THE EFFECT OF OPTIC ENUCLEATION ON THE ESTABLISHMENT AND MAINTENANCE OF THE CIRCADIAN RHYTHM IN NON-STRESS PITUITARY-ADRENAL FUNCTION IN THE FEMALE RAT

by

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INTRODUCTION

The role of the pituitary gland in maintaining the structure and secretory function of the adrenal cortex is well established. That these functions are mediated by adrenocorticotropic hormone (ACTH) has likewise been established. Regulation of ACTH release appears to be in large part under hypothalamic control, mediated by corticotropin releasing factor (CRF). CRF is presumed to be released at the median eminence into the hypothalamo-hypophyseal portal system by neurons located in the medial basal hypothalamus (MBH). This part of the brain includes the arcuate and ventromedial nuclei, and the medial part of the anterior hypothalamus. The investigations which played key roles in discovering these relationships are thoroughly reviewed by Yates and Maran (103).

The influence of factors in the external and internal environment upon the pituitary-adrenal system has been an active field of research for the last 30 years and much information has accumulated regarding its regulation and function. Much of this information is fragmented, however, and the conceptual approach of Liddle et al. (58) is useful in organizing what is known regarding the control of ACTH secretion and the effects of factors in the external and internal environments. This approach recognizes three features of the pituitary-adrenal system. First, in the absence of noxious or stressful stimuli, the behavior of this system is characterized by fluctuating levels of ACTH and plasma corticosteroids. These fluctuations are rhythmic with an approximate 24 hour period. For convenience, pituitary-adrenal function in the absence of overtly applied stressful stimuli will be referred to as non-stress activity. Second, the system is capable of responding markedly and rapidly to a variety of noxious or stressful stimuli. Third, the system is capable of responding

to its own end product, glucocorticoids, by way of a negative feedback loop.

The rhythmicity of environmental lighting has long been recognized to influence the rhythmic non-stress activity of the pituitary-adrenal system, as peak values of adrenocortical secretions or degradation products were found to correspond with light-dark transition in nocturnal animals and dark-light transition in diurnal animals (76, 15). The rhythm in this system was found to respond to shifts in lighting schedules or blinding (15). The effect of blinding on the non-stress rhythmic activity of the pituitary-adrenal system has for years been clouded by conflicting reports. The confusion has been, in part, due to inadequate techniques of assessing the non-stress activity in individual animals. The purpose of the studies reported in this thesis was to assess by means of serial sampling procedures the effects of blinding on the non-stress activity of the pituitary-adrenal system. Because previously reported studies suggested that the effects of blinding differ with the age at which blinding is performed, these studies included animals blinded at several ages from birth through adulthood.

To furnish a background for these studies, a historical review will be presented. This review will include brief coverage of concepts related to the control of stress-induced ACTH release and of feedback regulation of the pituitary-adrenal system. Information which bears on the development and control of rhythmic non-stress activity will be emphasized.

Stress-induced Changes in Pituitary-Adrenal Function

Since the discovery by Selye (87) that various noxious stimuli

result in adrenal hypertrophy, stress has been recognized as an important factor in causing ACTH release. Early studies by Hume and Wittenstein (46) and DeGroot and Harris (20) suggested that the intact hypothalamus was essential for normal stress-induced ACTH secretion. Harris (40) reviewed early investigations which utilized hypothalamic lesions and stimulation and suggested that there are two modes by which stress causes ACTH secretion. The first involves neural pathways activating hypothalamic secretion of a humoral mediator which traverses the hypothalamo-hypophyseal portal system to cause release of ACTH from the adenohypophysis. The second mechanism involves stressors which pass through the systemic circulation to induce ACTH secretion by an action on the hypothalamus and/or pituitary. Time has furnished support for these early concepts.

Because of the lack of neural connections from the hypothalamus to adenohypophysis, demonstration of a humoral mediator was necessary in order to support the neurosecretory model proposed by Harris (40). This was accomplished by use of tissue culture techniques which demonstrated the presence of a humoral hypothalamic factor capable of inducing ACTH release (33, 85). Partially purified or crude hypothalamic extracts containing an ACTH-releasing substance have since been prepared (94). Although not yet fully isolated or characterized chemically, this factor has been intensively studied and is termed CRF. Yates and Maran (103) have reviewed work in this field.

The neural substrate involved in the release of CRF has been the subject of many lesion studies (see reviews by Ganong (28); Yates and Maran (103)). McCann (59) demonstrated that large median eminence lesions abolished adrenal response to surgical stress in the rat. Because the median eminence is known to be the site of the primary capillary plexus

of the hypothalamo-hypophyseal portal system, this finding was taken to indicate that ACTH was released from the adenohypophysis in response to CRF released from neuronal terminals located within the median eminence. Lesion (9) and electrical stimulation (89) studies have shown that widespread areas of hypothalamus are involved in stress-induced CRF release. These areas include the ventral hypothalamus and preoptic anterior hypothalamic area (POAHA). Most CRF-producing neurons with terminals which end in the median eminence are believed to reside in the MBH (34, 80).

Stressors have been found to induce ACTH secretion by each of the modes suggested by Harris (40). The ACTH response to some stresses (sound, cold, and leg break) requires intact neural connections to the MBH. Other stressors (ether, immobilization, tourniquet, and subcutaneous formalin) do not require intact neural connections to the MBH, thereby suggesting stimulation of CRF-containing neurons or the pituitary directly by humorally-transmitted factors.

Isolation and ablation techniques have been utilized to delineate the neural substrate necessary to maintain the stress response to each of these stressors. Thus, Halász et al. (36) were the first to demonstrate that complete surgical isolation of the MBH is compatible with retention of the pituitary-adrenal response to ether stress. This investigation also demonstrated the capacity of the isolated MBH to maintain basal ACTH secretion. These findings led this group to conclude that there are two levels of control of ACTH secretion. One level is located within the MBH and is able to maintain basal ACTH secretion and responses to some stressors. The second level consists of extrahypothalamic structures which project to and influence the MBH and CRF responses to other stressors. It has since been demonstrated that rats with chronic

surgical isolation of the MBH respond to ether, and immobilization stresses by Feldman (25) and Palka et al. (74).

This same experimental preparation will not respond to stress requiring neural input such as sound, vibration or leg break (26, 43). The results of studies utilizing partial forebrain removal to isolate the MBH-pituitary unit are consistent with those obtained after chronic isolation of the MBH. ACTH responses to ether or immobilization stresses are abolished after chronic ablation of the MBH, thus supporting the assumption that CRF-producing neurons are necessary for these responses (24). More recently, it has been demonstrated that median eminence-pituitary islands (MEPI) or basal hypothalamic-pituitary islands (BHPI) also respond acutely to immobilization stress (81). The length of the period during which the acute MEPI preparation can respond to immobilization has not been determined. It may be that the median eminence contains CRF stores capable of release upon immobilization stress, or it may be that the median eminence maintains a vascular supply to the pituitary by which CRF of extraforebrain origin can gain access to the pituitary. As evidenced in preparations subjected to complete forebrain removal, including the median eminence, the pituitary appears capable of responding to some stimuli such as SC formalin (90) and a combination of ether-laparotomy stress (102) with an increase in ACTH secretion. Thus, it now appears that ACTH secretion may be increased by stressful stimuli via several mechanisms. One mechanism includes activation of CRF-containing neurons in the MBH by way of neural pathways. Another probably entails stimulation of these CRF-containing neurons directly by factors which are probably blood borne. In addition, it appears that some stimuli, perhaps blood borne, bypass the MBH and effect ACTH release by acting on the

median eminence or directly on the adenohypophysis. The mechanisms responsible for triggering pituitary-adrenal responses to stress in the absence of neural connections to the MBH are unknown.

Negative Feedback in Pituitary-Adrenal Function

It has long been acknowledged that secretion of ACTH is influenced by a negative feedback loop mediated by circulating glucocorticoids. The role played by this negative feedback in regulation of ACTH release under physiologic conditions is as yet uncertain and the anatomic sites at which feedback operates are likewise ill-defined (50, 103). The currently-accepted model for feedback regulation of stress-induced pituitary-adrenal function was formulated by Dallman and Yates (17) and includes rate- and level-sensitive components. The rate-sensitive component is believed to respond rapidly to increasing levels of corticoids. The level-sensitive component is apparently activated after an approximate 2-hour delay or "silent" period by steroid levels which have exceeded an as yet undetermined threshold for an as yet undetermined period of time. Jones et al. (48, 49) have presented evidence supporting the concept of rate- and level-sensitive elements in this feedback loop. The rhythmic non-stress secretion of ACTH appears more sensitive to negative feedback than does stress-induced ACTH release. Physiologic levels of circulating corticosterone suppress non-stress pituitary-adrenal function (107, 108) while leaving the response to several stressful stimuli apparently intact.

The site(s) at which corticoids regulate ACTH secretion is unclear, but the pituitary is presently felt to be a primary site of action (50, 103). However, there is some evidence that feedback effects are in part

mediated through the central nervous system. For example, some reports describe corticoid-induced alteration of CRF in blood (10) or the hypothalamus (14, 95). It has also been reported that systemic administration of cortisol to monkeys was more effective in inhibiting ACTH release produced by stimulation of the amygdala than that caused by stimulation of the hypothalamus, suggesting that a feedback-sensitive point is interposed between the amygdala and hypothalamus (60). It has been found that hippocampectomy prevents dexamethasone suppression of supination or injection stress in the PM but not AM (96), suggesting that the hippocampus is in some manner involved in feedback regulation of ACTH release. Allen and Allen (3) have reported that lesions in the amygdala or in the direct amygdalo-hypothalamic pathway block the hypersecretion of ACTH that follows adrenalectomy, suggesting again a role for this structure in feedback regulation. On the other hand, it has been found that non-stress pituitary-adrenal function can be suppressed by dexamethasone in rats with chronically-isolated MBH (74), or in rats with pituitary islands 24-h after complete forebrain removal (22). These reports imply that this dexamethasone suppression was exerted directly on the pituitary.

Non-Stress Pituitary-Adrenal Function

Early studies performed in humans not overtly stressed revealed fluctuations in the metabolized products of adrenocortical secretions and in plasma levels of those secretions (76, 7, 67). These fluctuations were discovered to be rhythmic and to have a period of approximately 24-h. Peak levels were found to occur in the early morning in close association with the sleep-wake transition and just prior to the assumption of daily

motor activity. Because of the approximately 24-h period, this rhythm is termed circadian. Evidence for such fluctuations in adrenal cortical function has since been described in all species studied including mice (12, 38), rats (32, 16), rabbits (82), cats (55), dogs (37), sheep (61), monkeys (62), and pigeons (8).

Most previous studies of the circadian rhythm in pituitary-adrenal function in experimental animals utilized separate groups of animals, with each group being sampled at one point during 24-h light-dark cycles. This approach yields rhythmic, smoothly rising and falling plasma corticosteroid levels. However, studies in the human based on the collection of serial blood samples every 20 to 30 minutes have yielded more detailed information (42, 57). Such serial sampling revealed relatively synchronous peaks of plasma levels of ACTH and corticosteroid which occur episodically throughout the day, with the majority of these peaks occurring in the period preceding awakening. Because the plasma half-life of corticosteroids is such that not all are removed from the blood at the time of day when there is a decreased interval between secretory peaks, corticosteroids in the plasma accumulate during the period which precedes awakening and results in the peak plasma levels seen with less frequent sampling.

Considerable effort has been expended to determine the mechanisms underlying the daily rhythmicity in circulating corticosteroid levels. Perkoff et al. (75) determined that the rate of removal of hydrocortisone from human plasma was constant throughout the day, therefore making alteration in metabolism an unlikely cause for diurnal fluctuations of circulating levels of corticosteroids. Because of the correlation between rhythmic changes in circulating ACTH and blood levels of gluco-

corticoids in humans (21, 5) and rats (64, 65, 79, 18), it is generally considered that adrenal cortical rhythmicity stems from fluctuations in ACTH secretion. Although current concepts hold that rhythmic secretion of ACTH is responsible for the circadian rhythm in adrenal cortical function, there is some evidence suggesting that fluctuations in adrenal sensitivity to ACTH play a role in this periodicity. Ungar et al. (93) found in studying mouse adrenals in vitro that there were diurnal fluctuations in adrenal sensitivity to ACTH. Nugent et al. (72) failed to find such variations in the human. More recently, two reports offer evidence from in vivo experiments performed in rats that adrenal sensitivity to ACTH may play a role in this rhythmicity. Thus, Meier (66) found that hypophysectomized rats implanted with pellets of ACTH and thyroxine demonstrated higher plasma levels of corticosterone in the afternoon than in the morning, and that reversal of the lighting regimen produced an apparent reversal of this pattern. On the basis of these data, Meier concluded that the circadian rhythm in adrenal cortical function is not dependent upon rhythmic ACTH secretion. Dallman et al. (18) reported that prepubertal rats treated with dexamethasome show greater response to ACTH administered in the afternoon than in the evening and postulated that such changes in sensitivity contribute significantly to rhythmic changes in adrenal cortical secretion. Confirmation of these experiments may necessitate revision of current views regarding the mechanisms responsible for adrenocortical rhythm. The rhythmic secretion of ACTH appears to be independent of feedback effects of glucocorticoids since circadian rhythmicity of ACTH is seen in Addisonian humans (31, 6) and adrenalectomized rats (13).

