OPIATES AND MONOAMINERGIC REGULATION OF LH RELEASE IN THE RAT

A thesis submitted to the University of Glasgow
in Candidature for the degree of

DOCTOR OF PHILOSOPHY

in the Faculty of Medicine

by

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from

The Institute of Physiology

The University, Glasgow.

November, 1988.

DECLARATION

I hereby declare that this thesis embodies the results of my own special work, that was carried out in the Reproductive Physiology Laboratory within the Institute of Physiology, The University of Glasgow, between November 1985 and October 1988.

This thesis does not include work forming part of a thesis presented successfully for a degree in this or another University.

A C K N O W L E D G E M E N T S

It is a great pleasure to express my deep gratitude to Dr. Des Gilmore for his guidance throughout the course of this study. My special thanks for his patience and constructive criticism in the preparation of this thesis.

I am very grateful to Professors Otto Hutter and Sheila Jennett for allowing me to work in the Institute and also for their recommendation for the Overseas Research Scholarship and University Scholarship that provided much appreciated financial support over the last three years.

My appreciation is also extended to Drs. Anthony Payne and Robin Orchardson for giving up their valuable time to read and pass judgement on this thesis.

For their assistance in completing parts of this work, I am thankful to Dominic Meek, Colin Brown, Ian Currie and Michael Bennett.

Much needed and greatly appreciated statistical advice was provided by Drs. Jay Rosenberg, Victor Moss, Frances Burton and especially Dr. A.M. Amjad.

I am also grateful to Sheila Byars, Robert Campbell, Alec Smith, Jim McGuire, Marion Kusel, Hilda Robison, Brenda Morris, Marjorie Wright, Marjorie Bilsborough, Peggy McCrossan and Sadie for their generous help at all times. My thanks are extended to Heather Johnston and Sheila Byars for "being there" with a helpful word of support when all else failed.

My thanks to Frank Fleming and all the staff of the Animal House for the speedy procurement and tender care of the animals.

Medical Illustrations also receive my appreciation for their preparation of the posters and slides used at the meetings at which parts of this work were communicated. I am also thankful to the Society for the Study of Fertility for providing the funds to attend these meetings.

I am indebted to Dr. Catherine Wilson and Amanda Leigh for providing the facilities for, and help in carrying out, the LH radioimmunoassay at St.George's Hospital Medical School, London.

I am also grateful to Roche Laboratories Inc., Neely, N.J. for the gift of levorphanol, the National Institution on Drug Abuse for the gift of SKF 10,047, Sterling-Winthrop Corporation, Guildford, Surrey for the gifts of cyclazocine and ketocyclazocine and Kali-Chemie, Hanover, West Germany for the gift of tifluadom.

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OPIATES AND HYPOTHALAMIC MONOAMINERGIC REGULATION OF LH RELEASE IN THE RAT

S U M M A R Y

Monoaminergic regulation of LH release appears to be influenced by the endogenous opioid peptides. Specific opioid receptors exist within the central nervous system and are richly concentrated within the hypothalamus. The present study was undertaken to investigate the effects of specific opiate agonists on hypothalamic monoaminergic activity.

In the first set of experiments, various opioid receptor agonists or their antagonist, naloxone, were administered intracerebroventricularly (icv) to short-term orchidectomized rats and blood samples were collected at pre-determined intervals. The animals were decapitated either at 20 minutes or at two-hours post-treatment, and the hypothalamus was surgically isolated.

In the second set of experiments, rats were treated with specific opioid receptor agonists, or their antagonist, during the early afternoon of pro-oestrus prior to the preovulatory LH surge. The animals were decapitated after an interval of two hours and the brains removed. Trunk blood was collected for LH measurement by radioimmunoassay. In one group of animals, the whole hypothalamus was dissected out; in another group, specific hypothalamic regions of the hypothalamus were isolated by a micropunch technique. Homogenates of either the whole or of specific hypothalamic

regions were prepared and concentrations of noradrenaline (NA), adrenaline (ADR), dopamine (DA), serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were measured using high performance liquid chromatography coupled to an electrochemical detector. NA and DA turnover rates were estimated by the blockade of the synthesis of these two amines using the enzyme-blocker alpha-methyl-para - tyrosine. 5-HT turnover was measured by obtaining the ratio between 5-HT and 5-HIAA.

The results are detailed below:

Table 1. Effects of opiates on plasma LH and the hypothalamic monoamine turnover in short-term orchidectomized rats.

Drugs	Receptor Properties	LH	Hypothalamic Turnover NA/DA/5-HT (15-25) / (5-9)
Naloxone	opiate antagonist	0	0/0/0
Morphine	mu-agonist	-	-/+/0
Cyclazocine	kappa agonist	-	-/0/-
SKF 10,047	sigma agonist	0	0/+/0

+ Increase, - Decrease, 0 No effect.

'n' Number of observations.

It was observed that the opiates, in general, caused a decrease in NA and 5-HT turnover and an increase in DA turnover in the hypothalamus of the short-term orchidectomized rat. The changes in hypothalamic DA turnover were not consistently associated with changes in LH levels.

Table 2. Effects of opiates on plasma LH and monoamine turnover in the whole or specific regions of the hypothalamus in pro-oestrous rats.

Drugs	LH	W.H.			SCH			MPO			AHA			ME			ARN			VMH		
		NA	DA	5-HT	NA	DA	5-HT	NA	DA	5-HT	NA	DA	5-HT	NA	DA	5-HT	NA	DA	5-HT	NA	DA	5-HT
Naloxone	+	+	0	0	0	0	0	+	+	0	0	0	0	0	+	0	0	+	0	0	0	0
Morphine	−	0	+	0	−	0	0	−	0	0	−	0	0	0	0	−	0	+	0	−	0	−
Tifluadom (κ-agonist)	−	+	0	−	−	0	−	0	+	0	0	0	−	0	0	0	0	0	0	−	+	−
SKF10,047	0	0	0	0	−	0	−	−	+	0	0	0	−	0	0	−	−	+	0	0	+	0

+ Increase, − Decrease, 0 No effect. (W.H.− Whole Hypothalamus)
(Number of observations, 5–24 animals in each group).

In the hypothalamic regions studied, opiate agonists either decreased or had no effect on NA turnover whereas the antagonist naloxone had the opposite effect. Surprisingly, DA turnover was increased by both the opiates and by their antagonist, naloxone. 5-HT turnover was significantly decreased by the opiates in most of the regions studied, but was unaffected by naloxone.

In conclusion, there exists a heterogenous group of opioid receptors within the hypothalamus which modulate monoamine neurotransmitters controlling LH release.

A B B R E V I A T I O N S

ADR - Adrenaline

AHA - Anterior Hypothalamic Area

ARN - Arcuate Nucleus

CNS - Central Nervous System

COMT - Catechol-O-methyltransferase

cAMP - cyclic Adenosine monophosphate

DA - Dopamine

DBH - Dopamine-β-hydroxylase

DMN - Dorsomedial Nuclues

DOPA - Dihydroxyphenylalanine

DOPAC - Dihydroxyphenylacetic Acid

EOP - Endogenous Opioid Peptide

FSH - Follicle Stimulating Hormone

GnRH - Gonadotrophin Releasing Hormone

5-HIAA- 5-Hydroxyindoleacetic Acid

5-HT - 5-Hydroxytryptamine (serotonin)

5-HTP - 5-Hydroxytryptophan

icv - intracerebroventricular

ip - intraperitoneal

LH - Luteinizing Hormone

LHRH - Luteinizing Hormone Releasing Hormone

MAO - Monoamine Oxidase

ME - Median Eminence

MHPG - 3-Methoxy, 4-hydroxyphenylglycol

MPO - Medial Preoptic Area

α-MPT - α-Methyl-p-tyrosine

NA - Noradrenaline

PAN - Paraventricular Nucleus

PNMT - Phenylethanolamine-N-methyltransferase

POA - Preoptic Area

PVN - Periventricular Nucleus

RIA - Radioimmunoassay

sc - subcutaneous

SCH - Suprachiasmatic Nucleus

SON - Supraoptic Nucleus

TRH - Thyrotrophin Releasing Hormone

VMH - Ventromedial Hypothalamus

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INTRODUCTION

The Central Control of Reproductive Processes

The understanding of the neuroendocrine mechanisms that control reproduction is complicated because of the involvement of several different neuronal systems within the brain. An attempt has been made here to briefly review the current understanding of the neuronal mechanisms involved in the release of LH. This includes the participation of the opioid, monoaminergic and gonadotrophin-releasing hormone (GnRH) neuronal systems and their interrelationship in controlling LH secretion. Before studying the actual involvement of these systems in the neuroendocrine regulation of LH, the anatomy of the hypothalamus and ventricular system of the brain will be briefly considered.

The Hypothalamus and Neuroendocrine Regulation

It has become increasingly evident that a major component of endocrine regulation lies within the brain, principally in the hypothalamus which has an immense anatomical and functional specialization (see Figure 1).

Hypothalamic neurones are formed from the ventral portion of the embryonic diencephalon. Once formed in the subventricular proliferative zone, hypothalamic neurones migrate to their final position and differentiate to form the nuclei of the adult hypothalamus. The hypothalamus develops ventrally to the dorsal thalamic and ventral thalamic derivatives of the diencephalon. It forms the lateral walls of the ventral part of the third ventricle. Rostrally, it is bounded by the lamina terminalis.

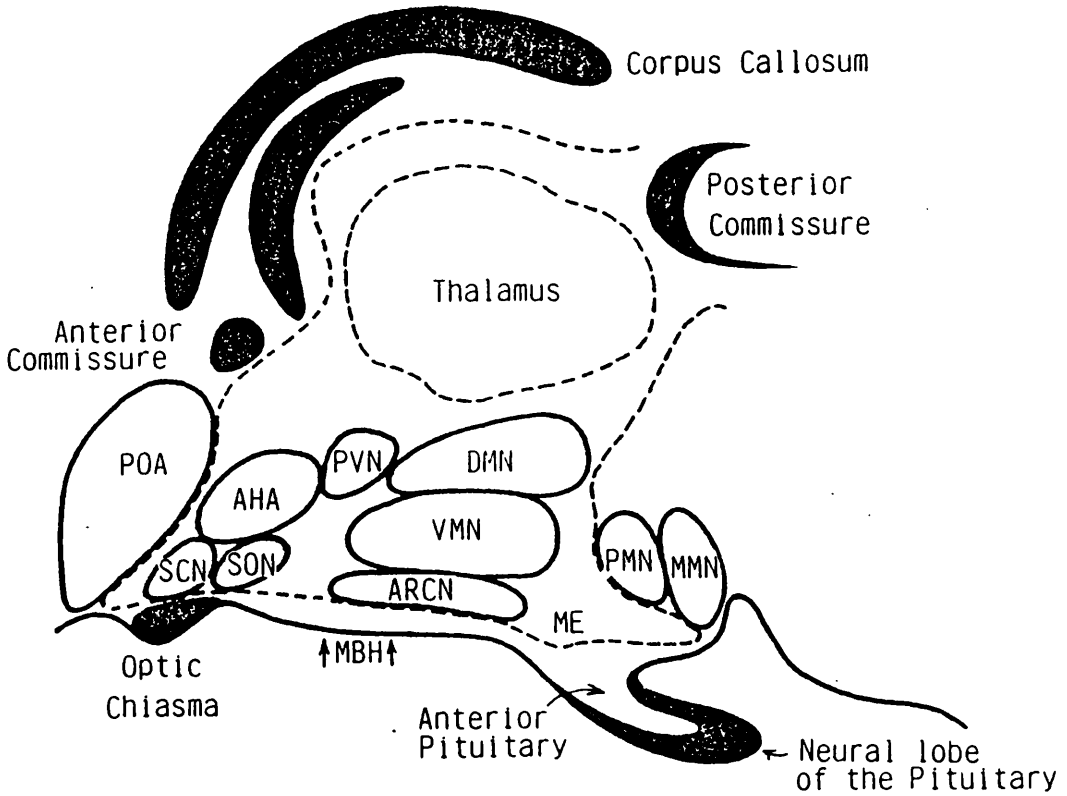


Fig. 1 Schematic diagram of the rat hypothalamus.
AHA: anterior hypothalamic area; ARCN: arcuate nucleus;
DMN: dorsomedial nucleus; ME: median eminence;
MMN: medial mammillary nucleus; PMN: premammillary nucleus;
POA: preoptic area; PVN: paraventricular nucleus;
SCN: suprachiasmatic nucleus; SON: supraoptic nucleus;
VMN: ventromedial nucleus.

By anatomical structure, the hypothalamus is split into two major subdivisions: a medial area and a lateral area (Raisman & Field, 1971). The medial area is predominantly nuclear in position. It is divided into three major groups:
a) *a rostral or anterior group of nuclei* which includes the medial preoptic nucleus (MPO), the anterior hypothalamic nucleus (AHA), the suprachiasmatic nucleus (SCH), the supraoptic nucleus (SON) and the paraventricular nucleus (PAN);
b) *a tuberal group of nuclei*, so named because of its association with the tuber cinereum (infundibulum), which consists of two very prominent nuclei: the ventromedial nucleus (VMH) and the dorsomedial nucleus (DMN). It also includes a much expanded component of the periventricular nucleus (PVN) and the arcuate nucleus (ARN). The ARN lies just above the median eminence (ME) and is adjacent to the third ventricle. There is also a large group of cells below and lateral to the VMH that has been termed the ventral tuberal area. This region contains clusters of large neurones, termed the lateral tuberal nuclei. The tuberal area is generally believed to be the region containing most of the neurones producing the various hypothalamic hormones. Caudal to the tuberal group of neurones lie;
c) *the posterior hypothalamic group of nuclei* containing the mammillary complex, the posterior hypothalamic nucleus, the supramammillary nucleus, and the tuberomammillary nucleus. In addition to these subdivisions, there is a narrow group of nerve cells lying just beneath the third ventricular ependyma, extending the entire length of the third ventricle.

These neurones are termed the periventricular system of the hypothalamus.

The lateral hypothalamic area is that region which forms the lateral border of the entire medial hypothalamic nuclear complex. The neurones of this area are scattered amongst axons of a large fibre bundle, the medial forebrain bundle. This is the major pathway by which all medial hypothalamic nuclei are interconnected with the rest of the brain.

The anatomical loci in the hypothalamus that exert a direct control of the adenohypophyseal function are collectively known as the "hypophysiotrophic area". The neurones of this area, referred to as tuberohypophyseal neurones, are composed of a cluster of cell bodies in anatomically defined nuclei which have origins as remote as the preoptic area (POA). The neurones of the hypophysiotrophic area produce a variety of peptide hormones. Some of these neurones terminate on capillary loops in the ME from where the neurosecretory products (neurohormones) are released into the portal circulation.

To date, five hypophysiotrophic hormones have been isolated: thyrotrophin releasing hormone (TRH), GnRH, somatostatin, corticotrophin releasing hormone and growth hormone releasing hormone.

The Median Eminence - Hypophyseal Portal System

The ME is the final common pathway for neurohumoral control of the anterior pituitary. It receives peptidergic

neurones of the tuberohypophyseal tract and their neuropeptides, the releasing and release-inhibiting hormones. From the substance of the ME, these hypophysiotrophic hormones are delivered to the portal capillaries (Jennes & Stumpf, 1986). The endothelium of these capillary loops is fenestrated, thus permitting macromolecules to enter without a functional blood brain barrier.

The ME contains specialized cells, the tanycytes, which extend from the lumen of the third ventricle to the outermost zone of the ME. Tanycytes may thus serve an important role in active transport in either direction between the ventricular compartment and the portal system.

In the portal blood, all of the known hypothalamic peptides and some biogenic amines are present in high concentrations. Tuberoinfundibular DA is secreted into the portal circulation via the ME. ADR and 5-HIAA, a 5-HT metabolite, are also present in the portal blood. It is suggested that biogenic amines, in addition to serving as neurotransmitters, may have a regulatory role on anterior pituitary function (Johnston, Gibbs & Negro-Vilar, 1983).

The Ventricular System of the Brain

The primitive ectoderm, which evolves into neural plate folds and the dorsal edges fuse to form the neural tube. The cerebral ventricles are developed from the lumen of this tube. At this stage, the nervous system is composed of a cylinder of cells surrounding a central cavity. The complex structure of the adult central nervous system is only a

series of permutations on this basic organization. These permutations occur largely as a consequence of differential rates of cellular proliferation along the neural tube and the growth of neuronal processes. With the growth of the brain, three primary and then five secondary brain vesicles are formed. The secondary vesicles are telencephalon, diencephalon, mesencephalon, pons and medulla which are the major subdivisions of the adult brain. The neural tube cavity persists in these as the lateral ventricles in the telencephalon, the third ventricle in the diencephalon, the cerebral aqueduct in the mesencephalon, and the fourth ventricle in the pons and the medulla (Figure 2). Cerebrospinal fluid (CSF) is formed by an active secretory process of the choroid plexus which is found in the lateral, third and fourth ventricles. Some interchange of water and solutes occurs between brain and CSF. The CSF formed in the ventricular system passes through it to emerge from the fourth ventricle. It circulates through the subarachnoid space and is removed over the convexity of the cerebral hemisphere from the arachnoid villi projecting into large dural venus sinuses.

The entire surface of the ventricular system is covered by a lining of modified neuroglial cells called ependyma. These cells are similar in all areas of the ventricular system except at the bottom of the third ventricle, where the specialized ependymal cells, the tanycytes, are found.

The third ventricle extends from the region of the optic chiasma rostrally (optic recess) to the mammillary bodies and

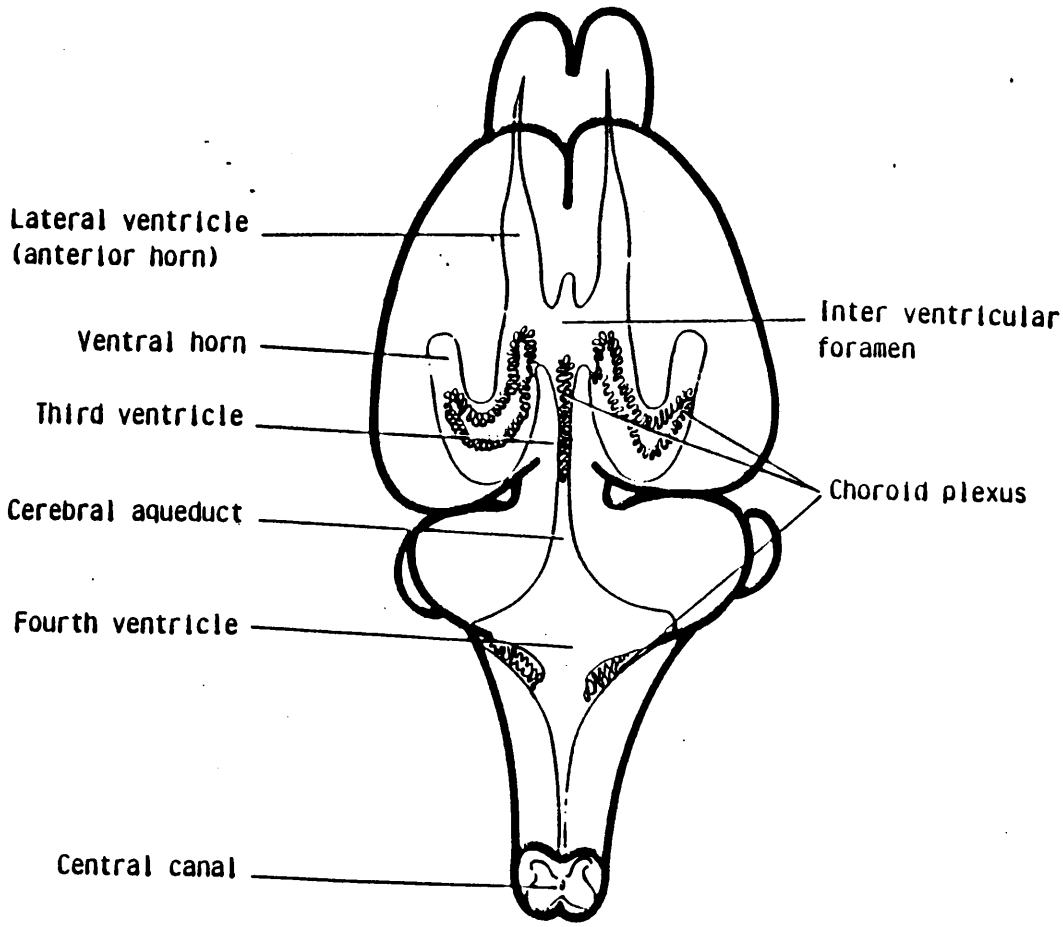


Fig.2 Showing the ventricular system of the rat brain (adapted from Zeman & Innes, 1963).

the cerebral aqueduct caudally. In addition to the optic recess, there is an infundibular recess - a ventral extension of the ventricle above the cerebral aqueduct. The walls of the third ventricle are made up predominantly of the thalamic and hypothalamic nuclei.

In the rat brain, it has been found that the average volume of the lateral ventricles is 43 µl, the third ventricle 38 µl and the fourth ventricle 10 µl (Levanger, 1971).

The Rat Oestrous Cycle

Rats are spontaneous ovulators and under controlled conditions ovulate every 4 to 5 days (see Feder, 1981). The oestrous cycle is brought about by a neural timing mechanism operating through the ME (Everett, 1977). It consists of a day of pro-oestrus followed by oestrus and then 2 or 3 days of dioestrus. These stages can be characterized by the cell types present in the vaginal epithelium which may be assessed by the taking of vaginal smears. Hormonal changes occurring at different stages of the cycle are summarized in the Figure 3.

During the period of late dioestrus the follicles grow and secrete oestradiol. The increased levels of this hormone in plasma reaches a peak on the morning of pro-oestrus. The high concentrations of oestradiol exerts a positive feedback effect and initiate an increase in gonadotrophin release during the afternoon of pro-oestrus. This increase is called the 'preovulatory gonadotrophin surge'. The high levels of

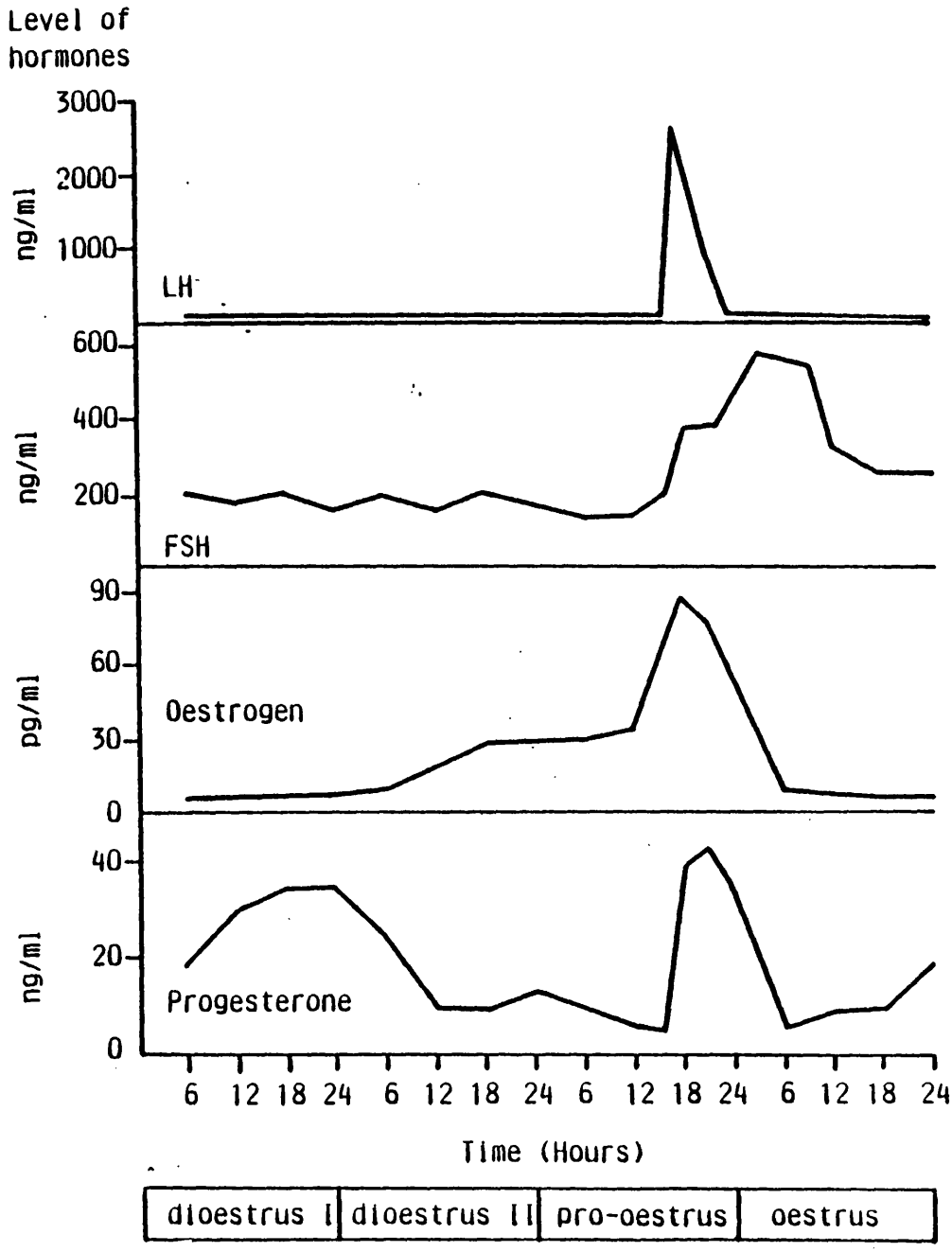


Fig. 3 Showing changes in plasma hormone levels during the 4-day Oestrous cycle in the rat (adapted from Takeo, 1984).

LH then inhibit further production of oestradiol, and at the same time stimulate an increase in progesterone secretion. The high levels of oestradiol are followed by a rise in progesterone levels which exert a positive feedback action on gonadotrophin release inducing and maintaining the preovulatory gonadotrophin surge. This in turn induces the rupture of mature follicles and thus ovulation. This stage is identified as oestrus. The ruptured follicles develop into secretory tissue (corpora lutea) capable of producing high levels of progesterone. In the normal cycle, the life span of the corpus luteum is only one day and can be seen by the transient rise in progesterone of luteal origin occurring on dioestrus I. The progesterone from the corpus luteum, and the low levels of oestradiol from the maturing follicles on dioestrus II, exert a negative feedback effect on GnRH levels during this period.

Oestrous cyclicity is under the regulation of the brain and in particular the hypothalamus (see Ramirez, Feder & Sawyer, 1984). During the afternoon of pro-oestrus, the anterior pituitary is stimulated by GnRH, which is transported to it via the hypothalamo-hypophyseal portal system, provoking discharge of the ovulatory quota of LH (see Ramirez, et al., 1984; Barr & Barraclough, 1978; Wise, Rance & Selmanoff, 1981). As oestradiol attains maximum plasma concentrations in the morning of pro-oestrus, the release of GnRH increases in amplitude and frequency (1 pulse/50 minutes at the pro-oestrous afternoon maximum). More detailed study of the GnRH stimulus that precedes release of surge levels of LH in conscious, unrestrained rats by use of the push-pull

perfusion technique, reveals that the neural GnRH apparatus is activated from a low level of GnRH release (10 fg/min) to a maximal secretory activity (50-70 fg/min) on the afternoon of pro-oestrus. There are intermediate levels of activity during dioestrus-I and dioestrus-II (Levine & Ramirez, 1980). In the afternoon of pro-oestrus, an initial increase in GnRH activity is observed around 14.00 to 15.00 hours. A much larger and longer burst in GnRH release occurring 2 to 3 hours later appears to coincide with the maximal secretion of LH from the pituitary. When the regimen of illumination is regulated, with lights on from 5.00 to 19.00 hours, the range of time during which the onset of the surge occurs is between 14.00 and 15.00 hours. Initially there is a slow rise in circulating LH levels to reach a plateau between 16.30 and 18.30 hours (Blake, 1976).

Two distinct modes of gonadotrophin release are observed during the oestrous cycle. Basal or episodic gonadotrophin release is reflected in the relatively low levels seen throughout most of the cycle and is essential for follicular development and oestradiol secretion. The frequency and amplitude of LH (Leipheimer, Bono-Gallo & Gallo, 1985) and follicle stimulating homone (FSH) (Culler & Negro-Vilar, 1987) pulses are dependent upon the time of day and the phase of oestrous cycle. The phasic pattern of the pre-ovulatory surge of LH leads to ovulation and the formation of the corpora lutea (see Barraclough & Wise, 1982; Feder, 1981).

Gonadotrophin - Releasing Hormone

The elucidation of the structure and the subsequent synthesis of hypothalamic GnRH, a decapeptide, by Schally and coworkers (1971) represented a milestone in the understanding of reproductive physiology. It is now established that the hypothalamus, particularly the ARN-ME region, produces and secretes GnRH, also known as luteinizing hormone-releasing hormone (LHRH). In the ME GnRH nerve terminals secrete the hormone directly into the portal capillaries, from which it is delivered by the portal circulation to the adenohypophyseal gonadotrophs.

The distribution of GnRH-producing neurones in the rat brain has been extensively investigated (Palkovits, 1973; Palkovits et al., 1974; see Figure 4). It seems that those GnRH neurones which project into the ME to exert a neurohumoral role are localized rostrocaudally from the diagonal band of Broca to the premammillary region in the pre-optic-tuberal pathway. Laterally they extend up to 2 mm from the midline in the preoptic region and the medial forebrain bundle (Kelly, Ronnekleiv & Eskay, 1982; King et al., 1982; Witkin, Paden & Silverman, 1982). A very high concentration of GnRH has been found in the ME; moderate levels have been detected in the ARN and small amounts in the SCH and preoptic regions (Palkovits, 1973; Palkovits et al., 1974). In the POA, the bulk of GnRH seems to reside in the supraoptic crest, a vascular structure that forms part of the rostral tip of the third ventricle (Kizer et al., 1976). The supraoptic crest and the other circumventricular organs have

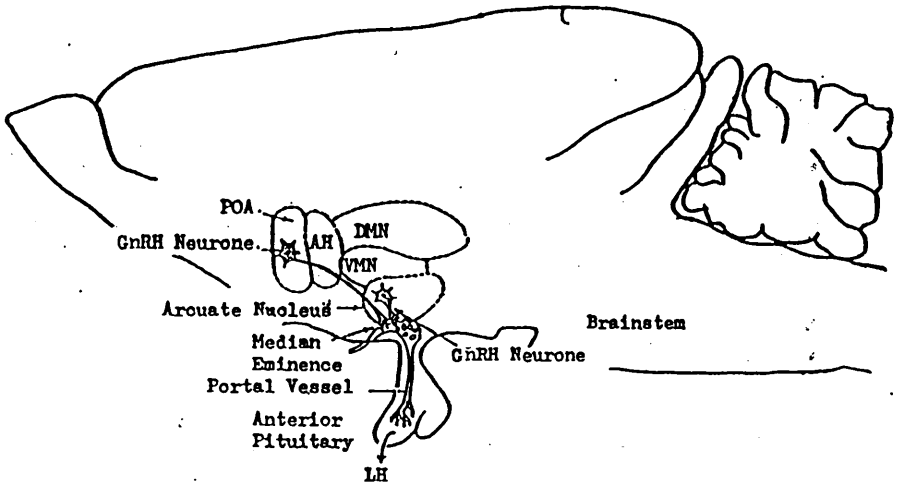


Figure 4. Anatomical distribution of GnRH neurones. POA, Preoptic Area; AH, Anterior Hypothalamus; DMN, Dorsomedial Nucleus; VMN, Ventromedial Nucleus.

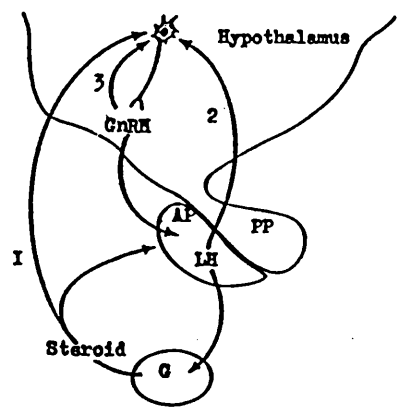


Figure 5. Humoral control of hormonal secretion. 1. Long Loop; 2. Short Loop and 3. Ultra-Short Loop. AP, Anterior Pituitary; PP-Posterior Pituitary; G-Gonad.

been found to be rich in GnRH which may be taken up from the CSF and disposed of, or secreted into the CSF to act on diverse areas of the brain. Shivers et al., (1983) reported that the major group of rat GnRH neurones occur in the POA and its vicinity (nucleus of the diagonal band, septum, AHA). The majority of GnRH fibres in the rat proceed from the POA to the ME. Most GnRH fibres originating in preoptic cells take a lateral course upon leaving that area, and travel along the medial forebrain bundle; then they turn medial to enter the MBH through the lateral retrochiasmatic area. Evidence gathered from lesion and hypothalamic deafferentation studies suggest that GnRH perikarya in the region of the retrochiasmatic area and elsewhere in the MBH are involved in the control of episodic LH release. Hence this region has been termed the "tonic pulse generating centre". Whereas GnRH neurones within a distinct narrow medial zone from the medial PVN caudally to the SCH region are thought to regulate the preovulatory gonadotrophin surge, hence termed the "surge generator region" (see Barraclough & Wise, 1982; Kalra & Kalra, 1983, 1985).

Hypothalamic GnRH binds to specific receptors on the adenohypophyseal gonadotroph and stimulates the synthesis and secretion of both LH and FSH (Conne et al., 1982). This secretory activity occurs intermittently in apparent synchrony with, and in response to, the pulsatile discharge of GnRH from the hypothalamus.Experimental data indicate that GnRH receptors on the gonadotroph change markedly under different physiological conditions. GnRH receptor numbers are highest when gonadotrophin responses to GnRH are maximal (Clayton & Catt, 1981). In the absence of steroid hormone feedback, e.g.,

after castration,the rise in GnRH numbers is induced by increased GnRH secretion. At the level of the pituitary, gonadal steroids exert their negative feedback effect at a post-GnRH receptor site rather than by directly altering the number of GnRH receptors (Clayton & Catt, 1981).

Release of both FSH and LH is stimulated by the hypothalamic peptide, GnRH. Administration of antiserum to GnRH or sodium pentobarbitone will abolish LH release but have no effect upon FSH secretion (Culler & Negro-Vilar, 1987; Berardo & De Paolo, 1986; see Everett, 1977). In addition, their release is reported to be dissociated in a number of physiological situations such as on the morning of oestrus (Smith, Freeman & Neill, 1975) and for the first few days post-ovariectomy (Berardo & De Paolo, 1986) when FSH is elevated but LH levels are basal. The release of LH seems to be completely dependent upon GnRH secretion, but GnRH is not the sole regulator of FSH secretion. McCann and his colleagues suggest that there is a separate FSH-releasing hormone that is produced in the PAN, AHA and surrounding ARN and ME to selectively regulate FSH release. Although the proposed FSH-RH has not yet been identified, there is some evidence for its existence (Lumpkins, De Paolo & Negro-Vilar, 1986).

Gonadal Steroid Hormones

Circulating gonadal steroid hormones exert a powerful influence on the regulation of reproduction by the brain. Gonadal steroids are apparently the primary hormonal signals

that drive and sustain the intricate balance within the closed feedback loop of the hypothalamo-pituitary-gonadal axis.

Feedback control of GnRH secretion

The neurosecretory activity of the hypothalamus is controlled by long, short and ultra-short loop feedback systems (Figure 5). In long-loop feedback, information is provided to the hypothalamic centres by hormone concentrations in the peripheral blood. In this way the hypothalamus obtains information concerning the amount of hypophysial trophic hormone (LH) in the circulation and adjusts its own releasing hormone production to the actual need. LH can influence its own release by a short loop feedback whereby the hormone-producing cells of the hypothalamus respond directly to pituitary LH. Ultra-short-loop feedback, by which GnRH regulates its own secretion, has been experimentally demonstrated within the hypothalamus.

Hypothalamic-pituitary-testicular axis

Testicular function is primarily under the control of the gonadotrophins, LH and FSH secreted by the anterior pituitary gland. Many of the neuromodulatory systems participate in the regulation of GnRH-secreting neurones by higher neural centres and by testicular steroids (Cicero et al., 1979; Miller et al., 1986; see Kalra, 1986). An important component in the regulation of the hypothalamic-pituitary-testicular axis is the negative feedback control of gonadotrophin secretion exerted by the testis. Testosterone

exerts a profound suppressive effect on both LH and FSH release (Cicero et al., 1979; see Cicero, 1980; Bhanot & Wilkinson, 1983; Herdon, Everard & Wilson, 1984). The site of the negative feedback effect of testosterone on gonadotrophin secretion is not entirely clear, but indirect evidence exists for an effect of this androgen at both the hypothalamic and pituitary level (Kalra & Kalra, 1983; Kalra, Sahu & Kalra, 1988).

Gonadectomy - Sexual Dimorphism

A striking sex difference exists between adult male and female rats in the initial increment in plasma LH levels after gonadectomy. LH levels begin to rise between 8-12 hours after orchidectomy, but do not increase until 3-4 days after ovariectomy when this surgery is performed on dioestrus 1 (see De Paolo, 1982). In the female rat LH and FSH are released in both a tonic and cyclic manner, while in the male rat these gonadotrophins are secreted in only a tonic fashion (Neill, 1972). The different dynamics of LH release after gonadectomy may be attributed, at least in part, to distinct differences in the overall response of the hypothalamic monoaminergic neurones to the removal of gonadal hormonal feedback. The delay in LH response to gonadectomy seen in the female rat may be due to a more prolonged retention of oestrogen in specific brain areas (Negro-vilar et al., 1984). Oestrogen treatment of male rats before orchidectomy delays the onset of increases in plasma LH levels after cessation of treatment (Justo & Negro-Vilar, 1979). It has also been suggested that the action of oestrogen is exerted within the

preoptico-hypothalamic region to ultimately inhibit the release of GnRH from the peptidergic neurones (Nigro-Vilar et al., 1984).

Neuroendocrine responses to Orchidectomy

Castration of adult male rats results in a rapid rise in plasma LH to a plateau within 16-24 hours (Ojeda & McCann, 1973, see Kalra, 1986). There is a further increase between days 5 and 15 after castration (Badger et al., 1978; Caraty et al., 1981) and a still further elevation after day 30 (Howland & Skinner, 1975; Sodersten et al., 1980; Linkie, Furth & Kourelakos, 1981). Plasma FSH levels also rise within 24 hours, continue to increase at a steady rate until day 8, and then at a slower rate until day 30 (Ojeda & McCann, 1973; Badger et al., 1978; Caraty et al., 1981) with a continuing gradual rise thereafter (Howland & Skinner, 1975; Linkie et al., 1981).

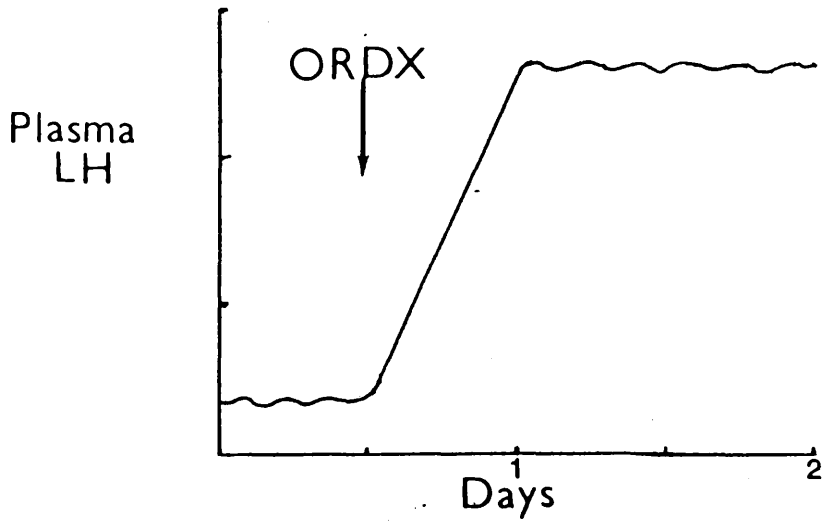


Figure 6. Orchidectomy (ORDX) and plasma LH.

Blockade of pituitary GnRH receptors with a GnRH-antagonist prevents the postcastration rise of both plasma LH and FSH (Condon et al., 1985). A postcastration rise in GnRH receptor numbers is induced by increased GnRH secretion (Clayton & Catt, 1981). The continuing rise of gonadotrophins after castration probably involves increased pituitary responsiveness to GnRH (Badger et al., 1978).

Castration has been reported to alter the dynamics of catecholamines in the brain in a variety of ways. (Simpkins, Kalra & Kalra, 1980; De Paolo, McCann & Negro-Vilar, 1982; Herdon, Evarard & Wilson, 1984; Al-Hamood et al, 1987; Gabriel et al., 1986a, b).

