Study of the pharmacological modification of neuroendocrine mechanisms controlling ovulation in the rat.

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STUDY OF THE PHARMACOLOGICAL MODIFICATION OF NEUROENDOCRINE MECHANISMS CONTROLLING OVULATION IN THE RAT

Being a thesis presented to the Council for National Academic Awards as partial fulfilment of the requirements for the degree of Doctor of Philosophy

by

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Declaration

While registered as a candidate for the award I confirm that I have not been a candidate for any other award of CNAA or of any University.

CONTENTS

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ANN WILSON STEWART

Abstract

Study of the Pharmacological Modification of Neuroendocrine Mechanisms Controlling Ovulation in the Rat

Ann W Stewart

The synthetic steroid, RMI 12,936 (17β-hydroxy-7α-methyl-androst-5-en-3-one) inhibits ovulation in the rat. This study investigated the mechanism of action of the drug. The results showed that administration at, or before 01:00h on proestrus, blocked the preovulatory LH surge which was restored by LHRH or oestradiol plus progesterone. Administration of oestradiol alone restored ovulation to only 43% of RMI 12,936 treated rats. The negative feedback effect of testosterone on LH release showed similar characteristics. Although RMI 12,936 was shown to be a potent androgen, the peripheral androgenic activity was found not to be correlated with its inhibitory effect on LH release. Instead, it was suggested that RMI 12,936 may act through antioestrogenic and antiprogestational activity.

Investigation of the site of action revealed effects a) at the ovarian level, inhibiting the biosynthesis of oestrogen and progesterone, b) at the adenohypophysial level, preventing full sensitization to LHRH and c) at the hypothalamic level, inhibiting noradrenergic and tryptaminergic neurotransmission. The first two are not the main sites of antiovulatory activity since administration of oestradiol plus progesterone, although they restore ovulation cannot restore full sensitization and secondly since RMI 12,936 injected into the third cerebral ventricle does not require to be transported to the ovary for effective ovulation blockade. The major site of action was therefore at the hypothalamic level where RMI 12,936 blocks the neural signal mediated by noradrenaline and triggered by oestrogen.

Based on the premise that RMI 12,936 has a similar mechanism of action to that of testosterone, it was proposed that the drug prevents full sensitization of the adenohypophysis by reducing the number of LHRH receptors. During this investigation, adenohypophysial sensitivity to LHRH altered. From observation throughout the year, a pattern emerged indicating the existence of seasonal variation in the mechanisms controlling LH release. This aspect requires further investigation.

PART I

GENERAL INTRODUCTION

Over the past 30 years a great deal of information has been learned about the processes leading to the ovulatory response. Briefly the preovulatory rise in plasma luteinizing hormone (LH) involves the positive feedback by the sex steroids secreted from the ovary. The steroids may act at the level of the hypothalamus to trigger a neural signal for release of the LH-releasing hormone (LHRH) and at the level of the adenohypophysis to increase its responsiveness to the releasing hormone. In turn, LHRH acts on the adenohypophysis to stimulate release of LH and at the same time further increases the sensitivity of the adenohypophysis. A positive feedback action on the hypothalamus by LHRH (ultra-short loop feedback) and LH (short loop feedback), each contribute to further increase in LH secretion. This cascade of events leads to a surge in LH secretion which acts on the ovary to rupture the ripened follicles, releasing the ova.

THE HYPOTHALAMUS

Levels of hypothalamic control

Isolation of the hypothalamo-hypophysial unit from the rest of the brain using lesioning, has shown that this unit is responsible for maintenance of basal secretion of LH (Halász, 1969). However ovulation is lost suggesting that another area must be responsible for this control. This led to the "dual control" hypothesis according to which neural control of the adenohypophysis occurs at two levels. One level represented by the hypophysiotrophic area, regulates basal secretion of LH from the adenohyphysis.

This has been illustrated by the finding that LH release is maintained at a very low rate after the anterior deafferentation of this region (Halász, 1969). The low rate of gonadotrophin release is sufficient to maintain the ovaries and prevent their atrophy. Implants of oestrogen into this area in amounts which have no effect systemically, inhibit LH secretion and cause gonadal atrophy indicating that this area is also the site of negative feedback action (Ramirez, Abrams & McCann, 1964).

The hypophysiotrophic area (fig. 1) a term coined by Halász, Pupp and Uhlarik (1962) for the half-moon shape region of the medial-basal hypothalamus (MBH), incorporates the anterior periventricular nucleus, the medial part of the retrochiasmatic area and ventromedial nucleus and the arcuate nucleus (AN).

A second level of control is represented by all other hypothalamic and extrahypothalamic areas involved in the control of ovulation, including the suprachiasmatic-preoptic area. Studies such as electrical or electrochemical stimulation of the POA which induced ovulation in rats in which spontaneous ovulation had been blocked by barbiturate anaesthesia (Terasawa & Sawyer, 1969; Cramer & Barraclough, 1971) indicated that this higher level of control is responsible for the cyclic release of LH. These areas are believed to be the sites of positive feedback of oestrogen since implants of small amounts of oestrogen into the POA in immature female rats induces premature onset of cyclic LH release, while the LH surge

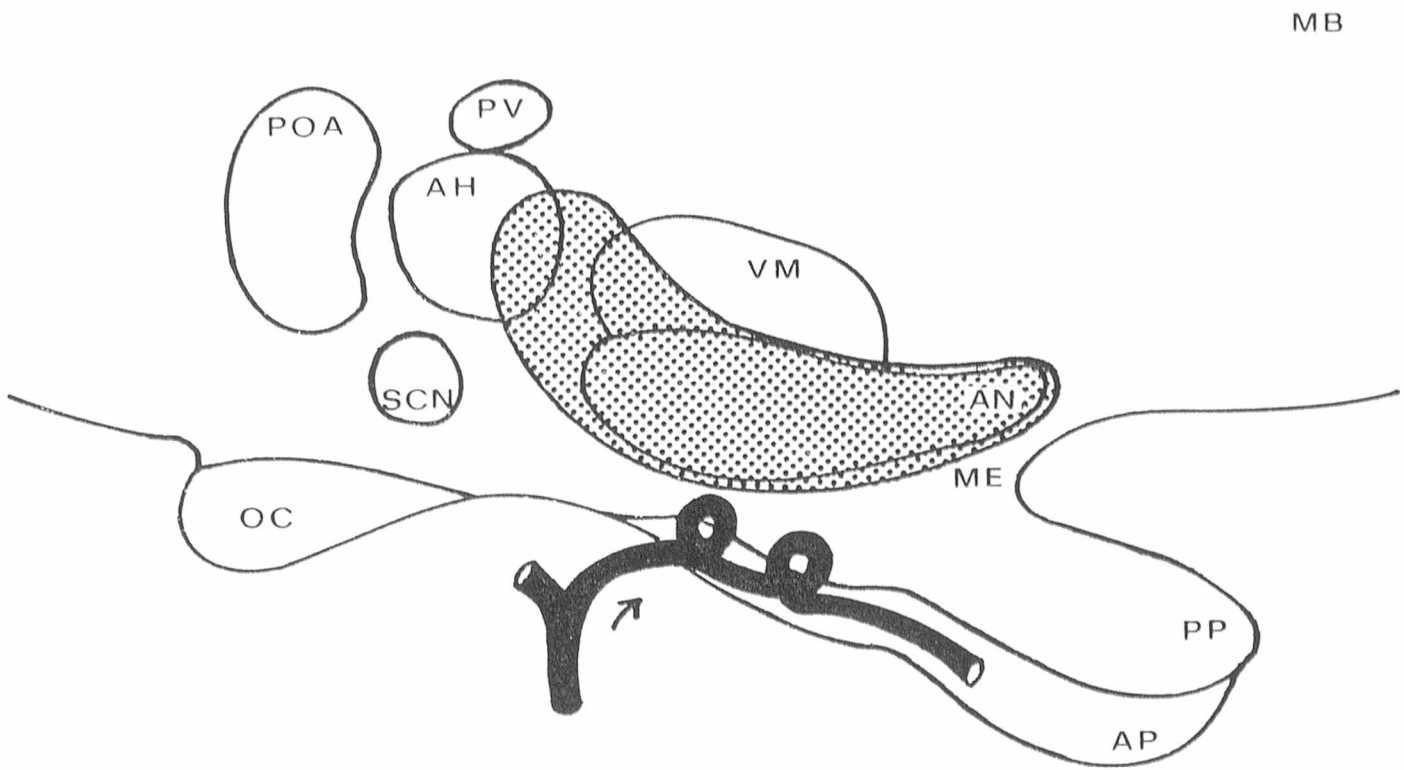


Fig. 1 Schematic representation of the anatomical relationship between the preoptic area (POA) and medial basal hypothalamus including the hypophysiotrophic area (stippled area). AH, anterior hypothalamus; AN, arcuate nucleus; AP, adenohypophysis; MB, midbrain, ME, median eminence; OC, optic chiasma; PP, neurohypophysis; PV, paraventricular nucleus; SCN, suprachiasmatic nucleus; VM, ventromedial nucleus.

which can be induced with progesterone in oestrogen-primed rats, cannot be induced if the suprachiasmatic area (SCN) is lesioned (McCann, 1974).

Localization of LHRH within the hypothalamus

Evidence gathered from studies using radioimmunoassay, bioassay and immunocytochemical techniques has demonstrated that LHRH is most concentrated in the median eminence (ME) (Palkovits, Arimura, Brownstein, Schally & Saavedra, 1974; Vale, Rivier, Palkovits, Saavedra & Brownstein, 1974; Wheaton, Krulich & McCann, 1975; Baker, Dermody & Reel, 1975; Sétáló, Vigh, Schally, Arimura & Flerko, 1975). It has been reported that LHRH is present within neurones originating from cell bodies in the MBH, particularly the AN, and in the septal-preoptic region, particularly the POA and SCN (Baker et al, 1975; Silverman, Krey & Zimmerman, 1979; Samson, McCann, Chud, Dudley & Moss, 1980). It was also reported that both neurones terminate in the ME in the immediate vicinity of the capillary loops at the beginning of the hypophysial portal system (Pelletier, Labrie, Puviana, Arimura & Schally, 1974; Goldsmith & Ganong, 1975), suggesting that on stimulation of the neurone, the hormone is released from the nerve terminals in the ME and passes into the portal vessels to be transported to the adenohypophysis. However in a recent study, it was shown that the main source of LHRH in the ME of the rat is in the septalmedial preoptic region and not the MBH (Merchenthaler, Kovács, Lovász & Sétálo, 1980). These authors claimed that the tuberoinfundibular tract does not contain LHRH axons as earlier studies had suggested. Therefore the

LHRH axons of the MBH originate from extrahypothalamic areas and approach the ME through the preoptic-infundibular LHRH tract (see fig. 2).

Significant concentrations of LHRH have also been found in the organum vasculosum of the lamina terminalis (OVLT) which is located at the rostral end of the third ventricle (Baker et al, 1975; Sétáló et al, 1975; Kiser, Palkovits & Brownstein, 1976). LHRH fibres to the OVLT appear to originate from the POA (Palkovits, Mezey, Amback & Kivovics, 1978).

The physiological significance of LHRH in the OVLT is unclear although the results of neural deafferentation and OVLT destruction suggest that it may be involved with ovulation (Halász & Gorski, 1967; Samson & McCann, 1979; Piva, Limonta & Marini, 1982). This role is supported by the fact that the LHRH content of the OVLT fluctuates during the different phases of the oestrous cycle(Wenger & Leonardelli, 1980) (see fig. 2).

The localization of LHRH just described indicates that these neurones would be in possible synaptic contact with a host of putative synaptic transmitters. Specific immunocytochemical studies have shown that the catecholamines and LHRH are synthesized and transported in distinct neural systems in the hypothalamus and that the terminals of many of these neurones end in close proximity to the portal vessels in the external layer of the ME (Hökfelt, Elde, Fuxe, Johansson, Ljungdahl, Goldstein, Luft, Efendic, Nilsson, Terenius, Ganten, Jeffcoate, Rehfeld, Said, Perez de la Mora, Possani, Tapia, Terran & Palacios, 1978). The neuroanatomy of the monoaminergic pathway is

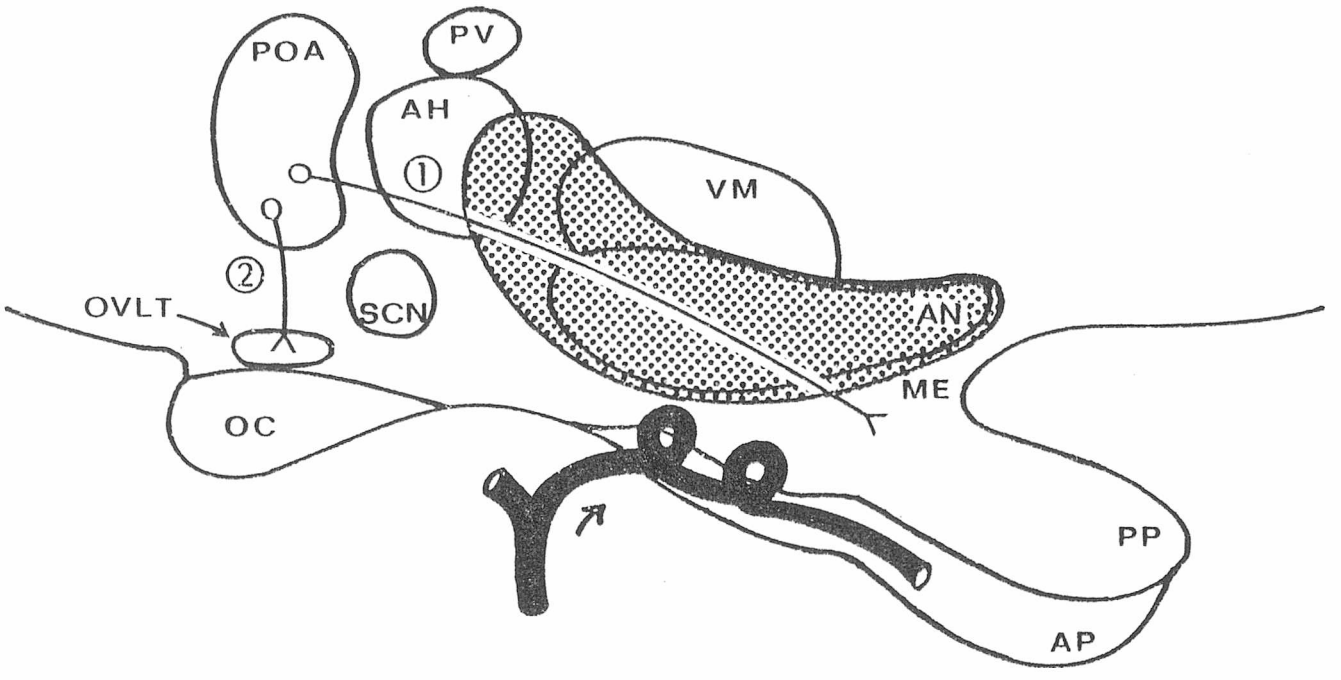


Fig. 2 Schematic representation of the LHRH pathways believed to be involved in the release of LH from the adenohypophysis. The area is as depicted in Fig. 1 with the exception of OVLT which represents the organum vasculosum of the lamina terminalis.

① The preopticoinfundibular LHRH tract.

② The preoptico-OVLT LHRH tract.

described in an excellent review by Palkovits (1978). Therefore this discussion shall concentrate only on the pathways specifically concerned with the control of ovulation.

Noradrenergic pathways

The ventral noradrenergic pathway (VNP) originating in the reticular formation in the midbrain and providing a dense input to the hypothalamus and basal telencephalon, is believed to be involved in the release of LHRH (see fig. 3). This is supported by evidence from studies where destruction of the tract results in reduction of LH release (Kitchen, 1974; Martinovic & McCann, 1977) and on NA administration, ovulation is restored (Tima & Flerkó, 1974). Also, electrochemical stimulation of this tract, resulted in ovulation in constant oestrous rats (Carrer & Taleisnik, 1970). There is some recent evidence which suggests that this pathway is not in fact mandatory in the normal regulation of LH secretion since destruction of the VNP with 6-hydroxydopamine produced only a temporary block of the preovulatory type of LH release (Clifton & Sawyer. 1979). It was concluded rather that NA plays a modulatory role in the control of ovulation. However since NA nerve terminals of the VNP are located in the same general region as the rostral LHRH neurones, in particular the POA, SCN, AN and ME, then it would seem likely that this catecholamine influences the release of LHRH from the ME.

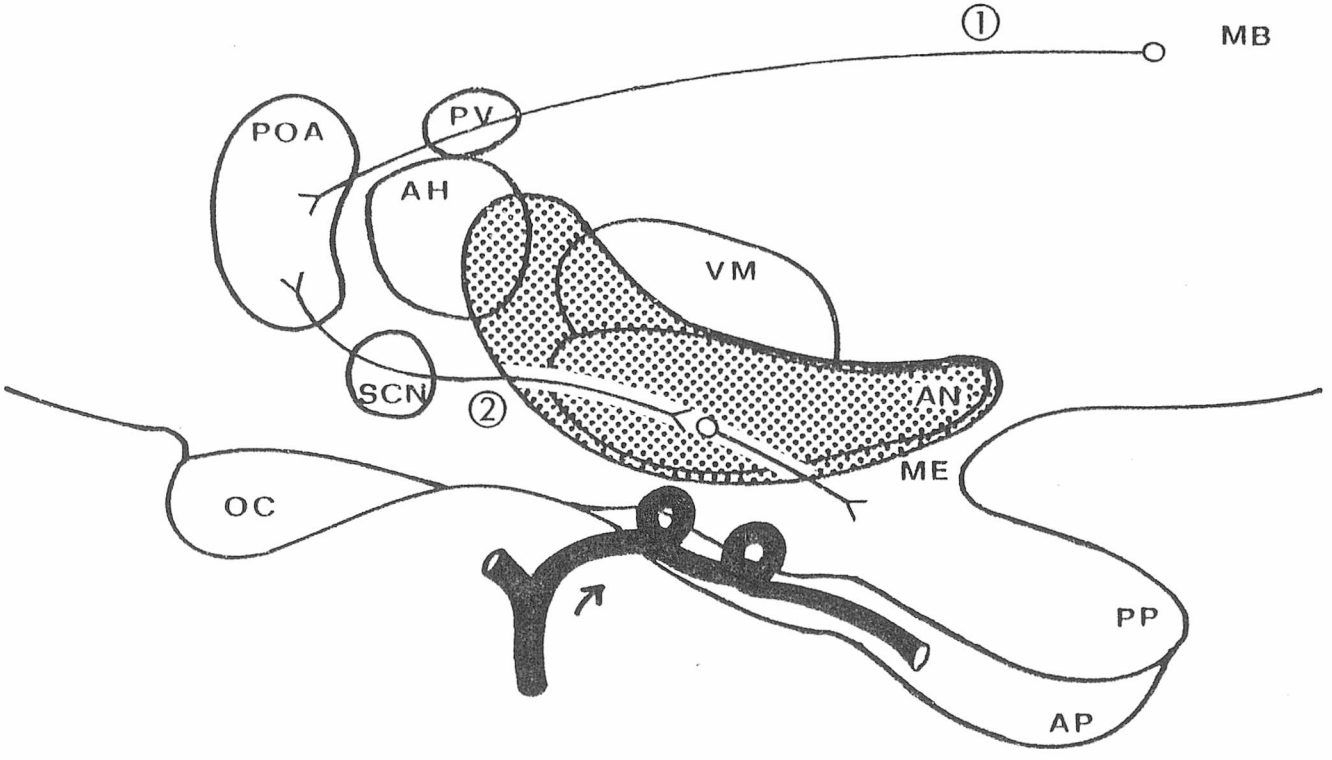


Fig. 3 Schematic representation of noradrenergic pathways believed to be involved in the control of ovulation. The area is as depicted in Fig. 1.

① The ventral noradrenergic pathway.

② The preoptico-tuberal noradrenergic pathway.

Dopaminergic pathways

A short intra-hypothalamic dopaminergic tract (see fig. 4) originates mainly from the arcuate and ventral periventricular nuclei and innervates the external layer of the ME (Fuxe & Hökfelt, 1969). The role of this tuberoinfundibular tract (TIDA) in the regulation of LH release is as yet unclear with evidence suggesting that it plays an inhibitory role (Miyachi, Mecklenburg & Lipsett, 1973; Gallo & Osland, 1976; Löfström, Eneroth, Gustafsson & Skett, 1977) while other evidence has been presented suggesting that it plays an excitatory role (Schneider & McCann, 1969; Kambēri, Mical & Porter, 1970; Bennet, Edwardson, Holland, Jeffcoate & White, 1975). Explanation of these discrepancies is not readily apparent although it is noteworthy that whenever there is a blockade of ovulation such as during pregnancy, pseudopregnancy and lactation, there is an activation of the TIDA neurones. On the other hand, the turnover of DA in the TIDA dopaminergic tract is reduced, preceding and during the preovulatory LH surge (Fuxe & Hökfelt, 1970). The findings of Hökfelt & Fuxe (1972) provide a good correlation between the TIDA system and the LH secretion. These authors reported that gonadal and certain anti-fertility steroids cause a dose dependent activation of this system suggesting that the effects observed are part of a feedback action of these hormones on the brain.

Anatomical considerations indicate that DA release from nerve terminals in the ME influences the release of LHRH

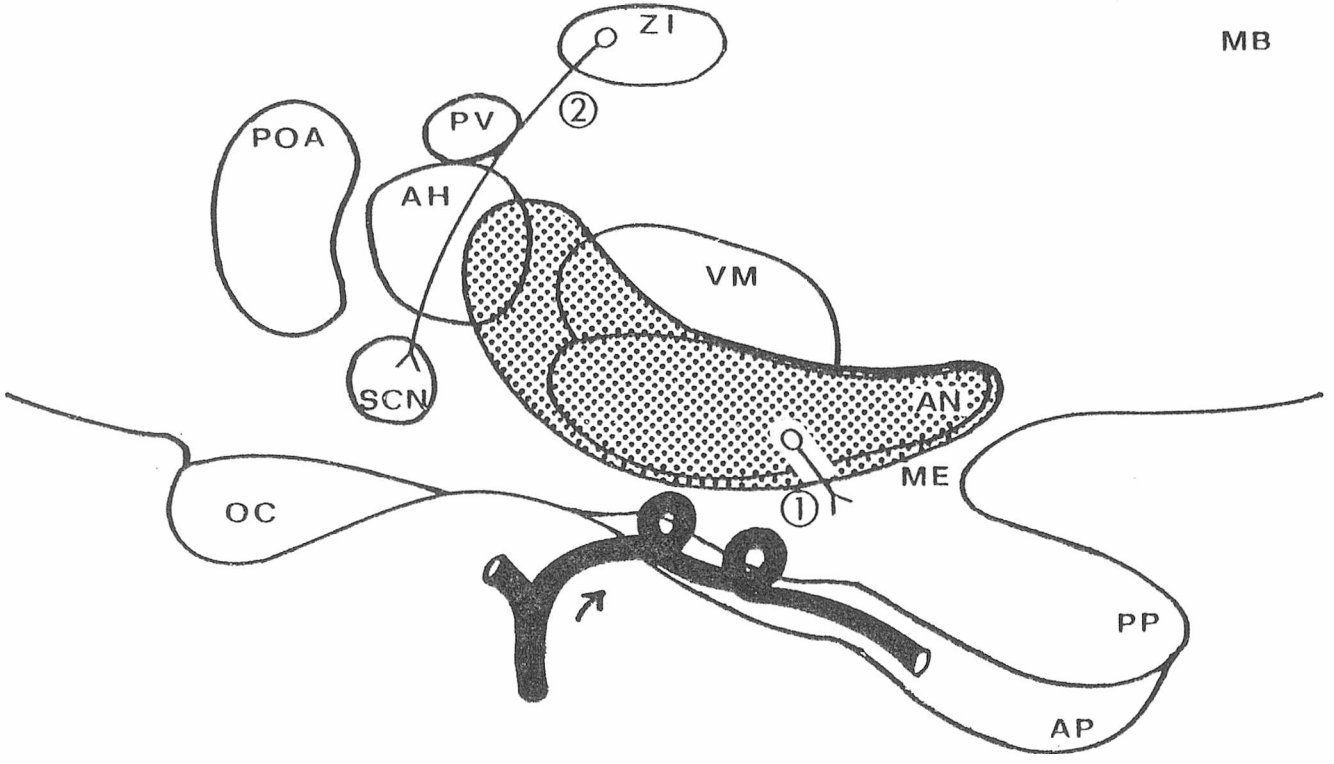


Fig. 4 Schematic representation of dopaminergic pathways believed to be involved in the control of ovulation. The area is as depicted in Fig. 1 with the exception of ZI which represents the zona incerta.

① The tuberoinfundibular dopaminergic pathway.

② The incertohypothalamic dopaminergic pathway.

probably by axoaxonic contact with LHRH terminals (Hökfelt & Fuxe, 1972). Histological evidence supports this hypothesis showing that the distribution pattern of LHRH, localized preferentially in the lateral perivascular region of the ME coincides well with that of DA terminals (Ajika & Hökfelt, 1973).

A second more recently discovered dopaminergic pathway must also be considered in the control of ovulation (Bjorklund, Lindvall & Nobin, 1975). This incerto-hypothalamic pathway (see fig. 4) extends from the zona incerta to the hypothalamus. The projection areas of these incerto-hypothalamic neurones signify an involvement of the system in the control of LH secretion. Pharmacological evidence has shown this pathway to be stimulatory to LH release (Kawakami, Manaka & Kawagoe, 1975; Naumenko & Serova, 1976).

Tryptaminergic pathways

The midbrain raphe region contains the cell bodies of an ascending 5'HT neuronal system that sends rostral projections to the hypothalamus and other forebrain areas. In the hypothalamus, the nerve terminals are found in the AN, ME, SCN and POA (Hamon, Javoy, Kordon & Glowinski, 1970; Saavedra, Palkovits, Brownstein & Axelrod, 1974) which are notably areas in which LHRH tracts are located. Electrochemical stimulation of the midbrain has been shown to block spontaneous ovulation as well as progesterone induced LH release in proestrus or ovariectomized, oestrogen-primed rats (Carrer & Taleisnik, 1970 and 1972). This

inhibitory effect was localized in particular to the dorsal raphe nucleus (DRN) which on electrical stimulation, resulted in inhibition of episodic release of LH (Arendash & Gallo, 1978). Drug induced increase in MBH 5'HT and electrical stimulation of the AN resulted in a block of spontaneous ovulation or a reduction in LH release (Kordon, 1969; Gallo & Moberg, 1977). These results imply that the MBH area is involved in the inhibitory effect of 5'HT on episodic LH secretion and suggest that the inhibitory 5'HT tract originates in the DRN and terminates in the AN/ME region (fig. 5).

Several investigators have found that instead of having an inhibitory effect on LH release and ovulation, 5'HT has in certain circumstances, a permissive or facilitatory effect (see role of 5'HT in the control of ovulation). Destruction of 5'HT neurones in the midbrain and in particular the DRN, led to a reduction in the circadian release of LH (Héry, Laplante & Kordon, 1978; Meyer, 1978). Because the SCN is very rich in 5'HT (Saavedra et al, 1974) and its destruction blocks spontaneous ovulation or oestrogen-induced LH release (Brown-Grant & Raisman, 1977; Kawakami, Arita & Yoshioka, 1980) it has been suggested (Kordon, 1978) that the permissive 5'HT tract originates in the DRN also but terminates in the POA/SCN region (fig. 5).

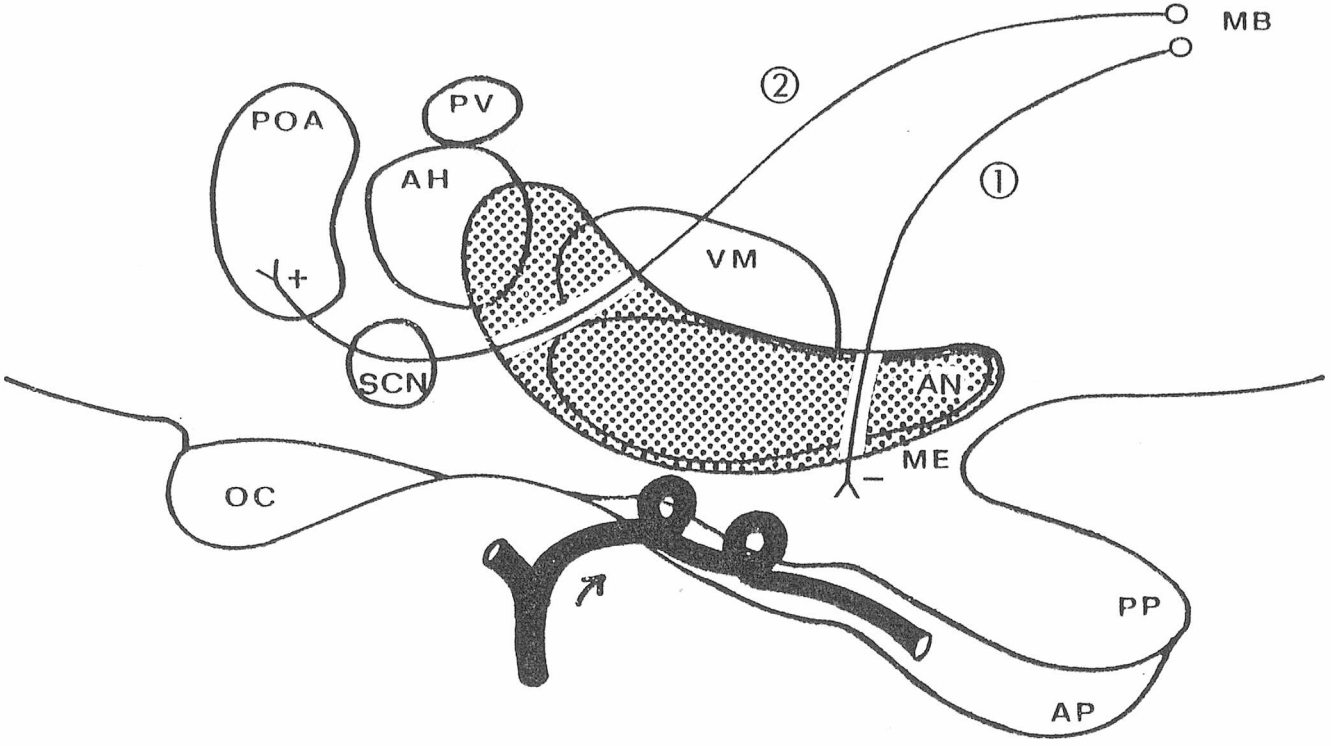


Fig. 5 Schematic representation of tryptaminergic pathways believed to be involved in the control of ovulation. The area is as depicted in Fig. 1.

① The dorsal-tuberal tryptaminergic pathway (inhibitory).

② The dorsal-preoptico tryptaminergic pathway (permissive).

PHARMACOLOGICAL EVIDENCE FOR CENTRAL NEUROTRANSMITTER INVOLVEMENT IN THE CONTROL OF OVULATION

There is little doubt that the release of LHRH and through it LH, are controlled by the central neurotransmitter systems, which according to present concepts include the noradrenergic, dopaminergic and tryptaminergic systems although other putative systems for instance cholinergic and histaminergic systems were also considered. This view is corroborated by recent research on the distribution of the neurotransmitters in the central nervous system which shows that they are in high concentrations in certain hypothalamic areas which also contain high concentrations of LHRH. It is further supported by copious experimental data on the effects of the individual transmitters or of any drugs which mimic or inhibit their action on the secretion of LH from the adenophypophysis.

The role of noradrenaline in the control of ovulation

The involvement of an adrenergic link in the release of ovulating hormone was suggested by the early work of Sawyer, Markee and Hollinshead (1947) who showed that the α-adrenergic blocking drug, dibenamine, would inhibit ovulation. However it was later shown that not all α-adrenergic blockers are effective (Sawyer, Markee & Everett, 1950) and stress or even direct action on the ovary may have accounted for their antiovulatory action (Moore, 1961; Ferrando & Nalbandov, 1969). Subsequently, reserpine, a drug which depletes hypothalamic stores of monoamines and α-methyl-p-tyrosine (α MPT), a drug which inhibits the synthesis of monoamines were also shown to

block ovulation (Coppola, Leonardi & Lippmann, 1966; Meyerson & Sawyer, 1968; Rubinstein & Sawyer, 1970; Craven & McDonald, 1971). Furthermore the rate of disappearance of NA after pretreatment with α MPT (which is also a measure of release) increases on proestrus when LH release is increased (Coppola, 1969). The results showing rises in plasma NA just before the spontaneous preovulatory LH surge also suggest an increase in release, as NA is thought to originate from increased noradrenergic neuronal activity, releasing NA into the cerebrospinal fluid and thence into the peripheral plasma (Nagle & Rosner, 1976; Rosner, Nagle, De Laborde,Pedroza, Badano, Figuero, Casas & Carril, 1976). Coppola and his coworkers showed that only those drugs that depleted amines within the brain were antiovulatory. Drugs acting solely as peripheral amine depletors had no effect on ovulation (Coppola et al, 1966; Lippman, Leonardi, Ball & Coppola, 1976; Coppola, 1968). More specific NA synthesis inhibitors such as diethyldithiocarbamate (DDC), FLA 63 and U14624 which are all dopamine-β-hydroxylase (DBH) inhibitors, inhibit the spontaneous preovulatory LH surge, the LH surge induced by electrochemical stimulation of the POA or by progesterone treatment in oestrogen primed ovariectomized rats (Kalra, Kalra, Krulich, Fawcett & McCann, 1972; Kalra & McCann, 1973). Inhibition of NA synthesis by U14624 has been shown to reduce peripheral plasma LHRH as well as LH (Kalra,1977). Dihydroxyphenyl-serine (DOPS) which is converted in the body directly to NA, by-passing DA, can reverse the effect of these NA synthesis inhibitors whereas dihydroxyphenylalanine (DOPA) which is converted preferentially to DA is less effective

(Hyyppä, Lehtinen & Rinne, 1971; Kalra et al, 1972; Sarkar & Fink, 1981). These experiments show that normal NA synthesis is required for the occurrence of a full LHRH surge.

Measurement of hypothalamic NA content and turnover showed them to be related to stages of the ovulatory cycle or to levels of gonadal steroids in the blood. The hypothalamic NA levels and turnover are higher on the day of proestrus compared to other days of the cycle (Stefano & Donoso, 1967; Zschaeck & Wurtman, 1973; Selmanoff, Pramik-Holdaway & Weiner, 1976; Kueng, Wirz-Justice, Menzi & Chappuis-Arndt, 1976; Löfström, 1977; Negro-Vilar, Chiocchio & Tramessani, 1977). The peak of NA activity occurs on the afternoon of proestrus, just before the LH surge (Kueng et al, 1976; Selmanoff et al, 1976). Further investigations have shown that during conditions of high LH secretion such as after ovariectomy, after oestrogen plus progesterone treatment or on proestrus, NA activity tends to be high. Under conditions where LH secretion is low ie dioestrus or in oestrogen-primed ovariectomized rats, NA activity tends to be low (Donoso & Stefano, 1967; Zschaeck & Wurtman, 1973; Everitt, Fuxe, Hökfelt & Jonsson, 1975; Löfström, 1977; Löfström et al, 1977; Munaro, 1977; Honma & Wuttke, 1980). Measurement of NA turnover rates in discrete hypothalamic nuclei and in the ME showed that ME NA turnover increased during the afternoon of proestrus. This increase coupled with the concomitant decline in ME LHRH levels and the rise in plasma LH levels suggest that increased NA release may

be important in initiating the preovulatory LH surge (Rance, Wise, Selmanoff & Barraclough, 1981).

Several investigators have studied the effects of intraventricular infusions of catecholamines on LH release and/or ovulation. NA was found to have a stimulatory effect on LH release in ovariectomized, oestrogen-, progesterone-primed rats, in oestrogen-primed rabbits, in rats made anovulatory by electrolytic lesions in the anterior hypothalamus and in male rats (Sawyer, Hilliard, Kanematsu, Scaramuzzi & Blake, 1974; Vijayan & McCann, 1978a; Kawakami & Ando, 1981). However a dual effect was demonstrated by Cramer and Barraclough (1978) where NA (3×10^{-6}M) inhibited LH secretion while lower doses of NA (3×10^{-8}M) potentiated the secretion of LH after electrical stimulation of the preoptic area.

In conclusion the majority of current evidence suggests that NA plays an excitatory role in secretion of LH.

The role of dopamine in the control of ovulation

In contrast to that of the central noradrenergic system, the role of the dopaminergic system in the regulation of LH secretion is highly controversial. There are two main bodies of thought. One is that DA plays an inhibitory role in the control of ovulation and the other that DA plays an excitatory role.

Evidence for an inhibitory role :
The existence of an inhibitory dopaminergic influence in

the control of LH secretion has been repeatedly claimed by Swedish investigators on the basis of observations using catecholamine fluorescence. These investigators pointed out that the turnover rate of DA in the ME of rats in various reproductive conditions is low when gonadotrophin secretion is high, as in castrates, or during the preovulatory LH surge. Conversely, DA turnover is high when gonadotrophin secretion is low, as in the lactating female rat or where sex steroids are exerting an inhibitory feedback effect (Fuxe & Hökfelt, 1970; Löfström et al, 1977).

Evidence from various pharmacological studies also support an inhibitory role for DA. Administration of DA or apomorphine to ovariectomized rats inhibited LH release (Drouva & Gallo, 1977; Vijayan & McCann, 1978b) whereas the administration of pimozide, a specific dopaminergic blocker, prevented this effect and in some cases enhanced the pulsatile release of LH (Gallo, 1978). Intrahypothalamic infusion of DA (80µg) blocked spontaneous ovulation in proestrous rats (Craven & McDonald, 1973). Moreover, Uemura and Kobayashi (1971) demonstrated that the rats implanted with DA into the AN-ME region showed prolonged anovulatory states while Miyachi, Mecklenburg and Lipsett (1973) using the ME - pituitary unit, demonstrated that DA could inhibit LH release *in vitro*. It was later reported that intraventricular DA was ineffective in inducing ovulation or stimulating LH release (Tima & Flerkó, 1974; Krieg & Sawyer, 1976).

Furthermore, intraventricular DA not only failed to stimulate LH release, but actually inhibited subsequent stimulation of LH release induced by NA in the rabbit (Sawyer et al, 1974).

Evidence for an excitatory role :

In vitro administration of DA to the ME stimulated LHRH secretion even when conversion of DA to NA was blocked by the prior administration of DDC (Negro-Vilar, Ojeda & McCann, 1979). Also the administration of L-dopa and lergotrile, a DA agonist, advanced the time of the LH surge in cyclic female rats (Wedig & Gay, 1974; Clemens, Tinsley & Fuller, 1977). Blockade of DA receptors by pimozide or γ-butyrolactone (GBL) inhibited ovulation (Beattie, Gluckman & Corbin, 1976). Studies involving intraventricular injection of DA showed that LH secretion was stimulated on administration of DA to ovariectomized, oestrogen-, progesterone-primed rats, oestrogen-primed ovariectomized rats, male rats and to female rats on proestrus (Kamberi, Mical & Porter, 1969; McCann, 1974; Vijayan & McCann, 1978b). It was also reported that intraventricular injection of DA stimulated release of LHRH into the portal vessels (Kamberi, Mical & Porter, 1970).

In vitro studies show that DA can stimulate LH release from the adenohypophysis when coincubated with hypothalamic tissue (Schneider & McCann, 1969; McCann, 1974) and can stimulate the release of LHRH from hypothalamic synaptosomes (Bennett et al, 1975) as well as from fragments of the mediobasal hypothalamus

(Rotsztein, Charli, Pattou & Kordon, 1977).

Recent studies may help to explain these divergent findings. Low doses of apomorphine were shown to inhibit LH release in ovariectomized rats while high doses were stimulatory (Becke & Wuttke, 1977). These authors suggested that high concentrations of DA or chronic administration leads to desensitization of the DA receptors and thence lack of inhibition of LH release. The stimulatory effect of the incerto-hypothalamic tract can now be expressed and hence DA has a stimulatory effect on LH release. Consequently, the results are compatible not only with the concept that DA may stimulate LHRH release but also that it may inhibit release of LHRH. However opposite results were found by Vijayan and McCann (1978b) and Kamberi and coworkers (1970) who found that low doses of DA stimulate LH secretion whereas high doses inhibit LH release.