Several lines of evidence implicate the brain in the control of rhythmic pituitary-adrenal function. This rhythm appears to be absent in comatose humans (75) and also appears disrupted in patients with pathology located in limbic, hypothalamic and midbrain structures (51). Subsequent studies in laboratory animals are consistent in indicating that the brain is responsible for the circadian rhythm in pituitary-adrenal function. Thus, the eyes and central neural connections were implicated when it was found that the rhythm could be phase shifted by changing the phase of rhythmic environmental lighting in mice (39) and rats (32) with intact visual systems but not in blind rats (16).

Experimentally-induced brain lesions have been consistent in indicating neural control of rhythmic ACTH secretion and useful for locating those regions and structures concerned with this control. Slusher (88) was the first to show that anterior hypothalamic lesions were capable of disrupting AM-PM differences in plasma corticosterone levels in the rat. Because these lesions did not alter stress responses, she suggested that anatomically-separate loci are responsible for neural control of stress-induced as opposed to rhythmic, non-stress pituitary-adrenal function. The next important contribution was the development of technique for surgically isolating the MBH (35) from adjacent brain. Complete isolation of the MBH (36) disrupted the AM-PM differences in steroid levels without abolishing responses to ether stress. It was further noted that anterior deafferentation of the MBH produced similar disruption while superior, lateral, or posterior deafferentation did not, suggesting that anterior neural connections to the MBH are essential for the maintenance of the circadian rhythm in non-stress pituitary-adrenal function. These findings precipitated a number of investigations dealing

with those areas of the brain with anterior connections to the MBH. Moberg et al. (68) reported that transection of the fornix in rats abolished diurnal variations in plasma corticosterone. However, Wilson and Critchlow (97) were unable to confirm, using serial blood samples, such a role for the hippocampo-fornix system; hippocampectomy or fornix transection were compatible with normal rhythmicity in pituitary adrenal function. It also appears that the septum is not the source of the anterior connections to MBH which are essential for this rhythm; ablation of the septum did not disrupt this rhythm as assessed by serial blood samples (98). The medial forebrain bundle also projects to the MBH, and it has been reported that transection of this pathway abolishes pituitary-adrenal rhythmicity (69).

The latter study assessed the effects of transection by killing groups of animals at 3-hour intervals. As discussed below, it is not possible without using serial blood sampling to determine whether a lesion causes free-running rhythms or loss of rhythmicity per se. Serial sampling has confirmed the importance of intact neural connections to the MBH. Serial sampling has demonstrated that isolation of the MBH abolishes the rhythm in individual rats but that they continue to demonstrate steroid peaks of normal amplitude which are asynchronous and lacking a 24-h cycle (100).

The lesion studies of Moore and Eichler (70) are of particular interest with regard to the control of rhythmic pituitary-adrenal function. They reported disruption of diurnal fluctuations of adrenal corticosterone concentrations in rats with large lesions involving the suprachiasmatic nuclei. This finding is of special interest because the retinohypothalamic tract extends from the ganglion cells of the retina to the suprachiasmatic

nucleus (71) and therefore provides an anatomical pathway by which light or lack of light might exert its influence on the pituitary-adrenal rhythm. Because Moore and Eichler (70) collected blood from separate groups of animals at 4 time points on one day, it is impossible to determine whether the suprachiasmatic lesions in fact abolished the rhythm or caused free-running by interrupting the neural connections which mediate the synchronizing influence of environmental lighting. Stephan and Zucker (91) demonstrated that suprachiasmatic lesions abolish circadian rhythms in drinking behavior and activity. These effects of lesions involving the suprachiasmatic nuclei have led to the concept that this part of the brain includes an endogenous "biological clock" driving these rhythms and that its activity is synchronized by light via projections from the retina. The recent findings of Wilson and Critchlow (100) and Gibbs (29) are consistent with this concept in that they suggest that rats blinded as adults have pituitary-adrenal rhythms which are free-running. Such preparations apparently have an intact clock mechanism, perhaps in the suprachiasmatic region, which cannot be synchronized by environmental light in the absence of connections from the retina. Perkoff et al. (75) and Aschoff and Wever (4) demonstrated that humans develop free-running circadian rhythms in plasma glucocorticoid concentrations and activity levels when deprived of rhythmic environmental lighting.

The relationship between the rhythm in pituitary-adrenal function and drinking, feeding and activity patterns has received considerable study in experimental animals and humans. Free-running adrenal and activity rhythms were found by Gibbs (29) to parallel each other in the rat. Other studies by Halberg (39), Orth and Island, and Aschoff and

Wever (4) in humans have indicated that in the presence of intact visual connections and normal light-dark cycles that light is dominant over activity as a synchronizer of corticosteroid rhythmicity. However, Johnson and Levine (47) and Krieger (53) have demonstrated in rats that restricting periods of food and water intake can serve to synchronize and to shift the plasma corticosterone rhythm out of phase with the lighting schedule. These findings indicate that under some conditions, drinking and feeding behavior are dominant over light as a synchronizer of the pituitary-adrenal rhythm.

Another major line of evidence implicating the brain in the control of pituitary-adrenal rhythmicity has been the study of hypothalamic content of CRF. Hypothalamic levels of CRF demonstrate diurnal variations (19, 44) and these variations parallel those in plasma ACTH and adrenal steroid concentrations. Because these fluctuations in CRF content persist in adrenalectomized and hypophysectomized rats (92), it appears that this rhythmicity is intrinsic to the CNS and not secondary to hormonal feedback. The age of appearance of plasma corticosteroid rhythmicity also correlates with the appearance of diurnal changes in hypothalamic CRF levels (45).

Consistent with the proposed neural origin of rhythmic pituitary-adrenal function, pharmacologic manipulation of several presumptive neurotransmitters is reported to influence this rhythm. Thus, alteration of CNS acetylcholine and serotonin levels can suppress plasma corticosteroid rhythmicity in the cat (55, 56) and rat (84).

The above review focused on information which points to CNS control of the circadian rhythm in pituitary-adrenal function. That which has been learned about the specific role of light and the visual system in

the control of this rhythm will be examined below in greater detail.

Most investigations concerned with the role of light have dealt with the effects of surgical removal of the eyes, exposure to constant light or dark, or manipulation of the phase of rhythmic lighting on pituitary-adrenal rhythmicity. Several investigators, Fiske and Leeman (27), Cheifetz et al. (13), Scheving and Pauly (86), and Krieger (52), studied the effects of constant light or constant dark and all reported that such conditions resulted in loss of pituitary-adrenal rhythmicity. Krieger (52) found that animals raised from birth under constant light or dark did not demonstrate rhythmicity. However, such animals demonstrated rhythmicity subsequently after exposure to light-dark cycles. Similarly, animals which had been placed in constant light or dark conditions at later ages resumed rhythmic function when exposed to conditions of rhythmic lighting. The significance of these findings is uncertain as none of the above mentioned investigations sampled a given animal repeatedly. As will be discussed below, data obtained in this manner are not adequate for determining whether constant light or dark abolishes this neuroendocrine rhythm.

For the most part, plasma corticosterone levels have been used as the endpoint for studying rhythmic pituitary-adrenal function in the rat. Two basic approaches have been used to collect blood for assay. The first, used in most experiments, utilizes serially independent sampling. This approach involves collecting blood from individual animals only once during the 24-h light-dark cycle and comparing the steroid levels in groups of such animals sampled at different times. The second approach utilizes serially dependent sampling, that is repeated sampling of individual animals at regular intervals. Serially

independent sampling generally offers less likelihood of stress-induced glucocorticoid secretion affecting subsequent samples, and it provides a reliable approach for obtaining adequate amounts of plasma for assay. However, in the presence of disrupted rhythmicity in individual rats of a group, serially independent technique is unable to differentiate between loss of fluctuations, loss of rhythmicity in persisting fluctuations or loss of synchronization of rhythms. An obvious limitation of the serially dependent sampling procedure is the need to limit blood loss by restricting the total number and frequency of sampling points. The frequency of sampling must be adequate for assessing 24-h rhythmicity but must not lead to feedback inhibition due to steroid surges associated with the stress of sampling. Within the constraints imposed by blood loss, it is also necessary to sample over a time span adequate to permit recognition of 24-h periodicity. Ideally, the method should permit sampling through several cycles in order to demonstrate rhythmicity.

As noted above, most studies have used serially independent sampling to study the effects of blinding on rhythmic pituitary-adrenal function. Despite the limitations of this approach, some useful information has been obtained. Critchlow et al. (16) found that whereas intact rats demonstrate an appropriate phase shift in the 24-h pattern of plasma corticosterone levels in response to a shift in the light-dark schedule, blind rats fail to show such a shift. Therefore, it appears that the eyes and central neural connections of the retinae are essential for the synchronization of this endocrine rhythm by environmental light. Because of the sampling procedure, however, it could not be determined whether blinding led to arhythmicity or to free-running

rhythms in individual rats. As a result, the important question as to whether the eyes are essential for rhythmicity per se was not resolved with this experiment. Others used the same sampling procedure, i.e., collecting blood from different groups of rats at different times during the 24 hour light-dark cycle, and observed apparent phase shifts in the pattern of corticosterone levels in blind rats in the absence of changes in the lighting regimen (83, 41, 70, 78). The presence of such a shift suggests that structures other than the eyes produce the rhythm and that another periodic cue from the environment is capable of entraining this rhythm in the absence of the eyes. Krieger's results, however, are discrepant with the above findings (52). She was the first to systematically study the effects of age of blinding on the establishment and maintenance of pituitary-adrenal rhythmicity, and she found no evidence of periodicity or phase-shift in rats blinded at 1, 14, or 30 days of age and studied at 80 days of age in an experiment which used serially independent sampling.

Because she recognized the inability of serially independent sampling to differentiate loss of fluctuations, loss of rhythmicity in persisting fluctuations or loss of synchronization within a group of individuals possessing rhythmicity, Krieger attempted in the same study to validate these findings utilizing a serially dependent method. In so doing she contributed an important approach for studying pituitary-adrenal rhythmicity in the rat . She described a method for rapidly obtaining blood samples from a tail vein in unanesthesized rats which permits sampling at 4-h intervals for 48-h. When applied to rats enucleated at 1 day of age and studied at 80 days of age, Krieger (52) found no evidence for rhythmic non-stress function. Fluctuations in circulating

corticosterone levels were observed, and these were of normal amplitude. However, the patterns were arhythmic and unsynchronized. Krieger concluded that the eyes and central visual connections are essential for the development of this circadian rhythm. The only other recent study of the effects of blinding rats at 1 day of age was performed by Ramaley (78). She used serial sampling, but at intervals of at least 4 days, and reported a 180° phase shift in the corticosterone rhythm when the animals were studied at 45 days of age. If such a phase shift were also observed using serial sampling at more frequent intervals, it would imply that rhythmicity was present in the blind rats and that the synchronization or production of this rhythmicity was mediated by an environmental cue other than light. These findings in conjunction with those of Krieger suggest that the circadian rhythm in pituitary-adrenal function appears transiently in rats blinded at 1 day of age, that it is entrained by periodic stimuli other than light and that it is lost by 80 days of age. If confirmed, such results would suggest that the visual system is not essential during development for the establishment of a rhythm but only for its maintenance.

Subsequently, Wilson and Critchlow (100) adopted the serially dependent sampling procedure to characterize the effects of complete isolation of the MBH on pituitary-adrenal rhythmicity in adult rats and found that this surgical procedure abolishes the corticosterone rhythm in individual rats. This finding implied that neural projections to the MBH are essential for rhythmicity and not just for synchronization. The arhythmic, asynchronous steroid patterns observed following MBH isolation were similar to those described by Krieger in rats blinded at 1 day of age. This similarity raised the possibility that the effects

of MBH isolation are due to interruption of the retino-hypothalamic pathways which project to the neural structures responsible for rhythmicity. This possibility was tested (100) and it was found that rats blinded as adults did not demonstrate loss of rhythmicity, as was the case with Krieger's neonatally-blinded rats or rats subjected to MBH isolation. Rather, the blinded rats showed evidence of free-running rhythms in plasma corticosterone levels. This result is consistent with the recent findings of Gibbs (29) who used the close association between corticosterone and activity rhythms to study the effects of blinding in adult rats. He concluded that both rhythms are free-running following removal of the eyes.

