This is to certify that the

dissertation entitled

HYPOTHALAMIC NEURAL PATHWAYS THAT MEDIATE PHOTOPERIODIC CONTROL OF REPRODUCTION IN THE SYRIAN HAMSTER (MESOCRICETUS AURATUS)

presented by

Michael Howard Brown

has been accepted towards fulfillment
of the requirements for

Doctor of Philosophy degree in Neuroscience/Psychology

Major professor

Date October 28, 1986

O-12771

HYPOTHALAMIC NEURAL PATHWAYS THAT MEDIATE PHOTOPERIODIC CONTROL OF REPRODUCTION IN THE SYRIAN HAMSTER (MESOCRICETUS AURATUS)

By

Michael Howard Brown

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Psychology and Neuroscience Program

1986

ABSTRACT

HYPOTHALAMIC NEURAL PATHWAYS THAT MEDIATE PHOTOPERIODIC CONTROL OF REPRODUCTION IN THE SYRIAN HAMSTER (MESOCRICETUS AURATUS)

By

Michael Howard Brown

Exposure of male golden hamsters to photoperiods providing less than 12.5 hours of light per day results in testicular regression within 8-12 weeks. This response is thought to be mediated by a multisynaptic neural pathway by which photic information is relayed from the retina through the suprachiasmatic hypothalamic nucleus (SCN), paraventricular hypothalamic nucleus (PVN), preganglionic sympathetic neurons in the spinal cord, and postganglionic sympathetic neurons of the superior cervical ganglion (SGC) to the pineal gland. Anatomical evidence for these pathways has been provided and ablation of the SCN, the PVN, the SCG, or the pineal prevents testicular regression in hamsters exposed to short days. The present investigation was designed to further explore the hypothalamic circuits involved in this phenomenon utilizing hypothalamic damage less severe than that employed by previous researchers.

Bilateral knife cuts placed in a horizontal plane ventral to, through, or dorsal to the PVN all prevented testicular regression in hamsters exposed to short days but affected neither the circadian pattern of wheel-running activity nor the phase relationship between this behavior and the light-dark cycle. Cuts that either produced unilateral damage or were anterior to the PVN had no effect on the testicular response to short days.

Other investigators have provided anatomical evidence that some paraventriculo-spinal fibers take a lateral course upon leaving the PVN while others exit the nucleus via a dorsal route. In the present investigation, injections of horseradish peroxidase (HRP) into the spinal cord of hamsters that received cuts dorsal to the PVN, that either were bilateral and prevented gonadal regression or were intentionally made on only one side, resulted in

labelled neurons in the PVN. Thus, while cuts dorsal to the PVN probably sever some paraventriculo-spinal axons and this may be the reason for their efficacy in abolishing photoperiodic responses, it appears that destruction of only a portion of these connections is sufficient to produce this effect.

Either knife cuts (present investigation) or electrolytic lesions (previous reports) in the area of the PVN damage, in addition to neurons of the PVN and their axons, fibers of possible relevance to photoperiodism that pass through the area. Therefore, intrahypothalamic injections of N-methyl-D,L-aspartate, a toxin that destroys some neurons but is thought not to damage fibers passing through the injection site, were used to test the possibility that the effect of the knife cuts and electrolytic lesions were due to damage to these fibers of passage. Unilateral injections of NMA near the PVN destroyed most of the neurons of the posterior portions of the PVN, and produced a statistically significant reduction in the number of PVN neurons labelled after injections of HRP into the spinal cord. Bilateral injections of NMA that were centered in or very near the PVN also produced substantial damage to the posterior part of that nucleus, and furthermore, prevented the testicular response to short days. Five of these animals received tests of sexual behavior and 4 of them were able to inseminate hormonally-primed, sexually receptive females. Injections of NMA that were centered more distant from the PVN produced either no, or minor damage to that nucleus and either no, or partial effects on the testicular response to short days, respectively. That injections ineffective in abolishing testicular regression produced little or no damage to that portion of the hypothalamo-hypophyseal axis responsible for the secretion of luteinizing hormone was suggested by the fact that the 10 animals that were exposed to short days for 28 weeks all exhibited complete spontaneous testicular recrudescence.

These results support the hypothesis that neural projections from the SCN to the PVN, and projections from the PVN to the spinal cord mediate gonadal regression in hamsters

exposed to short days. Furthermore, disruption of only a portion of the paraventriculo-spinal projections is sufficient to prevent photoperiod-dependent gonadal regression.

ACKNOWLEDGEMENTS

For all of her help with Experiment 3 and two "follow-up" experiments,. Lori Badura has practically earned a coauthorship. I'm sorry I couldn't give it to you, Lori. The graduate office won't accept coauthored dissertations, but thanks for all of your help.

Beth Dettmer-Radtke did a great job of keeping me supplied with chemical solutions and other necessities. Mark Griswald spent many long hours helping me mount tissue. The two of them did a great deal of work on two of my "side projects" while I was intently collecting data for the dissertation.

Ken Smithson, Kevin Grant, and Tim Youngstrom answered a multitude of my naive questions about photography and photomicrography.

The members of my dissertation and guideance committee, Drs. Glenn Hatton, John Johnson, and Cheryl Sisk, have provided many valuable comments and criticisms regarding the manuscript, the experiments, and neuroscience in general. Cheryl was kind enough to take the time to read, and comment, on a portion of the text while she was frantically preparing her grant proposal. I especially want to thank Glenn, and the Neuroscince Program for the use of much of the equipment that made this work possible.

Tony Nunez chaired my MA and PhD committies, Gave me free-run of the lab and virtually unlimited use of his grant funding, let me test my own ideas-even some of the wildest of them, and literally got me started in "this business" (as he himself would say).

Thanks to all of you.

This research was funded by NIH grant MH 37877 and A.U.R.I.G. funds to Tony Nunez.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Many mammalian species are photoperiodic. These animals show seasonal reproductive cycles in which reproductive competence is dependent on the length of the light portion of the light-dark cycle. In a long-day breeder such as the golden hamster (Mesocricetus auratus), adult gonadal development is maintained while the animal is exposed to a stimulatory, long-day photoperiod (i.e. >12.5 hr of light per 24 hr in the case of the golden hamster). When golden hamsters are exposed to nonstimulatory (short-day) photoperiods, the hypothalamo-hypophysial axis, through a mechanism involving pineal melatonin secretion, becomes hypersensitive to negative feedback from gonadal steroids (Tamarkin, et al., 1976). This leads to a reduction in the release of gonadotropins and regression of the gonads to a prepubertal state (i.e. gametogenesis ceases, steroid release decreases, and, in males, the testes show reduced size while, in females, ovaries exhibit proliferation of interstitial tissue and lack of corpora lutea (for review-see Reiter, et al., 1983). In male golden hamsters, 8-12 weeks of exposure to nonstimulatory photoperiod is generally considered to be sufficient for complete gonadal regression. Prolongation of exposure to nonstimulatory photoperiod after the occurrence of gonadal regression results in photorefractoriness followed by spontaneous reproductive recrudescence. Testicular recrudescence is preceded by increases in circulating titers of gonadotropins and prolactin and a reduction in sensitivity of the hypothalamo-hypophysial axis to negative feedback from gonadal steroids (Ellis and Turek, 1979; Matt and Stetson, 1979; Turek and Ellis, 1981) leading to spontaneous gonadal recrudescence which begins after 15-20 weeks of exposure to nonstimulatory photoperiod (Steger, et al., 1985). Hamsters exhibiting photorefractoriness fail to show gonadal regression upon further exposure to

nonstimulatory photoperiod but will regain sensitivity to photoperiod if exposed to a stimulatory photoperiod for 7-11 weeks (Stetson, et al., 1977).

The neural pathways that mediate this response have long been debated, the proposed circuit having been modified several times. The current model involves a long circuitous pathway begining at the retina. Some retinal ganglion cells send axons into the hypothalamus which terminate in the suprachiasmatic nuclei (SCN; Moore and Lenn, 1972; Pickard and Silverman, 1981), forming the retinohypothalamic tract. The SCN contain a circadian oscillator (for review, see Rusak and Zucker, 1979) and appear to be necessary for seasonal reproductive cycles since SCN lesions prevent gonadal regression in hamsters housed in short-day photoperiod (Rusak and Morin, 1976). The SCN in turn send axonal projections to the paraventricular nucleus (PVN) of the hypothalamus (Berk and Finkelstein, 1981; Silverman, et al., 1981; Stephan, et al., 1981). Lesions of the PVN prevent gonadal regression in hamsters exposed to nonstimulatory photoperiods (Bartness, et al., 1985; Lehman, et al., 1984; Pickard and Turek, 1984) as well as the nighttime rise in pineal melatonin production both in rats (Klein, et al., 1983) and hamsters (Lehman, et al., 1984). Some of the cells of the PVN send long descending fibers to the intermediolateral cell column of the spinal cord (Armstrong, et al., 1980; Don Carlos and Finkelstein, 1982; Swanson and Kuypers, 1980b). Among the areas innervated by these axons are the upper thoracic segments (T1-T3) which contain preganglionic sympathetic cell bodies that innervate the superior cervical ganglion (Rando, et al., 1981). Efferent fibers of the superior cervical ganglion (SCG) form the nervi conarii which provide sympathetic input to the pineal gland (Ariens Kappers, 1960). Interruption of the pathway by superior cervical ganglionectomy, central deafferentation of the SCG, or pinealectomy (Reiter and Hester, 1966) results in insensitivity to photoperiod as evidenced by lack of gonadal regression in hamsters exposed to short-day photoperiods.

Evidence implicating the PVN in relaying photoperiodic information to the spinal cord should not be regarded as conclusive yet. Among the efferent projections of the SCN are

fibers that course dorsally through and by the PVN (Stephan, et al., 1981). Some of these fibers continue coursing dorsally through the paraventricular thalamic nucleus to the habenula. A subset of these axons continues caudally through the mesencephalic periaqueductal grey, travelling at least as far caudally as the dorsal raphe nucleus. The habenula in turn, projects to the pineal (Dafny, 1983). The PVN also sends axons to the habenula (Conrad and Pfaff, 1976) as well as directly to the pineal (Buijs and Pevet, 1980; Guerillot, et al., 1982; Korf and Wagner, 1980; Moller and Korf, 1983). All of these pathways are disrupted by the electrolytic lesions of the PVN that have been used to abolish circadian rhythms of pineal serotonin N-acetyltransferase (NAT) activity and melatonin content in rats (Klein, et al., 1983), and gonadal regression in hamsters exposed to nonstimulatory photoperiod (Bartness, et al., 1985; Lehman, et al., 1984; Pickard and Turek, 1983). These direct projections from the brain to the pineal gland are not sufficient to maintain photoperiodically mediated seasonal reproductive cycles since superior cervical ganglionectomy abolishes the cycles (Reiter and Hester, 1966). Nevertheless, these alternative pathways may be necessary for normal circadian rhythms in the pineal and for gonadal regression in hamsters housed in short-day photoperiod.

Additionally, other hypothalamic areas send descending projections to autonomic regions of the brainstem and spinal cord (Don Carlos and Finkelstein, 1982; Swanson and Kuypers, 1980a). These other areas may also be involved in neural regulation of pineal function and photoperiodic reproductive cycles. A possibility that has not yet been ruled out is that the efficacy of PVN lesions may be due to interruption of fibers (from the SCN) passing through the lesion site to other areas.

To further explore the hypothalamic neural pathways involved in photoperiodic control of reproduction, the following experiments were performed. The specific goal of the research was to confirm the role of the PVN in photoperiodic regulation of reproductive cycles through the use of hypothalamic knife cuts, intrahypothalamic injections of neurotoxin, and neural tract tracing by the use of horseradish peroxidase

(HRP). Experiment 1 was designed to examine the role of fibers that project from the SCN to the PVN in testicular regression via placement of knife cuts between these two nuclei. In Experiment 2, knife cuts placed in the path of paraventriculo-spinal axons and injections of HRP into the spinal cord were used to investigate the role of projections from the PVN to the spinal cord in testicular regression. Intrahypothalamic injections of N-methyl aspartate, a neurotoxin thought to destroy neuronal cell bodies without affecting axons that pass through the injection site, were employed in Experiment 3 to test whether fibers that project dorsally from the SCN through the PVN are involved in testicular regression.

EXPERIMENT 1

As previously mentioned, the SCN contain a circadian oscillator (Rusak and Zucker, 1979) and send efferent projections to the PVN (Berk and Finkelstein, 1981; Silverman, et al., 1981; Stephan, et al., 1981). Bilateral electrolytic lesions of either nucleus abolish circadian rhythms of pineal NAT activity and melatonin content (Klein, et al., 1983; Lehman, et al., 1984; Moore and Klein, 1974) and prevent testicular regression in hamsters housed in short-day photoperiod (Bartness, et al., 1985; Lehman, et al., 1984; Pickard and Turek, 1983; Rusak and Morin, 1976). These results suggest that neural input to the PVN from the SCN is necessary for photoperiod-dependent reproductive cycles.

In the present experiment, knife cuts were placed in a horizontal plane aimed ventral to the PVN before the hamsters were placed in a short-day photoperiod. Knife cuts placed in the intended location should have interrupted projections from the SCN to the PVN without damaging either nucleus. Thus, the experiment was carried out to test whether fibers that project dorsally out of the SCN are necessary for testicular regression in hamsters housed in short days. To verify that the retinohypothalamic tract was not damaged by the knife cuts, some of the hamsters were given intraocular injections of HRP. Additionally, circadian locomotor activity patterns of some of the animals were monitored at the end of the experiment to test the ability of the animals to entrain to the light-dark cycle.

Methods

Subjects and Housing

Adult male golden hamsters (LVG/LAK; Charles River Breeding Laboratory, Newfield, NJ) were individually housed and given ad lib access to water and food. At the begining of the experiment, animals were housed in a long-day photoperiod (16 hrs light:8 hrs dark). Light intensity was approximately 15 foot candles.

Surgery

Twenty-five animals were assigned to one of two surgical conditions. Surgery was performed under Equithesin (4.5 ml/kg body weight) anesthesia. For placement of knife cuts, 10 hamsters were placed in a stereotaxic apparatus (Kopf Instruments) and an incision was made through the skin on the dorsal surface of the skull. A piece of skull (approximately 3 mm square) was removed, the superior sagittal sinus retracted, and a Scouten (Scouten, et al., 1981) retractable wire knife (Kopf Instruments) was lowered into the brain at the midline of the animal. Stereotaxic coordinates were 0.3 mm posterior to the bregma, 5.8 mm ventral to the sinus with the top of the incisor bar 2.0 mm below ear bar zero. With the cannula in position, the blade of the knife was extended 2 mm in a direction perpendicular to the midline of the animal in order to puncture the ependymal lining of the third ventricle. The knife was then rotated 90° counterclockwise (ie. until the blade was parallel to the midline) and then 180° clockwise then returned to the original position. With the outer guide cannula left in place, the blade was then retracted and the procedure repeated on the opposite side of the animal. Finally, the knife was rotated a full 360° degrees in each direction before the blade was retracted and the knife removed from the animal. Fifteen hamsters served as sham-operated control animals. These hamsters received the incision to expose the skull but no neural damage.

Procedure

Hamsters were returned to the long-day photoperiod and allowed to recover from surgery for one week. The 10 hamsters with knife cuts and nine of the sham-operated

control hamsters were then moved to another colony room and housed in a short-day photoperiod of 6 hrs light:18 hrs dark. The remaining six hamsters were kept in the original long-day photoperiod. After the short-day housed hamsters had been in that photoperiod for 13 weeks, the hamsters were again anesthetized with Equithesin and castrated. Paired testicular weights for each animal were recorded to the nearest 0.1 mg. Long-day housed control hamsters were sacrificed at the time of castration. Short-day housed control hamsters and hamsters that received knife cuts were returned to the short-day photoperiod and allowed to recover from surgery for two weeks. After that time, 3 control hamsters and 6 hamsters with knife cuts were placed in metal cages (21.5 x 11 x 11 cm) attached to activity wheels (35.5 cm in diameter x 14 cm wide). Photoperiod was maintained at 6 hrs light:18 hrs dark. Locomotor activity was monitored via optoelectronic activity counters. Each counter consisted of an infrared light emitting diode aimed at a phototransistor. The transistor and diode were placed in a position such that a cam on the axle of the wheel interrupted the light beam once during each wheel revolution. The phototransistors were connected to input channels of optoisolated input/output boards (Mullen Computer Products) inside a 64K Northstar Horizon computer which also contained a real-time clock board (Mountain Hardware, Inc.). Data were collected as number of wheel revolutions per 12-min interval (i.e. 120 intervals per 24-hr day) and stored on 5.25 in. floppy disks (Verbatim Corp.) every 24 hr. For visual presentation, the data were collapsed into 36-min intervals. For each 36-min interval in which the number of wheel revolutions exceeded 14, a dark rectangle was printed. A blank space was printed for each interval in which 14 or fewer revolutions occurred. Data were printed on a 24-hr time scale horizontally, with data for each day printed below data for the previous day. Hamsters were allowed to habituate to the recording conditions for 15 days, after which activity was monitored for 11 days. Visual inspection of the records was used to estimate phase angle (i.e. time between onset of activity and onset of darkness) of the activity patterns to the nearest 18 min.

Histology

After collection of locomotor activity data, eight of the hamsters that had received knife cuts were anesthetized and given bilateral intraocular injections of 6 µl of a 30% solution of HRP (Sigma Type VI). After a 24-hr survival period these animals were sacrificed by overdose of sodium thiamylal (Surital; Parke-Davis) and perfused transcardially with 5% saline containing Heparin (1000 U/l) followed by 4% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). The superphysiological concentration of saline was selected in an attempt to reduce enlargement of the third ventricle during histological processing of the tissue. Brains were removed and stored, refrigerated, for 48 hrs in 0.1M phosphate buffered sucrose solutions of increasing concentrations (5%, 10%, 15%, 20%; 6-12 hrs in each solution). In some cases a small amount of 4% glutaraldehyde was added to the first two sucrose solutions to improve fixation of the tissue. Frozen 50 µm thick sections were cut in an oblique coronal plane to include the SCN and the anterior part of the PVN in the same sections. Free-floating sections were reacted with tetramethylbenzidine (TMB) according to published procedures (Mesulam, et al., 1980). Alternate series of sections were mounted on chrome-alum subbed slides and stained with either cresylecht violet or pyronin Y. Two hamsters (Nos. 4 and 9) died before HRP injections were scheduled. Their brains were removed and stored for two weeks in 10% formalin, then sectioned and stained with cresylecht violet. Bright- and dark-field illumination at magnifications ranging from 25-100 X were used to verify the presence of TMB reaction product in the SCN. Selected sections were traced with the aid of a projecting microscope. From these tracings, schematic diagrams were made showing the location of the cuts at the point of greatest bilateral damage. The tracings were made without knowledge of the gonadal status of the individual animals. Finally, selected sections were photographed using Kodak Technical Pan Film 2145 and prints were made on Kodak F5 paper. Since the variance of testicular weight was higher for hamsters that received knife cuts than for each of the two groups of control hamsters, two-tailed Mann-

Whitney U tests were used for statistical comparisons of testicular weights between the three groups. In order to correct for multiple comparisons, the probability of incorrectly stating that differences exist, α, was divided equally among each of the 3 possible comparisons (Kirk, 1968). Thus, the critical value was 0.05/3 = 0.0166. In addition, when feasible (i.e., when U was an intergral number), exact probabilities associated with the obtained U values were reported either from tabled values (when n_1 and n_2 both < 9) or (when either n_1 or $n_2 \geq 9$) as estimated from the formula given by Kirk (1968). A two-tailed Mann-Whitney U test was also used for comparison of the phase angle of the activity patterns.

Results

In two hamsters (Nos. 6 and 7), the knife cuts produced unilateral damage to the hypothalamus dorsal to the PVN. In one case (No. 12) no evidence of gliosis or neural damage was detected. Seven hamsters had bilateral knife cuts that were placed in the intended anterior-posterior location. As illustrated in Figure 1, however, the dorso-ventral positioning of the knife cuts was quite variable. Knife cuts fell ventral to (No. 2), through (Nos. 9, 10, 11), or slightly dorsal to (Nos. 1, 4, 5) the PVN. A photomicrograph of a coronal section through the hypothalamus of a hamster (No. 1) representative of cases in which the cuts were dorsal to the PVN is presented in Figure 2.

As summarized in Figure 3, all seven of the hamsters with confirmed bilateral knife cuts had large testes (3.65 ± 0.44 g; mean ± SEM). The six sham-operated control hamsters that were housed in long-day photoperiod also had large testes (4.07 ± 0.20 g) and did not differ significantly from animals with knife cuts on this measure (U = 19, p = 0.836). On the other hand, the nine sham-operated control hamsters housed in short-day photoperiod had fully regressed testes (0.56 ± 0.06 g) which were statistically significantly different both from those of control hamsters that were housed in long days (U = 0, p = 0.00148) and from those of hamsters with bilateral knife cuts (U = 0, p = 0.00086). The three hamsters (Nos. 6, 7, and 12) in which knife cuts were either

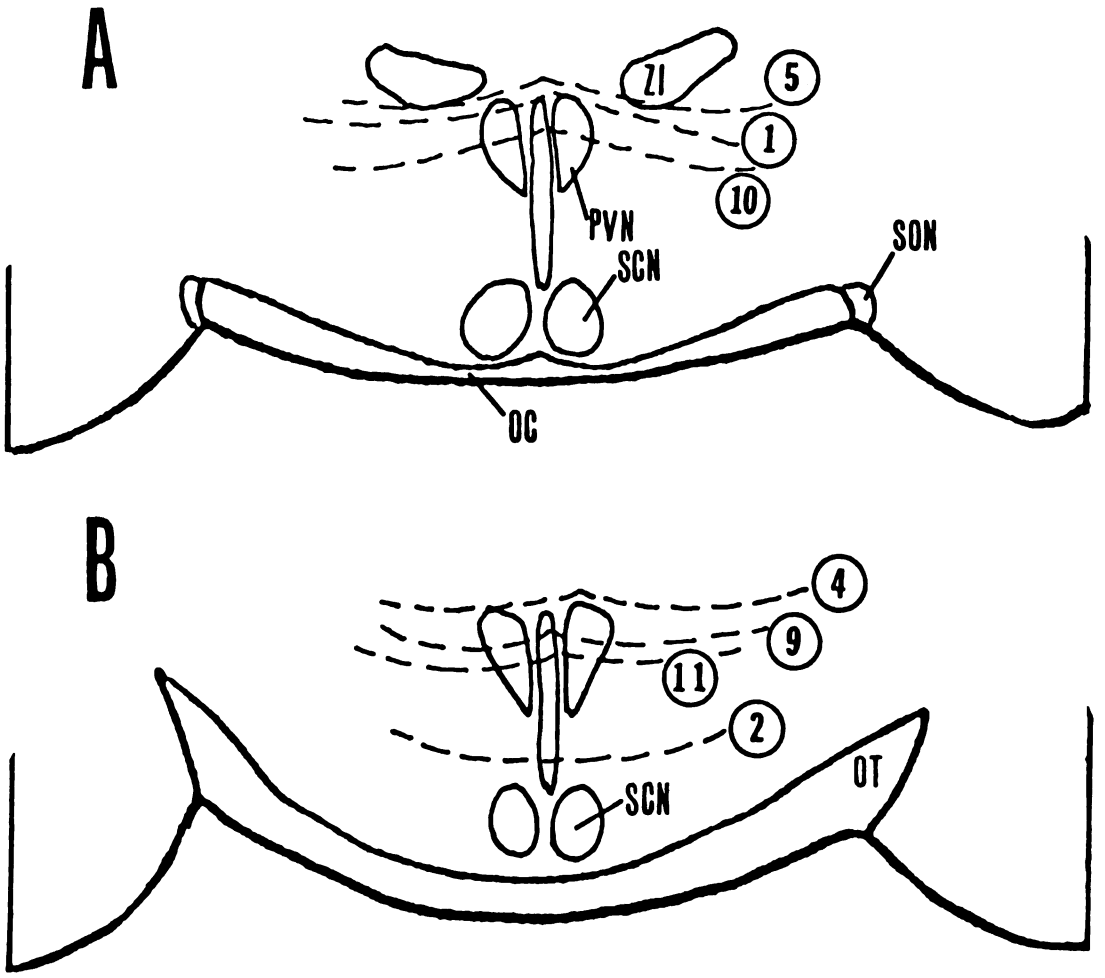

Figure 1: Schematic representation of knife cuts that produced bilateral damage to the hypothalamus. The cuts (dashed lines) are shown at the point of greatest bilateral damage for each case using drawings (A and B) modified from those published by Lehman et al. (1984). The individual cases are identified by numbers. Abbreviations are: OC, optic chiasm; OT, optic tract; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; ZI, zona incerta.

