J. steroid Biochem. Vol. 23, No. 5B, pp. 725–731, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

STIMULATION OF HYPOTHALAMIC LHRH LEVELS AND RELEASE BY GONADAL STEROIDS

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Summary—The inhibitory feedback effects of steroids on pituitary LH release are believed to be mediated via steroidal effects on the hypothalamic LHRH activity. We have examined the direct effects of individual steroids (T, DHT and E_2) on hypothalamic LHRH levels and on LHRH release *in vitro*. In castrated male rats, replacement of either steroid in physiological doses, resulted in augmentation of the MBH LHRH levels by steroidal action within the MBH. LHRH analyses of microdissected diencephalic nuclei revealed that this accumulation occurred exclusively in LHRH terminals in the ME. Careful examination of the time course of steroid action showed that whereas LH release was suppressed within hours of steroid treatment, the LHRH response occurred after 3–4 days of steroid exposure in 2-week castrated rats and 7–14 days in 8-week castrated rats. This temporal dichotomy in the LH and LHRH responses to steroid action was further substantiated by the differential effects of low, sub-physiological levels of steroids on these two responses. Very low levels of T or E_2 evoked maximal accumulation of the MBH LHRH, but LH release *in vivo* and the rate of LHRH release *in vitro* were not affected. Surprisingly, physiological levels of T which suppressed LH release concomitant with elevations in LHRH levels, augmented the *in vitro* rate of LHRH release. In fact, the LHRH release rate was found to be correlated with LHRH concentrations in hypothalami of intact, castrated and castrated rats treated with T. Thus it appears that in the hypothalamo–pituitary axis there are different thresholds of responsiveness to steroids. Apparently, the LHRH neurons, particularly the processes involved in LHRH accumulation are most sensitive to low levels of steroids; however, higher physiological levels of steroids are required to suppress pituitary LH release as well as to promote LHRH release. On the basis of our cumulative data, it is reasonable to speculate that steroid-induced accumulation of LHRH in the ME may not be a consequence of decrease in LHRH release, but may involve synthesis of the neurohormone.

INTRODUCTION

The restraining effect of gonadal steroids on the secretion of pituitary luteinizing hormone (LH) is beyond dispute [1–3]. It is generally believed that this inhibition of LH secretion may be the end result of steroidal action at the hypothalamic level to turn-off the release of lutenizing hormone releasing hormone (LHRH) into the hypophyseal portal veins. The demonstration of steroid concentrating cells [4, 5] and specific steroidal cytoplasmic and nuclear receptors [6] in the hypothalamus lends credence to this view. The fall in hypothalamic LHRH stores after castration [7–9] in association with LH hypersecretion has been generally considered to be a result of increased rate of LHRH release. Conversely, elevated LHRH stores seen in the hypothalami of gonad-intact rats [10] was assumed to be due to decrease of LHRH release induced by the endogenous circulating steroids. However, the concept that changes in the hypothalamic LHRH levels reflect the status of LHRH release is based on indirect evidence and is open to alternative interpretations. Attempts to determine the LHRH release rates in the hypophyseal portal blood samples [11–14] are fraught with technical complications including the profound effects of surgical stress and anaesthesia on secretion of the neurohormone. Our concerted efforts during the past few years to study the feedback effects of steroids in male rats have led to the formulation of an alternative hypothesis [15–19]. Our findings, indicate that gonadal steroids may augment neurosecretory activity to facilitate accumulation of LHRH and that this action of steroids at the hypothalamic level may not necessarily be associated with inhibition of pituitary LH release. The following account represents a summary of our findings on the sites and mode of action of the circulating gonadal steroids in male rats. We have described the effects of testosterone (T), 5α-dihydrotestosterone (DHT) and 17β-estradiol (E_2), on LHRH levels in the medial basal hypothalamus (MBH) and on pituitary LH secretion in male rats. Evidence will be presented to demonstrate that these two central effects of steroids may occur independently of each other and that, contrary to general belief, steroids may promote accumulation as well as release of LHRH.