Because blinding at 1 day of age reportedly precludes the development of the circadian pituitary-adrenal rhythm and free-running results when rats are blinded as adults, it appears that there is a critical stage of maturation, sometime between day 1 and adulthood, when the eyes are essential for the functional development of the neural mechanism responsible for rhythmicity. Once established, this mechanism apparently ceases to be dependent on visual input for rhythmicity per se and uses this input only for synchronization with environmental lighting. An intriguing anatomical correlate of this important maturational event was furnished when Campbell and Ramaley (11) reported that retinal projections to the suprachiasmatic nuclei are established at approximately 17-18 days of age, coincident with the appearance of pituitary-adrenal rhythmicity (77). As indicated above, lesions placed in the suprachiasmatic nuclei reportedly disrupt this rhythm (70) as well as rhythmic activity and drinking behaviors (91). These several findings form the basis for the current view that the suprachiasmatic

nuclei are importantly involved in the "biological clock" mechanism responsible for the generation of these physiological rhythms. It is therefore possible that blinding at 1 day of age abolishes the pituitary-adrenal rhythm in adults because it precludes the development of retino-hypothalamic connections which are essential for the functional maturation of the clock mechanism. Since removal of the eyes from adults appears compatible with retention of rhythmicity, even though free-running, it is also possible that once "imprinted" via the visual system during the "critical period" of development, the clock mechanism is capable of generating rhythmicity in the absence of the eyes. In that it implies at least transient functioning of the clock mechanism, the periodicity observed by Ramaley (78) in 45 day old rats which were blinded at 1 day of age is somewhat discrepant with this model. However, as discussed above, Ramaley's finding needs to be confirmed with serial sampling at reasonably frequent intervals before it constitutes a major obstacle to the above view.

The overall aim of the experiments presented in this thesis was to investigate the effects of age of blinding on rhythmic pituitary-adrenal function and to test some of the postulates included in the model discussed above. The first experiment was designed to determine whether the rhythmicity, with or without the phase shift, observed by Ramaley in rats blinded at 1 day of age and studied at 45 days is reproducible with serial sampling. The experimental question in this case is whether the mechanism responsible for rhythmicity functions even transiently in rats deprived of their eyes from birth. The second experiment was designed to compare systematically and concurrently the acute and chronic effects of blinding performed at different stages of development. One

group was blinded at 1 day of age in an attempt to replicate the findings of Krieger (52) and to verify that development of pituitary-adrenal rhythmicity is dependent upon connections and input from the visual system during some stage of postnatal maturation. Another group was blinded at 26 days of age to test the possibility that once established, the mechanism responsible for rhythmicity is refractory to the disruptive effects of blinding. This age represents a stage in development when the rhythm is reported to be newly established (2, 1, 77) and when retino-hypothalamic connections are newly formed (11). Finally, a group of rats was blinded at 60 days of age to study the effects of blinding young adults at a time when the rhythm and its sex-specific pattern is fully developed. It was expected that this group would confirm the previously cited findings of Wilson and Critchlow (100) that animals enucleated as adults retain rhythmic, albeit asynchronous, pituitary-adrenal function.

MATERIALS AND METHODS

A total of 56 Sprague-Dawley-derived female rats were obtained from Simonsen Laboratories (Gilroy, California). Of these animals, 22 were pregnant and delivered, as planned, seven days after arrival. Litters from these pregnant females provided all the animals blinded by optic enucleation at the age of 1 or 60 days and all the animals assigned to their respective intact and sham-operated control groups. The remaining 34 animals were weaned by Simonsen Laboratories at the age of 21 days and received in our laboratory at 22 days of age, four days prior to being blinded or placed in control groups at 26 days of age.

All of the animals shipped from Simonsen were raised from birth on a 12L-12D (L 0600-1800) lighting schedule with the light provided by overhead fluorescent fixtures. Once received, the animals were placed in an animal room having these lighting conditions. Temperature in the animal room was controlled at 26$\pm$ 2^{o}C. Purina chow and water were available at all times. Animals obtained as newborns from the timed pregnancies were raised in litters reduced to 8. Weaned at the age of 21 days, these animals provided all groups enucleated at 1 or 60 days of age and their controls. At 22 days of age, these animals were housed 6/cage. Those animals obtained from Simonsen at 22 days of age were likewise housed 6/cage. Equal numbers of optic enucleated, sham and intact controls were placed in each cage. After the age of 90 days all animals were housed 4/cage. Animals were assigned randomly to optic-enucleated, sham-operated or intact groups.

In view of the large number of rats involved in this experiment

and the need to limit the duration of sampling procedures, each of the experimental groups, i.e., rats blinded at either 1, 26, or 60 days of age and their respective controls, were divided into two cohorts. Although all animals in a given cohort were the same age, there was a 14-day difference in the age of the two cohorts. The two cohorts were housed in the same animal room, treated in the same manner, and subjected to the same environmental influences. A given cohort contained approximately half the animals enucleated at 1, 26, and 60 days of age and an equal number of sham-operated and intact controls for each age. Plasma corticosterone levels of each cohort were studied when it attained 12, 16, and 20 weeks of age. Data from the two cohorts were pooled only after statistical analyses indicated that they were homogenous. The first cohort included those animals in the first experiment which were used to study the effects of blinding at 1 day of age on pituitary-adrenal rhythmicity at 45 days of age.

Animals blinded at 1 day of age were anesthetized with cold (12 min at -15^{o} C.) prior to optic enucleation. A surgical microscope was used to visualize the eyes after incising the sealed orbital fissures. The extrinsic muscles and optic nerve were then transected and the globes removed. Sham-operation consisted of applying cold anesthesia and placing bilateral supraorbital incisions. Intact controls were not subjected to anesthesia or incisions. Animals optic enucleated at 26 or 60 days of age and their sham-operated controls were anesthetized with ether. After being anesthetized, the globes were removed under direct visualization following transection of the extrinsic muscles and optic nerves. Sham-operation for animals enucleated at 26 or 60 days of age consisted of ether anesthesia and bilateral supraorbital incisions. Intact controls were not

subjected to anesthesia or incisions.

The effect of optic enucleation on non-stress pituitary-adrenal function was studied by measuring plasma corticosterone levels in serially-obtained samples. The animals were placed in individual cages 3 days prior to the start of the sampling period. The animals were gentled by daily handling during these 3 days. Twenty-four hours prior to the start of sampling, a tail vein was longitudinally incised (2-3 mm) on each animal to be studied. The animals were provided adequate food and water for the duration of sampling, and the door to the animal room was locked 18-h before the sampling was started to minimize environmental disturbances. The room remained locked at all times between sampling sessions. Blood collection was performed in a laboratory immediately adjacent to the animal room where 0.5 ml of blood was obtained by gentle massage from the reopened tail vein incision. The rats were unanesthetized and lightly restrained during blood collection. All samples were obtained in EDTA-rinsed pipettes within 3-min of cage opening. Corticosterone levels in blood samples collected within 3-min of initiation of stress provide a valid estimate of non-stress pituitary-adrenal function (103). Blood samples were centrifuged and the plasma removed and frozen at -15^{o} C. until assayed. Krieger (52) demonstrated that blood samples can be obtained in this manner every 4-h over a 48-h period without obscuring the circadian rhythmicity of non-stress pituitary-adrenal function, and this finding was confirmed by Wilson and Critchlow (100).

The aim of the first experiment was to determine, using serial blood samples, whether rats blinded at 1 day of age demonstrate a circadian rhythm in pituitary-adrenal function at 45 days of age. To avoid

excessive blood loss in these young rats which weighed approximately 130g at time of sampling, blood samples were collected at 6-h intervals over a 24-h period. The second experiment was designed to compare, again using serial blood samples, the chronic effects of blinding at 1, 26, or 60 days of age on the pituitary-adrenal rhythm. In this case, blood samples were obtained at 4-h intervals for 44-h periods when the rats were 84, 112, and 142 days of age. To minimize sources of experimental error, this study was designed so that the several age groups were studied concurrently and sampled at identical ages. For the same reason and to minimize the effects of inter-assay variation, plasma corticosterone concentrations from all experimental groups were assayed together.

Plasma corticosterone concentrations were measured fluorometrically with the micro method of Glick et al. (30). Correction for residual fluorescence, approximately 6 ug/100 ml plasma, was not made. Sensitivity of the assay is such that 0.5 ng of corticosterone can be detected. The intra-assay coefficient of variation of a plasma pool with values in the normal working range of the assay is 9.2% while the inter-assay variation is 16.7%. 50 µl of plasma was used for each sample assayed.

To evaluate the homogeneity of the optic-enucleated rats in the second experiment and their similarity to sham-operated and intact controls, additional endpoints were studied. Autopsy was performed at approximately 40 weeks of age and adrenals, ovaries, uteri, and pituitaries were removed, cleaned and weighed. The skulls of animals blinded at one day of age were decalcified and sections taken through the orbit to ensure that optic enucleation had been achieved.

Statistical probabilities were determined by one- and two-way analyses of variance (ANOVA) for repeated measures (101). Where specified, correlation coefficients were calculated and used.

RESULTS

Fig. 1 shows the 24-h pattern in corticosterone levels in rats blinded at 1 day of age and sampled at 6-h intervals at 45 days of age. Included are the 24-h patterns of sham-operated and intact controls. These data represent the mean of individual values for each group at each time point and will subsequently be referred to as group data. Intact and sham-operated controls demonstrated significant fluctuations ($p < 0.05$) over the 24-h period,and peak concentrations of plasma corticosterone coincided with the time of light-dark transition. In contrast, the group data from enucleated rats failed to show significant fluctuation during the sampling period. Two-way analysis of variance (ANOVA) demonstrated no difference between the 24-h corticosterone patterns of intact and sham-operated controls but did demonstrate statistically significant differences between the 24-h pattern of blind rats and those of both control groups ($p < 0.05$). However, visual inspection of the data from individual enucleated rats suggested the presence of fluctuations consistent with a period of approximately 24-h. The 24-h corticosterone patterns of individual rats are presented in Fig. 3. To test the possibility that 24-h periodicity was retained in these blinded rats, the data from individual blinded rats were synchronized by aligning the highest steroid levels observed with the peaks that occurred in the control groups at 1800. The results of this synchronization are shown in Fig. 2. The group data after synchronization showed significant fluctuation with time ($p < 0.05$) and, furthermore, the pattern did not differ statistically from those of the intact and

sham-operated controls. These results suggest that the circadian rhythm in pituitary-adrenal function was present in these blinded rats but that the rhythms were free-running and not synchronized.

The effects of blinding at 1, 26 or 60 days of age on non-stress corticosterone levels at 84 days of age are shown in Figures 5, 6, and 7. As illustrated in the left panel of Fig. 5, the intact and sham-operated controls for the group blinded at 1 day of age demonstrated significant fluctuations ($p < 0.05$) in corticosterone levels during the 44-h sampling period. These fluctuations showed one peak on each of the 2 days of the experiment, and these peaks coincided with the time of the light-dark transition. No signigicant difference was observed between the patterns of these control groups. In contrast, ANOVA indicated that the group data from the rats blinded at 1 day of age did not demonstrate significant fluctuations in corticosterone concentrations during the 44-h period and that the steroid pattern of these blind rats was different from those of both groups of controls ($p < 0.05$). Thus, the group data from these blinded rats did not suggest persistence of rhythmicity in pituitary-adrenal function. However, inspection of the data from individual blinded rats (Fig. 4) suggested the presence of asynchronous 24-h rhythms. The possibility of persisting rhythms in the blinded rats was tested in two ways. The first method was similar to that used in the previous experiment and involved synchronizing the patterns from individual rats by aligning the highest steroid level observed on the first day of the experiment with the peak steroid levels found in the control groups at 1800. The results of this synchronization are shown in the right panel of Fig. 5. When this procedure

Figure 1. The effect of optic enucleation on non-stress plasma corticosterone in 45-day-old female rats which had been enucleated at 1 day of age. In this and the following figure on this page, lines connect group means of corticosterone levels at 6-h intervals over a 24-h period. Vertical lines indicate standard errors. The number of rats in each group is in parentheses. The solid bars represent dark periods.

Figure 2. The effect of synchronizing the individual non-stress plasma corticosterone patterns of the blind rats shown in Figure 1 so that the highest corticosterone level occurs at the same point in time as controls (0).

