THE ROLE OF ENDOGENOUS OPIOIDS AND BRAIN NEUROTRANSMITTERS IN THE GENERATION OF THE LH SURGE IN THE RAT

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Dedicated to my parents and to the memory of my grandfather.

CONTENTS

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ABSTRACT

It has been suggested that a multiplicity of neurotransmitter systems regulate the release of gonadotrophin-releasing hormone (GnRH) from their neurons in the hypothalamus which in turn control luteinising hormone (LH) secretion from the anterior pituitary. An example of such neurotransmitter systems is that secreting the endogenous opioid peptides which have a profound inhibitory influence on the LH secretion. Recently, it has been reported that a reduction in the activity of these peptides in the hypothalamus may be the initial neural stimulus for the generation of the pre-ovulatory LH surge which induces ovulation. Furthermore, this inhibitory opioidergic action may involve alterations in the activity of monoaminergic neurons which make synaptic contacts with the GnRH neurons in the hypothalamus. Naloxone-, the opioid antagonist, induced LH release can be prevented by prior administration of α-adrenergic blockers. The central noradrenergic system is believed to be an essential component of the GnRH pulse-generating mechanism. The present study was undertaken to determine (i) the modulating effects of specific μ-, κ- and δ-opioid receptor agonists and antagonists on hypothalamic monoaminergic content and on LH release and (ii) the inter-relationship between the opioid peptidergic and aminergic systems in the control of GnRH activity.

Intact female rats were employed in the first two sets of the experiments. Vaginal smearing was performed each morning and the morphology of the cells present used to identify the different stages of the oestrous cycle. Only those animals which had exhibited at least three consecutive oestrous cycles were selected for experimentation. On the day of pro-oestrus at around 12 noon, the right carotid artery was cannulated under urethane anaesthesia. Blood samples (200μl) were collected via the heparinised cannula at hourly intervals commencing at 13.00h. Animals were injected intraperitoneally (IP) with μ-, κ- and δ-opioid receptor agonists and antagonists at 13.00h. Controls received saline (1ml/kg, IP) alone. At 19.00h the rats were decapitated and the medial preoptic area (MPOA), suprachiasmatic nucleus (SCN), median eminence (ME) and arcuate nucleus (ARN) surgically isolated by micropunch. While attempting to achieve the objectives in Experiment I, it became clear that urethane itself abolished the expected pre-ovulatory LH surge. Therefore, in the second series of the experiments, blood samples (200μl) were collected from freely-moving conscious animals following cannulation of the right femoral artery under halothane anaesthesia. The animals

were allowed to recover prior to the blood sampling commencing at 13.00h. They were subsequently treated as in the first set of the experiments.

The third set of experiments involved intracerebroventricular (icv) administration and co-administration of various opioid agonists and antagonists to ovariectomised and steroid-primed rats on the afternoon of the anticipated LH surge. Serial blood samples were collected from the femoral artery of the ketamine-anaesthetised rats via an indwelling cannula. The decapitation and micropunch procedures were carried out as explained above.

The monoamine content of the specific hypothalamic regions was determined by high performance liquid chromatography with electrochemical detection. Plasma LH levels were measured by radioimmunassay.

In the urethane-anaesthetised rats, the expected pre-ovulatory surge was totally inhibited in all animals. Furthermore, concentrations of noradrenaline and its metabolite, 3,4-dihydroxyphenylglycol, were reduced in all the hypothalamic regions examined.

It was found that all three opioid agonists (diamorphine, U-50488H and DPDPE) administered to the conscious animals inhibited the pre-ovulatory surge on the afternoon of pro-oestrus. The inhibitory effects of diamorphine and DPDPE on LH release were negated by co-administration with naloxone. However, the κ-opioid antagonist, MR2266, failed to elevate plasma LH levels following its co-administration with U-50488H.

Suppression of the LH surge was consistently associated with decreased NA release and/or turnover in the MPOA, SCN, ME and ARN at 19.00h on the afternoon of pro-oestrus. These inhibitory effects of the μ-, κ- and δ-agonists on hypothalamic noradrenergic activity were prevented by their respective opioid antagonists. Naloxone enhancement of the pre-ovulatory LH surge occurred concomitantly with an increase in dopamine release in the MPOA, ME and ARN. Concentrations of serotonin and/or 5-hydroxyindoleacetic acid were selectively increased by naloxone at the time of the LH surge.

The inhibitory effects of all the opioid agonists on the hypothalamic monoamine content were confirmed following icv administration of diamorphine, DPDPE and

U-69593 to the ovariectomised and steroid-primed rats. Prevention of these suppressive effects on amine release and/or turnover by naloxone, MR2266 and ICI 154, 129 indicates that specific opioid receptor subtypes are involved in mediating these actions. However, the observations could not be correlated with the anticipated LH surge due to unexpected interference on this by ketamine anaesthesia.

In conclusion, the present results indicate that multiple opioid receptor subtypes are involved in the opioid suppression of the pre-ovulatory LH surge. It appears that opioid modulation of noradrenaline, dopamine and serotonin release and/or turnover within the specific areas of the hypothalamus via μ-, κ- and δ-opioid receptors is inhibitory at the time of the LH surge. This study supports the concept that an increase in the hypothalamic noradrenergic activity is a critical event in triggering the surge release of GnRH and LH. Both the dopaminergic and serotonergic neurotransmitter systems may also facilitate the pre-ovulatory LH surge, although their effects are thought to be of minor importance in this process. In view of the unexpected interference by both urethane and ketamine anaesthesia on plasma LH levels, it is recommended that use of general anaesthetics in neuroendocrine studies should be avoided if at all possible.

LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
5-HTP	5-hydroxytryptophan
6-OHDA	6-hydroxydopamine
ACh	Acetylcholine
ACTH	Adrenocorticotrophic hormone
ADH	Aldehyde dehydrogenase
ADR	Adrenaline
AHA	Anterior hypothalamic area
ARN	Arcuate nucleus
BSA	Bovine serum albumine
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CRF	Corticotropin releasing factor
DA	Dopamine
DHBA	3,4-dihydroxybenzylamine
DHPG	3,4-dihydroxyphenylglycol
DMN	Dorsomedial nucleus
DNAB	Dorsal noradrenergic bundle
DOMA	Dihydroxymandelic acid
DOPAC	3,4-dihydroxyphenylacetic acid
DPDPE	D-Pen^2,D-Pen^5-Enkephalin
DRN	Dorsal raphe nucleus
E_2	Oestradiol
EAA	Excitatory amino acid
ECD	Electrochemical detection
EDTA	Ethylenediamine tetra-acetic acid
EME	External layer of median eminence
EOP	Endogenous opioid peptide
EW	Egg white
FSH	Follicle stimulating hormone
GABA	γ-aminobutyric acid
GABA-T	GABA transaminase
GAD	Glutamic acid decarboxylase
GnRH	Gonadotrophin-releasing hormone (LHRH)

HA	Histamine
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
ICI 154,129	N, N, diallyl-Tyr-Gly-Gly (CH2S) Phe-Leu
ICI 174,864	N, N, diallyl-Tyr-Aib-Aib-Phe-Leu
ICV	Intracerebroventricular
IM	Intramuscular
IP	Intraperitoneal
IS	Internal standard
LC	Locus coeruleus
LH	Luteinising hormone
LHRH	Luteinising hormone-releasing hormone (GnRH)
MAO	Monoamine oxydase
MBH	Medial basal hypothalamus
ME	Median eminence
MHPG	3-methoxy,4-hydroxyphenylglycol
MMN	Medial mammillary nucleus
MPOA	Medial preoptic area
MPS	Modified physiological saline
MR1452	(-)-(1R, 5R, 9R)-5,9-Dimethyl-2-(3- furylmethyl)-2 hydroxy-6,7-benzomorphan methanesulfonate.
MR2266	(-)-5,9 alpha-Diethyl-2-(3-furylmethyl)-2'-hydroxy-6,7-benzomorphan.
MRN	Medial raphe nucleus
NA	Noradrenaline
NAL	Naloxone
NaOH	Sodium hydroxide
NM	Normetanephrine
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
NRS	Normal rabbit serum
NSB	Non-spesific binding
OVLT	Organum vasculosum of the lamina terminalis
OVX	Ovariectomised
P	Progesterone

PACAP	Pituitary adenylate cyclase-activating polypeptide
PCP	Phencyclidine
PeVN	Periventricular nucleus
PMN	Premammillary nucleus
PNMT	Phenylethanolamine-N-methyltransferase
POA	Preoptic area
POMC	Proopiomelanocortin
PPS	Precipitating serum
PVN	Paraventricualr nucleus
SCN	Suprachiasmatic nucleus
SD	Serum diluent
SEM	Standard error of the mean
SON	Supraoptic nucleus
SP	Substance P
TH	Tyrosine hydroxylase
TIDA	Tuberoinfundibular dopamine
U-50488H	trans-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl) cyclohexyl]-benzene-acetamide methane sulfonate.
U-69593	(5a, 7a, 8B)-(+)-N-methyl-N[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)-dec-7-8-yl] benzeneacetamide
VIP	Vasoactive intestinal peptide
VMN	Ventromedial nucleus
VNAB	Ventral noradrenergic bundle
ZI	Zona incerta

LIST OF FIGURES

Introduction

Materials and Methods

Experiment I

Experiment II

Experiment III

LIST OF TABLES

Introduction

Materials and Methods

Experiment I

Experiment II

Experiment III

INTRODUCTION

The understanding of how neuroendocrine mechanisms control gonadotrophin secretion is complicated because of the involvement of several different neurotransmitter/neuromodulator systems within the brain and of the modulatory effects of gonadal steroids. This chapter briefly reviews the current concept of the neuronal circuit involved in the central regulation of luteinising hormone (LH) release including the pre-ovulatory LH surge. This will include the participation of endogenous opioid peptides (EOPs), monoamines, amino acids, several other neuropeptides and gonadotrophin-releasing hormone (GnRH) neuronal systems and their interactions in controlling LH secretion at the hypothalamic level. However, before evaluating the role of these systems in the neuroendocrine regulation of LH release, the oestrous cycle of the rat, the anatomical organisation of the hypothalamo-pituitary axis and of the brain ventricular system will be briefly noted.

Oestrous Cycle of the Rat

The rat is a polyoestrous animal with approximately one year of sexually active life. As in the human, the rat is spontaneous ovulator but has a very short oestrous cycle of four or five days. Its oestrous cycle comprises of two or three days of dioestrus and one day each of pro-oestrus and oestrus. Metoestrus is sometimes seen between late oestrus and early dioestrus in five-day cycling rats and lasts only about six hours. Each of these stages is characterised by periodic histological changes in the epithelium of the uterus and the vagina, as well as specific behavioural changes. A classic description of the rat oestrous cycle was given by Long and Evans (1922).

Follicle stimulating hormone (FSH) is essential for follicular development and maturation. In general, it is secreted at low levels and in a pulsatile fashion throughout the oestrous cycle; however, there is a marked increase around the time of ovulation (Lumpkin *et al*, 1984). Concomitant with basal discharge of LH, the release of FSH from the anterior pituitary and the consequent rise in circulating levels bring about the final growth of the ripening follicles and their peak production of oestradiol (E_2) (Fortune, 1994). This brings the animal into heat, i.e. sexual receptivity. During the mid-follicular phase, E_2 and FSH together appear to stimulate production of LH receptors from granulosa cells, and therefore render the follicle sufficiently sensitive for LH to achieve ovulation and luteinisation (Zeleznik *et al*, 1974).

In early dioestrus plasma E_2 release from the Graafian follicle is low, but it increases gradually during late dioestrus. It finally peaks on the morning of pro-oestrus. This release of E_2, accompanying follicle maturation, is thought to be the initial stimulus for "the pre-ovulatory LH surge" from the anterior pituitary on the afternoon of pro-oestrus (Levine *et al*, 1985). The high levels of LH then inhibit further production of E_2, and at the same time stimulate an increase in progesterone (P) secretion. Indeed, this causal relationship has been clearly shown by the ability of antiserum to E_2 to suppress the surge (Ferin *et al*, 1970). P has no such important role in the LH surge. However, during the final maturation of follicles, a small amount of P is produced, which appears to facilitate ovulation (Espey, 1994).

The profiles of secretion of LH, FSH, E_2 and P during the oestrous cycle of the rat are illustrated in Figure 1.1.

The LH surge itself stimulates ovulation, which occurs after midnight between pro-oestrus and oestrus. The highly vascularised ovulatory follicles bulge from the ovarian surface. LH appears to combine with specific receptors on the surface of the Graafian follicle, stimulating adenyl cyclase, cyclic adenosine monophosphate production, and P output. P then induces synthesis or activation of a collagenase enzyme which, acting on the collagen framework of the follicle wall, increases distensibility and lowers breaking strength. Consequently, the rupture of the wall and release of the oocyte take place (Espey and Lipner, 1994). The role of P is crucial in this process since P antagonists delay or block ovulation (Sanchez-Criado *et al*, 1990). At this stage, coitus is necessary to maintain a fully functional corpus luteum whose life span is normally only one day in the rat. If coitus does not occur, the corpus luteum fails to become functional and only a small amount of P is secreted before luteal regression. As a result no luteal phase takes place and the oestrous cycle continues.

Gonadotrophin Secretion Profiles During The Oestrous Cycle

Secretion of FSH during the oestrous cycle of the rat consists of two different patterns; i.e. basal and surge release. The surge pattern of FSH secretion occurs during the pre-ovulatory period from the afternoon of pro-oestrus to the morning of oestrus and the basal pattern at other stages of the oestrous cycle (Noguchi *et al*, 1993).

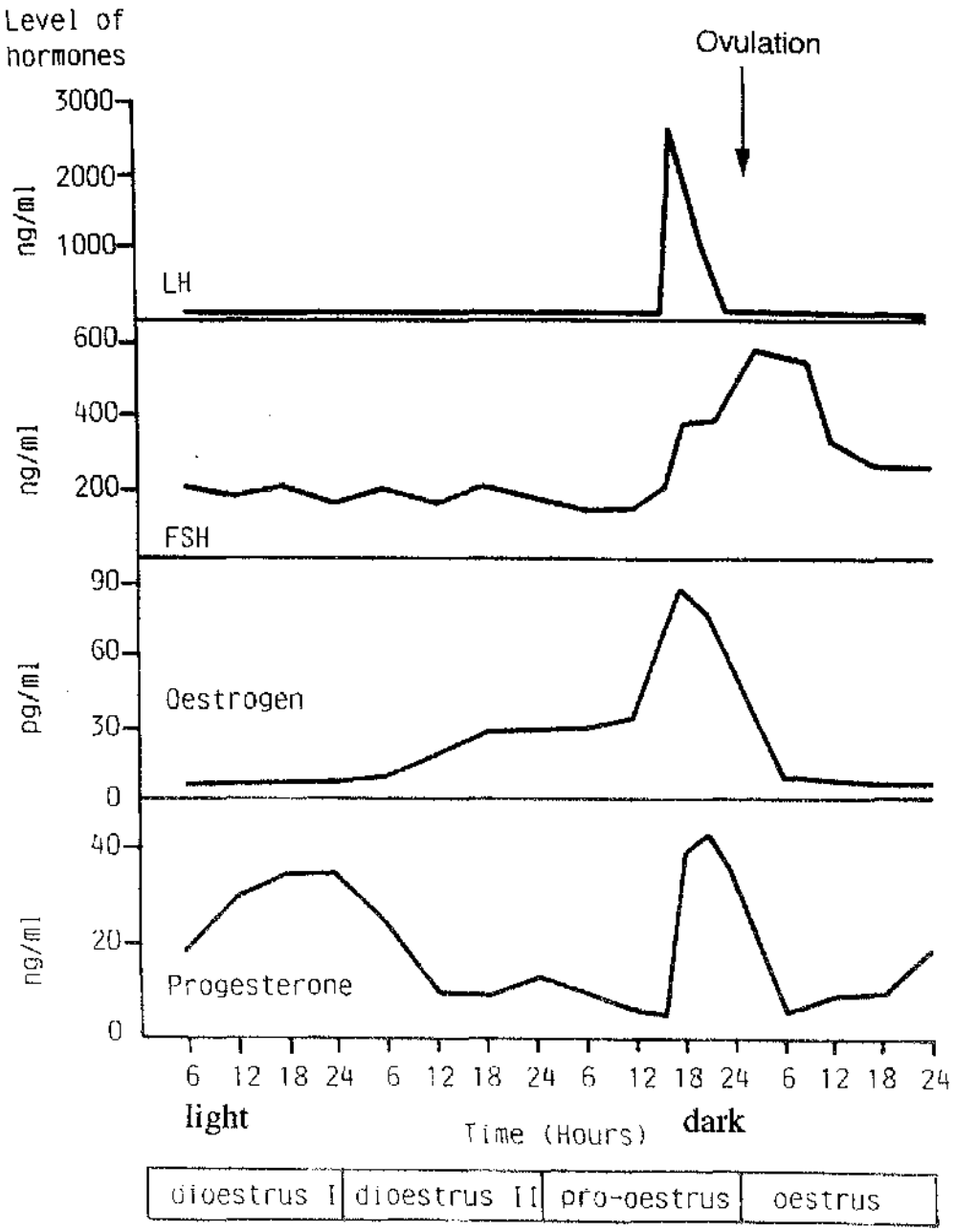


Figure 1.1. Schematic representation of the hormone changes in the systemic circulation of the rat throughout the 4-day oestrous cycle. Light phase occurs between 06.00h and 18.00h. Adapted from Takeo, 1984.

LH is released in a pulsatile manner during the rat oestrous cycle rather than at a steady rate (Gallo, 1981; Fox and Smith, 1985). The frequency and amplitude of these LH pulses depend upon the time of day (circadian) and the stage of the oestrous cycle (cyclic) (Leipheimer *et al,* 1985). There are two distinct patterns of LH release during the oestrous cycle; basal (or episodic) and surge (or phasic) release. LH pulses show little change on dioestrus day 1, day 2 and pro-oestrus before the LH surge while they are never seen during oestrus (Fox and Smith, 1985). During the transition from the episodic LH secretion to the LH surge, abrupt changes occur in the LH pulse frequency and amplitude, and this is described as the characteristic LH surge. When rats are kept in a lighting schedule of 14 hours light and 10 hours dark, the frequency and amplitude of the LH pulses increases on pro-oestrus. This initiates a linear rise in plasma LH levels approximately seven hours after the onset of the light. The increase in plasma LH levels continues and accelerates to a plateau some two to four hours later. It then drops rapidly but the decline is commonly interrupted by one or more rapid rises in plasma LH concentrations (Blake, 1976).

Acute timing of the LH surge and involvement of a neural signal in its control was first demonstrated in the spontaneously ovulating rat by Everett and his colleagues in a series of experiments (1949). Pharmacological suppression of this central stimulus consistently resulted in the blockade of the pre-ovulatory LH surge and hence of ovulation. The LH surge was delayed by 24 hours, but only if the drugs were administered during a particular time period. Thus the existence of a critical period of two to three hours on the afternoon of pro-oestrus, beginning at approximately 14.00h was indicated. After this time, the LH surge was found to be resistant to pharmacological blockade.

It has since become evident from a variety of experiments that the anatomical site of this neural stimulus lies within the hypothalamus. Indeed, a hypothalamic peptide (GnRH) with selective releasing action on LH and FSH, along with several other hypothalamic releasing factors have been identified in the mammalian hypothalami (Everett, 1994). The close functional relationship between GnRH and gonadotrophin secretion will be reviewed later in this chapter.

