AN ABSTRACT OF THE THESIS OF

Robert T. Zoeller for the degree of Doctor of Philosophy in Zoology presented on February 20, 1984. Title: The Role of Luteinizing Hormone-Releasing Hormone in the Neuroendocrine Control of Seasonal Reproduction in Male Rough-Skinned Newts, Taricha granulosa.

Abstract approved: Redacted for privacy
Frank L. Moore

One hypothesis proposed to explain how the endocrine system controls reproductive cycles in seasonally breeding animals is that the hypothalamus undergoes seasonal changes in sensitivity to negative feedback by gonadal steroids. The result is seasonal changes in the secretion of luteinizing hormone-releasing hormone (LHRH) and gonadotropin secretion. The present experiments were designed to test this hypothesis in a male amphibian, the rough-skinned newt.

To determine whether seasonal changes in testicular function are correlated with changes in LHRH secretion, LHRH was measured in specific brain areas and androgen and corticosterone were measured in plasma of male newts collected over a 13-month period. To determine whether the hypothalamus undergoes seasonal changes in sensitivity to negative feedback, the effect of castration and

testosterone (T) replacement on brain LHRH, plasma androgen and corticosterone concentrations were measured in male newts at the beginning and end of the breeding season.

The concentration of LHRH in the infundibulum (I), rostral hypothalamus (RH), and preoptic area (POA) fluctuated throughout the course of the reproductive cycle. LHRH was not detected in any brain area prior to the end of the breeding season when plasma androgen levels began to fall. Because the sensitivity of the pituitary-gonad axis did not change at the end of the breeding season, these results indicate that LHRH synthesis and release declines precipitously at the end of the breeding season.

Castration decreased and T replacement maintained LHRH concentrations in hypothalamic areas of male newts at the beginning of the breeding season, but not at the end of the breeding season. Thus, plasma androgens can influence brain LHRH concentrations. The sensitivity of the hypothalamus to negative feedback appears to be greater at the beginning of the breeding season, indicating that the abrupt decline in brain LHRH concentration before the end of the breeding season probably does not depend upon an increase in sensitivity of the hypothalamus to negative feedback.

The Role of Luteinizing Hormone-Releasing Hormone in the Neuroendocrine Control of Seasonal Reproduction in Male Rough-Skinned Newts, *Taricha granulosa*.

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed February 20, 1984

Commencement June, 1984

APPROVED

Redacted for privacy

Associate Professor of Zoology in charge of Major

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Date thesis is presented February 20, 1984

Typed by researcher for Robert T. Zoeller

ACKNOWLEDGEMENTS

For continuous and good-natured guidance in every aspect of this research, I thank Dr. Frank L. Moore. Dr. Moore also provided financial support in the form of graduate research assistantships for which I am very grateful. I would also like to thank my doctoral committee for their time and energy during my program. Members include Drs. Frederick Stormshak, Robert R. Becker, Phillip Brownell and Carl Bond. I would also like to acknowledge the help of Dr. Daniel M. Dorsa at the Veterans Administration in Seattle, Washington for teaching the punch technique to me and answering numerous questions about many techniques, and Endocrinology in general. In addition, Drs. Harold Spiess and William Ellinwood provided laboratory space at the Oregon Regional Primate Research Center for me to learn to iodinate LHRH. Dr. Stacia Sower at the University of New Hampshire, was also very helpful throughout the course of this study. Finally, I would like to thank Dr. Charles King and the Zoology department for further financial support.

Several faculty members in the department of Zoology provided assistance during the course of my program. Dr. Alfred Owczarzak was very helpful in the preparation of histological sections of newt brains. Drs. Chris Bayne, John Morris and John Rueben provided laboratory equipment necessary for processing brain tissue during the course of

this research.

Finally, many fellow graduate students provided support as the research progressed. Sandra Spielvogel helped in nearly every aspect of this research, including the preparation of manuscripts. Joan Whittier also provided needed technical assistance. Carl Boswell, Mary Yui, Brett and Cheryl Adams, Cathy Propper and Alice Weaver also contributed greatly to the academic environment from which I have benefitted.

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CHAPTER I: GENERAL INTRODUCTION

The research described in this dissertation was designed to investigate the role of luteinizing hormone-releasing hormone (LHRH) in the neuroendocrine control of seasonal reproduction in the urodele amphibian Taricha granulosa. To provide background information for these experiments, general aspects of the reproductive endocrinology of amphibians and mammals will be reviewed.

The rough-skinned newt is particularly well-suited as an experimental subject on which to study the role of LHRH in the neuroendocrine control of seasonal reproduction. Newts are abundant, they are easily maintained in the laboratory, and they survive surgical manipulations very well. There is also an extensive background of important information available on its life history, reproductive behavior, and reproductive physiology (Covell, 1923; Pimentel, 1952; Miller and Robbins, 1954; Pimentel, 1960; Oliver and McCurdy, 1974; Specker and Moore, 1980; Moore and Zoeller, 1979; Zoeller and Moore, 1982; Zoeller, Lais and Moore, 1983; Moore, 1983). Finally, amphibians occupy an important position in the evolutionary history of the vertebrates in that they are living representatives of the first vertebrates to leave an aquatic environment. Thus,

information about the reproductive endocrinology of amphibians is critical to understand the evolution of vertebrate reproduction.

Reproductive Endocrinology of Amphibians

Amphibians from the temperate zone commonly exhibit seasonal patterns of reproductive activity (see Lofts, 1974; Salthe and Mecham, 1974 for extensive reviews). Seasonal reproduction in male amphibians involves marked seasonal changes in testicular development, plasma androgen concentrations, plasma and pituitary gonadotropin levels, reproductive behavior, and secondary sexual characteristics (Miller and Robbins, 1954; Iwasawa and Asai, 1959; Bassu and Mondal, 1961; Lofts, 1964, 1974; Joly, 1971; Lofts et al., 1974; Salthe and Mecham, 1974; Tso and Lofts, 1977; Tanaka and Iwasawa, 1979; Specker and Moore, 1980; Licht et al., 1983). As reviewed below, the neuroendocrine control of seasonal changes in reproductive condition of amphibians appears to be very similar to that of other vertebrates.

As in mammals, amphibians possess two distinct gonadotropins that differentially regulate testicular steroidogenesis and spermatogenesis (reviewed by Licht et al., 1977; Callard et al., 1978). Two fractions showing chemical similarities to mammalian LH and FSH have been

isolated from pituitary glands of bullfrogs (Rana catesbeiana) and tiger salamanders (Ambystoma tigrinum) (Licht and Papkoff, 1974; Licht et al., 1975). In bullfrogs, presumptive LH stimulates steroid synthesis and secretion from testes in vivo and in vitro; presumptive FSH accelerates spermatogenesis in bullfrog testes in vivo (Muller, 1976, 1977a and b). Thus, testicular functions are regulated in amphibians by the pituitary gland.

The interactions of hormones within the hypothalamic-pituitary-gonad axis of amphibians is also similar to those of other vertebrates. Plasma androgens exert a negative feedback influence on plasma gonadotropin levels in amphibians. Castration results in hypertrophy of pituitary gonadotrophs or elevated plasma gonadotropin levels (Zahl, 1937; Copeland, 1943; Pisano, 1948; Rastogi and Chieffi, 1970; Cordier, 1953a, b; van Oordt, 1961a, b; Doerr-Schott, 1963; Licht and McCreery, 1983). In addition, castration results in hypertrophy of neurosecretory cells in specific areas of the hypothalamus (Dierickx et al., 1972). Androgen replacement reverses these effects of castration, and can produce antigonadal effects in intact animals (van Oordt, 1961a, b; Licht and McCreery, 1983). Thus, in amphibians as in mammals, the feedback effects of androgens on plasma gonadotropin levels appear to occur both at the level of the pituitary

gland and at the level of the hypothalamus.

It is well-documented for amphibians that the pituitary-gonad axis is under the stimulatory control of the hypothalamus. Amphibians possess a well-developed hypothalamo-hypophysial portal vascular system, and the integrity of this vascular system is required for maintenance of spermatogenesis (Green, 1966). For example, placement of a barrier between the pituitary and hypothalamus, or transplantation of the pituitary to an ectopic location, results in the diminution of gonadotropin-dependent stages of spermatogenesis (Jorgensen and Larsen, 1963; Dierickx, 1964, 1966). In addition, lesion studies have indicated that there are two gonadotropin control centers in the hypothalamus of amphibians (Dierickx, 1966, 1967a, b). One center, the nucleus infundibularis ventralis, is homologous to the mammalian medial basal hypothalamus, an important gonadotropin control center in mammals (Holmes and Ball, 1974). The other amphibian gonadotropin control center is the dorsal part of the preoptic area, which is also involved in gonadotropin secretion in mammals.

The stimulatory control of the pituitary-gonad axis by the amphibian hypothalamus appears to be mediated by LHRH. Extracts of amphibian hypothalamus stimulate gonadotropin release from amphibian and mammalian pituitaries (King and Millar, 1980; Thornton and

Geschwind, 1974), and contain a factor which is immunologically and chromatographically indistinguishable from mammalian LHRH (Deery, 1974; Alpert et al., 1976; King and Millar, 1980; Eiden and Eskay, 1980; Eiden et al., 1982; Branton et al., 1982). Administration of synthetic mammalian LHRH to various amphibian species elevates plasma concentrations of radioimmunoassayable LH and FSH (Daniels and Licht, 1980; McCreery et al., 1982; Licht et al., 1983; Licht and McCreery, 1983) and stimulates vitellogenesis, ovulation, spermatogenesis, spermiation, and steroidogenesis (Thornton and Geschwind, 1974; Licht, 1974; Vellano et al., 1974; Mazzi, 1978; Easley et al., 1979; Moore et al., 1982; Licht et al., 1983).

The anatomical distribution of immunoreactive (ir) LHRH in the amphibian brain also supports the contention that the hypothalamic control of the pituitary-gonad axis is mediated by LHRH. In general, irLHRH-containing cells are found in the septo-preoptic area, and fiber tracts project from the preoptic area to the median eminence where they terminate on capillaries of the hypothalamo-hypophysial portal vascular system (Alpert et al., 1976; Doerr-Schott and Dubois, 1976; Nozaki and Kobayashi, 1979; Kubo et al., 1979). Thus, the LHRH neuronal system of amphibians is a diffuse neuroendocrine system with cells distributed widely throughout the telencephalon and

ventral preoptic area and contained within several distinct nuclei (Doerr-Schott and Dubois, 1976). This diffuse pattern of irLHRH distribution also occurs in mammals (Bennett-Clarke and Joseph, 1982).

In summary, LHRH is widely accepted to mediate the hypothalamic control of the pituitary-gonad axis of amphibians. A factor which is immunologically and chromatographically indistinguishable from mammalian LHRH is localized in areas of the amphibian brain invoved with reproduction, and it can stimulate the release of gonadotropins from amphibian and mammalian pituitaries.

Reproductive Cycle of Rough-Skinned Newts

Rough-skinned newts exhibit marked seasonal changes in reproductive activity (Covell, 1923; Pimentel, 1952, 1960; Miller and Robbins, 1954; Oliver and McCurdy, 1974; Specker and Moore, 1980). In the Benton County, Oregon area, newts begin migrating to breeding ponds during rainy periods in November and December (Pimentel, 1952, 1960). Males migrating to ponds at this time develop secondary sexual characteristics typical of breeding males (e.g., smooth skin and enlarged tail fin), although true breeding probably does not occur until January or February (Pimentel, 1952; Zoeller, unpublished observations). Male newts continue to migrate to breeding ponds during rainy

periods throughout the winter months, and the densities of males in breeding ponds become very high (Miller and Robbins, 1954). The period of most intense breeding for rough-skinned newts occurs in March and April in the Benton County area. In late April and May, most newts leave the breeding pond to take up a terrestrial mode of life (Pimentel, 1952; Oliver and McCurdy, 1974).