Administration of testosterone to castrated rats, either at the time of castration or at any time interval thereafter, reduces gonadotrophin levels to normal (Clayton & Catt, 1981; Conne et al., 1982; Kalra, Sahu & Kalra, 1988). At the hypothalamus, testosterone has been shown to prevent the reduction in GnRH concentration in the MBH which normally occurs after castration (Kalra & Kalra, 1978, 1980); the testosterone probably acts by preventing GnRH release into the hypophysial portal system (Gross, 1980). Acute castration (4-24hours) is reported to increase the concentration of NA but not of DA in the ME. Testosterone has also been shown to reduce noradrenergic activity in the medial basal hypothalamus (Simpkins et al., 1980) and to augment dopaminergic activity in the ME (Simpkins, Kalra & Kalra, 1983). Pharmacological evidence indicates that the hypersecretion of LH after castration may be mediated by the

alpha- and beta-adrenergic system (Ojeda & McCann, 1973; Herdon et al, 1984; Al-Hamood et al., 1987).

The Aminergic System

The MBH is richly innervated by catecholaminergic terminals. Neuropharmacological studies of the effect of blockade and stimulation of biogenic amine receptors have led to an understanding that hypophysiotrophic hormone secretion is controlled by aminergic neurones. Many neurotransmitters have been identified in the brain. The catecholamines such as NA, ADR and DA, the indolemine, 5-HT, acetylcholine (ACh), histamine and gamma-aminobutyric acid (GABA) are all said to have neurotransmitter functions, but their physiological role remains to be fully understood. NA and 5-HT neurones from the midbrain come in contact with peptidergic neurones in the hypothalamus. DA cell bodies with their origin in the ARN send short axons to the ME. The role of these three monoamines influencing hypothalamic-pituitary function has been extensively studied. They produce biochemical inputs at the synaptic junction that triggers the action of hypothalamic peptidergic neurones following binding to the appropriate receptors.

The release of transmitters and their resulting actions are dependent on three important factors:

1. The degree of reuptake by presynaptic terminals and intraneuronal activation by monoamine oxidase;
2. The metabolism by catechol-O-methyl transferase (COMT), which rapidly inactivates some neurotransmitters at the synaptic junction; and

3. The comparative availability of receptors at pre- and postsynaptic sites.

The role of central catecholamines in the regulation of gonadotrophin release

The involvement of catecholamines in the regulation of LH release has been extensively studied (see Barraclough, Wise & Selmanoff, 1984; Barraclough & Wise, 1982; Kalra, 1986). Before considering their role individually in the regulation of LH release, the neuropharmacological properties will be briefly discussed.

Neurochemistry of Catecholamines

The biosynthesis and degradation of catecholamines has been reviewed by Moore & Johnston (1982). A diagrammatic representation of the neurochemical events occurring at the DA nerve terminals is shown in Figure 7.

The catecholamines are synthesized from the precursor amino acid L-tyrosine. This compound is actively taken up by DA neurones where it is converted to L-dihydroxyphenylalanine (DOPA) by the rate-limiting synthesizing enzyme, tyrosine hydroxylase. This enzyme is regulated, in part, by an end-product inhibitory process so that decreases in intraneuronal DA concentrations result in increased DA synthesis, and vice versa. By this mechanism, concentrations of DA within the nerve terminal remain fairly constant despite alterations in the amount of transmitter released. DOPA is rapidly decarboxylated to DA by L-aromatic amino acid decarboxylase

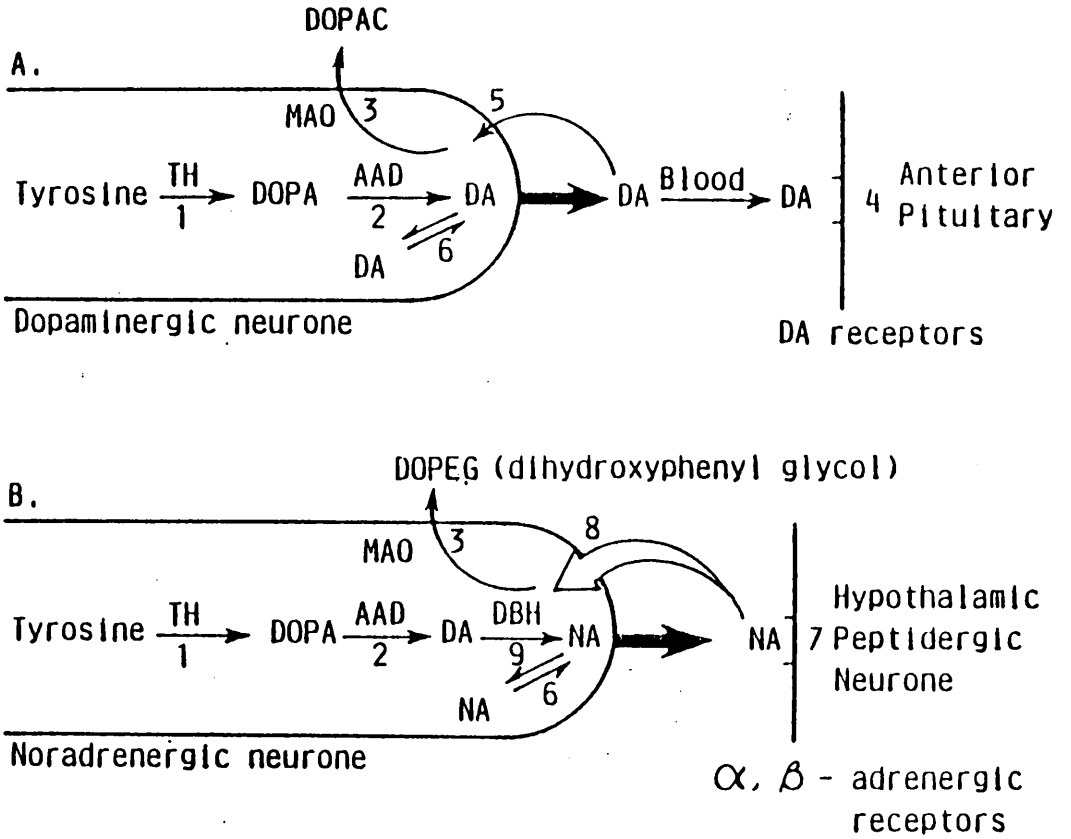


Figure 7. Schematic diagram of A. dopaminergic and B. noradrenergic nerve terminals and receptors depicting sites of drug action. Numbers represent the sites of drug action: 1. tyrosine hydroxylase inhibitors, 2. aromatic L-aminoacid decarboxylase inhibitors, 3. -monoamine oxidase inhibitors, 4. DA agonists and antagonists, 5. DA uptake inhibitors, 6. amine deplators, 7. NA agonists and antagonists, 8. NA uptake inhibitors and 9. DA-β-hydroxylase inhibitors. (adapted from Moore & Johnston, 1982).

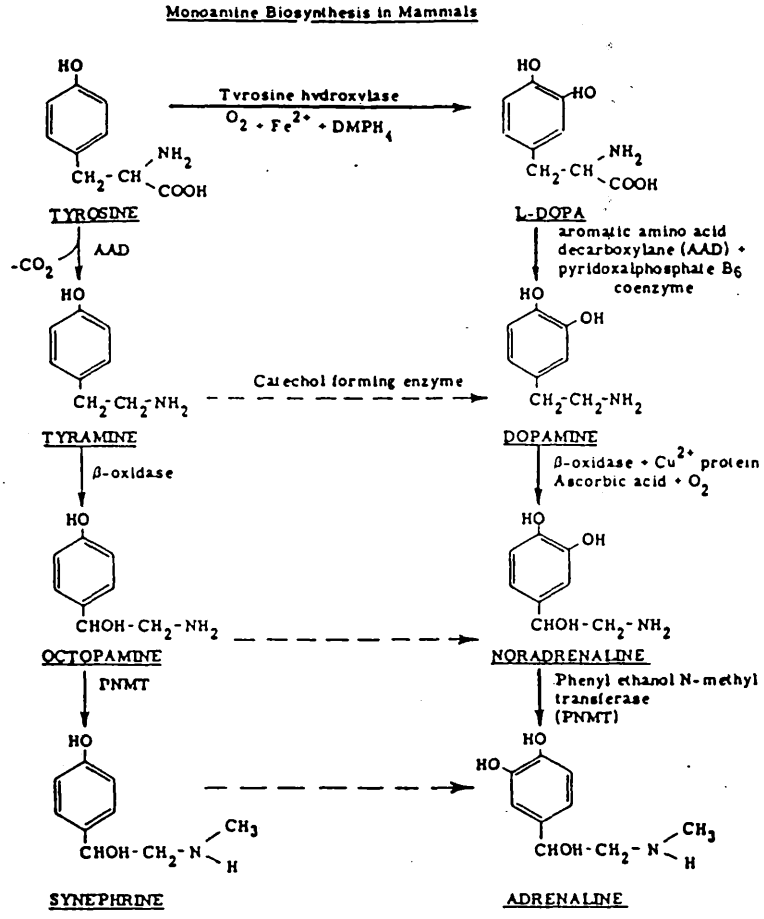


Figure 8. Metabolic Pathways of Catecholamines.

released from the nerve terminal in response to action potentials. In the ME the released DA may activate receptors on axon terminals of other neurones (e.g. those containing GnRH), but most DA terminals are believed to release DA into the primary plexus of the hypophysial portal blood.

DA neurones have a high affinity uptake mechanism which transports released DA back into the nerve terminal. In this way the actions of DA at pre- and postsynaptic receptors are terminated. Such a high affinity DA uptake system appears to be absent from tuberoinfundibular neurones (Demarest & Moore, 1979a; Annunziato et al., 1980). An uptake mechanism is probably not needed by these neurones since DA is rapidly removed from the region of the terminals by the blood. Tuberoinfundibular DA neurones also differ from nigrostriatal and other major ascending DA neurones in that they lack presynaptic autoreceptors which regulate the synthesis (and possibly release) of DA in the latter neurones (Demarest and Moore, 1979b).

In the NA nerve terminals, the synthesized DA is taken up by storage vesicles containing dopamine-B-hydroxylase (DBH), and converted to NA. It is then released in response to the arrival of nerve action potentials or to the actions of drugs. Within the synaptic cleft NA is free to interact with pre- or postsynaptic receptors. The action of this neurotransmitter at these receptors is terminated when the amine is actively transported back into the NA nerve terminal and oxidatively deaminated by intraneuronal monoamine oxidase (MAO). The deaminated products (3,4-dihydroxyphenylglycol is

the main metabolite in the brain) can then be further metabolized by catechol-O-methyltransferase which is localized in glial cells to 3-methoxy,4-hydroxyphenylglycol (MHPG) which is the metabolite that has generally been regarded to reflect NA neuronal activity, i.e., increased concentrations of MHPG reflect increased activity of NA neurones, and vice versa.

Estimation of catecholaminergic activity

The concentrations of catecholamines in nerve terminals do not change during periods of increased or decreased neuronal activity. It is believed, therefore, that synthesis of these amines keeps pace with release, and that degradation of amine transmitters occurs primarily after they have been released. Thus, synthesis, release and degradation are related in such a way that a change in one process is associated with reciprocal changes in the other processes. Hence, changes in any of these processes can be used to estimate the activities of aminergic neuronal systems.

There are several biochemical techniques that can be employed to estimate the activity of catecholaminergic neurones. These are primarily based upon the measurement of turnover which enables the simultaneous measurement of NA, ADR and DA neuronal activity.

Since the rates of synthesis of catecholamines are regulated at the step catalyzed by tyrosine hydroxylase, many estimations of catecholamine turnover are based upon *in vitro* and *in vivo* measurements of the activity of this enzyme.

Administration of an inhibitor of tyrosine hydroxylase, alpha-methyl-para-tyrosine, α-MPT, decreases the concentrations of these amines in the brain in an exponential manner, the rate being proportional to impulse activity in the neurones (Brodie et al., 1966). The exogenous administration of α-MPT permits the simultaneous estimation of NA, ADR and DA turnover(Spector et al., 1965). The method of Brodie et al.,(1966) has subsequently been applied by many workers (Hohn & Wuttke, 1979; Rance et al., 1981; De Paolo et al., 1982, Herdon et al., 1984; Coen & Coombs, 1983) to simultaneously derive turnover rates for both DA and NA.

The role of central noradrenaline in the control of gonadotrophin release

Noradrenergic neuronal system originate from two main brain regions; the locus coeruleus and the lateral tegmental region. A_4 and A_6 cell bodies originate from the locus coeruleus; A_1, A_2, A_5 and A_7 from the lateral tegmental region, which ranges from the caudal medulla to the caudal mesencephalon. Most hypothalamic nuclei including the supraoptic, MPO and ARN, and also the ME appear to be innervated by the lateral tegmental system (Jones and Moore, 1977). However, the PVN may also receive as much as 40% of its NA innervation from cells in the locus coeruleus. Axons from NA cell bodies in the lateral tegmental region ascend in the medial forebrain bundle to the level of the hypothalamus and then turn medially along the ventral surface of the brain to terminate in the ME; these neurones have been referred to as the reticuloinfundibular NA system (see Figure 9).

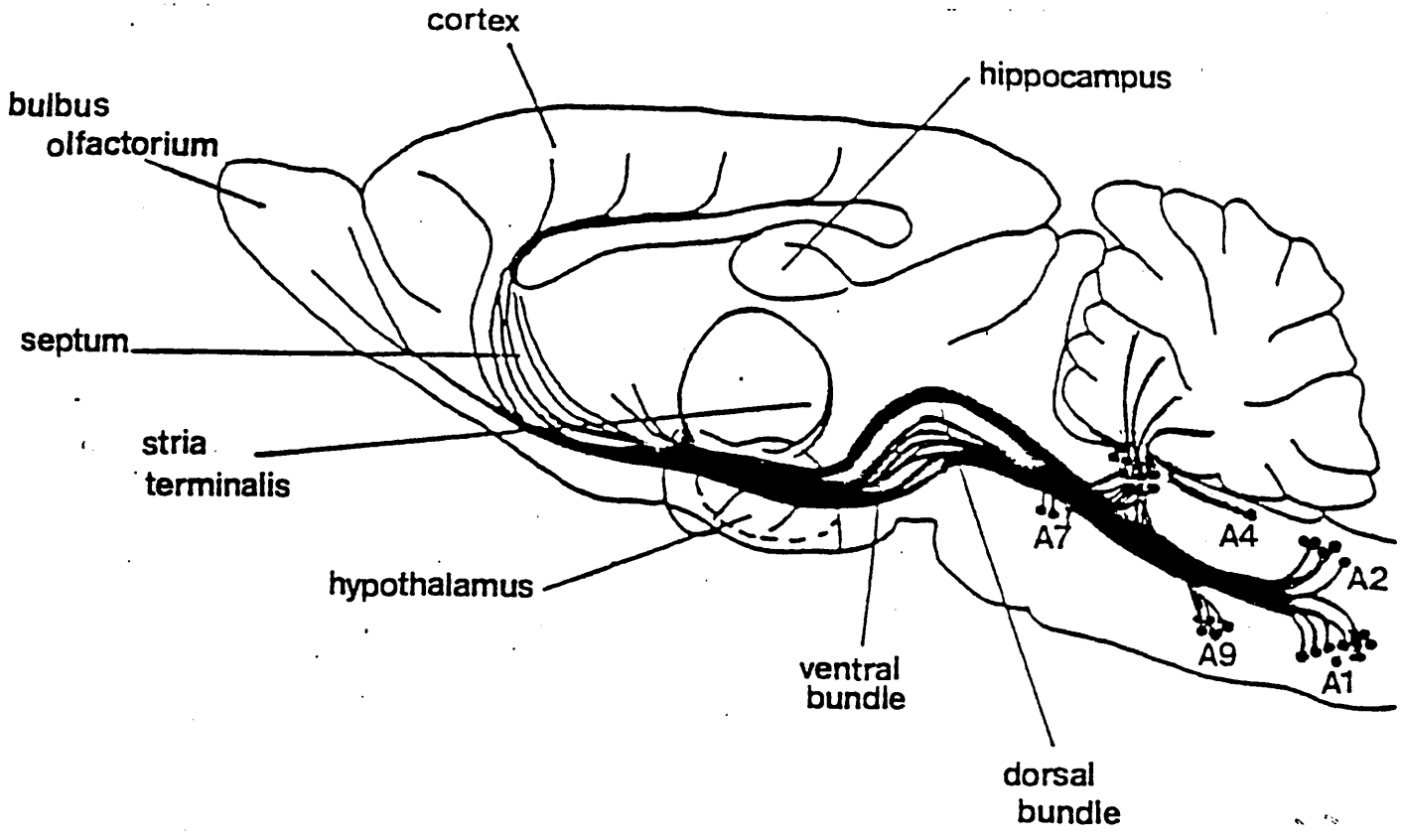


Figure 9. Schematic illustration of a saggital section through a rat brain depicting the two noradrenergic tracts innervating the hypothalamus; the ventral and dorsal tegmental pathways.

NA has been shown to act as an excitatory neurotransmitter (Renaud, 1988, Ching & Krieg, 1986; Miyake et al., 1983; see Kalra & Kalra, 1985; Ramirez et al., 1984; Barraclough & Wise, 1982). It has been demonstrated that NA consistently stimulates LH release in a dose-related fashion in oestrogen and progesterone-treated ovariectomized rats (Kalra & Gallo, 1983). Surprisingly, only a high dose of NA (10^{-4}M) has been shown to stimulate GnRH release *in vitro* from the ME of steroid-primed ovariectomized rats (Nigro-Vilar & Ojeda, 1978). In addition, NA induced ovulation has been demonstrated in a variety of experimental models (Sawyer 1975; Sawyer & Clifton, 1980) where the LH mechanisms were suppressed such as disruption of NA pathways, exposure to constant illumination, pentobarbitone blockade (Al-Hamood, Gilmore & Wilson, 1985; Gopalan, Meek & Gilmore, 1987; see Kalra & Kalra, 1983). Crowley et al., (1978 & 1982) have suggested an association between the increased NA activity in discrete sites within the hypothalamus and the spontaneous or steroid-induced LH surge. They observed that NA turnover in the ARN and ME was significantly elevated in association with stimulation of LH release by progestrone in oestrogen-primed rats (Crowley et al., 1978; Crowley, 1982). Likewise, Wise and co-workers (1981) noted that, in association with the peak LH levels at 15.00 hours induced by oestrogen in ovariectomized rats, there was increased NA turnover between 15.00 and 17.00 hours in the ME, ARN, POA and in the SCH. With additional progesterone administration to oestrogen-primed rats at 09.00 hours, elevated LH levels were seen at 12.00 hours and peak

levels at 15.00 hours. In these rats, NA turnover in the ME was increased at times when LH levels were either rising (12.00 hours) or had peaked (15.00 hours). A similar NA response in the ME was seen after the preovulatory LH surge had either progressed or peaked on pro-oestrus (Rance et al., 1981). It has been demonstrated that electrical stimulation of the locus coeruleus or ascending noradrenergic pathway stimulated LH release and induced ovulation (Gitler & Barraclough, 1987). Phentolamine, an alpha-adrenergic receptor blocker, has been shown to inhibit the action of NA in the ME of ovariectomized, steroid-primed rats (Negro-Vilar et al., 1979). Non-selective antagonists, phenoxybenzamine and phentolamine also blocked ovulation when infused on the morning of pro-oestrus (Al-Hamood et al., 1985). Further evidence suggests that this stimulatory effect of NA is mediated via $alpha_1$-adrenergic receptors (Drouva, Laplante & Kordon, 1982; Heaulme & Dray; 1984; see Kalra, 1986). Beta-agonists, isoprenaline and fenoterol are stimulatory to ovulation in pentobarbitone-treated rats when administered on the morning of pro-oestrus. Fenoterol could overcome the effect of pentobarbitone, stimulating ovulation even when administered on the afternoon of pro-oestrus. Beta-antagonists, propranolol and metoprolol stimulate ovulation only when administered on the afternoon of pro-oestrus (Al-Hamood et al., 1985).

Intracerebroventricular (icv) administration of NA is inhibitory to LH release in unprimed ovariectomized rats (Gallo & Drouva, 1979). Microinfusions of NA, and of phenylephrine and clonidine (alpha-agonists) or of

isoprenaline (a beta-agonist) into the third ventricle, significantly and acutely suppress the frequency of ultradian LH pulses in unanaesthetized, ovariectomized, unprimed rats (Leung et al., 1982). Chemical lesion of the ventral noradrenergic bundle depletes hypothalamic NA levels and disrupts the normal four day oestrous cycle with preovulatory LH release being much reduced (Hanke & Wuttke, 1979). In contrast, electrical stimulation of the dorsal noradrenergic bundle has been shown to abolish the preovulatory LH surge (Dotti & Taleisnik, 1982) indicating an inhibitory effect of NA in addition to its more widely recognized stimulatory one. Moreover, the blockade of ovulation, by electrical stimulation of NA cell groups in the brainstem on pro-oestrus, can be abolished by beta-adrenoceptor antagonists propranolol, but not by alpha receptor antagonists such as phenoxybenzamine (Dotti & Taleisnik, 1984). Beltramino & Taleisnik (1984) have reported that inputs from the nuclei of the posterior hypothalamus are inhibitory to LH release and can participate in determining the timing and magnitude of the preovulatory surge. They have demonstrated that the transverse cuts of the rat brain placed just in front of the mammillary bodies and caudal to the VMH result in an advance of the onset of the pre-ovulatory LH surge. Thus, it has been concluded that NA, acting via beta-adrenergic receptors, mediates the transmission of inhibitory impulses originating in the locus coeruleus nucleus. Ramirez et al., (1984) have speculated that during a normal oestrous cycle, the beta-adrenergic inhibitory component is quiescent in pro-oestrus thus helping to ensure a maximal LH surge, but

it becomes activated subsequently to restrict LH output at other stages of the cycle.

Kalra & Kalra (1983) have suggested a dual mode of NA involvement in controlling episodic LH secretion. According to this hypothesis, each LH pulse is evoked by the synchronous discharge of a "packet" of GnRH into the hypophysial portal vessels. The pulsatile GnRH release in turn, is tightly coupled with, and driven by, $alpha_1$-adrenergic receptor stimulation following an episodic discharge of NA in the proximity of GnRH neurones in the hypothalamus. As the NA discharge proceeds, at certain thresholds $alpha_2$-adrenergic receptors are activated to turn off further GnRH secretion. The abrupt shutting off of GnRH secretion may be due to a selective desensitization of adrenergic receptors as NA release proceeds uninterrupted. Thus it is possible to explain the episodic release of LH solely on the basis of the precisely tuned and temporally co-ordinated function of two different types of adrenergic receptors in the hypothalamus.

The role of central adrenaline in the regulation of gonadotrophin release

The role of ADR in controlling gonadotrophin release is less well understood (see Ramirez et al., 1984). ADR seems to play an important role in regulating the preovulatory LH surge (see Kalra, 1985, 1986; Fuller, 1983).

ADR-containing perikarya have been demonstrated to exist in only two brainstem areas. In the rat, the C1 cell group lies within the lateral reticular nucleus, about 500 µm

rostral to the A1 cell group. Other cells (C2 cell group) are scattered in the dorsomedial medulla oblongata mainly in and around the nucleus of the solitary tract, about 1500 µm rostral to the A2 cell group (see Moore & Johnston, 1982).

Phenylethanolamine-N-methyltransferase, PNMT (the enzyme responsible for the conversion of NA to ADR)-positive terminals have been identified in various hypothalamic nuclei including the ventrolateral region of the ARN and in the ME. Biochemical measurements have also revealed PNMT activity in the ME. Thus current evidence suggests that adrenaline-containing neurones project directly to the ME and also onto other neurones (eg.in the ARN) that, in turn, project to the ME. These fibres originate mainly from the C1 cell group.

The precise mode of participation of adrenergic neurones in transmission of the excitatory impulses which evoke the basal and cyclic release of LH release is poorly understood. Many studies have alluded to a predominant role for ADR in evoking the preovulatory LH surge (Adler et al., 1983; Coen & Coombs, 1983; Coombs & Coen, 1983; Crowley & Terry, 1981; Crowley, Terry & Johnson, 1982; Kalra, 1985).

PNMT-inhibitors block the preovulatory LH surge and also that induced by ovarian steroids in ovariectomized rats (Adler et al, 1983; Coen & Coombs, 1983; Crowley & Terry, 1981; Crowley, Terry & Johnson, 1982; Kalra, 1985). PNMT inhibitors may block LH release by decreasing ADR transmission and, in part, by antagonizing the activation of alpha$_1$-adrenoceptors (Kalra, 1985). ADR turnover, as

determined by the rate of decrease in ADR levels after administration of PNMT inhibitors, is reported to increase before and during the period of LH hypersecretion. Furthermore, Coen & Coombs (1983) noted an increase in the rate of ADR depletion in the POA when the preovulatory LH surge had peaked. A small increase in ADR turnover in the MBH and MPO has also been reported to occur during the preovulatory LH surge (Sheaves, Laynes & MacKinnon, 1985; Sheaves et al., 1984). However, because a parallel study on the time course of LH release was not performed, it is difficult to assess the significance of this rise in ADR turnover in evoking LH release.

The contribution of adrenergic neurones to the preovulatory LH discharge is also evident from another line of study. Surgical transection of brainstem projections into the hypothalamus drastically lower NA levels, whereas hypothalamic levels of ADR are only moderately reduced (Fuller, 1983; Fuller, Perry & Hemrick, 1980; Saavedra et al, 1983). Moreover, there is no apparent effect of these procedures on the occurrence of oestrous cycles and gonadotrophin secretion (Clifton & Sawyer, 1980).

The role of central dopamine in the regulation of gonadotrophin release

The role of DA in the regulation of gonadotrophin release is controversial since both stimulatory (see Vijayan, 1985; James et al., 1987; MacKenzie, James & Wilson, 1986) and inhibitory (Gallo, 1980) effects have been suggested in the control of LH. The variation in effect seems to depend

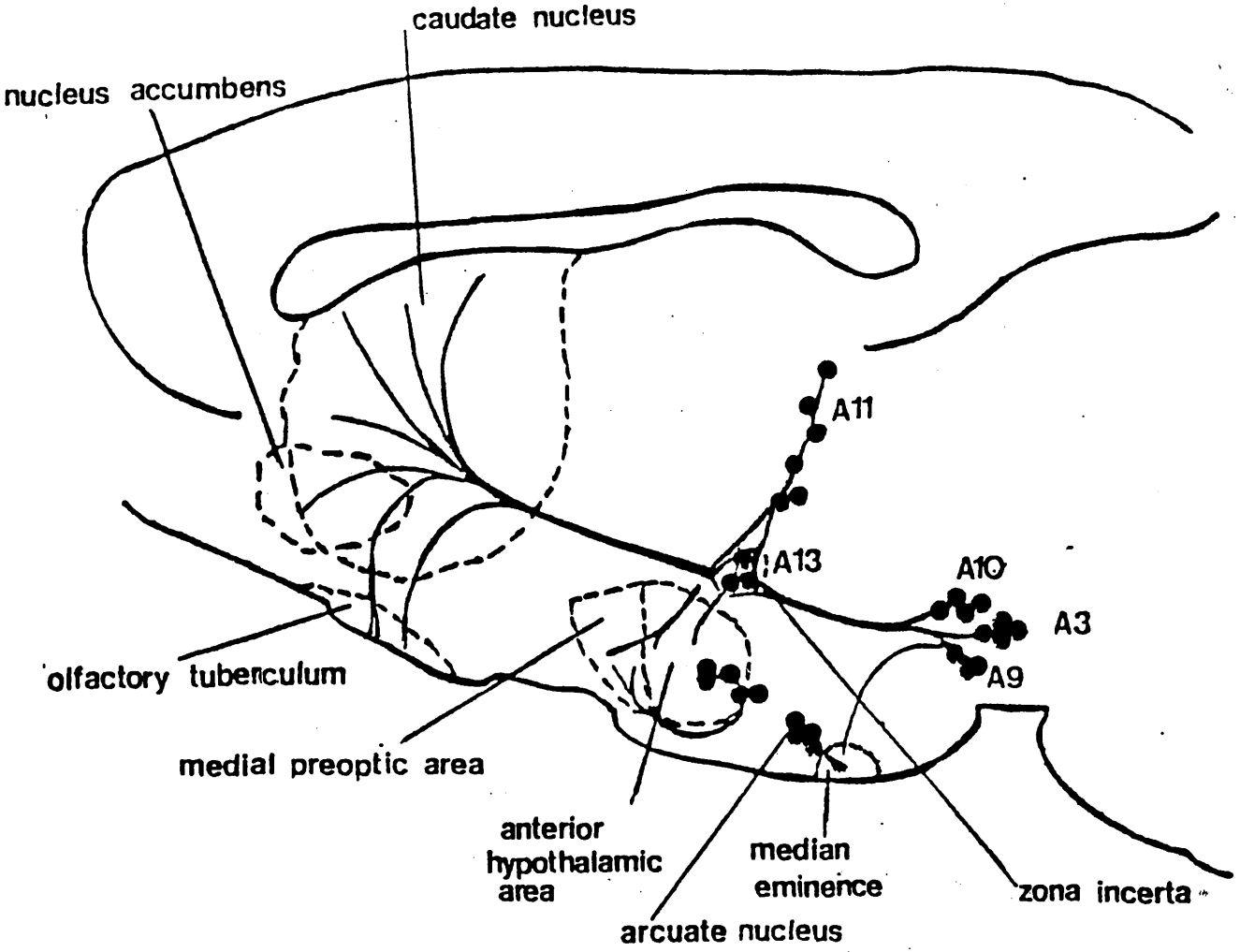


Figure 10. Schematic illustration of a saggital section through the rat brain depicting the two dopaminergic tracts innervating the hypothalamus; the tuberoinfundibular and the incertohypothalamic tracts.

on the endocrine state of the experimental model (Beck, Hank & Wuttke, 1978). In general, stimulatory effects seem to occur only in the adult animal and in the presence of steroids whereas in the absence of steroids increasing DA activity in the whole brain or in the hypothalamus produces an inhibition of gonadotrophin release (see Weiner & Ganong, 1978; Sarkar & Fink, 1981).

There are two anatomically distinct dopaminergic neuronal systems that innervate the hypothalamus; the tuberoinfundibular and the incertohypothalamic tracts (see Moore, 1987; Moore & Bloom, 1978). Cell bodies of the tuberoinfundibular neurones are located in the ARN and in the regions of the anterior PVN nucleus lying just dorsal to the former nucleus (A_{12}). DA terminals in the ME appear to originate from perikarya that are within the MBH (i.e., ARN and PVN).

The tuberoinfundibular DA neurones have short axons which project ventrally to terminate in the ME. Some DA neurones appear to terminate in close proximity to GnRH-containing nerve terminals (Sladek et al., 1978). Thus DA released from terminals of tuberoinfundibular neurones may alter the release of GnRH.

The incertohypothalamic tract, arising from the zona incerta projects into the MPO (Bjorklund et al., 1975). These neurones are thought to mediate endogenous inhibition of LH secretion, mimicked by the central action of the DA receptor agonists apomorphine (Drouva & Gallo, 1977) and DA (Gallo & Drouva, 1979). Despite this evidence, a

stimulatory role for DA in LH release has been favoured by other workers after demonstrating a positive relationship between DA and LH release (Negro-Vilar et al., 1982). Conflict existing in the literature concerning the action of DA could be due to the involvement of multiple dopaminergic pathways (the tuberoinfundibular, mesolimbic and incertohypothalamic neuronal systems) in the control of both the tonic and/or phasic release of LH.

DA does not act directly on the pituitary to affect LH release (Schneider & McCann, 1969), so any observed effects must be indirect. Icv administration of DA was observed to have no effect on LH secretion in ovariectomized rats (Schneider & McCann, 1970; Vijayan & McCann, 1978), whereas the systemic injection of the DA agonists apomorphine and piribedil inhibited LH secretion (Beck & Wuttke, 1977). In another study, icv infusion of DA has been reported to elevate LH levels on the morning of pro-oestrus (Schneider & McCann, 1970). Kamberi, Mical & Porter (1970) have provided evidence suggesting a stimulatory effect of DA on GnRH release both *in vitro* and *in vivo*. Incubation of hypothalamic synaptosomes or ME tissue with DA resulted in a dose-related increase in GnRH release which could be prevented by the DA receptor blocker, pimozide (Negro-Vilar et al., 1979). The stimulatory action of DA on LH release did not appear to be via uptake and conversion to NA since pretreatment with a NA-synthesis inhibitor, diethyldithiocarbamate, did not block the observed effect (Vijayan & McCann, 1978). Furthermore, apomorphine is not

taken up by aminergic terminals and has little inherent noradrenergic activity. Implantation of DA into the POA, containing incertohypothalamic dopaminergic neurones, stimulates LH release in ovariectomized oestrogen-primed rats (Kawakami et al, 1975). *In vitro* incubation of the MBH from oestrogen-primed ovariectomized rats with DA also results in GnRH release (Rotsztejn et al., 1976).

The turnover rates of DA in neurones of the MBH at different phases of the oestrous cycle, measured by Fuxe et al., (1978) showed that increased activity in DA neurones inhibits the release and/or synthesis of GnRH. Changes in DA turnover in specific hypothalamic nuclei during pro-oestrus in rats have been evaluated (Rance et al., 1981). Concurrent changes in GnRH and LH levels were also recorded. Between 9.00 and 12.00 hours on the morning of pro-oestrus, when GnRH levels are increasing in the ME, DA turnover rates in the MPO, SCH, ARN and ME are low, and peripheral gonadotrophin concentrations remain basal. Before and during the pro-oestrous gonadotrophin surge, ME GnRH declines and ME DA turnover rate is greatly increased (12.00 to 14.00 hours). During the interval (15.00 to 17.00 hours) in which LH and FSH are still rising to peak serum concentrations, DA turnover rates dramatically decline in the ME and ARN but not in the MPO.

Interruption of the preovulatory surge of LH by pentobarbital (Rubinstein & Sawyer, 1970) or AHA lesions (Tima & Flerko, 1974) was not reversed by icv administration of DA. Histochemical techniques used to monitor changes in

fluorescence intensity of the tuberoinfundibular DA system over the oestrous cycle (Ahren et al., 1971) suggest a decrease in the activity of this system occurs during pro-oestrus.

It appears that treatment of ovariectomized rats with gonadal steroids converts the inhibitory action of DA on LH to stimulation (Rotsztejn et al., 1976). Oestrogen treatment results in a reversal of the effects of electrical stimulation of the ARN (Gallo & Osland, 1976). In untreated ovariectomized rats stimulation of this region inhibits LH release, whereas in treated animals an increase in plasma LH levels is observed. In ovariectomized rats treated with oestrogen and progesterone, Vijayan & McCann (1978) observed that DA injected icv stimulate LH release. On the other hand, in a similar experimental model, Kreig & Sawyer (1976) did not find any effect. There are at least two sets of data which may explain the conflicting results obtained by the different techniques used to investigate the role of the central DA neurones in the control of gonadotrophin release; first, by supposing that the tuberoinfundibular tract is inhibitory and the incertohypothalamic tract is stimulatory; second by assuming that GnRH release may be affected by the actions of DA on two pharmacologically distinct receptor systems, the activation of which might have opposite effects.

The role of central 5-hydroxytryptamine in the control of GnRH release

The involvement of 5-HT in the regulation of LH release

has been extensively reviewed by Wilson (1979), Kalra & Kalra (1983; 1985). Evidence suggests that both inhibitory and stimulatory components may operate in the overall modulatory influence of 5-HT neurones on LH release.

Neurochemistry of 5-HT

Figure 11 diagrammatically represents the neurochemical events occurring at the serotoninergic nerve terminal.

5-HT is synthesized from the precursor amino acid tryptophan. This compound is actively transported into 5-HT neurones where it is hydroxylated to form 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase. This enzyme controls the rate-limiting step in the synthesis of 5-HT but the precise manner by which it is regulated is not completely understood. Tryptophan hydroxylase, unlike tyrosine hydroxylase in catecholaminergic neurones, is not tightly regulated by an end-product inhibitory mechanism. Furthermore, tryptophan hydroxylase is not saturated by concentrations of tryptophan that are normally found in the brain, so that raising or lowering brain concentrations of tryptophan can alter the saturation of the enzyme, and thus the rate of 5-HT synthesis (Fernstrom & Wurtman, 1971). Hence, alterations in the availability of 'free' tryptophan in the brain induced by dietary, hormonal, environmental, and/or pharmacological manipulations can influence brain 5-HT synthesis (see Ramirez et al., 1984). 5-HTP synthesized in 5-HT neurones is rapidly decarboxylated by AAD (thought to be the same enzyme that converts DOPA to DA) to 5-HT. Newly synthesized 5-HT, in

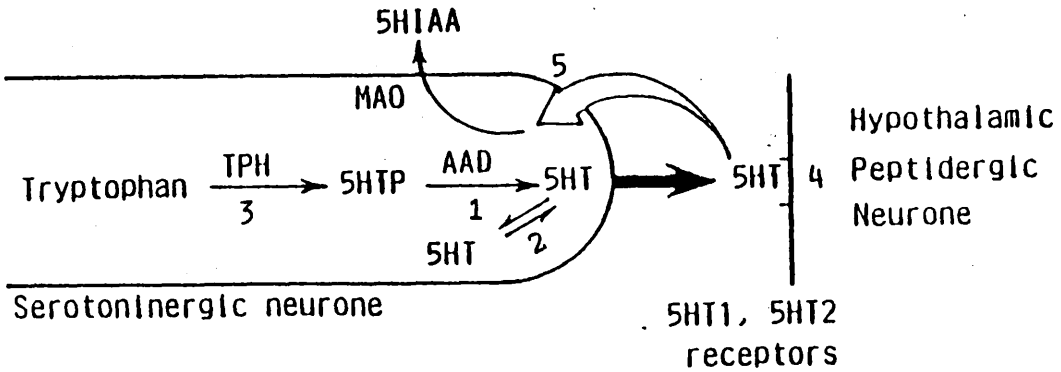


Figure 11(a). Schematic diagram of serotoninergic nerve terminals and receptors depicting sites of drug action. Numbers represent the sites of drug action. 1. aromatic L-amino acid decarboxylase inhibitors, 2. amine deplators, 3. tryptophan hydroxylase inhibitors, 4. serotonin agonists and antagonists and 5. serotonin uptake inhibitors (adapted from Moore & Johnston, 1982).

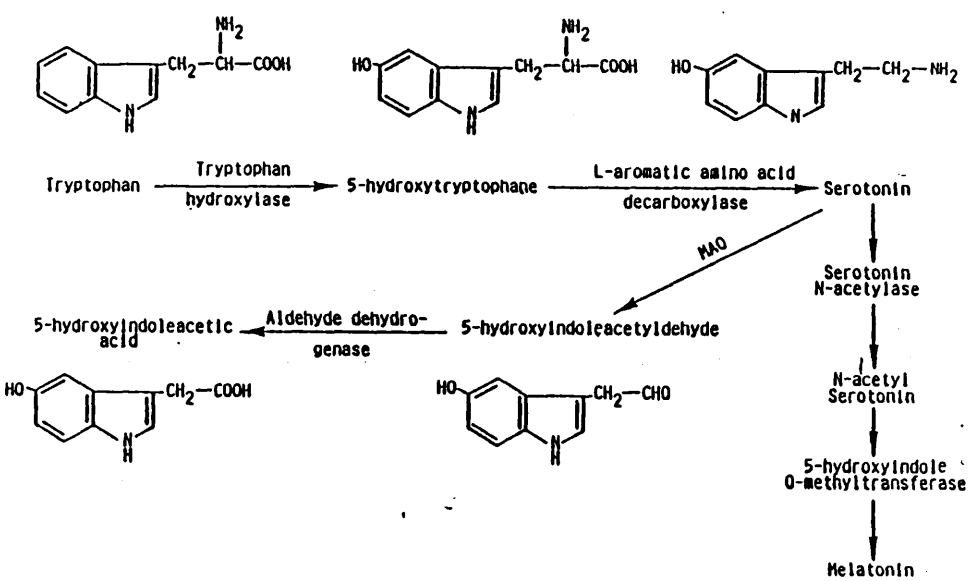


Figure 11 (b). Metabolic Pathways of Serotonin.

turn, may be stored in vesicles or released from the nerve terminals in response to the arrival of nerve impulses, electrical stimulation or drugs (Muller, Nistico & Scapagnini, 1977). Following its release, 5-HT is free to interact with postsynaptic 5-HT receptors. Activation of these receptors is terminated when 5-HT is either metabolized by extraneuronal MAO or is transported back into the nerve terminal by a stereospecific active uptake mechanism. Within the neurone 5-HT is oxidatively deaminated to form the end-product of 5-HT degradation, 5-HIAA (Figure 11). This acid metabolite is then removed from the brain by a probenecid-sensitive acid transport mechanism.

Estimation of Serotoninergic Activity

Although a relation between nerve impulse flow and turnover of 5-HT has been demonstrated (Carlsson et al., 1972), synthesis and degradation of 5-HT in the CNS may not be exclusively linked to 5-HT neuronal activity. Nevertheless, changes in the rate of synthesis and metabolism of this amine have been used to estimate the activity of 5-HT neurones. Of the methods available to measure the turnover of 5-HT, either the relative concentrations of 5-HT and 5-HIAA (Shannon et al., 1986) or the use of pargyline (a MAO-inhibitor) (Johnson & Crowley, 1984) are commonly employed.