Feedback Control of Gonadotrophin Secretion

The neurosecretory activity of the hypothalamus is controlled by long, short and ultra-short loop feedback systems. The best documented of these is the long-loop feedback mechanism in which the steroid hormones secreted from the gonads exert their regulatory effects at the level of the anterior pituitary gland and/or the hypothalamus. The short-loop feedback mechanism refers to an action of pituitary hormones to regulate their own secretions by acting within the hypothalamus to which they are delivered by retrograde-flow of the portal blood up to the pituitary stalk. The hypothalamic hormones also regulate their own secretions by the ultra-short-loop feedback mechanism (see Karsch, 1990).

Circulating gonadal steroid hormones (E_2, P and androgens) have powerful negative feedback effects on the tonic mode of GnRH secretion. However, E_2 exerts a positive feedback function by eliciting the surge release of GnRH in females. P also appears to enhance the LH surge on the afternoon of pro-oestrus in the rat (Lee *et al*, 1990). These steroids may act within the hypothalamus to modify the rate of GnRH secretion; they may act at the anterior pituitary gland to alter its response to the hypothalamic GnRH, or they may act upon both sites (Kalra, 1993). The gonadal steroids are apparently the primary hormonal signals that regulate and sustain the complicated balance within the closed feedback loop of the hypothalamo-pituitary-gonadal axis.

The Steroid-Primed Ovariectomised Rat as a Model for the Study of the Pre-ovulatory LH Surge

Ovariectomy removes the gonadal steroid feedback to the hypothalamus and anterior pituitary and results in the loss of oestrous cycles. As a consequence of this, increased amounts of gonadotrophins are secreted into the blood. The rise in hormone levels is delayed and occurs only after several days, depending on the circulating levels of E_2 at the time of ovariectomy.

Acute treatment of ovariectomised (ovx) rats with E_2 brings about a decrease of plasma LH levels, whereas chronic treatment of these animals with E_2 can stimulate a pre-ovulatory-type LH surge. Administration of P to E_2-primed ovx rats potentiates the afternoon LH surge. The E_2+P induced LH surges are similar in

timing, duration and magnitude to the pro-oestrous LH discharge. Moreover, this can be timed more reliably than the spontaneous surge of intact animals.

The ovx and steroid-primed animal model has been used extensively in investigating the neuroendocrine control of surge release of LH (See Ramirez *et al*, 1984; Kalra, 1993, Barraclough, 1994).

Anatomical Organisation of the Hypothalamo-Pituitary Axis

Hypothalamus

The hypothalamus is a part of the diencephalon lying ventral to the thalamus and forms the walls and floor of the third ventricle. The lamina terminalis, a thin complex structure of capillaries, neurons, glia and tanycytes, constitutes the midline rostral border of the hypothalamus and the rostral wall of the third ventricle. The floor of the third ventricle evaginates ventrally, caudal to the optic chiasm, to form the infundibular portion of the neurohypophysis known as the median eminence (ME). The adenohypophysis, arising as a dorsal evagination from the embryonic pharyngeal roof, surrounds all parts of the neurohypophysis and also forms the anterior lobe of the pituitary. Rostrally the hypothalamus is bounded by the lamina terminalis.

The hypothalamus may anatomically be divided into two major areas: a medial area and a lateral area. The areas are defined and named in accordance with the topographic region of the hypothalamus within which they lie (Bleier and Byne, 1985). A schematic diagram of the rat hypothalamus is shown in Figure 1.2.

Medial Area

The medial area is predominantly central in position, and it is itself subdivided into four groups:

1) Medial preoptic-anterior hypothalamic region

This is a rostral or anterior group of nuclei which includes;

a) Vascular organ of the lamina terminalis
b) Median preoptic nucleus (MnPO)
c) Medial preoptic area (MPOA)

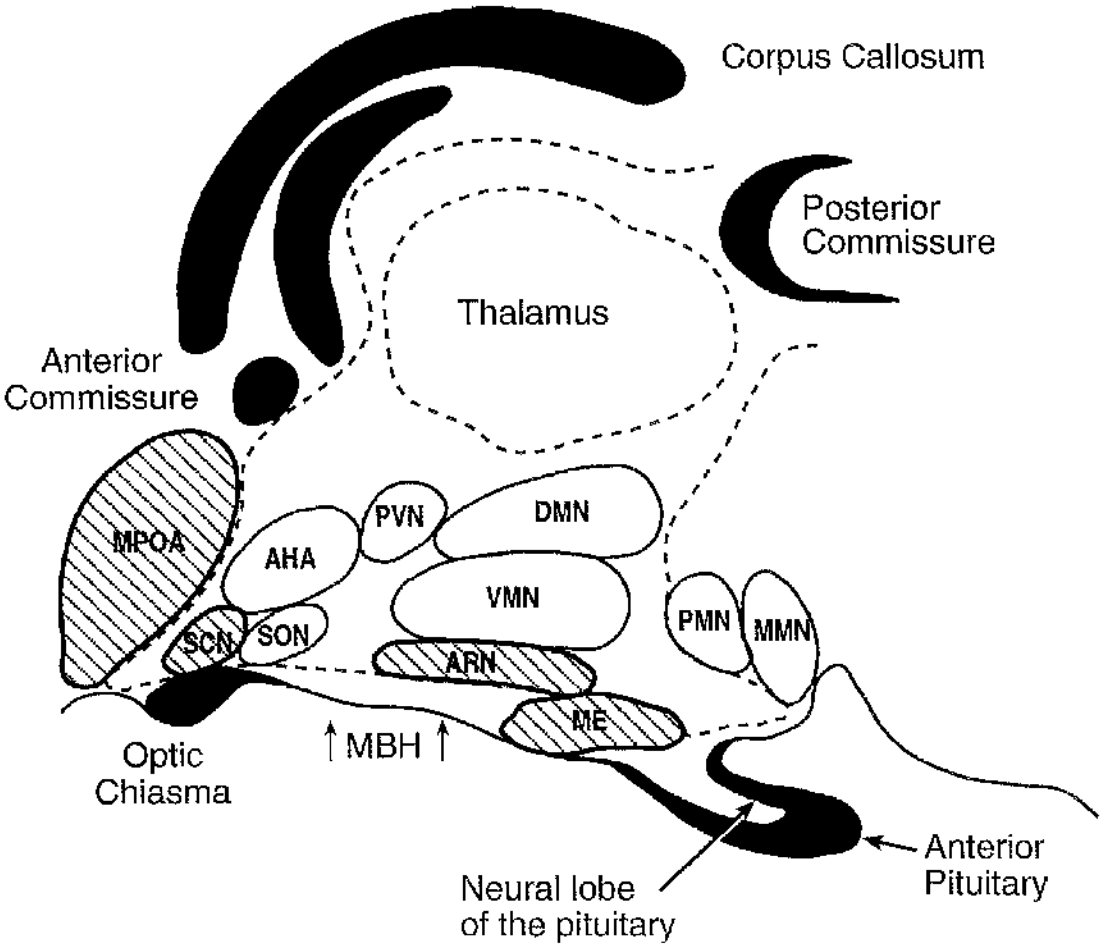


Figure 1.2. Schematic diagram of the rat hypothalamus and the pituitary gland. Those regions indicated in gray are the areas of interest in the present study.

AHA: Anterior hypothalamic area
ARN: Arcuate nucleus
DMN: Dorsomedial nucleus
MBH: Medial basal hypothalamus
ME: Median eminence
MMN: Medial mammillary nucleus
MPOA: Medial preoptic area
PMN: Premammillary nucleus
PVN: Paraventricualr nucleus
SCN: Suprachiasmatic nucleus
SON: Supraoptic nucleus
VMN: Ventromedial nucleus

d) Medial preoptic nucleus	(McPO)
e) Anterior hypothalamic nucleus	(AHy)
f) Anterior hypothalamic area	(AHA)
g) Suprachiasmatic nucleus	(SCN)
h) Lateral anterior nucleus	(LA)
i) Caudal anterior nucleus	(CA)
j) Paraventricular nucleus	(PVN)

2) Region of the tuber cinereum (Infundibulum)

The tuber cinereum is the middle part of the hypothalamus to which the pituitary gland is ventrally attached. As previously noted, the embryonic third ventricular floor evaginates ventrally and becomes the neurohypophysis.

a) Ventromedial nucleus	(VMN)
b) Dorsomedial nucleus	(DMN)
c) Perifornical nucleus	(PeF)
d) Area of the tuber cinereum	(TuC)
e) Dorsal tuberal nucleus	(DTu)
f) Tuberal magnocellular nucleus	(TMC)
g) Arcuate nucleus	(ARN)
h) Retrochiasmatic area	(RCh)

The VMN and DMN are very prominent in the tuber cinereum. The ARN lies just above the ME and is adjacent to the third ventricle. The region of the tuber cinereum is generally believed to be the area containing most of the neurons producing the various hypothalamic hormones.

3) Posterior or mammillary region and mammillary recess

The caudal third of the hypothalamus includes the posterior hypothalamic area, the mammillary complex, and two magnocellular groups.

a) Posterior hypothalamic area	(PHA)
b) Ventral premammillary nucleus	(PMV)
c) Dorsal premammillary nucleus	(PMD)
d) Submammillohypothalamic nucleus	(SMT)
e) Medial mammillary nucleus	(MMN)
f) Lateral mammillary nucleus	(LMN)

g) Supramammillary nucleus (SuM)
h) Nuclei gemini (Gem)
i) Caudal magnocellular nucleus (CMC)

In addition to these subdivisions, there is a narrow group of cells lying just beneath the third ventricular ependyma, extending the entire length of the third ventricle. These neurons are termed the periventricular system of the hypothalamus.

4) Dorsal region

The dorsal region overlies the anterior region and the tuber cinereum, and extends from the caudoventral border of the anterior commissure rostrally to the posterior hypothalamic area and mesencephalon caudally.

a) Dorsal hypothalamic area (DHA)
b) Dorsal nucleus (DN)
c) Paraventricular nucleus, dorsal division.

Lateral Area

This region of the hypothalamus lies lateral to the entire medial division of the hypothalamus and is the site at which the medial forebrain bundle enters the hypothalamus. This nerve tract contains the projections between the hypothalamus and the rest of the brain. The lateral hypothalamic area is also subdivided into three groups; 1) Preoptic-anterior region 2) Region of the tuber cinereum and 3) Posterior region.

The lateral hypothalamic area and supraoptic nucleus (SON) are located in the pre-optic anterior region. The ME lies directly below the ARN in the posterior region of the hypothalamus and is composed of two parts; an external layer and an internal layer. It contains few, if any, nerve cell bodies, but consists of axons and terminals of both hypothalamic and extrahypothalamic neurons, glial cells, and specialised ependymal cells called tanycytes. It is the final common pathway through which the neural control of the anterior pituitary hormone secretions are exercised.

Pituitary gland

The pituitary gland (hypophysis) is situated beneath the brain at the mesodiencephalic junction and formed by two distinct parts: the neurohypophysis

(posterior lobe) and the adenohypophysis (anterior lobe). The latter is further subdivided into the pars distalis, pars intermedia, and pars tuberalis.

The neurohypophysis is made up of neural tissue and is connected to the rest of the brain via the pituitary stalk. It contains terminals of neurons whose cell bodies reside in the hypothalamus. Thus there is a direct neuronal link between the posterior pituitary and the brain. This is an important mechanism for the secretion of hormones from the posterior lobe. The adenohypophysis is of glandular origin and surrounds the posterior lobe ventrally and laterally; it is composed of several types of hormone-secreting cells. Unlike the posterior pituitary, the anterior pituitary has no nerve fibres and terminals and so is not in direct neuronal contact with the hypothalamus. Instead, there is a vascular connection between it and the brain, "the hypothalamo-hypophyseal portal system". Figure 1.3. illustrates the anatomical relationship of the hypothalamus and the pituitary.

The hypothalamo-hypophyseal portal system

This system has its primary plexus in the external layer of the median eminence (EME). Its vessels course down the pituitary stalk and terminate in the secondary capillary plexus in the pars tuberalis. Most of the blood supplied to the anterior pituitary comes from this portal system. The capillaries deliver substances to the anterior lobe which are secreted by axons of the parvocellular neurosecretory cells terminating in the EME and which influence the release of the anterior pituitary hormones (Jennes and Stumpf, 1986). The endothelium in these capillary loops is highly fenestrated and flattened. Its basement is very thin, thus permitting macromolecules to enter without a functional blood brain barrier. Therefore, all of the known hypothalamic peptides and some biogenic amines are found in high concentrations in the portal blood. The tanycytes in the ME also extend the lumen of the third ventricle to the EME. They thus serve an important role in active transport in either direction between the ventricular compartment and the portal system (for review see Page, 1994).

The existence of a vascular connection between the hypothalamus and the pituitary gland was first recognised by Popa and Fielding (1930). This basic finding was subsequently substantiated and completed by Wislocki and King (1936), who also concluded that blood in this portal system was flowing from the hypothalamus toward the pituitary gland. In the following decade, Green and Harris (1947)

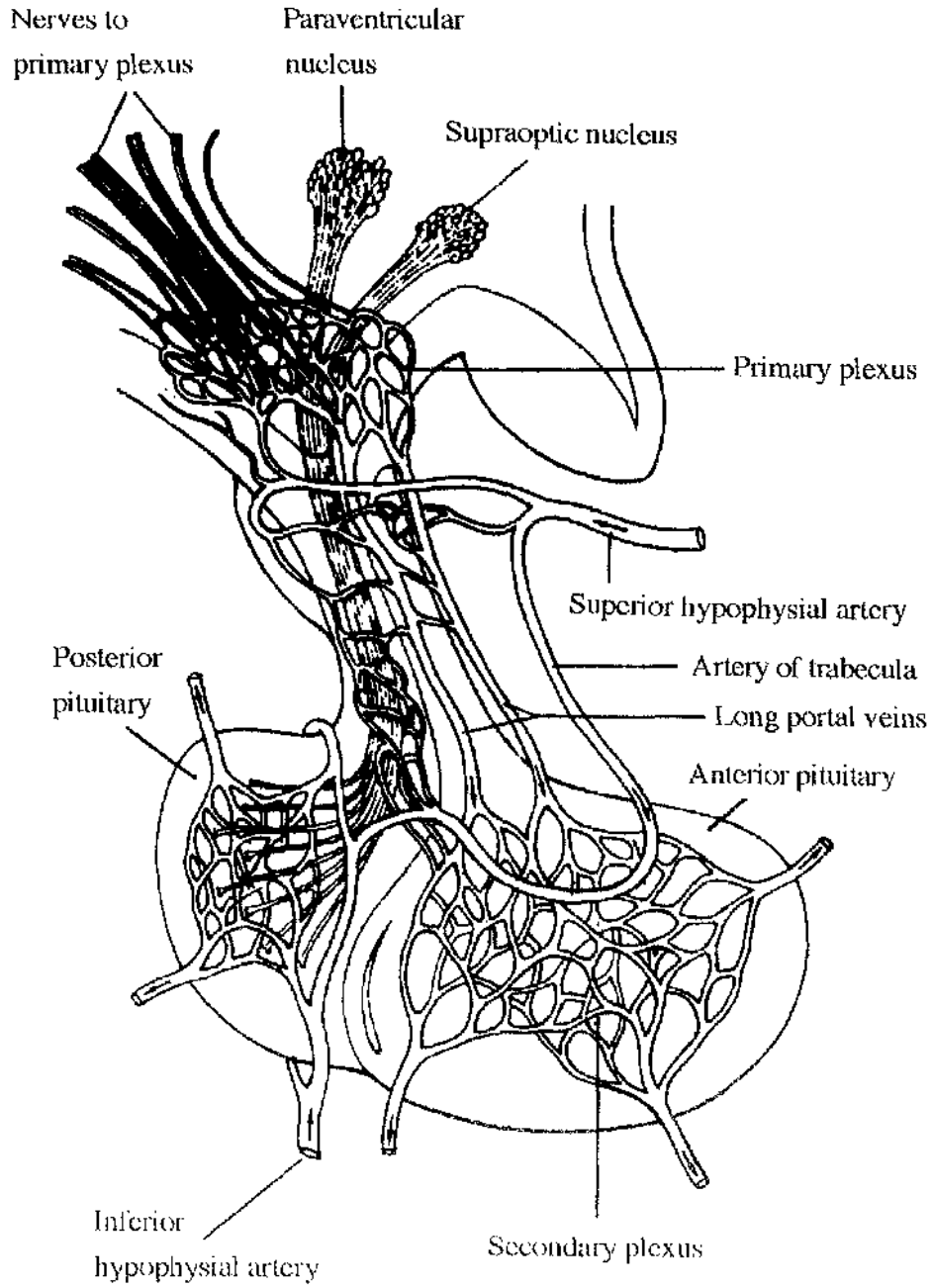


Figure 1.3. Diagram of the anatomical basis of vascular and neural communications between the hypothalamus and pituitary gland. Adapted from Guillemin, 1980.

realised functional implications of the portal circulation by demonstrating the downward direction of blood flow in the portal vessels of the living rat. More recently, it has been shown that, in addition, a small proportion of blood may actually flow up the stalk, thus providing a direct vascular link from the anterior pituitary back to the hypothalamus (Oliver *et al,* 1977). This retrograde blood circulation may be an important mechanism in the short-loop feedback regulation of gonadotrophin secretion, and it was first proposed by Halasz and Szentagothai (1960).

The Ventricular System of the Brain

The brain consists of lobular outgrowths from the walls of the fluid-filled neural tube. The central cavities of these outgrowths are called ventricles.

The embryonic neural tube originates from the primitive ectoderm, which then expands to form the cerebral ventricles. In early stages of fetal life, 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 organisation. These permutations occur as a consequence of a differential rate of cellular proliferation along the neural tube and the growth of neuronal processes.

With the growth of the brain, four brain vesicles are formed; 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. The central canal of the spinal cord is seen as a minute hole in the centre of the grey matter in transverse sections. It widens out anteriorly into the medulla oblongata to form the fourth ventricle. This narrows forward into a canal in the dorsal third of the mid-line of the midbrain as the cerebral aqueduct and continues as the third ventricle in the diencephalon. It then extends into each half of the cerebrum as the lateral ventricles through the interventricular foramen (Figure 1.4.).

