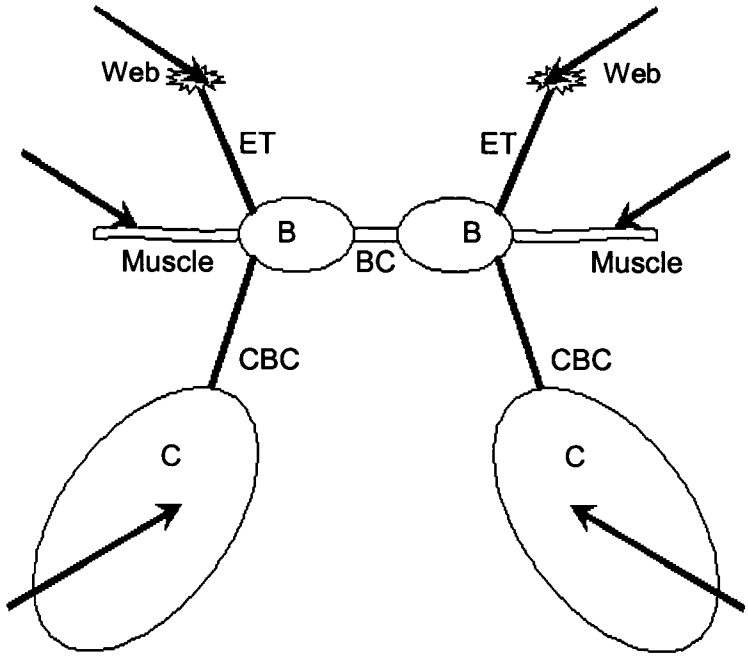


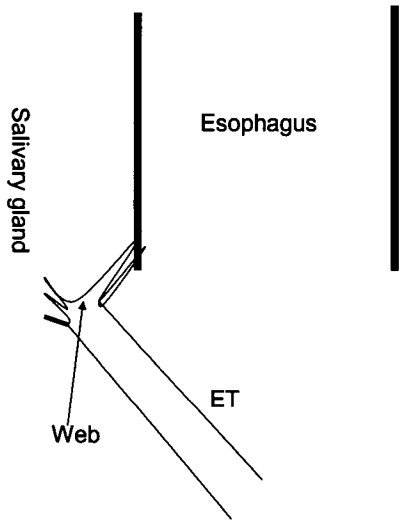
Figure 2.1 Pinning method for CE cell culture. Brains were pinned, ventral side up. The commissure between pedal ganglia was cut to pin the brain flatly. Red arrows represent the pins. The abbreviations are same as Figure 1.5.

Figure 2.2 Pinning method for neuron B5 cell culture. (A) Illustration of the critical way of pinning to isolate the single neuron B5. Ganglia were pinned with their dorsal sides up. The cerebral commissure was cut. Also, the esophagus was cut off. Red arrows represent the pins. The connectives linking the ET to the salivary glands and esophagus (called "web") should be pinned tightly. ET: esophageal trunk; B: buccal ganglion; BC: buccal commissure; CBC: cerebrobuccal connective; C: cerebral ganglion. (B) The *in situ* location of the "web".

A



B



CHAPTER 3 – RESULTS

Detection of anti-GnRH receptor immunoreactivity in *Helisoma* tissues

Western blot analysis was used to determine if GnRH receptor-like molecules are present in extracts of the *Helisoma* brain and penial complex. Using a polyclonal rabbit antibody against the third intracellular loop of the type I human GnRH receptor, one immunoreactive protein band with a molecular weight of approximately 58 kDa was consistently detected in crude extracts of the *Helisoma* penial complex (Figure 3.1). This antibody also reacted positively with five protein bands with molecular weights approximating 80, 74, 65, 58 and 37 KDa in *Helisoma* brain crude extracts (Figure 3.1). Similarly, a mouse monoclonal antibody raised against the extracellular N-terminal portion of the type I human GnRH receptor reacted positively with the *Helisoma* brain and penial complex extracts. Three bands of approximately 80, 74 and 58 kDa stained prominently in both *Helisoma* penial complex and brain extracts (Figure 3.1). Lightly stained bands of approximately 70 and 65 kDa were also detected in the *Helisoma* brain extracts, while the *Helisoma* penial complex extracts also yielded an additional lightly stained 70 kDa band (Figure 3.1). Extracts of goldfish pituitary cells, which are known to express GnRH receptors (Illing et al., 1999), also reacted positively

with these two antibodies. Both antibodies consistently stained two protein bands of approximately 72 and 58 kDa (Figure 3.1), while the monoclonal antibody also stained an additional 65 kDa band (Figure 3.1). Using normal rabbit serum or normal mouse serum in place of the primary antibodies, no positive staining was observed in *Helisoma* and goldfish tissue extracts (Figure 3.1).

Given that previous results with GnRH immunoreactivity have suggested that the CE and penial complex are likely targets for GnRH (Young et al., 1999), the tissue distribution of anti-GnRH receptor immunoreativity in *Helisoma* CE and penial complex was examined next. Immunohistochemical experiments were performed on wholemount brain and penial complex preparations using the rabbit polyclonal antibody mentioned above. Anti-GnRH receptor immunoreactivity was detected on some neurites that ran through the cerebrobuccal connective and penial nerve (Figure 3.2). On the other hand, specific GnRH receptor staining within the brain was difficult to detect, although faint staining was observed occasionally. An example of neuronal cell body staining in the CE is shown (Figure 3.2). Also, some tissues in the penial complex were positively stained with the rabbit polyclonal antibody. Not only was anti-GnRH receptor immunoreactivity revealed in deep tissues of the penis by confocal microscopy, but prominent anti-GnRH receptor immunoreactivity was also detected at the border of the penis

and vas deferens (Figure 3.3).

Establishment and characterization of CE neuronal cell culture

We hypothesized that the inability to demonstrate the presence of anti-GnRH receptor immunoreactivity in neurons consistently within the CE and other ganglia in wholemount brain preparations might have resulted from poor antibody penetration through the connective tissue sheath covering the *Helisoma* brain. If such is the case, one should be able to detect anti-GnRH receptor immunoreactivity in cultured neurons should these receptors be present. Since CE neurons are one of the hypothesized targets for GnRH (Young et al., 1999) and CE neuronal culture has not been previously established, a method for culturing CE cells was developed, followed by a characterization of the cultured CE neurons. Populations of dissociated CE neurons were cultured on day 0, *en masse*, in either DM or CM in order to determine the optimal culture conditions.

When cultured in DM, 31% of CE cells (26 of 83 cells from a total of 4 cultures) showed neurite outgrowth initiation; cell bodies of these neurons which showed neurite outgrowth initiation had an average diameter of 25.0 ± 1.3 μm (mean ± standard error of mean (SEM); n = 26) on day 1. Neurite outgrowth initiation and subsequent neurite elongation followed two typical patterns (Figure 3.4). In

Pattern 1, neurons (representing 4 of 26 neurons) initiated veil-like lamellipodia around the cell bodies and showed neurite outgrowth initiation on either day 1 or day 2; however, there was no subsequent neurite elongation beyond day 2, such that stable neurites did not form (Figure 3.4 A). Only projections of ≥ 10 μm are considered neurites since shorter projections are often unstable and get reabsorbed. In Pattern 2, neurons (representing 22 of 26 neurons) initiated neurite outgrowth and elongation by day 1 in culture. In these neurons, 83% of all neurites examined (n = 52 in 22 cells) stopped elongation by day 1, 8% stopped elongation by day 2, and 9% stopped by day 3 (Figure 3.4. B).

When cultured in CM, 83% of CE cells (67 of 81 cells in a total of 4 cultures) showed neurite outgrowth and cell bodies of neurons with neurite outgrowth had an average diameter of 26.1 ± 0.7 μm (mean ± SEM; n = 67) on day 1. There were also two similar outgrowth patterns in CE neurons cultured in CM as in DM (Figure 3.4 C, D). Four percent of neurons (3 of 67) showed a neurite outgrowth pattern that was consistent with Pattern 1 as described above, while 96% of neurons (64 of 67) formed actual neurites (Pattern 2). These neurons displayed neurite elongation by either day 1 (n = 62) or day 2 (n = 2). In neurons showing this outgrow pattern (total n=64), 53% of them stopped neurite elongation by day 1, 42% stopped by day 2, and 5% stopped by day 3. Neurons in which neurite

outgrowth had ceased could be identified based on the morphology of their growth cone (Figure 3.5). During active neurite outgrowth, filopodia extended from the active growth cone; when neurite outgrowth stopped, the filopodia disappeared and the inactive growth cone took on a club-shaped morphology and had a highly phase-bright appearance under phase-contrast objectives.

Four morphological groups of neurite-containing cells were identified; these were those containing one neurite (monopolar neurons), two neurites (bipolar neurons), three neurites (tripolar neurons) and four or more neurites (multipolar neurons) (Figure 3.6). Only neural projections longer than 10 μm were considered as neurites, since continuous observations confirmed that neural projections longer than 10 μm were never reabsorbed (n=52 neurites from 4 DM preparations; n=146 neurites from 6 CM preparations). The length of neurites at maximal elongation, usually measured within 3 days, was compared. CE neurons cultured in CM had neurites that, on average, were more than double the length of neurites from cells cultured in DM (Figure 3.7). On the other hand, there were no significant differences in the length of neurites between different morphological sub-populations of CE neurons cultured either in DM or in CM (Figure 3.8).

Cell survival at day 1 was also compared in separate cultures using the trypan blue dye exclusion test (Lewin et al., 1990). Based on their ability to

exclude the dye, 68% (52 of 77 cells from three cultures) and 92% (84 of 91 cells from five cultures) of CE cells were deemed to have survived in DM and CM, respectively (Figure 3.9). Interestingly, none of the dead cells showed any neurite outgrowth. Among the cells that survived, 44% (23 of 52) and 88% (74 of 84) showed neurite outgrowth in DM and CM, respectively. Based on the better neurite outgrowth performance and lower death rate of CE cells cultured in CM, only CE neurons cultured overnight in CM and containing neurites were used in subsequent experiments.

As a prelude to studying the effects of GnRH on CE neurons, the electrophysiological properties of CE neurons cultured for 1 day in CM were characterized. Intracellular recordings were used to examine the electrophysiological characteristics of CE neurons at rest and following hyperpolarizing current injections. Upon electrode penetration, resting membrane potential showed fluctuations, but generally ranged from -35 mV to -50 mV. Over one third of CE neurons (38%, 26 of 67) displayed spontaneous action potential activity on day 1; an example trace of which is shown in Figure 3.10. The ability to fire action potentials spontaneously was observed in at least some neurons in each of the four morphological sub-populations (14 of 32 monopolar neurons, 9 of 12 bipolar neurons, 1 of 12 tripolar neurons, and 2 of 11 multipolar neurons).

Fifty-one of the 67 CE neurons examined above were used in the study of input resistance, an important indicator of the integrated channel activities of the cell, when holding the membrane potential at -50 mV with constant hyperpolarizing current. Based on the membrane voltage responses to injected hyperpolarizing currents, input resistance was calculated. Results revealed that CE neurons, especially monopolar CE neurons, had a wide range of input resistances, from 20 to 1700 MΩ (Figure 3.11). However, most CE neurons (90%; 46 of 51) had input resistances below 1000 MΩ. All neurons with an input resistance greater than 1000 MΩ were from the monopolar sub-population (Figure 3.11). Overall, the average input resistance tended to decrease when the number of neurites increases, although no significant differences between groups were observed. 41% of CE neurons (21 of 51) also fired action potentials after the hyperpolarizing current injection.

Demonstration of anti-GnRH receptor immunoreactivity in cultured CE and B5 neurons

The ability of the two anti-mGnRH receptor antibodies to stain CE neurons and buccal neuron B5 cultured in CM and fixed on day 1 was next examined. Anti-GnRH receptor immunoreactivity was detected in 84% of CE cells in mass

culture (101 of 121 cells in 5 cultures) using the rabbit polyclonal antibody as the primary antibody (Figure 3.12). Similarly, 86% of CE cells in mass culture (71 of 83 cells in 4 cultures) stained with the mouse monoclonal antibody (Figure 3.12). All B5 neurons were stained positively with these two antibodies (3 of 3 single cell cultures for the polyclonal antibody and 3 of 3 single cell cultures for the monoclonal antibody) (Figure 3.13). Notably with cultured B5 neurons, which are larger in size than CE neurons, punctuate surface staining with the monoclonal antibody was easily observed while the polyclonal antibody also stained the neurites (Figure 3.13). Substitution of the primary antibody with normal sera produced no positive staining in cultured CE and B5 neurons (Figure 3.12 and Figure 3.13).

Effects of mGnRH on electrophysiological properties of cultured CE neurons

To examine the effects of mGnRH on electrophysiological properties of cultured CE neurons, input resistance and post-hyperpolarization rebound firing were measured. These tests were conducted right before vehicle applications, 2 minutes after bath application of vehicle and 2 minutes after bath application of mGnRH; mGnRH was applied immediately after the vehicle test. The two-minute delays in recording ensured that the application artifacts did not become a

confounding issue. Since CE neuron cultures likely represented a mixed population of cells, data were not pooled for statistical comparisons. Instead, a response to mGnRH treatment was considered to have occurred when the mGnRH-induced change in parameter amplitude was outside of the 95% confidence interval of that induced by all vehicle treatments for that particular parameter. The upper and lower limits of the 95% confidence intervals for changes in input resistance and firing (quantified according to the number of action potentials per burst) were -1.7% and 1.8%, and -2.7% and 8.5%, respectively. (Note that data were normalized against basal observations obtained prior to the vehicle application). Using these criteria, 96% of CE neurons tested (22 of 23 cells) responded to 6 μM mGnRH by changing input resistance and/or post-hyperpolarization firing. Of these 22 responders, all of them changed input resistance in response to mGnRH. Eight of them increased input resistance from 3% to 45%. Fourteen of them decreased input resistance from 7% to 91%. Among these, 8 cells also showed changes in firing (1 neuron increased both input resistance and firing, 3 neurons increased input resistance while decreasing firing, 2 neurons decreased input resistance and increased firing, and 2 neurons decreased both input resistance and firing). Whether particular response characteristics were associated with neuronal morphology or basal

electrophysiological properties was also examined. All four morphological sub-types were represented among responders to mGnRH (Figure 3.14). More than one category of actions (increase, decrease or no change in terms of both input resistance and firing) were also frequently observed within a single morphological sub-type after mGnRH application; however, all bipolar neurons increased input resistance while tripolar and multipolar neurons did not show changes in post-hyperpolarization rebound firing in response to mGnRH (Figure 3.14). In addition, another 2 features of interest were observed. First, mGnRH-induced increase in firing was only observed in monopolar neurons. Second, among responders of mGnRH, the basal input resistance range overlapped regardless of the direction of the change observed. Monopolar neurons responding to mGnRH with increases in input resistance had basal input resistance ranging from 40-820 MΩ while those responding with decreases in input resistance had input resistance around 20-1460 MΩ, respectively. Similarly, monopolar neurons that responded to mGnRH with increases in firing and those responding with decreases in firing also had overlapping ranges of basal input resistance values of 740-1420 MΩ and 560-1480 MΩ, respectively.

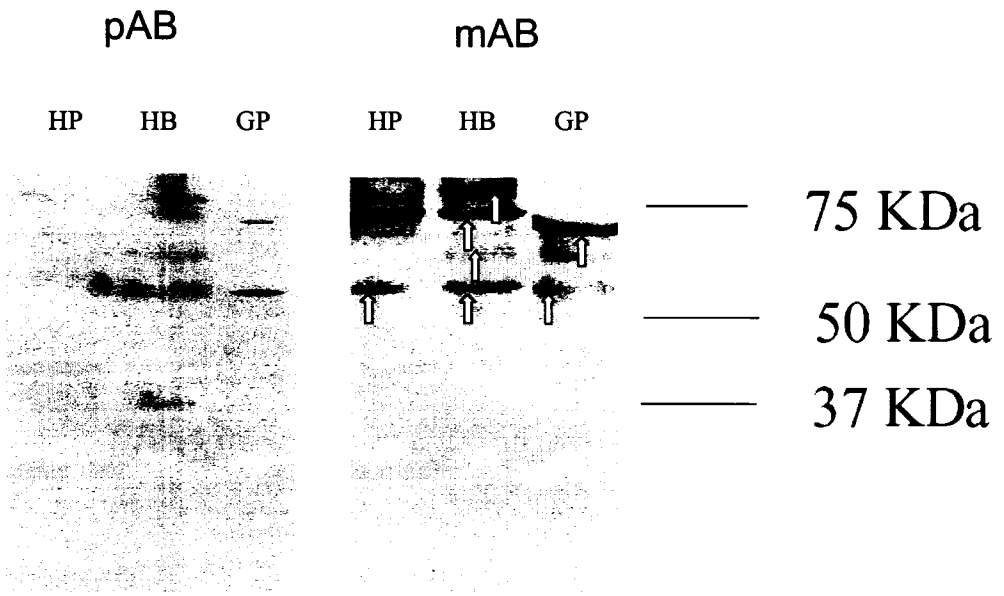
Effects of mGnRH on cultured B5 neurons

Neuron B5 survived and continued to grow neurites after intracellular recordings (Figure 3.15), suggesting that such recordings did not overtly and negatively affect cellular physiology. Single B5 neurons had an average resting membrane potential of -52.6 ± 0.7 mV (mean ± SEM) and input resistance of 176 ± 20 MΩ (mean ± SEM) following overnight culture in CM (n = 39 from 39 cultures). Vehicle controls, applied before mGnRH treatment, did not alter membrane potential and input resistance (n=17 from 17 preparations; Figure 3.16 and Figure 3.17). Applications of 10 and 50 μM mGnRH significantly reduced input resistance of B5 by 30 and 38%, respectively (n=11 from 11 preparations and 6 from 6 preparations respectively; Figure 3.17). On the other hand, neither concentrations of mGnRH had any effect on the membrane potential (n=11 from 11 preparations and 6 from 6 preparations, respectively; Figure 3.16). Applications of two sequential vehicle controls with the same time interval as in the mGnRH-treated groups were also examined in parallel cell preparations as external controls (n=7 from 7 preparations). These sequential vehicle treatments affected neither membrane potential nor input resistance in cultured B5 neurons (Figure 3.16 and Figure 3.17). Responses to depolarizing current as well as hyperpolarizing current injections were monitored to further investigate the

influence of mGnRH on excitability of B5 (Figure 3.18). Applications of vehicle control did not significantly affect both depolarization-induced and post-hyperpolarization rebound firing (data are quantified according to the number of action potentials per burst) (n=7 from 7 preparations and n=11 from 11 preparations respectively). Similarly, treatment with 10 μM mGnRH had no effect on these parameters (Figure 3.18).

The involvement of GnRH receptors in mediating mGnRH action on input resistance was next examined. Bath application of a mGnRH receptor antagonist ([D-pGlu^{1}, D-Phe^{2}, D-Trp^{3,6}] GnRH, 250 μM; Habibi, 1991) had no effect on input resistance (n=5 from 5 preparations), but its presence blocked the ability of a co-applied dose of 50 μM mGnRH to reduce input resistance in B5 neurons cultured for a day in CM (n=6 from 6 preparations; Figure 3.19).

Figure 3.1 Western blot analysis of anti-GnRH receptor immunoreactive molecules in extracts of the *Helisoma* penial complex (HP), *Helisoma* brain (HB) and goldfish pituitary cells (GP). Upper panels: representative results of five replicate experiments with a rabbit polyclonal anti-GnRH receptor antibody (pAB) and one of two experiments with a mouse monoclonal anti-GnRH receptor antibody (mAB). Lower panels: absence of bands when normal rabbit serum (NRS) and normal mouse serum (NMS) replaced the pAB and mAB, respectively. Clear arrows identify anti-GnRH receptor immunoreactive protein bands consistently detected by both pAB and mAB.