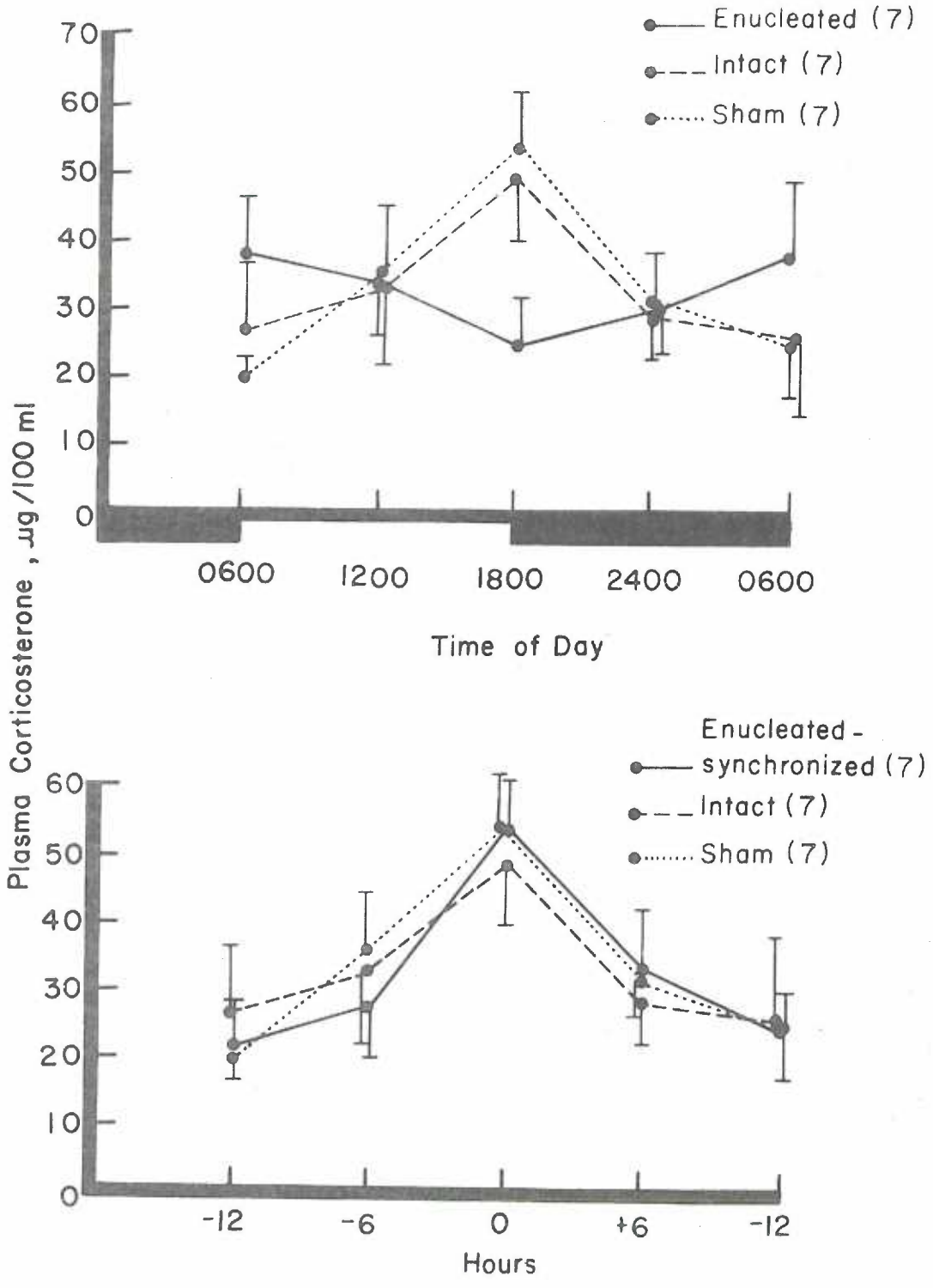
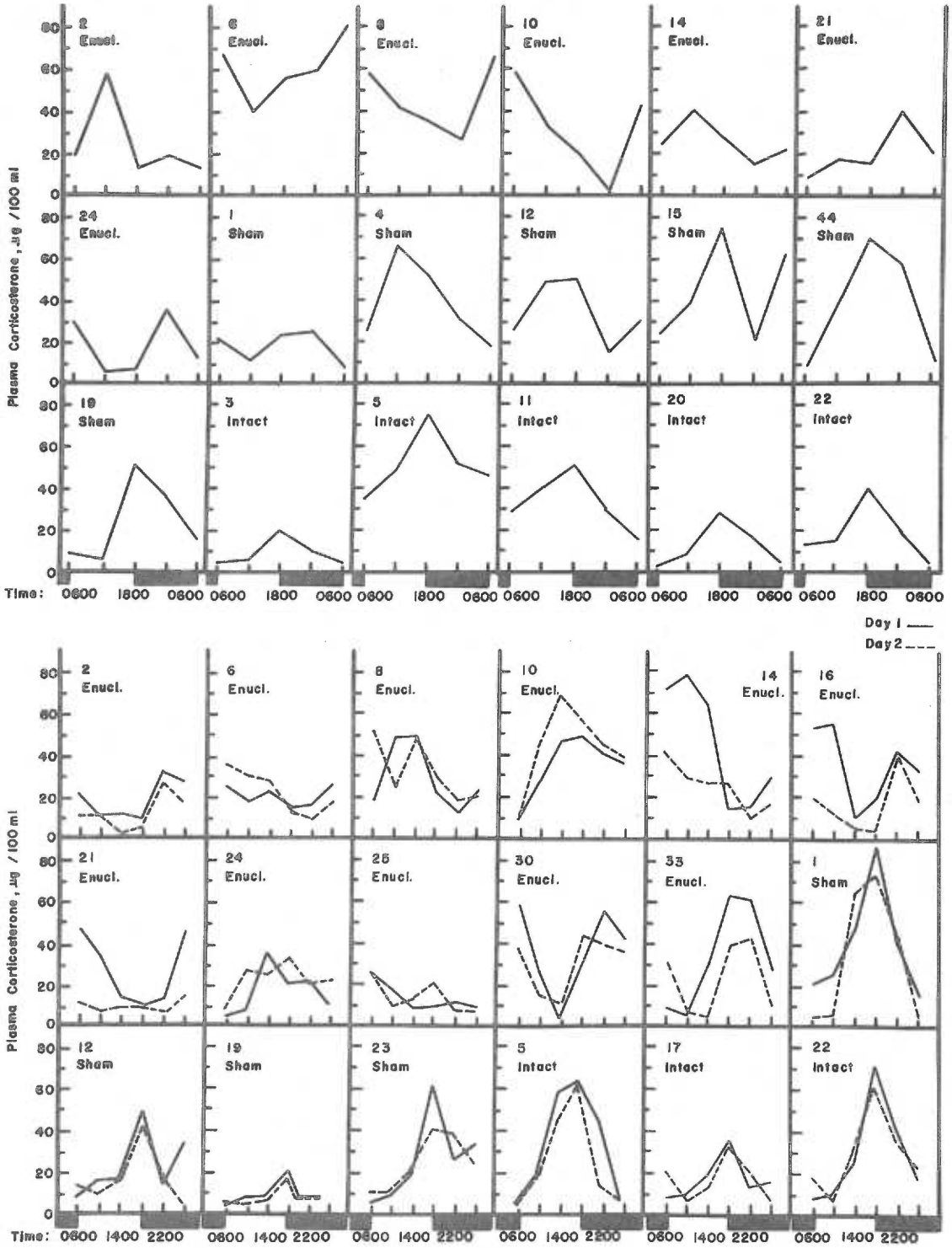

Enucleated (7)
Intact (7)
Sham (7)
70
60
50
40
30
20
10
0
0600
1200
1800
2400
0600
Time of Day
Plasma Corticosterone , µg/100 ml
Enucleated - synchronized (7)
Intact (7)
Sham (7)
60
50
40
30
20
10
0
-12
-6
0
+6
-12
Hours

Figure 3. Individual non-stress plasma corticosterone patterns of 45-day-old female rats which had been enucleated at 1 day of age. Representative sham-operated and intact control animals are included. The solid bars indicate dark periods.

Figure 4. Individual non-stress plasma corticosterone patterns of 84-day-old females enucleated at 1 day of age and representative sham-operated and intact control animals. The solid bars indicate dark periods.


Plasma Corticosterone, µg / 100 ml
2 Enucl.
6 Enucl.
8 Enucl.
10 Enucl.
14 Enucl.
21 Enucl.
24 Enucl.
1 Sham
4 Sham
12 Sham
15 Sham
44 Sham
19 Sham
3 Intact
5 Intact
11 Intact
20 Intact
22 Intact
Time: 0600 1800 0600
Day 1
Day 2
2 Enucl.
6 Enucl.
8 Enucl.
10 Enucl.
14 Enucl.
16 Enucl.
21 Enucl.
24 Enucl.
25 Enucl.
30 Enucl.
33 Enucl.
1 Sham
12 Sham
19 Sham
23 Sham
5 Intact
17 Intact
22 Intact
Time: 0600 1400 2200

was performed, the group pattern of rats enucleated at 1 day of age demonstrated significant fluctuation ($p < 0.05$) in corticosterone levels during the 44-h sampling period and there was no significant difference between the patterns of the blinded and control groups. As a second approach for assessing rhythmicity in these blinded rats, correlation coefficients were calculated between corticosterone levels on the first day and those at corresponding times on the second day for individual rats. The coefficients thus derived were averaged for each treatment group, and these were found to be not signigicant ($p > 0.1$) for all groups. Because of variability in steroid levels and an insufficient number of data points for each animal, this approach did not prove useful as an index of rhythmicity, even in intact and sham-operated controls. However, this method of analysis indicated that the relationships between steroid levels and time of day were comparable in the control and blinded groups; the results are therefore consistent with the interpretation that although they were unsynchronized, the steroid patterns in individual blind rats were similar to those in the control groups.

In this and most of the subsequent studies, the steroid peaks on the second day were somewhat lower than on the first day. The basis for the lower values on the second day of sampling are unknown, but similar differences appear in the data reported by Krieger (52).

Fig. 6 summarizes the effects of blinding at 26 days of age on non-stress pituitary-adrenal function at 84 days of age. As above, the intact and sham-operated controls showed significant fluctuations ($p < 0.05$) in steroid levels during the 44-h of sampling and the

Figure 5. The effects of optic enucleation on non-stress plasma corticosterone levels in 84-day-old female rats which had been enucleated at 1 day of age. In this and succeeding figures on this page, lines connect group means of corticosterone levels at 4-h intervals over a 44-h period. Vertical lines indicate standard errors. The number of rats in each group is in parentheses. The solid bars represent dark periods. The right panel demonstrates the effect of synchronizing the individual non-stress corticosterone patterns of the blind rats shown in the left panel so that the highest steroid level observed on the first day occurs at the same point in time (0) as controls.

Figure 6. The effect of optic enucleation on non-stress plasma corticosterone levels in 84-day-old female rats which had been enucleated at 26 days of age. The right panel demonstrates the effect of synchronization.

Figure 7. The effect of optic enucleation on non-stress plasma corticosterone levels in 84-day-old female rats which had been enucleated at 60 days of age. The right panel demonstrates the effect of synchronization.