The third ventricle is a high narrow slit with a rather thin floor, a membranous roof, and a massive lateral wall formed by predominantly thalamic and hypothalamic nuclei on each side. There are three recesses of the third ventricle; optic, infundibular and pineal. The first lies above the optic chiasma, the second extends through the infundibulum to the hypophysis, and the pineal recess passes

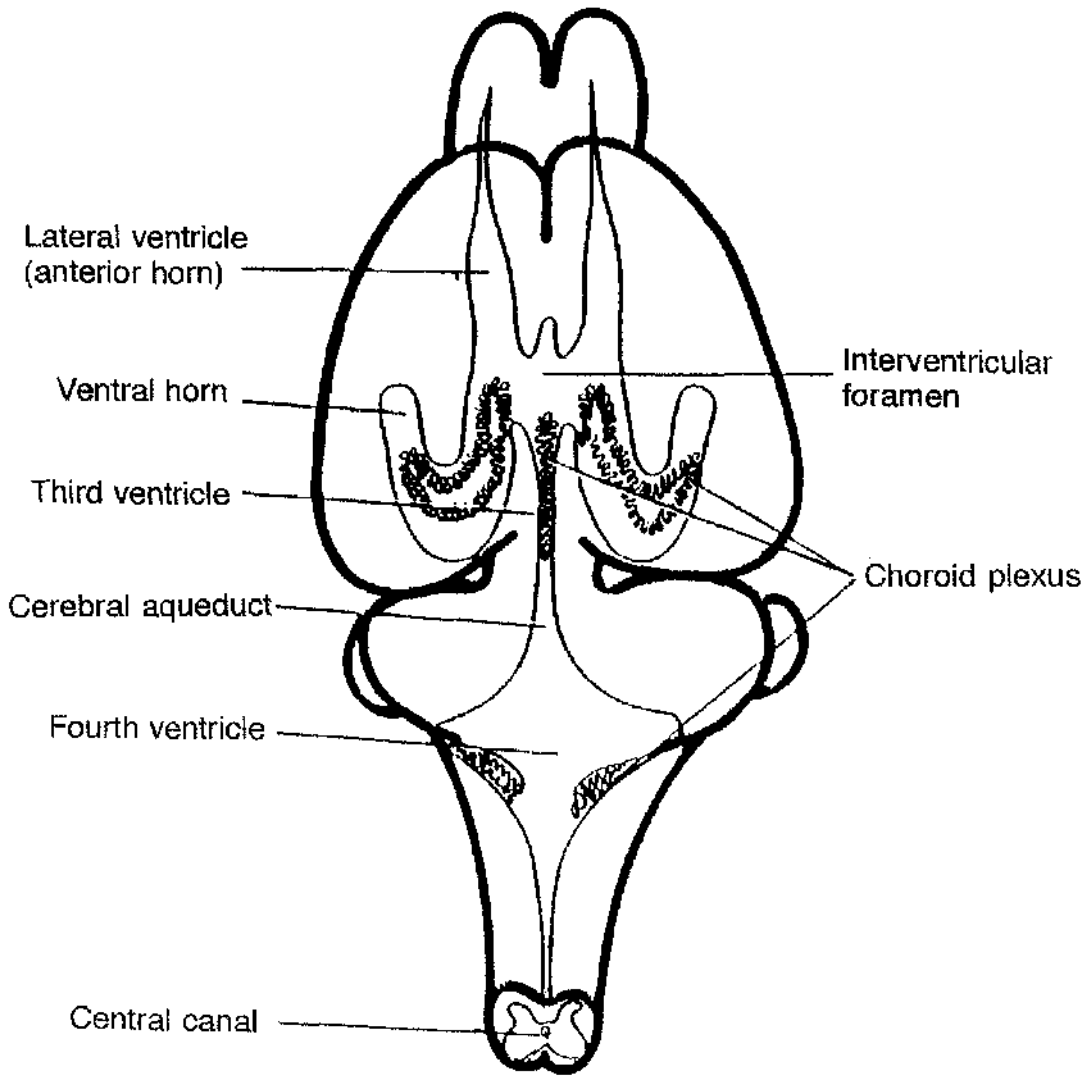


Figure 1.4. The ventricular system of the rat brain. Adapted from Zeman and Innes, 1963.

into the stalk of the pineal body. In front, the third ventricle communicates with the lateral ventricle on each side through the interventricular foramen (Zeman and Innes, 1963).

The entire surface of the ventricular system is covered by a lining of special differentiated neuroglial cells, with columnar ciliated epithelium, called ependyma. These cells are similar in all areas of the ventricular system except at the bottom of the third ventricle, where the specialised ependymal cells (the tanycytes) are found. The ependyma is continuous with the layer of short cuboidal epithelium which covers the choroid plexuses with their choroidal villi. The cerebrospinal fluid is initially formed by an active secretory process of the choroid plexuses within the ventricles. This fluid leaves the ventricular system through apertures in the posterior medullary velum. It circulates through the subarachnoid space of brain and spinal cord and is removed over the convexity of the cerebral hemisphere from the arachnoid villi projecting into large dural venous sinuses. Some interchange of water and solutes occurs between the brain and the cerebrospinal fluid.

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

Gonadotrophin-Releasing Hormone

The direct neurohumoral stimulus for the secretion of LH from the anterior pituitary is GnRH, also known as luteinising hormone-releasing hormone (LHRH). GnRH is released from the ME into the primary plexus of the hypophyseal portal blood system, where it is transported through portal veins to the second capillary bed in the anterior pituitary. Here it not only stimulates the secretion of LH, but, to a lesser extent, that of FSH as well.

The peptide sequence of GnRH was first determined from porcine hypothalami (Schally *et al,* 1971) and, soon afterwards from ovine hypothalami (Burgus *et al,* 1971) as the decapeptide: pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. Identification of the amino acid structure of this hypothalamic hormone then led to the production of synthetic GnRH, and it thus became available for clinical and research purposes. In most mammals, including the rat, only one molecular form of GnRH has been isolated, in spite of the characterisation of several molecular forms from non-mammalian vertebrate classes (King *et al,* 1988). However,

existence of a second molecular form of mammalian GnRH in the marsupial brain has recently been reported (King *et al,* 1989).

The distribution of GnRH-producing neurons in the rat brain has been extensively investigated (Palkovits *et al,* 1984). In the rat, high numbers of perikarya and fibres containing GnRH have been visualised in the MPOA, and high concentrations of GnRH itself have been found in the ARN, SCN and ME. In the latter two hypothalamic areas GnRH was restricted to fibres alone (Witkin *et al,* 1982; Shivers *et al,* 1983a; Palkovits *et al,* 1984). The GnRH fibres reach to the ME by more than one route (Merchanthaler *et al,* 1984; Silverman and Witkin, 1994). The major septo-preoptico-infundibular pathway begins as a midline bundle of GnRH which processes anteriorly near the level of the diagonal band of Broca (Silverman *et al,* 1994). This bundle bifurcates near the preoptic recess of the third ventricle. They both travel close the midline, one along the dorsal and the other along the ventral surface of the optic chiasm. The ventral bundle tends to run close to the surface of the brain, being covered only by the pia (Merchanthaler *et al,* 1984). Axons of the GnRH neurons in the more caudal and lateral aspects of the preoptic area and hypothalamus travel in or near the medial forebrain bundle and then turn medially near the level of the ME. These axons also project to the limbic system and circumventricular organs other than the ME, such as the organum vasculosum of the lamina terminalis (OVLT).

In addition to their major projections to the ME, GnRH neurons also project to several extrahypothalamic structures such as the midbrain, the hippocampus, the amygdala and other olfactory structures (Silverman *et al,* 1994). Coordination between both populations of GnRH neurons is achieved by intercellular communications through numerous collateral synaptic contacts between GnRH-producing neurons and putative GnRH receptors (Wetsel *et al,* 1992). Indeed, during the pre-ovulatory LH surge, all GnRH neurons have been shown to synchronise to initiate synthesis of this neuropeptide (Silverman and Witkin, 1994). This simultaneous firing of many, if not all, GnRH neurons is required for the pulsatile discharge of GnRH.

Hypothalamic GnRH binds to specific receptors on the adenohypophyseal gonadotroph and stimulates the synthesis and secretion of the gonadotrophins (Conne *et al,* 1982). This secretory activity occurs intermittently in apparent synchrony with, and in response to, the pulsatile discharge of GnRH from the

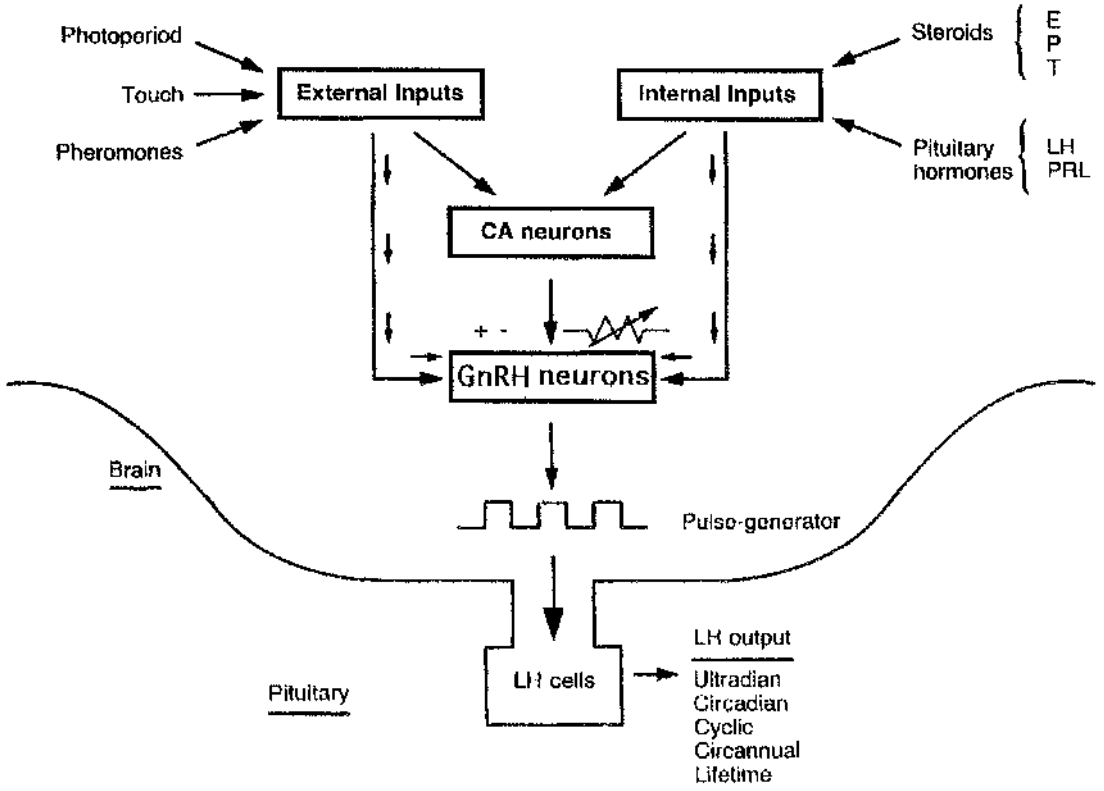


Figure 1.5. Schematic representation of the organisation of the neural circuitry regulating the secretion of GnRH from the ME. Short arrows represent interneuron pathways and continuous lines depict direct neural connections. From: Ramirez *et al*, 1984.

hypothalamus. GnRH receptor numbers on the gonadotroph increase over two-fold during dioestrus to reach a peak during the morning of pro-oestrus. This is followed by a rapid fall during the late afternoon of pro-oestrus to reach a nadir by oestrus (Childs *et al,* 1994). Experimental data indicate that E_2 increases pituitary responsiveness to the GnRH signal at the time of the pre-ovulatory LH surge. This is achieved by increasing GnRH receptor numbers, at least partially, by increasing the number of GnRH-receptive cells (Lloyd and Childs, 1988). In addition, the hypothalamic hormone itself may prime the gonadotrophs to increase their response to subsequent GnRH pulses (Mitchell *et al,* 1988).

Under the influence of gonadal steroids, a rapid increase in the hypothalamic GnRH content has been reported during the critical period on the afternoon of pro-oestrus (Kalra, 1986). GnRH levels in portal blood have been measured and well-correlated to the LH surge in anaesthetised (Sarkar *et al,* 1976) and conscious rats (Levine and Ramirez, 1982). These experiments clearly demonstrate that GnRH release is elevated on the afternoon of pro-oestrus and that this rise precedes the pre-ovulatory LH surge.

Existence of a "GnRH pulse generator" in the central nervous system has now been commonly accepted, since the pulsatile release of LH well corresponds with the episodic nature of GnRH (Clarke and Cummins, 1982; Kalra and Kalra, 1985; Leonhardt *et al,* 1991). The pulse generator appears to be contained within the hypothalamus (Karsch, 1990). This is in line with the recent evidence that multiunit activity of the GnRH neurons in the MBH increases in association with the LH surge (Kimura and Sano, 1995). Various neurotransmitter systems and the gonadal steroids, which convey information both from the external and internal environment, seem to be involved in modulating activity of the GnRH pulse generator. However, not much is yet known about how it operates.

As mentioned earlier, release of both FSH and LH is stimulated by the hypothalamic decapeptide, GnRH (Wise *et al,* 1979). Administration of antiserum to GnRH or sodium pentobarbitone abolish LH release, but have no effect upon FSH secretion (Culler and Negro-Vilar, 1987; Kovacs *et al,* 1993). In addition, their hormone profiles throughout the oestrous cycle are known to be dissociated. LH release seems to be completely dependent upon GnRH secretion, but GnRH is not the sole regulator of FSH release. It has been suggested by McCann and his colleagues that there may be a separate FSH-releasing hormone (FSH-RH) which

is produced in the paraventricular nucleus, anterior hypothalamic area and surrounding the ARN and ME to selectively regulate FSH release. However, this suggestion is controversial. Although there is some evidence for its existence (Mizunuma *et al,* 1983; Lumpkin *et al,* 1984), the proposed FSH releaser has not yet been identified.

NEUROTRANSMITTER SYSTEMS MODULATING GnRH RELEASE

It has recently become evident that GnRH neurons contain neither E_2 (Shivers *et al,* 1983b) nor P (Fox *et al,* 1990) receptors, and therefore the influence of the gonadal steroids on GnRH release must be indirect. There is now a growing evidence that a complex network of neuronal systems, which elaborate messenger molecules, regulate the release of GnRH from the hypothalamus (see Figure 1.5.). These neurotransmitter systems are also believed to mediate the feedback effects of the steroid hormones on GnRH release. This new theory is contrary to the earlier belief that the GnRH neurons initiate their own release (Estes *et al,* 1982).

The neuromodulatory systems in the central regulation of GnRH and hence LH release may be classified as 1) monoaminergic neurotransmitters, 2) amino acid neurotransmitters and 3) neuropeptides. However, most recently a gaseous neurotransmitter nitric oxide (NO) has emerged as a possible regulator as well.

Synthesis, storage, release and deactivation of neurotransmitters

Neurotransmitters are continually being synthesised, stored, released and deactivated. The amount of neurotransmitter stored in secretory vesicles can regulate its own rate of synthesis by a negative feedback loop. The concentration of transmitters recaptured by presynaptic reuptake mechanism also regulates formation of new neurotransmitters. This is accomplished by altering the levels of the enzymes necessary for their biosynthesis. Neural signals from other neurotransmitter systems can modulate each others synthesis as well.

Monoamines

In the first step of catecholamine synthesis, the amino acid, tyrosine, is converted to dopa in the nerve cell through the action of tyrosine hydroxylase (Figure 1.6.). Dopa is then converted to dopamine (DA) in the cell body by dopa decarboxylase,

and DA is transported to the nerve terminals in vesicles. In a dopaminergic neuron, biosynthesis does not proceed further. However, at a noradrenergic nerve terminal, DA is converted to noradrenaline (NA) in the synaptic vesicles by the action of dopamine-beta-hydroxylase. At an adrenergic nerve terminal, NA is converted to adrenaline (ADR) by a specific enzyme, phenylethanol amine N-methyltransferase (PNMT).

Serotonin or 5-hydroxytryptamine (5-HT) is synthesised from tryptophan, which is converted to 5-hydroxytryptophan (5-HTP) by the action of tryptophan hydroxylase. 5-HTP is then converted to 5-HT in the cytoplasm by tryptophan decarboxylase (see Figure 1.14.).

Acetylcholine (ACh) is derived from choline and acetyl coenzyme A through the action of choline acetyltransferase in the cholinergic nerve terminals (See Cooper *et al,* 1991).

Amino acid transmitters

Glutamate and aspartate are synthesised from glucose and other precursors in neurons and glial cells. GABA is formed from its precursor, glutamic acid, by the enzyme glutamic acid decarboxylase in the nerve terminals (Figure 1.7.).

Neuropeptides

Precursors for the neuropeptides are prepropeptides, which are synthesised in the endoplasmic reticulum of the cell body and packaged into secretory vesicles in the Golgi apparatus. Endogenous opioids, for example, are derived from three different precursors; proenkephalin, prodynorphin and proopiomelanocortin (POMC) (Akil *et al,* 1984). Synthesis of peptide neurotransmitters is completed in the synaptic vesicles, which are transported down to the nerve terminal for storage and release of the neuropeptides.

Release

Following postsynaptic stimulation by neurotransmitters, the neuron fires and releases its transmitters presynaptically into the next synaptic area. There, these neurotransmitters bind and stimulate their postsynaptic receptors. They cause ion channels to open and the nerve membrane is depolarised, i.e. sodium ions (Na^+) flow in and potassium ions (K^+) move out of the cell. This depolarisation causes a measurable change in the electrical activity of the cell, called an action potential

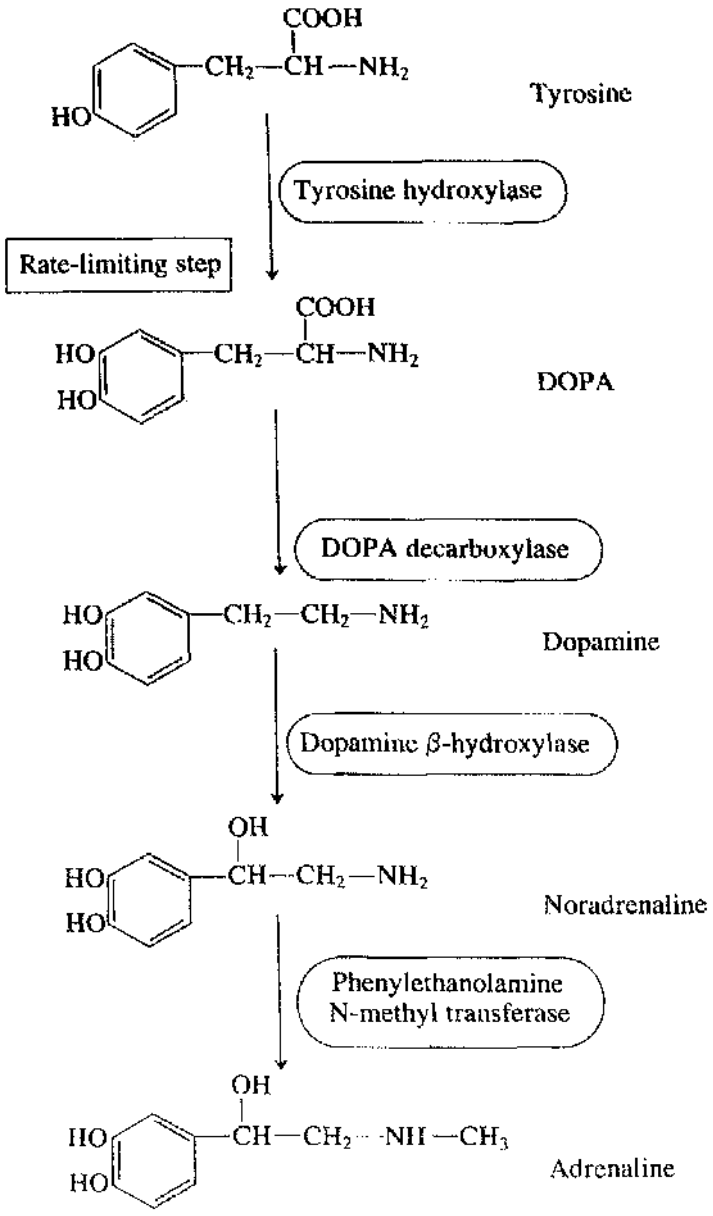


Figure 1.6. Biosynthesis of catecholamines. Adapted from Rang and Dale, 1991.

