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Title: ENDOCRINE REGULATION OF MALE SEXUAL BEHAVIOR IN ROUGH SKINNED NEWTS, TARICHA GRANULOSA: ROLE OF NEUROHORMONES

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To determine whether a homologous neurohormone, arginine vasotocin (AVT), can elevate the occurrence of clasping behavior in male rough-skinned newts (Taricha granulosa), intact, sexually inactive males were injected with either AVT or saline. The incidence of courtship was elevated in the group of males injected with AVT, but not in the group injected with saline. When the relative potency of AVT was compared with that of a heterologous but structurally similar neurohormone, arginine vasopressin (AVP), no difference was found in the minimum behaviorally effective dose.

Testicular androgens are known to influence the expression of sexual behaviors in male vertebrates. To determine whether androgens modify the behavioral effect of neurohormones, the effects of AVT on the incidence of clasping behavior was measured in castrated newts and compared to the effects in castrated, androgen-implanted newts. When castrated for only a short period of time (18 days), AVT increased the incidence of clasping behavior in unimplanted males, but not in androgen-implanted males. In contrast, when males were castrated for longer periods of time (33 and 54 days), AVT elevated the occurrence of clasping behavior in androgen-implanted males, but

not in unimplanted newts. The effect of AVT on the incidence of clasping behavior was also measured in hypophysectomized males to determine if the pituitary is required for newts to respond behaviorally to AVT. When injected with AVT, the incidence of courtship was elevated in hypophysectomized males, sham-operated males, and pituitary transplated males. These experiments support the hypothesis that endogenous neurohormones, in addition to androgens, influence the incidence of sexual behaviors in male T. granulosa.

Endocrine Regulation of Male Sexual Behavior in Rough-Skinned Newts, *Taricha granulosa*: Role of Neurohormones

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INTRODUCTION

The research described in this thesis investigates the endocrine regulation of male sexual behavior in the rough-skinned newt, Taricha granulosa. To provide background information for these experiments, some aspects of research concerning the hormonal control of male sexual behavior are reviewed for amphibians in this Introduction.

A comparison of studies from a variety of species reveals that the hormonal control of sexual behavior is remarkably similar among vertebrates. The extensive work with mammals has provided many insights into the hormonal control of sexual behavior in amphibians. Therefore, some research dealing with mammals will be reviewed here to provide a basis for comparison with amphibians.

The rough-skinned newt is particularly well suited for this type of study. It is easily maintained in captivity, and it readily exhibits normal mating behavior in the laboratory. Also, a good background of useful information is available on its life history, reproductive behavior, and reproductive physiology (Covell, 1923; Pimentel, 1952; Miller and Robins, 1954; Oliver and McCurdy, 1974; Moore and Muller, 1977; Moore, Specker and Swanson, 1977; Moore, 1978a and b; Specker, 1978). Because newts are also abundant in areas close to Corvallis, and because they survive surgical manipulations very well, they are excellent research animals.

As an experimental system, amphibians have an advantage over mammals in that they have a less complex nervous system and a less

complex behavioral repetoire (Kelley, 1978). Experiments dealing with amphibians are biologically important for two reasons; they provide information regarding the evolution of endocrine-dependent sexual behaviors, and they provide model systems which may prove valuable in understanding sexual behavior in other vertebrate classes, perhaps even humans.

Patterns and Measurements of Male Sexual Behavior

Knowledge of the normal behavioral responses of a species is a prerequisite for experiments designed to characterize the physiological control of these responses. Patterns of amphibian and mammalian sexual behaviors are described in this section, emphasizing behavioral patterns of the rough-skinned newt and the rat. Methods used to measure sexual behavior are also described because it is vital to understanding experiments designed to determine the underlying endocrine controls of these behaviors.

A multitude of species-specific behavioral patterns facilitate the sexual interaction between males and females and ultimately the union of sperm and egg. These behaviors are referred to variously as courtship, mating, and sexual behaviors. In the Amphibia, mating behaviors are highly species-specific (see review by Salthe and Mecham, 1974). However, a general pattern that is common to many urodeles (e.g., newts and salamanders) can be organized into stages (Salthe, 1967). The following is a description of sexual behaviors in T. granulosa as described by Pimentel (1952). These behaviors are organized into the stages of Salthe (1967).

In the initial stage of courtship (Stage A), the male becomes aware of a potentially receptive female conspecific by olfactory, visual, and tactile cues emmanating from her. The male then captures the female by clasping her with his forelimbs and hind limbs (Stage B). Male T. granulosa hold the female from a dorsal position, pressing his ventrum against her back. During this stage, which lasts for 3 to 48 hours, the male rubs the female's nares with his submandibular region containing hedonic glands. Her cloaca and inguinal regions are also palpated by movements of the male's hind limbs. This stage of courship is terminated when the male releases the female and slowly crawls over her (Stage C), his cloaca remaining not far from the female's head. In Stage D, the male deposits a spermatophore, a mucopolysaccharide stalk on top of which is located a sperm cap, which adheres to the substrate. In the final stage (Stage E), the female picks up the spermatophore in her cloaca, after having been directed or led to the appropriate position by the male. The spermatozoa are stored in a dorsal involution of the female's cloacal wall, the spermatheca, until egg-laying occurs. Covell (1923) and Pimentel (1952) suggested that egg laying in T. granulosa occurs over a period of four to five weeks. Eggs, laid singly, are fertilized before leaving the cloaca and are attached to aquatic vegetation (Oliver and McCurdy, 1974).

In order to measure the effects of experimental treatments on sexual behavior, there must be some assay for differences in the incidence of these behaviors. The presence of clasping behavior (Stage B of Salthe) is used to measure the initiation of sexual

activity in T. granulosa. Clasping behavior is readily identifiable under experimental conditions, and has been used in other investigations of this type (Moore and Muller, 1977; Moore, 1978a and b). In anurans, measures of sexual activity have included vocalizations (male calling and female release call), and the occurrence of clasping (e.g., Schmidt, 1966; Kelley and Pfaff, 1976; Diakow, 1978).

In mammals, an extensive volume of work has accumulated focusing on the hormonal control of sexual behavior. Because a great deal of this research has used the rat as the experimental system, the behavioral pattern and method of measuring these behaviors will be reviewed for this animal. Beach (1956), Leshner (1978), and Larsson (1979) have described the behavioral patterns of male rats. The behavioral patterns of other mammalian species have been described by Dewsbury (1979).

Within a few minutes after being presented with an estrous female, the male will mount the female. Mounting behavior in the male is recognized by an approach from the rear of the female, forepaw palpations of the stimulus animal by the male, and shallow pelvic thrusts. In a normal sexual encounter, intromission then occurs. Intromissive behavior is characterized by a mount terminated by a quick, deep pelvic thrust. After a number of intromissions, the ejaculatory pattern is seen. This is characterized by a mount terminated by a deep pelvic thrust that is maintained for several seconds while repeated flexures of the hindquarters occur.

These three components of sexual behavior in the rat (mounting, intromission, and ejaculation) are distinct behavioral responses.

Two aspects of each component, frequency and latency, are used to measure these behaviors. Mounting latency is the amount of time from presentation of the female to the first mount by the male. Mounting frequency is the number of mounts performed by the male before ejaculation occurs. Intromission latency is the time from presentation of the female to intromission, and intromission frequency is the number of intromissions per ejaculation. Ejaculation latency is found by measuring the time from first intromission to ejaculation, and the frequency is the number of ejaculations per sexual encounter.

Based on observations of the normal pattern of male sexual behavior in rats, Beach (1956) hypothesized that completing the entire copulatory pattern involves reaching two successive levels of excitement. This appears to be a two-step phenomenon which relies heavily on the activity of two at least partially independent physiological mechanisms. For mating responses to occur, Beach proposed that the male must first become sufficiently aroused to initiate contact with the female. This initial part of the total pattern was proposed to come under the control of the arousal mechanism (AM).

The main action of the AM is to increase the level of sexual arousal such that the male initiates copulation with the female. An important aspect of the AM is that if a male rat fails to copulate within the first five to ten minutes after presentation of an estrous female, he is very unlikely to do so at all.

Once intromission occurs, the copulatory mechanism (CM) comes into play. The function of the CM is to maintain sufficient

excitement to copulate until ejaculation occurs. This mechanism was proposed, in part, to explain the observation that sexually experienced male rats mount a female after the removal of the os penis, but because they are unable to achieve intromission, they soon become habituated to the female and lose interest.

There appears to be two mechanisms, at least for male rats, controlling the normal sequence of mating behaviors. Many studies of the endocrine control of sexual behaviors indicate that the AM is dependent upon androgens to a different extent than the CM (Batty, 1978; Leshner, 1978). This point will be discussed in greater detail later.

Effects of Castration and Androgen Replacement Therapy

Investigations of male amphibians and mammals indicate many apparent similarities regarding the endocrine control of sexual behavior. These investigations are reviewed for the Amphibia by Kelley (1978) and Kelley and Pfaff (1978) and for mammals by Leshner (1978) and Larsson (1978). The results of some of these studies are discussed in this section.