Neuroanatomy

Dahlstrom and Fuxe (1964) originally described nine major groups of 5HT-containing cells in the rat brainstem, designated B_1-B_9 (see Figure 12). Perikarya in at least four of these nuclear groups project to the forebrain. Most

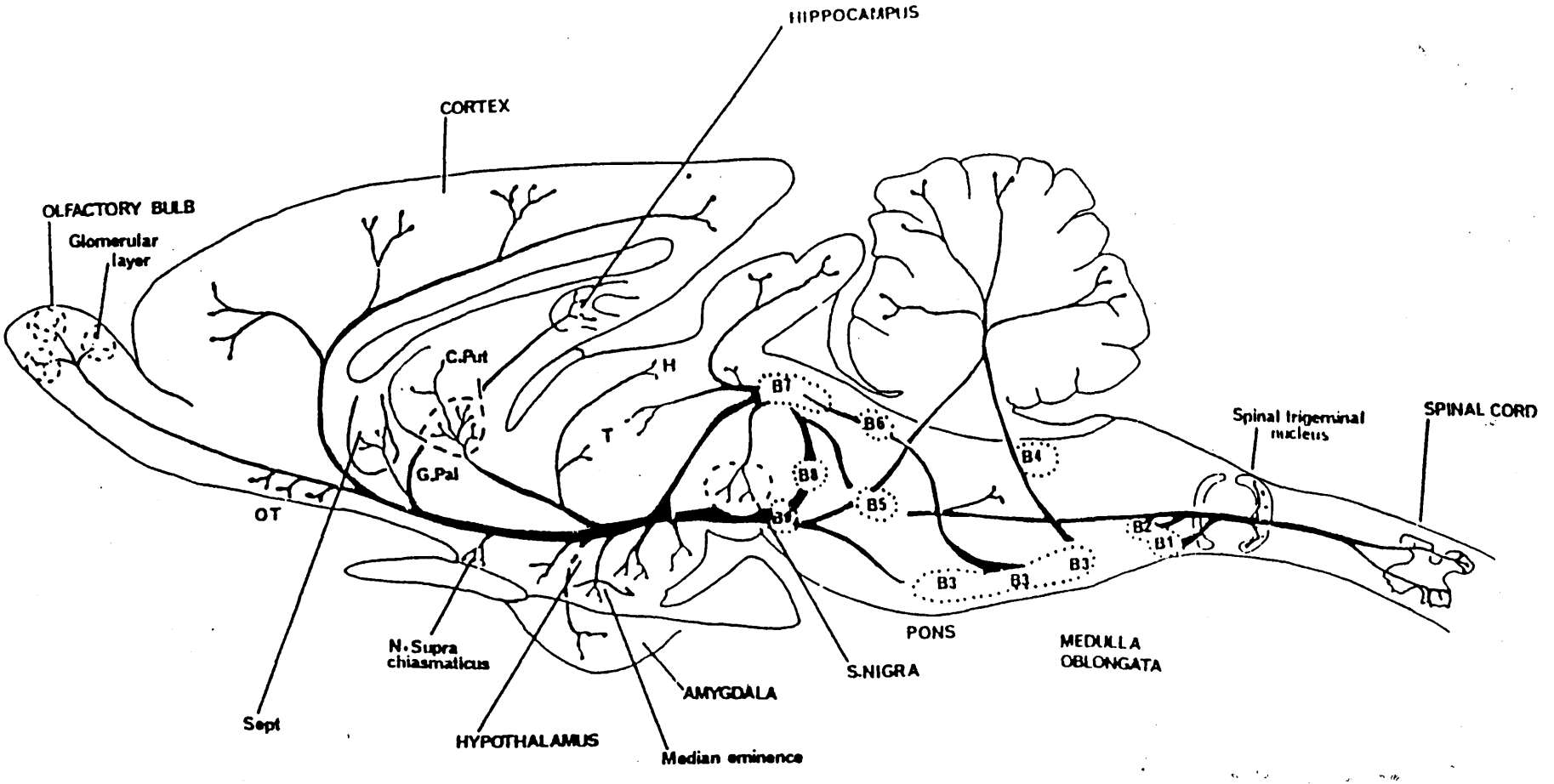


Figure 12. Schematic diagram representing the serotoninergic innervation of the hypothalamus from cell bodies (B1-9) located in the medulla oblongata, pons and mesencephalon, most of them within the so-called raphe nuclei (OT, olfactory tuberculum; sept, septum; C.Put, Caudate Putamens; T, Thalamus; H, Habenula).

evidence suggests that projections to the medial hypothalamus, including the ARN, PVN and ME, originate from perikarya in the dorsal raphe nucleus(B_7). More recently, 5-HT receptors have been visualized in the rat hypothalamus, being more concentrated in the POA and VMH (Beigon, Rainbow & McEwen, 1982). Cell bodies staining for both retrograde and fluorescent labels have been demonstrated in the dorsal and medial raphe nuclei and the medial lemniscus of the brain stem (Simerly, Swanson & Gorski, 1984). Villar, Chiocchio & Tramezzani (1984), using a similar technique, have shown that the 5-HT innervation of the ME originates from the rostral third of the dorsal raphe nucleus.

Neuropharmacological Evidence

Administration of 5-HT into the cerebral ventricles, inhibition of 5-HT synthesis with PCPA, or destruction of 5-HT neurones with 5,6- or 5,7-DHT have been reported to increase, decrease and produce no change in the basal secretion of LH (Gallo & Moberg, 1977; Wilson et al., 1977; see Wilson, 1979). In rabbits, icv infusion of 5-HT was shown to inhibit ovulation when given just before the expected pre-ovulatory LH surge (see Wilson, 1979). Nevertheless, the marked increases in serum LH concentrations that occur on the afternoon of pro-oestrus or in oestradiol-pretreated ovariectomized rats can be prevented by drugs which disrupt 5-HT neurotransmission (Weiner and Ganong, 1978; Baumgarten et al., 1978). Electrical stimulation of 5-HT neurones in the dorsal raphe nucleus inhibits LH secretion in ovariectomized rats (Arendash and Gallo, 1978; Gallo, 1980) and the inhibition of LH and GnRH release caused by stimulating the

ARN can be reversed by pre-treating animals with PCPA-an inhibitor of 5-HT synthesis (Gallo and Moberg, 1977).

Icv administration of 5-HT has been shown to elevate plasma LH levels (Porter, Mical and Cramer, 1971). Further in support of the stimulatory effect of 5-HT, blockade of 5-HT transmission by the administration of PCPA inhibited the GnRH surge in intact and steroid-primed ovariectomised rats, the effect of which could be reversed by the administration of 5-HTP or an agonist (see Wilson, 1979; Coen & McKinnon,1980; Iyengar & Rabii, 1983). 5-HT antagonists have also been shown to inhibit ovulation in intact and ovariectomized rats (Walker, 1980).

The turnover of 5-HT in the hypothalamus of female rats is reported to be greater at the time of the pro-oestrous preovulatory surge of LH than it is at the end of the surge (Walker, 1980). Furthermore, when the LH surge was prolonged by exposing the rats to light on the evening of pro-oestrus the hypothalamic 5-HT turnover remained high. These reports suggest that the pro-oestrous surge of LH is accompanied by and may be partially dependent upon 5-HT neuronal activity.

5-HT neurones may play a role in the feedback regulation of LH secretion by gonadal steroids. The castration-induced increases of serum LH concentrations and 5-HT concentrations in the VMH can be reversed by the administration of testosterone (Van de Kar et al., 1978). In ovariectomized rats a single ip or sc injection of oestradiol has been observed to reduce the serum concentration of LH without altering the 5-HT content in the ME; the administration of

progesterone caused a surge of LH and a marked increase in the concentration of 5-HT (Crowley et al., 1979). Fuxe et al., (1975) reported that the inhibitory feedback exerted by oestrogen on LH secretion was associated with an increase of 5-HT after the administration of a tryptophan hydroxylase inhibitor. Kizer et al., (1978) reported that tryptophan hydroxylase activity in the ME did not change following castration or the administration of testosterone.

A number of actions attributed to 5-HT appear to be mediated via 5-HT receptors which are divided into 5-HT_1, 5-HT_2 and 5-HT_3. The definite characterization of these receptors await the identification of specific antagonists.

The Opioid System

The discovery of opioid receptors in the brain (Goldstein, Lowney & Pal, 1971; Pert & Snyder, 1973) led to the search for endogenous opioids. In 1975, Hughes et al., identified two pentapeptides, met-enkephalin and leu-enkephalin. This was followed by the discovery of numerous larger opioid peptides such as beta-endorphin, dynorphin and many others (Li, Chung & Doneen, 1976). All these opioid peptides have analgesic activity and bind to opioid receptors within the brain and elsewhere. Chemical and structural relationships between these neuropeptides and their distribution in the rat brain have been extensively reviewed by Cox, 1982; Kalra, 1983; Morley, 1981; Bicknell, 1985; Atweh & Kuhar, 1983; Pfeiffer & Herz, 1984).

The multiplicity of opioid receptors was suggested by the physiological studies of Martin et al., (1976) who

concluded that there are atleast three different opioid receptors termed mu, kappa and sigma. One class of receptors called morphine or mu-receptors, preferentially bind opiates. Enkephalin or delta receptors preferentially bind enkephalin. Ketocyclazocine, a kappa receptor agonist, binds to a class of receptors that are insensitive to naloxone. Dynorphin represents the natural ligand, but both enkephalin and morphine possess high binding affinity to the putative kappa receptor. An endogenous ligand for the sigma receptor has not been described. The specificity of endogenous ligand for the receptor type is summarized in Table 1.

Table 1. Endogenous opioid ligands and their receptors.

Endogenous Ligand	**Receptor Type**
β-endorphin	Mu, Delta
Enkephalin	Delta
Dynorphin	Kappa
?	Sigma

Mu, delta, kappa and sigma receptors have been 'mapped' within the CNS.

These receptors are widely distributed throughout the brain, but are not uniform in number in the different areas. This would indicate multiple hormonal and/or transmitter roles with differences in contribution to neuronal activity in different areas subserving different functions. Indeed, the distribution of the opioid peptides in general is closely correlated to the distribution of opioid receptors (Bloom et

al., 1978; Sar et al., 1978). Of particular interest are the relatively high levels of both receptors and opioid peptides in the hypothalamus (see Kalra, 1986).

There are three families of opioid peptides in the CNS:

1. β-endorphin,
2. Enkephalins and
3. Dynorphin/neo-endorphins.

All three are derivatives of a large, common precursor molecule, the pro-opiomelanocortin.

β-endorphin is present both in hypothalamic and extrahypothalamic brain areas in various concentrations. Immunohistochemical studies (De Kloet, Palkovits & Mezey, 1981) have visualized β-endorphin in cell bodies in the ARN and in nerve fibres and terminals throughout the entire CNS. β-endorphin co-exists with β-LPH, ACTH and α-MSH in the same neurones in the ARN and the ventral premammillary region. Several β-endorphin-containing fibre bundles arising in the ARN have been reported (De Kloet et al., 1981). Fibres proceed from the MBH in rostral (to the ventral forebrain), dorsal (to the thalamus and the dorsal midbrain), lateral (to the amygdala) and caudal (to the lower brainstem) directions.

Enkephalins are derived from pro-enkephalin A and pro-enkephalin B. Pro-enkephalin A is the common precursor for methionine-enkephalin (met-enk) and leucine-enkephalin (leu-enk). Pro-enkephalin B is another precursor for leu-enk and a variety of opioids such as dynorphin 1-17 and alpha-neo-

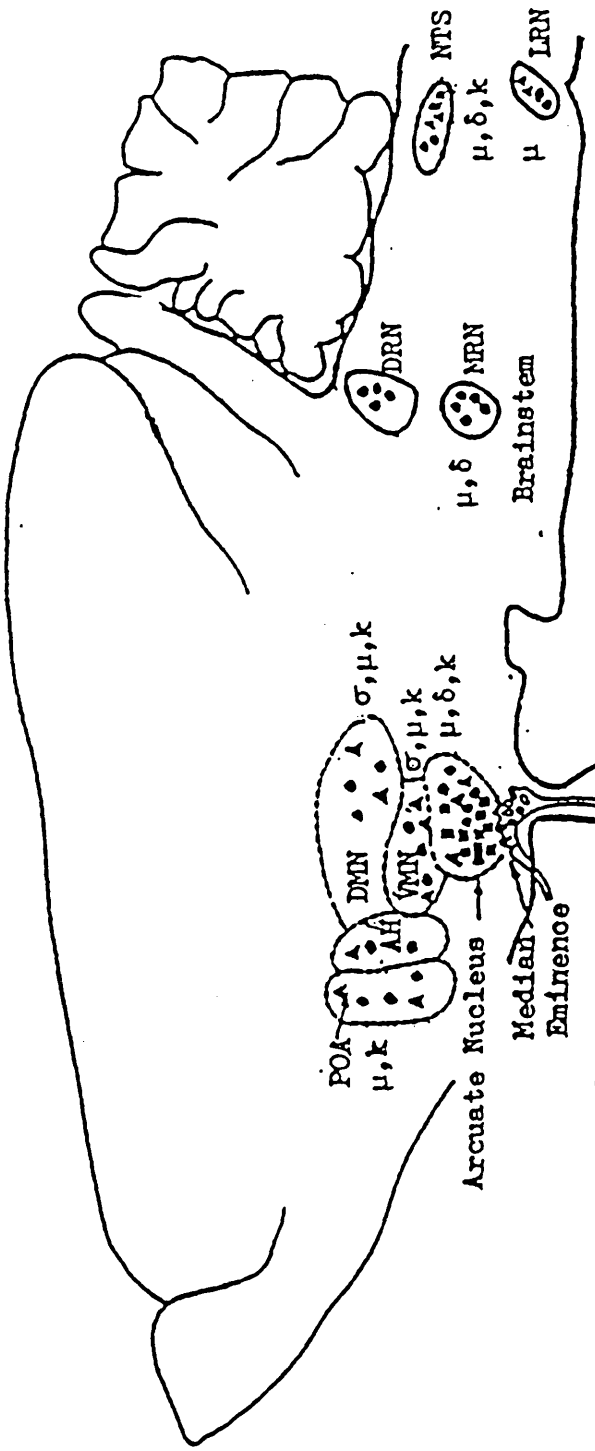


Figure 13. Distribution of Opioid Neurones and Receptors. ■ β-Endorphin-containing neurones. ● Enkephalin-containing neurones. ▲ Dynorphin-containing neurones. DRN; Dorsal Raphe Nucleus. MRN; Medial Raphe Nucleus. NTS; Nucleus Tractus Solitarius. LRN; Lateral Reticular Nucleus. μ,δ,σ,k; Specific opioid receptors described within adjacent region.

endorphin. Met-enk and leu-enk are widely distributed in the CNS. Enkephalins are present in measurable amounts in all major brain areas, the ratio of met-enkephalin versus leu-enkephalin varies from 1:1 to 6:1, being an average of 4:1 in the rat. The extremely high concentrations of both enkephalins in the globus pallidus is the most characteristic feature. Rich enkephalin innervation is found in the rostral forebrain, hypothalamus, amygdala, midline and periventricular thalamic nuclei and the lateral septal nucleus. The enkephalins are found exclusively in the neurohypophysis where they are associated with nerve fibres that also contain vasopressin and oxytocin.

Immunohistochemical studies have revealed that many hypothalamic nuclei contain groups of enkephalin cell bodies (Hokfelt et al., 1979). These include the PVN, MPO, PAN, VMH, the dorsal and ventral premammillary nuclei, the perifornical area, and the ARN. All hyothalamic nuclei appear to contain moderate to high concentrations of enkephalin fibres and terminals. In particular, immunofluorescence localizations have revealed enkephalin-positive nerve terminals within the external layer of the ME suggesting that this opioid peptide has a role in neuroendocrine regulation (see Figure 13).

The most dense network of dynorphins is found in the substantia nigra followed by the lateral POA and the nucleus accumbens, the substantia innominata and the entopeduncular nucleus.

Prior to the discovery of the endogenous opioid peptides, the actions of morphine on pituitary hormone

secretion had been described. Early research indicated that morphine would block ovulation in the rat (Barraclough & Sawyer, 1955). Although it was originally thought that this was a pharmacological action of morphine, it now appears likely that morphine interacts with opioid receptors in the brain to evoke these hormonal changes. Following the synthesis of the enkephalins and endorphins, a number of workers administered these systemically, or microinjected them into the brain ventricles to evoke a similar pattern of hormonal responses (Dupont et al., 1977; Cusan et al., 1977). The potency of enkephalin analogues parallels their potency in other indices of enkephalin action (Meltzer et al, 1978). That the action of morphine, enkephalins and endorphins are specific is suggested by the fact that their effects are all blocked by the opiate antagonist, naloxone.

Since the enkephalins have a hypothalamic distribution different from that of the endorphins (Elde and Hokfelt, 1979), the site of action of the various opioid peptides following their injection into the brain ventricle has not been established. It appears that one site of action is probably in the region of the ARN. This is the site of neuronal perykaria which synthesize proopiocortin and whose projections extend into various other regions of the brain. On the other hand, the enkephalinergic neurones seem to be located more in the periventricular region and in the rostral hypothalamus. Some of these neurones have cell bodies in the PAN and axons which extend to the neurohypophysis (Elde and Hokfelt, 1979).

After an acute injection of morphine in the normal male animal, LH levels begin to decline within 30–45 minutes reaching a peak level of depression approximately one hour later (Cicero et al., 1977). In the castrated male rat, however, a single injection of morphine produces a fall in serum LH levels within 10–15 minutes and the peak level of depression is attained 30 minutes afterwards (Cicero et al., 1979). The reason that LH levels fall somewhat more promptly after morphine administration in the castrated male than in the normal animal is probably related to the fact that LH levels are already markedly suppressed in the normal animal, as a result of the negative feedback effect of testosterone, and any further statistically significant decrease takes longer to be produced. In addition, this morphine-induced blockade of the postcastration-induced increase in serum LH can be readily overcome by the administration of GnRH.

Opiates have no direct effect on the secretion of gonadotrophin from the anterior pituitary (Cicero et al., 1977) and the gland contains very few opioid receptors (Simantov & Snyder, 1977; Atweh & Kuhar, 1983, see Bicknell, 1985). Thus opioids appear to inhibit gonadotrophin release by preventing the secretion of GnRH from the ME. Injection of the opioid antagonist naloxone will bring about a prompt rise in plasma gonadotrophins in many circumstances, suggesting that endogenous opioids are exerting a similar inhibitory influence (see Kalra & Kalra, 1984). It has been reported that intracranial implantation of naloxone in regions outside the hypothalamus fails to stimulate LH release (Kalra, 1981). On the other hand, implants containing minute amounts of

naloxone or intracerebral injection of naloxone anywhere in the MPO and extending to the ME-ARN stimulates LH release (Kalra, 1981). Furthermore, morphine pretreatment blocks the local effects of naloxone implants in eliciting GnRH release. The strict regional specificity of naloxone action, together with the fact that morphine blocks the local excitatory action of naloxone, point out that opiate receptors which influence LH release may be confined to a narrow neural zone in the preoptic-tuberal pathway (Everett, 1977; Kalra et al., 1981). A hypothalamic locus of action for the opiates has been suggested by the results from several other *in vivo* and *in vitro* studies in both rodents and humans (Cicero et al., 1977, 1979; Arita & Kimura, 1988, Allen et al., 1988; Elingboe et al., 1978).

The effects of opiates on the hypothalamic-pituitary-LH axis appear to be mediated by specific opioid receptors in the brain (Cicero, 1980; Cox, 1982; Wood, 1982; McDowell & Kitchen, 1987; Rance, 1983; Stojilkovic, Dufau & Catt, 1987). Administration of naloxone to the rat results in a sharp increase in plasma LH (Bruni et al., 1977; Cicero et al., 1979; Piva et al., 1986) suggesting the presence of an endogenous opioid-like substance that normally plays an inhibitory role in the secretion of LH. Furthermore, it has also been demonstrated that naloxone prevents the suppression of testosterone's feedback control of LH in castrated male rat (Kalra, Leadem, Kalra, 1984). These effects are not due to a direct receptor interaction between testosterone and naloxone, since it has been demonstrated that testosterone

and naloxone do not compete with one another for their respective binding sites in the hypothalamus (Cicero et al., 1979). Hence, it appears that testosterone is acting in some way through an opioid-containing system to suppress the secretion of LH.

The mechanism of action of opiates at the hypothalamic level may involve monoaminergic systems (Dyer et al., 1988; see Bicknell, 1985, Kalra, 1986). It has been suggested that opiates decrease NA and ADR activity (see Kalra, 1983). Prior NA and ADR depletion by diethyldithiocarbamate or bis-(4-methyl-1-homopiperanzinthylthiocarbonyl) disulfide (FLA 63) prevents the LH release elicited by naloxone administered either systemically or intracranially in the MPO or ME-ARN. These suggests the fact that sites of opiate-NA action may occur within the preoptic-tuberal pathway. It has been postulated that naloxone may promote NA discharge from the terminals in the preoptic-tuberal pathway. The increase in endogenous NA release, in turn, may excite GnRH discharge leading to enhanced LH release from the pituitary gland (see Kalra, 1983). It is further supported by the findings that naloxone could enhance the release of GnRH from hypothalamic fragments *in vitro* (Wilkes & Yen, 1981). Also, clonidine, a post-synaptic NA agonist, injected to overcome the inhibitory action of morphine in steroid-primed rats readily stimulates LH release (Kalra & Simpkins, 1981). Following morphine treatment, NA, and not DA, could induce LH release in steroid-primed ovariectomized rats (Kalra & Simpkins, 1981) supporting the inhibitory action of opiates on NA neurosecretion.

It appears that morphine or opioid peptides inhibit dopaminergic activity (Arita & Kimura, 1988, Meites et al., 1979; VanLoon et al.; 1980, Haskins et al., 1981) and augment serotoninergic turnover (Yarbrough et al, 1973; Van Loon et al., 1978; Garcia-Sepilla et al., 1978; Algeri et al., 1978; Johnson & Crowley, 1984).

The involvement of opiate-mediated monoaminergic regulation of LH release will be further considered in detail with the present results in the discussion part of this thesis.

Aims of the study:

This study was designed to further investigate

1. The involvement of opioidergic and monoaminergic systems in regulating the preovulatory luteinizing hormone (LH) surge in rats.

Three major experiments undertaken included:

a. The effects of specific adrenergic agonists and antagonists on hypothalamic noradrenaline (NA) and adrenaline (ADR) concentrations,

b. The actions of specific opiate receptor agonists and their antagonist, naloxone, on the hypothalamic content and the turnover of NA, ADR, dopamine (DA), serotonin (5-HT) and its metabolite, 5-hydroxy-indoleacetic acid (5-HIAA) and on plasma LH concentrations.

c. The effects of opiates on amine concentrations and turnover in specific hypothalamic nuclei.

2. The effects of specific opiate receptor agonists and their antagonist, naloxone, on the content and turnover of hypothalamic neurotransmitter concentrations and on plasma LH levels in short-term orchidectomized rats.

MATERIALS AND METHODS

Animals

The Sprague-Dawley rats were either purchased from Tuck & Sons, Battlesbridge, Essex or bred in the Animal Unit facilities at the Institute of Physiology, Glasgow University. Rats were housed in groups of 3 per cage under standardized conditions of temperature (21°C ± 1°C) and light/darkness rhythm (12h light : 12h darkness; lights on 06.00-18.00 hours), with free access to food and water.

Cages used were of transparent polycarbonate (macrolon) with a removable galvanised grill lid. The dimensions were approximately 42 x 25 x 20 cms. They were cleaned once a week when fresh sawdust was supplied.

Two different types of animal models were used during the course of this study.

1. Adult male rats

Animals weighing between 350-600g were used during this study. 5-12 rats were included in each experimental group.

i) Castration:

Rats were removed from the colony between 16.00 and 17.00 hours preceding the day of the experiment. They were weighed and colour-coded. Subsequently the animals were anaesthetized. While under deep ether anaesthesia the rat was secured to the dissecting board in a supine position and a midline incision made in the lower abdominal skin. The exposed subcutaneous fat was dissected out to reveal the abdominal muscle coat. The abdominal muscle was then cut on

one side of the midline incision to expose the viscera. Lateral to this position and superior to the viscera lie the epididymal fat pads. These were gently withdrawn to exteriorise the testes. Blunt dissection allowed identification of the spermatic artery, vein and the vas deferens. A haemostatic ligature was made around the spermatic cords and their associated blood vessels and divided just distal to the ligature. The tied ends were returned to the abdominal cavity and the muscle layer was sutured with surgical silk. The skin was closed using Michel suture clips (size 3).

Once removed, the testes were dissected free of connective tissue and fat and weighed. The normal combined weight of the two testes was about 3gms. Rats which had testes weighing less than 2.5gms were not used in the experiments, as testicular weight was used as an index of normal functioning of the brain. The castrated rats were returned to the Animal Unit until the next morning.

ii) **Intracerebroventricular Infusion:**

Stereotaxic Apparatus:

All infusions were performed using a 10 µl graduated Hamilton Syringe mounted upon a David Kopf stereotaxic equipment (Tujunga - California). Three dimensional construction of the frames of this instrument allows the fixation of the rat's head at three different points. An ear plug was fixed at the external auditory meatus on either side and the nose was adjusted to a position in which the upper

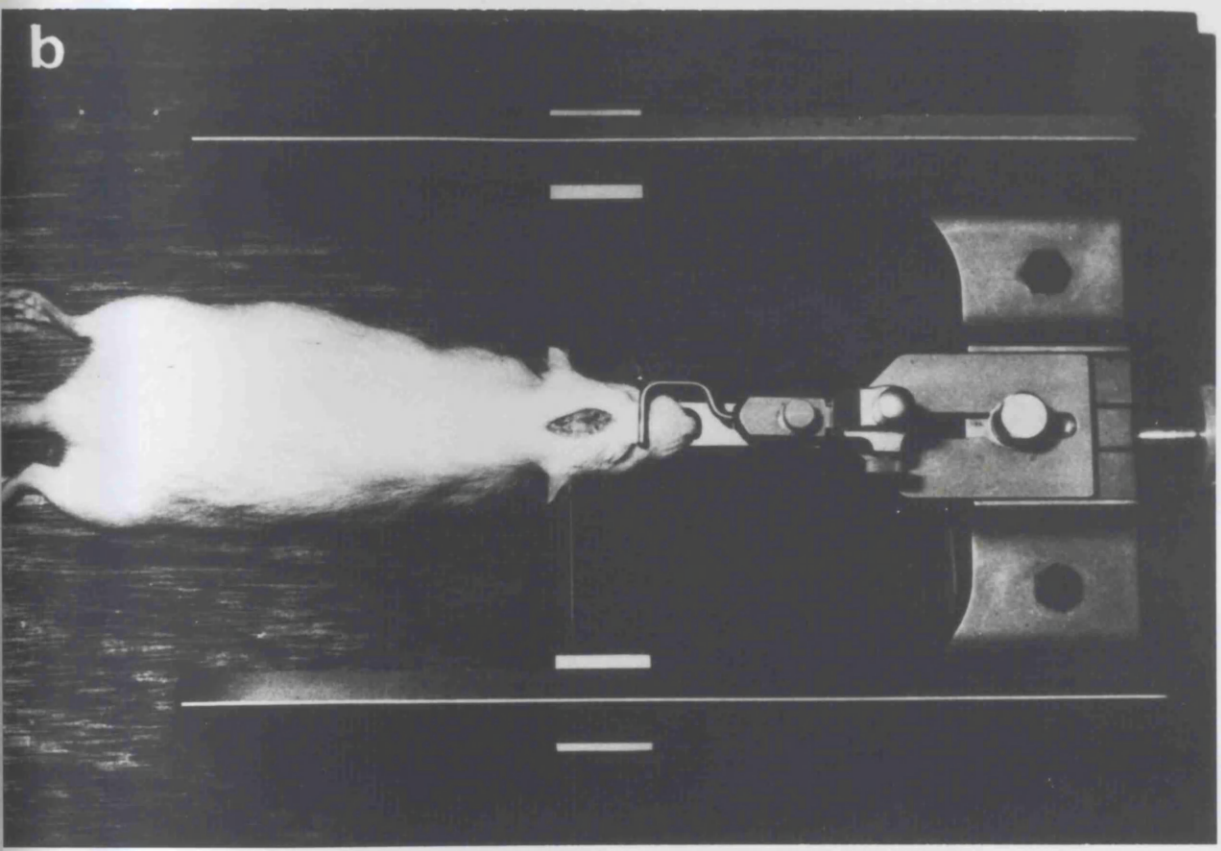


Figure 14a. Fixation of Rat in the Stereotaxic Apparatus for intraventicular infusion of pharmacological agents used in this study.

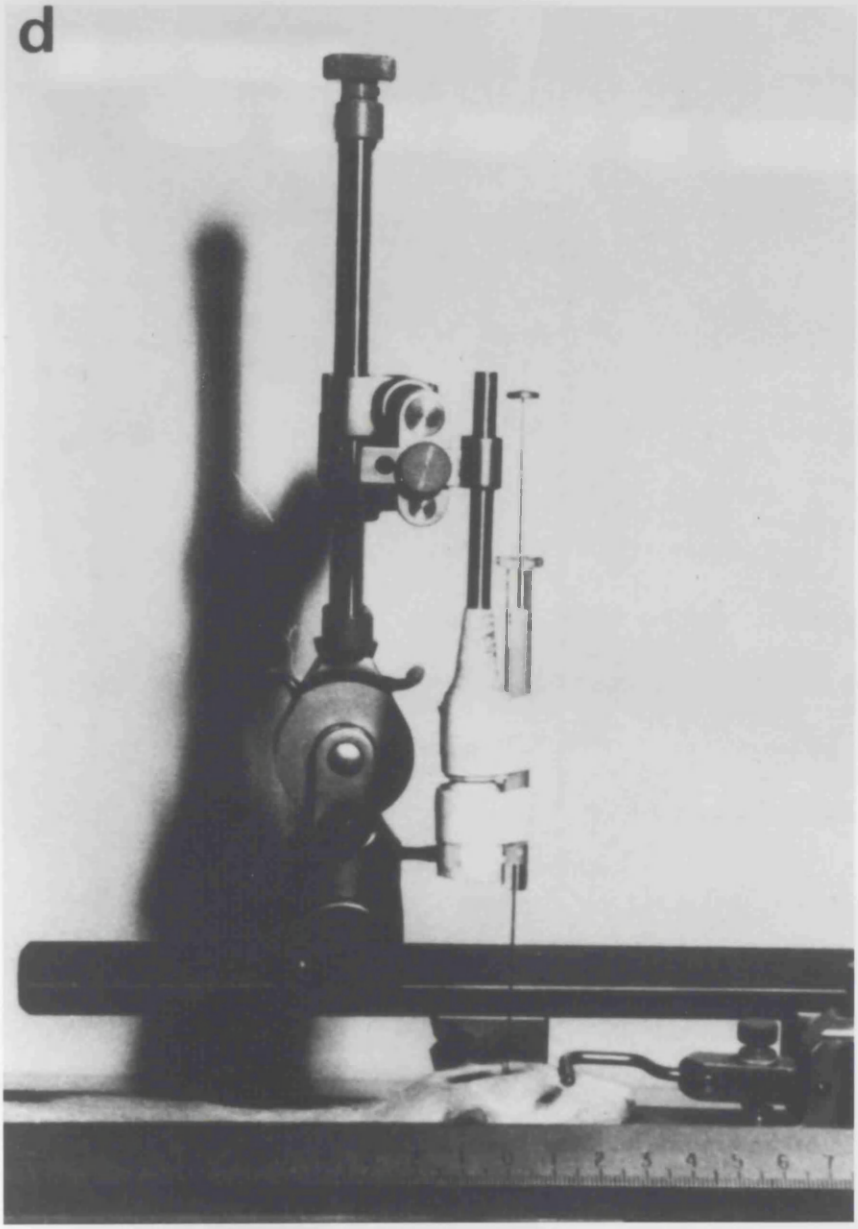


Figure 14b. Stereotaxic apparatus showing the intraventicular infusion technique.

incisor bar firmly supports the upper jaw at the gingival margin behind the incisor teeth and a clamp was lowered onto the nasal region. The interaural rostro-caudal fixation maintained a recognised position of the head during infusion (Figure 14 a,b). Thus the infusion apparatus could be moved anteriorly and posteriorly along the long axis of the skull and the underlying brain.

The placement of the syringe needle into the lateral ventricle was assessed with the aid of the De Groot Rat Atlas (1959) using bregma as the reference point. Bregma is where the frontal and parietal bones meet in the midline. The position of bregma according to the De Groot atlas was found to be approximately 5.9mm anterior to the vertical zero plane (A5.7 to A6.1) and 6.3mm above the horizontal zero plane (H + 6.1 to H 6.6)(Figure 15).

On the day following castration, the animals were anaesthetized with saffan (Althesin) (Glaxo, Ware, Herts) anaesthesia (2-3ml/rat i.p.). This steroid does not block preovulatory LH surge or GnRH surge in female rats (Sarkar, 1987). The anaesthetized rat was mounted on the stereotaxic apparatus. A midline incision was made in the scalp, the skin deflected and the membranous connective tissue scraped from the superior aspect of the skull. This revealed the bregma. The position of bregma was marked and the co-ordinates were noted. A hole was drilled at bregma using a 5mm dental burr to expose the superior saggital sinus from which a blood sample was collected (approximately 1 ml). The Hamilton syringe was filled with a freshly-made solution containing the drug under investigation, or with the vehicle

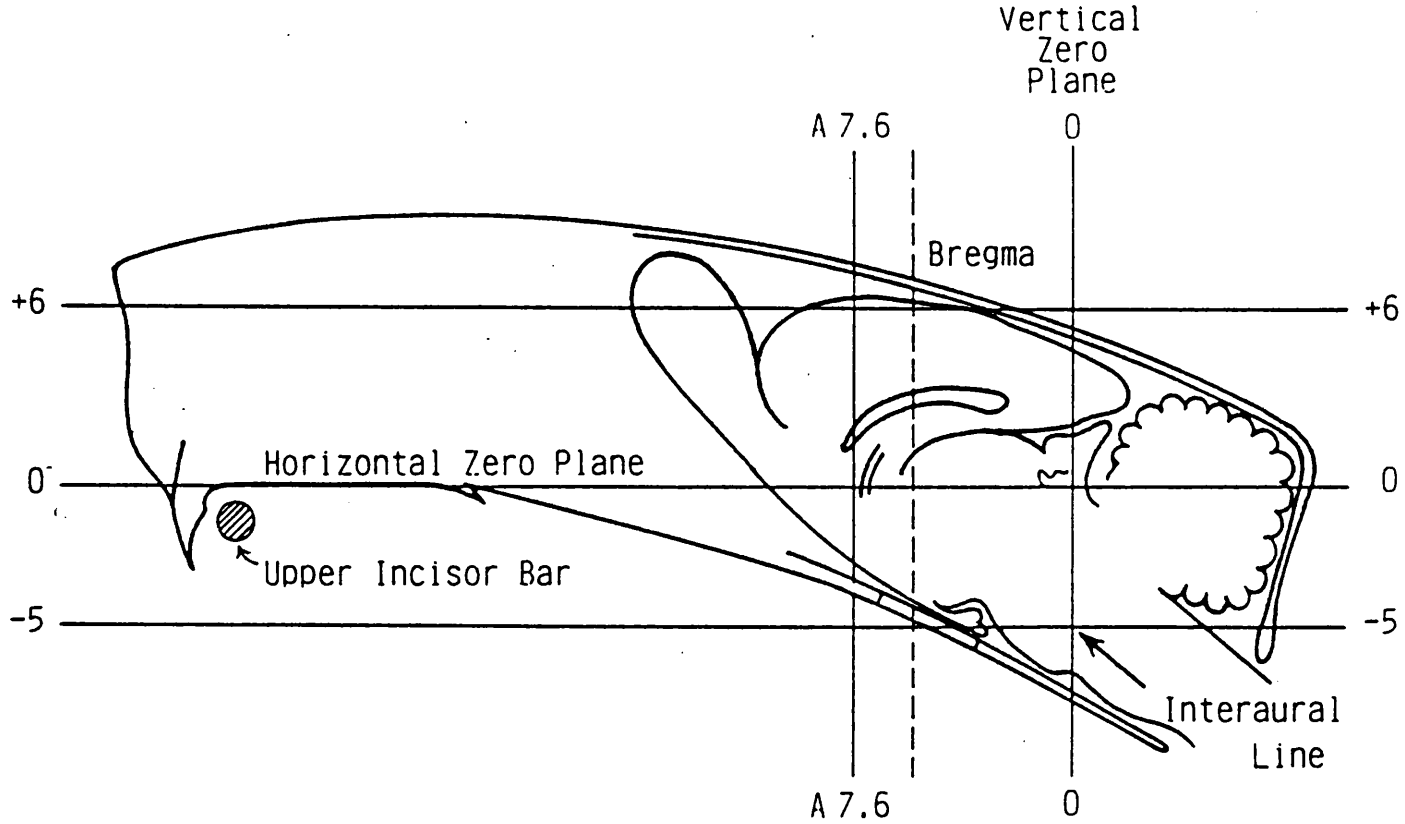


Fig 15 Mid-Sagittal section through the rat's head, illustrating the construction of the horizontal and vertical reference planes as it is in the stereotaxic equipment. (adapted from De Groot Rat Atlas, 1959).

alone. The infusion apparatus was placed on the stereotaxic frame and the needle tip lowered on to the brain surface and then advanced deeply to a pre-determined position (determined by reference to the De Groot Atlas). The solution was infused gently over a period of 60 seconds after which the syringe was raised and the infusion apparatus removed. Once the infusion had been completed, the animal was kept warm by wrapping in cotton wool. Blood samples were collected from the femoral vein at 15, 30, 60 and 90 minutes after the infusion. Immediately after each collection heparinized physiological saline (approximately 1ml) was infused via the femoral vein to compensate for the volume depletion. After the final blood collection, the heart was perfused with 10% formol saline and the brain removed from the skull and fixed in formol saline for subsequent histological verification of the needle positioning. Each blood sample collected was centrifuged at 4°C for 10 minutes at 3000 rpm. The plasma was snap frozen in liquid nitrogen and stored at -20°C until assayed for its LH levels at St.George's Hospital Medical School, London.

LH Radioimmunoassay:

Blood samples were placed in the relevant heparinized tubes and spun at 2000 rpm for 10 minutes at 4°C. The plasma was collected by aspiration and stored at -20°C. LH was measured in duplicate by a double antibody radioimmunoassay (RIA).

LH concentrations were determined by a heterologous double antibody RIA according to the modified method of

Naftolin and Corker (1971) as described by Kendle, Paterson & Wilson (1978). Ovine LH fraction LER-1056-C2 (provided by Professor L.E.Reichert Jr., Albany University, NY) was used for iodination by the chloramine T method and as the standard diluted to provide a range of 10 pg to 2560 pg per assay tube (covering 10 to 90% displacement of the label). The rabbit/rat LH antibody No. 15 (provided by Prof. G.D.Niswender, Colorado University) was used as the rat antiserum. The samples were counted on a LKB 1272 Glinigama Scintillation Counter with a 70% counting efficiency. LH concentrations were expressed as ng/ml in terms of the LER-1056-C2 standard (potency 1.73 times NIH-LH-S1). The intra- and interassay coefficients of variation were 9.8 and 9% respectively and the sensitivity of the assay was 2 ng/ml.

Histological Verification:

The brains fixed and stored in formol saline were rinsed with water. The frontal lobes and the hind brain were separated by coronal cuts. The dorsal portion of the brain was mounted on the specimen holder of a freezing microtome (Leitz-Wetzler) and then set at -80°C. The tissue was held firmly in place by encasement of the base in Cryo-Gel. 100 µm sections were cut and arranged on a slide in series. They were stained with thionin blue and examined under a dissecting microscope (x40 objective). The infusions were considered to be intraventricular if the tract of the needle was seen in the ventricle or if drops of blood were seen within its swollen cavity. 99-100% of the rats fulfilled one of these criteria.

Drugs

The pharmacological agents that were used in this experiment are listed in Al Hamood et al., (1987).

The above described methodology was employed in my thesis work mainly to enable me to become familiar with the various techniques involved, and also to finish some work that had already been commenced. However, a slightly modified method to that previously mentioned was applied in the reminder of the study.

Animals were castrated and the infusions were made as described above. The rats were kept under anaesthesia throughout the duration of the experiment. After infusion, the rats were divided into two groups. In the first group of animals, blood samples were collected at 0, 10 and 20 minutes after the drug administration, at which time the animals were decapitated by guillotine.

In the second group, the blood samples were collected at 0, 1 and 2 hours after the drug administration, at which time the animals were decapitated. The third and the final blood sample (at 20 minutes in the first group and two hours in the second group) was collected from the trunk at the time of decapitation. All the samples were centrifuged and the plasma stored at $-20^{\circ}C$ for LH radioimmunoassay.

Removal of the Brain and Isolation of the Hypothalamus:

The decapitated head was immediately placed on crushed ice. The skin lying over the skull was removed and the

occipital muscles from the occipital bone cut to reveal the foramen magnum. One blade of the scissors was cautiously introduced into the foramen. Keeping the blade parallel to the inner wall of the calvarium and as close to it as possible to avoid damaging the brain, cuts were made through the bone. These cuts were extended rostrally as far as bregma. After two parallel cuts had been made through the bone on each side of the skull, the bone was lifted forward and its rostral point of attachment separated. The closed scissors were then slipped under the frontal lobe and the brain lifted upwards. The optic and trigeminal nerves were cut, and the whole brain was coaxed gently out of the skull (Palkovits and Brownstein, 1983). The entire procedure was carried out within about 60 seconds to prevent *post mortem* degradation of the amines prior to the assay.