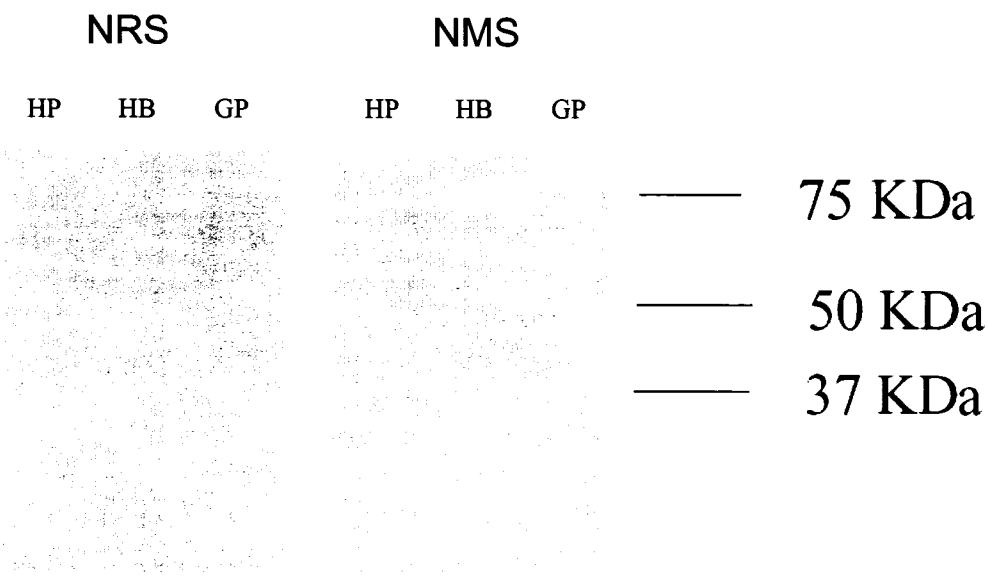
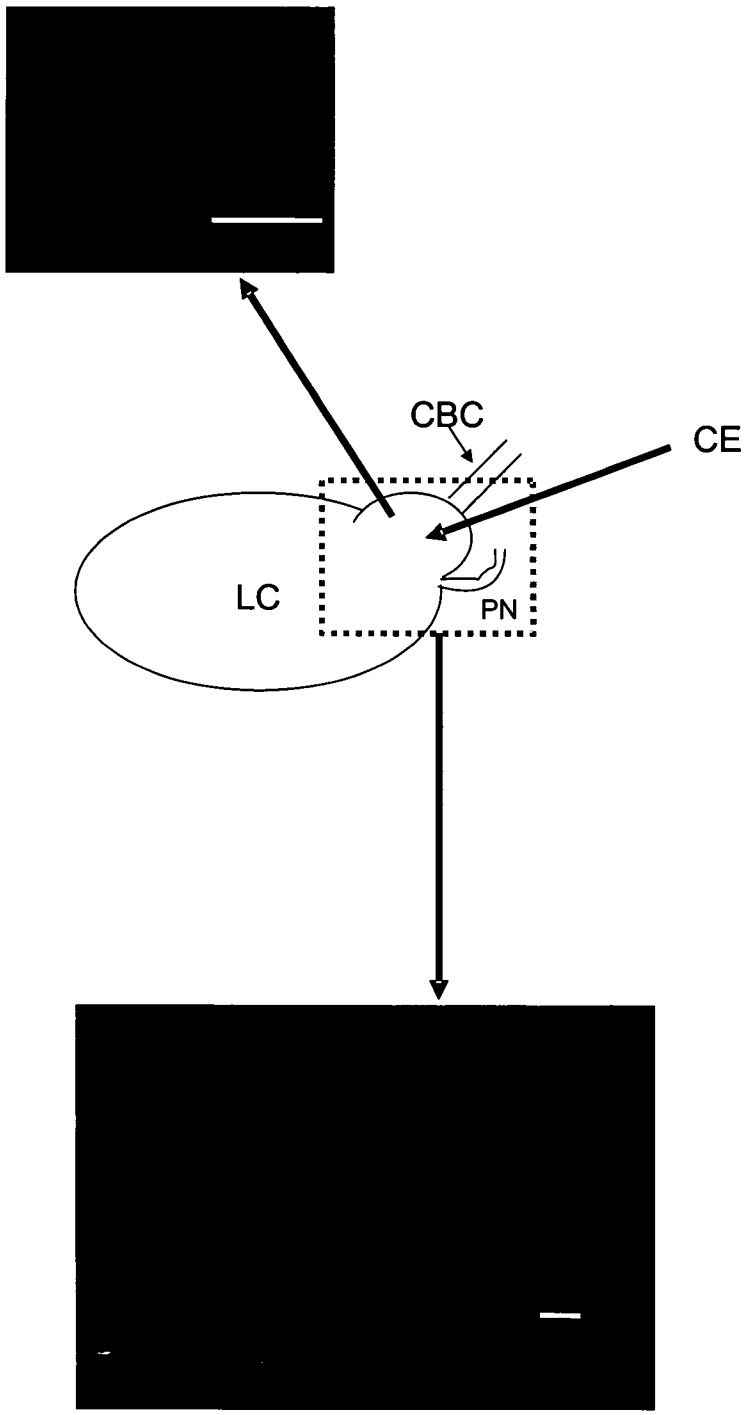


Figure 3.2 GnRH receptor immunofluorescence in the *Helisoma* CE in wholemount preparations. The rabbit polyclonal anti-GnRH receptor antibody was used as the primary antibody. A schematic diagram showing the left cerebral ganglion (LC), CE, cerebrobuccal connective (CBC), and penial nerve (PN), is presented as a reference. The upper panel shows the presence of weak GnRH receptor staining in some neuronal cell bodies within the CE. This staining was observed in 3 of 7 preparations. The lower panel demonstrates the presence of anti-GnRH receptor immunoreactivity in neurites within CBC and PN. Scale bar = 80 μm.



CE: cerebral extension; CBC: cerebrobuccal connective; PN: penial nerve; LC: left cerebral ganglia

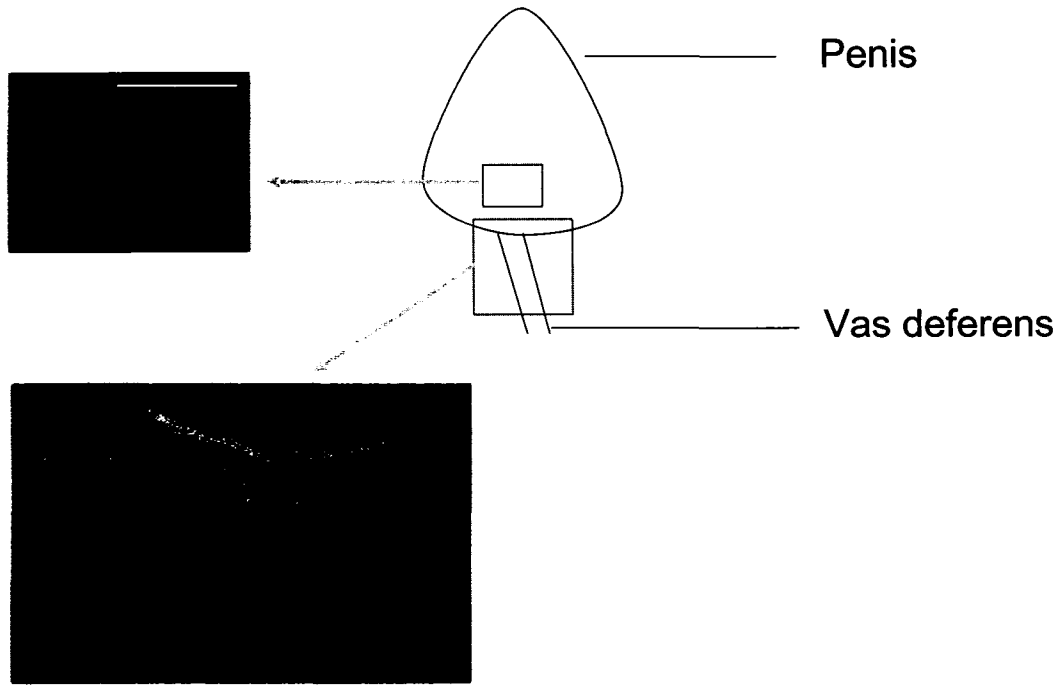


Figure 3.3 Anti-GnRH receptor immunoreactivity in the *Helisoma* penis. Staining with the rabbit polyclonal antibody was observed using confocal microscopy. A schematic diagram of the penis with vas deferens is presented as a reference. Selective cells in the deep tissues of the penis were stained, as revealed by a confocal section through the centre of the preparation (upper panel). GnRH receptor staining was also found in the area where the vas deferens entered the penis (lower panel). Scale bar = 200 μm.

Figure 3.4 Neurite outgrowth of CE neurons cultured in DM (A and B) and CM (C, D). Pictures were taken daily for three continuous days after plating. In both culture conditions, some neurons showed initiation of neurite outgrowth (less than 10 μm), but then stopped in this stage without further neurite extension (pattern 1, A & C). In other neurons, neurites continued to elongate following outgrowth initiation (pattern 2, B and D). Scale bar = 15 μm.

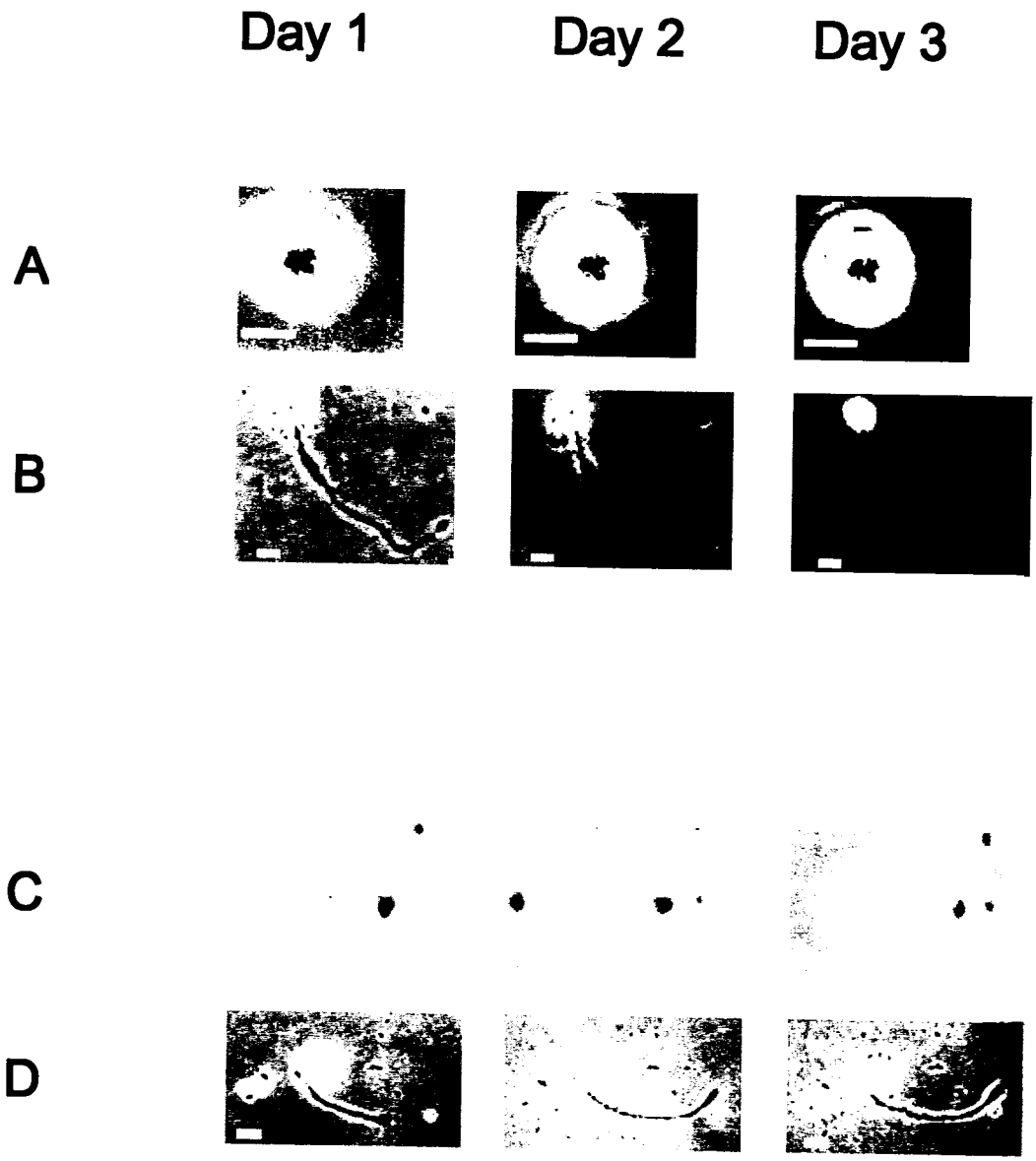
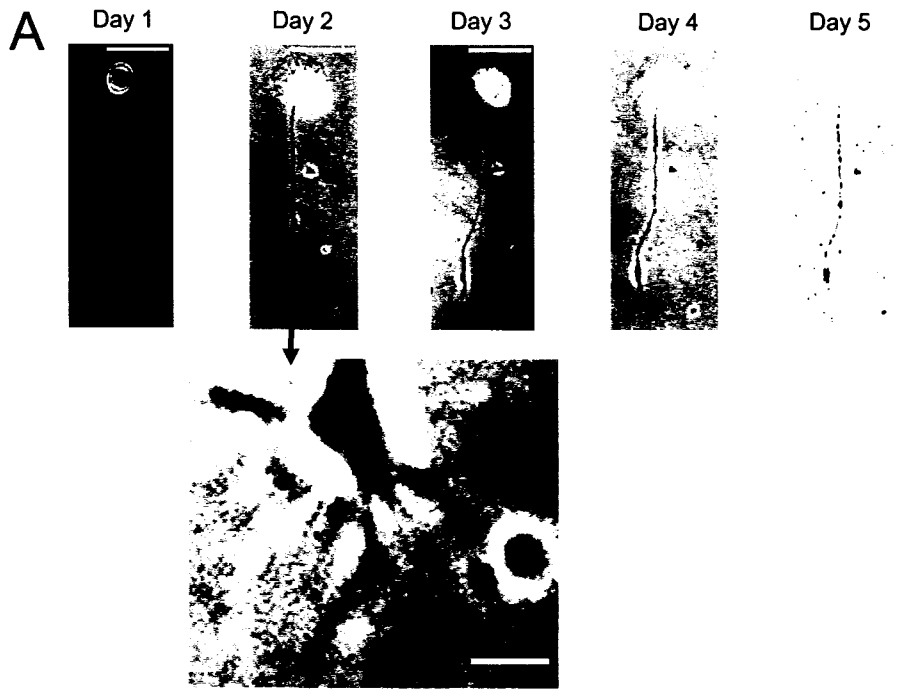


Figure 3.5 Neurite extension in CE neurons cultured in CM. Neurite outgrowth in two example CE neurons was observed over a five day period. Day 0 marked the day of plating. The neuron shown in the panel A did not show neurite elongation on day 1 but had a neurite with an active growth cone on day 2, as indicated by the presence of filopodia (inset). The neuron shown in the panel B had a neurite on day 1; however, on day 2, its growth cone had ceased to be active as indicated by the lack of filopodia and the presence of a phase bright halo around the stump of the growth cone under phase-contrast optics (inset). Scale bar = 60 μm for the pictures of neurons; scale bar = 10 μm for the pictures of growth cones.



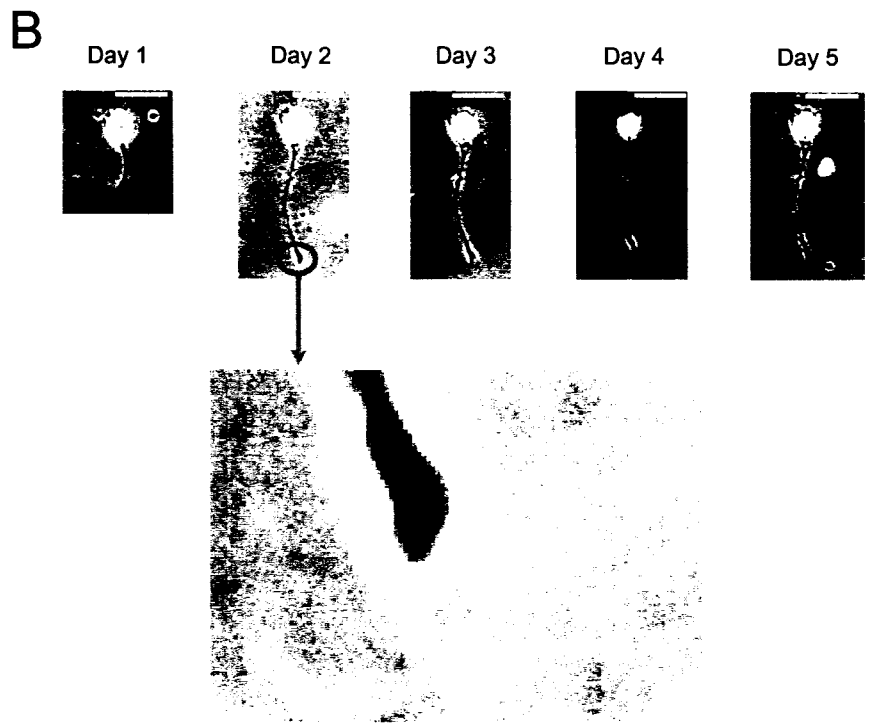
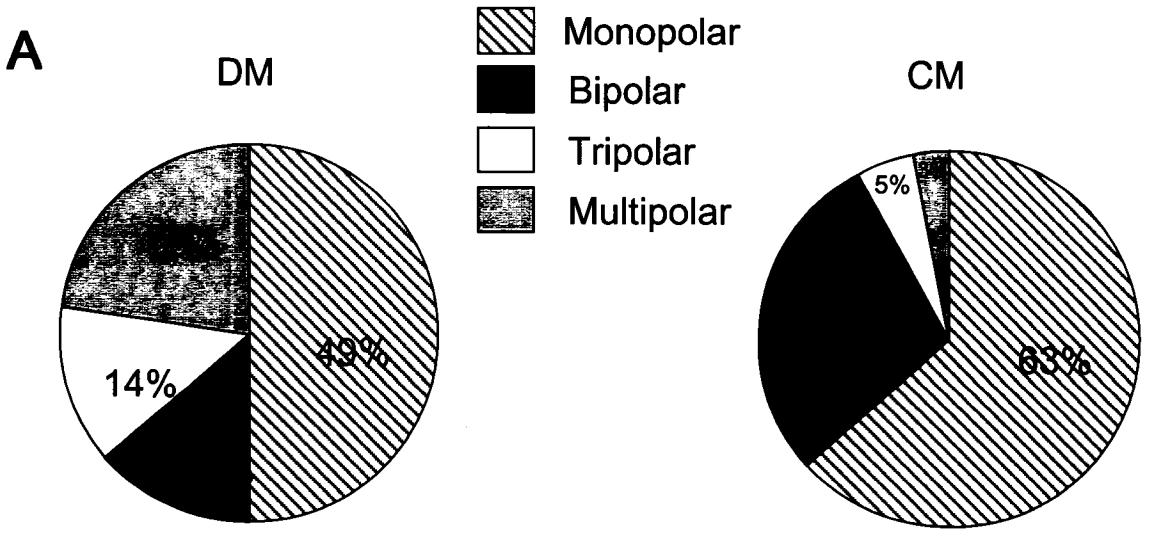
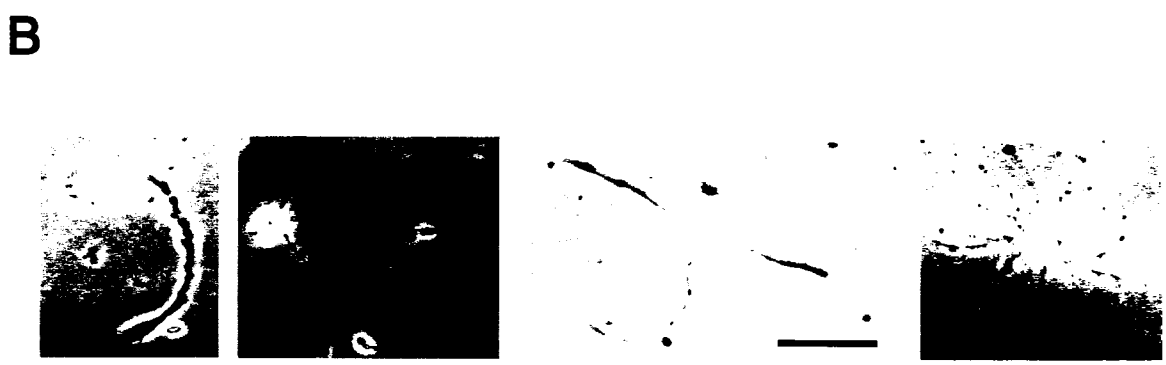


Figure 3.6 Morphological sub-populations of CE neurons that extended neurites in DM and CM. (A) Distribution of neurons of the 4 sub-populations in DM (left panel; n = 22 from 4 preparations) and CM (right panel; n = 98 from 6 preparations). The distribution in DM is significantly different from that found in CM ($P<0.05$; chi-squared). (B) Example pictures (from left to right) of monopolar, bipolar, tripolar, and multipolar CE neurons cultured in CM. Scale bar = 30 μm.





