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A Demonstration of Photoresponsiveness in Laboratory Rats using Whole Animal and Neuroendocrine Approaches

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A DEMONSTRATION OF PHOTORESPONSIVENESS IN LABORATORY RATS USING WHOLE ANIMAL AND NEUROENDOCRINE APPROACHES

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This thesis is submitted in partial fulfillment of

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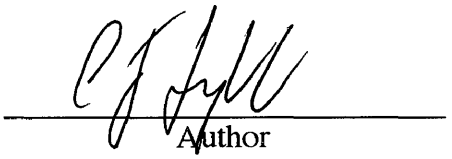

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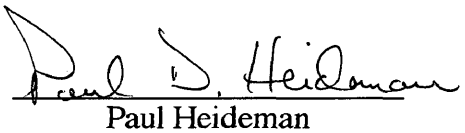

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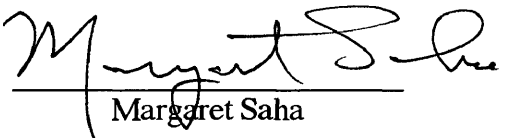

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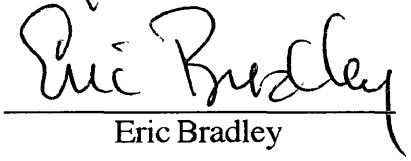

Eric Bradley

TABLE OF CONTENTS

page

ACKNOWLEDGMENTS iv

LIST OF TABLES v

LIST OF FIGURES vi

ABSTRACT vii

CHAPTER I. INTRODUCTION 2

CHAPTER II. REPRODUCTIVE PHOTORESPONSIVENESS IN THE FISCHER 344 LABORATORY RAT 16

CHAPTER III. CHANGES IN GNRH NEURONAL ABUNDANCE AND MORPHOLOGY IN REPRODUCTIVELY INHIBITED F344 RATS. 23

CHAPTER IV. EFFECTS OF SHORT DAY LENGTHS ON MELATONIN BINDING IN THE PARS TUBERALIS OF FISCHER 344 LABORATORY RATS 32

CHAPTER V. CONCLUSION AND FUTURE DIRECTIONS 40

LITERATURE CITED 43

TABLES 1-9 57

FIGURES 1-8 66

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LIST OF TABLES

Table Page

1. Comparison of testis size and body weight of Fischer 344 Rats (F344) in SD and LD. 57

2. Comparison of testis size and body weight of Harlan Sprague-Dawley Rats (HSD) in SD and LD. 58

3. Testicular mass and volume mass of Fischer 344 Rats (F344) following 6-18 days in SD or LD. 59

4. The effects of pinealectomy on body mass and testis size of rats reared in short days from 21 days of age. 60

5. GnRH neuronal abundance of Harlan Sprague-Dawley Rats (HSD) versus Fischer 344 (F344) Rats in SD. 61

6. GnRH neuronal abundance of Harlan Sprague-Dawley Rats (HSD) versus Fischer 344 (F344) Rats in LD. 62

7. GnRH neuronal abundance of Harlan Sprague-Dawley Rats (HSD) and Fischer 344 (F344) Rats LD versus SD. 63

8. GnRH soma size in SD versus LD treatments in Harlan Sprague-Dawley Rats (HSD) and Fischer 344 (F344) Rats. 64

9. Specific binding of IMEL in Pars Tuberalis of Fischer 344 (F344) rats after 2 weeks of photoperiod treatment. 65

LIST OF FIGURES

Figure Page

1. Schematic diagram showing environmental regulation of the reproductive axis. 66

2. Relationship between calipered testis length and actual testis length 67

3. Relationship between calipered testis width and actual testis width. 68

4. Relationship between calipered testis volume and actual testis volume 69

5. Coronal rat brain sections identifying the locations of GnRH cell soma categories 1-3. 70

6. Coronal rat brain sections identifying the locations of GnRH cell soma categories 4 and 5. 71

7. Staining of GnRH soma with the monoclonal antibody, both preoptic and diagonal band neurons are shown. 72

8. 2 - ^{125}I - melatonin (IMEL) binding in the pars tuberalis (PT) of Fischer 344 Rats (F344) maintained in LD and SD. 73

ABSTRACT

This study was conducted to describe the photoresponsive characteristics of the Fischer 344 (F344) male laboratory rat. First I compared the reproductive response of juvenile male F344 rats to short days (SD) (8:16, lights on at 0900) or long days (LD) (16:8, lights on at 0500). Exposure to SD for two weeks caused a 58% difference in the testis volume of SD rats when compared to the LD counterparts. This trend persisted for the 4 weeks; by 8 weeks of SD exposure there was no difference between SD and LD with regard to testis volume. The SD animals also displayed significantly lower body weight after 2 weeks of SD exposure. The differences in body weight persisted for 8 weeks. Pinealectomies performed on juvenile F344 rats abolished the reproductive responses caused by SD, showing that the F344 reproductive response to changes in daylength is mediated by the pineal gland.

Investigations at the cellular level examined the role of gonadotropin-releasing hormone (GnRH) neuronal abundance and morphology. Comparisons were made between F344 and a non-photoresponsive strain of laboratory rat, Harlan Sprague-Dawley (HSD), exposed to both LD and SD for 10 days. GnRH was detected using a monoclonal antibody for the peptide. No differences were observed in GnRH neuronal abundance or GnRH neuron morphology between the two strains or between treatment groups. The differences between F344 and HSD rats in photoperiodic response apparently is not due to differences in GnRH numbers or morphology.

To assess whether the F344 rats are typical of other seasonal rodents the distribution of melatonin receptors was examined. Seasonal rodents typically display a decrease in melatonin receptor levels in th pars tuberalis (PT) when exposed to SD. Using autoradiographic techniques with a radioligand for the melatonin receptor, 2 - ^{125}I - melatonin, melatonin receptor regulation was examined in the PT of F344 rats exposed to LD or SD for two weeks. No differences were detected in melatonin receptor density in the pars tuberalis.

Photoresponsiveness was demonstrated in male F344 laboratory rats; this marks the first time in which an unmanipulated laboratory rat displayed photoresponsiveness in a robust and repeatable fashion. F344 male laboratory rats appear to have adequate numbers of GnRH neurons when compared to other non-photosensitive strains of laboratory rats. It appears as though F344 laboratory rats are not typical of other seasonal rodents with regard to melatonin receptor regulation in the PT.

A DEMONSTRATION OF PHOTORESPONSIVENESS IN LABORATORY RATS

CHAPTER ONE

Introduction

Part One. *Photoperiodic Regulation of Reproduction*

The importance of reproduction

It is an axiom of evolutionary biology that all organisms are driven to reproduce successfully or to maximize their inclusive fitness. It can be argued that the most important act of an animal is reproduction. It is conceivable that if an individual is deficient in one aspect of its fertility, its genes will be rapidly eliminated from the population. It is clear that reproductive adaptations that enable reproduction to occur with greater efficiency will be favored by natural selection.

Environmental regulation of reproduction in mammals

The environment plays an important role in shaping a mammal's reproductive strategy. Due to the high costs of reproduction, mammals must be able to coordinate reproductive events with environmental changes in order to maximize reproductive success. Mistakes can result in reproductive failure. To reproduce optimally, a mammal needs to adjust to environmental conditions when making reproductive decisions. Natural selection favors adaptations that cause reproduction to occur in harmony with environmental variation (Bronson and Heideman, 1994). Bronson and Heideman (1994, p.555) described two strategies that mammals can adopt when adjusting to environmental variation. "First, some mammals react directly to seasonal changes in climate and food availability. These individuals will attempt to reproduce depending on the daily permissiveness of these environmental conditions. Conversely, a mammal could react to cues that predict oncoming periods of time when climatic and dietary conditions will permit successful reproduction. This latter strategy allows the individual

to prepare metabolically for the upcoming reproductive events.". The best known such cue that allows this latter strategy is photoperiod (Bronson and Heideman, 1994).

Photoperiod as a regulator of reproduction

Photoperiod is the predominant seasonal cue used by mammals that inhabit the temperate zone (Bronson and Heideman, 1994). It is in these regions that seasonal variation in the amount of daylight per day is most pronounced and therefore provides the most reliable information. Bronson and Heideman (1994) hypothesize that there are at least three general strategies by which mammals use photoperiod to regulate reproduction. First, a "critical" daylength could both initiate and terminate gonadal activity for those species that need to reproduce more than once a year. Second, if the individual relies on an endogenous timer to determine the length of the breeding or non-breeding season, it could use photoperiod to either initiate or terminate gonadal activity, but not both. Finally, an individual's reproductive cycle could be dictated by an endogenous circannual rhythm that is synchronized with seasonal climatic and dietary changes by photoperiod.

Current evidence suggests that the first strategy, in which a certain photoperiod both initiates and terminates the breeding season, does exist (Gorman and Zucker, 1997). However, this hypothesis assumes that animals ignore the changing length of the days that bracket their critical photoperiod. It has become increasingly clear that a mammal is not merely measuring daylength, but rather comparing a present daylength to a previous daylength (Gorman and Zucker, 1997; Horton, 1984; Lee and Zucker, 1988; Stetson and Watson-Whitmyer, 1981). Therefore, a certain daylength might be stimulatory if the daylength immediately preceding was shorter, while, in contrast, if the same daylength is preceded by a longer daylength, the animal would perceive that as an inhibitory signal.

The second photoresponsive strategy is used by the Syrian hamster, *Mesocricetus auratus*, which uses a critical daylength to terminate gonadal development but not to initiate gonadal development (reviewed by Bronson and Heideman, 1994). In this species, 12 hours or less of light results in gonadal regression. However, after 4-5 months of these inhibitory daylengths, the animals become refractory and become reproductively active as they undergo gonadal recrudescence. Gonadal recrudescence is a phenomenon in which an extended period of inhibitory daylengths stimulates gametogenesis. This phenomena is an advantage to the Syrian hamster in the wild, in that the gonads become active after 4-5 months of inhibitory daylengths. Therefore, the animal becomes sexually active well before the daylengths are greater than 12 hours.

The third strategy has been documented in only a few species (Bronson and Heideman, 1994). Kenagy (1980) showed that , when maintained in a constant daylength, ground squirrels display an endogenous circannual rhythm in testes size. In this case, photoperiod acts to synchronize individuals within a population (Bronson and Heideman, 1994).

It is clear that photoperiod can play a large role in the timing of reproductive events. Furthermore, photoperiod acts in concert with numerous other cues, including diet, social interactions, temperature, and other physical cues. These cues are somehow integrated at the level of the central nervous system and cause either a stimulation or inhibition of the animal's reproductive axis.

Hormonal control of reproduction

Information about external conditions is transduced and integrated by neural mechanisms before affecting reproductive status (Rissman, 1996). This integration of stimuli occurs in the hypothalamus, and the regulation of reproduction begins with inputs to the hypothalamic gonadotropin releasing hormone (GnRH) neurons (Figure 1).

GnRH is a decapeptide hormone that has been highly conserved throughout the vertebrate lineage. High performance liquid chromatography and radioimmunoassay have indicated that the form of GnRH present in mammals is also present in the order Dipnoi, with a 50% sequence identity (Sherwood et al., 1993). Therefore, it appears as though the mammalian form arose around 400 million years ago, before the evolution of the teleostian fishes (Sherwood et al., 1993). The conservation of GnRH structure reflects the importance of this peptide for normal reproductive function.

In mammals the GnRH neuronal population is comprised of approximately 1200-1400 neurons that are loosely distributed from the olfactory bulb to the mamillary body (Sagrillo et al., 1996). Approximately 25-50% of the GnRH neurons reside in the olfactory bulb and related structures (Wray and Hoffman, 1986). This subpopulation of GnRH cells is associated with the nervus terminalis and olfactory-related pathways (Silverman et al., 1994; Witkin and Silverman, 1983) and probably functions to coordinate olfactory stimuli with reproductive events (Sagrillo et al., 1996).

GnRH-like neurons have been identified in the cortex, the amygdala, and the midbrain, and in no area do they make up more than a few percent of the neuronal population (Silverman et al., 1994). It is possible that the diffuse location of the GnRH neurons maximizes the potential for input from other neuronal types containing different neurotransmitters. These types of interactions could be direct or indirect, but nevertheless affect the production and/or release of GnRH.

External stimuli are integrated at the level of the hypothalamus (Figure 1). The hypothalamus is therefore of great importance to those studying how environmental factors affect reproduction. However, only a few areas in the hypothalamus contain relatively high concentrations of GnRH neurons. More specifically, GnRH cell bodies are rare outside of the pre-optic area (POA) and the arcuate nucleus (AN); this has been confirmed by immunohistochemical staining for the GnRH peptide (Silverman et al., 1994). In contrast, GnRH terminals are more common and can be found throughout the

hypothalamus. One area with a high concentration of GnRH terminals is the median eminence (ME), as revealed by immunohistochemical staining.

The ME is a designation used to describe the base of the hypothalamus and the infundibulum, or stalk of the hypothalamus. This area is unique because of the confluence there of neural and blood-born messages that regulate adenohypophyseal function (McCann and Ojeda, 1996). It also contains a capillary plexus that is connected with the hypothalamic-pituitary portal system. The ME is therefore of special importance in the regulation of reproduction because of the integration that occurs at this site.

GnRH hormonal action occurs at the level of the adenohypophysis; it controls the secretion of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Figure 1). Both FSH and LH are synthesized by adenohypophyseal cells, the gonadotrophs, in response to GnRH binding to receptors on the adenohypophysis. The GnRH receptor is a heterotrimeric G-protein-coupled receptor (Stojilkovic et al., 1994). When GnRH is bound, a stimulation of multiple phospholipase activities occurs within the gonadotroph cell membrane (Stojilkovic et al., 1994). A significant amount of "cross-talk" occurs between the phospholipase pathways, which results in differential modulation of the inositol 1,4,5-trisphosphate and diacylglycerol signals. These two second messengers then causes an increase in the concentration of cytoplasmic calcium (Stojilkovic et al., 1994). The increase in cytoplasmic calcium causes a subsequent increase in synthesis and release of LH and FSH.