In amphibians, castration reduces the incidence of male sexual behaviors (Steinach, 1894; Shapiro, 1937; Dodd, 1960; Schmidt, 1966; Palka and Gorbman, 1973; Kelley and Pfaff, 1976; Moore, 1978a). Because amphibian testes are known to produce androgens, particularly testosterone and dihydrotestosterone (Moore and Muller, 1977), these studies indicate that the effect of castration on expression of sexual behaviors is to remove endogenous androgens.

A further indication of the importance of testicular androgens in amphibians is the general correlation between the level of plasma androgens and the season when sexual behaviors appear (Moore and Muller, 1977; Specker, 1978). Individual differences in the incidence of male courtship behavior, however, are not correlated with individual differences in plasma androgen concentrations. For example, Moore and Muller (1977) measured plasma androgen concentrations in freshly captured male newts in February (the height of the breeding season) and in June. Although androgen levels were generally higher in February than in June, there was no difference in the levels of plasma androgens between sexually active and inactive newts in either month.

Wada et al. (1976) measured plasma androgen concentrations in male Rana pipiens following injections of pituitary extracts. Because pituitary injections are known to elevate the incidence of sexual behaviors in some, but not all R. pipiens males (Palka and Gorbman, 1973), this experiment examined the hypothesis that plasma androgen levels are higher in males that respond to the injections. However, they found no difference in plasma androgen concentrations between male frogs that responded to the pituitary injection and males that did not respond. These studies indicate that although androgens may be necessary, in a permissive manner, for the expression of male sexual behavior, a specific serum level of androgen is not necessarily associated with these behaviors.

Because the testes of amphibians are known to secrete androgens, most notably testosterone, many investigators have studied the

effects of androgens on the sexual behavior of castrated males. These experiments have produced various results, depending on the species used and, perhaps, mode of administration of androgens. Many researchers have failed to influence sexual behaviors in amphibians with exogenous androgens (Wolf, 1939; Blair, 1946; Palka and Gorbman, 1973; Moore and Muller, 1977; Moore et al., 1977; Wada and Gorbman, 1977). In contrast, some researchers have reported success in restoring mating behavior in amphibians (Kelley and Pfaff, 1976; Moore, 1978a; Ruane, 1978).

Kelley and Pfaff (1976) found that castration abolished male sexual behaviors in Xenopus laevis, and that replacement therapy with implants of either testosterone or dihydrotestosterone restored these behaviors. Ruane (1978) reported similar findings for R. pipiens. These data are additional support for the hypothesis that androgens are an important hormonal component in the regulation of male sexual behaviors.

The experiments of Kelley and Pfaff (1976) demonstrate that a great deal of variation in individual responses to androgens exists. Specifically, the time necessary for clasping behavior to disappear after removal of an implant ranged from 6 to 30 days. Although these differences in the behavioral response to androgen replacement therapy may be explained by differences in the amount of androgens released by individual implants, it also may indicate a variation in the capacity of individual males to respond to androgens.

Moore (1978a) conducted an experiment that was designed to determine whether males differ in their response to androgen replacement

therapy. He found that androgens maintain clasping behavior in castrated male T. granulosa only if the animals were sexually active prior to castration. If males were sexually inactive prior to castration, androgen implants failed to influence the behaviors. Moore (1978a) concluded that the presence of testicular androgen is a necessary, but not sufficient, hormonal condition for expression of male sexual behaviors. He proposed that, in addition to androgen, these behaviors require the presence of some non-testicular factor.

Palka and Gorbman (1973) also concluded that male sexual behaviors in R. pipiens are influenced by some factor other than androgen. In contrast, they suggested that this factor is testicular in origin. Their conclusion was based on the observation that castrated frogs given injections of pituitary extracts continued to exhibit clasping behaviors if they were given implants of testes, but not if they were injected with testosterone propionate (TP). Wada and Gorbman (1977) later hypothesized that this second testicular factor causes an increased availability of androgens to pertinent behavior centers of the brain. This was inferred from the observation that intracranial implants of TP were effective in restoring sexual behaviors in castrated frogs, but intraperitoneal injections were not.

These studies of amphibians have employed castration and androgen replacement therapy, in addition to measurements of circulating hormones by radioimmunoassay, to demonstrate that androgens are active substances affecting male sexual behaviors. However, the evidence suggests that androgens alone may not

control sexual behaviors. It was hypothesized by Palka and Gorbman (1973) and Moore (1978) that the expression of male mating behaviors requires, in addition to androgens, some other factor. The nature and origin of this factor, if it exists, is unknown.

Moore (unpublished research) later tested several hormones in an attempt to demonstrate an affect on clasping behavior in male newts. Significantly, a single intraperitoneal injection of 25 µg arginine vasopressin (AVP) produced an immediate increase in the incidence of clasping behavior. This finding was the basis for the present investigation and will be discussed in detail later.

Many insights into the control of sexual behavior in amphibians have resulted from investigations in mammals. The effects of hormones on sexual behavior in mammals have been reviewed elsewhere by Young (1961), Phoenix et al. (1967), Lisk (1973), Hart (1974), Leshner (1978), and Larsson (1979). Certain aspects of the endocrine regulation of male sexual behaviors in mammals are reviewed here to provide a basis for comparison with the research on amphibians described above.

Castration lowers the incidence of mating behaviors in all mammalian species studied (Beach, 1944; Beach and Holtz, 1946; Beach and Holtz-Tucker, 1949; Grunt and Young, 1953; Feder, 1971). The time course for this reduction is different not only for different species, but also for the various components of sexual behaviors. For example, Davidson (1966) found that intromission latency in the rat was immediately increased by castration, whereas ejaculation latency was initially unaffected. Batty (1978) reported similar

findings for the house mouse, Mus musculus. These researchers hypothesized that the arousal mechanism proposed by Beach (1956) may be dependent upon androgens to a different extent than is the copulatory mechanism. Although other researchers have found the opposite to be true (e.g., Manning and Thompson, 1976), it illustrates that the effect of castration may depend upon which behavioral component is being monitored.

In general, these experiments imply that testicular androgens are important for the expression of male sexual behaviors in mammals. This conclusion is supported by a general correlation between the reproductive season and the level of androgens (Young, 1961; Robinson et al., 1975; Leshner, 1978). However, as is apparently true for amphibians, individual differences in the expression of sexual behaviors are not correlated with individual differences in circulating androgen levels. For example, Harding and Feder (1976) found that male guinea pigs, rated as being highly active in terms of sexual activity, had plasma androgen levels that did not differ from guinea pigs rated as being of low activity.

Experiments in which castrated animals are injected with testosterone have supported the conclusion that testosterone is the behaviorally active substance. Moreover, Beach and Holtz-Tucker (1949) demonstrated a dose-response relationship in rats between increased dosages of testosterone and the frequency of copulations per test. This dose-response relationship existed for concentrations of testosterone until the pre-castration level of sexual activity was reached. Further increments in the dose of

testosterone failed to produce a change in the level of sexual activity. Because individual differences in sexual activity existed before castration, these differences were restored by androgen replacement therapy. Similar results were found for guinea pigs (Harding and Feder, 1976), and individual differences in the level of sexual activity remained even after the concentration of circulating androgen had been equalized by castration and administration of the same dose of testosterone to all animals (see also Grunt and Young, 1952, and 1953; Beach and Fowler, 1959; Davidson, 1966; Davidson et al., 1971). Thus, not all individual differences in sexual behavior can be explained by differences in the level of plasma testosterone.

Numerous studies with mammals demonstrate that there is much interspecific variation in the effects of castration on male sexual activity. In the rat, various behavioral components are androgen-dependent to different degrees, and much intraspecific variation in the potency of androgens exists. Moreover, androgen administration in castrates elevates the level of sexual behavior only to the precastration level. These data indicate that while androgens are essential for the expression of male mating behaviors, individual differences in the level of sexual behavior expressed cannot be explained by differences in androgen levels alone, a conclusion somewhat similar to that for amphibians.

While these experiments demonstrate the importance of androgens in the regulation of male sexual behaviors in mammals, and identify testosterone as a behaviorally active hormone influencing these

behaviors, they have not characterized the entire hormonal prerequisite for the expression of sexual behaviors. Because so much information is available on the behavioral effects of androgens in mammals, the mechanism and site of action of androgens have been studied more extensively in mammals than in amphibians. This topic is reviewed next and compared with the few investigations that used amphibians.

Brain-Hormone Interactions

Androgens are thought to exert their effects on sexual behavior by influencing the brain. Studies focused on determining the neural substrates for androgen action in amphibians are reviewed by Kelley (1978) and Kelley and Pfaff (1978), and in mammals by Leshner (1978) and Larsson (1979). The mechanism by which sex hormones exert their effects on the brain is poorly understood. At present, only tentative models exist which are highly speculative (Beyer et al., 1979). This area of investigation is reviewed for mammals by Beyer et al. (1979). Some of these studies are reviewed here in order to provide a background of information relating to this investigation.