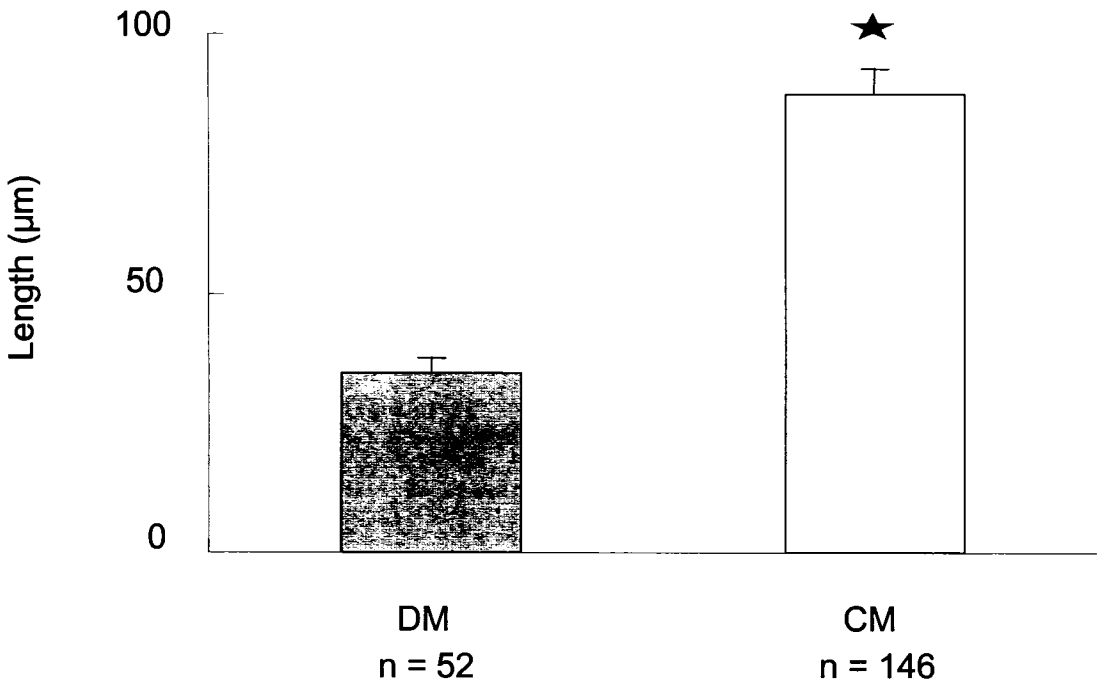


Figure 3.7 Comparison of maximal length of neurites by day 3 of culture in DM and CM. Data presented (mean ± SEM) are pooled observations from 22 neurons with neurites from 4 cultures in DM and 98 neurons with neurites from 6 cultures in CM. The "n" number presented refers to the number of neurites examined. Star denotes a significant difference between the culture conditions (*t*-test, $P<0.01$).

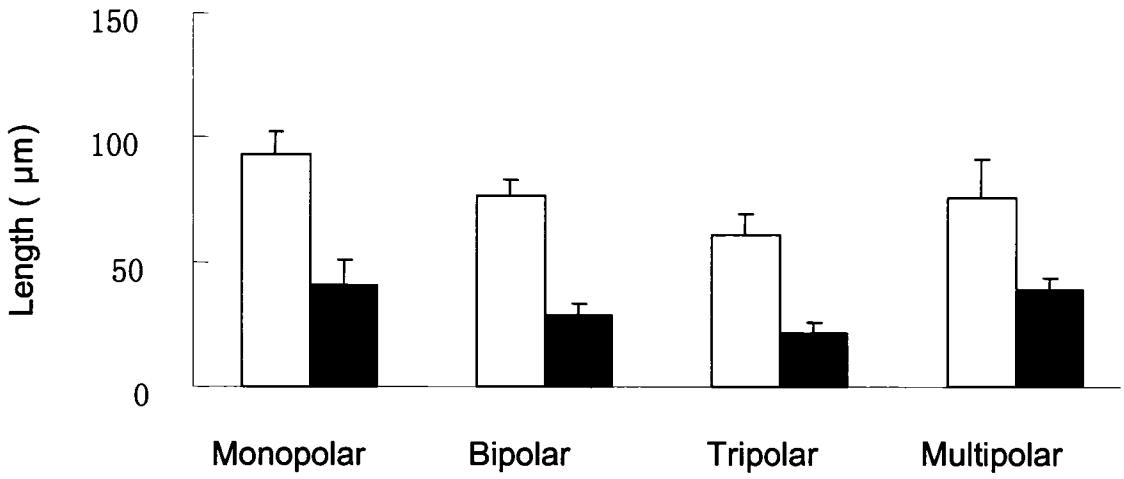


Figure 3.8 Neurite length as a function of the morphological sub-type. Maximal length (presented as mean ± SEM) of neurites by day 3 of culture in each of the four morphological sub-populations of CE neurons cultured in CM (white bars) and DM (dark bars). Data were obtained from 62 monopolar neurons, 28 bipolar neurons, 5 tripolar neurons and 3 multipolar neurons (13 neurites) observed in 6 different cultures in CM and from 11 monopolar neurons, 3 bipolar neurons, 3 tripolar neurons and 5 multipolar neurons (25 neurites) observed in 4 different cultures in DM. There were no significant differences in neurite length between morphological sub-types in either CM or DM (ANOVA; factorial analysis; $P>0.05$).

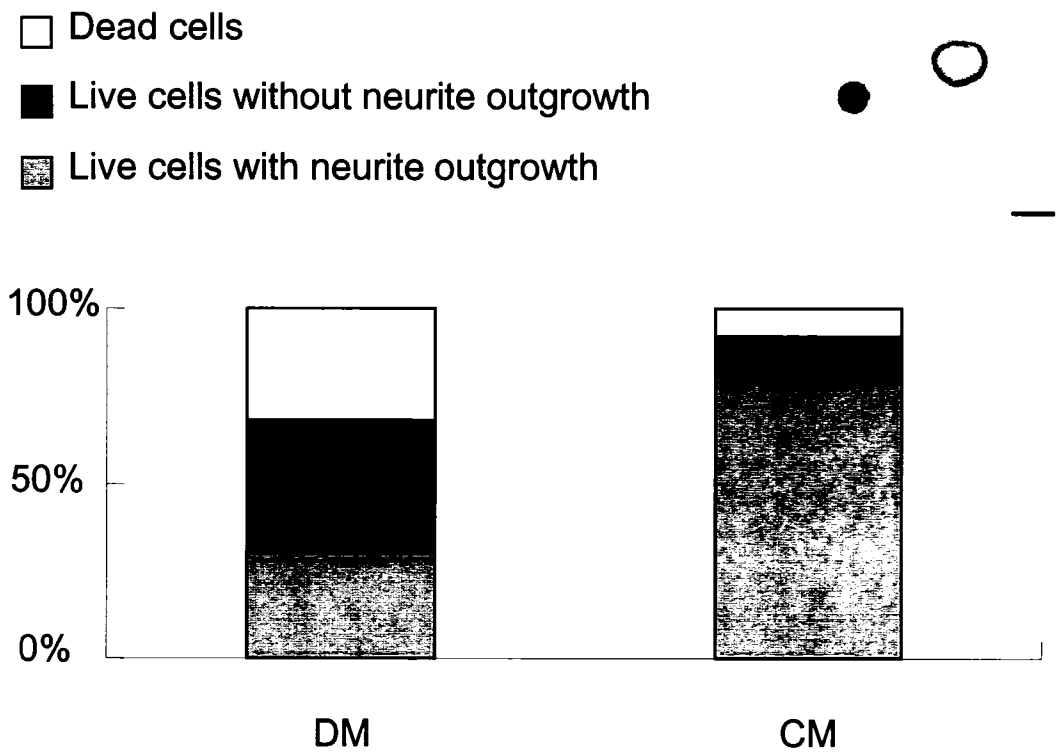


Figure 3.9 Percentage of live and dead cells at 1 day in culture in DM and CM as determined by the trypan blue exclusion test. The proportions of dead cells: live cells without neurite outgrowth: live cells with neurite outgrowth are significantly different between DM and CM cultures ($P<0.01$; chi-squared). Data presented were obtained from a total of 77 cells in 3 DM cultures and 91 cells in 5 CM cultures. Inset: a representative picture of trypan blue staining of two CE cells; the dead cell lost membrane integrity and was stained throughout the cell while the live cell was only stained on the cell membrane. Scale bar = 30 μm.

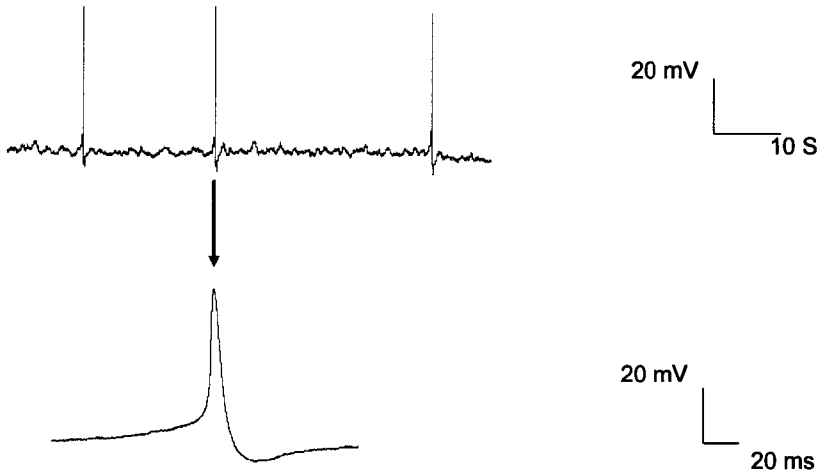
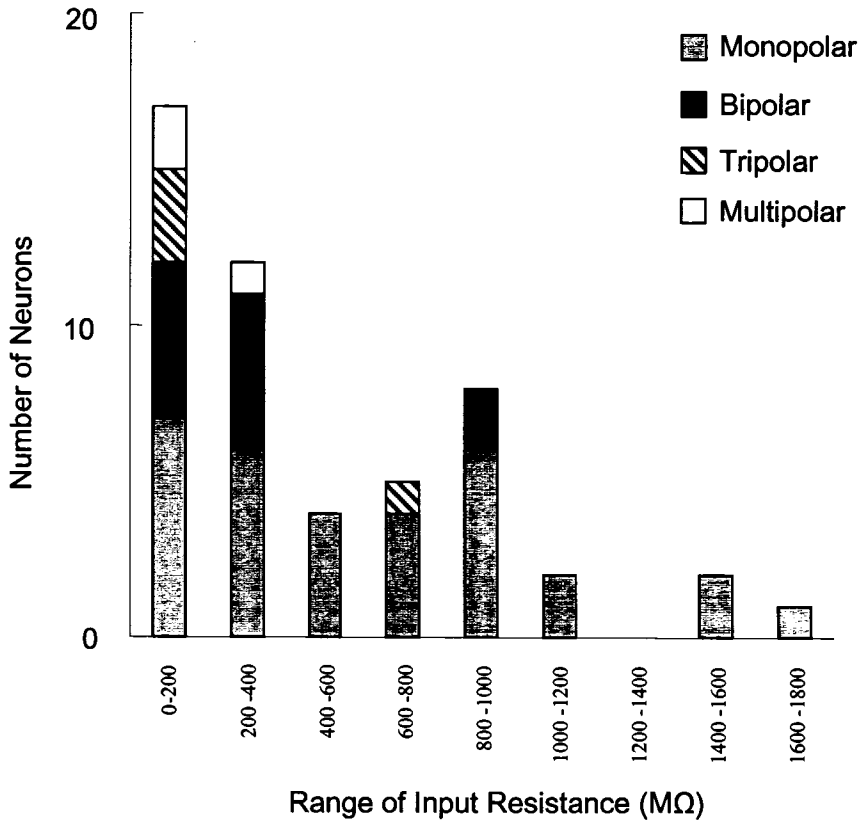


Figure 3.10 Example of spontaneous action potential activity in a CE neuron cultured in CM for 1 day. Bottom panel represents one action potential in the upper trace displayed on a magnified time scale.

Figure 3.11 Input resistance of CE neurons cultured in CM. Data from 51 CE neurons from 51 preparations. (A) Distribution of input resistance among the four different morphological sub-populations. Fisher's exact test revealed that there was a significant difference in input resistance distribution between monopolar and bipolar neurons ($P<0.01$). (B) Median and interquartile range (error bars) of input resistance in each of the four morphological sub-populations.

A



B

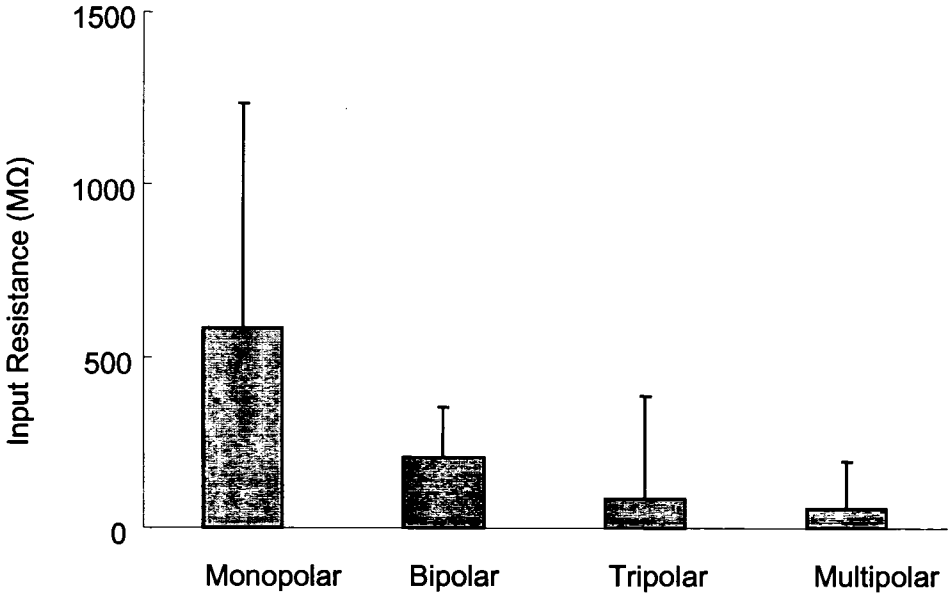


Figure 3.12 Anti-GnRH receptor immunoreactivity in CE neurons cultured in CM. Anti-GnRH receptor immunoreactivity was detected using the rabbit polyclonal antibody (pAB; A) or mouse monoclonal antibody (mAB; B) as the primary antibody. Negative controls were performed using normal rabbit serum (NRS; A) or normal mouse serum (NMS; B) in place of the primary antibody. Example differential interference contrast microscopy (DIC; left) and fluorescence (right) image pairs showing that not all CE neurons in any single culture stained with pAB (C) or mAB (D). Data shown are representative of results from at least four cultures. Scale bar = 30 μm.

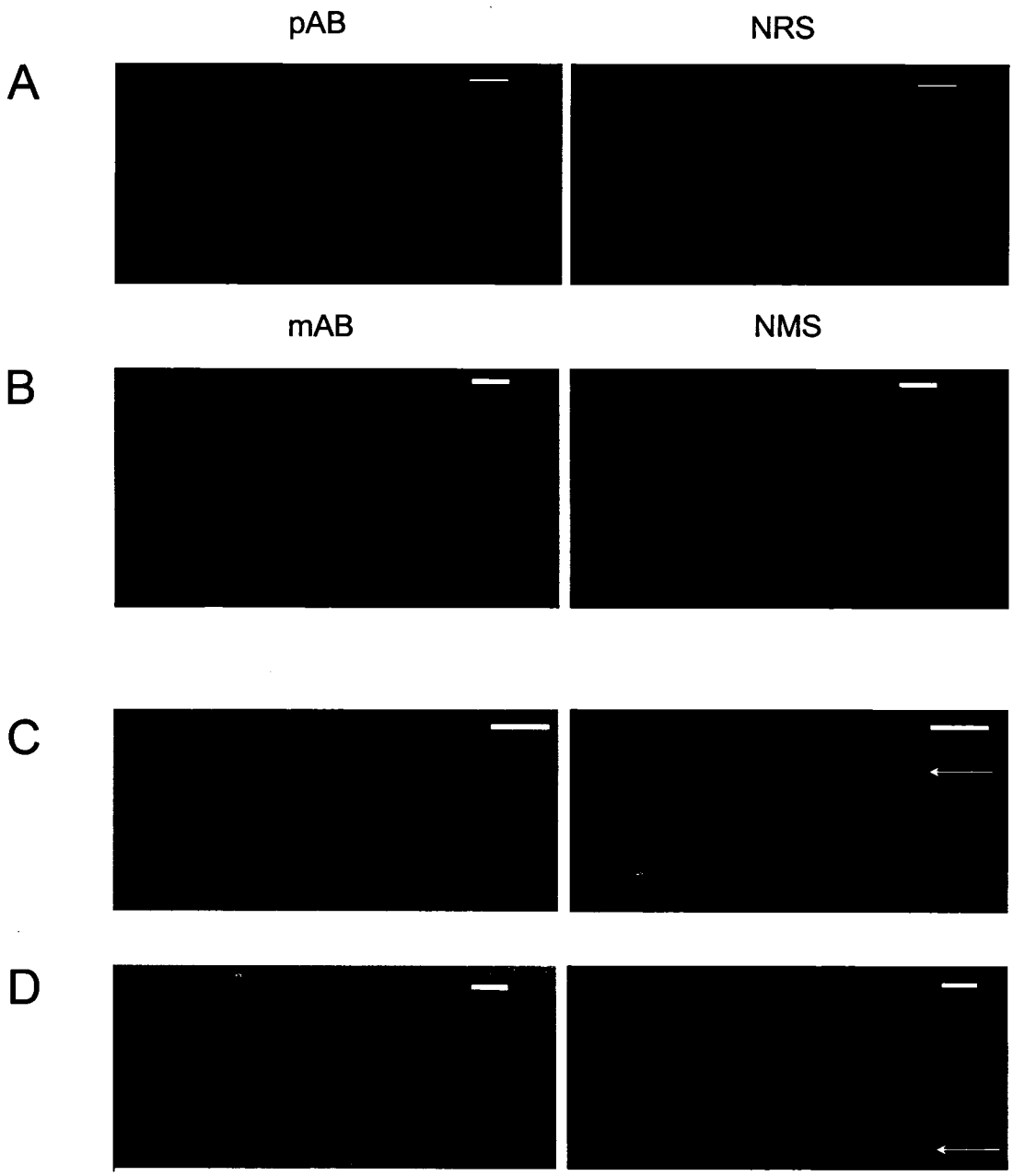
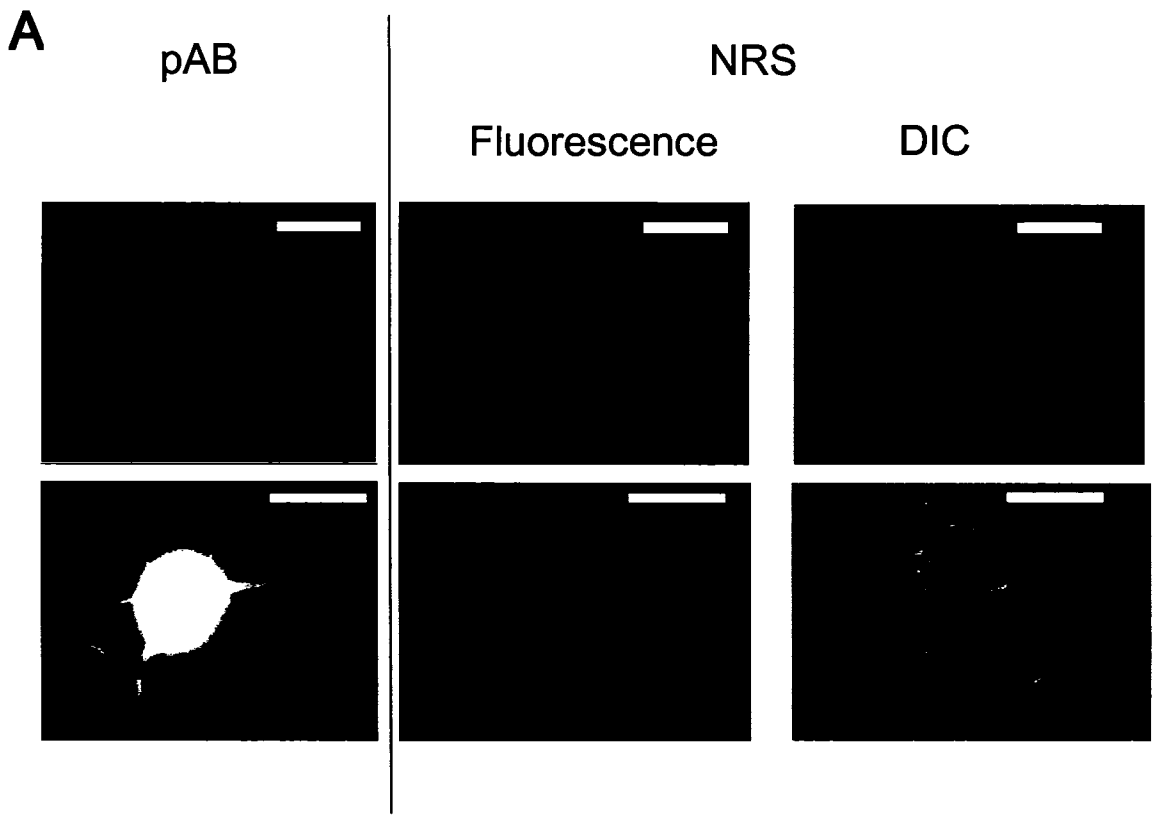


Figure 3.13 Anti-GnRH receptor immunoreactivity in buccal neuron B5 cultured in CM. Anti-GnRH receptor immunoreactivity was detected using the rabbit polyclonal antibody (pAB; A left panel) or mouse monoclonal antibody (mAB; B left panel) as the primary antibody. Negative controls were performed using normal rabbit serum (NRS; A middle and right panels) or normal mouse serum (NMS; B middle and right panels) in place of the primary antibody. Data shown are representative of results from 3 cultures. Scale bar = 60 μm.