In a male mammal, LH stimulates Leydig cell development and testosterone production at the level of the testes. In the female, LH stimulates final development of the follicle, ovulation, and the secretion of estrogen from the ovary. FSH functions to stimulate spermatogenesis in the male and aids in the development of the ovarian follicles in the female. More specifically, FSH is responsible in the female for the early

maturation of the follicle and then, in concert with LH, for the final maturation of the follicle (Johnson and Everitt, 1984).

Control of GnRH secretion

GnRH transcription is controlled by a variety of neural and humoral factors; these factors can be broken down into four broad categories: catecholamines, excitatory and inhibitory amino acids, peptides, and steroids. Using double-label ultrastructural techniques, GnRH neurons have been shown to receive synaptic input from afferent neurons containing norepinephrine (NE) (Chen et al., 1989b; Palkovits et al., 1982; Watanabe and Nakai, 1987), dopamine (DA) (Horvath et al., 1993; Kuljis and Advis, 1989; Nakai et al., 1985), serotonin (5HT) (Kiss and Halasz, 1985), gamma-aminobutyric acid (GABA) (Horvath et al., 1993; Leranth et al., 1985; Leranth et al., 1988a; Thind and Goldsmith, 1995; Witkin, 1992), glutamate (Goldsmith et al., 1994; Thind and Goldsmith, 1995), corticotropin-releasing hormone (CRH) (MacLusky et al., 1988), substance P (Tsuruo et al., 1991), neuropeptide Y (Tsuruo et al., 1990), proopiomelanocortin (POMC) (Leranth et al., 1988b), and ß-endorphin (Chen et al., 1989a). Many questions still exist as to the hierarchy that exists among these neurotransmitters for control of GnRH synthesis.

Catecholamine effects on GnRH mRNA levels have been well documented. NE, when administered through the third ventricle, causes a 50% increase in GnRH mRNA levels within 1-4 hours after administration (He et al., 1993). Use of the adrenergic α-1 receptor antagonist prazosin greatly reduces GnRH mRNA 24 hours after administration (Weesner et al., 1992). DA seems to have effects similar to those seen with NE. Administration of bromocriptine, a dopaminergic D2 receptor agonist, causes a 67% increase in GnRH mRNA after 2 weeks (Li and Pelletier, 1992). Use of haloperidol, a dopaminergic D2 receptor antagonist, caused a 31% decrease in GnRH mRNA after two weeks (Li and Pelletier, 1992). Similar effects are seen in GnRH

release as well. In the GT1 GnRH immortal neuronal cell lineage NE stimulates GnRH release in a dose-dependent fashion (Martinez de la Escalera et al., 1992).

Excitatory amino acids such as N-methyl-D-aspartate (NMDA) appear to be nonspecific stimulators of GnRH transcription. Within 15 minutes of an intravenous injection of NMDA an increase in GnRH mRNA can be seen (Petersen et al., 1991).

The data regarding the effects of GABAergic compounds on GnRH mRNA are controversial. It appears that GABA has a dual role in mediating control over hypothalamic reproductive hormones. GABA has an inhibitory role on LH secretion but an excitatory role in the regulation of GnRH release. These data come from studies in which GABA was infused into the ME (Vijayan and McCann, 1978). It has been proposed that GABAergic neurons in the POA mediate the negative feedback action of steroids (Demling et al., 1985; Flugge et al., 1986). Furthermore, the possibility exists that GABAergic neurons in the POA may directly inhibit GnRH release, since GABA-containing terminals synapse with GnRH-containing neurons (Leranth et al., 1985). This hypothesis has been supported by data gathered from studies on GT1 cells in which a biphasic response is seen in GnRH release. Administration of GABA causes a rapid stimulation followed by a delayed inhibition. The two different responses appear to be mediated by two different GABA receptors present on the GT1 cells.

As expected, control of GnRH secretion is inherently complex. In a majority of the aforementioned studies the only parameter measured was GnRH mRNA levels. Previously, it was assumed that GnRH transcription is closely correlated with GnRH release; if so, mRNA levels are indicative of serum hormone levels. However, in a recent study using immunocytochemical techniques, Korytko et al. (1995) suggested that GnRH release can be affected by inhibitory photoperiods in male deer mice, *Peromyscus maniculatus*. By measuring GnRH neuron soma area and staining density of the soma Korytko was able to show that short photoperiods cause an increase in the number of immunoreactive cell bodies. Furthermore, in those mice that display

testicular regression, GnRH soma area increases. Presumably, during short days there is a decrease in GnRH release from the cell body, which accounts for the increase in immunoreactivity. Additionally, those animals that are most reproductively responsive to short days show drastic changes in GnRH neuronal morphology. It appears that in the responsive animals, there is an actual swelling of the soma due to a lack of GnRH release.

It is clear that control of GnRH secretion occurs in the hypothalamus. The hypothalamus plays an integral role in a mammal's ability to interpret changes in daylength. However, the specific neuroendocrine inputs that mediate a mammals response to inhibitory photoperiods are unknown.

Part Two. ***How Mammals Detect Changes in Daylength***

The route by which mammals detect daylength has been well characterized (Tamarkin et al., 1985). Photic information impinges on the eye; this information then produces a stimulus that travels along the retino-hypothalamic tract. This tract terminates at a hypothalamic nucleus, the suprachiasmatic nuclei (SCN), and the impulse continues through this nucleus to another hypothalamic nucleus, the paraventricular nucleus (PVN). From there the impulse travels to the superior cervical ganglion (SCG) of the neck. Here, the signal becomes transformed from a strictly neuronal signal to one that is more endocrine in nature. Sympathetic nerve fibers from the SCG travel up to the pineal gland where they release NE from their terminals. The NE is then bound by β-adrenergic receptors on the pineal gland.

The pineal gland is the primary site for production of circulating melatonin. The rate of melatonin production is controlled primarily by one enzyme, N-acetyl transferase. The levels of this enzyme increase as NE is bound by β-adrenergic receptors on the pineal gland. The rise in N-acetyl transferase causes the conversion of

5-HT to melatonin inside the pinealocytes. It is assumed that melatonin production is tightly coupled to melatonin secretion.

Melatonin production is highest in the absence of light. Hence, the pattern of melatonin secretion closely mimics the daily light:dark cycle. Long durations of melatonin secretion are indicative of short days. If the secretion persists for a period longer than some threshold, reproduction becomes inhibited in photoresponsive rodents. This threshold is species-specific, but in most species it is produced by a night of 14 hours or more.

The melatonin receptor has a high affinity (KD < 200pM) for melatonin. The receptor is a heterotrimeric G-protein coupled receptor and binding of melatonin leads to the inhibition of adenylyl cyclase. The gene for the receptor was cloned in by Reppert et al. (1994); the gene encodes a 440 amino acid protein. To date three subtypes of the melatonin receptor have been identified; Mel1a, Mel1b and Mel1c. Recently, Liu et al. (1997) demonstrated distinct roles for the Mel1a and Mel1b receptors in the rodent SCN. Liu et al. suggest that these two receptor subtypes have different roles in mediating circadian responses to changes in daylength. It is not known whether this difference in action also plays a role in the reproductive responses to inhibitory daylengths.

The exact means by which melatonin affects reproduction remains unknown (Morgan et al., 1994). It is clear that melatonin must be acting at one, two, or all of the three levels in the hypothalamic-pituitary-gonadal axis. Because of the importance of the hypothalamus in regulating adenohypophyseal function, it is currently hypothesized that melatonin is acting indirectly on GnRH neurons of the hypothalamus. This hypothesis is supported by the findings that melatonin is taken up (Anton-Tay and Wurtman, 1969) and bound (Niles et al., 1976) in the hypothalamus. Experiments involving intracranial administration of melatonin in *Peromyscus leucopus* (Glass and Lynch, 1981; Glass and Lynch, 1982) found that target sites for melatonin's

antigonadal action are localized to the anterior hypothalamus and the medial preoptic area. Furthermore, electrolytic lesions of the anterior hypothalamus abolish the gonadal response to changes in photoperiod (Hastings et al., 1985; Rusak and Morin, 1976). However, interpreting the results from lesion studies is difficult. It is not clear whether the nuclei that were lesioned caused these effects or whether they are the result of damage to other adjacent nuclei.

The hypothalamus plays a central role in the body's regulatory processes, functioning as a regulatory center for physiological control. Specifically, the SCN and the PVN of the hypothalamus are integral nuclei involved in interpreting daylength. However, several additional nuclei have been implicated in the mammalian reproductive response to inhibitory daylengths. Consequently, identifying the roles of certain hypothalamic nuclei in the reproductive processes is inherently difficult due to the complexity and size of the neuronal systems that make their connections in the hypothalamus.

Part Three. *The Laboratory Rat as a Model for Photoresponsiveness*

Sexual maturation in male laboratory rats

The rat is born at a developmental stage comparable to 150 days of human gestational life (Tanner, 1974). The gestational period of the rat lasts for 22-23 days, and the first spermatozoa are seen in the lumen of the seminiferous tubules by 45 days of age (Clermont and Perey, 1957). The spermatozoa reach the vas deferens 13-14 days later (Clegg, 1960). Testicular descent occurs after day 15. Contrary to what is observed during the human juvenile period, male rodents do not display a postnatal period of testicular quiesence. In rodents, testicular development is initiated at a very early age.

Aubert et al. (1985) detected traces of GnRH in whole-brain extracts as early as gestational day 12. However, in that study, male and female tissue was pooled, and so

it is unknown whether sex differences exist during this early period of development. GnRH receptors are present in the adenohypophysis at gestational day 16 (Aubert et al., 1985). Ojeda and Urbanski (1994) suggest that this shows an active involvement of GnRH in the control of fetal pituitary function. The number of adenohypophyseal receptors increases in parallel with hypothalamic GnRH content, but with a phase delay of a few days (Ojeda and Urbanski, 1994).

Events occuring at the hypothalamic-adenohypophyseal axis

In the male rat, hypothalamic GnRH levels continue to rise throughout postnatal development (Desjardins, 1981; Payne et al., 1977). Similarly, the pituitary content of LH and FSH increases gradually with age, as does the responsiveness of the gland to GnRH stimulation (Chiappa and Fink, 1977; Dupon and Schwartz, 1971; Kragt and Ganong, 1968; Lisk, 1968). An analysis of the ontogeny of pituitary GnRH receptors has revealed a close correlation between the number of GnRH receptors and pituitary LH content. Both receptor number and pituitary LH appear to reach stable levels when the animals enter the peripubertal phase of development (30 days) (Chan et al., 1981; Duncan et al., 1983). When expressed as a concentration rather than by content, pituitary GnRH receptor levels show an increase during the first 4 weeks of life, reaching a peak at around 30 days, and then decline to the lower adult levels seen between 60 and 80 days of age (Chan et al., 1981). This decline during the latter part of sexual development is inversely correlated with rising serum testosterone levels and, therefore, suggests an increased negative-feedback action of testicular steroids on hypothalamic-pituitary function (Ojeda and Urbanski, 1994).

Data regarding LH levels during the pubertal transition in male rats are in disagreement (reviewed by Ojeda and Urbanski, 1994). However, there is a general consensus that sexual maturation in male rats is associated with an increase in FSH secretion. Serum FSH levels rise during postnatal life and reach a maximum usually

between 30 and 40 days of age. They then fall gradually, as serum testosterone concentrations increase, and attain relatively low adult levels.

Events occuring at the testis

LH receptors have been detected in the rat testis as early as gestational day 15.5 (Warren et al., 1984), and at this point the LH causes an increase in cAMP and testosterone production (Picon and Gangnerau, 1980). LH receptor numbers continue to increase and reach maximum levels around the time of birth (Ojeda and Urbanski, 1994). The Sertoli cells play a key role in the initiation of spermatogenesis, and they have been shown to possess FSH receptors as early as gestational day 17.5, reaching their maximum just before birth (Warren et al., 1984).

Pubertal changes in FSH and LH secretion precede the maturation of the testes in the rat. It is well established that FSH binds within the seminiferous tubules to facilitate spermatogenesis, whereas LH stimulates testosterone production by a direct action on the interstitial cells (Ojeda and Urbanski, 1994). Furthermore, FSH is able to up-regulate testicular LH receptors. Testicular responsiveness to LH is also enhanced by growth hormone and prolactin (Bartke, 1980; Zipf et al., 1978), both of which show a progressive rise during the pubertal transition. GnRH is also believed to play a direct role in testicular steroidogenesis. GnRH receptors have been revealed in the interstitial cells, and GnRH can directly inhibit steroidogenesis (Bourne et al., 1980), although the exact chemical identity of gonadal GnRH has yet to be identified (for reviews see Clayton, 1985; Clayton and Catt, 1981; Hsueh and Jones, 1981). In conclusion, testicular steroidogenesis appears to be regulated by two factors: the pattern of LH and FSH secretion and the responsiveness of the testes to these two hormones.

Activation of the hypothalmic-adenohypophyseal-testicular axis

It has been suggested that the number of pituitary GnRH receptors reflects, at least on a short-term basis, hypothalamic secretion of GnRH in the rat (Clayton, 1985; Clayton and Catt, 1981). "Since both the hypothalamic content of GnRH and the pituitary content of GnRH receptors begin to increase early in life, it is very probable that the developmental changes within the GnRH-releasing centers provide one of the earliest stimuli for initiating sexual maturation", (Wray and Hoffman, 1986 p.96). GnRH neurons undergo morphological changes as puberty approaches (Wray and Gainer, 1987; Wray and Hoffman, 1986). As puberty approaches, the GnRH neuronal population, which was previously comprised of neurons with smooth soma, becomes one in which the soma have many spiny processes. It is thought that the change in morphology reflects an increase in puberty-related synaptic inputs.