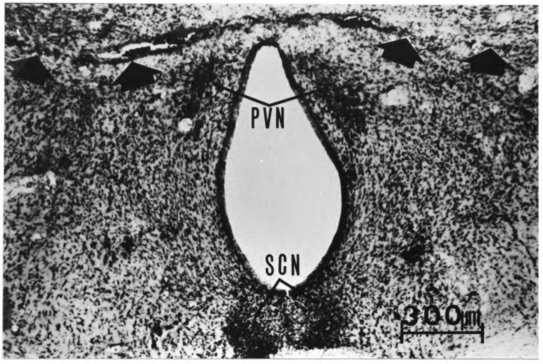

Figure 2: Photomicrograph of a coronal section (50 μm thick, pyronin Y stain) through the hypothalamus of a hamster (No. 1) in which the knife cut (indicated by arrows) produced bilateral damage just dorsal to the PVN. For abbreviations, see Figure 1.

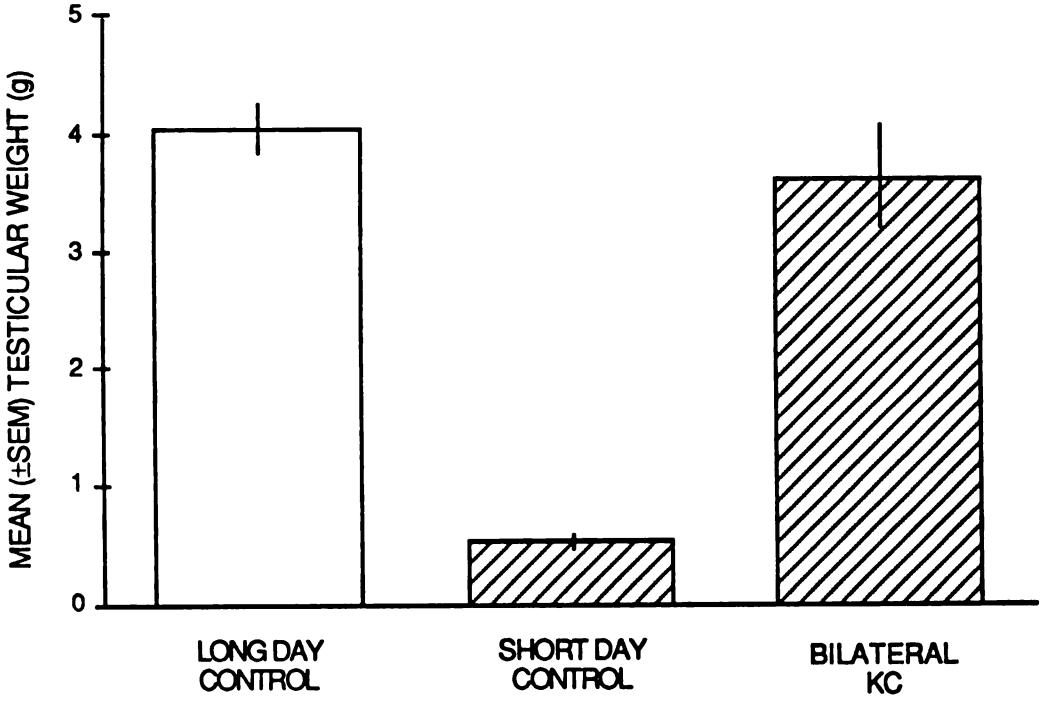

Figure 3: Mean (±SEM) testicular weights of the short-day housed hamsters with bilateral knife cuts (n = 7) and sham-operated hamsters kept in short days (shaded bar; n = 9) or long days (open bar; n = 6).

unilateral or undetectable also showed testicular regression (testicular weights were 0.45, 0.38, and 0.64 g, respectively) although they were not statistically compared with the other groups.

Microscopic examination of the brains revealed that HRP/TMB reaction product was present in the SCN of all eight hamsters that received injections of HRP. The distribution of reaction product was similar to that reported in intact hamsters after intraocular injection of HRP (Pickard and Silverman, 1981), indicating that the knife cuts did not damage the retinohypothalamic tract.

Each of the 6 hamsters with bilateral knife cuts and the 3 control hamsters for which activity patterns were monitored showed normal nocturnal patterns of activity. Figure 4 shows the activity record of a hamster (No. 2) in which the knife cut was ventral to the PVN. Onset of activity lagged behind onset of darkness (i.e., negative phase angle). Mean phase angle (± SEM) was -238.5 ± 34.0 min for the four hamsters with bilateral knife cuts (Nos. 2, 5, 10, 11) and -156.0 ± 6.0 min for control hamsters. Phase angle did not differ statistically between hamsters with knife cuts and control hamsters ($U = 2.5$, $p = 0.228$). One hamster in which the knife cut produced only unilateral damage to the hypothalamus (No. 7) and the hamster in which damage from the cut could not be detected (No. 12) showed phase angles of -306 min and -72 min, respectively.

Discussion

Knife cuts ventral to, through, or dorsal to the PVN prevented testicular regression in hamsters housed in nonstimulatory photoperiod without disrupting their circadian activity patterns. This effect of knife cuts ventral (Eskes and Rusak, 1985; Inouye and Turek, 1986) or dorsal (Inouye and Turek, 1986) to the PVN on the testicular response to short-day photoperiod has been independently shown by other investigators. Also, in rats, knife cuts placed ventral to the PVN but dorsal to the SCN fail to affect free-running circadian rhythms of locomotor activity and drinking behavior (Brown and Nunez, 1986).

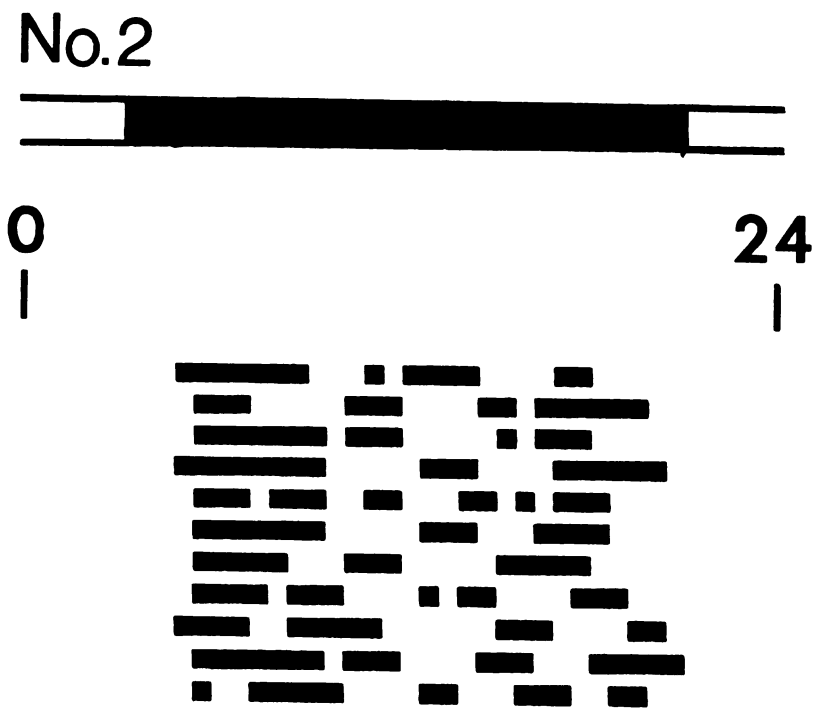

Figure 4: Activity record of a hamster (No. 2) that received a bilateral knife cut ventral to the PVN and did not show testicular regression after 13 weeks of exposure to short days. The black bar at the top represents the dark portion of the light-dark cycle.

It should be noted that sampling of testicular condition at only one point in time (via castration) does not permit evaluation of the possibility that the effect of the knife cuts was to affect the rate of testicular regression rather than to prevent this response. Therefore, in Experiment 2B (below) repeated sampling of testicular width of hamsters was used to test this possibility.

The knife cut ventral to the PVN probably prevented the testicular response to short-day photoperiod by interrupting neural input to the PVN from the SCN (Berk and Finkelstein, 1981; Silverman, et al., 1981; Stephan, et al., 1981). Efficacy of cuts through the PVN may have been due to interruption of SCN input to PVN cells dorsal to the cut, disruption of communication between cells within the PVN, and/or interruption of descending projections from the PVN to the thoracic spinal cord (Armstrong, et al., 1980; Don Carlos and Finkelstein, 1982; Swanson and Kuypers, 1980b).

Interpretation of the results of cuts dorsal to the PVN is less clear and at least two possible explanations exist. The SCN send fibers through and past the PVN to the habenula and other areas (Stephan, et al., 1981) and projections from the PVN to the habenula (Dafny, 1983) and the pineal (Buijs and Pevet, 1980; Guerillot, et al., 1982; Korf and Wagner, 1980; Moller and Korf, 1983) have been described. Thus, the dorsal cuts as well as the PVN lesions reported by other investigators (Bartness, et al., 1985; Klein, et al., 1983; Lehman, et al., 1984; Pickard and Turek, 1983;) should have interrupted these pathways from both the SCN and the PVN. Therefore, the loss of the gonadal response to short-day photoperiod might have been due to disruption of either of these pathways. Alternatively, the dorsal cuts may have blocked testicular regression by severing descending projections from the PVN to the thoracic spinal cord. Experiments 2 and 3 were conducted in order to further explore the neural pathways that mediate testicular regression in hamsters exposed to short days. Experiment 2 addressed the question of whether knife cuts dorsal to the PVN abolish testicular regression by interrupting paraventriculo-spinal projections. Experiment 3 was designed to evaluate the

possibility that the effects of knife cuts and electrolytic lesions in or near the PVN are due to interruption of fibers that pass through the area of the damage.

EXPERIMENT 2

The purpose of this experiment was twofold: first, to determine whether knife cuts dorsal to the PVN interrupt axons that project from the PVN to the spinal cord, and second; to replicate the results of Experiment 1 using repeated measurements of gonadal status. When the experiment was initiated, the exact course of fibers that project from the PVN to the spinal cord had not been described. Previous investigators had observed axons (e.g., Armstrong, et al., 1980) and axon collaterals (Hatton, et al., 1985) that project dorsally as well as those that project laterally from neurons of the PVN of rats, although limitations of the techniques that were used prevented the visualization of the full length of the longer of these fibers from the cell bodies of origin to the termination of the axons. Since these dorsally-projecting fibers from cells of the PVN had been reported, and since projections from the PVN to the spinal cord were known to exist both in rats (Armstrong, et al., 1980; Swanson and Kuypers, 1980b) and in hamsters (Don Carlos and Finkelstein, 1982), it seemed plausible that knife cuts dorsal to the PVN might prevent testicular regression by severing fibers that project from the PVN to the spinal cord. Further evidence that is consistent with this possibility was provided by Nance (1981), who reported that an obliquely parasagittal (or "frontolateral") unilateral knife cut prevented transport of bisbenzamide from the spinal cord to the PVN of rats. Nance interpreted this result as being due to disruption of fibers that project laterally from the PVN and terminate in the spinal cord. Careful inspection of the tracings of representative brain sections presented by the author, however, reveals that while the lower portions of these cuts were lateral to the PVN, the orientation of these cuts was such that the dorsalmost tips of the cuts were dorsal to the PVN and lay at the midline of the animal. Thus, the cuts that were effective in abolishing projections from the PVN to the spinal cord would have severed fibers that project dorsally as well as those that project laterally from the PVN. Therefore, given the knowledge that was available, it was not possible to determine whether the dorsal knife cuts that prevented testicular regression in Experiment 1 had severed

projections from the PVN to the spinal cord.

Part A: Do Knife Cuts Dorsal to the PVN Interrupt Paraventriculo-Spinal Projections?

Initially, unilateral horizontal cuts dorsal to the PVN were employed to further investigate the neural pathways destroyed by the cuts that abolish photoperiodic responses. These cuts were aimed at stereotaxic coordinates identical to those used in Experiment 1 and were followed by bilateral injections of HRP into the upper thoracic spinal cord. The procedure for these injections is explained in detail in Part B. Sections of the spinal cords were microscopically inspected to verify that the injections were bilateral. In addition an area of the lateral hypothalamus served as a control area to ensure that asymmetries in the distribution of labelled PVN neurons were not due to failure of the injections of HRP. Labelled neurons were counted in an area of the lateral hypothalamus represented in Figure 5. In order to show the location of knife cuts and the distribution of labelled neurons in the PVN, tracings were made of a series of sections through the PVN. In one case, the cut was placed dorsal to the anterior and medial portions of the PVN and passed through the dorsalmost part of the lateral portion (the lateral parvocellular division of the nucleus, according to the terminology of Swanson and Kuypers [1980b]). Similar to the knife cuts used by Nance (1981), this cut was in a position in which it should have severed most of the axons that project laterally, as well as most of those that project dorsally out of the PVN. As illustrated schematically in Figure 6, > 90% of the labelled PVN cells in this hamster were located contralateral to the knife cut. Conversely, labelled cells in the lateral hypothalamus were relatively evenly distributed between the two sides of the brain. The results of the cell counts indicated that 10 labelled cells were found in 7 sections through the control area ipsilateral to the cut while 12 were found on the contralateral side. These results indicate that the cut probably severed most of the axons that project from the PVN to the spinal cord. In another hamster (No. 128), an asymmetrical distribution of labelled PVN neurons was found. The cut in this hamster was more dorsal and centered more anterior than that in No. 122 and did not produce direct damage to the PVN. This cut was in a position such that it should have severed some of

Figure 5: Location of the area (indicated by hatching) of the lateral hypothalamus in which counts of HRP labelled cells were made. The drawings (A-C, rostral to caudal) of the hamster hypothalamus are a modification of those published by Malsbury (1977). Abbreviations are: ARC, arcuate nucleus; F, fornix; OT, optic tract; VMN, ventromedial nucleus.

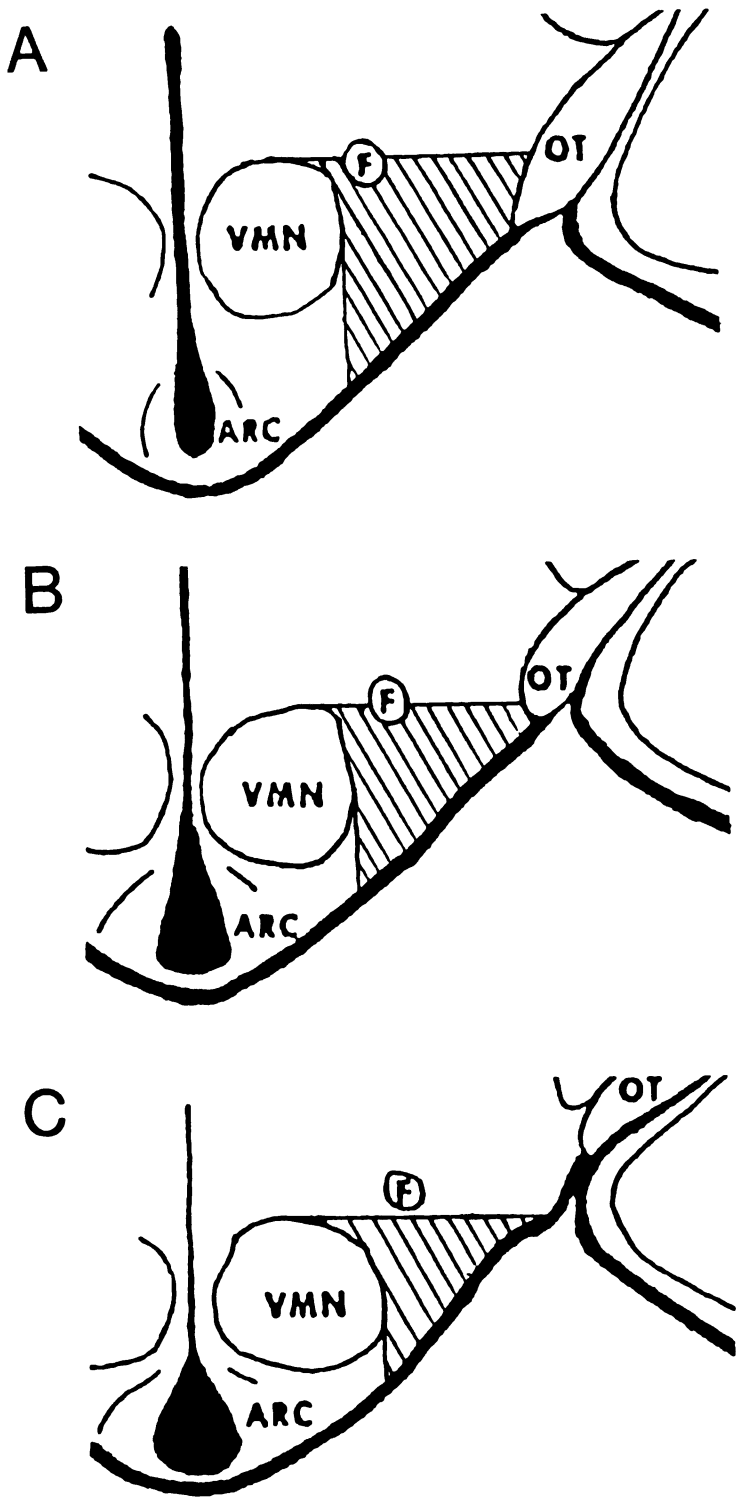

Figure 5

Figure 6: Schematic representation of the distribution of HRP-labelled neurons in three sections (at 240 μm intervals, A-C are rostral to caudal) through the paraventricular nucleus of a hamster (No. 122) that received a unilateral knife cut (indicated by arrows) before bilateral injections of HRP into the spinal cord. Note the reduction in the number of HRP-labelled cells ipsilateral to the cut. Abbreviations are: OC, optic chiasm; OT, optic tract; PVN, paraventricular nucleus; SCN suprachiasmatic nucleus; ZI, zona incerta; 3V, third ventricle. Each dot represents one labelled cell.

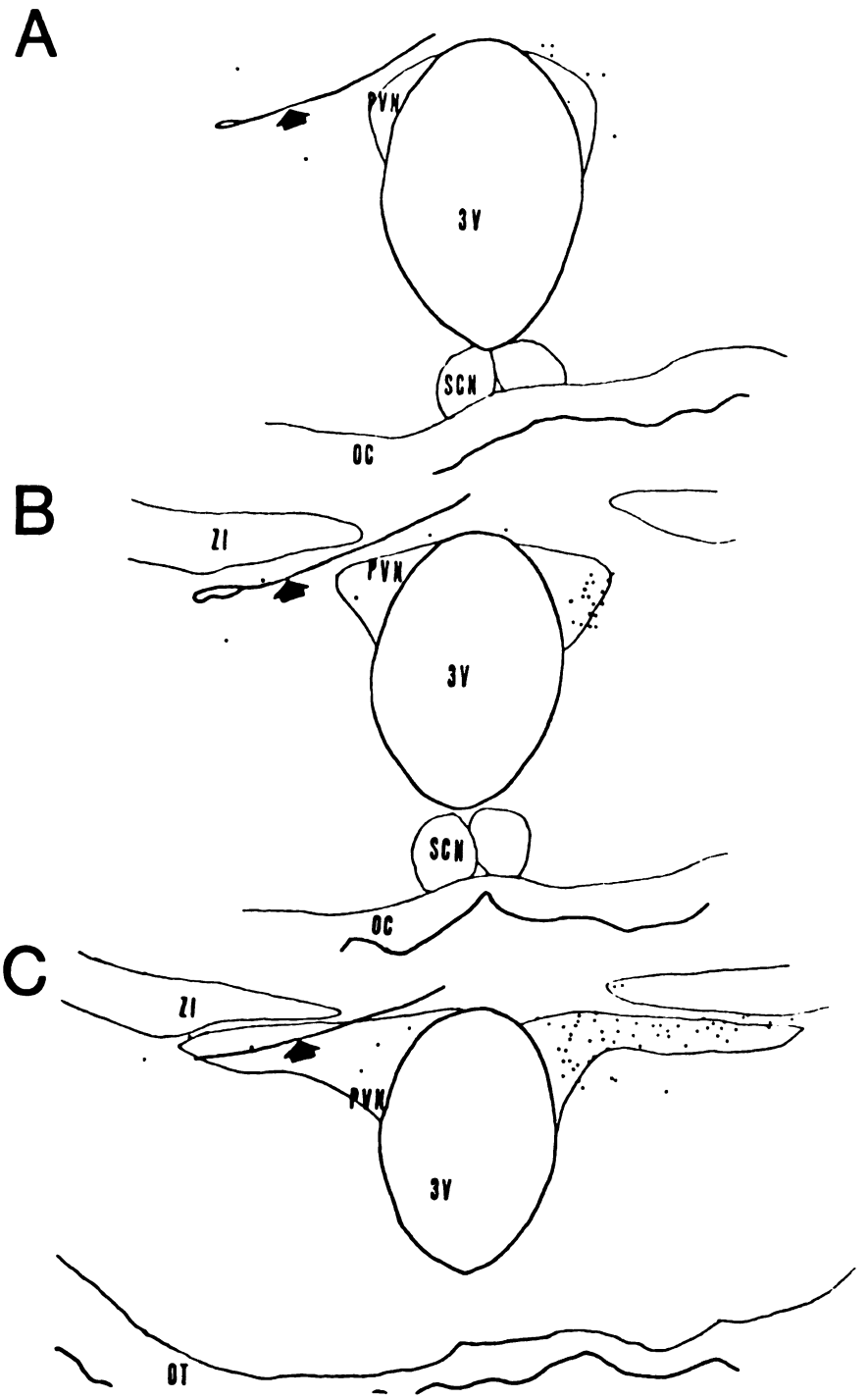

Figure 6

the fibers that project dorsally out of the PVN but probably did not damage axons that project laterally out of the nucleus. The location of the cut and the distribution of labelled neurons in this hamster are shown in Figure 7. The number of labelled neurons in 5 sections through the control area of the lateral hypothalamus of hamster No. 128 were 35 (ipsilateral to the cut) and 37 (contralateral to the cut). The small reduction in the number of labelled neurons in the PVN ipsilateral to the cut in this hamster indicates that some of the axons that project from the PVN to the spinal cord may have been severed by the cut but that many were left intact. In a third hamster, the cut was just dorsal to the PVN. As shown in Figure 8, the distribution of labelled PVN neurons was much more symmetrical in this hamster than in either of the two cases discussed above.

Although no strong conclusions can be drawn from these findings, these results, as well as the finding that bilateral cuts that were placed dorsal to the PVN prevented testicular regression (Experiment 1) are consistent with the idea that some paraventriculo-spinal fibers leave the nucleus in a dorsal direction while others take a lateral course upon leaving the PVN. In contrast to the results of dorsal cuts, bilateral parasagittal cuts placed lateral to the PVN fail to prevent testicular regression in hamsters exposed to nonstimulatory photoperiod (Larry Morin, personal communication). Thus, it appears that fibers that project laterally from the PVN are not critical for the testicular response to short days.