Another approach which supports this dual role of DA comes from experiments in which DA has been injected into specific areas of the brain. Injection into the AN-ME region has shown DA to be ineffective or inhibitory (Uemura et al, 1971; Porter, Kamberi & Ondo, 1972; Craven & McDonald, 1971) indicating that the tuberoinfundibular tract is inhibitory to LH release. This is further supported by studies which show that DA turnover in this region falls over proestrus (Lichtensteiger,1969) and that DA concentrations in the hypophysial portal blood also fall on proestrus (Cramer,

Parker & Porter, 1979). However DA implanted in the POA in oestrogen-primed ovariectomized rats induced an LH surge (Kawakami et al, 1975). Injection of DA into the zona incerta or periventricular gray of the caudal thalamus raised testosterone levels in immature intact male rats (Naumenko & Serova, 1976). These last two sites are areas of DA cell bodies of the incerto-hypothalamic tract which send axons to the AH. Similarly the POA is a site of dopaminergic nerve terminals of the same tract (Björklund et al, 1975). Thus it would appear that the incerto-hypothalamic tract is stimulatory to LH release while the TIDA tract is inhibitory. Activation of one or the other pathways by different experimental approaches may explain the conflicting results on the role of central dopaminergic neurones in the neural control of LH release. This concept of a dual role for DA on LH release is consistent with the existence centrally of two pharmacologically distinct DA receptors (Kebabian & Calne, 1979). The stimulatory effect of dopamine on LHRH (and thereby LH) release may be due to an action on DA receptors that are inhibited by haloperidol and stimulated by a high dose of apomorphine (Beck & Wuttke, 1977; Sarkar & Fink, 1981), while the inhibitory effect of DA on LHRH release may be due to an action on receptors that are inhibited by pimozide or domperidone and facilitated by a low dose of apomorphine (Beattie et al, 1976; Beck & Wuttke, 1977; Owens, Fleeger & Harms, 1980; Sarkar & Fink, 1981). This would suggest that the incerto-hypothalamic dopaminergic tract possess the former type of

receptor while the TIDA dopaminergic tract possesses the latter type.

It has been suggested that DA may not in fact play an active part in the induction of the preovulatory surge itself because at that time in the ovarian cycle both the turnover of DA in the ME and the concentration of DA in portal blood are at their lowest (Ben-Jonathan, Oliver, Weiner, Mical & Porter, 1977; Löfström, 1977) but that its main physiological role is in the control of prolactin secretion (see Krulich, 1979).

The role of 5'HT in the control of ovulation

There is considerable evidence to support the view that 5'HT plays an inhibitory role in the control of ovulation. In particular, systemic injections of large amounts of the amine (O'Steen, 1965), direct infusion of the transmitter into the third ventricle (Kamberi et al, 1970; Schneider & McCann, 1970), electrical stimulation of the dorsal raphe and arcuate nuclei and/or treatment with drugs that increase brain levels of 5'HT (Kordon, Javoy, Vassent & Glowinski, 1968; Lippman, 1968; Gallo & Moberg, 1977; Arendash & Gallo, 1978). However various other pharmacological studies have indicated that 5'HT has an excitatory role on ovulation. Raising 5'HT levels stimulates LH release and ovulation (Brown, 1966 and 1967; Wilson, Endersby & McDonald, 1974), lowering 5'HT with pCPA inhibits release of LH and prevents ovulation (Héry, Laplante, Pattou & Kordon, 1975; Wilson, Andrews, Hadley, Lemon & Yeo, 1977) and administration of pCPA to oestrogen-

primed ovariectomized rats abolishes the circadian variation of basal LH levels which is restored by subsequent administration of 5-hydroxytryptophan, a precursor of 5'HT (Héry, Laplante & Kordon, 1976).

5'HT displays a diurnal rhythm increasing daily in the afternoon (Dixit & Buckley, 1967; Quay, 1968; Wilson et al, 1977) and in the early evening (Héry, Rouer & Glowinski, 1972). Prevention of this diurnal increase in hypothalamic 5'HT activity by administration of pCPA (Wilson et al, 1977) or treatment with 5-hydroxytryptophan and pargyline, an antimetabolite, which obscures the circadian rhythm of the amine (Walker,1980b) both result in blockade of the LH surge and ovulation. These experiments show that the circadian rhythm in hypothalamic 5'HT content is essential for ovulation to occur.

Kordon and his coworkers have shown that the time course of the inhibitory and facilitatory effects of 5'HT are very different. Inhibition of the preovulatory LH surge is only possible when treatment is applied within a few hours of the critical period (Kordon et al, 1968) whereas in the case of the facilitatory effect of the amine, 5'HT inhibitors have to be given much earlier in order to interfere effectively with LH release (Héry et al, 1976). Walker (1980a,b) further investigated the temporal involvement of 5'HT in the control of ovulation using 5'HT receptor agonists and antagonists which give much more accurate information than 5'HT precursors and antimetabolites about the actual timing of 5'HT release. Cyproheptadine

and methysergide, 5'HT receptor antagonists, given at 15:30h on proestrus abolished phasic LH secretion and ovulation but were ineffective when administered at 08:00h on the same day. To the contrary, quizapine, a 5'HT receptor agonist, blocked the effect of cyproheptadine on gonadotrophin secretion, enhanced LH levels and induced premature ovulation. As a result of these findings Walker suggested that there is a positive correlation between phasic LH secretion and 5'HT neurotransmission which points to an active role for 5'HT in the phasic secretion of LH.

The experiments outlined provide evidence for a dual effect of 5'HT on LH release and ovulation. Kordon has postulated that the amine exerts thes effects via two different tryptaminergic pathways. A permissive system, passing via the SCN to act on the POA (Héry et al, 1978), which seems to control the rhythmic secretion pattern of a number of pituitary hormones including LH (Scapanini, Moberg, Van Loon, De Groot & Ganong, 1971) and an inhibitory one, which originates in the midbrain and acts on the ME (Kordon, 1969; Arendash & Gallo, 1978; Palkovits, 1978) to inhibit the basal secretion of LH.

Although there is considerable evidence showing that 5'HT can inhibit the basal secretion of LH, it is not yet clear whether the amine has this role under normal physiological conditions. Administration of pCPA had little effect on LH release in male rats (Donoso, Bishop,

Fawcett, Krulich & McCann, 1971) while manipulations of the 5'HT system with various pharmacological agents, did not alter the periodicity, rate or magnitude of the pulsatile LH secretion, suggesting that 5'HT is not an essential part of the neurotransmitter system regulating basal LH secretion (Gallo, 1980). Instead it was suggested that this inhibitory 5'HT system comes into play only under special circumstances eg in anovulatory conditions. The physiological significance of the facilitatory 5'HT system might be to regulate the phasic secretion of LH (Walker, 1980b) and to synchronise various endocrine and autonomous rhythms (Jouvet, 1969).

Other agents involved in the control of ovulation

Although considerable energy has been spent on the elucidation of the role of catecholamines and 5'HT in the control of ovulation, there is evidence to suggest a possible role for other classical and putative neurotransmitters. The drugs used in many of the investigations into the role of NA in LH secretion eg reserpine also affect transmission in central adrenaline systems. Therefore it is possi ble that the effects on LH secretion were due to interference of an adrenergic rather than a noradrenergic system. Adrenaline nerve terminals are present within the periventricular and medial basal hypothalamic areas providing an

anatomical basis for involvement in the control of LH secretion (Hökfelt, Fuxe, Goldstein & Johansson, 1974). It is noteworthy that adrenaline is the most potent catecholamine in stimulating LH release when administered intraventricularly (Rubinstein & Sawyer, 1970; Vijayan & McCann, 1978a). A selective inhibitor of adrenaline biosynthesis, SK & F64139, completely blocked the surge of LH induced by oestradiol plus progesterone, providing direct evidence for a role of adrenaline in the control of LH secretion (Crowley & Terry, 1981; Crowley, Terry & Johnson, 1982).

In the pioneering experiments of Sawyer and Everett in the 1940 s subcutaneous administration of large doses of atropine, a muscarinic antagonist, were shown to block ovulation (Everett, Sawyer & Markee, 1949; Sawyer, Everett & Markee, 1949). Later, administration of atropine both subcutaneously and intraventricularly, was shown to block LH release (Libertun & McCann, 1973) but the doses used in these experiments were very large and therefore raises the question as to the specificity of atropine. However a role for acetylcholine in the control of LH release was supported by further experiments in which intraventricular injection of acetylcholine or pilocarpine, a muscarinic agonist, resulted in elevation of plasma LH. This effect could be blocked by atropine (Meyerson & Palis, 1970; Vijayan & McCann, 1980).

Histamine nerve terminals are closely related to LHRH neurones in the ME suggesting a possible role for this amine in the control of LH secretion (Brownstein,

Saavedra , Palkovits & Axelrod, 1974). This suggestion is supported by pharmacological evidence which shows that intraventricular injection of histamine stimulates LH secretion in ovariectomized, oestrogen-progesterone-primed rats and intact female rats on proestrus but inhibits LH secretion in male rats (Donoso, 1978). Transmitter antagonists were unable to block the stimulatory actions of histamine suggesting that its actions are not mediated by cholinergic, noradrenergic or tryptaminergic mechanisms (Donoso, 1978).

The presence of amino acids in the hypophysis and hypothalamus suggests that they may participate in regulating the secretion of LH. The excitatory amino acids such as cysteic acid, glutamate and N-methylasparate have been found effective in stimulating LH secretion (Ondo, Pass & Baldwin, 1976; Price, Olney, Mitchell, Fuller & Cicero, 1978; Scheibel, Elsaser & Ondo, 1980). In general, intraventricular administration of inhibitory amino acids such as GABA and glycine, have no effect on LH release at low concentrations (Ondo et al, 1976) but at high concentrations have an inhibitory effect (Banzan & Donoso, 1982).

Several peptides localized in the hypothalamus have been shown to alter plasma LH levels (McCann, 1980). Intraventricular injections of cholecystokinin and gastin (gastrointestinal peptides), neurotensin and opioid peptides inhibit LH release. On the other hand vasoactive intestinal peptides and substance P stimulated release of the hormone. However the physiological significance

of these peptides in control of LH release is as yet to be determined although their presence in the hypothalamus makes a physiological role likely. In the case of the opioid peptides, recent investigations have led to the suggestion of opiates and opiate receptor antagonists exerting a modulatory effect on LH secretion (Cicero, Schainker & Meyer, 1979; Ieiri, Chen, Campbell & Meites, 1980) via noradrenergic neurones (Kalra & Simpkins, 1981) and suggests the existence of functional axoaxonic interactions between the opioid peptide and noradrenergic neurones occurring in the close vicinity of LHRH neurones (Kalra, 1981).

Prostaglandins also have been implicated in the release of LH from the adenohypophysis. Prostaglandins of the E series have been shown to stimulate LH release in pentobarbitone blocked rats on proestrus (Tsafriri, Koch & Lindner, 1973; Spies & Norman, 1973), in ovariectomized rats (Lau & Saksena, 1974) and in ovariectomized, oestrogen-primed rats (Ojeda, Jameson & McCann, 1977). Inhibitors of prostaglandin synthesis, indomethacin and aspirin, inhibit ovulation in intact rats on proestrus (Tsafriri et al, 1973) and in immature rats pretreated with pregnant mare serum (Orczyk & Behrman, 1972) and this effect of indomethacin can be reversed by administration of LH or a mixture of PGE_2 and $PGF_{2\alpha}$. In 1974 it was reported that adrenergic, dopaminergic, tryptaminergic and cholinergic blocking agents failed to block LH release caused by PGE_2 stimulation and so it was concluded that the prostaglandin stimulated LH release by action on LHRH neurones (Ojeda, Marms &

McCann, 1974). More recently it has been shown that blockade of prostaglandin synthesis *in vitro* will prevent the release of LHRH induced by NA indicating that noradrenergic control over LHRH is mediated by prostaglandins which in turn stimulate release of LHRH (Ojeda, Negro-Vilar & McCann, 1979) . Incubation of ME fragments from male rats with phentolamine, an α-receptor antagonist, resulted in inhibition of release of both PGE_2 and LHRH induced by NA. In contrast to phentolamine, the β-receptor antagonist propranolol, was ineffective (Ojeda, Negro-Vilar & McCann, 1982). It was concluded from these findings that NA stimulates release of PGE_2 and LHRH from nerve terminals of the ME by first interacting with an α-adrenergic receptor.

THE ROLE OF OESTROGEN AND PROGESTERONE IN THE CONTROL OF OVULATION

Recognition that plasma gonadotrophins and gonadal steroids are maintained in a state of dynamic equilibrium was first inferred from the finding that gonadectomy is followed by an increase in gonadotrophin secretion (Engle, 1929; Evans & Simpson, 1929). High dosage with sex steroids results in their decrease (Greep & Jones, 1950). This has since become commonly known as "negative feedback" and is responsible for maintaining tonic release of LH from the adenohypophysis.

Cyclic release of gonadotrophins is triggered by the positive feedback action of steroids. This is demonstrated nicely in experiments where ovulation can be blocked by MER-25, an oestrogen antagonist (Shirley, Wolinsky & Schwartz, 1968) or by antibodies to oestradiol (Ferin, Tempone, Zimmering & Vande Wiele, 1969) and in the latter case it could be restored with injections of diethylstilbœstrol, a synthetic oestrogen. Progesterone also has been shown to have a positive feedback effect on gonadotrophin secretion, inducing ovulation in persistent oestrous rats (Everett, 1943) and advancing ovulation with appropriately timed doses of progesterone (Everett, 1948). However, it has been suggested that the facilitatory effect of progesterone on LH release is dependent on previous exposure to oestrogen (Everett, 1948; Brown-Grant, 1969a). Likewise, oestrogen replacement alone failed to produce a rise in plasma LH on proestrus in adrenalectomized-ovariectomized rats (Mann & Barraclough, 1973). When

progesterone was administered to these rats, the LH surge was reinstated. The requirement of the presence of progesterone before a surge of LH is induced is further demonstrated in a study in which ovariectomy plus adrenalectomy, within 2-3h of the expected proestrus increase in LH secretion, abolishes the LH surge but is restored on administration of progesterone (Wilson, Hadley, Gilbert & McNeilly, 1978). It is noteworthy that the dramatic increase in progesterone plasma levels on proestrus follows the preovulatory surge of LH (Piacsek, Schneider & Gay, 1971) implying that it is not responsible for stimulation of the LH surge. This suggestion that progesterone does not play the major role in trigger of ovulation, is supported by the finding that injection of progesterone antibodies does not block ovulation in the rat although its peripheral effects are blocked (Ferin et al, 1969). Instead of having a physiological role in stimulating the preovulatory LH surge, progesterone has been suggested to be responsible for the timing of the critical period (Mann & Barraclough, 1973) and for limiting the LH surge to proestrus (Banks & Freeman, 1978; Schuiling, Pols-Valkhof & Zürcher, 1980). It has been postulated that oestrogen turns on a "memory centre" for the daily trigger of an LH surge (Legan & Karsch, 1975) and that progesterone limits this expression to one day (Freeman, Dupke & Croteau, 1976). Once oestrogen has prepared the hypothalamic-hypophysial system a decrease in the circulating level of this steroid is required for the full magnitude of the preovulatory surge of LH. This is demonstrated in studies in which peak LH levels were

reduced when the elevated serum oestradiol concentration was maintained during the surge (Turgeon, 1979).

The site at which feedback of the steroids was believed to act was first suggested in the Moore-Price (1932) theory of reproductive regulation and was at the level of the adenohypophysis. However further investigation of this site of steroid feedback was abandoned temporarily in favour of a central site of action which was suggested by the finding that steroid effects on gonadotrophin release could be suppressed by neural blocking agents (Sawyer et al, 1949; Everett & Sawyer, 1949). Later, using intrahypophysial implants of oestrogen to demonstrate the effects of steroids on gonadotrophin release, the potential significance of the adenohypophysis as a site of steroid feedback action was reasserted (Bogdanove, 1963; Weick & Davidson, 1970).

A number of investigators have attempted to determine the anatomical sites of the positive feedback action of oestrogen by employing several experimental strategies. These strategies include lesion techniques, implants of hormones in the brain and hypophysis and electrical stimulation. The results of these investigations have been conflicting with some workers concluding that oestradiol exerts its positive feedback effects on the AH-SCN-POA region (Kalra & McCann, 1975; Clemens, Smalstig & Sawyer, 1976; Kawakami, Yoshioka, Konda, Arita & Visessuvan, 1978) while other investigators favour the MBH (Davidson & Sawyer, 1961; Kanematsu & Sawyer, 1963; Palka, Ramirez & Sawyer, 1966; Billard & McDonald, 1973) with others

favouring the hypophysis (Weick & Davidson, 1970; Gersten & Baker, 1970). Moreover the interpretation of these results is complicated by the so-called "implantation paradox" (Bogdanove, 1964) as the effectiveness of the steroids implanted into the hypothalamus may result from transportation to the hypothysis by the portal circulation. Also the finding that LHRH can sensitize the hypophysis to subsequent exposure to the peptide (Castro-Vasquez & McCann, 1975) further complicates the interpretation of *in vivo* experiments aimed at dissociating hypothalamic and hypophysial sites of steroid action. Recent *in vitro* studies using rat adenohypophysial cells in culture have overcome these problems to a certain extent. These studies have shown that both oestrogen (Drouin & Labrie, 1981) and progesterone (Lagacé, Massicotte & Labrie, 1980; Turgeon & Waring, 1981) have a positive feedback action at the level of the hypophysis, increasing the responsiveness of the hypophysis to LHRH. In 1978, Goodman using a technique he claims removes the doubt of hypothalamic implants getting into the circulation and therefore possibly the hypophysis, showed that oestradiol cannot stimulate the LH surge when implanted into the adenohypophysis or the MBH, but when implanted into the medial POA induces LH release. On the basis of these results he reached the conclusion that the POA may be the primary site of the positive feedback action of oestradiol in the rat. This conclusion was supported by the findings of Honma and Wuttke (1980) who showed that NA turnover in the medial POA was high in proestrus and in ovariectomized, oestrogen-, progesterone-primed rats. These turnover rates paralleled high serum

LH levels. However, in a similar experiment to that of Goodman (1978), using progesterone-primed intact females instead of ovariectomized female rats, implants of oestradiol into the medial POA were not found to stimulate ovulation. This suggested that the POA may not be the primary site of positive feedback action of oestrogen (Döcke, 1980). In a recent study, the profiles of catecholamine turnover rates in the medial POA, SCN, ME and AN were examined and correlated with changes in ME LHRH and serum LH, oestradiol and progesterone (Rance et al, 1981). The results demonstrated increased NA turnover rates during the preovulatory LH surge, not only in the medial POA but also in the ME, SCN and AN. As a result of these findings, it was proposed that the stimulatory action of NA on LHRH release is not solely via its action within the medial POA but within the entire preoptico-suprachiasmatic tuberoinfundibular system, including the ME. Such a hypothesis would explain the conflicting results found in previous investigations into the positive feedback site of oestrogen and is supported by the presence of high affinity receptors for oestrogen in both the AH-SCN-POA and MBH regions (Pfaff & Keiner, 1973).

The results of investigations into the anatomical sites of positive feedback of progesterone have also been conflicting. There is some evidence to suggest that progesterone acts at the medial POA to stimulate LH release (Barraclough, Yrarrazaval & Hatton, 1964; Simpkins & Kalra, 1979). This was strongly disputed by other

investigators who considered that progesterone failed to affect the secretion of gonadotrophins in POA-lesioned rats because an intact POA is necessary to supply information to progesterone-sensitive neurones in the MBH and not because the POA itself is progesterone sensitive (Döcke & Dörner, 1969; Schuiling, van Dieten & van Rees, 1974). These authors argue that the MBH, rather than the POA, is a crucial site of progesterone action. The issue cannot be decided by uptake studies as progesterone receptors have been found in both the MBH and AH-SCN-POA (Sar & Stumpf, 1973; Blaustein & Wade, 1978). Recently it has also been shown that progesterone has a stimulatory action on LH release directly at the level of the adenohypophysis (Lagacé et al, 1980) and this site of action is supported by evidence which shows that receptors for progesterone are found here (Kato & Onouchi, 1979). In conclusion, although the precise hypothalamic site remains in conflict, it is now believed that the positive feedback action of the steroids occurs at both the adenohypophysial and hypothalamic levels (Snabes, Kelch & Karsch, 1977).

At the hypophysial level, the steroids increase the responsiveness of the adenohypophysis to LHRH (Aiyer & Fink, 1974; Aiyer, Fink & Greig, 1974; Higuchi & Kawakami, 1982). Oestrogen is the major steroid responsible for this effect with progesterone playing a facilitatory role (see Fink, Aiyer, Jamieson & Chiappa, 1975). At the hypothalamic level, oestrogen triggers a neural signal

which in turn stimulates release of LHRH and thereby LH. Current evidence points to NA as the neurotransmitter responsible for the release of LHRH (Löfström, 1977; Clifton & Sawyer, 1980; Honma & Wuttke, 1980; Kawakami & Ando, 1981; Rance et al, 1981).

SEQUENCE OF EVENTS LEADING TO OVULATION

The evidence reviewed in the foregoing sections has led to an understanding, although not complete, of the sequence of events leading to ovulation in the rat. These findings are summarized here. (The following times are approximate).

13:00h Day D1 LH and FSH act on the fresh set of follicles, stimulating growth which results in an increased secretion of ovarian oestrogen and progesterone.

09:00h Day D2 - 15:00h Day P Oestrogen levels continue to rise, increasing the adenohypophysial responsiveness to LHRH as they do so.

23:00h Day D2 - 03:00h Day P Oestrogen priming of neural tissues occurs at its sites of positive feedback.

14:00h Day P NA activity is highest on proestrus, peaking at this time. It is believed that oestrogen stimulates a neural signal, mediated by NA activity which in turn stimulates release of LHRH from the ME. These actions may involve opioid peptide and prostaglandin interneurones.

15:00h - 18:00h Day P Progesterone and LHRH stimulate the 2nd phase increase in adenohypophysial responsiveness to LHRH.

15:00h Day P A dramatic increase in plasma LH levels occurs. This increase in circulating LH levels results in a massive preovulatory swelling of follicles destined to ovulate.

15:00h - 17:00h Day P A sudden rise in plasma progesterone concentration follows the surge in LH and is responsible for the timing of the critical period, for completion of the final stages of follicular rupture and for limiting the LH surge to proestrus.

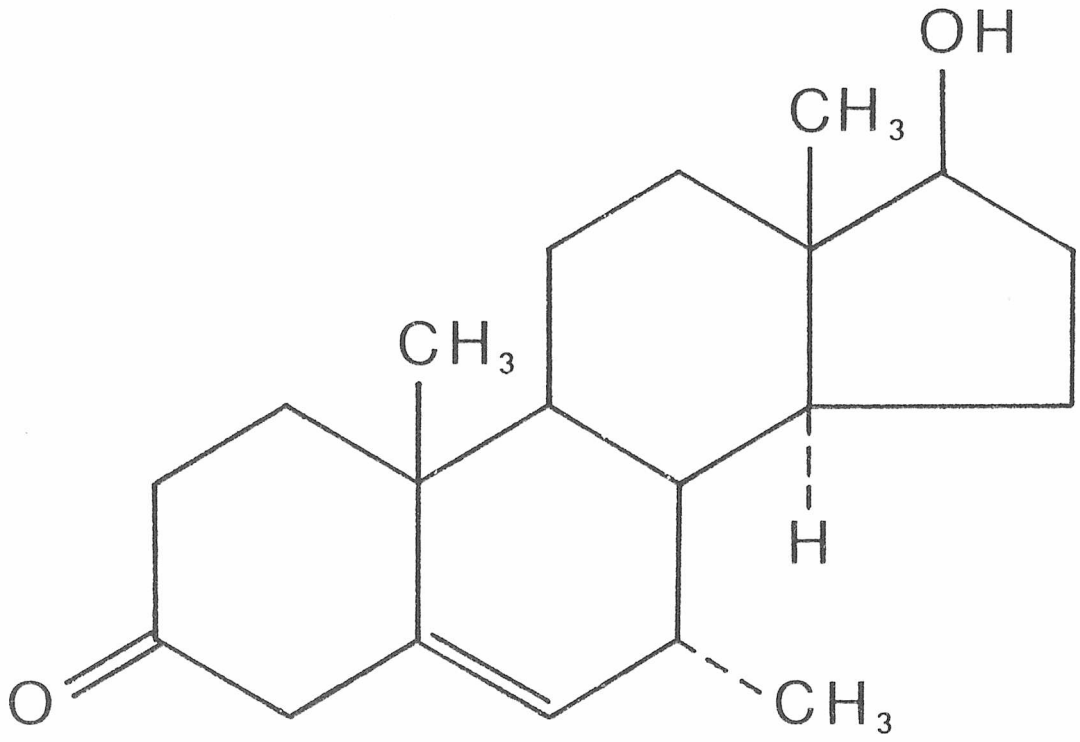
01:00h Day O Ovulation occurs.

RMI 12,936 : HISTORICAL BACKGROUND

Today few people can fail to have heard of the so-called 'Pill' with some 80 million women relying on its contraceptive efficacy. The present day contraceptive regime, generally a combination of an oestrogen with a progesterone, has a high contraceptive efficacy with a relatively low incidence of serious side effects. But this regime does involve for its effect, an almost continuous disruption of the physiology of the cycle which has led to growing fears regarding the long term hazards of this type of drug usage. Therefore a compound which would produce high contraceptive efficacy with a low incidence of side effects and only minor disruption of the menstrual cycle would, potentially, be a more desirable drug. Since progesterone is necessary for the maintenance of early pregnancy, drugs possessing antiprogestational activity were suggested as possible candidates to fill this role. Ideally the antiprogestin, administered immediately prior to menstruation would produce a brief antagonism of progesterone and induce menstruation whether or not a fertilized egg was present. Thus the drug need only be administered once during each cycle, with rapid excretion (in the ideal situation) leaving most of the cycle free of pharmacological interference.

17β-hydroxy-7α-methylandrost-5-en-3-one (RMI 12,936), since it was claimed by its discoverers (U.S. patent 3,844,621 issued 3rd September, 1974) to possess marked antiprogestational activity, was thought to have the potential as such an 'ideal' contraceptive drug (see fig. 6).

Fig. 6 Structure of RMI 12,936 (17β-hydroxy-7α-methylandrost-5-en-3-one)



Antifertility Activity

Most of the previous investigations concerning this drug have concentrated on its antifertility activity. Preliminary investigations (Kendle, 1975) showed that the compound is a very effective antifertility agent, terminating pregnancy when administered at any stage throughout pregnancy. Depending on the time of administration, implantation may be prevented by acceleration of egg transport, fetal resorption may be induced or the animals may abort. Administration of RMI 12,936 on Day 1 of pregnancy resulted in termination due to an expulsion of eggs from the reproductive tract plus a significant reduction in ovarian weight shown histologically to be due to luteal regression. Egg transfer experiments indicated that the compound also had a major effect on uterine receptivity. Administration on Day 8 of pregnancy resulted in fetal resorption associated with a significant ($P<0.01$) increase in ovarian weight shown histologically to be due to luteal hypertrophy. The possibility that this antifertility action of RMI 12,936 was due to an oestrogenic effect of the drug was investigated in the same study. This investigation revealed that the compound was similar in order of potency to a standard oestrogen, ethinyloestradiol, in antifertility tests but one thousandth less potent in the rat vaginal cornification test. Thus it was concluded that the antifertility action of RMI 12,936 cannot be explained by its weak oestrogenic activity.

Since the effect of the drug when administered on Day 8 of pregnancy was not reversed by exogenous or endogenous

progesterone, it was suggested that it acted as an antagonist of progesterone at the receptor level. This hypothesis was soon dismissed as the compound was shown to be incapable of displacing labelled progesterone from the specific progesterone binding protein in human (J. O'N. Johnston, personal communication to K.E. Kendle) and in guinea-pig (Bullock, Feil, Gupta, Demers & Bardin, 1978) cytosol.

In a study of biological activity, RMI 12,936 was reported to possess antifertility effects in pregnant hamsters, antidecidual activity in immature progesterone treated rats and androgenic activity in the rat (Grunwell,Benson, Johnston & Petrow, 1976). Investigation into whether the androgenic activity was related to its antifertility and antidecidual activities showed that the latter two activities were not a manifestation of androgenic activity although it should be noted that two species were used and therefore the differences may be a reflection of different metabolic rates.

In 1976, Kendle observed that 24h after administration of RMI 12,936, there was a significant reduction in the levels of plasma progesterone. On measurement 7 days later when luteal hypertrophy was apparent progesterone levels had returned to normal. The reduction in plasma progesterone was found not to be due to a reduction in enzymatic acitivity but was thought to be due to inhibition of progesterone synthesis (Hardy, Kendle, Lawrie & Omand, 1977). Further investigations gave rise to the hypothesis that RMI 12,936 inhibits ovarian synthesis of progesterone

in vivo by competing with its substrate, pregn-5-ene-3,20-dione for the enzyme, Δ^5 3-ketosteroid isomerase and is itself isomerized to 7α-methyltestosterone (Taylor & Kendle, 1978). But if the compound acted solely by inhibition of progesterone synthesis, it should be ineffective against exogenous progesterone. However, Kendle (1975) showed that the antifertility effect of RMI 12,936 was not reversed by exogenous progesterone and therefore the compound was presumed to be metabolized in the ovary to one or more substances which have different mechanisms of antifertility action from the parent compound, such as an inhibition of progesterone utilization. In a later study (Kendle, 1978), it was reported that RMI 12,936 failed to terminate pregnancy, maintained with progesterone, in rats ovariectomized on Day 9, 24h after RMI 12,936 administration. In contrast, ovariectomy performed on Day 10 to progesterone maintained preganant rats, 48h after RMI 12,936 administration, terminated pregancy. These results suggested that the presence of the ovary is required for at least 48h for RMI 12,936 to terminate pregancy. However it was possible that this finding was due to the sensitivity of the pregnant rat to the metabolite being greatest on Day 10, rather than the length of time for formation of effective concentrations of the active metabolite. This was clarified by the results of an experiment in which RMI 12,936 given on Day 9 was effective in terminating progesterone maintained pregnancy only when ovariectomy was performed 48h later. It was concluded from these results that the presence of the ovary for 48h is necessary to allow sufficient 'active' metabolite to be formed in order to produce this antifertility effect of

RMI 12,936.

On investigation into the identification of the 'active' metabolite, incubation of RMI 12,936 with ovarian homogenate was carried out. This yielded a product named Isomer 201 which was identifed by u.v. absorption and thin layer chromatography (Hardie et al, 1977) as 7α-methyltestosterone. Preliminary evaluation of this compound in antifertility and uterotrophic tests in rats showed that its actions and potency were similar to those of RMI 12,936. However, unlike the parent compound, Isomer 201 did show a cross reaction in the competitive protein binding assay for progesterone based on binding in human serum protein (Kendle, 1976), indicating that it is likely to be a competitive antagonist at the receptor level. It was concluded from these studies that RMI 12,936 does inhibit progesterone biosynthesis by acting as a competitive substrate for Δ^5-3 ketosteroid isomerase and in the process is itself metabolized to 7α-methyltestosterone which also possesses antifertility activity. If 7α-methyltestosterone is the 'active' metabolite, it should be able to terminate pregnancy in ovariectomized rats maintained with progesterone. However, like RMI 12,936 which requires the presence of the ovary for 48h in order to elicit its its antifertility effect, 7α-methyltestosterone was shown to be effective only in the presence of intact ovaries (Kendle, 1976) suggesting that it is not the active metabolite. Additonal evidence showed that the isomerization of RMI 12,936 to 7α-methyltestosterone *in vitro* was very rapid and therefore would not need the

presence of the ovary for 48h in order to be formed. It was therefore concluded that 7α-methyltestosterone is not the active compound responsible for the antifertility action of RMI 12,936 and therefore must undergo further metabolic change in the ovary.

In view of the enzyme systems present in the ovary it was considered likely that 7α-methyltestosterone would be further metabolized along one of the normal pathways to yield either 7α-methylandrostenedione by dehydrogenation or 7α-methyloestradiol by A ring aromatization. Investigation (Kendle, 1978) of the dehydrogenation pathway showed 7α-methylandrostenedione to be a significantly less potent antifertility agent ($P < 0.08$) than RMI 12,936 indicating that this metabolite is not the 'active' compound. Preliminary investigation of the aromatization pathway showed the aromatization inhibitors, androst-4-ene-3,6,17-trione and androst-1,17-dione to be ineffective in inhibiting the oestrogenic activity of RMI 12,936. It was concluded from these results that either aromatization is unimportant in the development of biological activity or that the 7α-methyl substrates had an even greater affinity than the inhibitors for the aromatization enzymes. Therefore the identity of the 'active' metabolite partly responsible for the antifertility activity of RMI 12,936 is at present unknown.

In summary, it has been suggested that although RMI 12,936 possesses androgenic and oestrogenic activities, that these activities are not responsible for its antifertility activity. Instead it was shown that its antiprogestational

activity is responsible for the antifertility activity of RMI 12,936 itself. This was a result of RMI 12,936 acting as a competitive substrate for Δ^5-3 ketosteroid isomerase, resulting in inhibition of progesterone synthesis. However this was not the sole mechanism of antifertility activity which was shown also to be a result of ovarian synthesis of an 'active' metabolite of RMI 12,936. This 'active' metabolite which is at present unidentified, is believed to act by inhibition of progesterone utilization.

Antiovulatory Activity

Not only has RMI 12,936 been shown to possess antifertility activity, it has also been shown to possess marked anti-ovulatory activity lasting for at least 2 weeks following a single dose (Kendle, Paterson & Wilson, 1978). RMI 12,936 blocked ovulation when administered at 16:00h on Day D2 but failed to block ovulation on the following day when administered on the morning of proestrus although it did block ovulation on the subsequent cycle. These results added support to the hypothesis that activity of the compound is due to a slowly forming metabolite (Kendle, 1978). Kendle and his coworkers showed that the compound did not produce this inhibition of ovulation by interfering with the capacity of the ovaries to respond to stimulation as they released ova in response to hCG stimulation (Kendle et al, 1978) nor with the follicles which were shown histologically to be normal (Geddes, Kendle, Shanks & Steven, 1979). The block of ovulation was however shown to be associated with a reduction in cyclic and tonic LH

secretion although the hypophysis retained the capacity to secrete sufficient LH to trigger ovulation in response to LHRH stimulation (Kendle et al, 1978). In these animals hypophysial sensitivity to LHRH was significantly lower than in a similar group in which ovulation was blocked by sodium pentobarbitone (Geddes et al, 1979). The anti-ovulatory activity of this compound could not be explained by its weak oestrogenic activity since oestrogen administered under similar conditions to RMI 12,936 did not block ovulation (Kendle et al, 1978). Its activity was however shown to be partly antiprogestational and partly antioestrogenic since ovulation could be restored partly by progesterone and fully by oestrogen plus progesterone administration (Kendle et al, 1978; Geddes et al, 1979). Surprisingly, oestrogen and progesterone replacement did not restore hypophysial sensitivity. It was suggested that this oestrogen/progesterone drug regime did not mimic physiological conditions and therefore could not restore full sensitivity of the hypophysis to LHRH.

There is considerable evidence showing that DA is a neurotransmitter which is involved in the influence of LH release (see general introduction). In order to determine whether inhibition of ovulation by RMI 12,936 could be attributed to hormone antagonism acting by modification of hypothalamic dopaminergic pathways, Geddes and coworkers (1979) looked at the effect of RMI 12,936 on DA levels during proestrus. They demonstrated the occurrence of a DA peak at 12:00h on proestrus which was suppressed by RMI 12,936 treatment. Administration of oestrogen did

not restore this hypothalamic DA peak and ovulation was not induced following administration of L-dopa in RMI 12,936 treated rats. It was concluded from these results that a hypothalamic dopaminergic pathway may have a non-essential role in the control of ovulation and therefore that RMI 12,936 does not block ovulation by this mechanism.

These preliminary investigations into the antiovulatory activity of RMI 12,936 have indicated that RMI 12,936 may block ovulation by exhibiting antioestrogenic and anti-progestational activity. The aim of this investigation was to confirm and extend these preliminary findings in order to test this hypothesis. The consequences to the sequence of events leading to ovulation (described earlier in this section), of prevention of oestrogen and progesterone activity are several.

At the adenohypophysial level oestrogen and progesterone are responsible for increasing the responsiveness (priming) of the gland to LHRH. A drug possessing antioestrogenic and antiprogestational activity might therefore be expected to prevent this hormonal priming action. Such a consequence of RMI 12,936 treatment has been reported (Geddes et al, 1979). In this investigation, the primary aim was to determine whether the antiovulatory activity of the drug was through prevention of oestrogen and progesterone activity at the adenohypophysial level and secondarily the mechanism by which the drug reduces sensitivity.

At the hypothalamic level, oestrogen is responsible for

priming its site of positive feedback action for release of LH and for triggering the neural signal for stimulation of LHRH release. A drug possessing antioestrogenic activity might therefore be expected to block the neural signal. The results of Geddes et al, (1979) have indicated that DA is not the neurotransmitter responsible for stimulating release of LHRH. Therefore this investigation aimed firstly to determine whether the hypothalamus is the site of RMI 12,936 anti-ovulatory activity by local administration of the drug. Subsequently the aim was to determine the effect of the drug on the levels, synthesis and breakdown of the hypothalamic neurotransmitters, NA and 5'HT in addition to DA. The priming of the positive feedback site for LH release might also be expected to be blocked by a drug possessing anti-oestrogenic activity. Such an effect of RMI 12,936 was also investigated in this study.

It has been demonstrated that androgens can inhibit LH release (Kingsley & Bogdanove, 1973; Drouin & Labrie, 1976). Therefore, since RMI 12,936 is an androstane derivative the final aim in this investigation was to determine the relationship of its androgenic activity with its antiovulatory activity.

PART 2

GENERAL METHODS

ANIMAL HUSBANDRY

Rats of the Sprague-Dawley strain were used in all experiments. The animals were housed under constant conditions of light (12h light from 08:00 - 20:00h and 12h dark from 20:00 - 08:00h) of intensity of 30 lumens, temperature (21± 1°C), humidity (50% + RH) and diet (Oxoid Pasturised Breeding diet for rats and mice). The animals were allowed to partake of food and water ad lib.

Vaginal smears of adult (at least 6 weeks old) virgin rats were examined microscopically to determine the regularity of the oestrous cycle. The different stages of the oestrous cycle were identified in the following way. The presence in the smear of (i) keratinised squamous cells only, indicated the stage of oestrus, designated Day O (ii) keratinised squamus cells, nucleated epithelial cells and polymorphonucleated leukocytes indicated the first day of diestrus, designated Day D1 (iii) nucleated epithelial cells and leukocytes indicated the second day of diestrus, designated Day D2 and (iv) nucleated epithelial cells only, indicated the stage of proestrus, designated Day P. Only those animals exhibiting at least two consecutive regular oestrous cycles were used in this investigation.

DETECTION OF OVULATION

On the expected day of oestrus, the animals were killed by cervical dislocation unless otherwise stated and the ovaries, oviducts and uteri were exposed by a mid-ventral abdominal incision. The oviduct was freed from the uterus by cutting

the uterus 2-4 mm from its tip. The oviduct could then be manipulated by the attached section of uterus, thus preventing damage to any of the eggs. Curved dissecting forceps were used to hold the uterine attachment and fine, pointed iridectomy scissors to straighten the oviducts, under a Nikon (type 102) dissecting microscope. Oviducts were then placed on a microscope slide, a coverslip placed over the tissue and normal saline (0.9% w/v sodium chloride solution) injected under the coverslip using a Pasteur pipette. The microscope slide was placed on a microscope (Vickers Instruments, magnification x 50) and the tissue examined for the presence of a swollen ampulla and freshly ovulated ova. The number of ova present were counted.

SERIAL BLOOD SAMPLING

The animals were placed in a hot box at approximately 45°C for a few minutes before insertion of the needle into the tail vein. A blood sample of 0.25-0.30 ml was then taken. This procedure was repeated at 30 min intervals over a 3½h period. Blood samples, originally collected in micro-centrifuge tubes (Bel-Art Products, Pequannock, N.J.), were allowed to clot. The samples were then centrifuged for 10 min at 1250g and the serum removed by pipettes to plastic tubes. The serum was stored at -20°C until assayed.