Specker and Moore (1980) demonstrated that testicular development and plasma concentrations of gonadal steroids fluctuate in relation to the migratory and behavioral patterns discussed above. Specifically, testis weight is highest, and plasma androgen levels are low, in August before migrations begin. During the fall, plasma androgen concentrations rise and the testes contain predominantly mature sperm and evacuated lobules. As winter progresses, plasma androgen concentrations remain elevated (above 30 ng/ml) and the testes become increasingly glandular in the regions of evacuated lobules. Toward the end of the breeding season (March and April), the vasa deferentia of male newts are filled with sperm (Zoeller, unpublished observation), but evacuated lobules in the testes regress and plasma androgen concentrations drop from year-high levels in March to low levels in May. Thus, the breeding season comes to an abrupt end in early May.

Testicular recrudescence begins in the spring (Specker and Moore, 1980). During the summer,

spermatogenesis continues and the testes reach their maximum weight in August. Plasma androgen concentrations are low during this period, and male newts do not possess any conspicuous secondary sexual characteristics.

The neuroendocrine control of these seasonal changes in male newts appears to be similar to that of other amphibians: the predominant form of LHRH in the newt brain is chromatographically indistinguishable from mammalian LHRH (Nancy Sherwood and F.L. Moore, unpublished data), and injection of synthetic mammalian LHRH into male newts can elevate plasma androgen concentrations and stimulate masculine sexual behavior (Moore et al., 1982). Thus, LHRH may be directly involved in the control of seasonal changes in testicular development, plasma androgen concentrations, and reproductive behavior of male newts.

The Research Problem

The experiments reviewed above demonstrate that seasonal reproductive cycles of many vertebrates are controlled by seasonal changes in pituitary function. These changes in pituitary function are controlled by seasonal changes in LHRH secretion. One hypothesis proposed to explain how the endocrine system is controlled to produce seasonal cycles is the so-called "gonadostat"

hypothesis (reviewed by Goodman and Karsch, 1981). This hypothesis proposes that seasonal changes in LHRH secretion are dependent upon seasonal changes in sensitivity of the hypothalamus to the negative feedback influence of gonadal hormones. The experiments described in this dissertation were designed to test the gonadostat hypothesis in male rough-skinned newts.

For any seasonally breeding vertebrate, three predictions can be made according to the gonadostat hypothesis. The first prediction is that there are seasonal changes in LHRH secretion that are correlated with seasonal changes in gonadal function and reproductive activity. The second is that gonadal hormones supress LHRH release (negative feedback), and third, that the sensitivity of the hypothalamus to the negative feedback effect of plasma steroids is greatest at the end of the breeding season.

These three predictions were tested in male newts by two experimental approaches. The first approach was to determine whether seasonal changes in LHRH secretion occur in male newts, and whether these changes are correlated with seasonal changes in gonadal function and reproductive activity. This study involved measuring LHRH concentrations in specific brain areas of male newts throughout the reproductive cycle and correlating these changes with changes in plasma androgen and corticosterone

levels. Luteinizing hormone-releasing hormone was measured in microdissected areas containing cell bodies (preoptic area), fiber tracts (rostral hypothalamus), nerve terminals (infundibulum), and an area devoid of LHRH as a control (dorsal habenulum) (see Appendix A for details). By comparing seasonal changes in the concentration of LHRH in these brain areas with seasonal changes in plasma steroid concentrations and other reproductive characteristics, predictions can be made about seasonal changes in LHRH secretion and the endocrine consequences of this secretion.

The second experimental approach was to determine whether gonadal hormones can exert a negative feedback effect on LHRH secretion and, if so, whether the sensitivity of the hypothalamus to negative feedback is greatest at the end of the breeding season. This study involved evaluating the effect of castration and gonadal hormone (testosterone) replacement on LHRH concentrations in specific brain regions of male newts collected at the beginning and end of the breeding season. Luteinizing hormone-releasing hormone was measured in microdissected brain areas correlated with changes in plasma steroid concentrations precipitated by castration and testosterone replacement. If plasma hormones exert a negative feedback influence on hypohalamic LHRH secretion, LHRH concentrations should decline in brain tissue in response

to castration. Gonadal hormone replacement should restore LHRH levels. If the sensitivity of the hypothalamus to negative feedback changes throughout the reproductive cycle, then the effect of castration and hormone replacement on LHRH levels should change throughout the reproductive cycle.

CHAPTER II

SEASONAL CHANGES IN HYPOTHALAMIC LHRH

INTRODUCTION

Seasonal changes in reproductive condition of male rough-skinned newts are marked, as in other amphibians, by changes in testicular function, plasma androgen levels, reproductive behavior, and secondary sexual characteristics (Pimentel, 1952; Specker and Moore, 1980; Salthe and Mecham, 1974). The neuroendocrine control of reproduction in male newts, and of amphibians in general, is similar to that of other vertebrates. The pituitary-gonad axis is controlled by the hypothalamus and luteinizing hormone-releasing hormone (LHRH) appears to mediate this control (Green, 1966; Jorgensen and Larsen, 1963; Dierickx, 1964 and 1966; Thornton and Geschwind, 1974; Daniels and Licht, 1980; King and Millar, 1980).

Administration of LHRH elevates plasma gonadotropin levels and stimulates gonadal function in amphibians; therefore, seasonal changes in plasma gonadotropin levels and gonadal function may reflect seasonal changes in endogenous LHRH secretion (Thornton and Geschwind, 1974; Takikawa et al., 1981; Licht et al., 1983). In the present study, we investigated this possibility in male newts by measuring hypothalamic LHRH concentrations and plasma androgen levels throughout the reproductive cycle.

MATERIALS AND METHODS

Animals Adult male rough-skinned newts, Taricha granulosa, were collected from Fathead Lake in Benton County, Oregon, at 4-6 wk intervals from March 6, 1982 to June 23, 1983. Only animals found in the pond were collected for use in this study, and no attempt was made to select animals with particular characteristics. This same population had been studied previously (Specker and Moore, 1980). Newts were transported to the lab in densities of no greater than 12 animals per 20-L of pond water. All animals were sacrificed within two hours of capture (between 12.00 and 14.00 h).

Processing of Brain Tissue Animals were decapitated and brain tissues immediately heated to 60-65^{o}C in a water bath for 10-13 min to decrease endogenous peptidase activity and increase the rigidity of brain tissue. Brains were then stored at 0^{o}C until dissection (within 10h), after which they were frozen to cryostat chucks on dry ice using Tissue Tek O.C.T. compound. Brains were sectioned in transverse plane (200 µm) in a cryostat (-11 to -15^{o}C), and the sections were frozen to a microscope slide and stored at approximately -80^{o}C in a plastic-sealed slide box until microdissection (within 24h).

Four areas of the brain were isolated (Fig. II.1). The identity of these areas was confirmed histologically, based on descriptions of the salamander brain by Herrick (1948). The infundibulum and rostral hypothalamus were cut from the base of the brain; the ventral preoptic area and dorsal habenulum were removed using a modified stainless steel hypodermic needle, 275 µm i.d. (Palkovits and Brownstein, 1982). After punching, tissues were immediately transferred to a polyethylene culture tube containing 200 µl ice-cold 1N acetic acid. The characteristics of microdissected brain regions are shown in table II.1.

Tissues were homogenized by sonication at 20 KHz for 20 sec., after which a 25 µl aliquot was removed for protein determination (Hartree, 1972). The remaining extract was centrifuged at 21,000 x g for 20 min, 150 µl of the supernatant was lyophylized and stored desiccated at -20^{o}C until assayed for LHRH by RIA. A second extraction of brain tissue with 200 µl 1N acetic acid yielded only 1.7±1.9% additional irLHRH. Therefore, only single extractions were performed for all samples.

LHRH Radioimmunoassay Each lyophylized sample was reconstituted in 200 µl assay diluent (0.02M sodium borate decahydrate, 0.004% thimerosol, 0.25% bovine gamma-globulin, pH 8.4). The antiserum (R#422 supplied by Dr. A. Arimura) was diluted to 1:12,000 in a final assay

volume of 400 µl. Assay standards were prepared in triplicate using synthetic mammalian LHRH (Beckman) at concentrations ranging from 6 to 200 pg/assay tube. Standards and samples were preincubated with antiserum at 4°C for 24 h before the addition of label (3,000 to 4,000 dpm ^{125}-I LHRH from New England Nuclear). Bound and free hormone were separated, after 72 h incubation at 4°C, with 400 µl 20% polyethylene glycol; the tubes were incubated for 15 min at 0°C and centrifuged at 2,500 x g for 20 min. The supernatant was immediately decanted and the tubes drained before counting the pellet in a Packard autogamma counter.

Serial, five-point dilutions of acid-extracted material from newt brain exhibited parallelism with the standard curve in two separate assays. In addition, the majority of immunoreactive LHRH extracted from newt brain is indistinguishable from synthetic mammalian LHRH on reverse-phase HPLC (Nancy Sherwood and F.L. Moore, unpublished data). The intraassay coefficient of variation for this assay was 6.23%; the interassay coefficient of variation was 20%. All samples from a specific brain area were analyzed, in duplicate, in a single assay. The sensitivity of the assay was 4.0 pg/assay tube.

Blood Collection and Steroid Radioimmunoassay

Trunk blood was collected in heparinized culture tubes

after decapitation, and plasma was stored at -20°C until assayed. Samples were extracted with 100% ethanol according to procedures of Gwosdow-Cohen et al. (1982). After centrifugation, separate aliquots of the supernatant were removed for androgen and corticosterone radioimmunoassays. The efficiency of this extraction procedure, as determined by recovery of steroid tracer, was 97% for androgen and 87% for corticosterone. The radioimmunoassay procedure and validation for plasma androgen of male newts has been described previously (Moore and Muller, 1977; Moore, Specker and Swanson, 1979). Because the antiserum (#250 anti-testosterone-11-BSA supplied by Dr. G.D. Niswender) reacts appreciably with T and DHT (Ismail et al., 1972), measurements are referred to as total androgens. The sensitivity of this assay was 0.6 ng/ml (5 pg/assay tube). The intraassay coefficient of variation was 7.63%; all samples were analyzed within a single assay.

Corticosterone was measured by RIA on non-chromatographed plasma using antiserum #377 (anti-corticosterone-3-BSA supplied by Dr. G.D. Niswender). Cross-reaction with cortisol, cortisone, progesterone, testosterone and 17B-estradiol is less than 1% (Gwosdow-Cohen et al., 1982). The assay was performed by dissolving the dried ethanol extract in 200 µl antiserum (1:3,000) and incubating with 100 ul ^{3}-H

corticosterone (Amersham; 6,000 dpm). Dextran-coated charcoal was used to separate bound from free hormone. The limit of sensitivity for the assay was 0.15 ng/ml or 10 pg/assay tube. All samples were analyzed within a single assay.

LHRH Injection On April 7, 1981, locally-collected male rough-skinned newts were injected with amphibian Ringer's, 1 ug, or 10 ug of LHRH, or 1ug or 10 ug LHRH antagonist (IBR No. 20061 from Dr. Karl Folkers). The procedure for intracerebroventricular (i.c.v.) injection of LHRH has been described previously (Moore et al., 1982). The tip of a microsyringe was inserted into the third ventricle through the parietal bone posterior to the epiphysis. A volume of 1 µl was injected during a 3- to 5-second period. Newts were not anesthetized during this procedure. Blood was collected 2 h after injection and processed for measurement of plasma androgen concentration by RIA as described above.

Statistical Analyses One-way analysis of variance (ANOVA) was performed, after square-root transformation, to examine differences among mean hormone levels throughout the year, and in response to LHRH injections. Bonferroni's t-test was used to test for differences between specific group means after ANOVA was run. Correlations were tested by linear regression analysis after $\log_{10}$ transformation.

RESULTS

The concentration of LHRH in the brain of male rough-skinned newts varied widely throughout the reproductive cycle (Fig. II.2). Some of these changes were associated with plasma androgen concentration, which was greatest during the breeding season and lowest during the summer (Fig. II.3).