Particular areas of the brain appear to be involved in the control of male sexual behaviors in both amphibians and mammals. These areas are thought to be stimulated by sex hormones. Lesions in the brain of frogs have shown that the preoptic area of the hypothalamus is essential for mating calling (Schmidt, 1968). Lesions placed in the preoptic area abolished mating calling while lesions placed in

in other parts of the brain did not affect normal calling. This conclusion is supported by the finding that electrical stimulation of the preoptic area stimulates normal mating calling (Schmidt, 1966, 1968, 1973, and 1974). Therefore, the preoptic area of the hypothalamus appears to be involved in the expression of sexual calling in a number of anuran species.

Autoradiographic studies provide further evidence that the preoptic area of the hypothalamus in male frogs is an important site of action of steroids (Kelley and Pfaff, 1975; Kelley et al., 1975; Morrell et al., 1975). Particular cells in the preoptic area concentrate radiolabelled steroids to a greater extent than areas outside the preoptic area. Other studies have demonstrated that androgens stimulate this area in R. pipiens (Wada and Gorbman, 1977; Ruane, 1978). Crystalline implants of androgen were most effective in restoring calling and clasping behaviors when placed in the preoptic area of castrated leopard frogs. Therefore, it appears that a particular area of the amphibian brain is involved in the expression of male sexual behaviors, and that this neural structure is stimulated by steroids.

Similar results have also been found for mammals. Lesions placed in the medial preoptic area abolish sexual behaviors (Heimer and Larsson, 1966/67), and electrical stimulation of this area stimulates these behaviors (Vaughan and Fisher, 1962). Further evidence is that cells of the preoptic area concentrate radiolabelled steroids (e.g., Pfaff, 1968; Pfaff and Keiner, 1973). This is thought to have biological meaning because implants of androgens

in the preoptic area stimulates male sexual behaviors (Davidson, 1966; Lisk, 1967).

The mechanism by which androgens affect these brain areas to stimulate sexual behaviors is poorly understood. Kawakami and Sawyer, (1959) showed that steroids modified the excitability of specific brain structures, suggesting that the synapse is the ultimate site of action for steroids. Because labelled androgens have been identified in the nucleus of these neurones, it is hypothesized that androgens affect the synapse by acting on the genome, perhaps by stimulating changes in protein synthesis (McEwen, 1976; Kato, 1977). Various kinds of evidence implies that androgens affect neurotransmitters to decrease the threshold for nerve transmission in neural pathways essential for the expression of sexual behavior (Beyer et al., 1979).

These studies provide some evidence from which models may be derived. One such model for the action of androgens on sexual behavior is that these steroids act upon the preoptic area of the hypothalamus to alter the level of critical enzymes involved in the synthesis, transport, metabolism, or action of neurotransmitters. This alteration is thought to sensitize or desensitize neurones which are excitatory or inhibitory in regulating sexual behaviors (Beyer et al., 1979). Very little solid evidence exists for this model. It was discussed here to emphasize that the affect of hormones on sexual behaviors must ultimately be explained in molecular terms.

Experiments designed to examine the specific chemical nature of steroids found in behavioral centers of the brain have revealed

that testosterone may not always be the active substance at the cellular level. Beach (1942) found that estrogen administration was effective in restoring sexual behaviors to castrated rats. Moreover, it was later found that some androgens, such as dihydrotestosterone, are not effective in restoring male sexual behaviors to castrated rats (Feder, 1971; Whalen and Luttge, 1971). Because androgens which are behaviorally active are capable of being converted to estrogen by a specific enzyme system in a process called aromatization (Naftolin *et al.*, 1972), the "aromatization" theory contends that testosterone is normally converted to estrogen by aromatases. Not all masculine sexual behaviors can be explained by conversion of testosterone to estrogen, however, because estrogen treatment to castrates does not restore the complete male sexual pattern (Larsson *et al.*, 1973; Johnson and Tiefer, 1974). Baum and Vreeburg (1973) and Feder *et al.* (1974) demonstrated that DHT administered with estrogen restored the complete pattern of sexual behavior in castrated male rats. Their hypothesis, based on this result, was that DHT maintains peripheral structures involved in copulation whereas estrogen acts on the central nervous system to affect sexual behaviors.

Although the aromatization theory is not supported in all species of mammals (e.g., Phoenix, 1973 and 1974; Alsum and Goy, 1974), evidence indicates that it is supported in at least some amphibians. Ruane (1978) restored sexual behaviors in castrated *R. pipiens* with intracranial implants of estrogen, or androgens that could be aromatized to estrogen. Kelley (1978) reported an overlap in the brain areas of *X. laevis* that concentrate testosterone and

those that concentrate estrogen, indicating that testosterone is converted to estrogen and concentrated by the appropriate neural cells. Significantly, Callard et al. (1978 and 1979) have found aromatase activity in the brain of Rana catesbeiana and Necturus maculosus.

In summary, various kinds of experimental evidence indicates many similarities in the neural control of sexual behaviors between amphibians and mammals. The preoptic area of the hypothalamus appears to be involved in the expression of sexual behaviors of both vertebrate classes. Moreover, this neural structure is stimulated by gonadal steroids. The particular steroid which acts at the cellular level, however, may not always be testosterone. The aromatization theory proposes that testosterone is converted to estrogen and that estrogen then acts at the cellular level to affect sexual behaviors.

The Research Problem

Experiments reported here were designed to test the hypothesis that neurohormones influence the occurrence of male sexual behaviors in T. granulosa. This hypothesis is based on the results of experiments discussed in this Introduction. These experiments demonstrate the following points: 1) castration reduces the incidence of sexual behavior, 2) the seasonal appearance of sexual behaviors coincides with a seasonal peak in the concentration of androgens in the plasma, 3) sexually active males have androgen levels similar to males that are not sexually active, 4) androgen replacement therapy maintains sexual behaviors in male newts that

are sexually active prior to castration, but not in males that are sexually inactive prior to castration, and 5) arginine vasopressin increases the incidence of sexual behaviors in castrated, androgen-implanted newts.

Based on these points, it is hypothesized that endogenous neurohormones affect the expression of clasping behavior in male T. granulosa in addition to androgens. If endogenous neurohormones influence the expression of male sexual behavior, it is predicted that an injection of neurohormone will elevate the occurrence of sexual behaviors, and that decreasing the availibility of endogenous neurohormones by removing its source will decrease the occurrence of clasping. Furthermore, it is predicted that the level of endogenous neurohormone, at the site of its action, is correlated with the presence of sexual behaviors.

The first two of these predictions were tested by experiments reported here. In Chapter 2, intact, sexually inactive males were injected with neurohormones in order to determine that a homologous neurohormone can elevate the incidence of clasping behavior in rough-skinned newts. In Chapter 3, the effects of neurohormones on the incidence of clasping behavior were measured in castrated newts implanted with androgens. These experiments were based on the observation that neurohormones influenced sexual behaviors in castrated males only if they were implanted with androgens (Moore, unpublished). The experiments were designed to examine the possibility that androgens influence the effects of neurohormones on the expression of sexual behaviors.

In Chapter 4, sexually active male newts were hypophysectomized and tested for their ability to respond to neurohormone injection by clasping a female. These experiments were designed to test the hypothesis that the pituitary is necessary for neurohormones to increase the incidence of courtship.

CHAPTER 2

Dependency of sexual behaviors on androgens is well documented for most male vertebrates. In amphibians, however, androgens are not thought to be the only hormone involved in the regulation of male sexual behaviors. Moore (1978a) hypothesized that some other factor is necessary for expression of these behaviors in the rough-skinned newt, T. granulosa. A similar hypothesis has been advanced for male R. pipiens (Palka and Gorbman, 1973).

Moore (1978a) based this hypothesis on experiments which demonstrate that androgens maintain sexual behaviors in castrated newts only if they were sexually active prior to castration. If animals failed to express clasping behavior before castration, androgens failed to influence this behavior.

Because arginine vasopressin (AVP) increased the incidence of clasping behavior in male newts, Moore (unpublished data) further hypothesized that neurohormones modulate male sexual behavior, in addition to androgen, in this species. He added that there may be a positive relationship between androgens and neurohormones in the control of sex behavior because a neurohormone increased sexual behavior only in androgen-implanted newts.

The following experiments were designed to examine the role of neurohormones in the expression of sexual behavior in male rough-skinned newts. In the first experiment, arginine vasotocin (AVT) was administered to intact, sexually inactive males in order to determine that a homologous neurohormone can elevate the incidence of clasping

behaviors. In experiment II, the relative potency of a heterologous neurohormone, arginine vasopressin (AVP), was compared to that of AVT, in order to provide further evidence that an endogenous neurohormone affects the expression of sexual behavior in male newts.

In Experiment III, plasma androgen concentrations were measured by radioimmunoassay in male newts that exhibited clasping behavior following an injection of AVP. These values were compared to androgen levels in males that failed to clasp a female following the injection. This experiment tested the hypothesis that adequate levels of androgens are required for neurohormones to increase the incidence of clasping in male rough-skinned newts.

In the final experiment, hematocrit values were measured in sexually active male newts, and compared to hematocrit values of sexually inactive males. This experiment examined the hypothesis that hematocrit values are inversely related to the endogenous level of AVT and, therefore, that endogenous AVT levels may be indirectly measured and compared to the occurrence of clasping.