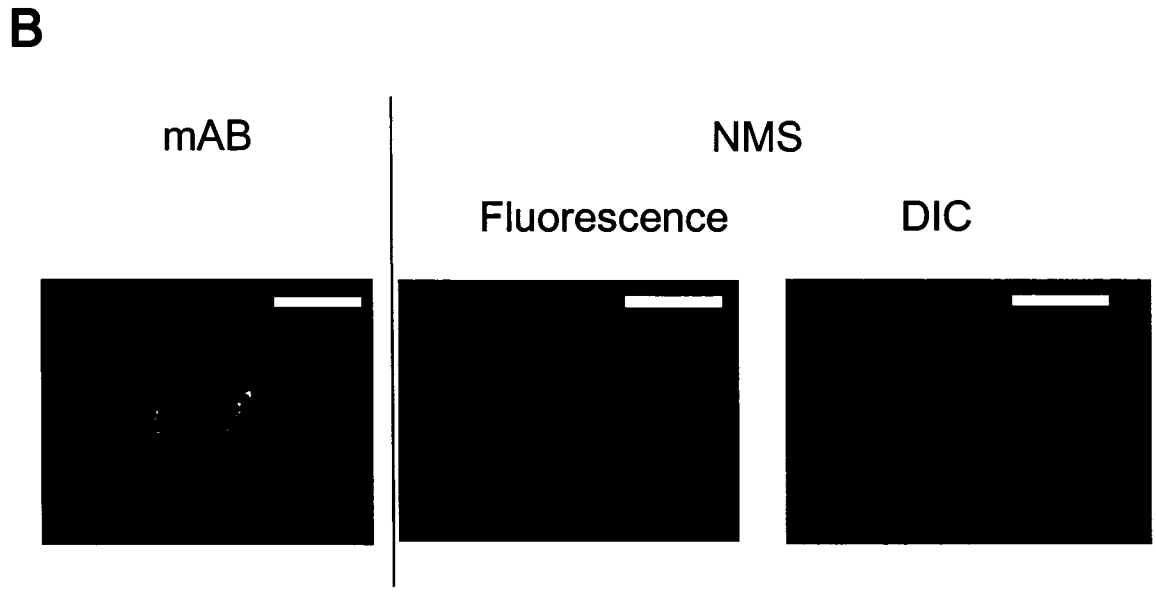
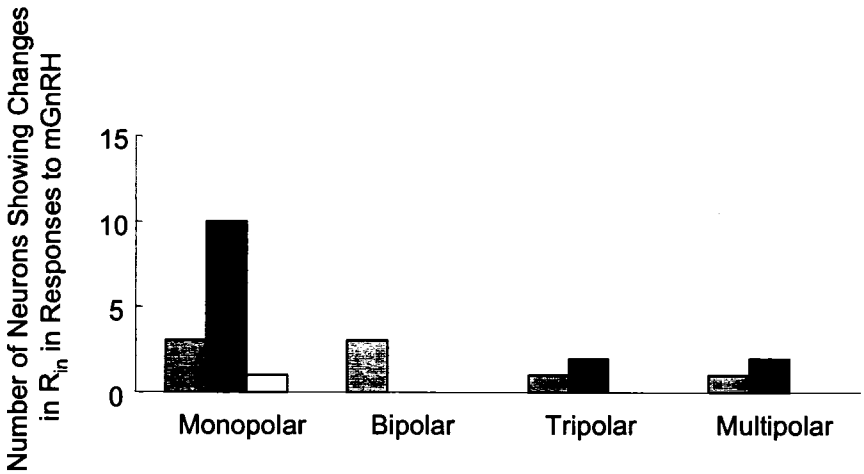
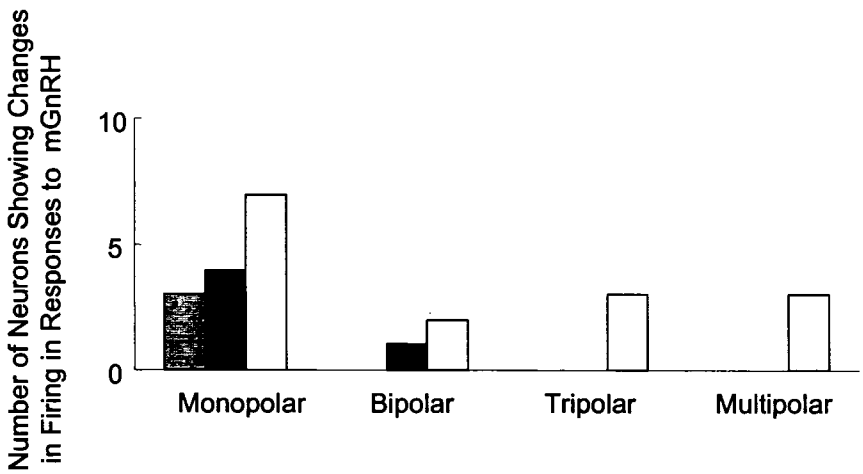


Figure 3.14 Summary of electrophysiological responses to bath application of 6 μM mGnRH in the four morphological sub-populations of CE neurons cultured in CM. Results on input resistance (R_{in}; upper panel) and post-hyperpolarization rebound firing (lower panel) represent data from 23 neurons from 23 cultures. Given that the CE likely contains multiple cell types, data are expressed as number of neurons that showed on increase (medium shade bars), decrease (dark bars) or no change (white bars) in response to GnRH.





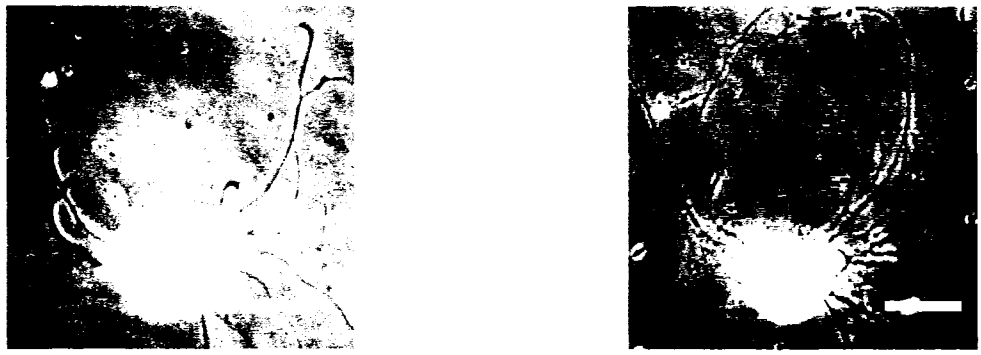


Figure 3.15 Phase-contract images showing a B5 neuron on the same day after intracellular recording (left panel) and two days after intracellular recording (right panel). Additional neurite branching was observed two days after intracellular recording (right panel). Scale bar = 60 μm.

Figure 3.16 Effect of mGnRH on membrane potential (MP) of neuron B5 cultured in CM. Results (mean ± SEM) of two sequential bath applications of vehicle (upper panel; n = 7 from 7 preparations), vehicle followed by 10 μM mGnRH (middle panel: n = 11 from 11 preparations), and vehicle followed by 50 μM mGnRH (lower panel; n = 6 from 6 preparations) are presented. No significant difference was demonstrated by ANOVA, repeated measures ($P>0.05$), in any of these experimental designs.

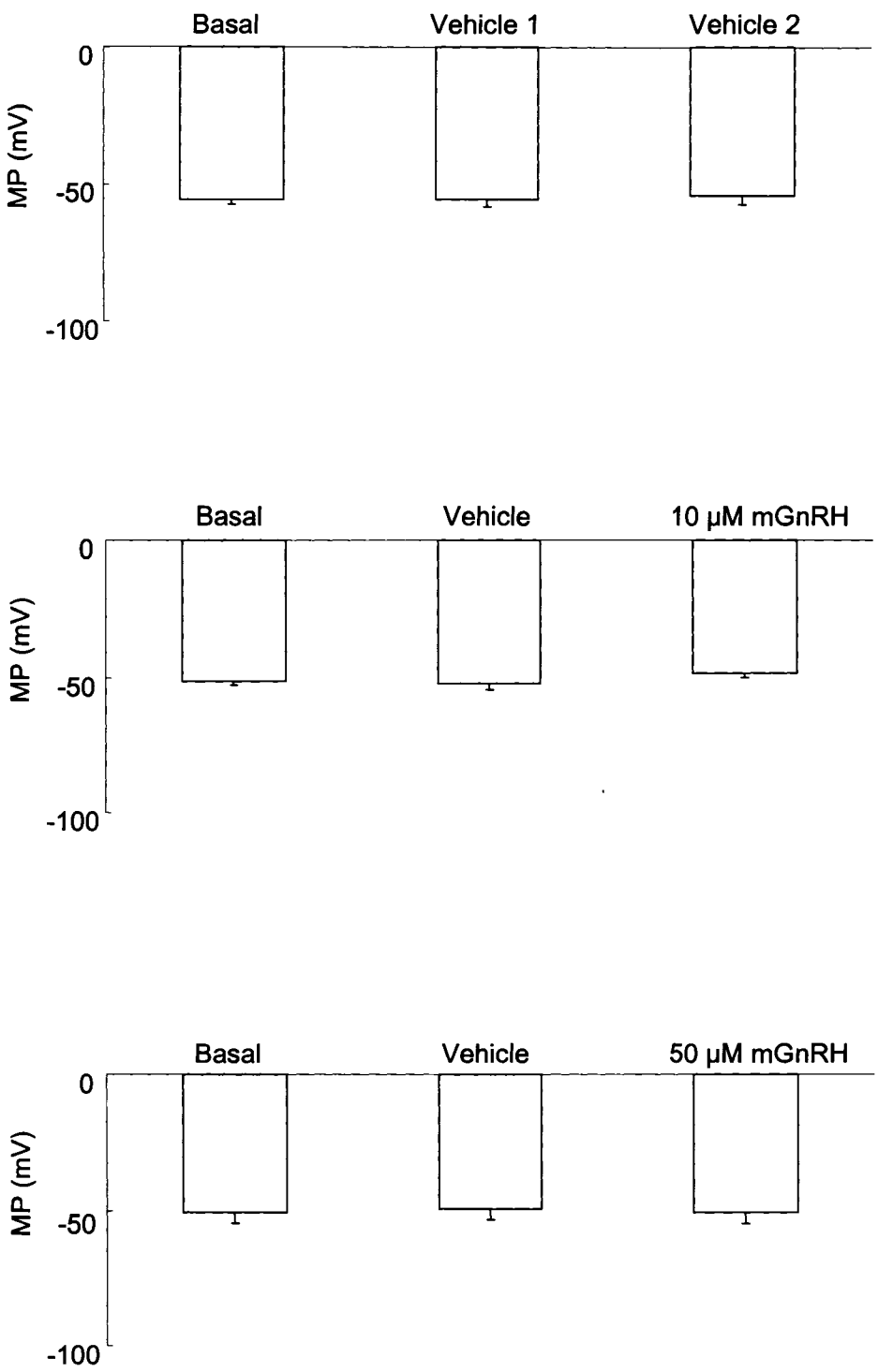


Figure 3.17 Effect of mGnRH on input resistance (R_{in}) in neuron B5 cultured in CM. (A) A representative recording in which bath-applied 50 μM mGnRH decreased the input resistance of neuron B5. I: injected current; MP: membrane potential. (B) Results (mean ± SEM) of two sequential applications of vehicle (upper panel; n = 7 from 7 preparations), vehicle followed by 10 μM mGnRH (middle panel; n = 11 from 11 preparations), and vehicle followed by 50 μM mGnRH (lower panel; n = 6 from 6 preparations) are presented. Asterisk indicates significant difference from both basal and vehicle-treated measurements (ANOVA, repeated measures, followed by Fisher's post hoc least significant difference [PLSD] test; $P<0.05$).

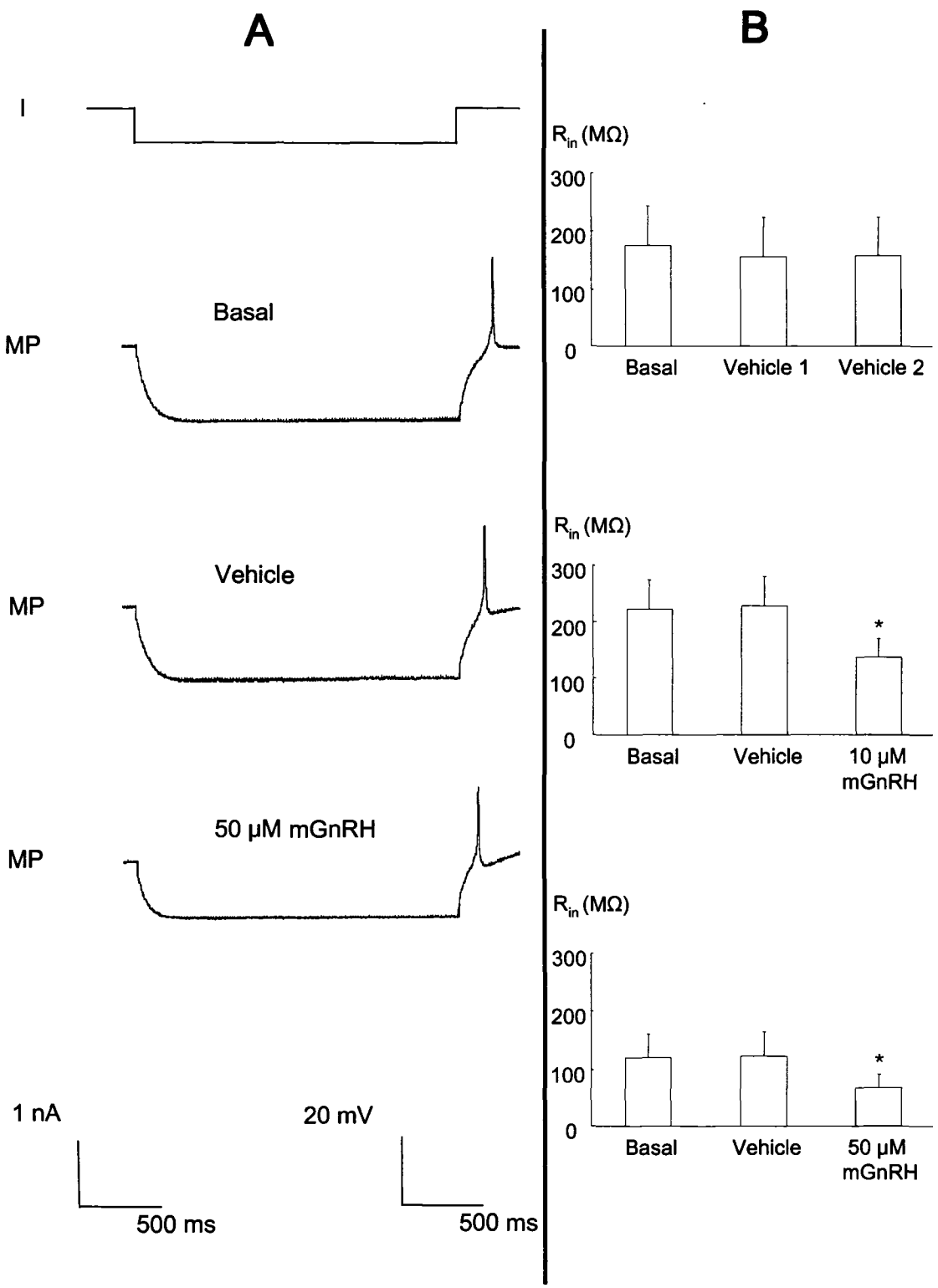
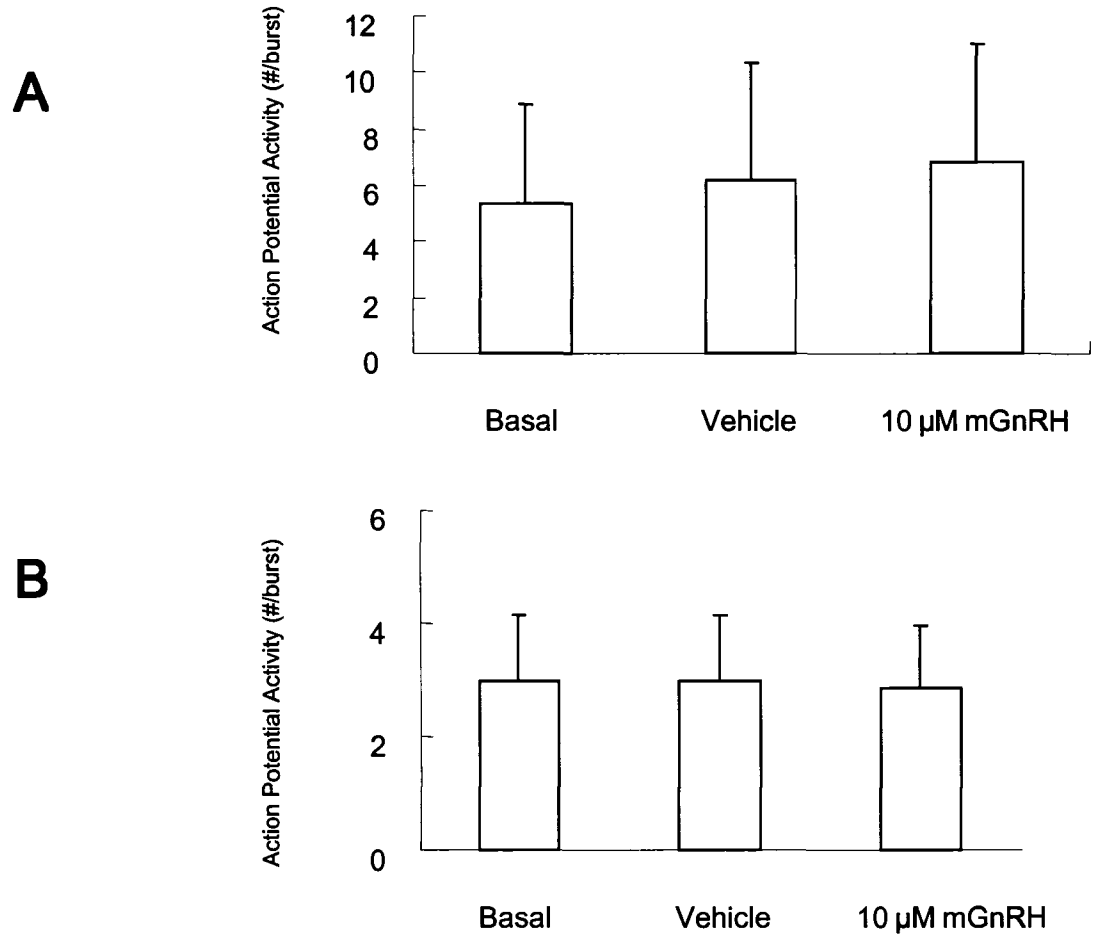


Figure 3.18 Effects of mGnRH on action potential activity of neuron B5 cultured in CM. Results (mean ± SEM) of two sequential bath applications of vehicle followed by 10 μM mGnRH are presented. (A) Post-hyperpolarization firing. Data are from 11 B5 neurons in 11 preparations. (B) Depolarization-induced firing. Data are from 7 B5 neurons in 7 preparations. (C) A representative recording. No significant difference was demonstrated by ANOVA, repeated measures ($P>0.05$), in either of these experimental designs.