SURGICAL PROCEDURES

The surface of the operating table was swabbed with a disinfectant solution (Cetrimide 1%, Chlorhexidine 0.1% in

70% ethanol). All instruments and suture materials were soaked in this solution before use.

OVARIECTOMY

The animal's fur was shaved on either side, below the rib cage. The animal was then placed in a large desicator and anaesthesia induced with ether. When the animal had ceased to move, it was placed on the operating table; anaesthesia was continued with an ether cone. The shaved area was cleansed with the disinfectant solution and an incision approximately 1.0 cm long made in the skin midway between the last rib and the knee. A second incision was made through the muscle layer and into the peritoneal cavity. Directly underneath the incision, the ovary could be seen, embedded in a mass of fat. The fat was withdrawn and the ovary separated and tied off with a cotton ligature. The ovary was then cut away and the uterus returned to the peritoneal cavity. The incision through the muscle was closed with continuous cotton sutures, using a small (No.11) round bodied curved suture and the skin closed with a suture clip. The ovary on the opposite side was then removed through a separate incision.

CASTRATION

After anaesthesia had been induced with ether, the animal was placed on the operating table on its back and the scrotal area was cleansed with the disinfectant solution. The testes were brought down from the abdominal area by

stroking down towards the scrotal area. An incision was made through the skin of the scrotum midway between the testes. Curved dissection forceps were used to expose and remove the testis on one side and then the other. The skin incision was then closed with two suture clips, making sure that the clips did not interfere with the function of the rectum and anus.

INTRAVENTRICULAR INJECTIONS

Pre-operative Calibration of the Stereotaxic Instrument

A 10µl SGE syringe (type 10A-RN) was used to inject the drug into the 3rd ventricle. The syringe was placed into a metal holder (see fig. 7) and checked to see if the needle was straight. This was performed by setting the ear bars of the instrument (see fig. 7) so that they were separated 1 to 2 mm at their tips. The needle was carefully lowered until its tip was midway between the tips of the ear bars. If the needle was perfectly straight, then continuing to lower it beyond this point as far as possible should not alter the distance between either ear bar tip and the needle. If on lowering the needle, its position relative to the ear bars did change, the syringe position was altered until the needle could be lowered between the two ear bar tips without changing its position relative to them.

When this adjustment in the lateral plane had been completed, the needle was then checked to see if it was straight in the

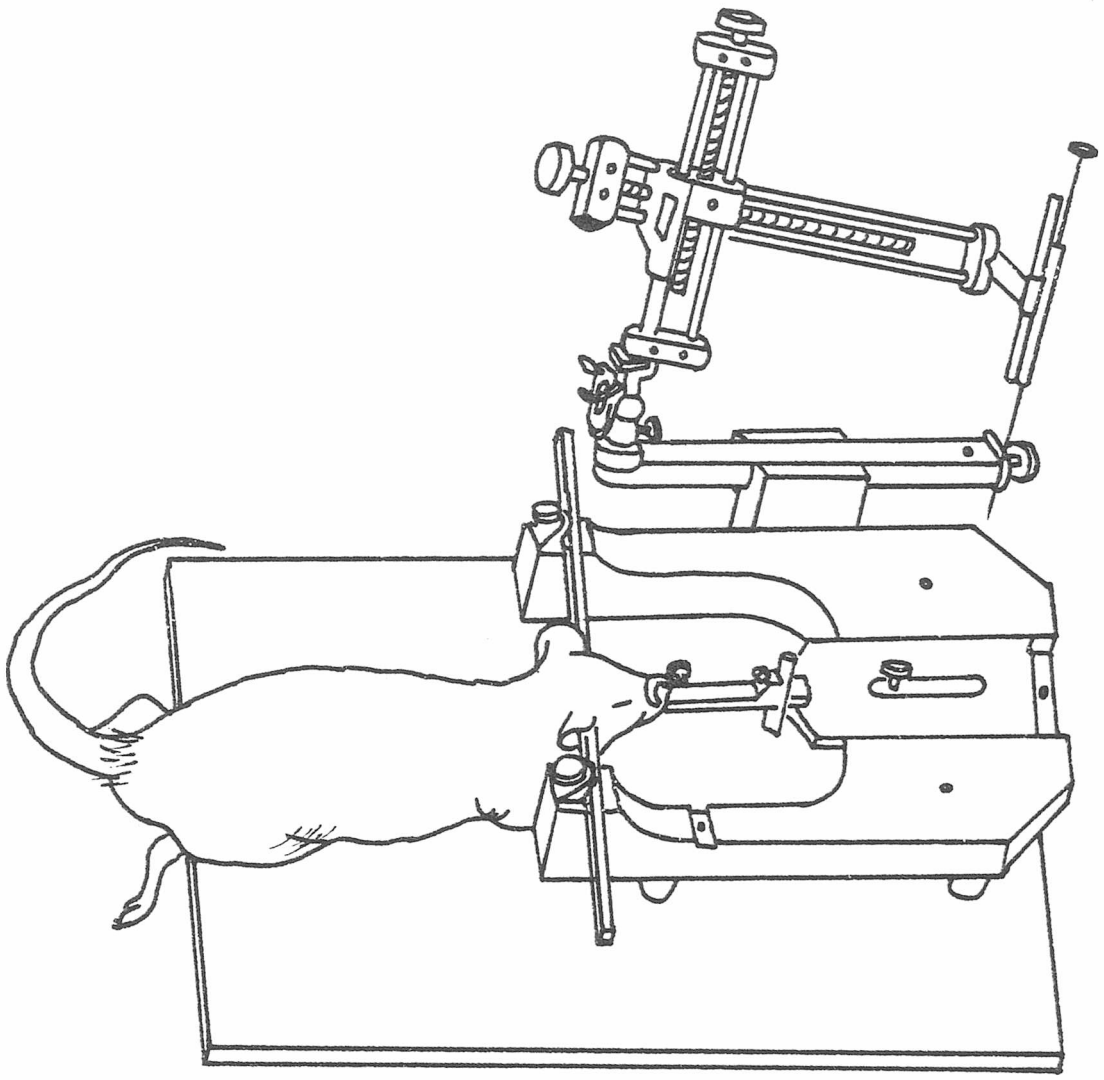


Fig. 7 Diagrammatic representation of stereotaxic apparatus. (See text for description).

anterior-posterior plane. This was achieved by centering the needle tip between the two ear bar tips while sighting down one of the ear bars and then lowering the needle as far as possible, making certain that the needle remained in the centre of the two ear bar tips throughout its full excursion in the vertical direction. If it did not, the syringe position was altered until it did. Once this step was completed, the first adjustment in the lateral direction was checked again and any appropriate adjustment made. (When the needle could be lowered without any variation in either the anterior-posterior direction or lateral direction simultaneously, the needle was straight).

Determination of Instrument Zero

Stereotaxic zero was determined by setting the ear bars so that the scale on each read 1.0 mm. The needle tip was then lowered until it was exactly in the middle of the space between the two ear bar tips. Now resting at stereotaxic zero, all three vernier scales (anterior-posterior, lateral and horizontal) on the stereotaxic instrument were read and recorded.

Arbitrary Horizontal Zero

The stereotaxic atlas referred to, was by De Groot (1972). This atlas has an arbitrary horizontal zero plane which passes through the anterior and posterior commissures which lie 5:0 mm above the interaural line (instrument horizontal zero). This plane requires that the animal's head is positioned in the instrument with the upper incisor bar adjusted to be 5.0 mm above the interaural line.

Calculation of Final Instrument Co-ordinates

The calculation of final instrument co-ordinates was accomplished with the following general formula: Atlas co-ordinate ± Instrument Zero Co-ordinate = Final Instrument Co-ordinate. This calculation gave the three numbers that the three vernier scales on the stereotaxic instruments would read when the needle tip was in the correct position.

Positioning of the animal in the instrument

The rat was positioned in the stereotaxic equipment (see fig. 7) by clamping one ear bar in place and retracting the other ear bar. The tip of the first ear bar was first inserted into one ear canal and then the other ear bar inserted into the contralateral ear canal and clamped into place. (The animals often provided some helpful clues as to when the bars were correctly positioned in the canal. Frequently the rat would blink reflexively or twitch the ipsilateral eye as the bar entered the canal). If the ear bars were properly inserted, the animal's head could be pivoted freely in a vertical direction about the ear bars but would not move in a horizontal direction. Also when the animal's head was examined from the front of the instrument, the head ought to appear straight (that is, the midline of the head ought to be perpendicular to the line formed by the two ear bars). If the animal's head was not properly placed into the instrument, often one eye would appear to bulge or one eye would be closed.

Once the ear bars were properly inserted, the entire head

was centred in the instrument. This was achieved by sliding both ear bars until their millimeter scales both had identical readings. When this was completed the final step was to place the animal's upper teeth over the upper incisor bar and gently push down on the nose until the top surface of the upper incisor bar rested on the upper surface of the rat's mouth just behind his upper incisors. The nose bar was then gently pulled forward until the incisor bar fitted tightly against the animal's teeth. Finally, the nose clamp was placed over the rat's nose and gently tightened.

Surgical Procedure

Prior to positioning in the instrument, the rat was anaesthetised and the head shaved and swabbed with the disinfectant solution. Once the animal had been correctly positioned in the instrument, an incision about 10 mm long in the anterior-posterior plane, was made with a scalpel blade.

The underlying connective tissue was scraped aside (using the scalpel blade) and the area swabbed with the disinfectant solution. The needle was moved 5.8 mm rostral to the instrument zero co-ordinate (position of the 3rd ventricle) according to De Groot's Stereotaxic Atlas. If the tip of the needle was within 0.1 mm of the bregma point then the rat had been positioned correctly in the instrument. The point was marked and the needle moved aside until a small hole had been made in the skull with a dental drill. The needle was returned to the correct co-ordinates and lowered

through the hole in the skull to the tip of the brain. The co-ordinates were noted and the needle lowered 8.5 mm. The drug was then slowly discharged and the needle withdrawn. The wound was swabbed until the blood flow ceased, the area cleansed and the skin sutured. This operative procedure took less than 15 min. The animals were left to recover until the expected day of oestrus when they were killed by cervical dislocation, the brains removed for histological verification and autopsied to determine whether ovulation had occurred.

Histological Verification of Stereotaxic Placements

Immediately after death, the brain was removed and fixed in 10% formalin. After a period of 24h, thin sections of the brain were made using a scalpel blade and the sections examined for evidence of a blood tract.

DISSECTION OF THE BRAIN: ISOLATION OF THE HYPOTHALAMUS

After cervical dislocation, the brain was rapidly removed and placed on an ice cooled glass plate with the caudal side of the brain facing upwards (fig. 8A). The hypothalamus was dissected from the rest of the brain as follows: first, vertical incisions were made (fig. 8A, 1 and 2) separating the anterior and posterior hypothalamus from the cerebral cortex and rhombencephalon, respectively. Second, vertical incisions were made (fig. 8B, 3 and 4) separating the hypothalamus from the cerebrum. The hypothalamus was lifted out of the brain by making an

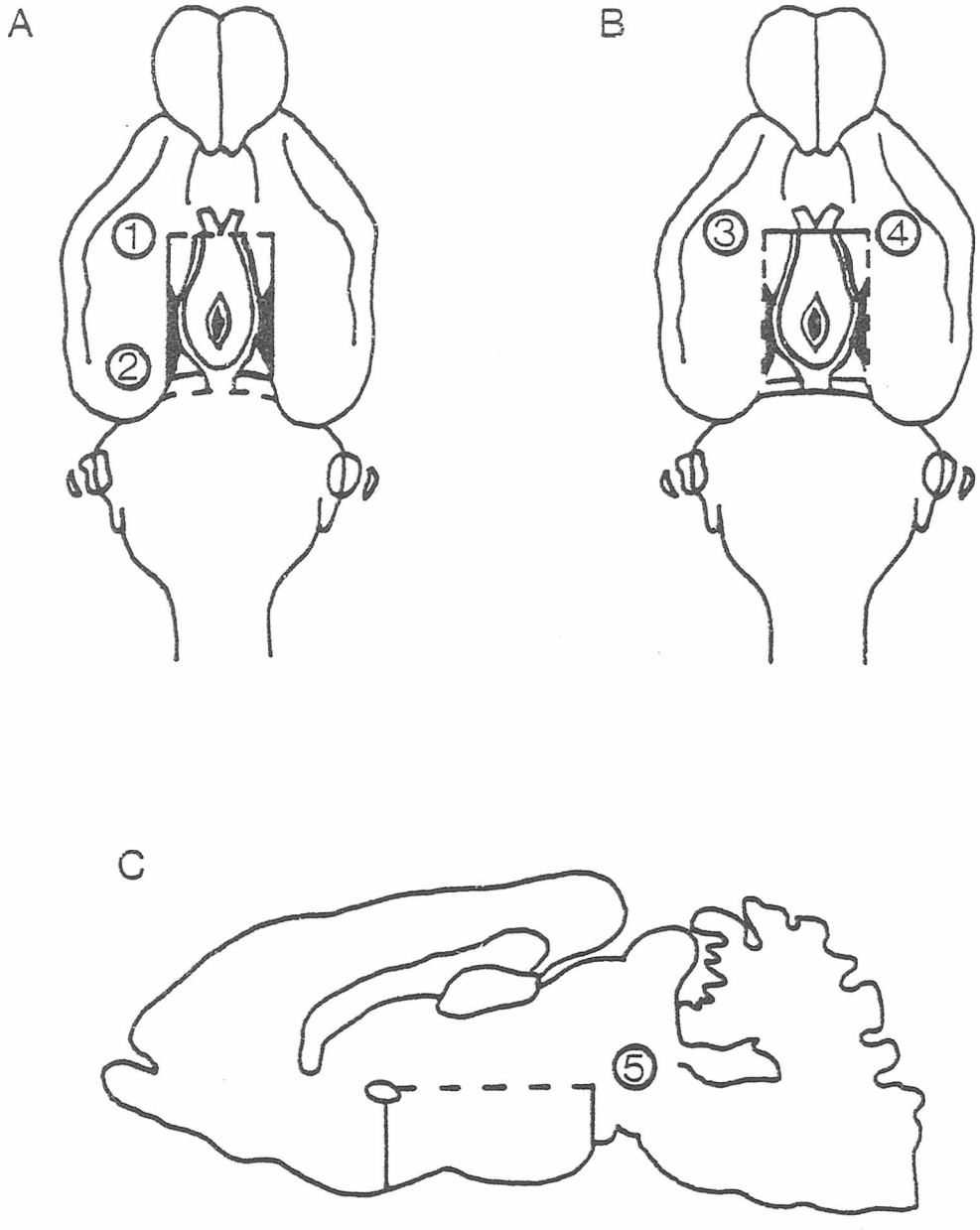


Fig. 8 Diagrammatic representation of the dissection procedure for isolation of the hypothalamus. Dotted lines indicate positions of incisions. (See text for explanation of the diagrams).

incision horizontally at the point of the anterior commissure (fig. 8C, 5). The hypothalamus was then weighed (mean weight 54.9 ± 0.5mg, n = 185) and stored at -20°C until assayed for its amine content as described in the subsequent two sections.

ASSAYS

LUTEINIZING HORMONE ASSAY

The LH assay was carried out at St George's Hospital Medical School. Serum LH concentrations were measured in triplicate by the double-antibody radioimmunoassay procedure as described by Naftolin and Corker (1971).

General Reagents

MPS: consisted of 0.01M sodium phosphate buffer (pH 7.0) in merthiolated saline. The merthiolated saline was prepared by dissolving 100mg thiomersal in 0.15M NaCl.

1% Egg White (EW): Prepared by dissolving 1g egg albumin in 100ml MPS. The material was then filtered through acetone washed glass wool and stored at 5°C. The pH was adjusted to pH 7.0 with 0.1N NaOH.

Serum Diluent (SD): prepared by adding 0.25% normal rabbit serum (NRS) to MPS containing 0.05M EDTA. The pH was adjusted to pH 7.0 with 1N NaOH and stored at 5°C.

Anti-serum (AS): prepared by diluting antiovine LH (provided by Dr G Niswender) in SD.

Precipitating Serum (PS): prepared by diluting donkey antirabbit serum (RD17, Wellcome Reagents Ltd.) 1:24 in 1% EW (pH 7.0).

Tracer LH (Label): ovine pituitary LH, LER-C_2-1056 (provided by Professor L E Reichert Jr.), was iodinated with ^{125}I (Amersham International Ltd.) by Dr C A Wilson using the chloramine T method.

Standards (Std): prepared by dissolving ovine pituitary LH in EW to give a stock solution of 7.5µg/ml. 100µl aliquots of the standard were frozen and stored at -20°C until required. For use, the stock solution was thawed and diluted with EW to cover a range of 10-90% displacement of label.

Pooled Plasma: a group of internal standards in the form of pooled plasma from control animals were used in this assay. Triplicate specimens were added to each assay to provide an index of the validification of the assay.

Sample: the blood samples were collected, allowed to clot and then centrifuged for 10 min at 750g. The serum was transferred to a clean container and stored at -20°C until assayed.

Serum Blank (SB): control sample in which the AS is replaced by SD.

Reference(R): control sample, containing the complete reaction mixture but no unlabelled LH.

The reagents were purchased from BDH unless otherwise stated.

Validation Details

The least detectable amount of LH in this assay was 20pg LER-C_2-1056 (potency 1.73 times NIH-LH-SI). The internal standards were used to calculate the intra- and inter-assay coefficients of variation which were 11 and 16% respectively.

Method

The serum blank, reference, standard and sample were prepared by addition of the sample, egg white, serum diluent and antiserum on Day 1, label on Day 2 and precipitating serum on Day 3 as shown in table 1. On the fourth day, the samples were centrifuged at 2500g for 30 min at 5°C until the precipitate was formed. The supernatant was aspiritated off and approximately 0.5ml of 1% egg white added and then mixed. The samples were again centrifuged at 2500g for 20-25 min at 5°C. The supernatant was aspirated off and the samples placed in the gamma counter (LKB 1270 Rack Gamma II) to be read for 60 sec each. For each hormone assay, the standard curve was linearized by means of logit transformation as described by Rodbard and Lewald (1970).

Table 1: Procedure followed for radioimmunoassay of LH. The concentrations are expressed in µl.

	DAY 1				DAY 2	DAY 3
	SERUM/Std	EW	SD	AS	LABEL	PS
LABEL	-	-	-	-	50	-
SB	50	100	50	-	50	50
R	-	150	-	50	50	50
Std	50	100	-	50	50	50
SAMPLE	50	100	-	50	50	50

SB = Serum Blank, R = Reference, Std = Standard, EW = Egg White,

SD = Serum Diluent, AS = Anti-serum, PS = Precipitating Serum.

DETERMINATION OF HYPOTHALAMIC AMINE LEVELS USING A FLUORESCENCE TECHNIQUE

The fluorescence technique used was adapted from those developed by Curzon and Green (1970), Shellenberger and Gordon (1971) and Cox and Perhach (1973).

Materials

n-butanol: acidified by adding 0.85ml concentrated HCl to 1 litre of n-butanol. The acidified n-butanol was stored at 4°C and kept a maximum of 2 days.

o-phthaldehyde (OPT): 0.004% (w/v) OPT in 1ON HCl. 0.01% (w/v) OPT in 1ON HCl.

0.5M phosphate buffer: prepared by dissolving 2.185g disodium hydrogen orthophosphate and 4.760g potassium dihydrogen orthophosphate in triple distilled water to give a volume of 100ml. The pH was adjusted to 7.4 with sodium hydroxide and stored at room temperature.

0.2M phosphate buffer/EDTA solution: prepared by making a 2 in 5 dilution of the 0.5M phosphate buffer and adding 9g of disodium edetate to 1 litre of the buffer. The pH was adjusted to 7.4 with 5N sodium hydroxide and stored at 4°C.

iodine reagent: prepared by adding 0.5g iodine plus 2g potassium iodide to 40 ml of triple distilled water and stored in the dark at room temperature.

alkaline sulphite reagent: prepared by adding 1ml of 25% anhydrous sodium sulphite to 9ml of 5N sodium hydroxide, less than one hour before use.

5N sodium hydroxide: prepared by adding 20g sodium hydroxide to 100ml of triple distilled water.

25% anhydrous sodium sulphite:prepared by dissolving 250mg anhydrous sodium sulphite in 1ml of triple distilled water. Stored in the dark at 4°C.

standard amine solutions: prepared by dissolving each amine (NA, DA, 5'HT and 5'HIAA) in 0.1N HCl to give stock solutions of 500µg/ml. These were frozen and stored at -20°C until required. For use, the stock solutions were thawed and diluted in either 1) 0.1N HCl to provide mixed standard solutions containing a) NA, DA, 5'HT and 5'HIAA for use as the internal standard and b) NA, DA and 5'HT for use as the external standards or in 2) 0.5M phosphate buffer to provide a standard solution containing 5'HIAA for use as the external standard.

All reagents were of analytical grade. Reagents and distilled water were stored in glass bottles with glass stoppers, since fluorescent contaminants are leached from plastic and rubber stoppers. Glassware was kept separate from that used in other procedures: after thorough washing it was rinsed exhaustively with tap water and then with glass-distilled water.

Method

Rats were killed by cervical dislocation and the brains rapidly removed. The hypothalamus was dissected out as previously described,weighed and stored at -20°C until use (maximum storage of 48h).

A. Extraction Procedure

Each tissue was homogenized in 3ml cold acidified n-butanol and then centrifuged for 10 min at 1000g. 2.5ml of the supernatant was transferred to a tube containing 5ml n-heptane plus* 1.5ml 0.1N HCl and shaken for 5 min. The samples were then centrifuged at 750g for 6 min.

At this stage 6ml of the organic phase was transferred to a clean test tube for 5'HIAA determination. 0.2ml of the acid phase was retained for 5'HT determination. These samples were stored at 4^{o}C until assayed.

B. Fluorophore Formation

Noradrenaline and Dopamine Determination: † 0.5ml of the acid phase was added to a test tube containing 1ml of 0.2M phosphate buffer/EDTA solution (pH 7.4). 0.5ml of the iodine reagent was added, mixed well and then left for 3 min exactly, whereupon 0.5ml alkaline sulphite reagent was added. The sample was mixed and then left for 5 min exactly when 0.3ml glacial acetic acid was added. The sample was then placed in a boiling water bath and after 3-4min, cooled on ice. NA fluorescence was then read on a Baird Spectrofluorimeter (Model FP100) at approximately 360/475nm.

*50µl of the internal standard solution (25, 50, 100 and 200ng) was added at this point to 1.45ml of 0.1N HCl, instead of 1.5ml 0.1N HCl.

†50µl of the standard solution of NA, DA and 5'HT was added at this point to 0.45ml of 0.1N HCl in place of 0.5ml of the acid phase, to form the external standard.

After NA determination the sample was returned to the boiling water bath for a further 35-40 min, cooled on ice and the DA fluorescence read from an ice bath, at approximately 320/375nm.
Tissue Blank: The same procedure was followed with the exception that the iodine reagent is added after the glacial acetic acid and not before the alkaline sulphite reagent.

5'Hydroxytryptamine Determination: *0.1ml of the acid phase was added to a test tube containing 0.6ml of 0.004% OPT in 10N HCl and mixed well. The sample was removed to a boiling water bath for 15 min, cooled on ice and the 5'HT fluorescence read at 360/470nm.
Tissue Blank: The same procedure is followed with the exception that the 0.004% OPT solution was added immediately before the reading.

5-Hydroxyindoleacetic acid Determination: 0.4ml of the 0.5M phosphate buffer (pH 7.4) was added to the test tube containing 5ml of the organic phase and shaken mechanically for 10 min. The sample was then centrifuged at 1000g for 6 min and +0.3ml of the aqueous phase transferred to a fresh test tube. To this, 0.7ml of the 0.01% OPT solution was added and the test tube shaken to mix the contents.

*50µl of the standard solution of NA, DA and 5'HT was added at this point to 50µl of 0.1N HCl in place of 0.1ml of the acid phase, to form the external standard.

+50µl of the standard solution of 5'HIAA was added at this point to 0.25ml of the 0.5M phosphate buffer in place of the aqueous phase, to form the external standard.

The sample was heated in a boiling water bath for 10 min and then cooled on ice. The 5'hydroxyindoleacetic acid (5'HIAA) fluorescence was read at approximately 360/470nm. Tissue Blank: The same procedure was followed with the exception that the 0.01% OPT solution was added immediately prior to the fluorescence reading.

All procedures were as far as possible, performed with the samples stored on ice. The concentration of amine present in each sample was read from a standard curve of internal standard concentration versus fluorescence intensity.

Validation Data

A. Intra-assay Coefficient of Variance

Five samples taken from a tissue pool were extracted and assayed as for the tissue samples. The individual results are displayed in table 2 and show a range of 5.3% to 11.4% coefficient of variance.

B. Inter-assay Coefficient of Variance

Five samples taken from a tissue pool were assayed for amine content in 5 separate assays. Table 3 shows the individual results and coefficient of variance ranging from 16.2% to 19.4%.

C. Limits of Detection

The limits of detection of the assay for each individual amine was calculated as the lowest concentration of internal standard which had fluorescence intensity of twice the reagent blank. Thus the limits of detection of the assay were 25ng for NA, DA, 5'HT and 5'HIAA.

Table 2: Intra-assay variation in the fluorescence amine assay.

		NA	DA	5'HT	5'HIAA
AMINE CONCENTRATION (ng/sample)	1	92.5	28	182	58
	2	97.5	28	166	76
	3	105.0	34	158	70
	4	105.0	34	170	76
	5	100.0	30	188	64
MEAN		100.0	30.8	172.8	68.8
S.D.		5.3	3.0	12.1	7.8
%		5.3	9.8	7.0	11.4

Table 3: Inter-assay variation in the fluorescence amine assay.

Assay Number	Amine concentration (ng/sample)			
	NA	DA	5'HT	5'HIAA
1	106	34	158	58
	106	34	166	76
2	130	36	156	64
	-	60	132	58
3	176	48	158	66
	154	42	144	60
4	142	45	200	62
	158	42	200	-
5	128	38	210	90
	128	34	-	-
MEAN	136.4	41.3	169.3	66.8
S.D.	23.5	8.2	27.4	11.1
%	16.9	19.9	16.2	16.6

D. Recovery

The percentage recovery of amine from this assay was calculated in the following way. Six equal samples were obtained from a tissue pool. A standard solution (50µl volume) containing 100ng each of NA, DA, 5'HT and 5'HIAA or 50µl of 0.1N HCl were added to these pooled tissue samples and assayed in three separate assays as previously described. The mean fluorescence intensity of the pooled tissue samples was subtracted from each of the samples containing the internal standards (table 4).

$$\text{The \% recovery} = \frac{X \times Z}{Y} \times 100$$

where X = fluorescence intensity of 100ng internal standard (standard plus tissue pool minus pooled tissue sample)

Y = fluorescence intensity of 100ng external standard

Z = correction factor for known loss of sample

For NA/DA, $Z = \frac{1.5}{0.5} = 3$

5'HT, $Z = \frac{1.5}{0.1} = 15$

5'HIAA, $Z = \frac{7.5}{5} \times \frac{0.4}{0.3} = 2$

The percentage recovery ranges from 68.7% for 5'HIAA to 102.6% for NA.

Table 4: Percentage recovery of amines in the fluorescence assay.

	X	Y	Z	% Recovery	Mean ± SEM
NA	20.5	59	3	104.2	102.6 ± 2.5
	12.0	34	3	105.9	
	14.0	43	3	97.7	
DA	21.0	63	3	100.0	91.6 ± 4.4
	17.0	57	3	89.5	
	16.5	58	3	85.3	
5'HT	17.0	338	15	75.4	79.9 ± 4.0
	15.0	294	15	76.5	
	17.5	299	15	87.8	
5'HIAA	33.0	90	2	73.3	68.7 ± 2.4
	23.0	68	2	67.6	
	29.0	89	2	65.2	

X = Fluorescence intensity of the internal standard (100ng) minus the pooled tissue sample.

Y = Fluorescence intensity of the external standard (100ng).

Z = Correction Factor for known loss of sample.

DETERMINATION OF HYPOTHALAMIC AMINE LEVELS USING ELECTRO-CHEMICAL DETECTION AFTER SEPARATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Apparatus

The liquid chromatograph consisted of a constant flow pump, Model 6000A (Waters Associates Ltd.) run at 1ml/min; an injection valve, Model 7120 (Rheodyne) equipped with a 20µl loop; a thin layer amperometric detector, Model LC-4 (Bioanalytical Systems) with a glassy carbon electrode as the working electrode, operated at 0.72V vs an Ag-AgCl reference electrode; and a Servoscribe potentiometric recorder, Model 524.20 (Belmont Instruments).

Materials

Trichloroacetic acid (TCA), purchased from Fisons Scientific Apparatus was of reagent grade. Acetonitrile, purchased from Rathburn Chemicals Ltd was of HPLC grade. Ethylenediaminetetra-acetic acid (EDTA) and sodium hydroxide (NaOH) were purchased from BDH Chemicals Ltd and were of analytical grade.

Chromatographic Technique

The column (125 x 5mm) was slurry packed at 9000 psi with Hypersil ODS 5µm (Shandon Southern Products Ltd).

The mobile phase consisted of 1% TCA, 12.5% acetonitrile and 0.1mM EDTA. The pH was adjusted to 3.5 by the addition of NaOH. Before use, the mobile phase was filtered through millipore paper (pore size 0.8µm) and degassed under a vacuum for 10 min.

Amine Assay

For assay of the amines, the frozen tissue samples were homogenized by hand in 300µl of the eluant. The homogenate was centrifuged for 10 min at 1250g and the supernatant transferred to a clean test tube. An aliquot of the supernatant was then injected on to the chromatographic column. The sensitivity of the detector was optimised for each peak of interest with typical settings of 10na/V (filter B) for NA, DA and adrenaline; 5na/V (filter B) for 5'HT and 5'HIAA.

Quantitations were performed from standard curves since recovery of the standard amines from a tissue pool was found to be approximately 100% indicating that there is no interference from the tissue. The standards were dissolved directly in the eluant to minimise alteration of the chromatograph baseline from homogenates. During analysis, standard solutions were injected intermittently (on average every sixth) with the samples and the concentrations of the samples were calculated based on the peak height of this standard solution.

Validation Data

A. Intra-assay Coefficient of Variance

Five samples taken from a tissue pool were extracted and assayed as previously described. The variance in results ranges from 2.4% for adrenaline to 31.5% for 5'HIAA. Table 5 shows the individual results.

Table 5: Intra-assay variation in the HPLC/ECD amine assay.

	NA	DA	5'HT	5'HIAA	ADR
Amine Conc. (ng/ 50µl)	4.8	2.6	3.8	1.4	2.1
	4.8	2.3	3.8	0.9	2.0
	4.7	2.1	3.4	0.6	2.15
	4.4	2.4	3.1	0.8	2.1
	4.4	2.5	3.1	0.9	2.1
MEAN	4.6	2.4	3.4	0.9	2.1
SD	0.2	0.2	0.4	0.3	0.05
%	4.3	8.0	10.2	31.5	2.4

B. Inter-assay Coefficient of Variance

This was not calculated as all the samples were assayed in the one assay.

C. Limits of Detection

The limits of detection calculated as the standard in which the baseline noise was not greater than 10% of the peak height of the standard was 400pg for NA, 5'HT and adrenaline; 225pg for DA; and 200pg for 5'HIAA.

D. Sensitivity of the Assay

The sensitivity of the assay for 4ng $\pm$ 0.5ng NA, DA, 5'HT, 5'HIAA and adrenaline was 10%, 7.5%, 6.25%, 5% and 10% respectively.

E. Recovery of the Amines

To four samples (0.5ml volume) taken from a homogenized tissue pool, 80ng standard (volume of 20µl) or 20µl eluant were added. A fifth sample (tissue blank) consisted of 0.5ml eluant plus 20µl of the standard (80ng). All five samples were assayed as previously described (see table 6). The percentage recovery was calculated by the following calculation.

$$\text{The \% recovery} = \frac{X-Z}{Y} \times 100$$

where X = Tissue + 80ng Standard

Y = 80ng Standard + Eluant

Z = Tissue + Eluant

Table 6: Percentage recovery of amines

Amine	X	Mean of X ± SEM	Y	Z	% Recovery
NA	33 34 34	33.7 ±0.3	20	15	93.5
DA	85 83 89	85.7 ±1.8	40	45	101.8
5'HT	31 32 34	32.3 ±0.9	18	15	96.1
5'HIAA	60 50 58	56.0 ±3.1	44	10	104.5
Adrenaline	49 50 49	49.3 ±0.3	19	32	91.1

X = Fluorescence intensity of sample containing tissue plus standard
Y = Fluorescence intensity of sample containing standard plus eluant
Z = Fluorescence intensity of sample containing tissue plus eluant

DRUGS USED

The following drugs were used in this study. Route of administration ( ) and commercial source are also given.

L-Adrenalin Bitartrate	(third cerebral ventricle)	Sigma
Althesin	(iv)	Glaxo
Anaesthetic Ether	(inhalation)	Duncan, Flockhart
5-Hydroxytryptamine	(third cerebral ventricle)	Sigma
Luteinizing Hormone Releasing Hormone	(ip)	Hoescht UK Ltd
αMethyl-para-tyrosine	(ip)	Sigma
L-Noradrenaline Bitartrate	(third cerebral ventricle)	Sigma
Oestradiol	(sc)	BDH
Pargyline	(ip)	Sigma
Pentobarbitone Sodium	(ip)	May & Baker
Progesterone	(sc)	BDH
17β Hydroxy-7α-methylandrost -5-en-3-one	(sc)	Donated by Merrell National Laboratories
Testosterone Propionate	(sc)	BDH

Oestradiol, progesterone, 17β hydroxy-7α-methylandrost-5-en-3-one (RMI 12,936) and testosterone were suspended in an aqueous vehicle containing 0.25% (w/v) sodium carboxymethyl cellulose and 1% (w/v) 'Tween 80'.

αMethyl-para-tyrosine (αMPT) was dissolved in 0.1N HCl and the pH adjusted to 5.5 - 6.0.

The anaesthetics, althesin (alphaxalone and alphadolone acetate) and ether were administered from stock.

The remaining drugs were dissolved or diluted in 0.9% saline to give the required concentration.

The routes of administration are as noted in parentheses unless otherwise specified. Drug dosages refer to the salt.

STATISTICAL ANALYSIS

Several statistical tests were employed in this investigation.

1) The *Student's t-test* was used to test the difference between two treatments whose responses were graded.
2) The *Welch test* (Welch, 1947) was used in place of the Student's t-test when variances could not be assumed to be equal and were not proportional to the mean.
3) The *Aspin-Welch test* is an extension of the former test and was used to test the difference between the synthesis or breakdown in control and drug treated animals (see Chapter 4). In order to do this, the levels of amine in control and in αMPT/pargyline treated animals were compared to the levels of amine in drug and in drug plus αMPT/ pargyline treated animals in the following way:

$$t' = \frac{(\bar{X}_1 - \bar{X}_2) - (\bar{Y}_1 - \bar{Y}_2)}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2} + \frac{S_3^2}{n_3} + \frac{S_4^2}{n_4}}}$$

and $$v = \frac{\left(\frac{S_1}{n_1} + \frac{S_2}{n_2} + \frac{S_3}{n_3} + \frac{S_4}{n_4}\right)^2}{\left[\frac{S_1^2}{n_1} \Big/ (n_1-1)\right] + \left[\frac{S_2^2}{n_2} \Big/ (n_2-1)\right] + \left[\frac{S_3^2}{n_3} \Big/ (n_3-1)\right] + \left[\frac{S_4^2}{n_4} \Big/ (n_4-1)\right]}$$

where $\bar{X}_1$ = mean amine level in controls

$\bar{X}_2$ = mean amine level in control + αMPT group

$\bar{Y}_1$ = mean amine level in RMI 12,936 group

$\bar{Y}_2$ = mean amine level in RMI 12,936 + αMPT group

S_1, S_2, S_3 and S_4 = standard deviation of $\bar{X}_1$, $\bar{X}_2$, $\bar{Y}_1$ and $\bar{Y}_2$ respectively.

n_1, n_2, n_3 and n_4 = number of animals in the groups $\bar{X}_1$, $\bar{X}_2$, $\bar{Y}_1$ and $\bar{Y}_2$ respectively.

t' = value of the t distribution for v degrees of freedom

v = number of degrees of freedom

4) The *Analysis of Variance* was used as a multiple comparison test.

5) The *Fisher Exact test* was used to test the difference between two treatments whose responses were quantal.

6) The *Probit Analysis test* gave the median effective dose and relative potency of treatments whose responses were quantal.

7) All fiducial limits are at 95%.

PART 3

DETAILED METHODS AND EXPERIMENTAL RESULTS

CHAPTER 1

Characteristics of the antiovulatory activity of RMI 12,936

INTRODUCTION

Previous investigations have provided information on the antiovulatory action of RMI 12,936. If administered at 16:00h on Day D2 in a dose level of 2mg/rat, RMI 12,936 will fully block ovulation but fails to do so if administered at 12:00h or later on the day of proestrus (Kendle et al, 1978). In the same study it was shown that the drug inhibits both cyclic and tonic release of LH. This RMI 12,936 block of ovulation can be reversed by the administration of LHRH at 16:00h on Day P or by the administration of 2µg oestradiol at 17:00h on Day D2 plus 2mg progesterone at 13:00h on Day P (Kendle et al, 1978; Geddes et al, 1979). The aim of this study was to confirm these results and extend knowledge of RMI 12,936 antiovulatory activity by:

i) correlating effects on LH with effects on ovulation in the same animals,

ii) establishing exact time limits for the effectiveness of RMI 12,936 administration and

iii) investigating the effect of RMI 12,936 on LH release in ovariectomized steroid treated rats.

METHODS

Expt. I Effect of RMI 12,936 on the preovulatory rise in plasma LH and on ovulation

Two groups of rats were dosed with 2mg of RMI 12,936 or 0.5ml of the vehicle at 16:00h on Day D2. Blood samples of approximately 0.25ml were collected from the tail vein

of each rat at 15:30h, 16:30h, 17:00h, 17.30h, 18:00h, 18:30h and 19:00h on Day P. The samples were then analysed for LH concentration as described in the general methods section. At autopsy on the following day between 09:00h and 12:00h, the oviducts were examined for the presence of ova.

Expt. II Reversal of the RMI 12,936 block of ovulation with LHRH

Two groups of animals were dosed with 2mg RMI 12,936 at 16:00h on Day D2 plus LHRH (0.125, 0.25, 0.5, 1.0 or 2.0µg in a dose volume of 0.5ml) or 0.5ml of the vehicle at 16:00h on Day P. The animals were sacrificed on the expected day of oestrus and the oviducts examined for the presence of ova.

Expt. III Time dependence of the antiovulatory activity of RMI 12,936

2mg RMI 12,936 was administered to a group of rats at various times throughout Days D2 and P (see table 10). A second group of animals were injected with 0.5ml of the vehicle at several of these times. Autopsy was between 09:00h and 12:00h on the expected day of oestrus when the numbers of animals ovulating were determined.

Expt. IV Effect of ovariectomy on RMI 12,936 activity

A group of 10 rats were ovariectomized. After a period of 3 weeks all 10 animals were treated with 2µg oestradiol at 12:00h (designated Day 1). Five of these animals were

dosed with 2mg RMI 12,936 at 16:00h on Day 2 and 5 with 0.5ml of the vehicle. On Day 3 at 13:00h all 10 rats received 0.5mg progesterone. Four hours later, the rats were killed by decapitation on a guillotine and blood samples collected for assay of LH.

Expt. V Reversal of the RMI 12,936 block of ovulation with oestrogen plus progesterone

Three groups of rats received 2mg RMI 12,936 at 16:00h on Day D2. The first group of rats also received 2µg oestradiol at 17:00h on Day D2 plus 2mg progesterone at 13:00h on Day P. The second group of rats were dosed with 0.5ml of the vehicle at the times of administration of the sex steroids in group one. These drug regimes were administered to animals in various months throughout the year (see table 12). The third group of rats also received 2µg oestradiol one hour after RMI 12,936 administration. This latter experiment was performed during the months of January, February and March in 1981. A fourth group of animals received 0.5ml of the vehicle in place of each of the steroids administered to group 1. All animals were autopsied on the expected day of oestrus and the presence of ova in the oviduct noted.