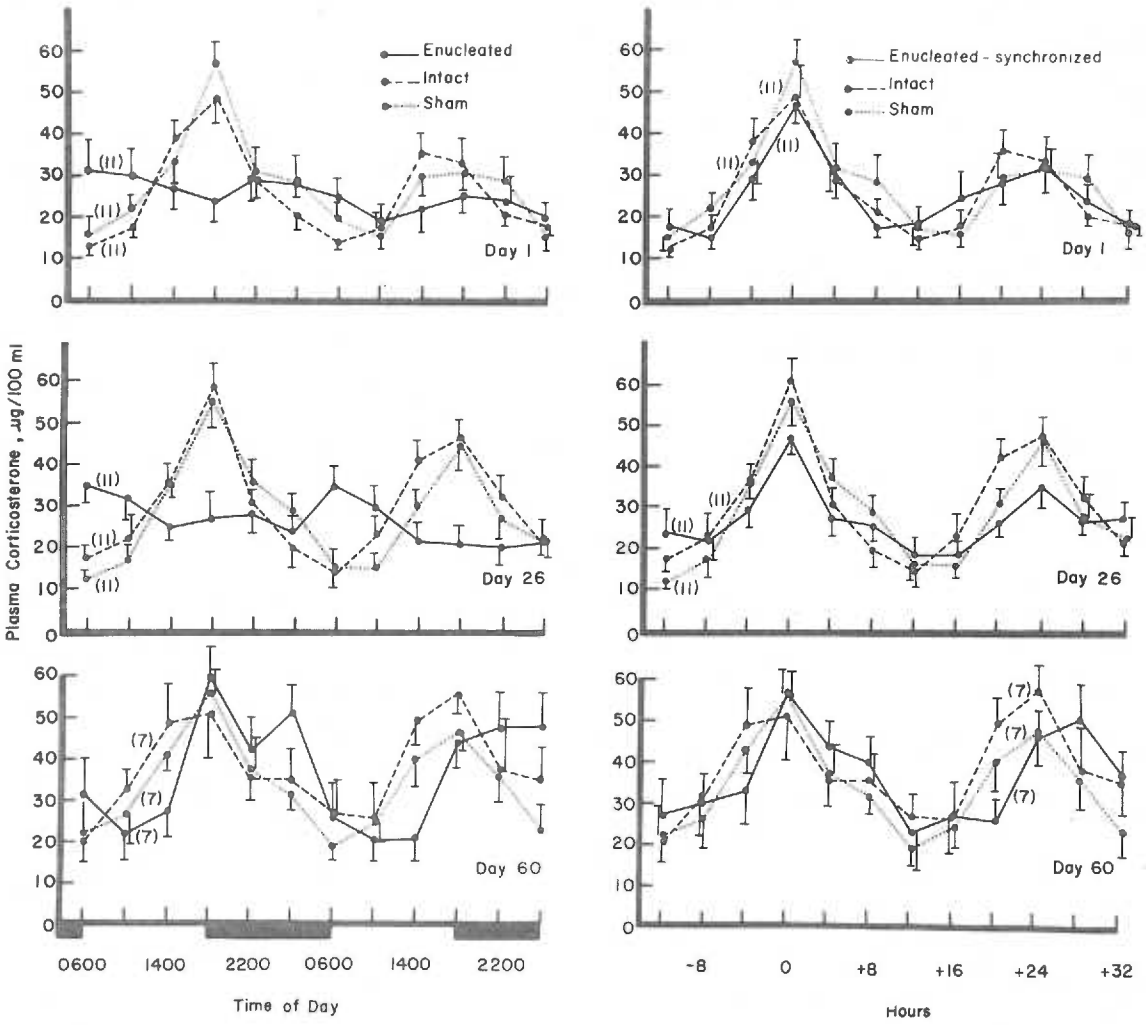
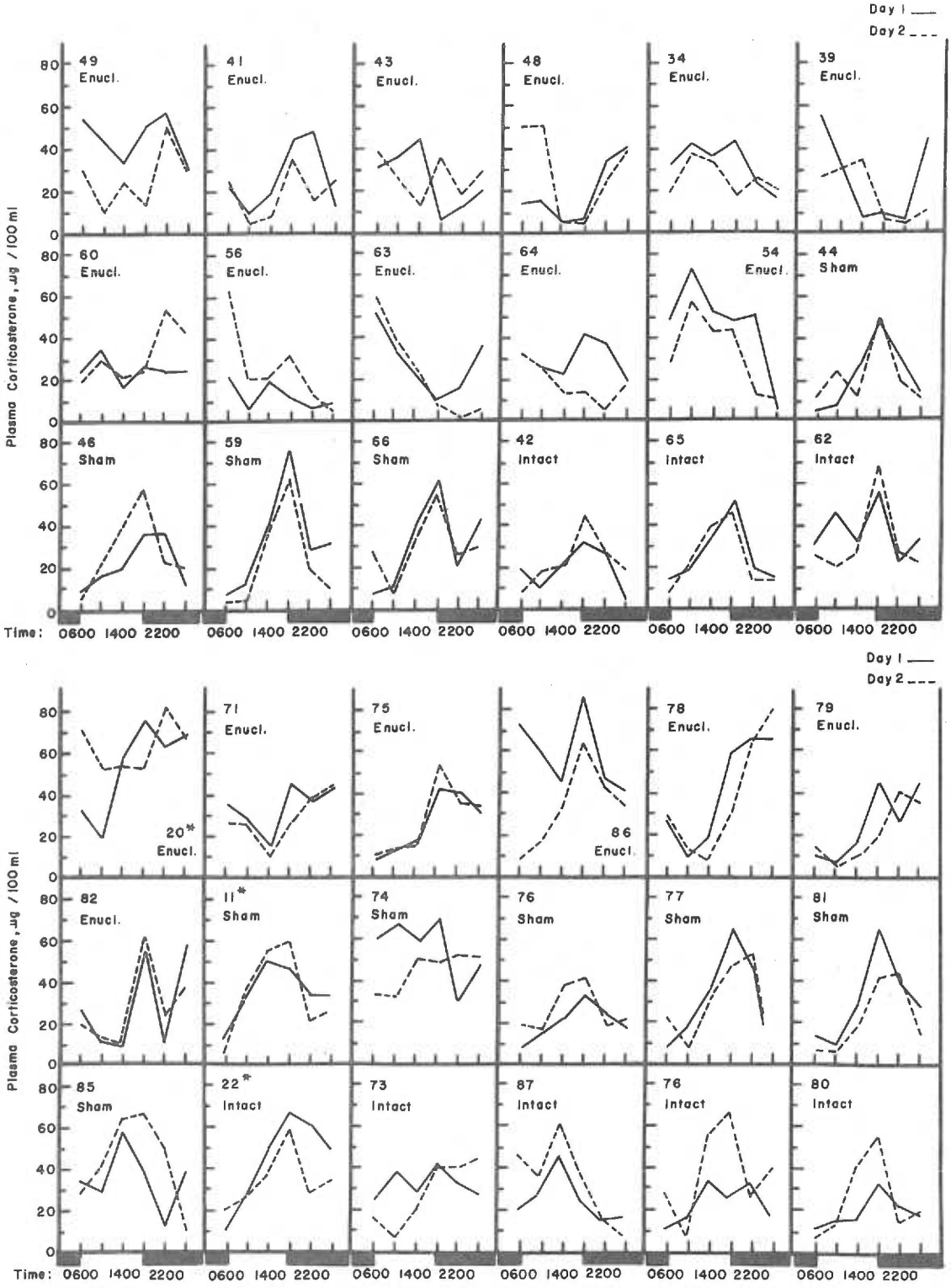

Enucleated
Intact
Sham
Enucleated - synchronized
Intact
Sham
Day 1
Day 1
Day 26
Day 26
Day 60
Day 60
Plasma Corticosterone, µg/100 ml
0600
1400
2200
0600
1400
2200
Time of Day
-8
0
+8
+16
+24
+32
Hours

Figure 8. Individual non-stress plasma corticosterone patterns of 84-day-old female rats enucleated at 26 days of age and representative sham-operated and intact control animals. The solid bars indicate dark periods.

Figure 9. Individual non-stress plasma corticosterone patterns of 84-day-old female rats enucleated at 60 days of age and representative sham-operated and intact control animals. The solid bars indicate dark periods.


Day 1
Day 2
Plasma Corticosterone, µg / 100 ml
0
20
40
60
80
49 Enucl.
41 Enucl.
43 Enucl.
48 Enucl.
34 Enucl.
39 Enucl.
60 Enucl.
56 Enucl.
63 Enucl.
64 Enucl.
54 Enucl.
44 Sham
46 Sham
59 Sham
66 Sham
42 Intact
65 Intact
62 Intact
Time: 0600 1400 2200
20* Enucl.
71 Enucl.
75 Enucl.
86 Enucl.
78 Enucl.
79 Enucl.
82 Enucl.
11* Sham
74 Sham
76 Sham
77 Sham
81 Sham
85 Sham
22* Intact
73 Intact
87 Intact
76 Intact
80 Intact

highest levels were observed in samples collected at 1800. Patterns in these two control groups did not differ statistically. Again, the group data for the blinded group suggested disruption of the rhythm in pituitary-adrenal function. One-way ANOVA did not demonstrate significant fluctuations in group data, and 2-way ANOVA demonstrated significant differences between the patterns of blind and control groups ($p < 0.05$). Visual inspection of the serially-dependent corticosterone patterns in enucleated rats (Fig. 8) revealed them to be similar to those of controls in that most had daily steroid peaks with a period of approximately 24-h between peaks. The peaks in enucleated animals were not, however, consistently found at 1800. Mean correlation coefficients did not vary significantly between blind and control groups. The results of synchronizing these group data are shown in the right panel of Fig. 6. Following this procedure, the pattern from the blind animals demonstrated fluctuations over time ($p < 0.05$) and it did not differ significantly from that of either control group. These data suggest the retention of unsynchronized rhythmicity.

The group data from animals enucleated at 60 days of age and studied at 84 days of age are presented in Fig. 7. In this case, the blinded as well as the control groups showed significant fluctuations ($p < 0.05$) with time, although the group patterns differed significantly ($p < 0.05$). The peak values in both groups of controls occurred at 1800 while those of enucleated animals appeared at 2200. These findings suggest a change in synchronization. The peaks in the patterns of individual rats (Fig. 9) did not appear to be as randomly distributed as in animals enucleated at 1 or 26 days. There was no difference between the correlation coefficients in the blinded and control groups. Synchronization produced a pattern

which showed significant fluctuations ($p < 0.05$) and which did not differ from those of controls (right panel, Fig. 7).

The effects of blinding at 1, 26, or 60 days of age on non-stress corticosterone levels at 112 days of age are shown in Figures 10, 11, and 12. As illustrated in the left panel of Fig. 10, the intact and sham-operated controls for the group enucleated at 1 day of age continued to demonstrate significant daily fluctuations ($p < 0.05$) in corticosterone levels. The peaks in steroid levels coincided with the light-dark transition and ANOVA indicated no significant difference between the patterns of these control groups. In contrast, the group data from the animals enucleated at 1 day of age did not demonstrate significant fluctuations during the 44-h sampling period, and the pattern from this group differed significantly from those of control groups ($p < 0.05$). The data from individual blinded animals, however, showed evidence of retained rhythmicity (Fig. 13) as was the case when these rats were studied at 84 days of age. Synchronization of the individual data as previously described yielded significant daily fluctuations ($p < 0.05$) in a pattern which did not differ significantly from those of controls. No statistical difference was found between the mean correlation coefficients of blinded and control groups.

Intact and sham-operated controls for animals enucleated at 26 days of age continued to demonstrate significant fluctuations at 112 days of age ($p < 0.05$) and their patterns of plasma corticosterone levels did not differ significantly over the 44-h period (left panel of Fig. 11). Consistent with previous findings, the blinded rats did not show significant fluctuations with time and their pattern differed

Figure 10. The effect of optic enucleation on non-stress plasma corticosterone levels in 112-day-old female rats which had been enucleated at 1 day of age. In this and succeeding figures on this page, lines connect group means of corticosterone levels at 4-h intervals over a 44-h period. Vertical lines indicate standard errors. The number of rats in each group is in parentheses. The solid bars represent dark periods. The right panel demonstrates the effect of synchronizing the individual non-stress corticosterone patterns of the blind rats shown in the left panel so that the highest corticosterone level observed on the first day occurs at the same point in time (0) as controls.

Figure 11. The effect of optic enucleation on non-stress plasma corticosterone levels in 112-day-old female rats which had been enucleated at 26 days of age. The right panel demonstrates the effect of synchronization.

Figure 12. The effect of optic enucleation on non-stress plasma corticosterone levels in 112-day-old female rats which had been enucleated at 60 days of age. The right panel demonstrates the effect of synchronization.