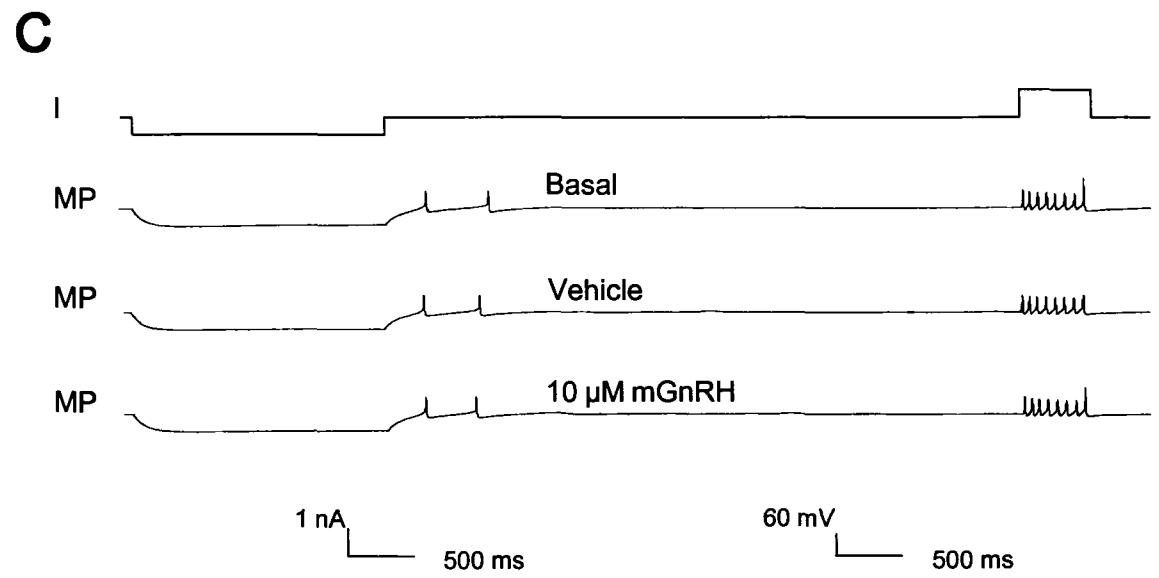
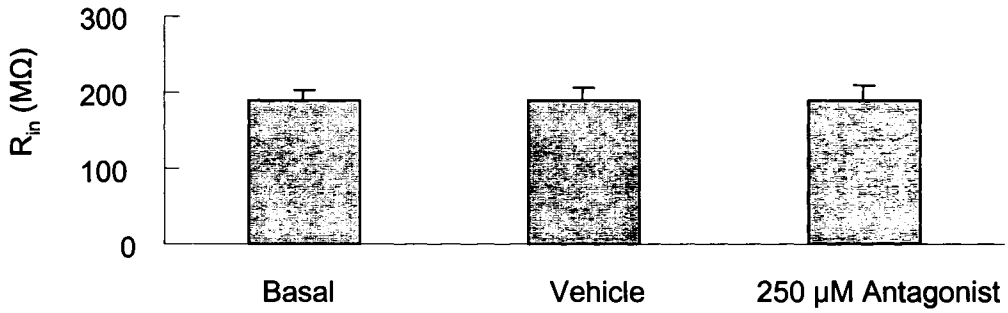
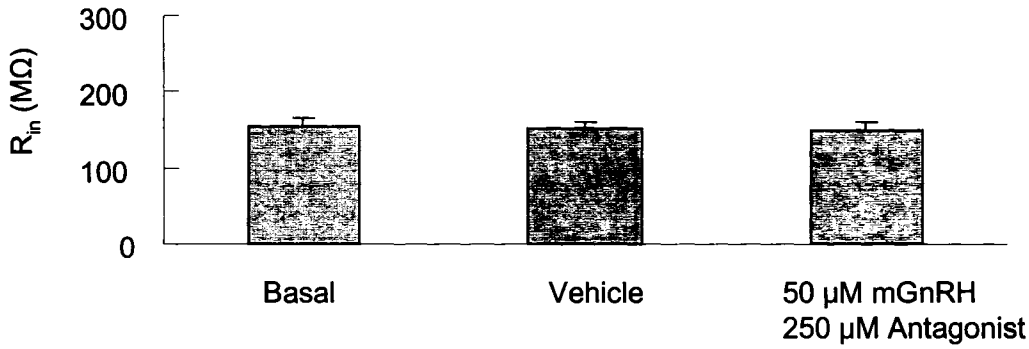
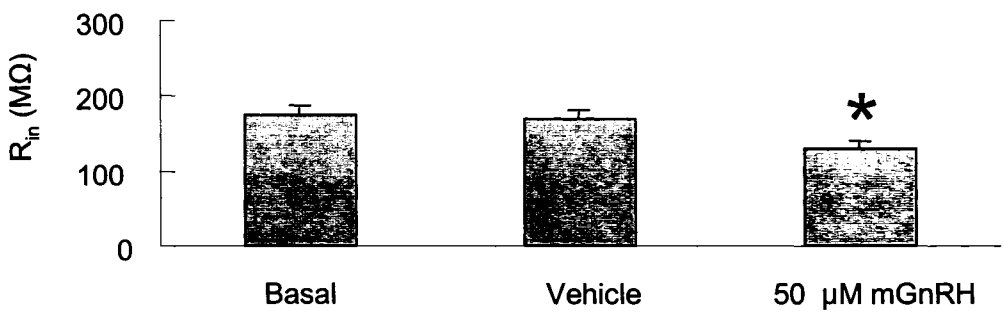


Figure 3.19 Effect of mGnRH receptor antagonist ([D-pGlu^1, D-Phe^2, D-Trp^{3,6}] GnRH) on input resistance (R_{in}) in neuron B5 cultured in CM. Results (mean ± SEM) of two sequential bath applications of vehicle followed by 250 μM antagonist (upper panel; n = 5 from 5 preparations), vehicle followed by co-solution of 250 μM antagonist and 50 μM mGnRH (middle panel; n = 6 from 6 preparations), and vehicle followed by 50 μM mGnRH (lower panel; n = 4 from 4 preparations; positive control). In the positive control, asterisk indicates significant difference from both basal and vehicle (ANOVA, repeated measures, followed by PLSD Fisher's test; $P<0.05$); however, no significant difference was demonstrated by ANOVA, repeated measures ($P>0.05$), in either the antagonist treatments or co-treatments by the mGnRH and antagonist.







CHAPTER 4 – DISCUSSION

Previous results on the localization of anti-GnRH immunoreactivity in brain and penial tissues (Goldberg et al., 1993; Young et al., 1999), as well as the ability of mGnRH to alter neurite outgrowth in cultured embryonic neurons and the electrophysiological responses of adult left parietal and visceral ganglia neurons in adult *Helisoma* (Goldberg et al., 1993), infer the presence of GnRH receptor(s) in this species. Several lines of evidence from this thesis also point to the presence of GnRH receptors in adult *Helisoma*. Western blot analyses using tissue extracts, as well as immunofluorescence experiments with wholemount tissues and cultured neurons, revealed the presence of GnRH receptor-like materials in nervous and reproductive tissues. In addition, these data provided insight into the possible molecular size and variety of *Helisoma* GnRH receptors. The ability of mGnRH to alter electrophysiological properties of cultured CE neurons and neuron B5 further confirmed the presence of bioactive GnRH receptor(s) in nervous tissues of this species. Taken together with other information available in the literature, results from this thesis also provide insights into the possible localization and function of the GnRH system in *Helisoma*. Furthermore, this thesis established, *de novo*, a culture system for CE neurons

and provided information on basic electrophysiological and morphological properties of isolated CE neurons in mass culture.

Basic characterization of CE neurons in culture

To facilitate studies of the presence of a GnRH receptor(s) and GnRH action in CE cells, a culture system for CE neurons was developed in the course of the thesis research. Regardless of whether CM or DM was used to culture CE cells, four morphological sub-populations of CE neurons were observed; these are mono-, bi-, tri- and mulitpolar neurons. Based on several criteria, including better cell viability in the trypan blue exclusion test, a higher percentage of cells showing initiation of neurite outgrowth, earlier initiation of neurite outgrowth and longer neurite length, the use of CM was judged to be better than DM in culturing CE neurons. CM may contain some neurotrophic factors derived and/or released from neurons and neuroglia during the preparation of the media which function to facilitate neurite outgrowth and neuronal metabolism (Wong et al., 1981; Wong et al., 1984). In CM conditioned by rat sciatic nerves, a potential sciatic nerve neurotrophic factor is present, which subsequently promotes neurite outgrowth and the survival of cultured neurons from chicken embryo (Villegas et al., 1995). CM conditioned by dorsal root ganglia also promotes neurite outgrowth, and a

similar effect can be duplicated by the addition of nerve growth factor (NGF), a neurotrophic factor, in unconditioned medium (Kitazawa and Shimizu, 2005). Interestingly, NGF also stimulates neurite outgrowth of *Lymnaea* neurons (Ridgway et al., 1993). Magoski and Bulloch (1998) showed that CM conditioned by *Lymnaea* brains contains a factor that promotes synapse formation between neurons. In *Helisoma*, although the presence of trophic factors in CM has been proposed earlier (Wong et al., 1981; Barker et al., 1982), these factors have yet to be revealed completely. Ahn and Goldberg (2003) demonstrated an HPLC peak from CM that has the same elution time as serotonin, which in low concentration (1-10 μM) increases the length of a proportion of neurites. Goldberg et al. (1992) also showed that serotonin increases the intracellular calcium level in *Helisoma* neurons to regulate neurite outgrowth. Therefore serotonin could be one of neurotrophic factors in CM. In addition, a protein synthesis inhibitor, anisomycin, added during the preparation of CM, can reduce the promoting effects on neurite outgrowth in 35% *Helisoma* neurons examined (Wong et al., 1984). Thus these neurotrophic factors may also contain protein components. Further comparative studies can use CE neurons cultured in DM and CM as a model to reveal potential trophic factors in CM.

In CE neurons grown in CM, a holding current was required to stabilize the

fluctuation in resting membrane potential. This fluctuation may be due to the intrinsic properties of CE neurons. Research has shown that voltage-gated sodium channels, calcium channels, and the oscillatory release of calcium from intracellular storage are involved in membrane potential fluctuations in ventricular cells, pars tuberalis cells, and T3-1 cells (Matsuda et al., 1982; Mason and Waring, 1985; Tiwari and Sikdar, 1998). Interestingly, in the classic vertebrate targets of GnRH, the gonadotrope cells, fluctuations of resting membrane potential of up to 15 mV were also recorded (Mason and Waring, 1985; Tiwari and Sikdar, 1998). Alternatively, this relative instability of membrane potential may be due to the differences in extracellular conditions between *in vivo* and *in vitro* situations. For example, the membrane potential of the squid axon becomes unstable when its environmental solution, sea water, was partially replaced by a NaCl solution (Matsumoto et al., 1978; Hayashi et al., 1980). In spite of the fluctuating resting membrane potential, the ability to exclude trypan blue and the fact that the resting membrane potential fluctuation was generally between -35 to -50 mV (i.e., fairly negative) together suggest that membrane integrity and normal electrophysiological functions were preserved in these cultured CE neurons. This conclusion is further supported by the ability of a portion of CE neurons to fire action potentials spontaneously and to exhibit post-hyperpolarization rebound

firing.

In addition to the observation that only a portion of CE neurons cultured in CM exhibit spontaneous and post-hyperpolarization rebound action potentials, these neurons also had a wide range of input resistance at rest. These variations in basic electrophysiological characteristics at rest, taken together with the presence of multiple morphological sub-populations in culture, suggest that CE neurons represent a non-homogeneous population. On the other hand, morphological characteristics are generally not a good predictor of basic electrophysiological properties, although the multipolar neurons had the lowest membrane input resistance values in the present study. This might reflect the greater total membrane surface area and thus greater total number of ion channels as the number of neurites increases.

Heterogeneity in a population of neurons from a single anatomical area is not uncommon. Dorsal raphe neurons contain both serotonergic and nonserotonergic neuronal sub-populations, and neurons in either sub-population have heterogeneous electrophysiological and pharmacological characteristics (Marinelli et al., 2004). Putative cholinergic neurons in the globus pallidus are also heterogeneous in terms of their electrophysiological properties (Aston-Jones et al., 1984). As revealed by many staining techniques, such as hematoxylin-basic

fuchsin-picric acid staining, purkinje neurons have also been shown to comprise a heterogeneous neuronal population (Khan, 1993). Interestingly, *Helisoma* cyberchron neurons, consisting of around 20 neurons which are electrically coupled and involved in feeding control, are also a heterogeneous population, displaying heterogeneous currents under the voltage clamp configuration (Merickel and Gray, 1980).

Basic characteristics of neuron B5 in culture

Isolated neuron B5 survived well in the present culture system as indicated by healthy neurite outgrowth even following transient impalement by sharp electrodes used in intracellular recordings. Neuron B5 in culture also displays similar resting membrane potential (-52.6 ± 0.7 mV) as reported *in situ* (around -50 to -60 mV; Berdan et al., 1993; Sekerli et al., 2004). On the other hand, rhythmic firing, such as those reported for *in situ* preparations and controlled by interneurons (Quinlan and Murphy, 1996), was not observed in isolated cultured neuron B5. The input resistance of neuron B5 is 176 ± 20 MΩ in present system, in which neuron B5 was cultured in CM and tested in day 1. On the other hand, Achee and Zoran (1997) reported that neuron B5 cultured in DM has input resistance of 73.3±15.8MΩ, which is around half that of the present

measurements. However, the number of days after cell plating when these measurements were made were not specified in the study by Achee and Zoran (1997), making direct comparisons of these two sects of *in vitro* measurements difficult. On the other hand, both sets of input resistance values from cultured neuron B5 also differ from that reported for B5 *in situ* (16 MΩ, single measurement; Berdan et al., 1993). Based on limited information available, it is hard to tell whether neuron B5 truly has different input resistance between *in situ* and cultured conditions. Nevertheless, it is very likely that culture conditions influence passive membrane properties as indicated by the difference in input resistance between this study and that by Achee and Zoran (1997).

GnRH receptors and GnRH receptor-like materials in *Helisoma*

In the Western blot analysis, a rabbit polyclonal antibody raised against the third intracellular loop of the human GnRH receptor type I reacted positively with one and five protein bands in extracts of *Helisoma* penial complexes and brains, respectively. In contrast, a mouse monoclonal antibody raised against the N-terminus of the human GnRH receptor type I reacted positively with four and five bands in extracts of *Helisoma* penial complexes and brains, respectively. These bands ranged from about 58 to about 80 kDa in molecular weight. In

addition, the polyclonal antibody also detected a 37 kDa band from *Helisoma* brain extracts. These immunoreactive bands all fell within the molecular weight range reported for a number of mGnRH receptors (from 37 to 75 kDa; Iwashita and Catt, 1985; Perrin et al., 1993; Santra et al., 2000; Mangia et al., 2002; Chien et al., 2004). Although the identity of the multiple anti-GnRH receptor immunoreactive bands revealed in *Helisoma* tissues has yet to be ascertained, they potentially represent different GnRH receptor isoforms in this species.

Among these anti-GnRH receptor immunoreactivity profiles, both antibodies consistently revealed the presence of a 58 kDa protein in *Helisoma* penial complex and brain tissues, and a 74 kDa protein in extracts of *Helisoma* brain. Both antibodies also revealed similar sized bands in the positive control, pituitary extracts from goldfish. Two GnRH receptor isoforms have been cloned from the goldfish and, interestingly, photo-affinity labeling suggests that goldfish pituitary GnRH receptors have molecular weights of around 51 and 71 kDa (Habibi et al., 1990; Illing et al., 1999). Thus, the 58 and 74 kDa proteins from *Helisoma* may be homologous to previously described GnRH receptors from goldfish.

Some of the anti-GnRH receptor immunoreactive bands that were not consistently revealed by both antibodies might be proteins that contained similar amino acid sequences and antigenic conformations as the immunogens used to

raise these two antibodies. However, whether any of the immunoreactive bands from *Helisoma* tissue extracts represent true GnRH receptors needs to be confirmed. This could be achieved by a combination of receptor cloning, functional expression, ligand binding and other studies as have been done for other chordate GnRH receptors (Kakar et al., 1992; Fujii et al., 2004; Ikemoto et al., 2004).

Within the last year, cloning and sequencing of two molluscan GnRH receptor cDNAs, from an oyster (Rodet et al., 2005) and an octopus (Kanda et al., 2006) respectively, have been reported. Strikingly, their expression in the gonads of these species is consistent with the discoveries that GnRH promotes DNA synthesis and steroidogenesis in gonadal cells of oysters and octopus, respectively (Pazos and Mathieu, 1999; Kanda et al., 2006). Interestingly, analysis of their predicted N-termini and the 3rd intracellular loops revealed that these regions share around 30-40% amino acid sequence similarity with sequences in the corresponding regions of the human GnRH receptor type I (Rodet et al., 2005; Kanda et al., 2006). These degrees of similarity are comparable to that for the two goldfish GnRH receptors with human GnRH receptor type I (Illing et al., 1999). The degree of amino acid similarity with human GnRH receptor type I for oyster GnRH receptor, octGnRH receptor, goldfish

GnRH receptor I, and goldfish GnRH receptor II are, respectively, N-terminus: 30, 34, 48 and 25%; and 3rd intracellular loop: 38, 28, 47 and 46%. These are in agreement with the ability of the two mGnRH antibodies to pick up immunoreactive protein bands in *Helisoma* and goldfish tissues. In addition, as in chordate GnRH receptors, the molluscan receptors contain sites for glycosylation (Rodet et al., 2005; Kanda et al., 2006), which may result in multiple isoforms of GnRH receptors (Cheng and Leung, 2000). This is also not at odds with the presence of multiple bands in *Helisoma* tissues revealed in this thesis.

Assuming that some, even if not all, of the immunoreactive bands detected in Western blot analyses of *Helisoma* tissues represent authentic GnRH receptors, then these data suggest that *Helisoma* has multiple GnRH receptors. Furthermore, the presence of non-identical anti-GnRH receptor immunoreactive bands in the *Helisoma* penial complex and brain extracts suggests the differential expression of GnRH receptors in different tissues in *Helisoma*. Although only one single GnRH receptor gene has thus far been cloned from each of oyster and octopus, the presence of multiple GnRH receptors in a single species and differential expression of different GnRH receptor isoforms among tissues have long been known in a variety of chordate species (Illing et al., 1999; Bogerd et al., 2002; Moncaut et al., 2005; Tello et al., 2005). For examples, although the two GnRH

receptors cloned from goldfish have some overlapping expression, only one of them is expressed in certain tissues, such as liver, ovary, and the area ventralis telencephali (Illing et al., 1999). In some species (e.g., tunicates, Kusakabe et al., 2003; cichlid fish, Chen and Fernald, 2006), the different GnRH receptors are each encoded by a different gene. On the other hand, alternate splicing (e.g., in bullfrogs, Wang et al., 2001) and/or post-translational modification, such as differential glycosylation as predicted by Cheng and Leung (2000), could also give rise to multiple GnRH receptor isoforms.

In chordates, it is generally accepted that multiple GnRH receptors co-evolved with multiple GnRH isoforms and these form functionally-distinct systems (Klausen et al., 2002; Millar et al., 2004). For example, two types of mammalians GnRH receptors (type I and type II) selectively bind their ligands, mGnRH and cGnRH-II, respectively (Li et al., 2005). To date, only one non-chordate GnRH has been identified, that of octGnRH; however, HPLC analysis of *Helisoma* brain extract revealed the presence of at least two anti-GnRH immunoreactive peaks (Goldberg et al., 1993). Furthermore, immunohistochemical and electrophysiological evidence suggest that more than one GnRH-GnRH receptor system also exist in another mollusk, *Aplysia*. Antisera against mGnRH and tGnRH-1 detected anti-GnRH immunoreactivity in neurons in

the *Aplysia* osphradium and brain, respectively; on the other hand, the osphradium and brain did not contain anti-tGnRH-1 and anti-mGnRH immunoreactivity, respectively (Tsai et al., 2003). Interestingly, the brain extracts containing anti-tGnRH-1 immunoreactivity, but no anti-mGnRH immunoreactivity, failed to stimulate LH release from mouse pituitaries (Tsai et al., 2003). In addition, bag cells, whose somata do not contain anti-GnRH immunoreactivity, selectively respond to cGnRH-II, but not tGnRH-1. All these findings indicate that more than one GnRH-GnRH receptor system is present in *Aplysia*.

In addition to the isolated octGnRH (Iwakoshi et al., 2002), another octopus GnRH-like molecular with same elution time as cGnRH-I has been revealed by HPLC (Di Cristo et al., 2002). Anti-cGnRH-I immunoreactivity has also been found in neuronal and reproductive tissues of octopus (Di Cosmo and Di Cristo, 1998). Moreover, the octGnRH receptor expressed in frog occytes can also recognize a chicken GnRH analogue, although it induces a lower response than octGnRH (Kanda, et al., 2006). All these observations suggest the presence of a second GnRH system in octopus which has yet to be identified. Taken together all the available information, it would not be surprising that *Helisoma* possesses more than one GnRH receptor sub-type.

Tissue distribution of GnRH receptors in *Helisoma*

Data from the Western blot analysis suggest the presence of GnRH receptor-like molecules in the brain and penial complex of adult *Helisoma*. Subsequent immunohistochemical studies with wholemount preparations of *Helisoma* penial complexes successfully demonstrated the presence of anti-GnRH receptor immunoreactivity in this organ. More specifically, anti-GnRH receptor immunoreactivity was localized in deep tissues within the head of the penis, as well as in the area where the vas deferens entered the penis. The presence of GnRH receptors in the male reproductive system in *Helisoma* has previously been predicted by the presence of anti-GnRH immunoreactivity in these tissues (Young et al., 1999).