RESULTS

Expt. I Effect of RMI 12,936 on the preovulatory rise in plasma LH and on ovulation

None of the rats treated with RMI 12,936 ovulated whereas all five of the control rats ovulated (table 7). During the time interval measured, no surges of LH were present

Table 7: Serum LH levels (ng/ml) in control (C) and in RMI 12,936 (R) treated rats at various times on Day P. Each row represents the LH serum levels from individual rats.

		LH CONCENTRATION (ng/ml)				
15:30h	C	*9.5	*6.0	*2.5	*4.9	*4.7
	R	2.1	3.0	3.3	n.d.	n.d.
16:30h	C	30.8	131.8	*6.5	*5.9	*5.3
	R	1.7	1.9	1.2	n.d.	0.7
17:00h	C	155.7	168.5	35.0	*7.7	*7.9
	R	1.2	1.3	1.5	0.5	n.d.
17:30h	C	160.0	167.0	28.7	*6.6	*7.4
	R	n.d.	0.9	1.0	0.8	n.d.
18:00h	C	103.4	142.4	109.8	*6.2	*6.7
	R	1.8	4.0	3.1	n.d.	n.d.
18:30h	C	94.4	47.4	138.7	*7.6	17.8
	R	0.7	3.6	2.2	n.d.	n.d.
19:00h	C	75.7	22.7	131.6	*11.6	90.2
	R	0.6	3.1	0.6	n.d.	n.d.
No. Ova Dectected	C	14	10	11	11	10
	R	0	0	0	0	0

n.d. - values were not detectable below 0.032 ng/ml

* - indicates levels of LH in control animals taken as pre-surge basal values.

in any of the rats treated with RMI 12,936 and the mean LH value in these animals (1.2 ± 0.2ng/ml) was significantly lower (Welch test, $P < 0.005$) than basal levels in controls (6.7 ± 0.5ng/ml). In the control animals, the peak of the LH surge occurred at a different time for each animal tested, with no surge of LH occurring during this time interval in one case (fig. 9a). The mean maximum LH value in the three animals which showed a peak during this time interval was 155.7 ± 8.9ng/ml (table 8).

Expt. II Reversal of the RMI 12,936 block of ovulation with LHRH

Ovulation can be fully restored to RMI 12,936 treated rats by administration of 2µg LHRH whereas 0.125µg LHRH fails to restore ovulation to any of the animals treated (table 9). The median effective dose (ED_{50}) calculated by probit analysis, of LHRH in reversal of the RMI 12,936 block of ovulation is 0.599µg with fiducial limits of 0.382 and 1.042.

Expt. III Time dependence of the antiovulatory activity of RMI 12,936

2mg RMI 12,936 blocked ovulation in all animals treated with the drug between 16:00h on Day D2 and 01:00h on Day P (see table 10). However administration of RMI 12,936 later than 01:00h on Day P, failed to block ovulation on the following day. All animals treated with the vehicle ovulated on the expected day of oestrus.

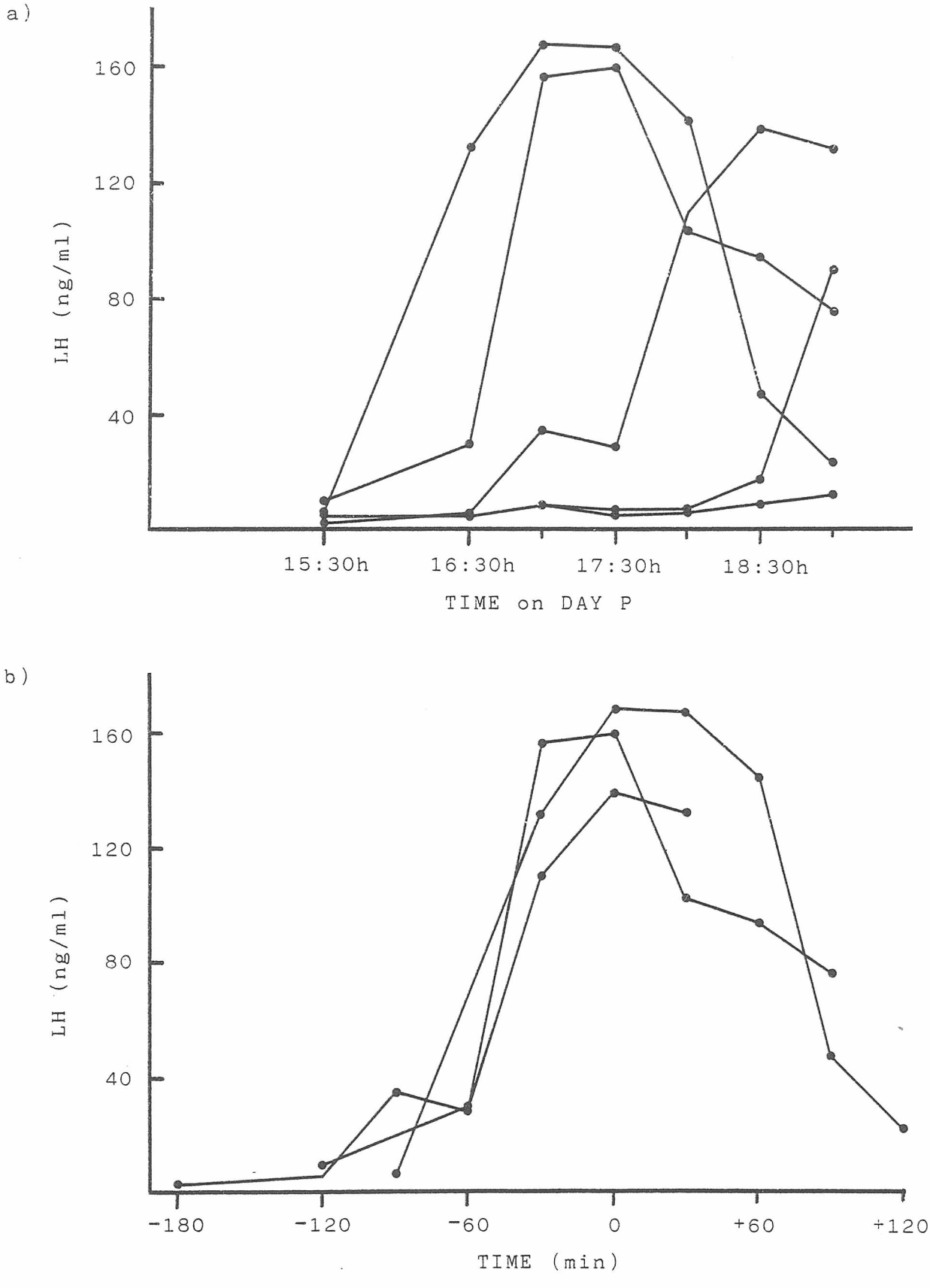


Fig. 9 Serum LH levels (ng/ml) in a) 5 individual control rats between 15:30h and 19:00h on Day P and b) 3 individual control rats where the maximum LH value is taken as time 0h.

Table 8: Serum LH levels (ng/ml) in individual control rats showing an LH peak between 15:30h and 19:00h on Day P. The maximum LH value in each rat is designated time 0h.

TIME (Min)	LH CONCENTRATION			MEAN	SEM
- 180	-	-	2.5	2.5	-
- 150	-	-	-	-	-
- 120	9.5	-	6.5	8.0	1.5
- 90	-	6.0	35.0	20.5	14.5
- 60	30.8	-	28.7	29.8	1.0
- 30	155.7	131.8	109.8	132.4	13.3
0	160.0	168.5	138.7	155.7	8.9
+ 30	103.4	167.0	131.6	134.0	18.4
+ 60	94.4	142.4	-	118.4	24.0
+ 90	75.7	47.4	-	76.6	14.2
+ 120	-	22.7	-	22.7	-

Table 9: Effect of various concentrations of LHRH administered at 16:00h on Day P on the RMI 12,936 block of ovulation.

LHRH (µg/rat)	No. of Rats Ovulating(%)
2.0	5/5 (100)
1.0	4/5 (80)
0.5	2/7 (29)
0.25	1/7 (14)
0.125	0/5 (0)

Table 10: The effect of 2mg RMI 12,936 on ovulation when administered at various times between 16:00h on Day D2 and 13:00h on Day P.

	Time of Administration	No. of Rats	No. Ovulating (%)
RMI 12,936	16:00h Day D2	5	0 (0)
	18:00h Day D2	5	0 (0)
	20:00h Day D2	5	0 (0)
	21:00h Day D2	5	0 (0)
	01:00h Day P	5	0 (0)
	03:00h Day P	5	5 (100)
	05:00h Day P	5	5 (100)
	07:00h Day P	5	5 (100)
	09:00h Day P	5	5 (100)
	11:00h Day P	5	5 (100)
	13:00h Day P	5	5 (100)
VEHICLE	16:00h Day D2	5	5 (100)
	20:00h Day D2	4	4 (100)
	01:00h Day P	5	5 (100)
	03:00h Day P	5	5 (100)
	07.00h Day P	4	4 (100)
	13:00h Day P	4	4 (100)

Expt. IV Effect of ovariectomy on RMI 12,936 activity

RMI 12,935 treatment did not significantly alter (Student's t-test) the LH serum levels in ovariectomized, oestrogen-, progesterone-primed rats (table 11).

Expt. V Reversal of the RMI 12,936 block of ovulation with oestrogen or oestrogen plus progesterone

Administration of 2µg oestradiol plus 2mg progesterone restored ovulation to the RMI 12,936 treated rats when the experiment was carried out during the months of January, February, March and April (table 12). When the experiment was performed during the months of July, August, September and October, the sex steroids failed to restore ovulation to any of the rats. However during the months of November and December, ovulation was restored to 30% and 40% of the animals respectively. The number of rats treated with RMI 12,936 which failed to ovulate after oestradiol plus progesterone administration during the months of November and December were significantly different ($P < 0.05$) from both months of July and August, and February and March (Fisher Exact test). All of the animals treated with RMI 12,936 and the vehicle, failed to ovulate at all times of the year whereas 97% of the controls ovulated (table 13).

In the third group of animals, treated with RMI 12,936 followed by oestradiol one hour later, 3 out of 7 rats ovulated (43%). This figure was significantly different from RMI 12,936 treatment (Fisher Exact test, $P = 0.017$).

Table 11: LH serum levels (ng/ml) in ovariectomized, oestrogen-, progesterone-primed rats which had been dosed with 2mg RMI 12,936 or its vehicle.

	VEHICLE	RMI 12,936
LH conc.	178.3	189.9
	183.3	160.4
	189.9	188.4
	200.0	189.8
	179.3	197.2
MEAN	186.2	185.1
SEM	4.0	6.4

Table 12: Seasonal effect of administration of 2µg oestradiol at 17:00h on Day D2 plus 2mg progesterone at 13:00h on Day P on rats treated with 2 mg RMI 12,936 at 16:00h on Day D2.

MONTH	YEAR	No. Ovulating	Total No. Ovulating (%)
January	1979	3/3	6/7 (86)*
	1980	1/1	
	1981	2/3	
February	1979	3/3	9/9 (100)*†
	1980	3/3	
	1981	3/3	
March	1979	2/2	10/11 (91)*†
	1980	5/5	
	1981	3/4	
April	1981	2/2	2/2 (100)
July	1979	0/19	0/19 (0)*†
August	1979	0/20	0/25 (0)*†
	1980	0/5	
September	1979	0/8	0/8 (0)
October	1980	0/6	0/6 (0)
November	1979	2/6	3/10 (30)
	1980	1/4	
December	1980	2/5	2/5 (40)

* Significantly different ($P < 0.05$) from the number of rats ovulating during November.

† Significantly different ($P < 0.05$) from the number of rats ovulating during December.

Table 13: Effect of RMI 12,936 or RMI 12,936 plus oestradiol on ovulation.

TREATMENT	MONTH	No. Ovulating	Total No. Ovulating (%)
0.5ml vehicle at 16:00h and 17:00h on Day D2 plus 0.5ml vehicle at 13:00h on Day P	January	6/6	98/102 (97)
	February	4/4	
	March	7/7	
	April	4/4	
	May	3/3	
	June	3/3	
	July	8/8	
	August	9/10	
	September	10/10	
	October	14/16	
	November	19/20	
	December	11/11	
2mg RMI 12,936 at 16:00h plus 0.5ml vehicle at 17:00h on Day D2 plus 0.5ml vehicle at 13:00h on Day P.	January	0/4	0/54 (0)
	February	0/5	
	March	0/8	
	April	0/4	
	May	0/3	
	June	0/2	
	July	0/6	
	August	0/5	
	September	0/3	
	October	0/4	
	November	0/5	
	December	0/5	
2mg RMI 12,936 16:00h plus 2µg Oestradiol at 17:00h on Day D2	January	1/3	3/7 (43)
	February	1/2	
	March	1/2	

Summary of Results

1) Administration of RMI 12,936 completely blocked the preovulatory LH peak which was observed in 4/5 controls between 15:30h and 19:00h on the day of proestrus. A complete block of ovulation was also observed in RMI 12,936 treated animals while all controls ovulated.

2) Ovulation was restored in RMI 12,936 treated animals by LHRH, the ED_{50} being 0.6µg given at 16:00h on Day P.

3) Ovulation blockade resulted when administration of RMI 12,936 was given at or before 01:00h on Day P but not when administration was at 03:00h or later.

4) Serum LH values obtained in ovariectomized rats treated with oestradiol and progesterone were unaffected by administration of RMI 12,936 and were similar to peak LH values obtained in control animals.

5) Ovulation was restored in RMI 12,936 treated rats with oestradiol and progesterone administration in experiments performed between February and April but not in exactly similar experiments performed between July and October. A progressive increase in response was seen between November and January.

6) Administration of oestradiol alone was partly effective in restoring ovulation in RMI 12,936 treated rats during the steroid sensitive period of January and March.

Discussion

The results obtained in this study confirm and extend previous observations on RMI 12,936 antiovulatory activity. Measurement of circulating LH levels during the expected time of the preovulatory LH surge showed that the anti-ovulatory activity of RMI 12,936 is associated with an inhibition of this proestrus rise in LH levels confirming the observations of Kendle and his coworkers (1978). It is interesting to note that although all five control rats ovulated, the preovulatory peak in LH plasma concentration occurred at a different time in each animal and that in one of the rats no LH peak was observed during the time interval measured. This highlights the variation which can occur between individual animals under these experi-mental conditions. This experiment extends previous findings in determining the incidence of ovulation in the same animals as were used for LH determinations. The results show that supression of the LH peak is associated with ovulation blockade while experimental conditions involving the stress of serial blood sampling do not affect ovulation in control animals. These procedures might however have contributed to the observed variation in the time of the LH peak.

Administration of LHRH restored ovulation to RMI 12,936 treated rats confirming that the antiovulatory effect is reversible (Kendle et al, 1978). Since release of quantities of LH sufficient to induce ovulation can be stimulated by LHRH, the mechanism inhibiting ovulation cannot be by inhibition of LH synthesis in the adeno-

hypophysis nor by interference with the mechanisms involved in release of LH from the adenohypophysis.

It has been reported that RMI 12,936 failed to block ovulation when administered at 12:00h on proestrus but was effective when administered at 16:00h on dioestrus II (Kendle et al, 1978), and therefore it was suggested that RMI 12,936 will block ovulation only if administered two days before the expected day of ovulation. In this study more detailed investigation has shown that the compound can be administered as late as 01:00h on proestrus and still produce a complete block of ovulation in all of the animals tested. Administration two hours later, at 03:00h, failed to block ovulation in any of the animals tested. It is therefore clear that RMI 12,936 must be administered at least 16h before the preovulatory LH surge (occurring at approximately 17:00h in this colony of rats) for it to be effective in blocking ovulation.

This finding raises two possibilities. Either that the antiovulatory activity of RMI 12,936 is dependent on a slowly forming metabolite or alternatively that it is dependent on the endocrine condition of the animal at the time of administration. The first possibility is strongly supported by the evidence from antifertility studies (see general introduction) which led to the hypothesis that the antifertility activity of RMI 12,936 is due to the activity of a slowly forming metabolite which requires the presence of an intact ovary for 48h.

To investigate whether ovarian metabolism of RMI 12,936 is necessary for its antiovulatory activity, the effect of the drug on plasma LH levels in ovariectomized, oestrogen-, progesterone-primed rats was measured. The results of this experiment showed that the plasma LH levels were not significantly altered by RMI 12,936 treatment in the absence of the ovary. However this finding could not be taken as an indication that ovarian metabolism is necessary, since oestrogen plus progesterone administration can reverse the inhibitory effect of RMI 12,936 on ovulation (Geddes et al, 1979). Therefore if RMI 12,936 is active in the ovariectomized rat, the steroids must be acting in this situation to reverse the inhibitory effect of RMI 12,936 on LH levels and so the result of this experiment leaves the question open.

The results of antifertility studies have demonstrated that pregnancy cannot be maintained in the intact RMI 12,936 treated rat by endogenous or exogenous progesterone (Kendle, 1975). This action was attributed to the active metabolite. If the active metabolite is responsible for the antiovulatory activity of RMI 12,936, then it would be expected that exogenous progesterone will not restore ovulation. However, the antiovulatory effect of the drug can be reversed partly by exogenous progesterone and fully by exogenous oestrogen and progesterone (Kendle et al, 1978; Geddes et al, 1979).

From these observations it can be concluded that the mechanisms behind the antifertility and antiovulatory activities of RMI 12,936 differ. In the latter case, the activity may be either due to the slow formation of a metabolite but one which is different from the active metabolite responsible for the antifertility effect of RMI 12,936 or it may not require the slow formation of an active metabolite but is instead dependent on altering the endocrine conditions of the animal for its antiovulatory effect.

It is well known that the effect of a steroid is dependent on the endocrine condition of the animal with both oestrogen and progesterone, having an inhibitory or a facilitatory effect on ovulation depending on the time of administration (see general introduction). Administration of progesterone late on the evening of dioestrus II (22:00h) was shown to block ovulation and prevent the proestrus rise of serum oestradiol (Kalra & Kalra, 1974). In view of the biphasic stimulatory feedback effects of oestrogen on ovulation (see general introduction), this observation raised the possibility that progesterone, by inhibiting the rise in oestrogen levels, prevented oestrogen concentrations reaching the threshold level sufficient to facilitate the neural trigger for preovulatory LH release. This mechanism of progesterone action gained support from the finding that exogenous oestrogen restored ovulation to the progesterone blocked rat but only when administered before 03:00h on proestrus (Kalra, 1975). The timing of effectiveness of oestrogen replacement parallels the change from inhibition to facilitation of progesterone on

LH release which was shown to occur between 21:30h on dioestrus II and 03:00h on proestrus (Brown-Grant, 1969b). Thus it can be concluded that progesterone, by exhibiting antioestrogenic activity, inhibits ovulation provided that insufficient priming of the CNS sites of oestrogen positive feedback have occurred. Once sufficient oestrogen priming has been achieved ie by 03:00h on proestrus, progesterone is incapable of inhibiting ovulation. This inhibitory mechanism of action is supported by the evidence of Aiyer and Fink (1974) who showed that the antioestrogen, ICI 46,474, is only effective in blocking ovulation when administered before 05:00h on proestrus. Since RMI 12,936 is also effective in blocking ovulation only when administered before 03:00h on proestrus, it is possible that it may also owe its antiovulatory activity to an antioestrogenic activity.

RMI 12,936 is known to inhibit progesterone synthesis (Kendle, 1976) probably by acting as an alternative substrate for Δ^5- 3 ketosteroid isomerase (Taylor & Kendle, 1978) and although oestrogen production has been shown not to be rate limited by enzymic isomerization of Δ^5- 3 ketosteroids (Csapo, Resch, Csapo & Salou, 1979) it is not unlikely that RMI 12,936 will also inhibit synthesis of oestrogen in the rat since significant reductions in oestrogen levels have been reported in the female golden hamster (Lau & Saksena, 1979). Therefore it is possible that RMI 12,936 blocks ovulation at least in part by acting in a similar manner to progesterone, preventing the second phase rapid elevation in systemic oestrogen. Hence RMI 12,936 would prevent oestrogen levels reaching

threshold for activation of the neural trigger for LHRH release. An alternative mechanism of antiovulatory action which would fit in with present evidence would involve antagonism of oestrogen utilization and therefore interference of the second phase priming action of oestrogen essential for preovulatory LH release.

If RMI 12,936 was to block ovulation by exhibiting an anti-oestrogenic effect either by inhibition of oestrogen synthesis or by competitive antagonism of oestrogen at the receptor level, exogenous oestrogen might be expected to restore ovulation. Oestradiol administration did restore ovulation but only to 43% of the animals. This could be interpretated as the oestrogen concentration not being quite sufficient in the cases where ovulation is not restored or that replacement did not mimic physiological conditions. For example, Kalra (1975) suggested that the hypothalamus requires continuous exposure to oestrogen until 03:00h on proestrus to ensure ovulation and this hypothesis was supported by an experiment which showed that a proestrus-like surge of LH could be attained in ovariectomized rats if a two-step treatment schedule with oestrogen was followed (Caligaris, Astrada & Taleisnik, 1971). It has also been demonstrated that to induce ovulation, oestrogen requires the presence of progesterone since oestrogen was incapable of inducing ovulation in ovariectomized-adrenalectomized rats (Mann & Barraclough, 1973). Since RMI 12,936 is known to inhibit progesterone synthesis, it is quite likely that both oestrogen and progesterone levels have been reduced in RMI 12,936 treated rats. This raises the possibility that

the antiovulatory activity is associated with an anti-progestational effect in conjunction with an anti-oestrogenic effect. This idea is supported by experiments in this and earlier studies (Kendle et al, 1978; Geddes et al, 1979) which showed that progesterone restored ovulation to 56% and that administration of both oestradiol and progesterone produced a full restoration of ovulation. Therefore it can be concluded that RMI 12,936 inhibits the preovulatory LH surge by exhibiting both antioestrogen and antiprogestational activity. In view of the sequence of events leading to ovulation, outlined in the general discussion, and the results of this study, it is proposed that RMI 12,936 blocks ovulation by preventing oestrogen priming of its CNS sites of positive feedback and by preventing oestrogen and progesterone priming of the adenohypophysis to LHRH.

Further results obtained in this study showed that reversal of the RMI 12,936 block of ovulation by oestrogen and progesterone was dependent on the time of year, with administration in January, February, March and April reversing the block whereas administration of the steroids between the months of July and October failed completely to restore ovulation. Ovulation did occur in some of the rats treated with the steroids during the months of November and December but these numbers were not significant. These findings suggest that the rats may be exhibiting seasonal variation in sensitivity of the mechanisms controlling LH secretion, displaying low sensitivity during the summer and autumn seasons which increases during the winter months to

attain greatest sensitivity during springtime. Seasonal variation has been demonstrated on various parameters of rats under controlled environmental conditions such as metabolic (Mikeska & Petrasek, 1977), male reproductive processes (Mock & Frankel, 1978) and puberty (Cohen & Mann, 1979). However little information is known about seasonal variation in female reproductive processes in the laboratory rat under controlled environmental conditions. Slama-Scemama and L'Heritier (1978) reported that the increase in plasma LH levels in immature female rats is larger in springtime than in winter and concluded from this that a seasonal variation in mechanisms controlling LH release may exist. These results are in agreement with the findings of this study and shows that seasonal variation in mechanisms controlling LH release exists in the adult rat also. It is interesting to note that this increase in sensitivity of the mechanisms controlling LH release in springtime, is in good agreement with the activation of metabolic processes (Golikov & Golikov, 1973) and parallels peaks of reproductive activity seen in the wild rat population (Davis, 1953).

Survey of the literature has revealed some conflicting results which may be explained by seasonal variation in mechanisms controlling LH release. In 1976, Krieg and Sawyer showed that intraventricular administration of 40µg NA produced very large increases in LH plasma levels (preinjection value of 60 $\pm$ 25ng/ml rising to maximum postinjection levels of 222 $\pm$ 44ng/ml) whereas Vijayan and McCann (1978b) showed a relatively small although also

significant increase in LH plasma levels (preinjection value of 1.7 ± 0.2ng/ml rising to maximum postinjection levels of 4.5 ± 0.6ng/ml) to the same dose of intraventricularly administered NA. Although the same strain of rats were used and similar experimental procedure followed, the response to NA differs considerably. It is also noteworthy that there is a very large (approximately 35-fold) difference in the basal LH values. In the light of the findings of this study it is suggested that the differences between the results of these similar experiments may be due to seasonal variation in the sensitivity of the mechanisms controlling LH release with the experiment of Krieg and Sawyer being conducted at a more sensitive period, perhaps during springtime and the experiment of Vijayan and McCann being conducted at less sensitive periods. This variation in plasma LH levels can also be demonstrated between the experiments of Schneider and McCann (1970) and Vijayan and McCann (1978b) although again the experimental procedures followed were similar.

Further evidence in support of this proposal of seasonal variation in the mechanisms controlling LH release has been obtained in a later part of this investigation. Results given in Chapter 2 show that in PB treated rats 0.15µg LHRH increased the levels of LH from a mean value of 6.0 to 138.1ng/ml when performed during the months of February and March, whereas in an earlier preliminary experiment performed during the months of June and July, 5µg LHRH only raised the LH values from a mean value of 0.69 to 1.04ng/ml. As this was a preliminary experiment the results are not presented in Chapter 2, but are given

in full in appendices V and VI. Likewise in RMI 12,936 treated rats LHRH was more effective in increasing the secretion of LH when the experiment was performed in early spring (1.0µg LHRH raised the LH levels from 0 to 101.8ng/ml) than in early summer (1.0µg LHRH raised the LH level from 1.62 to 1.93ng/ml). Thus in retrospect, this investigation has revealed that the mechanisms controlling release of adenohypophysial LH appear to have greater sensitivity during springtime than the rest of the year and indicates that a prospective study into seasonal variation would be of value.

In summary, the results of this study have shown that RMI 12,936 will block ovulation only if administered before 03:00h on proestrus suggesting that it interferes with the priming action of oestrogen. This block of ovulation is associated with an inhibiton of the pre-ovulatory LH surge and can be reversed by the administration of either LHRH or oestrogen plus progesterone. It was suggested that the mechanisms behind RMI 12,936's anti-ovulatory activity differ from those of its anti-fertility activity with the former effect possibly not requiring the formation of an active metabolite but is instead dependent on the endocrine condition of the animal. It also proposed that the antiovulatory activity of RMI 12,936 was associated with both an antioestrogenic and an antiprogestational effect with RMI 12,936 preventing oestrogen priming of its CNS sites of positive feedback and by preventing oestrogen and progesterone priming of the adenohypophysis to LHRH. Consideration of the evidence

outlined in this study suggests that a seasonal variation in mechanisms controlling the release of LH from the adenohypophysis exists in the female rat.

CHAPTER 2

The effect of RMI 12,936 on adenohypophysial responsiveness to LHRH

INTRODUCTION

The adenohypophysial responsiveness to LHRH increases dramatically between late dioestrus and the preovulatory LH surge on proestrus (Aiyer & Fink, 1974). This increase in responsiveness is essential for LHRH stimulation of sufficient LH secretion to trigger ovulation and the factors responsible for this increase are the gonadal steroids and LHRH (see general introduction).

Since it has been proposed that RMI 12,936 blocks ovulation by exhibiting antioestrogenic and antiprogestational activity, this investigation is aimed at testing the hypothesis that RMI 12,936 blocks ovulation partly by inhibition of steroid priming of the adenohypophysis to LHRH. Previous studies have shown that adenohypophysial sensitivity to LHRH was significantly lower in RMI 12,936 treated rats than in PB treated rats and that steroid replacement, although given in a dose regime known to restore ovulation, failed to restore full sensitivity to the adenohypophysis (Geddes et al, 1979).

This investigation is also aimed at extending previous work by:

(i) comparison of the methods of ovulation detection and measurement of serial LH levels, for determination of adenohypophysial sensitivity to LHRH.

(ii) determining the extent of RMI 12,936 inhibition of adenohypophysial sensitization by comparison with adenohypophysial sensitivity on dioestrus II

(iii) looking at the mechanisms involved in the RMI 12,936 inhibition of full sensitivity.

METHODS

Expt. I Effect of drug treatment on adenohypophysial sensitivity to LHRH

Three groups of rats received the following treatments: group A, 0.5ml vehicle at 16:00h and 17:00h on Day D2 + 0.5ml vehicle at 13:00h + 35mg/kg PB at 15:00h on Day P; group B, 2mg RMI 12,936 at 16:00h + 0.5ml vehicle at 17:00h on Day D2 + 0.5ml vehicle at 13:00h + 0.5ml saline at 15:00h on Day P; group C, 2mg RMI 12,936 at 16:00h + 2µg oestradiol at 17:00h on Day D2 + 2mg progesterone at 13:00h + 35mg/kg PB at 15:00h on Day P. A blood sample was collected from the tail vein of each rat (in a volume not exceeding 0.3ml) at 15:30h on Day P and after a period of 30 min, a dose of LHRH was administered (see table 14). After LHRH administration, blood samples were collected at half hourly intervals, the last being collected at 19:00h on Day P. A fourth group of rats received various doses of LHRH at 11:00h on Day D2 (see table 14). Blood samples were collected from each animal at 10:30h, 11:30h, 12:00h, 12:30h, 13:00h, 13:30h and 14:00h on Day D2.

The blood samples were assayed for LH concentration as described in the general methods section. At autopsy on the expected day of oestrus, the number of rats ovulating were counted. This experiment was performed during the months of January, February, March and April.

Expt. II Effect on adenohypophysial responsiveness to LHRH of PB administered between 13:30h and 15:00h on proestrus.

Three groups of rats were injected with 35mg/kg PB at 13:30h, 14:30h and 15:00h on Day P. At 16:00h, each animal received 0.5ml saline (ip) or 0.5ml LHRH in various concentrations (see table 15). Autopsy was performed between 09:00h and 12:00h on the expected day of oestrus in order to determine the number of animals which had ovulated. This experiment was performed during the months of April and May.

Expt. III Effect of serial administration of LHRH on adenohypophysial responsiveness to LHRH after various treatments.

Three groups of rats received the following pretreatment: group 1, 35mg/kg PB at 14:30h on Day P; group 2, 2mg RMI 12,936 at 16:00h plus 0.5ml vehicle at 17:00h on Day D2 plus 0.5ml vehicle at 13:00h plus 0.5ml saline at 14:30h on Day P; group 3, 2mg RMI 12,936 at 16:00h plus 2µg oestradiol at 17:00h on Day D2 plus 2mg progesterone at 13:00h plus 35mg/kg PB at 14:30h on Day P. Each group received 5ng/100g BW LHRH (iv) at 14:45h, 15:15h and 15:45h on Day P. A fourth group of rats received 35mg/kg PB at 14:30h plus 0.5ml saline instead of LHRH at 14:45, 15:15h and 15:45h on Day P. All four groups received LHRH in various concentrations (see table 16) at 16:ooh on Day P. On the following day, each animal was sacrificed between 09:00h and 12:00h and autopsied to determine the number of animals which had ovulated. This experiment was performed during the month of June.

RESULTS

Expt. I Effect of drug treatment on adenohypophysial sensitivity to LHRH

A. Measurement of LH levels

The figures for the circulating levels of LH in individual rats are displayed in appendices I to IV. For the purpose of determining the effect of drug treatment on adenohypophysial sensitivity to LHRH, the maximum increase in LH for individual rats in each group was used to calculate the mean maximum increase in LH for each treatment (table 14). From these results the relative potency of LHRH in each group of rats was calculated using analysis of variance.

The relative potencies of LHRH were 0.156 (fiducial limits of 0.124 to 0.198), 0.185 (fiducial limits of 0.137 to 0.234) and 0.041 (fiducial limits of 0.033 to 0.054) for groups of rats treated with RMI 12,936, RMI 12,936 plus gonadal steroids plus PB and Day D2 control rats respectively. These figures were calculated by taking the PB treated group as the standard with a potency of 1.0. The relative potency of LHRH in all three groups was significantly different from that of the PB treated group. There was no significant difference between the relative potencies of LHRH in RMI 12,936 and RMI 12,936 plus gonadal steroids plus PB treated groups. The relative potency of LHRH in Day 2 control rats was significantly different from the other three groups.

Table 14 : Effect of various pretreatments on adenohypophysial sensitivity to LHRH as measured by the maximum LH values in individual rats or by the percentage of animals ovulating.

PRETREATMENT	DOSE LHRH (µg)	TIME OF LHRH ADMINISTRATION	MEAN MAXIMUM LH VALUE (ng) ± SEM	NO. OVULATING (%)
DAY D2: 0.5ml vehicle at 16:00h and 17:00h. DAY P : 0.5ml vehicle at 13:00h + 35mg/kg PB at 15:00h.	0.05	16:00h on Day P	(15.1 ± 1.5)	0/10 (0)
	0.10		34.6 ± 13.5	2/10 (20)
	0.15		137.8 ± 10.7	9/10 (90)
	0.20		125.8 ± 18.4	5/5 (100)
DAY D2: 2mg RMI 12,936 at 16:00h + 0.5ml vehicle at 17:00h. DAY P : 0.5ml vehicle at 13:00h + 0.5ml saline at 15:00h.	0.125	16:00h on Day P	(5.9 ± 0.1)	0/5 (0)
	0.25		(16.7 ± 3.2)	1/7 (14)
	0.50		28.6 ± 5.1	2/7 (29)
	1.00		101.8 ± 49.1	4/5 (80)
	2.00		204.5 ± 10.5	5/5 (100)
DAY D2: 2mg RMI 12,936 at 16:00h + 2µg oestradiol at 17:00h DAY P : 2mg progesterone at 13:00h + 35mg/kg PB at 15:00h.	0.25	16:00h on Day P	(7.3 ± 0.2)	0/5 (0)
	0.375		28.2 ± 18.1	1/5 (20)
	0.50		40.7 ± 12.9	3/5 (60)
	1.00		125.9 ± 20.0	5/5 (100)
NO PRETREATMENT	1.25	11:00h on Day D2	(57.4 ± 14.6)	0/5 (0)
	2.50		63.0 ± 17.1	1/5 (20)
	3.75		132.8 ± 5.2	3/5 (60)
	5.00		136.4 ± 10.4	4/5 (80)
	6.25		156.0 ± 17.4	5/5 (100)

The figures in parentheses were omitted from statistical analysis in order to obtain linearity and parallelism.

B. Detection of Ovulation

The ED_{50} of LHRH for induction of ovulation was 0.117µg, 0.599µg and 0.468µg in rats treated with PB, RMI 12,936 and RMI 12,936 plus gonadal steroids plus PB respectively (table 14). The relative potency of LHRH was 0.189 in RMI 12,936 treated rats and 0.249 in RMI 12,936 + gonadal steroids+PB treated rats where the potency of LHRH in PB treated rats was taken as standard, designated as 1.0. These figures were significantly different from the standard but not from each other (fiducial limits of 0.085 to 0.361 and 0.165 to 0.333 respectively, where $P \leq 0.05$).

The ED_{50} of LHRH for induction of ovulation in Day D2 animals was 3.417µg and the relative potency of LHRH was 0.034 with fiducial limits of 0.024 and 0.053 where the potency of LHRH in PB treated animals was again taken as the standard. The relative potency of LHRH in Day D2 animals is significantly less than those of the other three treatments.

The ED_{50} and relative potency of LHRH were calculated using probit analysis.

Expt. II Effect on adenohypophysial responsiveness to LHRH of PB administered between 13:30h and 15:00h on proestrus

PB completely blocked ovulation when administered at 13:30h, 14:30h or 15:00h on Day P in animals treated with saline at 16:00h on Day P (table 15). The ED_{50} of LHRH required to restore ovulation to these animals was 0.408,

Table 15: Effect of PB administered at 15:00h, 14:30h and 13:30h on proestrus on adenohypophysial responsiveness to LHRH administered at 16:00h as measured by the percentage ovulating on oestrus.

Time of PB Administration	Dose LHRH (µg)	No. Ovulating (%)
15:00h	0:05 0.10 0.15 0.20	0/10 (0) 2/10 (20) 9/10 (90) 5/5 (100)
14:30h	0.10 0.20 0.375 0.50	0/6 (0) 3/6 (50) 4/5 (80) 5/5 (100)
13:30h	0.125 0.25 0.50 1.00 2.00	0/5 (0) 2/5 (40) 3/5 (60) 4/5 (80) 5/5 (100)

0.222 and 0.117 for animals treated with PB at 13:30h 14:30h and 15:00h respectively. The relative potencies of LHRH for each treatment were 0.274, 0.517 and 1.0 with fiducial limits of 0.108 to 0.674 and 0.312 to 0.874 showing that there is a significant difference ($P \leq 0.05$) between both the former two treatments and the latter treatment.

Expt. III Effect of serial administration of LHRH on adenohypophysial responsiveness to LHRH after various treatments

The number of animals ovulating in each group are as shown in table 16. The ED_{50} of LHRH required to restore ovulation to the group of animals treated with PB at 14:30h on Day P was 0.248µg and the relative potency was 0.462 with fiducial limits of 0.235 and 0.859. Serial administration of LHRH resulted in an increase in relative potency of LHRH (0.691 with fiducial limits of 0.509 and 1.036) but was not significant. This group had an ED_{50} of 0.170. The relative potencies of LHRH in the animals pretreated with RMI 12,936 (relative potency of 0.231 with fiducial limits of 0.118 and 0.430) or RMI 12,936 plus gonadal steroids plus PB at 14:30h (relative potency of 0.356 with fiducial limits of 0.180 and 0.658) increased, but not significantly as a result of serial administration of LHRH (see expt. I B results). The ED_{50} of LHRH in these groups were 0.497 and 0.322 respectively.

The relative potencies of LHRH were calculated from the potency of LHRH (arbitrarily designated as 1.0) in the

Table 16: Effect of serial administration of LHRH at 14:45h, 15:15h and 15:45h on Day P on adenohypophysial sensitivity to LHRH at 16:00h on Day P after various pretreatments.

PRETREATMENT	DOSE LHRH (µg)	NO. OVULATING (%)
Day P: 35mg/kg PB at 14:30h (CONTROL)*	0.10	0/5 (0)
	0.20	2/5 (40)
	0.40	4/5 (80)
	0.80	5/5 (100)
Day P: 35mg/kg PB at 14:30h	0.10	0/5 (0)
	0.20	4/5 (80)
	0.40	5/5 (100)
Day D2: 2mg RMI 12,936 at 16:00h + 0.5ml vehicle at 17:00h + Day P: 0.5ml vehicle at 13:00h + 0.5ml saline at 14:30h	0.20	0/5 (0)
	0.40	2/5 (40)
	0.80	4/5 (80)
	1.60	5/5 (100)
Day D2: 2mg RMI 12,936 at 16:00h + 2µg oestradiol at 17:00h + Day P: 2mg progesterone at 13:00h + 35mg/kg PB at 14:30h	0.10	0/5 (0)
	0.20	1/5 (20)
	0.40	3/5 (60)
	0.80	5/5 (100)

* Control group which received 0.5ml saline in place of LHRH at 14:45h, 15:15h and 15:45h on Day P.

standard group of animals which were pretreated with PB at 15:00h (see expt. I B results).

Summary of Results

1) The methods of a) serial LH measurement and b) detection of ovulation, used in this study to monitor the results of drug treatment on adenohypophysial sensitivity to LHRH were found to give similar results.

2) The adenohypophysial sensitivity to LHRH increased by approximately 30-fold between 11:00h on Day D2 (Day D2 control rats) and 16:00h on Day P (rats treated with PB at 15:00h, Day P).

3) RMI 12,936 treatment prevented full sensitization of the adenohypophysis to LHRH allowing only an approximate 5-fold increase ($P < 0.05$) to occur in these animals.

4) Administration of oestrogen and progesterone to RMI 12,936 treated rats produced a slight but not significant increase in adenohypophysial sensitivity.

5) Administration of PB earlier on the day of proestrus than the control at 15:00h, resulted in a progressive reduction in adenohypophysial sensitivity to LHRH. The relative potencies of LHRH were 1.0, 0.517 (fiducial limits of 0.312 to 0.874) and 0.274 (fiducial limits of 0.108 to 0.674) in PB, RMI 12,936 and RMI 12,936 plus gonadal steroids plus PB treated animals, respectively.