EFFECTS OF GONADAL STEROIDS ON LHRH LEVELS

Castration of male rats invariably resulted in depletion of LHRH stores in the MBH [7, 8, 15–19]. Although LH hypersecretion following gonad ablation was rapid, occurring within hours, the depletion of LHRH was far more gradual requiring 7–10 days [16]. That LHRH depletion was due to steroid-deprivation is evident from the fact that re-

placement of steroids in castrated rats restored the hypothalamic LHRH levels to the range found in intact rats. These early studies showed that long-term treatment of castrated rats with physiological levels of either T or its active metabolite, DHT, suppressed LH secretion and stimulated LHRH levels in the MBH. Interestingly, E_2 which is detectable in the circulation of intact male rats [17, 20], was just as effective as the androgens in eliciting the characteristic LH and LHRH responses. The temporal dissociation in the occurrence of the MBH LHRH and LH release responses, evident after castration, was also seen after steroid replacement. We found that while T, DHT or E_2-induced inhibition of LH release was observed within hours, the accumulation in MBH LHRH stores was much more sluggish, significant elevations occurred after 3–4 days [16, 17].

In view of the sluggish nature of the LHRH response, further studies were designed to examine the minimum duration of exposure to steroid required for manifestation of this response [18]. Rats castrated for 2 weeks were implanted s.c. with T containing Silastic implants. At varying times after implantation, the Silastic implants were removed in groups of rats and all the animals were decapitated 72 h after implantation. Results showed that supply of T for either 24, 48 or 56 h was not sufficient to augment the MBH LHRH levels above those in castrated controls. In fact, a minimum of continuous T exposure for 72 h was required to elicit the LHRH response in 2-week castrated rats. Recently, we have found that in long-term castrated rats this critical period of exposure to T to augment the MBH LHRH levels was dramatically prolonged to 14 days [19]. Interestingly, the minimal period of exposure to E_2 or DHT was extended only from 4 to 7 days. In fact, the temporal dichotomy in the LH and LHRH responses seen in short-term castrated rats was more apparent in long-term castrated rats since the time course of LH suppression was not altered; physiological levels of the steroids, which required 7–14 days to evoke the MBH LHRH response, were just as effective in promptly suppressing LH secretion in 8-week castrated rats as in 2-week castrates.

These series of studies provided further evidence that the sluggish MBH LHRH response to gonadal steroids may not be related to the rapid inhibition of LH secretion and were in disagreement with the hypothesis, espoused by many investigators, that steroid-induced suppression of LHRH release rate leads to accumulation of LHRH stores in the MBH [7, 8, 21]. On the other hand, the long time-lag seen between the LH and LHRH responses suggested that factors other than inhibition of LHRH release may contribute to augmenting the MBH LHRH concentrations. We have proposed the more likely possibility that LHRH accumulation may be due to acceleration of LHRH synthesis either by augmenting precursor LHRH protein formation in the LHRH perikarya or by increased processing and elaboration of immunoreactive and bioactive LHRH from the precursor in the nerve terminals [22, 23].

DIFFERENTIAL EFFECTS OF GONADAL STEROIDS ON HYPOTHALAMIC LHRH LEVELS AND LH SECRETION

To further strengthen our working hypothesis evolving from the above evidence which advocated that gonadal steroids directly augment elaboration of the neurohormone in the MBH, it was important for us to demonstrate that the steroid-induced increase in LHRH levels was not the result of decreased release rate. The unequivocal demonstration of steroid-induced LHRH accumulation in castrated rats in the presence of normal episodic LH secretion would provide strong evidence in favor of our hypothesis. These criteria were essentially established by our experiments aimed at determining the minimal effective levels of steroids required to elicit the LHRH response in castrated rats [17, 18].

Very low, sub-physiological serum concentrations of T in castrated rats were found to be just as effective as higher physiological concentrations in augmenting the MBH LHRH levels at 96 h (Fig. 1). However, although the higher doses of T (> 1 ng/ml) suppressed LH release, the low T concentrations did not adversely affect serum LH levels [18]. Essentially similar results were obtained with low doses of E_2. Maintenance of low serum E_2 concentrations (10 pg/ml) in castrated male rats failed to alter LH release, but these physiological E_2 levels were highly potent in restoring the MBH LHRH levels to the range of intact rats [17]. It is noteworthy that to raise the MBH LHRH levels, the minimum effective duration of exposure to these small doses of T or E_2, which do not suppress LH release, was the same as that with higher steroid doses which drastically inhibit LH release [16]. This demonstration of accumulation of LHRH in the presence of normal LH release in rats treated with very low levels of steroids constitutes powerful evidence in favor of the hypothesis of direct stimulation of LHRH elaboration by gonadal steroids.