There also exists the possibility that the rat pituitary becomes more responsive to GnRH stimulation as puberty approaches. This idea is supported by the fact that peak gonadotropin responses occur during the peripubertal period of develpoment (Debeljuk et al., 1972; Dullart, 1977). Furthermore, androgens have been shown to have direct effects on the pituitary gland; the studies of Nazian and Mahesh (1979) imply that testosterone can potentiate the pituitary response to GnRH in immature but not adult animals.

The laboratory rat as a model

For over a century the laboratory rat, *Rattus norvegicus*, has been used in scientific research. It has been the premier model system for the study of biomedical conditions ranging from cancer and autoimmune disorders to the study of infertility. Consequently, a great deal of knowledge about rat physiology has accumulated over the decades. An important reason for the use of the laboratory rat as a model system for many disease studies is the relative lack of individual variation within laboratory strains, because of high levels of inbreeding in laboratory rat strains. Researchers are

therefore able to eliminate a large portion of variation that normally exists in a wild population of animals by using the laboratory rat. Hence, identifying actual pathways and mechanisms is facilitated because the "noise" from data obtained in wild populations is eliminated.

Not suprisingly, much of our knowledge about mammalian reproduction has been obtained from experimentation on the laboratory rat. This knowledge ranges from the molecular interactions of compounds on the testes to specific neuronal pathways that integrate dietary conditions with reproductive status. Furthermore, the majority of the rat brain has been mapped histochemically. Therefore, our understanding of how specific neuronal systems interact with each other during reproductive events is probably better documented in the rat than in any other mammal.

Part Four. ***Significance***

The means by which photoperiod regulates reproduction in mammals remains unknown. The experiments conducted herein were an attempt to understand the underlying mechanisms of photosensitivity and to further knowledge of the neuroendocrinology of reproduction.

The use of this system will enable us to highlight mechanisms that may underlie individual variation within the mammalian brain. This knowledge could prove beneficial in the field of human health, such as furthering our understanding of why certain individuals respond to drug therapies, whereas others are resistant to these therapies.

Finally, by studying individual variation in the pathway mammals use to detect changes in daylength, we could elucidate how behavior evolves. By comparing a photoresponsive and non-photoresponsive strain we can first highlight the cellular mechanisms that cause this behavioral difference, and subsequently pinpoint the molecular and genetic mechanisms that produce these differences.

CHAPTER TWO

Reproductive Photoresponsiveness in the Fischer 344 Laboratory Rat

Introduction

The laboratory rat, *Rattus norvegicus*, has been considered a non-photoperiodic species because exposure to varying daylengths does not result in significant changes in the animal's reproductive status (Reiter and Sorentino, 1971; Wallen et al., 1987; Wallen and Turek, 1981). However, a photoperiodic response can be unmasked in laboratory rats by four experimental procedures: olfactory bulbectomy (Leadem and Blask, 1982; Nelson and Zucker, 1981; Reiter et al., 1971; Wallen et al., 1987), chronic food deprivation (Sorrentino et al., 1971), neonatal androgen treatment (Reiter et al., 1969; Vanecek and Illnerova, 1982; Wallen and Turek, 1981), and chronic exposure to exogenous testosterone (Wallen and Turek, 1981). These data indicate that the laboratory rat possesses the neuroanantomical connections that mediate sensitivity to photoperiod, but that these connection are normally not functional.

The majority of laboratory rat strains tested for reproductive photoresponsiveness show no significant alterations in reproductive function in response to shifts in photoperiod alone (Reiter et al., 1971; Sorrentino et al., 1971; Wallen et al., 1987; Wallen and Turek, 1981; Wray and Hoffman, 1986). However, recent work suggests that females of one strain may be truly photosensitive (Leadem, 1988). Leadem (1988) showed that female Fischer 344 (F344) rats, when blinded, displayed a 65% reduction in uterine weight and a 25% decrease in ovarian weight after 8 weeks. However, blinding, in which photic information is entirely lost, is not necessarily equivalent to short photoperiod treatment (Tamarkin et al., 1985).

In this study I evaluated photoresponsiveness in F344 rats by exposing males to different photoperiods. Additionally, because the pineal gland mediates reproductive

photoresponsiveness (Reiter, 1980; Reiter, 1993; Reiter et al., 1968; Reiter et al., 1969), I tested for an involvement of the pineal gland in this response, by pinealectomizing individuals and exposing them to short days.

Methods

EXPERIMENT 1

Thirty 21±2 day old male Fischer 344 (F344) rats (Charles River Labs, Raleigh NC) were weighed and placed in one of two treatments. Weight-matched groups of 15 were placed in short days (SD) (8:16; lights on at 0900) and in long days (LD) (16:8; lights on at 0500). These photoperiods were chosen because previous work by Wallen et al. (1987) indicated that more than 10 hours of light stimulates reproductive organs in testosterone-treated laboratory rats, while a photoperiod less than 8 hours of light produces testicular regression. All animals were held singly in polyethylene cages (36 x 24 x 19cm) in fan-ventilated photoperiod chambers (86 x 58 x 49cm), each holding five cages. Lighting was provided by two fluorescent bulbs (G.E. cool white flourescent bulbs - 20 watts) located along the width at the ceiling of each cage. Temperature was held at 23±2° C. Food (ProLab Rat-mouse-hamster 3000; PMI Feeds St.Louis, MO) and tap water were provided *ad libitum*.

Reproductive status was assessed at approximately 2-week intervals (after 17±2, 38±2, 52±2, 66±2, 84±2, and 100±2 days). Rats were lightly anesthetized with methoxyflurane (Pitman-Moore Inc., Mundelein, IL). External testis measurements were taken from the left testis. Length and width were each measured to the nearest 0.1mm using dial calipers, and measurements of body weight were taken. Reproductive status was assessed by two individuals, both blind with respect to treatment. Testis volume was calculated using the formula for a prolate spheroid [(l x w^2) x 0.523]. Testis length and width measured through the scrotum were highly correlated with length and width of excised testes (correlation analysis: $R^2 = 0.87$, P =

0.0001 and $R^2 = 0.89$, P= 0.0001, respectively; Figures 2, 3). Similarly, the estimate of testis volume was highly correlated with testis weight ($R^2 = 0.87$, P = 0.0001; Figure 4).

EXPERIMENT 2

In order to determine whether the F344 rats were truly more reproductively responsive to photoperiod than other strains, rats of the Harlan Sprague-Dawley (HSD) strain were tested for photosensitivity. The HSD strain was chosen because previous work had shown the capacity for photoresponsiveness in older testosterone treated males, but not in unmanipulated males (Wallen et al., 1987). Because of reports that young rodents may be more sensitive to photoperiod than adults (Johnston and Zucker, 1980; Nelson and Zucker, 1981),we tested young HSD rats under treatment conditions identical to the F344 rats in experiment 1.

EXPERIMENT 3

A third experiment was done to determine the time course of response to photoperiod and reproductive organ weight and development in LD and SD conditions in more detail. In this experiment, groups (N = 4-7) of 21±2 day-old male F344 rats were exposed to either LD (16:8, lights on at 0500) or SD (8:16, lights on at 0900) for 6, 10, 13, or 25 days. After the treatments, the rats were given an overdose of sodium pentobarbital and perfused transcardially with 4% formaldehyde in 0.1 M phosphate-buffered physiological saline (pH 7.4). The testes were then excised and weighed. A qualitative assessment of gametogenesis was made on the testes of the 10-day and 25-day treatment animals. 10μm cryostat sections of one testis from each rat were processed for hematoxylin and eosin staining. Testes were assigned a rank on the basis of the highest stage of the spermatogenic cycle (according to Figure 7.16 in Setchell, 1978) found in one cross section through the testis at its widest point, with observations blind with respect to photoperiod and duration of the treatment.

EXPERIMENT 4

In order to determine whether the effects of photoperiod were mediated by the pineal gland, I compared the responsiveness of pinealectomized and sham-operated young rats to those maintained in SD. 21±2 day-old male F344 rats born and raised in our animal facility (LD 16:8; lights on at 0500) were weight-matched and divided into two treatment groups, pinealectomized (PINX; N = 12) or sham operated (SHAM; N = 8). All operations were performed using isoflourane (Ohmeda Pharmaceutical Products Division Inc., Liberty Corner, NJ) delivered through a non-rebreathing anesthesia machine (Bickford Model 61010; Wales Center, New York, NY). Animals were placed in a stereotaxis apparatus, and pinealectomies were performed following the method of Waynforth (1992) with the following modifications. A #1/2 fissure burr attached to a dental drill was used to score a 3-4 mm circle above the intersection of the transverse sinus and the superior sagittal sinus. The disc of bone was gently removed to expose the dura matter. For the pinealectomy, the dura matter and sinus were punctured, blood was removed with suction, and the pineal gland was removed with fine-tip forceps. For the sham operations , the surgical procedure was identical, except that the pineal was not removed following puncture of the dura matter. Bleeding was stopped with Gel-Foam (Upjohn, Kalamazoo, MI) and the incision was closed with wound clips. All animals were then placed in SD (8:16, lights on at 0900) for 8 weeks. All animals were then anesthetized with an overdose of sodium pentobarbital and perfused transcardially with 4% paraformadlehyde in 0.1 M phosphate-buffered physiological saline (pH 7.4), after which their brains were removed and the completeness of surgery was assessed by inspecting the brain under a dissecting microscope. Data were discarded from one PINX animal with a partially intact pineal and one SHAM animal with a damaged pineal.

STATISTICAL ANALYSIS

Comparisons of gametogenesis (experiment 2) were conducted as Mann-Whitney U tests. In all other cases, data were analyzed by repeated measures ANOVA followed by pairwise comparisons of LD and SD (or PINX and SHAM) treatments at each age. Because there was no expectation for either increase or decrease in body weight in SD, pairwise comparisons of body weight were conducted as two-tailed t-tests. In contrast, as only suppression of reproductive function by SD was considered biologically important, pairwise comparisons of testis size were conducted as one-tailed t-tests. In all statistical tests, attained significance levels < 0.05 were considered significant. Analyses were performed using Statview+ Graphics (v 1.04 A) on a Power Macintosh 6100 computer. All means are presented with their standard errors.

Results

EXPERIMENT 1

After two weeks, SD animals had significantly smaller body weights and testis volumes ($P < 0.0001$, for both; Table 1). On average, the SD animals weighed 22% less than LD animals. SD testis volume was 42% less than the average LD volume. These differences persisted through ages 49 and 63 days, although the magnitude of the difference diminished (Table 1). After 8 weeks of SD, the difference in testis volume between LD and SD animals was no longer significant ($P< 0.67$). The differences between the LD and SD groups were not due to differences in body weight, as estimated testis volume divided by body weight showed the same trends. Significant differences in body mass persisted for the length of the study ($P < 0.0001$), with LD animals consistently heavier than the SD animals.

EXPERIMENT 2

There were no significant differences between groups of Harlan Sprague-Dawley rats raised in LD or SD (Table 2).

EXPERIMENT 3

After only 10 days of short photoperiod, the mean SD testis weight was 34% smaller than that in the LD group ($P < 0.02$; Table 3). At 13 and 18 days, the SD testis weight remained 33% smaller ($P < 0.08$, $P < 0.03$, respectively). While body weights were lighter in SD animals, this differences was not significant.

EXPERIMENT 4

Pinealectomy clearly altered the response to SD. SHAM animals in SD had significantly lower body weights, smaller testes, and smaller testes relative to body weight than PINX animals in SD ($P < 0.01$, $P < 0.005$, and $P < 0.05$, respectively). Testis volume of SHAM animals was significantly lower after 2, 4, and 6 weeks of photoperiod treatment, but the difference had disappeared at week 8 (Table 4). The difference in body weight between SHAM and PINX was significant after 4 weeks and persisted until the end of data collection at week 8 (Table 4). The surgery itself appeared to slow growth in both body weight and testis volume (compare Table 1 and Table 4). However, the relative magnitude of the difference in estimated testis volume between PINX and SHAM animals (~ 30%) was similar to that between LD and SD animals (~ 40%).

Discussion

The results presented here clearly show that adolescent male F344 rats are reproductively sensitive to photoperiod. Previous results indicated that blinding inhibited reproduction in prepubertal female F344 rats (Leadem, 1988). However, blinding may produce effects that differ from the more natural stimulus of a change in

photoperiod. In this study I was able to elicit a photoinhibitory response in a more physiologically relevant manner by exposing male prepubertal rats to SD.

Identification of an inhibitory photoperiod was an integral part of this study. Previous work by Wallen et al. (1987) on olfactory-bulbectomized rats indicated the existence of a critical amount of light that is photostimulatory to a laboratory rat. By exposing Harlan Sprague-Dawley rats to various daylengths, they concluded that daylengths greater than 10 hours cause a stimulation of the reproductive axis, whereas daylengths of less than 8 hours cause an inhibition of the reproductive axis. Inhibitory photoperiods less than 8 hours of light elicit similar responses to those that are obtained when the animal is blinded.

By exposing prepubertal male F344 rats to inhibitory photoperiods, we effectively delayed the onset of puberty. Two weeks of short photoperiod were sufficient to inhibit testicular development (Tables 1, 3, 4). Comparisons of testis volume at this stage showed that the SD values were 58% of the LD values (Table 1). The margin of difference between the two treatments gradually decreased, and after 8 weeks there was no significant difference between the two treatments. The reduction seen in both LD and SD testis measurements at week 8 is probably a measurement artifact. Differences in body weight were significant within 2 weeks and persisted for the entire length of the study (Table 1).