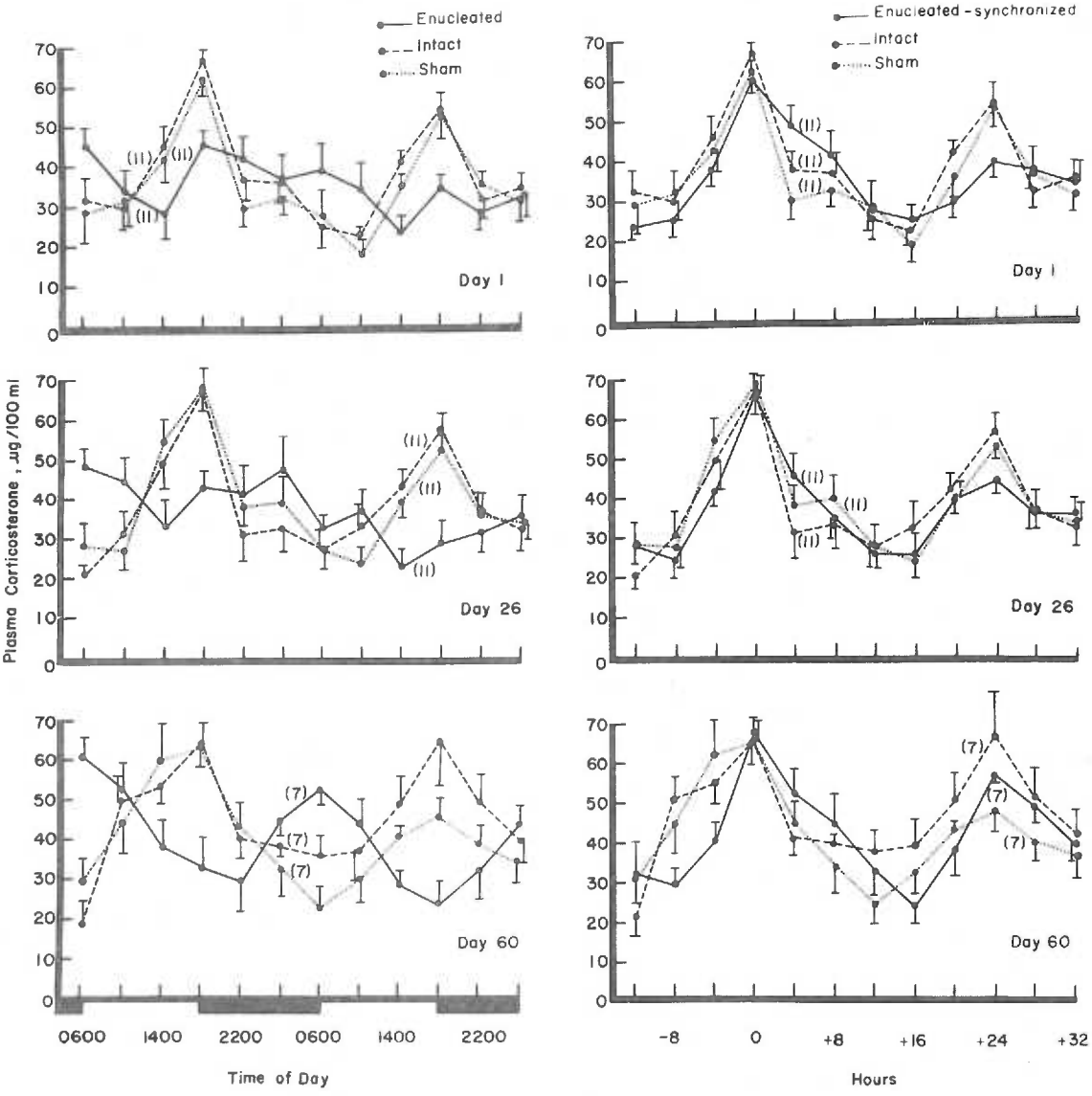
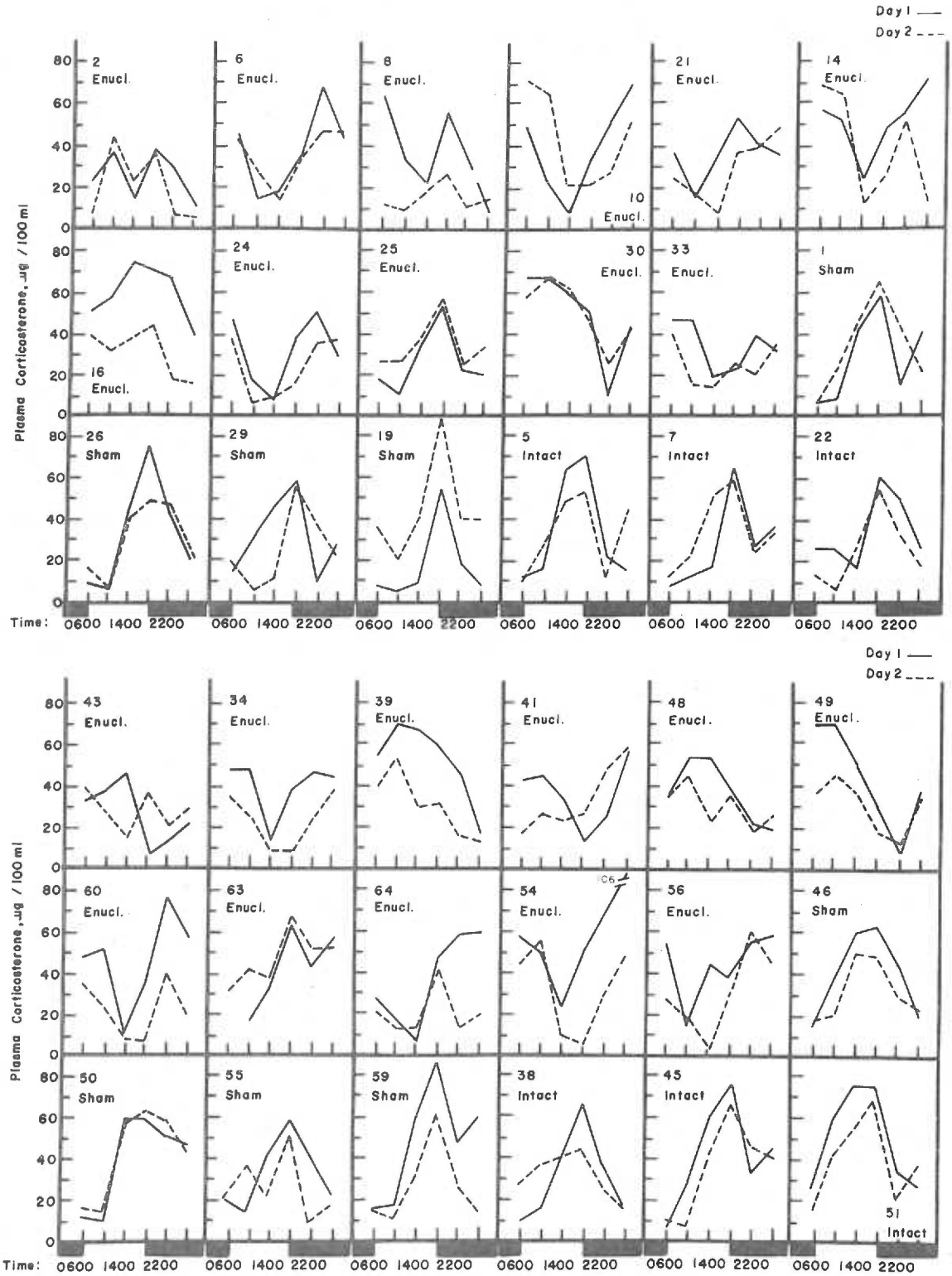

Enucleated
Intact
Sham
Enucleated - synchronized
Intact
Sham
Day 1
Day 1
Day 26
Day 26
Day 60
Day 60
Plasma Corticosterone, µg/100 ml
0600
1400
2200
0600
1400
2200
Time of Day
-8
0
+8
+16
+24
+32
Hours

Figure 13. Individual non-stress plasma corticosterone patterns of 112-day-old female rats enucleated at 1 day of age and representative sham-operated and intact control animals. The solid bars indicate dark periods.

Figure 14. Individual non-stress plasma corticosterone patterns of 112-day-old female rats enucleated at 26 days of age and representative sham-operated and intact control animals. The solid bars indicate dark periods.


Day 1 ——
Day 2 - - -
Plasma Corticosterone, µg / 100 ml
2 Enucl.
6 Enucl.
8 Enucl.
10 Enucl.
21 Enucl.
14 Enucl.
16 Enucl.
24 Enucl.
25 Enucl.
30 Enucl.
33 Enucl.
1 Sham
26 Sham
29 Sham
19 Sham
5 Intact
7 Intact
22 Intact
Time: 0600 1400 2200
43 Enucl.
34 Enucl.
39 Enucl.
41 Enucl.
48 Enucl.
49 Enucl.
60 Enucl.
63 Enucl.
64 Enucl.
54 Enucl.
56 Enucl.
46 Sham
50 Sham
55 Sham
59 Sham
38 Intact
45 Intact
51 Intact

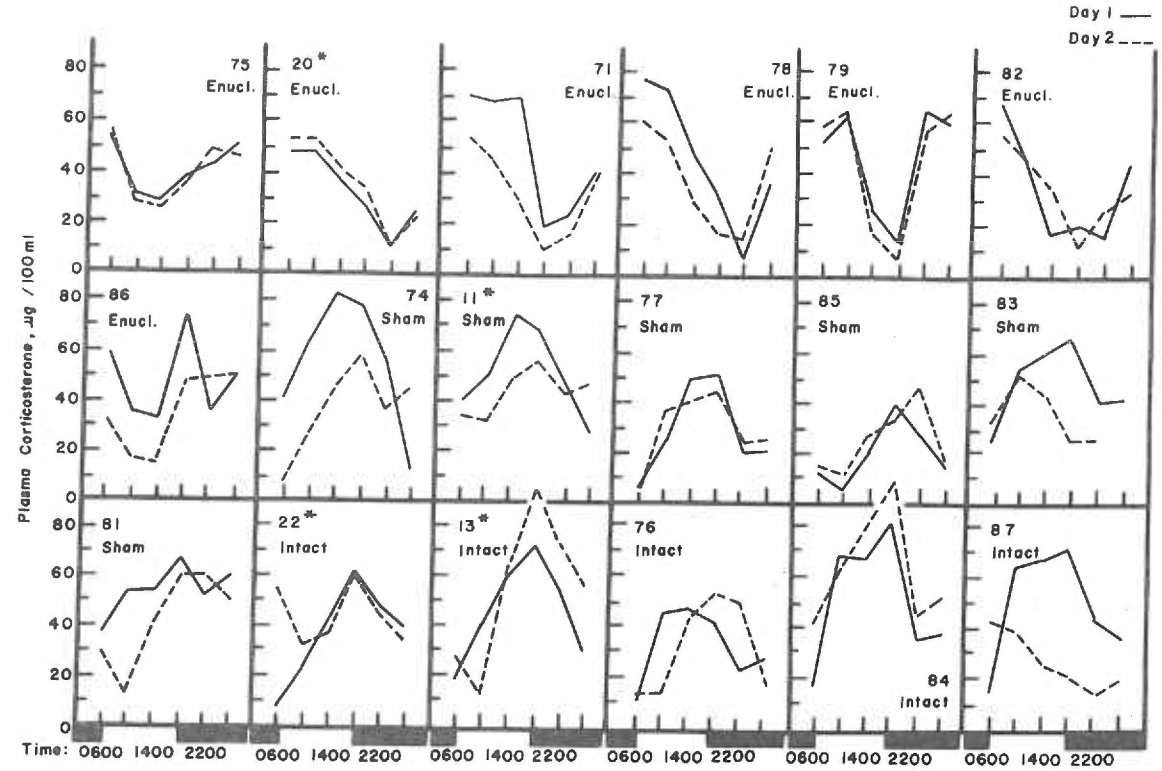
significantly from those of controls ($p < 0.05$). Again, the data from individual enucleated animals suggested rhythmic changes in corticosterone levels which had periods of approximately 24-h (Fig. 14). Synchronization of these individual patterns yielded group data (right panel Fig. 11) which demonstrated significant ($p < 0.05$) fluctuations and which did not differ from those of controls. Mean correlation coefficients were comparable in the experimental and control groups.