The hypothalamus was removed en bloc by a saggital cut medial to the temporal lobes and by a coronal cut rostral to both the optic chiasma and mammillary bodies. Once removed, the hypothalamic sample was immediately frozen in liquid nitrogen. It was then weighed and placed in a glass homogenizer, kept in ice. 600 µl of 0.1M hydrochloric acid containing 100 ng of an internal standard - IS (Dihydroxy benzyl amine - DHBA) was added and the tissue homogenized for a period of 60 seconds. HCl precipitates proteins and hence inactivates oxidising enzymes (monoamine oxidase), in addition to chemically stabilizing the amines. The homogenate was centrifuged at 4°C and the supernatant thus obtained either injected immediately on to the reversed-phase High-Performance Liquid Chromatography (HPLC) column coupled

to an electrochemical detector (ECD) or snap frozen in liquid nitrogen and stored at -80°C until assayed. However, all samples were analysed within a month of their preparation. 20 µl of the supernatant was injected on to the HPLC column in duplicates and the concentrations of NA, ADR, DA, 5-HT and its metabolite 5-HIAA were simultaneously measured.

The total content of the individual amine in each sample was divided by the wet weight of the hypothalamus and the concentration expressed as ng/gram wet weight of the tissue.

Histology:

As the brains were removed without fixation for hypothalamic sampling, a slightly different method had to be adopted to check for the position of infusion needle. After the separation of the hypothalamus, rest of the brain was frozen on ice. The hind brain was separated from the remaining tissue and the dorsal portion mounted on the specimen holder of a freezing microtome. 100 µm sections were cut and immediately checked for the needle placement under the dissecting microscope.

Opiate Drugs used in the Experiment:

Various opiate receptor agonists and the opiate antagonist, naloxone, were dissolved in physiological saline and administered in a 10 µl volume 18-20 hours postcastration.

Drug	Dose (μg/kg)	Receptor Subtype
Naloxone	30, 60	Opiate antagonist
Morphine	400, 800	Mu agonist
Levorphanol	400	Mu agonist
Cyclazocine	50	Kappa agonist
SKF 10,047	100	Sigma agonist
FK 33 824	2	Met-enkephalin analogue

Table 2. Names, dosages and the specific opiate receptor agonists and the antagonist used in the study.

Estimation of Turnover:

It has long been recognized that measurements of brain content or concentrations of amines do not provide a true index of their functional activity (Brodie et al., 1966). For this reason, turnover studies have been used to acquire more meaningful information on the dynamics of change in the aminergic system.

The turnover of the catecholamines, NA and DA, were measured using α-methyl-DL-para-tyrosine methyl ester HCl(α-MPT) (Sigma Chemicals, Poole, Dorset), a competitive inhibitor of tyrosine hydroxylase, to block the rate-limiting step in catecholamine biosynthesis (see Brodie et al., 1966). The rate of serotonin synthesis was measured by using the steady state kinetics in which rates of synthesis and loss are equal (Tozer, Neff & Brodie, 1966).

Experimental Design

The second group of animals, from which the blood samples were collected at 0, 1 and 2 hours used for the study of amine turnover. The rate of fall of biogenic amines was observed at two different intervals by blocking the synthesis of catecholamines by α-MPT.

a) **Control Group (Group 0):**

Rats were infused with one of the specific opiate receptor agonists or the opiate antagonist, naloxone, and decapitated after two hours of treatment. No α-MPT was given at any stage, and those thus served as controls.

b) **Group I:**

Rats were infused with one of the above mentioned drugs, and after an interval of one hour, the animals were injected with 1 ml of α-MPT (250 mg/kg, i.p.) dissolved in physiological saline. Thus the synthesis of catecholamines was blocked for an hour before the animals were decapitated.

c) **Group II:**

Immediately after infusion, in this group, the animals were given α-MPT thus blocking the catecholamine synthesis for two hours before decapitation.

Blood samples were collected at 0, 1 and 2 hours after drug administration and the plasma stored for LH assay. The brains were removed and the hypothalami separated. After homogenization and centrifugation, the supernatants analysed

for amine content using HPLC.

Calculation of turnover and rate constants

Catecholamines

Turnover rates and rate constants were calculated by the method of Brodie et al., 1966.

The values for the tissue levels of NA and DA were logarithmically transformed for calculation of linearity of regression, standard error of the regression coefficients and significance of differences between regression coefficients. A regression curve was obtained for the amine concentrations against time by computer (Glasgow V.M.E.).

The regression equation was of the form $y = mx + C$ with gradient m and intercept C on the Y axis.

$$\text{Equation of the line: } \ln[CA] = kt + \ln[CA]_0$$

where [CA] is the concentration of catecholamine, (ng/gm), $[CA]_0$ is the concentration of catecholamine at time = 0 (ng/gm), k is the rate constant of amine depletion (hr^{-1}), t is the time (hours).

The linear value of the intercept $\pm$ its standard deviation was calculated by taking the antilogarithms. The mean intercept was multiplied by k to obtain a turnover rate (TR), the unit of which was ng amine/gram tissue/hour. The standard deviation (SD) of the turnover was calculated by the

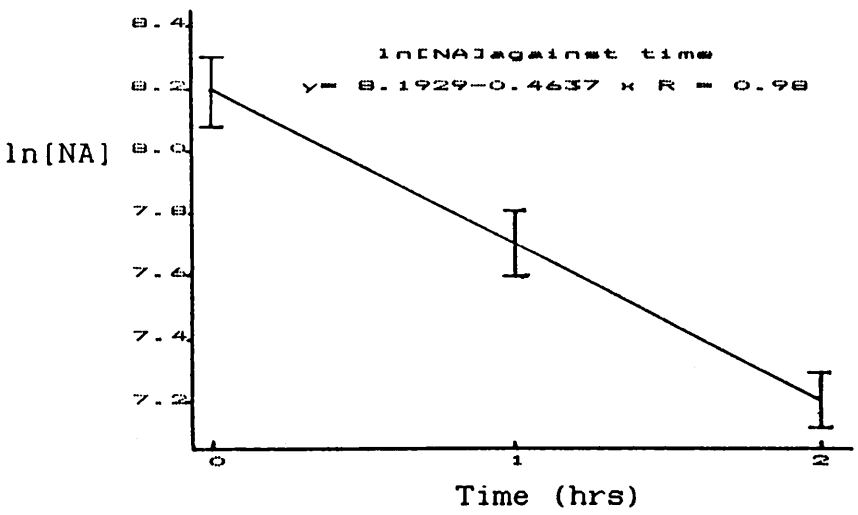
following formula (Hohn & Wuttke, 1979):

$$SD_{TR} = \sqrt{\left[(k)^2 (SD_{[CA]_o})^2 + 2(k)[CA]_o(\text{Covariance}) + (SD_k)^2 [CA]_o^2\right]}$$

where $SD_{[CA]_o}$ is the standard deviation of the intercept and SD_k is the standard deviation of the rate constant k; both values were obtained by the computer. The covariance was calculated from this expression,

$$\text{Covariance} = \frac{-\bar{X}\sigma^2}{\Sigma(X-\bar{X})^2}$$

where $\bar{X}$ is the mean of all observations of X (hours). σ^2 is the residual sum of squares divided by the degrees of freedom f_1 (where $f_1 = n-2$) of which both relate to the regression equation, and $\Sigma(X-\bar{X})^2$ is the sum of the squares about the mean, $\bar{X}$.



Regression Analysis of Amine Depletion

Serotonin

The turnover rate of 5-HT was calculated by taking the ratio between the content of 5-HT and its metabolite 5-HIAA.

Catecholamine Turnover: Statistical Analysis

Catecholamine turnover constants, initial tissue levels and turnover rates in the drug-treated groups were compared with the saline-treated groups. The degree of significance between the control and the drug-treated groups were determined by the Student's t-test with Fisher's adaption for multiple comparisons using the following formula:

$$t = \frac{\bar{X}_c - \bar{X}_t}{\sqrt{(se_c^2 + se_t^2)}}$$

where $\bar{X}_c$ is the mean value in the control group and se_c, the standard error of this value and $\bar{X}_t$, the mean value in the treated group and se_t, the standard error.

Serotonin Turnover: Statistical Analysis

The ratio between 5-HIAA and 5-HT was calculated for individual values obtained. The figures were converted to their percentage values. An unpaired Student's t-test was applied to calculate the level of significance between the control- and the treated-groups.

2. Adult female rats

3-4 month old rats weighing between 200-300g were purchased from Tuck and Sons, Battlesbridge, Essex. Upon their arrival in the Animal Unit, they were numbered and vaginal smears taken. The technique of smearing for the assessment of changes in the vaginal cytology is simple and rapid (Stockard & Papanicolaou, 1917). The smearing procedure involved swabbing of the vaginal lumen and examining the smear obtained under the x100 objective of a light microscope. Four different stages of the oestrous cycle were determined by the cell type present in the vaginal smear.

Stage of Cycle	Vaginal Smear
Dioestrus I	Nucleated epithelial cells and polymorphonuclear leucocytes with some keratinised squamous epithelial cells.
Dioestrus II	Nucleated epithelial cells and leucocytes.
Pro-oestrus	Nucleated epithelial cells.
Oestrus	Keratinised squamous cells only.

Table 3. Assessment of oestrous cyclicity by vaginal cytology.

The vaginal smears were monitored every day and only those rats showing three or more consecutive 4-day oestrous cycles were selected on the day of pro-oestrus for use in the experiment. The onset of pre-ovulatory LH surge occurs between 14.00 - 15.00 hours which reaches a peak at 16.30

hours on the day of pro-oestrus (Blake, 1976), and the GnRH surge slightly precedes the LH surge which it evokes (Sarkar et al., 1976). The central neurotransmitters have been shown to be involved in regulating this process (Cramer & Barraclough, 1978). Therefore, the specific adrenergic and the opiate agonists and antagonists were administered between 12.30 - 13.30 hours so that they would interfere with these surges and the animals were decapitated at 14.30 hours as it was anticipated that by this interval changes would have occurred in the amine concentrations and LH levels. Between 5-10 animals were used in each experimental group studied.

Three different types of experiments were used.

i) Effects of specific adrenergic agents on hypothalamic catecholamine levels

Rats were given pentobarbitone sodium (PB), 35mg/kg, i.p., at 13.30 hours on the afternoon of pro-oestrus to block the pre-ovulatory LH surge (see introduction). To determine the effects of stimulatory adrenergic agents, these were injected (i.p., 20mg/kg) in a 1ml volume of either physiological saline or 5% glucose between 14.00 and 14.30 hours. The animals were decapitated between 15.30 and 16.00 hours.

Adrenergic blocking agents were dissolved in 5% glucose and administered (i.p., 20mg/kg) to pro-oestrous rats at 13.30 hours. These animals had not previously been treated with PB. The rats were decapitated between 14.30 and 15.00 hours.

The trunk blood was collected at the time of decapitation, centrifuged and the plasma stored at -20°C for LH assay.

The brains were removed, hypothalami isolated and the samples prepared as already described. 20 µl aliquots of the supernatant was injected on to an HPLC column and the concentrations of NA and ADR were simultaneously analysed.

Drug	Receptor Subtype
Clonidine	α_2-agonist
Yohimbine	α_2-antagonist
Salbutamol	β_2-agonist
Fenoterol	β_2-agonist
ICI 118,551	β_2-antagonist

Table 4. Names and the specific adrenergic receptor agonists and antagonists used.

ii) Effects of opiates on the hypothalamic aminergic content:

Various opiate receptor agonists and the opiate antagonist, naloxone, were dissolved in physiological saline and administered in the dosages shown in Table 5, in a 1ml volume, at 12.30 hours on the afternoon of pro-oestrus.

Three different modes of injection were used: intraperitoneal, subcutaneous and intraventricular. Tifluadom, a kappa receptor agonist, was more potent when administered subcutaneously than when given intraperitoneally (Morley et al., 1983; Cooper et al., 1985). A similar mode of

injection was also used for SKF 10,047. Subcutaneous injections are known to cause discomfort and so were performed under light ether anaesthesia. However, a group of control animals treated in the same manner were used for comparison. The synthetic met-enkephalin analogue, FK 33,824 does not cross the blood-brain barrier (Wilkinson & Bhanot, 1982). Therefore, this drug was administered directly into the brain ventricles using the stereotaxic apparatus by the procedure already described. However, a control group treated in the same manner was also used. There were, thus, three different control groups, one treated intraperitoneally, the second treated subcutaneously and last group treated intracerebroventricularly.

Drug	Dose (mg/kg)	Mode	Receptor Subtype
Naloxone	10	ip	Opiate antagonist
Morphine	10,40	ip	Mu agonist
Levorphanol	10	ip	Mu agonist
cyclazocine	5	ip	Kappa agonist
Ketocyclazocine	5	ip	Kappa agonist
Tifluadom	10	sc	Kappa agonist
SKF 10,047	10	sc	Sigma agonist
FK 33,824	2,4	icv	Met-enkephalin analogue

Table 5. Names, doses and modes of injection and main receptor specificity of the opiate drugs used.

After an interval of two hours, the rats were

decapitated, brains removed and the hypothalami separated. The hypothalamic samples were homogenized and centrifuged at 4°C. The supernatant was analysed for NA, ADR, DA, 5-HT AND 5-HIAA using the HPLC.

FK 33,824, a superactive enkephalin analogue, had no significant effects at the two different doses studied and hence was omitted from further experimental studies.

Turnover studies:

The rats treated with specific opiate receptor agonists or the antagonist, naloxone, at 12.30 hours on the day of proestrus were given α-MPT (250 mg/kg, i.p.) at two different intervals.

Control (Group 0):

Pro-oestrous rats were treated with the drug at 12.30 hours and decapitated at 14.30 hours at which time trunk blood was collected for LH analysis. These animals served as controls as they were not injected with α-MPT at any stage before decapitation.

Group I:

Various opiate agonists or the antagonist, naloxone, were injected into pro-oestrous rats at 12.30 hours and α-MPT, a tyrosine hydroxylase blocker, was given at 13.30 hours to block the rate-limiting step of catecholamine biosynthesis. The animals were decapitated at 14.30 hours and the blood sample collected. Thus the synthesis of catecholamines was blocked for 60 minutes before

decapitation.

Group II:

α-MPT, dissolved in physiological saline was injected in a volume of one ml at 14.00 hours followed by the administration of specific opiate drugs. The animals were decapitated at 14.30 hours and the blood sample collected for LH determination. In this group, the catecholamine synthesis was blocked for 30 minutes.

The calculation of turnover of catecholamines, NA and DA using a blocker, and the indoleamine, serotonin using the steady state kinetics has been described in the earlier part of this section.

iii) The effects of opiates on biogenic amine levels in specific hypothalamic regions

Studying the effects of drugs on central neurotransmitter levels in the whole hypothalamus lacks specificity as there are several nuclei included within the hypothalamus. To study the action of the drugs under investigation on these nuclei, Palkovit's technique of punching the specific areas within the brain was developed, and the earlier work described in experiment 2 was repeated.

Brains were removed as previously described and were frozen immediately on dry ice in an upright position to ensure the brain remains symmetrical. Under these conditions there are no detectable changes in amine concentrations (Gaitonde, 1971). The frozen brains were wrapped tightly in

seacon film to prevent dehydration of the brain and stored at -80° C for a maximum of one month before analysis. Samples stored at -80° C displayed almost negligible degradation of any components (Kilpatrick et al., 1986).

Microdissection (Palkovits's) Technique:

The frozen brains were allowed to partially thaw to promote adherence. The hind brain and the anterior most portion of the forebrain were removed by coronal cuts. The remaining portion of the brain was mounted on the specimen holder of a freezing microtome which was then set at -70°C. The base of the tissue was held in place using Cryo-Gel. There are several landmarks in various parts of the brain which are of assistance in cutting the sections (see Palkovits & Brownstein, 1983). The first one of importance in punching the hypothalamic nuclei was the fusion of the corpus callosum. 100 μm sections were cut until this fusion was confirmed. Subsequently, serial 500 μm coronal brain slices were taken in the frontal plane and arranged on labelled slides (0-11) kept on dry ice. The last section (No. 12) was at the mamillary peduncles. Cut in this manner the whole of the hypothalamus which measured 4.2 mm in the rostral caudal diameter in a 200g rat, is contained within the last 8 (4-11) sections. The remainder of the brain was discarded. There were no punches made from the first four sections (0-3); they were merely cut to ensure the correct plane of sectioning. It was necessary to have ample space for correction of the sectioning plane. The sections numbered 4-11, which contained the areas of interest, were, in turn,

transferred on to a cold plate (set between -5°C to -20°C) under a dissecting microscope (x40 objective). Bilateral homologous tissue samples of suprachiasmatic nucleus (SCH), medial preoptic area (MPO), anterior hypothalamic area (AHA), median eminence (ME), ventromedial hypothalamus (VMH) and arcuate nucleus (ARN) were dissected from the sections using a stainless steel hypodermic needle with 0.7 mm or 0.9 mm internal diameter by cutting and sharpening the tip perpendicular to the long axis. A separate needle was used for each area and washed thoroughly with absolute alcohol between each brain dissection. The needle was always kept cold on dry ice before its use. The location of discrete brain regions were identified by reference to the Paxinos and Watson atlas (1982) in conjunction with section number and standard neuroanatomical landmarks such as the ventricle and midbrain (Palkovits, 1973) in a standardized manner. For a complete review of the methods and equipment required for microdissection the reader is referred to Palkovits & Brownstein (1983). To position the tip of the punch, the section was approached with the punch held at an angle of 45° to the surface of the section. When the tip of the needle was in the appropriate location, it was fixed with gentle pressure to the surface of the section and then the punch was brought into a vertical position and pressed into the brain slice. Finally, the punch was rotated to free the sample from the surrounding tissue and withdrawn. After a successful withdrawal of the sample, a sharp edged hole remained in the section (Figure 16) and the tissue disc was held within the needle. If tissue segments were retained in

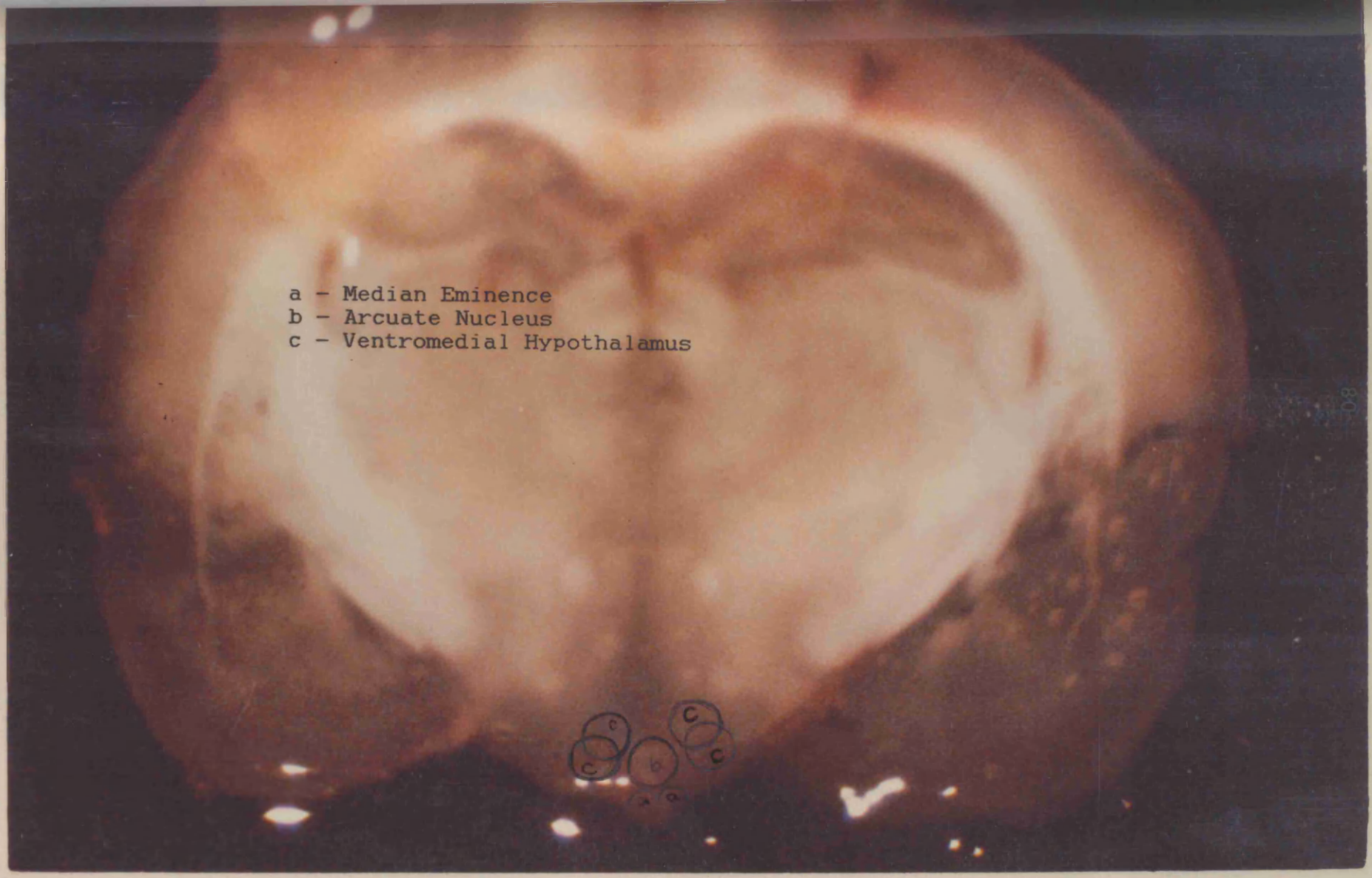


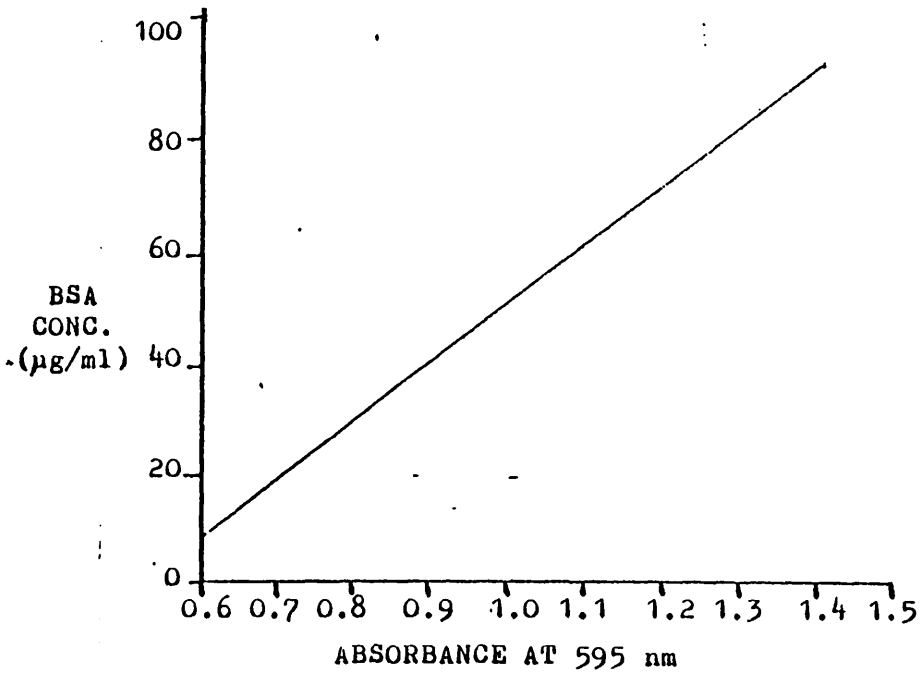
Figure 16. A coronal section of the rat brain (No. 8) showing various hypothalamic regions.

the punched area, or if the entire sample was left behind, the needle was replaced in its original position and the punching repeated. The tissue disc was pushed out of the punch into the bottom of the disposable polyallomer centrifuge tube and stored at -80° C without the addition of any fluid to increase the stability of the amines within the tissue. On the day of analysis, 75 µl of 0.1M hydrochloric acid containing 12.5 µg of DHBA as an internal standard was added to each tube and the tissue homogenized by sonication for about 5 seconds in a sonication bath. The homogenate was centrifuged at 4°C and the supernatant injected on to the HPLC column and analysed for NA, ADR, DA, 5-HT and 5-HIAA.

Protein Estimation:

The amount of brain tissue in the homogenate was estimated by its protein content. Since the protein content of the brain is fairly stable, the amount of protein in a brain extract is proportional to its tissue content. The protein pellet contained within the centrifuge tubes were stored at -80°C for protein determination by a modified comassie blue method of Bradford (1976) using Pierce reagents (Pierce, Chester, England, UK).

A standard curve of protein concentration versus absorbance was obtained by the reaction between known weights of bovine serum albumin (BSA-Sigma Chemical Co., Poole, Dorset) (2.5 - 100 µg) and Comassie Blue G250 Pierce protein assay reagent (Figure 17). The absorbance was read at 595 nm using an LKB Biochrom Ultrospec 4050 spectrophotometer.



Regression Equation:-

Protein Concentration= 110.417(Absorbance)-58.981

Correlation Coefficient= 0.959

Standard Error= 10.034

Figure 17. Standard curve of concentration of bovine serum albumin (BSA) versus absorbance at 595 nm immediately after reaction with Coomassie Blue G250

The sample homogenate was dispersed in 200 µl of 0.1N NaOH and incubated overnight at 4°C. The following morning, sample tubes were sonicated for 1-2 minutes. Duplicate 100 µl samples were transferred into two separate tubes and each tube was diluted with 900 ul distilled water. 1 ml of the assay reagent was added to the diluted samples and the absorbance read immediately against the blank at 595 nm.

The protein concentration (µg/ml) in each tube was calculated by substitution of its absorbance into the regression equation of the standard curve (see Figure 17). The protein content of the duplicate tubes were added together to give the total protein concentration of the original sample.

The total content of the individual amines in each sample was divided by the total amount of protein in that sample to give the concentration of each amine in the original tissue in pg/µg protein.

Turnover Estimation:

For the turnover estimation, the specific opiate agonists and the antagonist were limited to one in each receptor subtype studied. This is listed in Table 6.

The turnover studies described for pro-oestrous rats, where the whole hypothalamic amine concentrations were measured, was repeated to study the effects of opiates on specific hypothalamic nuclei. Here, the catecholamine synthesis was blocked for two intervals of 45 and 90 minutes.

Control animals were not treated with α-MPT, one set of animals received α-MPT at 13.45 hours. Another set of animals received α-MPT at 13.00 hours. The turnover calculations were similar to the one previously described.

Drug	Dose (mg/kg)	Mode	Receptor Subtype
Naloxone	10	ip	Opiate antagonist
Morphine	40	ip	Mu agonist
Tifluadom	10	sc	Kappa agonist
SKF 10,047	10	sc	Sigma agonist

Table 6. Names, doses, modes of injection and the receptor specificity of the drugs used.

Amine Analysis: High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ECD)

As all the monoamines including 5-HT and 5-HIAA were of interest, this study employed the more recent method of direct injection of sample supernatant into the high performance liquid chromatography system with electrochemical detection (HPLC-ECD), without prior extraction procedures. This reduces sample preparation time and eliminates the need for evaluation and calculation of recovery rates, thus increasing analysis efficiency and also reducing the possibility of technical errors.

There are, however, certain disadvantages with this direct method, such as the resulting large solvent front. This does not allow the analysis of some of the metabolites

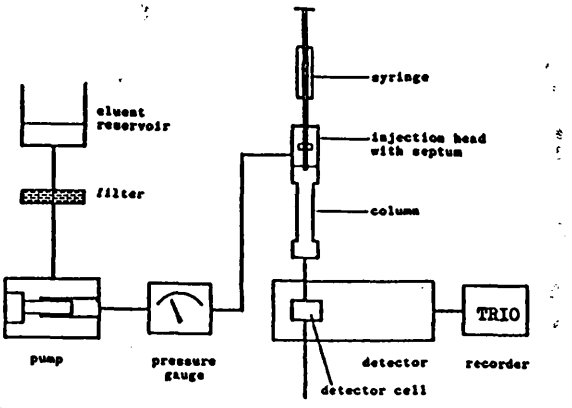
of NA which elute along with the solvent front. However, these metabolites are not of interest in this study as NA turnover was measured by the α-MPT depletion method. The second disadvantage is that the cell surface becomes coated with proteinaceous material very quickly reducing the sensitivity. This problem was alleviated by incorporating a precolumn into the HPLC system to remove residual microparticulate and/or dissolved proteinaceous material in the sample. In addition, the working electrode was frequently cleaned by alumina polishing or treated with chromium trioxide (200mg CrO_3, 0.3ml H_2O, 9.7ml H_2SO_4 for 30 seconds as described by Anton (1984) if responsiveness was severely impaired.

The simultaneous determination of NA, ADR, DA, 5-HT and 5-HIAA make considerable demands on the chromatographic system, as it entails the separation of compounds of different polarities such as basic (NA, ADR, DA and 5-HT) and acidic (5-HIAA). HPLC in the reverse phase mode, including ion pair offers selective separation. The composition and conditions of the mobile phase are the main determinants of solute separation.

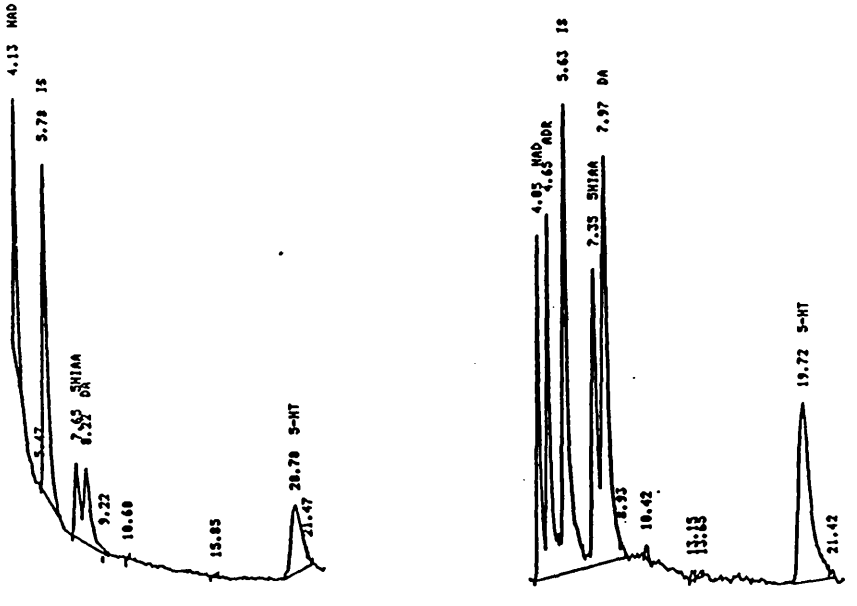
The main components of the HPLC system are schematically represented in Figure 18. The apparatus consisted of:

a) Column:

The column (Shandon Instruments, Runcorn, England) was packed with ODS (octadecyl silane) hypersil (5 μ) packing material using a column packing device. The octadecyl



Simplified diagram depicting HPLC-ECD. The sample is injected using a micro-syringe. The amines are separated by the column and detected electrochemically, whereupon the "TRIO" displays and prints a chromatogram.



Above are shown chromatograms which were plotted from a sample (left) and a standard (right). The computer is calibrated with the standard amine mixture, thereafter it identifies the internal standard (IS) in the sample and calculates the amounts of amines present.

Constituents of mobile phase.

Citric acid	6.74g
Sodium acetate	4.81g
Glacial acetic acid	1.15ml
Sodium EDTA	47mg
Sodium octane sulphonate	120mg
Tetra hydro furan	12ml
Methanol	50ml

Made up to 1 litre with distilled water.

Figure 18. High Performance Liquid Chromatography with Electrochemical Detection

hydrocarbon "tails" radiate from the silica particles, effectively forming a polar stationary phase. This forms a column environment suitable for reverse phase chromatography. An ion pairing agent (octane surphonic acid; HPLC grade, Fisons) was used to render the amines more lipophilic, this ensures their adequate column separation. The length of the column was 250mm long and 5mm in diameter. The column was protected by a precolumn which was hand-packed with the same material as was the column. The precolumn prevented the contaminated substances from the sample entering into the column itself thus increasing its life time. The precolumn often had to be cleaned by removing the top of the packing and replacing it with fresh ODS. This column was replaced by a shorter column (150 mm long and 4.6 mm wide; Beckman Instruments, England) to reduce the run time in the later part of the study. The packing material used was the same as in the other column. This also had a pre-column purchased from Beckman Instruments, England.

b) Pump:

A Gilson 302 pump capable of producing high pressure (up to 5000 psi) with a monometric module (Gilson, France) delivered the solvent through the column. The flow rate was set at 1.5 ml/minute during the analysis. The system was set to a low flow rate 0.15 ml/minute at other times.

c) Solvent:

An aqueous buffer constantly used consisted of 0.1 M sodium acetate, 0.1 M citric acid, 19 mM glacial acetic acid,

126 µm Na EDTA, 5% (v/v) methanol, 1.2% (v/v) tetrahydrofuran (THF) and 431 mM sodium octyl sulfate. This was adjusted to a pH of 4.9 with 10M sodium hydroxide to ensure complete ionisation of biogenic amine molecules. HPLC grade reagents and deionised water were used for the preparation of the solvent. The buffer was filtered under pressure through a millipore solvent clarification kit with 0.45 µm aqueous filters (Scotlab, Bellshill, Scotland) before use. This prevents any dust particles entering into the system. The solvent was then degassed with helium for 20-30 minutes. The occurrence of air bubbles could be eliminated (which increases the baseline noise) by this procedure. The solvent was recycled and renewed every 10-15 days.

d) Sample loading:

The sample was introduced on to the top of the pre-column through an injector valve (Rheodyne "7125", California). The valve was then turned from 'load' position to 'inject' position thereby diverting the solvent through the loop to carry the sample on to the pre-column. It was flushed with deionised water in both positions before the next sample was injected.

e) Detector:

To detect and quantitatively measure the separated compounds, an electrochemical detector was used.

The potentiostat system (detector) consists of a carbon working electrode, a platinum wire auxiliary electrode and a silver/silver chloride reference electrode.

The cell consists of a Plexiglass block divided in half by a 0.2 mm polytetra fluoroethylene (teflon) gasket. A flow cell with a volume of <1ml, containing the glassy carbon working electrode, is formed by a slit (1.6cm x 0.5cm) cut in the gasket.

Electrochemical detection is based on the principle that compounds capable of oxidation or reduction in an electrical field result in the passage of current and the magnitude of this current is a measure of the quantity of compound oxidised or reduced. Each chemical reaction has a threshold voltage related to its Redox Potential and the potentiostat, a form of feedback voltage control, allows this voltage to be set for the particular compound to be measured. This introduces some measure of selectivity into the system. For catechol- and indoleamines a +ve potential of 0.65 V was used.

An electronic controller provides the operating potential (+0.65 V) with reference to an Ag/AgCl reference electrode (sensitivity 1nA, time constant 5 sec). When the signal passes across the working electrode it is oxidised thus giving up electrons to it and thereby generating an oxidation current. Provided the flow rate, temperature and working electrode area remain constant, the current detected is directly proportional to the concentration of the amine passing across the electrode. The potentiostat amplifier maintains the constant preset potential across the electrochemical detector and amplifies the nanoampere oxidation current to provide a proportional output voltage

which is displayed on the recording integrator.

f) **Chromatogram:**

As oxidisable bands of each compound pass the detector, the current (and resultant voltage) rises and falls, as a function of time to yield a liquid-chromatography, electrochemical chromatogram (Figure 18). The first peak on the chromatogram was the solvent front representing the oxidation of hydrochloric acid which has the shortest retention time and leaves the column first. This was followed by NA, ADR, IS, 5-HIAA, DA and finally 5-HT. As 5-HT remains in the column longest, it is susceptible to band broadening and hence the peak is short and broad compared to the other five. The system enables the detection of amines both at picogram and nanogram levels and within the range of amounts assayed the relationship between amount of amine and response amplitude is linear. Chromatograms were analysed by Trivector computing integrator (Scotlab, Bellshill, Scotland) and quantified using the peak area ratio of IS to analyte method.

Standards:

Stock solutions of noradrenaline bitartrate, adrenaline bitartrate, 3-hydroxy tyramine (dopamine) hydrochloride, 5-hydroxytryptamine creatinine sulphate complex, 5-hydroxy indole-3-acetic acid and of internal standard, 3,4-dihydroxy benzylamine hydrobromide were prepared in 0.1M HCl. They were stored at 4°C and renewed every four weeks. The standard solution containing 2 ng/20 µl was made up every

day by diluting the stock standards with 0.1M HCl and used for calibrating the system for amine analysis.

RESULTS

Effects of intracerebroventricular administration of opiates on Hypothalamic Monoamine Content, turnover and Plasma LH in Short-term Castrated Rats

a) *Hypothalamic Monoamine content 20 minutes post-infusion:*

Treatment	Receptor properties	n	ng/gm wet weight of tissue			
			NA	DA	5-HT	5-HIAA
Saline	–	9	3704±138	474±53	1511±101	1136±105
Naloxone (30µg/kg)	opiate antagonist	8	3252±202	663±45*	–	–
Naloxone (60µg/kg)	" "	9	4323±195*	624±43	1715±251	1248± 98
Morphine (400µg/kg)	µ-agonist	12	2888±212*	364±71	–	–
Morphine (800µg/kg)	µ-agonist	10	3063±206*	374±50	1464±121	1108±101
Levorphanol (400µg/kg)	µ-agonist	6	2545±181**	552±39	2161±259*	1151± 85
Cyclazocine (50µg/kg)	k-agonist	5	2609±270**	363±45	2322±259*	631± 96*
SKF 10,047 (100µg/kg)	σ-agonist	7	3469±280	556±37	3089±337**	896± 67
FK 33,824 (2µg/kg)	Met-enk analogue	9	3238±202	513±43	1692±152	927±148

Table 7. Values are Mean ± SEM; Unpaired Student's t-test; Level of significance * $p<0.05$ and ** $p<0.01$. 'n' = Number of observations.

Morphine (400µg and 800µg/kg), levorphanol and cyclazocine significantly decreased, and naloxone significantly increased the NA content of the hypothalamus compared to the controls. On the other hand, DA levels were not significantly altered by any of the opiate-agonists administered; only naloxone (30µg/kg) brought about a significant elevation in DA concentrations. ADR levels were

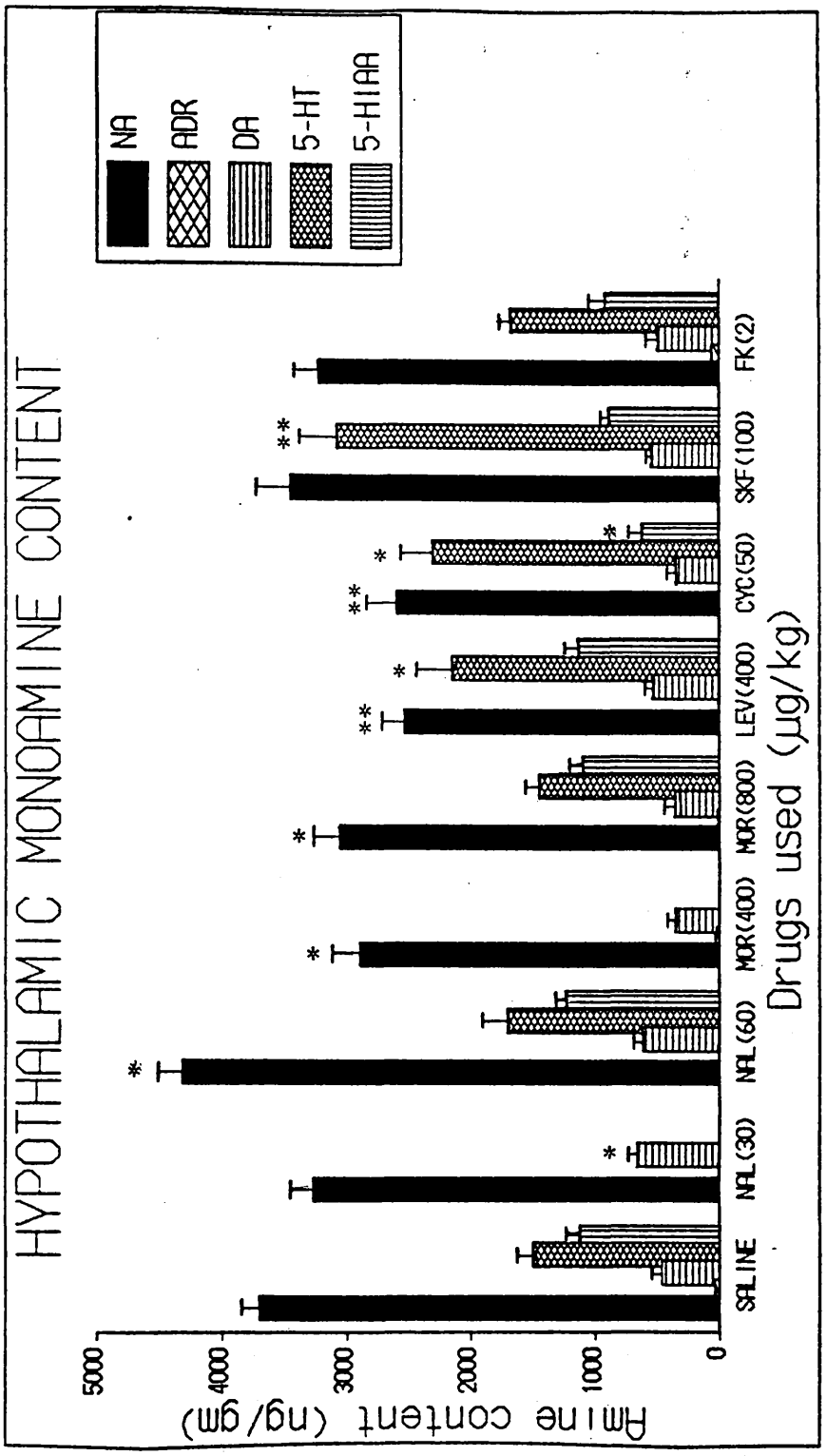

HYPOTHALAMIC MONOAMINE CONTENT
NA
ADR
DA
5-HT
5-HIAA
Amine content (ng/gm)
5000
4000
3000
2000
1000
0
SALINE
NAL(30)
NAL(60)
MOR(400)
MOR(800)
LEV(400)
CYC(50)
SKF(100)
FK(2)
Drugs used (µg/kg)

Figure 19

not significantly altered in any of the groups studied. SKF 10,047, cyclazocine and levorphanol caused a significant rise in 5-HT levels but neither morphine nor naloxone had a significant effect. Cyclazocine was the only opiate studied to cause 5-HIAA levels to decrease significantly. FK 33,824 had no significant effect on any of the amines measured.

b. Plasma LH levels 20 minutes post-infusion:

Treatment	Receptor properties	n	Plasma LH (ng/ml)		
			0 min.	10 mins.	20 mins.
Saline	- opiate	23	6.88±0.54	6.54±0.58	6.72±0.66
Naloxone (50µg/kg)	antagonist	9	6.94±0.94	7.78±0.84	8.90±1.18
Naloxone (60µg/kg)	" "	14	10.32±1.52	13.20±2.46	15.60±3.92
Morphine (400µg/kg)	µ-agonist	12	6.35±1.53	4.56±0.59	4.22±0.50
Morphine (800µg/kg)	µ-agonist	15	6.99±1.63	2.77±0.29*	2.51±0.3*
Levorphanol (400µg/kg)	µ-agonist	6	7.38±1.49	4.81±0.96	4.85±0.99
Cyclazocine (50µg/kg)	k-agonist	5	7.62±0.66	5.73±0.77	7.29±0.85
SKF 10,047 (100µg/kg)	σ-agonist	7	9.90±2.90	6.45±0.66	6.64±0.56
FK 33,824 (2µg/kg)	Met-enk analogue	9	5.91±0.41	5.85±0.5	5.56±0.68

Table 8. Values are Mean ± SEM; Paired Student's t-test confirmed by two-way analysis of variance. Level of significance * $p<0.05$. 'n' = Number of observations.

