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Title: The Role of Gonadotropin-Releasing Hormone in the Regulation of Courtship Behavior in the Male Red-Sided Garter Snake, *Thamnophis sirtalis parietalis.*

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What are the neurohormonal cues that regulate reproductive behavior in the red-sided garter snake, *Thamnophis sirtalis parietalis*? Prior to this thesis, a pheromonal system was known to be important for reproductive behavior in this species and a period of cold temperature was also essential for the expression of male sex behavior. No hormone or neurotransmitter was known to have any effect on male reproductive behavior.

This thesis investigates the role of gonadotropin releasing-hormone on the sex behavior of *Thamnophis sirtalis parietalis*. Using intracerebroventricular injections of gonadotropin-releasing hormone (GnRH) and some GnRH analogs, behavioral tests were run to determine the effect of these peptides on the response of males to either unmated females or the female sex attractiveness pheromone. Chicken-I GnRH, the native molecule in the brain of *Thamnophis sirtalis parietalis,* had no effect on male courtship behavior. The GnRH analog D-Phe^{2,6}, Pro^{3}-GnRH increased the time spent courting and decreased the latency to court when males were courting females, and increased the time spent courting when males were exposed to the female sex attractiveness pheromone.

Using immunocytochemistry, the neurons containing immunoreactivity to GnRH were mapped within the brain of the red-sided garter snake. GnRH neurons are widely distributed in the forebrains of reproductively active, male red-sided garter snakes. This distribution parallels the distribution of GnRH in other vertebrate species. The terminal nerve, a nerve that is present in all vertebrate classes and usually contains GnRH, was shown to exist in a reptile based on GnRH immunoreactivity. This had not been demonstrated in any other member of the class Reptilia.

Together these studies provide the first evidence that a hormonal factor, GnRH, is able to regulate sex behavior in the red-sided garter snake, and that GnRH is present in brain areas that are important in the control of reproductive behavior. These findings support the hypothesis that upon reception of the female sex attractiveness pheromone, the terminal nerve of the male red-sided garter snake releases or stimulates the release of GnRH and this will, in turn, stimulate the stereotyped sex behavior of the male.

The Role of Gonadotropin-Releasing Hormone in the Regulation of Courtship Behavior in the Male Red-Sided Garter Snake, *Thamnophis sirtalis parietalis.*

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PREFACE

This thesis is composed of manuscripts that are either submitted for publication or being prepared for submission. Chapter II has been submitted to the Journal of Comparative Neurology as Smith, M., Moore, F.L., and Mason, R.T., Neuroanatomical distribution of chicken-I gonadotropin-releasing hormone in the brain of the red-sided garter snake, *Thamnophis sirtalis parietalis*.

Portions of this work have also appeared as published abstracts: 1) Smith, M., Moore, F.L., and Mason, R. (1993) Neuroanatomical distribution of gonadotropin-releasing hormone in the red-sided garter snake. Western and Southwestern Regional Conference on Comparative Endocrinology, Boulder, CO. 2) Smith, M.T., Moore, F.L., and Mason R.T. (1992) Effect of GnRH and D-Phe^{2,6},Pro^{3}-GnRH on the sex behavior of male garter snakes. Amer. Zool. 410:67.

All research activities described in this thesis have been reviewed and approved by the Institutional Animal Care and Use Committee. All procedures and husbandry meet or exceed the standards delineated in the U.S. Public Health Service's Guide to the Care and Use of Laboratory Animals.

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The Role of Gonadotropin-Releasing Hormone in the Regulation of Courtship Behavior in the Male Red-Sided Garter Snake, *Thamnophis sirtalis parietalis.*

CHAPTER I: GENERAL INTRODUCTION

"Reptiles are abhorrent because of their cold body, pale color, cartilaginous skeleton, filthy skin, fierce aspect, calculating eye, offensive smell, harsh voice, squalid habitation, and terrible venom; wherefore their Creator has not exerted his power to make many of them."

Carl Linnaeus, 1797

Natural History of the Red-Sided Garter Snake

The red-sided garter snake, *Thamnophis sirtalis parietalis*, is the most northerly living reptile in North America, and perhaps the world, and has a range that extends into northern Canada (Logier and Toner, 1961). As a result of the cold temperatures found at these northerly latitudes, the red-sided garter snake is constrained to spend up to eight months of the year in hibernation (Gregory, 1976). In early May, the males of a given hibernaculum will emerge *en masse* and wait for the females to emerge (Gartska *et al.*, 1982). The females, who emerge one at a time over the course of the next 3-4 weeks, are courted by 10-100 males (Gartska *et al.*, 1982).

Mating behavior in the red-sided garter snake is characterized as having two major behavioral components: chin rubbing and caudocephalic waves (Noble, 1937). During chin rubbing behavior, the male will press his labial-mental area against the dorsal surface of the female and repeatedly traverse the length of her body (Gartska *et al.*, 1982). While chin rubbing is occurring, the male is constantly tongue-flicking the dorsal surface of the female's body with tongue flicks that are characterized as having a short duration with a short extension. Caudocephalic waves begin when the male aligns his body with the body of the female, either alongside it or on top of it, and begins rhythmic undulations of his

body (Pisani, 1976). These caudocephalic waves will continue until intromission (Gartska *et al.*, 1982).

The essential element by which males are attracted to females is a sex attractiveness pheromone that is a component of the female's skin lipids (Mason *et al.*, 1989). A series of monounsaturated, long chain methyl ketones are present in the skin of females and this substance elicits strong courtship behavior even after it has been extracted from the skin of females and applied to a paper towel (Mason *et al.*, 1989).

Males are able to detect this pheromone through the use of a sensory organ called the vomeronasal organ (Halpern, 1987). While the male is tongue-flicking the dorsal surface of the female, he transports this non-volatile sex attractiveness pheromone to his vomeronasal organ (Halpern, 1987). It is generally believed that both the olfactory and vomeronasal epithelium can be used to transduce chemical sensory information through parallel but completely separate pathways (Halpern, 1983). Indeed, it has been shown that these two structures have remarkably similar cell types, as well as the ability to regenerate sensory neurons (Halpern, 1987). It is generally thought that the olfactory system is involved with the transduction of relatively volatile chemical cues, whereas the vomeronasal organ is involved in the transduction of nonvolatile cues (Meredith and O'Connell, 1988).

It is known that the vomeronasal system is the most important sensory system for transduction of the garter snake chemosensory information to integration centers within the central nervous system (Halpern, 1987). The projections from the vomeronasal organ go to the accessory olfactory bulb, and then via the accessory olfactory tract to the nucleus sphericus (Halpern and Kubie, 1984). From the nucleus sphericus, projections extend to the stria terminalis, ventromedial nucleus of the hypothalamus, and the preoptic area (Halpern and Kubie, 1984). The hypothalamus and preoptic areas have been implicated in playing a role in the regulation of male sex behavior in the red-sided garter

snake. Lesions in the anterior hypothalamus-preoptic area cause an abrupt and immediate decline in the courtship behavior of adult, male garter snakes that are specific to this area (Friedman and Crews, 1985). Lesions in more anterior portions of the preoptic area cause a gradual decline in behavior, whereas lesions to the dorsal, ventral, or caudal hypothalamus-preoptic area cause no change in behavior relative to controls (Friedman and Crews, 1985). In addition, using 2-deoxyglucose labeling, Allen and Crews (1992) showed that male garter snakes who were actively courting females had a pronounced increase in 2-deoxyglucose uptake in the anterior hypothalamus-preoptic area. This uptake is indicative of increased neural activity.

The red-sided garter snake exhibits a dissociated reproductive tactic; when breeding activity is at its peak during May, the circulating levels of total androgenic steroid hormones are at their lowest (Krohmer *et al.*, 1987). Spermatogenesis is initiated after the breeding season and the sperm for the following breeding season is stored in the vas deferens over the winter (Krohmer *et al.*, 1987). In addition, Camazine *et al.* (1980) have shown that male courtship behavior of *Thamnophis sirtalis parietalis* is independent of the testes. Therefore, if the steroid hormones are not controlling the initiation and maintenance of reproductive behavior in the red-sided garter snake, what is?

Many factors have been investigated as possible regulators for male reproductive behavior including GnRH, arginine vasotocin, dopamine, epinephrine, norepinephrine, serotonin, thyrotropin-releasing hormone, melatonin, several steroid hormones, and a variety of metabolic effectors and electrolytes, none of which have been shown to have any effect on the mating behavior of the male red-sided garter snake (Gartska *et al.*, 1982). Of the many factors that have been postulated to control the reproductive behavior of the red-sided garter snake, only two have been shown to be essential for reproduction. The pheromone system, mentioned previously, is essential for reproduction, as is a period of cold exposure prior to the mating season (Gartska *et al.*, 1982). Cold exposure is the

only environmental variable that has been shown to be important for reproductive behavior (Crews *et al.*, 1984). Castration either just before spring emergence or prior to winter dormancy in the fall will not effect the intense courtship during the breeding season as long as the males have gone through a low-temperature dormancy period (Crews *et al.*, 1984). It is now interesting to note that the preoptic area is known to play a critical role in temperature regulation in vertebrates, and that lesions of this area result in mating deficits and that this area is active during courtship (Nelson *et al.*, 1984; Friedman and Crews, 1985; Allen and Crews, 1992). The neurohormonal correlates of reproductive behavior in the red-sided garter snake are currently unknown.

Gonadotropin releasing hormone

The decapeptide gonadotropin releasing hormone (GnRH) is one member of a family of peptides that plays an essential role in reproduction. Although the existence of this decapeptide had been postulated since the 1950's, its structure was not determined until the 1970's (Harris, 1950; Matuso *et al.*, 1971; Burgus *et al.*, 1972). Since the first structure determination of mammalian GnRH, the structures of six additional forms of GnRH have been determined: Chicken-I, chicken-II, salmon, catfish, dogfish, and lamprey (King and Millar, 1982; Sherwood, *et al.*, 1983; Ngamvongchon *et al.*, 1992; Sherwood *et al.*, 1986; Lovejoy *et al.*, 1992). The length of these molecules is identical (10 amino acids), and residues 1, 2, 4, 9, and 10 are conserved among the 7 structures (for review see Sherwood, 1987; Sherwood *et al.*, 1993).

GnRH perikarya and fibers are distributed widely in the brains, especially the forebrains, of many vertebrate classes, including teleosts, amphibians, birds, and mammals (Oka and Ichikawa, 1990; Muske and Moore, 1988; Millam *et al.*, 1993; and Silverman, 1988; for reviews see Muske, 1993; Sherwood *et al.*, 1993). In general, most vertebrates

have GnRH immunoreactive (ir) cell bodies located in the terminal nerve, septal structures, preoptic area, diagonal band of Broca, and the stria terminalis (For reviews see Sherwood *et al.*, 1993; Silverman, 1988; and Kuenzel and Blasher, 1991). GnRHir fibers are found in all brain areas in which cell bodies are located plus the circumventricular organs, projections to the olfactory bulbs, midbrain, amygdala, hippocampus, pallial structures, spinal cord, and the thalamus (For review see Muske, 1993; Sherwood *et al.*, 1993).

A prevalent theme in most vertebrate brains is that GnRH is usually present in two molecular forms that are anatomically segregated. This segregation is customarily seen as a class or species specific form in the forebrain, and the chicken-II form of the decapeptide in the midbrain (Muske, 1993). The ontogeny of the GnRH system gives insight into how the differential distribution of the two forms of GnRH are attained in the adult brain. The forebrain areas are populated with GnRH neurons by embryonic GnRH neurons migrating from the olfactory placode outside the brain into the forebrain during early development (Muske and Moore, 1988; Wray *et al.*, 1989; Schwanzel-Fukuda and Pfaff, 1989). These olfactory placode neurons emigrate to populate the terminal nerve, septal, and preoptic areas. The GnRH system in the midbrain appears to arise independently of the cells in the forebrain (Muske and Moore, 1990; Witkin, 1990). These midbrain neurons become immunoreactive to chicken-II GnRH prior to the cells of the forebrain's terminal nerve, septal, and preoptic systems becoming immunoreactive to the species specific form of GnRH (Muske and Moore, 1990; Muske, 1993). Further evidence that separates the two systems during development is that ablation of the olfactory placode does not effect the immunostaining of the midbrain cell population (Muske and Moore, 1990; Muske, 1993).

The classical function of GnRH is to regulate the hypothalamo-pituitary-gonad (HPG) axis (Hoffman *et al.*, 1992). This classical function involves the population of GnRH cells that are found in the preoptic area releasing GnRH into the median eminence

in a pulsatile fashion. GnRH then travels through the hypophyseal portal system to the anterior pituitary where it stimulates the gonadotrophs to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Hoffman *et al.*, 1992). These two peptides will then control a variety of functions including steroid secretion, gamete maturation, and ovulation (Hoffman *et al.*, 1992).