It could be argued that the apparent lack of effects of small concentrations of T or E_2 on LH secretion could be due either to subtle changes in the episodic pattern of LH secretion which were not detected by our one time-point sampling design or to increase in pituitary responsiveness to LHRH which may have compensated for any decrease in LHRH release. These two probabilities were experimentally tested [17, 18]. Frequent blood samples (every 5 or 10 min) were obtained from unrestrained, unanaesthetized castrated rats bearing T or E_2 containing implants. Analysis of LH levels revealed that low serum concentrations of T or E_2, which augment the MBH LHRH stores, did not alter either the LH pulse frequency or the amplitude of LH peaks as compared to castrated control rats. As the steroid concentrations were progressively increased, the LH pulse

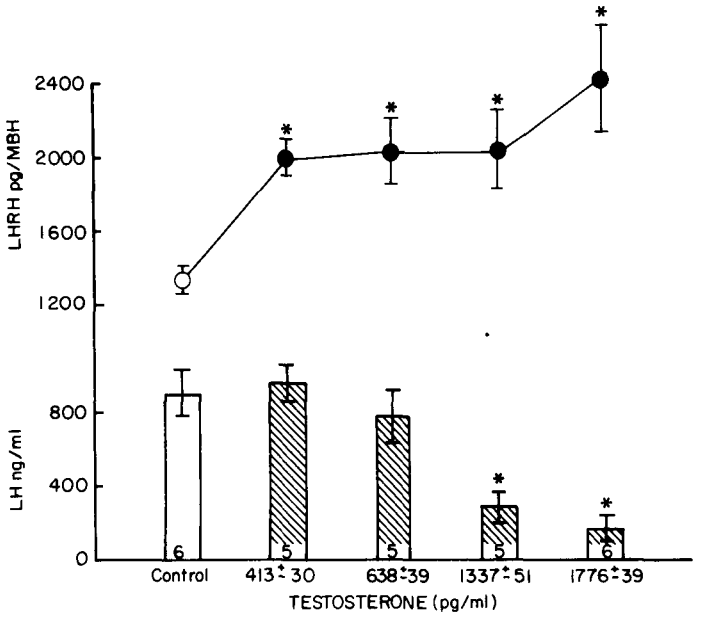

Fig. 1. Effects of varying doses of T on the MBH LHRH levels and serum LH levels. Castrated rats were implanted s.c. with T-containing Silastic implants of varying sizes to achieve a graded range of serum T levels as indicated on the abscissa. At 96 h after implantation the two lower doses of T were maximally effective in augmenting the MBH LHRH levels but, unlike the two higher T doses, they did not supress LH hypersecretion. $*P < 0.05$ vs castrated controls (From Ref. 18 with permission).

amplitude and mean plasma LH levels fell in a dose-related manner. Further, pituitary responsiveness to two doses of exogenous LHRH (10 and 60 ng) was tested in similarly treated rats. Results revealed that although low levels of E_2 did not alter the magnitude of LH released, as expected, the larger doses of E_2 (60–100 pg/ml) increased pituitary response to exogenous LHRH in association with decrease in LH release [17]. On the other hand, in agreement with numerous studies showing that androgens are capable of inhibiting the pituitary response to LHRH by a direct action at the pituitary level [24–26], our studies showed that both low and high T levels significantly attenuated LH release in response to LHRH [18]. These findings clearly showed that low doses of steroids, which effectively raised the MBH LHRH levels, neither altered any aspect of episodic LH secretion nor did they increase pituitary responsiveness to compensate for any decrease in endogenous LHRH reaching the pituitary gonadotrophs. On the contrary, decreased pituitary responsiveness in rats bearing small T implants, despite the presence of normal episodic LH secretion was suggestive of increased LHRH release. These studies also showed that the two central responses to steroids have differential thresholds of responsiveness. It was apparent that the MBH LHRH is far more sensitive to T and E_2 action than pituitary LH secretion since the LHRH response was elicited selectively by very low levels of these steroids.

EFFECTS OF GONADAL STEROIDS ON HYPOTHALAMIC LHRH RELEASE

On the basis of these demonstrations it appears unlikely that restoration of the MBH LHRH levels by gonadal steroids may be due solely to changes in LHRH release rates. However, as alluded to, there is no direct experimental evidence to show that androgens alter LHRH release rates. Attempts to directly assess the rate of LHRH release into the hypophyseal portal vessels have produced contradictory results [11–14]. More recently, the alternative *in vitro* perfusion method has been used by several investigators to assess the basal rate of LHRH release as well as release in response to secretogogues [27, 28]. We have compared the *in vitro* LHRH release rates from the hypothalami of intact, castrated and castrated rats treated *in vivo* with low or physiological doses of T [29].