The photoresponsiveness of the F344 rat can be abolished by pinealectomy. This extends Leadem's (1988) demonstration that the inhibitory effects of blinding can be abolished by pinealectomy. We found that PINX F344 males in SD had higher body weights and testis weights than their SHAM counterparts (Table 4). This result indicates that the photoresponsiveness of the F344 rat is mediated, at least in part, by the pineal gland. Clearly, the F344 rat is a photoresponsive strain of laboratory rat. The goal of the following experiments was to investigate the nature of this response on the cellular level.

CHAPTER THREE

Changes in GnRH Neuronal Abundance and Morphology in Reproductively Inhibited F344 Rats

Introduction

The ultimate causes of photoresponsiveness are clear. A mammal that is able to coordinate reproduction with favorable environmental conditions will be able to reproduce more efficiently, and this adaptive trait would be favored by natural selection. However, the proximate causes of photoresponsiveness remain unknown. Why do certain patterns of melatonin secretion cause reproductive inhibition in some individuals but fail to cause inhibition in others of the same species? One hypothesis is that individuals might differ in number or other characteristics of gonadotropin-releasing hormone (GnRH) neurons. There are between 1000-1200 GnRH neurons in the mammalian brain. Investigators have examined whether individuals need this entire GnRH system to be reproductively functional. Immunocytochemical studies using the hypogonadal mutant mouse *hpg*, which has an infantile reproductive tract as a result of a deficiency in the GnRH peptide (Cattanach et al., 1977), have shown that the homozygotes of this strain do not produce GnRH (Silverman et al., 1985). However, reproductive function can be restored in *hpg* homozygotes using fetal or neonatal septal-preoptic tissue implanted into the third ventricle of the adult. Immunocytochemical analysis of these implants has revealed that a single GnRH neuron can restore reproductive function (Gibson et al., 1984). Further evidence has shown that the neuron(s) of the graft must make connections with the median eminence (Gibson et al., 1984). Why, then, are over a thousand GnRH neurons present in a normal rodent brain?

A large segment of the GnRH neuronal population presumably serves to coordinate functions of distinct brain nuclei that are sometimes separated by relatively

large distances. Studies using the *hpg* mouse suggest that there is a baseline level for GnRH secretion that can stimulate reproduction. If GnRH levels fall below this level, reproduction is inhibited. It is possible that melatonin could inhibit GnRH neurons similarly in photoresponsive individuals and non-photoresponsive individuals. However, if non-responsive individuals have a greater number of GnRH neurons, and are therefore able to maintain a level of GnRH secretion that is above baseline, they might maintain fertility, while photoresponsive animals possessing fewer GnRH neurons, are inhibited to the point of halting reproductive activity. It may also be possible that Fischer 344 (F344) rats lack a subpopulation of GnRH neurons that are not inhibited by SD, whereas the Harlan Sprague-Dawley (HSD) rat does possess this population. Consequently the HSD rat remains in a functional reproductive condition. Therefore, the first goal of this experiment was to examine a photoresponsive strain of rat F344 and non-photoresponsive rat HSD for differences in GnRH abundance.

A second means by which investigators have assessed reproductive inhibition at the level of the hypothalamus is to look at the morphology of the GnRH neurons. Several investigators have hypothesized that inhibitory photoperiod initially reduces secretion of GnRH into the hypophyseal-portal circulation. This reduction in secretory activity of the GnRH neurons may cause an accumulation of the GnRH peptide within the neuron and a subsequent decrease in luteinizing hormone (LH) secretion from the adenohypophysis. This hypothesis is supported by immunocytochemical analysis of GnRH neurons of animals kept in short days that show an increase in staining density (Glass, 1986) and an increase in soma size of GnRH-containing neurons (Korytko et al., 1995; Urbanski et al., 1991). In addition, hypothalamic GnRH content has been shown to increase after short photoperiod exposure, as assessed by radio-immunoassy (RIA) (Glass et al., 1988; Hart et al., 1984; Kumar et al., 1982; Pieper, 1984; Versi et al., 1983). Furthermore, short-day exposure significantly reduces circulating levels of LH (Blank and Desjardins, 1986). This evidence suggests that GnRH release may be

modified by the inhibitory effects of photoperiod that are being transduced at the level of the hypothalamus. The second goal of this experiment was to determine whether changes in GnRH morphology and number are indicative of reproductive inhibition. It was hypothesized that GnRH neurons in individuals exposed to SD would show an increase in soma size relative to those in LD.

Methods

One group of F344 rats (N = 12) and one group of HSD rats (N = 13) were weight-matched and placed in either short days (SD) (8:16, lights on at 0900) or long days (LD) (16:8, lights on at 0500). Animals were held singly in polyethylene cages (36 x 24 x 19cm) in fan ventilated photoperiod chambers (86 x 58 x 49cm), each holding four cages, three of the chambers were on SD and three of which were on LD light cycles. Lighting was provided by two fluorescent bulbs (G.E. cool white flourescent bulbs, 20 watts) located along the width at the ceiling of each cage. Temperature was held at 23 ± 2° C. Food (Prolab Rat-Mouse-Hamster 3000; PMI Feeds, St.Louis, MO) and tap water were provided *ad libitum*.

The animals were exposed to treatments for 10 days and then were given an overdose of sodium pentobarbital, following which the animals were perfused transcardially with 10 ml of heparinized 0.1 M phosphate-buffered saline (0.02% sodium nitrite, 40 U/ml heparin in 0.1M PBS at pH 7.4). The saline perfusion was followed by perfusion of 180 ml of 4% paraformaldehyde in PBS. Once perfused, the brains were removed and placed in 5 ml of 4% paraformaldehyde and placed on a shaker at 4°C overnight. The brains were then sectioned at 75 μm using a vibratome. All coronal sections caudal to the corpus callosum and rostral to the mammilary body were processed for immunocytochemistry. Immediately after sectioning, three 10-minute rinses were done in 1.5 ml/well of 0.1 M PBS. Sections were then placed in a blocking solution (1.0 ml/well) consisting of 1.4% normal horse serum (135 μl/10ml),

and 0.2% Triton-X (20 µl/10ml) in 0.1M PBS for 20 minutes. Sections were incubated in a monoclonal antibody, mouse anti-GnRH (HU4H, kindly provided by Henryk Urbanski, University of Orgeon Primate Research Lab), at a concentration of 1:2000. The primary antibody solution consisted of the same solutes as the blocking solution, with the exception of the mouse anti-GnRH antibody. Sections were incubated in the primary antibody (1 ml/well) overnight on a shaker set at 170 rpm at 4°C. The following day, sections were transferred to a shaker at room temperature. After one hour, the sections were transferred to 0.01 M PBS for three 10-minute rinses. Sections were then placed in a solution of biotinylated horse anti-mouse antibody (1:200 horse anti-mouse, 1.4% normal horse serum, 0.2% Triton-X in 0.01 M PBS). After one hour, the sections were placed in three 10-minute rinses in 0.01 M PBS. Following the rinses, the sections were placed in a avidin-biotin-peroxidase complex (ABC kit, VectaStain, 0.5 ml/well) for one hour. Sections were then transferred to three 10 minute rinses in tris buffer gel solution (TBS). The sections were transferred to the chromagen solution (diaminobenzidine 0.2 mg/ml, 3% H_2O_2, in Tris buffer) for approximately 7 minutes. Sections were mounted on gelatin coated slides, dipped in xylene, and coversliped using permount. In this immunocytochemical experiment I neglected to perform a control, such as using the GnRH peptide to compete with the antibody.

DATA ANALYSIS

GnRH neuronal abundance

Analysis of GnRH neuronal abundance was performed using light microscopy with an Olympus CH-2 microscope using the 20x objective. In order to eliminate any bias during data collection, observations were conducted with the observer blind with respect to treatment.

The areas analyzed included: the medial portions of the diagonal band of Broca (DBB), the organum vasculosum of the lamina terminalis (OVLT), the lateral preoptic area (LPOA), the medial preoptic area (MPOA), the periventricular nucleus (PEVN), the suprachiasmatic nucleus (SCN), and the preoptic portion of the medial forbrain bundle (MFB). The total area analyzed was approximately 2000 μm in length. This 2000 μm area was chosen based on the studies by Silverman et al. (1987), in which a retrograde tracer was injected into a 2000 μm surrounding the median eminence of rats. It was found that only those neurons in this area had their terminals in the median eminence.

To facilitate comparison between HSD and F344 rats and between treatment groups, brain sections were placed to one of five categories. The five categories were defined based on the presence of anatomical landmarks. Category one consisted of those sections in which the corpus callosum had not yet fused between the two brain hemispheres, the anterior commissure had not fused and was represented by one oval in each hemisphere, and the optic nerves were separated (Figure 5). Category two consisted of those sections in which the corpus callosum had become fused, the ovals of the anterior commisure had become larger and closer, and the optic chiasm had recently appeared (Figure 5). Category three was a 300 μm long region encompassing the optic chiasm. In the brains in which the optic chiasm was missing (N = 11), I was forced to estimate this position by examining those brains in which the optic chiasm was present and counting sections posterior to it the next significant anatomical landmark, bifurcation of the optic tract was visible. It was found that the optic tract separated between 7-9 sections posterior to the optic chiasm or 525 μm-675 μm (Figure 5). Category four consisted of those sections that possessed the most posterior portions of the optic chiasm (where the optic chiasm had become broad and flattened), and continued back to the point at which the optic tract bifurcated (see figure 6). The fifth and final category consisted of those sections in which the optic tract had split. The

categories corresponded to discrete neuronal populations. Category one was primarily DBB. Category two was DBB and POA. Category three was comprised of DBB, POA, and MFB. Category four consisted of neurons in the POA and MFB and category five was primarily composed of neurons in the MFB.

Neurons were counted manually. This method required focusing up and down through the section to record the neurons in the most interior part of the section. Interior neurons did not stain as darkly as those near the cut surface, but they were clearly detectable.

A one-way ANOVA was performed using Statview SE + Graphics (Abacus Concepts, Inc., Berkeley, CA). In the case where more than one section corresponded to a particular group, an average was taken and that average used in the statistical analysis.

GnRH cell soma size

Analysis of GnRH cell soma area was performed on neurons located in the DBB. These are the only brain nuclei for which differences have been seen in soma size as a result of SD exposure (Korytko et al., 1995). For each animal in which there were GnRH neurons present in the DBB (N = 9 for F344, N = 11 for HSD), each neuron was measured. Measurements were performed using NIH Image Version 1.60 with an LG-3 Scientific Frame Grabber PCI Version (Scion Corporation, Frederick MD) on a Power Macintosh 7600/132 using a Leitz Laborlux S microscope (Leitz, Portugal) using the 20x objective with an mti 65 camera (Dage-MTI, Inc., Michigan City, IN). Each neuron was outlined using the "free-hand" preference and the outline was then measured for the area. Values for neurons from the DBB for each animal were averaged. A one-way ANOVA was performed using Statview SE + Graphics (Abacus Concepts, Inc., Berkeley, CA).

Results

The quality of specific staining was excellent (Figure 7). There were no significant differences in GnRH neuronal abundance between the HSD and F344 rats (Tables 5-7). Furthermore, there were no differences between different photoperiod treatments within the strains.

There were no significant differences seen between the HSD and F344 rats with regard to GnRH neuron cell soma area (Table 8). Again, there were no differences within the strains with regard to photoperiod treatment.

Discussion

There were no significant differences in the relative abundance of GnRH neurons between HSD and F344 rats. There was a trend present in that F344 rats had more GnRH soma in the MFB/POA than their HSD counterparts (P=0.0693, Table 5). This may be due to the inhibitory effects of short days whereby a photoresponsive animal would accumulate GnRH in the soma, consequently making it easier to visualize GnRH neurons.

The F344 LD animals tended to have larger GnRH cell soma areas (Table 8). In HSD rats the numbers of neurons were nearly equal in SD and LD (Table 8). Overall, F344 rats had larger cell somas regardless of treatment. The results allow me to reject my original hypothesis that SD would cause a retention of the GnRH peptide within the cell soma thereby causing it to increase in size (Korytko et al., 1995).

The fact that short days cause an increase in GnRH cell soma has been reported in both male deer mice (*Peromyscus maniculatus*) (Korytko et al., 1995), and Syrian hamsters (*Mesocrcetus auratus*) (Urbanski et al., 1991), although Urbanski et al. (1991) did not discuss where in the brain these differences were observed. In both of the aforementioned experiments the analysis was performed in sexually mature animals;

hence, it is conceivable that neuronal changes that occur during the pubertal transition are not comparable.

It is possible that the changes in GnRH neuronal morphology are too subtle to be detected by the methods of analysis used here. Wray and Hoffman (1986) used immunocytochemical techniques to look at the contours of the GnRH soma in peripubertal HSD rats. They found an identical number of GnRH immunoreactive cells (~1300) in all brains regardless of age (2-90 days post-natal). However, differences were observed in the contours of the soma. They noticed that as animals matured sexually, GnRH neurons possessed more "peduncular-like dendritic spines" or "sessile-like dendritic spines" (Wray and Hoffman, 1986). Such subtle differences were not detected between strains or between treatments in this study.

There are two avenues that were not pursued in this experiment that could have proven useful. First, RIA on hypothalamic extracts would allow measurement of actual levels of the GnRH peptide. This would provide information about differences between the two strains of rats in GnRH synthesis and release. Second, an autoradiographical study using radiolabeled iodonated GnRH might answer questions as to whether one strain was more sensitive to circulating GnRH levels. By using the radioligand, GnRH receptor levels could be compared between strains and between treatments.

In summary, if indeed there are no differences between the two strains, these results suggest that the sensitivity to changes in photoperiod is not due to differences in number or distribution of GnRH neurons. The effects of SD could be regulating pituitary sensitivity to the GnRH peptide. It is possible that photoperiodic effects cause a down regulation in pituitary GnRH receptors, thereby making the pituitary less sensitive to levels of GnRH. Additionally, it may be that photoperiodic effects cause a change in the secretory pattern of hormones from the pituitary. The work by Bockers et al. (1996) suggest that this latter scenario is indeed possible. Melatonin appears to alter the secretory activity of cells in the pars tuberalis (Bockers et al., 1996).