METHODS AND MATERIALS

Experimental animals

Male newts, Taricha granulosa, were collected from Fathead Lake, approximately 24 km northwest of Corvallis. Animals were collected with a dipnet and transported to the laboratory in ten-liter polystyrene buckets containing pond water.

Experimental animals were maintained in containers of dechlorinated water and held in a controlled-environment chamber. Lighting was provided by two 40-watt flourescent bulbs. During the course of an experiment, animals were kept at a constant temperature and photoperiod. The exact settings approximated those of the lake at the time of collection. Because the collection date for each experiment differed, temperature and photoperiod settings were different for each experiment. Before each experiment, animals were weighed and individually marked by toe clipping.

Female T. granulosa also were captured from Fathead Lake, and were maintained during the experiments in plastic tanks filled with dechlorinated water. Females were held in the same environmental chamber as the males.

Test of Male Sexual Activity

The expression of clasping behavior was used as a test of male sexual activity. Clasping behavior is an initial component of courtship in which the male presses his ventrum against a female's back by firmly holding the female with his forelimbs and hind limbs.

To test for this behavior, "sexually attractive" females were placed in a container (as described for each experiment) with experimental males for 60 minutes. The ratio of males to females was held constant for the particular experiment. During this time, all experimental males were exposed to the same females. At five-minute intervals, males that were clasping a female were removed from the test tank, recorded as being sexually active, and placed in a separate container. Females were not removed during the test. When the test was ended, all experimental males and test females were returned to their original containers. Depending on the specific experiment, these tests were repeated at various intervals after the experimental treatment.

Since the sexual attractivity of female T. granulosa varies greatly among individual newts (Moore, 1978b), the following methods were used to insure that stimulus females were sexually attractive. Before each test of male sexual activity, a pretest was conducted to select sexually attractive females. Females were placed in a tank with males; those that were clasped by a male were operationally classified as sexually attractive. Experimental males were never used during the pretest. In some experiments, an injection of 500 or 1000 µg progesterone to intact female newts was used to increase sexual attractivity (Moore, 1978b). Progesterone was suspended, using the standard method of steroid suspensions, in 0.1 ml amphibian Ringer's, and injected intraperitoneally 24 to 30 hours before using them in a test of male sexual activity.

Experimental procedures

Experiment I

Thirty male newts were collected on September 30, 1978 in order to determine whether an injection of AVT affects the incidence of clasping behavior in intact newts. None of these males were in amplexus at the time of collection. Newts were held at 18°C in ten-liter plastic containers.

On the day after collection, males were tested to determine whether they were sexually active. Mating tests took place in a large polystyrene tank containing about 20 liters of dechlorinated water. Males were placed in the tank for one hour before the test began, in order to allow them to acclimate to the test conditions. At the beginning of a test, sexually attractive females were placed in the tank where they remained for the duration of the test. Clasping males were removed from the tank 30 and 60 minutes after the test began. Because only sexually inactive newts were needed, males that clasped a female during the initial test were released, whereas males that never clasped a female were used in the experiment.

These males were then assigned to one of two groups, ten animals in each group. One group was injected with 25 µg AVT (from Calbiochem) in 0.1 ml saline (amphibian Ringer's), and the other group was injected with 0.1 ml saline. To determine the effect of AVT on the incidence of clasping behavior, mating tests were performed at 0, 1, and 8 hours after injection. All AVT-injected males were weighed 2, 5, 9, 19, and 24 hours after injection.

Experiment II

Male newts were collected on October 10, 1978. They were held in a large basin (165 x 65 cm) that contained approximately 100 l dechlorinated water. Temperature was held at 10°C, the temperature of the pond at the time of collection, and photoperiod was 12L:12D.

To obtain sexually inactive males, a mating test was conducted within an hour of the injection. Fifty females were placed in the basin that contained the experimental males for 60 minutes. The occurrence of clasping was observed at five-minute intervals and, at these times, clasping males were removed from the basin and released. At the end of the test, sexually inactive males were assigned to one of two hormone groups, arginine vasotocin (AVT) or arginine vasopressin (AVP) (both from Sigma), or a control group. Within each of the two hormone groups, males were further assigned to one of four subgroups corresponding to one of four dose levels: 0.1, 1.0, 10, or 100 µg. Control animals received 0.1 ml saline. Each animal then received a single intraperitoneal injection of their respective treatment and a mating test was conducted at 0, 4, 8, 16, and 24 hours after injection. During each mating test, the occurrence of clasping behavior was observed at five-minute intervals for one hour. At each observation, clasping males were removed from the basin and held in a separate container for the duration of the mating test.

Experiment III

The design of this experiment is based on the observation that not all males exhibit clasping behaviors following injection of

neurohormone. Because October is known to be a season of high individual variation in plasma androgen concentration (Specker, 1978), the behavioral effect of AVT may occur only in males that have a detectably higher level of plasma androgens. For this experiment, male newts were collected on October 22, 1978. The animals were held in the laboratory as described for Experiment II. Only sexually inactive newts were used in this experiment, and this was determined as described in Experiment II. Within one hour of selecting sexually inactive newts, 20 males were injected intraperitoneally with 0.1 ml saline containing 63 µg AVP (Sigma). To control for the possibility that neurohormones alter the concentration of androgen in the plasma, a group of ten males were injected with 0.1 ml saline.

The effect of AVP on sexual behavior was determined by a single mating test, conducted 8 hours after the injection. The procedure for this mating test was described in Experiment II. Within an hour of this test, the newts were decapitated and blood was collected in chilled, heparinized centrifuge tubes. The blood was centrifuged; plasma was collected and stored at $-20^{\circ}C$ until assayed. The procedure for radioimmunoassay of plasma androgens is described in Appendix A. Hematocrit values were also determined.

Experiment IV

In a pilot experiment, hematocrit values were shown to be lower in males injected with a neurohormone. Because exogenous neurohormone also elevates the occurrence of clasping, this experiment was designed to examine whether hematocrit values are depressed in intact, sexually

active male newts. For this experiment, newts were collected on October 27, 1978. Some of these males were in amplexus when captured. The animals were maintained as described in Experiment II. On the day after collection, males were tested to determine which ones would exhibit clasping behavior. Blood was then collected from the caudal vein in the tail from both sexually active and inactive newts. Hematocrit values were then calculated.

Statistical Analysis

Chi-square values were calculated using the number of different males that exhibited clasping behavior after injection of neurohormone, and compared with the appropriate saline-injected group. Fisher exact tests were also calculated for these data, but because the two statistical tests yielded the same results, only chi-square values are reported. A one-way analysis of variance was used to determine if plasma androgen levels were different in Experiment III.

RESULTS

Experiment I

The intraperitoneal injection of 25 µg AVT increased the incidence of clasping behavior in intact male newts (Table 1). The number of males which exhibited clasping behavior was significantly higher for animals injected with AVT compared to those injected with saline ($\chi^2=6.67$; df=1; $p<0.01$). This elevation in the incidence of clasping behavior, however, was not seen until eight hours after the injection, at which time five of ten AVT-injected males, and none of the saline-injected males, clasped a female. At one and two hours after the injection, the incidence of clasping behavior was similar for the AVT-injected males and the saline-injected males.

Body weights did not change significantly during the 24 hours after injection.

Experiment II

The largest dose of AVT and of AVP (100 µg) significantly increased the incidence of clasping behavior over that of the controls. Seven of ten males injected with 100 µg AVT and six of ten males injected with 100 µg AVP clasped a female in at least one mating test, whereas only two of ten saline-injected males exhibited clasping behaviors (Table 2).

Elevation of sexual activity by AVT and by AVP was greatest at eight hours after injection, at which time 60% of the males were sexually active , whereas no saline-injected male clasped. Lower

Table 1. Experiment I of Chapter 1: Effect of arginine vasotocin on the incidence of clasping behavior in intact male Taricha granulosa.

Treatment	N[1]	Hours after injection		
		0[2]	1	8
Arginine vasotocin 25µg	10	2	0	5[3]
Saline 0.1ml	10	0	0	0

[1] refers to the number of animals in each experimental group.

[2] represents the number of intact males exhibiting sexual behavior during each test.

[3] represents a significant elevation in incidence of clasping behavior over that of controls ($\chi^2=6.67$; df=1; $p<0.01$). All animals were sexually inactive prior to injection.

Table 2. Experiment II of Chapter 1: Comparison of two different neurohormones at four different dose levels on the incidence of clasping behavior in male newts, Taricha granulosa.[1]

Treatment	N[2]	Hours after injection					Σ[3]
		0	4	8	16	24	
Arginine-8 vasopressin							
100µg	10	30	30	60[4]	0	0	60[5]
10µg	9	0	11	11	0	0	11
1µg	9	0	11	11	0	0	11
0.1µg	8	0	0	0	0	0	0
Arginine-8 vasotocin							
100µg	10	20	40	60[4]	40	20	70[6]
10µg	10	10	0	0	0	10	20
1µg	10	20	0	10	10	10	20
0.1µg	10	10	10	10	0	0	20
Saline							
0.1ml	10	10	10	0	0	0	20

[1]percent of intact males exhibiting sexual behavior during each test.

[2]refers to the number of animals in each experimental group.