In agreement with the Western blot results of this study suggesting neuronal and reproductive localizations of GnRH receptors, previous studies on tissue distributions of anti-GnRH immunoreactivity suggest that GnRH receptors are likely present in the brain, ovotestis, penial complex, prostate gland, and female reproductive glands (Young et al., 1997; Young et al., 1999). On the other hand, immunohistochemistry with wholemounts of *Helisoma* brains proved to be a less ideal method to map the distribution of GnRH receptors in the brain in this thesis. The presence of a connective tissue sheath covering the *Helisoma* brain probably

acted as a barrier to antibody penetration, resulting in inconsistencies in staining. Various enzymatic digestion and detergent treatment protocols were tried to improve antibody penetration, but these were not very successful and potentially also disrupted the integrity of cell surface GnRH receptors. Nevertheless, some staining of neuronal cell bodies was occasionally seen in cerebral and buccal ganglia, and more consistently in neuronal projections along the penial nerve and connectives between ganglia, especially the cerebrobuccal connective. These observations suggest that GnRH receptors are expressed on at least some *Helisoma* neurons in cerebral and buccal ganglia. That some CE neurons and buccal neurons possess GnRH receptors is strongly suggested by the ability of both antibodies to stain around 85% of CE and 100% of B5 neurons in culture, as well as by the ability of mGnRH to alter electrophysiological properties in 96% of CE and 100% of B5 neurons in culture. Although one cannot be absolutely sure that both antibodies targeted the exact same population of CE neurons or that CE neurons which stained positively with the two GnRH receptor antibodies were the ones that would respond to mGnRH, the fact that 96% of CE neurons responded to mGnRH bode well for the hypothesis that a great majority, if not all, anti-GnRH receptor immunoreactive CE neurons would respond to mGnRH. Whether GnRH receptors are also present on CE cells that did not sustain neurite elongation or

on other buccal ganglia neurons has not been ascertained in the present study.

Influence of mGnRH on CE and B5 neurons

Results from electrophysiological recordings in this thesis support the hypothesis that *Helisoma* CE neurons and B5 neurons have functional GnRH receptors since application of mGnRH caused changes in electrophysiological properties in 96% of CE neurons and 100% of B5 neurons; furthermore, the effects of mGnRH on B5 were inhibited by a mGnRH antagonist. Although previous data suggest that mGnRH altered B5 activity in buccal ganglia preparations (Garofalo et al., unpublished), the present study represents the first time that direct GnRH actions on isolated B5 and CE neurons have been demonstrated. However, whether GnRH also acts via interneurons to indirectly alter the electrophysiological activities of B5 and CE neurons remains a possibility that should be further investigated. In agreement with the hypothesis that CE neurons represent a heterogeneous population of neurons, application of mGnRH elicited a number of electrophysiological responses in CE neurons. Responses to mGnRH application in CE neurons include both increases and decreases in input resistance, as well as both increases and decreases in post-hyperpolarization rebound firing. In contrast, in B5 neurons, mGnRH consistently reduced input

resistance but had no effects on resting membrane potential, post-hyperpolarization rebound firing and depolarization-induced firing.

The ionic currents targeted by mGnRH that resulted in these effects have not been directly examined in this thesis, but some speculations can be made based on previous findings. GnRH inhibits M-type potassium current in sympathetic neurons (Jones, 1987). In gonadotropes, GnRH triggers inward sodium currents and N-type calcium currents (Heyward, et al., 1995) and increases calcium-dependent, apamine-sensitive, small conductance potassium current (SKca) via increases in intracellular calcium (Tse and Hille, 1992). GnRH is also known to increase chloride current in *Xenopus* oocytes expressing rat GnRH receptor or octGnRH receptor (Yoshida et al., 1989; Kanda et al., 2006). In *Aplysia*, GnRH reduces firing activity (number of action potentials during afterdischarge) in bag cell neurons (Zhang et al., 2000). Zhang et al. (2000) speculated that GnRH might reduce cell excitability by hyperpolarizing the membrane potential. On the other hand, GnRH increases firing activity of *Aplysia* white neurons (Seaman et al., 1980). Thus, channel activities in response to GnRH may differ between different cells even within the same organism.

In *Helisoma* neurons, if GnRH can also trigger or inhibit currents by regulating the activity of channels, the input resistance and firing activity will

consequently change. Input resistance will decrease with more channel opening, and increase with more channels closing. Usually, increased inward sodium current will induce action potential activity through depolarization, whereas increased outward potassium current will reduce firing through hyperpolarization. Chloride currents may cause either hyperpolarization or depolarization depending on chloride ion ratio between the intracellular and extracellular environments (Kyrozis and Reichling, 1995). In addition, changes in ion channel activities would also result in changes in the kinetics and shape of the action potentials. If GnRH induces an increase in sodium current by promoting channel opening as in previous study (Heyward, et al., 1995), changes in the time of the peak of the action potential may not be observed due to the fast inactivation of sodium channels (Nicholls et al., 1992) but the rate of rise and the amplitude of the action potential may increase. If more calcium channels become open, a broader action potential may also result since many calcium channels (e.g. N-type calcium channel) have long opening time and are not rapidly inactivating (Nicholls et al., 1992). Calcium currents not only affect the firing through depolarization but also alter intracellular calcium levels, which regulate other channel activities, such as Ca^{2+}-sensitive potassium channels (Johnson and Chang, 2000). Generally speaking, many potassium channels are not fast-inactivation (Nicholls et al.,

1992). Increases in outward potassium current will reduce the repolarization time and increase the magnitude and duration of the afterhyperpolarization (undershoot). In particular, activation of voltage-independent SKca channels as in gonadotropes would be expected to cause prolonged afterhyperpolarization, which can last for a few seconds, and increase the intervals between action potential firing (Lancaster and Nicoll, 1987; Hirschberg et al., 1999; Maylie et al., 2004). Increases in voltage-dependent, calcium-activated big conductance potassium channels (BKca) have similarly been reported to contribute to fast hyperpolarization in some systems (Faber and Sah, 2002). On the other hand, increases in BKca currents may have minimal effects on duration of the afterhyperpolarization since these channels deactivate rapidly (Brenner et al., 2005). Changes in chloride currents may either increase or decrease the firing depending on choloride equilibrium potential, which is determined by ion ratio between the intracellular and extracellular environment (Eckert and Randall, 1983).

In *Helisoma* neurons, if GnRH can also trigger or inhibit currents by regulating the activity of channels, the input resistance and firing activity will consequently change. Input resistance will decrease with more channel opening, and increase with more channels closing. Usually, increased inward sodium

current will induce action potential activity through depolarization, whereas increased outward potassium current will reduce firing through hyperpolarization. Chloride currents may cause either hyperpolarization or depolarization depending on chloride ion ratio between the intracellular and extracellular environments (Kyrozis and Reichling, 1995). In addition, changes in ion channel activities would also result in changes in the kinetics and shape of the action potentials. If GnRH induces an increase in sodium current by promoting channel opening as in previous study (Heyward, et al., 1995), changes in the time of the peak of the action potential may not be observed due to the fast inactivation of sodium channels (Nicholls et al., 1992) but the rate of rise and the amplitude of the action potential may increase. If more calcium channels become open, a broader action potential may also result since many calcium channels (e.g. N-type calcium channel) have long opening time and are not rapidly inactivating (Nicholls et al., 1992). Calcium currents not only affect the firing through depolarization but also alter intracellular calcium levels, which regulate other channel activities, such as Ca^{2+}-sensitive potassium channels (Johnson and Chang, 2000). Generally speaking, many potassium channels are not fast-inactivation (Nicholls et al., 1992). Increases in outward potassium current will reduce the repolarization time and increase the magnitude and duration of the afterhyperpolarization

(undershoot). In particular, activation of voltage-independent SKca channels as in gonadotropes would be expected to cause prolonged afterhyperpolarization, which can last for a few seconds, and increase the intervals between action potential firing (Lancaster and Nicoll, 1987; Hirschberg et al., 1999; Maylie et al., 2004). Increases in voltage-dependent, calcium-activated big conductance potassium channels (BKca) have similarly been reported to contribute to fast hyperpolarization in some systems (Faber and Sah, 2002). On the other hand, increases in BKca currents may have minimal effects on duration of the afterhyperpolarization since these channels deactivate rapidly (Brenner et al., 2005). Changes in chloride currents may either increase or decrease the firing depending on choloride equilibrium potential, which is determined by ion ratio between the intracellular and extracellular environment (Eckert and Randall, 1983).

In this study, the ability of GnRH to alter input resistance in CE neurons and B5 neurons is consistent with the idea that GnRH modulates the activities of ion channel in *Helisoma* neurons. However, unlike previous reports using other vertebrate and invertebrate model systems (Heyward et al., 1993; Zhang et al., 2000), no obvious changes in the firing frequency and action potential kinetics have been detected. Admittedly, I was unable to precisely examine these

parameters since the chart recorder used did not have the sensitivity to resolve very small changes in time-dependent events. On the other hand, no obvious changes in these parameters were detected when signals were monitored visually through the oscilloscope during recording. However, once again, the resolution of the oscilloscope during recording may not have been optimized for detection of subtle changes. Thus, the ionic currents underlying the observed GnRH-induced changes in electrophysiological properties in CE neurons and B5 neurons remain unknown. Interestingly, N-like calcium currents have been found in neuron B5 (Wang et al., 1993). In addition, many neurosecretory factors, such as 5-HT and FMRF amide, can activate potassium channels in B5 (Bahls et al., 1992; Achee and Zoran, 1997) though calcium-activated potassium channels have not been detected in B5 neurons. In future studies, the rising, falling and undershoot phases of action potentials could be examined in more detail to gain an insight into the ionic currents affected by GnRH. Pharmacological tools, such as the blockage of SKca with apamine (Tse and Hille, 1992) and BKca by iberiotoxin (Brenner et al., 2005), could be employed to further identify the types of channels involved. In addition, patch clamp can be applied to monitor specific channel activities under GnRH action to elucidate the identity of the currents affected by GnRH.

Intracellular signal transduction mechanisms of GnRH actions in vertebrate cells support that GnRH has diverse effects on electrophysiological properties. The PLC/Ca^{2+}/PKC pathway is a major postreceptor signaling pathway for vertebrate GnRH receptors (Millar et al., 2004). Activation of different PKC isozymes can result in further diversity in GnRH signaling (Klausen et al., 2002). Activation of the MAPK pathway is also associated with GnRH receptor signaling (Sim et al., 1995). Changes of membrane electrophysiological properties depend on changes in membrane channels. PKC, activated in the PLC pathway, can regulate a number of membrane channels, including sodium, potassium, calcium and chloride channels (Fathallah et al., 1997; Fathallah and Harvey, 1998; Lo and Numann, 1999; Curia et al., 2004). PIP_2, the substrate of PLC, is an important membrane second messenger regulating many cation channels. It increases the activity of epithelial sodium channels (Yue et al., 2002), and stabilizes open states of many potassium channels (Baukrowitz et al., 1998; Shyng and Nichols, 1998; Loussouarn et al., 2003). It also regulates some calcium permeable cation channels (Zhainazarov and Ache, 1999; Rohacs et al., 2005). Its hydrolysis may reduce channel activities generally (Suh and Hille, 2005), as well as reducing M-currents specifically (Winks et al., 2005). Also, activated MAPK can change the activities of multiple channel types, such as sodium, potassium and calcium

channels (Fitzgerald, 2000; Ross and Armstead, 2003; Wittmack et al., 2005). Differential expression of membrane ion channels in different cells may result in a diversity of electrophysiological responses during PKC, PIP_2, or MAPK signaling in GnRH action. In addition, calcium and CAM-dependent kinases (Ca^{2+}/CAMKs), potentially activated by GnRH stimulation of the PLC pathway, can change the activity of many receptor channels, such as N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) channels, adding further to the diversity of responses and effects (Kitamura et al., 1993; Carvalho et al., 1998). The possible presence of more than one GnRH receptor in CE neuronal population may also be the basis of the diversity of mGnRH-induced changes in electrophysiological properties observed in this study.

In this culture system, neuron B5 displays similar resting membrane potential (-52.6 ± 0.7 mV) as reported *in situ* (around -50 to -60 mV, Berdan et al., 1993; Sekerli et al., 2004). Also, it is not surprising that the typical firing pattern of neuron B5 which is controlled by subunits of interneurons *in situ* (Quinlan and Murphy, 1996) was not recorded in this single neuron culture system. Although Berdan et al. (1993) reported that the input resistance of one B5 neuron *in situ* is 16 MΩ, no statistical data about input resistance of neuron B5 was reported. Based on limited information, it is hard to tell whether neuron B5 has different

input resistance between *in situ* and in this culture system. Achee and Zoran (1997) reported that neuron B5 cultured in DM has input resistance of 73.3±15.8MΩ, although the detailed days after cell plating were not specified; however, the input resistance of neuron B5 is 176 ± 20 MΩ in this system, in which neuron B5 was cultured in CM and tested in day 1. Thus, culture conditions may influence passive membrane properties as indicated by the difference in input resistance. This difference may due to different extracellular environment cues or different intrinsic cell properties which might be altered by different culture conditions. At this point, I would not exclude the possibility that possible changes of cell properties induced by the cell culture condition will affect the response to GnRH. Therefore, direct effects of GnRH on neuron B5 *in situ* could be performed for future research, when techniques become available to effectively block interactions between neurons *in situ*.

Possible physiological functions of the GnRH-GnRH receptor system in *Helisoma*

This thesis established the presence of GnRH receptor-like materials on CE neurons, the head of the penis and areas around its junction with the vas deferens, and the penial nerve. In addition, mGnRH was also able to alter electrophysiological properties of CE neurons. Previously, anti-GnRH

immunoreactivity has also been localized to the CE neuropil, penial nerve and the musculature surrounding the vas deferens (Young et al., 1999). Taken together, these results support the speculation that snail CE neurons project to the penis and release GnRH to control functions of the male reproductive tract. In addition, these data also suggest that CE neurons themselves are targets of GnRH action. These observations strongly indicate that the GnRH-GnRH receptor system functions as an important regulator of reproduction in *Helisoma*.

Information from other molluscan and invertebrate systems also supports the idea of a reproductive function for the GnRH-GnRH receptor system in *Helisoma*. GnRH has been shown to increase DNA synthesis in gonads of bivalves (Pazos and Mathieu, 1999). GnRH can promote spermatogenesis and vitellogenesis in scallops (Nakao et al., 2005; Osada et al., 2005), and stimulate spawning in snails (Young et al., 1997), chitons (Gorbman et al., 2003) and tunicates (Adams et al., 2003). GnRH and its analogue also stimulate steroidogenesis in octopus and coral (Kanda et al., 2006; Twan et al., 2006). GnRH alters the patterns of action potentials that trigger egg-laying hormone release from *Aplysia* bag cell neurons, suggesting potential GnRH regulation of egg laying and sexual hormone release (Zhang et al., 2000). Moreover, in line with the discovery of anti-GnRH receptor immunoreactivity in the *Helisoma* reproductive tract, the expression of GnRH

receptor mRNA has been demonstrated in the ovary, testis, oviduct, and vas deferens of octopus (Kanda et al., 2006). Thus, the GnRH-GnRH receptor system is likely to be an important regulator of reproduction in mollusks, including *Helisoma*.

One scenario based on current information derived from *Helisoma* may be that brain GnRH neurons outside the CE area project neurites which 1) have secondary branches in the CE area to release GnRH to modulate the activities of some GnRH-positive, as well as GnRH-negative, CE neurons that innervate male reproductive tract, and 2) enter the penial nerve to innervate the male reproductive tract and regulate its function. Since anti-GnRH immunoreactivity was located in the muscle layer of vas deferens (Young et al., 1999), it is speculated that GnRH may cause vas deferens contraction. In addition to these neurotransmitter/modulator actions, GnRH may also act as a neuroendocrine factor to regulate reproduction. Snails possess an open circulatory system, in which blood circulates into the spongy hemocoel in which tissues are bathed (Lutz, 1985). Therefore, there is the possibility that snail neurosecretory factors can be released directly into blood, transported to their target organs and work as classic endocrine hormones. Data from tunicates and *Aplysia* support the hypothesis that GnRH is directly secreted into the blood stream to act as a hormone since

anti-GnRH immunoreactive nerve fibers run into blood sinuses in tunicates (Powell et al., 1996) and anti-GnRH immunoreactivity was found in cell-free blood of *Aplysia* (Zhang et al., 2000).

The neuron B5 is one of several important motor neurons controlling food intake in *Helisoma* (Murphy et al., 1985; Murphy, 2001). In this study, B5 was shown to possess functional GnRH receptor(s). In addition, application of GnRH to buccal ganglia preparations alters the firing activity of neuron B19, another buccal neuron that regulates the feeding behavior (Ramakrishnan and Murphy, 2003). These identified buccal neurons may be innervated by a set of unidentified buccal neurons that stained for GnRH (Young et al., 1999) and/or by interneurons receiving input from GnRH neurons. A recent report also demonstrated the expression of GnRH receptor mRNA in the buccal ganglia, esophagus, crop, stomach, salivary gland and radular retractor muscle of octopus (Kanda et al., 2006). The octGnRH can dose-dependently stimulate the retractor muscle of the octopus radula (Kanda et al., 2006), which helps collect food particles by a rasping or licking action. Taken together, these results suggest that the GnRH-GnRH receptor system also plays a role in regulating feeding in *Helisoma* and other mollusks.

Interestingly, known molluscan GnRH receptors highly resemble AKH

receptors and the possibility that these molluscan receptors recognize AKH cannot be discounted at present (Rodet et al., 2005). AKH and other related neuropeptides, including APGWamide, not only share a common molecular ancestor, but some of them also have known functions as regulators of reproduction and feeding in mollusks (Li et al., 1992; van Golen et al., 1995b; Henry et al., 1997; Martinez-Perez et al., 2002). In gastropods, APGWamide regulates muscles in the reproductive tract (van Golen et al., 1995b; de Boer et al., 1997; Henry et al., 1997) and neurons in the central pattern generator responsible for feeding (Elliott and Vehovszky, 2000). Whether cross binding with GnRH receptors may explain some of these previous reported functions of adipokinetic hormone and APGWamide are not known, but in view of other results in the literature, data from the present thesis strongly suggest that GnRH and GnRH receptors need to be considered as one of many neuropeptide systems regulating feeding and reproduction in mollusks.

Future studies

One important area of future research has to be the identification of *Helisoma* GnRH receptors followed by their functional validation. Since the GnRH receptor protein is apparently present in *Helisoma* and may share amino acid sequence

similarity with the mammalian GnRH receptor, especially in the N-terminus and the 3rd intracellular loop as demonstrated in this thesis, degenerate primers can be designed to clone the cDNA for the *Helisoma* GnRH receptor. Also, primers can be designed based on the sequence of the known molluscan GnRH receptors in octopus and oyster, respectively. Functional tests can be done by expressing the potential mRNA of *Helisoma* GnRH receptor and other known GnRH receptors in frog oocytes and then testing for GnRH responses, as in the case of the identified octGnRH receptor (Kanda et al., 2006).

Further GnRH and GnRH receptor distribution studies with *Helisoma* tissue sections, together with functional studies, would also go a long way towards contributing to an understanding the physiology of the GnRH-GnRH receptor system in this animal. One approach could be to establish an *in vitro* bioassay system using muscles around the junction area of the penis and vas deferens where both anti-GnRH (Young et al., 1999) and anti-GnRH receptor immunoreactivities were found. GnRH effects on muscle contraction in the reproductive tract can be tested using similar assays to those developed for studies of *Lymnaea* reproductive peptides (van Golen et al., 1995a; van Golen et al., 1995b; van Golen et al., 1996; de Boer et al., 1997; de Lange et al., 1997) and octGnRH actions on radular retractor muscle in octopus (Kanda et al., 2006).

Summary

The data from this thesis support the main hypothesis that GnRH is functional in the adult pond snail, *Helisoma*. Evidence also suggests that multiple GnRH receptor isoforms may exist in this species. In addition, the present results indicate that the GnRH-GnRH receptor system may be one of several neuropeptide systems involved in the regulation of reproduction and feeding in this species. Since the control of reproduction and feeding are also important functions of GnRH in vertebrates (Klausen et al., 2002; Kauffman et al., 2005), these ideas also provide insight into the functional conservation of the GnRH-GnRH receptor system in the evolution of both protosome and deuterostome lineages. Furthermore, this thesis research developed a novel culture system of CE neurons and provided the first characterization of CE neurons in culture. These results will form an important basis for any further research using cultured CE cells as a model.

REFERENCES

Abe H, Oka Y (2000) Modulation of pacemaker activity by salmon gonadotropin-releasing hormone (sGnRH) in terminal nerve (TN)-GnRH neurons. J Neurophysiol 83:3196-3200.

Achee N, Zoran M (1997) Serotonin-induced modulation of excitability in an identified *Helisoma trivolvis* neuron. J Exp Biol 200:1537-1548.

Adams BA, Tello JA, Erchegyi J, Warby C, Hong DJ, Akinsanya KO, Mackie GO, Vale W, Rivier JE, Sherwood NM (2003) Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, *Ciona intestinalis*. Endocrinology 144:1907-1919.

Ahn KC, Goldberg JI (2003) Initiation, characterization and serotonergic modulation of neurite outgrowth in serotonergic cerebral neuron C1 from *Helisoma trivolvis*. Soc Neurosci Abstr: Program No. 884.4. New Orleans, Louisiana, USA (November 8-12, 2003).