One of the first clues to the possibility that GnRH may have diverse functions was the demonstration that GnRH cell bodies and fibers were widely distributed in brain areas that did not control the HPG axis. In fact, one study has shown that GnRH cell bodies in the septal-preoptic-hypothalamic region send only 50-70% of their axons to the median eminence, and the remaining axons are widely distributed throughout the brain (Silverman *et al.*, 1987). Many of the diverse functions of GnRH seem to center on reproduction, signifying that reproduction may be a conserved function of this molecule (Sherwood *et al.*, 1993). It has been demonstrated that the sex behavior of the newt (*Taricha granulosa*), voles (*Microtus canicaudus*), rats (*Rattus rattus*), frogs (*Xenopus laevis*), chameleons (*Anolis carolinensis*), and horses are all enhanced by GnRH administration (Moore *et al.*, 1982; Boyd and Moore, 1985; Moss and McCann, 1973; Kelly, 1982; Alderete *et al.*, 1980; McDonnell, 1988). It has also been shown that GnRH levels change in specific brain areas in response to reproductive cues. The GnRH concentration in the posterior olfactory bulb of female voles (*Microtus ochrogaster*) increases in response to a male urine cue; the GnRH concentration of the mast cells in the habenula of the ring dove (*Streptopelia roseogrisea*) increases following two hours of courtship; and the GnRH concentration of the terminal nerve in the newt (*Taricha granulosa*) increases in response to mating (Dluzen and Ramirez, 1981, 1987; Zhuang *et al.*, 1993; Propper and Moore, 1991).

Few studies have explored the functions and/or distributions of GnRH in reptiles. GnRH injections into estrogen primed female anoles (*Anolis carolinensis*) causes an

increase in receptivity, and GnRH implants into female Iguanas will increase male courtship (this may involve upregulation of some visual or chemical mating cue) (Alderete *et al.*, 1980; Phillips and Lasley, 1987). The rat snake (*Elaphe climacophora*) has been shown to have a well defined GnRHir in the septal-preoptic system and hippocampus; the chameleon (*Chameleon chameleo*) has GnRHir in the midbrain for the salmon form of the decapeptide; and the alligator (*Alligator mississippiensis*) has been shown to have immunoreactivity to both the chicken-I and chicken-II forms of GnRH (Nozaki *et al.*, 1984; Bennis *et al.*, 1989; Lovejoy *et al.*, 1991). The red-sided garter snake has never been examined for GnRH immunoreactivity and the one study that looked at the effects of GnRH on male sexual behavior found no indication that it was important for the regulation of male courtship (Gartska *et al.*, 1982).

The terminal nerve

The terminal nerve (or nervus terminalis) was first described by Fritsch in the late 1800's in sharks, and has since been described in a variety of vertebrates including humans. The terminal nerve lies, in general, as a loose plexus that courses along the ventromedial aspect of the brain, and makes many connections within the forebrain, including the medial and lateral septal nuclei, olfactory tubercle, and the preoptic area (Muske, 1993).

The neurons that contribute fibers to the terminal nerve are found singly or in ganglionated clusters along the course of this nerve. The largest ganglion is customarily referred to as the terminal nerve ganglion and is usually located at the transition between the olfactory bulbs and the telencephalon. The terminal nerve ganglion in some species of fish, including the goldfish, *Carassius auratus*, is located at this transition, and the projections of these terminal nerve fibers run peripherally from the olfactory nerve and centrally into the ventromedial forebrain (Oka and Ichikawa, 1990; Bartheld and Meyer,

1986). In an amphibian, the rough-skinned newt (*Taricha granulosa*), the terminal nerve ganglion is located caudal to the olfactory bulbs, and in mammals, terminal nerve perikarya occur as single cells or ganglionated clusters along the length of the terminal nerve (Muske and Moore, 1988; Schwanzel-Fukuda and Silverman, 1980).

In addition to the neuroanatomical position and sites of projection, certain neurotransmitters are characteristic of the terminal nerve. The terminal nerve of most vertebrates is identifiable by GnRHir, and cartilaginous fishes, bony fishes, amphibians, and mammals all have GnRHir in the terminal nerve (Sherwood *et al.*, 1993). It is not surprising that the terminal nerve stains positively for GnRH, since during the development of the GnRH forebrain system the terminal nerve is the course along which the GnRH neurons that will eventually populate the septal and preoptic structures must migrate (Muske and Moore, 1988; Wray *et al.*, 1989; Schwanzel-Fukuda and Pfaff, 1989). In addition to GnRHir, other peptides have been localized to the terminal nerve. In the tench, *Tinca tinca*, many perikarya and fibers are labeled using an antibody to substance P, and in the bullfrog, *Rana catesbeiana*, and tiger salamander, *Ambystoma tigrinum*, the terminal nerve stains positively for acetyl cholinesterase (Alonso *et al.*, 1989; Wirsig and Getchell, 1986). Finally, choline acetyltransferase and vasoactive intestinal peptide immunoreactivity have been identified in the terminal nerve of fetal and neonatal rats (Schwanzel-Fukuda *et al.*, 1986).

The function of the terminal nerve is, at present, unknown. The original hypothesis concerning terminal nerve function was that, due to the correlation between the terminal nerve's projections and neurobehavioral studies, the terminal nerve might mediate the response to sex pheromones in the goldfish (Demski and Northcutt, 1983). This hypothesis has not been supported and it is now clear that the medial olfactory tract and not the terminal nerve is mediating the response to goldfish sex pheromones (Fujita *et al.*, 1991). In the golden hamster, *Mesocricetus auratus*, the terminal nerve is closely

associated with the vomeronasal nerve (a nerve that is known to carry important chemosensory reproductive information) and transection of the terminal nerve in the male results in specific mating deficits and reduces the males attraction to the odor of the female (Wirsig, 1987). This transection of the terminal nerve did not alter the plasma testosterone levels in males and Wirsig (1987) concluded that the terminal nerve may function to potentiate the effects of semiochemical cues during social or sexual encounters, or during the appropriate hormonal states. Subsequently, Wirsig-Wiechmann (1993) looked at the testosterone surge that is commonly seen in male golden hamsters, *Mesocricetus auratus,* after exposure to a vaginal smear of female hamsters. Lesions of the terminal nerve had no effect on pheromonally induced testosterone surges in male hamsters, and it was concluded that the terminal nerve is not necessary for this pheromonally mediated neuroendocrine reflex (Wirsig-Wiechmann, 1993). Electrophysiological studies on the Bonnethead shark, *Sphyrna tiburo,* showed that there was no interaction between the terminal nerve and the olfactory bulb affecting the sensitivity of either for chemical cues (Meredith and White, 1987). Finally, a possible function that has been recently proposed is that the terminal nerve-GnRH system functions as a neuromodulator. This is based on the evidence that the system has wide projections within the central nervous system like other neuromodulators (serotonin, norepinephrine, dopamine, and histamine), and that it has a rhythmic, spontaneous oscillatory activity that may be modulated by hormonal, pheromonal, and environmental stimulus (Oka and Matsushima, 1993). In short, the function of the terminal nerve is still unknown, although many hypotheses have been suggested.

Hypothesis

I propose to explore the hypothesis that upon reception of the female sex attractiveness pheromone, the terminal nerve of the male red-sided garter snake releases or stimulates the release of GnRH and this will, in turn, stimulate the stereotyped sex behavior of the male.

The second chapter will explore the neuroanatomical location of the terminal nerve in *Thamnophis sirtalis parietalis.* Even though few reptiles have been examined for GnRHir, no reptile has ever stained positively for GnRH in the terminal nerve. This fact seems especially problematic given that during development the GnRH neurons that are seen in the septal-preoptic system of some reptiles must migrate along the terminal nerve and that the terminal nerve has been known to exist in reptiles since 1913 (Muske and Moore, 1988; Wray *et al.*, 1989; Schwanzel-Fukuda and Pfaff, 1989; Johnston, 1913).

The third chapter will explore the direct effects of GnRH on male sex behavior. Using both chicken-I GnRH and a GnRH antagonist, the male response to both unmated females and the female sex attractiveness pheromone are examined. This is the first study to determine that GnRH administration can enhance reproductive behaviors in the red-sided garter snake, *Thamnophis sirtalis parietalis.*

CHAPTER II: NEUROANATOMICAL DISTRIBUTION OF CHICKEN-I GONADOTROPIN-RELEASING HORMONE IN THE BRAIN OF THE RED-SIDED GARTER SNAKE.

Abstract

Immunocytochemistry was used to investigate the neuroanatomical distribution of the chicken-I form of gonadotropin-releasing hormone (cGnRH-I) in reproductively active, male, red-sided garter snakes (*Thamnophis sirtalis parietalis*). Cell bodies with cGnRH-I immunoreactivity (ir) were found in the hypothalamus, medial preoptic area, nucleus of the diagonal band of Broca, and the terminal nerve ganglion. Fibers containing cGnRH-Iir were distributed in the following brain areas: within the olfactory bulb, fibers were found in the internal plexiform, mitral and glomerular cell layers, as well as the terminal nerve; within the forebrain, fibers were observed in the diagonal band of Broca, rostral and lateral septum, lateral pallium, retrobulbar region pars dorsomedialis, medial preoptic area, hypothalamus, median eminence, nucleus accumbens, hippocampal commissure, posterior dorsal ventricular ridge, thalamus and amygdala; within the midbrain fibers were found in the interpeduncular nucleus and the stratum album periventricular of the optic tectum. This study shows that the distribution of cell bodies for cGnRH-Iir in this reptile is consistent with the distribution of immunoreactivity for cGnRH-I in birds and mammalian GnRH in amphibians and mammals. This is also the first study to show that the terminal nerve in a reptile contains GnRH immunoreactivity.

Introduction

Members of the gonadotropin-releasing hormone (GnRH) family of decapeptides are found in the brains of vertebrates, frequently with more than one form in a given species (Sherwood, 1987; Sherwood *et al.*, 1993). In roughskin newts (*Taricha granulosa*), mice (*Mus musculus*), and other species, studies suggest that during embryonic development GnRH-containing neurons migrate from the olfactory placode to specific positions in the forebrain, namely in the terminal nerve (TN), septal, and pre-optic areas (Muske and Moore, 1988; Wray *et al.*, 1989; Schwanzel-Fukuda and Pfaff, 1989). Other evidence indicates that the GnRH containing cell bodies in the midbrain do not originate embryonically in the olfactory placode (Muske and Moore, 1990; Witkin, 1990; Muske, 1993; Northcutt and Muske, 1991). There are many unanswered questions concerning the functions and neuroanatomical distributions of GnRH cells in the forebrain and midbrain.

GnRH-containing cells in the septal and preoptic areas send fibers to the median eminence and regulate gonadotropin release in the anterior pituitary. Release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary will, in turn, control a variety of reproductive functions including the synthesis of gonadal steroids, gametogenesis, and ovulation (Hoffman *et al.*, 1992).

GnRH, however, appears to have other functions besides regulating FSH and LH secretion. It has been demonstrated that GnRH administration can enhance the sex behavior of newts (*T. granulosa)*, voles (*Microtus canicaudus*), rats (*Rattus rattus*), frogs (*Xenopus laevis*), green anole lizards (*Anolis carolinensis*), and horses (Moore *et al.*, 1982; Boyd and Moore, 1985; Moss and McCann, 1973; Kelly, 1982; Alderete *et al.*,

1980; McDonnell *et al.*, 1989). It has also been shown that GnRH levels change in specific brain areas in response to reproductive cues. The GnRH concentration in the posterior olfactory bulb of female voles (*Microtus ochrogaster*) increases in response to a male urine cue; the number of mast cells with GnRHir in the medial habenula of ring doves (*Streptopelia roseogrisea*) increases following two hours of courtship; and the GnRH concentration in the terminal nerve of *T. granulosa* increases in response to mating (Dluzen and Ramirez 1981, 1987; Zhuang *et al.*, 1993; Propper and Moore, 1991). These extra-hypothalamic changes in GnRH, as well as the direct effects of GnRH on behaviors, indicate that GnRH may function as a neurotransmitter in the brain. There is evidence that in the sympathetic ganglia of the bullfrog GnRH functions as a neurotransmitter, regulating potassium channels and the slow excitatory post-synaptic potential (Jan *et al.*, 1979; Jones, 1987).

The distribution of GnRH in the brain is much wider than would be predicted if GnRH only controlled gonadotropin release. In general, in the forebrain system, GnRHir is found in cell bodies in the TN, septal structures, preoptic area, diagonal band of Broca, and the stria terminalis (for reviews see Silverman, 1988; Kuenzel and Blasher, 1991). In the midbrain, GnRHir cell bodies are found in the midbrain tegmentum, oculomotor complex, and the posterior tubercle of the vertebrates that have been studied (For review see Muske, 1993). Prior to the current study, immunocytochemical studies described the distribution of GnRHir in two species of reptiles. Using antisera for mammalian GnRH, the rat snake (*Elaphe climacophora*) was found to have GnRHir cell bodies in the medial septo-preoptic region and hippocampus (Nozaki *et al.*, 1984). Using antisera for salmon GnRH, the chameleon (*Chameleon chameleo*) was found to have GnRHir cell bodies in the midbrain tegmentum bordering the nucleus fasciculus longitudinalis medialis and along the dorsolateral part of the oculomotor nucleus (Bennis, *et al.*, 1989). Neither study of

reptiles described GnRHir in the TN, even though the TN exists in reptiles (Johnston, 1913).

Using HPLC, the alligator (*Alligator mississippiensis*) has been shown to have immunoreactivity to both the chicken-I and chicken-II forms of GnRH (Lovejoy *et al.*, 1991). Sherwood and Whittier (1988) found that the red-sided garter snake (*T. s. parietalis*) has one major HPLC peak that elutes with the same retention time as, and cross-reacts with antisera to chicken-I GnRH. Our working hypothesis concerning the forms of GnRH found in the reptilian brain is that both chicken-I and chicken-II GnRH are present, and that the distribution of chicken-I GnRH in the forebrain that is similar to that of salmon GnRH in teleosts, mammalian GnRH in amphibians and mammals, and chicken-I GnRH in birds (Oka and Ichikawa, 1990; Muske and Moore, 1988; Silverman, 1988; Millam *et al.*, 1993; for review see Muske, 1993).