LHRH In general, the pattern of seasonal changes in irLHRH concentration observed in the infundibulum was very similar to that observed in the rostral hypothalamus. LHRH immunoreactivity was not detected in the infundibulum or rostral hypothalamus of animals collected in March or April. The concentration of infundibular irLHRH was significantly greater in animals collected in June and February (4.5±0.4 ng/mg protein, respectively) than in animals collected in September through January (range of 3.3±0.3 to 3.6±0.3 ng/mg protein). The concentration of irLHRH in the rostral hypothalamus was also greatest in June (4.3±0.6 ng/mg protein) and February (2.4±0.7 ng/mg protein). However, there was an additional peak in irLHRH in the rostral hypothalamus in September (2.7±0.7 ng/mg protein). The seasonal pattern of irLHRH in the infundibulum was positively correlated with that observed in the rostral

hypothalamus, although the amount of irLHRH in the infundibulum was always greater than that in the rostral hypothalamus.

The seasonal pattern of irLHRH in the preoptic area was different from the pattern observed in the two caudal areas of the hypothalamus. LHRH immunoreactivity was not detected in the preoptic area of animals collected in March or April, but peak levels were observed in animals collected in June. Although this pattern is similar to that in the two caudal brain areas, irLHRH levels in the preoptic area remained at peak levels throughout the summer (range of 5.4±1.2 to 8.0±0.6 ng/mg protein) and were significantly lower in October (1.8±0.3 ng/mg protein) than in December or January (4.0±0.9 and 3.9±1.0 ng/mg protein). Finally, the concentration of irLHRH in the preoptic area of animals collected in February (8.0±1.7 ng/mg protein) was at a peak, similar to that observed in other brain areas of February- collected animals. Thus, the seasonal pattern of irLHRH concentrations in the preoptic area was similar to the pattern observed in the infundibulum and rostral hypothalamus from December to June, but was very different from the pattern observed in the caudal brain areas for the remainder of the year.

Plasma Hormones Androgen concentrations were lowest in plasma from animals collected in May and June

(2.1±1.1 and 3.7±1.3 ng/ml, respectively) and highest in plasma from animals collected in October through March (range of 26.2±8.3 to 38.1±3.8 ng/ml; Figure II.3). Animals collected in April, July and September had plasma androgen concentrations intermediate between the highest and lowest levels (less than 7.7±3.5 ng/ml).

Corticosterone concentrations were lowest in plasma from animals collected in October through March (range of 1.1±0.2 to 1.5±0.3 ng/ml) and highest in plasma from animals collected in June and July (5.6±2.3 and 5.5±2.5 ng/ml, respectively). Animals collected in April, May and September had plasma corticosterone levels intermediate between the highest and lowest values (2.3±0.5 to 2.9±2.2 ng/ml), although these levels were not significantly different from either the highest or lowest values.

Correlations Among Plasma Hormones and irLHRH There was a significant negative correlation between plasma androgen and corticosterone concentrations when comparing group means for all collection dates ($r=-0.79$; $p<0.01$).

There was a positive correlation between the concentration of corticosterone in plasma and the content of irLHRH in the preoptic area when comparing means for those groups with corticosterone levels above 2.0 ng/ml

(March through October; $r=0.81$; $p<0.05$). In addition, there was a significant positive correlation between the concentration of irLHRH in the infundibulum and that in the rostral hypothalamus ($r=0.79$, $p<0.01$). No other significant correlation was detected between irLHRH and plasma hormones, or between irLHRH levels among various brain regions.

LHRH Injections 10 ug LHRH significantly elevated plasma androgen concentrations (Table II.2). Plasma androgen concentrations tended to be elevated in animals injected with 1 ug LHRH, but this was not statistically significant. There were no significant differences among the other treatment groups.

DISCUSSION

The results of this study demonstrate that irLHRH concentrations in the brain of male rough-skinned newts fluctuates throughout the reproductive cycle (Fig. II.2). These data support the conclusion that seasonal changes in LHRH secretion are an essential component of the neuroendocrine control of the reproductive cycle of male newts. The possibility that this conclusion is true for other amphibians as well is supported by the observation

that hyopthalamic irLHRH levels measured at four times of year in female Xenopus laevis are positively correlated with ovarian weight (King and Millar, 1979).

The most striking feature of the seasonal pattern of irLHRH observed in the present study was that irLHRH declined in all brain areas from highest values in February to undetectable levels in March and April (Fig. II.2). Several lines of evidence indicate that this decline in irLHRH reflects a decline in both the synthesis and release of LHRH. The strongest evidence for this interpretation is that plasma androgen concentrations declined from over 30 ng/ml in February to less than 10 ng/ml in April (Fig. II.3). This drop in plasma androgen concentration was not caused by decreased sensitivity of the pituitary-gonad axis to LHRH stimulation, because injections of LHRH elevated plasma androgen concentrations equally well in April- and November-collected newts (Table II.2 and Moore et al., 1982). Thus, the decline in plasma androgens in March and April is due to a decline in release of LHRH, resulting in reduced stimulation of the pituitary-gonad axis. The hypothesis that LHRH synthesis is also low during March and April is supported by the observation that irLHRH was undetectable in the area of cell bodies (POA, Fig. II.2). Because these changes in irLHRH concentrations occur in animals just prior to the end of the breeding season, these results indicate that a

drop in both synthesis and release of LHRH in male newts is the endocrine event triggering the yearly end of reproductive activity.

Seasonal changes in LHRH secretion also may be an essential component of the neuroendocrine control of seasonal reproduction in vertebrates other than amphibians. Seasonal changes in gonadotopin secretion have been described extensively in birds and mammals (e.g., Matt, 1980; Lincoln and Short, 1980). In addition, several studies have shown that the sensitivity of the pituitary to exogenous LHRH does not change throughout the reproductive cycle (Pickard and Silverman, 1979), indicating that changes in gonadotropin levels are the result of changes in LHRH secretion.

Several other aspects of the present study provide additional evidence that LHRH controls reproductive activity of male rough-skinned newts. For example, the concentrations of irLHRH in the infundibulum and rostral hypothalamus of male newts were elevated in May and June, and remained high, but below peak levels, throughout the summer and fall (Fig. II.2). This period of elevated irLHRH in the infundibulum corresponds with testicular recrudescence; the percentage of mature sperm in the testis increases from less than 10% in May to greater than 60% by September, and testicular weight is highest in August (Specker and Moore, 1980). Thus, the resumption of

testicular development in the spring appears to be associated with an increase in LHRH secretion.

An unexpected finding was that the concentration of irLHRH in the infundibulum and rostral hypothalamus, but not in the preoptic area, were positively correlated throughout the reproductive cycle (Fig. II.2). Specifically, irLHRH levels in the POA remained elevated during the summer and dropped significantly only in October, when irLHRH levels did not change in the infundibulum. These results indicate that the concentration of LHRH in different parts of the LHRH neuronal system of male newts is independently controlled. This independent control may reflect differential regulation of LHRH within distinct cellular compartments (e.g., cell bodies versus terminals), or differential regulation of LHRH among functionally separate neuronal systems.

In conclusion, the results of this study demonstrate that male rough-skinned newts exhibit seasonal changes in irLHRH in specific areas of the hypothalamus. The hypothesis that seasonal changes in hypothalamic LHRH reflect changes in LHRH release is supported by the observation that the sensitivity of the pituitary-gonad axis to exogenous LHRH does not change at a time of year when LHRH is undetectable in the hypothalamus and plasma androgens are declining. There are no reports for

amphibians regarding the physiological mechanisms that regulate seasonal changes in LHRH secretion. In seasonally breeding mammals, LHRH stimulation of the pituitary-gonad axis is apparently influenced by the negative feedback effects of gonadal steroids (see reviews by Goodman and Karsch, 1981; Turek and Ellis, 1981). Studies described in the following chapter investigate whether plasma steroids can influence LHRH concentrations in male rough-skinned newts, and if so, whether there are seasonal changes in the sensitivity of the hypothalamus to the effects of these steroids.

Table II.1. Characteristics of Brain Regions Microdissected for LHRH Determination.

Region dissected	Punches/brain[a]	Brains pooled for RIA[b]	Protein content (µg ± SEM)
Infundibulum	3	1	5.40 ± 0.21
Rostral Hyp.	2	2	3.39 ± 0.13
Dorsal Hab.	10	2	1.86 ± 0.07
Ventral POA	5	2	1.22 ± 0.06

a. The number of transverse sections from which brain areas were isolated.
b. The number of individual areas, from separate animals, pooled for RIA.

Table II.2. The Effect of LHRH and LHRH Antagonist on Plasma Androgen Concentrations of Male Rough-Skinned Newts.

Untreated	Saline	1µgLHRH	10µgLHRH	1µg#60	10µg#60
2.50 (7)	2.14 (5)	4.63 (5)	12.68[a] (5)	2.22 (3)	0.61 (4)

Numbers represent mean plasma androgen values for the group of (n) animals. The mean standard error, derived from the 1-way ANOVA table, was 0.323.
a. Value is significantly different ($p<0.05$) from the mean value observe in the untreated group.

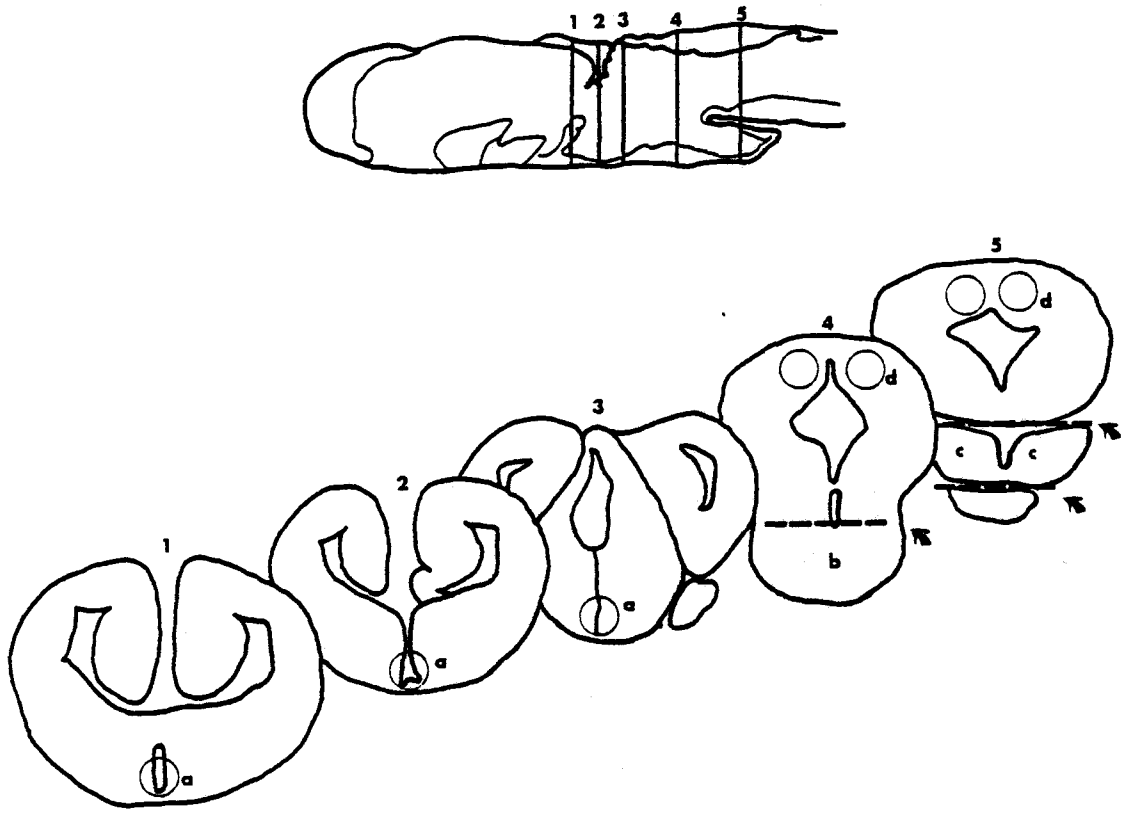

Figure II.1. Brain areas of male rough-skinned newts microdissected for measurement of LHRH. Diagrammatic representation of newt brain in mid-sagittal plane (above), with representative sections (numbered bars and corresponding cross sections below) from which brain areas were isolated. Circles represent the size of the punch needle in the a) ventral preoptic area, b) rostral hypothalamus, c) infundibulum, and d) dorsal habenulum. See text for details of microdissection procedure.