There are numerous sites where photoperiod could act upstream from the GnRH neuronal population. There are many neurotransmitter systems that affect GnRH secretion, as mention in the introduction to this chapter. One possible candidate is the dopaminergic system. Krajnak (1995) demonstrated the SD causes a decrease in tyrosine hydroxylase levels inside the dopaminergic neurons. This is the rate limiting enzyme in dopamine sythesis. Hence, a decrease in dopaminergic input to the GnRH neuronal system could lower GnRH secretion.

CHAPTER FOUR

Effects of Short Daylengths on Melatonin Binding in the Pars Tuberalis of Male Fischer 344 Laboratory Rats

Introduction

Mammals maintain circadian, or daily, cycles in locomotion, feeding, drinking, plasma corticosterone, growth hormone, heart rate, and sleep-wake cycles using an endogenous rhythm controlled by cells in the suprachiasmatic nucleus (SCN) (Moore, 1981). However, the action of the SCN alone cannot control the seasonal reproductive rhythms that some mammals show. Many environmental stimuli can elicit changes in a mammal's rhythms, including dietary factors and changes in the physical environment. However, the most reliable environmental stimulus is photoperiod (Bronson and Heideman, 1994). By using photoperiod as a cue, mammals can achieve a precisely timed seasonal cycle.

The means by which changes in photoperiod are transduced into neurochemical changes was reviewed in Chapter 1. Briefly, changes in photoperiod produce changes in the pattern of melatonin secretion. The duration of melatonin secretion is directly related to the amount of light in a day (Morgan et al., 1994). Since melatonin is produced in the absence of light, longer durations of melatonin secretion are indicative of short days. If melatonin secretion persists for a period longer than some threshold, then reproduction becomes inhibited. This threshold is species-specific, but in most species it is produced by a night of 14 hours or more.

Preliminary attempts to identify the sites where melatonin might be acting have used the radioligand 2 -^{125} I-melatonin. Using this technique, melatonin binding has been characterized in the following mammalian species: laboratory rats (Gauer et al., 1994; Weaver et al., 1989; Williams, 1989), Syrian hamsters (Vanecek and Jansky,

1989; Weaver et al., 1989; Williams et al., 1989), Siberian hamsters (Duncan et al., 1989; Weaver et al., 1989), the European hamster (Gauer et al., 1992), ferrets (Weaver et al., 1990), domestic rabbits (Stankov et al., 1992), domestic sheep (Bittman and Weaver, 1990; Morgan et al., 1989), ground squirrels (Stanton et al., 1991), and several primates (Stankov et al., 1993). It is clear from all these studies that the only brain/pituitary area that shows binding in all of the photoresponsive species is the pars tuberalis (PT) of the adenohypophysis; the only species that does not display binding in the PT is humans (Weaver et al., 1993). In each species in which the PT binds 2 -^{125}I-melatonin, the PT display the most intense binding of any brain region . The melatonin receptors in the PT have a high affinity and specificity for their ligand (Vanecek et al., 1987; Williams and Morgan, 1988). It therefore appears that the PT may play a significant role in mediating a mammal's response to changes in daylength. However, the physiological role of the PT has yet to be elucidated.

In the rat, it has been argued that melatonin has a stimulatory role on GnRH secretion by inhibiting the negative feedback loop of PT LH. Nakazawa et al. (1991) suggests that melatonin is able to reduce the effects of hormone negative feedback in the PT. This theory is consistent with findings that the density of melatonin receptors in the PT decreases in sexually inactive mammals (Masson-Pevet and Gauer, 1994). Therefore, in sexually active mammals, where there is an increase in melatonin receptors in the PT, GnRH release would be enhanced.

The objective of this study was to examine melatonin receptor regulation in the F344 rat.

Methods

Male F344 rats 21±2 days old obtained from our breeding colony were weighed and placed in one of two treatments. Weight-matched groups of 6 were placed in short days (SD) (8:16, lights on at 0900) and in long days (LD) (16:8, lights on at 0500). All

animals were held singly in polyethylene cages (36 x 24 x 19cm) in photoperiod controlled rooms, one LD and one SD. Temperature was held at 23 ± 2°C. Food (Prolab Rat-Mouse-Hamster 3000; PMI Feeds, St.Louis, MO) and tap water were provided *ad libitum.*

The rats remained in each of the photoperiod treatments for 14 days, when they were euthanized individually (1500-1630 hours) with CO_2 , weighed, and deacapitated. The brain was then rapidly removed from the skull. A small pair of dissecting scissors were inserted into the foramen magnum, with the tips of the scissors angled towards the skull to avoid damaging the brain. The skull was then cut along the lateral margins to create a flap. This flap was peeled forward to expose the brain. The fifth cranial nerve and pituitary stalk were cut with fine scissors. The optic nerves were then severed approximately 2 mm in front of the optic chiasm. Finally, the olfactory bulb was cut and the brain was dislodged from the skull. Brains were placed in a beaker containing 75 ml of 2-methylbutane (Sigma Chemical Company, St. Louis, MO), and the beaker was surrounded by dry ice and kept at -15°C to -20°C in a styrofoam cooler. After 2 minutes in 2-methylbutane, the brains were wrapped in aluminum foil and placed in 5 ml plastic sample vials (Nalgene, Rochester, NY). Brain removal averaged 3:15 minutes and was no greater than 4 minutes. Brains were then stored at -80°C for up to 10 days.

Prior to sectioning, each brain was allowed to equilibrate in the cryostat -12°C to -15°C for fifteen minutes. Brains were then mounted on metal chucks using Triangle Biomedical Sciences Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC). 20μm sections were cut and thaw-mounted onto gel-coated slides. To keep the slides cold, slides were kept in the bottom of the cryostat when sections were not being mounted. Only sections from the anterior comissure to the most posterior portion of the mammillary body were saved, and of this sample every other section was discarded. Of

those sections retained, every third section was was reserved to test for non-specific binding.

After sectioning, the slides with brain sections were vacuum dried, and stored overnight at 4°C. The slides were then placed in a slide box with dessicant, sealed with electrical tape, and stored at -80°C.

Autoradiography was performed using the method outlined by Duncan et al. (1989, 1992). Slides were allowed to equilibrate to room temperature for 20 minutes and then placed in wire slide racks. Slides were pre-incubated for 1 hour in 50 mM Tris-HCl, 0.1% bovine serum albumin (BSA), and 4 mM $CaCl_2$ at pH 7.4. After the pre-incubation period, the slides were placed on stainless steel rods inside Rubbermaid tubs. Paper towels moistened with distilled H_20 were placed in the bottom of the tubs to provide humidity during the incubation. Two solutions were prepared for the incubation. The first solution contained 50 mM Tris-HCl, 0.01% BSA, and 4mM $CaCl_2$ at pH 7.4. To this was added a solution of 200 pM 2- ^{125}I-melatonin (Specific Acitvity = 2000 ci/mmol, Amersham Life Sciences). The second solution, used for non-specific binding, was composed of 40ml of 50mM Tris-HCl, 0.01% BSA, 4 mM $CaCl_2$ at pH 7.4, and 1 mM melatonin solution. Specific and non-specific binding measurements were carried out in separate tubs.

The solutions were pipetted on to the brain sections (75 μl/brain section) and incubated for one hour. Following incubation, the slides were rinsed in the Tris-BSA buffer two times for 10 minutes each at 0°C. The slides were then dried on a slide warmer at 45°C for one hour. Dried slides were loaded into Fisher Series XC autoradiography cassetes. X-ray film (Kodak X-OMAT AR, Fisher Scientific, Pittsburgh, PA) was placed on top of the slides and the cassettes closed. For the analysis of the PT the film was exposed for 10 days.

Film was developed using a standard darkroom protocol. The slides were then processed for standard cresyl violet staining.

DATA ANALYSIS

Data analysis was performed using NIH Image Version 1.60 with an LG-3 Scientific Frame Grabber PCI Version (Scion Corporation, Frederick MD).The software was run on a Power Macintosh 7600/132 using an Olympus BH-2 microscope attahced to a Sony DXC-960MD 3CCD color video camera. Illumination was held constant during the entire analysis. Prior to each measurement of absorbance, the illumination was checked using a microscale standard in which there was a known density value.

Those sections containing the PT were analyzed. Presence of the PT was confirmed with cresyl violet stain under examination with a light microscope (Olympus CH-2). Approximately 50% of the central area in which iodonated melatonin had bound was analyzed. This was done to limit counting to areas of film directly above the PT. Binding was measured from five sections per animal and the absorbance values were then averaged. Absorbance was also measured for the cortical areas in the sections where PT binding was seen; this measurement served as one control. A second control was obtained by measurement of the absorbance of the PT region in the adjacent section that had been reserved for non-specific binding.

Absorbance values were converted into femtomoles/milligram of protein using the method described by Nazarali et al. (1989). Initially, microscale standards were used to establish a standard curve of disintegrations per minute/milligram protein versus absorbance (Figure 7). Using this curve the dpm/mg protein was interpolated for a particular PT section. The activity of the IMEL (Ci/mmol) solution was used to calculate femtomoles/milligram protein.

In two brains, cresyl violet staining of the sections indicated that the PT had been torn away; therefore, these brains were not included in the data analysis. A one-way ANOVA was performed using Statview SE + Graphics (Abacus Concepts, Inc.,

Berkeley, CA) on body weight before and after photoperiod treatment, and on PT binding.

Results

There were no significant differences found between treatment groups in melatonin receptor density in the PT (P=0.9210) (Table 9, Figure). Differences in body weight were significant after 2 weeks of photoperiod treatment (P=0.0488).

Discussion

Only recently has the role of the PT begun to be elucidated. Cells of the PT express melatonin receptors more densely than any other adenohypophyseal cell type (Bockers et al., 1997). Anatomically, PT cells form a cover around the hypophyseal stalk and median eminence such that coronal sections of the PT are ring-like in appearance. These cells have been associated with the transmission of photoperiodic stimuli to the endocrine system. However, their principal secretory products have not been identified.

The results reported here indicate that the F344 male laboratory rat differs from seasonal rodents that display seasonal differences in PT melatonin receptor density. There are two possible explanations for this phenomenon. It is possible that F344 rats differ in the neuroendocrine pathways that underlie melatonin receptor regulation. It may also be true that the changes seen in PT melatonin receptor density in other species are not associated with reproductive responses that occur with changes in photoperiod. Several authors have suggested that the changes seen in PT melatonin receptor density are due to action on prolactin secretion that cause other seasonal physiological changes such as molt and torpor (Williams et al., 1997).

The second possible explanation is that the reproductive changes that F344 rats display when exposed to short days may not be large enough to cause recognizable differences in PT

melatonin receptor levels. Although F344 rats are photoresponisve, the degree to which they exhibit inhibition is not as robust as it is in other seasonal rodents (for a review see Bronson and Heideman, 1994). Furthermore, the autoradiographic assay employed may not have been sensitive enough to detect subtle differences in receptor levels that might elicit changes in reproductive status. There is a large degree of variability in my results, as indicated by the standard errors. I suspect this is due to a methodological flaw in my protocol. The slides were not perfectly horizontal and, consequently, the sections received varying coverage by the IMEL solution during incubation.

Recently, a study by Bockers et al. (1996) on ovine PT may have revealed how endocrinological functions are affected by cells of the PT. Using immunocytochemistry, Northern blot analysis, and in situ hybridization for several adenohypophyseal hormones and their subunits (thyrotropin (TSH), follicle stimulating hormone (FSH), luteinizing hormone (LH), common alpha-chain, growth hormone (GH), corticotropin (ACTH), prolactin (PRL), alpha- and gamma-melanocyte stimulating hormone, beta-lipotropin). They compared the secretion of these hormones in animals taken from short photoperiods and long photoperiods. Using immunocytochemical techniques, they found that the only peptide present in SD animals was the common alpha-chain. Immunocytochemical techniques failed to detect any of these peptides in the long day animals. Northern blot analysis with the antisense oligonucleotides showed that the mRNA for prolactin was expressed throughout the PT; beta-LH and beta-FSH were found only in the caudal part of the PT. Interestingly, no seasonal influence was observed on gene transcription or translation. Bockers et al. concluded, " . . . that ovine PT cells are capable of expressing different mRNAs of adenohypophyseal hormones and that a mRNA pool for hormone subunits indicates that hormone synthesis is mainly regulated at the translation level and that secretion of hormones may be primarily constitutive", (Bockers et al., 1996).

Recently, studies in laboratory rats have shown that melatonin receptor regulation in the PT is not typical of other seasonal rodents. Recio et al. (1996) induced photoresponsiveness in male Wistar laboratory rats using subcutaneous testosterone-filled capsules. They hypothesized

that testosterone could cause photoresponsiveness by increasing the sensitivity of PT melatonin receptors to endogenous melatonin. The enhanced sensitivity would presumably lead to receptor downregulation after chronic exposure to longer melatonin levels, resembling the change in seasonal breeders exposed to SD. Recio et al. (1996) were able to induce a SD reproductive response in the testosterone-treated animals, as shown by a significant decrease in testis weight. However, the reproductive response was not accompanied by a decrease in PT melatonin receptors. The testosterone did cause a decrease in PT melatonin receptors in both LD and SD when compared with controls. It appears that in male Wistar rats, testosterone regulates PT melatonin receptors independently of photoperiod. It appears that neither F344 rats, nor Wistar rats undergo changes in melatonin receptor levels in the PT in SD or LD. This suggests that the laboratory rat is not typical of other seasonal rodents. It is possible that the seasonal regulation of melatonin receptors in PT is not involved in the reproductive responses to photoperiod; if so we should not expect to see these types of changes in laboratory rats.