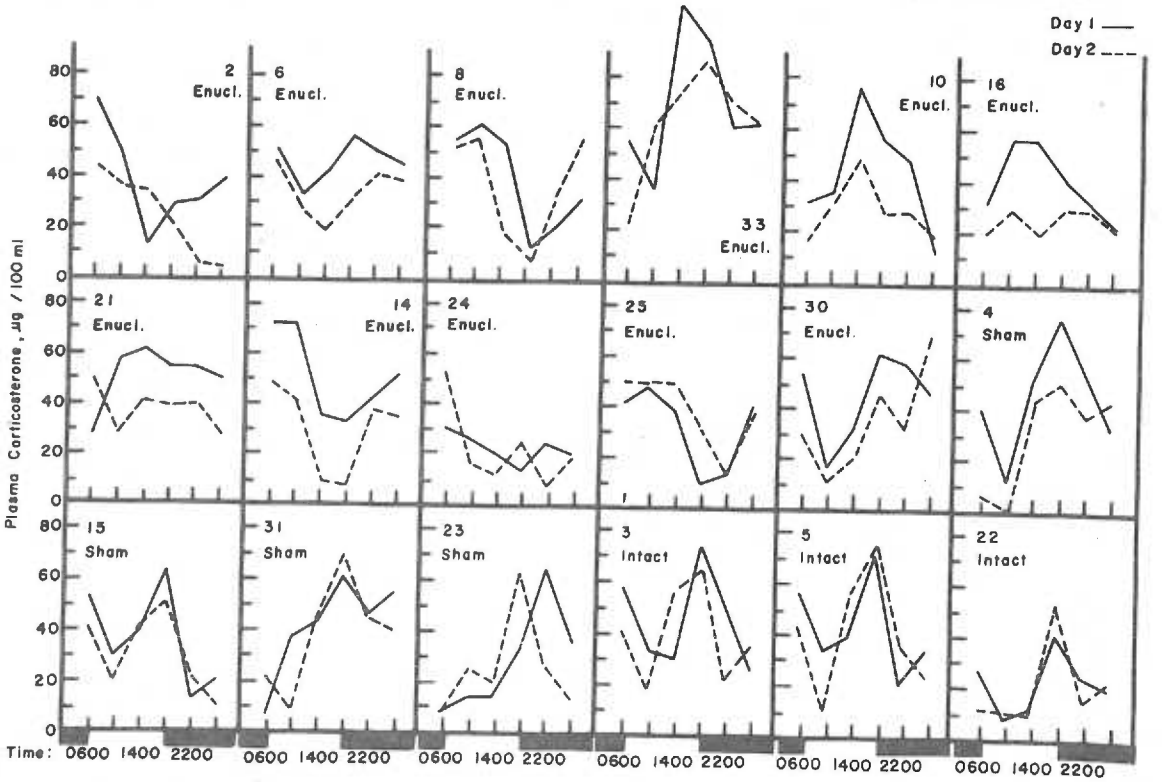
The group data from animals enucleated at 60 days of age, and studied at 112 days of age are presented in the left panel of Fig. 12. The control groups showed peaks at light-dark transition and demonstrated fluctuations in steroid levels ($p < 0.05$), and these patterns did not differ statistically. Interestingly, the blinded animals also showed fluctuations ($p < 0.05$), but the pattern differed significantly from those of controls. The steroid levels of the blinded animals showed peaks at 0600 on each of the two days, suggesting some synchronization and perhaps a phase shift. The individual blinded animals from this group (Fig. 15) showed levels of corticosterone which appeared to fluctuate with a period of approximately 24-h. Synchronization of the steroid patterns yielded group data which retained daily fluctuations ($p < 0.05$) but the 44-h pattern no longer differed from those of controls. The results of synchronization are presented in the right panel of Fig. 12. Mean correlation coefficients did not differ significantly between intact and control groups.

Final sampling was performed at 142 days (20 weeks) of age, and the group data from this study are shown in Figures 17, 18, and 19. As previously, the intact and sham-operated controls for those animals

Figure 15. Individual non-stress plasma corticosterone patterns of 112-day-old female rats enucleated at 60 days of age and representative sham-operated and intact control animals. The solid bars indicate dark periods.

Figure 16. Individual non-stress plasma corticosterone patterns of 142-day-old female rats enucleated at 1 day of age and representative sham-operated and intact control animals. The solid bars indicate dark periods.


Day 1 ——
Day 2 - - -
Plasma Corticosterone, µg / 100 ml
75 Enucl.
20* Enucl.
71 Enucl.
78 Enucl.
79 Enucl.
82 Enucl.
86 Enucl.
74 Sham
11* Sham
77 Sham
85 Sham
83 Sham
81 Sham
22* Intact
13* Intact
76 Intact
84 Intact
87 Intact
Time: 0600 1400 2200


Day 1 ——
Day 2 - - -
Plasma Corticosterone, µg / 100 ml
2 Enucl.
6 Enucl.
8 Enucl.
33 Enucl.
10 Enucl.
16 Enucl.
21 Enucl.
14 Enucl.
24 Enucl.
25 Enucl.
30 Enucl.
4 Sham
15 Sham
31 Sham
23 Sham
3 Intact
5 Intact
22 Intact
Time: 0600 1400 2200

enucleated at 1 day of age had fluctuations ($p < 0.05$) in plasma corticosterone levels which had periods of approximately 24-h and the peaks occurred at the light-dark transitions (left panel, Fig. 17). The patterns of the two control groups did not differ significantly. The group data for the blinded animals did not show such fluctuations and their pattern differed from those of controls ($p < 0.05$). Individual animals enucleated at 1 day of age continued to show apparent rhythmic fluctuations in corticosterone concentrations with a 24-h period (Fig. 16), and synchronization produced a pattern which had significant fluctuations ($p < 0.05$) and which was comparable to those of the control groups (Fig. 17) Mean correlation coefficients were similar in the blinded and control groups.

The group data for animals enucleated at 26 days of age and their controls are illustrated in Fig. 18. Intact and sham controls demonstrated significant fluctuations ($p < 0.05$) in steroid levels over time and the patterns were comparable over the 44-h period. The blinded animals did not demonstrate such fluctuations and their patterns differed significantly from those of controls ($p < 0.05$). The data from individual blinded rats are presented in Fig. 20. Synchronization of the individual patterns yielded the results shown in the right panel of Fig. 18; the group data now showed significant fluctuations ($p < 0.05$) and they did not differ significantly from those of controls. Testing for rhythmicity by means of correlation coeffieients suggested that the patterns of blinded and control animals were comparable.

The group data for animals enucleated at 60 days of age and sampled at 142 days of age are shown in the left panel of Fig. 19.

Both control groups demonstrated fluctuations ($p < 0.05$) consistent with a 24-h rhythm and these did not differ significantly. At this age the group data from these enucleated animals for the first time lacked significant fluctuations with time. Although their pattern differed significantly from those of controls ($p < 0.05$), the data from individual rats (Fig. 21) continued to suggest the presence of 24-h rhythms; these apparent rhythms seemed randomly distributed in phase. Testing for rhythmicity via synchronization yielded a pattern that was statistically comparable to those of controls (right panel, Fig. 19) over the 44-h period. Mean correlation coefficients were also similar in the experimental and control groups.

Autopsy data revealed significant differences in the weights of anterior pituitaries, with those of enucleated animals weighing more ($p < 0.05$) than those of controls. Posterior pituitary, uterine, ovarian and adrenal weights did not differ significantly in the enucleated and the sham-operated or intact control groups.

Figure 17. The effect of optic enucleation on non-stress plasma corticosterone levels in 142-day-old female rats which had been enucleated at 1 day of age. In this and succeeding figures on this page, lines connect group means of corticosterone levels at 4-h intervals over a 44-h period. Vertical lines indicate standard errors. The number of rats in each group is in parentheses. The solid bars represent dark periods. The right panel demonstrates the effect of synchronizing the individual non-stress corticosterone patterns of the blind rats shown in the left panel so that the highest corticosterone level observed on the first day occurs at the same point in time (0) as controls.

Figure 18. The effect of optic enucleation on non-stress plasma corticosterone levels in 142-day-old female rats which had been enucleated at 26 days of age. The night panel demonstrates the effect of synchronization.

Figure 19. The effect of optic enucleation on non-stress plasma corticosterone in 142-day-old female rats which had been enucleated at 60 days of age. The right panel demonstrates the effect of synchronization.

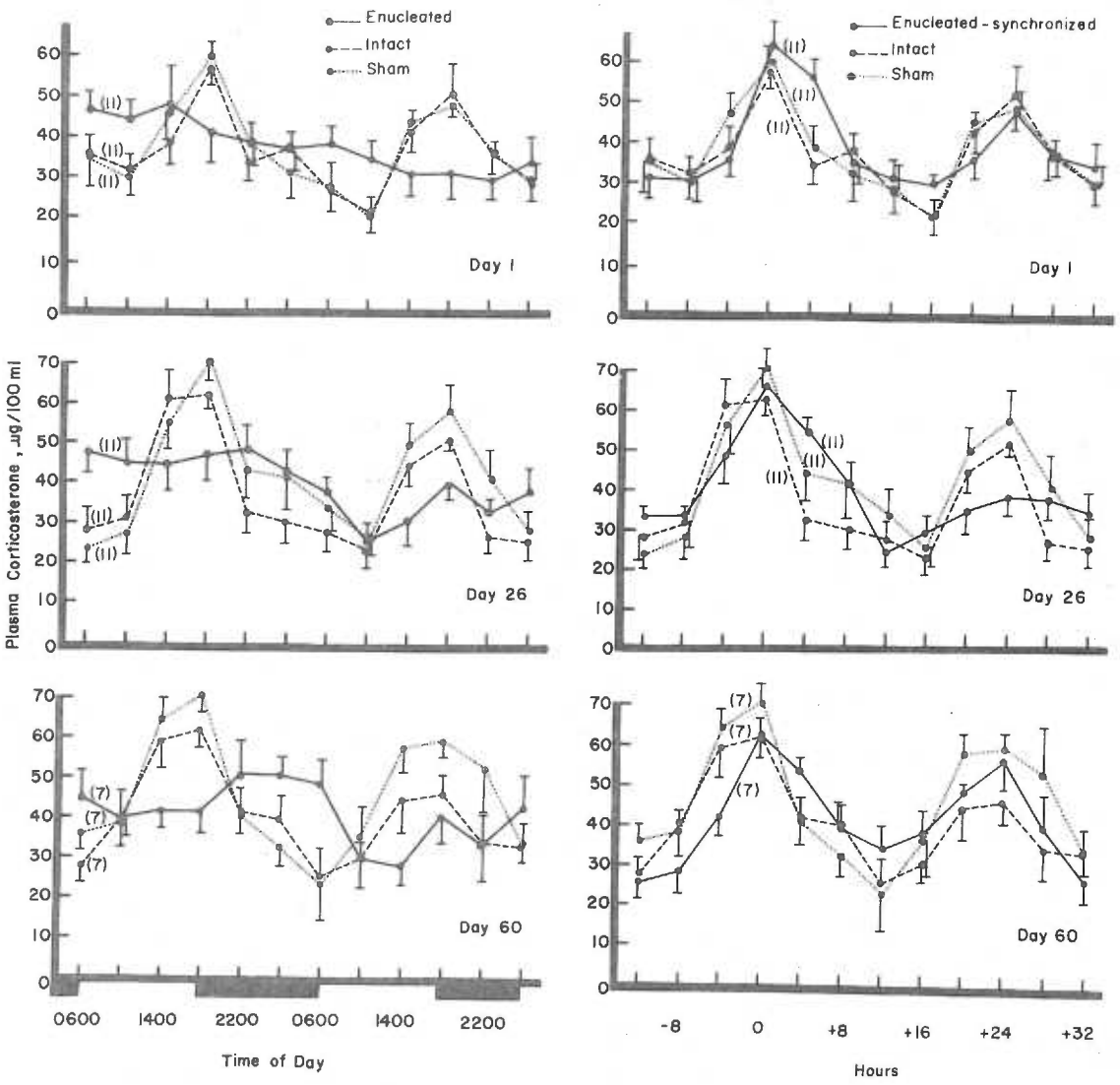
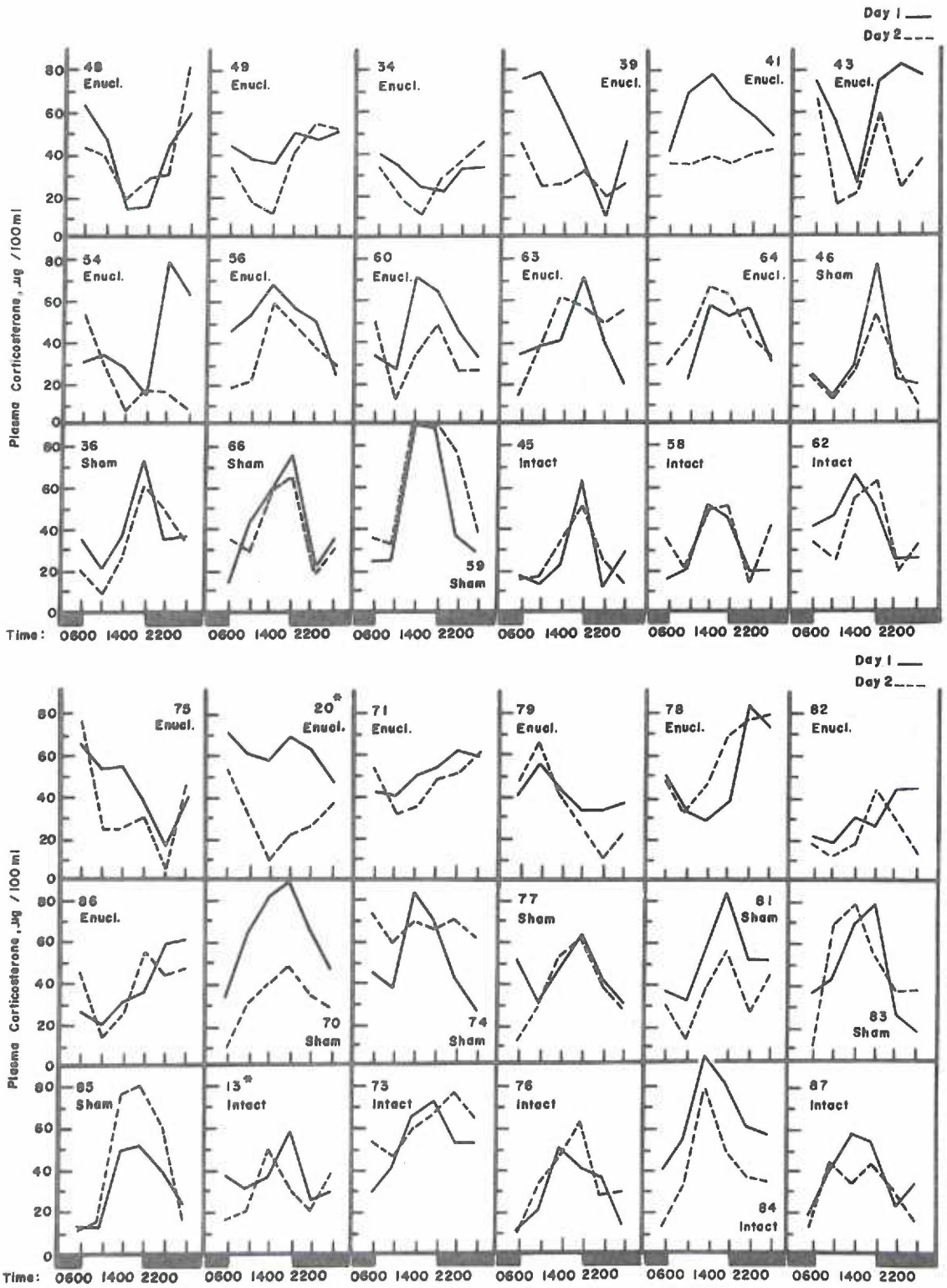