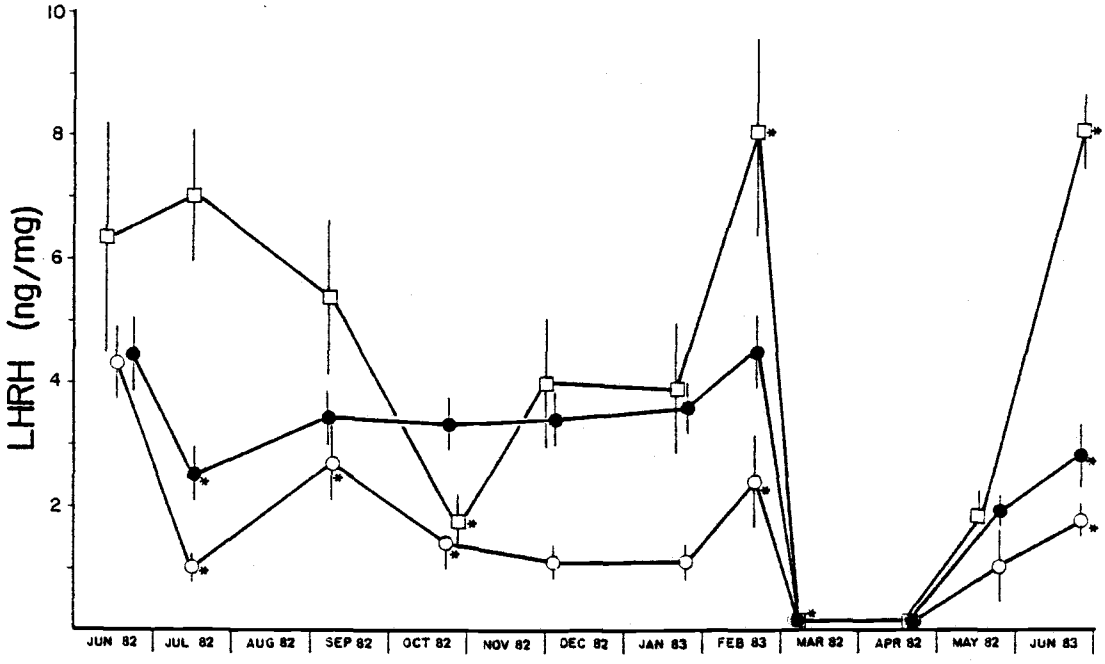

Figure II.2. Seasonal changes in LHRH concentration in microdissected brain areas of male rough-skinned newts. Newts were collected from a single population over a period ranging from March, 1982 to June, 1983 (abscissa). Points represent mean (±SE) LHRH concentration expressed as ng LHRH/mg protein (ordinate) in the infundibulum (●), rostral hypothalamus (○), and ventral preoptic area (□). Means for samples from the infundibulum are plotted along the abscissa according to date of collection. Some points from other brain areas are offset along the abscissa for graphic representation of the standard errors.
*Represents a significant difference ($p<0.05$) in LHRH concentration when compared to the previous mean within the same brain area.

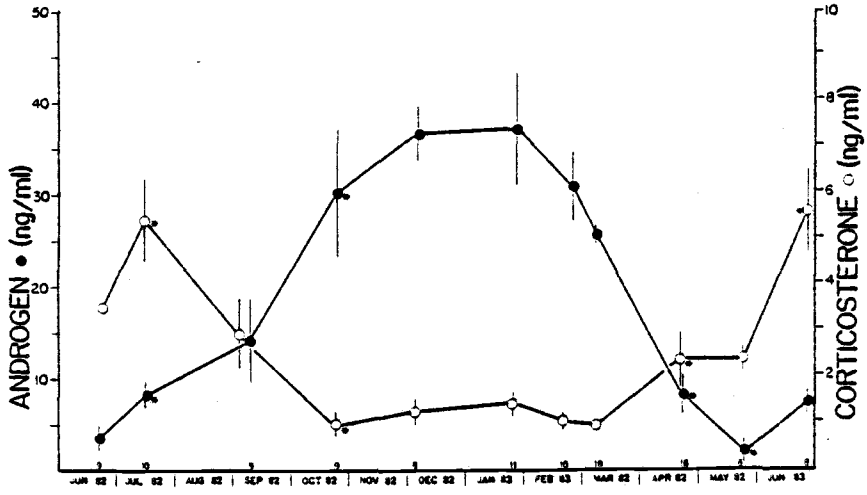

Figure II.3. Seasonal changes in plasma androgen and corticosterone concentrations of male rough-skinned newts. Newts were collected over a period ranging from March, 1982 to June, 1983 (abscissa). Points represent mean (±SE) androgen (●) and corticosterone (○) concentrations expressed as ng/ml plasma (ordinate). Plasma androgen concentrations are plotted along the abscissa according to date of collection. Some points representing plasma corticosterone are offset for graphic representation of standard errors. The number of animals for each collection from which plasma hormones and brain LHRH were obtained are plotted along the abscissa. *Represents a significant difference ($p<0.05$) in plasma steroid concentration when compared to the previous sample.

CHAPTER III

SEASONAL CHANGES IN HYPOTHALAMIC SENSITIVITY TO ANDROGEN FEEDBACK

INTRODUCTION

The rough-skinned newt (Taricha granulosa) is a seasonally breeding amphibian exhibiting marked changes in gonadal function, plasma hormone concentrations, reproductive behaviors, and secondary sexual characteristics (Pimentel, 1952; Oliver and McCurdy, 1974; Specker and Moore, 1980). Changes in reproductive condition of male newts are typical of those seen in other amphibians, and other vertebrates in general, and appear to be controlled by hypothalamic luteinizing hormone-releasing hormone (LHRH) (Joly, 1971; Lofts, 1974; Tanaka and Iwasawa, 1979; Licht et al., 1983; Lincoln and Short, 1980). Extracts of hypothalamus from male rough-skinned newts possess a factor which is indistinguishable from mammalian LHRH using radioimmunoassay and reverse-phase HPLC systems (Sherwood and Moore, unpublished), and mammalian LHRH can elevate plasma androgens in male newts (Moore et al., 1982). In addition, the amount of immunoreactive LHRH in the hypothalamus changes in relation to the reproductive cycle of male newts (Chapter II).

The mechanism(s) controlling seasonal changes in LHRH

activity in male rough-skinned newts is unknown. In seasonally breeding mammals, seasonal changes in the negative-feedback effect of gonadal hormones may play a central role in the neuroendocrine control of seasonal changes in reproductive condition (Goodman and Karsch, 1981; Turek and Ellis, 1981). For example, in the golden hamster, changes in photoperiod can alter the sensitivity of the hypothalamus to negative feedback by plasma steroids (e.g., Pickard and Silverman, 1979). The present experiments were conducted to determine whether plasma steroids can influence the concentration of LHRH in several regions of the brain of male rough-skinned newts, and if so, to determine whether the sensitivity of hypothalamic LHRH to the feedback influence of plasma androgens changes during the breeding season.

MATERIALS AND METHODS

Animals Adult male rough-skinned newts, Taricha granulosa, were collected locally at the beginning (October 27, 1982) and end (May 20, 1982) of the breeding season. Newts were maintained in an environmental chamber: 10 males per 15-liter tank, 12L:12D photocycle at 10-12^{o}C. Chopped beef heart and fresh water were provided every other day. Care was taken to avoid unnecessarily stressing the animals.

Procedure Males were acclimated to laboratory conditions for 10 days before assignment to the following experimental groups: intact males (untreated), sham-castrated males, castrated males implanted with an empty Silastic capsule ("unimplanted castrates"), and castrated males implanted with a testosterone-filled Silastic capsule. Hypothalamic LHRH, plasma androgen, and plasma corticostrone concentrations were measured at 5 and 30 days after treatment.

On the day of surgery (Day 0), newts that were to be sham-castrated or castrated were anesthetized by immersion in 0.1% ethyl m-aminobenzoate methanesulfonate (from Sigma). Testes were removed through a right lateral incision and Silastic capsules placed in the abdominal cavity prior to closure with a 9-mm autoclip (Clay Adams). Capsules, either empty or containing approximately 3 mg testosterone (Sigma), were prepared by cutting 5-mm segments of Silastic tubing (1.47 x 1.96 mm; i.d. x o.d.; from Dow Corning) and sealing the ends with Dow Medical Elastomer (Moore, 1981). To control infection, all animals were immersed in a solution containing penicillin, streptomycin and chloramphenocol for one hour on the day of surgery.

Processing of Brain Tissue On days 5 and 30, animals were decapitated and brains heated in a 60-65^{o}C water bath for 10-13 min to decrease

endogenous peptidase activity and increase the rigidity of the tissue. Tissues were stored at 0°C until dissection (less than 24 h). Brains were frozen to cryostat chucks using dry ice, sectioned at -11 to -15°C and stored at -75 to -78°C until microdissection (within 6 weeks). The region of the brain between the accessory olfactory bulb and the anterior commissure was sectioned at 300 µm; the region between the anterior commissure through the diencephalon was sectioned at 200 µm.

Five areas of the brain were isolated (Fig. III.1). The identity of each area was confirmed histologically, based on the description by Herrick (1948). The infundibulum and rostral hypothalamus were cut from the base of the brain sections; the medial septal area, ventral preoptic area and dorsal habenulum were removed using a modified hypodermic needle (275 µm i.d.) as described by Palkovits and Brownstein (1982). The characteristics of microdissected brain regions are shown in Table III.1.

Tissues were placed in ice-cold 1N acetic acid, homogenized by sonication at 20 KHz for 20 sec., and a 25 µl aliquot was removed for protein determination (Hartree et al., 1972). The remaining extract was centrifuged at 21,000 x g for 20 min, and 150 µl of the supernatant was lyophylized and stored desiccated at -20°C until

assayed for LHRH by RIA. A second extraction of brain tissue with 200 µl 1N acetic acid yielded only 1.7±1.9% additional irLHRH. Therefore, only single extractions were performed for all samples.

LHRH Radioimmunoassay Samples were reconstituted in 200 µl assay diluent (0.02M sodium borate decahydrate, 0.004% thimerosol, 0.25% bovine gamma- globulin, pH 8.4). The antiserum (R#422 supplied by A. Arimura) was diluted to 1:12,000 in a final assay volume of 400 µl. Assay standards were prepared in triplicate using synthetic mammalian LHRH (Beckman) at concentrations ranging from 3 to 200 pg/assay tube. Standards and samples were preincubated with antiserum at 4°C for 24 h before the addition of label (3,000 to 4,000 dpm ^{125}I-LHRH from New England Nuclear). Bound and free hormone were separated, after 72 h incubation at 4°C, with 400 µl 20% polyethylene glycol; the tubes were incubated for 15 min at 0°C and centrifuged at 2,500 x g for 20 min. The supernatant was immediately decanted and tubes drained before counting the pellet in a Packard autogamma counter.

Serial, five-point dilutions of acid-extracted material from newt brain exhibited parallelism with the standard curve in two separate assays. In addition, the majority of immunoreactive LHRH extracted from newt brain is indistinguishable from synthetic mammalian LHRH on reverse-phase HPLC (Nancy Sherwood and F.L. Moore,

unpublished data). The intraassay coefficient of variation for this assay was 6.2%; the interassay coefficient of variation was 20.0%. All samples from a specific brain area were analyzed, in duplicate, in a single assay. The sensitivity of the assay was 4.0 pg/assay tube.