Hypothalami of castrated rats released LHRH at a markedly reduced rate (10.4 ± 0.4 pg/10 min) as compared to that of intact rats (24.0 ± 0.8 pg/10 min) over a 6 h perfusion period (Fig. 2). Treatment *in vivo* of castrated rats with small T implants, which significantly raise the MBH LHRH levels without altering normal episodic LH secretion [18], failed to alter LHRH release rate *in vitro* (not shown). In contrast, hypothalami of rats treated *in vivo* with physiological levels of T, which raise the MBH LHRH levels concomitant with decrease in serum LH levels, released significantly higher amount of LHRH

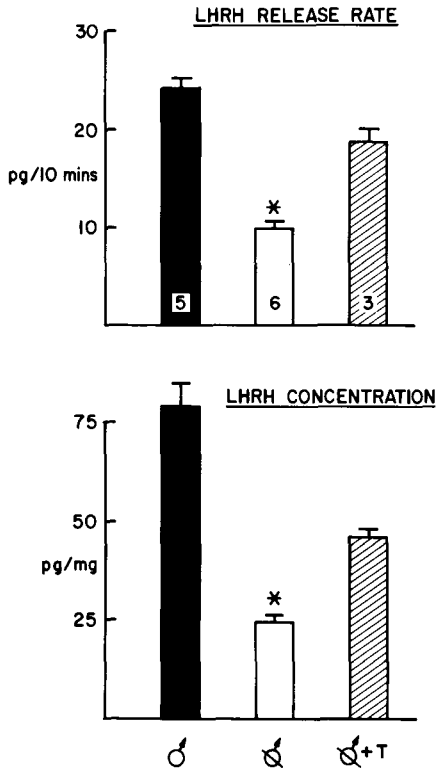

Fig. 2. The *in vitro* LHRH release rates from the hypothalami of intact, castrated and castrated rats treated with physiological levels of testosterone for 96 h. Six hypothalami were pooled for each *in vitro* perifusion. The lower panel depicts the LHRH concentrations of the hypothalami at the end of 6 h of perifusion. The LHRH release rates *in vitro* were found to be correlated with the LHRH concentrations. $^*P < 0.05$ vs the other 2 groups.

in vitro, as compared to that from castrated rats. In fact, the LHRH release rates were found to be correlated with hypothalamic LHRH concentrations (Fig. 2). These surprising results showed that the T-induced accumulation of LHRH in the MBH and increased LHRH output have differential thresholds of response. Accumulation of LHRH was more sensitive to low levels of serum T than augmentation of LHRH output which was seen only with the higher T dose [29].

Augmented LHRH release *in vitro* from the hypothalami of rats showing suppressed LH release is intriguing. These findings can be reconciled if one considers the probability that at higher doses androgens not only promote accumulation of LHRH in the MBH but may also cause increased release of LHRH. The suppression of LH release in these rats may be accounted for by the androgen-induced decrease in pituitary responsiveness to LHRH [18, 24–26] and by decrease of pituitary LHRH receptors [30–32]. Whether steroids similarly augment LHRH release *in vivo* has not been established yet. However, these *in vitro* results are in agreement with the preliminary report of Dluzen and Ramirez[33] who measured LHRH levels in perfusate of the ME using the push–pull cannula technique in unanaesthetized, unrestrained rats; the *in vivo* LHRH release rate was found to be lower in castrated rats as compared to that in intact rats.

SITE(S) OF GONADAL STEROID ACTION IN THE BRAIN

Recent extensive immunocytochemical studies have mapped the distribution of LHRH perikarya and their projections in the brain. Small, fusiform LHRH containing neurons are distributed rostrocaudally along the mid-line from the diagonal band of Broca to the pre-mammillary region [34–36]. Although the majority of immunopositive LHRH cells are concentrated rostrally in the diagonal band of Broca, stria terminalis and the medial preoptic area (MPOA) [34, 35], a few LHRH perikarya were also seen in parts of the MBH, viz. the retrochiasmatic area (RCA), the cell-poor zone and lateral arcuate area (ARC); interestingly a few neurons were also visualized in the caudal aspect of the median eminence (ME) [36]. Projections of these LHRH neurons converge into the ME. In the external layer of the lateral aspect of the ME, LHRH axons terminate on capillaries of the primary portal plexus of the pituitary.