Anctil M (2000) Evidence for gonadotropin-releasing hormone-like peptides in a cnidarian nervous system. Gen Comp Endocrinol 119:317-328.

Aston-Jones G, Shaver R, Dinan T (1984) Cortically projecting nucleus basalis neurons in rat are physiologically heterogeneous. Neurosci Lett 46:19-24.

Bahls FH, Richmond JE, Smith WL, Haydon PG (1992) A lipoxygenase pathway of arachidonic acid metabolism mediates FMRFamide activation of a potassium current in an identified neuron of *Helisoma*. Neurosci Lett 138:165-168.

Barker DL, Wong RG, Kater SB (1982) Separate factors produced by the CNS of the snail *Helisoma* stimulate neurite outgrowth and choline metabolism in cultured neurons. J Neurosci Res 8:419-432.

Barker JL, Ifshin MS, Gainer N (1975) Studies on bursting pacemaker potential activity in molluscan neurons. III. Effects of hormones. Brain Res 84:501-513.

Barran PE, Roeske RW, Pawson AJ, Sellar R, Bowers MT, Morgan K, Lu ZL, Tsuda M, Kusakabe T, Millar RP (2005) Evolution of constrained gonadotropin-releasing hormone ligand conformation and receptor selectivity. J Biol Chem 280:38569-38575.

Batticane N, Morale MC, Gallo F, Farinella Z, Marchetti B (1991) Luteinizing hormone-releasing hormone signaling at the lymphocyte involves stimulation of interleukin-2 receptor expression. Endocrinology 129:277-286.

Baukrowitz T, Schulte U, Oliver D, Herlitze S, Krauter T, Tucker SJ, Ruppersberg

JP, Fakler B (1998) PIP_2 and PIP as determinants for ATP inhibition of K_{ATP} channels. Science 282:1141-1144.

Berdan RC, Easaw JC, Wang R (1993) Alterations in membrane potential after axotomy at different distances from the soma of an identified neuron and the effect of depolarization on neurite outgrowth and calcium channel expression. J Neurophysiol 69:151-164.

Bogerd J, Diepenbroek WB, Hund E, van Oosterhout F, Teves AC, Leurs R, Blomenrohr M (2002) Two gonadotropin-releasing hormone receptors in the African catfish: no differences in ligand selectivity, but differences in tissue distribution. Endocrinology 143:4673-4682.

Brenner R, Chen QH, Vilaythong A, Toney GM, Noebels JL, Aldrich RW (2005) BK channel beta4 subunit reduces dentate gyrus excitability and protects against temporal lobe seizures. Nat Neurosci 8:1752-1759.

Brownlee DJ, Fairweather I, Johnston CF, Smart D, Shaw C, Halton DW (1993) Immunocytochemical demonstration of neuropeptides in the central nervous system of the roundworm, *Ascaris suum* (Nematoda: Ascaroidea). Parasitology 106:305-316.

Bulloch AG, Kater SB (1982) Neurite outgrowth and selection of new electrical connections by adult *Helisoma* neurons. J Neurophysiol 48:569-583.

Cariello L, Romano G, Spagnuolo A, Zanetti L, Fasano S, Minucci S, Di Matteo L, Pierantoni R, Chieffi G (1989) Molecular forms of immunoreactive gonadotropin-releasing hormone in hypothalamus and testis of the frog, *Rana esculenta*. Gen Comp Endocrinol 75:343-348.

Carvalho AL, Duarte CB, Faro CJ, Carvalho AP, Pires EV (1998) Calcium influx through AMPA receptors and through calcium channels is regulated by protein kinase C in cultured retina amacrine-like cells. J Neurochem 70:2112-2119.

Castro-Fernandez C, Conn PM (2002) Regulation of the gonadotropin-releasing hormone receptor (GnRHR) by RGS proteins: role of the GnRHR carboxyl-terminus. Mol Cell Endocrinol 191:149-156.

Chen CC, Fernald RD (2006) Distributions of two gonadotropin-releasing hormone receptor types in a cichlid fish suggest functional specialization. J Comp Neurol 495:314-323.

Chen HF, Jeung EB, Stephenson M, Leung PC (1999) Human peripheral blood mononuclear cells express gonadotropin-releasing hormone (GnRH), GnRH receptor, and interleukin-2 receptor gamma-chain messenger ribonucleic acids that are regulated by GnRH *in vitro*. J Clin Endocrinol Metab 84:743-750.

Cheng KW, Leung PC (2000) The expression, regulation and signal transduction pathways of the mammalian gonadotropin-releasing hormone receptor. Can J Physiol Pharmacol 78:1029-1052.

Chien CH, Chen CH, Lee CY, Chang TC, Chen RJ, Chow SN (2004) Detection of gonadotropin-releasing hormone receptor and its mRNA in primary human epithelial ovarian cancers. Int J Gynecol Cancer 14:451-458.

Cone RD (2000) The corticotropin-releasing hormone system and feeding behavior--a complex web begins to unravel. Endocrinology 141:2713-2714.

Cook H, Berkenbosch JW, Fernhout MJ, Yu KL, Peter RE, Chang JP, Rivier JE (1991) Demonstration of gonadotropin releasing-hormone receptors on gonadotrophs and somatotrophs of the goldfish: an electron microscope study. Regul Pept 36:369-378.

Corp ES, Greco B, Powers JB, Marin Bivens CL, Wade GN (2001) Neuropeptide Y inhibits estrous behavior and stimulates feeding via separate receptors in Syrian hamsters. Am J Physiol Regul Integr Comp Physiol 280:R1061-1068.

Curia G, Aracri P, Sancini G, Mantegazza M, Avanzini G, Franceschetti S (2004) Protein-kinase C-dependent phosphorylation inhibits the effect of the

antiepileptic drug topiramate on the persistent fraction of sodium currents. Neuroscience 127:63-68.

Curia G, Aracri P, Sancini G, Mantegazza M, Avanzini G, Franceschetti S (2004) Protein-kinase C-dependent phosphorylation inhibits the effect of the antiepileptic drug topiramate on the persistent fraction of sodium currents. Neuroscience 127:63-68.

Davidson JS, Wakefield IK, Millar RP (1994) Absence of rapid desensitization of the mouse gonadotropin-releasing hormone receptor. Biochem J 300:299-302.

de Boer PA, ter Maat A, Pieneman AW, Croll RP, Kurokawa M, Jansen RF (1997) Functional role of peptidergic anterior lobe neurons in male sexual behavior of the snail *Lymnaea stagnalis*. J Neurophysiol 78:2823-2833.

de Lange RP, van Golen FA, van Minnen J (1997) Diversity in cell specific co-expression of four neuropeptide genes involved in control of male copulation behaviour in *Lymnaea stagnalis*. Neuroscience 78:289-299.

Di Cosmo A, Di Cristo C (1998) Neuropeptidergic control of the optic gland of *Octopus vulgaris*: FMRF-amide and GnRH immunoreactivity. J Comp Neurol 398:1-12.

Di Cristo C, Paolucci M, Iglesias J, Sanchez J, Di Cosmo A (2002) Presence of

two neuropeptides in the fusiform ganglion and reproductive ducts of *Octopus vulgaris*: FMRFamide and gonadotropin-releasing hormone (GnRH). J Exp Zool 292:267-276.

Di Matteo L, Vallarino M, Pierantoni R (1996) Localization of GnRH molecular forms in the brain, pituitary, and testis of the frog, *Rana esculenta*. J Exp Zool 274:33-40.

Eckert R, Randall D (1983) Animal Physiology. pp 89-228. San Francisco, California, USA: W.H. Freeman and Company.

Elliott CJ, Vehovszky A (2000) Comparative pharmacology of feeding in molluscs. Acta Biol Hung 51:153-163.

Elliott CJH, Susswein AJ (2002) Comparative neuroethology of feeding control in molluscs. J Exp Biol 205:877-896.

Faber ES, Sah P (2002) Physiological role of calcium-activated potassium currents in the rat lateral amygdala. J Neurosci 22:1618-1628.

Fang YQ, Wang H, Zhang CL (1991) Correlation of annual change of luteinizing hormone-releasing hormone (LH-RH) with gonadal development in amphioxus. Sci China B 34:814-822.

Fann MC, Wan WC, Wang PS (1990) Existence of a gonadotropin-releasing hormone-like factor in the grass shrimp (black tiger prawn, *Penaeus*

monodon). Chin J Physiol 33:169-178.

Fathallah H, Sauvage M, Romero JR, Canessa M, Giraud F (1997) Effects of PKC α activation on Ca^{2+} pump and K_{Ca} channel in deoxygenated sickle cells. Am J Physiol 273:C1206-1214.

Fernald RD, White RB (1999) Gonadotropin-releasing hormone genes: phylogeny, structure, and functions. Front Neuroendocrinol 20:224-240.

Fitzgerald EM (2000) Regulation of voltage-dependent calcium channels in rat sensory neurones involves a Ras-mitogen-activated protein kinase pathway. J Physiol 527:433-444.

Fujii Y, Enomoto M, Ikemoto T, Endo D, Okubo K, Aida K, Park MK (2004) Molecular cloning and characterization of a gonadotropin-releasing hormone receptor in the guinea pig, *Cavia porcellus*. Gen Comp Endocrinol 136:208-216.

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

Goldberg JI, McCobb DP, Guthrie PB, Lawton RA, Lee RE, Kater SB (1988) Characterization of cultured embryonic neurons from the snail *Helisoma*. In: Cell Culture Approaches to Invertebrate Neuroscience (Beadle DJ, Lees G,

Kater SB, eds), pp 85-108. San Diego, California, USA: Academic Press Limited.

Goldberg JI, Mills LR, Kater SB (1991) Novel effects of serotonin on neurite outgrowth in neurons cultured from embryos of *Helisoma trivolvis*. J Neurobiol 22:182-194.

Goldberg JI, Mills LR, Kater SB (1992) Effects of serotonin on intracellular calcium in embryonic and adult *Helisoma* neurons. Int J Dev Neurosci 10:255-264.

Gorbman A, Whiteley A, Kavanaugh S (2003) Pheromonal stimulation of spawning release of gametes by gonadotropin releasing hormone in the chiton, *Mopalia* sp. Gen Comp Endocrinol 131:62-65.

Grens KE, Greenwood AK, Fernald RD (2005) Two visual processing pathways are targeted by gonadotropin-releasing hormone in the retina. Brain Behav Evol 66:1-9.

Habibi HR (1991) Homologous desensitization of gonadotropin-releasing hormone (GnRH) receptors in the goldfish pituitary: effects of native GnRH peptides and a synthetic GnRH antagonist. Biol Reprod 44:275-283.

Habibi HR, Peter RE, Hazum E (1990) Photoaffinity labeling of pituitary gonadotropin-releasing hormone receptors in goldfish (*Carassius auratus*). Biol Reprod 43:1006-1011.

Habibi HR, Peter RE, Nahorniak CS, Milton RC, Millar RP (1992) Activity of vertebrate gonadotropin-releasing hormones and analogs with variant amino acid residues in positions 5, 7 and 8 in the goldfish pituitary. Regul Pept 37:271-284.

Harris D, Bonfil D, Chuderland D, Kraus S, Seger R, Naor Z (2002) Activation of MAPK cascades by GnRH: ERK and Jun N-terminal kinase are involved in basal and GnRH-stimulated activity of the glycoprotein hormone LH-subunit promoter. Endocrinology 143:1018-1025.

Hauser F, Sondergaard L, Grimmelikhuijzen CJ (1998) Molecular cloning, genomic organization and developmental regulation of a novel receptor from *Drosophila melanogaster* structurally related to gonadotropin-releasing hormone receptors for vertebrates. Biochem Biophys Res Commun 249:822-828.

Hayashi H, Hirakawa K (1980) Nitella fluctuation and instability in the membrane potential near threshold. Biophys J 31:31-43.

Henry J, Favrel P, Boucaud-Camou E (1997) Isolation and identification of a novel Ala-Pro-Gly-Trp-amide-related peptide inhibiting the motility of the mature oviduct in the cuttlefish, *Sepia officinalis*. Peptides 18:1469-1474.

Heyward PM, Chen C, Clarke IJ (1993) Gonadotropin-releasing hormone

modifies action potential generation in sheep pars distalis gonadotropes. Neuroendocrinology 58:646-654.

Heyward PM, Chen C, Clarke IJ (1995) Inward membrane currents and electrophysiological responses to GnRH in ovine gonadotropes. Neuroendocrinology 61:609-621.

Hirschberg B, Maylie J, Adelman JP, Marrion NV (1999) Gating properties of single SK channels in hippocampal CA1 pyramidal neurons. Biophys J 77:1905-1913.

Ho HN, Chen HF, Chen SU, Chao KH, Yang YS, Huang SC, Lee TY, Gill TJ, 3rd (1995) Gonadotropin releasing hormone (GnRH) agonist induces down-regulation of the $CD3^+CD25^+$ lymphocyte subpopulation in peripheral blood. Am J Reprod Immunol 33:243-252.

Holz RW, Hlubek MD, Sorensen SD, Fisher SK, Balla T, Ozaki S, Prestwich GD, Stuenkel EL, Bittner MA (2000) A pleckstrin homology domain specific for phosphatidylinositol 4, 5-bisphosphate (PtdIns-4,5-P_2) and fused to green fluorescent protein identifies plasma membrane PtdIns-4,5-P_2 as being important in exocytosis. J Biol Chem 275:17878-17885.

Ikemoto T, Enomoto M, Park MK (2004) Identification and characterization of a reptilian GnRH receptor from the leopard gecko. Mol Cell Endocrinol

214:137-147.

Illing N, Troskie BE, Nahorniak CS, Hapgood JP, Peter RE, Millar RP (1999) Two gonadotropin-releasing hormone receptor subtypes with distinct ligand selectivity and differential distribution in brain and pituitary in the goldfish (*Carassius auratus*). Proc Natl Acad Sci U S A 96:2526-2531.

Iwakoshi E, Takuwa-Kuroda K, Fujisawa Y, Hisada M, Ukena K, Tsutsui K, Minakata H (2002) Isolation and characterization of a GnRH-like peptide from *Octopus vulgaris*. Biochem Biophys Res Commun 291:1187-1193.

Iwakoshi-Ukena E, Ukena K, Takuwa-Kuroda K, Kanda A, Tsutsui K, Minakata H (2004) Expression and distribution of octopus gonadotropin-releasing hormone in the central nervous system and peripheral organs of the octopus (*Octopus vulgaris*) by *in situ* hybridization and immunohistochemistry. J Comp Neurol 477:310-323.

Iwashita M, Catt KJ (1985) Photoaffinity labeling of pituitary and gonadal receptors for gonadotropin-releasing hormone. Endocrinology 117:738-746.

Jimenez-Linan M, Rubin BS, King JC (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.

Johnson JD, Chang JP (2000) Function- and agonist-specific Ca^{2+} signalling: the requirement for and mechanism of spatial and temporal complexity in Ca^{2+} signals. Biochem Cell Biol 78:217-240.

Jones JE, Pick RR, Davenport MD, Keene AC, Corp ES, Wade GN (2002) Disinhibition of female sexual behavior by a CRH receptor antagonist in Syrian hamsters. Am J Physiol Regul Integr Comp Physiol 283:R591-597.

Jones SW (1987) Chicken II luteinizing hormone-releasing hormone inhibits the M-current of bullfrog sympathetic neurons. Neurosci Lett 80:180-184.

Kageyama H, Takenoya F, Kita T, Hori T, Guan JL, Shioda S (2005) Galanin-like peptide in the brain: effects on feeding, energy metabolism and reproduction. Regul Pept 126:21-26.

Kakar SS, Jennes L (1995) Expression of gonadotropin-releasing hormone and gonadotropin-releasing hormone receptor mRNAs in various non-reproductive human tissues. Cancer Lett 98:57-62.

Kakar SS, Musgrove LC, Devor DC, Sellers JC, Neill JD (1992) Cloning, sequencing, and expression of human gonadotropin releasing hormone (GnRH) receptor. Biochem Biophys Res Commun 189:289-295.

Kanda A, Takahashi T, Satake H, Minakata H (2006) Molecular and functional characterization of a novel gonadotropin-releasing-hormone receptor

isolated from the common octopus (*Octopus vulgaris*). Biochem J 395:125-135.

Kater SB, Mattson MP (1988) Extrinsic and intrinsic regulators of neurite outgrowth and synaptogenesis in isolated, identified *Helisoma* neurons in culture. In: Cell Culture Approaches to Invertebrate Neuroscience (Beadle DJ, Lees G, Kater SB, eds), pp 1-32. San Diego, California, USA: Academic Press Limited.

Kauffman AS, Rissman EF (2004) The evolutionarily conserved gonadotropin-releasing hormone II modifies food intake. Endocrinology 145:686-691.

Kauffman AS, Wills A, Millar RP, Rissman EF (2005) Evidence that the type-2 gonadotrophin-releasing hormone (GnRH) receptor mediates the behavioural effects of GnRH-II on feeding and reproduction in musk shrews. J Neuroendocrinol 17:489-497.

Khan MA (1993) Histochemical and ultrastructural investigation of heterogeneous Purkinje neurons in mammalian cerebellum. Cell Mol Biol Res 39:789-795.

King JA, Millar RP (1995) Evolutionary aspects of gonadotropin-releasing hormone and its receptor. Cell Mol Neurobiol 15:5-23.

Kitamura Y, Miyazaki A, Yamanaka Y, Nomura Y (1993) Stimulatory effects of

protein kinase C and calmodulin kinase II on N-methyl-D-aspartate receptor/channels in the postsynaptic density of rat brain. J Neurochem 61:100-109.

Kitazawa A, Shimizu N (2005) Differentiation of mouse embryonic stem cells into neurons using conditioned medium of dorsal root ganglia. J Biosci Bioeng 100:94-99.

Klausen C, Chang JP, Habibi HR (2001) The effect of gonadotropin-releasing hormone on growth hormone and gonadotropin subunit gene expression in the pituitary of goldfish, *Carassius auratus*. Comp Biochem Physiol B Biochem Mol Biol 129:511-516.

Klausen C, Chang JP, Habibi HR (2002) Multiplicity of gonadotropin-releasing hormone signaling: a comparative perspective. Prog Brain Res 141:111-128.

Kraus S, Levy G, Hanoch T, Naor Z, Seger R (2004) Gonadotropin-releasing hormone induces apoptosis of prostate cancer cells: role of c-Jun NH_2-terminal kinase, protein kinase B, and extracellular signal-regulated kinase pathways. Cancer Res 64:5736-5744.

Kusakabe T, Mishima S, Shimada I, Kitajima Y, Tsuda M (2003) Structure, expression, and cluster organization of genes encoding

gonadotropin-releasing hormone receptors found in the neural complex of the ascidian *Ciona intestinalis*. Gene 322:77-84.

Lancaster B, Nicoll RA (1987) Properties of two calcium-activated hyperpolarizations in rat hippocampal neurones. J Physiol 389:187-203.

Lewin A, Tal Z, Zohav E, Schenker JG (1990) Ultrarapid freezing and thawing of hamster oocytes. Morphologic parameters, trypan blue staining and sperm penetration assay for evaluating survival. J Reprod Med 35:136-140.

Li JH, Choe H, Wang AF, Maiti K, Wang C, Salam A, Chun SY, Lee WK, Kim K, Kwon HB, Seong JY (2005) Extracellular loop 3 (EL3) and EL3-proximal transmembrane helix 7 of the mammalian type I and type II gonadotropin-releasing hormone (GnRH) receptors determine differential ligand selectivity to GnRH-I and GnRH-II. Mol Pharmacol 67:1099-1110.

Li KW, Smit AB, Geraerts WP (1992) Structural and functional characterization of neuropeptides involved in the control of male mating behavior of *Lymnaea stagnalis*. Peptides 13:633-638.

Licht P, Tsai PS, Sotowska-Brochocka J (1994) The nature and distribution of gonadotropin-releasing hormones in brains and plasma of ranid frogs. Gen Comp Endocrinol 94:186-198.

Lin XW, Lin HR, Peter RE (1993) Growth hormone and gonadotropin secretion in

the common carp (*Cyprinus carpio* L.): *in vitro* interactions of gonadotropin-releasing hormone, somatostatin, and the dopamine agonist apomorphine. Gen Comp Endocrinol 89:62-71.

Lo CF, Numann R (1999) PKC modulation of minK current involves multiple phosphorylation sites. Ann N Y Acad Sci 868:431-433.

Loussouarn G, Park KH, Bellocq C, Baro I, Charpentier F, Escande D (2003) Phosphatidylinositol-4,5-bisphosphate, PIP_2, controls KCNQ1/KCNE1 voltage-gated potassium channels: a functional homology between voltage-gated and inward rectifier K^+ channels. Embo J 22:5412-5421.

Lutz PE (1985) Invertebrate Zoology. pp 239-322. Don Mills, Ontario, Canada: Addison-Wesley Publishing.

Magoski NS, Bulloch AG (1998) Trophic and contact conditions modulate synapse formation between identified neurons. J Neurophysiol 79:3279-3283.