Results published after these initial findings were obtained have provided more direct evidence that some paraventriculo-spinal fibers project dorsally out of the PVN. Following injection of the plant lectin, PHA-L, into the PVN of rats, Luiten, et al. (1985) were able to trace two bundles of labelled fibers from the PVN through the dorsolateral funiculus of the spinal cord to the intermediolateral cell column at the thoracolumbar level of the spinal cord. The two bundles were reported to be composed of approximately equal numbers of fibers. Bundle number 1 takes a dorsal course upon leaving the PVN and travels through the thalamus and mesencephalic periaqueductal grey. Bundle number 2 leaves the PVN in

Figure 7: Schematic representation of the distribution of HRP-labelled neurons in three sections (at 240 μm intervals, A-C are rostral to caudal) through the paraventricular nucleus (PVN) of a hamster (No. 128) that received a unilateral knife cut (indicated by arrows) before bilateral injections of HRP into the spinal cord. Note that the cut was more dorsal and centered more anterior than that in hamster No. 122 (Figure 5). Note also that the reduction in the number of HRP-labelled cells ipsilateral to the cut was less substantial than that in hamster No. 122.

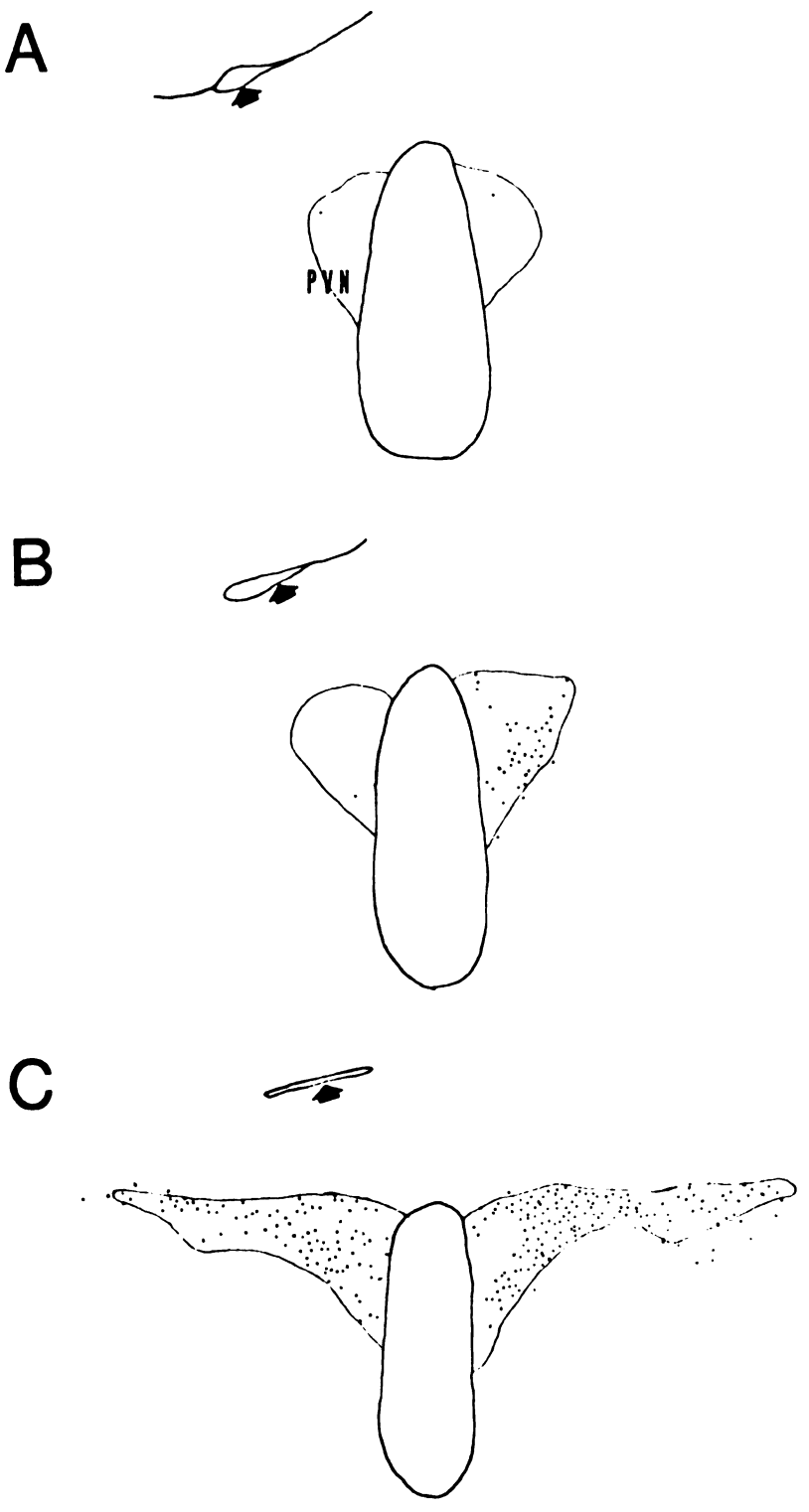

Figure 7

Figure 8: Schematic representation of the distribution of HRP-labelled neurons in three sections (at 240 μm intervals; A-C, rostral to caudal) through the paraventricular nucleus (PVN) of a hamster (No. 123) that received a unilateral knife cut (indicated by arrows) before bilateral injections of HRP into the spinal cord. Note that the cut was dorsal to the PVN and failed to produce a reduction in the number of HRP-labelled cells in the PVN ipsilateral to the cut.

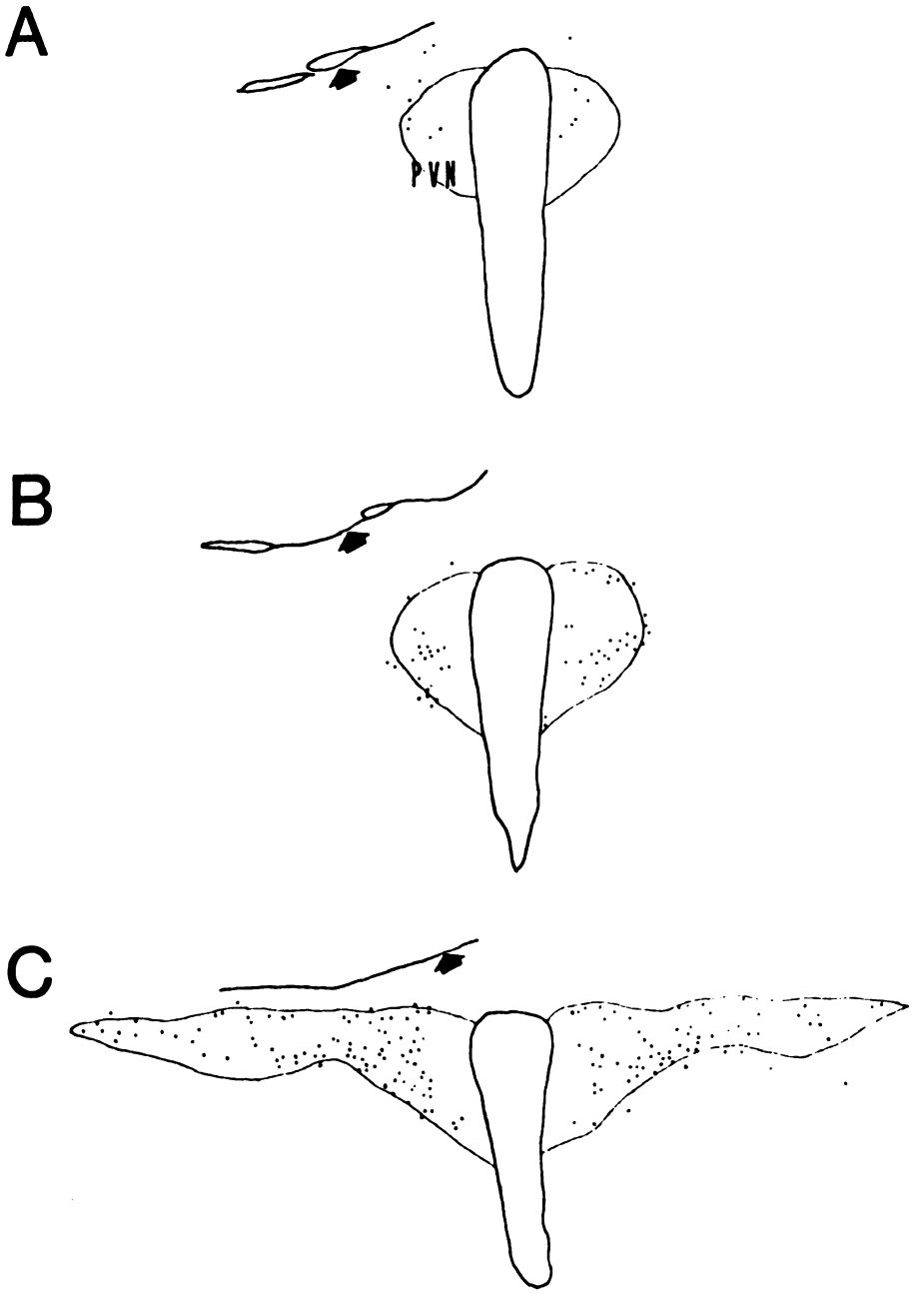

Figure 8

a lateral and caudal direction turning caudally at the boundry between the internal capsule and the medial forebrain bundle. At the level of the pons, fibers from the two bundles merge and continue coursing caudally. Preliminary results obtained using PHA-L indicate that paraventriculo-spinal fibers in the hamster may follow a course similar to that reported in the rat (Youngstrom and Nunez, 1986). Provided that similar pathways exist in the hamster, it would appear that the knife cut through the dorsal PVN prevented transport of HRP into the PVN by severing both bundle 1 and bundle 2. More dorsally placed knife cuts would have spared most or all of the fibers in bundle 2 and hence, failed to have as dramatic an effect on the retrograde transport of the enzyme into the PVN. Furthermore, some of the fibers of bundle 1 may arise as collateral branches of axons of bundle 2. If a sizeable proportion of these fibers are collateral branches of the same parent axon, then transection of only one of these two bundles would not be likely to produce a dramatic reduction in the number of neurons labelled after injections into the spinal cord. While Luiten, et al. did not address the issue of axon collaterals, Hatton, et al. (1985) have observed axons of magnocellular PVN neurons that give rise to collateral branches. While most of these branches terminated within the hypothalamus, some of them apparently left the plane of the sections without showing a terminal thickening and thus, may have projected to extrahypothalamic sites. Therefore, while the possibility that some of the fibers in bundle 1 and bundle 2 may arise as collateral branches of the same parent axon is speculative, evidence of branching of axons of PVN neurons has been reported.

Since the results of Luiten, et al. (1985) provided evidence that the dorsal cuts in Experiment 1 probably interrupted bundle 1 and that similarly placed unilateral cuts would not sever all of the fibers that project from the PVN to the spinal cord, the strategy involving unilateral cuts and spinal injections of HRP was abandoned. Work in progress (Youngstrom and Nunez) using PHA-L, should provide direct evidence for the course of fibers that project from the PVN to the spinal cord in hamsters. The use of PHA-L allows direct visualization of axons and, therefore, is a more powerful technique for elucidating

the course of fiber tracts than placing knife cuts in their path. Instead, Part B of the present experiment was focused on replicating the results of Experiment 1 and determining whether it is possible to prevent testicular regression by severing some but not all of the PVN projections to the spinal cord (i.e., bundle 1 but not bundle 2).

Part B: Paraventriculo-Spinal Projections and the Testicular Response to Short Days.

Since testicular condition was measured only once in Experiment 1, it was not possible to determine whether the knife cuts had prevented testicular regression or affected the rate of testicular regression. It is remotely possible that the animals with confirmed bilateral cuts showed an accelerated testicular regression followed by a rapid spontaneous gonadal recrudescence. It is also possible that the effect of the cuts was to slow the rate or delay the onset of testicular regression. Although 8-12 weeks of exposure to short days or blinding is generally considered sufficient to induce complete testicular regression in hamsters, there may be conditions under which regression is delayed in some animals. In one report (Eskes and Zucker, 1978) 3 out of 9 hamsters that were housed in activity wheels showed little or no testicular regression 13 weeks after blinding. Two of these 3 animals showed signs of testicular regression 17 weeks after blinding. Thus, since it is possible to delay gonadal regression, it was important to replicate Experiment 1 using repeated measurements of testicular condition in order to evaluate the time-course of gonadal regression or lack thereof. Moreover, the use of repeated measurements on individual animals facilitated assessment of the possibility that partial knife cuts might cause partial effects such as delayed gonadal regression.

Bilateral knife cuts were made dorsal to, or through the PVN of hamsters before the animals were exposed to short days. Gonadal status of the animals was monitored via biweekly measurement of testicular width. At the end of the experiment, horseradish peroxidase was injected bilaterally into the spinal cord of hamsters in which the cuts had prevented testicular regression to determine whether any projections from the PVN to the spinal cord still existed in these animals.

Methods

Subjects and housing conditions were similar to those reported in Experiment 1.

Procedure

Twelve hamsters were given bilateral knife cuts aimed dorsal to the PVN as described in Experiment 1. Ten animals served as sham-operated controls. All of these animals were housed in long-day photoperiod before and for a week after surgery. All of the hamsters with knife cuts and five of the sham-operated control hamsters were then moved to a short-day photoperiod while five sham-operated hamsters served as long-day photoperiod control animals. After four weeks of housing in short days, the animals were anesthetized with methoxyflurane (Metofane; Pittman-Moore, Inc.) and testicular length and width were recorded to the nearest 0.1 mm using hand-held calipers. This procedure was repeated biweekly for 12 weeks (i.e. until the hamsters had been exposed to the nonstimulatory photoperiod for 16 weeks). Three hamsters with knife cuts were sacrificed after 12 weeks of exposure to short days. At the completion of data collection, the 9 remaining hamsters with knife cuts and the 10 sham-operated hamsters were anesthetized and castrations were performed as previously described. The 9 animals with knife cuts were allowed to recover from this surgery for one week before they were transferred to activity wheel cages and their activity patterns monitored as in Experiment 1. One hamster failed to drink after being placed in the activity wheel and was sacrificed before the completion of data collection. As a result, wheel-running activity data were recorded for only 8 of the hamsters. Phase angles of their activity patterns were estimated from 11-day segments of the activity records as described in Experiment 1.

Bilateral injections of HRP into the spinal cord were performed in 5 animals under Equithesin anesthesia. An incision was made through the skin on the dorsal surface of the animal. With the aid of a dissecting microscope, adipose, muscular, and connective tissue were then dissected and retracted to expose the vertebral column. A 10-µl Hamilton syringe with a 32-gauge needle was used to inject 2 µl of a 30% solution of HRP (Sigma

Type VI) into the spinal cord between the seventh cervical (C7) and first thoracic (T1) vertebrae. The injections were bilateral and were made midway between the midline and the lateral edge of the spinal cord. Injections were made over a 1-min period and the needle left in place for approximately 1 min after the injection. Following the injections of HRP, the hamsters were allowed to survive 48 hr before perfusion-fixation as described in Experiment 1. Hamsters that did not receive injections of HRP were perfused with 0.9% saline followed by 10% formaldehyde.

Histology

For animals that had received injections of HRP, frozen oblique coronal sections of the brains (30-40 μm thick) and transverse sections of the spinal cords (30-80 μm thick) were reacted with TMB as in Experiment 1. For each animal, alternate sections of the brain and spinal cord were counterstained with pyronin Y. Other series of brain sections were counterstained with cresylecht violet while the other series of spinal sections were either counterstained with cresylecht violet or dehydrated in ethanol and coverslipped unstained. One hamster died before HRP injections were scheduled. Brains of the hamsters that did not receive HRP injections were stored in 10% formalin with 30% sucrose until sectioning. Alternate sections were mounted on slides and stained with cresylecht violet. Microscopic examination was used to verify the extent and location of knife cuts and the presence or absence of labelled cells. A drawing tube attached to the microscope was used to trace selected sections. From these tracings, schematic diagrams were made to illustrate the extent and location of the knife cuts at the anterior-posterior level of the PVN and, where applicable, the distribution of PVN neurons containing HRP reaction product. Statistical comparisons were made using two-tailed Mann-Whitney U tests, corrected for multiple comparisons, when necessary, as in Experiment 1.

Results

In 5 animals, the knife cuts failed to produce the intended bilateral damage to the hypothalamus throughout the rostro-caudal level of the PVN. In 3 of these 5 hamsters

(Nos. 102, 107, 108) the cuts produced asymmetrical damage that was mostly or entirely anterior to the PVN. In one hamster (No. 109), the cut produced damage dorsal to the anterior part (approximately 1/3) but not the more posterior portions of the PVN. No evidence of the knife cut was seen in the last hamster (No. 116). In all of these cases, the cuts failed to prevent testicular regression. Mean testicular width of these hamsters was 7.14 ± 0.64 mm after 12 weeks of exposure to short days. For the 4 of these animals that were allowed to survive for 16 weeks of exposure to short days (Nos. 102, 107, 108, 116), mean testicular weight at that time was 0.964 ± 0.1199 g. No clear evidence for partial effects of misplaced or asymmetrical cuts was found, however, one of these animals showed evidence of spontaneous testicular recrudescence during the experiment. For hamster No. 108, testicular width reached a minimum of 5.7 mm at week 10 of exposure to short days and grew to 9.1 mm by week 16 as shown in Figure 9.

Microscopic evaluation of the brains indicated that in 7 animals, the cuts produced bilateral damage to the hypothalamus through most or all of the rostro-caudal level of the PVN. In one of these cases (No. 103), the knife cut produced a bilateral mechanical lesion of the PVN. In the other six hamsters that received bilateral damage to the hypothalamus, the cuts were centered anterior to the cuts made in Experiment 1 (and anterior to the intended location). In all six of these cases (see Figure 10) the cuts did, however, produce bilateral damage through (No. 114) or dorsal to (Nos. 104, 105, 111, 115, 118) the PVN through most or all of its anterior-posterior extent. All 7 of these animals with damage in or dorsal to the PVN maintained large testes (i.e., $\geq$10.6 mm in width), as shown in Figure 11. Mean testicular width after 12 weeks of exposure to short days was 12.26 ± 0.278 mm. Two of these hamsters were sacrificed after 12 weeks of exposure to short days. Mean paired testicular weight for the remaining 5 animals (Nos. 103, 105, 111, 114, and 115) was 3.776 ± 0.4048 g after 16 weeks of exposure to the nonstimulatory photoperiod. In addition, the five sham-operated control hamsters housed in long-day photoperiod all maintained adult gonadal size, showing a mean testicular width

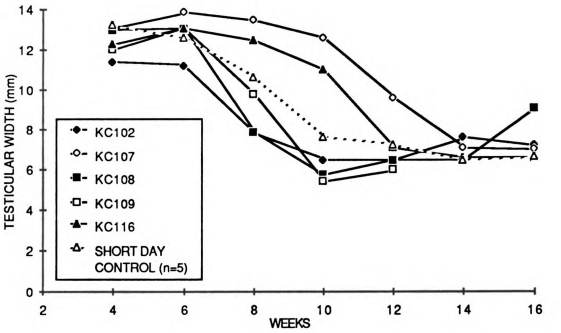

Figure 9: Testicular widths of individual animals in which the knife cuts failed to produce the intended bilateral damage dorsal to or through the PVN (solid lines) and mean testicular width of control hamsters (n = 5) exposed to short days (broken line).

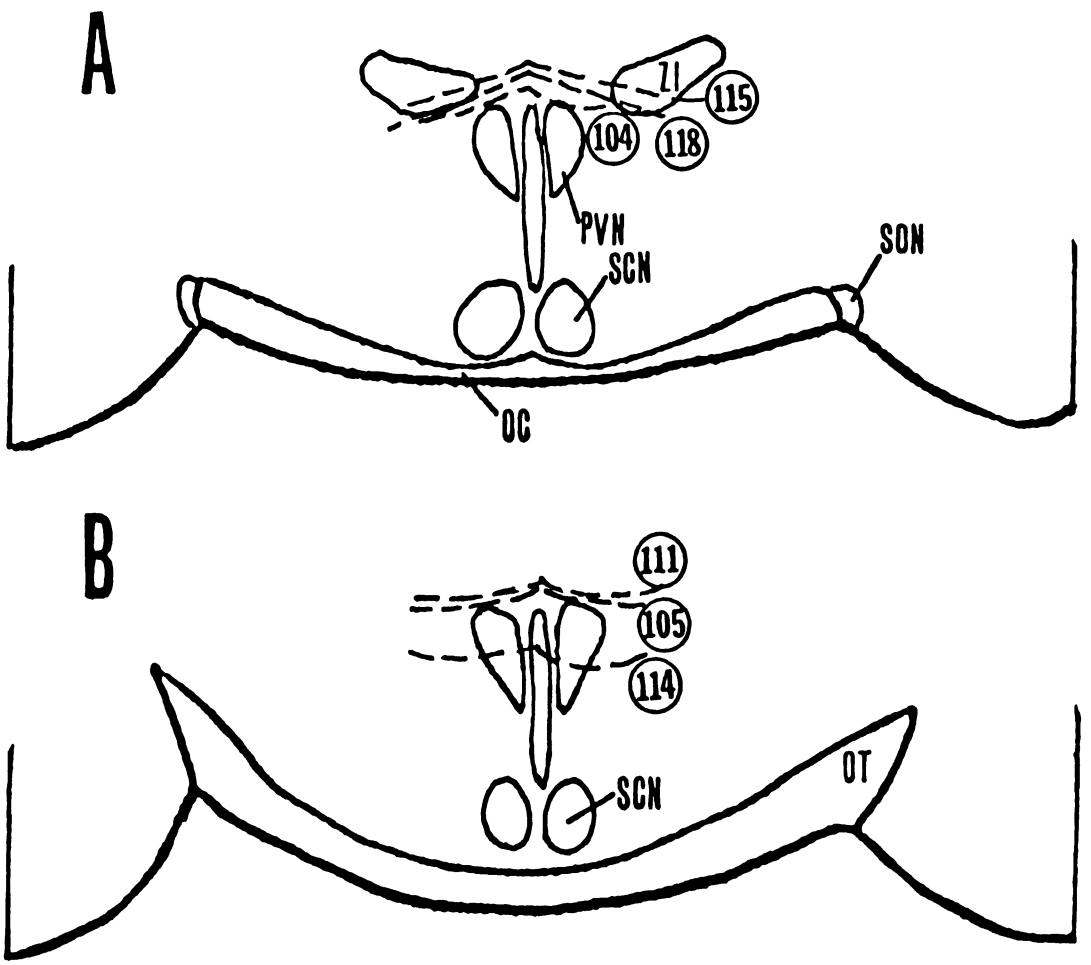

Figure 10: Schematic representation of the location of knife cuts that produced bilateral damage to the hypothalamus in Experiment 2. The cuts are drawn to show their location and extent in sections (A or B) through the PVN although in most cases the cuts were centered anterior to the nucleus. In one hamster (No. 103, not shown, the cuts produced a bilateral mechanical lesion of the PVN). Abbreviations are as in Figure 1.