Blood Collection and Steroid Radioimmunoassay

Trunk blood was collected in heparinized culture tubes after decapitation, and plasma was stored at -20°C until assayed. Samples were extracted with 100% ethanol using the procedures of Gwosdow-Cohen et al. (1982). After centrifugation, aliquots of the supernatant were removed for use in the androgen and corticosterone assays. The efficiency of this extraction procedure, determined by recovery of steroid tracer, was 97% for androgen and 87% for corticosterone. The radioimmunoassay procedure and validation for plasma androgen of male newts has been described previously (Moore and Muller, 1977; Moore, Specker and Swanson, 1979). Because the antiserum (#250 anti-testosterone-11-BSA supplied by Dr. G.D. Niswender) reacts appreciably with T and DHT (Ismail et al., 1972), measurements are referred to as total androgens. The sensitivity of this assay was 0.6 ng/ml or 5 pg/assay tube. The intraassay coefficient of variation was 7.63%; all samples from a single season were analyzed within a

single assay.

Corticosterone was measured by RIA on non-chromatographed plasma using antiserum #377 (anti-corticosterone-3-BSA supplied by Dr. G.D. Niswender). Cross-reaction with cortisol, cortisone, progesterone, testosterone and 17B-estradiol is less than 1% (Gwosdow-Cohen et al., 1982). The assay was performed by dissolving the dried ethanol extract in 200 µl antiserum (1:3,000) and incubating with 100 µl ^{3}-H corticosterone (Amersham; 6,000 dpm). Dextran-coated charcoal was used to separate bound from free hormone. The limit of sensitivity for the assay was 0.15 ng/ml or 10 pg/assay tube. All samples from a single season were analyzed within a single assay.

Statistical Analyses Differences among means were detected using a 2-way analysis of variance (ANOVA). Bonferroni's t-test was used to detect individual differences between means.

RESULTS

Castration reduced and testosterone replacement maintained plasma androgen levels of male newts at the beginning and end of the breeding season (Fig. III.3). These endocrine manipulations precipitated changes in irLHRH levels that depended on the area of the brain and

time of year, as well as on the duration of treatment (Fig. III.2).

Spring

Plasma Steroids On day 30, plasma androgen levels in castrated, unimplanted males were significantly lower than those of all other groups. Plasma androgen levels in testosterone (T)-implanted males remained elevated and unchanged throughout the 30 days of the experiment. Plasma androgen levels in intact (untreated) males were not different from those of castrated, unimplanted males on day 5 (Fig. III.3), and both were significantly higher than those observed in sham-operated males.

On day 5, plasma corticosterone levels were significantly higher in unimplanted and T-implanted castrates than in the other two treatment groups. On day 30, however, plasma corticosterone was elevated only in unimplanted castrates; corticosterone levels in T-implanted castrates were no different from those observed in untreated and sham-operated controls. Plasma corticosterone levels in untreated males were identical to those observed in sham-operated males on both days 5 and 30 (Fig. III.3).

Brain irLHRH: Spring On day 5, irLHRH was significantly higher in the infundibulum of intact and

unimplanted, castrated males than in the infundibulum of T-implanted castrates and sham-operated controls. On day 30, however, irLHRH in the infundibulum of intact and unimplanted, castrated males was significantly lower than their respective day 5 levels, so that irLHRH concentrations were similar in all 4 treatment groups.

The concentrations of irLHRH in the rostral hypothalamus were not significantly different among the 4 treatment groups on either days 5 or 30. Similarly, there were no significant differences in irLHRH concentrations in the preoptic area among the 4 treatment groups on day 5. On day 30, however, the concentration of irLHRH in the preoptic area of unimplanted castrates was significantly higher than that of intact and sham-operated males. This resulted from a decline, on day 30, in the concentration of irLHRH in the preoptic area of intact and sham-operated males, but not in the unimplanted castrates.

The concentration of irLHRH in the septal area of T-implanted castrates and sham-operated males was significantly higher than that of intact and castrated, unimplanted males on day 5. These differences did not occur on day 30.

Fall

Plasma Steroids

Plasma androgen concentrations

were high (above 40 ng/ml) in untreated males in the fall. There were no significant differences in androgen levels among the four treatment groups on day 5, although androgen levels tended to be lower in unimplanted castrates. On day 30, however, the concentration of plasma androgens was significantly lower in unimplanted castrates than in any other treatment group. There were also no significant differences in plasma corticosterone concentrations among the treatment groups on either days 5 or 30.

Brain irLHRH: Fall The concentration of irLHRH in the infundibulum of unimplanted castrates was significantly lower than that of all other treatment groups on day 5. On day 30, however, the concentrations of irLHRH in the infundibulum of unimplanted and T-implanted castrates were similar, and both were significantly lower than that observed in intact animals.

In the rostral hypothalamus, the irLHRH concentration of unimplanted castrates was significantly lower than that of intact males on day 5. On day 30, the concentration of irLHRH in the rostral hypothalamus of castrated, unimplanted males was similar to that observed in T-implanted castrates, and irLHRH in both groups were significantly lower than that observed in the sham-operated controls.

The irLHRH concentration in the preoptic area of

intact males was significantly higher than that observed in sham-operated and unimplanted castrates on day 5. The amount of irLHRH in the preoptic area of T-implanted castrates was similar to that of intact males, and was significantly higher than that observed in unimplanted castrates. On day 30, however, the concentration of irLHRH in the preoptic area of intact males was similar to that in sham-operated males and these were significantly higher than the concentration of irLHRH in both unimplanted and T-implanted castrates.

No irLHRH was detected in the septal area of any treatment group in the fall.

Comparison of Fall and Spring Effects

Plasma androgen levels in untreated controls were much higher in the fall than in the spring (40 vs 1 ng/ml). However, plasma androgen levels decreased significantly, in unimplanted castrates from day 5 to day 30 at both times of year.

In the fall, brain irLHRH tended to be higher in intact animals on day 30 than on day 5. In the spring, however, irLHRH tended to be lower on day 30. Also in the spring, irLHRH levels were not different in unimplanted castrates from that in untreated males in any brain area on day 5. Conversely, in the fall, irLHRH was significantly lower in unimplanted castrates than in

untreated controls at both 5 and 30 days after treatment. T-implanted males in the spring had lower concentrations of brain irLHRH than that of intact males and unimplanted castrates on day 5; in the fall, T-implanted males had higher irLHRH concentrations in all brain areas. By day 30, the T-implanted castrates had irLHRH levels that were not different from that in unimplanted castrates, whether this represented a decrease (fall) or an increase (spring) in irLHRH compared to their respective day 5 levels.

DISCUSSION

These experiments were designed to test whether plasma androgens can influence the concentraton of irLHRH in the hypothalamus of male rough-skinned newts, and whether this influence changes throughout the reproductive cycle. The results show that plasma androgens can influence the concentration of LHRH in the brain of male newts, and that this influence changes throughout the reproductive cycle. Castration reduced and testosterone replacement maintained LHRH concentrations in male newts at the beginning of the breeding season, but not in males at the end of the breeding season (Fig. III.2). This observation indicates that plasma androgens can exert a negative-feedback influence on LHRH secretion, and that the sensitivity of the hypothalamus to androgen feedback

changes from spring to fall. However, these results do not support the hypothesis that the reproductive cycle of male newts is controlled by changes in hypothalamic sensitivity to androgen feedack, because LHRH concentrations were not alterred by castration or testosterone replacement in newts at the end of the breeding season.

Hypothalamic irLHRH was reduced by castration and maintained by testosterone replacement in fall-collected newts, indicating that plasma androgens can exert a negative-feedback effect on LHRH. This interpretation is consistent with studies on other amphibians in which castration results in elevated plasma gonadotropin levels, hypertrophied pituitary gonadotrophs and hypothalamic neurosecretory cells, and testosterone replacement reverses these effects (van Oordt, 1961a, b; Dierickx et al., 1972; Licht and McCreery, 1983). The results of the present experiment are also consistent with experiments in mammals in which the negative feedback action of plasma androgens on the hypothalamic-pituitary axis is well-documented (review by Kalra and Kalra, 1983). Specifically, hypothlamic irLHRH is decreased by castration and maintained by androgen replacement in reproductively active mammals (Shin and Howitt, 1976; Campbell and Ramaley, 1978; Pickard and Silverman, 1979; Kalra and Kalra, 1980, 1981, 1983). Thus, the

similarities between results of the present experiment and experiments with other vertebrates support the interpretation that plasma androgens exert a negative feedback influence specifically on LHRH activity in reproductively active male newts.

The observation that castration influenced hypothalamic irLHRH in sexually active, not sexually regressed, male newts is also similar to a study of male golden hamsters (Pickard and Silverman, 1979). In this study, castration alterred hypothalamic irLHRH concentrations in sexually active, not sexually regressed animals. The critical question in the present, and other, studies is whether the failure of castration and testosterone replacement to alter hypothalamic irLHRH concentrations in sexually regressed animals reflects an increase or decrease in hypothalamic sensitivity to negative feedback.

In male rough-skinned newts, the failure of castration and testosterone replacement to alter hypothalamic irLHRH concentrations in sexually regressed animals may reflect a decrease in hypothalamic sensitivity to negative feedback. Male newts captured in the spring for the present experiment exhibited high levels of irLHRH when compared to fall-collected newts (Fig. III.2). Furthermore, irLHRH concentrations declined significantly over the course of the 30-day experiment. Neither

castration nor testosterone replacement influenced the level of hypothalamic irLHRH or the pattern of change over the course of the experiment, indicating that the low levels of androgens present after castration had no effect on LHRH activity in spring-collected males. Field-collected male newts also exhibit elevated levels of irLHRH in the spring, and irLHRH concentrations decline by 50% during this time (Chapter II), indicating that lab-acclimated animals exhibited patterns of LHRH changes that occur in field-collected animals.

The conclusion that plasma androgens exert a negative-feedback influence on hypothalamic LHRH activity is dependent upon measuring changes in LHRH concentrations as a result of castration, and reversing these effects with testosterone replacement. Since no changes in LHRH concentrations were precipitated by castration in sexually regressed animals, there is no direct evidence for a feedback relationship during this time of year.

The present study also demonstrates that there are differences in the effects of castration and testosterone replacement on irLHRH concentrations in different brain regions of male newts. In spring-collected males, irLHRH declined over the course of the 30-day experiment in all brain areas, and castration prevented this decline specifically in the preoptic area (Fig. III.2). Thus, the concentration of irLHRH in the preoptic area of male newts

appears to be regulated independently of that in the infundibulum and rostral hypothalamus. This hypothesis is supported by the observation that seasonal changes in irLHRH concentrations in the preoptic area of male newts differ from those observed in the infundibulum and rostral hypothalamus (Chapter 2).

One hypothesis is that, in male rough-skinned newts, plasma corticosterone is involved in the regulation of irLHRH levels specifically in the preoptic area. Elevated plasma corticosterone is associated with increased preoptic LHRH in castrated males (present study) and in field-collected newts examined in the summer (Chapter 2). Although corticosterone has not been reported previously to influence brain LHRH levels, gonadal steroids have been reported to alter the specific pattern of development of LHRH immunostaining in platyfish (Schreibman et al., 1983), and differences in the effect of castration and testosterone replacement on irLHRH concentrations among various brain regions have been described for mammals (Kalra and Kalra, 1980).

In both spring- and fall-collected newts, the effect of testosterone replacement on hypothalamic irLHRH was seen only on day 5, even though plasma androgen levels did not change over the course of the 30-day experiment. Thus, the duration of testosterone treatment produced different effects on hypothalamic irLHRH concentrations.