In this paper, we describe the distribution of GnRH in the brain of the red-sided garter snake, *T. s. parietalis*, using a highly specific form of the chicken-I GnRH antibody.

Materials and Methods

Reproductively active, adult male red-sided garter snakes (*Thamnophis sirtalis parietalis*) were collected in Manitoba, Canada during early May. Males were transported back to a field station and were housed in outdoor pens measuring 1.2m x 1.2m x 0.9m. Each pen contained approximately 100 males and 3-5 unmated females. Courting males were removed from the back of an unmated female and sacrificed by rapid decapitation within 24 hours of capture. Brains were immediately removed and fixed for 24 hours in a fixative that contained 4% paraformaldehyde, 3% sucrose, 7.5% saturated picric acid solution in 0.1M sodium phosphate buffer. Brains were then cycled through two washes of 12 hours each in 0.1M sodium phosphate buffer. Finally, brains were washed for 12 hours with, and subsequently stored in, 0.1M sodium phosphate buffer that contained 30% sucrose and 0.1% sodium azide.

Several GnRH antisera were screened for this study (EL-14, Stiener 540, Arimura 720, King 5348, and Millam's chicken I and II antibodies). Based on sensitivity, specificity, and previous work showing that cGnRH-I exists in the brain of the red-sided garter snake, the chicken-I antibody (generously supplied by J. Millam, University of California, Davis) was chosen for these studies (Millam *et al.*, 1993; Sherwood and Whittier, 1988). This chicken-I antibody has a low degree of cross-reactivity for other forms of GnRH: It is 1000 times more specific for chicken-I than chicken-II in immunocytochemistry, and has less than 0.5% cross-reactivity for mammalian, salmon, or lamprey GnRH in radioimmunoassay (Millam *et al.*, 1993). Fixed brains (N=7) were frozen and embedded in Tissue-Tek®O.C.T. Compound. Sections were cut at 25 μm in the transverse (N=6) or horizontal (N=1) plane in a cryostat that was maintained at -16°C and thaw-mounted serially onto sets of four gelatin coated slides. Slides were stored at -20°C until immunocytochemistry was performed. Immunocytochemistry

utilized an avidin-biotin immunoperoxidase protocol (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Sections were washed twice with phosphate buffered saline (PBS), and then serially exposed to 1% H_2O_2 in PBS, 10% normal goat serum, primary antibody (diluted 1:4000 in 0.1% Triton-X-100 in PBS), biotinylated goat anti-rabbit IgG, and an avidin-biotin-horseradish peroxidase complex. Sections were washed twice in PBS between each of the steps. Immunoreactive GnRH was visualized with a solution that contained 0.01% H_2O_2, 0.025% diaminobenzidine, and 0.04% $NiCl_2$ for 5-7 minutes. The reaction was terminated through washing two times in dechlorinated H_2O. All antibody incubations were performed at 4°C and other incubations at room temperature. Sections were counterstained with 1% methyl green, dehydrated, and mounted with cover slips.

Control experiments consisted of incubation of the chicken-I antibody with PBS alone or PBS with 100μM cGnRH-I (Sigma Chemical Co., St. Louis, MO) prior to staining. Additional controls for non-specific binding were replacement of either the primary or secondary antibody with incubation buffer. Specific immunoreactive staining was completely absent when the primary antibody was preincubated with 100 μM cGnRH-I, and when the primary or secondary antisera was replaced with PBS (Fig. 2a, 2b).

Nomenclature and identifications of neuroanatomical structures were based on descriptions of the ophidian telencephalon and tract tracing studies of the visual system (Halpern, 1980; Dacey and Ulinski, 1986).

Results

Distribution of cGnRH-Iir cell bodies and fibers

Olfactory bulb

cGnRH-Iir fibers appeared to be extensively beaded and scattered throughout the olfactory bulb in all layers, except the internal granular layer. Fibers in the olfactory bulb were typically short in 25 μm transverse sections suggesting the fibers were oriented rostrocaudally. The most rostral fibers are located in the internal plexiform layer at the transverse level where the vomeronasal nerve is still dorsal and medial, surrounding the medial portion of the olfactory bulb. Caudally, in the region where the olfactory ventricle is located at the lateral margin of the olfactory bulb, fibers are found in the mitral and glomerular layers of the olfactory bulb, as well as in the medial olfactory tract. In the most caudal aspect of the olfactory bulb, some fibers are seen scattered throughout the lateral pallium. A small series of cGnRH-Iir fibers are seen in nearly all sections and are located at the ventromedial aspect of the olfactory bulb. These fibers, located just beneath the pial surface, are in the terminal nerve (Fig. 2c, 2d, 2e).

Telencephalon

A common feature of cGnRH-Iir fibers in the brain of *T. s. parietalis*, especially in the telencephalon, is that the fibers are concentrated around the ventricles and along the surface of the brain.

In the rostral telencephalon, a few cGnRH-Iir fibers were observed in the dorsal pallium. cGnRH-Iir fibers were observed consistently in the retrobulbar region pars

dorsomedialis, a region in the dorso-medial aspect of the rostral telencephalon that is characterized as having a triangular shape in cross section with the cGnRH-Iir fibers having an outward radiation from a point just dorsal to the ventricle (Halpern, 1980). Also in the dorsal telencephalon, cGnRH-Iir fibers were found in the medial forebrain bundle, typically extending from just inside the ventromedial aspect of the brain dorsally to the ventral edge of the C-shaped ventricle. Short, beaded fibers are seen in the rostral septum at the most rostral extent of the telencephalon. As the rostral septum separates caudally into the lateral and medial septum, the majority of the cGnRH-Iir fibers are concentrated in the lateral septum, and extend from the medial preoptic area to the optic chiasm (Fig 3b).

cGnRH-Iir fibers in the TN extended along the ventromedial aspect of the telencephalon. The TN ganglion is found just proximal to the olfactory bulb (Fig. 2c, 2d, 2e). This ganglion consists of 2-5 unipolar or bipolar cGnRH-Iir cells per hemisphere. Caudal to the terminal nerve ganglion, there is a cGnRH-Iir fiber tract that extends ventromedially to the nucleus of the diagonal band of Broca (nDB). The nDB contains the most cGnRH-Iir cell bodies in the brain of *T. s. parietalis*. This nucleus had 5-10 unipolar or bipolar cells per hemisphere that were surrounded by many darkly immunoreactive fibers (Fig. 3a).

cGnRHir cell bodies were also found in the medial preoptic area which is lateral to the rostral most aspect of the third ventricle and dorsal to the optic tract (Fig 3e). Fibers with cGnRH-Iir in the medial preoptic area are darkly staining and extend into the lateral preoptic area. Ventral to the rostral end of the third ventricle is the suprachiasmatic nucleus (SCN) which extends rostrocaudally and had cGnRH-Iir fibers oriented dorso-ventrally in a region of presumed terminals. This is the end point for the fiber tract that extends from the terminal nerve, through the terminal nerve ganglion, to the nucleus of the diagonal band of Broca, and caudally to the SCN. Darkly stained cGnRH-Iir fibers and

presumed nerve terminals were seen in the median eminence at the transition between the caudal end of the nucleus sphericus and the rostral margin of the optic tectum.

In the amygdala, at the level of the medial preoptic area, cGnRH-Iir fibers extended from near the optic tract down into the amygdala proper (Fig. 3d). The cGnRH-Iir fibers found in the amygdala were some of the smallest diameter fibers seen throughout the brain.

The two other forebrain areas that showed accumulations of cGnRH-Iir fibers were the nucleus accumbens (See Warner, 1947) and the nucleus of the hippocampal commissure. Short cGnRH-Iir beaded fibers with a rostrocaudal orientation were found in the nucleus accumbens at the level of the lateral septum. The nucleus of the hippocampal commissure, located above the third ventricle and medial to the lateral septum, had a dense accumulation of cGnRH-Iir fibers present in an area that has very closely spaced unstained cell bodies (Fig. 3c). Finally, dark cGnRH-Iir fibers that were oriented dorsoventrally and abutted the pallium were occasionally observed in the posterior dorsal ventricular ridge.

In the caudal forebrain there were two main areas for dense staining: the hypothalamus and the thalamus. The hypothalamus had many cGnRH-Iir fibers distributed throughout its length in close association with the third ventricle (Fig. 4a). Dense fiber staining was present along the medial aspect of the hypothalamus, and the number of fibers stained decreased caudally until the level of the optic tectum. At this level cGnRH-Iir fibers were restricted ventrally to the median eminence. The thalamus had occasional cGnRH-Iir fibers that were confined to the medial half of each thalamic hemisphere.

Midbrain

Two areas in the midbrain contained cGnRH-Iir fibers: the optic tectum and the interpeduncular nucleus. Staining in the optic tectum was confined to the stratum album periventricular region, where there were several beaded fibers that ran in close proximity to and following the contours of the ventricle (Fig 4b). There were several beaded fibers in the interpeduncular nucleus, an area which is ventral to the medial longitudinal fasiculus.

Discussion

This immunocytochemical study reveals the neuroanatomical distribution of cGnRH-Iir cells and fibers in the brain of the red-sided garter snake, *T. s. parietalis.* Using an antibody directed against cGnRH-I we found that the distribution of immunoreactive cGnRH-I parallels the forebrain distribution of GnRH in other vertebrates; i.e. the TN-septal-preoptic distribution of mammalian GnRH in mammals and amphibians, of chicken-I GnRH found in birds, and of salmon GnRH found in teleosts (Muske, 1993). This forebrain GnRH system is thought to include GnRH neurons that originate in the olfactory placode during embryonic development and then migrate into the forebrain to populate the TN, septal, and preoptic structures (Muske, 1993). To our knowledge, this is the first description of GnRH staining in the terminal nerve of any reptile.

Staining in the olfactory bulb of the red-sided garter snake was diffuse and widespread. cGnRH-Iir fibers were seen in all layers: the internal plexiform layer, mitral layer, glomerular layer, the medial olfactory tract, the lateral pallium, and the terminal nerve. This pattern is similar to the pattern seen in teleost fishes. In the dwarf gourami, *Colisa lalia,* cGnRH-Iir fibers are widespread in the olfactory bulbs and present in all cytoarchitectonic layers (Oka and Ichikawa, 1990). Salmon, *Oncorhynchus masou,* have a more restricted pattern of GnRHir, with staining for salmon GnRH being found in the olfactory bulb and in an area between the olfactory bulb and olfactory nerve (Amano *et al.*, 1991). GnRH staining in the olfactory bulb was reported in one other reptile, the rat snake, *E. climacophora*, but this immunoreactivity was not described in detail (Nozaki *et al.*, 1984). In the rat, GnRHir for mammalian GnRH is seen in the accessory olfactory bulb in the form of fibers, and input of GnRHir fibers is also seen in the external plexiform layer (Merchanthaler, 1984; Witkin and Silverman, 1983). In primates, GnRHir fibers are

localized to the main olfactory bulb and lateral olfactory tract in the rhesus monkey, *Macaca mulatta*, and in the external plexiform layer of the squirrel monkey, *Sciureus saimiri* (Witkin, 1985). The function of GnRH in the olfactory bulb remains unknown.

The TN in *T. s. parietalis* is located on the ventral surface of the brain just below the pial layer, and extends in a rostro-caudal extent from the middle of the olfactory bulb back to the TN ganglion. The TN ganglion is located at the transition between the olfactory bulb and the telencephalic hemispheres and from here, terminal nerve fibers extend caudally back to the nucleus of the diagonal band of Broca. The TN ganglion (as localized by GnRHir) in some species of fish is also located at this transitional area. In the dwarf gourami, *C. lalia*, the TN ganglion is found at the transition between the olfactory bulb and the telencephalic hemispheres (Oka and Ichikawa, 1990). In the goldfish, *Carassius auratus*, the projections of these TN fibers run peripherally from the olfactory nerve and centrally into the ventromedial forebrain, and in the platyfish, *Xiphophorus maculatus*, the nucleus olfactoretinalis, the homologue to the terminal nerve ganglion, is located at the boundary between the ventral telencephalon and the olfactory bulb (Bartheld and Meyer, 1986; Schreibmann and Margolis-Nunno, 1987). In amphibians, the terminal nerve runs along the midline from the olfactory nerve implantation cones to the medial wall of each hemisphere, before dividing into two main branches at the level of the mid-telencephalon. One branch remains on the ventral brain surface and projects medially, and the other projects dorsocaudally along the olfactory tubercle to the medial septal nucleus (Muske and Moore, 1988). In the roughskin newt, *T. granulosa,* the terminal nerve ganglion is located caudal to the olfactory bulbs, a pattern that is similar to that in *T. s. parietalis* (Muske and Moore, 1988). In mammals, TN perikarya occur as single cells or in ganglionated clusters along the length of the TN, which extends from the vomeronasal system, along the ventromedial aspect of the brain, back to the medial septum (Schwanzel-Fukuda and Silverman, 1980).