6) Serial administration of LHRH to rats treated with PB at 14:30h or RMI 12,936 or RMI 12,936 plus gonadal steroids plus PB induced a slight but not significant increase in adenohypophysial sensitivity to LHRH.

DISCUSSION

Two different methods of calculating the effect of various drug treatments on adenohypophysial responsiveness to LHRH were employed in this study. The method involving detection of ovulation was compared to the other method which involved serial measurement of the LH concentration over a period of 3½h in individual rats in order to determine whether the former is a feasible method of calculating adenohypophysial sensitivity to LHRH. The latter method may be considered the more accurate as there is normally a large safety margin with more LH secreted than is necessary to induce ovulation (Greig & Weisz, 1973; Turgeon & Barraclough, 1973). Therefore ovulation may occur even though adenohypophysial sensitivity to LHRH and hence LH secretion have been reduced. In this situation the method of detection of ovulation would be rendered inaccurate. However it was demonstrated in this study that use of both methods gave very similar results showing that the above consideration is of minor importance in this study. The fiducial limits of the relative potencies of LHRH were slightly narrower in the method where LH concentrations were measured than in the method using detection of ovulation and in some investigations this may be of importance in showing a significant

difference between groups.

The disadvantages of the blood sampling method are that it is time consuming, expensive and there is a risk of possible stress on the rat from the procedure of blood sampling. It was however shown that serial blood sampling in control rats does not prevent ovulation (see Chapter 1).

Taking all these factors into consideration, the method using detection of ovulation as a means of determining changes in adenohypophysial responsiveness to LHRH appears to be adequate and is less time consuming and less expensive than the method of measurement of serial LH levels.

The results of this study show that there is an approximately 30-fold increase in sensitivity of the adenohypophysis to LHRH between 10:30h to 14:30h on Day D2 and 15:30h to 19:00h on Day P. This increase is comparable with that found by Aiyer and his coworkers who demonstrated an approximate 40-fold increase in sensitivity between 13:30h on Day D2 and 18:00h on Day P (Aiyer et al, 1974). In the same study they observed a 2-phase rise in responsiveness of the adenohypophysis to LHRH. The first phase consisted of a long slow increase (about 8-fold) in sensitivity and was due to a gradual rise in oestradiol levels. The second phase consisted of a rapid increase (about 5-fold) in sensitivity and was thought to be due to progesterone secreted in response to the increasing levels of circulating LH. This second phase increase in sensitivity was later shown to be also due to the self-priming action of LHRH on the adenohypophysis (Fink, Chiappa & Aiyer, 1976).

In confirmation of previous findings (Geddes et al, 1979) treatment with RMI 12,936 was shown to prevent full sensitization of the adenohypophysis to LHRH. To test the hypothesis that RMI 12,936 blocks ovulation by acting in part to inhibit steroid priming of the adenohypophysis, the responsiveness of the adenohypophysis to LHRH was determined after oestradiol and progesterone replacement. This experiment showed that steroid replacement did not significantly increase sensitivity although this drug regime could restore ovulation to the RMI 12,936 blocked rat since the experiment was performed during the time of year of high adenohypophysial sensitivity. The sensitivity of the adenohypophysis under these conditions is therefore sufficient for ovulation to occur, indicating that reduced sensitivity is not the mechanism by which RMI 12,936 blocks ovulation and hence the hypothesis is false.

However RMI 12,936 does prevent full sensitization of the adenohypophysis and it could do this by the following methods: (i) by preventing the sensitization of the adenohypophysis by oestrogen (ii) by preventing the sensitization of the adenohypophysis by progesterone and (iii) by preventing LHRH self-priming of the adeno-hypophysis. Since the first phase increase in sensitivity begins early on the morning of dioestrus II, RMI 12,936 cannot prevent oestrogen sensitization of the adenohypophysis occuring before its administration at 16:00h on dioestrus II. It is noteworthy that the sensitivity in the RMI 12,936 treated rat is approximately 5 times greater than that in the dioestrus II control rats, nearly the amount by which

the first phase increase in sensitivity is reported to increase. This supports the suggestion that RMI 12,936 does not interfere with most of the first phase oestrogen sensitization of the adenohypophysis. Oestradiol and progesterone replacement to RMI 12,936 treated rats increases adenohypophysial sensitivity to LHRH slightly but not significantly. This is an increase in sensitivity of about 7-fold from dioestrus II control rats which may reflect completion of the first phase increase in adenohypophysial sensitivity to LHRH and facilitation by progesterone. However oestrogen and progesterone administration provides only a slight increase in sensitivity and therefore prevention of their secretion cannot account for the full mechanism by which RMI 12,936 prevents sensitization. Irreversible inhibition of their effects however may do so.

To test whether the third mechanism is plausible, PB was administered to rats at various times during proestrus. This experiment showed that the sensitivity of the adenohypophysis during proestrus increases with time which is in agreement with the observations of other investigations (Aiyer et al, 1974; Fink et al, 1975; Fink et al, 1976). The fact that the change in sensitivity is similar in conscious compared with anaesthetized animals suggests that PB does not significantly alter the responsiveness of the adenohypophysis to LHRH (Aiyer et al, 1974). Instead it is believed that PB has a central site of action in blocking ovulation resulting in a block of the spontaneous surge of LHRH on proestrus (Kalra, Simpkins & Kalra, 1980;Sherwood, Chiappa, Sarkar & Fink, 1980).

Therefore if PB prevents LHRH release and also reduces the sensitivity of the adenohypophysis if administered earlier on proestrus, then this reduction in sensitivity between 13:30h and 15:00h can be explained by inhibition of a self-priming action of LHRH. The sensitivity of the adenohypophysis at 13:30h is approximately the same as in RMI 12,936 treated rats. This suggests that in these animals the increase in sensitivity due to LHRH ie the 2nd phase increase, is not occurring. This hypothesis was tested by administration of a series of small, self-priming, doses of LHRH. The results showed that serial administration of LHRH increased the sensitivity of the adenohypophysis in rats treated with PB at 14:30h to approximately that of rats treated with PB at 15:00h. Although this increase was not significant, the data suggest that LHRH self-priming has occurred in these animals. In a similar experiment, Fink and his coworkers (1976) demonstrated the self-priming effect of LHRH by showing that serial administration of LHRH in a total dose of 5ng/100g BW over a 90 min period produced a significant increase in plasma LH concentrations. A small increase in adenohypophysial sensitivity to LHRH also occurred in RMI 12,936 and RMI 12,936 plus gonadal steroid treated rats after serial administration of LHRH. These results suggest that LHRH self-priming was occurring to a small extent in these animals, although the increase was not significant, and that full adenohypophysial sensitivity has not been restored to these animals. A possible explanation for the poor response to LHRH release

may be a reflection of the time of year this experiment was performed ie during the beginning of the period of reduced sensitivity in the mechanisms controlling LH release. However since sensitizing doses of LHRH can partly restore adenohypophysial sensitivity to that at 15:00h on proestrus, it is suggested that RMI 12,936 prevents full sensitization by inhibiting LHRH release and thereby its self-priming effect on the adenohypophysis. Hence these findings support the hypothesis that in RMI 12,936 treated rats the 2nd phase increase in adenohypophysial sensitivity due to the self-priming effect of LHRH is not occuring. If this hypothesis is correct, administration of oestrogen and progesterone, in a dose regime which restores ovulation, would be expected to stimulate release of LHRH and thereby promote LHRH self-priming action. However an increase in adenohypophysial responsiveness to LHRH does not occur on oestrogen and progesterone administration. It may be that the physiological conditions have not been met in this experiment and so LHRH self-priming does not occur. On the other hand it may be that RMI 12,936 prevents LHRH self-priming, not only by inhibiting release of LHRH but also by reducing the number of LHRH receptors present in the tissue. This hypothetical mechanism is discussed further in Chapter 7 and in the general discussion.

In summary, it has been shown that the method of detection of ovulation is a reliable way to measure the results of drug treatment on adenohypophysial sensitivity to LHRH. Using this technique it was demonstrated that RMI 12,936, although it prevents full sensitization of the adenohypophysis to LHRH, does not block ovulation by this action

since administration of oestrogen plus progesterone which is known to restore ovulation, does not significantly increase adenohypophysial sensitivity. Therefore the hypothesis that RMI 12,936 blocks ovulation partly by reducing adenohypophysial sensitivity to LHRH was shown to be false. The results of the investigation into the mechanism by which the drug acts to prevent full sensitization suggested that it was not by prevention of oestrogen and progesterone priming of the adenohypophysis. Instead it was suggested that the drug prevents LHRH self-priming action by blocking LHRH release and more importantly it acts by reducing the levels of LHRH receptors.

CHAPTER 3

The effect on ovulation of centrally administered RMI 12,936

INTRODUCTION

When injected peripherally, RMI 12,936 will produce a complete block of ovulation and it has been proposed that this block is associated with an antioestrogenic and antiprogestational activity (see Chapter 1). Investigation of the site of action of this drug has shown that although RMI 12,936 prevents full sensitization of the adenohypophysis to LHRH, the level of responsiveness to LHRH is sufficient to allow enough LH to be released for ovulation to occur. Thus it was concluded that the adenohypophysis is not the sole nor the major site of RMI 12,936 antiovulatory activity.

After further investigation (see Chapter 2) it was proposed that the compound prevents full sensitization partly by inhibiting release of LHRH, suggesting a central site of antiovulatory action. There is considerable evidence showing that oestrogen has a positive feedback action at the level of the hypothalamus (see general introduction) where it acts to trigger a neural signal for release of LHRH. Therefore it is conceivable that RMI 12,936 may antagonise oestrogen action at the site of positive feedback action, blocking the stimulus for a neural signal and thereby LHRH release, LH release and ovulation. This study was designed to determine whether RMI 12,936 can inhibit ovulation by acting at a site in the central nervous system.

METHODS

Four groups of rats were anaesthetized with 1ml/kg althesin between 15:40h and 14:00h on Day D2. The rats were then injected with the vehicle or 30µg, 10µg, 3µg, 1.0µg or 0.3µg RMI 12,936, each in a total volume of 2µl, into the third ventricle as described in the general methods section.

For comparison with the potency of centrally administered RMI 12,936, a further three groups of rats were injected subcutaneously with 0.5ml of the vehicle or 30µg, 10µg or 3µg RMI 12,936.

To determine whether a central injection of RMI 12,936 is effective in blocking ovulation when administered on proestrus, two groups of rats were anaesthetized with 1ml/1kg althesin between 09:00h and 09:30h on Day P. The rats were then injected with 30µg RMI 12,936 or the vehicle both in a volume of 2µl into the third ventricle.

All animals tested were sacrificed on the expected day of oestrus and their oviducts removed and examined for the presence of ova.

RESULTS

The ED_{50} of RMI 12,936 in blocking ovulation when injected into the third ventricle on Day D2 was 2.148µg and when injected subcutaneously on Day D2 11.621µg (see table 17). The relative potency of RMI 12,936 when administered intraventricularly was 5.943 (fiducial limits of 30.199 and 1.924) and is significantly different from the relative potency of subcutaneously administered RMI 12,936, designated

Table 17: Effect on ovulation of RMI 12,936 when administered intraventricularly (IV) or subcutaneously (sc).

Treatment	Dose of Drug Administered at		No. of Animals Ovulating (%)
	14:00-14:30h, Day D2	09:00-09:30h, Day P	
VEHICLE (IV)	0µg	-	7/7 (88)
RMI 12,936 (IV)	-	30µg	5/5 (100)
RMI 12,936 (IV)	30µg	-	0/5 (0)
RMI 12,936 (IV)	10µg	-	0/5 (0)
RMI 12,936 (IV)	3µg	-	3/8 (38)
RMI 12,936 (IV)	1µg	-	4/5 (80)
RMI 12,936 (IV)	0.3µg	-	5/5 (100)
VEHICLE (sc)	0µg	-	5/5 (100)
RMI 12,936 (sc)	30µg	-	0/5 (0)
RMI 12,936 (sc)	10µg	-	4/6 (67)
RMI 12,936 (sc)	3µg	-	5/5 (100)

1.0. The statistical test used to determine the ED_{50} and relative potency of RMI 12,936 was probit analysis.

Intraventricular administration of 30µg RMI 12,936 produced a 100% block of ovulation when administered on Day D2 at 16:00h but failed to block ovulation in any rat when administered between 09:00 and 09:30h on Day P (table 17).

Summary of Results

1) RMI 12,936 blocks ovulation when administered intraventricularly at 16:00h on Day D2. Administration by the same route at 09:00h - 09:30h on Day P fails to block ovulation.

2) Comparison of the antiovulatory potency of RMI 12,936 when administered by different routes, shows that it has relative potencies of 5.943 and 1.0 when administered intraventricularly and subcutaneously respectively.

DISCUSSION

It has been proposed that the antiovulatory activity of RMI 12,936 may be expressed through a central site of action. The results of this study have shown that RMI 12,936 is effective in blocking ovulation when injected into the third ventricle and that it achieves this at doses significantly lower than those which are effective when administered peripherally. Since the drug is more

potent (approximately 6 times) when administered intra-ventricularly, it can be concluded that it has a central site of action.

It is possible that steroids implanted in the hypothalamus can be readily distributed to the adenohypophysis by the portal circulation (Palka et al, 1966) and therefore it is difficult to ascertain precisely the site of action of the drug following its injection into the third ventricle. However, it is likely that administration of as small a concentration of drug as 300ng would act at a site near the ventricle. These results in conjunction with those that suggest that the adenohypophysis is not the major site of antiovulatory action of the drug (see Chapter 2), support the hypothesis that RMI 12,936 has its major site of action at the level of the hypothalamus.

The data presented in this study also show that when administered on proestrus, directly into the third ventricle, RMI 12,936 is ineffective at blocking ovulation. This ineffectiveness on proestrus occurred on subcutaneous administration also (Chapter 1) implying that the same mechanism of action is common to both routes of administration.

From the results obtained earlier in this investigation (see Chapter 1), it was proposed that RMI 12,936 blocks ovulation by exhibiting antioestrogenic and antiprogestational activity. However, since the drug required to be administered at least 16h before the preovulatory LH surge in order to block oestrogen and progesterone activity, it was questionable

whether RMI 12,936 acted directly or was first metabolized in the ovary to a more active form as demonstrated in antifertility studies. Although the first possibility was favoured, the latter could not be ruled out. However, in the light of the finding that RMI 12,936 can block ovulation by a direct action at the central level, it can be concluded that ovarian metabolism cannot be taking place. Therefore the drug cannot depend on ovarian metabolism for its antiovulatory activity.

It has previously been suggested that the mechanism of RMI 12,936 antioestrogenic and antiprogestational activity is either by inhibition of both oestrogen and progesterone biosynthesis or by antagonism of oestrogen and progesterone utilization. The major site of oestrogen and progesterone biosynthesis is in the ovary although the adrenal cortex also contributes to the synthesis of both steroids. Therefore, since it has been shown in this study that RMI 12,936 can block ovulation by a direct action at the level of the brain, it cannot be producing this effect by inhibition of peripheral steroid biosynthesis.

There is considerable evidence showing that oestrogen and progesterone exert a positive feedback action at the level of the hypothalamus (see general introduction). Therefore it is conceivable that RMI 12,936, administered centrally, may produce its antiovulatory effect directly at the site of steroid positive feedback action. There are several possible mechanisms by which RMI 12,936 could achieve this. It could interfere with steroid-receptor interaction

possibly by reducing the number of receptors in the tissue or by blocking the receptors. Since oestrogen and progesterone can maintain high levels of LH in ovariectomized - RMI 12,936 treated rats and restore ovulation to intact rats treated with RMI 12,936, then its mechanism of action must be reversible by oestrogen and progesterone. This favours RMI 12,936 action by antagonism with oestrogen and/or progesterone at the receptor level.

Consideration of the information gathered on RMI 12,936 mechanism of antiovulatory action indicates that it may have a dual action. It may act by prevention of peripheral oestrogen and progesterone biosynthesis and secondly, by reversible blockade of oestrogen and/or progesterone receptors at the central level.To test whether it exhibits both an antioestrogenic and antiprogestational activity at the central level, these gonadal steroids could be administered separately or together to see if they can restore ovulation to rats injected intraventricularly with RMI 12,936.

Although RMI 12,936 does not depend on ovarian metabolism for its antiovulatory activity, it may be metabolized in the hypothalamus to a more active form. Recent evidence has suggested that metabolites may mediate some of the effects of steroids in neuroendocrine tissues. Certain metabolites, particularly 5α-dihydroprogesterone, have progesterone-like effects on gonadotrophin regulation (Nuti & Karavolas, 1977) and ovulation (Sridharan, Meyer & Karavolas, 1974), but not on uterine parameters(Karavolas, Hodges & O'Brien,

1976). The biological effects of the 5α-reduced progestins on neuroendocrine parameters may be analagous to androgen action in certain target tissues in which the 5α-reduced androgens, particularly 5α-dihydrotestosterone mediate some of the biological effects of testosterone after *in situ* conversion (Wilson & Gloyna, 1970). It has also recently been shown that oestrone and oestradiol can be converted to 2- and 4- hydroxylated derivatives in brain tissues (Fishman & Norton, 1975; Paul & Axelrod, 1977). These findings have directed attention to the possible role of these catecholoestrogens in brain function, showing that they influence LH secretion after systemic or intracranial administration (Parvi & Ellendorf, 1975; Martucci & Fishman, 1979; Rodriguez-Sierra & Blake, 1982). The mechanisms underlying the neuroendocrine action of catecholoestrogens are unclear. The findings that catechol-oestrogens bind to cytosol oestrogen receptors and possess uterotrophic and enzyme-inducing activities suggest that they are acting as classical oestrogens (Davies, Naftolin, Ryan, Fishman & Siu, 1975; Martucci & Fishman, 1977; Merriam, MacLusky, Picard & Naftolin, 1980). On the other hand, the action of the catecholoestrogens in the central nervous system could be linked to their interaction with the biogenic amines. They inhibit the biological inactiv-ation of the catecholamines by Catechol-*O*-methyl transferase (Breuer & Köster, 1975) and inhibit tyrosine hydroxylase, the rate limiting enzyme of catecholamine biosynthesis (Lloyd & Weisz, 1978). Thus by these means, they could decrease catecholamine turnover and content in the central

nervous system. However there is convincing evidence to indicate that this is not the mechanism by which they regulate LH release (Fishman & Martucci, 1979; Blake, Fuxe, Andersson, Rodriguez-Sierra, Eneroth, Gustafsson & Agnati, 1981; Jellinck, Krey, Davis, Kamel, Luine, Parsons, Roy & McEwen, 1981). It is possible that the catechol-oestrogen could activate presynaptic NA receptors or directly alter the firing of LHRH-containing neurones through a change in the ionic permeability of the neural membrane (Blake et al, 1981). Therefore a possible mechanism of RMI 12,936 antiovulatory activity may be through prevention of the formation of catecholoestrogens.

In summary, RMI 12,936 was shown to block ovulation when administered directly into the third ventricle in a concentration significantly lower than is effective on subcutaneous administration. This finding supported the hypothesis that the hypothalamus is the major site of RMI 12,936 antiovulatory activity. The drug does not depend on ovarian metabolism in order to elicit this effect. On subcutaneous administration it was proposed that the drug has a dual action : 1) it inhibits peripheral oestrogen and progesterone biosynthesis and 2) it acts directly at the central level to prevent oestrogen and/or progesterone from expressing their activity.

CHAPTER 4

The effect of RMI 12,936 on hypothalamic amine levels, synthesis and breakdown. Detection by a fluorescence method.

INTRODUCTION

A central site of RMI 12,936 antiovulatory activity was suggested by Geddes et al (1979) who showed that the drug inhibits a surge of DA occurring at 12:00h on proestrus. This central site of drug action was supported by the finding (Chapter 3) that RMI 12,936 can block ovulation by a direct action at the hypothalamic level.

In an earlier study, it was shown that RMI 12,936 acts through antioestrogenic and antiprogestational activity (Chapter 1). Such an effect at the hypothalamic level would result in block of the neural signal for LHRH release and therefore ovulation. Central noradrenergic, dopaminergic and tryptaminergic pathways have all been implicated in the control of ovulation (see general introduction). Therefore the aim of this study was to determine whether RMI 12,936 blocks ovulation by interfering with these hypothalamic neurotransmitters. The measurement of brain levels of amines do not provide a true index of functional amine activity (Carlsson, 1964) and for this reason synthesis and breakdown of the amines have also been measured in this study in order to obtain more meaningful information on the dynamics of the amines. A synthesis inhibitor, αMPT, and an inhibitor of breakdown, pargyline, were used to determine the synthesis and breakdown of NA/DA and 5'HT respectively.

METHODS

Six groups of rats were dosed with 2mg RMI 12,936 or 0.5ml

of the vehicle at 16:00h on Day D2. Four of these groups received either 200mg/kg αMPT or 150mg/kg pargyline, four hours prior to death. The animals were killed by cervical dislocation at 11:00h, 15:00h or 19:00h on Day P, the brains removed and the hypothalami isolated, weighed and stored at -20°C for not more than 24h.

The hypothalamic concentrations of NA, DA, 5'HT and 5'HIAA were measured using the fluorescence technique described in the general methods section. Significance between amine levels was tested using the Aspin-Welch Test (see Statistical Analysis in the general methods section).

Calculation of turnover rate as described by Brodie and his colleagues is not justified in the present study because steady state kinetics, an assumption of the method, is not applicable here where the amine levels are not constant (Brodie, Costa, Dlabac, Neff & Smookler, 1966). Furthermore, Pirke and Spyra (1982) have pointed out that the decrease in catecholamine concentration in tissue is not a simple linear logarithmic function which means that the turnover rate calculated depends on the time chosen for measurement. These latter authors proposed a method for statistical analysis of turnover rather than turnover rate but also assumed steady state kinetics. In this chapter a method of calculating synthesis and breakdown has been described which does not depend on these two parameters being

equal. Statistical comparison was carried out as described in the general methods section under 'Statistical Analysis'.

Calculation of Synthesis and Breakdown

The total amount of synthesis and breakdown of the amines over a 4h period were calculated rather than rates of synthesis and breakdown as there was insufficient data to calculate the rates. Where there was no significant difference (Aspin-Welch Test) between the amine levels in non-αMPT treated and αMPT treated animals, it was considered that the total amount of synthesis or breakdown over the 4h period was zero. If a significant difference was detected the following calculations for synthesis and breakdown were made:

Total synthesis over 4h = y-z ng/g

Total breakdown over 4h = x-z ng/g

where, x = NA/DA conc. in the control group at 0h

y = NA/DA conc. in the control group 4h after x

z = NA/DA conc. in the αMPT treated group 4h after x

The same criteria were applied for non-pargyline and pargyline treated animals. However, the calculations for synthesis and breakdown were slightly different since pargyline inhibits breakdown of 5'HT whereas αMPT inhibits the synthesis of NA and DA.

Here Total synthesis over 4h = z-x ng/g

Total breakdown over 4h = z-y ng/g

where, x = 5'HT conc. in the control group at 0h

y = 5'HT conc. in the control group 4h after x

z = 5'HT conc. in the pargyline treated group 4h after x

RESULTS

The effect of RMI 12,936 on hypothalamic NA, DA, 5'HT and 5'HIAA levels are shown in table 18. The effect of the drug on total synthesis and breakdown of NA, DA and 5'HT over a 4h period are shown in table 19. The concentration of hypothalamic NA in control rats is significantly greater at 15:00h than at 11:00h ($P < 0.05$) or 19:00h ($P < 0.005$) on Day P (fig. 10). This mid-afternoon rise in NA levels on Day P is suppressed by RMI 12,936 treatment ($P < 0.005$). RMI 12,936 treatment also causes a slight but significant increase in hypothalamic NA at 11:00h on Day P ($P < 0.05$). This pattern in hypothalamic NA levels in both control and RMI 12,936 treated animals is reflected by the changes in synthesis and breakdown of NA between 11:00h and 15:00h and 15:00h and 19:00h on Day P (fig. 11). In controls, the total synthesis between 11:00h and 15:00h is significantly greater than the total synthesis between 15:00h and 19:00h

Table 18: Hypothalamic levels (µg/g) of NA, DA, 5'HT and 5'HIAA on proestrus after various treatments, using the fluorescence technique. The values are Mean ± SEM with the number of animals per group in parenthesis.

Amine	Treatment	11:00 h	15:00 h	19:00 h
NA	Control	1.04 ± 0.09 (10)*	1.30 ± 0.05 (10)	0.95 ± 0.06 (12)**
	Control + αMPT	-	0.80 ± 0.06 (10)	0.77 ± 0.04 (10)
	RMI 12,936	1.28 ± 0.06 (8)†	0.95 ± 0.07 (13)††	0.92 ± 0.07 (8)
	RMI 12,936 + αMPT	-	1.02 ± 0.16 (10)	0.65 ± 0.04 (10)
DA	Control	0.74 ± 0.07 (9)	0.85 ± 0.03 (10)	0.85 ± 0.07 (12)
	Control + α MPT	-	0.82 ± 0.04 (10)	0.88 ± 0.02 (10)
	RMI 12,936	0.71 ± 0.03 (8)	0.91 ± 0.04 (13)	1.28 ± 0.10 (8)††
	RMI 12,936 + α MPT	-	0.81 ± 0.03 (10)	0.93 ± 0.06 (10)
5'HT	Control	0.36 ± 0.03 (10)	0.32 ± 0.03 (10)	0.32 ± 0.02 (7)
	Control + Pargyline	-	0.90 ± 0.03 (7)	1.09 ± 0.05 (10)
	RMI 12,936	0.38 ± 0.05 (6)	0.35 ± 0.01 (13)	0.38 ± 0.02 (6)†
	RMI 12,936 + Pargyline	-	0.83 ± 0.03 (6)	0.77 ± 0.03 (13)
5'HIAA	Control	0.60 ± 0.07 (9)	0.50 ± 0.07 (8)	0.68 ± 0.05 (8)*
	RMI 12,936	0.68 ± 0.23 (4)	0.55 ± 0.06 (13)	0.61 ± 0.26 (6)

* $P < 0.05$, ** $P < 0.005$ Control value is significantly different (Aspin-Welch test) from the control value at 15.00h.

† $P < 0.05$, †† $P < 0.005$ RMI 12,936 value is significantly different from the control value at the same time interval.

Table 19: Synthesis and breakdown (µg/g) of NA, DA and 5'HT in control and RMI 12,936 treated animals on proestrus.

		11:00 - 15:00 h		15:00 - 19:00 h	
		Synthesis	Breakdown	Synthesis	Breakdown
NA	Control	0.50	0.24†	0.18***	0.53*†††
	RMI 12,936	0···	0	0.27	0.30·
DA	Control	0	0	0	0
	RMI 12,936	0.10	0	0.35··	0
5'HT	Control	0.54	0.58	0.77***	0.77***
	RMI 12,936	0.45	0.48·	0.42···	0.39···

* $P < 0.05$, *** $P < 0.005$ Control value significantly different (Aspin-Welch Test) from control value at 11:00 - 15:00h.

† $P < 0.05$, ††† $P < 0.005$ Control breakdown value significantly different (Aspin-Welch Test) from control synthesis value at the same time inerval.

· $P < 0.05$, ·· $P < 0.01$, ··· $P < 0.005$ RMI 12,936 value significantly different from control value at the same time interval.

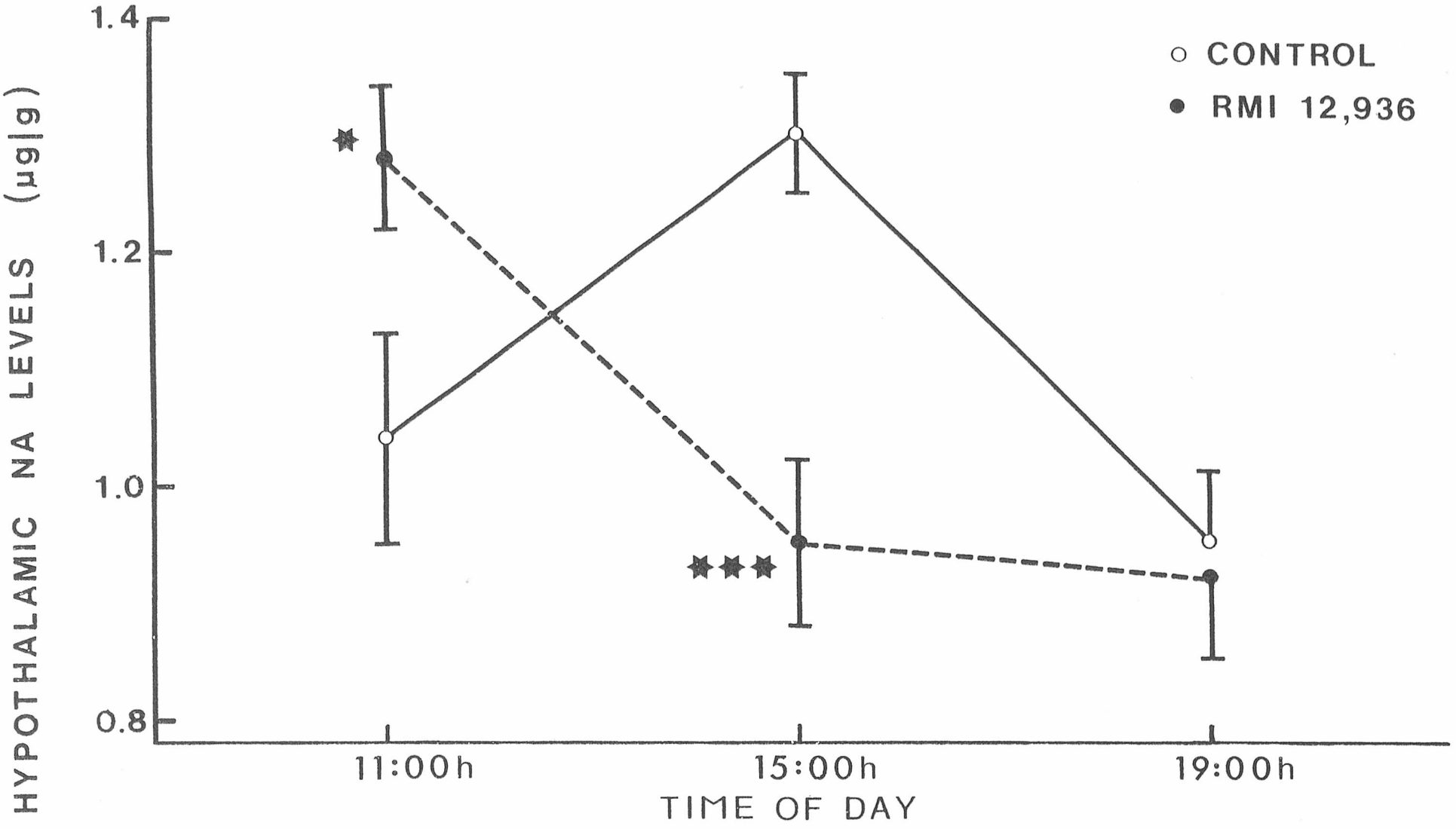


Fig. 10 The effect of RMI 12,936 on the hypothalamic levels of NA (µg/g) at 11:00h, 15:00h and 19:00h on proestrus. * $P < 0.05$, *** $P < 0.005$ RMI 12,936 value is significantly different (Aspin-Welch test) from the control value.

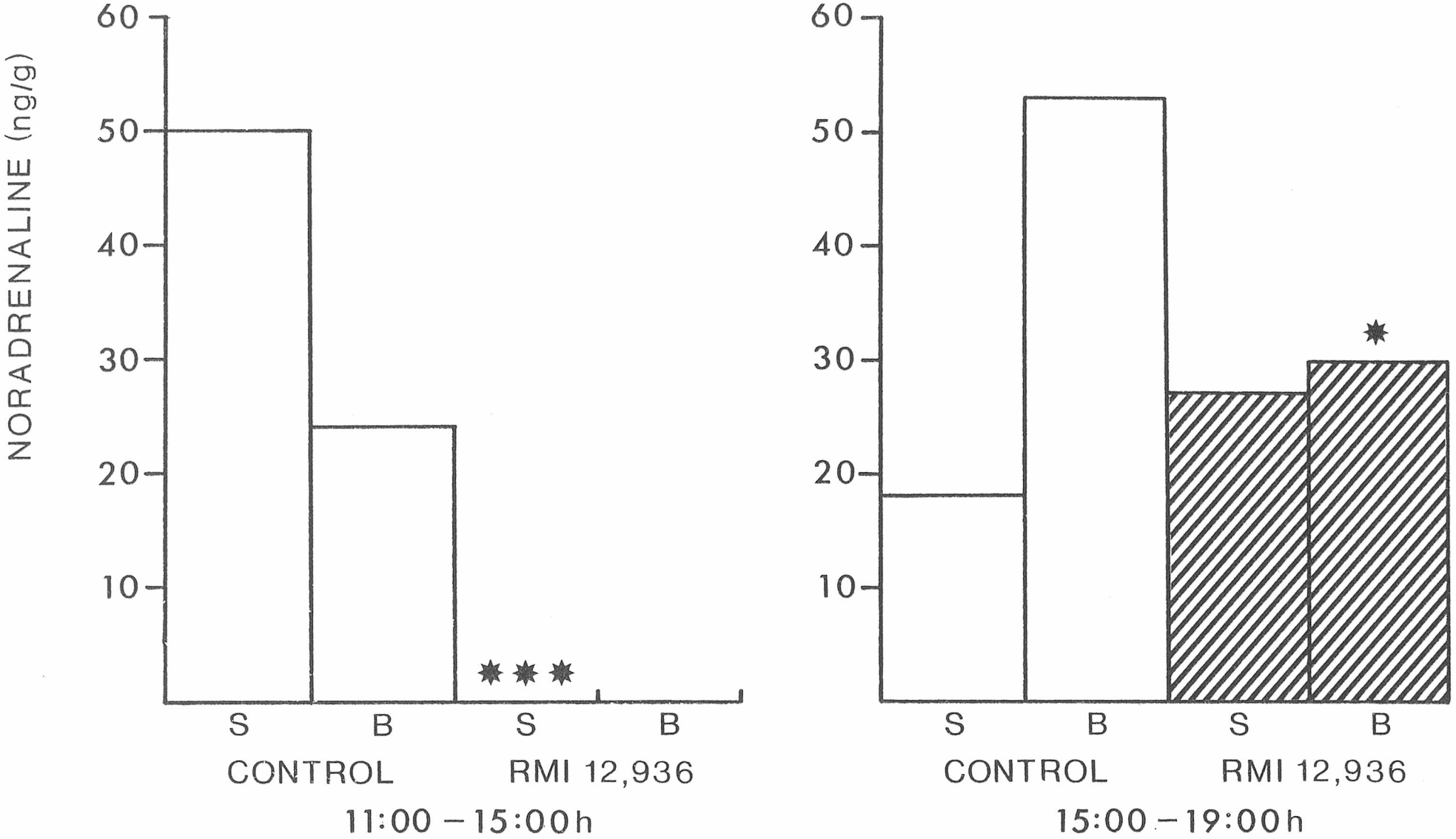


Fig. 11 The effect of RMI 12,936 on the synthesis and breakdown of hypothalamic NA (ng/g) between 11:00h and 15:00h; and between 15:00h and 19:00h on proestrus. * $P < 0.05$, *** $P < 0.005$ RMI 12,936 value is significantly different (Aspin-Welch test) from the control value.

($P < 0.005$) and the total breakdown between 11:00h and 15:00h ($P < 0.05$). The total breakdown between 15:00h and 19:00h is significantly greater than the total breakdown between 11:00h and 15:00h ($P < 0.05$) and the total synthesis between 15:00h and 19:00h ($P < 0.005$). In RMI 12,936 treated rats, NA synthesis between 11:00h and 15:00h and NA breakdown between 15:00h and 19:00h are significantly lower ($P < 0.005$ and $P < 0.05$, respectively) than in controls.

The hypothalamic concentrations of DA and 5'HT in control rats (figs. 12 and 14 respectively) showed only slight variations during Day P which were not significant. RMI 12,936 treatment caused a significant increase in both DA ($P < 0.005$) and 5'HT ($P < 0.05$) levels at 19:00h on Day P. Again the pattern of hypothalamic DA and 5'HT levels throughout proestrus is reflected by the changes in synthesis and breakdown of the amine. The data indicate that no DA is being synthesized or broken down in control animals between 11:00h and 19:00h on proestrus but after RMI 12,936 treatment, there is an increase in DA synthesis which at 19:00h achieves significance ($P < 0.01$) from the control value (fig.13).

There is no significant difference between synthesis and breakdown of 5'HT in both control and RMI 12,936 treated animals throughout proestrus (fig. 15). There is, however, a significant increase ($P < 0.005$) in both synthesis and breakdown of 5'HT between 15:00h and 19:00h in control rats. This increase in 'turnover' is suppressed ($P < 0.005$) by RMI 12,936 treatment. RMI 12,936 treatment also suppresses

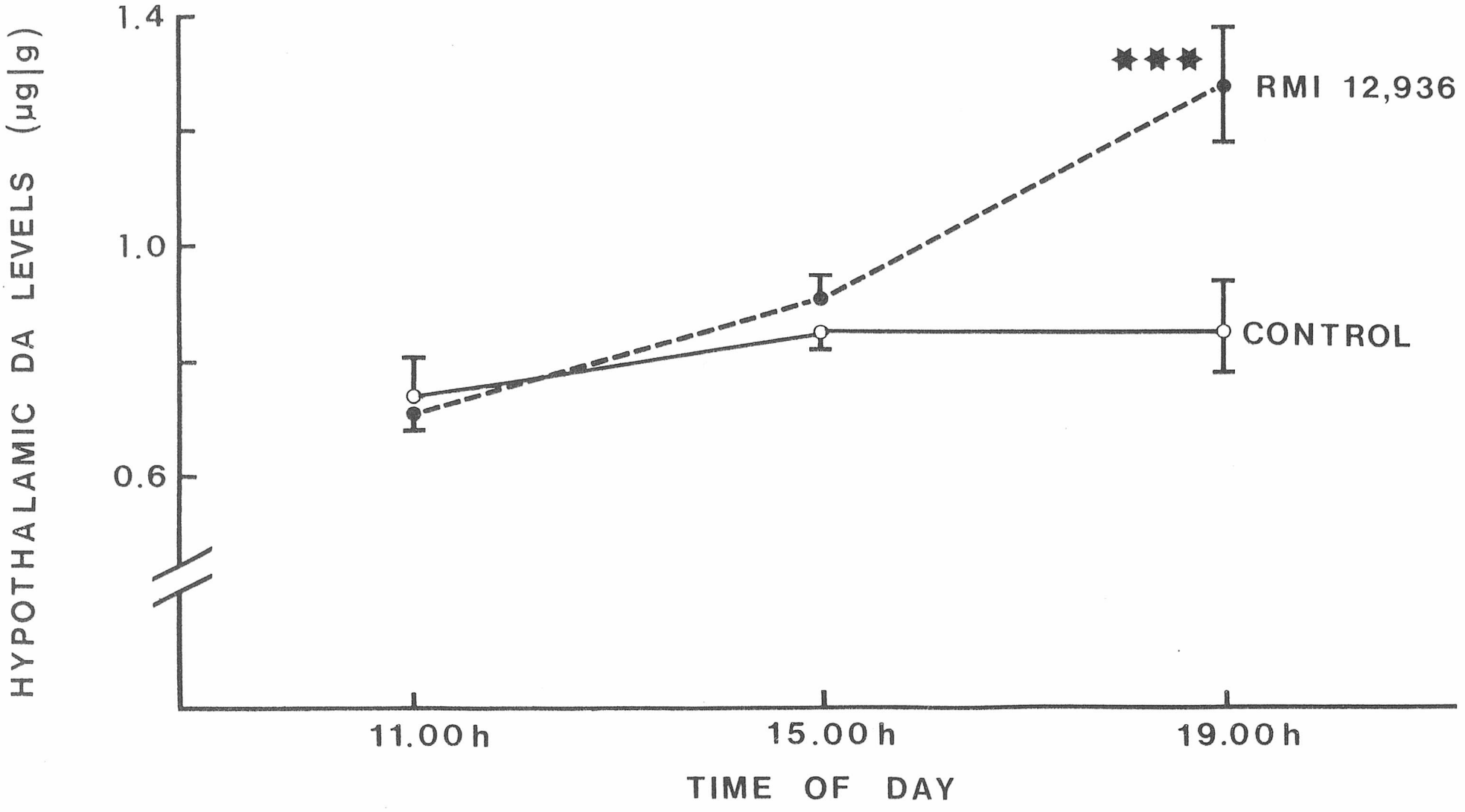


Fig. 12 The effect of RMI 12,936 on the hypothalamic levels of DA (µg/g) at 11:00h, 15:00h and 19:00h on proestrus.
***P<0.005 RMI 12,936 value is significantly different (Aspin-Welch test) from control value.