Several studies report that Silastic capsules of testosterone can have a potent but sometimes temporary negative feedback effect on plasma gonadotropins of mammals: testosterone implants initially decreased gonadotropin levels, but after several weeks levels rose even though circulating androgens remained constant (Aafjes et al., 1978; Ellis and Turek, 1980). In male newts, testosterone implants initially (5 days) inhibit the ability of a single injection of arginine vasotocin (AVT) to stimulate clasping behavior of male newts, whereas later (30 days) the implants maintained the ability of AVT to stimulate clasping behavior. The mechanism underlying the apparent neuroendocrine compensation to sustained concentrations of plasma androgens in newts, and other species, remains to be determined.

In conclusion, the present results demonstrate that castration and testosterone replacement can influence hypothalamic irLHRH in male rough-skinned newts and that this influence changes throughout the reproductive cycle. However, the mechanisms that alter the sensitivity of hypothalamic irLHRH to gonadal steroids, including the environmental cues initiating these events, remain to be investigated. The present experiments also demonstrate that the effect of castration on irLHRH concentrations is different among the various brain areas investigated.

This observation indicates that different populations of LHRH neurons may be independently regulated in male newts. Independent regulation of distinct populations of LHRH neurons could provide a mechanism by which the LHRH neuronal system subserves different functions in male newts (e.g., sexual behavior and gonadotropin secretion) or where reproduction could be influenced by different cues (e.g., stress and season).

Table III.1. Characteristics of Brain Regions Microdissected for LHRH Determination.

Region dissected	Punches/ brain[a]	Brains pooled for RIA[b]	Protein content (µg ± SEM)
Infundibulum	3	1	7.35 ± 0.19
Rostral Hyp.	2	2	7.83 ± 0.21
Dorsal Hab.	10	2	1.85 ± 0.12
Ventral POA	5	2	2.29 ± 0.10
Septal Area	7	2	2.14 ± 0.14

a. The number of transverse sections from which brain areas were isolated.
b. The number of individual areas, from separate animals, pooled for RIA.

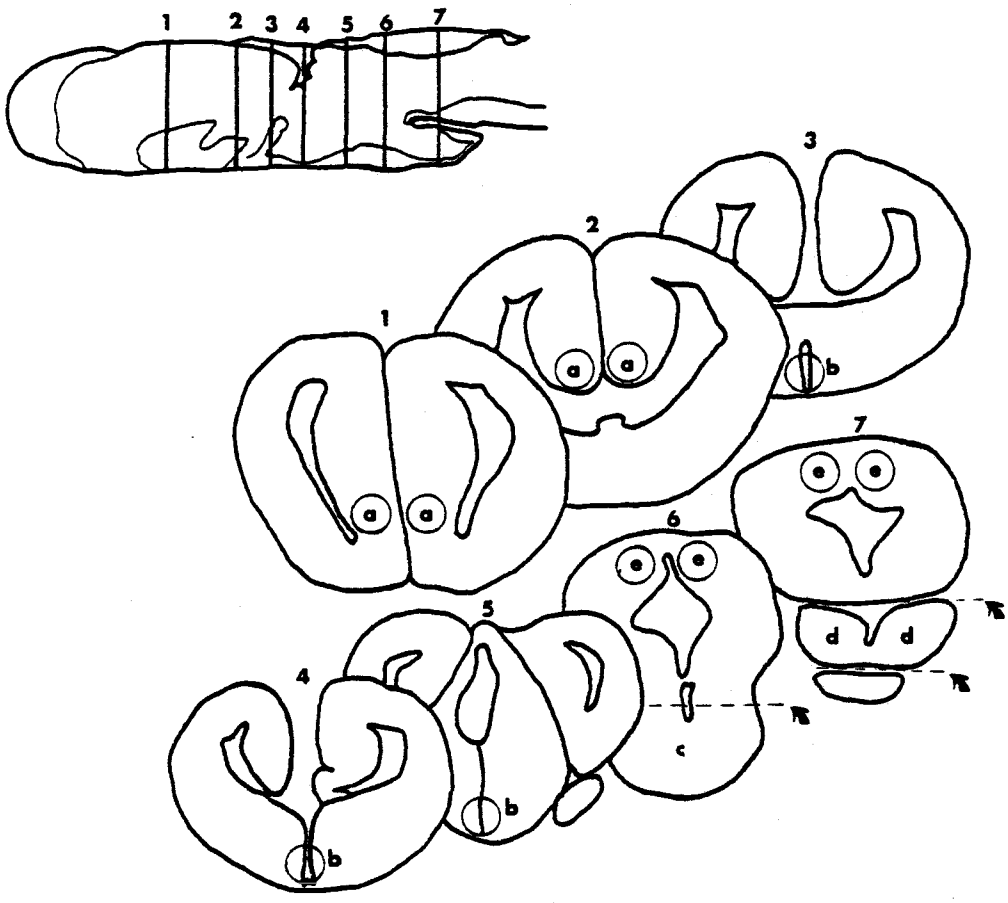

Figure III.1. Brain areas of male rough-skinned newts microdissected for measurement of LHRH. Diagrammatic representation of a newt brain in mid-sagittal plane (above), with representative sections (numbered bars with corresponding cross-sections below) from which brain areas were isolated. Circles represent the size of the punch needle in the a) septal area, b) ventral preoptic area, c) rostral hypothalamus, d) infundibulum, and e) dorsal habenulum. See text for details of the microdissection procedure.

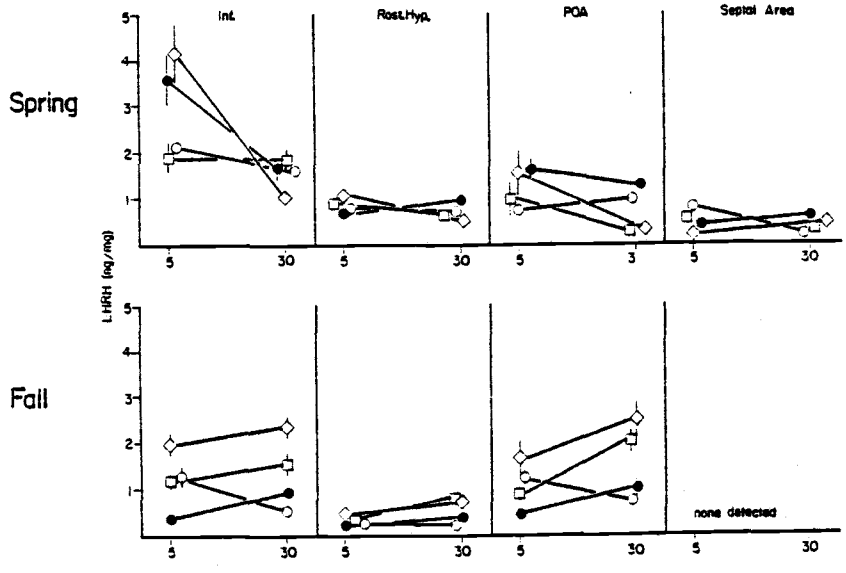

Figure III.2. The effect of castration and testosterone replacement on LHRH concentrations in the brain of male rough-skinned newts. Points represent mean (±SE) LHRH concentration expressed as ng/mg protein (ordinate) at 5 and 30 days after castration (abscissa). LHRH levels were measured in the infundibulum (Inf.), rostral hypothalamus (Rost.Hyp.), ventral preoptic area (POA), and septal area of male newts collected in the spring and fall. Experimental groups include intact males (◇), sham-castrated males (□), castrated males implanted with an empty Silastic capsule (●), and castrated males implanted with a testosterone-filled Silastic capsule (○).

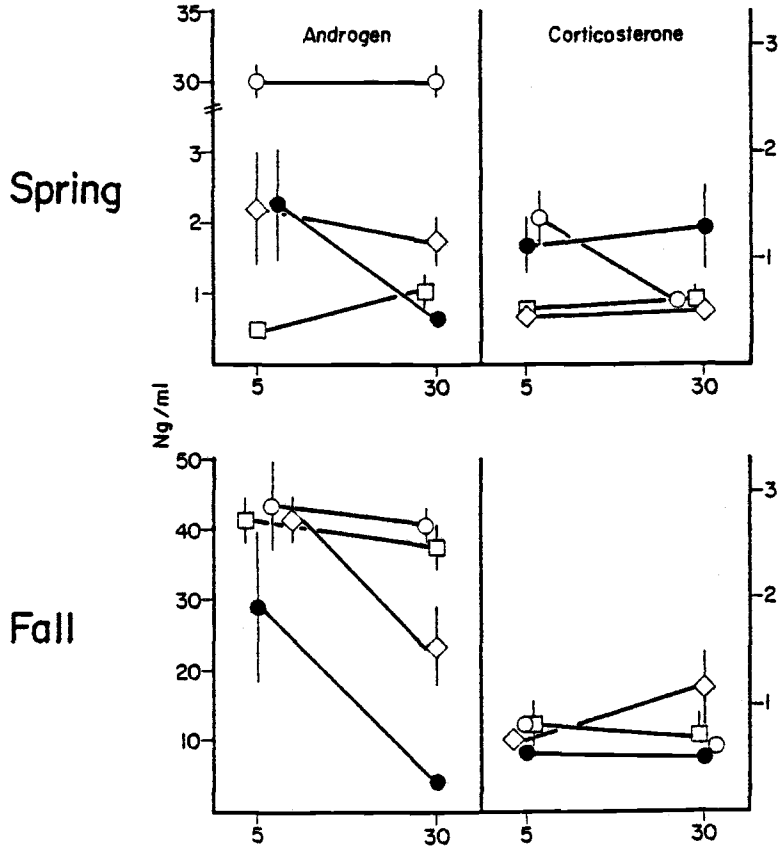

Figure III.3. The effect of castration and testosterone replacement on plasma androgen and corticosterone concentrations in male rough-skinned newts. Points represent mean (±SE) steroid concentrations expressed as ng/ml plasma (ordinate) at 5 and 30 days after castration (abscissa). Steroid levels were measured in newts collected in the spring and fall. Experimental groups include intact males (◇), sham-castrated males (□), castrated males implanted with an empty Silastic capsule (●), and castrated males implanted with a testosterone-filled capsule (○).

CHAPTER IV

GENERAL DISCUSSION

The experiments described in this dissertation demonstrate that there are seasonal changes in the concentration of irLHRH in the hypothalamus of male rough-skinned newts, and that these changes are associated with seasonal changes in reproductive condition. In addition, these experiments demonstrate that plasma androgens exert a feedback influence on hypothalamic LHRH activity. If the seasonal cycle of irLHRH were controlled by changes in hypothalamic sensitivity to steroid feedback, one would predict that the sensitvity of the hypothalamus to androgen feedback would be greater at the end of the breeding season. However, the present experiments indicate that the sensitivity of the hypothalamus to androgen feedback is greater at the beginning of the breeding season than at the end of the breeding season. Thus, seasonal changes in the concentration of hypothalamic irLHRH in male newts may result from extrinsic or intrinsic processes (e.g., endogenous circannual rhythms or environmental cues) that alter the activity of the LHRH neuronal system directly.

In amphibians in general, light, temperature, and humidity are the major environmental cues affecting reproduction (see review by Salthe and Mecham, 1974).

However, there are many conflicting results among studies on amphibians, and different environmental cues have been reported as the major factors affecting reproduction within the same species (Anderson, 1967). Although the extrinsic cues affecting reproduction in male rough-skinned newts are poorly understood, it is possible that different environmental cues affect different aspects of the hypothalamic-pituitary-gonad axis. For example, hypothalamic LHRH activity could be influenced predominantly by changes in photoperiod, whereas the pituitary-gonad axis could be influenced predominantly by temperature changes.