Autoradiographic studies have shown a striking similarity between the location of steroid-concentrating cells and LHRH neurons in the brain. Within the hypothalamus, steroid-concentrating cells have been found in the ARC and dorsally and laterally to it in the ventromedial and dorsomedial nuclei, as well as in the stria terminalis, the MPOA and anterior hypothalamic areas [4–6].

Despite the overlapping distribution of peptidergic and steroid-concentrating cells, it was intriguing that the steroid-induced augmentation of LHRH stores occurred exclusively in the MBH—a fragment encompassing the ME, RCA, ventromedial hypothalamus, and parts of the anterior hypothalamic area. Following castration or steroid replacement, LHRH levels remained unchanged in the rostral POA fragment which included areas where the majority of LHRH perikarya are reported [16–18]. This apparent specificity of action prompted us to attempt to identify the specific central site where steroids may act to augment LHRH levels [15, 40]. Immediately after castration, implants containing crystals of T or DHT were placed intracranially at rostral sites such as the MPOA which has been shown to contain LHRH perikarya involved in regulation of LH release. These implants caused no change in LHRH levels either locally in areas surrounding the implant or caudally in the MBH. However, similar androgen implants in the MBH prevented the post-castration depletion of the MBH-LHRH levels without altering those of the rostral POA. Furthermore, these MBH T implants did not prevent the post-castration hypersection of LH, indicating that the local concentration of T in the

MBH was sufficient to block the LHRH response to castration, but was perhaps below the threshold to suppress pituitary LH release [15].

Similar attempts to identify the central site of E_2 action were somewhat complicated by the fact that circulating levels of E_2 in the male rat are in the picogram range which is a fraction of the normal levels of T (nanograms) or of DHT (in hundreds of picograms) [17, 37]. Since these picogram quantities of E_2 are highly effective centrally in eliciting the MBH LHRH response, slight diffusion of the intracranially implanted steroid from the site of implant, would result in the transport of optimal quantities of E_2 to remote sites. Thus, we found that E_2 implants in the central amygdaloid nucleus, medial septal area, MPOA or the MBH were successful in preventing the castration-induced decrease in the MBH LHRH stores. However, when diffusion of E_2 into the systemic circulation was neutralized by treatment with E_2-antiserum, implants in the amygdala and septal areas were no longer effective. These results substantiated the suspicion that optimal levels of E_2, not detectable by direct measurement, were reaching the hypothalamus by systemic route. On the other hand, intraneural diffusion of E_2 was found to be the contributing factor for the effectiveness of E_2 implants in the MPOA. We measured large amounts of E_2 in the MBH and pituitary for up to 14 days after E_2 implantation in the MPOA. By markedly reducing the amount of E_2 in the MPOA implants, we later demonstrated the ineffectiveness of these implants to raise the MBH LHRH levels [22, 23].

Thus, after applying this extensive spectrum of approaches, it was apparent that the three steroids display a remarkable regional specificity of action; they augmented LHRH stores only when placed in the MBH. When the distribution pattern of steroid concentrating cells in the MBH is considered it is apparent that there may be a distinct group of these neurons in the MBH (ARC, ventromedial hypothalamus, etc.) which by direct or indirect synaptic links communicate with peptidergic neurons to promote accumulation of LHRH in the MBH. The recent demonstrations that LHRH perikarya themselves do not concentrate E_2 are in agreement with this view [38].

Since the majority of LHRH perikarya are located rostrally with their projections terminating in the ME, our findings raised the interesting question of the site in the peptidergic neurons where these steroid-induced changes in LHRH levels may occur. If the steroids indeed promote synthesis of the neurohormone then the action of steroid concentrating neurons could occur at the level of the perikarya, axons, terminals or dendrites. This link may promote either precursor LHRH formation, transformation of the precursor to the immunoactive form or accelerate transport of the precursor proteins along the fibers to the ME. Since the number of LHRH neurons found in the rat brain is small and