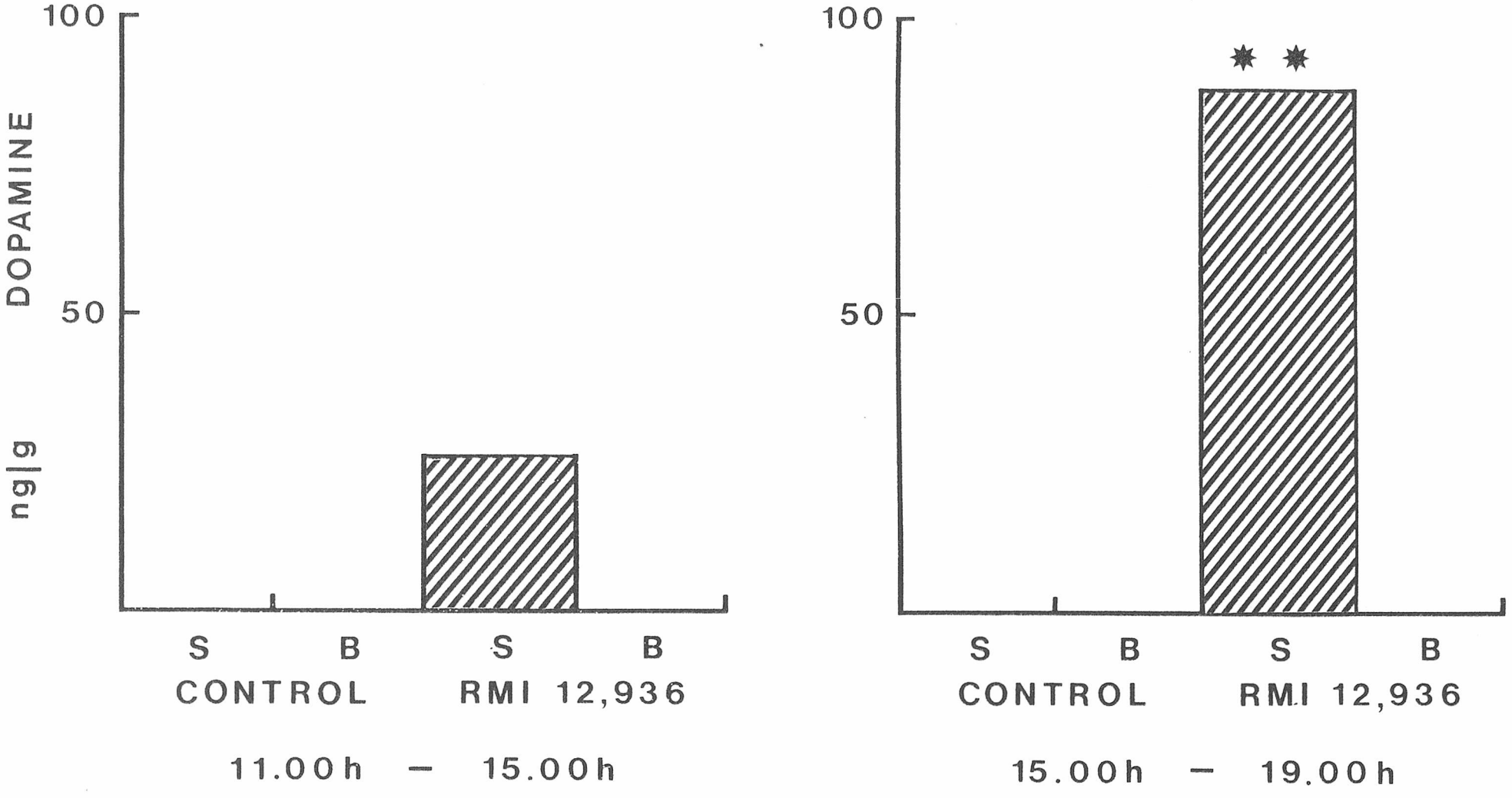


Fig. 13 The effect of RMI 12,936 on the synthesis and breakdown of hypothalamic DA (ng/g) between 11:00h and 15:00h; and between 15:00h and 19:00h on proestrus. ** $P < 0.01$ RMI 12,936 value is significantly different (Aspin-Welch test) from the control value.

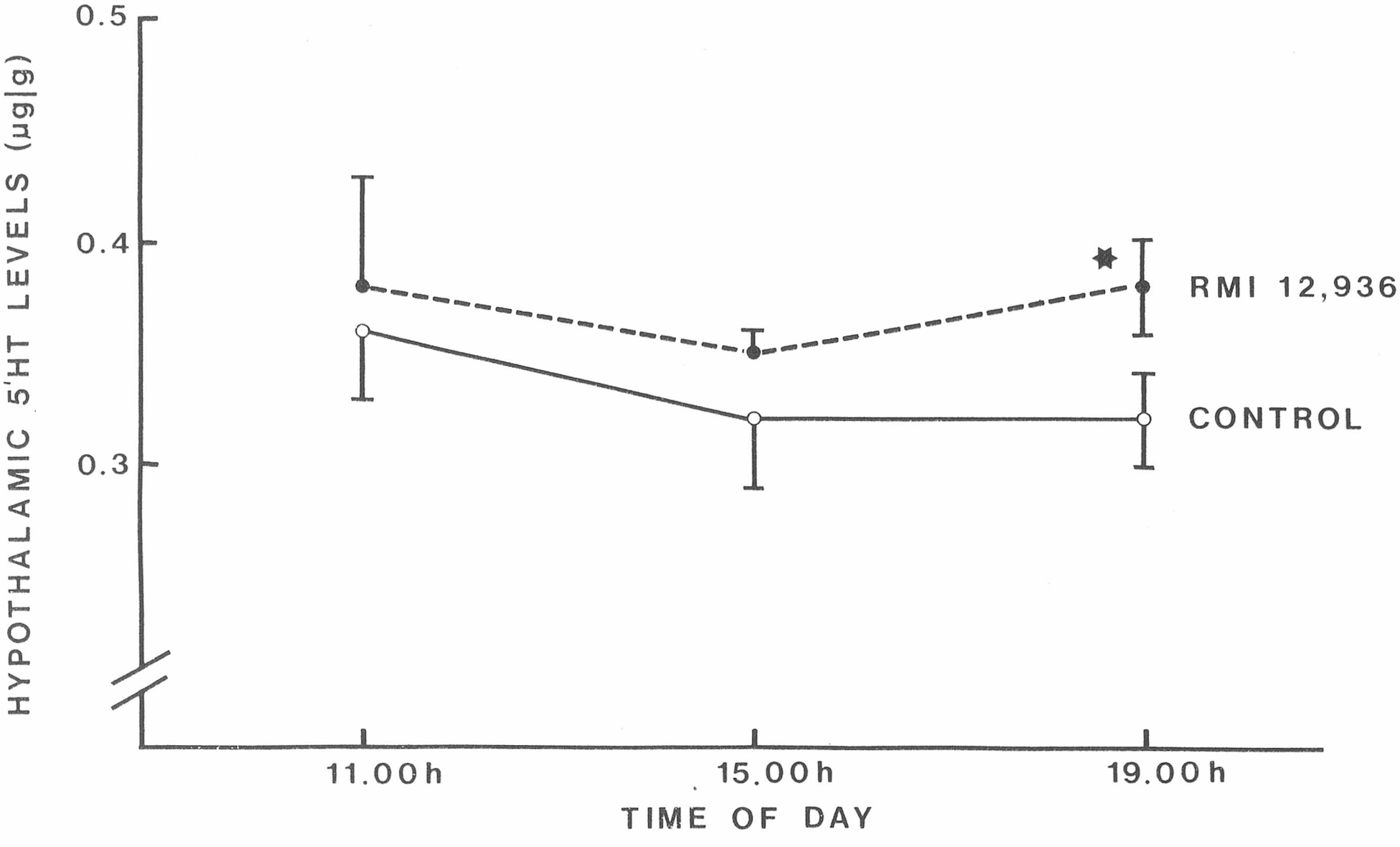


Fig. 14 The effect of RMI 12,936 on the hypothalamic levels of 5'HT (µg/g) at 11:00h, 15:00h and 19:00h on proestrus.
*$P < 0.05$ RMI 12,936 value is significantly different (Aspin-Welch test) from control value.

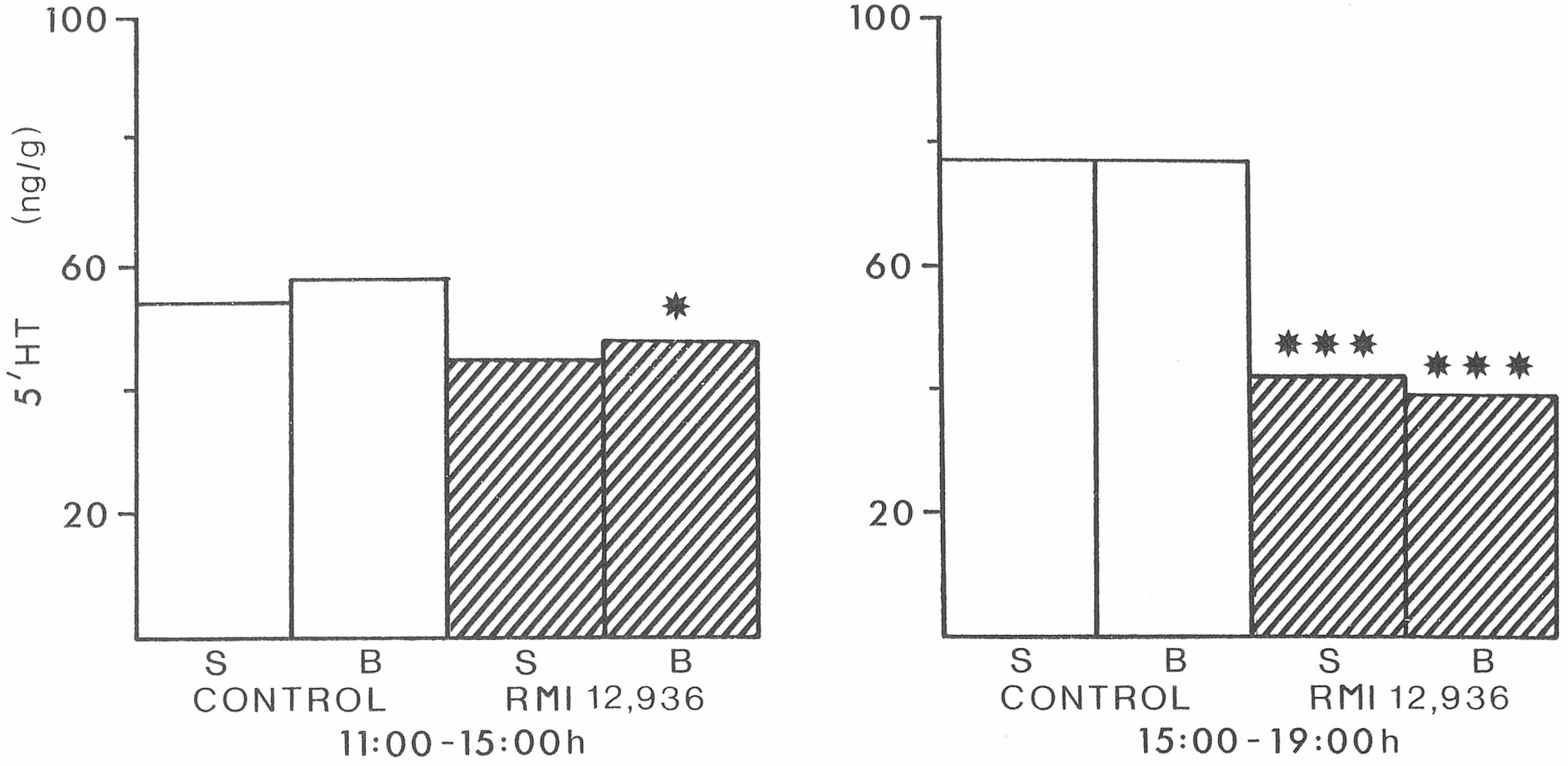


Fig. 15 The effect of RMI 12,936 on the synthesis and breakdown of hypothalamic 5'HT (ng/g) between 11:00h and 15:00h; and 15:00h and 19:00h on proestrus. *P < 0.05, ***P < 0.005 RMI 12,936 value is significantly different (Aspin-Welch test) from the control value.

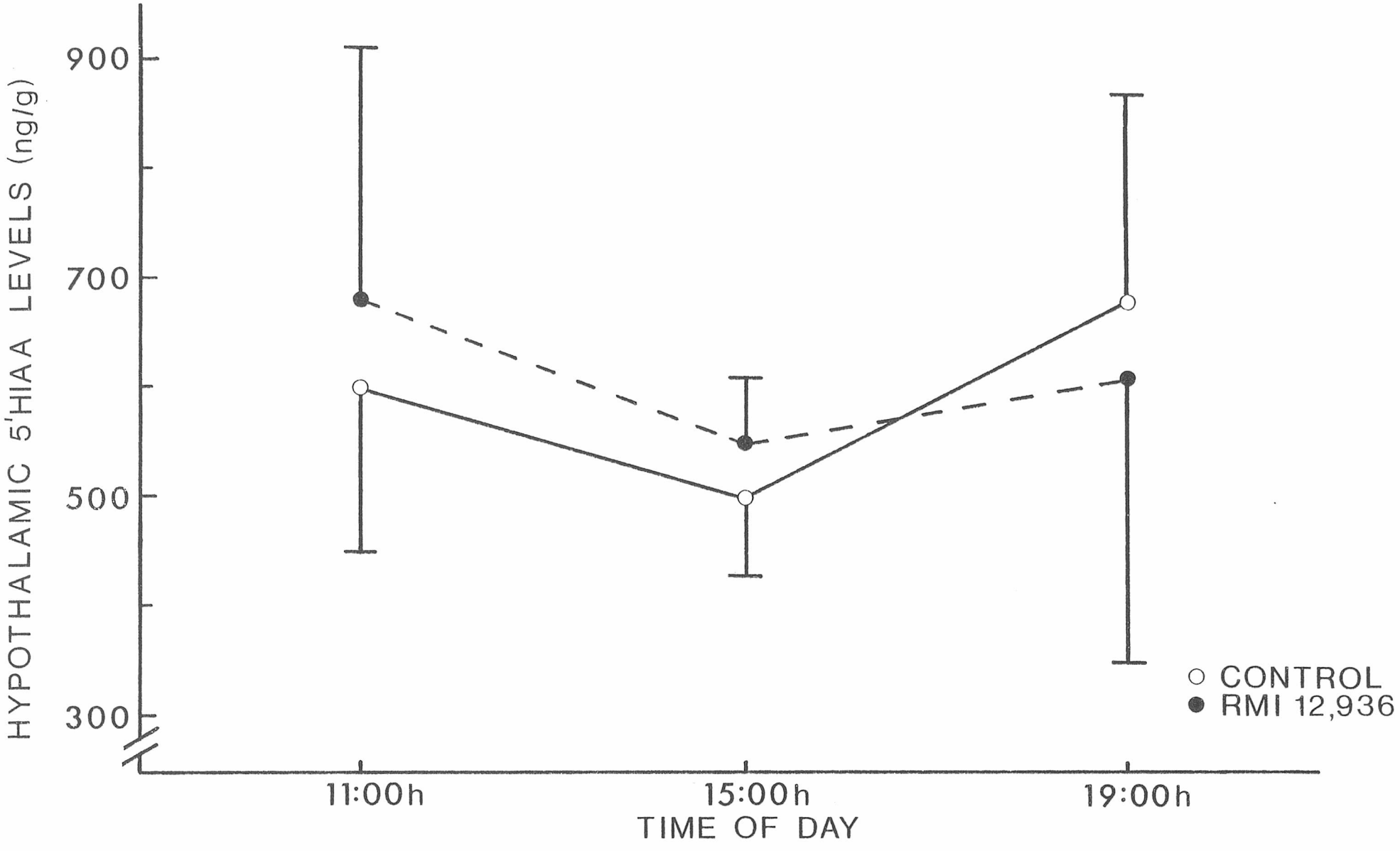


Fig. 16 The effect of RMI 12,936 on the hypothalamic levels of 5'HIAA (μg/g) at 11:00h, 15:00h and 19:00h on proestrus. No significant difference between control and RMI 12,936 values (Aspin-Welch test).

5'HT breakdown between 11:00h and 15:00h ($P < 0.05$).

The hypothalamic levels of 5'HIAA in control animals rose significantly ($P < 0.05$) at 19:00h. There is no significant difference between 5'HIAA levels in control and RMI 12,936 treated animals (fig. 16).

Summary of Results

1) The hypothalamic levels of NA in control animals, were greatest at 15:00h on Day P. This rise in NA levels was reflected by a significant ($P < 0.05$) increase in synthesis between 11:00h and 15:00h. In RMI 12,936 treated animals these rises in concentration and synthesis were blocked ($P < 0.005$). The hypothalamic levels of NA were significantly greater ($P < 0.05$) at 11:00h in RMI 12,936 treated rats than in controls.

2) There was no significant change in control hypothalamic DA levels throughout Day P. Total synthesis and breakdown between 11:00h and 15:00h and 15:00h and 19:00h was negligible. In RMI 12,936 treated rats the levels of DA increased during Day P, achieving significance at 19:00h ($P < 0.005$). This increase was reflected by a significant increase in DA synthesis between 15:00h and 19:00h ($P < 0.01$).

3) The hypothalamic levels of 5'HT in control animals remained constant throughout Day P. Both synthesis and breakdown of 5'HT were significantly greater ($P < 0.005$) between 15:00h and 19:00h than 11:00h and 15:00h. These

increases in synthesis and breakdown were inhibited ($P < 0.005$) by RMI 12,936 treatment. There was also a small but significant ($P < 0.05$) increase in 5'HT levels at 19:00h.

4) In control animals, the hypothalamic levels of 5'HIAA rose significantly ($P < 0.05$) at 19:00h. The levels of 5'HIAA in RMI 12,936 treated animals were not significantly different from control values.

DISCUSSION

There have been a considerable number of investigations into the neural involvement in the control of ovulation (see general introduction). These investigations have led to the generally held view that NA plays an excitatory role in the control of ovulation. Unlike the noradrenergic system, there still remains some controversy over the roles that DA and 5'HT play in control of LH release. Some investigators believe them to have an inhibitory effect on LH release while others believe them to have an excitatory effect. However it is not yet clear whether they play a physiological role in the trigger of ovulation.

In this study, hypothalamic NA levels in control animals were shown to peak at 15:00h on proestrus and this increase was reflected by a greater amount of synthesis than breakdown. These results agree with previous observations where NA activity has been shown to increase during proestrus, peaking immediately prior to the preovulatory LH surge (Kueng et al, 1976); Fuxe et al, 1977). In RMI 12,936 treated rats,

this NA peak was suppressed, indicating that the steroid has an inhibitory effect on the noradrenergic system. Since it is believed that an excitatory noradrenergic tract is involved in the trigger of ovulation, it would seem likely that RMI 12,936 inhibits ovulation as a result of this inhibitory effect on NA. RMI 12,936 also produced a significant increase in NA levels at 11:00h on proestrus.

In contrast to NA, hypothalamic DA levels in control animals remained constant throughout proestrus and the total synthesis and breakdown over the 4h intervals were negligible. These results indicate low activity in the hypothalamic dopaminergic system preceding and during the preovulatory LH surge. Low DA activity during proestrus has also been reported by other workers (Fuxe & Hökfelt, 1970; Löfstöm, 1977). These results support the view that DA does not play an active part in the induction of the preovulatory LH surge. RMI 12,936 treatment resulted in a gradual increase in DA synthesis which led to a significant increase in hypothalamic DA levels, indicating that it has an excitatory effect on the hypothalamic dopaminergic system. This conclusion is contradictory to the claims of Geddes and coworkers (1979) who showed that DA levels peak at 12:00h on proestrus and that this surge is depressed after RMI 12,936 treatment. Although a peak in dopamine levels was observed at mid-day by another group of workers (Negro-Vilar et al, 1977) other investigations (in agreement with the present study) did not reveal any change in activity throughout proestrus

(Fuxe & Hökfelt, 1970; Selmanoff et al, 1976; Löfström, 1977). On closer inspection of the results obtained by Geddes et al (1979), it was discovered that the control levels of DA were raised in only two out of the eight animals tested. These higher levels may have been a reflection of the assay system used which has a reputation for unreliability.

Therefore, ignoring these two higher levels, the control levels remain constant throughout proestrus. The results obtained by Negro-Vilar and his coworkers may be a reflection of the area of the hypothalamus measured (ME). In other studies, an increase in DA turnover was demonstrated in the ME (Rance et al, 1981) but not in the POA or MBH (Honma & Wuttke,1980; Rance et al, 1981). In this study, the amine content of the whole hypothalamus was measured whereas Negro-Vilar measured the amine content of the ME alone. Therefore the use of gross dissection methods which include considerable amounts of hypothalamic tissue outside the TIDA system, may disguise any changes occurring in the ME alone.

Since it has been reported that DA can block ovulation (see general introduction), it is possible that the drug acts to inhibit ovulation by stimulating activity in an inhibitory pathway, possibly the intra-hypothalamic TIDA dopaminergic tract.

Measurement of hypothalamic 5'HT levels in control animals showed that the levels remain constant throughout proestrus

but that immediately preceding the preovulatory LH surge, both synthesis and breakdown of 5'HT increased. This increase in hypothalamic 5'HT turnover before the preovulatory LH surge has also been reported by Walker (1980a). Kordon has postulated that 5'HT has a dual effect on release of LH: an inhibitory effect on basal secretion of LH which probably is a result of direct action on the LHRH neurones in the ME; and control over the rhythmic secretion pattern of LH, involving facilitation of 5'HT diurnal rhythm (Héry et al, 1978). Therefore the observed increase in 5'HT activity in this study, may be expression of diurnal activity (Dixit & Buckley, 1967; Quay, 1968). Inhibition of the diurnal rise in 5'HT with p-chlorophenylalanine, a synthesis inhibitor, blocks ovulation in immature rats treated with pregnant mare serum (Wilson et al, 1977) demonstrating that hypothalamic 5'HT activity is essential for ovulation to occur. This is further substantiated by the work of Walker (1980a,b) who demonstrated that abolition of the 5'HT circadian rhythm blocks the phasic secretion of LH. Moreover, interference with the pattern of 5'HT activity during the afternoon of proestrus either with drug treatment or by lengthening the light period, results in a similar effect on the preovulatory LH surge. This apparent positive correlation between 5'HT neurotransmission and phasic LH secretion indicates an active role for 5'HT in the control of ovulation.

In this study, RMI 12,936 treatment suppresses the increase in 5'HT 'turnover' and therefore may block ovulation by

interference of 5'HT diurnal activity and hence regulation of the phasic secretion of LH. Another feature of the facilitatory action of 5'HT upon LH release is its different time course from the inhibitory effect. Inhibition of the LH ovulatory surge is only possible when treatment is applied within a few hours of the'critical period' of ovulation control (Kordon et al, 1968). In the case of the facilitatory effect of the amine, 5'HT inhibitors have to be given much earlier in order to interfere effectively with LH release (Héry et al, 1976). RMI 12,936 has to be administered before 03:00h on proestrus in order to block ovulation. This time course of drug activity agrees with an inhibitory action on the early facilitatory effect of 5'HT on ovulation and suggests that the gonadotrophin response to 5'HT may be oestrogen dependent.

There is also a small but significant increase in 5'HT concentration at 19:00h on proestrus. This increase in 5'HT levels may be a result of an imbalance in RMI 12,936 induced reduction in synthesis and breakdown. 5'HIAA, a metabolite of 5'HT, is often used, as it is in this study, as a measure of 5'HT turnover. The increase in hypothalamic 5'HIAA levels at 19:00h in control animals on proestrus is a reflection of an increase in 5'HT turnover and confirms the results obtained with pargyline. There is a small reduction in 5'HIAA levels at 19:00h on proestrus in RMI 12,936 treated rats when compared to the control but the large standard deviations obtained prevent any

firm conclusions being drawn. However, from the results obtained with 5'HT measurement, it can be concluded that the RMI 12,936 block of ovulation is associated with a reduction in 5'HT activity.

Consideration of the effect of RMI 12,936 on hypothalamic amine levels indicates that although NA is probably the neurotransmitter responsible for the trigger of ovulation, any one of its effects on the amines could result in a block of ovulation. Prior investigation into the anti-ovulatory activity of RMI 12,936 has led to the hypothesis that it acts through an antioestrogenic and antiprogestational activity. The hypothalamic sites of positive feedback of oestrogen and progesterone are reported to be in the MBH and AH-SCN-POA region, areas which receive a rich catecholaminergic innervation. Many of these areas also receive 5'HT innervation (see general introduction). These findings provide an anatomical basis for gonadal steroid-monoamine interaction. It has also been reported that administration to rats of oestrogen and progesterone, which elevates plasma LH levels, increased hypothalamic NA and 5'HT but not DA turnover (Munaro, 1977; Crowley, O'Donohue, Muth & Jacobowitz, 1979; Honma & Wuttke, 1980; Wilson, Bonney, Everard, Parrott & Wise, 1982). Therefore antagonism of oestrogen activity might be expected to prevent these responses. This is supported by the finding that the reduction in LHRH secretion,following PB treatment, was associated with a fall in hypothalamic NA neurotransmission but not with DA activity (Kalra et al, 1980). Similarly, the antiovulatory activity of RMI 12,936

is associated with an inhibition of hypothalamic NA and 5'HT neurotransmission plus stimulation of hypothalamic DA neurotransmission. These results are inkeeping with the hypothesis that RMI 12,936 acts to prevent stimulation of the neural signal and thus ovulation by preventing oestrogen and progesterone stimulatory activity.

In summary, the hypothesis that RMI 12,936, acting through antioestrogenic and antiprogestational activity, blocks the neural signal for trigger of ovulation was tested. This study showed that the antiovulatory activity of the drug is associated with an inhibitory effect on hypothalamic NA levels and synthesis. This effect is compatible with the hypothesis, if the neural signal is through stimulation of an excitatory noradrenergic pathway. The antiovulatory activity of RMI 12,936 was also shown to be associated with changes in hypothalamic 5'HT and DA levels, synthesis and breakdown. Either of these effects could also result in inhibition of ovulation.

CHAPTER 5

The effect of RMI 12,936 on hypothalamic amine levels. Detection by electrochemical detection after separation using high performance liquid chromatography.

INTRODUCTION

In the previous study the pattern of hypothalamic amine levels in control and in drug treated animals was recorded during proestrus using a fluorescence technique. It was shown that RMI 12,936 alters the levels and/or synthesis and breakdown of all three biogenic amines. The rise in concentration of NA at 15:00h and the increase in synthesis and breakdown of 5'HT between 15:00h and 19:00h were inhibited by RMI 12,936 treatment. On the other hand the synthesis of DA between 15:00h and 19:00h and the levels of DA and 5'HT at 19:00h were increased significantly. From this evidence various conclusions on the mechanisms of antiovulatory activity of the drug were drawn. However because of possible inadequacies in the fluorescence technique (see discussion), verification of the results was preferred.

The importance of recording biogenic amine activity in regions of the brain has necessitated the development of analytical assays capable of measuring simultaneously, minute amounts of the endogenous amines. Micromethods for determination of biogenic amines have been developed based on fluorescence (Westerink & Korf, 1977), gas chromatography-mass spectrometry (Takahashi, Yoshioka, Yoshiue & Tamura, 1978) and radioenzymatic methods (Argiolas & Fadda, 1978). Recent developments have led to the separation of the amines by high performance liquid chromatography combined with electrochemical detection (HPLC/ECD) (Ikenoya, Tsuda, Yamano, Yamanishi, Yamatsu, Ohmae, Kawabe, Nishino & Kurahashi, 1978). In

this study, measurement of hypothalamic amine levels was based on the latter technique. However work performed in our laboratories and by Asmus and Freed (1979) led us to use trichloroacetic acid rather than the more usual ion-pairing reagent, sodium octylsulphate, for the separation of catecholamines.

METHODS

Two groups of rats were dosed with 0.5ml of the vehicle or 2mg RMI 12,936 at 16:00h on Day D2. The animals were killed by cervical dislocation at 11:00h, 15:00h and 19:00h on Day P, the brains removed and the hypothalami isolated, weighed and stored at -20^{o}C for two weeks.

The hypothalamic concentrations of NA, DA, 5'HT and 5'HIAA were measured using the HPLC/ECD method previously described in the general methods section. The Aspin-Welch test was used to test for significance between concentrations.

RESULTS

The concentration of hypothalamic NA in control rats on proestrus was greatest at 15:00h. This concentration was significantly greater ($P<0.01$) than the concentration at 19:00h (fig.17 ; table 20). In RMI 12,936 treated rats, this rise in NA levels at 15:00h was inhibited ($P<0.05$).

The hypothalamic levels of DA in control animals, did not vary significantly throughout proestrus nor were they

Table 20: Hypothalamic levels (µg/g) of NA, DA, 5'HT and 5'HIAA in control and RMI 12,936 treated animals on proestrus after measurement by HPLC/ECD. The values are Mean ± SEM with six animals per group.

		11:00h	15:00h	19:00h
NA	Control	1.32 ± 0.18	1.98 ± 0.18	1.07 ± 0.14*
	RMI 12,936	1.35 ± 0.13	1.27 ± 0.12 †	1.45 ± 0.16
DA	Control	1.03 ± 0.10	1.22 ± 0.12	1.06 ± 0.09
	RMI 12,936	1.02 ± 0.06	1.04 ± 0.11	0.99 ± 0.05
5'HT	Control	0.34 ± 0.05	0.39 ± 0.04	0.29 ± 0.05
	RMI 12,936	0.34 ± 0.05	0.49 ± 0.10	0.37 ± 0.05
5'HIAA	Control	0.25 ± 0.01	0.97 ± 0.55	0.33 ± 0.16
	RMI 12.936	0.13 ± 0.02	0.17 ± 0.02	0.16 ± 0.03

* $P < 0.01$ Control value significantly different (Aspin-Welch Test) from control value at 15:00h

† $P < 0.05$ RMI 12,936 value significantly different from control value

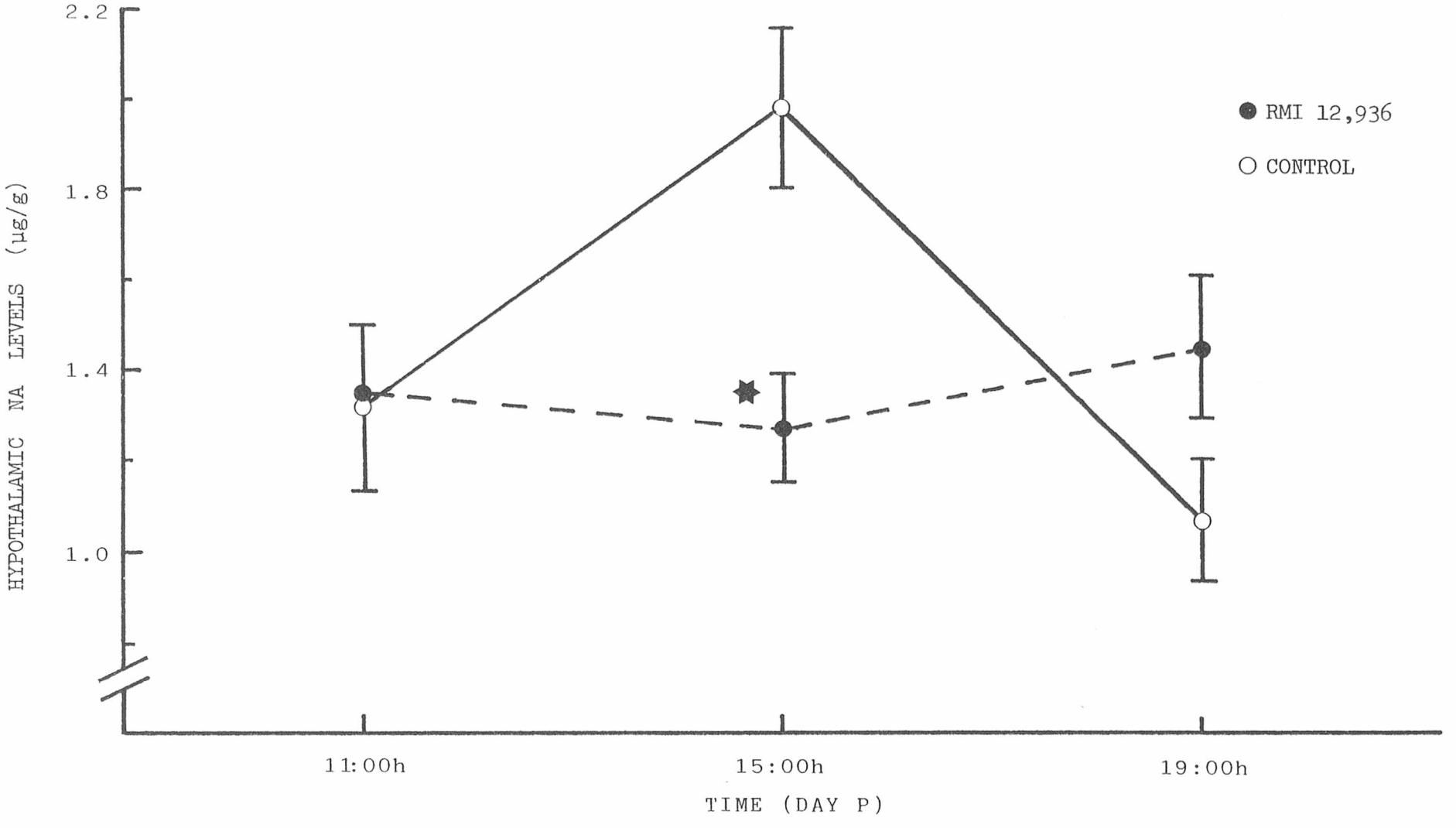


Fig. 17 The effect of RMI 12,936 on the hypothalamic levels of NA (as detected by the HPLC/ECD method) at 11:00h, 15:00h and 19:00h on proestrus. * $P < 0.05$ RMI 12,936 value significantly different (Aspin-Welch test) from the control value.

significantly different from the RMI 12,936 treated animals (table 20).

There was no significant difference in the hypothalamic levels of 5'HT in control animals throughout proestrus. There was an increase (although not significant) in the levels of 5'HT at 15:00h and 19:00h on proestrus in RMI 12,936 treated animals (table 20).

The hypothalamic levels of 5'HIAA in control rats rose at 15:00h on proestrus although the concentration was not significantly different from those at 11:00h and 19:00h. In RMI 12,936 treated rats, the hypothalamic 5'HIAA levels did not alter significantly throughout proestrus and there was no significant difference between 5'HIAA levels in control and RMI 12,936 treated rats (table 20).

Summary of Results

1) The hypothalamic levels of NA in control animals, were greatest at 15:00h on Day P. This figure was significantly greater ($P<0.01$) than the concentration at 19:00h. In RMI 12,936 treated rats, this rise in NA levels was inhibited ($P<0.05$).

2) The hypothalamic levels of DA in control animals, remained constant throughout Day P. RMI 12,936 treatment did not alter these DA levels.

3) The hypothalamic levels of 5'HT in control animals, remained constant throughout Day P. After RMI 12,936 treatment there was an increase in the level of 5'HT at 15:00h and 19:00h on Day P, although not significant.

4) There was no significant change in the hypothalamic levels of 5'HIAA in control animals throughout Day P. RMI 12,936 treatment did not significantly alter these levels.

DISCUSSION

In this study, the technique of amine measurement using HPLC/ECD was used to verify the results obtained in the previous chapter by fluorescence assay. This HPLC/ECD technique was found to have various advantages over the fluorescence technique.

(1) The limits of detection are far lower (in order of 50 to 100 x) than in the florescence assay. The determination of hypothalamic amine levels by the fluorescence method involves working close to the detection limits for this assay, whereas use of the HPLC/ECD method involves assay of amounts well above detection limits.

(2) The loss of precision is minimised in the HPLC/ECD technique since this technique only involves one step before measurement whereas in the fluorescence technique there are several steps before measurement and consequently more risk of loss, which may vary between samples.

(3) It is possible to detect and measure quantitatively adrenaline, simultaneously with NA, DA, 5'HT, and 5'HIAA whereas in the fluorescence technique, adrenaline cannot be discriminated from NA. In the latter method,

false levels of NA may be recorded as a consequence.

(4) For routine use, HPLC/ECD is a far simpler method to use than fluorescence which involves many steps and thus is a more likely source of operator error. The disadvantage of this HPLC/ECD method compared to fluorescence is that measurement of 5'HIAA is unreliable due to interference causing loss of baseline stability in this region. However further development of this HPLC/ECD technique should allow reliable measurement of 5'HIAA to be incorporated into this assay.

The data obtained on the pattern of hypothalamic amine levels in control animals throughout proestrus and the effect of RMI 12,936 on these levels generally agrees with the results obtained with the fluorescence technique. The hypothalamic NA levels in control animals were greatest at 15:00h on proestrus. After RMI 12,936 treatment this rise in NA levels was suppressed. These results agree with those obtained with the fluorescence technique, verifying that the block of ovulation by the drug is associated with suppression of a NA peak at 15:00h on proestrus. In controls, the hypothalamic DA and 5'HT levels remained constant throughout proestrus. RMI 12,936 treatment caused an increase in the level of 5'HT at 19:00h as observed with the results of the fluorescence technique. However, due to the higher SEM this increase which is of a similar magnitude to that shown in the results of the fluorescence technique, is not statistically significant. It was suggested in the previous chapter that this drug

induced increase in 5'HT level at 19:00h on proestrus, was a result of an imbalance in the reduction of synthesis and breakdown which was deemed to be the important effect of the drug.

Contrary to the results obtained using the fluorescence technique, the DA levels did not alter significantly at 19:00h after RMI 12,936 treatment. A possible explanation for this discrepancy may come from previous observations on seasonal variation in the sensitivity of the mechanisms controlling ovulation (see Chapter 1). The experiment in which the amines were measured using HPLC/ECD was performed during the months of July and August, the period of low sensitivity. On the other hand the experiment in which the fluorescence technique was used, was performed during the months of January to May, the period of high sensitivity. Since RMI 12,936 had no effect on the DA levels when administered during the less sensitive period of the year, it is suggested that the reduced sensitivity of the hypothalamus to steroid triggers during this period, prevents RMI 12,936 from producing its full effect. It was also suggested in the previous chapter that the stimulatory effect of RMI 12,936 on DA, is not necessary for the inhibition of ovulation. This supposition is confirmed by the results of this study which show that RMI 12,936 blocks ovulation when the levels of DA remain constant.

The large intra-assay variation of 5'HIAA using the HPLC/ ECD technique prevents any conclusion from being drawn as to the changes in 5'HIAA levels throughout proestrus

(and thereby 5'HT) and also the effect that RMI 12,936 treatment has on them.

In conclusion, the results obtained on hypothalamic NA and 5'HT levels verify those obtained using the fluorescence technique. Therefore it can be concluded that the anti-ovulatory activity of RMI 12,936 is associated with changes in these amine levels and synthesis and breakdown.
This evidence supports the suggestion that the major effect of RMI 12,936 antiovulatory activity is through inhibition of an excitatory noradrenergic pathway although the inhibitory effect on 5'HT activity will facilitate inhibition of ovulation. However the results on the effect of RMI 12,936 on hypothalamic DA levels differ. It was suggested that this discrepancy was a result of seasonal variation and that stimulation of a dopaminergic pathway is not necessary for the block to occur.

CHAPTER 6

Intraventricular administration of monoamines to RMI 12,936 and to PB treated rats.