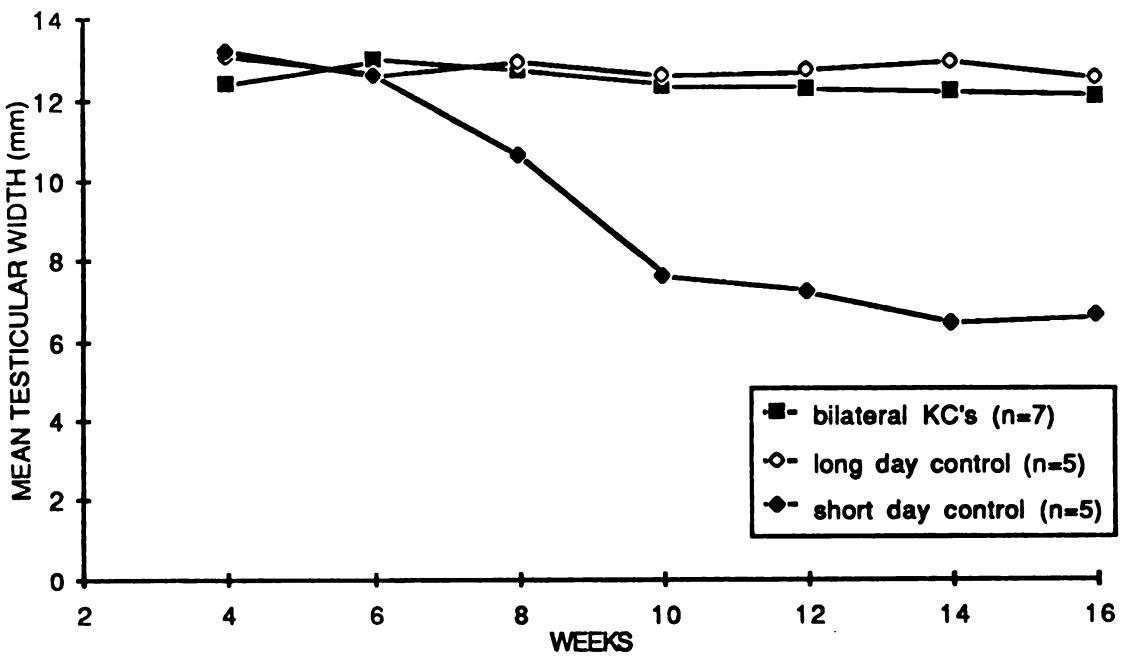

Figure 11: Mean testicular width for the 7 hamsters with bilateral knife cuts (KC) through or dorsal to the PVN, for 5 sham-operated control hamsters that were housed in short-day photoperiod, and 5 sham-operated hamsters that were housed in long-day photoperiod. Data for one hamster (No. 103) in which the knife cut produced a mechanical lesion of the PVN are included with data for the other hamsters that received knife cuts. Two of the hamsters that received knife cuts were sacrificed early, hence, the last two data points (weeks 14 and 16) do not include data for them.

of 12.7 ± 0.094 mm at week 12 and a mean paired testicular weight of 4.728 ± 0.0670 g at week 16 of exposure of short-day housed animals to that photoperiod. In contrast, control hamsters that were housed in short days all showed complete testicular regression, exhibiting a mean testicular width of 7.22 ± 1.034 mm at week 12 and a mean paired testicular weight of 0.751 ± 0.0755 g at week 16 of exposure to the nonstimulatory photoperiod. As illustrated in Figure 11, the average testicular width of control hamsters housed in short days for 12 weeks was statistically significantly different both from that of control hamsters housed in long days ($U = 0$, $p = 0.008$) and from that of the 7 hamsters with extensive bilateral damage through or dorsal to the PVN ($U = 1.5$, $p < 0.01$), while the latter two groups did not differ statistically from one another ($U = 9.5$, $p = 0.202$). Similarly, at week 16 the average paired testicular weight of the control hamsters housed in short days was statistically significantly different from that of control animals housed in long days and from that of the 5 hamsters with bilateral knife cuts through or dorsal to the PVN ($U = 0$, $p = 0.008$, for each comparison) while the difference between the latter two groups did not reach statistical significance ($U = 5$, $p = 0.15$).

All 8 of the hamsters for which locomotor activity was recorded exhibited normal nocturnal patterns of activity. In all of these cases, the circadian activity pattern showed normal entrainment to the light-dark cycle and a negative phase angle. Mean phase angle was -315 ± 33.27 min for hamsters with bilateral knife cuts that prevented testicular regression (Nos. 105, 111, 114, and 115) and -283.5 ± 11.33 min for hamsters in which the knife cuts were asymmetrical (Nos. 102, 107, 108) or undetectable (No. 116) and failed to prevent testicular regression. The difference in average phase angle between these two groups did not reach statistical significance ($U = 4$, $p = 0.342$).

Bilateral injections of HRP were made in 5 of the 7 hamsters that received knife cuts that prevented testicular regression. In one of these animals, labelled cells were not found in either the PVN or in the lateral hypothalamus, thus, it appears that there was no transport of the enzyme into the hypothalamus. In 4 of these animals, however, HRP

injections and TMB reactions resulted in unequivocal labelling of cells in the PVN. In three hamsters in which the knife cuts were dorsal to the PVN, a number of labelled neurons were found in the PVN. The locations of the knife cuts and distributions of labelled neurons in two animals, representative of these cases, are presented in Figures 12 (No. 115) and 13 (No. 118). A darkfield photomicrograph showing the knife cut dorsal to, and labelled neurons in the PVN of one of these hamsters (No. 118) is presented in Figure 14. The knife cut in the fifth hamster that received injections of HRP (No. 114) passed through the PVN. A few labelled neurons were found in the PVN of this animal (data not shown) both ventral and dorsal to the cut.

Discussion

Knife cuts through or dorsal to the PVN again prevented testicular regression in hamsters exposed to short days but failed to affect their entrained circadian activity rhythms, thus confirming the results of Experiment 1. There was no evidence that testicular regression had begun in any of these animals by week 16 of exposure to short days. These results indicate that the bilateral cuts disrupted the neural pathways that mediate effects of photoperiod on the reproductive system but did not affect the neural mechanisms responsible for the generation and entrainment of circadian activity rhythms. Furthermore, these activity patterns all showed relatively large negative phase angles. Elliot (1976) reported that intact hamsters housed in long days (11.5-18 hrs of light/ 24 hrs) show activity patterns in which the active phase of the rhythm is compressed and the phase angle of the rhythm varies with the length of the photoperiod. With increasing length of the dark portion of the light-dark cycle, the active phase of the activity rhythm becomes decompressed, reaching an asymptote at a photoperiod of about 11.5 hr. Further increases in the length of the dark portion of the light-dark cycle have little effect on the length of the active phase of the rhythm and, moreover, the onset of activity always falls about 12 hrs before the termination of darkness. Thus, in hamsters housed under a light-dark cycle, circadian rhythms are entrained by the onset of light. All 8 of the animals for

Figure 12: Schematic representation of the distribution of HRP-labelled neurons in three sections (at 240 μm intervals; A-C, rostral to caudal) through the paraventricular nucleus (PVN) of a hamster (No. 115) that received a bilateral knife cut (indicated by arrows) that prevented testicular regression. This animal received bilateral injections of HRP into the spinal cord and was sacrificed 48 hr later. Note that the cut was dorsal to the PVN and failed to prevent labelling of cells in the PVN.

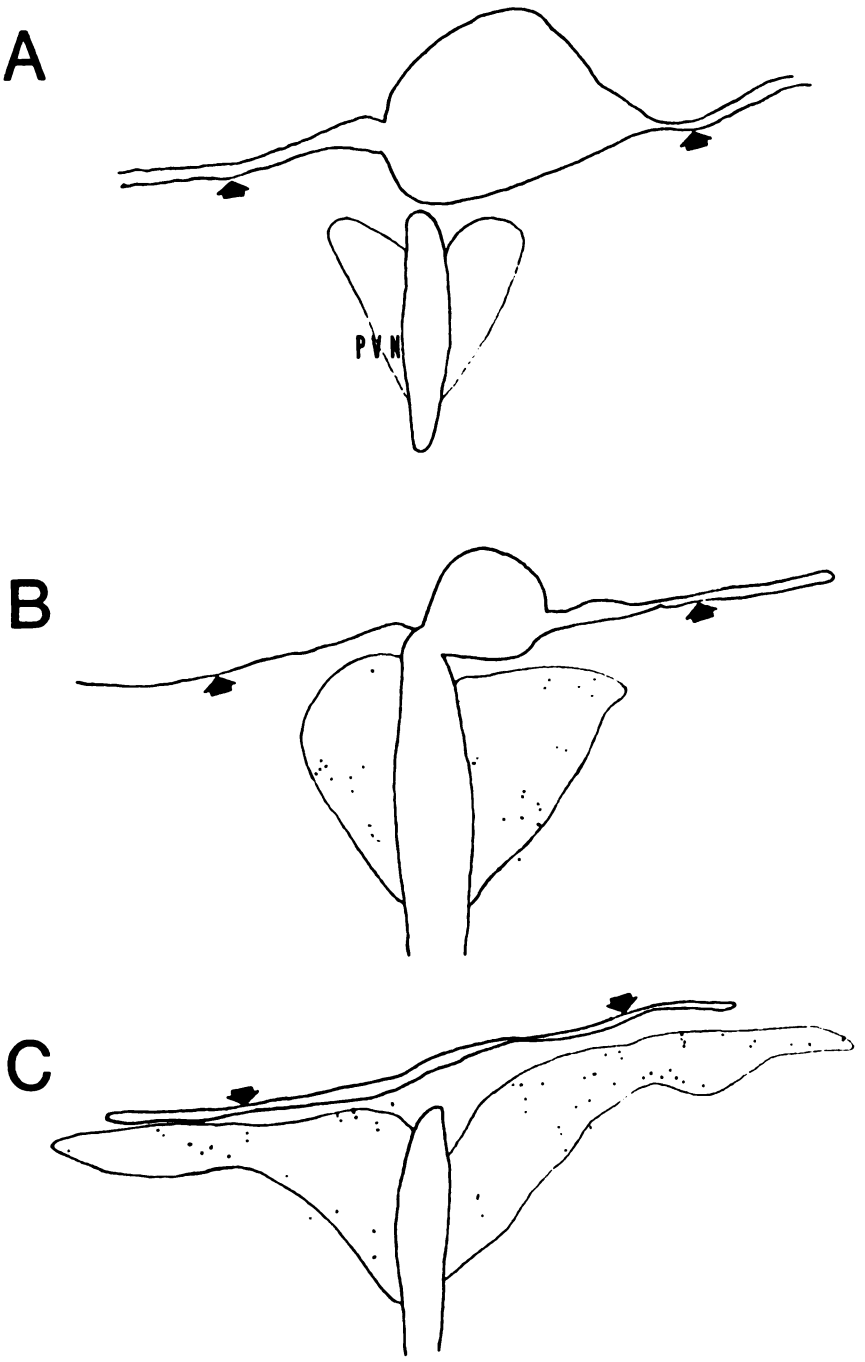

Figure 12

Figure 13: Schematic representation of the distribution of HRP-labelled neurons in three sections (at 240 μm intervals; A-C, rostral to caudal) through the paraventricular nucleus (PVN) of a hamster (No. 118) that had received a bilateral knife cut (indicated by arrows) that prevented testicular regression before receiving bilateral injections of HRP into the spinal cord. Note that the cut was dorsal to the PVN and failed to prevent labelling of cells in the PVN.

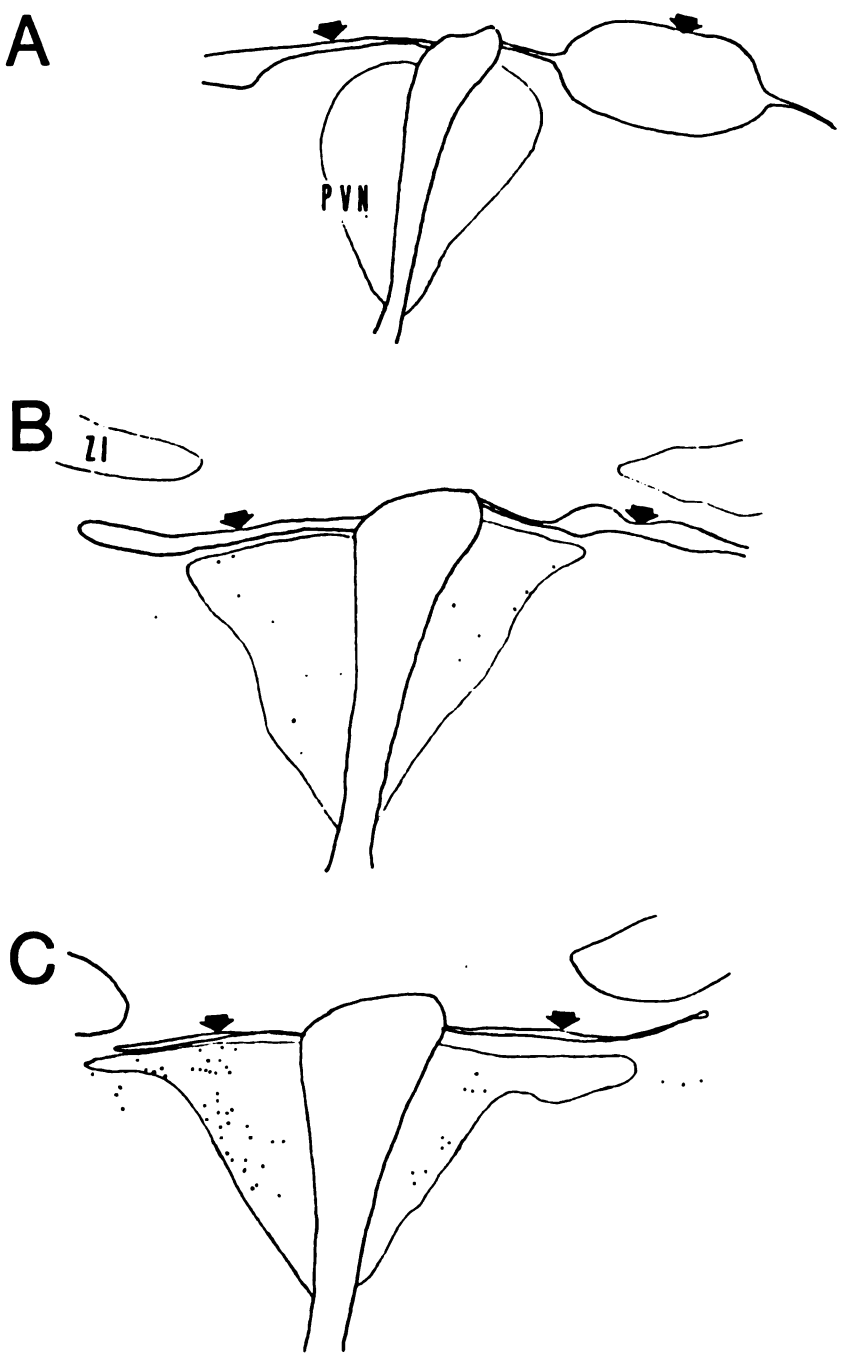

Figure 13

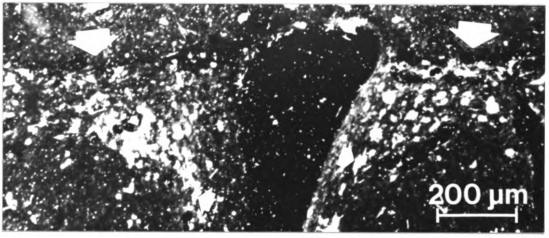

Figure 14: Darkfield photomicrograph of a section (30 µm thick, counterstained with pyronin Y) through the hypothalamus of a hamster (No. 118) in which a bilateral knife cut (indicated by large arrows) just dorsal to the PVN prevented testicular regression but did not prevent labelling of PVN neurons (indicated by small arrows) after injections of HRP into the spinal cord.

which activity patterns were monitored in the present experiment showed phase angles within the range reported by Elliot (1976) for hamsters housed in a photoperiod of 6 hrs light:18 hrs dark. Thus, while the reproductive system of hamsters with cuts through or dorsal to the PVN responded as if the animals were exposed to long days, the animals responded behaviorally as if they were exposed to short days.

In 3 hamsters, cuts dorsal to the PVN prevented testicular regression but failed to prevent retrograde transport of HRP from the spinal cord to the PVN. This indicates that some paraventriculo-spinal fibers were spared by the cuts and suggests that destruction of all paraventriculo-spinal fibers is not necessary to prevent the gonadal response to short-day photoperiod. These cuts should have interrupted all of the fibers of bundle 1 but probably did not sever all of the fibers of bundle 2.

In contrast to the results seen in hamsters with bilateral cuts, exposure of animals with asymmetrical or misplaced knife cuts to short days resulted in testicular widths ≤ 7.0 mm in all cases. Furthermore, the length of time required for animals with these cuts to attain testicular widths of ≤ 7.0 mm was within the range of the time required for control hamsters to reach the same criterion. Thus, there was no evidence of partial effects of these cuts.

In contrast to the results of dorsal cuts, parasagittal cuts aimed lateral to the PVN fail to block testicular regression in hamsters housed in nonstimulatory photoperiod (Larry Morin, personal communication). Taken together, these results suggest that bundle 1 mediates responses of the pineal and the gonads to photoperiod while bundle 2 does not. Bundle 2 appears likely to be involved in other autonomically mediated responses. For example, parasagittal knife cuts placed in the path of bundle 2 result in hyperphagia and obesity in rats (Gold, et al., 1977). However, it appears that bundle 1 is also involved in the regulation of energy balance since knife cuts placed in a coronal plane through the central grey of the thalamus or midbrain and transecting the path of bundle 1 (Weiss and Leibowitz, 1985) abolish the feeding response that is normally elicited in satiated, intact

rats by injection of norepinephrine or clonidine into the PVN (Leibowitz, 1978; Weiss and Leibowitz, 1985). Further evidence implicating bundle 2 in the regulation of energy balance is the finding that electrical stimulation centered at a site lateral to the anterior PVN, in or near bundle 2 evokes an initial decrease followed by a nearly 1° C rise in the temperature in interscapular brown adipose tissue (BAT [Brown, 1986]). Both in rats (Tulp, et al., 1982) and hamsters (Bartness and Wade, 1984; Wade, 1982, 1983), dietary composition influences metabolic efficiency (i.e. the proportion of ingested calories that are diverted to energy storage depots) and thermogenesis. Furthermore, photoperiod also influences metabolic efficiency and thermogenesis in hamsters (Bartness and Wade, 1984; Campbell, et al., 1983; Wade, 1983). These effects are mediated in part by brown adipose tissue (Campbell, et al., 1983; Tulp, et al., 1982). While descending neural pathways to BAT have not yet been fully mapped, recent evidence suggests that the PVN may be involved in the regulation of thermogenesis (Bartness, et al., 1985).

It is interesting to note that in one case a cut dorsal to the anterior 1/3 of the PVN failed to prevent testicular regression. The anterior-posterior placement of the cut in this animal is reminiscent of that of a partial electrolytic lesion reported by Lehman, et al. (1984). The lesion was reported to have "spared most of the mid- and caudal PVN" while destroying the more anterior portion of the nucleus. Blinding of the animal resulted in substantial but incomplete testicular regression. Taken together, these results hint that the anterior part of the PVN is not critical for testicular regression in hamsters exposed to nonstimulatory photoperiod.

EXPERIMENT 3

In addition to their projections to the PVN, the SCN send fibers dorsally through and by the PVN to the habenula, periventricular thalamus and the mesencephalic central grey (Stephan, et al., 1981). Therefore, it is possible that electrolytic lesions of the PVN and knife cuts through and dorsal to the nucleus block testicular regression by interrupting fibers that do not terminate in the PVN. To test whether cells of the PVN are, in fact, involved in the gonadal response to short-day photoperiod, the present set of experiments employed intrahypothalamic injections of N-methyl-aspartate (NMA), a neurotoxin which destroys cell bodies while apparently sparing fibers that pass through the injection site (Hastings, et al., 1985; Olney and Price, 1983).

Part A: Are Neurons of the Paraventricular Nucleus Sensitive to the Toxic Effects of N-Methylaspartate?

Injections of NMA, a structural analog of the putative neurotransmitter aspartate, into the lateral hypothalamus in the region of the medial forebrain bundle destroy lateral hypothalamic neurons without affecting dopamine or serotonin content of the basal ganglia (Hastings, et al., 1985b). Thus, the toxin appears to selectively destroy neurons without affecting axons that pass through the site of the injection. While magnocellular neurons of the PVN appear to be insensitive to the toxic effects of NMA, evidence suggests that parvocellular neurons of the PVN are destroyed by the toxin (Olney and Price, 1983). Furthermore, a recent report indicates that kainic acid, a structural analog of glutamate, destroys parvocellular but spares magnocellular neurons of the PVN (Zhang and Ciriello, 1985).

Hastings, et al. (1985a) reported that injections of NMA into the anterior hypothalamus, in an area dorsolateral to the SCN, prevented testicular regression and suspension of vaginal cyclicity in hamsters exposed to nonstimulatory photoperiod. These authors reported that the injections that blocked the effects of exposure to short days had caused a loss of cells in the anterior hypothalamic area (AHA) dorsolateral to the SCN without affecting either the SCN or the PVN. The fact that the animals in the study by Hastings, et al. exhibited normal circadian rhythms of locomotor activity indicates that the SCN were functionally intact. Furthermore, other investigators have reported that neurons of the SCN are resistant to the toxic effects of kainic acid (Peterson and Moore, 1980). However, since the figures presented by Hastings, et al. indicate that the site of the injections that blocked gonadal regression included at least part of the PVN, it is suprising that they reported that the injections did not affect neurons within the PVN.

In order to determine whether neurons of the PVN are sensitive to the toxic effects of NMA, the present experiment employed unilateral intrahypothalamic injections of NMA.

These intrahypothalamic injections were followed by bilateral injections of HRP into the spinal cord to determine whether the toxin kills cells that project to the spinal cord.

Methods

Male hamsters were housed in long days as described in Experiment 1. Hamsters (n = 5) were anesthetized with Equithesin (4.5 to 5 ml/kg body weight) and placed in a stereotaxic apparatus. A 1-µl Hamilton syringe was then used to inject N-methyl-D,L-aspartate (NMA; Sigma Chemical Co., St. Louis, MO) dissolved in artificial cerebrospinal fluid (ACSF) on one side of the brain. The constituents of the ACSF were 130 mM NaCl, 25 mM $NaHCO_3$, 5mM Na_2HPO_4, 30 mM KCl, 8 mM $MgCl_2$, and 13 mM $CaCl_2$ (Dohanich and Clemens, 1981). The injection volume (0.5 µl), concentration of NMA (0.12 M), and pH of the solution (7.0) were chosen so as to replicate parameters used by Hastings, et al. (1985). For two animals in which the injections were aimed at the PVN, stereotaxic coordinates were 0.8 mm posterior to the bregma, 6.0 mm ventral to the superior sagittal sinus and 0.5 mm lateral to the midline with the top of the incisor bar 2.0 mm below the ear bars. For the rest of the animals (n = 3) the injections were aimed at the AHA, and the anterior-posterior stereotaxic coordinate was changed to 0.2 mm anterior to the bregma. To reduce backflow of the solution up the needle tract, injections were made over a 2-min period and the needle was left in place for 5 min after the injection.

Animals were allowed to survive for at least 6 days before injections of HRP were made into the spinal cord as described in Experiment 2B. The hamsters were allowed to survive for 48 hr after the injections of HRP and then sacrificed using an overdose of Equithesin. Perfusions and reaction and staining of the tissue were performed as in Experiments 1 and 2. Thickness of the sections was 30 µm for the brains and 40-80 µm for the spinal cords. Microscopic evaluation of the tissue was performed to determine the locations of the NMA injections and to visualize the HRP reaction product in the brain and spinal cord. For quantification of loss of cells that project to the spinal cord, labelled neurons in the PVN on each side of the brain were counted using the pyronin-Y stained

sections through the nucleus (8 sections/brain). Cell counts were made by two investigators and labelled cells from 8 sections taken at random were independently counted by both investigators to test the reliability of the method. Although the variance in the number of HRP-labelled cells differed between the two sides of the brain, the Student's *t* test for independent samples (2-tailed probabilities) was used for statistical comparisons. For comparisons involving groups of equal sizes, the outcome of the Student's *t* test is not seriously affected by differences in variance between groups (e.g.; see Boneau, 1960). Thus the Student's *t* test, which is more powerful than the Mann-Whitney *U* test for detecting differences between groups, was used.