The neuroanatomical pattern of GnRH staining in the terminal nerve appears to be similar in fish, amphibians, reptiles, and mammals, although which chemosensory nerve is associated with the terminal nerve may vary. In the red-sided garter snake, the terminal nerve is more closely associated with the vomeronasal nerve, and the vomeronasal nerve is known to carry important reproductive information that is used in the courtship behavior in this species (Kubie *et al.*, 1978). The close association of the vomeronasal nerve and the terminal nerve, as well as the fact that GnRH analogs have been shown to be effective in eliciting courtship behavior in male red-sided garter snakes (Smith *et al.*, unpublished), raises the possibility that one of the terminal nerve's functions is the initiation or maintenance of reproductive behavior in this species by release of GnRH after reception of a pheromonal cue. The evidence concerning the TN's role in pheromonal transduction is unclear. Some studies show that TN transection leads to mating deficits and other studies show that there is no effect of terminal nerve lesions on parameters associated with sex behavior (Wirsig, 1987; Wirsig-Wiechmann, 1993, Fujita *et al.*, 1991).

The telencephalon of *T. s. parietalis* had more areas with cGnRH-I immunoreactivity than any other brain subdivision examined. Like other vertebrates that have been examined, *T. sirtalis* had dark staining for cGnRH-I fibers in the rostral septum, the nucleus of the diagonal band of Broca, the medial pre-optic area, and the median eminence, with cell bodies found in the nucleus of the diagonal band of Broca and the medial preoptic area (Nozaki and Kobayshi, 1979; Silverman, 1988; Millam *et al.*, 1993). Another characteristic that appears to be shared with other vertebrates is a loose plexus of GnRHir fibers that are found in the periventricular region of the third ventricle at the level of the hypothalamus (Yellon *et al.*, 1990).

Dense fiber staining was seen in the amygdala of the red-sided garter snake; these fibers usually abutted the lateral pre-optic area and then extended down into the amygdala. Staining in the amygdala is a common feature in other vertebrates including the guinea pig

and rhesus monkey, *M. mulatta,* which have mammalian GnRH localized in this area (Leonardelli and Poulain, 1977; Silverman *et al.*, 1982). cGnRH-Iir fibers were also seen in the nucleus of the hippocampal commissure of the red-sided garter snake. Hippocampal GnRHir has been observed in mammals for the mammalian form of the peptide and in birds for the chicken-I form of the peptide (Barry *et al.*, 1985; Millam *et al.*, 1993). In addition, the rat has been shown to possess GnRH receptors in the hippocampus (Leblanc *et al.*, 1988). The fact that GnRHir and receptors have been localized in the hippocampus suggests the question: Is GnRH involved in the process of learning and memory?

cGnRH-I immunoreactivity in the midbrain of *T. sirtalis* was restricted to two areas: the stratum album periventricular layer (SAP) of the optic tectum and the interpeduncular nucleus. Immunoreactivity for GnRH in the optic tectum is a feature that is seen in certain fish species, but it is not confined to the SAP layer as we observed in the red-sided garter snake. Amano *et al.* (1991) have shown that the masu salmon has immunoreactivity to salmon GnRH seen in the deep layers of the optic tectum. Oka and Ichikawa (1990) report that the dwarf gourami has GnRH immunoreactivity in the stratum album centrale and the stratum griseum centrale. These areas both receive retinotectal terminals and it has been proposed that GnRH may play a role in the regulation of visual processing. Merchanthaler *et al.* (1984) studying the distribution of mammalian GnRH in the rat brain have demonstrated that rats have GnRH immunoreactivity that is seen in the interpeduncular nucleus (IPN), and Rastogi *et al.* (1990) have seen IPN GnRHir in the brain of the edible frog, *Rana esculenta.* Few studies have shown GnRHir in the midbrain of reptiles. Bennis *et al.* (1989) looked at the distribution of salmon GnRH in the brain of the chameleon and showed GnRHir localized in the anterior midbrain tegmentum and the fasiculus longitudinalis medialis (MLF). Although the IPN is immediately ventral to the MLF, we observed no immunoreactivity that extended into the MLF.

It is also interesting to note what was not found in this study. Using a chicken-II GnRH antibody, we were not able to detect any cGnRH-IIir in any structure, including the midbrain. In many vertebrate species there are two populations of GnRH cells: the GnRH containing cells that are part of the TN-septo-preoptic system, and cells that are found in the midbrain (for review see Muske, 1993). As a general rule, the telencephalic structures vary in which GnRH form is present across species and the cells that are found in the midbrain are immunoreactive to chicken-II. Why we did not identify any chicken-II cells or fibers is not known although several possibilities exist: it may be that our antibody was not sensitive enough, or that chicken-II GnRH is either not present or is present in too low a concentration to be detected.

In conclusion, this study indicates that cGnRH-Iir is widespread in the forebrains of reproductively active, male, red-sided garter snakes, and that the terminal nerve in a species of reptile contains immunoreactive GnRH. We believe that the TN-septal-preoptic system that we have seen in the red-sided garter snake is homologous to the TN-septal-preoptic system that is seen in other vertebrate classes and that this system is most closely related to the system found in birds due to the specific presence of cGnRH-Iir.

Table II.1. Abbreviations of brain regions found in *Thamnophis sirtalis parietalis*.

A: amygdala
AC: anterior commissure
ADVR: anterior dorsal ventricular ridge
AOT: anterior olfactory tract
DB: diagonal band of Broca
DC: dorsal cortex
DP: dorsal pallium
ep: external plexiform layer

gl: glomerular cell layer
H: hypothalamus
ig: internal granular layer
ip: internal plexiform layer
IP: interpeduncular nucleus
L: lateral pallium
LFB: lateral forebrain bundle
lPOA: lateral preoptic area
m: mitral cell layer
MC: medial cortex
ME: median eminence
MFB: medial forebrain bundle
MLF: medial longitudinal fasiculus
MOT: medial olfactory tract
mPOA: medial preoptic area
NA: nucleus accumbens
nDB: nucleus of the diagonal band of Broca
NHC: nucleus of the hippocampal commissure
NS: nucleus sphericus
OC: optic chiasm
OT: optic tract
Ot: olfactory tubercle
OV: olfactory ventricle
PDVR: posterior dorsal ventricular ridge
PT: pretectal nucleus
RDM: retrobulbar region pars dorsomedialis
SCN: suprachiasmatic nucleus
Sd: dorsal septum
Sl: lateral septum
Sm: medial septum

VnN: vomeronasal nerve

Layers of the optic tectum

SAC: stratum album centrale
SAP: stratum album periventriculare
SFGS: stratum fibrosum et griseum superficiale
SGC: stratum griseum centrale
SO: stratum opticum

Table II.1, Continued

Sr: rostral septum
T: thalamus
TN: terminal nerve
TNg: terminal nerve ganglion

Fig. II.1. Distribution of cGnRH-Iir cell bodies and fibers in the brain of *Thamnophis sirtalis parietalis*. Camera lucida drawings depicting the distribution of GnRHir fibers and cell bodies in *Thamnophis sirtalis parietalis*. Cell bodies are described with large dots and fibers are described by small dots.

Figure II.1

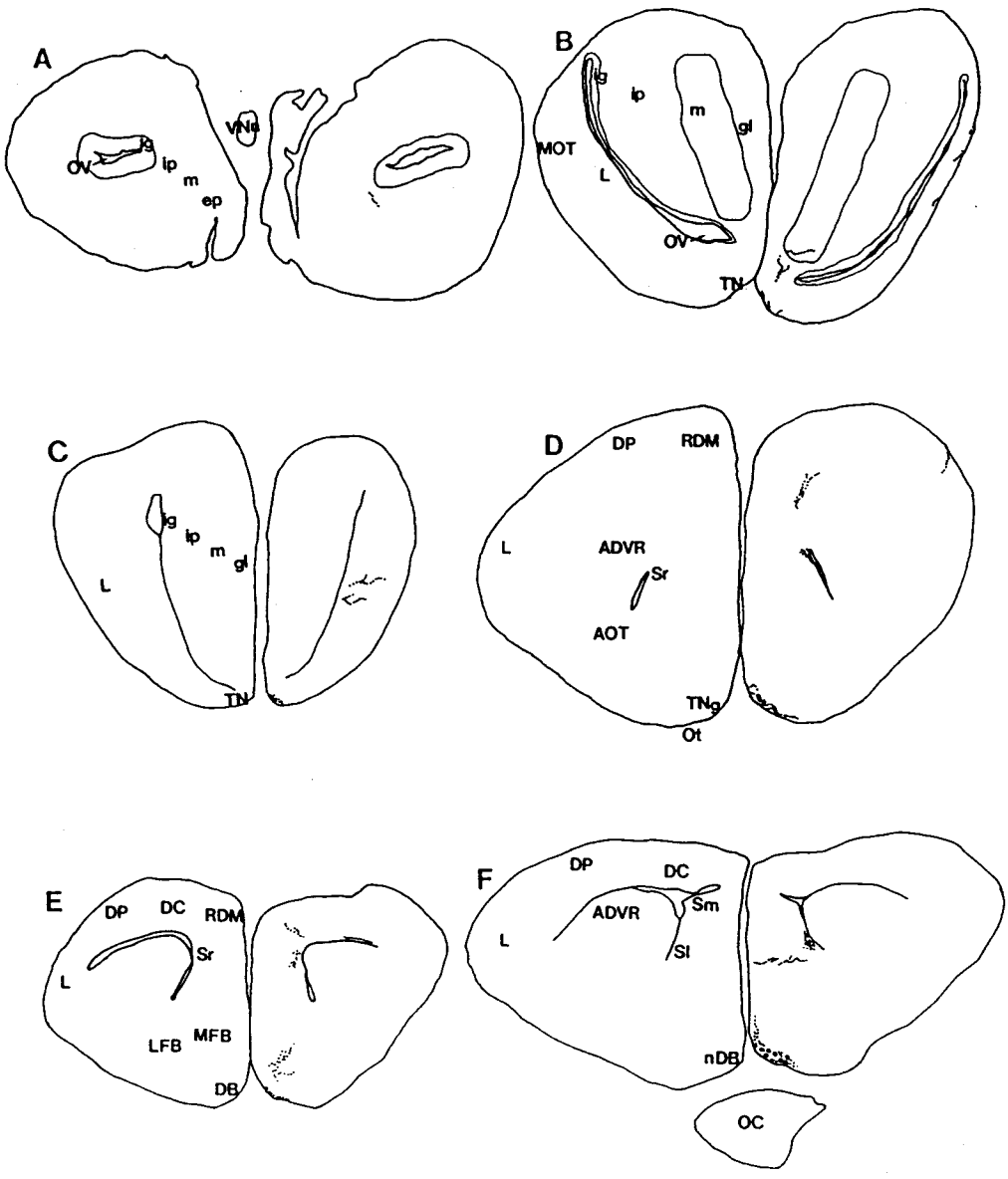
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Fig. II.1, Continued