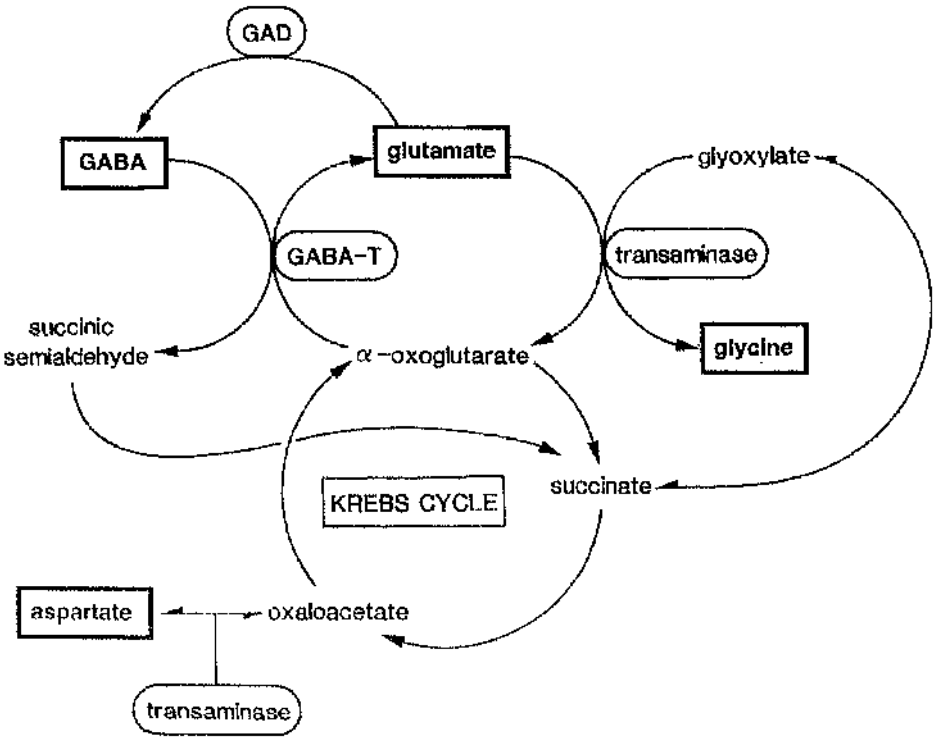


Figure 1.7. Metabolism of neurotransmitter amino acids in the brain. Neurotransmitter substances are marked with thick black boxes.

which travels along the axon until the nerve terminal. When depolarisation of the nerve terminal occurs, the secretory vesicles fuse with the cell membrane, and under the influence of calcium ions (Ca^{+}) release their transmitters into the synaptic area. After these pre- and postsynaptic events, the cell returns to its resting potential until stimulated by another neurotransmitter to fire again. The release of these messenger molecules involves a number of complex biochemical actions which are explained in detail elsewhere (Rang and Dale, 1991). A schematic diagram of neurotransmitter release is shown in Figure 1.8.

Deactivation

After the neurotransmitter has stimulated its postsynaptic receptor, it is deactivated by two degradation mechanisms. The first is reuptake (endocytosis) by receptors on the presynaptic cell which take the neurotransmitter into vesicles where it is reused or deactivated. Up to 80% of the monoamines released into synapse are degraded in this way. The amino acid neurotransmitters are also deactivated by presynaptic reuptake. ACh is rapidly broken down by acetylcholine esterase. The two degradation products, acetic acid and cholin may then be picked up by the presynaptic neuron and used to synthesise new transmitter molecules.

The second way of degradation is catabolism of the neurotransmitters by specific enzymes within the synapse. The monoamines are deactivated by two different enzymes, catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) (Figures 1.9. and 1.10.). Peptidergic transmitters are deactivated by peptidase enzymes (McKelvy, 1986).

1) Monoaminergic Neurotransmitters

Noradrenaline

Considerable amount of literature suggests that NA may be the major neurotransmitter in initiation of the LH pre-ovulatory surge which directly stimulates the GnRH neurons (See Ramirez *et al*, 1984; Barraclough, 1994). The neurotransmitter role of NA in mediating this process may be complicated due to its interactions with the other neurotransmitter systems and its diffuse pathways.

There are two major noradrenergic pathways within the central nervous system (CNS) which project to the hypothalamus. The cell bodies of the noradrenergic

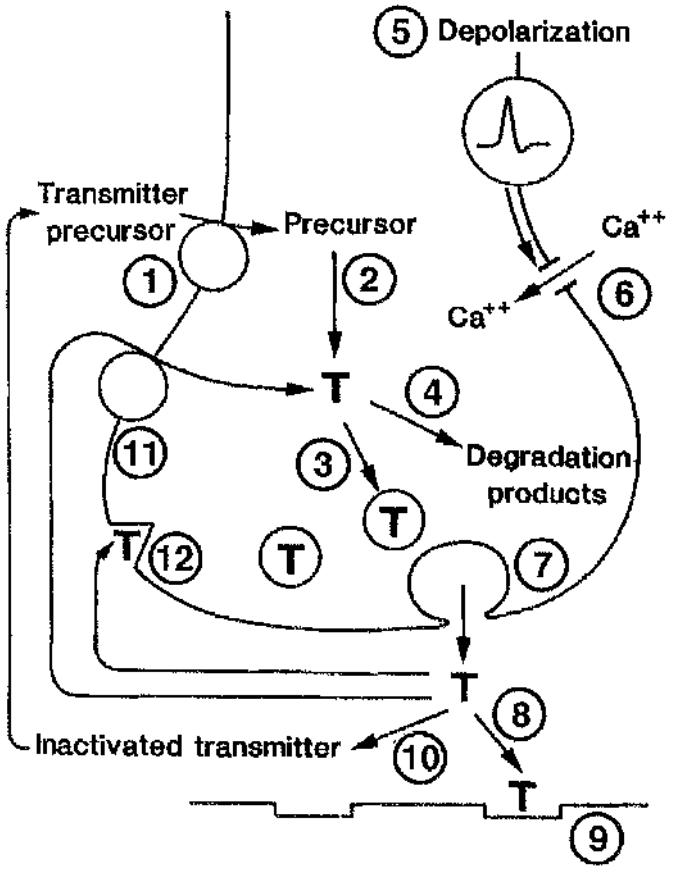


Figure 1.8. The main processes involved in synthesis, storage and release of aminergic transmitters (including NA, DA, 5-HT and ACh): **1**) Uptake of transmitter precursors by nerve terminals, **2**) Synthesis of transmitter in nerve terminals, **3**) Storage of transmitter in releasable form, **4**) Degradation of surplus transmitter within nerve terminals, **5**) Depolarisation of nerve terminal by propagated action potential, **6**) Influx of Ca^{++} in response to depolarisation, **7**) Release of transmitter in quantal packets, **8**) Diffusion of transmitter to post-synaptic receptors, **9**) Interaction with receptors and production of post-synaptic effect, **10**) Inactivation of transmitter within synaptic cleft. T: Transmitter or neurotransmitter. Adapted from Rang and Dale, 1991.

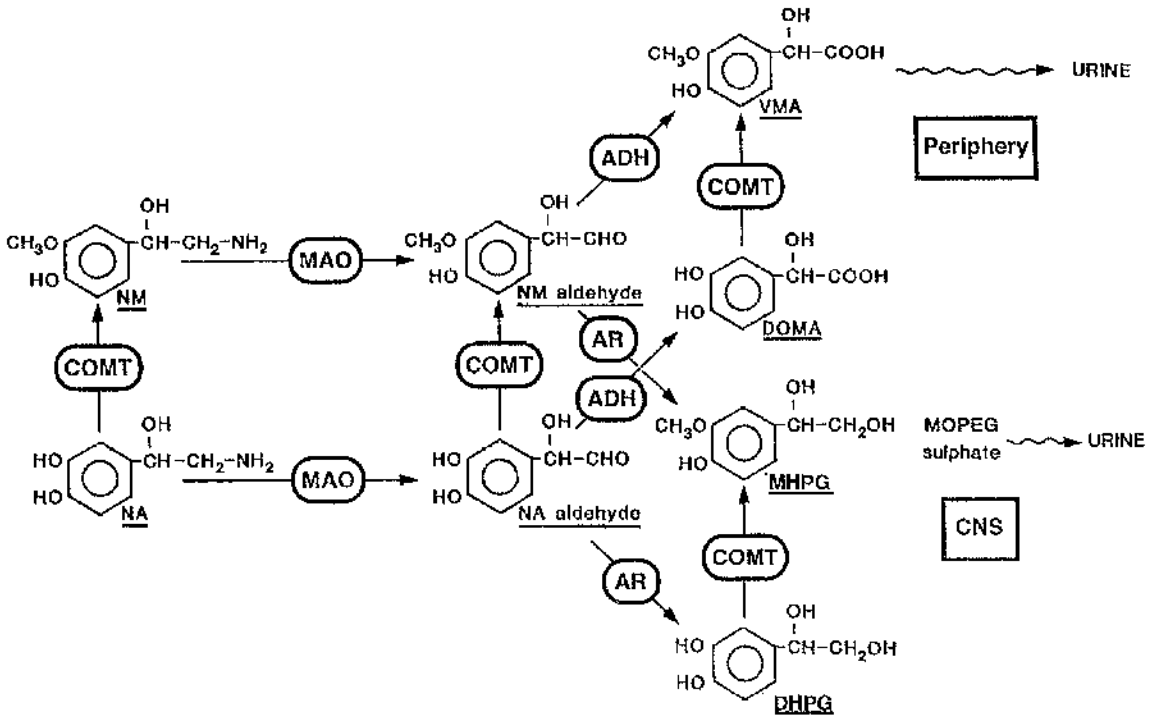


Figure 1.9. The main pathways of NA metabolism in the brain and periphery. Adapted from Rang and Dale, 1991.

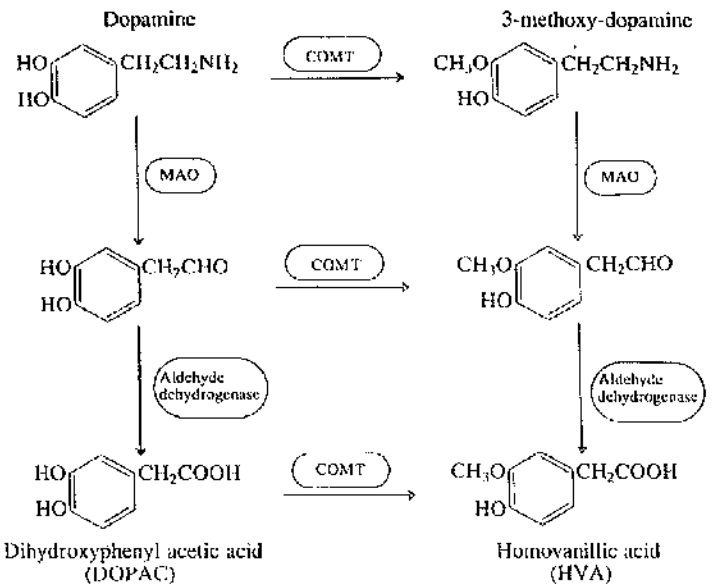


Figure 1.10. The main pathways of DA metabolism in the brain. Adapted from Rang and Dale, 1991.

neurons are found exclusively in the pons and medulla. The locus coeruleus (LC) appears to contain the most prominent cluster of NA neurons whose axons pass through the cerebral cortex, limbic system, the hypothalamus, the cerebellum and the spinal cord. The major projection of LC axons ascends in a dorsal noradrenergic bundle (DNAB) transversing the midbrain tegmentum which innervates the MPOA and ME of the hypothalamus, the cortex, the hippocampus and the cerebellum (Jones and Moore, 1977). At the caudal diencephalon the LC axons descend to enter the ventral noradrenergic bundle (VNAB) which provides a diffuse innervation of the hypothalamus including the MPOA, SCN, ME and ARN, and the hippocampus and the other parts of the forebrain (See Figure 1.11. for NA pathways).

NA nerve terminals and GnRH neurons are found in close proximity in the MPOA and to a lesser extent in the ME and ARN (McNeill and Sladek, 1978). Furthermore, catecholamine neurons have been shown to innervate GnRH perikarya in the MPOA (Watanabe and Nakai, 1987; Leranth *et al,* 1988a) and other hypothalamic areas (Chen *et al,* 1989). Thus, these neuroanatomical sites provide evidence for direct functional connections between the two systems.

Based on the relative affinities of adrenergic agonists and antagonists, adrenergic receptors are divided into two main classes; α- and β-adrenoreceptors. These are further subdivided into α_1- and α_2- and β_1- and β_2- adrenoreceptors on the same criteria. All four adrenoreceptor types and subtypes have been visualised in the hypothalamus (Leibowitz *et al,* 1982). The α-adrenoreceptors have been found in highest concentrations in the MPOA, ME and ARN with β-adrenoreceptors (predominantly β_2) being most common in the MPOA.

It has been recognised that blockade of NA adrenoreceptors or suppression of NA synthesis or release disrupts spontaneous pre-ovulatory LH surges while intracerebroventricular (icv) infusion of this neurotransmitter stimulates GnRH secretion in cyclic rats (Barraclough and Wise, 1982; Ramirez *et at,* 1984; Al-Hamood *et al,* 1985). During the E_2-induced LH surge in ovx rats, increased NA turnover rate in the MPOA, SCN, ME and ARN occurs concomitant with a rise in circulating levels of LH (Wise *et al,* 1981). Conversely, suppression of increased NA turnover by administration of phenobarbital or lesion of the LC in the brainstem results in failure of the pre-ovulatory LH surge on pro-oestrus (Kalra and Simpkins, 1981; Rance and Barraclough, 1981; Franci and Rodrigues, 1985).

There is evidence for a relationship between the noradrenergic activity of the hypothalamus and the oestrous cycle of the rat. During the oestrous cycle, NA turnover follows a cyclic fluctuation that reaches its highest level during the afternoon of pro-oestrus. This is intimately correlated with GnRH and the pre-ovulatory LH discharge (Rance and Barraclough, 1981). A decrease in the hypothalamic activity of MAO during the afternoon of pro-oestrus has also been reported (Honma and Wuttke, 1980).

Tyrosine hydroxylase (TH) is the rate-limiting enzyme for the synthesis of catecholamines (see Figure 1.6). One index of increased activity within the noradrenergic neurons is a rise in TH mRNA levels. Noradrenergic nerve terminals in the vicinity of GnRH neurons are known to originate almost exclusively from the A1 and A2 noradrenergic cell groups located in the brainstem. There is an increase in the TH activity of the NA neurons from the A1 and A2 region (Liaw *et al*, 1992) as well as of the NA terminals in the MPOA (Herbison *et al*, 1990) at the time of the afternoon LH surges. This increased TH activity is accompanied by a rise in hypothalamic NA release. *c-fos* is an immediate early gene (Sagar *et al*, 1988). Immunocytochemical detection of its protein product, Fos, has now been used extensively as a marker for neuronal activation (for review see Hoffman *et al*, 1993). Jennes *et al* (1992) has shown a significant increase in the Fos immunoreactivity of the A1 and A2 noradrenergic neurons on the afternoon of pro-oestrus compared with that at dioestrus. These biochemical studies indicate that an increased neuronal activity within these cells occurs at a time when hypothalamic NA release and turnover rates increase and plasma LH levels begin to rise.

Inhibitory effects of NA on pulsatile LH release have also been reported. Infusion of NA directly into the MPOA (Leipheimer and Gallo, 1985) or into the third ventricle and electrical stimulation of the ascending noradrenergic tracts (Bergen and Leung, 1987) or LC (Dotti and Taleisnik, 1984) inhibit LH secretion. This inhibition is predominantly through a reduction in LH pulse frequency. It should be noted that the rat models employed in these studies were mainly ovx and not steroid-primed. However, ventricular administration of NA, β- (isoproterenol) or α-adrenoreceptor agonists (phenylephrine and methoxamine) have been found to be effective in disrupting the P-induced GnRH release in ovx rats (Bergen and Leung, 1988). It thus appears that NA exerts a dual action on GnRH release

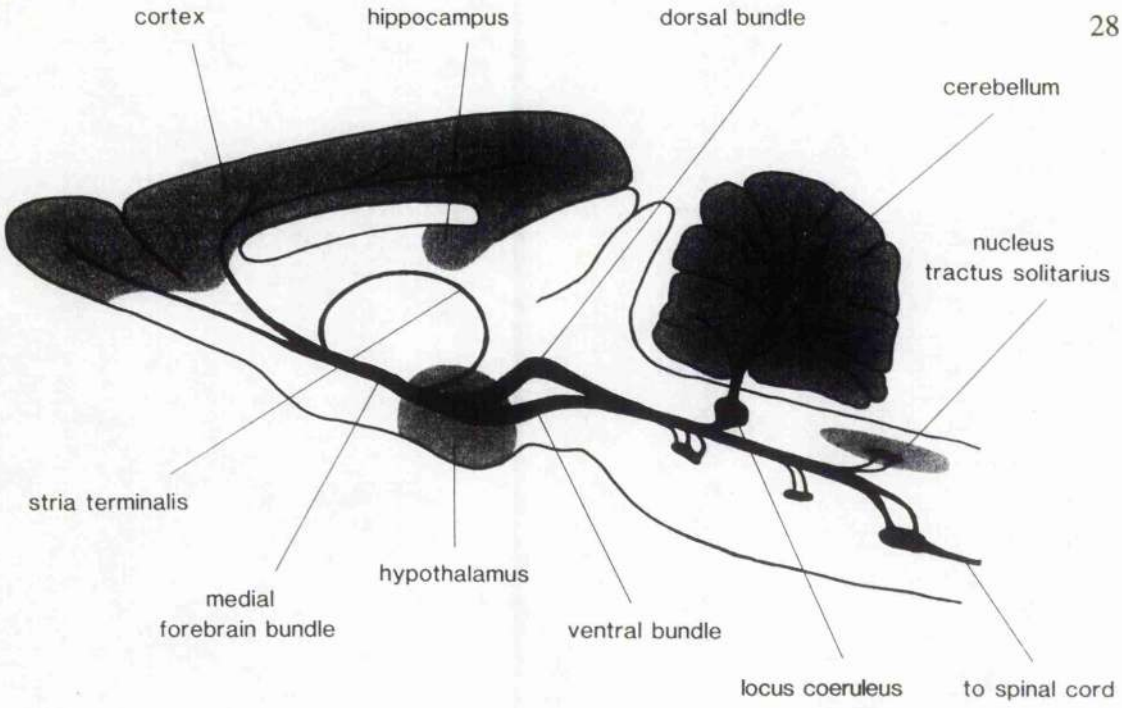


Figure 1.11. Schematic illustration of a saggital section through the rat brain showing the noradrenergic projections to the hypothalamus. The main groups of perikarya and fibre tracts are shown in black. Grey areas represent the location of axon terminals. Adapted from Rang and Dale, 1991.