Both at 10 and 20 minutes after the treatment with the various opiate agonists or the antagonist, naloxone, plasma LH concentrations were little altered. The higher dose of morphine was the only drug to cause a significant decrease in

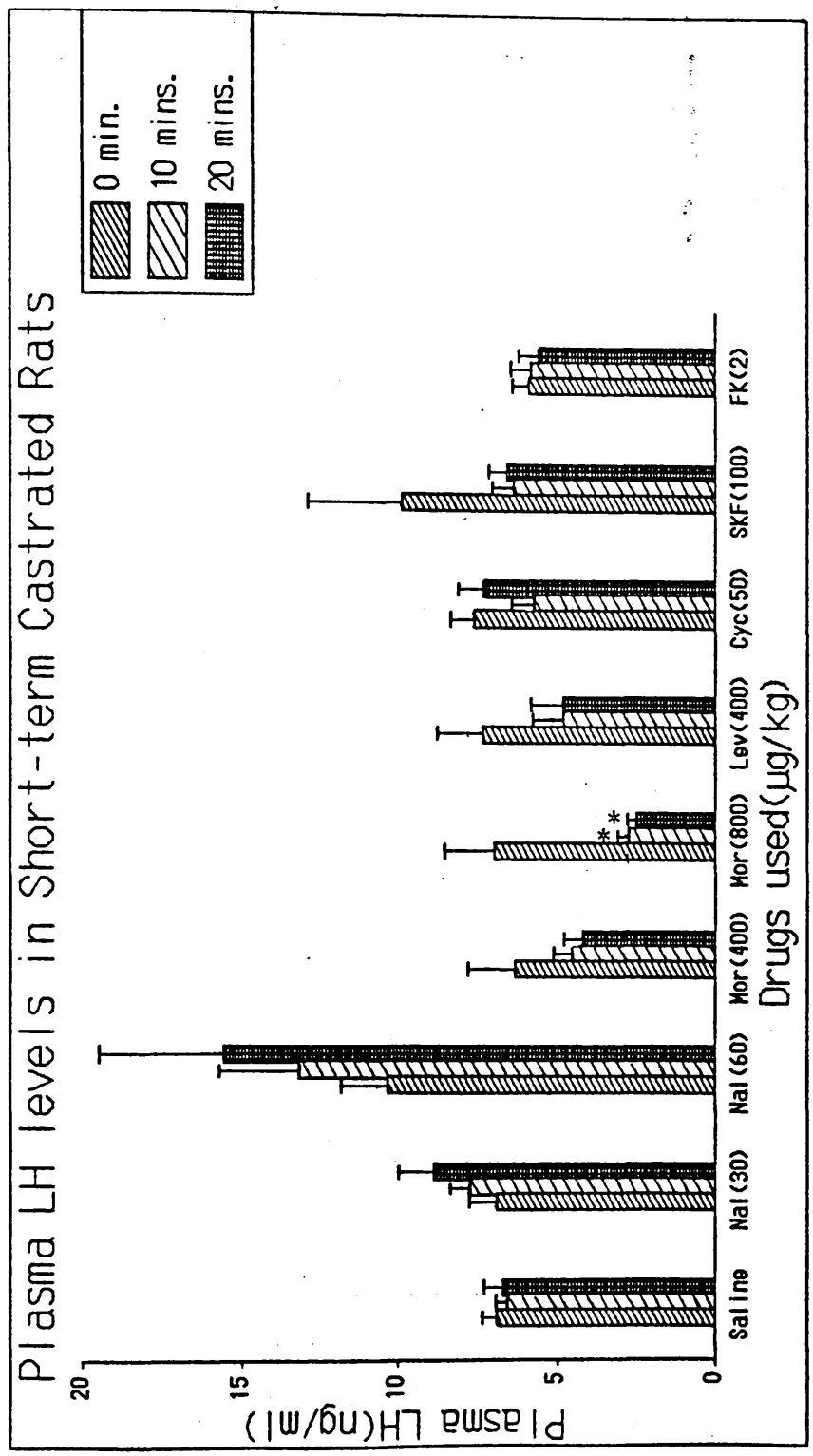

Plasma LH levels in Short-term Castrated Rats
0 min.
10 mins.
20 mins.
Plasma LH(ng/ml)
20
15
10
5
0
**
Saline
Nal(30)
Nal(60)
Mor(400)
Mor(800)
Lev(400)
Cyc(50)
SKF(100)
FK(2)
Drugs used(μg/kg)

Figure 20

the plasma LH content. Both doses of naloxone slightly increased the LH levels; but, the rises were not significant.

c. Hypothalamic NA concentration and turnover two-hours post-infusion:

Treatment	Receptor properties	n	k±SEM (per hour)	TR±SEM (ng/g/hr)	C±SEM (ng/g)
Saline	-	23	0.42±0.01	1412±23	3361±21
Naloxone (40µg/kg)	opiate antagonist	15	0.44±0.01	1600±21	3677±19
Morphine (800µg/kg)	µ-agonist	20	0.21±0.01***	561±31***	2670±42**
Cyclazocine (50µg/kg)	k-agonist	16	0.17±0.02***	419±46***	2540±64**
SKF 10,047 (100µg/kg)	σ-agonist	15	0.42±0.02	1342±71	3225±85

Table 9. The rate constant (k±SEM), turnover (TR±SEM) and the initial concentration (C±SEM) of hypothalamic NA. Level of significance * $p<0.05$; ** $p<0.01$ and *** $p<0.001$. 'n' = Number of observations.

d. Hypothalamic DA concentration and turnover two-hours post-infusion:

Treatment	Receptor properties	n	k±SEM (per hour)	TR±SEM (ng/g/hr)	C±SEM (ng/g)
Saline	–	23	0.28±0.01	96±5	344±06
Naloxone (60µg/kg)	opiate antagonist	15	0.15±0.02	51±8	340±10
Morphine (800µg/kg)	µ–agonist	20	0.66±0.01***	401±10***	608±09***
Cyclazocine (50µg/kg)	k–agonist	16	0.29±0.02	168±14	578±16***
SKF 10,047 (100ug/kg)	σ–agonist	15	0.69±0.02***	468±19***	679±19***

Table 10. The rate constant (k±SEM), turnover (TR±SEM) and the initial concentration (C±SEM) of hypothalamic DA . Level of significance * $p<0.05$; ** $p<0.01$ and *** $p<0.001$. 'n' = Number of observations.

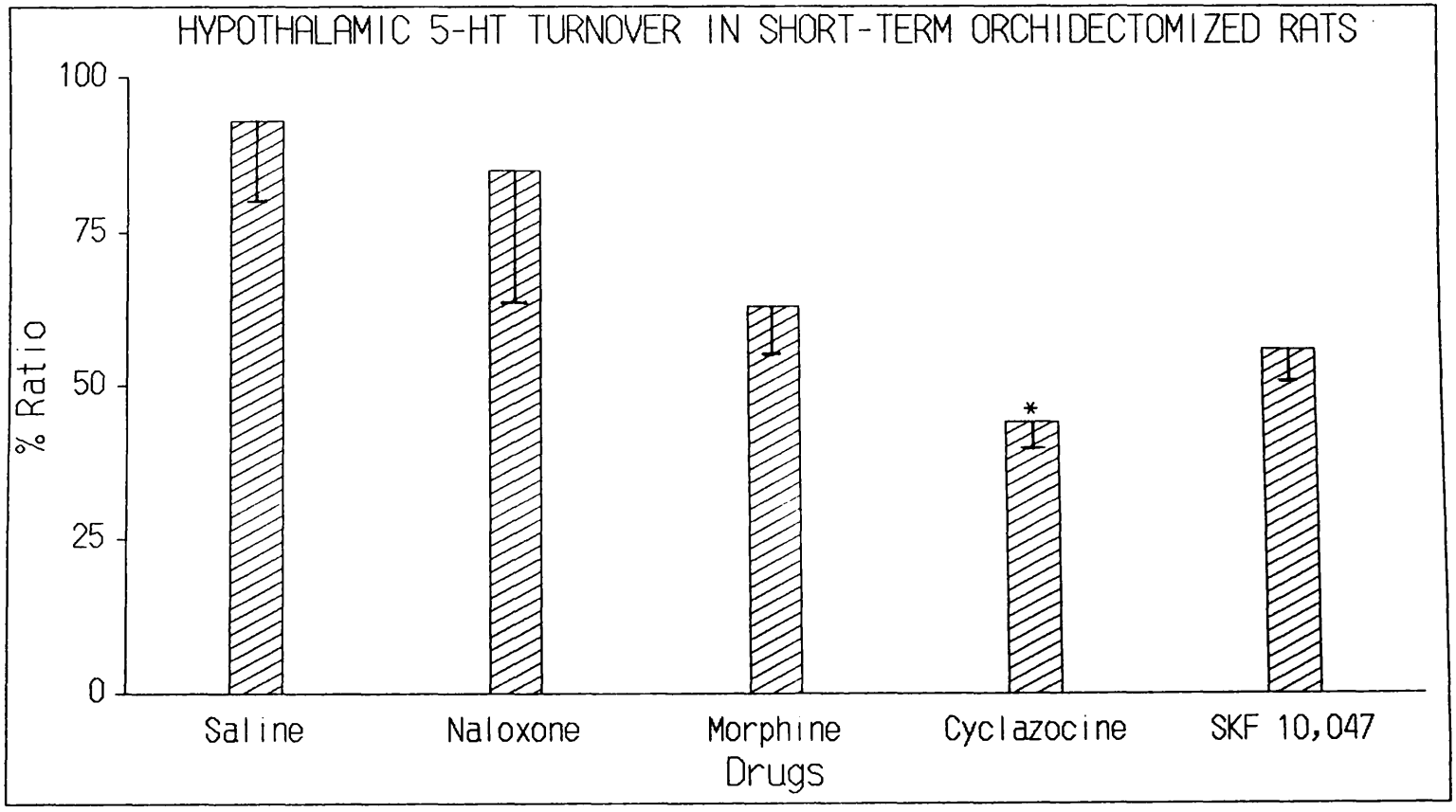
It was observed that the NA content of the hypothalamus and its turnover rate was very significantly decreased in both morphine and cyclazocine-treated animals. SKF 10,047 and naloxone, both, had no significant effect on either the NA content or its turnover. On the other hand, both morphine and SKF 10,047 brought about a highly significant rise in the hypothalamic DA content and its turnover. There was a highly significant rise in the rate constant in both these groups. Cyclazocine also caused a significant rise in the DA content of the hypothalamus, but there was no significant change observed in its turnover. Naloxone again did not significantly alter either the DA content or turnover.

e. Hypothalamic 5-HT concentration and turnover two-hours post- infusion:

Treatment	Receptor properties	n	5-HT C±SEM (ng/g)	5-HIAA C±SEM (ng/g)	5-HT TR±SEM (% Ratio)
Saline	–	5	1670±213	1458±190	93±13
Naloxone (60µg/kg)	opiate antagonist	5	2789±249*	2124±358	85±21
Morphine (800µg/kg)	µ-agonist	9	2911±111**	1177±172	63±08
Cyclazocine (50µg/kg)	k-agonist	6	1644±262	1720±159	44±04*
SKF 10,047 (100µg/kg)	σ-agonist	5	1920±283	0988± 60	56±05

Table 11. Values are Mean ± SEM; unpaired Students' t-test; level of significance * $p < 0.05$; ** $p < 0.01$. 'n' = Number of observations. C, Concentration; TR, Turnover.

The results are summarized in Table 11 and Figure 21. 5-HT content in the hypothalamus was significantly elevated by both naloxone and morphine, but had no significant effect on its turnover rates. Cyclazocine brought about a significant decrease in the turnover of 5-HT, the content of which was not significantly altered. SKF 10,047 had no effect on either the content or turnover of 5-HT. There was no significant change observed in the hypothalamic 5-HIAA levels in any of the groups studied.


HYPOTHALAMIC 5-HT TURNOVER IN SHORT-TERM ORCHIDECTOMIZED RATS
100
75
50
25
0
% Ratio
*
Saline
Naloxone
Morphine
Cyclazocine
SKF 10,047
Drugs

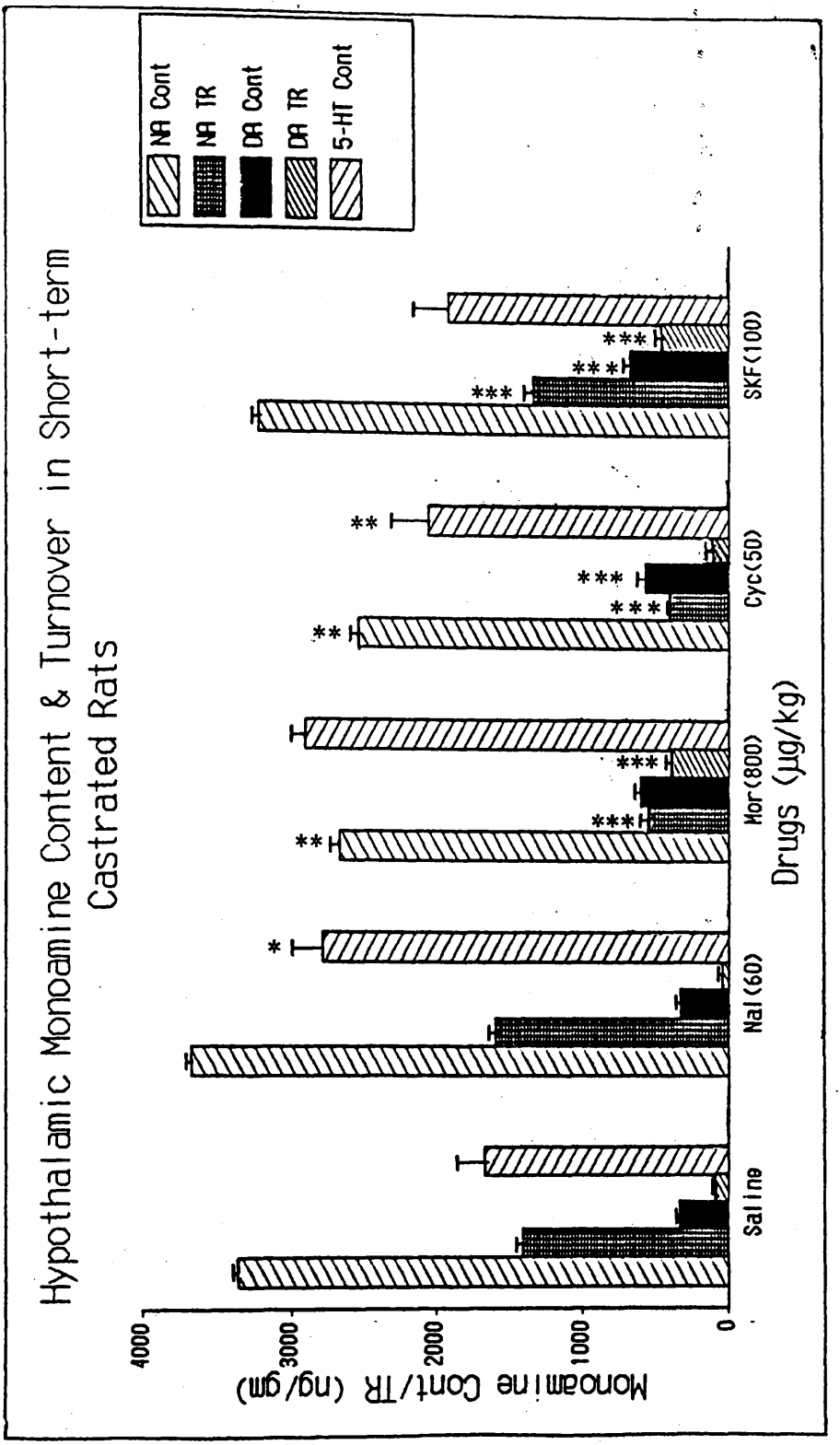

Hypothalamic Monoamine Content & Turnover in Short-term Castrated Rats
NA Cont
NA TR
DA Cont
DA TR
5-HT Cont
Monoamine Cont/TR (ng/gm)
4000
3000
2000
1000
0
Saline
Nal(60)
Mor(800)
Cyc(50)
SKF(100)
Drugs (μg/kg)

Figure 21

f. Plasma LH levels two-hours post-infusion:

Treatment	Receptor properties	n	Plasma LH (ng/ml)		
			0 hour	1 hour	2 hours
Saline	- opiate	14	6.57±1.06	5.03±0.72	4.32±0.67
Naloxone (60µg/kg)	antagonist	5	6.73±0.41	5.56±0.82	12.26±3.17
Morphine (800µg/kg)	µ-agonist	8	8.45±0.97	4.14±0.34*	4.74±0.56*
Cyclazocine (50µg/kg)	k-agonist	6	9.15±1.22	4.65±0.46*	4.89±1.03*
SKF 10,047 (100µg/kg)	σ-agonist	5	8.05±1.03	8.16±1.22	4.44±0.96

Table 12. Values are Mean ± SEM; Paired Student's t-test confirmed by two-way analysis of variance. Level of significance * $p<0.05$. 'n' = Number of observations.

Both morphine and cyclazocine brought about a significant decrease in circulating LH levels both at one hour and two hours after infusion. In contrast neither naloxone nor SKF 10,047 caused any significant alterations in circulating LH levels.

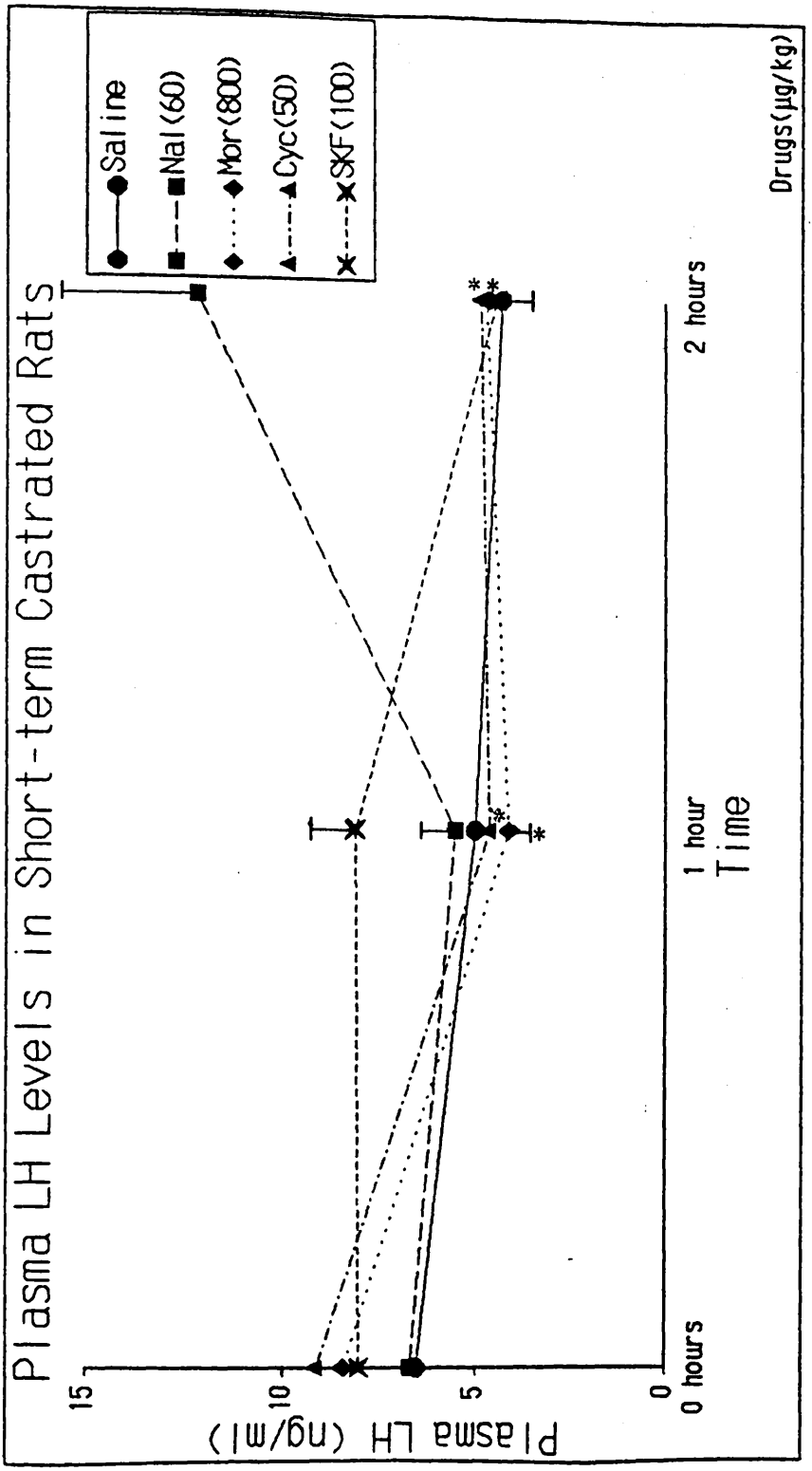

Plasma LH Levels in Short-term Castrated Rats
Saline
Nal(60)
Mor(800)
Cyc(50)
SKF(100)
Drugs(µg/kg)
Plasma LH (ng/ml)
15
10
5
0
0 hours
1 hour
2 hours
Time

Figure 22

g. Plasma LH levels two-hours post-infusion: Effect of α-MPT for one hour:

Treatment	Receptor properties	n	Plasma LH (ng/ml)		
			0 hour	1 hour Receive α-MPT	2 hours
Saline	– opiate	5	6.43±0.27	3.75±1.25	2.91±0.31***
Naloxone (60μg/kg)	antagonist	8	6.78±0.98	5.94±1.21	5.04±1.41
Morphine (800μg/kg)	μ-agonist	6	7.01±0.74	4.72±0.75	2.61±0.34***
Cyclazocine (50μg/kg)	k-agonist	5	6.31±0.58	3.81±0.33	1.87±0.22**
SKF 10,047 (100μg/kg)	σ-agonist	5	6.70±0.42	9.34±1.95	3.17±0.25***

Table 13. Values are Mean±SEM. Paired Student's t-test confirmed by two-way analysis of variance. Level of significance * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. 'n' = Number of observations.

α-MPT treatment one hour after the intraventricular infusion of various specific opiate receptor agonists caused a highly significant decrease in the levels of plasma LH. The control animals which received α-MPT also showed a very highly significant fall in their LH levels. Naloxone prevents the fall normally induced by α-MPT.

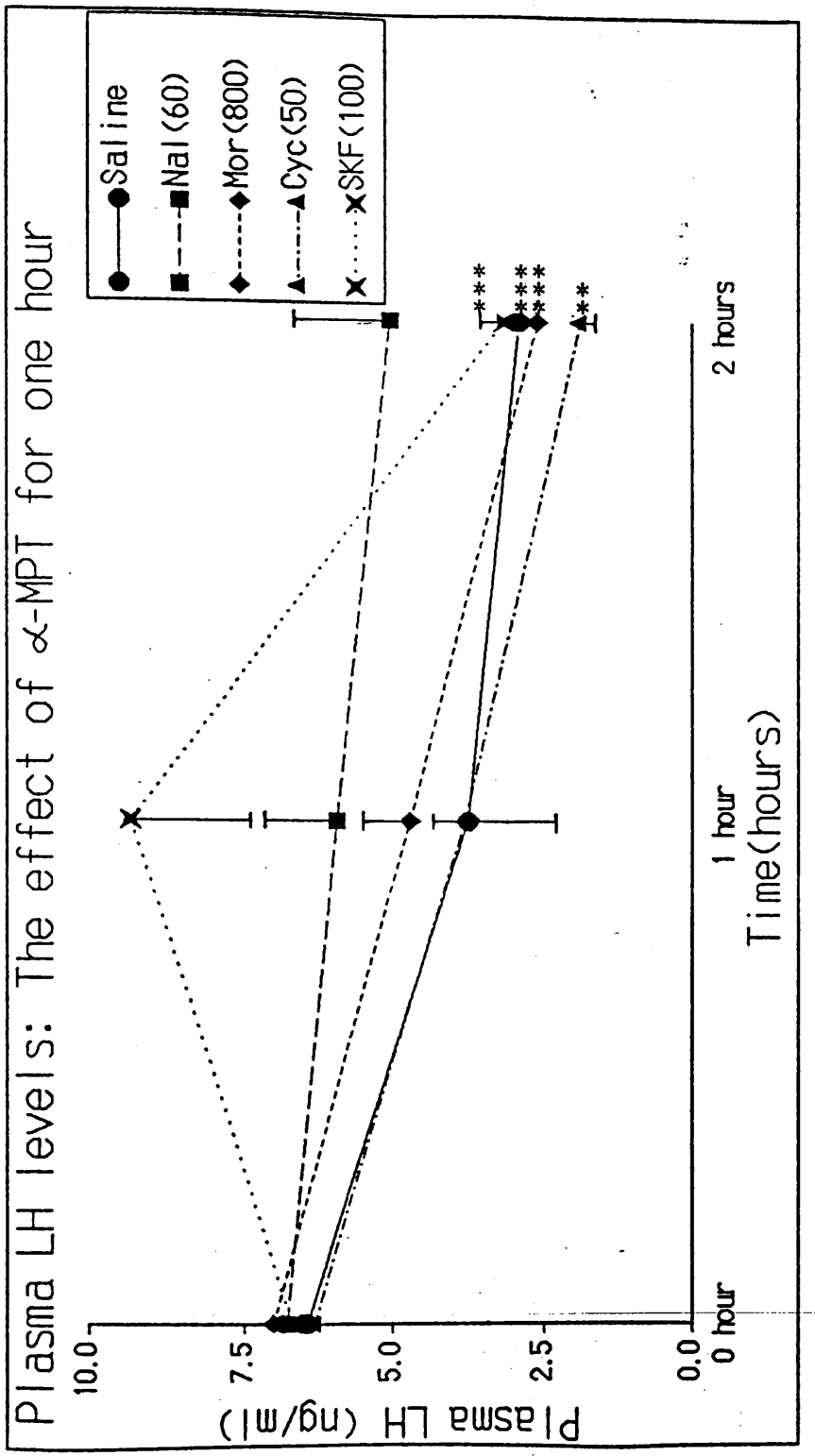

Plasma LH levels: The effect of α-MPT for one hour
Saline
Nal(60)
Mor(800)
Cyc(50)
SKF(100)
Plasma LH (ng/ml)
10.0
7.5
5.0
2.5
0.0
0 hour
1 hour
2 hours
Time(hours)
***
***
***
**

Figure 23

h. *Plasma LH levels: Effect of α-MPT for two hours:*

Treatment	Receptor properties	n	Plasma LH (ng/ml)		
			0 hour receive α-MPT	1 hour	2 hours
Saline	– opiate	5	8.00±0.97	3.00±0.30*	1.70±0.27**
Naloxone (60μg/kg)	antagonist	5	6.08±0.88	5.87±1.35	3.20±0.63
Morphine (800μg/kg)	μ-agonist	6	7.29±1.12	2.55±0.63*	4.69±1.11*
Cyclazocine (50μg/kg)	k-agonist	5	8.20±0.84	4.33±0.92*	3.07±0.83**
SKF 10,047 (100μg/kg)	σ-agonist	5	7.13±0.85	5.36±1.39	5.17±1.23

Table 14. Values are Mean±SEM; paired Student's t-test confirmed by two-way analysis of variance). Level of significance * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. 'n' = Number of observations.

It was observed that α-MPT administration to both the control animals and those receiving morphine and cyclazocine caused a significant decrease in their plasma LH levels both at one hour and two hours post-treatment. However, circulating LH levels were not significantly changed in those animals treated with SKF 10,047 and with naloxone.

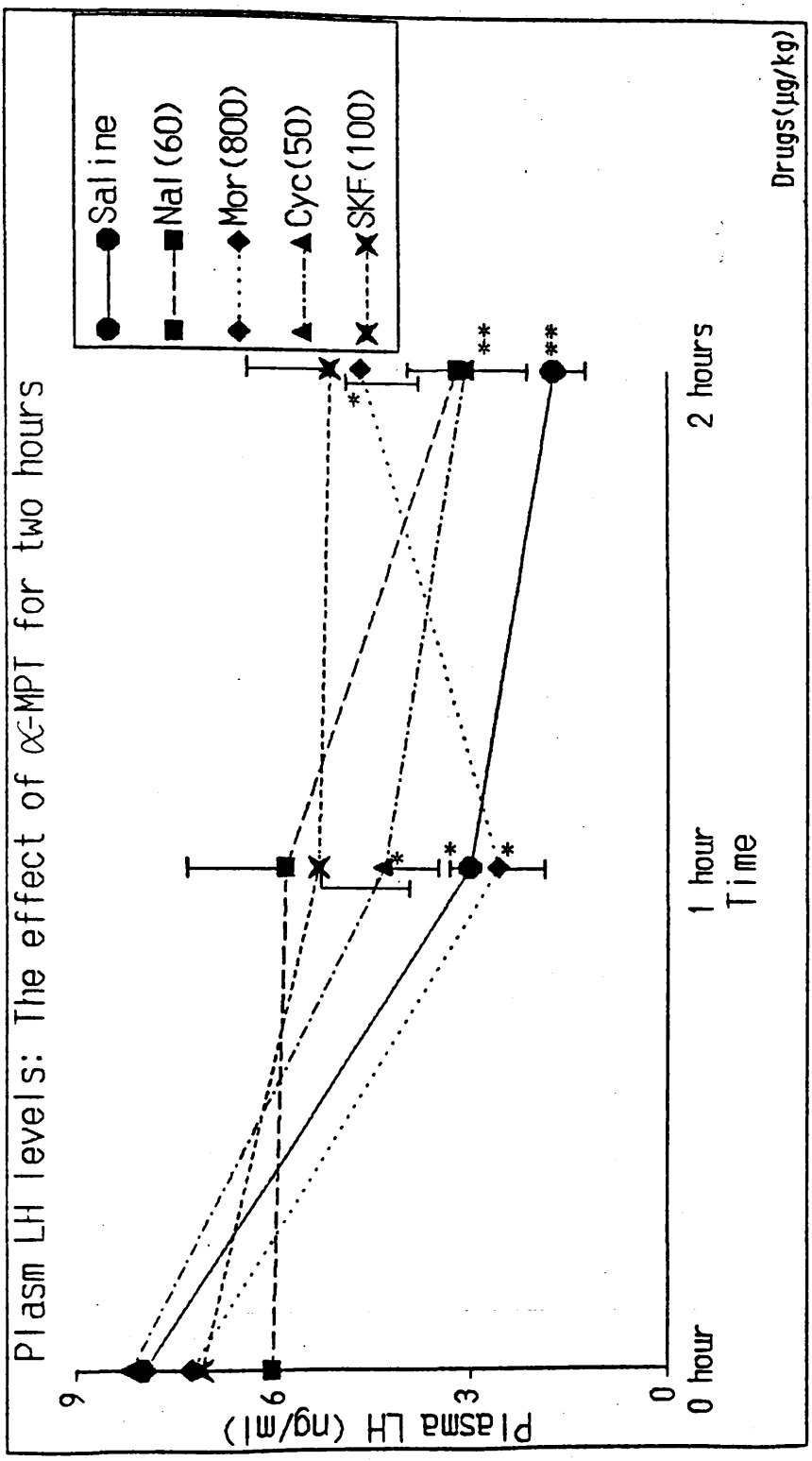

Plasm LH levels: The effect of α-MPT for two hours
Saline
Nal(60)
Mor(800)
Cyc(50)
SKF(100)
Drugs(μg/kg)
Plasma LH (ng/ml)
6
3
0
0 hour
1 hour
2 hours
Time
*
**

Figure 24

Effects of Opiates on the Hypothalamic Monoamine Concentration and Turnover in Pro-oestrous Rats

a. *Hypothalamic NA concentration and turnover:*

Treatment	Receptor properties	n	k±SEM per 30min	TR±SEM ng/g/30min	C±SEM ng/g
Saline ip	-	18	0.20±0.01	0750±37	3752±44
Naloxone (10mg/kg ip)	opiate antagonist	28	0.39±0.01**	1955±54***	5014±66**
Morphine (10mg/kg ip)	μ-agonist	10	0.58±0.04***	1949±161*	3361±106
Morphine (40mg/kg)	μ-agonist	15	0.21±0.01	0492±31	2345±36***
Levorphanol (10mg/kg ip)	μ-agonist	14	0.48±0.03*	1308±77	2724±49***
Cyclazocine (5mg/kg ip)	k-agonist	15	0.37±0.01**	1474±53**	3984±31
Ketocyclazocine (5mg/kg ip)	k-agonist	15	1.22±0.03***	5265±148***	4316±67
FK 33,824 (4μg/kg iv)	met-enk analogue	12	0.33±0.03	1189±128	3605±83
Saline sc	-	18	0.19±0.01	0713±45	3752±53
Tifluadom (10mg/kg sc)	k-agonist	19	0.38±0.01**	1397±41**	3677±42
SKF 10,047 (10mg/kg sc)	σ-agonist	20	0.31±0.01	1140±52	3678±49

Table 15. The rate (k±SEM), turnover(TR±SEM) and the initial concentration of NA (C±SEM) in the hypothalamus. Level of significance * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

It was noted that the higher dose of morphine and levorphanol very significantly decreased the hypothalamic NA concentration. No significant changes were observed in its turnover rates. Naloxone significantly increased both the concentration and the turnover of NA. Morphine (10mg/kg), cyclazocine, ketocyclazocine and tifluadom had no significant effect on the hypothalamic NA concentration, but brought

about a significant rise in its turnover rate. Neither SKF 10,047 nor FK 33,824 significantly altered the concentration nor the turnover of hypothalamic NA.

b. Hypothalamic DA concentration and Turnover:

Treatment	Receptor properties	n	k±SEM per 30min	TR±SEM ng/g/30min	C±SEM ng/g
Saline i.p.	-	18	0.87±0.04	305±19	351±14
Naloxone (10mg/kg i.p.)	opiate antagonist	28	0.90±0.02	386± 9	428± 6
Morphine (10mg/kg i.p.)	μ-agonist	10	2.57±0.07***	1266±67***	493±22
Morphine (40mg/kg i.p.)	μ-agonist	15	0.82±0.02	514±18*	626±14***
Levorphanol (10mg/kg i.p.)	μ-agonist	14	0.64±0.03	277±16	433± 9
Cyclazocine (5mg/kg i.p.)	k-agonist	15	1.03±0.04	351±15	340± 7
Ketocyclazocine (5mg/kg i.p.)	k-agonist	15	0.90±0.03	297±10	330± 6
FK 33,824 (4μg/kg i.v.t.)	met-enk analogue	12	1.18±0.02	439±10	372± 4
Saline sc	-	18	0.66±0.04	212±16	320±14
Tifluadom (10mg/kg i.m.)	k-agonist	19	0.52±0.02	225± 9	433± 8
SKF 10,047 (10mg/kg i.m.)	σ-agonist	20	0.31±0.01	135± 6	437± 6

Table 16. The rate (k±SEM), turnover (TR±SEM) and the initial concentration of DA (C±SEM) in the hypothalamus. Level of significance * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. 'n' = Number of observations.

The concentration, turnover and the rate constant of DA in the hypothalamus are summarized in Table 16 (Figure 25). Except morphine, none of the other groups brought about any significant changes in their DA activity.

c. Hypothalamic 5-HT Concentration and Turnover:

Treatment	Receptor properties	n	5-HT C±SEM (ng/g)	5-HIAA C±SEM (ng/g)	5-HT TR ±SEM (% Ratio)
Saline ip	–	7	2122±164	1510±160	72±06
Naloxone (10mg/kg ip)	opiate antagonist	6	2012±265	1214±290	56±10
Morphine (10mg/kg)	μ-agonist	5	1644±111	1177±172	71±10
Morphine (40mg/kg ip)	μ-agonist	6	4209±178***	3259±142***	78±04
Levorphanol (10mg/kg ip)	μ-agonist	5	944± 69***	0788± 94**	85±10
Cyclazocine (5mg/kg ip)	k-agonist	7	2075±215	1481± 53	79±09
Ketocyclazocine (5mg/kg ip)	k-agonist	6	1814±103	1531± 58	87±07
FK 33,824 (4μg/kg iv)	Met-enk analogue	5	2474±160	1503±213	62±09
Saline sc	–	9	2869±246*	1693±187	59±04
Tifluadom (10mg/kg sc)	k-agonist	5	2469±200	2083±123	89±09*
SKF 10,047 (10mg/kg sc)	σ-agonist	9	4321±224**	2518±219*	59±05

Table 17. Values are Mean±SEM; unpaired Students' t-test; level of significance * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$. 'n' = Number of observations. C, Concentration, TR, Turnover.

From the above table, it can be seen that, morphine (40mg/kg), and SKF 10,047 caused a significant rise in the hypothalamic 5-HT and 5-HIAA levels, but had no significant effect on their turnover. The lower dose of morphine, FK 33,824 and naloxone, on the other hand, did not significantly alter the hypothalamic content of 5-HT, 5-HIAA

or their turnover. An unusual observation was that levorphanol brought about a significant decrease in the levels of hypothalamic 5-HT and 5-HIAA. Saline subcutaneously injected into the controls caused a significant elevation in the hypothalamic content of 5-HT when compared to those controls which received saline intraperitoneally (Gopalan, Brown & Gilmore, 1988). Cyclazocine and ketocyclazocine did not have a significant effect on the levels of 5-HT and 5-HIAA and their turnover. Tifluadom was the only k-agonist to increase the hypothalamic turnover of 5-HT significantly.