CHAPTER FIVE

Conclusion and Future Directions

The experiments reported in Chapter 2 clearly indicate that F344 rats are a photoresponsive strain. The mechanism through which F344 laboratory rats are photoresponsive remains unclear. This marks the first instance in which an unmanipulated laboratory rat displayed robust photoresponsiveness. Furthermore, it is clear that a secretion(s) of the pineal gland is responsible for this sensitivity. Presumably, melatonin is the compound responsible, and experiments are currently being conducted to test melatonin's role. The F344 strain has the potential to advance our knowledge of photosensitivity and environmental regulation of the pubertal transition.

Historically, the F344 strain has shown heightened pituitary sensitivity to serum steroid levels (Piroli et al., 1996). The strain originated in the early part of this century and was developed by individuals investigating cancer at Columbia University. It is unclear what role steroids might play in the photoresponsiveness of F344 rats. Photoresponsiveness in other strains of laboratory rats can be unmasked using chronic steroid treatments suggesting that F344 rats maintain photoresponsiveness due to a heightened sensitivity to steroids. Thus, photoresponsiveness may be the consequence of selection in the laboratory for this trait

It would be useful to extend the GnRH immuncytochemistry experiments. Wray and Hoffman (1987) showed that GnRH soma morphology changes as rats progress through puberty. Soma that were once smooth become spiny; this level of analysis was not performed in our study. Secondly, androgen receptor levels should be compared between F344 and HSD rats; as this might be a reason for the differences between the strains. Furthermore, an examination of GnRH receptor levels should be performed using autoradiography. To examine whether one strain is more sensitive to GnRH, or perhaps one strain has a brain region more

populated with GnRH receptors. There are no clear differences in GnRH secretion between F344 and HSD rats, but perhaps differences do exist regarding GnRH receptors and their regulation.

The autoradiography performed using ^{125}I-melatonin (IMEL) binding should be repeated. There are several methodological issues that need to be resolved. A major source of "noise" in our sample may have come from differences in the volume of IMEL solution over each brain section. Although attempts were made to keep 75μl of IMEL solution on each individual brain section, it was clear that not all the slides were on a perfectly level plane and therefore the IMEL solution tended to pool at one end of the slide. Effort was made to prevent this, but it is possible that some sections received only a brief incubation in the IMEL solution and therefore displayed lower binding. It is clear that further refinement of our technique is needed.

In addition to repetition of the F344 IMEL experiment, a comparison of melatonin receptor levels between HSD and F344 rats should be performed. An interesting component to this experiment would be to induce photosensitivity in the HSD animals to a level that is comparable to the reproductive inhibition that is seen in F344 rats; then perform the autoradiography on melatonin receptors. If heightened steroid sensitivity is the route by which F344 rats maintain photoresponsiveness this experiment would test that theory.

It is also necessary to examine the roles of other brain nuclei that possess melatonin receptors, such as the dorsomedial nucleus of the hypothalamus. There is evidence that this nucleus plays a role in the response to photoperiod (Maywood and Hastings, 1995). It is also an area abundant in steroid receptors and may therefore have the ability to alter the effects of steroid negative feedback in the hypothalamic-pituitary-gonadal axis.

The experiments reported herein were an attempt to identify the neuroendocrine mechanisms that may differ between photoresponsive and non-photoresponsive laboratory rats. We have eliminated one possible source of this variation. In terms of overall GnRH neuron abundance and cell soma area there are no differences. However, we have not yet to

elucidated the means by which melatonin is exerting its inhibitory influence on reproduction. As a result of this work the questions that need to be asked have become better defined.

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Table 1. Comparison of testis size and body weight of Fischer 344 Rats (F344) in SD and LD

		Approximate Age (days)				
		38	**52**	**66**	**84**	**100**
		*	*	*	*	*
Body weight (g)	F344 **LD**	108.8 ± 0.99	178.8 ± 1.60	230.6 ± 1.82	265.3 ± 2.02	290.3 ± 2.37
	F344 **SD**	96.4 ± 1.61	142.5 ± 2.26	195.5 ± 2.76	238.8 ± 2.44	273.5 ± 2.61
		*	*	*		
Testis volume (mm^3)	F344 **LD**	589.4 ± 30.18	949.5 ± 32.29	1102.2 ± 41.84	872.0 ± 33.64	1295.3 ± 40.55
	F344 **SD**	340.8 ± 37.37	611.1 ± 38.33	879.6 ± 27.49	851.5 ± 34.05	1304.4 ± 46.66
		*	*	*		
Testis $^{vol.}/_{weight}$	F344 **LD**	5.4 ± 0.27	5.3 ± 0.19	4.8 ± 0.16	3.3 ± 0.12	4.5 ± 0.14
	F344 **SD**	3.5 ± 0.34	4.3 ± 0.21	4.5 ± 0.13	3.6 ± 0.14	4.8 ± 0.18

SD = short days (8:16, lights on at 0900)

LD = long days (16:8, lights on at 0500)

* - statistically significant

Table 2. Comparison of testis size and body weight of Harlan Sprague-Dawley Rats (HSD) in SD and LD.

		Approximate Age (days)				
		38	**52**	**66**	**84**	**100**
Body weight (g)	HSD **LD**	137.0 ± 3.27	236.2 ± 4.78	296.7 ± 3.97	338.7 ± 4.39	353.9 ± 4.57
	HSD **SD**	136.1 ± 3.61	234.0 ± 5.70	301.0 ± 5.4	341.8 ± 6.00	351.4 ± 6.21
Testis volume (mm^3)	HSD **LD**	546.2 ± 22.47	1049.0 ± 25.03	1106.0 ± 23.63	1095.1 ± 31.41	891.3 ± 19.27
	HSD **S D**	532.6 ± 27.48	1032.1 ± 40.15	1154.1 ± 24.02	1129.6 ± 17.21	908.2 ± 16.19
Testis vol./weight	HSD **LD**	4.0 ± 0.12	4.5 ± 0.10	3.7 ± 0.07	3.2 ± 0.09	2.5 ± 0.07
	HSD **SD**	3.9 ± 0.14	4.4 ± 0.11	3.8 ± 0.10	3.3 ± 0.07	2.6 ± 0.07

S D = short days (8:16, lights on at 0900)

L D = long days (16:8, lights on at 0500)

Table 3. Testicular mass and volume mass of Fischer 344 Rats (F344) following 6-18 days in SD or LD.

		Days in Treatment			
		6	**10**	**13**	**18**
Body weight (g)	F344 **LD**	52.0 ± 9.43	77.0 ± 9.49	90.4 ± 8.42	116.1 ± 5.20
	F344 **SD**	58.7 ± 1.66	68.7 ± 6.47	82.6 ± 3.77	103.9 ± 4.33
		(P < 0.50)	(P < 0.13)	(P < 0.26)	(P < 0.10)
Combined Testis weight (g)	F344 **LD**	0.56 ± 0.04	0.67 ± 0.04	0.79 ± 0.07	1.25 ± 0.12
	F344 **SD**	0.46 ± 0.03	0.45 ± 0.06	0.53 ± 0.11	0.84 ± 0.12
		(P < 0.15)	(P < 0.02)	(P < 0.08)	(P < 0.03)

SD = short days (8:16, lights on at 0900)

LD = long days (16:8, lights on at 0500)

Table 4. The effects of pinealectomy on body mass and testis size of Fischer 344 (F344) rats reared in short days from 21 days of age.

		Approximate Age (days)			
		35	**49**	**64**	**78**
Body weight (g)	**Sham**	95.5 ± 3.01	139.3 ± 6.54	190.2 ± 8.7	224 ± 20
	Pinx	99.3 ± 2.00	156.3 ± 3.55	209.6 ± 5.2	246 ± 15
		(P=0.1483)	(P=0.0132)	(P=0.0293)	(P=0.0057)
Testis volume (mm^3)	**Sham**	280.8 ± 35.36	509.2 ± 32.65	712.4 ± 54	910 ± 55
	Pinx	381.0 ± 14.94	703.5 ± 32.15	802.5 ± 30	920 ± 65
		(P=0.0066)	(P=0.0007)	(P=0.0726)	(P=0.3432)
Testis volume/wt	**Sham**	2.92 ± 0.34	3.66 ± 0.17	3.72 ± 0.16	4.1 ± 0.09
	Pinx	3.84 ± 0.15	4.49 ± 0.15	3.83 ± 0.11	3.7 ± 0.07
		(P=0.0088)	(P=0.0018)	(P=0.2908)	(P=0.0041)

Table 5. GnRH neuronal abundance: Harlan Sprague-Dawley (HSD) rats versus Fischer 344 (F344) rats in short days (8:16).

		Category				
		1	**2**	**3**	**4**	**5**
		DBB	**DBB/POA**	**DBB/POA/MFB**	**MFB/POA**	**MFB**
Number of Soma	**HSD**	6.84±1.08 (N=6)	21.41±3.02 (N=6)	51.00±3.02 (N=5)	15.23±1.82 (N=5)	5.61±0.79 (N=5)
	F344	7.49±0.85 (N=5)	21.69±1.36 (N=6)	55.16±4.96 (N=5)	20.46±1.75 (N=6)	3.94±0.46 (N=6)
		(P=0.6576)	(P=0.9339)	(P=0.4913)	(P=0.0693)	(P=0.893)

DBB = diagonal band of Broca

POA = preoptic area

MFB = medial forebrain bundle

Table 6. GnRH neuronal abundance: Harlan Sprague-Dawley (HSD) rats versus Fischer 344 (F344) rats in long days (16:8).

		Category				
		1	**2**	**3**	**4**	**5**
		DBB	**DBB/POA**	**DBB/POA/MFB**	**MFB/POA**	**MFB**
Number of Soma	**HSD**	5.08±0.83 (N=5)	19.36±2.05 (N=5)	51.75±6.84 (N=4)	14.07±2.25 (N=4)	4.21±0.42 (N=6)
	F344	6.31±1.17 (N=4)	20.85±1.26 (N=6)	52.40±4.57 (N=5)	17.20±2.08 (N=5)	3.62±0.49 (N=6)
		(P=0.4086)	(P=0.5358)	(P=0.937)	(P=0.3432)	(P=0.3868)

DBB = diagonal band of Broca

POA = preoptic area

MFB = medial forebrain bundle

Table 7. GnRH neuronal abundance: Fischer 344 (F344) and Harlan Sprague-Dawley (HSD) long days versus short days.

		Category				
		1	**2**	**3**	**4**	**5**
		DBB	**DBB/POA**	**DBB/POA/MFB**	**MFB/POA**	**MFB**
Number of Soma	**F344 SD**	7.49±0.85	21.69±1.36	55.16±4.96	20.46±1.75	3.94±0.46
	F344 LD	6.31± 1.17	20.85±1.26	52.40±4.57	17.20±2.08	3.62±0.49
		(P=0.428)	(P=0.6594)	(P=0.6935)	(P=0.2576)	(P=0.6444)
Number of Soma	**HSD SD**	6.84±1.08	21.41±3.02	51.00±3.02	15.23±1.82	5.61±0.79
	HSD LD	5.08±0.83	19.36±2.05	51.75±6.84	14.07±2.25	4.21±0.42
		(P=0.2417)	(P=0.6044)	(P=0.9129)	(P=0.6968)	(P=0.1327)

DBB = diagonal band of Broca

POA = preoptic area

MFB = medial forebrain bundle

SD = short days (8:16, lights on at 0900)

LD = long days (16:8, lights on at 0500)

Table 8. Diagonal band (DBB) GnRH soma size in short day versus long day treatments in Harlan Sprague-Dawley rats(HSD) versus Fischer 344 rats (F344).

Strain	Treatment	n	Average DBB soma size (μm^2)
F344	SD	5	164.00±9.90
F344	LD	4	193.00±20.26 (P=0.215)
HSD	SD	6	147.87±16.14
HSD	LD	5	141.76±11.63 (P=0.7747)
HSD	SD	6	147.87±16.14
F344	SD	5	164.00±9.90 (P=0.4404)
HSD	LD	5	141.76±11.63
F344	LD	4	193.00±20.26 (P=0.0552)

Table 9. Specific Binding of IMEL in Pars Tuberalis (PT) in Fischer 344 rats (F344) rats after 2 weeks of photoperiod treatment.