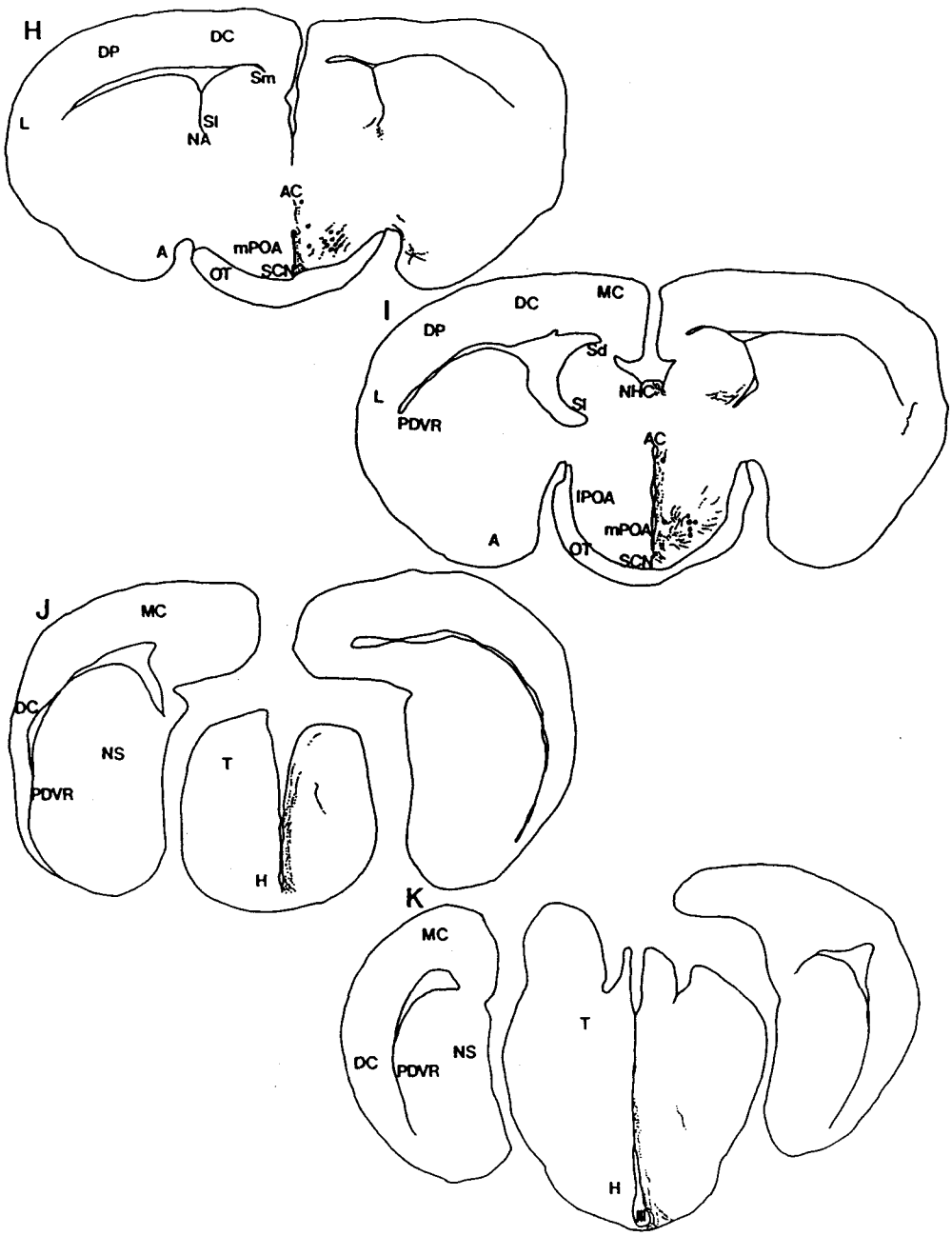


Fig. II.1, Continued

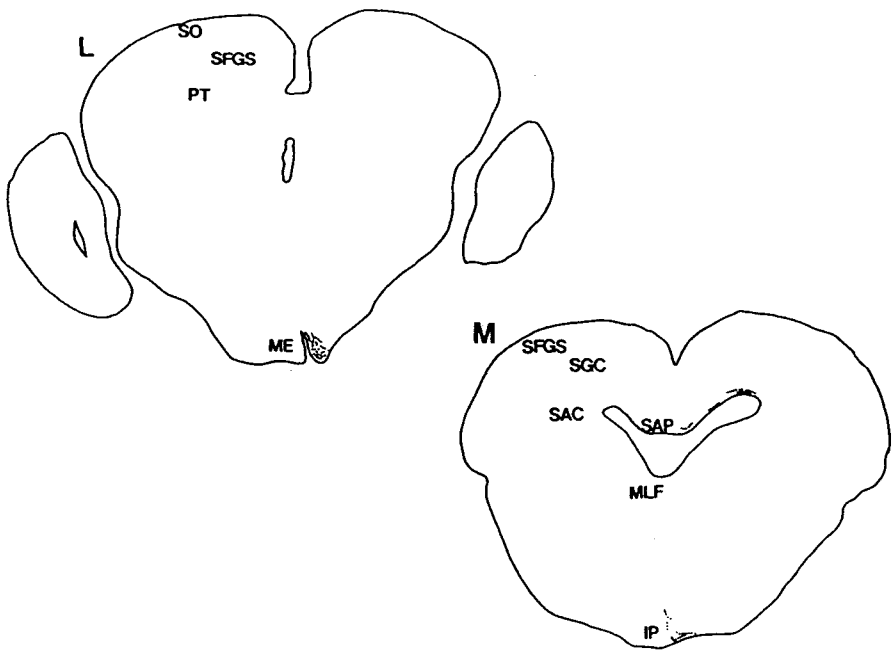


Fig. II.2. Control experiments demonstrating the specificity of the antibody and cGnRH-Iir in the terminal nerve. A comparison between adjacent sections showing the nucleus of the diagonal band of Broca where the antibody was preabsorbed with 100μM chicken-I (A) or treated normally (B) for immunocytochemistry. Scale bar=100μm for A and B. C. A section through the transition between the olfactory bulb and the telencephalon showing some of the cells that comprise the terminal nerve ganglion. Immunoreactive cells and fibers are present and highlighted with arrows. Scale bar=100μm. D. The same section as C, but showing a close-up view of the cells and fibers. Note that one cell is bipolar and that the other is unipolar. Scale bar=20μm. E. A terminal nerve ganglion cell from another animal. This cell projects lateral to the long axis of the brain. Many fibers were observed extending at a right angle to the direction the nerve tract is projecting. Scale bar is the same as in D.

Fig. II.2

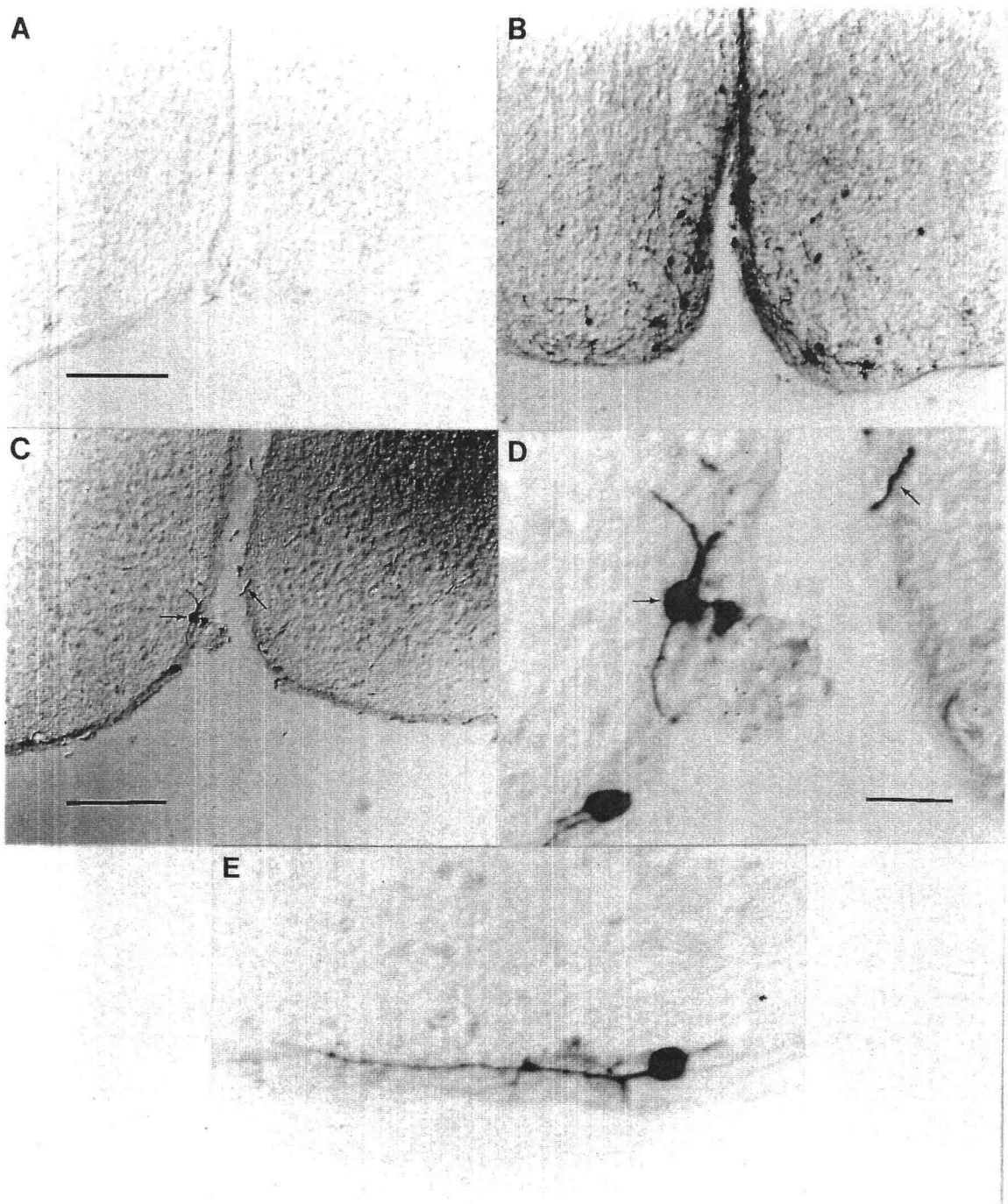


Fig. II.3. cGnRH-Iir in the nucleus of the diagonal band of Broca, lateral septal nucleus, nucleus of the hippocampal commisure, amygdala, and medial preoptic area. A. GnRH immunoreactivity in the nucleus of the diagonal band of Broca. This was one of the most intense areas for GnRH staining with many cell bodies and fibers found throughout this nucleus. Scale bar=100μm. B. GnRH staining in the lateral septal nucleus. Two fibers are highlighted by arrows, one of which is found on the medial margin of the lateral ventricle. Scale is the same as A. C. Dark staining of fibers in the nucleus of the hippocampal commissure. Note the two beaded fibers that are highlighted by arrows. Scale bar=20μm. D. A very long, beaded fiber in the amygdala. Arrows denote the course of the fiber. Scale is the same as C. E. GnRHir in the medial preoptic area. The staining in this area was not as intense as that seen in the nucleus of the diagonal band of Broca, but was consistently present in all animals. Two regions of many staining cells are located lateral to the third ventricle, with fibers found throughout the cell bodies. Arrows identify cells that stain for GnRH in this region. Scale bar=100μm.

Fig. II.3

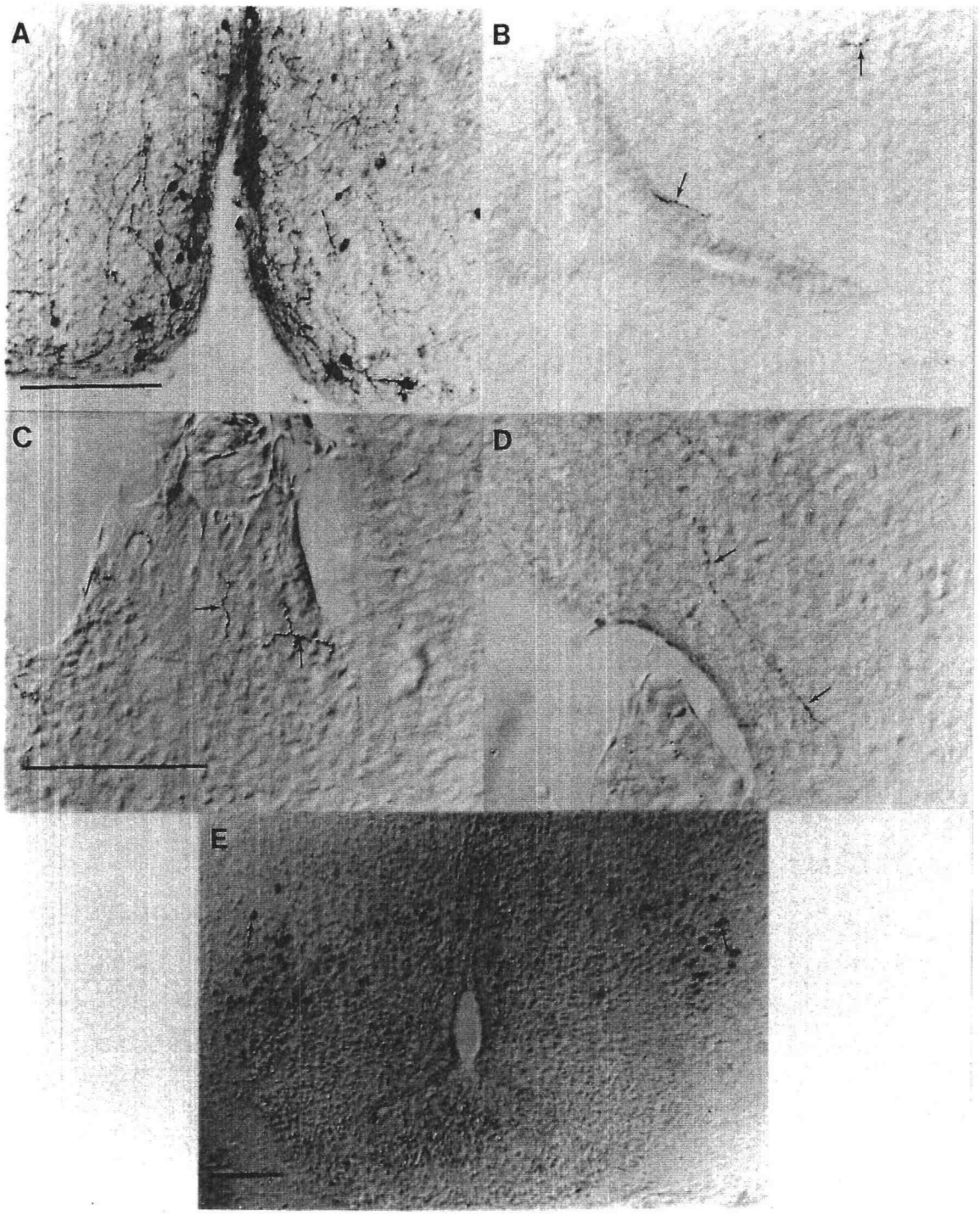
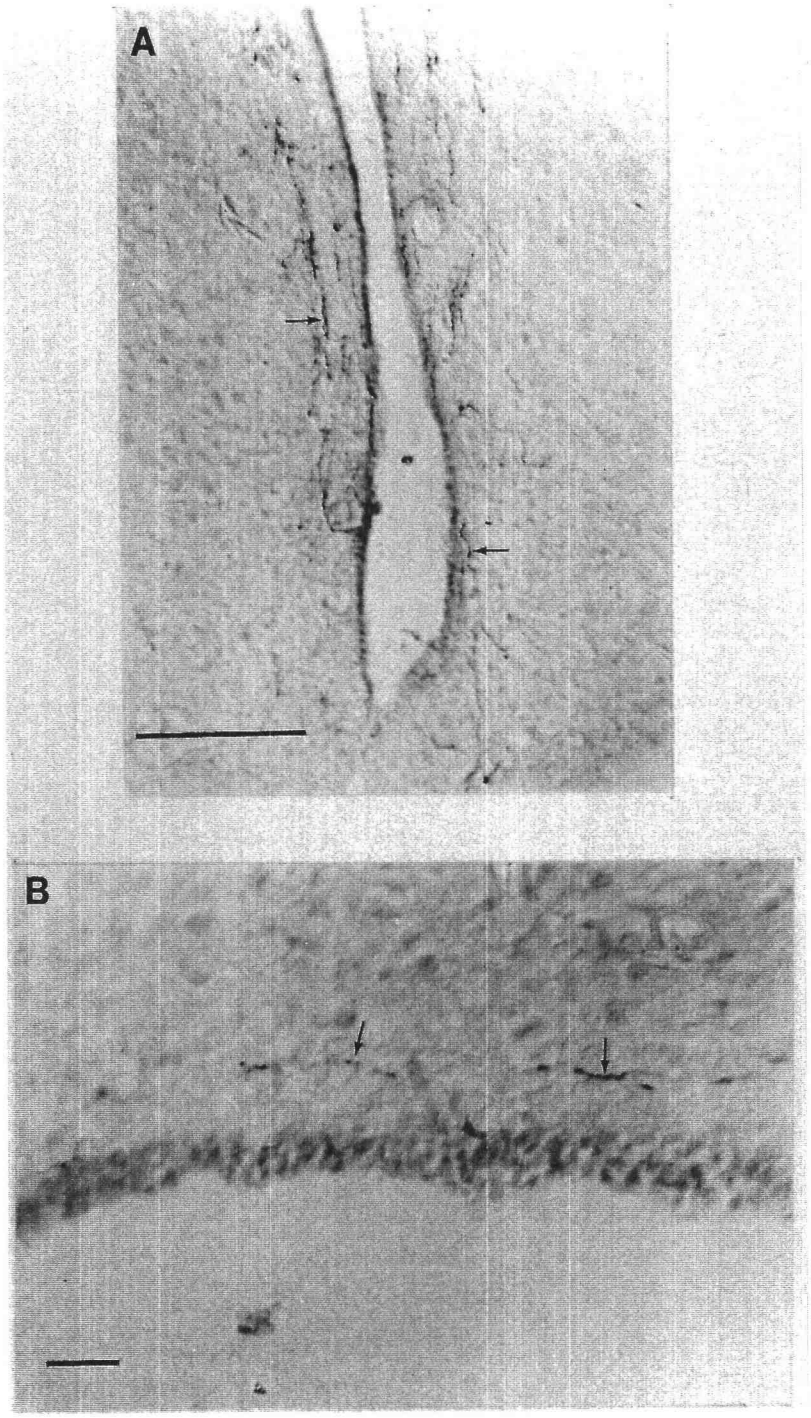