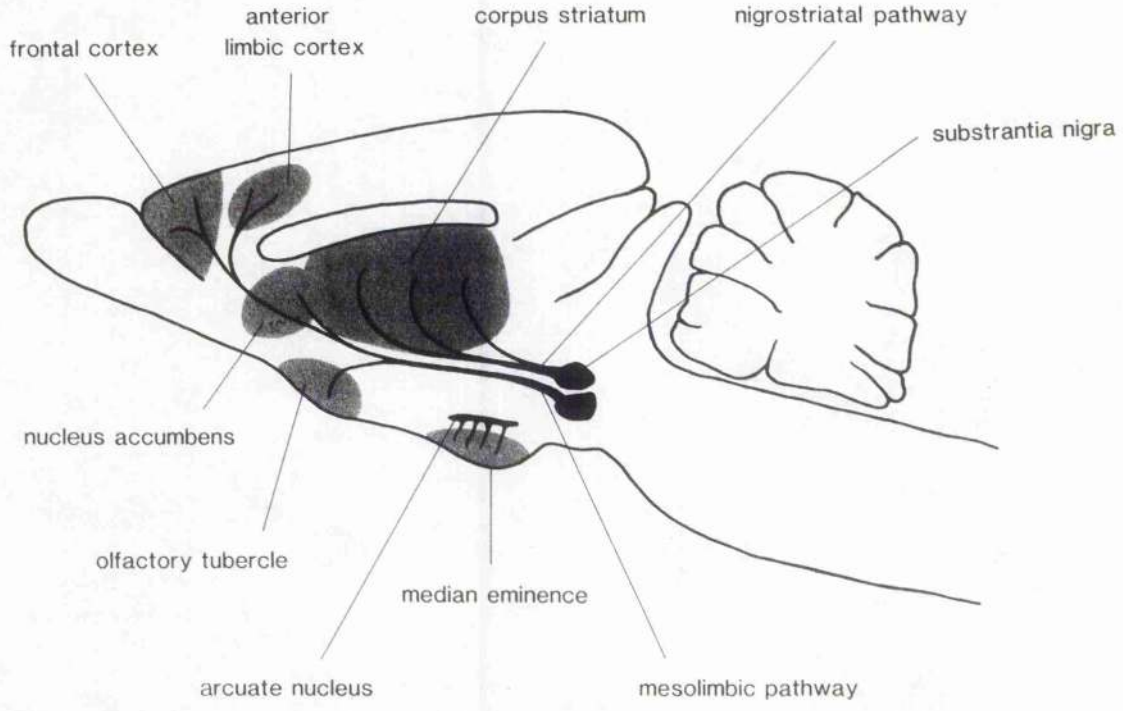


Figure 1.12. Schematic illustration of a saggital section through the rat brain showing the dopaminergic projections to the hypothalamus. The main groups of perikarya and fibre tracts are shown in black. Grey areas represent the location of axon terminals. Adapted from Rang and Dale, 1991.

depending upon the steroidal milieu so that NA is stimulatory in the presence of E_2 but inhibitory in its absence (Ramirez *et al,* 1984; Dyer *et al,* 1985).

These stimulatory effects of NA are mediated by α-adrenoreceptors (Gallo and Kalra, 1983) particularly by α_1-receptor subtypes in the anterior hypothalamic-preoptic area and MBH (Ramirez *et al,* 1984). NA acting via β-adrenoreceptors modulates the transmission of inhibitory impulses originating in the LC (Dotti and Taleisnik, 1984), on LH release in ovx E_2-primed rats. The β_2-adrenergic receptor sites may become de-activated on the late afternoon of pro-oestrus, since only the stimulation of β_2-adrenoreceptors in the afternoon with a high dose of fenoterol (a selective β_2-receptor agonist) is effective in inducing ovulation along with the well-documented α-adrenergic stimulatory components (Al-Hamood *et al,* 1985); this could mask the possible inhibitory action of the β_1-adrenergic system. Moreover, it has also been shown that electrical stimulation of the LC or of the medullary A1 noradrenergic cell group in E_2-primed ovx animals markedly amplifies the surge release of LH in the MPOA, and this amplifying action can be blocked by α- (predominantly α_1-) but not β-adrenoreceptor antagonists (Gitler and Barraclough, 1988). On the other hand, α-adrenergic mechanisms have been implicated the inhibitory action of NA on pulsatile GnRH release (Bergen and Leung, 1988).

α-adrenoreceptors have been visualised on GnRH neurons in the MPOA and ME. These neurons show high affinity for NA (Al-Damluji *et al,* 1993). It may thus indicate that NA released within brain regions could directly affect GnRH neuronal activity. Moreover, NA can stimulate GnRH release from ME fragments *in vitro* (Negro-Vilar *et al,* 1980).

In addition to direct modulatory effects of NA on GnRH neurons in inducing LH secretion, and thereby ovulation, indirect effects of NA have also been reported via putative interneurons interposed between NA and GnRH neurons (Jennes *et al,* 1982; Barraclough, 1994). Noradrenergic terminals within the hypothalamus possess a mixed population of opioid receptors. There is a variety of evidence indicating that the EOPs can modulate the activity of NA neurons acting at pre-synaptic nerve terminals (Van Vugt *et al,* 1982; Bicknell, 1985). Ovarian steroids may reactivate NA neurons by removing the tonic inhibitory effect of the opioid peptides (Crowley, 1988). The modulatory effects of opioids on GnRH and LH release will be reviewed later in this chapter.

A good candidate for neurons which could alter the responsiveness of GnRH neurons are GABA interneurons. GnRH cells receive GABAergic innervation (Leranth *et al,* 1988b) and GABA receptor agonists suppress LH surges in steroid-primed ovx rat (Adler and Crowley, 1986). A pre-synaptic modulatory role of GABA on the NA nerve terminals has been shown (Demling *et al,* 1985). Perhaps this inhibitory transmitter may affect the activity of the GnRH neurons both via its direct synaptic connections and indirectly by modulating the presynaptic release of NA. It could also modulate GnRH neuronal responsiveness to NA (Barraclough, 1994). Conversely, NA can influence GABA release in the hypothalamus whose excitatory action is mediated by α-adrenoreceptors (Herbison *et al,* 1990) and particularly α_1-subtypes (Kim *et al,* 1991). Thus, these indirect effects provide a simple explanation for the inhibitory action of NA on both basal and surge release of LH (Adler and Crowley, 1986) depending upon the steroid environment (Herbison *et al,* 1989).

A serotonergic involvement has also been suggested to affect NA concentrations, at least in the DNAB. This belief is based on the inhibitory effect of electrical stimulation of the DNAB on steroid-induced LH secretion. Indeed, in the midbrain, NA neurons have been found close to serotonergic neurons (Morello and Taleisnik, 1985).

Gonadal steroids are known to influence catecholamine transmission in the brain (Barraclough and Wise, 1982). The catecholaminergic neurons have been shown to possess E_2 receptors (Sar, 1984; Lehman and Karsch, 1993). The steroid hormones may thus exert their feedback effects on the GnRH neurons through the brain noradrenergic system. It is believed that the role of NA in the operation of the GnRH surge generator is dependent on priming effects of E_2 or E_2 plus P. These hormones may determine whether the noradrenergic input is excitatory or inhibitory on the LH surge (Ramirez *et al,* 1984). All these findings provide further evidence for the crucial role of the ovarian steroids, particularly E_2, in the mediation of GnRH release. They not only elevate the releasable pool size of GnRH in the ME, but they are also essential for the increased release of NA during the early afternoon of pro-oestrus.

In view of all these reports, there can be little doubt of the influence of NA on GnRH secretion. It is generally believed that NA is an essential component of the

GnRH pulse generator and may be the triggering neural stimulus for the pre-ovulatory LH surge.

Dopamine

There is a large body of evidence implicating central dopaminergic mechanisms in the neuroendocrine regulation of GnRH release and the LH surge (Ramirez *et al,* 1984; McKenzie *et al,* 1988).

There have been conflicting reports on the modulatory role of the dopaminergic system on GnRH release suggesting both stimulatory (McKenzie *et al,* 1988; Sanghera *et al,* 1991a) and inhibitory (Tasaka *et al,* 1985; Kerdelhue *et al,* 1989) actions of DA in rats (For review see Ramirez *et al,* 1984). Dopaminergic neurons exert an important stimulatory effect on the pulsatile release of LH, since administration of DA antagonists such as pimozide or haloperidol, reduces the LH surge and ovulation when given to pro-oestrous rats (MacKenzie *et al,* 1988). Infusion of DA or dopaminergic receptor agonists into the zona incerta (ZI) elevates plasma LH levels and advances the LH surge in the intact or ovx and steroid-primed rats (McKenzie *et al,* 1984; James *et al,* 1987). Lesions of the medial ZI have disrupted the LH surge (Sanghera *et al,* 1991a). Moreover, there is a temporal relationship between the onset of LH pulses and increases in DA concentrations in the ME of ovx rats (Negro-Vilar *et al,* 1982). However, infusion of DA receptor agonists such as apomorphine into the third ventricle has also been reported to cause a marked inhibition of the episodic LH release (Drouva and Gallo, 1977).

These dual effects of DA on GnRH release could be due, at least in part, to a complex network of dopaminergic neurons in the hypothalamus where DA may have both direct and indirect actions. Four distinct groups of DA-containing cell bodies, which were originally designated as A11 through A14 (Bjorklund *et al,* 1975), appear to innervate the hypothalamus (See Figure 1.12. for DA pathways). Neurons of the tuberoinfundibular dopaminergic tract (TIDA or A12) originate in the ARN and PeVN and project to both the external and the internal layers of the ME (Lindvall and Bjorklund, 1982), where they are found to be in close apposition to the GnRH terminals (Jennes *et al,* 1983). The TIDA appears to exert an inhibitory effect upon GnRH release (Rose and Weick, 1986). Furthermore, DA turnover rates were found to be significantly lower in the ME compared to the other stages of the oestrous cycle (Rance and Barraclough, 1981). Indeed, a decrease in

the TIDA dopaminergic activity has been associated with the pre-ovulatory LH surge (Hiemke *et al,* 1985).

The incertohypothalamic dopaminergic tract consists of cell bodies in the thalamus (A11) which run to the ZI, where another dopaminergic cell group (A13) projects terminals to the PVN and anterior hypothalamus. A third group of incertohypothalamic dopaminergic cells in the PeVN (A14) sends terminals to the preoptic area (Bjorklund *et al,* 1975; Chan-Palay, 1984). Synaptic contacts between dopaminergic and GnRH neurons in the MPOA have been demonstrated (Leranth *et al,* 1988a). Several studies have suggested that DA may have stimulatory effects in the control of LH secretion in these brain areas (Kawakami *et al,* 1975; MacKenzie *et al,* 1988).

Receptors for DA have been characterised and divided into two main subtypes, DA_1 and DA_2, based on their ability to stimulate adenylate cyclase activity (Dumbrille-Ross *et al,* 1985). A DA_3 receptor subtype has also been identified in the midbrain; however, its function in controlling GnRH release is unknown. Dopaminergic receptors have been visualised in the hypothalamus (Leibowitz *et al,* 1982). The ME and ARN appear to contain a moderate density of these receptors, while the SCN and MPOA are sparsely populated. It has been found that DA_1 receptors stimulate (Stoof and Kebabian, 1984) whereas DA_2 receptors inhibit (Onali *et al,* 1985) adenylate cyclase activity. Indeed, the stimulatory effect of DA on GnRH release in the ZI has been observed to be exerted via DA_1 but not DA_2 post-synaptic receptors (James *et al,* 1987). More recent evidence has emerged to favour this view that DA directly stimulates GnRH release via DA_1 receptors *in vitro* (Escalara *et al,* 1992). Hence, the nature of the receptor subtype involved may be another factor to explain the diverse actions of DA in the central control of LH release.

TH-containing immunoreactive cells are known to concentrate P (Fox *et al,* 1990) and E_2 (Sar, 1984) receptors in the hypothalamus, while neither E_2 (Shivers *et al,* 1983b) nor P (Fox *et al,* 1990) are found on the GnRH-immunoreactive neurons in the rat brain. E_2 and P can affect the quantity and enzymatic activity of TH in neurons of the ME (Wang and Porter, 1986). Furthermore, E_2+P treatment has a positive feedback effect on the TH neurons (Sanghera *et al,* 1991b), and the DA-producing neurons of the TIDA region are found to be targets for E_2 and P (Morrell *et al,* 1989). Thus, it appears that raising DA activity in the hypothalamus can

either stimulate or inhibit LH secretion depending on the endocrine state of animal. In the presence of steroids DA is excitatory (Vijayan, 1985) whilst being inhibitory in their absence (Sarkar and Fink, 1981) on GnRH release. However, opposite findings have also been reported. E_2 treatment may elevate the contents and turnover rates of DA in the hypothalamus, which in turn inhibits pulsatile GnRH release (Tadokoro *et al,* 1986).

There are also reports of interactions between the dopaminergic system and other modulatory systems in the control of LH release. β-endorphin axon terminals have been visualised to make contact with DA cell bodies and dendrites throughout the hypothalamus and lateral part of the ZI, suggesting that the dopaminergic system may mediate, at least in part, the inhibitory effects of opioids on gonadotrophin secretion (Horwarth *et al,* 1992a). Increased opioid peptidergic activity before the critical period of pro-oestrus is concomitant with enhanced hypothalamic DA release (Leadem and Kalra, 1985) that suppresses the pre-ovulatory LH surge and ovulation (Kalra and Kalra, 1985). Conversely, morphine, a μ-opioid receptor agonist, has been found to stimulate DA turnover in the hypothalamus (Gopalan *et al,* 1989a). Furthermore, activity of the TIDA dopaminergic neurons is increased following the activation of δ-opioid receptors (Manzanares *et al,* 1993). However, there are opposite findings in the literature as well indicating that activation of both μ- and κ-opioid receptors inhibits DA release from the MBH slices and β-endorphin decreases the turnover rate of hypothalamic DA in the TIDA tract (Wilkes and Yen, 1980; van Loon *et al,* 1980; Heijna *et al,* 1991). DA neurons have also been shown to impinge on GABAergic neurons (Leranth *et al,* 1988b) which may operate as interneurons in the control of GnRH secretion. These reports thus provide evidence for an indirect action of DA in LH release.

Dopaminergic activity in the hypothalamus varies during the oestrous cycle (Honma and Wuttke, 1980; Osterberg *et al,* 1987). DA may be stimulatory in the MPOA and SCN (Kawakami *et al,* 1975) and the ZI (MacKenzie *et al,* 1988) but inhibitory in the ARN and ME (He *et al,* 1994) to GnRH release. It would appear that both a decrease in inhibitory dopaminergic activity and an increase in its stimulatory activity contribute to the stimulation of the LH release. However, the central dopaminergic transmission is probably not the crucial neural signal in initiation of the pre-ovulatory LH surge under physiological conditions.

Serotonin

The hypothalamus is extensively innervated by serotonergic projections from the midbrain raphe nuclei (Steinbusch, 1984). Serotonergic fibres have been visualised in the MPOA, MBH, SON, PVN, and all layers of the ME (Sawchenko *et al,* 1983; Steinbusch, 1984). Their anatomical distribution overlaps with GnRH perikarya and processes in the MPOA, MBH, OVLT and septo-preoptic region (Jennes *et al,* 1982; Kiss and Halasz, 1985). This spatial correlation between 5-HT and GnRH fibres also exists in the ME (See Figures 1.13. and 1.14. for serotonergic pathways and biosynthesis, respectively).

Serotonergic receptors are divided into two major classes; 5-HT_1 and 5-HT_2. Additional receptor subtypes for 5-HT and further subdivisions have been documented based on binding affinity of selective agonists and antagonists (Frazer *et al,* 1990). Populations of 5-HT receptors have been demonstrated in the MPOA, ARN and ME in addition to several other hypothalamic areas (Biegon *et al,* 1982; Villar *et al,* 1984).

Both a stimulatory and inhibitory role for 5-HT in the regulation of GnRH and LH release have been reported (For review see Vitale and Chiocchio, 1993; Kordon *et al,* 1994). Icv infusion of 5-HT into female rats inhibits GnRH release (Schneider and McCann, 1970). Activation of the medial raphe nucleus (MRN) blocks the LH surge and ovulation (Morello and Taleisnik, 1985). On the other hand, there is a bulk of evidence suggesting that 5-HT is excitatory to GnRH release (Walker, 1983; James *et al,* 1989; Dow *et al,* 1994). Administration of a 5-HT agonist (quipazine) stimulates LH secretion. Accordingly, depletion of hypothalamic 5-HT content or impairment of the serotonergic transmission abolishes the LH surge (Walker, 1980). *In vitro*, 5-HT stimulates GnRH release from the ME fragments (Vitale *et al,* 1986). The hypothalamic serotonergic content fluctuates during the oestrous cycle (Honma and Wuttke, 1980) and an increase in its activity occurs in association with the pre-ovulatory LH surge on pro-oestrus (Vitale *et al,* 1987; Kerdelhue *et al,* 1989). Furthermore, 5-HT binding sites increase in the hypothalamus on the day of pro-oestrus; the increases appear to be modulated by E_2 (Williams and Uphouse, 1989).

This dual effect of the serotonergic system on the release of GnRH could correspond to different distribution and action of the two raphe nuclei. The dorsal

raphe nucleus (DRN) sends terminals to the PeVN, ARN and ME, whereas fibres from the MRN terminate in the MPOA and lateral hypothalamic area. Injection of a 5-HT agonist into the MRN blocks the LH surge and lesions of the DRN in the rat result in a decreased pro-oestrous LH surge and blockade of ovulation (Morello and Taleisnik, 1985; Morello *et al,* 1992). These experimental data thus suggest that DRN-ME 5-HT projections may exert a facilitatory influence while MRN is inhibitory to the LH surge.

Administration of steroids can alter 5-HT synthesis, turnover and release (Johnson and Crowley, 1986). This may provide explanation for the different effects of 5-HT in the regulation of LH release. Steroid treatment increases 5-HT synthesis in the MPOA (King *et al,* 1986). In ovx rats, 5-HT has a negative influence on LH release, which is converted to a stimulatory effect by pre-treatment of the animals with E_2. Electrical stimulation of the DRN in ovx rats inhibits LH release, however, the same stimulus elevates plasma LH levels in the presence of E_2 (Kitts and Johnson, 1986). *In vitro,* P enhancement of the E_2-induced LH surge in ovx rats can be blocked by 5-HT antagonists indicating that their excitatory action on the GnRH neurons is expressed by 5-HT (King and Kang, 1988). Thus, it would appear that 5-HT facilitates the LH surge in the presence of the gonadal steroids.

Another important aspect of steroid modulated-serotonergic neurotransmission is the density of brain 5-HT receptors. The positive feedback effect of the ovarian steroids on the serotonergic system appears to involve 5-HT_2, not 5-HT_1 receptors (Johnson and Kitts, 1991). Stimulatory actions of 5-HT on GnRH release have been attributed to the activation of 5-HT_2 receptor subtypes (Lenahan *et al,* 1987; Tanaka *et al,* 1993; Dow *et al,* 1994). Hormone-induced alterations of 5-HT binding sites may be one of the factors that modulate the final effect of 5-HT on the LH secretory mechanism.