INTRODUCTION

Earlier in this investigation it was shown that RMI 12,936 is an effective antiovulatory agent not only on peripheral administration but on central administration also. Furthermore, the compound was more potent following administration by the latter route. This suggested that the major site of antiovulatory activity may be at the central level. As a result of this finding it was proposed that RMI 12,936 acts at this level by preventing expression of oestrogen activity at its site of positive feedback action and thereby blocks the neural signal for trigger of ovulation.

On further investigation the block of ovulation was shown to be associated with certain changes in hypothalamic amine levels and turnover. The most profound effect of RMI 12,936 treatment was a suppression of NA levels at 15:00h on proestrus which was reflected by an inhibition of NA synthesis between 11:00h and 15:00h on the same day. It also suppressed an increase in 5'HT synthesis and breakdown between 15:00h and 19:00h on proestrus. As a result of these findings, it was proposed that RMI 12,936 blocks ovulation by inhibiting an excitatory noradrenergic signal for release of LHRH. It was also suggested that RMI 12,936 inhibition of 5'HT diurnal activity would result in a block of ovulation. If these changes in hypothalamic NA and 5'HT levels are important in producing the RMI 12,936 block of ovulation, then restoration of ovulation by replacement with exogenous amines would confirm this proposed mechanism of action. It has previously been reported that intraventricular administration of NA to

ovariectomized, ovariectomized, oestrogen-, progesterone-primed rats, female rats after hypothalamic lesioning, oestrogen-primed rabbits and male rats all significantly increased the plasma LH levels or induced ovulation (Kamberi et al, 1970; Sawyer et al, 1974; Tima & Flerkó, 1975; Krieg & Sawyer, 1976; Vijayan & McCann, 1978a; Kawakami & Ando, 1981). The aim of this study was to determine whether the RMI 12,936 block of ovulation can be reversed by intra-ventricular injection of NA or 5'HT.

METHODS

Expt I Intraventricular injection of amines to RMI 12,936 treated rats

Each rat received 2mg RMI 12,936 at 16:00h on Day D2 and 1ml/kg althesin at 14:55h on Day P. These animals were split into four groups and 2µl saline, 2µl NA (40µg, 80µg), 5'HT (80µg) or NA + 5'HT (40µg + 40µg) were injected into the third ventricle (as described in the general methods section) between 15:00h and 15:30h on Day P. A further group of rats were treated with 1ml/kg althesin at 14:55h on Day P followed by 2µl saline injected into the third ventricle. The amines were freshly prepared in isotonic saline at pH 6.8 and used within 30 min. The rats were autopsied on the expected day of oestrus when the number of animals ovulating was determined.

Expt II Intraventricular injection of amines to pentobarbitone treated rats

A. 35mg/kg PB was administered to each rat at 14:55h on proestrus. Immediately following this each group (of not less than 3 rats per group) were injected intraventricularly with either NA (20µg, 40µg, 100µg), 5'HT (20µg, 40µg), NA plus 5'HT (20 + 20µg, 40 + 40µg), Adr (20µg) or saline (pH 5.5) in volumes of 2µl. The amines were freshly prepared in isotonic saline, adjusted to pH 5.5, and used within 30 min.

B. Each rat (3 rats/group) received 35mg/kg PB at 14:55h on Day P. This was immediately followed by infusion of NA (20µg,40µg) in a volume of 10µl over a 30 min period. NA was freshly prepared in isotonic saline, adjusted to pH 5.5, and used within 30 min.

C. A group of rats (not less than 4 rats per group) received 35mg/kg PB at 14:55h on Day P immediately followed by an intraventricular injection of 2µl NA (40µg) or 2µl saline (pH 6.8). NA was freshly prepared in isotonic saline at pH 6.8 and used within 30 min.

All animals were sacrificed on the expected day of oestrus between 09:30h and 10:30h and the oviducts removed and examined for the presence of ova.

RESULTS

Expt I Intraventricular injection of amines to RMI 12,936 treated rats

Of those rats which had received 1ml/kg althesin at 14:55h on Day P followed immediately by an intraventricular injection of saline, all five ovulated. Administration of RMI 12,936 at 16:00h on Day D2 in addition to this treatment blocked ovulation in all 4 rats tested. Intraventricular injection of NA, 5'HT and NA + 5'HT all failed to restore ovulation to the RMI 12,936 blocked rats. In each case, out of the four animals which were successfully injected with the amine or combination of amines, none ovulated.

Expt II Intraventricular injection of amines to pentobarbitone treated rats

A. None of the PB treated rats which were injected with NA, 5'HT, NA + 5'HT, Adr or saline (pH 5.5) ovulated.

B. Infusion of NA (20µg, 40µg) over a 30 min period failed to restore ovulation to the PB blocked rats.

C. Ovulation remained blocked in all PB treated rats injected intraventricularly with NA or saline (pH 6.8).

Summary of Results

1) 1ml/kg of the anaesthetic, althesin, does not interfere with ovulation when administered at 14:55h on Day P. Pretreatment with RMI 12,936 results in a block of ovulation. Intraventricular administration to this latter group of animals, of NA, 5'HT or NA + 5'HT at approximately 15:00h on Day P, failed to restore ovulation.

2) 35mg/kg PB administered at 14:55h on Day P, blocks ovulation. Intraventricular administration of NA, 5'HT, NA + 5'HT or Adr at approximately 15:00h on Day P failed to restore ovulation.

3) Infusion into the third ventricle of 10µl NA, between 15:00h and 15:30h on Day P, failed to restore ovulation to the PB-blocked rat.

4) Intraventricular injection at 15:00h on Day P, of saline or NA at pH 5.5 and 6.8, failed to restore ovulation to the PB-blocked rat.

DISCUSSION

It has been proposed that RMI 12,936 blocks ovulation by preventing the positive feedback action of the steroids on the hypothalamus, resulting in inhibition of the neural signal for trigger of ovulation. The results of the previous studies (Chapters 4 and 5) have indicated that the neural signal inhibited by RMI 12,936 administration

was probably of noradrenergic origin although it also had an inhibitory effect on a tryptaminergic pathway. Therefore, if administration of these amines restore ovulation to the RMI 12,936 treated rats, it will confirm this hypothesis. However, this study clearly shows that intraventricular injection of NA, 5'HT or a combination of NA and 5'HT, under these experimental conditions, cannot restore ovulation to the RMI 12,936 treated animals. A possible explanation for these results is that the mechanism of antiovulatory activity of RMI 12,936 does not involve inhibition of a neural signal along a noradrenergic or a tryptaminergic pathway. An alternative possibility is that the mechanisms by which oestrogen stimulates ovulation is probably a more complex procedure than solely by trigger of a neural signal which initiates a series of events, culminating in ovulation. For example, it has been reported that oestrogen can induce the release of prostaglandins (see Craig, 1975), putative neuro-transmitters which have been implicated in the control of LHRH release and ovulation (see general introduction). Furthermore, administration of a prostaglandin synthetase inhibitor prevented NA stimulation of ovulation, indicating that prostaglandins mediate the noradrenergic control over LHRH secretion (Ojeda et al, 1979). Oestrogen is believed to trigger a noradrenergic signal which stimulates release of LHRH via prostaglandins. Therefore antagonism of this steroid's activity by RMI 12,936, may not only result in block to ovulation by inhibition of the neural signal but also by reduction of prostaglandin release.

Thus administration to the RMI 12,936 blocked rat, of NA alone would be insufficient to restore ovulation. Instead NA plus prostaglandin or prostaglandin itself would be necessary. A third possible explanation is that the experimental design is inadequate to test the hypothesis that RMI 12,936 blocks ovulation by inhibition of a noradrenergic or a tryptaminergic pathway. However the first two possibilities cannot be considered until the third possibility has been satisfied since the lack of restoration of ovulation in the RMI 12,936 treated rats by intraventricular injection of the amines may be due to the physiological conditions not being mimicked.

To confirm whether this experimental model is adequate to monitor the effects of the amines, restoration of ovulation was attempted with intraventricular administration of NA, 5'HT, NA plus 5'HT or adrenaline to PB-blocked rats. The PB-blocked rat was used as it is widely quoted that intraventricular administration of these amines, restored ovulation (Hökfelt & Fuxe, 1972; McCann & Moss, 1975; Gnodde & Schuiling, 1976; Krieg & Sawyer, 1976; Sawyer, 1977; Simpkins, Huang, Advis & Meites, 1979). However, in this study the PB-block of ovulation could not be restored by administration of the amines. On closer inspection of the sources of reference it was found that all the authors referred to one paper detailing the work of Rubinstein and Sawyer (1970). Examination of their results showed that neither NA (Fisher exact test, $P = 0.60$) nor 5'HT (Fisher exact test, $P = 0.69$)

significantly increased the number of rats ovulating and that only adrenaline (Fisher exact test, P = 0.05) significantly increased the percentage of ovulators. Although basically these results did agree with the findings in this study (with the exception of adrenaline), the fact that a few of the rats in each case did ovulate whereas in this study none did, remained puzzling. The technique of amine administration into the third ventricle used by Rubinstein and Sawyer differed from the original experiments in this study in that the amines were infused into the third ventricle in a volume of 10µl over a 30 min period whereas in this study the amines were infused in a volume of 2µl over a 2 min period. However this difference in technique was shown not to be of importance since in a further experiment where NA was infused in a volume of 10µl over a 30 min period, the animals still failed to ovulate. In support of this, it has been reported in other studies that NA can stimulate LH release after infusion in a volume of 2µl over a 2 min period (Kamberi et al, 1970; Krieg & Sawyer, 1976; Vijayan & McCann, 1978a; Kawakami & Ando, 1981). Also in this study, the amine solutions, injected into the third ventricle of RMI 12,936 and one group of PB treated rats, were freshly prepared in isotonic saline (pH 6.8). This differed from the procedure followed by Rubinstein and Sawyer who prepared the solutions in isotonic saline, adjusted to pH 5.5 to protect the amine from rapid oxidation. To evaluate whether the latter procedure was necessary for the amine solutions to induce ovulation,

the amines were prepared in isotonic saline adjusted to pH 5.5 and injected into the third ventricle of PB treated rats. None of the rats treated in this way ovulated showing that the pH of the amine solution was not the critical factor. Again this was supported by the results of several studies which showed that injection of isotonic saline at pH 6.8 or 5.5 did not alter plasma LH levels whereas injection of NA at both pH 6.8 and 5.5 significantly increased the levels of LH (Kamberi et al, 1970; Krieg & Sawyer, 1976; Vijayan & McCann, 1978a, Kawakami & Ando, 1981).

A satisfactory explanation for the differences in results may come from the fact that the PB-block of ovulation in the experiments of Rubinstein and Sawyer was not in fact a complete one. In rats treated with PB plus saline, 25% ovulated. The authors suggested that this was a result of experimental procedures although they do not provide any evidence to support their claim that PB blocks ovulation 100% when injected at this time. It is more likely that PB was injected during the critical period and not before it, which would explain why 25% of the rats ovulated after PB plus saline treatment. Thus the rats ovulating after NA or 5'HT administration could have been due to an incomplete block of ovulation. Furthermore it leaves the effect of adrenaline on restoration of ovulation questionable also. In support of the findings in this study, it has been reported that neither NA nor 5'HT, administered intraventricularly, were effective at restoring ovulation to PB-blocked rats (Raziano,

Cowchock, Ferin & Vande Wiele, 1971).

Although the administration of amines in this study did not restore ovulation to the PB or RMI 12,936 treated rats, intraventricular injection of NA has been shown to produce a sufficiently large increase in plasma LH levels to induce ovulation (Sawyer et al, 1974; Tima & Flerkó, 1974; Kawakami & Ando, 1981). In these studies the experimental models used differed from those used in this study. However in other experiments, intraventricular injection of NA even in steroid primed animals, produced only a relatively small although significant increase in plasma LH (Kamberi et al, 1970; Vijayan & McCann, 1978a). This increase raised the plasma LH to levels ranging from 2ng/ml to 20ng/ml which are insufficient to trigger ovulation (Greig & Weisz, 1973). Therefore it is possible that in this study the plasma levels of LH increased as a result of intraventricular injection of amines, but not in sufficient quantities to induce ovulation - the criterion used to determine the effect of amine administration on PB and RMI 12,936 treated rats. It is noteworthy that ovulation was also the criterion used by the other investigators when looking into the effect of intraventricular administration of monoamines on PB-blocked rats (Rubinstein & Sawyer, 1970; Raziano et al, 1971). Neither group measured plasma LH levels and therefore may also have missed a stimulatory effect on this parameter. It should also be noted that the experiments performed in this study were executed during the months of May to October. According to previous experience (see Chapter 1)

this is the least sensitive period in the mechanisms controlling release of LH from the adenohypophysis and therefore may provide partial explanation for the amines not restoring ovulation to the PB or RMI 12,936 treated rats. However, since it is apparent that intraventricular injection of, certainly NA, increases the plasma LH levels, sufficiently in some cases to induce ovulation, then it must be concluded that the design of this study is inadequate to test the effect of the amines on adenohypophysial release of LH. A more useful design would have included measurement of the plasma LH levels after amine administration in addition to the criterion of ovulation.

It is therefore not possible to evaluate the first and second explanations suggested at the beginning of this discussion, until further experiments have been completed. However, it seems unlikely that the changes in hypothalamic amine levels, associated with RMI 12,936 treatment (see Chapters 5 and 6) are not part of the mechanism by which the compound blocks ovulation. Also the second possibility in which RMI 12,936 produces a more complex block of ovulation which cannot be reversed by administration of amines alone, cannot be ruled out as a possible mechanism of action.

In summary, intraventricular injection of NA, 5'HT or a combination of these amines, could not restore ovulation to the RMI 12,936 treated rats. It was concluded that this could not be taken as an indication that the changes in hypothalamic amine levels associated with RMI 12,936 treatment are not a result of its mechanism of action in

blocking ovulation since administration of these amines could not restore ovulation to PB-blocked rats.

CHAPTER 7

The androgenic activity of RMI 12,936

INTRODUCTION

The results of the previous studies have given rise to the hypothesis that the mechanism of antiovulatory activity of RMI 12,936 is via its antioestrogenic and antiprogestational activity. It has been concluded that this antiovulatory activity does not involve the production of the slowly forming metabolite which appears to contribute to the antifertility activity but the possibility of some metabolic transformation cannot be ruled out. RMI 12,936 itself is a potent androgen (Grunwell et al, 1975) and is metabolised in the ovary to 7α-methyltestosterone (Kendle, 1976; Hardy et al, 1977; Taylor & Kendle, 1978) which is also a potent androgen (Grunwell et al, 1975). Therefore this androgenic activity of RMI 12,936 or its metabolite could be responsible for its antiovulatory activity.

In recent years ovarian androgens have been implicated in female reproductive function in control of follicular growth (Beyer, Cruz, Gay & Jaffe, 1974) and maturation (Louvet, Harman, Schreiber & Ross, 1975; Kumari, Datta, Das & Roy, 1978), continued secretion of FSH (Gay & Tomacari, 1974) and are essential substrates for follicular oestrogen production (Wotiz, Davis, Leman & Gut, 1956). The results of Mori and his coworkers indicate that androgen may also be involved in the ovulatory process of follicular rupture (Mori, Suzuki, Nishimura & Kambegawa, 1977). It has also been demonstrated that androgens can influence LH release through a negative feedback action at both the hypothalamic and hypophysial levels (Motta, Piva & Martini, 1973; Kingsley & Bogdanove, 1973; Drouin & Labrie, 1976).

Therefore the aim of this study is to test the hypothesis that the antiovulatory activity of RMI 12,936 is associated with its androgenicity.

METHODS

Expt. I Androgenic potency of RMI 12,936 compared to testosterone.

Three week old male rats were castrated and dosed with testosterone or RMI 12,936 in dose levels of 1mg/kg and 3mg/kg daily for ten consequtive days. At the end of this period the animals were killed and their seminal vesicles and prostate glands isolated as described in the general methods section and then weighed. Relative potencies were calculated by analysis of variance.

Expt. II Antiovulatory potency of testosterone compared to RMI 12,936

At 16:00h on Day D2, two groups of rats were dosed with RMI 12,936 or testosterone in various concentrations (see table 22). A further two groups of animals were dosed with 2mg RMI 12,936 or 10mg testosterone at 11:00h on Day P. Autopsy was performed between 09:00h and 12:00h on the expected day of oestrus when the numbers of animals ovulating were determined. The median effective doses and potency of RMI 12,936 and testosterone in inhibiting ovulation were calculated by using probit transformation.

Expt. III Reversal of RMI 12,936- and testosterone-induced inhibition of ovulation

At 16:00h on Day D2, two groups of rats were dosed with 2mg RMI 12,936 and two groups with 10mg testosterone. One group of RMI 12,936 and one group of testosterone treated rats received 2µg oestradiol at 17:00h on Day D2 and 2mg progesterone at 13:00h on Day P. The remaining two groups were dosed with LHRH in various concentrations (see table 24) at 16:00h on Day P. Autopsy was performed between 09:00h and 12:00h on the expected day of oestrus, when the numbers of animals ovulating were determined. The median effective doses and potencies of LHRH in restoring ovulation to the steroid-blocked rats were calculated using probit transformation. This experiment was performed during the months of January and February.

RESULTS

Expt. I Androgenic potency of RMI 12,936 compared to testosterone

The weights of the seminal vesicles and the prostate glands, taken from RMI 12,936 treated rats are not significantly different from those treated with testosterone (table 21). The relative potencies of RMI 12,936 in increasing the weights of the seminal vesicles and the prostate glands are 1.35 (fiducial limits of 2.20 and 0.91) and 1.36 (fiducial limits of 2.58 and 0.83) respectively. Testosterone was taken as the standard, with a potency of 1.0. Therefore the androgenic potency of RMI 12,936 is slightly but not significantly greater than that of testosterone (fig. 18).

Table 21: The weights (mg) of the seminal vesicles and prostate glands of castrated rats dosed daily for 10 consequtive days with testosterone or RMI 12,936.

TREATMENT	Wt. Seminal Vesicles (mg)	Wt. Prostate Gland (mg)
1mg Testosterone	29.2	44.6
	34.8	47.2
	34.5	58.3
	46.2	61.4
MEAN	36.18	52.88
SEM	3.58	4.11
SD	7.16	8.22
3mg Testosterone	116.7	84.0
	134.8	111.0
	205.3	127.2
	153.4	115.1
MEAN	152.55	109.33
SEM	19.11	9.12
SD	38.26	18.23
1mg RMI 12,936	37.5	63.9
	50.0	58.4
	58.2	56.9
	48.4	71.3
MEAN	48.53	62.63
SEM	4.26	3.26
SD	8.51	6.52
3mg RMI 12,936	195.6	103.9
	157.8	116.6
	217.1	105.7
	196.9	147.7
MEAN	191.85	118.48
SEM	12.37	10.14
SD	24.74	20.28

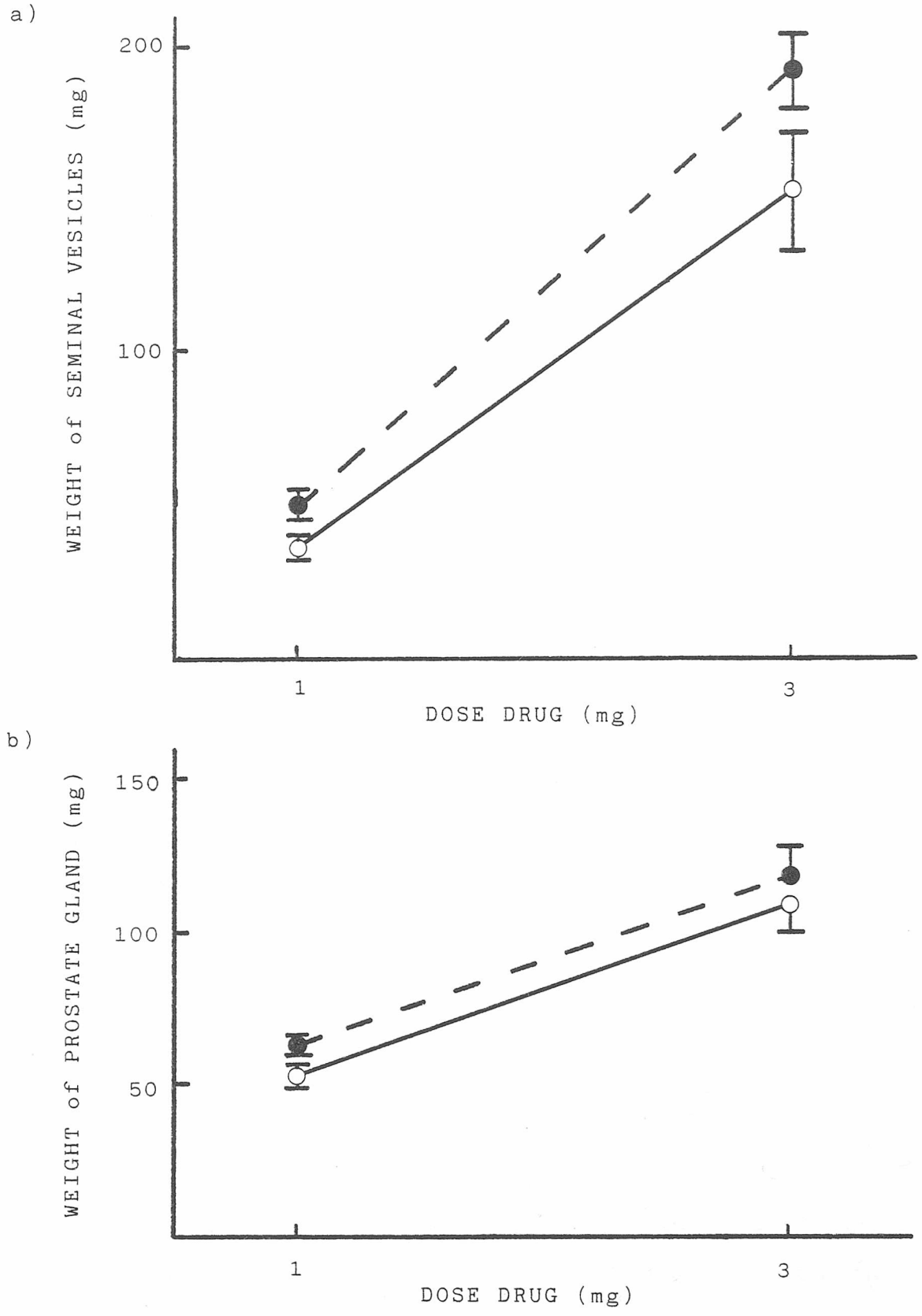


Fig. 18 Androgenic activity of testosterone (open circle, solid line) and RMI 12,936 (closed circle, dotted line).

Expt II Antiovulatory potency of testosterone compared to RMI 12,936

The ED_{50} for inhibition of ovulation is 10.67µg for RMI 12,936 and 81.12µg for testosterone (table 22). The relative potency of testosterone is 0.173 with fiducial limits of 1.868 and 0.038, where the potency of RMI 12,936 is taken as 1.0. This approximate 6-fold difference in antiovulatory potency is not significant.

2mg of RMI 12,936 and 10mg of testosterone both failed to inhibit ovulation when administered at 11:00h on Day P (table 23).

Expt III Reversal of RMI 12,936- and testosterone-induced inhibition of ovulation

The ED_{50} of LHRH required to restore ovulation in RMI 12,936 treated rats is 0.599µg and in testosterone treated rats is 3.781µg (table 24). The relative potency of LHRH in testosterone treated rats is 0.15 with fiducial limits of 0.111 and 0.203 and is significantly different from the relative potency of LHRH in RMI 12,936 treated rats, designated as 1.0.

Administration of 2µg oestradiol plus 2mg progesterone restored ovulation to 100% RMI 12,936 treated rats and 75% testosterone treated rats (table 24). There was no significant difference (Fisher Exact test) between RMI 12,936 and testosterone treated rats dosed with the sex steroids.

Table 22: The antiovulatory potencies of RMI 12,936 and testosterone when administered at 16:00h on Day D2.

Dose RMI 12,936 (mg/rat)	No. Rats	No. Ovulating (%)	Dose Testosterone (mg/rat)	No. Rats	No. Ovulating (%)
2.0	5	0(0)	2.0	5	0(0)
1.0	5	0(0)	1.0	-	-
0.5	5	0(0)	0.5	5	0(0)
0.125	5	0(0)	0.125	5	2(40)
0.031	9	0(0)	0.031	5	4(80)
0.008	5	4(80)	0.008	5	5(100)
0.002	5	5(100)	0.002	-	-

Table 23: The effect of RMI 12,936 and testosterone on ovulation when administered at 11:00h on Day P.

TREATMENT	DOSE (mg/rat)	NO. OF RATS	NO. OVULATING (%)
RMI 12,936	2	5	5 (100)
TESTOSTERONE	10	5	5 (100)

Table 24: The effect of administration of LHRH and oestrogen plus progesterone on RMI 12,936- and testosterone- induced inhibition of ovulation.

Treatment	No. Ovulating (%)
A. 2mg RMI 12,936 +	
2µg oestradiol + 2mg progesterone	4/4 (100)
0.125µg LHRH	0/5 (0)
0.25µg LHRH	1/7 (14)
0.5µg LHRH	2/7 (29)
1.0µg LHRH	4/5 (80)
2.0µg LHRH	5/5 (100)
B. 10mg Testosterone +	
2µg oestradiol + 2mg progesterone	3/4 (75)
2.0µg LHRH	0/5 (0)
4.0µg LHRH	3/5 (60)
8.0µg LHRH	5/5 (100)

Summary of Results

1) The androgenic potency of RMI 12,936 was slightly greater (approximately 1.4 times) than testosterone, although not significantly.

2) The antiovulatory activity of RMI 12,936 is greater (approximately 6 times) than testosterone, although not significantly.

3) Both RMI 12,936 and testosterone failed to block ovulation when administered at 11:00h on Day P.

4) LHRH induced ovulation in animals treated with 2mg RMI 12,936 or 10mg testosterone. LHRH is significantly more potent at inducing ovulation in RMI 12,936 treated rats (relative potency of 1.0) than in testosterone treated rats (relative potency of 0.15; fiducial limits of 0.111 and 0.203).

5) Administration of oestradiol plus progesterone during the months of January and February, restored ovulation to both RMI 12,936 and testosterone treated rats.

DISCUSSION

The evidence obtained previously in this investigation has shown that RMI 12,936 blocks ovulation through an anti-oestrogenic and antiprogestational activity. However the mechanism by which the drug achieves this is as yet unknown.

Comparison of some of the characteristics of the RMI 12,936 induced block of ovulation with those of a potent androgen, testosterone, suggests that these two drugs may act in a similar manner to block ovulation. RMI 12,936 blocks ovulation when administered at 16:00h on the second day of diestrus but not when administered at 11:00h on proestrus. The block of ovulation can be reversed by administration of LHRH or oestradiol plus progesterone. The same characteristics are found in the block of ovulation induced by testosterone.

Since testosterone is a potent androgen, a test was performed to determine whether the antiovulatory activity of these drugs is associated with their androgenic activity. The results of this experiment showed that RMI 12,936 was approximately 6 times more potent an antiovulatory agent than testosterone but only approximately 1.4 times more potent an androgenic agent. This finding shows that the antiovulatory activity is not associated with the drug's androgenic activity since the relative potencies differ.

In a recent study the antiovulatory activity of 7α-methyltestosterone was compared to that of RMI 12,936 and although not significant, it was shown to possess approximately 50% of the potency of RMI 12,936 (Harper, Honours project RGIT). This finding, in addition to those reported in this study, suggests that the order of potency of antiovulatory activity for these related compounds is RMI 12,936 < 7α<methyltestosterone testosterone and therefore differs from the order of androgenic potency reported by

Grunwell and his coworkers (1975). These results provide additional support for the finding in this study that the antiovulatory activity of these compounds is not a direct result of their androgenic properties. This finding is also supported by the work of Zanisi, Motta and Martini (1973) which showed that the relative order of androgenic potency of testosterone and its metabolites differed from that of its effect on LH levels in castrated male rats. Therefore it was concluded that peripheral androgenic activity of a given steroid does not correlate with its ability to inhibit LH secretion. However, this does not preclude the possibility of the mechanism of the antiovulatory activity of RMI 12,936 and testosterone being the same.

It has been reported that testosterone acts at both the hypothalamic (Motta et al, 1973) and adenohypophysial levels (Kingsley & Bogdanove, 1973; Drouin & Labrie, 1976) to regulate the negative feedback of LH release. Similarly, it has been shown earlier in this investigation that RMI 12,936 also acts at both levels (see Chapters 2 and 3). Furthermore, it has been reported that testosterone exerts its inhibitory action on LH release at the hypothalamic level at a dose lower than that active at the hypophysial level (Ferland, Drouin & Labrie, 1976), a characteristic which is similar to that of RMI 12,936 (Chapter 3). Incubation of androgens with adenohypophysial cells in culture, results in a marked inhibition of the LH response to LHRH (Drouin & Labrie, 1976) and this has been found to be at least in part due to a reduction of the number of LHRH receptors (Giguere, Lefebvre

& Labrie, 1981). Similarly, RMI 12,936 reduces the responsiveness of the adenohypophysis to LHRH. From the results of an earlier study (Chapter 2), it was proposed that RMI 12,936 achieves this effect by preventing release of LHRH through antagonism of oestrogen and possibly progesterone activity and therefore inhibits LHRH self-priming action. However, it was demonstrated that this could not be the full mechanism of action because administration of oestrogen and progesterone could not reverse the block. A mechanism of action which could fulfil this criteria would be reduction of the number of LHRH receptors present in the adenohypophysis. Such a mechanism of action for RMI 12,936 is possible since RMI 12,936 has similar antiovulatory characteristics to that of testosterone which reduces adenohypophysial sensitivity to LHRH in this manner. The median effective dose of LHRH necessary to restore ovulation to RMI 12,936 treated rats is 0.599µg and 3.781µg to testosterone treated rats. This is an approximate 6½-fold difference in sensitivity which roughly corresponds to the 5-fold difference in drug concentration, indicating that both steroids may reduce adenohypophysial responsiveness to LHRH to the same extent. Further support for this hypothesis comes from the observation that administration of oestrogen will not reverse the inhibitory effect of testosterone on adenohypophysial sensitivity to LHRH (Debeljuk, Vilchez-Martinez, Arimura & Schally, 1974; Drouin, Lagacé & Labrie, 1976). This finding led to the discovery that oestrogen and testosterone modify adenohypophysial

sensitivity by different mechanisms. Oestrogen increases the sensitivity to LHRH by a direct stimulatory effect on LH-secreting cells (Drouin et al, 1976) while testosterone reduces the number of LHRH receptors (Giguere et al, 1981). These different mechanisms of steroid action would explain why administration of oestrogen cannot restore full sensitivity to testosterone treated rats. Similarly, oestrogen and progesterone administration cannot restore full sensitivity to RMI 12,936 treated rats indicating that RMI 12,936 also modifies adenohypophysial sensitivity by a different mechanism from these gonadol steroids. This observation provides further support for a similar mechanism of adenohypophysial action of RMI 12,936 to that of testosterone. However it must be noted that this reduced number of receptors are sufficient for ovulation to occur in response to LHRH stimulation directly or indirectly through oestrogen and progesterone administration. Therefore in agreement with previous studies (see Chapter 2) it can be concluded from these results that an action at the adenohypophysial level, probably by reduction of LHRH receptors, cannot be the only mechanism of antiovulatory action of these drugs.

Testosterone concentrating neurones have been detected in areas of the hypothalamus known to be involved in the feedback control of LH such as the POA and the AH (Sar & Stumpf, 1975). These areas are rich in catecholaminergic and tryptaminergic innervation (Grant & Stumpf, 1975) providing an anatomical basis for gonadal hormone - monoamine interaction. However, little is known regarding the effects of androgens on neurotransmitter activity. It has been reported that castration of male rats causes a

reduction in NA turnover in various brain regions and replacement with testosterone increases NA and TIDA DA activity (Fuxe, Hökfelt & Nilsson, 1969; Fuxe & Hökfelt, 1970). More recently in castrate rats it has been demonstrated that subcutaneous injection of testosterone or injection directly into the MBH causes a reduction in NA turnover (Simpkins, Kalra & Kalra, 1980). In an earlier study in this investigation it was shown that RMI 12,936 also, has an inhibiting effect on hypothalamic NA synthesis and content (see Chapters 4 and 5). This effect is believed to be associated with the drug's antiovulatory activity. Since oestrogen triggers the neural signal for ovulation, believed to be mediated by an excitatory noradrenergic pathway, the evidence that testosterone and RMI 12,936 have an inhibitory effect on such a pathway suggests that they block ovulation by preventing oestrogen from stimulating the neural signal. However this mechanism has not yet been proved. It has been suggested that testosterone acts by conversion to an oestrogen (Naftolin, Ryan, Davies, Reddy, Flores, Petro, Kuhn, White, Takaoka & Wolin, 1975). It is possible that the relatively large dose of androgen, by aromatization to an oestrogen has an antiovulatory effect. However oestrogen replacement would be expected to exacerbate the situation instead of restoring ovulation as is the case. It has also recently been demonstrated that the action of testosterone on electrical stimulation of the amygdala is independent of its aromatization or reduction (Parvizi & Ellendorf,1980). Therefore this mechanism of action seems unlikely.

It has also been reported that conversion of testosterone into dihydrotestosterone and 5α-androstan-3 ,17 -diol, is a crucial step for the hormone to exert its negative feedback effect on LH secretion (Martini, Celotti, Massa & Martini, 1978). At the hypothalamic level testosterone is converted to these more active metabolites (Massa, Stupnicka, Kniewald & Martini, 1972) which may act on androgen receptors to inhibit the neural signal for ovulation. It has been reported that oestrogen can modulate the negative feedback effect of testosterone on LH secretion by modifying its rates of conversion to these metabolites (Martini et al, 1978). Therefore on oestrogen administration it might be expected that the conversion of testosterone to its more active metabolites would be reduced, resulting in a reduction in the inhibitory effect of testosterone. Indeed administration of oestrogen plus progesterone completely restored ovulation. It is noteworthy that administration of oestrogen alone to RMI 12,936 treated animals restored ovulation to only 50% of the animals. However RMI 12,936 itself possesses anti-progestational activity (Kendle, 1976) and therefore administration of progesterone together with oestrogen restores ovulation to 100% of RMI 12,936 treated animals. This mechanism of action falls down when applied to the effect of testosterone at the level of the adenohypophysis. If testosterone, through conversion to its metabolites, reduces the adenohypophysial sensitivity to LHRH by reducing the number of LHRH receptors, then administration of oestrogen and progesterone ought to increase the sensitivity. However if testosterone acts in the same manner as RMI 12,936 where the

increase in sensitivity is not significant, it would indicate that conversion to dihydrotestosterone and 5α-androstan-3α, 17β-diol is not crucial for reduction of adenohypophysial sensitivity.

An alternative explanation for this apparent discrepancy is that RMI 12,936 and testosterone act on hypothalamic androgen receptors to produce a negative feedback on LH release via a noradrenergic pathway. This inhibitory input overrides the excitatory input from oestrogen but on oestrogen and progesterone replacement the balance is readjusted to allow oestrogen excitatory input to be dominant and ovulation to be restored.

In summary, it was concluded that RMI 12,936 antiovulatory activity is not dependent on its androgenic potency. It was suggested that it may act in a similar manner to that of testosterone in inhibition of ovulation since both compounds have similar antiovulatory properties.

PART 4

GENERAL DISCUSSION

This investigation has been aimed at confirming previous findings on RMI 12,936 antiovulatory activity and at extending these findings to investigate the mechanism of action of this antiovulatory agent. Initial studies (see Chapter 1) confirmed the findings that RMI 12,936 blocks the preovulatory surge of LH which can be restored by administration of LHRH or oestradiol plus progesterone (Kendle et al, 1978; Geddes et al, 1979). It was also previously shown that RMI 12,936 is effective at blocking ovulation if administered at 16:00h on dioestrus II but not if administered after 12:00h on proestrus (Kendle et al, 1978). This work was confirmed and extended to show that the time limits of change from inhibition to permissiveness are narrower, with RMI 12,936 effective at blocking ovulation on or before 01:00h on proestrus but not when administered on or after 03:00h on proestrus. Two possible explanations for this finding were suggested. 1) In order to produce its antiovulatory activity, RMI 12,936 requires to be metabolized in the ovary to an active compound as demonstrated in antifertility studies (Kendle, 1978). 2) RMI 12,936 inhibits ovulation by interfering with the hormonal conditions of the animal and therefore must be administered before these hormones have produced their effects. The first possibility was tested by determining whether RMI 12,936 was effective at reducing steroid-induced plasma LH levels in the absence of the ovary ie. in ovariectomized, oestrogen-, progesterone-primed rats. No significant change in the plasma levels of LH were observed, suggesting that the

drug does depend on ovarian metabolism for its activity to be expressed. However, oestrogen plus progesterone are able to reverse the block of ovulation in intact RMI 12,936 treated rats and therefore must be capable of reversing the RMI 12,936 block in ovariectomized rats. Clarification of the question was obtained on intraventricular administration of the drug (see Chapter 3). RMI 12,936, administered by this route, was effective at a dose significantly lower than that effective on subcutaneous administration. Therefore at this low dose, RMI 12,936 cannot be transported to the ovary for metabolism to an active form which produces the antiovulatory activity. This study shows that ovarian metabolism is not necessary for RMI 12,936 to express its antiovulatory activity.

The second possible explanation for the timing of RMI 12,936 antiovulatory activity finds support from the results of several studies (Brown-Grant, 1969; Aiyer & Fink, 1974; Kalra & Kalra, 1974; Kalra, 1975). These studies showed that the presence of oestrogen is necessary up to at least 22h before ovulation for sufficient priming of central oestrogen positive feedback sites. Once sufficient oestrogen priming has been achieved ie. by 03:00h on proestrus, administration of progesterone or ICI 46,474, an antioestrogen, are incapable of blocking ovulation. It was suggested that progesterone, administered before 03:00h on proestrus, blocks ovulation by inhibiting the rise in circulating oestrogen levels. Since RMI 12,936 is effective in blocking ovulation only when administered

before 03:00h on proestrus, it was proposed that it may owe its antiovulatory activity to an antioestrogenic activity. To test this hypothesis, oestrogen was administered to rats 1h after RMI 12,936 in order to see if it could restore ovulation. However only 43% of the animals ovulated. It has been demonstrated that to induce ovulation, oestrogen requires the presence of progesterone (Mann & Barraclough, 1973). Since RMI 12,936 is known to inhibit progesterone synthesis (Kendle, 1976), it would seem likely that administration of progesterone is required in addition to oestrogen. This idea was supported by the finding that administration of both oestrogen and progesterone restores ovulation to 100% of RMI 12,936 treated animals. As a result of these findings, a new hypothesis was proposed: RMI 12,936 inhibits ovulation by exhibiting antioestrogenic and antiprogestational activity. To produce these effects, its mechanism of action could be by inhibition of steroid biosynthesis or by inhibition of the expression of steroid activity at its site of positive feedback action.

SITE OF ANTIOVULATORY ACTIVITY OF RMI 12,936

From the possible mechanisms of antioestrogenic and antiprogestational activity of RMI 12,936, the drug's sites of action would be at 1) the ovarian level, or 2) the adenohypophysial level and 3) the hypothalamic level. The major site of oestrogen and progesterone biosynthesis is in the ovary. Therefore RMI 12,936 may

act at the level of the ovary to inhibit oestrogen and progesterone biosynthesis (see Chapter 3). It has previously been reported that RMI 12,936 inhibits progesterone biosynthesis (Kendle, 1976). Therefore to determine whether it has a similar effect on oestrogen biosynthesis the circulating levels of oestrogen could be measured in control and in RMI 12,936 treated rats. However, this mechanism of action cannot be involved in the block of ovulation produced by centrally administered RMI 12,936 as doses too low to be effective when administered peripherally, were effective on central administration.

A site of positive feedback action of the steroids on LH release is at the adenohypophysial level (see general introduction). At this level, oestrogen primes the 1st phase increase in adenohypophysial sensitivity to LHRH while progesterone is partly responsible for the 2nd phase increase (Aiyer et al, 1974). Examination of the effect of RMI 12,936 on adenohypophysial sensitivity revealed that it prevented full sensitization to LHRH (Chapter 2). However oestrogen and progesterone did not restore full sensitivity to LHRH nor even significantly increased it although such a regime was capable of restoring ovulation to RMI 12,936 treated rats. This finding showed that the adenohypophysis was not the sole nor the major site of RMI 12,936 antiovulatory action.