[3]refers to the total number of different animals exhibiting clasping behavior in at least one test.

[4]represents a significant elevation in the incidence of clasping behavior over that of the controls ($\chi^2=8.57$; df=1; $p<0.01$).

[5]does not represent a significant elevation in the incidence of clasping behavior over that of the controls.

[6]represents a significant elevation in the incidence of clasping behavior over that of the controls ($\chi^2=5.05$; df=1; $p<0.05$).

doses of AVT and of AVP were ineffective in elevating the number of sexually active newts over that of the control group.

Experiment III

Arginine vasopressin significantly increased the number of males that clasped a female during the one-hour mating test. Fifty-five percent of the males exhibited clasping behavior when exposed to sexually attractive females eight hours after the injection of AVP, compared to 10% of the saline-injected animals (χ^2=5.63; df=1; $p<0.02$).

No significant difference in plasma androgen level was seen, as determined by a one-way analysis of variance after logarithmic transformation of the data (F=2.55; df=2, 28; ns). However, a possible trend is indicated. The 11 AVP-injected males which exhibited clasping behavior had a mean plasma androgen concentration higher than that of the AVP-injected males which did not express clasping behavior (37.75±10.28 vs 17.95±5.73 ng/ml; mean±SE). Furthermore, saline-injected males had a mean plasma androgen concentration of 15.96±3.99 ng/ml.

Hematocrit values were lower in the AVP-injected males. The mean hematocrit value for AVP-injected males was 9.5±0.8 compared to 21.3±2.2 (mean±SE) in the saline-injected males (t=6.21; df=29; $p<0.001$). However, there was no difference in hematocrit values between those males which clasped a female and those that did not (9.09±1.22 vs 10.0±1.03).

Experiment IV

No difference in hematocrit values was found between male newts that were sexually active and those that were not. The mean hematocrit value for sexually active males was 30.5±2.4 (n=8), compared to 31.2±3.3 (mean± SE, n-9) in sexually inactive newts. This does not represent a significant difference (t=0.15; ns).

DISCUSSION

Results from these experiments support the hypothesis that male sexual behavior in T. granulosa is influenced, at least in part, by neurohormones. Injections of either AVP or AVT elevated the incidence of clasping behavior in intact males that had expressed no clasping behavior prior to the injection. This finding agrees with that of Moore (unpublished), inasmuch as an injection of neurohormone elevated clasping behavior in castrated, androgen-implanted male newts. These data indicate that endogenous neurohormones may play a role in the endocrine regulation of male sexual behavior in amphibians.

Neurohormones have also been demonstrated to influence mating behavior in some fish. Wilhelmi et al. (1955) reported that injections of neurohormones stimulated the spawning reflex in the killifish, Fundulus heteroclitus. The spawning reflex is a characteristic S-shaped flexure of the fish's body during which gametes are expelled. Pickford and Strecker (1977) showed that AVT is more potent in enhancing the spawning reflex than either AVP or oxytocin. It is difficult, however, to assess the relevance of this finding to the results with Taricha. Not only are the experimental subjects different, but the spawning reflex in killifish is a completion behavior, analogous to spermatophore deposition in newts.

In amphibians, Diakow (1978) found that AVT inhibits the release call of Rana pipiens females. Since unreceptive female frogs emit

a release call when they are clasped by a male, the inhibition of this call by AVT indicates an induction of female sexual behavior. Diakow's hypothesis was that AVT causes an increase in water uptake by female frogs, and that subsequent abdominal distention inhibits the release call. This hypothesis was supported by implanting a balloon intraperitoneally into unreceptive females. Inflation of the balloon inhibited the release call.

In Experiment II, AVP was found to be as effective as AVT in elevating the number of males that clasped a female. The apparent equality in the potency of these two neurohormones may have resulted from the failure to determine the smallest effective dose for each hormone. Because 25 µg AVT was effective in elevating the number of sexually active newts in Experiment I, whereas 10 µg AVT was not effective in Experiment II, a dose-response study comparing doses of AVT and AVP between 10 µg and 25 µg may clarify this point.

Previous studies of neurohormone action using urodele amphibians have been concerned primarily with water-balance effects. A comparison with doses used to induce antidiuresis is instructive here in that at least some indication of a physiological range may be obtained. Bentley and Heller (1964) found for Triturus alpestris that 7 µmoles/kg body weight caused a significant decrease in urine flow. The behaviorally effective dose of AVT used in Experiment I corresponds to 1.57±0.52 µmoles/kg (mean±SE; n=10). Although this value is well within the range for an antidiuretic response in T. alpestris, further study is needed to determine if the behavioral effect of neurohormones in Taricha is physiological.

Androgens are known to modulate sexual responses in many male amphibians. This is especially illustrated by experiments in which castration abolishes male sexual behaviors (cited earlier). However, experiments in which androgens are administered to castrates have yielded seemingly contradictory results. In particular, androgens have been shown to maintain sex behavior in male newts only if the animals were sexually active prior to castration. If the animals were sexually inactive prior to castration, androgens failed to affect their behavior (Moore, 1978a). This apparent contradiction in the effects of androgens on male sexual behavior may be due to differences in levels of endogenous neurohormones among individual newts.

Experiment III was designed to examine whether endogenous androgen concentrations influence the effect of neurohormones on the incidence of clasping behavior. Although none of the plasma androgen values were significantly different among the groups, the trend in the data indicates that AVP-injected males that clasped a female may have had higher plasma androgen levels than either of the other two groups. Repeating this experiment during a time of year when androgen levels are more uniform (e.g., February) is needed to determine if the trend in these data is meaningful.

Because the mean androgen concentration in the AVP-injected males that displayed clasping behavior was nearly twice that of the other two groups, it is possible that AVP increased the concentration of androgens only in this group. If this is true, perhaps another factor is involved, besides androgens and neurohormones, in

regulating male sexual behavior. However, it would be difficult to test this hypothesis.

The apparent increase in androgen levels may also have been caused by clasping behavior itself, regardless of the injection. This is unlikely however, because Moore and Muller (1977) failed to detect a change in plasma androgen concentrations in male newts that had clasped a female for different lengths of time, indicating that clasping behavior is not associated with elevated levels of plasma androgens.

Basic neurohormones, which include AVT and AVP, have been most studied for their effects on water-balance and vasopressor activity. In Experiment I, body weights were measured in AVT-injected males in order to determine if the injection resulted in water uptake or retention. No measurable increase in body weight was detected during the period of study. This indicates that AVT did not increase water uptake or retention. Therefore, the behavioral effect of neurohormones is probably not mediated by abdominal distention caused by water uptake as proposed by Diakow (1978). Pickford and Strecker (1977) also found no evidence to support the hypothesis that neurohormones induce the spawning reflex response in killifish via a water-balance effect.

Hematocrit values were apparently depressed by the injection of neurohormone in Experiment III. Although blood pressure was not measured in these animals, it is conceivable that the depressed hematocrit values were accompanied by an increase in blood pressure. Therefore, the behavioral effect of neurohormones in Taricha may

be mediated by their vasopressor effects.

In summary, the experiments described in this chapter demonstrate that administration of exogenous neurohormones can elevate the incidence of clasping behavior in intact male newts. Although a heterologous neurohormone (AVP) was found to be of similar potency as a homologous one (AVT), the least effective dose was not determined for either hormone. Although the dosages of hormones used in these experiments were within the range for a water-balance effect in other urodele amphibians, a water-balance effect was not seen in Taricha, as indicated by the lack of a detectable increase in body weight after injections of AVT. However, since hematocrit values were depressed by AVP in Experiment III, the behavioral effect of neurohormones may be mediated by a vasopressor or other osmoregulatory action.

CHAPTER 3

The results of experiments described in Chapter 2 support the hypothesis that neurohormones can influence sexual behaviors in male T. granulosa. Moore (unpublished) hypothesized that androgens exert a positive influence on the behavioral effect of neurohormones. This hypothesis was based on the observation that, following an injection of AVP, androgen-implanted castrates clasped a female, whereas unimplanted castrates failed to display clasping behavior.

The following experiments were designed to examine whether androgens influence the effects of neurohormones on the incidence of clasping behavior in T. granulosa. This was accomplished by experimentally controlling the concentration of androgens in the males by castration and androgen replacement therapy. The effects of neurohormone injection on the incidence of clasping behavior was measured in newts exposed to different levels of plasma androgens at various time intervals after castration.

METHODS AND MATERIALS

Experimental Animals

Locally collected male newts, T. granulosa, were maintained during the experiments as described for Experiment II of Chapter 2. Tests of male sexual activity were also conducted as described in Chapter 2.

All males used in these experiments were determined to be sexually active by a standard mating test. After these tests, males were bilaterally castrated using the following techniques. Animals were anesthetized by immersion in a 0.1% solution of MS-222 (ethyl m-amino benzoate methane sulfonic acid, from Sigma). The abdominal cavity was opened with a 5 to 10 mm right lateral incision, the testes removed and steroid capsules implanted. The incision was closed using one or two 9-mm autoclips (Clay-Adams).