Although this hypothesis is speculative, there are several lines of circumstantial evidence supporting the idea that the hypothalamic-pituitary-gonad axis of male newts is differentially controlled by separate environmental cues. In particular, hypothalamic LHRH activity appears to decline dramatically in March and April (see Chapter 1). It is unlikely that this decline in irLHRH concentration is related to temperature changes because changes in irLHRH concentrations were not associated with temperature changes at other times of year (Chapter 1). However, the pituitary-gonad axis remains active during March and April, as indicated by continually elevated plasma androgen concentrations, in spite of the decline in LHRH activity. Thus, hypothalamic LHRH

activity appears to be regulated independently of the activity of the pituitary-gonad axis, providing a mechanism whereby different environmental cues may influence specific components of the hypothalamic-pituitary-gonad axis.

The present experiments also demonstrate that, when compared to other brain regions, the preoptic area exhibits a different seasonal cycle of irLHRH, and also responds differently to castration and testosterone replacement. The concentration of irLHRH reached a year-high peak in all hypothalamic areas in June. However, irLHRH levels remained high throughout the summer and early fall in the preoptic area but decreased significanly in other brain areas during this period (see Chapter 2). In addition, long-term castration (30 days) in the spring did not influence irLHRH concentrations in the infundibulum, rostral hypothalamus or medial septal area, but prevented irLHRH levels from declining in the preoptic area over the course of the experiment (Chapter 3). These differences among brain areas in the seasonal cycle of irLHRH and in the response to castration are associated with elevated plasma corticosterone concentration. Specifically, plasma corticosterone was elevated in males collected during the summer and early fall, and also in males castrated for 30 days in the spring.

The hypothesis that corticosterone can influence the level of irLHRH specifically in the preoptic area is supported by recent studies in which experimentally-elevated plasma corticosterone was associated with greatly elevated irLHRH levels specifically in the preoptic area (Moore and Zoeller, unpublished data). The functional significance of a causal relationship between plasma corticosterone and preoptic LHRH remains to be determined. However, one possibility is that plasma corticosterone mediates the inhibitory effect of stress on masculine sexual behavior by acting on the LHRH system of the preoptic area. The preoptic area is well-known to be an important neural substrate subserving the expression of masculine sexual behavior in amphibians (see review by Kelley, 1978), and intracerebroventricular injections of LHRH into male newts can stimulate clasping behavior (Moore et al., 1982).

The present results also indicate that there are seasonal differences in the effect of "stress" on hypothalamic irLHRH concentrations. In particular, sham-castration in the spring was associated with a significant decrease in hypothalamic irLHRH 5 days after surgery, but this was not observed in the fall (Figs. III.2 and III.3). In addition, the 40-fold increase in plasma androgen concentration supplied by testosterone-filled Silastic capsules in spring-collected

males was associated with a significant decrease in hypothalamic irLHRH, indicating that this high level of plasma androgen may have induced a stress-response in these animals. Seasonal differences in the effect of stress on various reproductive parameters have been observed in other amphibians. In bullfrogs, for example, the effect of confinement on plasma corticosterone concentrations changes throughout the reproductive cycle (Licht et al., 1988). The present results are unique, however, by indicating that specific plasma hormones, such as corticosterone, may influence the LHRH neuronal system of male newts in specific brain areas.

The seasonal changes in irLHRH levels seen in specific brain areas (Chapter 2) may represent changes in the mean levels of irLHRH in these areas, or changes in the time of a daily peak in irLHRH concentrations. Although circadian changes in irLHRH have not been demonstrated for amphibians, they have been described for some mammals (Kerdelhue, 1977; Kerdelhue et al., 1977; Kerdelhue et al., 1981). These studies show that circadian changes in irLHRH levels occur in areas of nerve terminals (medial basal hypothalamus), not in areas of cell bodies (preoptic area), indicating that there are circadian changes in irLHRH secretion, not synthesis (Kerdelhue et al., 1981). The seasonal changes in the preoptic area described in the present study may result

from circadian or seasonal changes in rates of synthesis, or from changes in irLHRH activity of terminals in the preoptic area not previously described for amphibians.

The observation that there are differences in the seasonal and experimentally-induced changes in irLHRH levels among specific brain areas also may be interpreted within the context of recent chromatographic evidence that there are at least two chemically distinct forms of irLHRH in the brain of male rough-skinned newts (Nancy Sherwood and F.L.Moore, unpublished data). Specifically, LHRH immunoreactivity was extracted from newt brains and chromatographed using reverse-phase HPLC. One form of newt immunoreactive LHRH was indistinguishable from mammalian LHRH; the other form was indistinguishable from teleost LHRH (Sherwood et al., 1983). These results raise the intriguing possibility that the LHRH neuronal system of male newts consists of distinct systems characterized by chemically distinct forms of LHRH. These different forms of LHRH could functionally separate the LHRH neuronal system (e.g., the control of sexual behavior versus that of the pituitary-gonad axis).

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APPENDICES

APPENDIX A

Histological Study of the Newt Brain

To develop the technique for microdissection of specific brain areas of male newts, it was important to define the anatomy of the newt brain so that brain areas could be correctly microdissected. The procedures described below were used to develop this "punch map" for newt brains.

Procedure for Brain Fixation

Several male newts were deeply anesthetized by immersion in 1% MS222 (ethyl m-aminobenzoate methanesulfonate, from Sigma). The heart was exposed by removing the pectoral girdle; care was taken to avoid rupturing major blood vessels. A 25 guage "butterfly" syringe needle was inserted into the conus arteriosus and clamped in place with a small hemostat. Phosphate-buffered paraformaldehyde (see Appendix B) was slowy injected into the arterial supply via the conus arteriosus; the ventral abdominal vein was also severed and fixative was injected until the veinous fluid ran clear for several minutes. After fixation *in situ* , the brain was removed from the cranium and immersed in fresh fixative for 12 hours. During the dissection, it was

important to remove the dura mater to allow for proper dehydration and infiltration of paraffin.

Preparation of Paraffin-Embedded Brains

After fixation, whole brains were passed through the following solutions for 12 hours each:

a. 70% ETOH
b. 80% ETOH
c. 95% ETOH
d. 100% ETOH
e. 100% Chloroform (Xylene clears the tissues too quickly and they become brittle)
f. Paraffin-saturated chloroform

Following 12 hours of immersion in paraffin-saturated chloroform, tissues were placed in 50-52^{o}C paraffin. Because heating causes excessive shrinkage of brain tissue, the temperature of the paraffin bath was kept at 50-52^{o}C. The paraffin was changed three times at 1.5 hour intervals. During this time, the infiltrating tissue was placed for short periods (5-10 min) in a vacuum to remove air bubbles.

Following infiltration, fresh paraffin was poured

into a cubical mold[1]; when a thin crust had formed on the face of the mold, an infiltrated brain was removed from the infiltration paraffin and pressed into the crust of the mold. The block was cooled at room temperature for 24 hours.

Serial transverse sections were taken at both 10 and 15 microns (several brains each). Ribbons were floated onto a 43°C water bath and mounted on clean slides (20 sections per slide). After staining, the tissues were mounted under a cover slip using Permount (Fisher).

Procedure for Staining Paraffin-Embedded Brain Sections

Brain sections were stained for nissl-substance using buffered cresyl violet. This procedure allowed visualization of cell bodies, but also provided a good brackground stain. The following protocol was used for all serial sections:

a. Xylene----------3 min
b. Xylene----------2 min
c. 100% ETOH----------2 min
d. 95% ETOH----------2 min
e. 70% ETOH----------2 min

[1] G.L. Humason, 1979: Animal Tissue Techniques , W.H. Freeman and Co., p43

f. Distilled water----------2 min

g. Buffered Cresyl Violet (Appendix B)-10 min

h. Aqueous Cresyl Violet (Appendix B)---1 min

i. 70% ETOH----------10 dips

j. 95% ETOH----------2 min

k. 100% ETOH----------2 min

l. 100% ETOH----------2 min

m. Xylene----------2 min

n. Xylene----------2 min

o. Mount

APPENDIX B

Preparation of Fixative

1. Under a hood, dissolve 40g paraformaldehyde in 100 ml distilled water by heating the mixture to 65°C with stirring.

2. Add a few drops of 40% NaOH until the solution becomes clear. Allow the solution to cool.

3. Prepare a 0.2M solution of dibasic sodium phosphate (1.7805 g in 50 ml distilled water).

4. Prepare a 0.2M solution of monobasic sodium phosphate (1.38 g in 50 ml distilled water).

5. Prepare the 0.1M solution phosphate buffer by mixing 30.5 ml 0.2M dibasic sodium phosphate with 19.5 ml 0.2M monobasic sodium phosphate and diluting to 100 ml with distilled water.

6. Add 100 ml buffer to 100 ml paraformaldehyde solution.

Preparation of Buffered Cresyl Violet

1. Prepare 100 ml buffer: 94 ml 0.1M acetic acid and 6 ml 0.1M sodium acetate.

2. Add 100 ml buffer to 6 ml aqueous cresyl violet.

Preparation of Aqueous Cresyl Violet

1. Add 0.2g Cresyl violet acetate (Sigma) to 150 ml distilled water.

APPENDIX C

PUNCH TECHNIQUE FOR AMPHIBIANS

Dissection and Sectioning Newt Brains

Male newts were sacrificed by decapitation, and the head was immediately placed in a 60-65°C water bath for 10-13 min. After heating, the head was cooled to 0°C and stored until dissection. The following protocol was used to dissect the brain from the cranium; care was taken to remove the brain without damage. The following protocol for dissection was found to minimize structural damage to the brain.

1. Remove lower jaw with large sissors.

2. Orient head so that the ventral surface is up and the caudal end to the left.

3. Stabilize the head with long, curved forceps with points on the prootic area (Figure C.1).

4. With curved tip of a potter's tool, clear away muscle and tooth rows from the roof of the mouth.

5. Cut through and remove the premaxilla and prevomers, exposing the anterior tip of the parasphenoid.

6. Separate the atlas from the occipital condyle using the straight tip of the potter's tool. Be sure to remove excess spinal cord if it did not break off during dissection.

7. Remove the parasphenoid by hooking the curved tip of the potter's tool beneath the anterior tip of the bone and lifting gently. The parasphenoid will break in at least one place before it comes off. It is very important to firmly anchor the head with the forceps for this step. At the same time, gently squeeze the tips of the forceps together so that when the parasphenoid is removed, the brain case does not break apart, destroying the brain.

8. Slide the straight point of the potter's tool between the brain and the pterygoid. This severs the optic nerves and allows the prevomers to be moved away from the brain without tearing the preoptic area.

9. Free the anterior tip of the brain from the olfactory nerves with the straight point of the potter's tool. This is done by scraping the dark (olfactory) tissue away from the anterior tip of the brain, but be careful not to damage the dura mater.

10. Press on the stabilizing forceps to open the brain case, especially at the parietal area. This step exposes the cranial nerves.

11. Gently sever the cranial nerves with the straight tip.

12. Grasp the anterior tip of the brain by the dura mater with watch maker's forceps and pull the brain forward out of the brain case. This can only be done if the cranial nerves are severed.

13. Freeze the brain to a cryostat chuck using O.C.T. compound. Make sure that the resin completely covers the brain and that the brain is oriented at a 90^{o} angle to the chuck.

14. Cut the brain in a cryostat at 300 um to the anterior commissure, then 200 um throughout

the rest of the brain.

Newt Brain Areas Microdissected

Camera lucida tracings were made from frozen sections cut in a cryostat as described above (Figure C.2). Brain areas were first identified in histological preparations of newt brain as described in Appendix A. Processing of brain tissue is described in Figure C.3. Brains were dissected from the cranium after heating to 60-65^{o}C for 10-13 minutes. After dissection, the brains were embedded in O.C.T. compound and sectioned in a cryostat at -12^{o}C. The procedure for microdissecion involved placing the slide containing frozen brain sections on a freezing stage beneath a dissecting microscope and removing specific areas with a modified stainless steel hypodermic needle (275 um i.d.). The resultant tissues were immediately transferred to a polyethylene culture tube containing 200 ul 1N acetic acid at 0^{o}C. Tissues were homogenized by sonication at 20 KHz for 20 seconds, after which a 25 ul aliquot was removed for protein determination. The remaining extract was centrifuged at 21,000 x g for 20 minutes and 150 ul of the supernatant was lyophylized and stored desiccated at -20^{o}C until assayed for LHRH by RIA.