The hypothalamus is also a site of steroid positive feedback action for release of LH (see general introduction). At this level oestrogen primes its site of positive feedback

action (Kalra, 1975) and triggers a neural signal believed to be mediated by NA. This neural signal stimulates release of LHRH and thereby LH, culminating in ovulation. Investigation into the timing of RMI 12,936 antiovulatory activity (Chapter 1) indicated that this phenomenon was due to inhibition of oestrogen priming of its central sites of positive feedback. Since it was shown that the drug does not block ovulation by inhibition of oestrogen and progesterone biosynthesis, it implies that RMI 12,936 has a direct action at the level of the hypothalamus. To test this, the potency of RMI 12,936 on intraventricular administration was compared to that on subcutaneous administration. This comparison showed that the drug was significantly more potent when administered centrally indicating that this was its major site of antiovulatory action.

If RMI 12,936 does block the central expression of oestrogen activity, it would be expected that the neural signal triggered by oestrogen, would also be blocked. Measurement of hypothalamic amine levels during proestrus showed that the drug's antiovulatory effect was associated with an inhibitory effect on the hypothalamic noradrenegic system (Chapters 4 and 5). The rise in NA levels at 15:00h was suppressed in RMI 12,936 treated animals. This finding supports the hypothesis that RMI 12,936 blocks the expression of the central action of oestrogen and thereby a neural signal mediated by NA. The study also showed that the drug had an inhibitory effect on the hypothalamic tryptaminergic

system. Such an effect could, by preventing the diurnal rise in 5'HT activity, also block ovulation (Dixit & Buckley, 1967; Quay, 1968; Wilson et al, 1977). Involvement of DA in the drug induced block of ovulation was discounted since no change in hypothalamic DA levels could be detected after RMI 12,936 treatment in one set of experiments (Chapter 5). The small change that occurred in the other set of experiments was put down to seasonal variation and was not associated with a block of ovulation. From these results, it was proposed that the inhibitory effect on the tryptaminergic system facilitates inhibition of ovulation produced by RMI 12,936 antioestrogenic activity and thereby block of the noradrenergic signal. The dopaminergic system does not play a role in the block of ovulation.

As further conformation of the hypothesis, NA and two other amines were administered intraventricularly to RMI 12,936 blocked rats, in an attempt to restore ovulation (Chapter 6). However due to an inadequate experimental model, no conclusions could be drawn. The design of a future experiment would require incorporation of measurement of plasma LH levels in addition to ovulation, before and after intraventricular amine administration.

The precise hypothalamic site of RMI 12,936 antiovulatory activity is probably at the positive feedback site of oestrogen. However the precise site of oestrogen positive feedback is controversial. Numerous studies have shown

that oestrogen acts on the AH-SCN-POA and thereby has a stimulatory influence on adenohypophysial function (Barraclough et al, 1966; Bishop, Kalra, Fawcett, Krulich & McCann, 1972; Clemens et al, 1976; Goodman, 1978; Kawakami et al, 1978) whereas other studies have demonstrated that the steroids act on the MBH to stimulate LH release (Palka et al, 1966; Weick & Davidson, 1970; Billard & McDonald, 1973; Döcke, 1980). The issue cannot be decided by uptake studies, because oestrogen receptors have been found in both the MBH and the AH-SCN-POA (Pfaff & Keiner, 1973; Sar & Stumpf, 1973; Blaustein & Wade, 1978). Neither can it be decided by localization of noradrenergic innervation since both regions receive a rich innervation (see general introduction). In a recent study, increased NA turnover rates during the preovulatory LH surge, were demonstrated not only in the medial POA but also in the ME, SCN and AN (Rance et al, 1981). As a result of these findings, it was proposed that the stimulatory action of NA on LHRH release is within the entire preoptico - suprachiasmatic tuberoinfundibular system, including the ME. This suggests that oestrogen may also act at all of these sites to stimulate release of NA. This hypothesis may be confirmed or contested by the results of an investigation into the effects of RMI 12,936 on amine levels and turnover in discrete hypothalamic areas, particularly the AN, ME, POA, SCN and AH and the subsequent effect of oestrogen administration.

THE MECHANISM OF ANTIOVULATORY ACTIVITY OF RMI 12,936

It has been established that RMI 12,936 blocks ovulation by exhibiting antioestrogenic and antiprogestational activity. However the mechanism by which it does this is as yet unproven.

A. Mechanism of action at the level of the hypothalamus

At the level of the hypothalamus, it has been proposed that RMI 12,936 prevents oestrogen from stimulating a neural signal mediated by NA. This may be achieved in two ways. 1) Inhibition of oestrogen biosynthesis or 2) inhibition of the expression of oestrogen activity. Although the first possibility is plausible (Chapter 1), the discovery that the drug can block ovulation by a direct action at the level of the brain, rules this out as the sole mechanism of action.

The second method encompasses a large number of possible mechanisms of action. The comparisons of antiovulatory activity made between RMI 12,936 and testosterone may help to resolve this. In this study (Chapter 7), many similarities of action were observed. Both drugs were effective at blocking ovulation when administered at 16:00h on dioestrus II but not at 11:00h on proestrus and ovulation could be restored by the administration of LHRH or oestradiol plus progesterone. In addition,

it has been reported that testosterone can reduce hypothalamic NA turnover, a phenomenon associated with RMI 12,936 antiovulatory activity. Furthermore, the relative antiovulatory potencies of these drugs did not correlate with their relative androgenic potencies. As a result of these findings, it was proposed that these two antiovulatory agents have a similar mechanism of action, independent of their classic androgenic activity. The mechanism of negative feedback action of testosterone on gonadotrophin release is the subject of ongoing research. From the information presently known, testosterone may act through one of the following mechanisms of action.

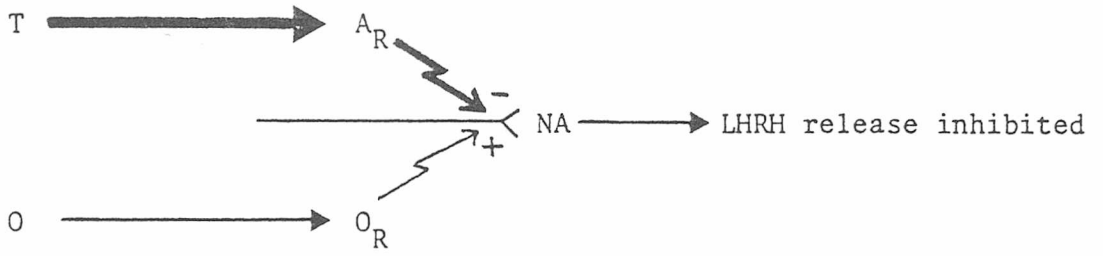
1) Testosterone is aromatized to oestradiol which then binds to oestrogen receptors.
2) Testosterone is aromatized to oestradiol which is then hydroxylated to form a catecholoestrogen.
3) Testosterone binds directly to androgen receptors.
4) Testosterone is reduced to dihydrotestosterone or 5α-androstan-3α,17β-diol which then bind to androgen receptors.

The transformation of testosterone to oestradiol by aromatization has been implicated in the rat brain for the activation of male sexual behaviour (Christensen & Clemens, 1974) and for the defeminizing aspects of testosterone action in the sexual differentiation of the brain (Vreeburg, van der Vaart & van der Schoot, 1977).

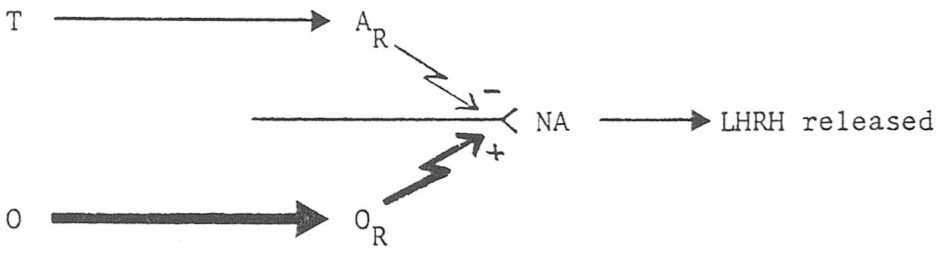
If testosterone also relies on aromatization to oestradiol in order to exhibit its negative feedback action on LH release, the oestrogen receptors would need to be inhibitory to LH release. Since oestrogen priming of the hypothalamus is important up to approximately 03:00h on the morning of proestrus for its stimulatory effect on LH release (Kalra, 1975), it may be that oestrogen is changing the balance of oestrogen receptor types from predominantly inhibitory for its negative feedback action to predominantly excitatory, for its positive feedback action. This model fits in with the observation that testosterone is only effective at inhibiting ovulation when administered on diestrus II and not on the morning of proestrus. However it does not adequately explain why oestrogen administration one hour after testosterone, should restore ovulation and not facilitate the inhibitory effect. The second model involves further conversion to a catecholoestrogen. In recent studies, catecholoestrogens have been reported to influence LH secretion (see Fishman, 1981) and therefore it was proposed that they may play an intermediary role in the control of oestradiol over LH release. However, the work of Krey and his coworkers provides convincing evidence against these two hypothetical models (Krey, Lieberburg, Roy & McEwen, 1979). They demonstrated that aromatization is not a prerequisite for the negative feedback effect of testosterone on LH release in the male rat, as the administration of ATD, a steroid aromatase inhibitor, does not alter the effect of the

drug on LH release. Furthermore, they showed that administration of an androgen antagonist, flutamide, resulted in a block of the inhibitory effect on LH release with no change in oestrogen-receptor binding. These findings demonstrate that testosterone does not have its negative feedback effect through aromatization to oestradiol but by binding to androgen receptors (models 3 and 4). Since testosterone has an inhibitory effect on hypothalamic NA turnover (Simpkins et al, 1980), it is possible that testosterone-receptor interaction may result in an inhibitory effect on the neural signal triggered by oestrogen. This would involve the presence of oestrogen and androgen receptors in close proximity. Mapping studies have revealed a pattern of androgen-sensitive neurones (Sar & Stumpf, 1977) which overlaps to some extent with that of the oestrogen-sensitive neurones (Pfaff & Keiner, 1973) in areas rich in noradrenergic innervation (Grant & Stumpf, 1975). It is even possible that oestrogen and androgen receptors exist in the same neurones in some of these sites. The following model (fig. 19) might explain the mechanism of action of testosterone. Before 03:00h on proestrus,oestrogen binding at excitatory receptor sites has not reached a maximum and therefore testosterone-receptor binding is dominant, resulting in an inhibitory effect on LH release. After 03:00h, the stimulatory effect of oestrogen has reached its threshold and cannot be overcome. On oestrogen and progesterone administration, the number of oestrogen-receptor interactions increases, shifting the balance back towards

a) BEFORE 03:00h DAY P



b) AFTER 03:00h DAY P



c) PLUS OESTROGEN and PROGESTERONE

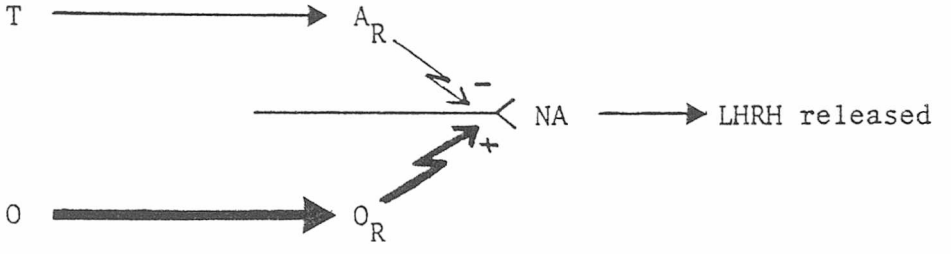


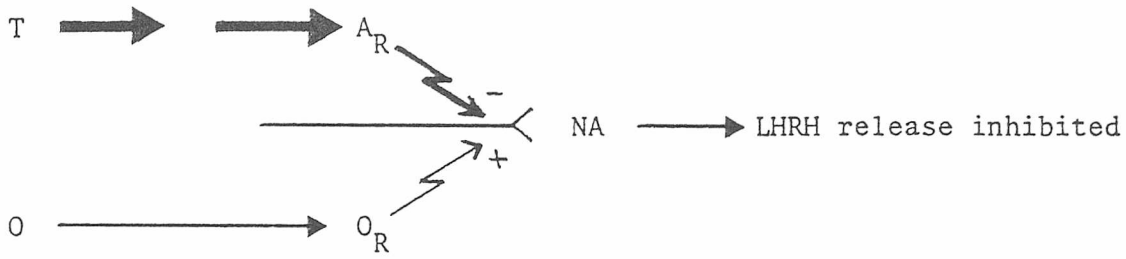
Fig. 19 Hypothetical model for the mechanism of antiovulatory action of testosterone when administered a) before 03:00h Day P b) after 03:00h Day P and c) before 03:00h Day P plus the subsequent addition of oestrogen and progesterone.
T = Testosterone, A_R = Hypothalamic Androgen Receptor, O = Oestrogen, O_R = Hypothalamic Oestrogen Receptor, NA = Noradrenaline.

oestrogen and with the facilitatory effect of progesterone, the excitatory effect on the neural signal becomes dominant again.

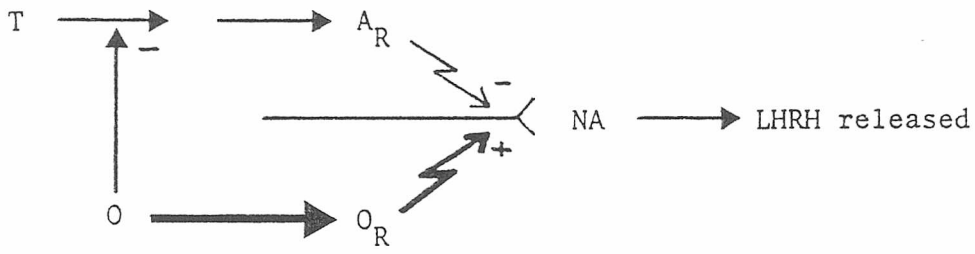
This third model may be extended to form the fourth model in which testosterone does not bind directly to androgen receptors but is firstly converted into dihydrotestosterone or 5α-androstan-3α,17β-diol. In a recent study, it was reported that this conversion is a crucial step for the negative feedback effect of testosterone on LH release (Martini et al, 1978). Furthermore, it was reported that oestrogen can modulate this effect by modifying the rates of conversion. This finding provides a more simplified mechanism of anti-ovulatory action (fig. 20). Testosterone is reduced to metabolites which bind to androgen receptors, producing an inhibitory effect on the neural signal. Before 03:00h on proestrus, the oestrogen levels are still rising and therefore do not interfere greatly with the rate of conversion. After 03:00h the levels of oestrogen have risen sufficiently to produce a substantial reduction in rate of testosterone conversion allowing the excitatory effect of oestrogen to become dominant again. On oestrogen administration the rate of conversion is reduced, lessening the inhibitory influence of testosterone over NA turnover. This effect combined with a facilitatory effect of progesterone is sufficient to allow oestrogen to trigger the neural signal. Therefore, based on the premise that RMI 12,936

MODEL 4

a) BEFORE 03:00h DAY P



b) AFTER 03:00h DAY P



c) PLUS OESTROGEN and PROGESTERONE

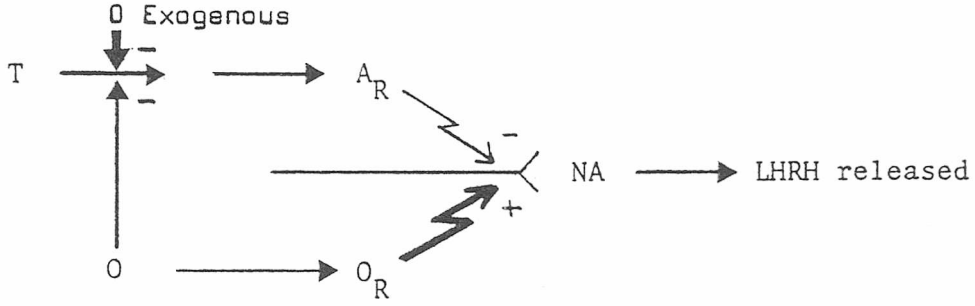


Fig. 20 Hypothetical model for the mechanism of antiovulatory action of testosterone when administered a) before 03:00h Day P b) after 03:00h Day P and c) before 03:00h Day P plus the subsequent addition of oestrogen and progesterone.

T = Testosterone, A_R = Hypothalamic Androgen Receptor,

O = Oestrogen, O_R = Hypothalamic Oestrogen Receptor,

NA = Noradrenaline

has the same mechanism of antiovulatory action as testosterone, it is proposed that it is reduced in the hypothalamus to metabolites which bind to androgen receptors and act as described in model 4.

It was previously concluded that the drug's antiovulatory activity was not dependent on its androgenic activity whereas this latter hypothetical model is based on the assumption that the antiovulatory agent possesses an affinity for androgen receptors. This conclusion was based on the peripheral androgenic activity of these drugs and not on their affinity for hypothalamic androgen receptors. However, the characteristics of hypothalamic androgen receptors may differ slightly from peripheral androgen receptors resulting in androgens having different affinities for each receptor type. This concept may explain the differences in relative potencies of various androgens with regard to their androgenic potency on the prostate and seminal vesicles compared to their negative feedback effect on LH release (Zanisi et al, 1973). To test the hypothesis that RMI 12,936 acts through interaction with an androgen receptor, an experiment could be designed to determine whether the inhibitory effect of the drug on LH release is reduced by the administration of flutamide, an androgen antagonist.

Although the similarities in antiovulatory activity between RMI 12,936 and testosterone suggest that they have the same mechanism of action, it is possible that they both prevent oestrogen from triggering the neural signal for LHRH release by different mechanisms. For example, RMI 12,936

may compete with oestrogen for oestrogen receptors, blocking the stimulatory effect of oestrogen. Such an action would result in the same antiovulatory characteristics. This mechanism of action is perhaps less likely but may initially be tested by determining the affinity of RMI 12,936 for oestrogen receptors.

B. Mechanism of action at the level of the adenohypophysis

RMI 12,936 also has an inhibitory effect on adenohypophysial sensitization. Although this was shown to be of minor importance to its antiovulatory action, the mechanism by which it prevents full sensitization was investigated (Chapter 2). The findings of this investigation suggested that the drug did not produce this effect by inhibiting oestrogen and progesterone sensitization of the adeno-hypophysis. Instead the data suggested that it acted by preventing the 2nd phase increase in sensitivity produced by LHRH self-priming. RMI 12,936 could achieve this through its antioestrogenic activity since a block to oestrogen stimulation of the neural trigger would result in reduced LHRH secretion. However this mechanism could not be the sole answer as administration of oestrogen and progesterone which might be expected to restore normal secretion of LHRH, failed to restore full adenohypophysial sensitivity. In addition, it was proposed that RMI 12,936 acted directly at the level of the adenohypophysis to reduce the number of LHRH receptors. This hypothetical mechanism of action arose from the premise that testosterone and RMI 12,936 have similar mechanisms of action (Chapter 7).

It was reported that testosterone reduces adenohypophysial sensitivity at least partly by reducing the number of LHRH receptors present in the tissue (Giguere et al, 1981). To test this hypothesis, the effect *in vitro* of RMI 12,936 on the number of LHRH receptors present in the adenohypophysis, should be determined. Additional support for this hypothesis comes from the finding that oestrogen has a different mechanism of action for modulating adenohypophysial sensitivity to LHRH (Drouin et al, 1976). Thus administration of oestrogen would not be expected to reverse the effect of these drugs. This result has been reported in the literature in the case of testosterone (Debeljuk et al, 1974; Drouin et al, 1976) and in this study in the case of RMI 12,936 (Chapter 2). It must be noted however that this reduced number of receptors in testosterone and RMI 12,936 treated rats remains sufficient for ovulation to occur in response to LHRH stimulation directly or indirectly through oestrogen and progesterone administration.

In conclusion, it has been found that RMI 12,936 is a potent antiovulatory agent which posseses antioestrogenic, anti-progestational and androgenic activity. The results indicate that the drug inhibits the preovulatory surge in plasma LH by blocking the neural signal induced by oestrogen for stimulation of LHRH release. It was proposed that the mechanism by which RMI 12,936 blocks the neural signal is a dual mechanism: a) it blocks the biosynthesis

of oestrogen and progesterone and b) the drug itself or a metabolite, binds to androgen receptors present in the hypothalamus, to produce an inhibitory effect on the noradrenergic pathway involved in the positive feedback effect of oestrogen on LH release. The precise site of action of the drug may lie within the preoptico-suprachiasmatic tuberoinfundibular regions.

Changes in the sensitivity of the adenohypophysis to LHRH were observed in this investigation (see Chapter 1). From experiments performed throughout the year a pattern emerged indicating that a seasonal variation in the mechanisms controlling the release of LH from the adeno-hypophysis exists in the female rat. Consequently, seasonal variation should be taken into consideration when interpreting the results of experiments concerned with the control of ovulation.

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APPENDIX I

Serum LH levels (ng/ml), means plus SEM, in rats bled at various times on Day P after treatment with 35mg / kg PB at 15:00h on Day P. Each row represents LH serum levels from individual rats.

1530h	1600h	1630h	1700h	1730h	1800h	1830h	1900h	
3.3		3.8	7.6	10.2	14.1	10.6	8.9	-
3.1		3.6	2.8	1.9	2.9	3.5	3.9	-
3.4		3.5	3.6	3.6	1.6	2.8	19.7	-
1.2	0.05	3.2	2.3	4.0	5.4	14.5	16.8	-
nd	µg	7.6	3.3	8.6	13.3	8.3	17.1	-
2.7	LHRH	2.3	3.9	7.1	3.4	11.6	2.6	-
9.9		8.3	16.6	15.3	8.1	8.9	1.6	-
3.7		12.2	14.6	15.2	10.9	3.9	1.9	-
2.2		15.6	13.5	1.4	11.8	3.8	2.4	-
2.4		20.7	15.9	11.3	3.5	8.8	2.5	-
3.2	MEAN	8.1	8.4	7.9	7.5	7.7	7.7	0/10
0.8	SEM	2.0	1.9	1.6	1.5	1.3	2.3	
13.7		76.8	110.2	129.5	69.2	31.8	13.4	+
14.7		39.9	71.3	98.5	72.1	38.9	14.9	+
2.9		17.6	11.4	15.5	25.8	6.2	3.5	-
3.3	0.10	10.5	3.7	20.5	16.2	3.6	13.9	-
3.3	µg	3.2	3.8	14.0	10.5	1.6	3.6	-
1.1	LHRH	2.7	3.9	1.5	1.4	3.3	1.7	-
3.6		16.0	1.2	10.2	2.4	15.3	2.3	-
2.3		12.4	3.0	8.8	2.6	3.2	3.1	-
2.8		2.5	13.2	3.5	14.1	2.0	1.6	-
3.3		2.0	2.2	2.9	2.7	11.7	1.9	-
5.1	MEAN	18.4	22.4	30.5	21.7	11.8	6.0	2/10
1.5	SEM	7.4	11.8	14.2	8.5	4.2	1.8	
3.9		7.7	29.2	128.2	92.8	29.3	11.6	+
6.4		21.4	94.5	107.9	52.7	24.2	15.2	+
5.5		24.0	140.6	144.0	125.2	56.4	20.2	+
3.3	0.15	16.5	126.1	150.2	100.0	6.3	16.3	+
6.0	µg	±3.7	53.0	77.8	10.1	10.0	7.0	-
1.8	LHRH	77.4	165.5	175.2	135.0	86.3	37.8	+
3.5		77.7	128.1	120.8	80.5	40.9	15.4	+
11.6		71.0	149.7	188.2	121.2	78.8	30.1	+
14.9		81.9	162.2	168.5	151.3	75.2	21.3	+
3.3		20.0	112.7	104.0	30.3	13.5	4.8	+
6.0	MEAN	41.1	116.2	136.2	89.9	42.1	18.0	9/10
1.3	SEM	9.9	14.4	11.0	14.7	9.6	3.2	
3.3		22.0	83.7	143.9	93.3	21.8	10.6	+
7.0	0.20	6.5	7.9	55.8	31.4	13.4	11.8	+
4.2	µg	6.5	74.6	129.5	83.1	21.8	13.0	+
5.0	LHRH	18.1	128.7	149.8	84.9	31.8	19.2	+
5.4		13.4	132.6	151.8	92.6	26.1	5.4	+
5.0	MEAN	13.3	85.5	125.8	77.1	23.0	12.0	5/5
0.6	SEM	3.1	22.6	18.4	11.6	3.0	2.2	

nd, the concentration of LH in the sample was undetectable.
+, presence of ova in ampullae; -, absence of ova in ampullae.

APPENDIX II

Serum LH levels (ng/ml), means plus SEM, in rats bled at various times on Day P after treatment with 2mg RMI 12,936 at 16:00h on Day D2. Each row represents the LH serum levels from individual rats.

1530h	1600h	1630h	1700h	1730h	1800h	1830h	1900h	
6.2		5.1	4.5	4.7	5.7	6.1	6.3	-
5.2	0.125µg	5.1	4.3	4.8	5.1	5.6	5.3	-
5.4		5.4	5.6	5.0	4.9	5.8	6.1	-
4.8	LHRH	4.8	5.1	5.3	4.2	5.6	5.4	-
4.7		5.3	4.3	5.6	5.3	5.4	5.9	-
5.3	MEAN	5.1	4.8	5.1	5.0	5.7	5.8	
0.3	SEM	0.1	0.3	0.2	0.2	0.1	0.2	0/5
n.d.		10.1	25.5	25.0	15.7	n.d.	1.5	+
n.d.	0.25µg	12.0	24.6	23.0	13.8	14.3	7.9	-
n.d.		6.0	13.2	4.8	4.1	9.7	5.1	-
n.d.	LHRH	4.6	18.5	8.5	6.3	n.d.	1.5	-
n.d.		2.5	5.5	n.d.	n.d.	5.0	2.7	-
6.3		6.0	2.5	4.2	2.5	4.4	3.2	-
3.7		3.8	23.5	6.2	3.7	6.2	5.9	-
1.4	MEAN	6.4	16.2	10.2	6.6	5.7	4.0	
1.0	SEM	1.3	3.5	3.7	2.2	1.9	0.9	1/7
n.d.		23.5	57.7	25.1	6.3	5.2	4.5	+
n.d.		16.8	25.3	23.4	9.2	4.6	n.d.	-
n.d.	0.5µg	18.2	27.4	26.7	6.3	4.8	n.d.	+
n.d.		19.0	20.9	12.0	4.2	15.3	4.1	-
n.d.	LHRH	18.1	29.1	13.0	7.2	4.2	2.0	-
6.2		5.0	2.3	2.1	5.2	18.0	5.9	-
5.9		5.2	5.4	5.6	5.9	22.1	5.4	-
1.7	MEAN	15.1	24.0	15.4	6.3	10.6	3.1	
1.1	SEM	2.7	6.9	3.7	0.6	2.9	0.9	2/7
n.d.		15.3	44.6	37.5	10.1	5.3	2.1	+
n.d.	1.0µg	18.1	10.4	7.9	n.d.	n.d.	n.d.	-
n.d.		23.4	95.6	50.5	n.d.	6.2	4.8	+
n.d.	LHRH	27.2	197.8	291.6	31.9	17.8	26.3	+
n.d.		28.0	59.0	29.2	11.8	5.8	5.8	+
	MEAN	22.4	81.5	83.3	10.8	7.0	7.8	
n.d.	SEM	2.5	32.1	52.5	5.8	2.9	4.7	4/5
1.9		91.9	205.0	175.1	105.7	30.2	13.0	+
3.2	2.0µg	44.2	163.2	216.4	95.4	28.1	14.7	+
5.3		19.5	105.7	171.1	91.9	27.3	9.1	+
n.d.	LHRH	50.5	183.8	234.3	97.9	33.7	23.5	+
3.2		88.7	188.9	195.8	107.8	47.4	16.6	+
2.7	MEAN	59.0	169.3	198.5	99.7	33.4	15.4	
0.9	SEM	13.8	17.2	12.1	3.0	3.7	2.4	5/5

n.d., the concentration of LH in the sample was undetectable.
+, presence of ova in ampulla; -, absence of ova in ampulla.

APPENDIX III

Serum LH levels (ng/ml), means plus SEM, in rats bled at various times on Day P after treatment with 2mg RMI 12,936 at 16:00h plus 2µg oestradiol at 17:00h on Day D2 plus 2mg progesterone at 13:00h plus 35mg/kg PB at 15:30h on Day P. Each row represents the LH serum levels from individual rats.

1500h	1600h	1630h	1700h	1730h	1800h	1830h	1900h	
5.6		4.2	4.7	4.0	5.4	4.8	7.4	-
6.0	0.25µg	4.3	4.5	3.6	5.6	6.7	6.4	-
5.2		6.8	5.5	7.2	6.4	7.3	6.1	-
5.6	LHRH	7.6	5.9	7.8	4.7	7.9	6.2	-
7.6		6.8	5.7	7.2	6.0	6.6	4.9	-
6.0	MEAN	5.9	5.3	6.0	5.6	6.7	6.2	
0.4	SEM	0.7	0.3	0.9	0.3	0.5	0.4	0/5
4.8		4.7	4.5	8.4	6.4	5.3	4.5	-
4.4	0.375µg	6.7	12.3	8.3	6.4	4.8	4.5	-
4.3		6.8	10.1	5.6	4.4	4.2	2.9	-
3.8	LHRH	5.2	9.4	4.2	4.3	3.1	4.2	-
3.1		23.7	100.7	69.5	19.1	2.7	3.1	+
4.1	MEAN	9.4	27.4	19.2	8.1	4.0	3.8	
0.3	SEM	3.6	18.4	12.6	2.8	0.5	0.3	1/5
6.0		13.7	53.0	77.9	10.1	10.0	7.0	+
3.4	0.5µg	6.0	7.5	8.6	14.6	8.0	5.1	-
3.6		13.9	39.0	51.4	15.2	5.2	5.6	+
4.4	LHRH	11.6	48.5	50.8	20.1	6.0	7.3	+
5.7		4.4	8.7	5.7	n.d.	n.d.	3.5	-
4.6	MEAN	9.9	31.3	38.9	12.0	5.8	5.7	
0.5	SEM	2.0	9.8	13.9	3.4	1.7	0.7	3/5
4.7		8.6	39.0	132.8	76.7	14.3	15.0	+
3.6	1.0µg	10.4	59.3	118.2	43.7	9.1	7.4	+
5.1		11.1	63.9	147.0	54.7	9.2	14.3	+
5.5	LHRH	10.3	16.0	55.7	4.8	9.5	7.1	+
5.9		13.0	135.6	175.7	136.9	50.2	33.1	+
5.0	MEAN	10.7	62.8	125.9	63.4	18.5	15.4	
0.4	SEM	0.7	20.1	20.0	21.8	8.0	4.7	5/5

n.d., the concentration of LH in the sample was undetectable.
+, presence of ova in ampulla; -, absence of ova in ampulla.

APPENDIX IV

Serum LH levels (ng/ml), means plus SEM, in rats bled at various times on Day D2. Each row represents the LH serum levels from individual rats.

1030h	1100h	1130h	1200h	1230h	1300h	1330h	1400h	
5.4		3.7	28.4	44.6	20.0	5.1	6.9	-
5.4	1.25µg	9.4	57.9	69.2	21.5	4.0	4.6	-
5.3		11.7	62.9	56.4	27.5	15.3	5.9	-
7.0	LHRH	6.4	10.9	7.3	7.1	4.7	4.7	-
4.6		16.8	80.7	99.5	33.1	16.1	6.3	-
5.5	MEAN	9.6	48.2	55.4	21.8	9.0	5.7	
0.4	SEM	2.3	12.5	15.1	4.4	2.7	0.4	0/5
n.d.		1.8	n.d.	45.7	24.5	n.d.	n.d.	-
n.d.	2.5µg	19.5	104.9	124.0	5.9	7.9	19.3	+
21.2		18.5	27.2	23.7	19.8	6.0	31.3	-
n.d.	LHRH	43.3	49.4	10.6	31.8	2.6	8.0	-
5.2		17.3	72.3	66.6	28.7	28.9	n.d	-
5.3	MEAN	20.1	50.8	54.1	22.1	9.1	11.7	
4.1	SEM	6.6	18.1	19.9	4.5	5.1	6.0	1/5
5.6		27.4	85.7	121.4	73.2	25.6	5.1	-
28.6	3.75µg	71.4	129.1	133.9	71.0	22.0	24.4	-
25.1		19.8	103.2	131.6	30.3	30.2	6.7	+
27.7	LHRH	21.1	120.3	125.6	62.0	20.1	n.d.	+
n.d.		26.7	123.5	151.6	118.4	43.1	31.6	+
17.4	MEAN	33.3	112.4	132.8	71.0	28.2	13.6	
6.1	SEM	9.6	7.9	5.2	14.1	4.1	6.1	3/5
n.d.		n.d.	30.0	130.3	109.7	114.7	16.1	+
2.0	5.0µg	3.1	42.2	143.1	118.6	68.4	25.4	+
n.d.		7.8	93.8	134.0	143.6	31.5	25.8	+
n.d.	LHRH	31.9	98.7	81.8	100.6	54.4	n.d.	-
n.d.		4.1	124.4	163.7	132.5	52.2	25.4	+
0.4	MEAN	9.4	77.8	130.6	121.0	64.2	18.5	
0.4	SEM	5.8	17.9	13.5	7.7	13.9	5.0	4/5
n.d.		7.3	129.9	175.6	119.5	81.5	47.0	+
n.d	6.25µg	17.9	99.3	169.8	116.2	25.9	28.6	+
n.d.		25.7	135.4	199.8	140.9	75.0	74.0	+
3.2	LHRH	19.1	134.2	169.1	145.6	54.2	25.5	+
6.2		7.7	107.9	144.9	81.6	22.8	29.8	+
n.d.		25.5	76.1	76.5	49.6	37.3	18.3	+
1.6	MEAN	17.2	113.8	156.0	108.9	49.5	37.2	
1.1	SEM	3.3	9.7	17.4	15.1	10.2	8.3	6/6

n.d., the concentration of LH in the sample was undetectable.
+, presence of ova in ampulla; -, absence of ova in ampulla.

APPENDIX V

Serum LH values (ng/ml) in rats bled at various times on Day P after treatment with 35mg/kg PB at 15:00h on Day P plus the mean of LH values pre- LHRH injection and the mean of maximum LH values in each rat post- LHRH injection. Each row represents LH serum levels from individual rats.

1530h	1600h	1630h	1700h	1730h	1800h	1830	1900h
0.40		0.37	0.29	1.61	0.61	0.43	0.77
0.32	0.01µg	0.90	1.38	0.27	0.20	1.41	0.60
0.15	LHRH	0.09	0.61	0.46	0.20	0.80	0.50
0.04		0.50	0.83	0.31	0.51	0.26	0.66
n.d.		0.08	2.68	0.75	0.44	0.44	0.30
The mean ± sem of pre- LHRH injection LH values is 0.19 ± 0.07							
The mean maximum ± sem of post- LHRH injection LH values is 1.47 ± 0.34							
0.92		0.23	0.81	1.21	1.97	0.96	0.47
0.73	0.10µg	0.77	0.73	0.26	0.92	0.94	0.05
0.61	LHRH	0.94	0.85	0.71	0.81	0.85	1.07
0.62		0.25	0.80	0.01	1.18	2.82	0.50
0.77		0.90	0.09	0.34	0.81	0.66	0.60
The mean ± sem of pre- LHRH injection LH values is 0.73 ± 0.06							
The mean maximum ± sem of post- LHRH injection LH values is 1.52 ± 0.38							
0.03		0.89	0.70	0.26	0.50	1.01	0.29
0.03	1.00µg	n.d.	0.28	0.05	0.10	0.29	1.38
0.71	LHRH	0.47	0.61	1.21	1.21	0.45	0.34
0.85		0.45	0.94	0.83	0.06	0.66	0.61
0.80		0.34	0.64	1.04	0.70	0.60	0.08
The mean ± sem of pre- LHRH injection LH values is 0.48 ± 0.19							
The mean maximum ± sem of post- LHRH injection LH values is 1.12 ± 0.08							
0.92		0.03	0.03	0.17	0.75	0.07	0.20
1.01	5.00µg	0.71	0.89	0.92	0.40	1.61	0.92
n.d.	LHRH	0.70	1.45	0.67	0.94	0.29	0.73
0.90		0.94	0.92	0.87	0.48	0.60	0.44
0.61		0.16	0.22	n.d.	0.47	0.32	0.37
The mean ± sem of pre- LHRH injection LH values is 0.69 ± 0.18							
The mean maximum ± sem of post- LHRH injection LH values is 1.04 ± 0.21							

n.d., the concentration of LH in the sample was undetectable.

APPENDIX VI

Serum LH values (ng/ml) in rats bled at various times on Day P after treatment with 2mg RMI 12,936 at 16:00h on Day D2 plus the mean of LH values pre- LHRH injection and the mean of maximum LH values in each rat post- LHRH injection. Each row represents LH serum levels from individual rats.

1530h	1600h	1630h	1700h	1730h	1800h	1830h	1900h
n.d.		1.43	n.d.	n.d.	1.60	1.51	1.17
1.14	0.01µg	1.24	1.01	n.d.	1.65	n.d.	n.d.
1.39	LHRH	0.47	1.04	1.51	1.79	0.29	0.25
0.41		0.81	n.d.	n.d.	1.56	1.43	1.17
0.24		n.d.	1.56	0.37	0.99	0.22	1.17
The mean ± sem of pre- LHRH injection LH values is 0.64 ± 0.26							
The mean maximum ± sem of post- LHRH injection LH values is 1.63 ± 0.04							
0.57		0.14	0.51	0.96	0.79	1.56	1.79
1.31	0.1µg	1.47	0.99	1.07	n.d.	n.d.	0.30
1.47	LHRH	n.d.	0.28	1.43	1.56	0.35	n.d.
n.d.		1.51	n.d.	1.43	n.d.	1.43	n.d.
n.d.		n.d.	1.47	n.d.	1.24	0.26	1.31
The mean ± sem of pre- LHRH injection LH values is 0.68 ± 0.09							
The mean maximum ± sem of post- LHRH injection LH values is 1.56 ± 0.06							
2.91		1.14	0.83	1.04	0.55	n.d.	n.d.
1.07	1.0µg	1.14	1.39	2.19	0.53	1.39	n.d.
1.56	LHRH	0.06	1.69	0.24	2.19	0.66	2.07
0.66		1.04	1.27	1.69	2.13	0.36	1.27
1.90		0.86	1.47	n.d.	1.65	n.d.	2.01
The mean ± sem of pre- LHRH injection LH values is 1.62 ± 0.38							
The mean maximum ± sem of post- LHRH injection LH values is 1.93 ± 0.20							
1.56		1.43	n.d.	2.01	1.31	1.43	2.53
1.47	5.0µg	-	1.24	1.04	0.65	n.d.	1.47
n.d.	LHRH	0.74	1.56	-	0.31	1.47	1.06
1.24		1.43	1.31	0.96	1.47	2.07	1.47
n.d.		2.07	1.01	1.14	1.31	1.11	0.33
The mean ± sem of pre- LHRH injection LH values is 0.87 ± 0.34							
The mean maximum ± sem of post- LHRH injection LH values is 1.94 ± 0.19							

n.d., the concentration of LH in the sample was undetectable.

APPENDIX VII

A. As partial fulfilment for the degree of PhD, the following programme of study was undertaken:

1) Attendance at the B.Sc (Pharmacy) course in statistics and computing.
2) Attendance at the Institute course in basic programming.
3) Attendance at the B.Sc (Hons) Pharmacy course in reproductive physiology and pharmacology.
4) A course of guided reading in experimental design and statistics with tutorials by a statistician.
5) A course of guided reading on reproductive pharmacology with tutorials with the director of studies.

B. Additional advanced study included extensive reading of relevant research literature and attendance at and participation in meetings of the Society for the Study of Fertility.

C. Seminar : In the School of Pharmacy, RGIT, June 1981.
'The effect of RMI 12,936 on hypothalamic neurotransmitter activity in the rat.'

Communication : Summer meeting of the Society for the Study of Fertility, July 1981, Edinburgh.
'The effect of RMI 12,936 on hypothalamic neurotransmitter activity in the rat.'