Maney DL, Richardson RD, Wingfield JC (1997) Central administration of chicken gonadotropin-releasing hormone-II enhances courtship behavior in a female sparrow. Horm Behav 32:11-18.

Mangia A, Tommasi S, Reshkin SJ, Simone G, Stea B, Schittulli F, Paradiso A (2002) Gonadotropin releasing hormone receptor expression in primary

breast cancer: comparison of immunohistochemical, radioligand and Western blot analyses. Oncol Rep 9:1127-1132.

Mann DR, Lunn SF, Akinbami MA, Samuel K, Waterfall M, Fraser HM (1999) Effect of neonatal treatment with a GnRH antagonist on development of the cell-mediated immune response in marmosets. Am J Reprod Immunol 42:175-186.

Marchetti B, Guarcello V, Morale MC, Bartoloni G, Palumbo G, Jr., Raiti F, Cutuli N, Farinella Z, Scapagnini U (1990) A physiological role for the neuropeptide luteinizing hormone-releasing hormone (LHRH) during the maturation of thymus gland function. Int J Neurosci 51:287-289.

Marinelli S, Schnell SA, Hack SP, Christie MJ, Wessendorf MW, Vaughan CW (2004) Serotonergic and nonserotonergic dorsal raphe neurons are pharmacologically and electrophysiologically heterogeneous. J Neurophysiol 92:3532-3537.

Martin SM, Spencer AN (1983) Neurotransmitters in coelenterates. Comp Biochem Physiol C 74:1-14.

Martinez-Perez F, Becerra A, Valdes J, Zinker S, Arechiga H (2002) A possible molecular ancestor for mollusk APGWamide, insect adipokinetic hormone, and crustacean red pigment concentrating hormone. J Mol Evol

54:703-714.

Mason WT, Waring DW (1985) Electrophysiological recordings from gonadotrophs. Evidence for Ca^{2+} channels mediated by gonadotrophin-releasing hormone. Neuroendocrinology 41:258-268.

Matsuda H, Noma A, Kurachi Y, Irisawa H (1982) Transient depolarization and spontaneous voltage fluctuations in isolated single cells from guinea pig ventricles. Calcium-mediated membrane potential fluctuations. Circ Res 51:142-151.

Matsumoto G, Tasaki I, Inoue I (1978) Oscillatory subthreshold responses and potential fluctuation observed in squid giant axons. J Phys Soc Jpn 44:351-352.

Maylie J, Bond CT, Herson PS, Lee WS, Adelman JP (2004) Small conductance Ca^{2+}-activated K^+ channels and calmodulin. J Physiol 554:255-261.

Melamed P, Gur G, Elizur A, Rosenfeld H, Sivan B, Rentier-Delrue F, Yaron Z (1996) Differential effects of gonadotropin-releasing hormone, dopamine and somatostatin and their second messengers on the mRNA levels of gonadotropin II βsubunit and growth hormone in the teleost fish, tilapia. Neuroendocrinology 64:320-328.

Merickel M, Gray R (1980) Investigation of burst generation by the electrically

coupled cyberchron network in the snail *Helisoma* using a single-electrode voltage clamp. J Neurobiol 11:73-102.

Middleton LM, Harvey RD (1998) PKC regulation of cardiac CFTR Cl^- channel function in guinea pig ventricular myocytes. Am J Physiol 275:C293-302.

Millar R, Lowe S, Conklin D, Pawson A, Maudsley S, Troskie B, Ott T, Millar M, Lincoln G, Sellar R, Faurholm B, Scobie G, Kuestner R, Terasawa E, Katz A (2001) A novel mammalian receptor for the evolutionarily conserved type II GnRH. Proc Natl Acad Sci U S A 98:9636-9641.

Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR (2004) Gonadotropin-releasing hormone receptors. Endocr Rev 25:235-275.

Miyamoto K, Hasegawa Y, Nomura M, Igarashi M, Kangawa K, Matsuo H (1984) Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. Proc Natl Acad Sci U S A 81:3874-3878.

Mohamed JS, Khan IA (2006) Molecular cloning and differential expression of three GnRH mRNAs in discrete brain areas and lymphocytes in red drum. J Endocrinol 188:407-416.

Moncaut N, Somoza G, Power DM, Canario AV (2005) Five

gonadotrophin-releasing hormone receptors in a teleost fish: isolation, tissue distribution and phylogenetic relationships. J Mol Endocrinol 34:767-779.

Morita M, Hall F, Best JB, Gern W (1987) Photoperiodic modulation of cephalic melatonin in planarians. J Exp Zool 241:383-388.

Murphy AD (1990) An identified pleural ganglion interneuron inhibits patterned motor activity in the buccal ganglia of the snail, *Helisoma*. Brain Res 525:300-303.

Murphy AD (2001) The neuronal basis of feeding in the snail, *Helisoma*, with comparisons to selected gastropods. Prog Neurobiol 63:383-408.

Murphy AD, Kater SB (1980) Differential discrimination of appropriate pathways by regenerating identified neurons in *Helisoma*. J Comp Neurol 190:395-403.

Murphy AD, Lukowiak K, Stell WK (1985) Peptidergic modulation of patterned motor activity in identified neurons of *Helisoma*. Proc Natl Acad Sci U S A 82:7140-7144.

Muske LE, Moore FL (1994) Antibodies against different forms of GnRH distinguish different populations of cells and axonal pathways in a urodele amphibian, *Taricha granulosa*. J Comp Neurol 345:139-147.

Nakamura S, Kijima A, Parhar I, Aida K, Osada M (2005) Immunolocalization of GnRH neurons in the cerebral and pedal ganglion of the scallop, *Patinopecten yessoensis*. Abstract of the 15th International Congress of Comparative Endocrinology:102 (Abstract No. P4.13). Boston, MA, USA (May 22-27, 2005).

Nakao R, Nakamura S, Kijima A, Osada M (2005) Involvement of GnRH-like peptide and estradiol-17βin vitellogenin synthesis in the ovary of scallop, *Patinopecten yessoensis*. Abstract of the 15th International Congress of Comparative Endocrinology:103 (Abstract No. P4.23). Boston, MA, USA (May 22-27, 2005).

Naor Z, Benard O, Seger R (2000) Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor. Trends Endocrinol Metab 11:91-99.

Nicholls JG, Martin AR, Wallace BG (1992) From Neuron To Brain. pp 31-387. Sunderland, Massachusetts, USA: Sinauer Associates.

Ohno T, Imai A, Furui T, Takahashi K, Tamaya T (1993) Presence of gonadotropin-releasing hormone and its messenger ribonucleic acid in human ovarian epithelial carcinoma. Am J Obstet Gynecol 169:605-610.

Osada M, Nakamura S, Kijima A (2005) Involvement of GnRH neuron in the

spermatogenial proliferation of the scallop, *Patinopecten yessoensis*. Abstract of the 15th International Congress of Comparative Endocrinology:108 (Abstract No. 6.7). Boston, MA, USA (May 22-27, 2005).

Pakkenberg B, Gundersen HJ (1997) Neocortical neuron number in humans: effect of sex and age. J Comp Neurol 384:312-320.

Pati D, Habibi HR (1998) Presence of salmon gonadotropin-releasing hormone (GnRH) and compounds with GnRH-like activity in the ovary of goldfish. Endocrinology 139:2015-2024.

Pati D, Habibi HR (2000) Direct action of GnRH variants on goldfish oocyte meiosis and follicular steroidogenesis. Mol Cell Endocrinol 160:75-88.

Pazos AJ, Mathieu M (1999) Effects of five natural gonadotropin-releasing hormones on cell suspensions of marine bivalve gonad: stimulation of gonial DNA synthesis. Gen Comp Endocrinol 113:112-120.

Perrin MH, Bilezikjian LM, Hoeger C, Donaldson CJ, Rivier J, Haas Y, Vale WW (1993) Molecular and functional characterization of GnRH receptors cloned from rat pituitary and a mouse pituitary tumor cell line. Biochem Biophys Res Commun 191:1139-1144.

Peter RE, Habibi HR, Chang JP, Nahorniak CS, Yu KL, Huang YP, Marchant TA

(1990) Actions of gonadotropin-releasing hormone (GnRH) in the goldfish. Prog Clin Biol Res 342:393-398.

Piquette GN, LaPolt PS, Oikawa M, Hsueh AJ (1991) Regulation of luteinizing hormone receptor messenger ribonucleic acid levels by gonadotropins, growth factors, and gonadotropin-releasing hormone in cultured rat granulosa cells. Endocrinology 128:2449-2456.

Powell JF, Reska-Skinner SM, Prakash MO, Fischer WH, Park M, Rivier JE, Craig AG, Mackie GO, Sherwood NM (1996) Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. Proc Natl Acad Sci U S A 93:10461-10464.

Price CJ, Goldberg JI (1993) Serotonin activation of a cyclic AMP-dependent sodium current in an identified neuron from *Helisoma trivolvis*. J Neurosci 13:4979-4987.

Quinlan EM, Arnett BC, Murphy AD (1997) Feeding stimulants activate an identified dopaminergic interneuron that induces the feeding motor program in *Helisoma*. J Neurophysiol 78:812-824.

Quinlan EM, Gregory K, Murphy AD (1995) An identified glutamatergic interneuron patterns feeding motor activity via both excitation and inhibition. J Neurophysiol 73:945-956.

Quinlan EM, Murphy AD (1991) Glutamate as a putative neurotransmitter in the buccal central pattern generator of *Helisoma trivolvis*. J Neurophysiol 66:1264-1271.

Quinlan EM, Murphy AD (1996) Plasticity in the multifunctional buccal central pattern generator of *Helisoma* illuminated by the identification of phase 3 interneurons. J Neurophysiol 75:561-574.

Ramakrishnan S, Murphy AD (2003) Modulation of the central pattern generator by the neuropeptide GnRH, in the snail *Helisoma*. Soc Neurosci Abstr: Program No. 500.18. New Orleans, Louisiana, USA (November 8-12, 2003).

Rao LV, Cleveland RP, Ataya KM (1993) GnRH agonist induces suppression of lymphocyte subpopulations in secondary lymphoid tissues of prepubertal female mice. Am J Reprod Immunol 30:15-25.

Ridgway RL, Syed NI, Bulloch AG (1993) *In vitro* evidence for multiple neuritogenic factors in the central nervous system of pulmonate molluscs. Acta Biol Hung 44:109-113.

Rodet F, Lelong C, Dubos MP, Costil K, Favrel P (2005) Molecular cloning of a molluscan gonadotropin-releasing hormone receptor orthologue specifically expressed in the gonad. Biochim Biophys Acta 1730:187-195.

Rohacs T, Lopes CM, Michailidis I, Logothetis DE (2005) $PI(4,5)P_2$ regulates the activation and desensitization of TRPM8 channels through the TRP domain. Nat Neurosci 8:626-634.

Ross J, Armstead WM (2003) Differential role of PTK and ERK MAPK in superoxide impairment of K_{ATP} and K_{Ca} channel cerebrovasodilation. Am J Physiol Regul Integr Comp Physiol 285:R149-154.

Saleuddin ASM, Khan HR, Ashton M-L (1992) Immunocytochemical localization of FMRFamide in the central nervous system and the kidney of *Helisoma duryi* (Mollusca): Its possible antidiuretic role. Cell Tissue Res 24:179-189.

Santra S, Rao VS, Shanker YG, Rao AJ (2000) Cloning and characterization of bonnet monkey GnRH receptor. Mol Hum Reprod 6:415-421.

Seaman RL, Lynch MJ, Moss RL (1980) Effects of hypothalamic peptide hormones on the electrical activity of *Aplysia* neurons. Brain Res Bull 5:233-237.

Sekerli M, Del Negro CA, Lee RH, Butera RJ (2004) Estimating action potential thresholds from neuronal time-series: new metrics and evaluation of methodologies. IEEE Trans Biomed Eng 51:1665-1672.

Sherwood NM, Sower SA, Marshak DR, Fraser BA, Brownstein MJ (1986) Primary structure of gonadotropin-releasing hormone from lamprey brain. J

Biol Chem 261:4812-4819.

Shyng SL, Nichols CG (1998) Membrane phospholipid control of nucleotide sensitivity of K_{ATP} channels. Science 282:1138-1141.

Sim PJ, Wolbers WB, Mitchell R (1995) Activation of MAP kinase by the LHRH receptor through a dual mechanism involving protein kinase C and a pertussis toxin-sensitive G protein. Mol Cell Endocrinol 112:257-263.

Staubli F, Jorgensen TJ, Cazzamali G, Williamson M, Lenz C, Sondergaard L, Roepstorff P, Grimmelikhuijzen CJ (2002) Molecular identification of the insect adipokinetic hormone receptors. Proc Natl Acad Sci U S A 99:3446-3451.

Stefano AV, Vissio PG, Paz DA, Somoza GM, Maggese MC, Barrantes GE (1999) Colocalization of GnRH binding sites with gonadotropin-, somatotropin-, somatolactin-, and prolactin-expressing pituitary cells of the pejerrey, *Odontesthes bonariensis*, *in vitro*. Gen Comp Endocrinol 116:133-139.

Steiner FA, Felix D (1989) Effects of hypothalamic releasing hormones and biogenic amines on identified neurones in the circumoesophageal ganglia of the water snail (*Planorbis corneus*). Comp Biochem Physiol C 92:301-307.

Suh BC, Hille B (2005) Regulation of ion channels by phosphatidylinositol

4,5-bisphosphate. Curr Opin Neurobiol 15:370-378.

Tanriverdi F, Gonzalez-Martinez D, Silveira LF, Hu Y, Maccoll GS, Travers P, Bouloux PM (2004) Expression of gonadotropin-releasing hormone type-I (GnRH-I) and type-II (GnRH-II) in human peripheral blood mononuclear cells (PMBCs) and regulation of B-lymphoblastoid cell proliferation by GnRH-I and GnRH-II. Exp Clin Endocrinol Diabetes 112:587-594.

Tello JA, Rivier JE, Sherwood NM (2005) Tunicate gonadotropin-releasing hormone (GnRH) peptides selectively activate *Ciona intestinalis* GnRH receptors and the green monkey type II GnRH receptor. Endocrinology 146:4061-4073.

Terakado K (2001) Induction of gamete release by gonadotropin-releasing hormone in a protochordate, *Ciona intestinalis*. Gen Comp Endocrinol 124:277-284.

Tilly JL, LaPolt PS, Hsueh AJ (1992) Hormonal regulation of follicle-stimulating hormone receptor messenger ribonucleic acid levels in cultured rat granulosa cells. Endocrinology 130:1296-1302.

Tiwari JK, Sikdar SK (1998) Voltage gated Na^+ channels contribute to membrane voltage fluctuation in αT3-1 pituitary gonadotroph cells. Neurosci Lett 242:167-171.

Tsai PS, Maldonado TA, Lunden JB (2003) Localization of gonadotropin-releasing hormone in the central nervous system and a peripheral chemosensory organ of *Aplysia californica*. Gen Comp Endocrinol 130:20-28.

Tse A, Hille B (1992) GnRH-induced Ca^{2+} oscillations and rhythmic hyperpolarizations of pituitary gonadotropes. Science 255:462-464.

Tsutsui H, Yamamoto N, Ito H, Oka Y (1998) GnRH-immunoreactive neuronal system in the presumptive ancestral chordate, *Ciona intestinalis* (Ascidian). Gen Comp Endocrinol 112:426-432.

Twan WH, Hwang JS, Lee YH, Jeng SR, Yueh WS, Tung YH, Wu HF, Dufour S, Chang CF (2006) The presence and ancestral role of gonadotropin-releasing hormone in the reproduction of scleractinian coral, *Euphyllia ancora*. Endocrinology 147:397-406.

Van der Kraak G, Rosenblum PM, Peter RE (1990) Growth hormone-dependent potentiation of gonadotropin-stimulated steroid production by ovarian follicles of the goldfish. Gen Comp Endocrinol 79:233-239.

van Golen FA, Li KW, Chen S, Jimenez CR, Geraerts WP (1996) Various isoforms of myomodulin identified from the male copulatory organ of *Lymnaea* show overlapping yet distinct modulatory effects on the penis muscle. J Neurochem 66:321-329.

van Golen FA, Li KW, de Lange RP, Jespersen S, Geraerts WP (1995a) Mutually exclusive neuronal expression of peptides encoded by the FMRFa gene underlies a differential control of copulation in *Lymnaea*. J Biol Chem 270:28487-28493.

van Golen FA, Li KW, de Lange RP, van Kesteren RE, van der Schors RC, Geraerts WP (1995b) Co-localized neuropeptides conopressin and ALA-PRO-GLY-TRP-NH_2 have antagonistic effects on the vas deferens of *Lymnaea*. Neuroscience 69:1275-1287.

Villegas GM, Haustein AT, Villegas R (1995) Neuronal differentiation of PC12 and chick embryo ganglion cells induced by a sciatic nerve conditioned medium: characterization of the neurotrophic activity. Brain Res 685:77-90.

Wang L, Oh DY, Bogerd J, Choi HS, Ahn RS, Seong JY, Kwon HB (2001) Inhibitory activity of alternative splice variants of the bullfrog GnRH receptor-3 on wild-type receptor signaling. Endocrinology 142:4015-4025.

Wang R, Pang PK, Wu L, Shipley A, Karpinski E, Harvey S, Berdan RC (1993) Neural effects of parathyroid hormone: modulation of the calcium channel current and metabolism of monoamines in identified *Helisoma* snail neurons. Can J Physiol Pharmacol 71:582-591.

White RB, Eisen JA, Kasten TL, Fernald RD (1998) Second gene for

gonadotropin-releasing hormone in humans. Proc Natl Acad Sci U S A 95:305-309.

White RB, Fernald RD (1998) Ontogeny of gonadotropin-releasing hormone (GnRH) gene expression reveals a distinct origin for GnRH-containing neurons in the midbrain. Gen Comp Endocrinol 112:322-329.

Winks JS, Hughes S, Filippov AK, Tatulian L, Abogadie FC, Brown DA, Marsh SJ (2005) Relationship between membrane phosphatidylinositol-4,5-bisphosphate and receptor-mediated inhibition of native neuronal M channels. J Neurosci 25:3400-3413.

Wittmack EK, Rush AM, Hudmon A, Waxman SG, Dib-Hajj SD (2005) Voltage-gated sodium channel Nav1.6 is modulated by p38 mitogen-activated protein kinase. J Neurosci 25:6621-6630.

Wong RG, Barker DL, Kater SB, Bodnar DA (1984) Nerve growth-promoting factor produced in culture media conditioned by specific CNS tissues of the snail *Helisoma*. Brain Res 292:81-91.

Wong RG, Hadley RD, Kater SB, Hauser GC (1981) Neurite outgrowth in molluscan organ and cell cultures: the role of conditioning factor(s). J Neurosci 1:1008-1021.

Yang Y, Zhou LB, Liu SQ, Tang JF, Li FY, Li RY, Song HD, Chen MD (2005)

Expression of feeding-related peptide receptors mRNA in GT1-7 cell line and roles of leptin and orexins in control of GnRH secretion. Acta Pharmacol Sin 26:976-981.

Yoshida S, Plant S, Taylor PL, Eidne KA (1989) Chloride channels mediate the response to gonadotropin-releasing hormone (GnRH) in *Xenopus* oocytes injected with rat anterior pituitary mRNA. Mol Endocrinol 3:1953-1960.

Young KG (1998) The gonadotropin-releasing hormone-immunoreactive neuronal system of the freshwater snails, *Helisoma trivolvis* and *Lymnaea stagnalis*: possible involvement in reproduction. M.Sc. thesis, University of Alberta, Edmonton, Alberta, Canada.

Young KG, Chang JP, Goldberg JI (1999) Gonadotropin-releasing hormone neuronal system of the freshwater snails *Helisoma trivolvis* and *Lymnaea stagnalis*: possible involvement in reproduction. J Comp Neurol 404:427-437.

Young KG, Zalitach R, Chang JP, Goldberg JI (1997) Distribution and possible reproductive role of a gonadotropin-releasing hormone-like peptide in the pond snail, *Helisoma trivolvis*. Soc Neurosci Abstr 23:696.15. New Orleans, Louisiana, USA (October 25-30, 1997).

Yu WH, Arisawa M, Millar RP, McCann SM (1989) Effects of the

gonadotropin-releasing hormone associated peptides (GAP) on the release of luteinizing hormone (LH), follicle stimulating hormone (FSH) and prolactin (PRL) *in vivo*. Peptides 10:1133-1138.

Yue G, Malik B, Eaton DC (2002) Phosphatidylinositol 4,5-bisphosphate (PIP_2) stimulates epithelial sodium channel activity in A6 cells. J Biol Chem 277:11965-11969.

Zhainazarov AB, Ache BW (1999) Effects of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate on a Na^+-gated nonselective cation channel. J Neurosci 19:2929-2937.

Zhang L, Wayne NL, Sherwood NM, Postigo HR, Tsai PS (2000) Biological and immunological characterization of multiple GnRH in an opisthobranch mollusk, *Aplysia californica*. Gen Comp Endocrinol 118:77-89.