Fig. II.4. cGnRH-Iir concentrated around the third ventricle in the hypothalamus and in the stratum album periventricular region of the optic tectum. A. As seen in this photomicrograph, GnRHir was often confined to regions that surround the ventricles. This figure shows short beaded GnRH fibers around the third ventricle at the level of the hypothalamus. Scale bar=100μm. B. GnRHir in the stratum album periventricular region of the optic tectum. This was the most caudal area that had staining for chicken-I GnRH. Two fibers are highlighted by arrows. Scale bar=20μm.

Fig. II.4



CHAPTER III: THE EFFECT OF CHICKEN-I GnRH AND D-Phe^{2,6}, Pro^{3}-GnRH ON MALE COURTSHIP BEHAVIOR IN THE RED-SIDED GARTER SNAKE, *Thamnophis sirtalis parietalis.*

Abstract

Behavioral studies were used to investigate the central effects of chicken-I GnRH and D-Phe^{2,6}, Pro^{3}-GnRH, a GnRH antagonist, on the courtship behavior of the male red-sided garter snake, *Thamnophis sirtalis parietalis*. Intracerebroventricular (ICV) injections of chicken-I GnRH had no effect on time spent courting or latency to court when experimental males were exposed to unmated females or when experimental males were exposed to the female sex attractiveness pheromone. ICV injections of D-Phe^{2,6}, Pro^{3}-GnRH caused a significant decrease in latency to court and a significant increase in time spent courting when experimental males were exposed to unmated females. When males injected with D-Phe^{2,6}, Pro^{3}-GnRH were exposed to the female sex attractiveness pheromone, it caused a significant increase in time spent courting compared to saline injected controls. This is the first study to demonstrate that any hormone or neuropeptide can enhance sexual behaviors in *Thamnophis sirtalis parietalis*.

Introduction

The red-sided garter snake, *Thamnophis sirtalis parietalis*, is the most northerly living reptile in North America and perhaps in the world (Logier and Toner, 1961). As a result of the extreme winter temperatures at these northerly latitudes, the red-sided garter snake is constrained to spend up to eight months of the year in hibernation (Gregory, 1974). In early May, the males of a given hibernaculum will emerge *en masse* and wait for the females to emerge (Gartska *et al.*, 1982). The females, who emerge singly or in small groups over the course of the next 3-4 weeks, are courted by anywhere from 10-100 males (Gartska *et al.*, 1982).

One component of courtship behavior in the red-sided garter snake is chin rubbing behavior that is coupled with an increased tongue flicking rate (Mason *et al.*, 1989). This chin rubbing behavior is characterized by the male rubbing his chin up and down the back of the female while constantly tongue flicking her dorsal surface (Gartska *et al.*, 1982). This tongue flicking behavior has been shown to transport nonvolatile sex pheromones sequestered on the female's dorsal surface to the male's vomeronasal organ (Noble, 1937). This sex attractiveness pheromone, a suite of lipids that are components of the integumental skin lipids, is the fundamental constituent by which males are attracted to females (Mason *et al.*, 1989). This pheromone, a series of long chain, saturated and monounsaturated methyl ketones, is present in the skin of females and this substance elicits strong courtship behavior from males even after it has been extracted from the skin of females and applied to a paper towel (Mason *et al.*, 1989). If this pheromone is not present on the female's skin or if the male is unable to detect it, the males will not exhibit courtship behavior (Halpern, 1987).

Many neurotransmitters and hormones have been investigated as possible regulators of courtship behavior in the red-sided garter snake, but only two parameters have been shown to be essential for reproduction. The pheromone system, mentioned previously, is

essential for reproduction, as is a period of cold exposure prior to the mating season (Gartska *et al.*, 1982). The present study describes a series of experiments designed to investigate the role of GnRH in the initiation and maintenance of male sex behavior in *Thamnophis sirtalis parietalis.*

Members of the gonadotropin-releasing hormone (GnRH) family of decapeptides are found in the brains of most vertebrates (Sherwood, 1987). One function of GnRH is to regulate the hypothalamo-pituitary-gonad (HPG) axis by stimulating gonadotrophs of the anterior pituitary to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which, in turn, control a variety of functions including steroid secretion, gamete maturation, and ovulation (Hoffman *et al.*, 1992).

The effects of GnRH are not strictly limited to interactions with the HPG axis. It has been demonstrated that the sex behavior of the roughskin newt (*Taricha granulosa)*, voles (*Microtus canicaudus*), rats, frogs (*Xenopus laevis*), anoles (*Anolis carolinensis*), and horses are all modulated by exposure to GnRH (Moore *et al.*, 1982; Boyd and Moore, 1985; Moss and McCann, 1973; Kelly, 1982; Alderete *et al.*, 1980; McDonnell *et al.*, 1989). It has also been shown that GnRH levels change in specific brain areas in response to reproductive cues. The GnRH concentration in the posterior olfactory bulb of female voles (*Microtus ochrogaster*) increases in response to a male urine cue, ring doves (*Streptopelia roseogrisea*) show an increase in GnRHir in the mast cells of the medial habenula following two hours of courtship, and in an amphibian, *T. granulosa*, there is an increase in the terminal nerve concentration of GnRH in response to mating (Dluzen and Ramirez 1981, 1987; Propper and Moore, 1991). Finally, GnRH can function as a neurotransmitter in the sympathetic ganglia of the frog where it elicits a late, slow excitatory post-synaptic potential (Jan *et al.*, 1979; Jones, 1987). This study was designed to look at the direct effects of GnRH administration on the sex behavior of male

Thamnophis sirtalis parietalis, i.e. those effects that do not involve activation of the HPG axis.

Materials and Methods

Reproductively active, adult, male red-sided garter snakes (*Thamnophis sirtalis parietalis*) were collected in Manitoba, Canada during early May. Males were transported back to a field station and were housed in outdoor pens measuring 1.2 x 1.2 x 0.09m. Each pen contained approximately 100 males and 3-5 unmated females. A courting male was removed from the back of an unmated female, a small hole was drilled into the skull, and 2µl of test solution containing 1, 10, 100, 1000 ng of chicken-I GnRH or D-Phe^{2,6}, Pro^{3}-GnRH was injected over the course of 2 seconds into the third ventricle using a Rainin peristaltic pump and a drawn out glass micro-pipette. The third ventricle injection site was confirmed by sectioning the brains (N=5) of animals that, after being anesthetized, had india ink injected into the same site. Many GnRH agonists and antagonist were tested in these studies, with the results of chicken-I GnRH and D-Phe^{2,6}, Pro^{3}-GnRH presented here. Chicken-I GnRH has been shown to be the major form of GnRH in the brain of *T. s. parietalis* through a study that analyzed antibody cross-reactivity and HPLC retention times (Sherwood and Whittier, 1988). D-Phe^{2,6}, Pro^{3}-GnRH is a potent GnRH receptor antagonist for the mammalian form of the peptide (Humphries *et al.*, 1978). The test males were then exposed to one of two testing regimes.

Experiment 1

The first testing regime compared the responses of saline injected control males (N=16), males injected with chicken-I GnRH (N=32, 8 animals/treatment), or D-Phe^{2,6}, Pro^{3}-GnRH (N=32, 8 animals/treatment), to courting an unmated female. After injection, the test male was introduced into an arena that contained an unmated female and another courting male. The other male was present because of facilitated courtship, a

phenomenon whereby one male will court more vigorously when another male is also courting (Joy and Crews, 1985). Latency to court and time spent courting were recorded over a 5 minute test period. Test males had to show both chin rubbing behavior and caudocephalic waves to be scored as exhibiting sex behavior. The unmated female and auxiliary male were changed once after half of the males had been tested.

Experiment 2

In this testing regime, the males were tested in the absence of visual cues, and the behavioral response of chicken-I GnRH and D-Phe^{2,6}, Pro^{3}-GnRH was observed in response to the female pheromone alone. Female sex attractiveness pheromone was extracted as described previously by washing females in hexane (Mason *et al.*, 1989). The hexane washes from 5 females were reduced in volume to a more concentrated form. Ten ml of this concentrated pheromone was applied to a paper towel on one half of a testing arena, with 10 ml of hexane being applied to a paper towel on the other half of the testing arena. The solvent from both sides of the arena was allowed to evaporate prior to testing.

As in the previous experiment, saline injected controls (N=16), 1, 10, 100, 1000ng of chicken-I GnRH (N=32, 8 animals/treatment) or D-Phe^{2,6}, Pro^{3}-GnRH (N=32, 8 animals/treatment) was injected into the third ventricle, with time spent courting and side preference being recorded over a 5 minute test period.

Experiment 3

Male and female red-sided garter snakes were brought back to Oregon State University and tested to determine if chicken-I GnRH or D-Phe^{2,6}, Pro^{3}-GnRH could initiate mating behavior during August, a month where no mating behavior is observed in this species. As with the previous experiments, a small hole was drilled into the skull, and

2μl of test solution containing 1, 10 μg of chicken-I GnRH (N=9/group) or D-Phe^{2,6}, Pro^{3}-GnRH (N=8/group) was injected over the course of 2 seconds into the third ventricle using a Rainin peristaltic pump and a drawn out glass micro-pipette. Females were estrogen primed to increase attractivity with 40μg estradiol benzoate/75 gm body weight/day for 3 days prior to testing with the males. After injection, test males were introduced into the testing arena that contained the estrogen primed female and another male. Latency to court and time spent courting were recorded over a five minute test period.

Statistics

All data was analyzed by one-way analysis of variance, followed by the Tukey multiple range test where appropriate. When variance was not equal between groups the data was natural log transformed and analysis was done on the transformed data. Only one data set did not conform to the assumptions for parametric analysis and the time spent courting data for D-Phe^{2,6}, Pro^{3}-GnRH in experiment 1 was analyzed using Kruskal-Wallis one-way analysis by ranks. All of the data analysis was done using the Statgrafics® computer software program.

Results

Experiment 1

Chicken-I GnRH had no effect at any concentration when looking at the latency to court an unmated female ($p=0.54$, fig. 1). There was also no significant effect of any concentration of chicken-I GnRH on time spent courting ($p=0.37$, fig. 1).

D-Phe^{2,6}, Pro^{3}-GnRH significantly decreased the latency to court an unmated female ($p=0.002$, fig. 2). The behavioral response to the 1000ng antagonist injection significantly reduced the latency to court when compared to the saline injected controls ($p<0.05$, fig. 2). The 100ng injection also significantly decreased latency to court when compared to the 1ng injection ($p<0.05$, fig. 2). The time spent courting data for D-Phe^{2,6}, Pro^{3}-GnRH showed no significant differences between any of the treatments and the saline injected control animals. The time spent courting when 1000ng of D-Phe^{2,6}, Pro^{3}-GnRH was injected was statistically different from the males that were injected with 1ng of D-Phe^{2,6}, Pro^{3}-GnRH. ($p<0.05$, fig. 2).