5-HT metabolism may also influence other neurotransmitter systems which are known to participate in the neuroendocrine regulation of LH secretion. Serotonergic innervation of the TIDA dopaminergic neurons in the ZI and ARN has been reported (Bosler *et al,* 1984; Willoughby and Blessing, 1987). The serotonergic mediation of LH secretion may depend, at least partly, upon noradrenergic transmission (Gopalan *et al,* 1989b). Effects of opioid peptidergic system on the pre-ovulatory LH surge may also involve an interaction with the serotonergic system (Gopalan *et al,* 1989b). Recent evidence has suggested that in

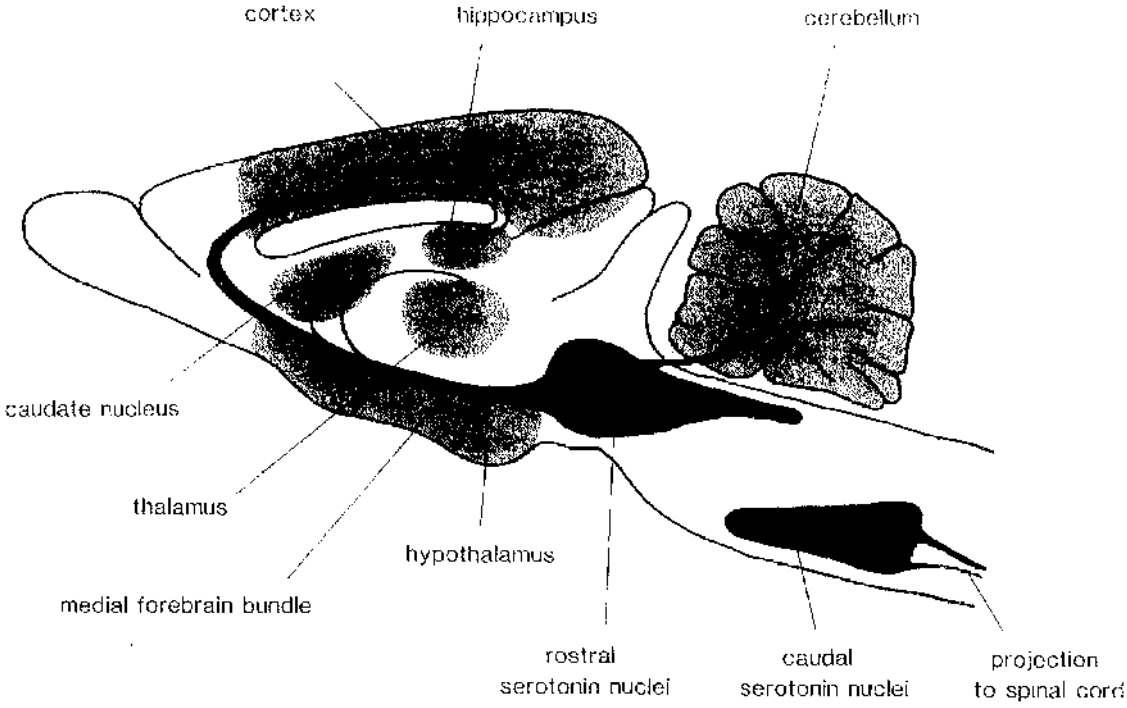


Figure 1.13. Schematic illustration of a saggital section through the rat brain showing the serotonergic projections to the hypothalamus. The main groups of 5-HT cell bodies and fibre tracts are shown in black. Grey areas represent the location of axon terminals. Adapted from Rang and Dale, 1991.

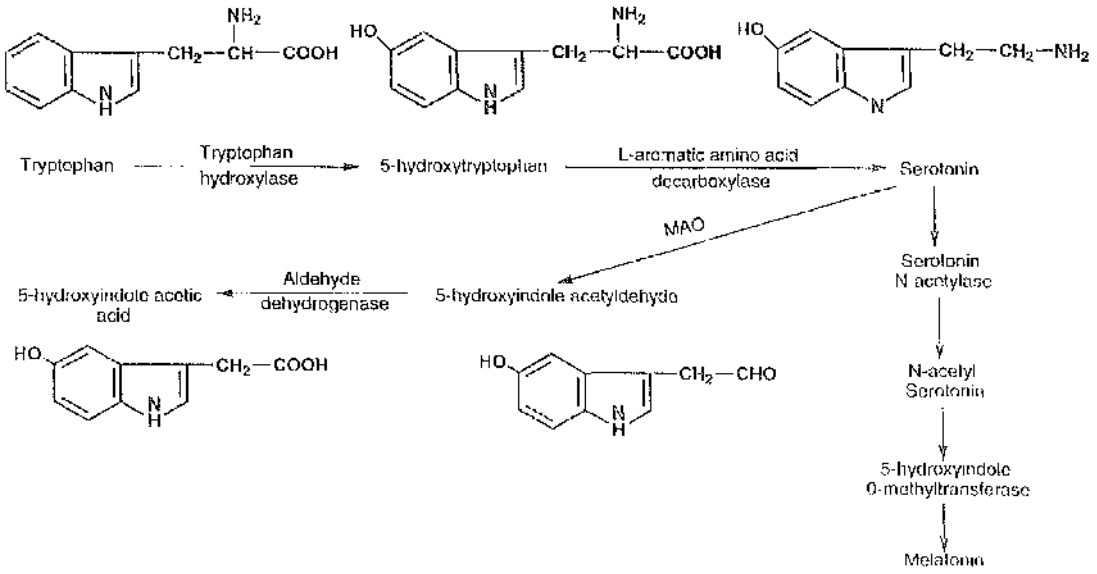


Figure 1.14. Metabolic pathways of the biosynthesis and breakdown of 5-HT. From Johnston, 1990.

the DRN, EOPs may pre-synaptically inhibit 5-HT release (Pinnock, 1992). Inhibitory effects of 5-HT on LH release have been reported to be mediated by GABAergic neurons (Morello *et al,* 1989). It thus appears that effects of 5-HT may be either direct or indirect on GnRH release.

The existence of anatomically separate excitatory and inhibitory serotonergic systems (Morello *et al,* 1992), temporal factors (Walker 1983), steroid hormone milieu (Johnson and Crowley, 1986) and interactions with other modulatory systems may all contribute to determining the direction of 5-HT influence on LH release.

Adrenaline

Involvement of ADR as a neurotransmitter in the central regulation of LH release has been proposed. Icv administration of exogenous ADR stimulates GnRH and LH release (Swartz and Moberg, 1986). Inhibitors of PNMT have been shown to suppress the pre-ovulatory or the steroid-induced LH surge in ovx and pro-oestrous rats (Crowley and Terry, 1981; Coen and Gallo, 1986). Moreover, a decrease in the hypothalamic ADR content was observed concomitantly with the abolition of the LH surge in the same animals. An increase in the hypothalamic ADR turnover on the afternoon of pro-oestrus has been reported (Coen *et al,* 1985). ADR has also been described as the most potent catecholamine in stimulating the LH release (Rubinstein and Sawyer, 1970). Thus, these findings would indicate an essential excitatory neurotransmitter role for ADR in the central control of LH release (Kalra and Kalra, 1985).

The presence of adrenergic nerve terminals in the hypothalamus has been demonstrated (Hokfelt *et al,* 1977). These projections are of extrahypothalamic origin and arise from adrenergic cell bodies in the brainstem. Reportedly, the hypothalamus contains the highest content of ADR in the brain (Hokfelt *et al,* 1984). However, the existence of ADR as a classical neurotransmitter in the rat brain has been questioned. It has been claimed that nerve terminals containing all the enzymes required for ADR biosynthesis are not necessarily adrenergic (Sved, 1989). ADR may be synthesised by other cells in the hypothalamus and then taken up into catecholamine nerve terminals. Indeed, there is evidence to favour the view that this catecholamine may be a co-neurotransmitter with NA in the noradrenergic nerve terminals or a postsynaptic metabolite of NA catabolism

(Mefford, 1987). Therefore, the effects of adrenergic agonists and antagonists on GnRH release could be a result of activating the adrenoreceptors on the noradrenergic system or by altering the NA metabolism itself.

Histamine

Histamine (HA) has been suggested as a neurotransmitter in the mammalian CNS (Schwartz *et al*, 1991). The brain histaminergic system has been implicated in the neuroendocrine regulation of LH secretion (Knigge and Warberg, 1991). The modulatory actions of HA are mediated through pharmacologically well-characterised H_1 and H_2 receptors and H_3 autoreceptors.

HA is found in high concentrations in the hypothalamus with the highest levels observed in the preoptic region, SCN, ME and ARN. HA-containing nerve terminals have also been visualised in close proximity to GnRH-producing cells or nerve terminals (Panula *et al*, 1989). Increases in plasma LH levels have been reported following icv administration of HA to ovx, steroid-primed (Libertun and McCann, 1976) and pro-oestrous rats (Donoso, 1978). HA stimulates GnRH release from the steroid-treated rat MBH *in vitro* (Jorgenson *et al*, 1989). Furthermore, the histaminergic system is involved in elevation of LH release in response to ovarian steroids (Moguilevsky *et al*, 1989). Conversely, E_2 activates the release of both HA and NA, which results in the amplification of GnRH secretion. However, the HA action appears to be independent from that of NA (Ohtsuka *et al*, 1989). The pharmacological evidence outlined above seems to indicate that HA may have a facilitatory role in the induction of LH release. However, the modulatory role of HA in the physiological regulation of the LH surge is still unclear and awaits further investigations for a convincing argument to be made.

Acetylcholine

ACh receptors are divided into two main classes; muscarinic and nicotinic. The population of ACh muscarinic receptors fluctuates in the MPOA throughout the oestrous cycle, being highest at pro-oestrus when sexual behaviour normally occurs, and rats mate (Olsen *et al*, 1988). Administration of a cholinergic muscarine agonist, bethanechol, increases the apparent number of E_2 binding sites in a dose-dependent manner in the whole hypothalamus of the female rat, but not in

the male (Lauber and Whalen, 1988). Furthermore, in an *in vitro* study P has been found to inhibit hypothalamic and pituitary muscarinic receptors, but oestrogenic compounds do not (Klangkalya and Chan, 1988). Thus, the muscarinic receptors may be involved in the induction of E_2 receptors in the hypothalamus.

The existence of ACh nicotinic receptors in the brain has been shown. Exposure of dioestrous rats to acute intermittent nicotine results in a dose- and time-dependent lowering of plasma LH levels (Anderson *et al,* 1988). However, very little is known about the neuroendocrine function of the nicotinic receptors.

The hypothalamus receives cholinergic projections from cell bodies located in the other brain areas (Hokfelt *et al,* 1977; Hokfelt *et al,* 1984). An important mechanism of cholinergic system in regulating GnRH secretion has been suggested. Administration of atropine (a muscarinic receptor antagonist) blocks GnRH-induced LH release (Casanueva *et al,* 1986). However, a modulatory function of ACh on the GnRH system has not yet been established. Sexual behaviour in female rats is influenced by an E_2-mediated cholinergic mechanism in which the MPOA and VMH appear to be involved in ACh regulation of lordosis (Dohanich and Clemens, 1981). Thus although ACh may be an important neurotransmitter in control of oestrous behaviour, but it may have little or no involvement in the central control of the pre-ovulatory LH surge.

2) Amino acid neurotransmitters

γ-Aminobutyric Acid

GABA has been known to act on the CNS to influence GnRH/LH release (Kordon *et al,* 1994).

GABA and the rate-limiting enzyme for its synthesis, glutamate decarboxylase (GAD), are found in high concentrations in the hypothalamus (Vincent *et al,* 1982). GABAergic neurons are extensively distributed throughout the hypothalamo-pituitary system (Sakaue *et al,* 1988). A dense plexus of GAD-containing nerve terminals in the external layer of the ME has been shown (Vincent *et al,* 1982). Direct connections have been visualised in the MPOA between GABA-immunoreactive axons and GnRH-containing neurons (Leranth *et al,* 1988b).

Furthermore, the hypothalamic GABAergic neurons have been found to contain E_2 receptors (Flugge *et al,* 1986). The rich GABAergic innervation of the hypothalamus is consistent with the belief that GABA may be involved in the neural circuitry which controls LH secretion.

Receptors for GABA are divided into two subtypes; $GABA_A$ and $GABA_B$ receptors, which mediate both pre- and post-synaptic actions of GABA in the CNS (Akema and Kimura, 1993).

Central GABAergic neurotransmission exerts an inhibitory influence on the GnRH neurons which in turn results in the suppression of LH release (Kordon *et al,* 1994). Pharmacologically-induced increase of endogenous GABA levels in the hypothalamus inhibits LH release in ovx rats (Donoso, 1988). Infusion of either GABA or the $GABA_A$ receptor agonist, muscimol, in regions containing GnRH neurons suppresses the pulsatile release of LH in ovx rats (Fuchs *et al,* 1984; Herbison and Dyer, 1991). Similarly, muscimol and the $GABA_B$ agonist, baclofen, have been shown to block the surge release of LH in ovx and steroid-primed rats (Donoso, 1988; Adler and Crowley, 1986). Activation of $GABA_A$ but not $GABA_B$ receptors has been seen to cause a rapid decrease in GnRH gene expression in the preoptic and anterior hypothalamic area concomitant with the inhibition of LH secretion (Leonhardt *et al,* 1995). Furthermore, release rates of GABA in the preoptic-anterior hypothalamic area are markedly reduced prior to the E_2-induced LH surge in ovx rats (Jarry *et al,* 1988). Thus, this spontaneous decrease in hypothalamic GABAergic activity may transiently relieve the GnRH neurons from a strong inhibitory action of GABA and may lead to pulsatile release of GnRH. Indeed, decreased GABA activity in the MPOA, MBH and LC have been found to precede an E_2-induced LH surge in ovx rats (Seltzer and Donoso, 1992).

In addition to the direct effects of GABA on GnRH release, central GABAergic neurons may interact with the other neuromodulator systems. Stimulation of A1 noradrenergic input projecting to the MPOA results in GABA release in ovx rats when E_2 levels are relatively low, but not when they are high (Herbison *et al,* 1989). E_2 has a determining role on this interaction via E_2 receptors on the GABA neurons (Flugge *et al,* 1986). Thus, it appears that GABAergic neurons in the MPOA may function as inhibitory interneurons between noradrenergic terminals and GnRH neurons. Furthermore, expression of the triggering neural signal of

NA for the pre-ovulatory LH discharge has been attributed to a reduction in the hypothalamic GABAergic activity (Jarry *et al,* 1991). GABAergic modulation of the noradrenergic system has also been shown (Diez-Guerra *et al,* 1987; Horwarth *et al,* 1992b). Activation of GABA neurons with muscimol and baclofen causes a fall in NA turnover in the MPOA and MBH in association with a sustained decrease in serum LH levels (Fuchs *et al,* 1984; Demling *et al,* 1985; Adler and Crowley, 1986). Microinjection of the GABA transaminase inhibitor into the LC and MPOA decreases plasma LH levels, suggesting that GABA may affect the noradrenergic function in discrete brain areas (Donoso, 1988). Furthermore, GABA may modulate GnRH neuronal responsiveness to NA and its withdrawal at the time of the LH surge would allow a greater pre-synaptic release of NA (Barraclough, 1994).

An interaction between GABA and DA systems in the hypothalamus has also been reported. In the ZI, GABA suppresses the stimulatory action of the incertohypothalamic DA tract upon GnRH neurons, which in turn inhibits LH secretion (Wilson *et al,* 1990). Icv infusion of muscimol decreases the DA turnover in the MPOA, MBH and anterior hypothalamic area (Fuchs *et al,* 1984). Surprisingly, a stimulatory neurotransmitter role has been attributed to GABA on GnRH release both *in vivo* (Kalra and Kalra, 1985; Masotto and Negro-Vilar, 1986) and *in vitro* (Hales *et al,* 1994). GABA may have a stimulatory effect on GnRH release in the ARN and ME (Masotto and Negro-Vilar, 1986). This is believed to be a consequence of its negative modulatory action on the activity of the inhibitory dopaminergic system in these hypothalamic regions (Nikolarakis *et al,* 1988). Furthermore, GABAergic inhibition of the β-endorphin neurons in the MPOA may contribute to its stimulatory action by eliminating the opioidergic suppression of GnRH release (Horwath *et al,* 1992b). Interactions between GABA action and the serotonergic system has also been reported to influence LH release (Morello *et al,* 1989).

These inhibitory effects of GABA on the steroid-induced LH surge are exerted via $GABA_A$ and $GABA_B$ receptors (Wilson *et al,* 1990; Akema and Kimura, 1993). $GABA_B$ receptors are further implicated in the neural suppression of the responsiveness of the GnRH system to NA (Hartman *et al,* 1990). On the other hand, stimulatory GABA action, if any, is mediated only by $GABA_A$ receptor types (Nikolarakis *et al,* 1988).

In summary, GABA is generally believed to be an inhibitory neurotransmitter in the CNS acting either directly or indirectly on GnRH neurons.

Excitatory Amino Acids

Recently, excitatory amino acid (EAA) neurotransmission has been suggested as an essential component of the neuroendocrine regulation of the LH release from the anterior pituitary (Bourguignon *et al,* 1989; Brann and Mahesh, 1994). The mediation of EAAs neurotransmission in the CNS is achieved primarily by glutamate and aspartate. However, cysteic and quinolinic acid may also function as excitatory neurotransmitters. Glutamate, aspartate and cysteic acid have been shown to increase LH secretion following icv or systemic administration (Ondo *et al,* 1976; Scheibel *et al,* 1980), respectively. Quinolinic acid has also been suggested to stimulate LH release through a 5-HT-dependent mechanism (Johnson and Crowley, 1986). The postulated stimulatory role of the EAAs on LH release has recently been reinforced by *in vitro* studies (Brann and Mahesh, 1991).

The site of action of the EAAs in regulating gonadotropin secretion resides at a suprapituitary level (Arslan *et al,* 1988). Immunocytochemical studies have revealed the presence of the EAAs, such as glutamate and aspartate, in large concentrations in several hypothalamic nuclei, including the MPOA, ME, ARN and SCN (van den Pol *et al,* 1990). Furthermore, glutamate-immunoreactive terminals have been shown to form synapses with GnRH neurons in the lamina terminalis and the ARN.

Receptors for EAAs are classified in two major classes; NMDA and non-NMDA receptors (Brann and Mahesh, 1991), and the latter is further subdivided into kainate and quisqualate receptors (Watkins and Olverman, 1987). These receptors types have been identified in various hypothalamic regions (Cotman *et al,* 1987).

Infusion of a specific agonist, NMDA into the MPOA stimulates LH release (Ondo *et al,* 1988; Pohl *et al,* 1989). Accordingly, NMDA receptor antagonists prevent the steroid-induced LH surge in ovx rats (Urbanski and Ojeda, 1990). In addition to the NMDA, kainate receptor agonists have also been demonstrated to enhance the release of LH in steroid-treated ovx rats (Brann and Mahesh, 1991). NMDA, kainate and glutamate have also been reported to elicit GnRH release from hypothalamic fragments incubated *in vitro* (Bourguignon *et al,* 1989; Lopez *et al,*

1992). This stimulation of GnRH secretion is enhanced by the E_2+P pre-treatment of ovx rats (Carbone *et al,* 1992) which indicates that ovarian steroids potentiate the GnRH response to NMDA. Indeed, the stimulatory influence of the EAAs on both GnRH and LH release appear to be dependent on the steroid milieu, since NMDA inhibited the LH release in ovx animals while it potently enhanced the LH secretion in pro-oestrous rats (Brann and Mahesh, 1991).

The major site of NMDA action on GnRH release appears to be the MPOA where the GnRH cell bodies reside whereas glutamate and kainate act primarily at the ARN-ME which are the sites of the GnRH nerve terminals (Brann and Mahesh, 1994). Interestingly, NMDA has been found to facilitate sexual receptivity through stimulating GnRH release (Hsu *et al,* 1993).

In addition to the direct effects of the EAAs, there may be interactions with the other neuromodulator systems as well. The EAAs act upon the noradrenergic neurons in the brainstem to influence hypothalamic GnRH release (Blandina *et al,* 1992). Conversely, noradrenergic modulation of the amino acid neurotransmission in GnRH and LH release has also been shown (Barraclough and Wise, 1982). Recent work by Rettori *et al* (1993) has suggested that NO is an important mediator of glutamate stimulation in GnRH release *in vitro*.

Although EAAs neurotransmission in the CNS has been suggested as an important stimulatory component in the regulation of GnRH release and the pre-ovulatory LH surge, their involvement in this mechanism has not yet been fully assessed.

3) Neuropeptides

Endogenous Opioid Peptides

A large number of studies have indicated a neuromodulator role for opioid peptidergic mechanisms in the central control of gonadotrophin release.