Enucleated
Intact
Sham
Enucleated - synchronized
Intact
Sham
Day 1
Day 1
Day 26
Day 26
Day 60
Day 60
Plasma Corticosterone, µg/100 ml
0600
1400
2200
0600
1400
2200
Time of Day
-8
0
+8
+16
+24
+32
Hours

Figure 20. Individual non-stress plasma corticosterone patterns of 142-day-old female rats enucleated at 26 days of age and representative sham-operated and intact control animals. The solid bars indicate dark periods.

Figure 21. Individual non-stress plasma corticosterone patterns of 142-day-old female rats enucleated at 60 days of age and representative sham-operated and intact control animals. The solid bars indicate dark periods.


Day 1 ——
Day 2 - - -
Plasma Corticosterone, µg / 100 ml
48 Enucl.
49 Enucl.
34 Enucl.
39 Enucl.
41 Enucl.
43 Enucl.
54 Enucl.
56 Enucl.
60 Enucl.
63 Enucl.
64 Enucl.
46 Sham
36 Sham
66 Sham
59 Sham
45 Intact
58 Intact
62 Intact
Time: 0600 1400 2200
75 Enucl.
20* Enucl.
71 Enucl.
79 Enucl.
78 Enucl.
82 Enucl.
86 Enucl.
70 Sham
74 Sham
77 Sham
81 Sham
83 Sham
85 Sham
13* Intact
73 Intact
76 Intact
84 Intact
87 Intact

DISCUSSION

These experiments were designed to clarify the role played by light and the central retinal connections in the establishment and maintenance of the circadian rhythm in pituitary-adrenal function. The results suggest that the eyes and central connections of the retinae are not essential during post-natal life for the development and maintenance of such rhythmicity in female rats. Thus, these results indicate that the age of blinding is not an important variable. The visual system, however, does appear to be necessary for the synchronization of this rhythm with environmental light. The eyes also appear, under the conditions used in these experiments, to be essential for between-animal synchronization of individual rhythms because optic enucleation produced rhythms in individual rats which were asynchronous and free-running. Therefore, these results suggest that rhythmic pituitary-adrenal function in blind rats is not entrained by such periodic environmental cues as activity, feeding and drinking behaviors of normal rats in the same or adjacent cages.

The principal aim of the first experiment was to determine, using serially dependent sampling, whether rats blinded at 1 day of age show a synchronized circadian rhythm at the age of 45 days. As discussed in the introduction, Ramaley (78) reported such animals to have a circadian rhythm which was 180^{o} out of phase with controls at 45 days. Taken in conjunction with Krieger's (58) report that animals blinded at 1 day of age lack a rhythm at 80 days of age, the findings of Ramaley suggest that the mechanisms responsible for this rhythmicity function at least transiently and that the eyes and their central connections are not

necessary for the initiation of function by these mechanisms. The results of the present experiment agree partially with those reported by Ramaley. Group data for the 24-h period of the study demonstrated that the blind rats had a pattern of corticosterone concentrations that differed significantly from those of controls. However, there was no indication of a synchronized 24-hour periodicity in the blinded animals, nor was a phase shift evident. Unlike the intact and sham-operated controls, the steroid levels in the blind rats did not show significant variation with time, suggesting that blinding at 1 day of age disrupted the pituitary-adrenal rhythm. The nature of this disruption was indicated by the steroid patterns of individual rats. These patterns showed fluctuations in steroid levels which were comparable in amplitude and period with those of controls, but the peaks and troughs appeared to demonstrate free-running rhythms which were asynchronous between animals. This possibility was supported by the effects of aligning steroid peaks of individual rats. This manipulation produced a group pattern which did not differ significantly from that of either control group. While a higher frequency and a longer duration of sampling in this experiment might have produced more convincing results, the similarity in steroid patterns in the control and blinded groups suggest that rhythmic pituitary-adrenal function was present in the blind rats and that this rhythm had a period of approximately 24-h.

The results of this experiment differ from those of Ramaley in that the peaks and troughs were randomly distributed within the group of blinded animals, so there was no evidence of between-animal synchronization. The basis for this discrepancy is unknown. In view of the

apparent free-running rhythms noted in the present study, it is surprising that Ramaley observed evidence of a rhythm using a serially independent sampling procedure. It is possible, however, that the AM-PM differences in steroid levels which she observed were indicative of free-running rather than synchronized rhythms. On the other hand, it is possible that the rats in Ramaley's experiment were entrained by an environmental cue which was not effective and/or not available in the present study. Despite the above differences, these results and those of Ramaley are consistent in suggesting that the circadian rhythm in pituitary-adrenal function persists in 45 day old rats blinded at 1 day of age. This experiment also demonstrated that 45 day old rats can be studied in a serially dependent manner every 6-h for 24-h without obscuring fluctuations in plasma corticosterone levels.

The results of the second experiment are in agreement with those of the first and offer further evidence that optic enucleation at 1 day of age is compatible with the development and long term function of the mechanisms responsible for rhythmic pituitary-adrenal function. Thus, it was demonstrated that rats blinded at 1 day of age showed an approximate 24-h periodicity in plasma corticosterone levels when studied at 84, 112 and 142 days of age. As in the first experiment, comparison of the steroid patterns of the blinded group with those of controls at each of the above ages showed that there were marked and significant differences. The group data from blind rats did not show the AM troughs and PM peaks in steroid levels which characterized the patterns of both control groups and there was no evidence of a synchronized phase shift in that analysis of variance indicated that steroid

levels did not vary significantly with time. As previously, the relatively flat, intermediate steroid pattern in the blind group did not appear representative of the patterns in individual rats, and this was verified by the effects of aligning the peaks of these patterns. Such alignment produced patterns which on each occasion showed significant fluctuations with time and which did not differ significantly from those of controls. Although of limited value, comparisons of correlation coefficients were consistent in suggesting that the steroid fluctuations in the blind rats were similar to those of control groups. Because these rats were studied on three occasions for 44-h at 4-h intervals and similar data were obtained in each study, these results suggest that the eyes are not essential in the post-natal period for the development and long-term function of the mechanisms which drive this circadian rhythm. That this rhythm is apparently free-running suggests that the eyes are necessary only as a Zeitgeber for the synchronization with environmental light and that other cues from the environment are incapable of entraining the rhythm. It is of particular interest that activity patterns of intact rats in the same and adjacent cages were ineffective in this regard. Similar findings were reported by Wilson and Critchlow (100). In suggesting retention of rhythmicity, even though free-running, these findings in rats blinded at 1 day of age are clearly discrepant with those of Krieger (52). Krieger also used serial sampling and found that rhythmicity was abolished in rats blinded at 1 day of age and studied at 80 days of age. The basis for this discrepancy is unknown.

As discussed in the Introduction, Campbell and Ramaley (11)

reported that retinal connections to the suprachiasmatic nuclei are established by approximately 17 days of age, and these investigators stressed the potential causal relationship between this maturational event and the initiation of rhythmic pituitary-adrenal function which is presumed to occur at approximately 18-19 days of age (77). The results obtained in these experiments do not support such a relationship. The presence of free-running rhythms in rats blinded at 1 day of age suggest that retino-hypothalamic connections are not necessary for circadian rhythmicity *per se*. However, the present data are compatible with the possibility that these connections, when established, allow environmental light to synchronize an endogenous rhythm produced by a biological "clock" mechanism located in or near the suprachiasmatic nuclei. Because all of the studies which have explored the age at which pituitary-adrenal rhythmicity is established have used serially independent sampling, it is possible that the diurnal fluctuations recorded in the group data reflect the beginning of synchronization of previously free-running rhythms rather than the appearance of rhythmicity *per se*. Until serially dependent sampling is used in neonatal and preweanling rats,it will not be possible to ascertain the age of onset of rhythmic function and the onset of synchronization.

The results obtained from the rats blinded at 26 or 60 days of age are comparable to those from rats blinded at 1 day of age. Whereas it was anticipated, based on Krieger's previous findings (52) and the interesting anatomical correlate described by Campbell and Ramaley (11), that the effects of blinding at 1 day of age might differ from blinding at 26 or 60 days of age, such was not the case. These findings support

the conclusion that blinding, regardless of the age at which it is performed, results in a free-running rhythm. The only difference of note observed in animals blinded at an older age appeared in those animals enucleated at 60 days of age. When studied at 84 days of age, the group data from these animals showed patterns which retained significant fluctuations with time which were still synchronized appropriately with the light-dark cycle; the steroid pattern from this group did not differ statistically from those of controls. This finding may be attributed to the fact that only 24 days elapsed between optic enucleation and blood sampling. This may be insufficient time for rhythms of individual rats with slightly different periods to drift out of phase and to become randomized with respect to the 24-h light-dark cycle. Similar observations were made by Wilson and Critchlow (100) at 3 weeks after blinding adult female rats; by 10 weeks, their blinded rats appeared to have free-running rhythms.

The group data for rats enucleated at 60 days of age demonstrated a tendency to peak at 0600 when studied at 112 days of age. This raised the possibility that an environmental stimulus was synchronizing these animals in the absence of visual imput. Because this effect was seen only in those animals enucleated at 60 days of age, and not in those enucleated at 1 or 26 days of age, it was possible that there was a difference in the ability of an environmental stimulus to synchronize which was dependent on the age of enucleation. Subsequent results suggested that if such were the case, the effect was transient because animals enucleated at 60 days of age demonstrated random distribution in the peaking of their rhythm. These data and those of Wilson and

Critchlow (100) suggest that between 52 and 70 days are necessary for free-running to become evident in rats blinded as adults.

An interesting point which emerged from the present studies and from those of Wilson and Critchlow (100) concerns the inability of other cues from the environment to synchronize rhythmic pituitary-adrenal function in the female rat. Although temperature and lighting conditions were maintained relatively constant throughout these experiments, it is likely that many other periodic stimuli from the environment were available to these rats. Most importantly, the blinded rats were caged with intact and sham-operated controls and were thus exposed to the cues associated with normal periodic feeding, drinking and activity behaviors. These experiments indicate that exposure to these rhythmic behaviors is not sufficient to synchronize rhythmic pituitary-adrenal function.

SUMMARY AND CONCLUSIONS

These studies demonstrate that individual rats optic enucleated at 1, 26, or 60 days of age have periodic fluctuations in plasma corticosterone levels. The peaks of the fluctuations were of normal amplitude. Although group patterns suggested that blinding disrupted the normal circadian rhythm in plasma corticosterone levels, patterns of individual rats indicated that an approximate 24-h rhythmicity was present. The data suggest that these rhythms are free-running. Thus, the eyes and their central connections appear under the conditions used in these experiments to be essential for synchronization to the light-dark cycle and for synchronization between blinded animals. The other environmental cues available to the blinded rats were incapable of establishing between-animal synchronization of the steroid rhythm. These results were obtained in all blinded groups, regardless of age of optic enucleation, provided sufficient time was allowed for the free-running rhythms to become randomized in phase. It is concluded that female rats have at birth the neural substrate required to develop and maintain circadian rhythmicity in pituitary-adrenal function. The eyes are not essential in this regard, but they are necessary to allow the 24-h light-dark cycle to function as a Zeitgeber for the pituitary-adrenal rhythm.

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