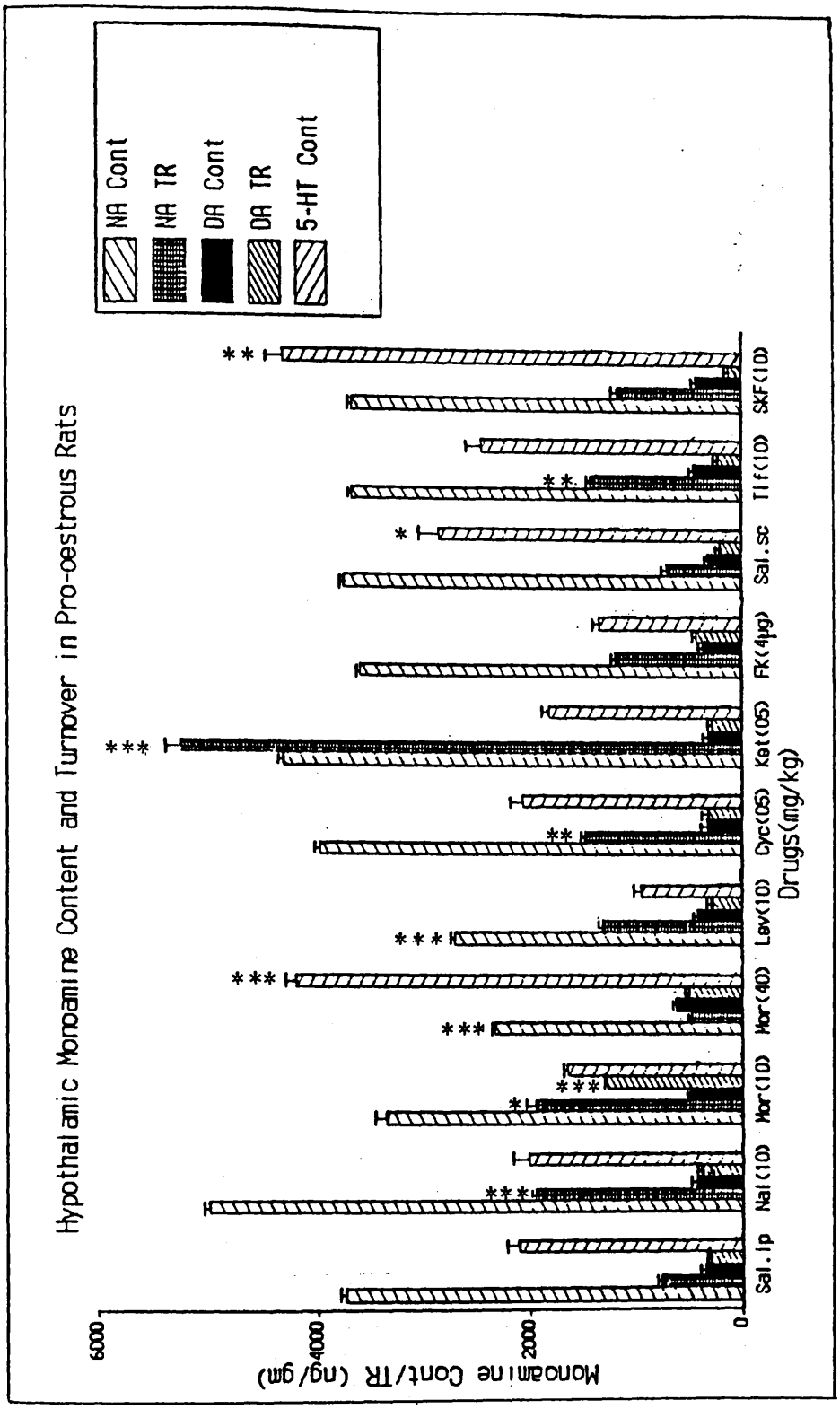

Hypothalamic Monoamine Content and Turnover in Pro-oestrous Rats
NA Cont
NA TR
DA Cont
DA TR
5-HT Cont
Monoamine Cont/TR (ng/gm)
6000
4000
2000
0
Sal.ip
Nal(10)
Mor(10)
Mor(40)
Lev(10)
Cyc(05)
Ket(05)
FK(4µg)
Sal.sc
Tif(10)
SKF(10)
Drugs(mg/kg)

Figure 25

d. Plasma LH levels in Pro-oestrous Rats (with or without ∝-MPT):

Treatment	Receptor properties	Plasma LH (ng/ml) ∝-MPT 0 mins.	∝-MPT 30 mins.	∝-MPT 60 mins.
Saline ip	–	4.74±0.62 (27)	1.50±0.07*** (5)	3.20±0.44 (10)
Naloxone (10mg/kg ip)	opiate antagonist	11.34±2.04** (32)	1.33±0.09*** (5)	6.97±0.65 (5)
Morphine (10mg/kg ip)	μ-agonist	2.76±0.38* (5)	–	2.10±0.32 (5)
Morphine (40mg/kg ip)	μ-agonist	2.35±0.39** (17)	1.66±0.17* (5)	2.06±0.10* (5)
Levorphanol (10mg/kg ip)	μ-agonist	4.92±2.04 (20)	–	4.03±1.55 (5)
Cyclazocine (5mg/kg ip)	k-agonist	4.91±0.60 (17)	–	2.56±0.37 (5)
Ketocyclazocine (5mg/kg ip)	k-agonist	6.06±1.71 (19)	–	1.94±0.29* (5)
FK 33,824 (2μg/kg iv)	Met-enk analogue	1.82±1.68 (9)	–	–
FK 33,824 (4μg/kg iv)	Met-enk analogue	3.09±0.76 (7)	–	1.11±0.41 (5)
Saline sc	–	3.89±0.38 (20)	1.40±0.2*** (5)	2.39±0.43* (5)
Tifluadom (10mg/kg sc)	k-agonist	2.19±0.21** (22)	1.70±0.10 (5)	1.78±0.07 (5)
SKF 10,047 (10mg/kg sc)	σ-agonist	4.04±0.54 (22)	2.00±0.13** (5)	2.21±0.57 (5)

Table 18. Values are Mean ± SEM; Unpaired Student's t-test confirmed by two-way analysis of variance. Level of significance * $p<0.05$ ** $p<0.01$ and *** $p<0.001$. (n)= Number of observations.

The plasma LH concentration was significantly decreased by both doses of morphine and tifluadom. This effect was significantly increased by naloxone. On the other hand, levorphanol, cyclazocine, ketocyclazocine, both doses of FK 33,824 and SKF 10,047 failed to cause any significant changes

levorphanol, cyclazocine, ketocyclazocine, both doses of FK 33,824 and SKF 10,047 failed to cause any significant changes in the levels of plasma LH. α-MPT administered 30 minutes prior to decapitation brought about a significant decrease in the concentration of plasma LH in all groups studied except the tifluadom-treated group. α-MPT administered one hour prior to decapitation showed a similar decrease in LH levels in the groups treated with morphine, ketocyclazocine and saline (sc). Levorphanol was the only opiate which did not cause any significant changes in the plasma LH levels either in the control group or after α-MPT treatment for one hour.

Plasma LH levels in pro-oestrous rats
Plasma LH (ng/ml)
0
5
10
15
Sal.ip
Nal (10mg/kg) ip
Mor (10mg/kg) ip
Mor (40mg/kg) ip
Lev (10mg/kg) ip
Cyc (5mg/kg) ip
Ket (5mg/kg) ip
FK (2ug/kg) ivt
FK (4ug/kg) ivt
Sal.sc
Tif (10mg/kg) sc
SKF (10mg/kg) sc
Treatment
*
**
**

Figure 26

Effects of Opiates on Monoamine Concentration and Turnover within the Specific Hypothalamic regions in Pro-oestrous Rats

i) NA and DA concentration in the SCH, MPO, AHA, ME, ARN and VMH:

Area	Saline (control) [13]	Levorphanol (μ-agonist) 10mg/kg [7]	Cyclazocine (k-agonist) 5mg/kg [7]	Ketocyclazocine (k-agonist) 5mg/kg [7]
SCH				
NA	64.32± 8.36	37.07±4.88*	64.40± 6.91	40.8±4.15*
DA	6.79± 1.21	12.44±3.02	21.49± 5.87	09.48±2.4
MPO				
NA	78.98± 4.86	53.73±3.02**	91.29± 7.83	80.05±9.32
DA	7.24± 1.45	13.12±2.29	20.41± 4.61	16.00±4.04
AHA				
NA	72.10± 8.70	41.46±2.75*	79.50±10.70	58.20±3.68
DA	5.26± 0.62	10.83±2.43	11.77± 4.49	11.77±4.49
ME				
NA	108.40±15.30	52.68±5.96*	75.50±11.70	68.30±9.12
DA	28.50± 2.85	17.46±3.52**	62.00±11.80	17.18±5.02*
ARN				
NA	98.77± 8.36	48.65±3.00***	72.18± 7.99	73.10±7.34
DA	7.54± 1.58	10.32±1.94*	15.94± 2.75*	10.94±2.52*
VMH				
NA	89.27± 6.23	53.34±4.04***	106.70±13.40	61.78±3.24**
DA	10.40± 2.46	17.51±4.86	15.96± 3.23	7.24±2.14

Table 19. Values are Mean ± SEM (pg/μg); unpaired Student's t-test. Level of significance * $p<0.05$; ** $p<0.01$ and *** $p<0.001$. [n] = Number of observations.

Levorphanol caused a significant decrease in the NA content of all six regions studied. DA content was

significantly decreased only in the ME. Cyclazocine did not cause any significant changes in the NA concentration of any hypothalamic area studied; DA levels, however, were significantly increased in the ARN. Ketocyclazocine brought about a significant decrease in the NA concentration of the SCH and VMH. It also brought about a significant fall in the DA concentration of the ME.

ii) NA Concentration and Turnover:

Suprachiasmatic nucleus (SCH)

Treatment	Receptor properties	n	k±SEM per 45min	TR±SEM pg/µg/45min	Conc±SEM pg/µg
Saline ip	-	24	0.48±0.02	30.45±1.34	63.43±1.98
Naloxone (10mg/kg ip)	opiate antagonist	24	0.65±0.02	46.96±1.69	72.24±1.62
Morphine) (40mg/kg ip)	µ-agonist	22	0.27±0.02	12.44±1.10*	46.10±0.88*
Saline sc	-	22	0.50±0.01	30.47±1.04	60.95±0.96
Tifluadom (10mg/kg sc)	k-agonist	22	0.28±0.01*	13.08±0.62**	46.06±0.6**
SKF 10,047 (10mg/kg sc)	σ-agonist	21	0.15±0.02**	07.53±0.93***	48.91±1.03

Table 20A. The rate constant (k±SEM), turnover(TR±SEM) and the initial concentration of NA (Conc±SEM) in the SCH. Level of significance * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. 'n' = No. of observations.

Medial Preoptic Area (MPO)

Treatment	Receptor properties	n	K±SEM per 45min	TR±SEM pg/µg/45min	Conc±SEM pg/µg
Saline ip	-	24	0.34±0.01	25.06±1.00	73.70±1.42
Naloxone (10mg/kg; ip)	opiate antagonist	24	0.45±0.01	47.06±1.33**	104.58±1.38**
Morphine (40mg/kg; ip)	µ-agonist	22	0.21±0.01	11.13±0.80*	52.98±0.62**
Saline sc	-	22	0.47±0.01	31.66±1.07	67.36±1.15
Tifluadom (10mg/kg; sc)	k-agonist	22	0.27±0.02	17.89±1.56	66.02±1.60
SKF 10,047 (10mg/kg; sc)	σ-agonist	22	0.23±0.01**	16.82±0.97*	74.44±1.06

Table 20B. The rate (k±SEM), turnover(TR±SEM) and the initial concentration of NA in the MPO. Level of significance * $p < 0.05$ and ** $p < 0.01$. 'n' = No. of observations.

Anterior Hypothalamic Area (AHA)

Treatment	Receptor properties	n	K±SEM per 45min	TR±SEM pg/µg/45min	Conc±SEM pg/µg
Saline ip	-	24	0.26±0.02	18.97±1.54	72.97±1.49
Naloxone (10mg/kg; ip)	opiate antagonist	24	0.36±0.01	27.07±0.90	75.19±0.91
Morphine (40mg/kg; ip)	µ-agonist	22	0.04±0.01*	01.82±0.39*	45.6 ±0.6***
Saline sc	-	22	0.18±0.01	13.02±1.00	71.52±1.11
Tifluadom (10mg/kg; sc)	k-agonist	22	0.20±0.02	13.20±1.59	66.00±1.89
SKF 10,047 (10mg/kg; sc)	σ-agonist	22	0.31±0.02	25.50±1.87	82.27±2.07

Table 20C. The rate constant (k±SEM), turnover(TR±SEM) and the initial concentration of NA in the AHA. Level of significance * $p < 0.05$ and *** $p < 0.001$. 'n' = Number of Observations.

Median Eminence (ME)

Treatment	Receptor properties	n	k±SEM per 45min	TR±SEM pg/µg/45min	Conc±SEM pg/µg
Saline ip	-	24	0.65±0.01	48.39±4.08	74.44±1.67
Naloxone (10mg/kg; ip)	opiate antagonist	24	0.50±0.01	41.13±1.19	82.27±1.26
Morphine (40mg/kg; ip)	µ-agonist	22	0.31±0.02**	16.76±1.35	54.05±1.50
Saline sc	-	22	0.22±0.01	18.38±0.67	83.93±0.86
Tifluadom (10mg/kg; sc)	k-agonist	22	0.43±0.01**	24.74±0.61	57.40±0.56***
SKF 10,047 (10mg/kg; sc)	σ-agonist	22	0.21±0.02	11.07±1.13	52.46±1.12***

Table 20D. The rate (k±SEM), turnover(TR±SEM) and the initial concentration of NA (Mean±SEM) in the **ME**. Level of significance ** $p < 0.01$ and *** $p < 0.001$. 'n' = Number of observations.

Arcuate Nucleus (ARN)

Treatment	Receptor properties	n	K±SEM per 45min	TR±SEM pg/µg/45min	Conc±SEM pg/µg
Saline ip	-	24	0.49±0.01	45.90±1.58	93.69±1.72
Naloxone (10mg/kg; ip)	opiate antagonist	24	0.53±0.01	55.43±1.71	104.58±2.13
Morphine (40mg/kg; ip)	µ-agonist	22	0.5 ±0.01	34.36±0.89	68.72±1.03*
Saline sc	-	22	0.41±0.01	36.54±1.10	89.12±1.33
Tifluadom (10mg/kg; sc)	k-agonist	22	0.60±0.02	42.49±1.44	70.81±1.49
SKF 10,047 (10mg/kg; sc)	σ-agonist	22	0.29±0.01	16.70±0.80**	57.40±0.73***

Table 20E. The rate constant (k±SEM), turnover(TR±SEM) and the initial concentration of NA in the **ARN**. Level of significance * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. 'n' = Number of observations.

Ventromedial Nucleus (VMH)

Treatment	Receptor properties	n	k±SEM per 45min	TR±SEM pg/µg/45min	Conc±SEM pg/µg
Saline ip	-	24	0.55±0.01	55.27±1.23	100.45±1.23
Naloxone (10mg/kg ip)	opiate antagonist	24	0.39±0.02	42.03±2.47	107.77±2.86
Morphine (40mg/kg ip)	µ-agonist	22	0.10±0.02***	07.01±1.50***	70.81±1.66**
Saline sc	-	22	0.31±0.01***	32.75±0.70**	105.64±0.68
Tifluadom (10mg/kg sc)	k-agonist	22	0.14±0.01*	11.05±1.10***	80.64±1.2***
SKF 10,047 (10mg/kg sc)	σ-agonist	22	0.28±0.01	27.30±0.24	97.51±0.42

Table 20F. The rate constant (k±SEM), turnover(TR±SEM) and the initial concentration of NA in the VMH. Level of significance * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. 'n' = Number of observations.

The NA content and its turnover in the six hypothalamic regions studied i.e., SCH, MPO, AHA, ME ARN and VMH are summarized in Table 20(A-F) and Figure (27a-f). Morphine caused a significant decrease in the NA content and turnover in the SCH, MPO, AHA and VMH. The NA content alone was significantly decreased in the ARN. Tifluadom significantly decreased the content and turnover of NA in both the SCH and VMH and decreased the NA content alone in the ME but had no significant effects in the MPO, AHA and ARN. SKF 10,047 brought about a significant decrease in both the content and the turnover of NA in the ARN, but decreased turnover alone in the SCH and MPO and content alone in the ME. It did not cause any significant changes in either the AHA or the VMH. On the other hand, naloxone significantly increased both the

concentration and the turnover of NA in the MPO. There were no significant changes observed in any other region studied.

iii) *DA Concentration and Turnover:*

SCH

Treatment	Receptor properties	n	K±SEM per 45min	TR±SEM pg/µg/45min	Conc±SEM pg/µg
Saline ip	-	24	1.01±0.02	7.10±0.25	7.03±0.20
Naloxone (10mg/kg ip)	opiate antagonist	24	1.09±0.03	9.35±0.28	8.58±0.09
Morphine (40mg/kg ip)	µ-agonist	22	0.89±0.03	7.27±0.22	8.17±0.29
Saline sc	-	22	0.98±0.03	8.98±0.32	9.11±0.37
Tifluadom (10mg/kg sc)	k-agonist	22	1.01±0.04	13.87±0.67	13.74±0.61
SKF 10,047 (10mg/kg sc)	σ-agonist	21	1.09±0.05	14.10±0.76	12.94±0.74

Table 21A.The rate constant (k±SEM), turnover(TR±SEM) and the initial concentration of DA (conc ±SEM) in the SCH. 'n' = Number of observations.

MPO

Treatment	Receptor properties	n	K±SEM per 45min	TR±SEM pg/µg/45min	Conc±SEM pg/µg
Saline ip	-	24	0.71±0.03	05.14±0.30	07.24±0.30
Naloxone (10mg/kg; ip)	opiate antagonist	24	1.04±0.02	15.79±0.47***	15.18±0.34***
Morphine (40mg/kg; ip)	µ-agonist	22	0.36±0.03	02.39±0.14	06.69±0.22
Saline sc	-	22	0.65±0.04	03.74±0.09	05.75±0.24
Tifluadom (10mg/kg; sc)	k-agonist	22	0.93±0.02	11.97±0.31***	12.94±0.31***
SKF 10,047 (10mg/kg; sc)	σ-agonist	22	1.01±0.02*	19.49±0.46***	19.30±0.41***

Table 21B. The rate constant(k±SEM), turnover(TR±SEM) and the initial concentration of DA (conc ±SEM) in the **MPO**. Level of significance * $p < 0.05$ and *** $p < 0.001$. 'n' = Number of observations.

AHA

Treatment	Receptor properties	n	K±SEM per 45min	TR±SEM pg/µg/45min	Conc±SEM pg/µg
Saline ip	-	24	0.69±0.02	3.60±0.06	5.26±0.13
Naloxone (10mg/kg; ip)	opiate antagonist	24	0.59±0.03	3.09±0.11	5.26±0.20
Morphine (40mg/kg; ip)	µ-agonist	22	0.43±0.01*	3.37±0.08	7.92±0.12
Saline sc	-	22	0.53±0.02	2.63±0.13	5.00±0.15
Tifluadom (10mg/kg; sc)	k-agonist	22	0.28±0.02	1.98±0.12	7.03±0.14*
SKF 10,047 (10mg/kg; sc)	σ-agonist	22	0.26±0.03	1.77±0.22	6.82±0.03

Table 21C. The rate constant(k±SEM), turnover(TR±SEM) and the initial concentration of DA (conc ± SEM) in the **AHA**. Level of significance * $p < 0.05$. 'n' = Number of observations.

ME

Treatment	Receptor properties	n	K±SEM per 45min	TR±SEM pg/µg/45min	Conc±SEM pg/µg
Saline ip	-	24	0.62±0.02	17.67±0.63	28.50±0.58
Naloxone (10mg/kg ip)	opiate antagonist	24	1.72±0.04***	74.61±3.49**	43.38±1.77
Morphine (40mg/kg ip)	µ-agonist	22	0.86±0.02	16.60±0.62	19.3 ±0.49*
Saline sc	-	22	0.7 ±0.01	23.65±0.65	33.78±0.58
Tifluadom (10mg/kg sc)	k-agonist	22	0.65±0.01	26.41±0.58	40.45±0.53
SKF 10,047 (10mg/kg sc)	σ-agonist	22	1.05±0.05	40.40±2.88	38.47±2.14

Table 21D. The rate constant (k±SEM), turnover(TR±SEM) and the initial concentration of DA (conc ± SEM) in the **ME**. Level of significance * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. 'n' = Number of observations.

ARN

Treatment	Receptor poroperties	n	K±SEM per 45min	TR±SEM pg/µg/45min	Conc±SEM pg/µg
Saline ip	-	24	0.73±0.03	05.5 ±0.34	07.54±0.32
Naloxone (10mg/kg ip)	opiate antagonist	24	1.14±0.03	18.01±0.76**	15.80±0.56**
Morphine (40mg/kg ip)	µ-agonist	22	1.06±0.03	14.41±0.75*	13.60±0.58
Saline sc	-	22	0.69±0.03	04.31±0.29	06.23±0.29
Tifluadom (10mg/kg sc)	k-agonist	22	0.85±0.03	09.5 ±0.40	11.13±0.35
SKF 10,047 (10mg/kg sc)	σ-agonist	22	1.05±0.04	15.78±0.21**	15.03±0.61**

Table 21E. The rate constant (k±SEM), turnover (TR±SEM) and the initial concentration of DA (conc ±SEM) in the **ARN**. Level of significance * $p < 0.05$ and ** $p < 0.01$. 'n' = Number of observations.

VMH

Treatment	Receptor properties	n	K±SEM per 45min	TR±SEM pg/µg/45min	Conc±SEM pg/µg
Saline ip	-	24	0.91±0.02	08.54±0.32	09.39±0.27
Naloxone (10mg/kg ip)	opiate antagonist	24	1.1 ±0.03	11.42±0.52	10.38±0.38
Morphine (40mg/kg ip)	µ-agonist	22	0.61±0.02*	05.38±0.20	08.85±0.19
Saline sc	-	22	0.42±0.01***	02.95±0.11***	07.03±0.10
Tifluadom (10mg/kg sc)	k-agonist	22	0.69±0.03	07.33±0.32**	10.59±0.29*
SKF 10,047 (10mg/kg sc)	σ-agonist	22	0.95±0.04*	10.49±0.66*	11.02±0.54

Table 21F. The rate constant (k±SEM), turnover (TR±SEM) and the initial concentration of DA in the VMH. Level of significance * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. 'n' = Number of observations.

The content and the turnover of DA studied in various specific hypothalamic regions are summarized in Tables 21(A-F) and Figures 27(a-f). The interesting observation made was that in the SCH there were not any significant changes in either the content or the turnover of DA after opiate treatment. Morphine brought about a significant decrease in the DA content of the ME. This opiate also caused a significant rise in DA turnover in the ARN. It failed to have a significant effect in the SCH, MPO, AHA and VMH. Tifluadom significantly increased both the content and turnover of DA in the MPO and VMH and the content alone in the AHA. This drug did not affect the DA content or its turnover in the SCH, ME and ARN. SKF 10,047 caused a significant rise in both the content and the turnover of DA in the MPO and ARN;

the turnover alone was increased in the VMH. SKF 10,047 did not bring about any significant changes in either the DA concentration or its turnover in the SCH, AHA and ME. Naloxone, which was expected to reverse the effects of the opiates brought about a significant rise in the concentration and the turnover of DA in both the MPO and ARN and of the turnover alone in the ME; no significant changes were observed after naloxone treatment in DA concentration and turnover in the SCH, AHA and VMH.

iv) 5-HT Concentration and Turnover:

SCH

Treatment	Receptor properties	n	5-HT Conc±SEM (ng/g)	5-HIAA Conc±SEM (ng/g)	5-HT TR±SEM (% Ratio)
Saline ip	-	13	25.81±3.62	18.63±3.52	54±07
Naloxone (10mg/kg ip)	opiate antagonist	13	18.18±4.63	13.81±2.90	91±12
Morphine (40mg/kg ip)	μ-agonist	12	16.90±3.35	13.87±1.68	120±32
Levorphanol (10mg/kg ip)	μ-agonist	7	18.62±4.41	09.36±1.26	66±17
Cyclazocine (5mg/kg ip)	k-agonist	7	09.34±4.33*	32.66±7.23	157±51
Ketocyclazocine (5mg/kg ip)	k-agonist	7	20.49±2.46	17.80±6.04	83±28
Saline sc	-	12	41.03±4.98*	06.98±1.96*	26±11
Tifluadom (10mg/kg sc)	k-agonist	12	17.98±2.12**	25.34±2.94***	166±44*
SKF 10,047 (10mg/kg sc)	σ-agonist	12	16.68±2.95**	12.22±2.78	81±13*

Table 22A. Values are Mean±SEM; unpaired Students' t-test; level of significance * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$. 'n' = Number of observations. Conc, Concentration, TR, Turnover.

MPO

Treatment	Receptor properties	n	5-HT Conc±SEM pg/µg	5-HIAA Conc±SEM pg/µg	5-HT TR (% Ratio) ±SEM
Saline ip	-	7	22.22±2.42	17.58±0.99	86±11
Naloxone (10mg/kg; ip)	opiate antagonist	6	21.83±6.76	18.49±5.61	156±73
Morphine (40mg/kg; ip)	µ-agonist	6	18.05±3.68	13.86±1.23	91±18
Levorphanol (10mg/kg; ip)	µ-agonist	5	17.76±3.05	21.50±5.34	123±17
Cyclazocine (5mg/kg; ip)	k-agonist	7	27.00±3.15	23.43±2.16	92±13
Ketocyclazocine (5mg/kg; ip)	k-agonist	6	20.92±5.40	32.79±7.45	188±23**
Saline sc	-	9	16.91±2.59	12.34±1.62*	91±18
Tifluadom (10mg/kg; sc)	k-agonist	5	16.62±2.85	14.94±2.30	99±18
SKF 10,047 (10mg/kg; sc)	σ-agonist	9	21.81±4.13	14.62±3.62	83±16

Table 22B. Values are means ± SEM; unpaired Students' t-test; level of significance * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$. 'n' =Number of observations.

AHA

Treatment	Receptor properties	n	5-HT Conc±SEM pg/µg	5-HIAA Conc±SEM pg/µg	5-HT TR (% Ratio) ±SEM
Saline ip	–	7	32.47±4.46	24.29±2.80	85±14
Naloxone (10mg/kg; ip)	opiate antagonist	6	23.92±3.58	25.95±2.42	125±21
Morphine (40mg/kg; ip)	µ-agonist	6	31.68±3.58	28.80±2.13	96±07
Levorphanol (10mg/kg; ip)	µ-agonist	5	29.35±5.06	22.52±3.43	79±07
Cyclazocine (5mg/kg; ip)	k-agonist	7	16.53±3.12*	24.94±2.63	118±31
Ketocyclazocine (5mg/kg; ip)	k-agonist	6	26.21±2.33	28.55±4.05	111±14
Saline sc	–	9	48.21±4.19*	23.07±3.15	48±06*
Tifluadom (10mg/kg; sc)	k-agonist	5	21.42±2.30**	25.80±1.93	130±14***
SKF 10,047 (10mg/kg; sc)	σ-agonist	9	29.87±3.27*	38.25±5.12*	130±13*

Table 21C. Values are means ± SEM; unpaired Students' t-test; level of significance * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$. 'n' = Number of observations.

ME

Treatment	Receptor properties	n	5-HT Conc±SEM pg/µg	5-HIAA Conc±SEM pg/µg	5-HT TR (% Ratio) ±SEM
Saline ip	–	7	37.92±4.21	17.10±2.06	116±54
Naloxone (10mg/kg; ip)	opiate antagonist	6	24.34±5.30	15.68±1.43	65±13
Morphine (40mg/kg; ip)	µ-agonist	6	25.98±6.05	19.83±4.41	78±10
Levorphanol (10mg/kg; ip)	µ-agonist	5	16.08±1.34*	18.33±6.91	174±66
Cyclazocine (5mg/kg; ip)	k-agonist	7	22.74±5.83	20.70±4.66	126±40
Ketocyclazocine (5mg/kg; ip)	k-agonist	6	20.75±4.66*	26.45±7.94	157±69
Saline sc	–	9	24.87±3.32	13.18±2.44	56±08
Tifluadom (10mg/kg; sc)	k-agonist	5	23.89±4.54	14.27±3.70	69±20
SKF 10,047 (10mg/kg; sc)	σ-agonist	9	20.62±3.80	18.56±2.91	99±13*

Table 22D. Values are means ± SEM; unpaired Students' t-test; level of significance * $p < 0.05$. 'n' = Number of observations.

ARN

Treatment	Receptor properties	n	5-HT Conc±SEM pg/µg	5-HIAA Conc±SEM pg/µg	5-HT TR (% Ratio) ±SEM
Saline ip	-	7	26.46±2.05	16.87±3.79	68±17
Naloxone (10mg/kg; ip)	opiate antagonist	6	20.16±5.66	18.31±3.59	121±26
Morphine (40mg/kg; ip)	µ-agonist	6	22.74±4.11	19.78±3.24	98±14
Levorphanol (10mg/kg; ip)	µ-agonist	5	26.32±0.88	20.01±4.06	81±23
Cyclazocine (5mg/kg; ip)	k-agonist	7	16.79±4.65	32.69±6.1	203±26**
Ketocyclazocine (5mg/kg; ip)	k-agonist	6	25.47±4.46	28.15±4.44	141±34
Saline sc	-	9	23.37±3.66	22.91±2.53	148±32
Tifluadom (10mg/kg; sc)	k-agonist	5	27.40±4.12	18.98±2.32	81±17
SKF 10,047 (10mg/kg; sc)	σ-agonist	9	19.71±1.99	13.75±1.41*	71±05

Table 22E. Values are means ± SEM;. unpaired Students' t-test; level of significance * $p < 0.05$ and ** $p < 0.01$. 'n' = Number of observations.

VMH

Treatment	Receptor properties	n	5-HT Conc±SEM pg/µg	5-HIAA Conc±SEM pg/µg	5-HT TR (% Ratio) ±SEM
Saline ip	-	7	34.78±4.03	18.40±2.09	54±04
Naloxone (10mg/kg; ip)	opiate antagonist	6	31.30±4.17	19.99±4.36	75±15
Morphine (40mg/kg; ip)	µ-agonist	6	25.96±3.39	27.25±2.29*	135±32*
Levorphanol (10mg/kg; ip)	µ-agonist	5	27.75±4.54	23.96±4.61	86±13
Cyclazocine (5mg/kg; ip)	k-agonist	7	39.13±5.99	28.97±4.86	75±06
Ketocyclazocine (5mg/kg; ip)	k-agonist	6	27.78±5.78	29.58±6.27	175±65
Saline sc	-	9	34.07±2.82	20.66±2.03	64±07
Tifluadom (10mg/kg; sc)	k-agonist	5	25.60±4.71	24.64±3.04	126±30
SKF 10,047 (10mg/kg; sc)	σ-agonist	9	36.82±5.09	28.72±5.88	75±06

Table 22F. Values are means ± SEM; unpaired Students' t-test; level of significance * $p < 0.05$. 'n' = Number of observations.

The 5-HT content and turnover in specific hypothalamic nuclei are summarized in Tables 22(A-F) and Figures 27(a-f). Tifluadom and SKF 10,047 brought about a significant decrease in the 5-HT content and an increase in the turnover in the SCH. Tifluadom also brought about a very significant rise in 5-HIAA levels in this region. Cyclazocine was the only other opiate to significantly decrease the 5-HT content of the SCH. However, there were no significant changes observed in its turnover. Saline, injected subcutaneously

into the controls, caused a significant rise in the 5-HT content but a significant decrease in 5-HIAA levels in the SCH compared to those controls which received saline intraperitoneally.

None of the treatments caused any significant changes in the 5-HT content of the MPO. However, in this region the turnover of 5-HT was very significantly increased by ketocyclazocine. The controls which received saline subcutaneously, again had significantly reduced levels of 5-HIAA in the MPO although the content and turnover of 5-HT were not significantly altered.

Within the AHA, cyclazocine, tifluadom and SKF 10,047 all caused a significant decrease in 5-HT content. Both tifluadom and SKF 10,047 caused an increase in 5-HT turnover in the AHA. However, SKF 10,047 brought about a significant increase in 5-HIAA levels in this region. Those controls which received saline subcutaneously had an increase in the 5-HT content and a decrease in turnover of 5-HT in the AHA.

The turnover of 5-HT in the ME was significantly increased by SKF 10,047; its content was decreased by levorphanol. Ketocyclazocine caused a significant fall in the content of 5-HT in the ME; no significant changes were observed in either the 5-HIAA content or in the 5-HT turnover in this region.

The 5-HT content of the ARN was not significantly altered by any of the drugs administered. However, 5-HT turnover was significantly increased by cyclazocine, and 5-HIAA levels were significantly reduced by SKF 10,047.

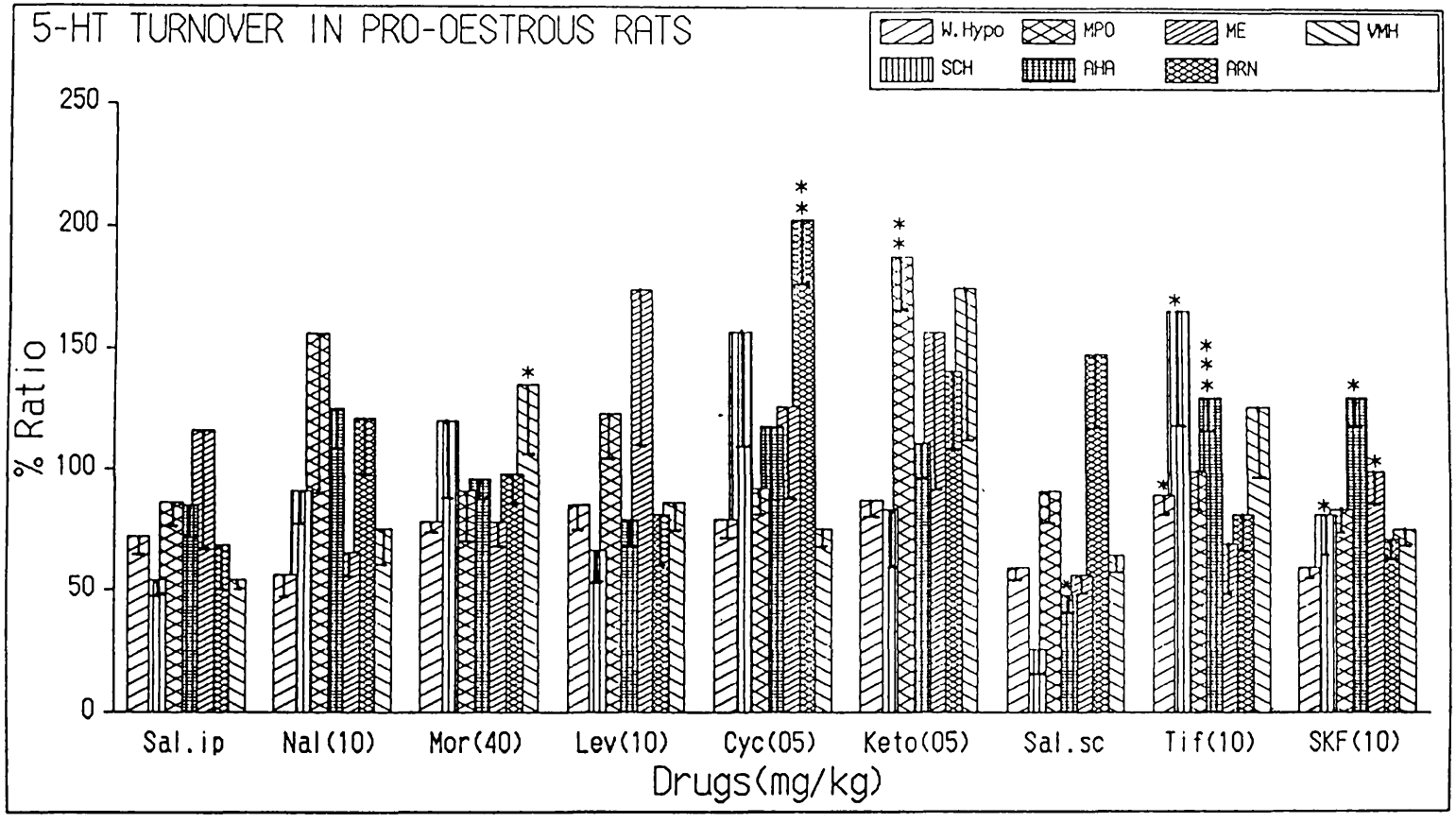

5-HT TURNOVER IN PRO-OESTROUS RATS
W. Hypo
MPO
ME
VMH
SCH
AHA
ARN
250
200
150
100
50
0
% Ratio
Sal.ip
Nal(10)
Mor(40)
Lev(10)
Cyc(05)
Keto(05)
Sal.sc
Tif(10)
SKF(10)
Drugs(mg/kg)

Figure 27

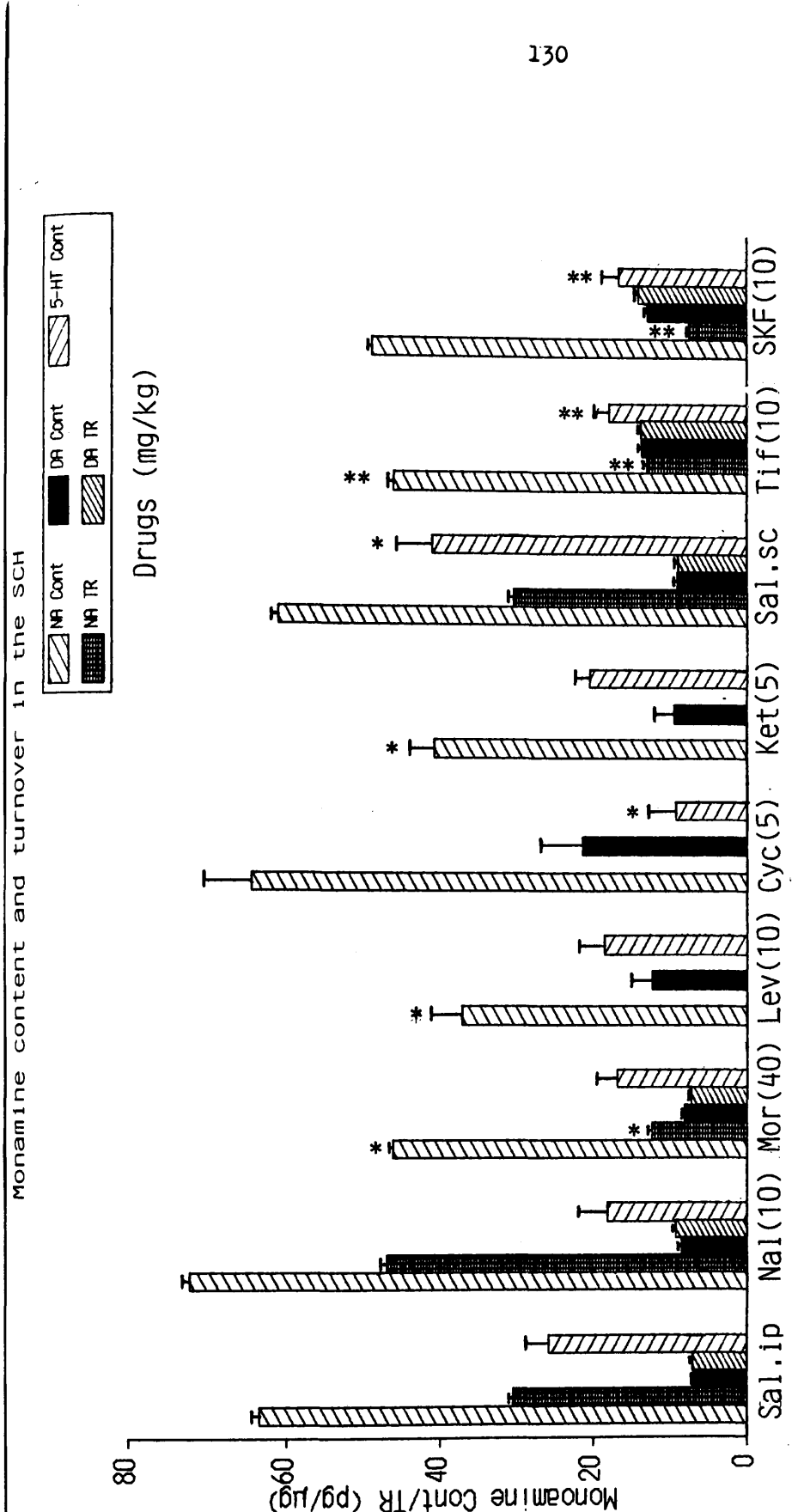

Monamine content and turnover in the SCH
NA Cont
NA TR
DA Cont
DA TR
5-HT Cont
Drugs (mg/kg)
Monoamine Cont/TR (pg/µg)
0
20
40
60
80
Sal.ip
Nal(10)
Mor(40)
Lev(10)
Cyc(5)
Ket(5)
Sal.sc
Tif(10)
SKF(10)

Figure 27(a)