The opium alkaloid, morphine, was first isolated from the poppy, *Papaver somniferum*, by Serturner in 1803 and has long been known to have widespread effects on many physiological systems (Bernard, 1864). There had long been convincing evidence that the administration of morphine during the critical period on the day of pro-oestrus could block the pre-ovulatory LH surge and ovulation in

rats (Barraclough and Sawyer, 1955). However, it was not until 1971 that a stereospecific binding site for morphine in the brain was identified (Goldstein *et al,* 1971). Two years later opioid receptors in the CNS were discovered (Pert and Snyder, 1973). The existence of an endogenous substance in the brain, acting as an agonist at opioid receptor sites, was then described (Hughes *et al,* 1975). The isolation and characterisation of the first EOPs, the leu- and met-enkephalins was reported by Hughes *et al* (1975). EOPs have since been extensively studied. Three major classes of EOPs have now been defined; the endorphins, enkephalins and dynorphin/neo-endorphins. These opioid peptides are derived from three different precursors; POMC, pro-enkephalin A and pro-enkephalin B or pro-dynorphin, respectively (Hollt, 1983). The major opioid product of POMC processing is β-endorphin. Several other less common EOPs have been identified in the brain (for review see Palkovits, 1988; Bonavera *et al,* 1994).

There have been numerous investigations into the opioid receptors and their widespread distribution throughout the brain (see Table 1.1.). Activation of these receptors generally results in an inhibition of cellular activity and a corresponding decrease in neurotransmitter release (Schoffelmeer *et al,* 1992). Three major types of opioid receptors, mu (μ), kappa (κ) and delta (δ) have been pharmacologically characterised by radioligand binding and functional analyses (Leslie, 1987; Mansour *et al,* 1988). Recent cloning studies have confirmed the presence of these three receptor subtypes, which are structurally homologous but encoded by separate genes (Chen *et al,* 1993; Yasuda *et al,* 1993). Two other opioid receptor subtypes, sigma (σ) and epsilon (ε), have also been postulated (Hollt, 1983; Su *et al,* 1986; Holtzman, 1989). There is also further support for additional subdivisions of the μ- and κ-opioid receptor classes (Dougall, 1988) on the basis of the ability of various μ- and κ-agonists and antagonists to bind with high affinity to these receptors.

The μ-opioid receptors are characterised as having high affinity for morphine-like drugs and for several EOPs, especially β-endorphin (Leslie, 1987). The μ-opioid receptors are widely distributed in the brain and high numbers of the μ receptor sites have been visualised in the hypothalamus, including the MPOA, SCN, ME, AHA and ARN (Loughlin *et al,* 1995). Several raphe nuclei, the midbrain dopaminergic cells and the LC noradrenergic neurons have also been shown to contain moderate levels of μ-opioid receptor subtypes (Thompson *et al,* 1993; Loughlin *et al,* 1995). The κ-opioid receptors are characterised as those sites

which have high affinity for benzomorphan drugs such as U-50488H and U-69593 (Vonvoigtlander *et al,* 1983) and for certain endogenous opioids, particularly dynorphin A (1-17). High numbers of κ sites have been found in many hypothalamic regions, including the ME, lateral hypothalamic area, MPOA, DMN, PeVN, ARN and SCN and some also in the raphe nuclei, the brainstem and several midbrain structures (Hoffman *et al,* 1989; Loughlin *et al,* 1995). The δ-opioid receptors are characterised as having high affinity for the endogenous opioid peptide, enkephalin (Leslie, 1987) and enkephalin analogues like DPDPE (Mosberg *et al,* 1983). The δ-opioid receptors have a more restricted distribution than those of μ- and κ-opioid receptors. The density of δ-opioid binding has usually been described as being low to moderate throughout the rat hypothalamus and the highest concentrations of these receptors are located in the SCN, ARN and MPOA (Mansour *et al,* 1988; Desjardins *et al,* 1990). Dense numbers of δ-opioid receptors have been found in several other brain areas including the brain stem and the midbrain. The distribution of the δ-opioid receptors correlates well with that of enkephalin-containing axon terminals (Desjardins *et al,* 1990).

The σ-receptor subtype was originally defined by Martin *et al* (1976), based on the unique effects of SKF 10,047, the prototypical agonist for the σ-receptors. However, the opioid nature of these receptor subtypes has been brought into question as cocaine and pharmacologically-related compounds also display affinities for the σ-opioid subtypes (Sharkey *et al,* 1988). Furthermore, a non-opioid σ-receptor has been found in the guinea-pig myenteric plexus (Roman *et al,* 1988). However, more recent evidence has shown that the σ-receptor exists and is distinct from the non-opioid σ-receptor (Holtzman, 1989). These σ-opioid receptors have been visualised by autoradiographic localisation studies in high density in the hypothalamus (Largent *et al,* 1986). Although they have been originally classified as a subtype of opioid receptors, many *in vivo* effects of SKF 10,047 are not blocked by the opioid antagonists, naloxone and naltrexone (Katz *et al,* 1985).

EOPs are widely distributed throughout the CNS, particularly in the limbic system. There are at least three separate opioid neuronal networks in the brain; an enkephalin family, a β-endorphin family and a dynorphin family (Watson *et al,* 1982). High concentrations of β-endorphin and leu- and met-enkephalin have been demonstrated in the ME, SCN, MPOA, ARN and PVN as well as in many other hypothalamic regions and extrahypothalamic sites. In the SCN and MPOA,

the enkephalins have been found to be contained in the perikarya of opioid peptidergic neurons (Hokfelt *et al,* 1977). In the ME, the enkephalins (Watson *et al,* 1982; Williams and Dockray, 1983) and β-endorphin (Mezey *et al,* 1985) are present only in fibres. Enkephalin-containing perikarya and fibres (Hokfelt *et al,* 1977; Watson *et al,* 1982; Williams and Dockray, 1983) and β-endorphin-containing cells (Mezey *et al,* 1985; Jirikowski *et al,* 1986) have been visualised in the ARN. Dense concentrations of dynorphin cells and fibres are present in the ARN and corresponding projections innervate the ME (Fallon and Leslie, 1986; Loughlin *et al,* 1995). Dynorphin-containing cells and fibres are also found in the lateral hypothalamic area, preoptic area, SCN, PeVN, DMN, VMN along with some other hypothalamic and extrahypothalamic areas, including the raphe nuclei, dopaminergic midbrain structures and the brainstem (Hokfelt *et al,* 1977; Loughlin *et al,* 1995). In addition to these well known EOPs, some other less common EOPs have also been demonstrated in several parts of the hypothalamus (for review see Palkovits, 1988). Furthermore, there is also evidence for the co-existence of two or more endogenous peptides within the same neuronal perikarya or even in the same secretory granule (Chan-Palay *et al,* 1984; Griffond *et al,* 1993). Indeed, β-endorphin, ACTH and α-MSH have been shown to be all present in the same population of ARN neurons (Mezey *et al,* 1985).

Opioid peptides depress spontaneous neuronal activity and hence neuronal transmission by direct action on the neuron and the underlying basis for this effect is a naloxone-reversible hyperpolarisation of the cell membrane (Henderson, 1983). For ligands of μ-, κ- and δ-opioid receptors, the inhibitory hyperpolarisation results from activation of a calcium-sensitive potassium conductance (Williams *et al,* 1982; Grudt and Williams, 1995; Lagrange *et al,* 1995). This inhibitory opioid influence appears to involve activation of potassium conductance, a decrease in calcium conductance and inhibition of adenylyl cyclase activity (Grudt and Williams, 1995). The reduction of both spontaneous electrical activity and the amount of neurotransmitter released is a common feature of opioid action (Grossman and Dyer, 1989).

β-endorphin is one of the most important and biologically active endogenous peptides in the CNS. It has an important role in the control of the anterior pituitary function, particularly in the stimulation of prolactin and the inhibition of LH release (Van Vugt *et al,* 1982; Genazzani *et al,* 1990). β-endorphin inhibits GnRH secretion by preventing its release from the ME (Bicknell, 1985; Bonavera *et al,*

1994). It is generally agreed that β-endorphin, as well as other EOPs, act at the hypothalamic level and have no direct effect at the pituitary level, on LH secretion (Parnet *et al,* 1990). Indeed, the anterior pituitary has been reported to be relatively poor in opioid receptors (Khachaturian *et al,* 1985). It is therefore most likely that the endogenous opioid control of LH release is caused by actions and/or interactions within the brain; in particular, by modulating the secretion of GnRH into the hypophyseal portal system.

EOPs have a profound inhibitory influence on GnRH and LH release (Kalra, 1993; Barraclough, 1994; Kordon *et al,* 1994). There is considerable overlap in the distribution of EOPs and GnRH innervation in the preoptic-tuberal pathway (Witkin *et al,* 1982) and direct synaptic connections between opioid peptidergic and GnRH neurons in the MPOA have been shown (Leranth *et al,* 1988a). Acute and chronic administration of morphine suppresses the release of GnRH and therefore blocks ovulation (Barraclough and Sawyer, 1955; Mehmanesh *et al,* 1988). Similarly, both morphine and β-endorphin can block the pre-ovulatory LH surge and hence ovulation when administered on the day of pro-oestrus (Leadem and Kalra, 1985). Infusion of antisera to β-endorphin or to dynorphin into the third ventricle elevates plasma LH levels (Schultz *et al,* 1981). Studies by several groups have indicated that naloxone is able to overcome the suppressive opioidergic influence on GnRH secretion and its administration is associated with GnRH release in rats (Piva *et al,* 1985; Allen and Kalra, 1986; Brown *et al,* 1994). Furthermore, chronic morphine treatment brings about a supersensitivity to naloxone (Cicero *et al,* 1983). The administration of naloxone on the morning of pro-oestrus advances the LH surge by restraining the inhibitory opioid action (Allen and Kalra, 1986). In view of these results it seems that a decrease in the hypothalamic opioid peptidergic tone before the critical period on the day of pro-oestrus may be the neural stimulus to initiate the pre-ovulatory LH surge.

β-endorphin concentrations in the hypophyseal portal blood vary during different stages of the oestrous cycle (Sarkar and Yen, 1985). In the hypothalamus largest variations in the concentration of β-endorphin occurs in the ME, and fluctuations are also evident in the MPOA and ARN. The plasma levels of β-endorphin have been found to be minimal on the morning and afternoon of pro-oestrus (Parnet *et al,* 1990). However, it has also been suggested that β-endorphin levels are high on the morning whereas the lowest concentration is seen on the late afternoon of

the pro-oestrus (Sarkar and Yen, 1985). Furthermore, the hypothalamic β-endorphin content fluctuates with a diurnal rhythm, showing a significant nocturnal increase that is abolished by the suppression of the dark phase of the day-night cycle (Genazzani *et al,* 1987). The circadian changes in the hypothalamic β-endorphin content are dependent upon the steroid hormone status of animals, since they are elevated by gonadectomy and restored following chronic E_2 replacement (Genazzani *et al,* 1990). Thus the ovarian steroids appear to exert a priming effect on this endogenous neuronal rhythm.

There is ample evidence to show that ovarian steroids are potentially important in modulating the output of the EOPs (Kalra, 1993). A considerable number of β-endorphin, enkephalin and dynorphin-immunoreactive neurons in the hypothalamus have been demonstrated to contain E_2 and P receptors (Jirikowski *et al,* 1986; Fox *et al,* 1990). Specific implantation of E_2 in the MPOA of dioestrous rats increases β-endorphin levels in the ARN-ME axis (Sturzebecher *et al,* 1988). During chronic exposure to opioids, E_2 administration causes an exaggerated pro-oestrous-like LH surge in E_2 plus P-treated ovx rats (Gabriel *et al,* 1986). E_2 has a time-specific inhibition on β-endorphin levels on the afternoon of pro-oestrus (Lustig *et al,* 1988). Indeed, the hypothalamic opioidergic tone decreases in response to increased circulating E_2 levels on the afternoon of pro-oestrus. These modulatory effects of steroids on β-endorphin release may be dependent on the amount and the duration of exposure to the steroids rather than on their simple presence or absence. Moreover, steroid hormones may activate LH release by reducing the responsiveness of the LH secretory mechanism to the tonic inhibitory influence of EOPs (Berglund *et al,* 1988). More recent studies have indicated that POMC gene expression changes significantly during the oestrous cycle as well. Increased POMC mRNA levels are sustained until 10.00h on pro-oestrus, thereafter a steady decrease occurs, reaching nadir levels by 18.00h (Wise *et al,* 1990). The regulation of the POMC gene expression is steroid-dependent because ovariectomy abolishes and E_2 plus P treatment reconstitutes the diminution in mRNA levels (Wise *et al,* 1990; Bohler *et al,* 1991). Ovarian steroids also up-regulate pro-enkephalin and pro-dynorphin gene expression and immunoreactivity (Langub and Watson, 1992). Thus, it would appear that opioid peptidergic neurons are targets for steroid hormones.

The effects of EOPs on GnRH and LH release have been extensively investigated by using selective opioid agonists and antagonists in order to reveal the nature of

opioid receptor subtypes involvement in the control of the pre-ovulatory LH surge and ovulation. Activation of μ-opioid subtypes inhibits GnRH neuronal activity and thereby its release *in vitro* (Lagrange *et al,* 1995). Administration of a long-acting μ-opioid agonist, Duromorph, suppresses the LH surge (Brown *et al,* 1994). Both κ- and μ- receptor agonists tonically inhibit LH secretion in a dose-related manner following central infusion (Pfeiffer *et al,* 1987). The latter effect can, however, be blocked by a highly specific μ-receptor antagonist β-funaltrexamine, which suggests that opioid suppression of LH release is predominantly mediated via μ-receptors. The number of opioid receptors within the brain is not constant throughout the oestrous cycle. In the whole brain of cycling rats the density of μ-opioid receptors significantly increases on the morning of pro-oestrus (Limonta *et al,* 1989). This is followed by a progressive decline in the number of μ-receptors on the afternoon of the same day. Furthermore, the decrease in the μ-binding sites observed on the afternoon of pro-oestrus coincides with the beginning of the LH surge and may be linked to the increase in serum E_2 and P levels (Maggi *et al,* 1993). These observations thus provide an explanation why μ-opioid receptor antagonists, naloxone and β-funaltrexamine have failed to elevate LH secretion in long term ovx rats owing to the lack of a steroid milieu (Pfeiffer *et al,* 1987).

There is also evidence for the involvement of κ-opioid receptors in the opioid control of LH secretion. The highly specific κ-agonist, tifluadom is as effective as morphine in reducing plasma LH concentrations on the early afternoon of pro-oestrus (Gopalan *et al,* 1989a; Brown *et al,* 1994). In agreement with these observations, κ-agonists have also been seen to inhibit GnRH release *in vitro* (Leposavic *et al,* 1991). There are contradictory reports for the participation of σ- and δ-opioid receptors in the regulation of LH secretion. Only high doses of the σ-receptor agonist, N-allynormetazocine hydrochloride, have been found to induce a fall in serum LH levels (Brown *et al,* 1994). However, the significance of σ-opioid receptors in LH release is poorly understood because of the lack of specific ligands for these receptors and awaits further investigations. Selective δ-opioid receptor agonists have been shown to suppress GnRH and LH secretion on the day of pro-oestrus (Leadem and Yagenova, 1987). However, their involvement in this process has also been questioned since a specific δ-opioid antagonist fails to elevate plasma LH levels (Leposavic *et al,* 1991). Hence, it appears that multiple opioid receptors may be involved in regulating LH release (Pechnick *et al,* 1985).

Although EOPs may directly inhibit GnRH release by a presynaptic inhibitory action, there is a large volume of evidence indicating that EOPs may have indirect effects on GnRH secretion by interfering with the other neuromodulator systems (For review see Kalra, 1993; Barraclough, 1994). The modulatory actions of EOPs on GnRH and LH release appear to involve central monoaminergic transmission (Leadam *et al,* 1985; Nishihara *et al,* 1991). Immunocytochemical studies have illustrated a degree of overlap in the distribution of opioid peptidergic perikarya and NA neurons and terminals (Kordon *et al,* 1994). Moreover, noradrenergic terminals within the hypothalamus possess a mixed population of opioid receptors. Naloxone-induced LH secretion can be prevented by prior administration of α-adrenoreceptor antagonists (Van Vugt *et al,* 1981; Kalra and Simpkins, 1981; Al-Hamood *et al,* 1985) and dopamine-β-hydroxylase inhibitors (Kalra and Crowley, 1982). Naloxone has been shown to stimulate NA release within the hypothalamus (Nishihara *et al,* 1991). Furthermore, icv administration of α-adrenoreceptor agonists can enhance LH release in morphine-treated rats (Kalra and Gallo, 1983). Ionophoretically applied naloxone elevates the response of GnRH neurons in the MPOA to stimulation of the VNAB (Dyer *et al,* 1988). Activation of μ- and δ-opioid receptor subtypes inhibit NA release from slices of the MPOA (Diez-Guerra *et al,* 1987). Thus, it would appear that NA axon terminals in discrete hypothalamic areas may be presynaptically inhibited by EOPs and this interaction may be of importance in the regulation of the LH release.

Many of the central catecholamine cell groups have been shown to contain one or more peptides (Hokfelt *et al,* 1984). Coexistence of TH and met-enkephalin in a considerable proportion of the A1 NA cell group has also been reported (Ceccatelli *et al,* 1989). Therefore, these observations would point to the possibility that EOPs may exist as co-transmitters in the NA nerve terminals.

Pharmacological evidence has implicated a role for ADR in mediating the opioid peptidergic influence on GnRH and LH release since central administration of ADR synthesis inhibitors suppress naloxone-induced LH secretion (Kalra and Crowley, 1982).

In addition to the interaction between the NA and EOP systems, there is also evidence that the EOPs can alter the modulatory effects of the dopaminergic system on GnRH release. Systemic administration of morphine reduces the rate of synthesis and turnover of DA in the ME by inhibiting activity of the TIDA neurons

(Alper *et al,* 1980). Similarly, morphine reduces the DA content in the hypophyseal portal blood (Reymond *et al,* 1983). The TIDA neurons are tonically inhibited in diestrous rats by κ-, but not by a μ-opioid receptor mechanism (Manzaranes *et al,* 1992). *In vitro*, the stimulation of both κ- and μ-opioid receptors with their respective agonists results in an inhibition of evoked DA release from the MBH (Heijna *et al,* 1991). Furthermore, activation of α_2-adrenoreceptors inhibits DA release indirectly by elevating the release of EOPs which in turn reduce the evoked DA release via κ-opioid receptors (Werling *et al,* 1988; Schofelmeer *et al,* 1988). Naloxone by itself has been shown to stimulate DA and 3,4-dihydroxyphenylacetic acid (DOPAC) efflux from the TIDA dopaminergic neurons within the hypothalamus (Wilkes and Yen, 1980). The dopaminergic TIDA neurons are also believed to be responsible, at least in part, for the regulation of prolactin release. Inhibition of the TIDA DA turnover and release into the hypophyseal portal system results in a decrease in plasma LH levels (Van Loon *et al,* 1980). These effects also account for the prolactin-stimulating action of opioid peptides since a decrease in portal blood DA concentrations correlates well with morphine-induced prolactin secretion (Arita and Porter, 1984).