Steroid capsules were prepared by packing about 3 mg testosterone (17β-hydroxyandrost-4-en-3-one), dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), or cholesterol (from Sigma) into a 1 cm segment of Silastic tubing (Dow Corning; I.D.=0.64 mm, O.D.= 1.19 mm). The ends were sealed with silicon adhesive.

Experimental Procedures

Male newts were collected on February 8, 1979. Two days later, the animals were tested for the presence of clasping behavior; the sexually active males were assigned to one of the experimental groups. All animals were castrated and were implanted with either one capsule

of testosterone plus one capsule of dihydrotestosterone or two capsules of cholesterol (20 males per group). All experimental animals appeared healthy throughout the experimental period.

The presence of clasping behavior was tested 18 days after castration; none of the males were determined to be sexually active. Males were then given a single intraperitoneal injection of either 25 µg AVT in 0.1 ml saline, or 0.1 ml saline. Animals in each implant group were equally divided into two subgroups. One subgroup received AVT, the other received saline. The occurrence of clasping was tested at 0, 2, 4, and 9 hours after injection.

Experiment II

In Experiment I, AVT influenced sexual behaviors only in cholesterol-implanted males. In order to determine whether the cholesterol implant influenced the effect of AVT on the incidence of mating behavior, male newts were collected on March 3, 1979. They were tested on the same day for the expression of clasping behavior and 30 sexually active males were castrated. These males received one of the following treatments: two capsules of testosterone plus two capsules of dihydrotestosterone, four capsules of cholesterol, or no implants (10 males per group). All animals appeared healthy throughout the experimental period.

Five days after castration, the newts were tested for the presence of clasping behavior. They were then injected intraperitoneally with either 25 µg AVT or 0.1 ml saline. Each of the three implant groups was divided equally between males that received

AVT and those that received saline. The occurrence of clasping behavior was measured at 0, 1, 4, and 18 hours after injection.

Experiment III

The first two experiments described in this chapter demonstrated that AVT influenced the incidence of clasping behavior in castrated newts that had received no androgen implants, but not in androgen-implanted newts. This finding contradicts the finding of Moore (unpublished) in which AVT affected sexual behaviors only in androgen-implanted newts. However, the animals in Moore's experiment had been castrated for 30 days, whereas animals reported here were castrated for only 18 days. Experiment III was designed to examine the possibility that the apparent inhibition of the behavioral effect of AVT by androgens occurs for only a short period of time after castration.

For this experiment, male newts were collected on March 17, 1979. On the following day, the animals were tested for the presence of clasping behavior. Sixty sexually active males were then castrated, and received one of three treatments: four capsules of testosterone plus four capsules of dihydrotestosterone, one capsule of testosterone plus one capsule of dihydrotestosterone, or no implants (20 males per group).

A single intraperitoneal injection of either 25 μg AVT in 0.1 ml saline or 0.1 ml saline was given 33 and 54 days after treatment. Each of the three implant groups was divided equally between those animals that received AVT and those that received saline.

Animals injected with AVT on day 33 were again injected with AVT

on day 54. The same was true for animals injected with saline. To determine whether injection of AVT on day 33 affected the animal's response to AVT on day 54, males that received AVT on days 33 and 54 received saline on day 63, whereas males that received saline on days 33 and 54 were injected with AVT on day 63. After the injection on each of these days, the animals were tested for expression of clasping behavior for three consecutive hours.

Statistical Analysis

Because male newts in this chapter were sexually active prior to castration, some males exhibited clasping behavior before injection of AVT or saline. Therefore, chi-square values were calculated using the number of males in a particular group that exhibited clasping behavior before the injection and compared to the number of males that displayed courtship behaviors after the injection. Fisher exact tests were also calculated for these data. Because the two statistical tests yielded the same results, only chi-square values are reported.

RESULTS

Experiment I

The injection of AVT increased the incidence of clasping behavior in castrated males implanted with cholesterol, but not in males implanted with androgens. Only one of ten androgen-implanted males clasped a female after the injection of AVT (ns), whereas seven of ten cholesterol-implanted males clasped a female after the injection (χ^2=7.5; df=1; $p<0.01$). None of the saline-injected males, in either implant group, exhibited clasping behavior, and no animal clasped a female during the test that preceded the injection.

Experiment II

Five days after castration, administration of 25 µg AVT increased the incidence of clasping behavior in castrated newts that had received no implants, but not in males implanted with androgens or cholesterol (Table 3). In the group that was castrated but given no implants, one male was sexually active prior to injection of AVT, whereas all five males displayed clasping behavior after the injection (χ^2=6.67; df=1; $p<0.01$). None of the androgen-implanted males clasped a female before the injection of AVT, and only two of five males exhibited clasping afterward (ns). Likewise, no other group showed a significant increase in the incidence of clasping behavior after injection of either AVT or saline.

Table 3. Experiment II of Chapter 2: Effects of arginine vasotocin in male newts (Taricha granulosa) castrated and implanted with androgens for a short period of time.[1]

Injection	N[2]	Implant type		
		Androgens	Cholesterol	Unimplanted
Arginine-8 vasotocin	5			
Before injection		0	2	1
After injection		2	4	5[3]
Saline	5			
Before injection		0	0	0
After injection		3	1	2

[1]The occurrence of clasping was measured at 0, 1, and 4 hours after injection. Number of males which exhibited clasping behavior during at least one test.

[2]refers to the number of animals in each experimental group.

[3]Represents a significant elevation in the incidence of courtship over that before injection. ($\chi^2=6.67$; df=1; $p<0.01$.)

Experiment III

When injected with AVT 33 and 54 days after castration, the incidence of clasping behavior was elevated in the androgen-implanted males, but not in the unimplanted males (Table 4). In the group of males implanted with a low dose of androgen, six of ten AVT-injected males displayed clasping behavior 33 days after castration, whereas none clasped a female during the test before the injection (χ^2=8.57; df=1; $p<0.01$). For the group of males implanted with a high dose of androgen, five of ten AVT-injected males exhibited clasping behavior on day 33, whereas none clasped a female prior to the injection (χ^2=6.67; df=1; $p<0.01$). No other group showed a significant elevation in the incidence of clasping behavior after an injection of either AVT or saline.

On day 54, a significantly greater number of males implanted with a low dose of androgens displayed clasping behavior after injection of AVT than in the test before the injection (χ^2=9.89; df=1; $p<0.01$). Within the group of males implanted with a high dose of androgen, a significant elevation in the incidence of clasping behavior was also seen after injection of AVT (χ^2=8.57; df=1; $p<0.01$). No other group showed a significant elevation in the incidence of clasping behavior after injection of either AVT or saline on day 54.

On day 63, six of nine androgen-implanted males clasped a female after injection of AVT; none had clasped a female in the test before the injection (χ^2=10.47; df=1; $p<0.01$). None of the 11 saline-injected males clasped a female before or after injection.

Table 4. Experiment III of Chapter 2: Effects of arginine vasotocin in male newts (Taricha granulosa) castrated and implanted with androgens for a long period of time.[1]

Injection	N[2]	Implant Type		
		Low Androgens	High Androgens	Unimplanted
DAY 33				
Vasotocin	10			
Before injection		0	0	0
After injection		60*	50*	30
Saline	10			
Before injection		0	10	10
After injection		0	10	10
DAY 54				
Vasotocin	10			
Before injection		10	0*	0
After injection		80*	60*	20
Saline	10			
Before injection		20	10	0
After injection		50	10	0
DAY 63				
Vasotocin	9			
Before injection		0		
After injection		67*		
Saline	11			
Before injection		0		
After injection		0		

[1] percent of intact males exhibiting sexual behavior during at least one test.

[2] refers to the number of animals in each experimental group.

* significant increase in the incidence of clasping: $p(\chi^2)<0.01$.

DISCUSSION

Results of these experiments indicate that androgens influence the effect of neurohormones on the incidence of clasping behavior in male rough-skinned newts. Furthermore, the influence of androgens on the behavioral effect of neurohormones changed during the 54 days after castration that the experiment was conducted. When injected five and 18 days after castration, AVT increased the incidence of courtship behavior in cholesterol implanted and unimplanted males, but not in androgen-implanted males. In contrast, when AVT was injected 33 and 54 days after castration, the incidence of clasping behavior was elevated in androgen-implanted males, but not in unimplanted males. These data support the hypothesis that endogenous neurohormones influence male sexual behavior in T. granulosa in conjunction with androgens.

In the first experiment, AVT increased the incidence of courtship behavior only in cholesterol-implanted males. It seems reasonable to propose that androgens abolished the behavioral effect of AVT in this experiment. This conclusion is based on the observation that cholesterol-implanted males in Experiment I received the same treatment as androgen-implanted males, up to and including steroid implants (cholesterol instead of androgens), yet AVT increased the incidence of clasping behavior only in cholesterol-implanted males. However, this result may have been due to the presence of cholesterol or the absence of androgens. Experiment II supports the conclusion that the absence of androgens facilitated the response to AVT, because

the incidence of clasping behavior was elevated in unimplanted newts, but not in the implanted groups.

In contrast, when AVT was injected 33 and 54 days after castration, the incidence of courtship behavior was elevated only in the androgen-implanted males. This implies that castration ultimately abolishes the animal's capacity to exhibit sexual behavior after injection of AVT. Because androgen-implanted males displayed clasping behavior, however, it is suggested that androgens ultimately maintain the animal's capacity to exhibit sexual behavior.