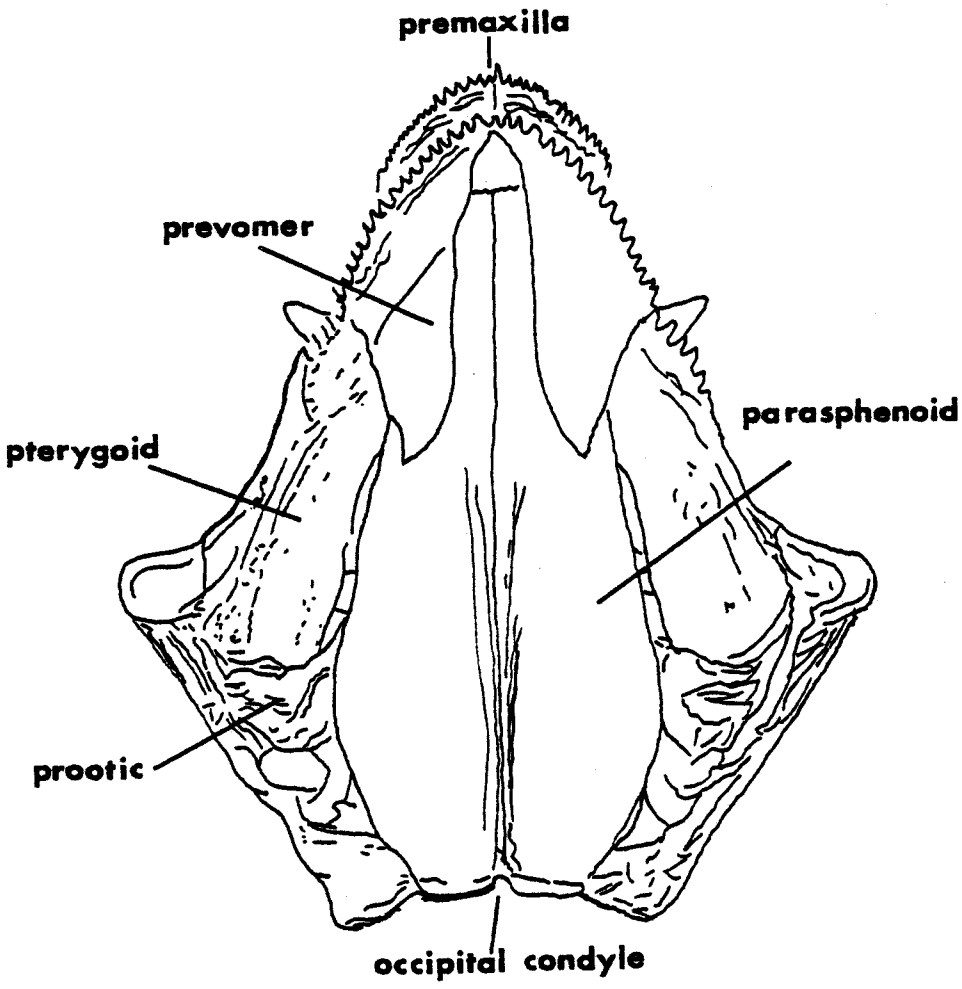

Figure C.1. Diagrammatic representation of bones forming the floor of the brain case in salamanders.

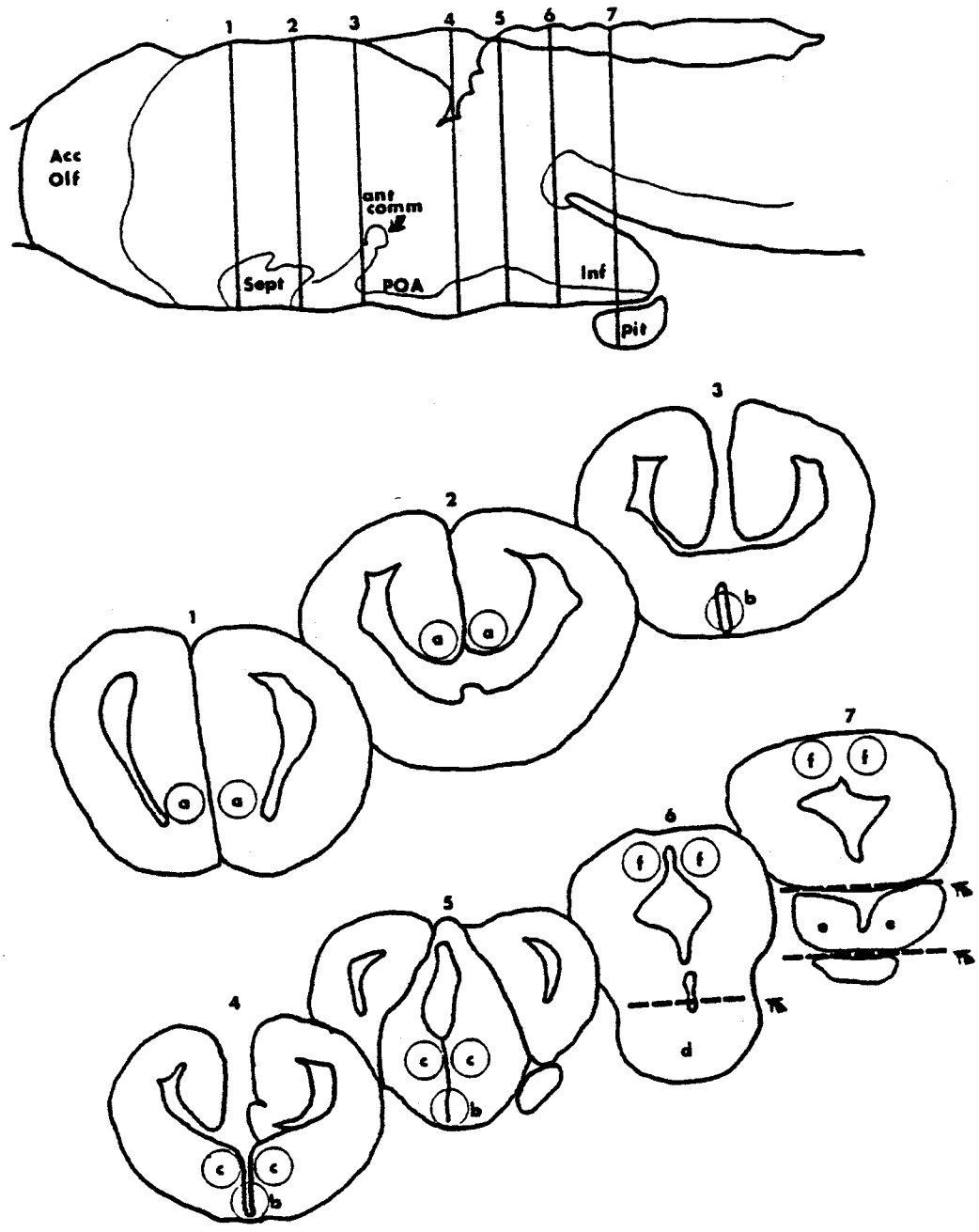

Figure C.2. Brain areas of male rough-skinned newts isolated for measurement of LHRH. Diagrammatic representation of the newt brain in mid-sagittal section (above), with representative sections (numbered bars and corresponding cross sections below) from which brain areas were isolated. Circles represent the size of the punch needle in the a) septal area, b) ventral preoptic area, c) dorsal magnocellular area, d) rostral hypothalamus, e) infundibulum, and f) dorsal habenulum.

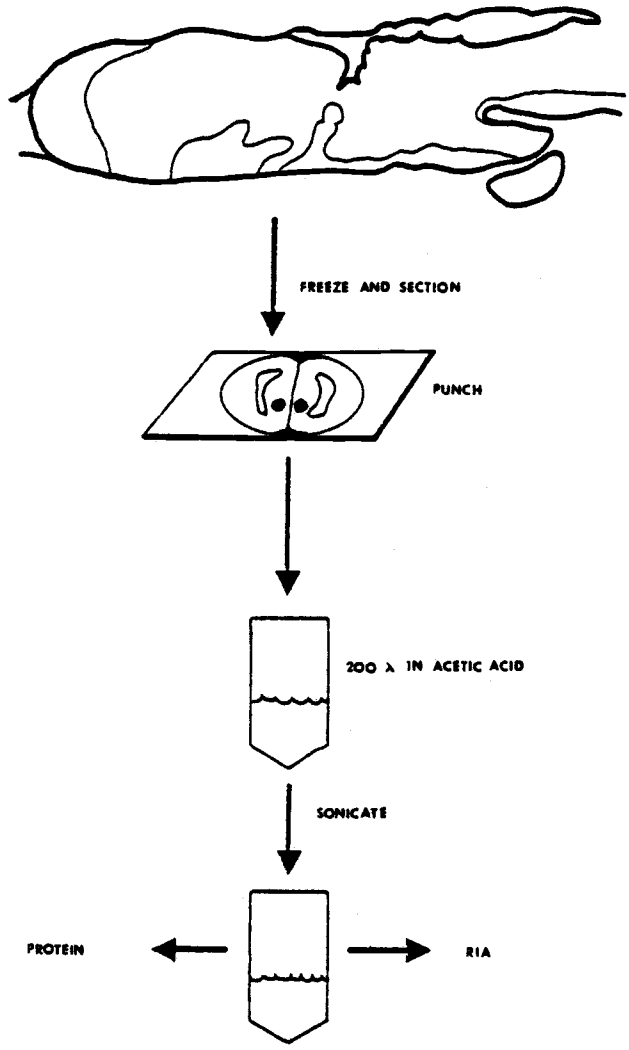

Figure C.3. Procedure for processing brain tissue from rough-skinned newts isolated by the micropunch technique. Tissues were extracted by sonication in 1N acetic acid. After removing an aliquot for protein determination, the solution was centrifuged and the supernatant was lyophilized. The resulting powder was reconstituted in buffer and radioimmunoassay performed for LHRH.

APPENDIX D

PROTEIN DETERMINATION

This procedure is a modification of Hartree (1972)[1]. The color is proportional to protein over a wider range than the Lowry method and is less sensitive to KCl which causes precipitation in the standard method. However, there is interference by Triton X-100 and sucrose. The following protocol is a modification for low volumes.

Solutions

A. 1g K-Na tartrate and 50g sodium carbonate dissolved in 250 ml 1N NaOH and diluted to 500 ml with distilled water.

B. 2g K-Na tartrate and 1g cupric sulfate dissolved in 90 ml water and 10 ml 1N NaOH.

C. 1 volume of Folin-ciocalteau reagent diluted with 15 volumes water.

[1] E.F. Hartree (1972) Anal. Biochem. 48, 422-427.

Procedure

1. Dilute 15 to 300 ug protein to 100 ul water.

2. Add 90 ul solution A and heat to 50°C for 10 min.

3. Cool to room temperature*, and add 10 ul solution B and incubate for 10 min.

4. Add 300 ul solution C forcefully and immediately vortex**.

5. Heat at 50°C for 10 minutes.

6. Cool to room temperature*, and read versus blank at 650 or 750 nm.

7. Wash cuvette with 6N HCl to avoid etching.

*Tubes should be cooled quickly by removing to a 25 C water bath.

**Vortex gently because foaming will cause errors in the reading.

Standards

Stock solution is 300 ug/ml BSA in double distilled

water.

a. 600 ul stock in 600 ul water (15 ug/tube)

b. 600 ul A + 600 ul water (75ug/tube)

c. 800 ul B + 400 ul water (50ug/tube)

d. 600 ul C + 600 ul water (25ug/tube)

e. 400 ul D + 600 ul water (10ug/tube)

f. 400 ul E + 400 ul water (5ug/tube)

g. 100 ul water (BLANK)