Results

The NMA injections were centered near the PVN in 4 hamsters. In 3 of these animals, the injections were centered dorsolateral to the PVN while in the fourth hamster the injection was centered ventrolateral to the PVN and dorsolateral to the SCN. The center of the injection sites (i.e., termination of the needle tracks) in these hamsters are represented schematically in Figure 15. In a fifth hamster, the injection site was near the midline, rostral to the preoptic area and just dorsal to the diagonal band of Broca.

Microscopic examination of the brains of the hamsters in which injections were centered near the PVN indicated a loss of neurons in the posterior PVN ipsilateral to the injection site. Neurons in the anterior PVN of these animals, however, were spared. Moreover, the loss of HRP/TMB labelled neurons was particularly dramatic (see Figure 16). As shown in Figure 17, counts of labelled neurons revealed a statistically significant loss of labelled cells ipsilateral to the NMA injections ($t = 3.07$, $df = 6$, $p < 0.05$). In these 4 hamsters, the PVN contralateral to the injection site contained an average of $89.65 \pm 3.18\%$ of the total number of labelled cells. For the sections that were evaluated by two investigators, the independent estimates were in good agreement with $91.68 \pm 2.79\%$ and $89.92 \pm 2.64\%$ of the total number of labelled cells found on the uninjected side of the brain. The difference between the two estimates did not reach

Figure 15: Schematic representation of the locations of the centers of the unilateral injections of NMA in 4 hamsters that received bilateral injections of HRP into the spinal cord after the NMA injections. The drawings of the hamster hypothalamus (A-C are rostral to caudal) are modifications of those published by Malsbury (1977). Abbreviations are: ARC, arcuate nucleus; F, fornix; OT, optic tract; PV, paraventricular nucleus; SC, suprachiasmatic nucleus; SO, supraoptic nucleus.

Figure 16: Photomicrographs of a series of sections (30 μm thick, counterstained with pyronin Y) at 180 μm intervals (A-D, rostral to caudal) through the PVN of a hamster (No. 380) that received a unilateral injection of NMA just dorsolateral to the PVN prior to bilateral injections of HRP into the spinal cord. Note that in the most anterior section (A) there does not appear to be any loss of unlabelled cells on the injected (left) side; however, the number of both HRP-labelled, and unlabelled cells on the injected side is substantially reduced in the more posterior sections (B-D).

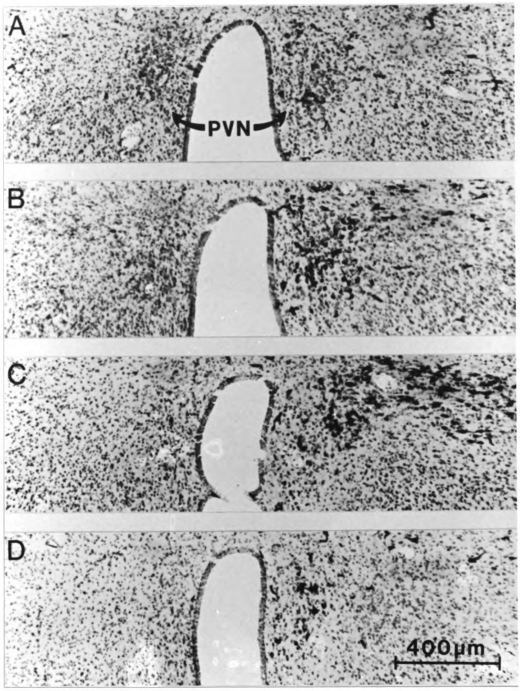

Figure 16

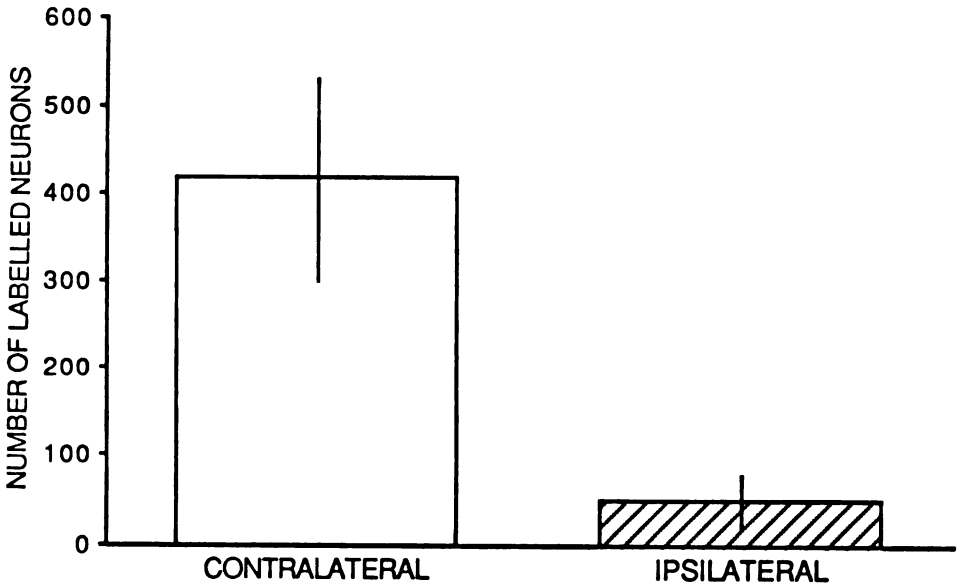

Figure 17: Mean (±SEM) number of HRP-labelled neurons in the PVN ipsilateral and contralateral to the site of injection of NMA. The centers of the sites of the NMA injections in the four hamsters were represented in Figure 15.

statistical significance ($t = 0.49$, $df = 14$, $p > 0.05$). In contrast, the hamster in which the NMA injection was centered near the midline showed a more symmetrical pattern of labelled neurons in the PVN (47.18 vs 52.82%).

Discussion

These results support earlier reports that intrahypothalamic injections of the aspartate analog, NMA (Olney and Price, 1983), and the glutamate analog, kainic acid (Zhang and Ciriello, 1985), destroy parvocellular but spare magnocellular neurons of the PVN. Furthermore, since unilateral injections of the toxin resulted in a statistically significant reduction in the number of PVN neurons containing HRP reaction product after injections of the enzyme into the spinal cord, the present results extend previous findings in demonstrating that neurons that project from the PVN to the spinal cord are among the cells that are sensitive to the toxic effects of NMA. Since all of the injection sites and needle tracks were outside of the PVN, it is not likely that the loss of cells in the PVN was due to direct mechanical damage produced by the needle. The use of unilateral injections of NMA permitted utilization of the uninjected side of each brain as a control in evaluating the damage produced by the NMA. This procedure circumvents some of the problems associated with comparisons of number of HRP labelled cells across animals (e.g., differential transport or enzymatic activity between cases). Similar results have recently been reported independently by other investigators. Although the amount of destruction was not quantified, Hastings and Herbert (1986) reported that injections of NMA destroyed parvocellular PVN neurons that project to the spinal cord (as determined by injections of true blue into the spinal cord), but not axons (as observed by the Holmes silver stain method) or magnocellular neurons within the PVN.

In summary, the present results indicate that intrahypothalamic injection of NMA should serve as an effective tool in studying the function of neurons that project from the PVN to the spinal cord.

Part B: Axon-Sparing Chemical Lesions of the Paraventricular Nucleus: Confirmation of the Role of the PVN in Photoperiod-Dependent Seasonal Reproductive Cycles.

Since the results of Experiment 3A indicated that NMA does kill neurons of the PVN and particularly neurons that make paraventriculo-spinal connections, a further experiment was executed in which bilateral injections of NMA were used to test whether neurons of the PVN are involved in the testicular response to exposure to short days. In some of the animals, injections were aimed at the PVN in order to maximize neuronal destruction in that nucleus. In additional animals, the injections were aimed at the AHA dorsolateral to the SCN in an attempt to replicate the results of Hastings, et al (1985a). In addition to the use of testicular width and weight as measures of gonadal function, ability of some of the animals to inseminate female hamsters was tested.

Methods

Subjects and housing conditions were the same as reported in Experiment 1.

Surgery

Under Equithesin anesthesia, hamsters received one of three types of stereotaxically-placed injections. Bilateral injections of NMA (0.5 µl of 0.12 M NMA dissolved in ACSF, pH 7.0) were aimed at either the AHA or the PVN, while control injections of (0.5 µl of) the ACSF vehicle were aimed at the PVN. To prevent accidental contamination, separate syringes were used for injections of the toxin and the ACSF vehicle. The injection procedure was as described in Experiment 3A. Stereotaxic coordinates for injections aimed at the PVN were 0.8 mm posterior to the bregma, ± 0.5 mm lateral to the midline, and 6.1 mm ventral to the superior sagittal sinus, For injections aimed at the AHA, stereotaxic coordinates were 0.2 mm anterior to the bregma, 0.5 mm lateral to the midline, and 6.0 mm ventral to the sinus. In all cases the top of incisor bar was positioned 2.0 mm below ear bar zero.

Procedure

Hamsters that received bilateral injections of NMA (n = 27) or ACSF (n = 13) were returned to long-day photoperiod and allowed to recover from surgery for approximately 1 week. These animals were then transferred to short-day photoperiod. Five hamsters that did not receive injections were individually housed in long-day photoperiod to serve as control animals for that photoperiod. Begining 4 weeks after the hamsters were moved to short-day photoperiod and their testicular width and length recorded to the nearest 0.1 mm biweekly as reported in experiment 2B. For testicular measurements, the animals were injected with Ketamine HCl (Vetalar, Parke-Davis; 10 mg/100 g body weight).Surgeries were performed on two batches of hamsters staggered 6 weeks apart. Some of the animals from the second batch were used in an experiment (described below) to determine whether hamsters that have been experimentally induced to maintain adult gonadal size during exposure to short days will show sexual behavior when presented with the opportunity to do so. Because this experiment required testing of the animals after they should have shown testicular regression, the length of time between transfer of the hamsters to short days and sacrifice of the animals varied between the two batches. After 14 weeks of exposure to short days, 10 hamsters that had received injections of NMA (8 that had exhibited testicular regression and 2 that had failed to do so), and 4 of the control hamsters housed in short days were anesthetized with Equithesin and castrated. Paired testicular weights for these animals were obtained to the nearest 0.1 mg. These hamsters were sacrificed by an overdose of the anesthetic after castration. Hamsters that had received injections of NMA were perfused as described below. Of the 31 hamsters that were allowed to survive beyond week 14 of the experiment, 17 were sacrificed at week 19. The 17 hamsters that were sacrificed at that time included 2 that exhibited complete testicular regression and 5 that showed no, or only partial testicular regression after injections of NMA, 5 control hamsters that were housed in short days and 5 control hamsters that were housed in long days. These animals were anesthetized and their testes and seminal vesicles

removed. Paired weights of each organ were recorded to the nearest 0.1 mg. These hamsters were then given an overdose of the anesthetic and animals that had received injections of NMA were perfused as described below.

The remaining 14 hamsters (10 that received NMA injections and 4 that received control injections) had all shown complete testicular regression. These hamsters were allowed to survive until week 25 of the experiment. From week 19 to week 25, the width of the right testis of each of these hamsters was recorded biweekly as described above in order to determine whether these animals were capable of exhibiting testicular recrudescence. One of the hamsters that had received NMA injections died between week 25 and week 28 of the experiment. The brain of this animal was removed and stored in 10% formaldehyde. At week 28, testicular and seminal vesicle weights for the 13 remaining animals were obtained, the animals sacrificed, and perfusions performed on hamsters that had received injections of NMA, as described below.

Histology

After castrations had been performed, the hamsters that had received injections of NMA were perfused transcardially with 0.9% saline followed by 10% formaldehyde. Brains were removed and stored in 10% formaldehyde with 30% sucrose. Frozen sections, 40 μm thick, were cut through the preoptic area and hypothalamus. Alternate sections were mounted on slides, stained with cresyl violet and coverslipped. In order to facilitate evaluation of the tissue without knowledge of the gonadal response of individual animals, identification numbers of the animals were reassigned at this time by an investigator who did not participate in the histological analysis.Microscopic examination (at magnifications of 25 to 400 X) of the brains was performed in order to determine the extent and location of the damage produced by the NMA injections. In performing this analysis, the amount of neuronal loss in the PVN was noted. For each animal, tracings of the location of the center of the injection sites (i.e., termination of the needle track) were made via the use of a drawing tube attached to the microscope. These tracings were then

used to identify the locations of the centers of the injection sites on a standard set of drawings of the hamster hypothalamus that were modifications of the drawings published by Malsbury (1977). These hamsters were then divided into three groups based on the site and extent of neuronal damage. Hamsters in the first ("complete" damage) group showed extensive loss of neurons from the PVN while animals in the second (partial damage) group showed substantial but partial loss neurons from the PVN and animals in the third (no damage) group showed either minimal or no loss of neurons from the PVN. After this histological evaluation was performed, reproductive responses of the animals that had received injections of NMA were compared with those of control hamsters. Statistical comparisons of testicular widths and testicular and seminal vesicle weights between hamsters that had shown extensive damage to the PVN and the two control groups, and between the two groups of animals that were allowed to show testicular recrudescence were made using Mann-Whitney U tests corrected for multiple comparisons, when necessary, as described in Experiment 1. The time of sacrifice of hamsters from each of the 3 histologically-defined groups and each of the 2 control groups are presented in Table 1.

Behavioral Testing

During weeks 16 and 17 of exposure of the hamsters to short days, reproductive competence of 5 hamsters in which injections of NMA had produced complete or partial insensitivity to short days was verified via behavioral testing of the ability of these animals to deliver sperm. Under Equithesin anesthesia, female hamsters were ovariectomized through a single abdominal incision. Estrogen replacement was delivered via Silastic capsules implanted subcutaneously on the ventral surface of the animal. Each capsule consisted of a segment of Silastic medical grade tubing (Dow Corning) 3.175 mm O. D., 1.575 mm I. D. filled with crystalline estradiol benzoate (Steraloids, Inc.) and sealed on each end with wooden plugs covered with Silastic sealer. The distance between plugs was 3-5 mm. Sexual behavior of the 5 male control hamsters exposed to long days and the 5

Table 1: Number of hamsters sacrificed at each of three times and number of hamsters that were used in the sexual behavior tests.

Group (Type of Damage)	Number	Number of Animals Sacrificed Week 14	Week 19	Week 29	Number Used in Behavioral Tests
Long Day Control	5	0	5	0	5
Short Day Control	13	4	5	4	0
NMA-Injected	27	10	7	10	5
("Complete" Damage)	(5)	(1)	(4)	(0)	(4)
(Partial Damage)	(6)	(2)	(3)	(1)	(1)
(No Damage)	(16)	(7)	(0)	(9)	(0)

male hamsters that maintained the largest testes after injections of NMA were tested in glass aquaria containing a small amount of bedding. A dim red incandescent bulb, turned on before the onset of darkness and left on overnight, provided illumination (and a romantic atmosphere) during the behavioral observations. Testing sessions for the control hamsters began at the begining of the dark portion of the light-dark cycle while testing of the animals that received injections of NMA began 8 hr after the begining of the dark portion of the light dark cycle, in an attempt to minimize differences due to testing the two groups during different phases of their circadian activity patterns. Since Elliot (1976) found that hamsters housed in photoperiods of 18 and 6 hr of light begin to show wheel running activity 2 hr before and 6 hr after the onset of darkness, respectively, the testing sessions for each group began 2 hr after the average onset of activity as measured by wheel running. In order to induce sexual receptivity, female hamsters were given subcutaneous injections of 0.5 mg of progesterone (Sigma) in 0.05 ml of sesame oil, 4 hr before the begining of the testing session. For each test, a male was placed in an aquarium (51 x 26 x 30 cm) and allowed to habituate to the testing conditions for 5 min before introduction of the female. Behavior of the animals was observed for 20 min and the aquaria were washed and bedding replaced between tests. In the first test (week 16), sexual behavior was not quantified but exhibition of mounting behavior was noted for each animal. One week later, sexual behavior of the animals was again tested and recorded orally on a Panasonic F35 cassette tape recorder. For quantification, behavioral measures were scored as latency to show first mount and first intromission after introduction of the female, and number of mounts during which intromission did not occur and number of intromissions exhibited during the 20-min testing session. Statistical comparisons between the two groups were performed using Student's t test for independent samples (two-tailed probabilities). The rationale for the use of this test was as discussed in Experiment 3A. Since all of the hamsters failed to ejaculate during the 20-min testing sessions, ability of the animals that received NMA injections to ejaculate was tested by pairing these animals

with sexually receptive females overnight on the day before they were sacrificed. Five of the ovariectomized females received subcutaneous injections of progesterone (0.5 mg in 0.05 ml of sesame oil) 4 hr before termination of the dark portion of the light-dark cycle. Each of these animals was placed in a cage with a male shortly before the end of the light portion of the light-dark cycle. The following morning vaginal smears were obtained from the females by insertion of a cotton swab, moistened with distilled water, into the vagina. Fluid from the swab was deposited onto a microscope slide. As a test of the ability of these hamsters to exhibit spermatogenesis, a small amount of seminal fluid was removed and placed onto microscope slides following castration of the animals. Vaginal and testicular smears were microscopically examined for evidence of spermatozoa.

Results

As shown in Figure 18, all 13 of the control hamsters that were housed in short days showed complete testicular regression by week 14 of the experiment while the 5 control hamsters that were housed in long days maintained large testes for the duration of the experiment. Mean testicular widths for these two groups were 5.89 ± 0.17 and 12.9 ± 0.43 mm, respectively. This difference was statistically significant ($U = 0$, $p = 0.00138$).

Effects of NMA: Histological Results and Testicular Responses

The sites of the injections of NMA typically showed a zone of dense gliosis along the path of the needle surrounded by a region of less dense gliosis and loss of neurons. In 5 hamsters, the NMA injections caused a substantial loss of neurons from the PVN, bilaterally. As illustrated schematically in Figure 19, the injections were centered just dorsal or dorsolateral to the PVN in 3 (Nos. 1, 2, 3) of these hamsters and within the boundaries of the PVN in 2 additional animals (Nos. 23, 28). In all 5 of these hamsters, neurons in the anterior PVN, for the most part, survived the injections. In contrast, the posterior and lateral portions of the PVN were completely, or almost completely destroyed by the NMA injections, thus the bilateral pattern of damage in these animals was similar to

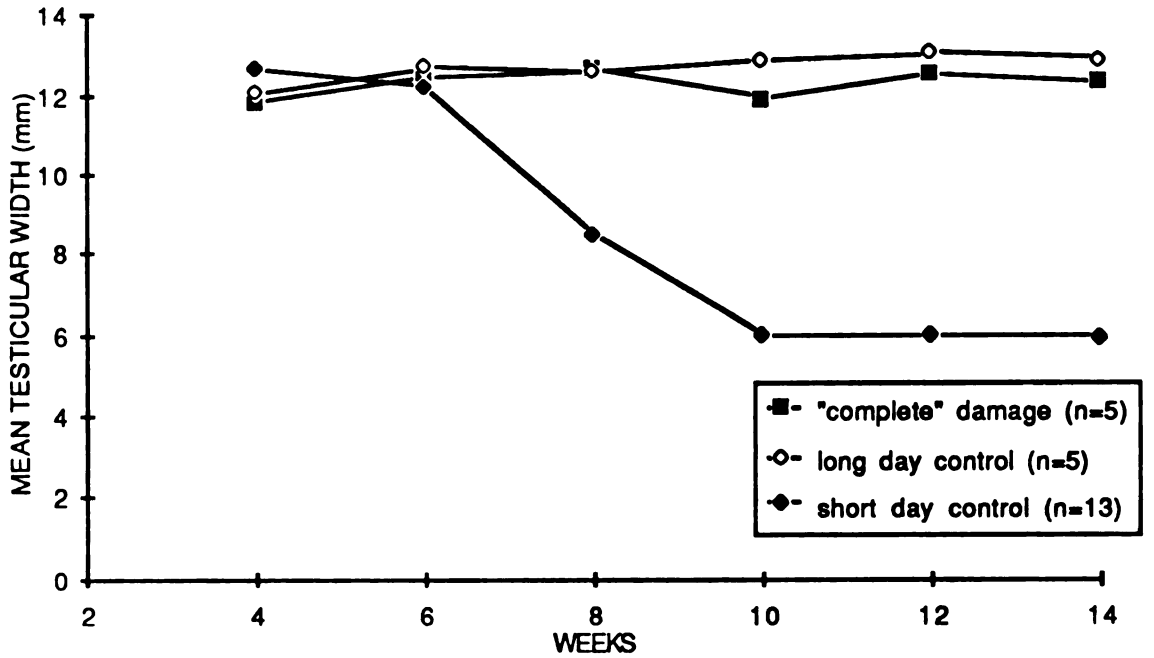

Figure 18: Mean testicular width of 13 control hamsters that were housed in short days after receiving injections of ACSF, 5 unoperated control hamsters that were housed in long days, and 5 hamsters that were housed in short days after receiving injections of NMA that caused "complete" damage to the PVN.