In order to give direction to further research, it is hypothesized that androgen replacement therapy initially inhibits the effects of AVT on sexual behavior, but later maintains a male's capacity to display clasping behavior when injected with AVT. One possible explanation for this is that androgens are involved in a negative feedback mechanism which alters the effect of neurohormone injection on the incidence of sexual behavior.

If this is true, then castration must initially increase, and androgen replacement therapy initially decrease, some factor(s) which affects the ability of neurohormones to influence male sexual behavior. The change in the influence of androgens on the behavioral effect of neurohormones after 18 days may be explained in one of two ways. If androgen implants release smaller quantities of hormone over time, perhaps the negative feedback action of androgens is uncoupled while sufficient quantities of androgens remain in the behavioral centers of the central nervous system to permit the expression of clasping. Quantitative measurements of plasma androgen concentration by

radioimmunoassay in androgen-implanted animals would clarify this point.

Alternatively, perhaps the set-point for the negative feedback mechanism is elevated by androgen implants. This may occur because implants probably release androgens at a constant rate, whereas androgens are released from the testes, at least in some mammals, in a pulsatile fashion (Bartke and Dalterio, 1975; Katongole et al., 1974). Therefore, even if implants are not releasing androgens in a pharmacological dose at any one point in time, the average level over a long period of time may be pharmacological. This constant exposure to a single level of androgens over a long period of time may elevate the set-point for the proposed negative feedback mechanism. Therefore, the animals would have compensated for the artificially high level of androgen, allowing them to exhibit clasping behavior. This would explain why there is a change in the influence of androgens on the behavioral effect of AVT over time after castration. However, this hypothesis is highly speculative and much work is needed to determine its usefulness

Castration appeared to sensitize experimental males to the effect of AVT, compared to the effect of AVT in intact males in Chapter 2. Castrated males in this chapter were found to clasp a female within an hour after injection of neurohormone, whereas the intact males in Chapter 2 did not clasp a female until eight hours after the injection. However, the experiments described in this chapter were conducted three to four months later than those in

Chapter 2, and the enhanced sensitivity could reflect a seasonal change in the sensitivity of newts to neurohormones. The apparent change in sensitivity could also be due to the fact that newts in Chapter 2 were sexually inactive prior to injection, whereas newts in this chapter were sexually active prior to castration and injected with neurohormones only after they no longer exhibited clasping behavior. An experiment in which both castrated and intact sexually inactive newts are injected with AVT is needed to clarify this point.

In summary, these experiments imply that the relationship between neurohormones and androgen on affecting the incidence of clasping behavior in male newts is more complex than originally hypothesized. Androgens appeared to initially inhibit the behavioral effect of neurohormones, although they ultimately maintained the capacity of newts to clasp a female when injected with neurohormones. A negative feedback action of androgens on some factor which affects the action of neurohormones on sexual behavior was proposed to explain this apparent contradiction.

CHAPTER 4

Results from experiments described in Chapter 2 and Chapter 3 demonstrate that an injection of neurohormones can induce clasping behavior in sexually inactive male newts. Furthermore, when castrated for only a short period of time, AVT increased the incidence of courtship behavior in newts that had received no androgen implants at the time of castration, but not in males that had received androgens. In contrast, when males were castrated for longer periods of time, AVT elevated the occurrence of clasping behavior in androgen-implanted males, but not in unimplanted males.

One hypothesis explaining the differential effect of androgens on the behavioral response to AVT is that androgens are involved in a negative feedback mechanism on some factor, which in turn, alters the effect of neurohormones on the incidence of clasping behavior.

In light of the known negative feedback relationship between androgens and pituitary gonadotropins (see Turner and Bagnara, 1976), it seemed reasonable to suspect that the pituitary may be involved in the influence of androgens on the behavioral effect of AVT. The following experiments were designed to examine whether the pituitary gland is necessary for AVT to influence the incidence of clasping behavior in castrated newts.

METHODS AND MATERIALS

Experimental Animals

Locally collected male newts, T. granulosa, were maintained during the experiments as described in Chapter 3. Tests of male sexual activity, and castration, were also conducted as in Chapter 3.

Prior to hypophysectomy, male newts were anesthetized in a 0.1% solution of MS-222 (from Sigma Chemical Co.). The pituitary gland was exposed by cutting a small hole (about 2 mm) in the parasphenoid bone. A dental drill equipped with a piece of sharpened brass tubing (outer diameter 2 mm) was used to cut this hole. The pituitary was removed with a pair of curved microforceps and the wound was sealed with dental cement. In sham operations, this procedure was duplicated but the pituitary was not removed. Success of the operation was verified by dark-background adapting the animals for 24 hours. Melanophores in the tail fail to expand (background adapt) in animals that were successfully hypophysectomized. Only those males determined to be hypophysectomized were used in these experiments.

In animals that received a pituitary implant, freshly excised pituitaries were inserted into a subcutaneous pocket, formed by a sharpened probe, in the gular region. The wound was sealed with either carboxylate cement (Experiment I), or "New Skin" (Natcon Chemical Co.) (Experiment II).

Experimental Procedures

Experiment I

Male newts were collected on March 16, 1979. On April 3, 1979, the animals were determined to be sexually active by a standard mating test, then were castrated. Half of these newts were also hypophysectomized, the others were sham-operated. Five days after the operations, males were injected intraperitoneally with 25 µg AVT or 0.1 ml saline. Clasping was measured 0, 1, 3, 6, and 8 hours later.

Seven days after surgery, each hypophysectomized male received a freshly excised pituitary from a donor male. Donor males were intact and sexually active (as determined by a pretest), and had not been used in any experiment prior to the operation. On the following day, pituitary-implanted males were injected with either 0.1 ml saline or 25 µg AVT. Mating tests were conducted for three consecutive hours. No animals died and all appeared healthy during the experimental period.

Experiment II

Male newts were collected on March 6, 1979. On the following day, 20 sexually active males were castrated according to the techniques described in Chapter 3. These males then received one Silastic capsule of testosterone plus one capsule of dihydrotestosterone.

Sixty-six days after castration, the animals received one of three treatments: hypophysectomy (n=7), sham hypophysectomy (n=6), or hypophysectomy plus transplantation of the pituitary to the

gular region (n=7). One animal in the first and third groups died, but all other newts appeared healthy throughout the course of the experiment.

Ten days after the operations, the animals were given an injection of 25 µg AVT in 0.1 ml saline or 0.1 ml saline. Each experimental group was equally divided between males that received AVT and those that received saline. Twenty-four hours later, the injection groups were reversed and AVT and saline were again injected. Mating tests were conducted for three consecutive hours after each injection.

Statistical Analysis

Because male newts in this chapter were sexually active prior to castration, some males exhibited clasping behavior before injection of AVT or saline. Therefore, chi-square values were calculated using the number of males in a particular group that exhibited clasping behavior before the injection and compared to the number of males that displayed courtship behaviors after the injection. Fisher exact tests were also calculated for these data. Because the two statistical tests yielded the same results, only chi-square values are reported.

RESULTS

Experiment I

Injection of AVT increased the incidence of courtship behavior only in males that were sham hypophysectomized. Five of six sham-operated males clasped a female after injection of AVT, whereas none had clasped a female prior to the injection (χ^2=8.57; df=1; $p<0.01$). The incidence of courtship was not affected in any other group after injection of either saline or AVT (Table 5).

Experiment II

The incidence of clasping behavior was elevated in hypophysectomized males and in pituitary-transplanted males. Although none of the animals clasped a female during the test prior to the injections, six of six hypophysectomized males (χ^2=12.00; df=1; $p<0.001$), and four of six pituitary-transplanted males (χ^2=6.00; df=1; $p<0.02$) clasped a female after injection of AVT (Table 6). In sham-operated males, injection of AVT did not significantly elevate the incidence of courtship behaviors over that of the pre-injection level. Small sample size, however, is probably responsible for this result. No saline-injected animal clasped a female before, or after, the injections

Table 5. Experiment I of Chapter 4: Effect of hypophysectomy and arginine vasotocin injections on the induction of clasping behavior in castrated newts.[1]

Treatment	N[2]	Percent
Hypophysectomy		
Arginine vasotocin	8	25
Saline	8	0
Sham-operate		
Arginine vasotocin	6	83[3]
Saline	6	0
Pituitary-implant		
Arginine vasotocin	8	37
Saline	8	0

[1] percent of experimental males exhibiting sexual behavior during at least one test. None of the animals clasped a female in the pretest.

[2] refers to the number of animals in each experimental group.

[3] represents a significant elevation in the incidence of courtship behavior over the preinjection level: $\chi^2=5.48$; df=1; $p<0.01$.

Table 6. Experiment II of Chapter 4: Effect of hypophysectomy, androgen replacement therapy, and arginine vasotocin injections on the induction of sexual activity in castrated newts.[1]

Treatment	N[2]	Percent
Hypophysectomy		
Vasotocin	6	100[3]
Saline	6	0
Sham-operate		
Vasotocin	6	50
Saline	6	0
Pituitary-transplant		
Vasotocin	6	67[4]
Saline	6	0

[1] percent of experimental males exhibiting sexual behavior during at least one test. None of the animals clasped a female in the pretest.