Experiment 2

Chicken-I GnRH had no effect on side preference when tested with only the vomeronasal cues from females present ($p=0.47$, fig. 3). All of the test males spent greater then 50% of their time on the pheromone side of the test arena, with the saline injected animals spending 63% of tested time on the pheromone side of the test arena compared with 86% for the 1000ng injected animals. This was not statistically different at the 0.05 level. Time spent courting the female pheromone wash showed no significant differences in any of the groups injected with chicken-I GnRH when compared to saline injected controls ($p=0.08$, fig 3). In three of the groups, the 1, 10, and 100ng of chicken-I

GnRH, the test animals did not court at all. In two of the groups, the saline injected control males and the 1000ng antagonist injected males, the animals spent 2% and 4%, respectively, of the total time courting.

D-Phe^{2,6}, Pro^{3}-GnRH did not increase the amount of time spent on the pheromone side of the test arena ($p=0.30$, fig. 4). A large variance in the 1ng group of this data set made the values not significantly different. The analog, D-Phe^{2,6}, Pro^{3}-GnRH, did cause an increase in courtship on the female pheromone side of the test arena ($p=0.04$, fig. 4). The 1000ng group spent significantly more time courting the female pheromone wash than the saline-injected controls and the 1ng injected animals. Using these doses, there appeared to be a threshold effect with the saline-injected animals and the first three analog concentrations not being statistically different, but with a statistically significant increase in percent of time spent courting the female pheromone wash when 1000ng of analog was injected.

Experiment 3

None of the males in the 1 or 10μg chicken-I GnRH or the 1 or 10μg D-Phe^{2,6}, Pro^{3}-GnRH groups spent any time courting the estrogen primed female (Data not shown). Sex behavior was not expressed by any male in any of the three groups.

Discussion

This study shows that the administration of a form of GnRH can alter the sex behavior of the male red-sided garter snake. D-Phe^{2,6}, Pro^{3}-GnRH administration decreases the latency to court and increase the time males spend courting unmated females. In addition, D-Phe^{2,6}, Pro^{3}-GnRH administration also increases the time spent courting when only the pheromonal cues from females are present to the test male. Chicken-I GnRH administration has no effect on courtship behavior using these testing paradigms at these dosages. This is the first study to show that any hormonal factor can effect sex behavior in *T. s. parietalis*.

Male sex behavior was not affected by administration of chicken-I GnRH (cGnRH-I) in either the unmated female or pheromone alone testing paradigms. GnRH has been shown to modulate sex behavior in a variety of species, with the evidence for rapid effects of GnRH coming from work on rats (Sakuma and Pfaff, 1980). GnRH can potentiate lordosis behavior when systemically administered to estrogen primed, ovariectomized rats (Moss and McCann, 1973; Pfaff, 1973). The site of action for the rapid effects of GnRH on sex behavior appears to be the dorsal half of the midbrain central grey (Sakuma and Pfaff, 1980). When GnRH is administered to the midbrain central grey, there was an increase in the lordosis reflex within 5 minutes that persists for approximately 2 hours; an antibody to GnRH eliminates the lordosis reflex in 90 minutes and this persists for up to 12 hours (Sakuma and Pfaff, 1980). This effect was specific for the midbrain central grey and control injections to the superior colliculus were ineffective at eliciting a response (Sakuma and Pfaff, 1980). Immunocytochemical data suggests that the mid-brain population of GnRH neurons in many vertebrates is immunoreactive to the chicken-II (cGnRH-II) form of the GnRH molecule (for review see Muske, 1993). It remains to be

determined if injection of cGnRH-I into the midbrain or injection of cGnRH-II could modulate sex behavior in *T. s. parietalis* .

Latency to court and time spent courting in male *T. s. parietalis* were significantly modulated with administration of D-Phe^{2,6}, Pro^{3}-GnRH. This peptide increased the time males spent courting and decreased the latency to court when males were tested with unmated females. This peptide has been shown to be an antagonist in mammals for mammalian GnRH (Humphries et al., 1978), and a partial antagonist in goldfish at 10^{-7} or 10^{-6}M with a more pronounced ability to antagonize salmon GnRH than chicken-II GnRH (Habibi, 1991). It is not known whether this peptide functions as an agonist or an antagonist for the receptor that is specific for chicken-I GnRH.

There is a small body of literature that describes the effect of GnRH antagonists on male sex behavior or physiology. Administration of a GnRH antagonist to adult male, rhesus monkeys (*Macaca mulatta*) will lead to decreased LH and testosterone secretion, decreased pituitary responsiveness to GnRH, and will ultimately lead to a decrease in male sexual behavior within one week (Wallen *et al.*, 1991). A GnRH antagonist given to neonatal, male rats will lead to a transient infertility in adults (Kolho and Huhtaniemi, 1989), and if given to immature male rats there will be an inhibition of sexual development (Van den Dungen *et al.*, 1989). In general, it appears that administration of GnRH antagonists will lead to a depression in traits associated with male sexual behavior. To our knowledge, this is the first study that shows administration of a GnRH antagonist for a mammal or fish will increase sexual behavior in another vertebrate. This raises the possibility that D-Phe^{2,6}, Pro^{3}-GnRH may function as an agonist in some nonmammalian vertebrates, including *T. s. parietalis.*

Chicken-I GnRH (cGnRH-I) appears to be the major form of GnRH in the brain of *T. s. parietalis* (Sherwood and Whittier, 1988), and cGnRH-I immunoreactivity has been localized to the median eminence and infundibulum in reproductively active males of this

species (Smith *et al.*, Submitted). cGnRH-I would presumably control the release of the one ophidian gonadotropin molecule that appears to function like FSH (Licht *et al.*, 1979) and, in turn, this gonadotropin would control testosterone release.

There is also evidence for other forms of GnRH in the brain of *T. s. parietalis* (Sherwood and Whittier, 1987). It may be that the stimulatory effect of D-Phe^{2,6}, Pro^{3}-GnRH that we observed is occurring through another GnRH receptor subtype. It has been demonstrated that GnRH receptors with similar pharmacology are differentially regulated depending on the tissue (Ban *et al.*, 1990). It is also possible that D-Phe^{2,6}, Pro^{3}-GnRH is able to induce the dimerization that is needed for typical agonist functionality, since it has been shown that both agonists and some antagonists are able to induce the same responses of receptor clustering and internalization (Hazum *et al.*, 1983).

Although D-Phe^{2,6}, Pro^{3}-GnRH was able to enhance male sex behavior during the breeding season, it failed to initiate courtship behavior during the non-breeding season. Apparently the regulatory system by which D-Phe^{2,6}, Pro^{3}-GnRH is enhancing courtship behavior during the breeding season is composed of more than one component and that D-Phe^{2,6}, Pro^{3}-GnRH alone is insufficient for the full expression of male sexual behavior during the non-breeding season. These additional components can be either environmental factors or other neurohormones/peptides that, in concert, regulate the expression of male sex behavior. A strong possibility is that the prior exposure to cold temperatures during hibernation is the other causative factor that is missing during the non-breeding months since it has been demonstrated that a period of cold temperature is essential for expression of sexual behavior (Gartska *et al.*, 1982). It has also been noted that there are strong seasonal differences both qualitatively and quantitatively in the skin lipids of female garter snakes (Mason *et al.*, 1987), and the change in the pheromonal component of the females skin lipids may lead the males to be reproductively ineffective during the non-breeding months due to a failure of the females to produce the pheromone over background skin

lipid levels. Finally, there may be a seasonal change in the vomeronasal organ's responsiveness to the sex pheromone. Males may be unable to detect the sex-attractiveness pheromone due to a combination of decreased vomeronasal responsiveness and a change in the pheromonal component of the skin lipids.

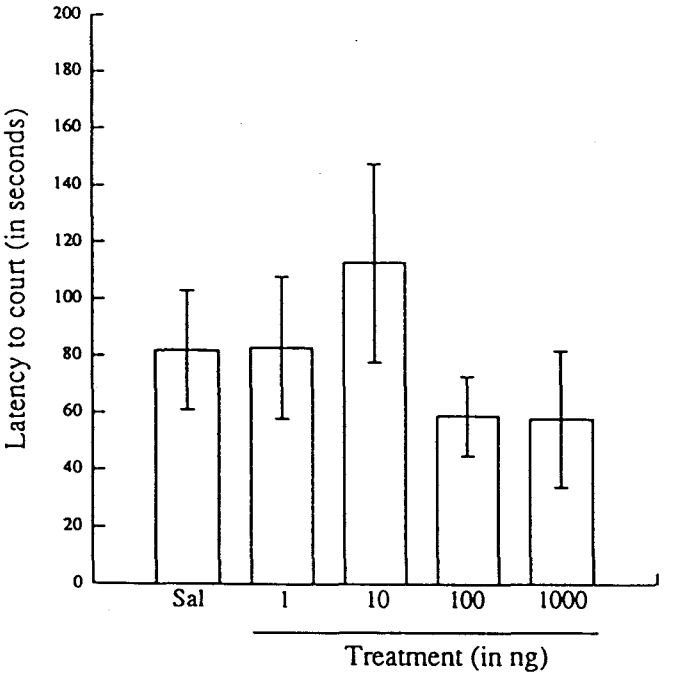
How is this GnRH analog enhancing male courtship behavior in the red-sided garter snake? In the male courting female tests, D-Phe^{2,6}, Pro^{3}-GnRH caused males to find females more quickly which led to a significant decrease in the latency to court over saline injected controls. This peptide may be functioning to lower the threshold of responsiveness to sensory information in general or to specifically increase the sensitivity of the vomeronasal system. In young, mature rats, it has been shown that a GnRH analog, D-Trp^{6}-GnRH, may enhance visual discrimination (Nauton *et al.*, 1992). Investigation of the effects of GnRH on testosterone-treated geldings, showed no effect on sex behavior in the absence of testosterone, but when testosterone was present the horses appeared to fixate on vomeronasal cues as evidenced by an increased flehmen responsiveness (McDonnell *et al.*, 1988). Increased reliance on vomeronasal cues due to a GnRH analog injection, may explain the decrease in latency to court in the red-sided garter snake since courtship in this species is highly dependent on vomeronasal information (Halpern, 1987).

In conclusion, we have demonstrated that D-Phe^{2,6}, Pro^{3}-GnRH administration is capable of enhancing male reproductive behavior in the red-sided garter snake. This is the first time that any hormone or peptide has been shown to influence reproduction in this species and this study shows that the GnRH system is involved in the initiation of courtship behavior in *T. s. parietalis*.

Fig. III.1. Effect of chicken-I GnRH on latency to court and time spent courting an unmated female. A. Effect of chicken-I GnRH on experimental male's latency to court an unmated female (N=8 for each group). B. Effect of chicken-I GnRH on the time spent courting an unmated female during a five minute test period (N=8 for each group).

Fig. III.1

A. Effect of Chicken-I GnRH on latency to court



B. Effect of Chicken-I GnRH on time spent courting

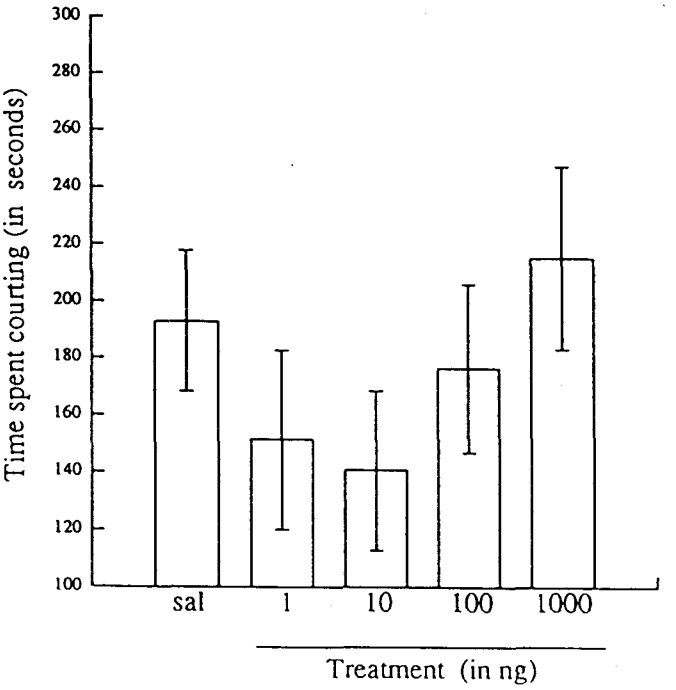
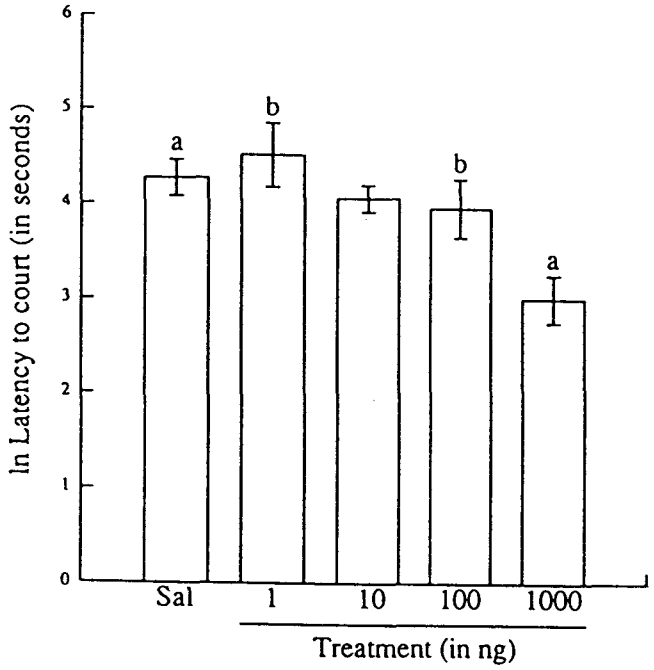


Fig. III.2. Effect of D-Phe^{2,6}, Pro^{3}-GnRH on latency to court and time spent courting an unmated female. A. Effect of D-Phe^{2,6}, Pro^{3}-GnRH on an experimental male's latency to court an unmated female (N=8 for each group). a's are different at the $p<0.05$ level and b's are different at the $p<0.05$ level. B. Effect of D-Phe^{2,6}, Pro^{3}-GnRH on time spent courting an unmated female during a five minute test period (N=8 for each group). a's are different at the $p<0.05$ level.