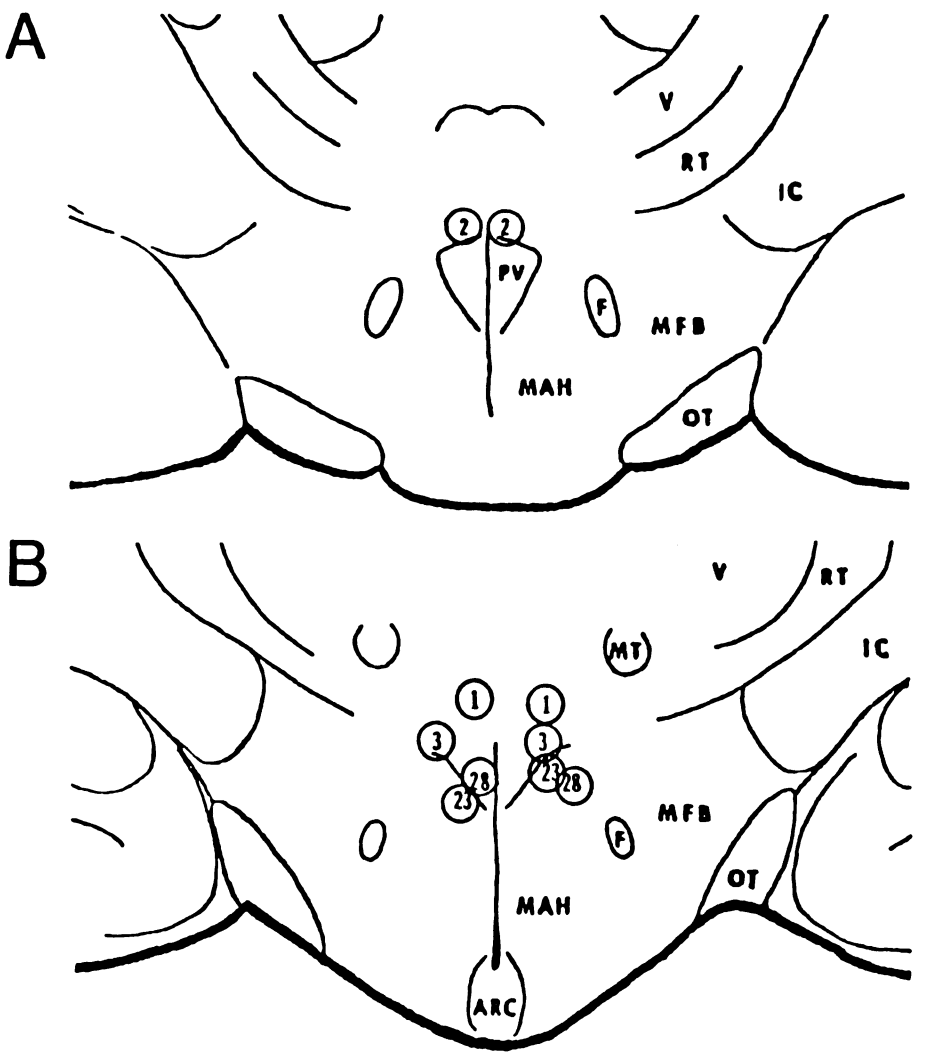

Figure 19: Schematic representation of the locations of the centers of the injections of NMA that produced "complete" bilateral destruction of the PVN. The pairs of circled numbers represent the bilateral injection sites in individual hamsters. The drawings of the hamster hypothalamus (A-B, rostral to caudal) are modifications of those published by Malsbury (1977). Abbreviations are: ARC, arcuate nucleus; F, fornix; IC, internal capsule; OT, optic tract; MAH, medial anterior hypothalamus; MFB, medial forebrain bundle; MT, mammillo-thalamic tract; PV, paraventricular nucleus; RT, reticular thalamic nucleus; V, ventral thalamic nucleus.

the unilateral pattern of damage in the hamsters that received unilateral injections just dorsolateral to the PVN in Experiment 3A. In 4 cases a small proportion of cells in the posterior and periventricular parts of the nucleus survived after the injections. In all 5 of these cases there was minor damage to the area of the dorsal hypothalamus surrounding the PVN, as well as the ventral thalamus including the zona incerta. Photomicrographs of a series of sections through the area of the PVN of a hamster representative of these cases are presented in Figure 20. All 5 of these hamsters maintained adult testicular size for the duration of the experiment. Mean testicular widths of these animals are presented in Figure 18. After 14 weeks of exposure to short days, the average testicular width of these animals (12.3 ± 0.29 mm) was statistically different from that of the control hamsters that were housed in short days ($U = 0$, $p = 0.00138$) but not from that of control hamsters that were housed in long days ($U = 8$, $p = 0.420$). One of these hamsters (No. 1) and 4 of the control hamsters that were housed in short days were sacrificed after week 14 of the experiment. Testicular weight of the hamster that sustained "complete" damage to the PVN was 2.54 g while the mean testicular weight of the control hamsters was 0.445 ± 0.042 g. Five control hamsters that were housed in each of the two photoperiods and 4 hamsters that had sustained "complete" damage to the PVN were sacrificed at week 19 of the experiment. Mean testicular weights for these groups were 2.679 ± 0.168 g for hamsters that sustained "complete" damage to the PVN, 3.403 ± 0.137 g for the hamsters that were housed in long days and 0.837 ± 0.124 g for the control hamsters that were housed in short days. Testicular weight of the control hamsters housed in short days was statistically significantly different both from that of control hamsters housed in long days ($U = 0$, $p = 0.008$) and from that of hamsters that showed "complete" damage to the PVN ($U = 0$, $p = 0.016$). The difference in testicular weight between the animals that sustained "complete" damage to the PVN and hamsters that were housed in long days did not reach statistical significance ($U = 2$, $p = 0.064$). Mean seminal vesicle weights for hamsters that

Figure 20: Photomicrographs of a series of 40 μm thick sections (160 μm intervals, cresyl violet stain) through the hypothalamus of a hamster (No. 3) representative of those cases in which the loss of neurons of the PVN, following bilateral injections of NMA, was judged to be "complete" damage. Notice that while PVN neurons are visible in the more anterior sections (A and B), there was a substantial loss of neurons from the posterior PVN (C and D). The arrows in D indicate the gliosis surrounding the needle tracks.

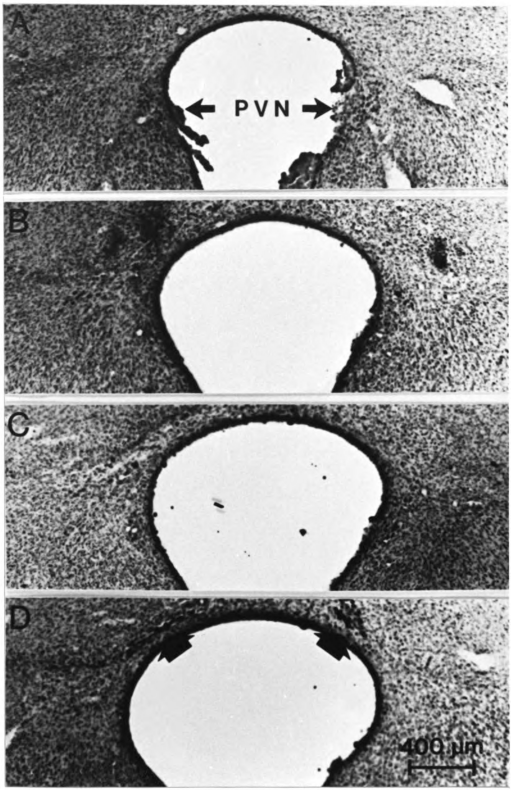

Figure 20

sustained "complete" damage to the PVN, and for control hamsters housed in long, and short days were 1.020 ± 0.339 , 1.412 ± 0.198, and 0.125 ± 0.023 g, respectively. On this measure, control hamsters that were housed in short days differed statistically both from control hamsters housed in long days ($U = 0$, $p = 0.008$) and from hamsters that showed "complete" damage to the PVN ($U = 0$, $p = 0.016$), while the difference between animals that sustained "complete" damage to the PVN and hamsters that were housed in long days did not reach statistical significance ($U = 7$, $p = 0.556$). Mean testicular and seminal vesicle weights of these groups of hamsters are presented in Table 2.

In 6 hamsters that received NMA injections, a loss of neurons which was independently judged by each of 2 investigators to be partial damage to the PVN was found. As shown in Figure 21, the injections of NMA were centered dorsal to the PVN in 5 of these hamsters and dorsal to the SCN in the other animal. Testicular widths of individual hamsters in this group are shown in Figure 22 while testicular and seminal vesicle weights are given in Table 2. In one hamster (No. 15), the loss of PVN neurons on one side of the brain was comparable to that in hamsters that sustained "complete" damage, while the contralateral PVN showed a less extensive loss of neurons. This hamster maintained large testes for the duration of the experiment, showing a testicular width of 12.1 mm (see Figure 22) and a testicular weight of 2.330 g when sacrificed at week 14. The hamster (No. 4) in which the NMA injections were centered dorsal to the SCN showed substantial but partial damage to the PVN bilaterally and exhibited partial testicular regression. Testicular width of this hamster at week 14 was 9.2 mm (see Figure 22). The other hamsters that showed partial damage to the PVN included one (No. 8) that showed "complete" damage to the PVN on one side and no damage to the contralateral PVN, and 3 hamsters (Nos. 7, 14, 21) that showed partial damage to the PVN on each side. As shown in Figure 22, the injections of NMA failed to prevent testicular regression in all of these animals.

Table 2: Testicular and seminal vesicle weights*.

Group	Week of Sacrifice	n	Testicular Weight (g)	Seminal Vesicle Weight (g)
Short Day Control	14	4	0.445±0.042	-----
"Complete" Damage	14	1	2.540	-----
Partial Damage	14	2	2.330 0.6104	-----
No Damage	14	7	0.587±0.057	-----
Short Day Control	19	5	0.837±0.124	0.125±0.023
Long Day Control	19	5	3.403±0.137	1.412±0.198
"Complete" Damage	19	4	2.679±0.168	1.020±0.339
Partial Damage	19	2	0.6280 1.4676	0.1375 0.4888
Short Day Control	29	4	3.695±0.104	1.643±0.166
No Damage	29	9	3.389±0.266	1.180±0.199
Partial Damage	29	1	3.885	1.907

*Weights are reported as group mean±SEM, where feasible. For hamsters that received partial damage to the PVN and the one hamster that was sacrificed at week 14 after receiving "complete" damage to the PVN, data for individual animals are reported.

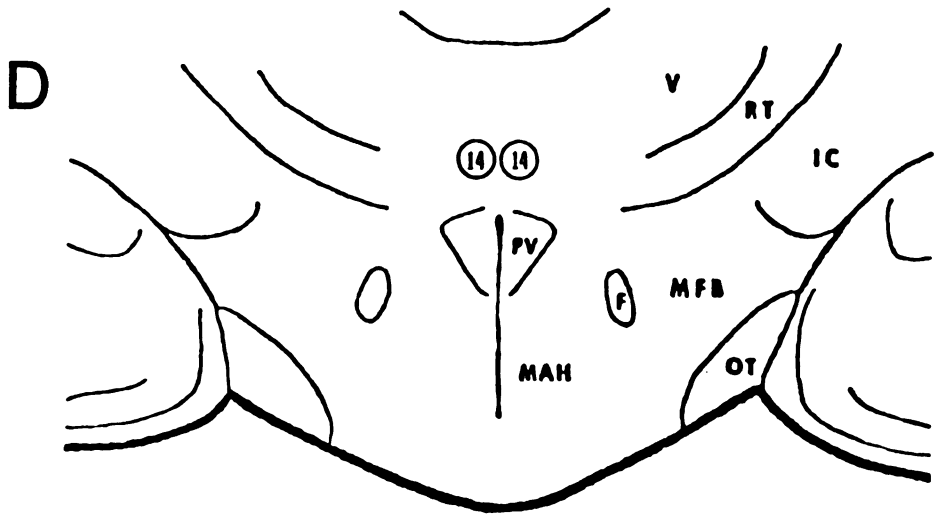

Figure 21: Locations of the centers of the injections of NMA that produced partial damage to the PVN. The drawings of the hamster hypothalamus (A-D, rostral to caudal) are modifications of those published by Malsbury (1977). Abbreviations are: F, fornix; IC, internal capsule; OT, optic tract; MAH, medial anterior hypothalamus; MFB, medial forebrain bundle; PV, paraventricular nucleus; RT, reticular thalamic nucleus; SC, suprachiasmatic nucleus; SO, supraoptic nucleus; V, ventral thalamic nucleus.

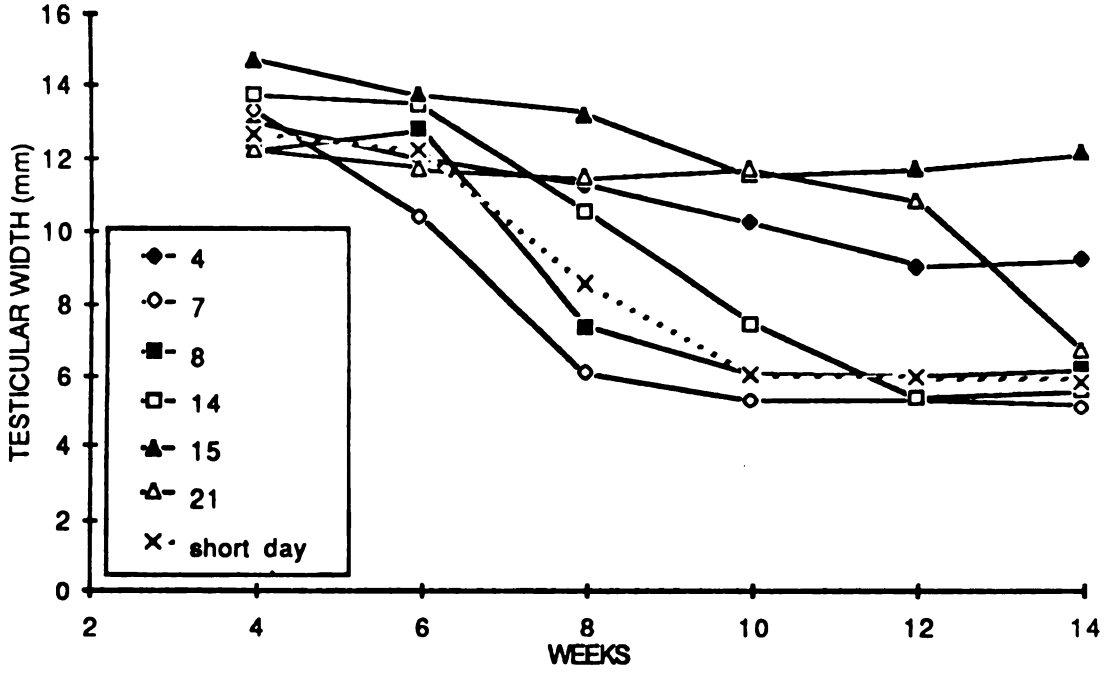

Figure 22: Testicular widths of individual hamsters in which the injections of NMA (presented in Figure 21) produced partial damage to the PVN.

In 7 of the hamsters that were sacrificed at week 14 of the experiment, the injections of NMA did not cause damage to the PVN. The centers of the injection sites in these animals are represented in Figure 23. As shown in that figure, the placement of the injections in these hamsters was quite variable, ranging from just dorsal to the anterior commissure at the level of its decussation across the midline, to the central region of the thalamus at the anterior-posterior level of the PVN. In each case, gliosis and loss of neurons were seen in the area surrounding the injection site. All of these hamsters showed complete testicular regression. Mean testicular width of these animals at week 14 was 5.71 ± 0.45 mm. Mean testicular weight for this group (see Table 2) was 0.587 ± 0.057 g.

Of the hamsters that were sacrificed at week 19 of the experiment, 2 animals (Nos. 14 and 21) showed partial damage to the PVN (discussed above) and complete testicular regression. Testicular widths (Figure 22) of these animals at week 14 were 5.6 mm (No. 14) and 6.7 mm (No. 21). At the time of sacrifice, one of these hamsters showed evidence of having begun to exhibit spontaneous testicular recrudescence. Testicular weights of these animals were 0.6280 and 1.4676 g, while the corresponding seminal vesicle weights were 0.1375 and 0.4888 g (Table 2). This observation led to a major change in procedure. The remaining animals (10 that had received NMA injections and 4 control animals) had all shown complete testicular regression and had been scheduled to be sacrificed at week 19. These animals were allowed to survive an additional 9 weeks and their testicular widths recorded biweekly. While this manipulation further complicated the experimental design, it permitted a physiological assay of the ability of these animals to secrete LH and FSH and, by implication LHRH. In one of these hamsters (No. 8), the injections caused partial damage to the PVN (discussed above). In the other 8 hamsters that were sacrificed at week 28 and the hamster that spontaneously died after week 25, the injections did not cause damage to the PVN. The locations of the centers of the NMA injection sites that did not damage the PVN, represented in Figure 24, were similar to the locations of the injections

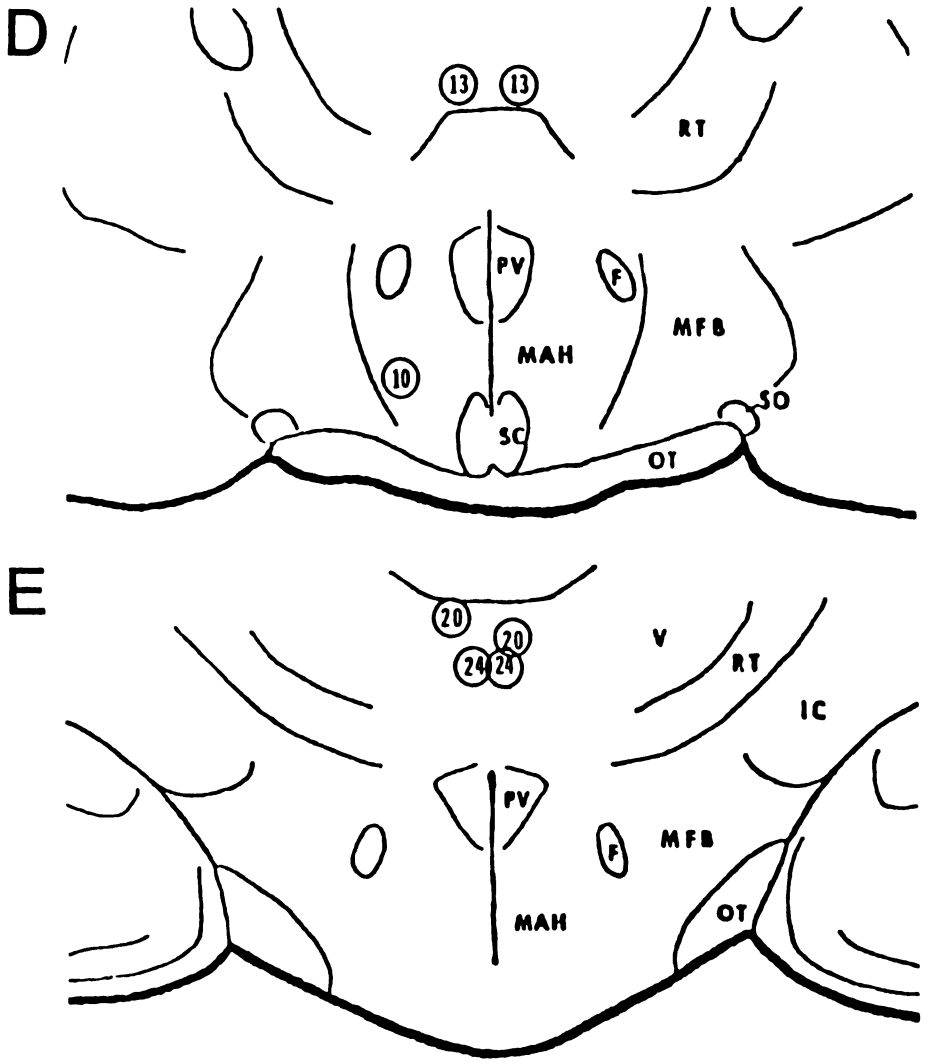

Figure 23: Locations of the centers of the injections of NMA that did not damage the PVN or prevent testicular regression in hamsters that were exposed to short days for 14 weeks. The drawings of the hamster hypothalamus (A-E, rostral to caudal) are modifications of those published by Malsbury (1977). Abbreviations are: AC, anterior commissure; F, fornix; IC, internal capsule; OC, optic chiasm; OT, optic tract; MAH, medial anterior hypothalamus; MFB, medial forebrain bundle; MPO, medial preoptic area; PV, paraventricular nucleus; RT, reticular thalamic nucleus; SC, suprachiasmatic nucleus; SM, stria medullaris; SO, supraoptic nucleus; V, ventral thalamic nucleus.

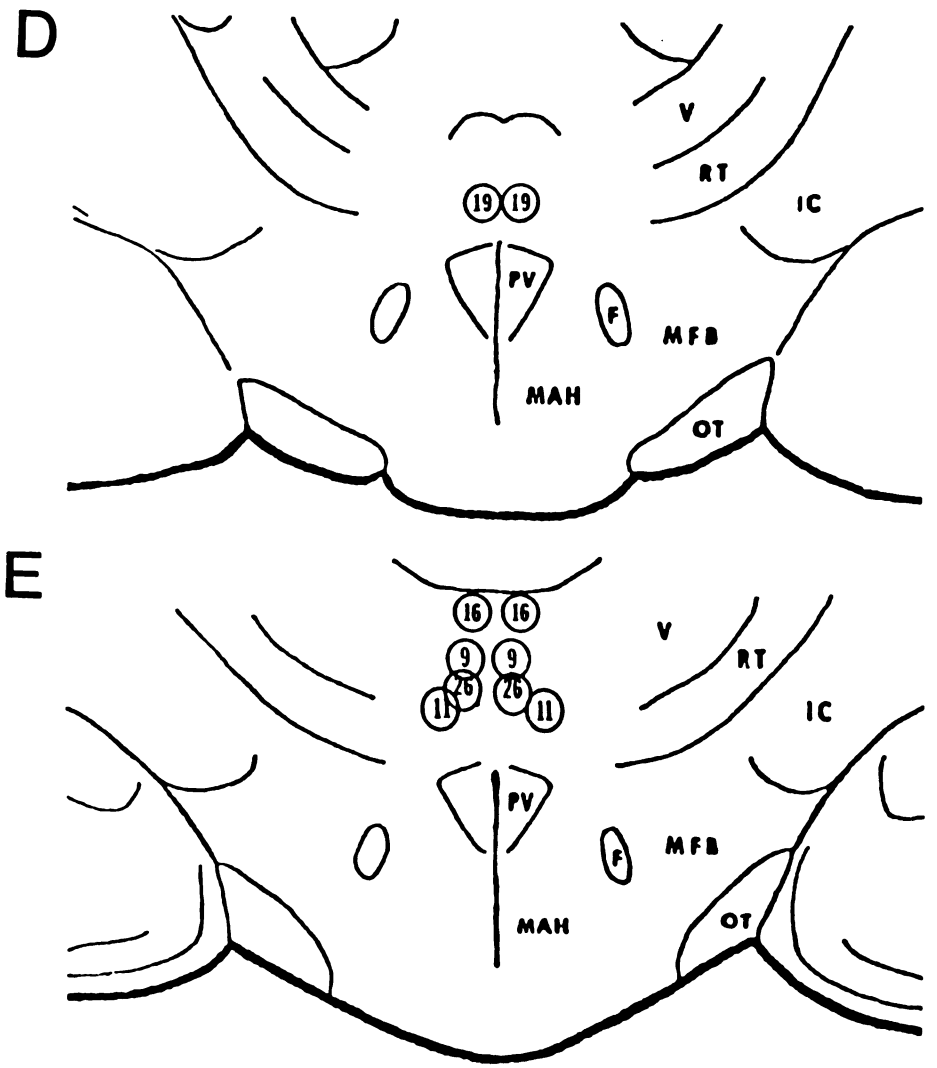

Figure 24: Locations of the centers of the injections of NMA that did not damage the PVN of hamsters that were exposed to short days for 25-28 weeks. The drawings of the hamster hypothalamus (A-E, rostral to caudal) are modifications of those published by Malsbury (1977). Abbreviations are: AC, anterior commissure; F, fornix; IC, internal capsule; OC, optic chiasm; OT, optic tract; MAH, medial anterior hypothalamus; MFB, medial forebrain bundle; MPO, medial preoptic area; PV, paraventricular nucleus; RT, reticular thalamic nucleus; SC, suprachiasmatic nucleus; SM, stria medullaris; SO, supraoptic nucleus; V, ventral thalamic nucleus.

that did not damage the PVN of hamsters that were sacrificed at week 14. In Figure 25 a photomicrograph of a section just caudal to the center of the injection site of one of these hamsters is presented. As shown in that figure, the injection caused substantial gliosis and loss of neurons in the central part of the thalamus, but did not damage the PVN. As illustrated in Figure 26, all 9 of these hamsters as well as the control hamsters that were sacrificed at week 28 showed complete testicular regression by week 14 and complete testicular recrudescence by week 25. Mean testicular widths of control hamsters and hamsters that received NMA injections that did not damage the PVN were 6.10 ± 0.21 and 6.34 ± 0.37 mm at week 14 and 12.03 ± 0.47 and12.38 ± 0.18 mm at week 25. The difference between these two groups was not statistically significant at either of these two times ($U = 15$, $p = 0.64552$, at week 14; $U = 18$, $p = 1.0$, at week 25). Furthermore, neither the difference in testicular weight (3.389 ± 0.266 vs 3.695 ± 0.104 g, $U = 11$, $p = 0.28014$) nor the difference in seminal vesicle weight (1.18 ± 0.199 vs 1.643 ± 0.166 g, $U = 6$, $p = 0.06432$) between the 8 hamsters that survived until week 28 after receiving NMA injections that did not damage the PVN and the control group reached statistical significance (see Table 2). The hamster that had shown partial damage to the PVN also exhibited complete testicular regression and recrudescence, having shown testicular widths of 6.2 mm at week 14 and 13.1 mm at week 25, and testicular and seminal vesicle weights of 3.885 g and 1.907 g at week 25.