[2] refers to the number of animals in each experimental group.

[3] represents a significant elevation in the incidence of courtship behavior over the preinjection level: $\chi^2=12.00$, df=1; $p<0.001$.

[4] represents a significant elevation in the incidence of courtship behavior over the preinjection level: $\chi^2=6.00$; df=1; $p<0.02$.

DISCUSSION

These experiments were designed to examine whether the pituitary gland is necessary for AVT to influence the incidence of clasping behavior in rough-skinned newts. The results of Experiment I support the hypothesis that the pituitary is required for an injection of AVT to influence the incidence of clasping in male newts. When injected five days after the operation, AVT increased the incidence of courtship behavior in sham-operated males but not in hypophysectomized males. If the absence of the pituitary gland was the reason these males failed to exhibit clasping behavior following the injection of AVT, then implanting a pituitary should have restored their capacity to respond behaviorally to the neurohormone. However, this did not occur in Experiment I. Injections of AVT did not elevate the occurrence of clasping in pituitary-implanted newts.

There are numerous explanations for these results. Perhaps the pituitary is required to remain in connection with the hypothalamus for AVT to affect behaviors. On the other hand, perhaps behavioral centers in the hypothalamus were damaged during the surgical removal of the pituitary. Another explanation is that hypophysectomy causes a physiological stress which abolishes the behavioral response to neurohormones. Experiment II was conducted to clarify these points.

Results of Experiment II, in contrast to Experiment I, demonstrate that the pituitary is not necessary for AVT to affect the occurrence of clasping behavior. AVT increased the incidence of

clasping behavior in hypophysectomized males and pituitary-transplanted males. Although the injection of neurohormone did not significantly affect clasping in the sham-operated males, this was probably due to the small sample size.

Comparison of these two experiments is difficult because males in Experiment I were castrated and hypophysectomized at the same time, whereas males in Experiment II were castrated 66 days before hypophysectomy and had received androgen implants at the time of castration.

Androgen replacement therapy over a long period of time may cause an increase in some factor from the pituitary which influences the behavioral effect of neurohormones. Therefore, a sufficient amount of this factor may have been present at the time of the injection of neurohormone such that the behavioral effect was seen. This speculation is not likely, however, because this hypothesized factor would have to be present at least ten days after removal of the pituitary.

Results of Experiment II also agree with published results of similar findings in Fundulus. Macey et al. (1974) reported that AVT induces the spawning reflex response in hypophysectomized, castrated fish. However, since they abolished this response with lesions in the preoptic area, they hypothesized that AVT may act directly on this brain center. This intriguing hypothesis remains to be tested in Taricha.

In summary, the pituitary was not found to be necessary for injections of AVT to affect the incidence of clasping behavior under

the conditions of the experiment. This indicates that the influence of AVT on the incidence of courtship behaviors in Taricha is not mediated by a factor released from the pituitay gland.

GENERAL DISCUSSION

Results from experiments described in the preceding chapters demonstrate that administration of exogenous neurohormones elevate the incidence of clasping behavior in male rough-skinned newts, T. granulosa. Injections of either AVT or AVP elevate the incidence of courtship behavior in intact males, as well as restore these behaviors to castrated newts. Furthermore, androgen implants influence the action of an AVT injection on the incidence of clasping behavior in castrated newts. Moore (1978a) and Palka and Gorbman (1973) hypothesize that another factor, besides androgens, is involved in the endocrine regulation of male sexual behavior in amphibians. Data reported here support the hypothesis that an endogenous neurohormone is the proposed second factor.

Wada and Gorbman (1977) hypothesized that the testes of R. pipiens secrete something that promotes transfer of testosterone into the sexual behavior centers of the brain. This hypothesis was based on the observation that systemic injections of testosterone propionate failed to influence the incidence of clasping behavior, whereas intracranial implants did increase the incidence of clasping. In light of these studies, in addition to the observation that neurohormones are known to selectively alter the permeability of some membranes (see Bentley, 1976), Moore hypothesized that neurohormones in Taricha may alter the permeability of the blood-brain barrier to androgen, thus promoting the uptake of the steroids into the sexual behavior centers of the brain. Preliminary evidence

indicates that AVT does influence the uptake of androgen in Taricha brain tissue (Moore, Crews, and Baum, unpublished research). Other hypotheses exist, however. For example, AVT may influence the incidence of sexual behavior by affecting enzyme systems that alter the rate of aromatization of androgen to estrogen, or that modify the ratio of testosterone to dihydrotestosterone in situ. However, these hypotheses are very speculative and no solid evidence exists to support one over another.

Neurohormones are also implicated in the hormonal control of behavior in other vertebrate classes. In addition to the evidence discussed previously concerning killifish (Wilhelmi et al., 1955; Pickford and Strecker, 1977) and R. pipiens females (Diakow, 1978), there is also evidence that neurohormones influence behavior in mammals. Delanoy et al. (1978) reported that intracerebroventricular injections of AVT, AVP, and other neurohypophysial peptides into mice modify the amount of time spent in performing activities such as grooming, foraging, and ingesting. deWied and Gispen (1977) found that neurohormones affect learning behavior in rats.

The effect of neurohormones on sexual behavior in mammals has not received much attention. However, AVT, which is thought to be synthesized in the ependymal cells lining the pineal stalk in adult mammals (Pavel, 1971), has been found to affect the pituitary-gonad axis (Pavel et al., 1973), perhaps by acting on the hypothalamus. Since there is evidence that male sexual behavior in mammals is not controlled solely by androgens, it is interesting to speculate that

a neurohormone such as AVT, found in every vertebrate from cyclostomes to humans, is involved in the control of sexual behavior even in mammals. Clearly, this area of research offers many opportunities.

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APPENDICES

APPENDIX A

PROCEDURE FOR RADIOIMMUNOASSAY OF PLASMA ANDROGENS

Plasma androgen concentrations were measured using the procedure that was originally validated for plasma from newts by Specker (1978). This radioimmunoassay is based on the procedure from Louis et al. (1973), with modifications to accomodate the low plasma volumes available from individual newts. The antiserum, anti-testosterone-11-BSA serum (#S250 received from Gordon Niswender), reacts appreciably with testosterone (17β-hydroxyandrost-4-en-3-one) and dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) according to Louis et al. (1973). Since these two steroids are present in this species (Moore and Muller, 1977) and were not chromatographically separated, measurements represent both of these androgens.

Plasma samples were extracted in duplicate using 25 µl of sample. To calculate extraction efficiency, ^{3}H-testosterone tracer (2150 dpm in 10 µl ethanol) was added to the sample. All tubes were vortexed for 10 seconds and allowed to equilibrate at room temperature for 30 minutes. Plasma was extracted using 2 ml redistilled benzene-hexane (1:2). Following vigorous vortexing (30 seconds), the tubes were frozen at -20°C for a minimum of one hour to allow for freezing of the aqueous phase. For calculation of the extraction efficiency, 500 µl of the organic phase was then transferred to scintillation vials, air-dried at 40°C and counted in 10 ml toluene based scintillation fluid (formula in Appendix B). Each sample was corrected for extrac-

tion efficiency; the average extraction efficiency was 95.7±1.8% (mean±SE).

Two sets of standards were prepared. Each set contained 0, 25, 50, 100, 250, 500, 1000, and 2000 pg testosterone per 100 µl absolute ethanol. They were air-dried at 40°C and included in each assay.

The antiserum (100 µl), diluted 1:24,000 with 0.1% gelatin in 0.01M phosphate-buffered saline (GPBS, pH=7), was added to each tube and the contents vortexed. Following a 20-minute incubation at room temperature, ^{3}H-testosterone (43,137 dpm in 100 µl 0.1% GPBS) was added to all tubes and vortexed. The tubes were then incubated for 16 to 22 hours at 5°C.

At the end of this second incubation period, the tubes were placed in an ice bath for five minutes. One ml dextran-charcoal (0.25 g Dextran T-70 (Pharmacia)/l, 2.5 g Neutralized Norit (Sigma)/l in GPBS.) was added to incorporate the free testosterone into a precipitate. After a 10-minute incubation in an ice bath, the tubes were centrifuged at 3000 rpm (2500 x g) for ten minutes at 4°C. A fraction of the supernatant (0.7 ml) was added to 7 ml toluene: Triton X-100 scintillation fluid (formula in Appendix B) and counted for ten minutes or 10,000 counts in a liquid scintillation spectrometer. The average binding for the antiserum was 54.7±4.2% (mean±SE) of which 2.3% was non-specific. The sensitivity of the assay, the smallest amount significantly different from zero (as determined by Specker, 1978) was 10 pg/tube or 0.33 ng/ml.

APPENDIX B

SCINTILLATION FLUID FORMULAE

Toluene-based Scintillation fluid

5.0 g 2.5-diphenyl oxazole (PPO)/1 toluene (technical grade)

Triton Scintillation Fluid

7.0 g PPO/1 toluene and Triton-X 100 (2:1)