Fig III.2

A. Effect of D-Phe^{2,6}, Pro^{3}-GnRH on latency to court



B. Effect of D-Phe^{2,6}, Pro^{3}-GnRH on time spent courting

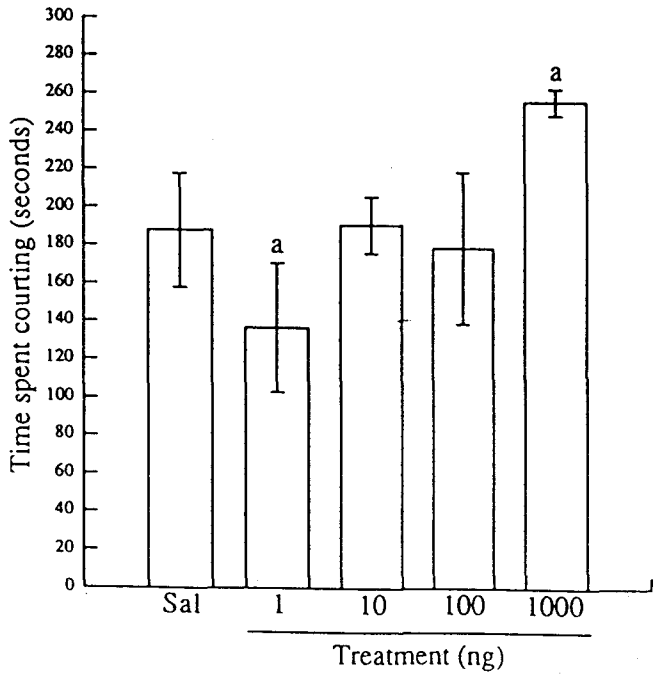
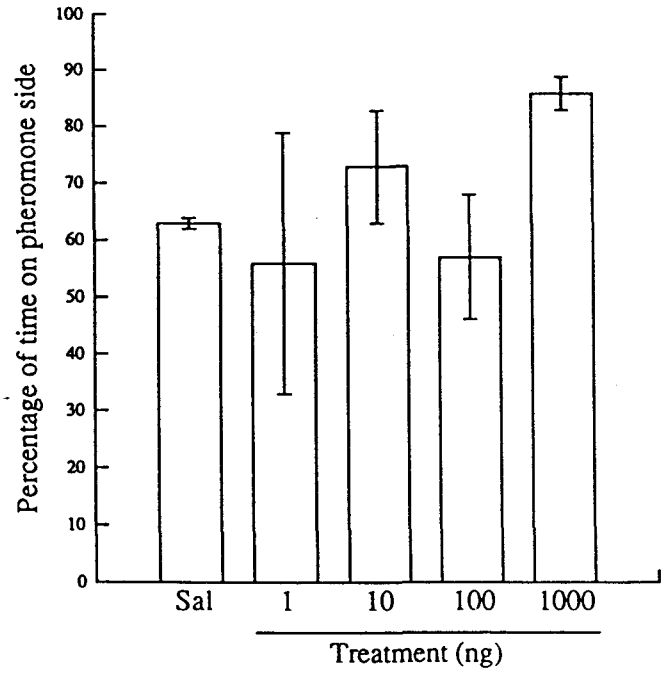


Fig. III.3 Effect of chicken-I GnRH on side preference and time spent courting the female sex attractiveness pheromone. A. Effect of chicken-I GnRH on the side preference of an experimental male to the side of the test arena with the female sex attractiveness pheromone versus a hexane control (N=8 for each group). B. Effect of chicken-I GnRH on the time that the test male spent courting the female pheromone wash (N=8 for each group).

Fig. III.3

A. Effect of Chicken-I GnRH on pheromone side preference



B.

Effect of Chicken-I -GnRH on time spent courting a female pheromone wash

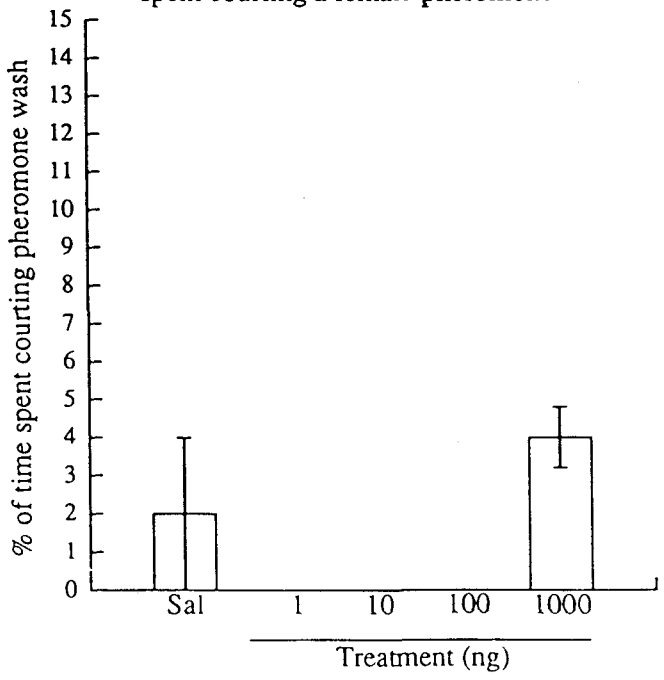
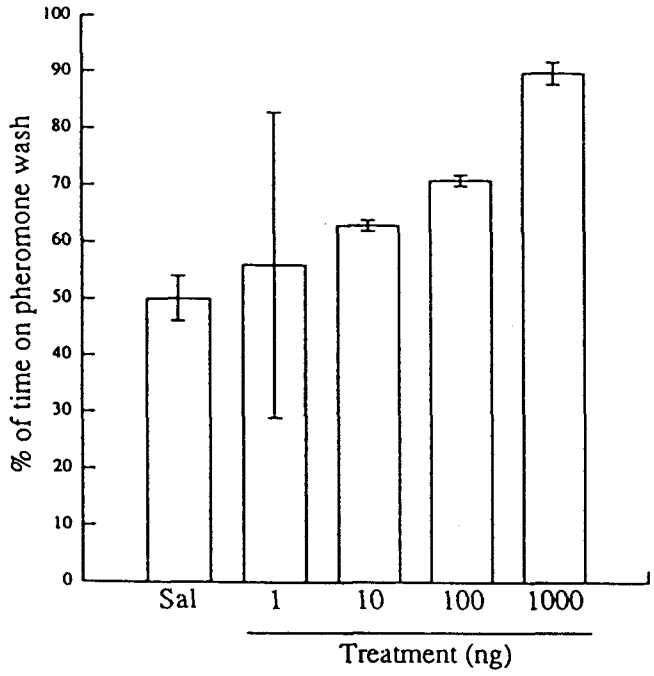


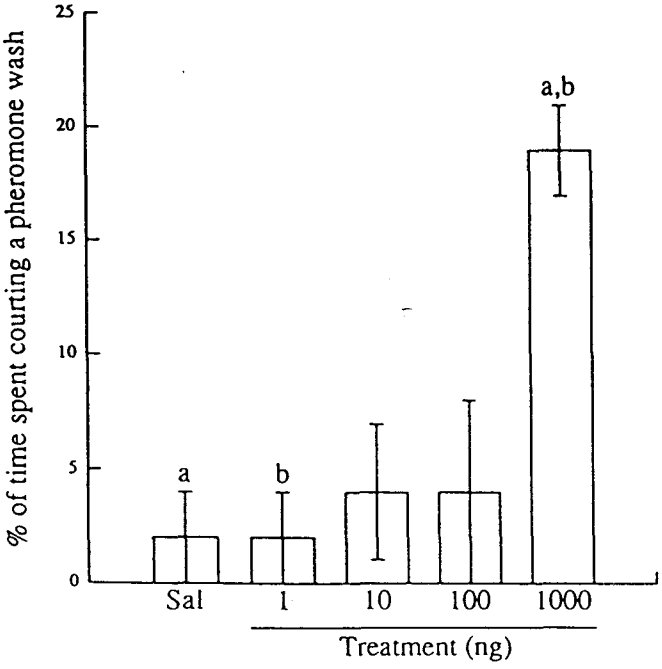
Fig. III.4. Effect of D-Phe^{2,6}, Pro^{3}-GnRH on side preference and time spent courting the female sex attractiveness pheromone. A. Effect of D-Phe^{2,6}, Pro^{3}-GnRH on the side preference of an experimental male to the side of the test arena with the female sex attractiveness pheromone versus a hexane control (N=8 for each group). B. Effect of D-Phe^{2,6}, Pro^{3}-GnRH on the time that the test male spent courting the female pheromone wash (N=8 for each group). a's are different at the $p<0.05$ level and b's are different at the $p<0.05$ level.

Fig III.4

A. Effect of D-Phe^{2,6}, Pro^{3}-GnRH on pheromone side preference



B. Effect of D-Phe^{2,6}, Pro^{3}-GnRH on time spent courting a female pheromone wash



CHAPTER 4: CONCLUSIONS

Summary

The original hypothesis was that upon reception of the female sex attractiveness pheromone, the terminal nerve of the red-sided garter snake releases or stimulates the release of GnRH and this will, in turn, stimulate the stereotyped sex behavior of the male.

Chapter 2 explored the neuroanatomical distribution of GnRH, with the specific aim being to determine if the red-sided garter snake had a terminal nerve that was immunoreactive to the chicken-I form of the GnRH molecule. The red-sided garter snake has a forebrain system of GnRH that is homologous to the forebrain GnRH systems of other vertebrates. Using an antibody directed against cGnRH-I, a form that is known to be present in the brain of *Thamnophis sirtalis parietalis*, we were able to show that the distribution of immunoreactive cGnRH-I parallels the forebrain distribution of GnRH in other vertebrates; i.e. the TN-septal-preoptic distribution of mammalian GnRH in mammals and amphibians, of chicken-I GnRH found in birds, and of salmon GnRH found in teleosts (Sherwood and Whittier, 1988; Muske, 1993). In addition, to our knowledge, this is the first description of GnRH staining in the terminal nerve of any reptile, although the terminal nerve has been previously described based on anatomical location (Johnston, 1913). In addition to demonstrating the homology of the forebrain GnRH system in a reptile, this is the first demonstration of the terminal nerve being immunoreactive to GnRH in the class Reptilia. We have also shown that the complete terminal nerve-septal-preoptic system is developed in a reptile, thus showing that the ontogeny of the GnRH system in reptiles may parallel the ontogeny seen in other vertebrates.

Chapter 3 explores the ability of chicken-I GnRH or D-Phe^{2,6}, Pro^{3}-GnRH to modulate courtship behavior in the male red-sided garter snake. Intracerebroventricular

(ICV) injections of chicken-I GnRH had no effect on time spent courting or latency to court an unmated female and did not effect time spent courting the female sex attractiveness pheromone. ICV injections of D-Phe^{2,6}, Pro^{3}-GnRH had no effect on time spent courting unmated females when control animals were compared to treated animals, but significantly decreased latency to court unmated females and significantly increased time spent courting the female sex attractiveness pheromone. This is the first study to show that any hormonal factor can enhance courtship behavior in the male red-sided garter snake.

It is interesting to note that injection of a GnRH analog is capable of eliciting courtship behavior in the red-sided garter snake when injected into a third ventricle site that lies directly above the preoptic area, that the preoptic area contains immunoreactive GnRH neurons, and that the temperature sensitive sites are located within the preoptic area (Nelson *et al.*, 1984). It appears that the preoptic area is one of the important sites for the regulation of reproductive behavior in *Thamnophis sirtalis parietalis*.

Future considerations

Although I have shown that GnRH is involved with reproductive behavior in the red-sided garter snake, and that the red-sided garter snake has a terminal nerve, the hypothesis that the terminal nerve either releases or causes the release of GnRH remains to be tested. The direct release of GnRH is the more easily testable hypothesis; through the use of the Palkovits brain punch technique followed by radioimmunoassay, it would be possible to determine if the GnRH concentration of the terminal nerve was changing in response to either exposure to the sex attractiveness pheromone or unmated females. Recent evidence that an inhibitory pheromone has been found in females would provide another opportunity to address whether terminal nerve release of GnRH can affect sexual

motivation (Mason and Smith, unpublished). Determining if exposure to this pheromone would leave TN GnRH levels above that of control males would provide further evidence for the role GnRH plays in regulating sex behavior.

Given that it was not possible to elicit male mating behavior in the off season with GnRH analogs, other factors must be involved in the regulation of male sex behavior. It would be interesting to know the relative roles that other factors may play with respect to initiation of mating behavior. For example, if the preoptic area is involved with the regulation of sex behavior, are the temperature sensitive neurons the most important factor for the timing or initiation of reproductive behavior with GnRH modulating their activity? The red-sided garter snake is an excellent model with which to explore the interrelationship of pheromones and the initiation of sex behavior. This model system will continue to yield new insights into the sensory control of reproductive behavior and how neurohormonal factors influence this association.

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