		Weight (time 0) (g)	**Weight(14days) (g)**	**Specific Binding PT (fmol/mg protein)**
Treatment	**LD**(N=4)	52.9±4.07	123.08±4.00	25.42±13.12
	SD(N=7)	51.1±3.45 (P=0.7521)	110.11±3.62 (P=0.0488)	26.95±8.53 (P=0.9210)

SD = short days (8:16, lights on at 0900)

LD = long days (16:8, lights on at 0500)

Figure 1. Schematic diagram showing environmental regulation of the reproductive axis

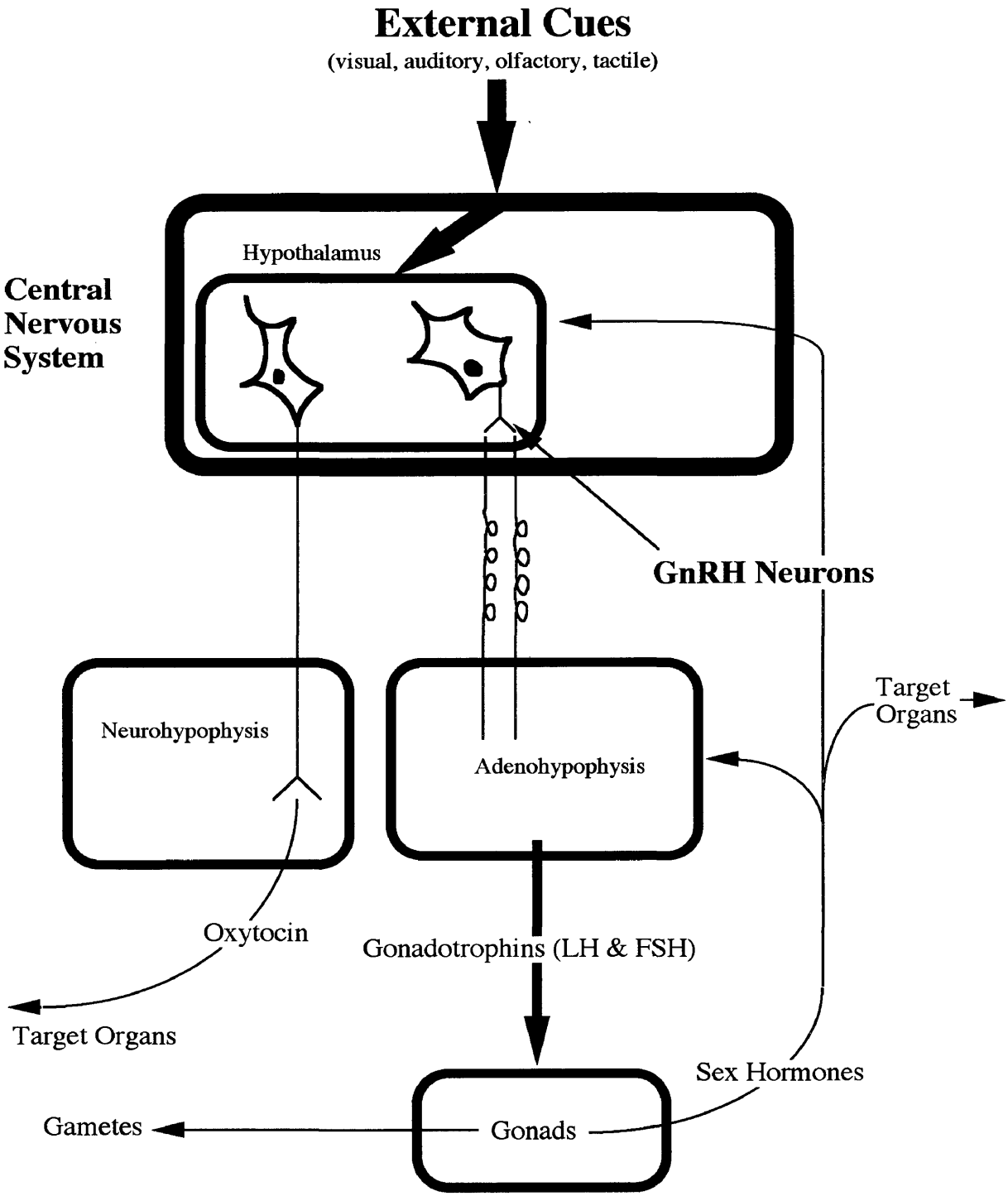


Figure 2. Relationship between calipered testis length and actual testis length

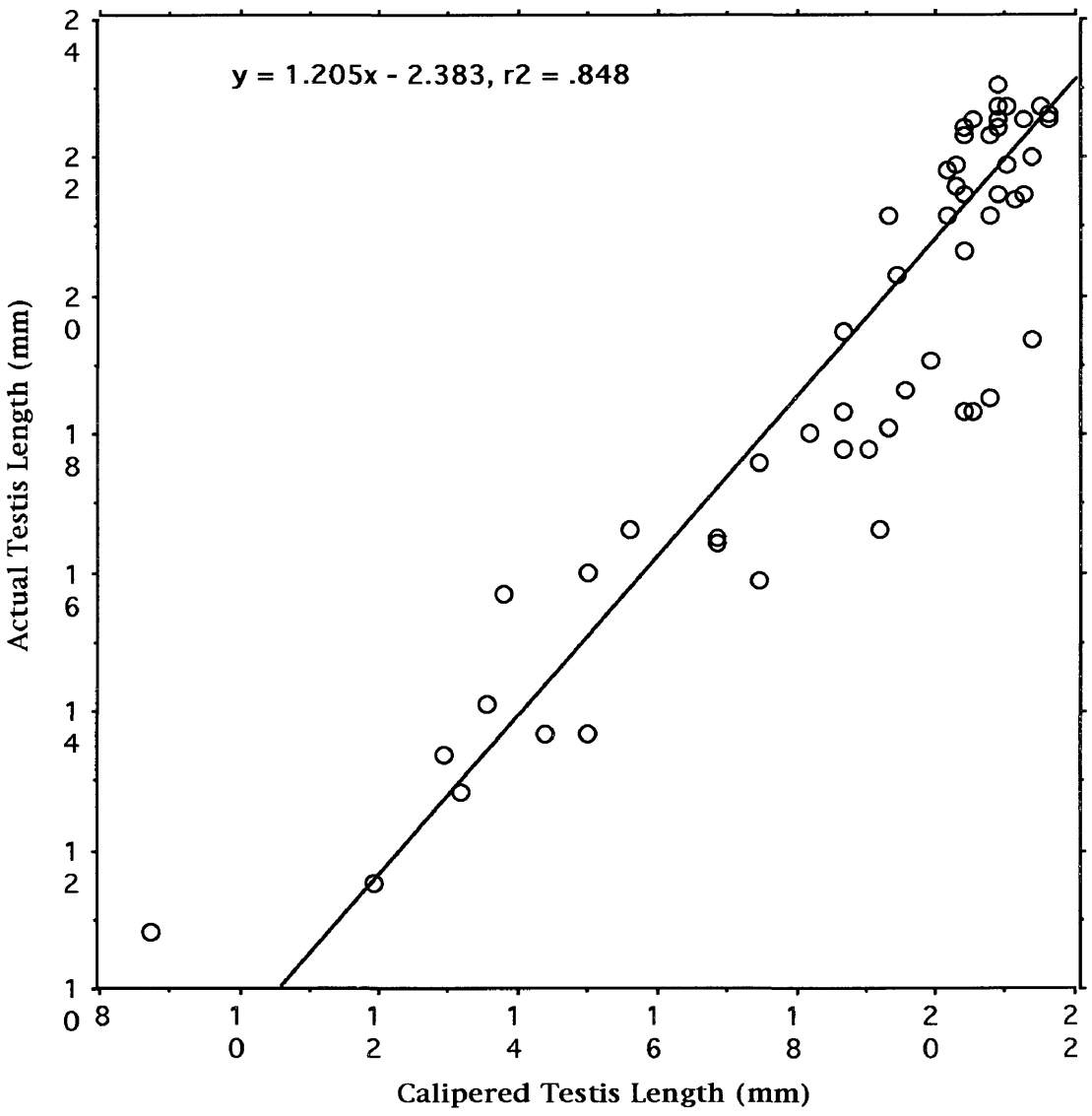


Figure 3. Relationship bewteen calipered testis width and actual testis width

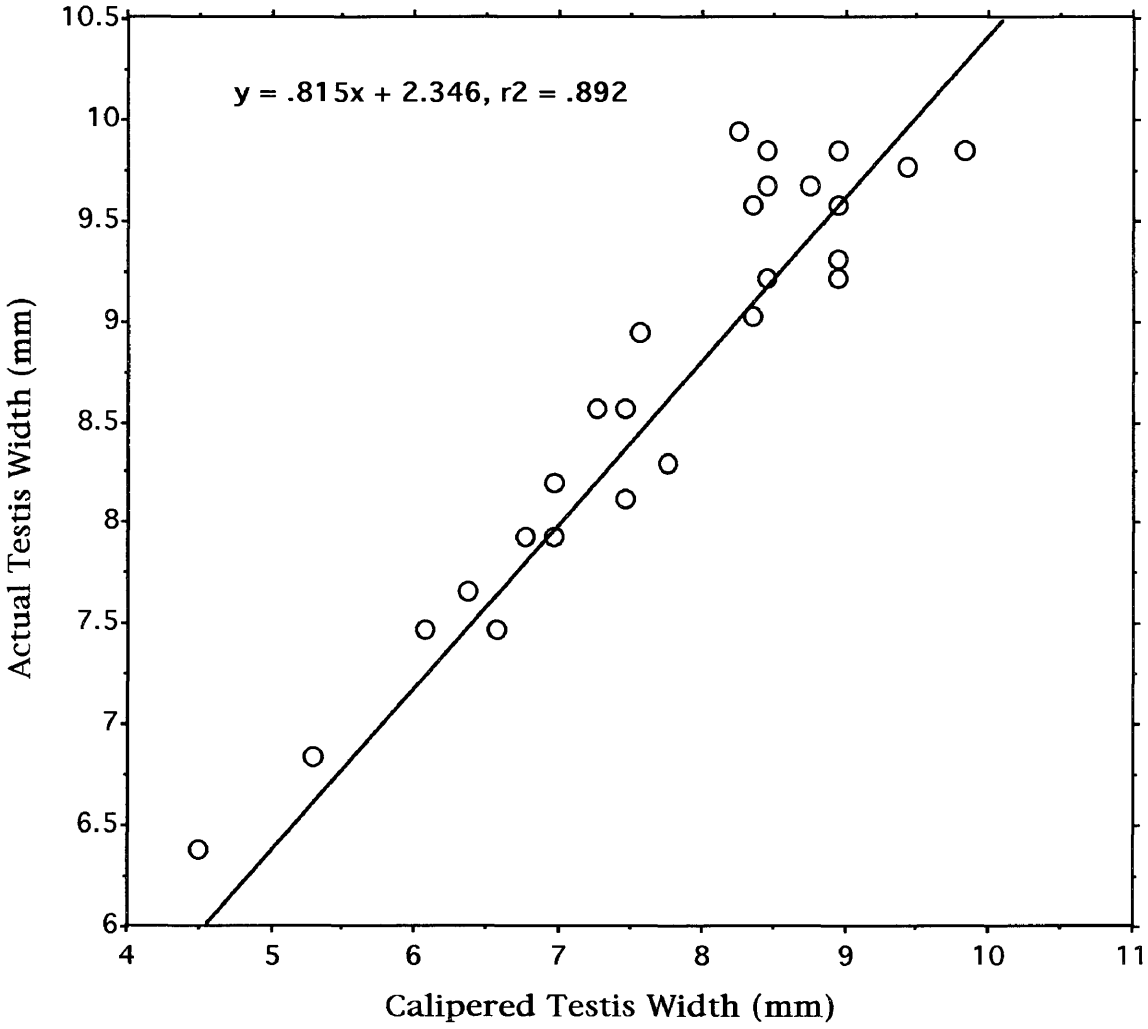


Figure 4. Relationship between calipered testis vol. and actual testis vol.

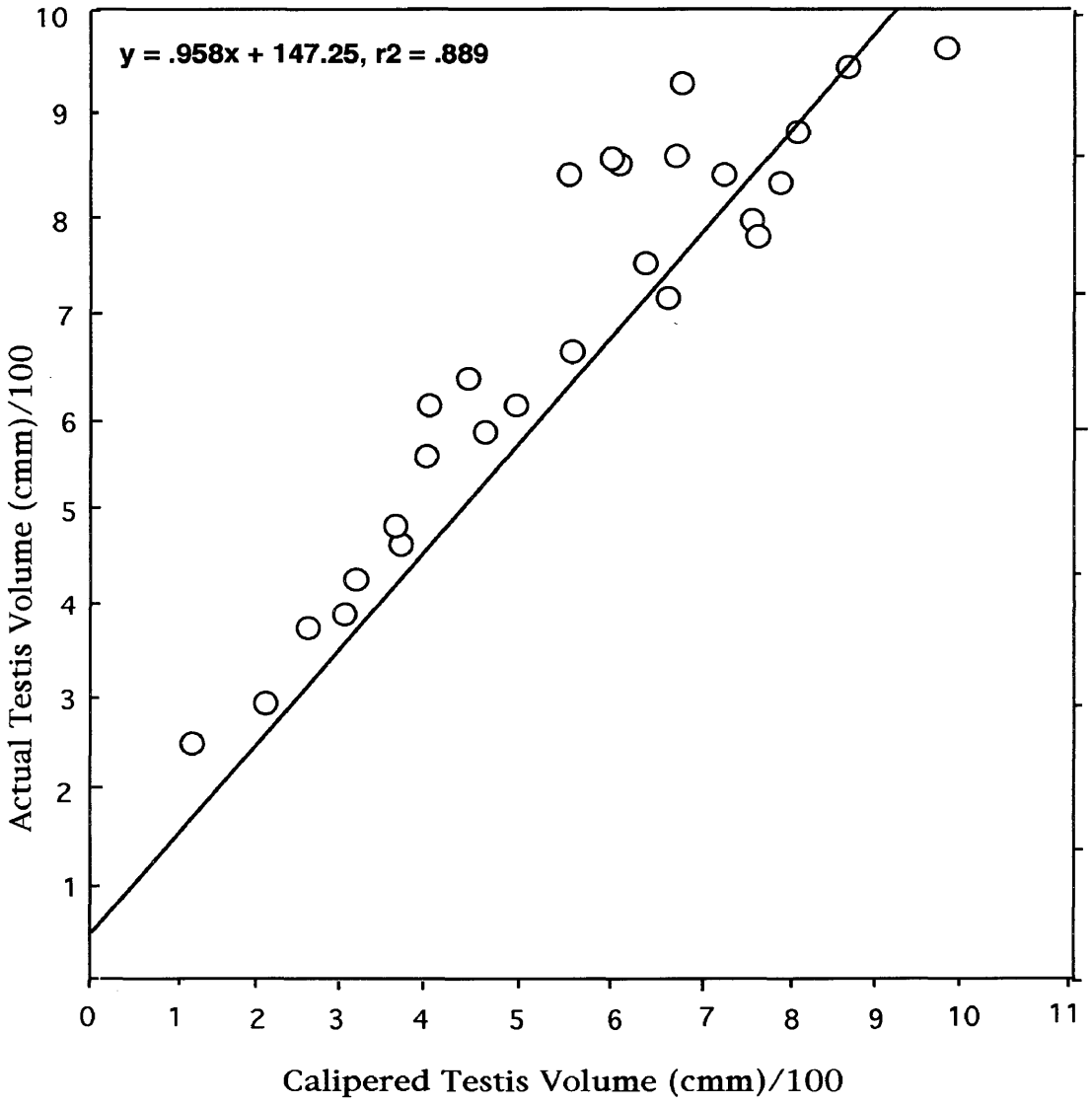


Figure 5. Coronal rat brain sections identifying the locations of GnRH cell soma (Categories 1-3) Redrawn from Silverman (1987).