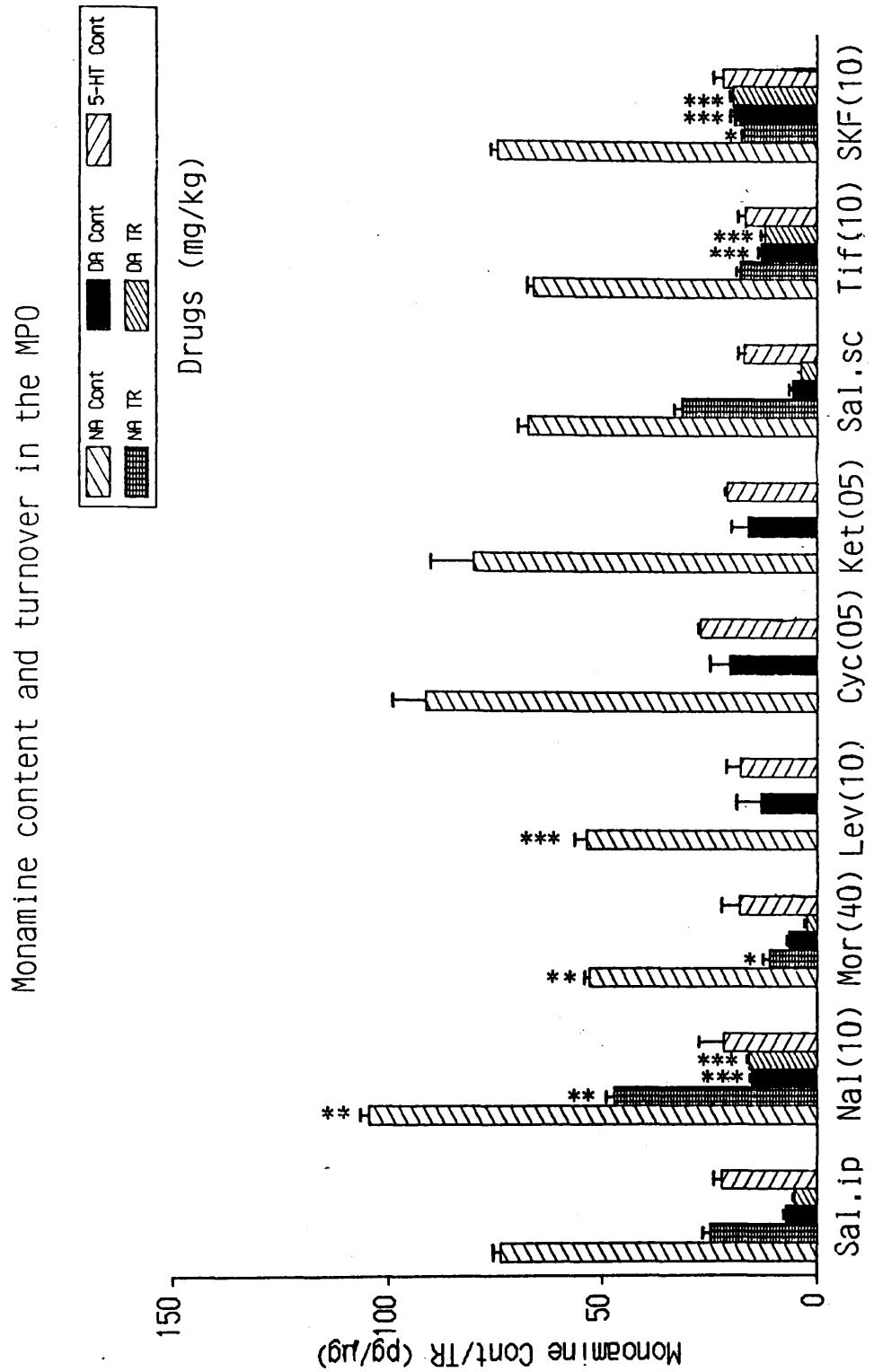

Monamine content and turnover in the MPO
NA Cont
NA TR
DA Cont
DA TR
5-HT Cont
Drugs (mg/kg)
Monoamine Cont/TR (pg/µg)
150
100
50
0
Sal.ip
Nal(10)
Mor(40)
Lev(10)
Cyc(05)
Ket(05)
Sal.sc
Tif(10)
SKF(10)

Figure 27(b)

Monamine content and turnover in the AHA

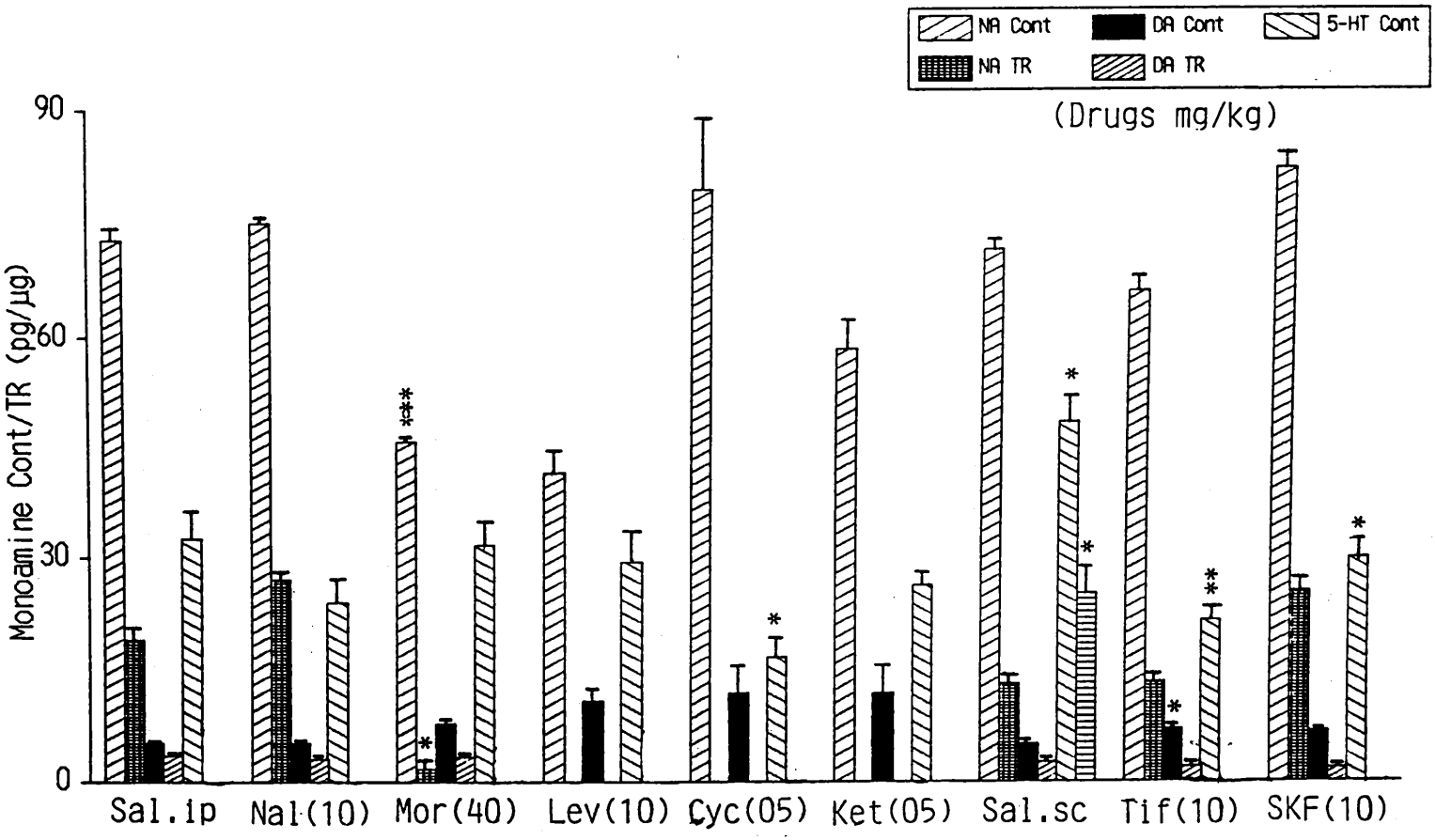


Figure 27(c)

Monamine content and turnover in the ME

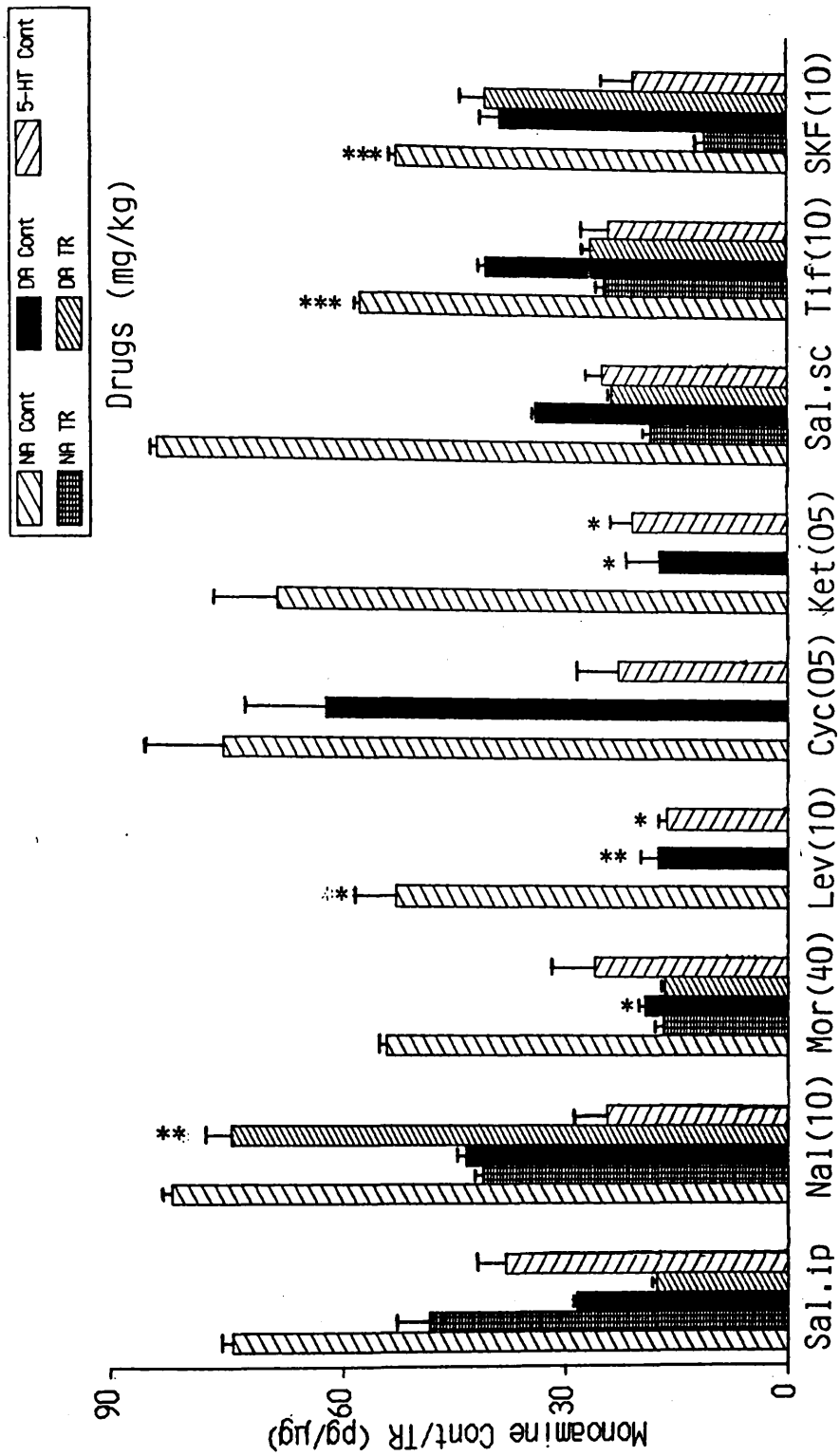


Figure 27(d)

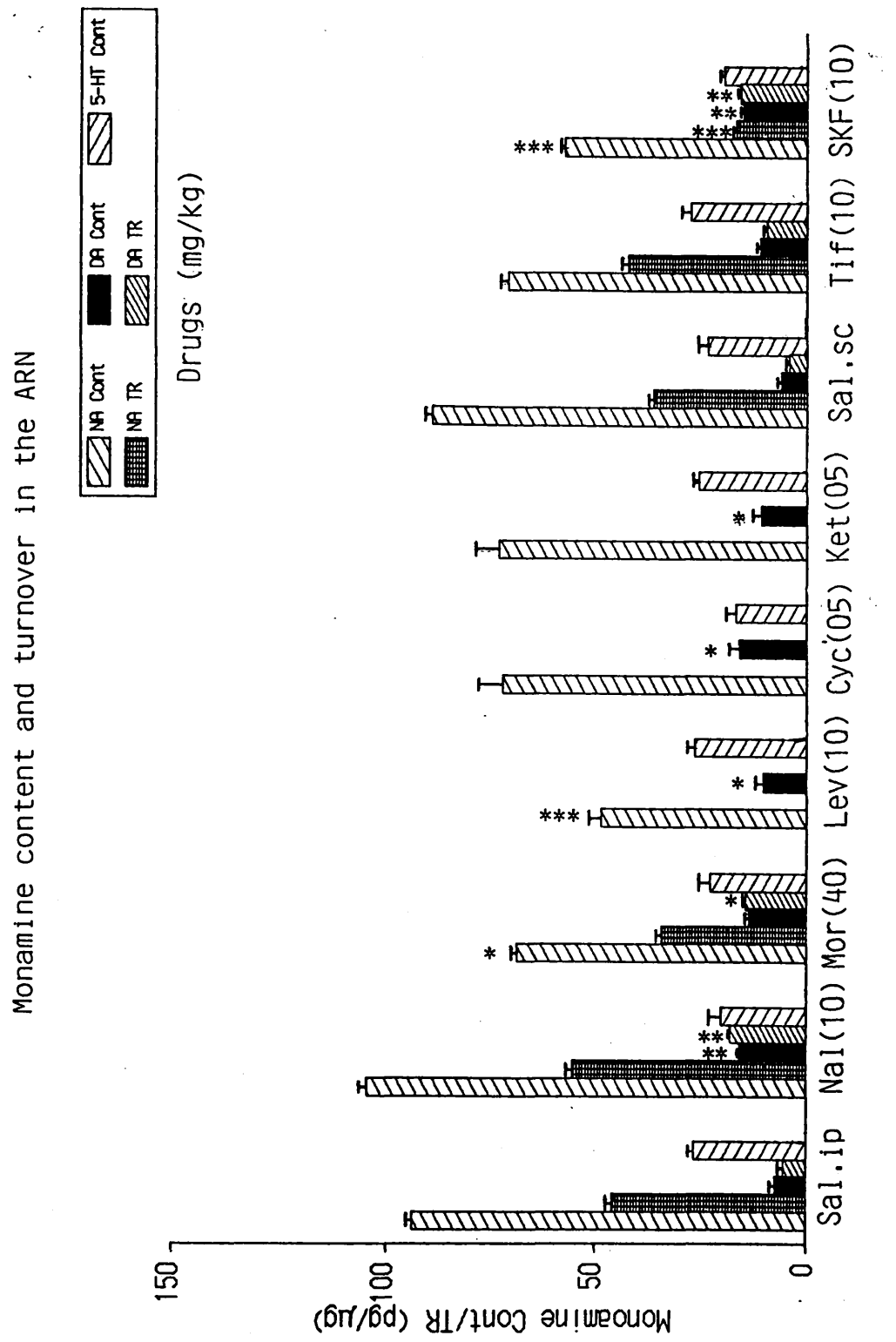

Monamine content and turnover in the ARN
NA Cont
NA TR
DA Cont
DA TR
5-HT Cont
Drugs (mg/kg)
Monoamine Cont/TR (pg/µg)
150
100
50
0
Sal.ip
Nal(10)
Mor(40)
Lev(10)
Cyc(05)
Ket(05)
Sal.sc
Tif(10)
SKF(10)

Figure 27(e)

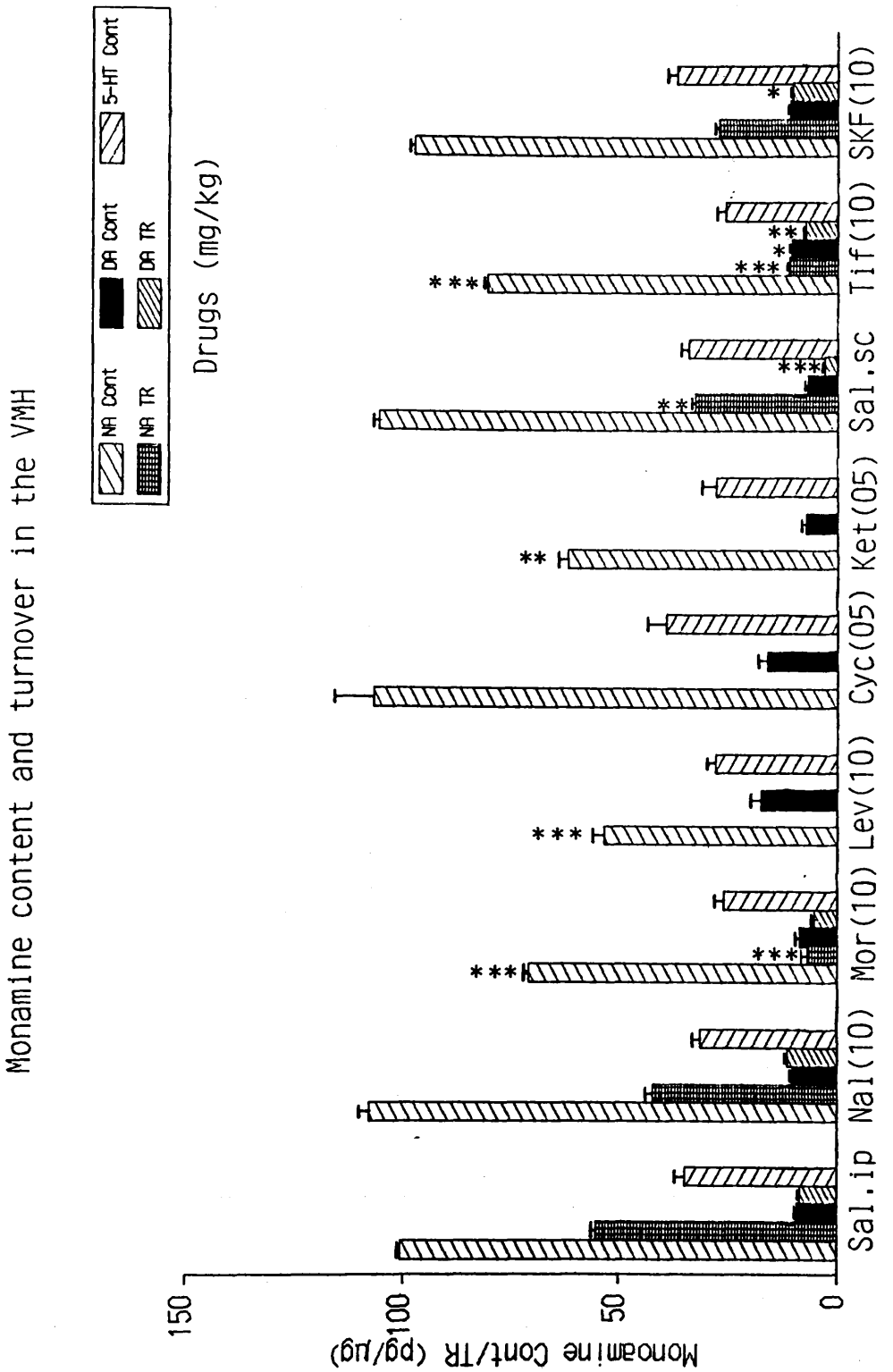

Monamine content and turnover in the VMH
NA Cont
NA TR
DA Cont
DA TR
5-HT Cont
Drugs (mg/kg)
Monoamine Cont/TR (pg/µg)
150
100
50
0
Sal.ip
Nal(10)
Mor(10)
Lev(10)
Cyc(05)
Ket(05)
Sal.sc
Tif(10)
SKF(10)

Figure 27(f)

Within the VMH, morphine caused a significant rise in the content of 5-HIAA and 5-HT turnover. The content of 5-HT in this region was not significantly altered in any of the groups studied.

It was interesting to note that naloxone did not cause any significant changes in either the levels or turnover of 5-HT and 5-HIAA in the six specific hypothalamic regions studied.

v) Plasma LH levels in Pro-oestrous Rats (with or without α-MPT):

Treatment	Receptor properties	Plasma LH (ng/ml) α-MPT 0 mins.	α-MPT 45 mins.	α-MPT 90 mins.
Saline ip	-	4.74±0.62 (27)	1.36±0.09*** (6)	1.72±0.11*** (5)
Naloxone (10mg/kg ip)	opiate antagonist	11.34±2.04** (32)	1.47±0.04*** (6)	1.64±0.08*** (5)
Morphine (40mg/kg ip)	μ-agonist	2.35±0.39** (5)	1.43±0.21 (5)	1.45±0.45* (5)
Saline sc	-	3.89±0.38 (20)	1.56±0.06*** (5)	1.44±0.11*** (5)
Tifluadom (10mg/kg sc)	k-agonist	2.19±0.21** (22)	1.48±0.15* (5)	1.41±0.14* (5)
SKF 10,047 (10mg/kg sc)	σ-agonist	4.04±0.54 (22)	1.45±0.19*** (5)	1.54±0.17** (5)

Table 23. Values are Mean ± SEM; unpaired Student's t-test confirmed by two-way analysis of variance. Level of significance * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.
(n) = Number of observations.

The plasma LH levels were significantly decreased compared to the saline-injected controls by both morphine and tifluadom. In contrast naloxone significantly increased the levels of LH. SKF 10,047 did not significantly alter LH concentration in the plasma from those in the controls. Treatment with α-MPT for both 45 minutes and 90 minutes caused an overall fall in LH content in the plasma.

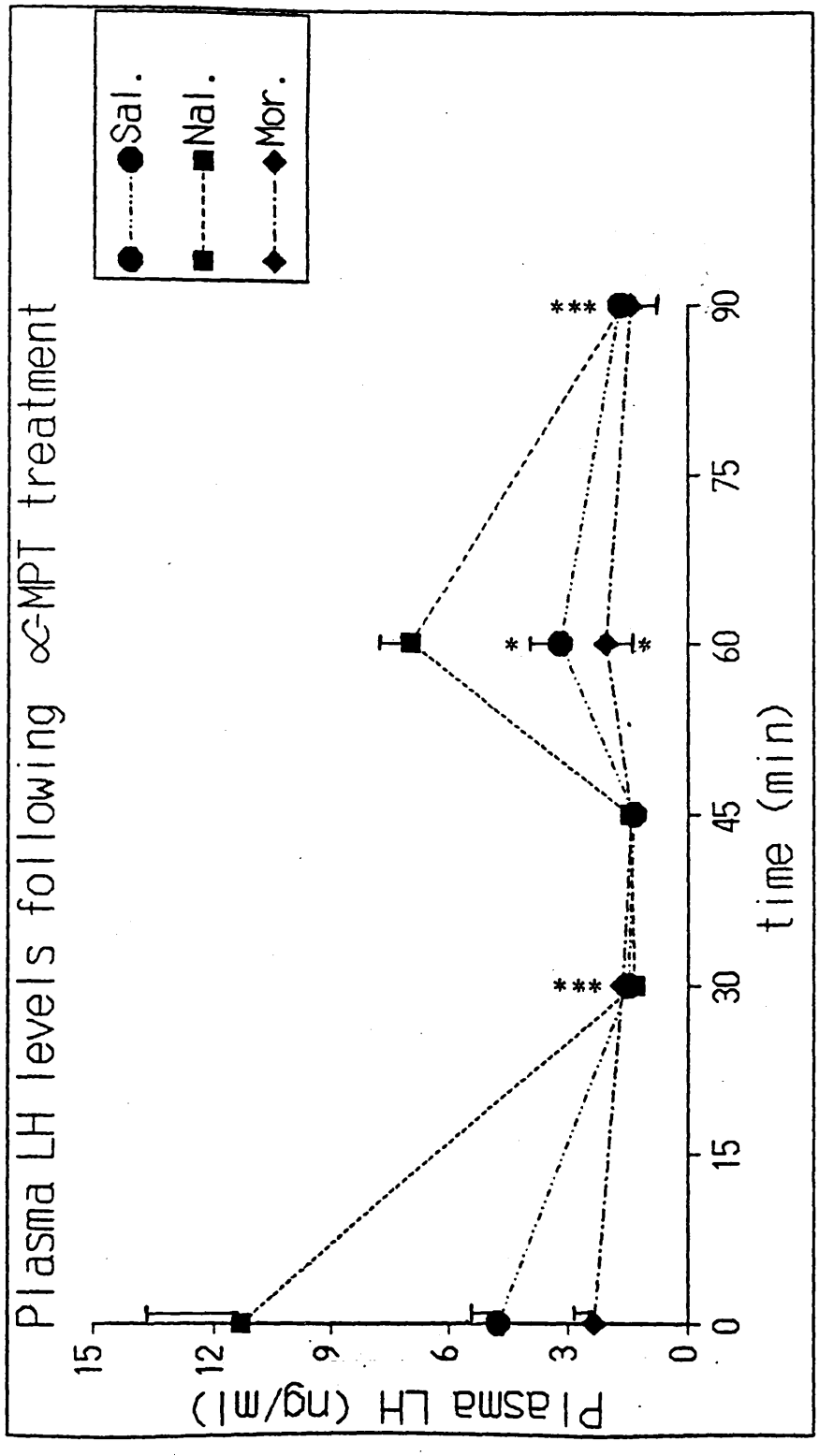

Plasma LH levels following ∝-MPT treatment
Sal.
Nal.
Mor.
Plasma LH (ng/ml)
15
12
9
6
3
0
0
15
30
45
60
75
90
time (min)
***
*
*
***

Figure 28(a)

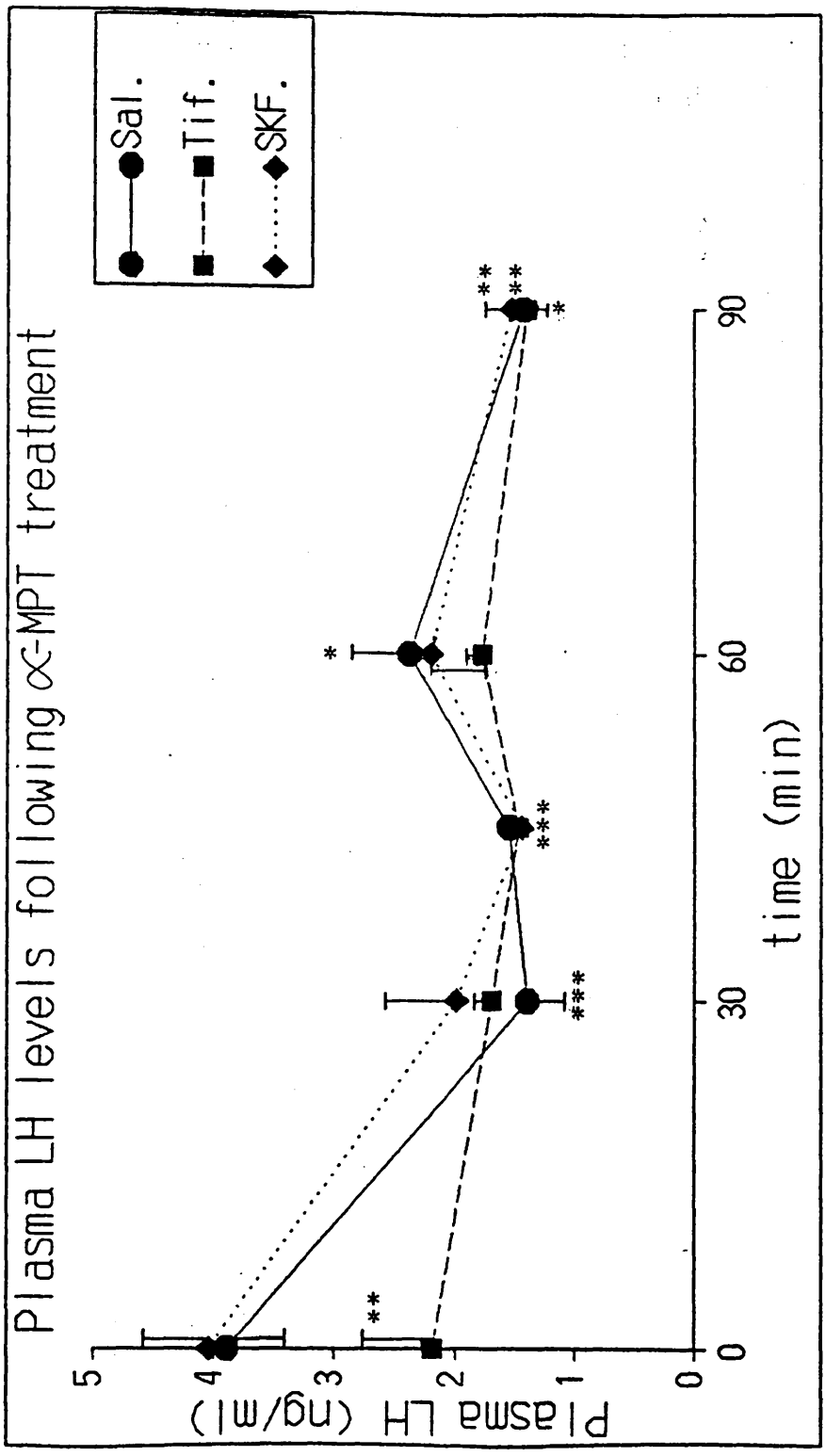

Plasma LH levels following α-MPT treatment
Sal.
Tif.
SKF.
Plasma LH (ng/ml)
time (min)
0
1
2
3
4
5
0
30
60
90
*
**
***

Figure 28(b)

DISCUSSION

Numerous peptidergic and monoaminergic neural elements present in the hypothalamus influence pituitary gonadotrophic function. However, there is very little information available about how these various structures are interconnected. Among the different peptides isolated and characterized, the opioids represent a significant group. They are widely distributed within the CNS and their very high concentration within the hypothalamus (Stojilkovic et al., 1987) suggests that they may be important in neuroendocrine regulation (see Kalra, 1986). The precise mechanism of action i.e., whether opiates act directly on the release of hypothalamic GnRH or interfere with other modulatory transmitters is a subject of current research.

It has been shown that opiates do not directly alter the release of LH from the anterior pituitary (see Grossman & Rees, 1983; Bicknell, 1985) instead they appear to inhibit gonadotrophin secretion by preventing the discharge of GnRH at the hypothalamic level (Ching, 1983; see Kalra, 1983). The effect of opiates on GnRH secretion is controversial. *In vitro* studies have shown that exposure of GnRH terminals to opiates and opioid peptides causes little change in basal GnRH release into the medium (Rotsztejn et al., 1978; Drouva et al., 1981). These observations are not in agreement with numerous *in vivo* studies, where GnRH levels promptly decline (Ching, 1983) after opiate administration. However, a large body of evidence suggests that the effects of opioids on GnRH neurones are indirect and are probably mediated via opioid interaction with monoaminergic neurones (Adler & Crowley,

1984; Lookingland & Moore, 1985; Miller, Clifton & Steiner, 1985).

There is a great deal of evidence to indicate that the physiological effects of opioid peptides are exerted through their interaction with specific binding sites present in the brain identified as the opioid receptors (Cox, 1982; Wood, 1982; Lutz et al., 1985; Schoffelmeer, Hogenboom & Mulder, 1987; Zukin et al., 1988). Different classes such as mu, delta, kappa and sigma receptor types have been described (Iyengar, Kim & Wood, 1986; Wood, 1980). Additionally, each of the endogenous opioids apparently binds to more than one opioid receptor type (Cox, 1982, Stojilkovic et al., 1987). Therefore, in order to understand further the influence of the endogenous opioid peptides on the neuroendocrine control of LH secretion, it is important to define what effect activation of each of the opioid receptor types has on this hormone. Though this issue has been addressed (Leadem & Kalra, 1985; Pfeiffer et al., 1983), it is only recently that agonists have been synthesized that exhibit sufficient binding selectivity to distinguish between several major opioid receptor sites. James and Goldstein (1984) developed a method of assessing this selectivity and have surveyed a number of opioid agonists. They define a selective opioid as any ligand that exhibits binding to its preferred receptor type by at least 100-fold over the other opioid receptor types.

The present study was undertaken to investigate the effects of specific opioid receptor agonists and antagonist

on the hypothalamic monoaminergic systems in regulating LH release in the rat.

The Effects of Opiates on Monoamine Content, Turnover and Plasma LH levels in short-term Orchidectomized Rats

The present study provides firm evidence that drugs acting on central opioid receptors alter amine content and turnover in the hypothalamus. Opiates have previously been reported to alter catecholamine and indoleamine turnover in the rat brain (Akabori & Barraclough, 1986, Clark et al., 1988; Deyo, Swift & Miller, 1979; Yarbrough, Buxbaum and Sanders-Bush, 1973; Johnson & Crowley, 1984). The results reported here suggest that morphine decreases hypothalamic NA levels immediately after infusion. This effect is seen up to the maximum interval studied (i.e., two hours post-infusion), at which time the turnover of this amine is also decreased. The evidence, as provided by this study, suggests that morphine inhibits the synthesis of NA, in addition to reducing its rate of release. Furthermore, it is proposed that these effects are mediated by two different receptors, mu_1 and mu_2. Mu_1 receptors have been characterized as causing a profound membrane hyperpolarisation on binding opiates (Williams & North, 1984), Mu_2 receptors are coupled to adenylate cyclase and mediate mu effects other than those acting through the mu_1 receptor (Pasternak, 1986). Additionally, a decrease in intracellular levels of cyclic adenosine monophosphate (cAMP), as mediated by the mu_2 receptor, could alter neurone biochemistry leading to a

reduction in the initial tissue content of NA. The existence of both mu_1 and mu_2 receptors on noradrenergic axon terminals within the area studied would be sufficient to explain the observed effects of morphine.

None of the opiates tested in this study had any significant effect on hypothalamic DA concentrations at 20 minutes post-infusion. However, both the content and turnover of hypothalamic DA were very significantly increased two hours following morphine administration. Acute administration of morphine has been reported to increase hypothalamic DA concentrations (deWeid et al., 1974), but to have either no effect or merely cause a slight increase in the whole brain DA concentration (Gauchy et al., 1973; deWeid et al., 1974) in the rat. DA is the most likely prolactin release inhibitory factor (PIF) (Tuomisto and Mannisto, 1985), the increase in prolactin secretion which occurs after morphine treatment is hence incompatible with elevated DA release.

Little information is available on the effects of morphine on 5-HT levels in the hypothalamus (see Meites et al., 1979) although acute morphine administration has been reported to increase 5-HT turnover in the whole brain of rats (Haubrich & Blake, 1973; Yarbrough, Baxbaum & Sanders-Bush, 1971). In the present study, while morphine did not cause any significant changes in the hypothalamic 5-HT content at 20 minutes post-treatment, a significant increase in 5-HT levels was seen after an interval of two hours.

Treatment	Plasma LH 10 min/20 min	Monoamine Content (20minutes post-infusion)			
		NA	DA	5-HT	5-HIAA
Saline					
Naloxone (30µg/kg)	0/0	0	+	0	0
Naloxone (60µg/kg)	0/0	+	0	0	0
Morphine (400µg/kg)	0/0	–	0	0	0
Morphine (800µg/kg)	–/–	–	0	0	0
Levorphanol (400µg/kg)	0/0	–	0	+	0
Cyclazocine (50µg/kg)	0/0	–	0	+	–
SKF 10,047 (100µg/kg)	0/0	0	0	+	0
FK 33,824 (8 µg/kg)	0/0	0	0	0	0

Table 24. Plasma LH levels and hypothalamic monoamine content at 20 minutes post-infusion. (+ = Increase; – = decrease; 0= No change).

The majority of the data available suggest that mu receptors are specifically involved in the control of gonadotrophin secretion (Schluz et al., 1981; Pfeiffer et al., 1983; Panerai et al., 1985). However, it is also possible that other types of receptors (kappa and delta) might also participate in such control (Kato et al., 1982; Iyengar et al., 1986; Leadem & Kalra, 1985, 1987). Further,

an interaction between hypothalamic GnRH neurones and kappa receptors has been implicated by the work of Marko (1982).

The effects of cyclazocine on hypothalamic monoamine levels were in some respects similar to those of morphine, but cyclazocine had no effect on DA turnover rate, although the content of DA was significantly increased. This opiate also brought about a rise in hypothalamic 5-HT levels at 20 minutes following infusion. It has been suggested that cyclazocine is not a pure kappa receptor agonist, but also has partial agonist/antagonist activity at other opioid receptor types (Wood, 1983). Furthermore, cyclazocine has been shown to possess a high affinity for the mu receptor (Zukin & Zukin, 1981). The action of this opiate is dose-dependent. At higher doses the antagonist activity predominates and at lower doses it acts as an agonist (Wood et al., 1982). The dose used in this study (50µg/kg) would suggest an agonistic action of cyclazocine.

The effects of SKF 10,047 (N-allylnor-phenazocine), a prototype sigma receptor agonist, differed from those of morphine and cyclazocine in that it had no significant effect on either hypothalamic NA levels or turnover. Although the 5-HT content of the hypothalamus was initially raised (i.e., 20 minutes post-infusion), this was not maintained at two hours. Both hypothalamic DA content and turnover were significantly increased after SKF 10,047 infusion, a similar effect to that seen in the morphine-treated group (Wood et al., 1982). The drug used in this study was DL SKF 10,047; the D isomer is a potent sigma-agonist, whereas the L isomer

is a potent mu-antagonist (Compton et al., 1987). The effects of this drug are thus also of a mixed agonist-antagonist type. The rise in DA content and turnover in the hypothalamus is suggestive of sigma-agonist activity, whereas the absence of changes in hypothalamic NA activity (similar to naloxone) seems to be a mu-antagonist effect. SKF 10,047 has likewise been shown to possess antagonistic properties at both mu and kappa receptor sites, but displays a powerful sigma agonism (Compton et al., 1987). Mu and kappa effects on DA turnover have been demonstrated in this study, characterized by a significant increase in the rate of amine release and synthesis. If SKF 10,047 had antagonised endogenous mu and kappa activity, a stimulatory effect on NA and 5-HT turnover would have been witnessed. It therefore appears that the antagonist effects of the drug are small or may be suppressed by some other factors involved in this regulation.

The mode of action of SKF 10,047 remains obscure. However, the sigma-agonist phencyclidine (PCP) has been shown to inhibit the uptake of DA by synaptic transmitter storage vesicles (Ary & Komiskey, 1979). Such an action *in vivo* would decrease intraneuronal amine oxidation, decreasing axon terminal levels of neurotransmitter. It is possible that SKF 10,047, acting via the sigma receptor, may exert similar effects within noradrenergic and serotoninergic neurones. Although the deficit would tend to be compensated by subsequent synthesis, a depleted storage capacity could render this ineffectual. Hence, it is possible that SKF

10,047 may exert its effects on NA and 5-HT levels via such a mechanism.

The most powerful stimulatory effect on DA turnover observed in this study was that exerted by SKF 10,047. A marked increase in both the amine depletion rate and the initial tissue concentrations resulted in a five-fold increase in turnover. The qualitative differences in the actions of morphine, for example, and SKF 10,047, cannot be determined by measurements of turnover. Other studies have revealed that the behavioural manifestations of SKF 10,047 treatment (Martin, 1983) are very similar to those exhibited by the administration of centrally active DA agonists (Seeman, 1980). Although SKF 10,047 increased DA turnover in a manner indistinguishable from that of morphine, it is suggested that SKF 10,047 does not act to dissociate DA synthesis from release (Martin, 1983). Hence, the large turnover would correspond to an increased rate of secretion of DA from nerve terminals.

Naloxone, which largely acts as a mu antagonist (Folders et al., 1963), is also known to bind to other opioid receptors with varying affinity (Martin et al., 1976; Pfeiffer & Herz, 1982). This antagonist, although causing an initial rise in the hypothalamic NA content, i.e., at 20 minutes after administration, did not have a persistent effect. There were no changes observed in the content or turnover rates of NA or DA in the hypothalamus at two hours post-treatment. However, naloxone did bring about a rise in

Treatment	Plasma LH 1hr/2hrs	Amine Content/Turnover (2 hours post-infusion)		
		NA Cont/TR	DA Cont/TR	5-HT Cont/TR
Saline				
Naloxone (60µg/kg)	0/0	0/0	0/0	+/0
Morphine (800µg/kg)	-/-	-/-	+/+	+/0
Cyclazocine (50µg/kg)	-/-	-/-	+/0	0/-
SKF 10,047 (100µg/kg)	0/0	0/0	+/+	0/0

Table 25. Plasma LH levels and monoamine content and turnover two hours post-infusion. (+ = Increase, - = Decrease; 0 = No change).

the 5-HT content of this region. Morphine had a similar effect on the hypothalamic 5-HT content. The effect of the antagonist naloxone was thus unexpected, and contradicts the findings of previous workers (see Martin, 1983). The present results would imply that naloxone may have activated a stimulatory opioidergic input to the hypothalamic 5-HT neurones. The existence of such an input has been postulated by others, who have demonstrated the stimulation of LH release by icv morphine (Motta & Martini, 1982; Piva et al., 1986). Furthermore, naloxone may elicit morphine-like analgesia when administered icv (Dickensen, Le

Bars & Besson, 1981). These observations preclude a precise interpretation of the mode of action of naloxone in this investigation. Nevertheless, many central and peripheral axon terminals have inhibitory autoreceptors (e.g. alpha$_2$-adrenoceptors); if opioidergic neurones are no exception naloxone may in certain situations cause a disinhibition of these neurones leading to effects indistinguishable from those of opioid administration.