Behavioral Tests

During the first testing session, the 5 hamsters that received NMA injections (Nos. 2, 3, 4, 23, and 28) and the 5 control hamsters all showed mounting behavior. During the second testing session, all of the males again showed mounting behavior. The hamster (No. 4) that had exhibited partial testicular regression failed to show intromission while the other 9 hamsters all displayed this aspect of sexual behavior. None of the hamsters ejaculated during the 20-min testing session. As illustrated in Figures 27 and 28, however, the animals that received NMA injections were less sexually competent than control

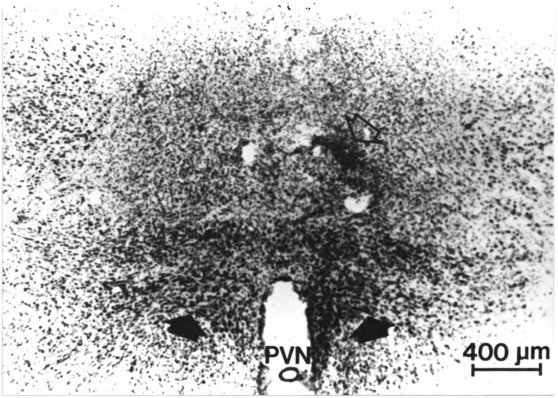

Figure 25: Photomicrograph of a section (40 µm thick, cresyl violet stain) through the brain of a hamster in which the NMA injections were centered in the thalamus approximately 750 µm dorsal to the PVN. The gliosis and loss of neurons is typical of cases in which the NMA injections were centered in the thalamus. Note that the damage did not extend to the PVN. The open arrow indicates the zone of dense gliosis surrounding the center of the NMA injection site.

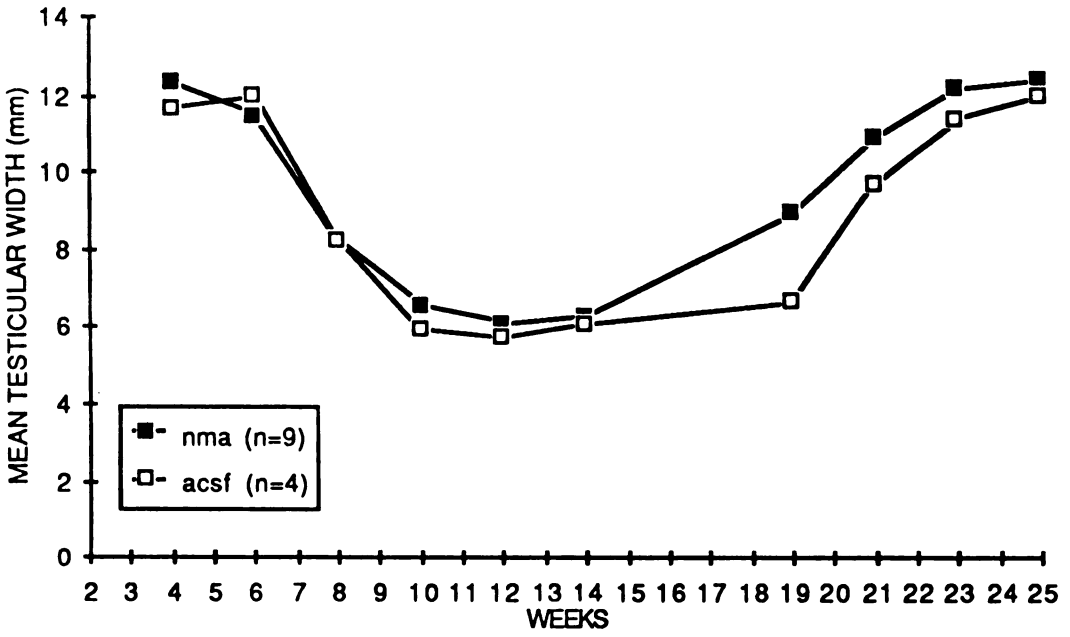

Figure 26: Testicular widths of the 9 hamsters for which the injection sites were presented in Figure 24 and for 4 control hamsters that were exposed to short days for 25- 28 weeks.

hamsters. While the average number of mounts did not differ statistically ($t = 0.28$, df = 8, $p > 0.05$) between the animals that received NMA injections (5.8 ± 1.9) and control hamsters (5.2 ± 0.04), hamsters that received injections of NMA showed a longer latency to mount (173.0 ± 31.1 vs 93.0 ± 15.3 sec, $t = 2.31$, df = 8, $p < 0.05$), fewer intromissions (27.8 ± 1.7 vs 42.4 ± 2.6, $t = 4.41$, df = 8, $p < 0.01$), and a longer latency to intromit (272.5 ± 53.8 vs 132.3 ± 9.8 sec, $t = 2.89$, df = 8, $p < 0.05$) than control animals. Although none of the hamsters showed the full complement of male sexual behavior during the sessions in which they were observed, 4 out of the 5 that had received NMA injections were capable of delivering sperm. Microscopic examination of the vaginal smears revealed that intact spermatozoa, as well as detached tails were present in the vaginae of the females that were paired with these males. No evidence of spermatazoa was found in the vaginal smear obtained from the female that was paired with the hamster (No. 4) that had shown partial testicular regression. Intact spermatozoa were found in the testicular fluid obtained from each of the 5 hamsters that received NMA injections.

Discussion

In 6 animals, injections of NMA produced a substantial loss of neurons from the posterior PVN as judged independently by two investigators. As reported in Experiment 3A, and by other investigators using NMA (Olney and Price, 1983) or kainic acid (Zhang and Ciriello, 1985), the magnocellular neurons of the anterior portion of the PVN were not destroyed by injections of the toxin. In all six of these cases, the injections of NMA prevented testicular regression when the hamsters were exposed to short days. In 4 of these animals, two measures of testicular function, in addition to weight and size, were used to confirm that the gonads of these animals had remained active. Since seminal vesicle weight is dependent on circulating concentrations of gonadal steroids (Ellis and Turek, 1979), the large seminal vesicles of these animals indicates that their testes were secreting androgens. Moreover, the presence of spermatozoa in the vaginal smears obtained from the 4 female hamsters that were paired overnight with these animals

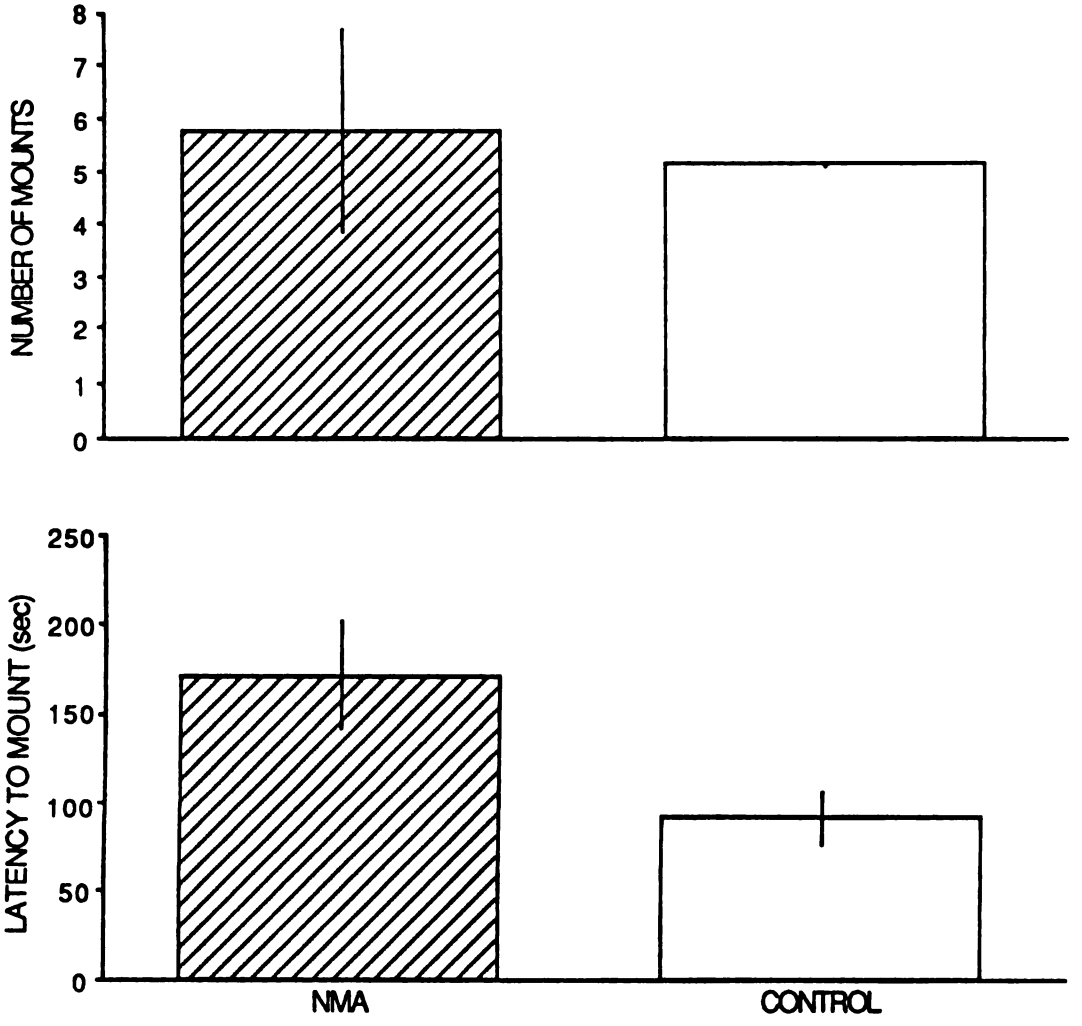

Figure 27: Mean number of mounts (without intromissions) and latency to mount exhibited by hamsters that received injections of NMA (n = 5) that caused either complete (n = 4) or partial (n =1) damage to the PVN, and unoperated control hamsters that were housed in long days. While the number of mounts did not differ statistically between groups, the difference in latency to mount was statistically significant ($p < 0.05$).

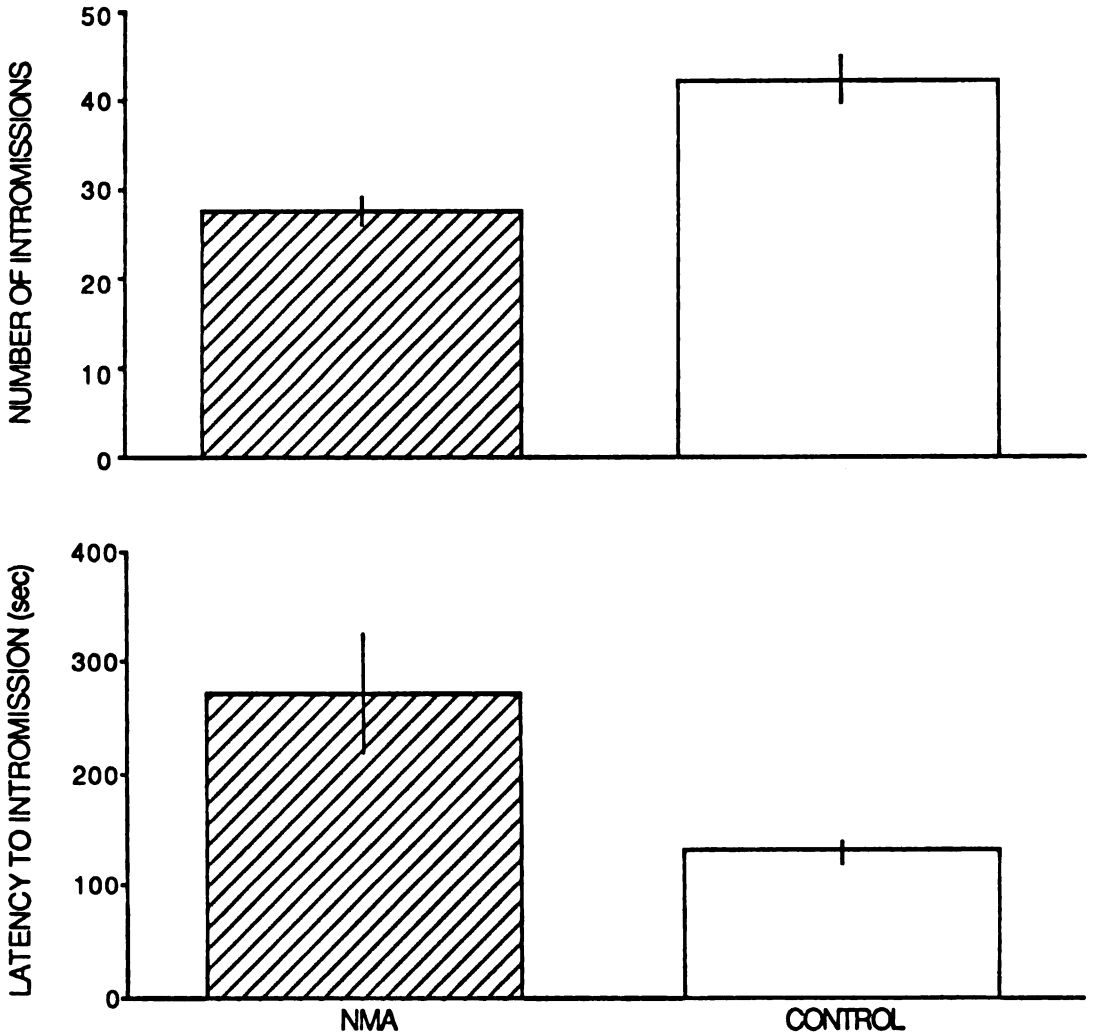

Figure 28: Mean number of intromissions and latency to intromit exhibited by hamsters that received injections of NMA (n = 5) that caused either complete (n = 4) or partial (n =1) damage to the PVN, and unoperated control hamsters that were housed in long days. Statistically significant differences between groups were obtained both for number of intromissions ($p < 0.01$) and for latency to intromit ($p < 0.05$).

indicates that they were able to produce sperm and inseminate females. While limitations of the techniques that were used prohibited assessment of the viability of the spermatozoa, the results suggest that reproductive competence of the animals was maintained.

Some of the hamsters received injections that were aimed at the area of the anterior hypothalamus which Hastings, et al. (1985) reported that NMA-induced damage of prevented reproductive regression in male and female hamsters exposed to short days. Since the majority of the injections in the present experiment were centered outside of this area, it was not possible to compare these results with those of Hastings, et al. In one notable case (No. 4), however, the injections were centered dorsolateral to the SCN approximately midway between that nucleus and the PVN and within the area of neuronal loss reported by Hastings, et al. (see their figure 1). The injections in this animal resulted in a loss of neurons from the anterior hypothalamus similar to that reported by Hastings, et al. and a partial loss of neurons from the PVN. In agreement with previous reports (Peterson and Moore, 1980), no evidence of damage was seen in the SCN of this animal. Exposure of this hamster to short days resulted in partial testicular regression. Furthermore, there was no evidence of spermatozoa in the vaginal smear obtained from the female hamster with which this animal was paired overnight. Microscopic examination of the testicular fluid obtained from this animal after castration revealed that spermatozoa were present. Thus, although this hamster may have been able to produce sperm, he failed to deliver it. Of the injections that produced damage to the PVN, this one was centered the farthest from the PVN. Since the glial scar could be traced from the center of the injection site to the PVN, it is likely that the damage to the PVN was due to backflow of the toxin along the path of the needle.

While injections of NMA that produced "complete" damage to the PVN were centered either within the PVN or ≤ 400 µm dorsal (not corrected for shrinkage of the tissue during histological processing) to it, injections that were centered ≥ 520 µm dorsal to the PVN and injections that were centered anterior to the PVN failed to produce damage to that

nucleus. Injections that were dorsal to the PVN and ≤ 500 μm from that nucleus produced effects that ranged from "complete", to partial, to no damage to the PVN. In one animal (discussed above), injections dorsal to the SCN, and ventral and anterior to the PVN produced partial damage to the PVN. In one additional hamster, the injection on one side of the brain was dorsal to the SCN while the contralateral injection was in the dorsal aspect of the preoptic area. The injections in this hamster produced no or only very little loss of cells from the PVN.

Injections that did not produce damage within the PVN failed to prevent testicular regression. Furthermore, 9 of these hamsters were exposed to short days for 25 weeks and all of them showed complete spontaneous testicular recrudescence. The ability of these animals to show complete testicular recrudescence implies that the portion of the hypothalamo-hypophysial axis responsible for the secretion of LH was not destroyed by the injections of NMA. While the location of the LHRH neurons responsible for the secretion of LHRH into the hypothalamo-hypophysial portal system is not specifically known, the cells that are currently thought to be the most likely candidates lie in a ventral position through the preoptic area and hypothalamus. Thus, that in each of the animals in which NMA injections did not damage the PVN the injections were centered dorsal to PVN (n = 10) in the thalamus, or in the dorsal aspect of the septum or preoptic area (n = 5), and (with the exception of one animal in which the injection on one side was dorsolateral to the SCN while the injection on the contralateral side was in the dorsal aspect of the preoptic area) in no case was a loss of cells within the ventral hypothalamus evident, support the argument that the injections in these animals probably did not cause significant damage to the LHRH neurons that regulate release of gonadotropins from the pituitary. Research in progress (Brown, Badura, and Nunez) using hamsters exposed to long days after receiving injections of NMA should add further support to the argument that NMA-induced neuronal damage similar to that reported here does not induce gonadal regression.

As mentioned previously, partial destruction of the posterior PVN prevented testicular regression in one hamster and partially prevented regression in another animal. In one additional hamster in which partial destruction of the PVN was found, testicular regression was delayed. Testicular width of this hamster was 10.5 mm at week 12 and 7.5 mm at week 14. The 3 other hamsters that received partial damage to the PVN all showed complete testicular regression within the range of time required for control hamsters to do so. Thus, the effects of partial destruction of paraventriculo-spinal projections on the testicular response to short days were quite variable, ranging from no effect to delay or partial prevention, to complete prevention of the response. Evidence for effects of partial destruction of the PVN and its connections with the spinal cord on the testicular response to short days has been reported previously. As mentioned above, Lehman, et al. (1984) reported that a partial lesion of the PVN, which destroyed the anterior PVN but spared most of the mid and caudal part of that nucleus, partially prevented testicular regression. Moreover, in a study of the effects of knife cuts ventral and dorsal to the PVN on testicular regression in hamsters housed in short days, Inouye and Turek (1986) reported variable effects of their sham-surgical procedure. Their knife cuts were made with a non-retractable wire knife that extended 1.5 mm perpendicularly from a stainless steel cannula. In order to avoid damaging the superior sagittal sinus, the authors lowered the knife into the brain at a point lateral to the midline, pushed the knife to the midline, and lowered the blade to a point ventral to the PVN. This procedure was reported to result in "variable damage" to areas dorsal to the PVN (e.g., hippocampus, thalamus, and habenular area) and either prevented or partially prevented testicular regression in 7 out of 12 animals subjected to this procedure. Moreover, that bilateral cuts (present study) placed dorsal to the PVN in an area in which they should have completely severed bundle 1 but not bundle 2, prevented testicular regression but not labelling of PVN neurons after injections of HRP into the spinal cord (Experiments 1 and 2) supports the argument that testicular regression can be prevented by severing only a portion of the paraventriculo-spinal projections. Since the

pattern of secretion of melatonin, gonadotropins, and other endocrine hormones in these animals is not known, it is not possible to determine the mechanism by which misplaced or partial lesions partially prevent testicular regression. It is possible, however that these lesions affect the release of gonadotropins.

Although injections of NMA in the dose used in this investigation have been reported not to damage monoaminergic axons that pass through the lateral hypothalamus (Hastings et al., 1985b), toxic effects of NMA on axons of SCN neurons have not been directly tested. However, converging evidence from experiments on the function of the SCN indirectly suggests that these axons are probably insensitive to the toxicity of NMA. Knife cuts that create an "island" of tissue containing the SCN and sever all of the efferent projections of that nucleus are equivalent to SCN lesions (Stephan and Zucker, 1974) in that they abolish circadian rhythms of locomotor activity, sleep, brain temperature, and drinking behavior (Stephan and Nunez, 1977). Knife cuts that partially isolate the SCN, severing fibers that project dorsally, laterally, and caudally, but not rostrally from the SCN abolish the nocturnal pattern of water intake of rats housed under a light-dark cycle (Nunez and Stephan, 1977). Thus, disruption of a large proportion of the axons that project from the SCN causes a disruption of behavioral circadian rhythms. Further evidence regarding disruption of axons of SCN neurons comes from a comparison of the effects of intrahypothalamic injections of NMA with effects of injections of colchicine. Intracerebral injections of colchicine temporarily disrupt microtubule-dependent axoplasmic transport (Dahlstrom, 1968). Injections of colchicine have been used to produce temporary behavioral deficits (Avrith and Morgenson, 1978; Willis and Smith, 1983). Bilateral injections of colchicine (1, 2, or 4 μg in 0.2 μl of vehicle) just dorsolateral to the SCN temporarily disrupt circadian rhythms of drinking behavior in blinded rats for up to 12 days, after which the rhythms return (Brown and Nunez, unpublished observation). Injection of NMA (0.5 μl of 0.15 M) at the same site had no effect on the drinking rhythm. Furthermore, the NMA injection sites that were reported by Hastings, et al.

(1985a) to prevent gonadal regression in hamsters included, and appear to have surrounded (see their figure 2), the SCN but did not affect the circadian activity patterns of the animals. Since manipulations that are known to affect axons (i.e., knife cuts and injections of colchicine) disrupt behavioral circadian rhythms while injections of NMA at sites that include the SCN do not, these results indirectly suggest that axons of SCN neurons are not damaged by injections of NMA. Furthermore, Hastings and Herbert (1986) recently reported seeing intact axons (stained via the Holmes silver method) both within the PVN and coursing dorsally through the site of damage produced by injections of NMA into the PVN. Thus, it appears that the injections of NMA that produced substantial damage to the PVN and prevented testicular regression in the present experiment probably did so without destroying axons that have been reported to travel from the SCN through or near the PVN to the habenula and other structures (Stephan, et al., 1981). Therefore, the effects of electrolytic lesions or knife cuts in or near the PVN on the testicular response to short days are probably not due to incidental damage to these fibers of passage. Work in progress (Brown, Badura, and Nunez), using knife cuts aimed just ventral to the habenula, may provide further evidence to support the argument that projections from the SCN and the PVN to the habenula are not involved in testicular regression in hamsters exposed of short days.

A recent report has provided evidence in support of the argument that knife cuts (Experiments 1 and 2), injections of NMA (present experiment), and electrolytic lesions in or near the PVN (Bartness, et al., 1985; Lehman, et al., 1984; Pickard and Turek, 1983) prevent testicular regression by interrupting the neural pathway from the SCN to the pineal and not the pathway by which pineal melatonin influences release of gonadotropins. Injections of NMA that completely destroyed the parvocellular portions of the PVN prevented the nighttime rise in pineal melatonin content (Hastings and Herbert, 1986). Similarly, electrolytic lesions of the PVN also prevent the nocturnal increase in melatonin content of the pineal (Klein, et al., 1983; Lehman, et al., 1984). Further research (e.g.,

testing of hamsters with electrolytic lesions of the PVN for sensitivity to systemic injections of melatonin) is needed to confirm that the insensitivity to short days seen in hamsters with damage to the PVN is not due to insensitivity to melatonin. Similarly, further research (e.g., subhabenular knife cuts or electrolytic lesions of the habenula) is needed to confirm that effects of damage to the PVN are not due to disruption of projections from the PVN to the habenula (Conrad and Pfaff, 1976) or pineal (Buijs and Pevet, 1980; Guerillot, et al., 1982; Korf and Wagner, 1980; Moller and Korf, 1983). However, the most parsimonious explanation for the effects of electrolytic lesions, injections of NMA or knife cuts in or near the PVN on the testicular response to short days is that all of these manipulations disrupt the multisynaptic neural pathway from the SCN to the pineal gland.