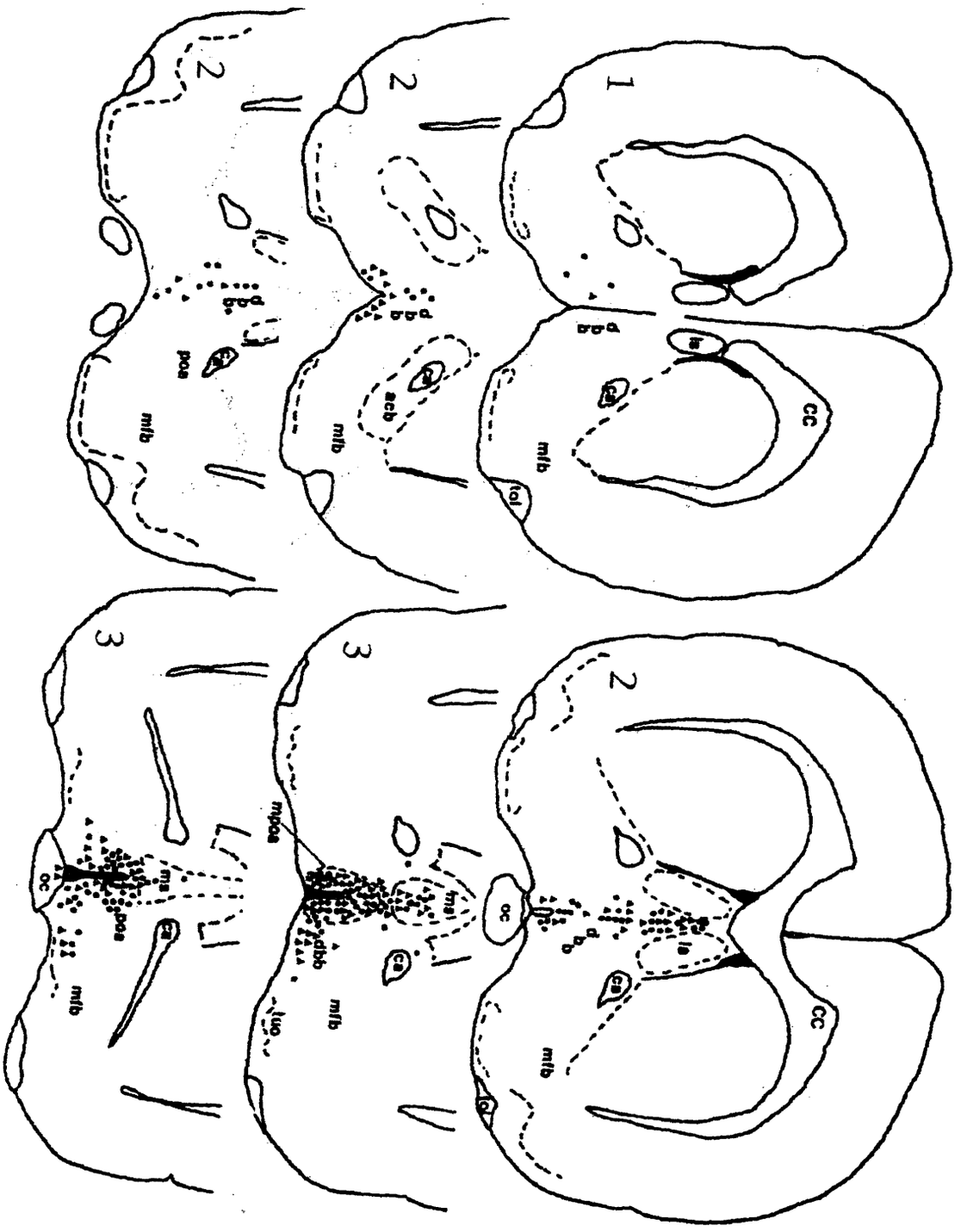


Figure 6. Coronal rat brain sections identifying the locations of GnRH cell soma (Categories 4 & 5) Redrawn from Silverman (1987).

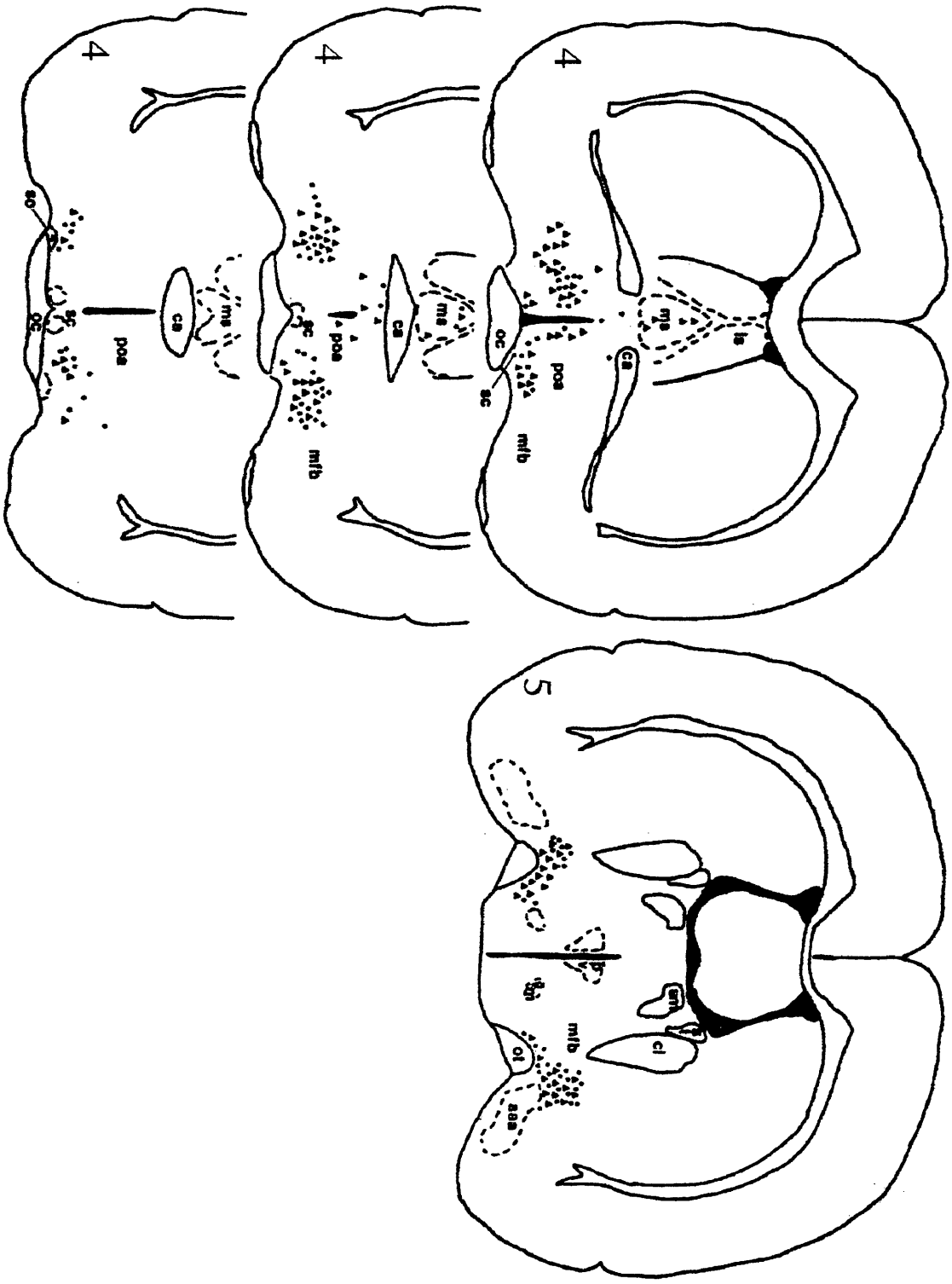


Figure 7. Staining of GnRH soma with the monoclonal antibody, both preoptic and diagonal band neurons are shown.

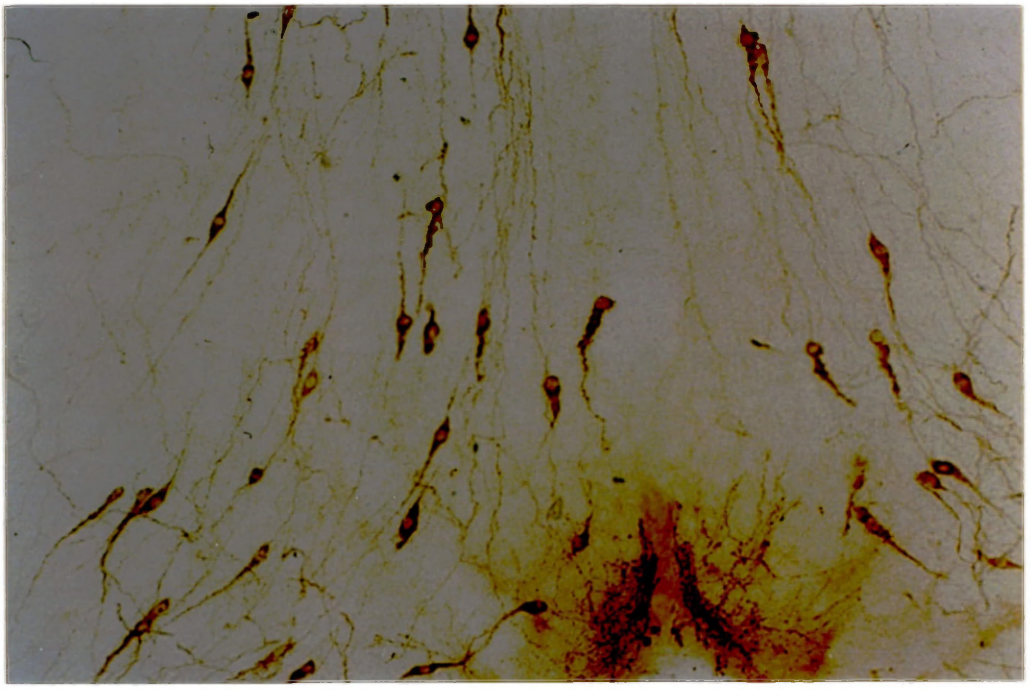
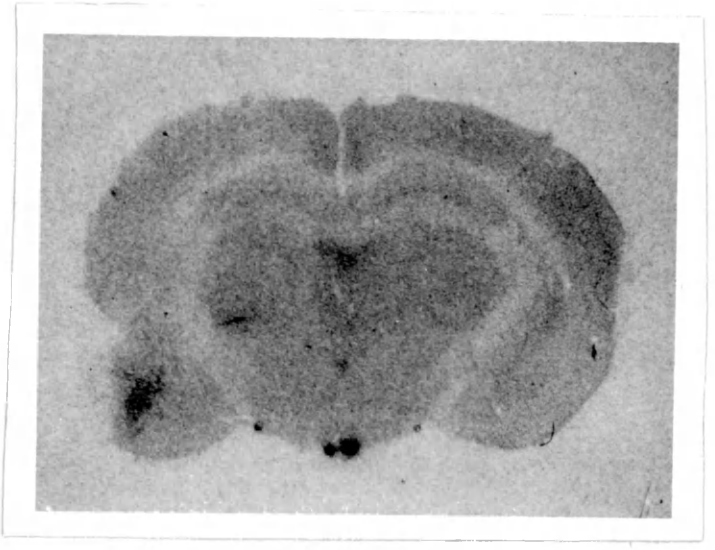
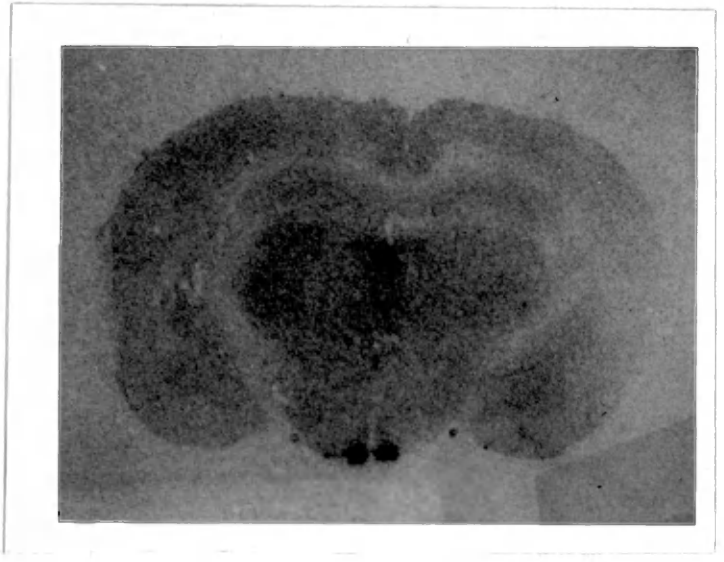


Figure 8. 2 - ^{125}I - melatonin binding in the pars tuberalis (PT) of Fischer 344 (F344) rats maintained in long days (LD) (16:8, lights on at 0500) and short days (8:16, lights on at 0900); top is LD and bottom is SD.





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