Morphine has been found to lower LH levels in intact male rats (Cicero et al., 1979; see Cicero, 1980). Naloxone administration sharply increases serum LH levels (Cicero et al., 1979; Kalra & Simpkins, 1981; Van Vugt et al., 1981, Piva, et al., 1986), suggesting an inhibitory role of endogenous opioids on the hypothalamic-pituitary-LH axis. However, in the present study, it was found that naloxone failed to cause any significant changes in circulating LH levels and the effect of SKF 10,047 was similar. The effects of naloxone seen during the present study could perhaps be related to the steroidal manipulation created in this particular experimental model. The animals were treated with the opiates or their antagonist, naloxone, between 18-20 hours post-castration. It could be predicted therefore, that in spite of removing gonadal steroid production by castration, the diminishing residual levels of testosterone could explain why the effects of naloxone differed from those recorded by others. Morphine has been shown to mimic the effects of testosterone in exerting its inhibitory action on LH release at the hypothalamic level (Miller et al., 1986). The highly significant changes observed in hypothalamic

catecholaminergic activity at two hours after treatment with morphine would also imply that the traces of testosterone might have enhanced its activity and decreased the antagonistic effect of naloxone. Only future studies at differing intervals post-castration would provide more evidence towards this hypothesis. Further, long-term gonadectomy has been shown to alter the number of opioid receptors in the CNS (Cicero et al., 1987). However, there are no reports suggesting a change in the number of opioid receptors in the short-term castrated rat. It could be assumed therefore, that such a change in number occurring immediately after castration could result in a decreased antagonistic activity of naloxone.

The stimulatory role of NA in the control of LH release is well documented (Adler et al., 1983; Kalra & Gallo, 1983; see Kalra, 1986). This was further confirmed by the present findings; the decrease in hypothalamic NA content and turnover observed after morphine and cyclazocine administration most likely caused the significant fall seen in LH levels. Furthermore, the drugs which failed to cause any significant changes in noradrenergic turnover within the hypothalamus also did not alter circulating LH levels in the animals. Thus in the naloxone- and SKF 10,047-treated animals in which there was no significant alteration in the hypothalamic NA content or turnover, there was also no change in circulating LH levels.

α-MPT, an inhibitor of tyrosine hydroxylase, used to study catecholaminergic activity in the hypothalamus, itself

brought about a significant decrease in LH levels in the controls and in the morphine- and cyclazocine-treated groups.

Treatment	Plasma LH		
	Control 1hr/2hrs	α-MPT for 1 hour 1hr/2hrs	α-MPT for 2 hrs 1hr/2hrs
Saline	0/0	0/-	-/-
Naloxone (60µg/kg)	0/0	0/0	0/0
Morphine (800µg/kg)	-/-	0/-	-/-
Cyclazocine (50µg/kg)	-/-	0/-	-/-
SKF 10,047	0/0	0/-	0/0

Table 26. Effects of α-MPT on plasma LH levels one and two hours post-infusion. - = Decrease 0 = No change.

However, α-MPT treatment to the groups which had earlier received naloxone did not led to an alteration in their LH levels. Although SKF 10,047 caused a significant decrease in circulating LH one hour after α-MPT treatment, no similar change was seen two hours after α-MPT administration. The acute depletion of NA caused by the injection of α-MPT could be responsible for the reduction seen in LH levels (Herdon et al., 1984). The effects of naloxone and SKF 10,047 on circulating LH would indicate that these two drugs might somehow sustain noradrenergic activity, thus, not affecting their LH levels. If DA was involved in the α-MPT- mediated

decrease in plasma LH, then one would have expected LH levels also to decrease in the SKF 10,047-treated animals. This is because this drug caused a very high increase in the hypothalamic content of DA similar to that brought about by morphine and cyclazocine. However, it is difficult to predict what effect the increased DA activity observed in the opiate-treated groups had on LH release. Both a stimulatory and an inhibitory action for DA have been claimed by other investigators (James et al., 1987; Gallo, 1980).

In this study, although changes in NA content and turnover could be correlated with changes observed in LH levels, the same was not true for DA. While the increased hypothalamic DA content and turnover seen in the morphine- and cyclazocine-treated groups would be suggestive of an inhibitory role for DA on LH release, SKF 10,047 which significantly increased the turnover of DA, brought about no alterations in LH levels to the animals treated with it. Rotsztejn et al., (1978) observed that although morphine and other endogenous opioid peptides had no effect on basal GnRH secretion, these agents significantly suppressed the DA-induced stimulation of GnRH release from hypothalamic slices *in vitro*. Consequently, these investigators suggested that the interaction between opiates and DA may occur postsynaptically at the level of the GnRH nerve endings. There is little evidence to show that opiates affect the response of GnRH neurones to DA or adrenergic agonists *in vivo* (Kalra & Gallo, 1983; Kalra & Simpkins, 1981). Although DA was found to excite GnRH release from the ME of male rats *in vitro* (Negro-Vilar & Ojeda, 1978; Negro-Vilar,

Ojeda & McCann, 1979; Schneider & McCann, 1969), icv injection produced little stimulation of GnRH-induced LH release *in vivo* (Kalra & Kalra, 1983). Another body of evidence suggests that opiates acting at the level of the ME do not affect GnRH release directly, but rather interfere with the response of neurosecretory nerve endings to DA stimulation (Gallo, 1980). The stimulatory action of DA on *in vitro* GnRH release could be inhibited by DA antagonists, such as pimozide and haloperidol (see Seeman & Guttman, 1987; Barnett, 1986). Attempts have been made to correlate changes in LH secretion induced by steroid feedback mechanisms with changes in DA activity (Gallo, 1980, Simpkins, Kalra & Kalra, 1980, 1983). Gunnet, Lookingland & Moore (1986) have reported that DA levels were increased in the MPO, DMN and median zona incerta following castration. Testosterone replacement brought DA levels back to normal. Two weeks following orchidectomy, the DA turnover in the MPO was decreased. Subsequent administration of testosterone to these animals, in doses that reduce LH levels to intact values, increased the DA turnover. The present findings that morphine and cyclazocine increased DA turnover may be compared to those showing increased DA turnover in the MPO after testosterone administration, as both morphine and testosterone exert similar effects on LH in the castrated rat (see Cicero, 1980). However, Simpkins et al., (1980, 1983) reported that local injections of testosterone into the MPO of castrated rats failed to modify LH secretion. Thus, the effects of increased dopaminergic activity on LH release is not clear.

The observed decrease in hypothalamic 5-HT turnover by treatment with cyclazocine seen in the present study would suggest a stimulatory effect of 5-HT on LH release (Walker, 1983). Apart from a direct action on kappa receptors, it is likely that the effect of cyclazocine on 5-HT turnover is at least partly mediated by the action of this drug at other opioid receptor sites. Specifically, cyclazocine has been shown to possess a high affinity for the mu receptor where it acts as a competitive antagonist (Martin, 1983). For this reason, the drug-induced diminution of 5-HT turnover observed in this study may partly be due to the blockade of stimulatory opioidergic innervation (Johnson & Crowley, 1984). SKF 10,047 also caused a fall in 5-HT turnover although it failed to reach significant levels.

The effects of opiates and their antagonist, naloxone, on both monoamine activity and LH levels could be, at least partly, explained by the existing steroidal feed-back mechanism and its manipulation by castration and opiate administration. The gonadal steroids exert powerful negative feedback effects on LH secretion. Part of this influence is exerted via the hypothalamus and probably includes an opioidergic component (Schulz et al., 1981; Gabriel et al., 1983; Bhanot & Wilkinson, 1984). As reported earlier, morphine administration can mimic both the acute and chronic effects of this steroid on the hypothalamic-pituitary-LH axis of the castrated male rat (Cicero et al., 1979, 1980; Van Vugt et al., 1982). The parallel actions of morphine and

steroids suggest a common mechanism of action (Miller et al., 1986). Low concentrations of testosterone (400-600pg/ml serum), which fail to inhibit LH release in orchidectomized rats, were maximally effective when these rats concurrently received continuous opiate stimulation by morphine (Gabriel et al., 1983). It has been found that the effects of endogenous and exogenous opiates on LH release disappear coincident with the removal of gonadal steroid feedback at 7 days post-castration (Bhanot & Wilkinson, 1983). Naloxone is no longer able to stimulate LH secretion in these animals. These findings suggest the endogenous opioid peptide neurones may not only affect GnRH release, but may also influence neurosecretory events modulated by steroids to promote GnRH accumulation in the ME nerve terminals (Kalra & Kalra, 1983).

In conclusion, it was found that opiates modulate hypothalamic monoaminergic activity in the short-term orchidectomized rats. The reduced LH levels caused by morphine and cyclazocine could be related to their causing a decreased hypothalamic NA turnover. The rise in DA turnover brought about by SKF 10,047 failed to alter LH levels. Surprisingly, naloxone had no significant effect on either the amine turnover or LH release.

The effects of Opiates on Hypothalamic Monoaminergic activity and LH levels Prior to the Pre-ovulatory LH surge in the Rat

In the present study, it was found that naloxone treatment prior to the pre-ovulatory LH surge (12.30 hours on the afternoon of pro-oestrus) led to an increase in basal LH levels. This stimulatory action of naloxone on LH release in female rats has been widely reported (Gabriel, Simpkins & Kalra, 1983; Piva et al., 1985; Allen & Kalra, 1986; Petraglia et al., 1986). Recently, it has been demonstrated that with prolonged naloxone treatment it is possible to evoke a premature surge of LH on the day of dioestrous II when the LH levels are basal (Allen & Kalra, 1986). This naloxone-induced surge resembles the normal preovulatory LH surge observed on the afternoon of pro-oestrus. This also implies the involvement of inhibitory opioid regulation of preovulatory LH surge in rats.

The results presented here indicate that hypothalamic noradrenergic activity is greatly increased by naloxone administration. Many others have also reported a stimulatory action of naloxone on NA content and/or turnover within the hypothalamus during the afternoon of pro-oestrus (Akabori & Barraclough, 1986). Earlier studies have shown that injections of NA into the third ventricle elicit LH release in ovariectomized steroid-primed rats (Krieg & Sawyer, 1976). The stimulatory action of naloxone on hypothalamic NA content and turnover, as reported in this investigation, is further strengthened by the results of previous pharmacological

studies showing that naloxone-induced LH release is prevented by inhibitors of noradrenergic and/or adrenergic synthesis (Kalra, 1981; Kalra & Crowley, 1982; Kalra & Simpkins, 1981; Van Vugt et al., 1981).

The catecholaminergic systems that are activated by naloxone may, in turn, enhance the release of GnRH (Kalra, Crowley & Kalra, 1987; Simpkins et al., 1980; Wilkes & Yen, 1981). This is well supported by the findings of Blank & Roberts (1982), who reported that GnRH antagonists could block naloxone-induced elevation of serum LH in immature female rats. Furthermore, Leadem et al., (1985) reported that naloxone infusion within minutes increased the release of GnRH from the MBH-POA *in vitro*. Both *in vivo* and *in vitro* studies have convincingly demonstrated that stimulation of LH release by NA may be the result of hypersecretion of GnRH into the hypophyseal portal system (Krieg & Ching, 1982; Negro-Vilar & Ojeda, 1978; Negro-Vilar et al., 1979). There is further evidence indicating an increased release of endogenous NA during the afternoon of pro-oestrus into the ME region which is in close proximity with GnRH terminals (McNeill & Sladek, 1978; Ajika, 1979). The rise in NA in the ME region is correlated with the initiation of the LH surge. The release of GnRH into portal blood is increased several fold by electrical stimulation of the ME, POA and SCH, areas which are known to contain the high concentration of GnRH-containing cell bodies and fibres (POA-SCH) and nerve terminals (ME). Stimulation of the AHA, which is separated by less than 1mm from the POA, results in only a very slight increase in GnRH release into the portal blood. Furthermore,

stimulation of the hippocampus and amygdala does not bring about any GnRH release into the portal blood (see Fink, 1986).

Further investigations into the effects of naloxone on amines in specific hypothalamic regions showed that naloxone significantly increased the content and turnover of NA in the MPO alone. This is supported by the findings of Honma & Wuttke (1980) who have concluded that the stimulatory action of NA on GnRH release is via its action within the MPO.

Rance et al., (1981) suggest that increased catecholaminergic activity within the ME is important in initiating the preovulatory LH surge. Further, they have demonstrated the existence of a clear temporal relationship between increased ME catecholamine turnover and a rise in serum gonadotrophins between 12.00 and 14.00 hours on the afternoon of pro-oestrus. Rance and his co-workers also propose that NA may act within the entire POA-SCH tuberoinfundibular system including the ME in influencing the preovulatory gonadotrophin surge. In support of this claim they demonstrated that NA turnover rates were increased, not only in the MPO, but also in the ME, SCH and ARN between 15.00 and 17.00 hours on the afternoon of pro-oestrus, at which time LH levels are continuing to rise (Barraclough et al., 1979). Increased NA turnover in crucial sites along the POA-tuberal pathway has been reported by many others (Crowley et al., 1978; Wise, Rance & Barraclough, 1981).

In an elegant study, Kalra (1981) reported that

intracranial implantation of naloxone in regions outside the hypothalamus failed to elicit LH release. However, implants containing naloxone, or intracerebral injection of naloxone anywhere in the narrow medial zone within the MPO and extending caudally to the ME-ARN, readily stimulated LH secretion. Morphine pretreatment blocked these local effects of the naloxone implants. Furthermore, naloxone implants into the VMH & DMN, where GnRH perikarya and axons have been visualized (Kelly, Ronnekleiv & Eskay, 1982; King et al., 1982; Witkin, Paden & Silverman, 1982), were ineffective in stimulating LH release. The strict regional specificity of the action of naloxone, together with the fact that morphine blocked the local excitatory effect of naloxone on GnRH secretion, indicate that opioid receptors which influence LH secretion may be confined to a narrow neural zone in the POA-tuberal pathway.

In the present study, 10mg/kg morphine was seen to increase turnover of NA within the hypothalamus. This effect of morphine was unexpected. However, Pang, Zimmerman & Sawyer (1977), observed that a dose of 10mg/kg morphine, administered subcutaneously to normal female rats during the critical period of the day of pro-oestrus, enhanced the pre-ovulatory surge of LH. These authours also found that only a dose of morphine 6 times higher (i.e., 60mg/kg) was capable of depressing the LH peak on the day of pro-oestrus. In addition, small doses of morphine administered to immature gonadotrophin-treated rats apparently increased pituitary LH output (Hulse & Coleman, 1983). In the present study, using 10mg/kg morphine, however, LH levels were significantly

decreased in spite of their increased NA turnover within the hypothalamus. It should be noted here that in these animals, the hypothalamic DA turnover was also greatly increased. It is suggested, therefore, that increased dopaminergic activity may be inhibitory to LH release. On the other hand, the higher dose (40 mg/kg) of morphine caused a significant decrease in the hypothalamic NA content. Circulating LH levels were also decreased in these animals. Due to the somewhat ambiguous results obtained with the lower dose of morphine, only the higher dose was used to estimate monoaminergic activity within specific hypothalamic regions. Among the isolated hypothalamic areas, morphine (40mg/kg) was seen to reduce NA turnover in the SCH, MPO, AHA and VMH. Furthermore, the reduction seen in the ME was not significant because of the huge standard errors. These could have arisen from the technique of removing semilunar punches to isolate this region, and thus inconsistencies in the amount of tissue collected (Figure 16). The opiate-induced decrease in NA content/turnover has been widely reported (Dyer & Grossman, 1988; Dyer et al, 1988). Diez-Guerra et al., (1986) also demonstrated that morphine could inhibit electrically stimulated NA release from slices of rat MPO *in vitro*. Similarly, Akabori & Barraclough (1986) have shown that morphine decreases noradrenergic activity within the MPO and ME of ovariectomized, oestrogen-primed rats. Morphine administration has also been shown to decrease NA levels in the whole brain and hypothalamus of rats (deWeid et al., 1974; van Ree et al., 1976).

The characteristic effects of all the kappa receptor agonists investigated on the whole hypothalamus were increased NA turnover. The differential effects of the mu and kappa opiate agonists on the NA content of the whole hypothalamus are however consistent with recent results which have demonstrated that mu, but not kappa agonists, suppress LH release after their icv infusion into the long-term ovariectomized rat (Pfeiffer et al., 1987).

Treatment (mg/kg)	W.H.	Specific Hypothalamic Regions					
		SCH	MPO	AHA	ME	ARN	VMH
Nal (10)	+	0	+	0	0	0	0
Morp(40)	0	-	-	-	0	0	-
Tifl(10)	+	-	0	0	0	0	-
SKF(10)	0	-	-	0	0	-	0

Table 27. Effects of opiates and their antagonist, naloxone, on NA turnover in the whole hypothalamus (W.H.) and in specific regions within the hypothalamus of pro-oestrous rats. (+ = Increase; - = Decrease and 0 = No effect).

Within the specific hypothalamic areas it was surprising to find that none of these agonists caused a significant rise in either NA content or turnover. The highly selective kappa agonist, tifluadom (Burkard, 1984), caused a significant decrease in NA turnover in the SCH and VMH. These results, therefore, contradict those that were obtained from the studies upon the whole hypothalamus. Using the entire

hypothalamic homogenate to study aminergic activity in the region has certain drawbacks (Palkovits & Brownstein, 1983; Kilts & Anderson, 1986). Firstly, it is not possible to localize the effects of a particular drug and secondly, its effects might differ in specific areas. Only further studies in areas not included in this study may explain these differential effects caused by the kappa agonists.

The sigma agonist, SKF 10,047, had no significant effect either upon plasma LH levels or on NA turnver within the whole hypothalamus. This finding is similar to that observed in the short-term orchidectomized rats. However, when specific regions within the hypothalamus were examined in pro-oestrous rats, SKF 10,047 did bring about a significant decrease in NA turnover within the SCH, MPO and VMH.

The synthetic met-enkephalin analogue, FK 33,824, has been shown to cause a decrease in LH levels in various experimental models (Bhanot & Wilkinson, 1982; Hill, Marbach & Scherrer, 1980; Kato et al., 1982; Leadem & Kalra, 1985) which does not cross the blood-brain barrier (Bhanot & Wilkinson, 1982; Hill, 1980). Peripheral administration of this drug reduces circulating LH levels, suggesting its action is within the ME which lies outside the blood-brain barrier (Bhanot & Wilkinson, 1982). Central administration of this drug has also been found to be effective in decreasing LH levels (Kato et al., 1982). However, in the present study, icv administration of FK 33,824 (both 2 µg and 4 µg/kg) had no significant effect upon plasma LH levels. Also, both the NA and DA content within the brain were unaffected by FK

33,824. The action of this opiate on specific hypothalamic regions were not studied as the injury from the icv mode of injection could have interfered with the punch technique.

Treatment (mg/kg)	W.H.	Specific Hypothalamic Regions					
		SCH	MPO	AHA	ME	ARN	VMH
Nal (10)	0	0	+	0	+	+	0
Morp(40)	+	0	0	0	0	+	0
Tifl(10)	0	0	+	0	0	0	+
SKF(10)	0	0	+	0	0	+	+

Table 28. Effects of opiates and their antagonist, naloxone, on DA turnover in the whole hypothalamus (W.H.) and in specific regions within the hypothalamus of pro-oestrous rats. (+ = Increase; - = Decrease and 0 = No change).

Only the opiate morphine brought about a significant increase in the whole hypohalamic turnover of DA in the pro-oestrous rat. Its antagonist, naloxone, had no significant effect on this. These findings are similar to those observed in the short-term castrated rats.

When specific hypothalamic regions were studied, morphine caused a significant decrease in DA content within the ME, similar to the findings of Akabori & Barraclough (1986). Naloxone caused an increase in turnover of DA in the same region. Both naloxone and morphine were seen to bring about a significant increase in DA turnover in the ARN.

This finding was unexpected, and may be due to an increase in mu receptor levels in the pro-oestrous rats as reported by Casulari et al., (1987). The dose of naloxone given may have failed to overcome the increased activity of the endogenous opioid peptides.

Tifluadom, SKF 10,047 and naloxone all caused an increase in DA turnover in the MPO region. This could be due to both kappa and sigma agonists acting as antagonists in the MPO region probably exerting this effect via a different receptor type, most likely the mu receptors. Similar effects were seen in the short-term orchidectomized rats, where the antagonist properties of the sigma agonist predominated, and neither SKF 10,047 nor naloxone had any effect on hypothalamic NA and 5-HT turnover.

The increase in DA turnover observed after administration of the opiates was also seen when the antagonist naloxone was given. This would suggest an increased opioidergic activity within the hypothalamus during the afternoon of pro-oestrus. Indeed, a large body of evidence exists in favour of this hypothesis. On the afternoon of pro-oestrus there is an increase in concentration of brain mu receptors (Casulari et al., 1986), and an elevation of beta-endorphin in the portal plasma (Sarkar & Yen, 1985). The levels of leu-enkephalin and dynorphin have been found to be significantly higher in the hypothalamus and anterior pituitary on the afternoon of pro-oestrus (Suda et al., 1986). In the SCH, ME and ARN beta-endorphin concentrations were seen to be increased

significantly during the same period (Barden et al., 1981; Knuth et al., 1983; Hulse et al., 1984). These reports indicate an increase in intrinsic opioidergic tone during the afternoon of pro-oestrus. This increased tone may overcome the effects of the administered naloxone. This conclusion is further supported by the present findings regarding the effects of opiates on 5-HT turnover. One or more of the opiate agonists caused a significant increase in 5-HT turnover in all the hypothalamic regions studied, but 5-HT turnover was not significantly affected by naloxone.

The present study confirms the stimulatory activity of NA on LH release. Recently, Dyer & Grossman (1988) have reported that the opioid peptides regulate noradrenergic transmission in the MPO thereby influencing the neurones controlling the release of LH. The richly innervated region of the MPO, receiving inputs from the ventral noradrenergic neurones, seems to be the major site regulating the release of LH (Dyer & Grossman, 1988). In the present study, it was found that naloxone increased the turnover of NA in the MPO and this was correlated with an increase in plasma LH levels. The opposite effect was seen when morphine was administered. These findings support the theory that the action of mu receptors is limited to the MPO-tuberal pathway (Kalra, 1981). This is further supported by the fact that kappa and sigma agonists did not produce the same effect in the MPO. SKF 10,047, although decreasing NA turnover in the MPO failed to affect LH levels. Tifluadom, on the other hand, decreased LH levels without affecting NA turnover in the MPO,

but reduced noradrenergic activity in the VMH and SCH.

The reports so far available are not consistent with regard to the effects of DA on LH secretion. Rotsztejn et al., (1978) reported that DA induced the release of GnRH from fragments of MBH *in vitro*. However, Kalra (1985) was unable to bring about GnRH release by DA in intact pro-oestrous rat whether or not they had been pretreated with morphine. In the present study, both agonists and antagonist drugs increased DA turnover, but they differed in their effects on LH levels further supporting the conclusion that DA turnover may not significantly influence LH release.

Treatment (mg/kg)	W.H.	Specific Hypothalamic Regions					
		SCH	MPO	AHA	ME	ARN	VMH
Nal (10)	0	0	0	0	0	0	0
Morp(40)	0	0	0	0	0	0	+
Tif1(10)	+	+	0	+	0	0	0
SKF(10)	0	+	0	+	+	0	0

Table 29. Effects of opiates and naloxone on 5-HT turnover in the whole hypothalamus (W.H.) and in specific regions within the hypothalamus in pro-oestrous rats. (+ = Increase; - = Decrease and 0 = No effect).

An inhibitory action of 5-HT on LH secretion has been widely reported (Yarbrough, Buxbaum & Sanders-Bush, 1973; Kamberi, Mical & Porter, 1970; Pilotte & Porter, 1979). The

daily periodicity in LH levels has been shown to be associated with neuronal activity in the SCH where there is a rich concentration of 5-HT neurones (see Consolazione et al., 1982). In the present study, both tifluadom and SKF 10,047 increased 5-HT turnover in the SCH and AHA. However, only tifluadom brought about a significant decrease in circulating LH levels. Morphine did not have any significant effect on 5-HT turnover in the SCH, but increased this in the VMH. Furthermore, naloxone did not have any significant effect on 5-HT turnover in any of the regions studied. It can therefore be concluded that 5-HT neurotransmission may be mediated by opioid receptors other than those of the mu type. Studies using pure kappa and sigma antagonists when such drugs become available, would further elucidate the exact role of 5-HT in the regulation of LH release.

Thus, in conclusion, the mu agonist morphine decreased NA turnover in the SCH, MPO, AHA & VMH. Naloxone increased NA turnover in the MPO alone suggesting that NA turnover in the MPO region is mediated by mu receptors. Since kappa and sigma agonists also decreased NA turnover in the regions studied it is concluded that there exists a heterogenous group of receptors mediating this. None of the opiates had any significant effect on NA turnover in the ME which is the final neuronal pathway. This would therefore indicate that the opiate-mediated effects on NA turnover occur at higher levels.

Since both opiate agonists and naloxone increase DA

turnover but do not have consistent effects on circulating LH levels it could be concluded that changes in DA turnover do not play a major role in the regulation of LH release.

Both kappa and sigma agonists significantly increased 5-HT turnover in the SCH and AHA. This effect was not seen after treatment with the mu agonist morphine, nor with naloxone. Thus it may be concluded that increased 5-HT turnover may be mediated by opioid receptors other than those of the mu type.

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APPENDIX II

[*From the Proceedings of the Physiological Society*, 19–20 *June* 1987
Journal of Physiology, **392**, 16*P*, 1987]

The use of high-performance liquid chromatography with electrochemical detection to examine the effects of opiates on rat brain catecholamine levels

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Currently we are employing high-performance liquid chromatography with electrochemical detection (h.p.l.c.–e.c.d.) to examine how opiates might alter gonadotrophin release in the rat through their effects on brain amine levels. Both pro-oestrous female and short-term castrated male rats are being utilized. The latter are operated upon under ether anaesthesia. Varying doses of morphine sulphate and its antagonist, naloxone, are being administered to groups of animals either I.P., or stereotaxically into the brain ventricles under Saffan (alphaxolone 0·9%, w/v; alphadolone acetate 0·3%, w/v) anaesthesia (2–3 ml/rat given I.P.). At pre-determined intervals the rats are killed by decapitation and the brains removed on ice. Hypothalamic and cortical extracts are prepared as previously described (Gopalan, Meek & Gilmore, 1987), and applied directly to a h.p.l.c. column. For each sample we are able simultaneously to measure the content of noradrenaline, adrenaline, dopamine, serotonin and 5-hydroxyindole acetic acid. The concentration of each amine present in the tissue is determined by a Trivector chromatography computing integrator using peak area as the basis for calculation. The run time for each sample is usually 1 h. The traces from up to 16 injected samples can be stored in the memory of the integrator and recalled for display at any time during or after the analysis. Differences in hypothalamic amine concentrations between the experimental groups and the controls is providing information on the role of the aminergic systems in the regulation of gonadotrophin secretion.

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GOPALAN, C., MEEK, D. R. & GILMORE, D. P. (1987). *Med. Sci. Res.* **15**, 309–310.

Med. Sci. Res., 1987; 15, 309-310

The effect of specific adrenergic agents on hypothalamic catecholamine levels during the pre-ovulatory LH surge in the rat

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The brain catecholaminergic system appears to play an important role in regulating the pre-ovulatory LH surge. Pharmacological suppression of these central neurotransmitters prior to the "critical" period on the day of pro-oestrus consequently results in blockade of the LH surge and ovulation [1, 2]. Recently we have investigated the effect of intraventricular infusions of various adrenoceptor agonists and antagonists on ovulation in the cycling female rat [3].

The present study was carried out to obtain more information about the participation of the catecholamines in the initiation of the pre-ovulatory LH surge. Various pharmacological agents acting on specific adrenergic receptor subtypes were administered (by i.p. injection) to rats on the day of pro-oestrus. Effects of these procedures on hypothalamic adrenaline (ADR) and noradrenaline (NA) levels were then measured by high performance liquid chromatography with electrochemical detection (HPLC-ED).

Materials and methods: Female Sprague-Dawley rats (Tuck and Sons, Battlesbridge, Essex) weighing 250-300 g were maintained under conditions of controlled temperature (21 ± 1°C) and 12 h light: 12 h darkness (lights on 06:00 – 18:00). Food and water were available *ad libitum*. Vaginal smears were monitored each morning and only those rats showing three or more consecutive 4-day oestrous cycles were selected, on the day of pro-oestrus, for use in this study.

Rats were given pentobarbitone sodium (PB), 35 mg/kg, at 13:30 on the afternoon of pro-oestrus to block the pre-ovulatory LH surge. To determine the effects of stimulatory adrenergic agents, these were administered in either 1 ml of 0.9% saline or 5% glucose between 13:30 and 14:30. Adrenergic blocking agents were given at 13:30 in 1 ml of 5% glucose to pro-oestrous rats not previously treated with PB. The drugs administered were clonidine (an α_2-adrenergic agonist), yohimbine (an α_2-adrenergic antagonist), salbutamol and fenoterol (β_2-adrenergic agonists) and ICI 118, 551 (which is (2RS, 3RS)-3-isopropylamino-1-(7-methylindan-4-yloxy) butan-2-ol) (a β_2-adrenergic antagonist). Controls received either 0.9% saline or 5% glucose (1 ml).

The animals were decapitated between 14:30 and 16:00. The brains were rapidly removed and the hypothalamus surgically isolated on ice in 0.1 M HCl containing 3,4-dihydroxy-benzylamine (DHBA) as an internal standard. Although catecholamines are known to be subject to oxidative degradation, they have been found to be stable for at least 12 h at room temperature in 0.1 M HCl [4]. The hypothalamic samples were centrifuged and the supernatants stored at −25°C until assayed.

Twenty μl aliquots of the supernatant were injected onto a reverse phase HPLC column with an electrochemical detector and the concentration of ADR and NA present simultaneously measured. The mobile phase used was citrate-acetate buffer with 5% (v/v) methanol, 2% (v/v) tetrahydrofuran and 100 mg/l sodium octyl sulphate.

Results and discussion: The results are summarized in Table 1. β_2-adrenergic agonists are known to overcome the pentobarbitone block to ovulation [3]. Our results indicate that the β_2-agonists fenoterol and salbutamol both increase the ADR content of the hypothalamus whereas the β_2 antagonist ICI 118, 551 lowers it. Previously, exogenously administered ADR has been shown to be more potent than NA in evoking ovulation in the pro-oestrous rat [5]. Our findings would also support the view recently put forward [6] that endogenous ADR plays a major role in controlling the pre-ovulatory LH surge.

The α_2-adrenergic agonist clonidine has previously been found both to prevent ovulation [3] and to inhibit LH release when given to ovariectomized unprimed rats [7]. Although our results demonstrated that clonidine causes a highly significant decrease in the hypothalamic ADR content, it is also seen to bring about a significant increase in NA levels in this same brain region. It is probable that the former effect is responsible for the inhibitory action of clonidine on ovulation and LH release.

The α_2-antagonist yohimbine has been shown to have no clear-cut effect on ovulation [3]. This is understandable as our results have indicated that although this drug significantly reduces the hypothalamic ADR content, it does not significantly alter NA levels in this region.

In summary, our findings would support the previously postulated roles for both ADR and NA in the events leading to the pre-ovulatory LH surge in the pro-oestrous rat.

Table 1: Noradrenaline and adrenaline content in the hypothalamus (ng/g wet weight of tissues; means ± SEM)

Treatment	Adrenergic receptor type	Dose (mg/kg)	No. of rats	Noradrenaline	Adrenaline
Glucose (5%)		–	9	1062 ± 130	276 ± 85
Glucose (5%) + PB		–	6	678 ± 91[a]	30 ± 26[a]
Normal Saline + PB		–	8	1160 ± 110	173 ± 86
Clonidine† + PB	α_2-agonist	20	6	1575 ± 120[b]	6 ± 4[c]
Yohimbine	α_2-agonist	20	5	1166 ± 119	37 ± 13[a]
Salbutamol + PB	β_2-agonist	20	5	851 ± 271	767 ± 112[c]
Fenoterol + PB	β_2-agonist	20	5	1212 ± 165[d]	526 ± 104[c]
ICI 118, 551	β_2-agonist	20	6	1012 ± 124	17 ± 15[a]

† *Dissolved in saline (other drugs dissolved in 5% glucose). In comparison with 5% glucose,* $p < {}^{a}0.05$; *in comparison with saline + PB,* $p < {}^{b}0.05, {}^{c}0.01$; *in comparison with 5% glucose + PB,* $p < {}^{a}0.05, {}^{c}0.01$

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We are grateful to Glaxo Group Research Ltd., Ware, Herts for the gift of salbutamol, to Boehringer Ingelheim Ltd., Bracknell, Berks for the gift of fenoterol and ICI, Macclesfield, Cheshire for the gift of ICI 118, 551. The HPLC equipment was purchased on a grant to D.P.G. from the Scottish Hospitals Endowments Research Trust.

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Paper received: 12th February, 1987, amended 23rd February, 1987

The effects of opiates on the hypothalamic aminergic content during the pre-ovulatory LH surge in the rat

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There is now a general consensus that both catecholamines and indoleamines play an important role in the pre-ovulatory release of luteinizing hormone releasing hormone (LHRH) [1]. Pharmacological suppression of central neurotransmission prior to the critical period on pro-oestrus has consistently resulted in blockade of the pre-ovulatory LH surge and ovulation [2].

Morphine has long been known to inhibit gonadotrophin release [3–5] by its action upon the central nervous system. Furthermore, peptidergic neurones containing the endogenous opioids β-endorphin, enkephalin and dynorphin have been identified in close proximity to the LHRH neurones in the preoptic area of the rat hypothalamus [6]. It has now been established that several different classes of opiate receptor subtypes exist within the mammalian brain. The present study was undertaken to investigate how specific opiate receptor agonists and antagonists (see Table 1) might alter monoamine concentrations in the rat hypothalamus and thus affect the pre-ovulatory LHRH and LH surges. High performance liquid chromatography with electrochemical detection (HPLC-ECD) was employed to measure amine levels in the hypothalamus.

Materials and methods: Female Sprague-Dawley rats (200–250 g) were purchased from Tuck and Sons, Battlesbridge, Essex and housed in a temperature (21 ± 1°C) and light-controlled room (lights on 06:00 –18:00). Food and water were supplied *ad libitum*. Daily vaginal smears were taken and only rats which had shown at least two consecutive 4-day cycles were used in this study. The rats were injected with the specific receptor agonist or antagonist dissolved in 1 ml of physiological saline at 12:30 on the afternoon of pro-oestrus (prior to the pre-ovulatory LH surge). After an interval of 2 h, the animals were decapitated and the brains rapidly removed on ice. Hypothalamic extracts were prepared as previously described [7].

20 μl samples of the supernatant were injected on to a reverse phase HPLC column and the concentrations of noradrenaline (NA), adrenaline (ADR), dopamine (DA), serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA) were simultaneously measured. The solvent used was a citrate-acetate buffer with 5% (v/v) methanol, 1.2% (v/v) tetrahydrofuran and 100 mg/l sodium octyl sulphonate. The flow rate was set at 1.5 ml/min.

Results and discussion: The results are summarized in Table 1. It is apparent that although the lower dose of morphine, a mu receptor agonist, raised DA levels in the hypothalamus, it did not significantly alter levels of the other amines present in this region. In contrast, the higher dose of morphine and levorphanol, another mu receptor agonist, significantly lowered the hypothalamic content of both NA and 5-HT while the mu receptor antagonist naloxone significantly increased hypothalamic NA levels. Both cyclazocine and ketocyclazocine (kappa receptor agonists) brought about very significant increases in the hypothalamic indoleamine content, but only the latter significantly affected NA levels in this region. Another kappa receptor agonist, tifluadom, was the only opiate to significantly lower ADR levels in the hypothalamus, while at the same time it caused the DA content to be significantly elevated. SKF 10,047 (a sigma agonist) significantly raised levels of DA, 5-HT and 5-HIAA.

The endogenous opioids have been shown to modulate the action of a variety of neurotransmitters in the central nervous system, in particular, the biogenic amines NA, DA and 5-HT [8]. NA is known to play an excitatory role in LHRH and LH release, and by decreasing the hyothalamic content of this neurotransmitter morphine is able to block ovulation in pro-oestrous rats [9]. In this study, we noted that the decrease in hypothalamic NA that occurred after morphine and levorphanol treatment could be reversed by naloxone. On the other hand, kappa and sigma receptor agonists failed to reduce hypothalamic NA levels; ketocyclazocine even significantly raised these.

Acute administration of morphine (10 mg/kg) has been shown to increase the brain turnover of DA [10]. Our studies using a similar dose of this opiate brought about a significant elevation in DA levels.

5-HT has been reported to have a permissive action on the pre-ovulatory gonadotrophin surge in rat. Numerous studies indicate that morphine can affect the actions of 5-HT on gonadotrophin release. It has been suggested that the endogenous opioid peptides stimulate 5-HT turnover in the preoptic area of the hypothalamus [11]. Such a theory is sup-

Table 1: Biogenic amine concentrations in the rat hypothalamus (ng/g wet weight of tissue; means ± SEM)

Treatment	*n*	Opiate receptor type	Dose (mg/kg)	NA	ADR	DA	5-HT	5-HIAA
Physiological saline	9			1455 ± 86	27 ± 6	188 ± 13	782 ± 62	643 ± 69
Morphine	5	Mu-agonist	10	1856 ± 185	70 ± 26	309 ± 40**	996 ± 68	714 ± 105
Morphine	13	Mu-agonist	50	1078 ± 69**	13 ± 3	257 ± 29	416 ± 41**	377 ± 32**
Naloxone	8	Mu-antagonist	10	1830 ± 132*	25 ± 6	226 ± 28	763 ± 75	446 ± 106
Levorphanol	10	Mu-agonist	10	1114 ± 70*	15 ± 2	234 ± 21	543 ± 44*	567 ± 74
Cyclazocine	10	Kappa-agonist	5	1650 ± 62	22 ± 3	212 ± 15	1258 ± 130**	898 ± 32**
Ketocyclazocine	10	Kappa-agonist	5	1752 ± 80*	21 ± 2	221 ± 24	1099 ± 62**	928 ± 31**
Physiological saline[a]	8			1444 ± 93	23 ± 3	174 ± 10	1048 ± 79	650 ± 66
SKF 10,047[a]	10	Sigma agonist	10	1514 ± 63	29 ± 7	275 ± 13**	1530 ± 92**	941 ± 74*
Tifluadom[a]	9	Kappa agonist	10	1517 ± 64	8 ± 3*	237 ± 18*	907 ± 74	765 ± 45

[a]*Administered subcutaneously; all others administered intraperitoneally.* n = *number of animals in each treatment group. In comparison with the control value,* $p < : *0.05; **0.01$ *(unpaired t test).*

ported by the effects observed on hypothalamic indoleamine levels after the administration of cyclazocine, ketocyclazocine and SKF 10,047.

One unexpected finding of the study was that hypothalamic 5-HT levels were significantly higher in the controls injected subcutaneously than in those given saline intraperitoneally. Subcutaneous injections are likely to result in some discomfort and for this reason they were always administered to rats which had first been placed under light ether anaesthesia. However, ether inhalation is known to cause mild stress and stress itself has been shown to lead to an increase in brain 5-HT levels [12].

Although, at present, little information is available on the effects of specific opiate receptor agonists on biogenic amine concentrations in the brain, studies on LH levels during the pre-ovulatory surge indicate that activation of mu, kappa and sigma receptors inhibit LH release [12, 13]. This inhibition may involve the participation of NA, DA and 5-HT containing neuronal systems which are affected by exogenous opiates.

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We are grateful to Roche Laboratories Inc., Neely, NJ for the gift of levorphanol the National Institute on Drug Abuse for the gift of SKF 10,047, Sterling-Winthrop Corporation, Guildford, Surrey for the gifts of cyclazocine and ketocyclazocine and Kali-Chemie, Hannover, West Germany for the gift of Tilfluadom. The HPLC equipment was purchased on a grant to D.P.G. from the Scottish Hospitals Endowments Research Trust. C.G. was supported by an Overseas Research Scholarship and a University of Glasgow Postgraduate Scholarship and C.H.B. by a grant from the Dale/Rushton Fund of the Physiological Society.

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Paper received: 11th November, 1987; amended 26th November, 1987

APPENDIX II

PRESENTATIONS

1. *Gopalan,C., Meek,R.M.D. & Gilmore,D.P.* (1986)

The study of specific adrenergic agents on brain neurotransmitter levels during the pre-ovulatory LH surge in the rat.

The winter meeting of the Society for the Study of Fertility held at Sutton Bonington.

2. *Gopalan,C. & Gilmore,D.P.* (1987)

Effects of opiates on hypothalamic amine levels in the male rat.

Annual Conference of the Society for the Study of Fertility held at York.

3. *Gopalan,C. & Gilmore,D.P.* (1987)

Opiate involvement in the hypothalamic neurotransmitter control of the pre-ovulatory LH surge in the rat.

International Conference of Brain Opioid Systems in Reproduction held at Babraham, Cambridge.

4. *Gilmore,D.P. & Gopalan,C.* (1987)

The use of high-performance liquid chromatography with electrochemical detection to examine the effects of opiates on rat brain catecholamine levels.

Physiological Society Meeting held at Glasgow.

5. *Gopalan,C., Currie,I.S. & Gilmore,D.P.* (1988)

Opiates and hypothalamic amine turnover in castrated rats.

Annual Conference of the Society for the Study of Fertility held at Edinburgh.

6. *Brown,C.H., Gopalan,C. & Gilmore,D.P.* (1988)

The effects of opiates on biogenic amine levels in specific hypothalamic areas during the pre-ovulatory LH surge in the rat.

Annual Conference of the Society for the Study of Fertility held at Edinburgh.