GENERAL DISCUSSION

These results are consistent with, and support the model of photic and circadian regulation of the autonomic neural and endocrine systems wherein the SCN act as a circadian oscillator to regulate the timing of various functions. Mammalian circadian rhythms persist in constant environmental conditions and are generated by one or more circadian oscillators (Rusak and Zucker, 1979). The light-dark cycle acts as a zeitgeber (time-giver) to entrain circadian rhythms. Ablation of the SCN abolishes several circadian rhythms including adrenal corticosterone content (Moore and Eichler, 1972), pineal serotonin N-acetyltransferase activity (Moore and Klein, 1974), and locomotor activity and drinking behavior (Stephan and Zucker, 1972), as well as seasonal and ovulatory cycles (Rusak and Zucker, 1979).

Through their axonal projections to other neural structures, the SCN produce seasonal and circadian (and other) cycles of various end points. Thus, while axons that project dorsally from the SCN to the PVN and other structures are not critical for the generation or entrainment of behavioral circadian rhythms (Experiment 1; Brown and Nunez, 1986; Eskes and Rusak, 1985), they appear to be necessary for photoperiod-dependent reproductive cycles (Experiment 1; Eskes and Rusak, 1985; Inouye and Turek, 1986) and photic and circadian regulation of pineal melatonin secretion (Lehman, et al., 1984; Klein, et al., 1983; Moore and Klein, 1974). Similarly, results of experiments using retrochiasmatic knife cuts (in a coronal plane caudal to the SCN) indicate that axons that project caudally from the SCN are not critical for the generation of circadian rhythms of water intake (Nunez and Stephan, 1977), feeding (Nishio, et al., 1979), or locomotor activity (Nunez and Casati, 1979); but appear to be necessary for such endocrine cycles as daily surges of prolactin during pseudopreganancy (Freeman, et al., 1974), estrous cycles (Nunez and Casati, 1979), and circadian rhythms of adrenal corticosterone content (Moore and Eichler, 1974). Therefore, at least 2 distinct functional subpopulations of SCN axons, controlling different endocrine outputs, exist.

In contrast to circadian regulation of endocrine function, which can be abolished by selective interruption of axonal projections from the SCN, more severe disruption of "suprachiasmatico-fugal" projections is required to abolish behavioral circadian rhythms. Thus, complete isolation of an "island" of tissue containing the SCN (Stephan and Nunez, 1977); or knife cuts that partially isolate the SCN, severing axons that project dorsally, laterally, and caudally, but not rostrally from the SCN (Nunez and Stephan, 1977) abolish behavioral circadian rhythms. In contrast, more secective knife cuts placed in bilateral parasagittal planes lateral to the SCN (Nunez and Stephan, 1977), a coronal plane either rostral (Nunez and Stephan, 1977) or caudal to the SCN (Nishio, et al., 1979; Nunez and Casati, 1979; Nunez and Stephan, 1977), or a horizontal plane dorsal to the SCN (Experiment 1; Brown and Nunez, 1986; Eskes and Zucker, 1985) fail to affect behavioral rhythms.

Regarding the functional role of the PVN and paraventriculo-spinal projections, several interesting questions are raised by the present (and related) findings. It is very difficult to resist the temptation to ask the teleological question: Why do 2 bundles of paraventriculo-spinal projections exist ? Related questions that are easier to answer empirically include:

Are the fibers of bundles 1 and 2 collateral branches of the same parent axons ?

Do bundles 1 and 2 arise from distinct functional and/or anatomical subnuclei ?

What functions does bundle 2 serve ?

In addition to mediating photoperiodic regulation of the pineal gland and reproduction, what other functions does bundle 1 serve ?

Is there a distinct subpopulation of PVN neurons that participates in photoperiodic control of reproduction ?

While the answers to these questions are currently unknown, future research may provide some of the answers. As mentioned above, evidence exists that bundle 1 might mediate the feeding behavior that is elicited by microinjections of norepinephrine into the

PVN (Weiss and Leibowitz, 1986) and bundle 2 may be involved in the regulation of energy balance (Gold, et al., 1977). Another question that merits investigation is whether the PVN and bundles 1 and 2 are involved in nonreproductive responses to photoperiod. Exposure of Syrian hamsters, and other mammalian species, to short days results in a constellation of adaptations in addition to changes in reproductive capacity. Many of these adaptations enhance the ability of the animal to survive the "real world" conditions of low ambient temperatures and dwindling food supplies during winter. Examples of seasonal cycles in non-reproductive functions which are dependent on photoperiod and the pineal gland include: the shedding of antlers by deer during winter and spring months (Gross, 1969; Brown, et al., 1978); thyroid activity (Vriend, and Reiter, 1977; Vriend, et al., 1979, 1982); fur color and fur density in Siberian hamsters (Duncan and Goldman1984a, 1984b; Duncan, et al., 1985; Hoffman, 1978).

Photoperiodic responses that are not dependent on the pineal also exist. Hamsters show adaptations that are dependent on photoperiod, directly related to energy balance, and independent of the pineal. Exposure of Siberian hamsters to cold ambient temperatures results in increased thermogenesis (as measured by resting O_2 consumption). Siberian hamsters kept in short days show enhanced capacity for thermogenesis, and animals that have been kept in short days for 2 months are able to maintain normal body temperature during subsequent exposure to ambient temperatures of -41° C (Heldmaier, et al., 1981). Syrian hamsters housed in short days exhibit increases in body weight gain, carcass lipid content, feed efficiency (i.e., weight gain per Kcal of food ingested), thermogenic capacity (i.e., norepiniphrine-stimulated increase in O_2 consumption), and show growth of brown fat pads (Bartness and Wade, 1984), a major site thermogenesis (Foster and Frydman, 1978). These effects are exaggerated in hamsters fed a high fat diet (Bartness and Wade, 1984). These effects of short days are mimicked by peripheral injections of melatonin given to hamsters housed in long days but, suprisingly, are not prevented by pinealectomy (Bartness and Wade, 1984). Increased feed efficiency and body weight gain are also seen

in hamsters fed a high fat diet (Wade, 1982) and housed in long days, and the effects of photoperiod and diet on these measures are greater in female than in male hamsters (Wade, 1983). Recent evidence suggests that the PVN may be involved in the effects of diet and photoperiod on energy balance and thermogenesis since electrolytic lesions of the PVN enhance high fat diet-induced, but prevent short photoperiod-induced obesity in hamsters (Bartness, et al., 1985).

Thus, some commonalities (e.g., projections from the retina to the SCN and from the SCN to the PVN) may exist in the neural pathways that regulate reproductive and those that mediate nonreproductive responses to photoperiod. However, there must also exist differences between these pathways since metabolic responses to photoperiod are independent of the pineal gland. In conclusion, while the neural pathways that mediate pineal-dependent photoperiodic regulation of reproduction have been a "hotbed" of scientific investigation in recent years, the neural pathways that mediate photoperiodic regulation of thermogenesis and body energy balance and other nonreproductive responses, some of which may be pineal independent (?) remain largely uncharted territory.

LIST OF REFERENCES

LIST OF REFERENCES

Ariens Kappers, J. The development of topographical relations and innervations of the epiphysis cerebri in albino rat. Z Zellforsch 52: 163-215, 1960.

Armstrong, W.E., S. Warach, G.I. Hatton, and T.H. McNeill. Subnuclei in the rat hypothalamic paraventricular nucleus: a cytoarchitectural, horseradish peroxidase and immunocytochemical analysis. Neuroscience 5: 1931-1958, 1980.

Avrith, D. and G. Morgenson. Reversible hyperphagia and obesity following intracerebral microinjections of colchicine into the ventromedial hypothalamus of the rat. Brain Res 153: 99-107, 1978.

Bartness, T.J., E.L. Bittman, and G.N. Wade. Paraventricular nucleus lesions exaggerate dietary obesity but block Photoperiod-induced weight gains and suspension of estrous cyclicity in syrian hamsters. Brain Res Bull 14: 427-430, 1985.

Bartness, T.J. and G.N. Wade. Photoperiodic control of body weight and energy metabolism in Syrian hamsters (Mesocricetus auratus): role of pineal gland, melatonin, gonads, and diet. Endocrinology 114: 492-498, 1984.

Berk, M.L. and J.A. Finkelstein. An autoradiographic determination of the efferent projections of the suprachiasmatic nucleus of the hypothalamus. Brain Res 226: 1-13, 1981.

Boneau, C.A. The effects of violations of assumptions underlying the t test. Psychol Bull 57: 49-64, 1960.

Brown, M.H. Hypothalamic regulation of thermogenesis: Stimulation of the paraventricular nucleus elicit thermal responses in brown adipose tissue. Soc Neurosci Abstr 12: 1453, 1986.

Brown, M.H. and A.A. Nunez. Hypothalamic circuits and circadian rhythms: effects of knife cuts vary with their placement within the suprachiasmatic area. Brain Res Bull, 16: 705-711, 1986.

Brown, R.D., R.L. Cowan and J.F. Kavanaugh. Effect of pinealectomy on seasonal androgen titers, antler growth and feed intake in white-tailed deer. J Animal Sci 47: 435-440, 1978.

Buijs, R.M. and P. Pevet. Vasopressin- and oxytocin-containing fibers in the pineal gland and subcommissural organ of the rat. Cell Tiss Res 205: 11-17, 1980.

Campbell, C.S., J. Tabor, and J. Davis. Small effect of brown adipose tissue and major effect of photoperiod on body weight in hamsters (Mesocricetus auratus). Physiol Behav 30: 349-352, 1983.

Conrad, L.C.A. and D.W. Pfaff. Efferents from medial basal forebrain and hypothalamus in the rat II: an autoradiographic study of the anterior hypothalamus. J Comp Neurol 169: 221-262, 1976.

Dahlstrom, A. Effect of colchicine on transport of amine storage granules in sympathetic nerves of rat. Eur J Pharmacol 5: 111-113, 1968.

Dafny, N. Evidence that the rat pineal has direct neuronal connections via the pineal stalk. Exp Neurol 79: 858-861, 1983.

Dohanich, G.P., and L.G. Clemens. Brain areas implicated in cholinergic regulation of sexual behavior. Horm Behav 15: 157-167, 1981.

Don Carlos, L.L. and J.A. Finkelstein. Hypothalamic projections to the spinal cord in the hamster. Soc Neurosci Abstr 8: 218, 1982.

Duncan, M.J. and B.D. Goldman. Hormonal regulation of the seasonal pelage color cycle in the Djungarian hamster, Phodopus sungorus. I. Role of the gonads and pituitary. J Exptl Zool 230: 89-95, 1984a.

Duncan, M.J. and B.D. Goldman. Hormonal regulation of the seasonal pelage color have different critical daylengths in the Djungarian hamster, Phodopus sungorus. I. Role of prolactin. J Exptl Zool 230: 97-103, 1984b.

Duncan, M.J., B.D. Goldman, M.N. DiPinto and M.H. Stetson. Testis function and pelage color cycle in the Djungarian hamster, Phodopus sungorus. I. Role of prolactin. Endocrinology 116: 424-430, 1985.

Elliott, J. A. Circadian rhythms and photoperiodic time measurement in mammals. Fed Proc Fed Am Soc Exptl Biol 35: 2339-2346, 1976.

Ellis, G.B. and F.W. Turek. Time course of the photoperiod-induced change in sensitivity of the hypothalamic-pituitary axis to testosterone feedback in castrated male hamsters. Endocrinology 104: 625-630, 1979.

Eskes G.A. and B. Rusak. Horizontal knife cuts in the suprachiasmatic area prevent gonadal responses to photoperiod. Neurosci Lett 61: 261-266, 1985.

Eskes, G. A. and I. Zucker. Photoperiodic regulation of the hamster testis: Dependence on circadian rhythms. Proc Natl Acad Sci USA 75: 1034-1038, 1978.

Foster, D.O. and M.L. Frydman. Nonshivering thermogenesis in the rat. II. Measurements of blood flow with microspheres point to brown adipose tissue as the dominant site of the calorigenesis induced by noradrenaline. Can J Physiol Pharmacol 56: 110-122, 1978

Freeman, M.E., M.S. Smith, S.J. Nazian and J.D. Neill. Ovarian and hypothalamic control of the daily surges of prolactin during pseudopregnancy in the rat. Endocrinology 94: 875-882 , 1974.

Gold, R.M., A.P. Jones, P.E. Sawchenko and G. Kapatos. Paraventricular area: Critical focus for a longitudinal neurocircuitry mediating food intake. Physiol Behav 18: 1111-1119, 1977.

Gross, R. Photoperiodic control of antler cycles in deer. I. Phase shift and frequency changes. J Exptl Zool 170: 311-324, 1969.

Guerillot, C., A. Pfister, J. Muller, and C. Da Lage. Recherche de l'origine des fibers nerveuses extraorthosympathiques innervant l'epiphyse du rat (etude du transport retrograde de la peroxydase de raifort). Reprod Nutr Dev 22: 371-378, 1982.

Hastings, M.H., A.C. Roberts, and J. Herbert. Neurotoxic lesions of the anterior hypothalamus disrupt the photoperiodic but not the circadian system of the Syrian hamster. Neuroendocrinology 40:316-324, 1985a.

Hastings, M.H., P. Winn, and S.B. Dunnett. Neurotoxic amino acid lesions of the lateral hypothalamus: A parametric comparison of the effects of ibotenate, N-methyl-D,L-aspartate and quisqualate in the rat. Brain Res 360: 248-256, 1985b.

Hastings, M.H. and J. Herbert. Neurotoxic lesions of the paraventriculo-spinal projection block the nocturnal rise in pineal melatonin synthesis in the Syrian hamster. Neurosci Lett 69: 1-6, 1986.

Hatton, G.I., P. Cobbett, and A.K. Salm. Extranuclear axon collaterals of paraventricular neurons in the rat hypothalamus: Intracellular staining, immunocytochemistry and electrophysiology. Brain Res Bull 14: 123-132, 1985.

Hoffman, K. Effects of short photoperiod on puberty, growth, and molt in the Djungarian hamster (Phodopus sungorus). J Reprod Fert 54: 29-35, 1978.

Hoffman, K. The influence of photoperiod and melatonin on testis size, body weight, and pelage colour in the Djungarian hamster (Phodopus sungorus). J Comp Physiol 85: 267-282, 1973.

Inouye, S.-I.T. and F.W. Turek. Horizontal knife cuts either ventral or dorsal to the hypothalamic paraventricular nucleus block testicular regression in golden hamsters maintained in short days. Brain Res, 370: 102-107, 1986.

Kirk, R.E. Experimental Design: Procedures for the Behavioral Sciences. Belmont, CA: Brooks/Cole, 1968.

Klein, M.N., R. Smoot, J.L. Weller, S. Higa, S.P. Markey, G.J. Creed, and D.M. Jacobowitz. Lesions of the paraventricular nucleus area disrupt the suprachiasmatic-spinal cord circuit in the melatonin rhythm generating system. Brain Res Bull 10: 647-652, 1983.

Korf, H.W. and U. Wagner. Evidence for a nervous connection between the brain and the pineal organ in the guinea pig. Cell Tiss Res 209: 505-510, 1980.

Lehman, M.N., E.L. Bittman, and S. Winans-Newman. Role of the hypothalamic paraventricular nucleus in neuroendocrine responses to daylength in the golden hamster. Brain Res 308: 25-32, 1984.

Leibowitz, S.F. Paraventricular nucleus: A primary site mediating adrenergic stimulation of feeding and drinking. Pharmacol Biochem Behav 8: 163-175, 1978.

Luiten, P.G.M., G.J. ter Horst, H. Karst, and A.B. Steffens. The course of paraventricular hypothalamic efferents to autonomic structures in medulla and spinal cord. Brain Res 329: 374-378, 1985.

Malsbury, C.W. A schematic stereotaxic atlas of the golden hamster brain. Catalog of Selected Documents in Psychology 7: 67, 1977.

Matt, K.S. and M.H. Stetson. Hypothalamic-pituitary-gonadal interactions during spontaneous testicular recrudescence in golden hamsters. Biol Reprod 20: 739-746, 1979.

Mesulam, M.-M., E. Hegarty, H. Barbas, K.A. Carlson, E.C. Gower, A.G. Knapp, M.B. Moss, and E.J. Mufson. Additional factors influencing sensitivity in the tetramethyl benzidine method for horseradish peroxidase neurohistochemistry. J Histochem Cytochem 28: 1255-1259, 1980.

Moller, M. and H.W. Korf. The origin of central pinealopetal nerve fibers in the Mongolian gerbil as demonstrated by the retrograde transport of horseradish peroxidase. Cell Tiss Res 230: 273-287, 1983.

Moore, R.Y. and V. B. Eichler. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Res 42: 201-206, 1974.

Moore, R.Y. and D.C. Klein. Visual pathways and the central neural control of a circadian rhythm in pineal serotonin N-acetyltransferase activity. Brain Res 71: 17-33, 1974.

Moore. R.Y. and N.J. Lenn. A retinohypothalamic projection in the rat. J Comp Neurol 146: 1-14, 1972.

Nance, D. W. Frontolateral hypothalamic knife cuts: Interruption of paraventricular efferents to the thoracolumbar cord. Brain Res Bull 7: 581-584, 1981.

Nishio, T.S., S. Shiosaka, H., H. Nakagawa, T. Sakumoto and K. Satoh. Circadian feeding rhythm after hypothalamic knife-cut isolating suprachiasmatic nucleus. Physiol Behav 23: 763-769, 1979.

Nunez, A.A. and M.J. Casati. The role of efferent connections of the suprachiasmatic in the control of circadian rhythms. Behav Neural Biol 25: 263-267, 1979

Nunez, A.A. and F.K. Stephan. The effects of hypothalamic knife cuts on drinking rhythms and the estrous cycle of the rat. Behav Biol 20: 224-234, 1977.

Olney, J.W. and M. T. Price. Excitotoxic amino acids as neuroendocrine research tools. Methods in Enzymology 103: 379-392, 1983.

Peterson, G.M. and R.Y. Moore. Selective effects of kainic acid on diencephalic neurons. Brain Res: 202: 165-182, 1980.

Pickard G.E. and A.J. Silverman. Direct retinal projections to the hypothalamus, piriform cortex and accessory optic nuclei in the golden hamster as demonstrated by a sensitive anterograde horseradish technique. J Comp Neurol 196:155-172, 1981.

Pickard, G.E. and F.W. Turek. The hypothalamic paraventricular nucleus mediates the photoperiodic control of reproduction but not the effects of light on the circadian rhythm of activity. Neurosci Lett 43: 67-72, 1983.

Rando, T.A., C.W. Bowers, and R.E. Zigmond. Localization of neurons in the rat spinal cord which project to the superior cervical ganglion. J Comp Neurol 196:73-83, 1981.

Reiter, R.J. and R.J. Hester. Interrelationships between the pineal gland, the superiorcervical ganglion, and the photoperiod in the regulation of the endocrine systems of hamsters. Endocrinology 79: 1168-1170, 1966.

Reiter, R.J., B.A. Richardson, and T.S. King. The pineal gland and its indole products: Their importance in the control of reproduction in mammals. In: The Pineal Gland, edited by R. Relkin. New York: Elsevier Biomedical, 1983.

Rusak, B. and L.P. Morin. Testicular responses to photoperiod are blocked by lesions of the suprachiasmatic nucleus in the golden hamster. Biol Reprod 15: 366-374, 1976.

Rusak, B. and I. Zucker. Neural regulation of circadian rhythms. Physiol Rev 59: 449-526, 1979.

Scouten, C.W., C.F. Cegavske, and L. Rozboril. A versatile carrier for simply constructed wire knives and microsyringes. Physiol Behav 26: 1115-1119, 1981.

Silverman, A. J., D.L. Hoffman, and E.A. Zimmerman. The descending afferent connections of the paraventricular nucleus of the hypothalamus (PVN). Brain Res Bull 6: 47-61, 1981.

Steger, R.W., K. Matt, and A. Bartke. Neuroendocrine regulation of seasonal reproductive activity in the male golden hamster. Neurosci Biobehav Rev 9: 191-201, 1985.

Stephan F.K., K.J. Berkley, and R.L. Moss. Efferent connections of the rat suprachiasmatic nucleus. Neuroscience 6: 2625-2641, 1981.

Stephan, F.K. and A.A. Nunez. Elimination of circadian rhythms in drinking, activity, sleep, and brain temperature by isolation of the suprachiasmatic nuclei. Behav Biol 20: 1-18, 1977.

Stephan, F.K. and I. Zucker. Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hyothalamic lesions. Proc Natl Acad Sci USA 69: 1583-1586, 1972.

Stetson, M.H., M. Watson-Whitmyre, and K.S. Matt. Termination of photorefractoriness in golden hamsters-photoperiodic requirements. J Exptl Zool 202: 81-88, 1977.

Swanson, L.W. and H.G.J.M. Kuypers. A direct projection from the ventromedial nucleus and retrochiasmatic area to the medulla and spinal cord of the rat. Neurosci Lett 17: 307-312, 1980a.

Swanson, L.W. and H.G.J.M. Kuypers. The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and the organization of projections to the pituitary, dorsal vagal complex and spinal cord as demonstrated by retrograde fluorescence double labelling methods. J Comp Neurol 194: 555-570, 1980b.

Tamarkin, L., J.S. Hutchison, and B.D. Goldman. Regulation of serum gonadotropins by photoperiod and testicular hormone in the Syrian hamster. Endocrinology 99:1528-1533, 1976.

Tulp, O.L., R. Frink, and E. Danforth. Effects of cafeteria feeding on brown and white adipose tissue cellularity, thermogenesis, and body composition in rats. J Nutr 112: 2250-2260, 1982.

Turek, F.W. and G.B. Ellis. Steroid-dependent and steroid-independent aspects of the photoperiodic control of seasonal reproductive cycles in male hamsters. In: Biological Clocks in Seasonal Reproductive Cycles. edited by B.K. Follett and D.E Follet. Bristol, England: John Wright and Sons, 1981, pp. 251-260.

Vriend, J. and R.J. Reiter. Free thyroxiod index in normal, melatonin treated and blind hamsters. Hormone Metabol Res 9: 231-234., 1977.

Vriend, J., R.J. Reiter and G.R. Anderson. Effects of the pineal and melatonin on thyroid activity of male golden hamsters. Gen Comp Endocr 38: 189-195., 1979.

Vriend, J., B.A Richardson, M.K. Vaughn, L.Y. Johnson and R.J. Reiter. Effects melatonin on thyroid physiology of female hamsters. Neuroendocrinology 35: 79-85., 1982.

Wade, G.N. Obesity without overeating in golden hamsters. Physiol Behav 29: 701-707, 1982.

Wade, G.N. Dietary obesity in golden hamsters: reversibility and effects of sex and photoperiod. Physiol Behav 30: 131-137, 1983.

Weiss, G.F., and S.F. Leibowitz. Efferent projections from the paraventricular nucleus mediating α_2-noradrenergic feeding. Brain Res 347: 225-238, 1985.

Willis, G.L. and G.C. Smith. Short-term effects of intrahypothalamic colchicine. Brain Res 276: 119-125, 1983.

Youngstrom, T.G. and A.A. Nunez. Anatomical features of the hypothalamic circuits that mediate responses to photoperiod on the golden hamster. Soc Neurosci Abstr 12: 212, 1986.

Zhang, T.-X. and J. Ciriello. Kainic acid lesions of paraventricular nucleus neurons reverse the elevated arterial pressure after aortic baroreceptor denervation in the rat. Brain Res 358: 334-338, 1985.