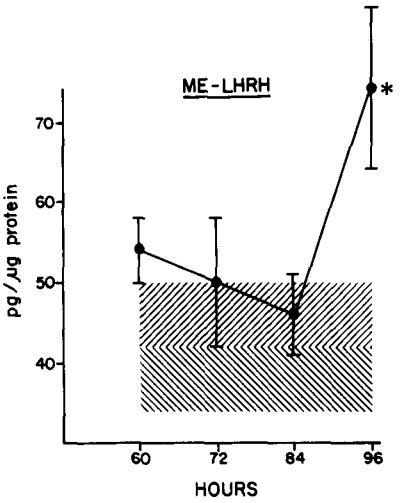

Fig. 3. LHRH concentrations (pg/μg proteins) in the microdissected median-eminence of castrated rats at 60–96 h after implantation of Testosterone. The shaded area depicts the average LHRH concentrations and 95% confidence intervals in the ME of castrated controls. The ME LHRH concentration following T treatment remained within the castrated range up to 84 h followed by an abrupt and significant increase at 96 h. (* <0.01) (From Ref. 40 with permission).

they are distributed widely, it has been technically difficult to utilize the technique of radiolabeled precursor protein incorporation for assessing the synthetic activity of these neurons. An alternative approach has been the microdissection technique of Palkovits[39] for isolation and identification of specific diencephalic nuclei which may show characteristic LHRH changes at intervals after gonadal steroid treatment.

We isolated 9 relevant nuclei from the preoptic tuberal pathway of castrated rats at 60, 72, 84 and 96 h after the s.c. implantation of T [40]. As expected, these implants produced physiological serum T levels of around 2 ng/ml, suppressed LH levels to those seen in intact rats, but there was no immediate effect of T on LHRH concentrations in any of the regions examined. In fact, 8 of the 9 regions known to contain LHRH perikarya and fibers, viz. the medial and septal POA, suprachiasmatic nuclei, RCA and lateral ARC, or fibers alone such as ARC and anterior hypothalamic areas displayed no change in LHRH concentrations at any time after T treatment. The ME was the only region at which significant elevations in LHRH were detected at 96 h (Fig. 3). It is interesting that the ME LHRH concentrations remained within the range seen in castrated control rats at 60, 72 and 84 h after T implantation concomitant with suppressed LH levels. An additional 12 h of exposure to T resulted in a dramatic and significant increase in the ME LHRH concentrations. This observation of a discrete temporal event in the ME between 84 and 96 h, in response to T treatment despite rapid inhibition of LH release, supported our view that augmentation of LHRH levels was not due

to inhibition of LHRH release, but resulted from either new synthesis or increased rate of processing of precursor LHRH into the immunoactive form within nerve terminals in the ME [40]. This steroid-induced accumulation of LHRH specifically in the ME is in agreement with the earlier immunocytochemical studies of Gross in male rats [9] and our studies in steroid-treated ovariectomized rats [41]. Cumulatively these lines of evidence favor the view that T-induced LHRH increase may occur within nerve terminals in the ME. These findings, taken together with the results of intracranial implantation of steroids clearly suggest that steroid-concentrating cells in the MBH exclusively may participate in elicitation of the ME LHRH response.

CONCLUSIONS

Although indirect, the evidence discussed here suggests that gonadal steroids may direct increased synthesis and storage of LHRH in the basal hypothalamus. Detailed examination of the time course and central site of steroidal action revealed that steroids may augment the appearance of the biologically and immunologically active decapeptide predominantly in the axons and terminals in the ME region. It is apparent that LHRH neurons are extremely sensitive to changes in steroid titers particularly those biochemical processes which increased LHRH levels. Since we found no effect of low steroid levels on LHRH release (both LH levels as well as LHRH output *in vitro*), it is reasonable to presume that these concentrations may primarily regulate the formation and storage of LHRH. On the other hand, on the basis of *in vitro* data it appears that physiological levels of T not only augment LHRH synthesis, but may also promote release into the hypophyseal portal vessels. Thus, our studies indicate that LHRH production may occur at two levels: the gonadal steroid-independent level, perhaps intrinsic to the peptidergic neurons, as seen in castrated rats, and the gonadal steroid-dependent level which may not be related to suppression of LHRH release since the steroids appear to directly augment appearance of new neurohormone in the MBH [2]. Conclusive proof for this hypothesis must await direct analysis of LHRH synthesis with radiolabeled precursor proteins incorporation and also reliable measurements of LHRH release rates *in vivo*.

Acknowledgements—These studies were supported by a grant from the National Institutes of Health, HD 11362. Thanks are due to Ms Sally McDonell for typing the manuscript.

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