On the contrary, stimulatory actions of EOPs on the dopaminergic system have also been documented. Administration of morphine activates the incertohypothalamic dopaminergic neurons and increases the DA turnover (Gopalan *et al,* 1989a) and concentrations of DOPAC (Alper *et al,* 1980; Lookingland and Moore, 1985) concomitant with the inhibition of the LH release. These effects of morphine may be reversed by the opioid antagonists, naloxone and naltrexone. Furthermore, recent evidence has shown that morphine enhances DA neuronal activity within the incertohypothalamic DA neurons by increasing TH mRNA levels without affecting plasma LH levels (He *et al,* 1994; Barraclough, 1994). Morphine-induced increases in DA release and activity in the incertohypothalamic system seem somehow surprising since this pathway has been suggested to be stimulatory to GnRH and LH release as explained earlier in this chapter. It is evident from the above studies that EOPs differentially affect activity of various DA-containing neurons and pathways in the brain, particularly in the hypothalamus.

High numbers of the various opioid receptors have been detected in the raphe nucleus (Mansour *et al,* 1988). In the DRN, activation of κ-, but not μ-opioid receptors decreases the response of 5-HT-sensitive neurons to electrical stimulation (Pinnock, 1992). Thus, EOPs may have an inhibitory effect at the pre-synaptic

5-HT nerve terminals in this brain region. In addition, morphine has been shown to stimulate LH release in the presence of exogenous 5-HT while having inhibitory influence in its absence (Lenahan *et al,* 1987). There may be an important interaction between the opioid and serotonergic systems in controlling LH secretion.

Thus, it would appear that the inhibitory effects of EOPs on GnRH secretion may be mediated in a large part by the central noradrenergic, but to some extent also by the dopaminergic and serotonergic systems.

On the other hand, both the serotonergic and the dopaminergic (George and Kertesz, 1986) systems may modulate the activity of the hypothalamic pro-enkephalin neurons. Activation of the α-adrenergic system stimulates the accumulation of met-enkephalin in the ME (George *et al,* 1990). Long term DA agonist treatment of male rats reduces β-endorphin immunoreactivity in the MBH and ME, two brain areas where β-endorphin perikarya and terminals, respectively, are located (Locatelli *et al,* 1983). However, DA agonists increase β-endorphin concentrations in the hypothalamus of females rats. What becomes obvious from these studies is that there may be a reciprocal interactions between catecholamine, indolamine and EOP systems in the hypothalamus.

Recently, intermodulatory actions of opioid peptidergic systems both on EAAs (Bonavera *et al,* 1993) and neuropeptide Y (NPY) (Xu *et al,* 1993) in the central regulation of LH release have also been reported. Corticotropin releasing factor (CRF) has been implicated in the modulation of the inhibitory effects of EOPs. CRF-induced inhibition of LH secretion may be mediated by opioid peptidergic mechanisms since opioid antagonists eliminate this inhibitory influence of CRF (Akema *et al,* 1996). Hence, in addition to the monoaminergic mediation, some amino acid and peptidergic neurotransmitters may also mediate the effects of EOPs in the GnRH neurosecretory mechanisms.

CNS Region	μ	κ	δ
Telencephalon			
Frontal cortex	+++	++	+
Piriform cortex	++	++	++
Entorhinal cortex	++	++	++
Amygdala			
Central nucleus	0	0	++
Medial nucleus	+++	++	++
Lateral nucleus	++++	+++	+++
Hippocampal formation			
Hippocampus	+++	++	+
Dentate gyrus	+++	+	+
Olfactory tubercle	+	+++	+++
Nucleus accumbens	++++	++++	+++
Caudate-putamen	++++	++++	+++
Globus pallidus	+	+	+
Medial septum	+++	+	+
Bed nucleus stria terminalis	++	++	+++
Preoptic area	+	+	++++
Diencephalon			
Hypothalamus			
Supraoptic nucleus	0	0	++
Paraventricular nucleus	0	0	++
Arcuate nucleus	0	0	++
Ventromedial nucleus	0	+	+++
Dorsomedial nucleus	+	0	+++
Lateral hypothalamic area	+	0	++
Thalamus			
Periventricular area	0	0	+++
Central-medial nucleus	++++	+	++
Reuniens nucleus	+++	+	++
Medial habenula	+++	+	+++
Mesencephalon			
Interpeduncular nucleus	++++	+++	+++
Substantia nigra			
Pars compacta	+++	0	0
Pars reticulata	++	+	+
Ventral tegmental area	++	0	+
Periaqueductal grey	+	0	++
Superior/Inferior colliculi	++++	+	++
Dorsal raphe nucleus	++	0	++
Pons/medulla			
Parabrachial nucleus	+++	0	++
Nucleus raphe magnus	++	0	+
Nuc. retic. gigantocellularis	+	0	+
Nucleus tractus solitarius	++++	+	+++
Lateral reticular nucleus	+	0	+
Spinal trigeminal nucleus	+++	0	++
Spinal Cord			
Substantia gelatinosa	+++	+	++

Table 1.1. Regional distribution of opioid receptors in the rat brain (very dense: ++++; dense: +++; moderate: ++; low: +; undetectable; 0). From: Mansour *et al*, 1988).

Neuropeptide Y

Neuromodulator actions of NPY in the central control of LH release have been extensively studied (for review see Kalra, 1993; Kordon *et al,* 1994).

NPY-containing neurons show an extensive distribution pattern in the rat brain. NPY cells and nerve terminals have been found in the MBH, ARN, SCN and the preoptic area along with the other hypothalamic regions (Horwath *et al,* 1992b). Synaptic contacts between the NPY perikarya and GnRH neurons in the lateral aspect and internal zone of the ME have also been visualised (McDonald *et al,* 1989).

NPY exerts both stimulatory and inhibitory effect on pulsatile discharge of LH in ovx rats depending upon the steroid hormone milieu. Infusion of NPY into the third ventricle in ovx hormonally-untreated rats produces a dramatic and sustained inhibition of LH secretion, while it transiently increases plasma LH levels and advances the LH surge in steroid-primed ovx rats (Crowley *et al,* 1987; McDonald *et al,* 1989). NPY may mediate the positive feedback effects of the ovarian steroids since NPY-producing neurons have been shown to possess E_2 receptors (Sar *et al,* 1990). *In vitro,* NPY stimulates the release of GnRH from the MBH of steroid-primed ovx and pro-oestrous rats (Crowley and Kalra, 1987; Besecke and Levine, 1994). Accordingly, icv treatment with antiserum to NPY prevents the LH surge in steroid-treated rats (Wehrenberg *et al,* 1989). Thus, NPY would appear to have an important role in the stimulation of the episodic LH surge on the day of pro-oestrus. Indeed, its levels in the ME reach a peak nearly one hour prior to anticipation of the impending LH surge (Sahu *et al,* 1989). Furthermore, NPY gene expression is increased in the MPOA and MBH during the pre-ovulatory LH surge (Bauer-Dantoin *et al,* 1992; Sahu *et al,* 1995).

The gonadal steroid-dependent effects of NPY on GnRH release are remarkably similar to those of NA (Kalra, 1993). There is a variety of evidence suggesting that NPY and NA or ADR coexist intraneuronally in the brainstem and also in the diencephalon (Lundberg *et al,* 1982; Hokfelt *et al,* 1984). It has further been reported that NPY may act on the GnRH neurons as a co-transmitter with NA (Everitt *et al,* 1984). Thus, NPY and the adrenergic system in the hypothalamus may play a concerted role in evoking the LH surge (Allen *et al,* 1987).

There may also be interplay between NPY and the hypothalamic dopaminergic systems since NPY has been shown to increase genetic expression of TH in the TIDA neurons in the rat (Hong *et al,* 1995). Immunopositive NPY nerve terminals synapse on the β-endorphin-containing cells in the ARN (Horwath *et al,* 1992b). This anatomical relationship may imply that NPY modulates secretion of β-endorphin in the hypothalamus.

In addition to a neurotransmitter/neuromodulator role in the hypothalamus, NPY may also function as a neurohumoral signal to amplify LH release from the anterior pituitary. NPY potentiates the GnRH-induced LH response from dispersed pituitary cell cultures (Crowley and Kalra, 1987). A similar potentiation of LH to GnRH by NPY has also been observed *in vivo* (Bauer-Dantoin *et al,* 1992).

In view of the evidence outlined above, one would conclude that NPY plays an important role in the regulation of LH release in the rat either independently or in concert with the adrenergic system.

Neurotensin

Neurotensin-containing cell bodies are found in the anterior periventricular system, PVN, preoptic area, SCN and the ARN along with other some other brain areas (Jennes *et al,* 1982). The majority of the neurotensin-positive cells in the ARN terminate in the ME (Merchenthaler and Lennard, 1991).

The major nonreproductive endocrine functions of neurotensin are stimulation of insulin secretion, inhibition of glucagon secretion and modulation of gastrointestinal motility (see Kordon *et al,* 1994).

High concentrations of neurotensin and its abundant binding sites in the hypothalamus (Moyse *et al,* 1987) are suggestive of this neuropeptide's participation in the pre-ovulatory discharge of LH. Neurotensin has been reported to suppress LH levels in long term ovx rats (Vijayan and McCann, 1979). However, it has also been claimed that neurotensin stimulates LH release when infused directly into the MPOA (Ferris *et al,* 1984). In agreement with the latter report, infusion of neurotensin antiserum directly into the diagonal band of the broca, MPOA and lateral preoptic area significantly decrease the steroid-induced LH surge (Alexander *et al,* 1989). Neurotensin immunopositive cells have been

demonstrated to be E_2 receptive (Herbison and Dyer, 1991) and, therefore, upregulation of neurotensin secretion by the ovarian steroids may account for the dual actions of this neuropeptide on the LH release.

Neurotensin-containing neurons show an overlapping distribution with catecholamines in the hypothalamus (Hokfelt *et al,* 1984). This neuropeptide stimulates DA and NA release within the hypothalamus (Gudelsky *et al,* 1989). Furthermore, neurotensin-induced LH surge can be blocked by dopaminergic and α-adrenergic receptor antagonists (Akema and Kimura, 1989). It is therefore possible that catecholamines mediate the excitatory effects of neurotensin on LH release.

Galanin

Galanin is widely distributed in the brain and its immunoreactive perikarya are abundant in the MPOA, SCN, PeVN, PVN, ME and ARN (Melander *et al,* 1986a). Moreover, galanin has been visualised to co-localise with several other neuropeptides such as GnRH, neurotensin as well as GABA and TH (Melander *et al,* 1986b; Merchenthaler *et al,* 1990). Immunocytochemical and autoradiographic studies have demonstrated that a large population of galanin-immunopositive cells in the hypothalamus concentrate E_2 (see Kalra, 1993). Galanin may thus mediate the positive feedback effects of steroidal milieu on the GnRH secretory systems.

Current evidence suggest a role for galanin in initiation of the pre-ovulatory LH surge. Administration of galanin stimulates LH release in a dose-dependent manner in steroid treated ovx rats (Sahu *et al,* 1987). Intraventricular infusion of a galanin antagonist, galantide, blocks the steroid-induced LH surge in ovx rats (Xu and Sahu, 1993). A marked increase in the ME-ARN concentrations of galanin has been reported to occur after treatment with E_2 concomitant with the LH surge (Gabriel *et al,* 1990). Furthermore, galanin has been shown to stimulate GnRH release from ME-ARN fragments *in vitro* (Lopez *et al,* 1991).

Although these studies appear to indicate a stimulatory influence of galanin in the regulation of the LH secretion, the significance of its involvement in this process has yet to be determined.

Substance P

Neurons containing substance P (SP) are widely distributed throughout the hypothalamus, especially in the MPOA, ARN and ME (Makara *et al,* 1986; Tsuruo *et al,* 1991).

It has been suggested that SP acts as a neuromediator in the control of LH release, at least in part, at the level of the MPOA (Picanco-Diniz *et al,* 1990). SP-containing projections make synaptic contact with GnRH neurons in the septo-preoptic area of the rat (Tsuruo *et al,* 1991). It has been shown that extrinsic SP stimulates LH secretion by affecting the hypothalamic GnRH neurons (Jarry *et al,* 1988). Endogenous SP exerts a tonic stimulatory effect on GnRH release (Arisawa *et al,* 1990). In addition, it elevates GnRH secretion from the MBH *in vitro* and this stimulatory LH response appears to be E_2-dependent (Ohtsuka *et al,* 1987). Consistent with the latter observation, levels of SP have been found to fluctuate in discrete hypothalamic areas throughout the oestrous cycle of the rat (Jarry *et al,* 1988). Central administration of SP exerts little effect on LH release in unprimed and ovarian steroid-primed ovx rats (Kalra, 1993). On the other hand, inhibitory effects of SP have also been reported, icv (Kerdelhue *et al,* 1982) or peripheral (Battmann *et al,* 1991) administration of SP inhibits cyclical GnRH release in rats on the day of pro-oestrus. These results are contradictory since Kerdelhue and co-workers have also shown that SP concentrations are the highest in the MPOA, ME and ARN during the entire course of the pre-ovulatory LH surge compared to the other stages of the oestrous cycle (Parnet *et al,* 1990). Hence, these results would point to an excitatory influence of the hypothalamic SP on GnRH and LH release.

In addition to its neuromodulatory effects at the hypothalamic level, SP also exists in the anterior pituitary and its concentrations and binding sites vary depending upon the ovarian steroidal milieu (Mikkelsen *et al,* 1989). Recently, it has been shown that SP stimulates LH release *in vitro* from anterior pituitary cells obtained from female rats (Shamgochian and Leeman, 1992). These findings would thus suggest a paracrine/autocrine role for SP in the regulation of LH release.

In view of the data reported so far, it could be inferred that SP may have only a minor involvement in the central control of LH release.

Vasoactive Intestinal Peptide

The existence of vasoactive intestinal peptide (VIP) in the hypothalamus has been reported, specifically in neurons located in the SCN and PVN (Mezey and Kiss, 1985; Koves *et al,* 1991). VIP-containing cell bodies located in the PVN project to the ME, whereas those of the SCN send nerve terminals to the preoptic area (Mezey and Kiss, 1985). In the SCN, VIP-containing fibres have been observed in apposition to a substantial proportion of the GnRH neurons suggesting a direct functional role for VIP in the circadian control of LH secretion (van der Beek *et al,* 1994).

A single class of VIP receptors has been described and its distribution in several brain areas has been shown (Kordon *et al,* 1994).

Infusion of VIP into the third ventricle considerably attenuates the magnitude of the steroid-induced LH surge in ovx rats (Weick and Stobie, 1992). This inhibitory action appears to be exerted within the hypothalamus. Accordingly, administration of VIP into the MPOA has also inhibited the anticipated LH surge in the ovx and steroid-primed rat (Kimura *et al,* 1987). The effects of VIP on the LH surge appear to be independent of the inhibitory influence of the endogenous opioids since administration of naloxone does not prevent the inhibiton of pulsatile LH secretion by VIP (Kimura *et al,* 1987). However, VIP has also been suggested to stimulate GnRH release *in vitro* from the ME synaptosomes and the perifused MBH (Samson *et al,* 1981; Ohtsuka *et al,* 1988), respectively. Furthermore, it has been reported that the VIP content of the SCN, ME and PVN does not change during the LH surge (Watanobe *et al,* 1991). Thus, it is possible that the stimulatory and inhibitory effects of VIP may be expressed through different hypothalamic sites.

In summary, this neuropeptide may act to suppress GnRH secretion during the LH surge. However, its significance in the regulation of GnRH release has not yet been fully established.

Pituitary Adenylate Cyclase-Activating Polypeptide

Pituitary adenylate cyclase-activating polypeptide (PACAP) stimulates LH release both in the rat anterior pituitary cell culture, *in vitro* (Schomerus *et al,* 1994) and

from the anterior pituitary, *in vivo* (Sawangjaroen and Curlewis, 1994). This polypeptide is also able to interact synergistically with GnRH in producing this effect (Culler and Paschall, 1991).

Immunohistochemical studies have demonstrated the presence of PACAP immunoreactive nerve terminals in the rat ME (Koves *et al,* 1991). These fibres are in close contact with the hypophyseal portal capillaries. High-affinity specific binding sites for PACAP have also been demonstrated in the rat hypothalamus and anterior pituitary (Koves *et al,* 1991).

Taken the above findings suggest that PACAP may function both as a hypophysiotropic factor and as a neurotransmitter within the hypothalamus. However, more work has yet to be carried out to elucidate the involvement of this neuropeptide in the regulation of LH secretion.

Nitric Oxide

NO is a novel messenger molecule and enzymatically formed from L-arginine by nitric oxide synthase (NOS) in the presence of oxygen (Palmer *et al,* 1987). It is a short-lived free radical which rapidly diffuses in all directions in the tissue. Recently, Bredt *et al* (1990) have isolated the enzyme (NOS) and shown by immunocytochemistry its wide distribution in the brain. A large population of NOS-containing neurons, termed NOergic neurons, has also been found in the hypothalamus.

NO has recently been implicated in the regulation of several neuroendocrine functions including that of GnRH release (for review see McCann and Rettori, 1996). Intraventricular infusion of a NOS inhibitor, N^G-monoethyl-L-arginine, cause a cessation of LH but not FSH pulses, indicating that NO is required for pulsatile GnRH secretion (Rettori *et al,* 1993). NO stimulates GnRH release from ARN-ME fragments *in vitro* (Moretto *et al,* 1993). Furthermore, it has been suggested that NO is involved in ovulatory mechanisms since administration of NOS inhibitors significantly reduce the rate of ovulation in rats both *in vivo* and *in vitro* (Bonello *et al,* 1996).

Intermodulatory actions of NO have also been reported. Inhibition of NOS activity suppresses the excitatory amino acid stimulation of GnRH release *in vivo*

(Bonavera *et al,* 1993) indicating that NO may modulate the stimulatory effects of EAAs on LH release. Similarly, NO may also mediate the stimulatory effects of NA on LH release. Furthermore, *in vitro* studies have shown that NO may control GABA release since inhibitory effects of GABA on GnRH secretion can be prevented by NOS inhibitor (Seilicovich *et al,* 1995; McCann and Rettori, 1996). Therefore, it appears that NO may not only be involved in the stimulation, but also in the inhibition of GnRH release.

Although the evidence presented so far would point to a neuromodulatory role for NO, its participation in the regulation of GnRH and LH release has not yet been well established.

Summary

In view of the review presented so far, it is clear that the secretion of LH, including the pre-ovulatory LH surge, is regulated by a complex neural circuitry involving several classes of neurotransmitters/neuromodulators. These neuromessengers modulate the LH release by altering the activity of the GnRH pulse generator within the hypothalamus.

The neurotransmitters which appear to be of paramount significance in this neural network are NA, EOPs, DA, 5-HT, GABA and NPY. There is a complex interaction between these neurons with the effects of some being mediated by others (see Figure 1.5).

AIMS

The present study was designed to examine:

(i) the involvement of μ-, κ- and δ-opioid receptors in the central regulation of the LH surge in the rat. To date, there have been relatively few attempts to study the significance of the δ-opioid subtypes in the control of LH release.

(ii) role of monoamines, particularly NA, in the generation of the pre-ovulatory LH surge.

(iii) the inter-relationship between the opioid peptidergic and aminergic systems in the control of LH release.

(iv) neurochemical changes in the preoptic-tuberal pathway, where GnRH cell bodies (MPOA) and nerve terminals (SCN, ARN and ME) are found, at the time of the LH surge.

To achieve these aims, specific opioid receptor agonists and antagonists were utilised. Their effects on plasma LH levels and on the aminergic content and/or turnover within selected hypothalamic regions were investigated in the urethane-anaesthetised rat on the late afternoon of pro-oestrus.

When the initial findings indicated that the urethane anaesthesia totally abolished the anticipated pre-ovulatory LH surge, a modified method for blood sampling from freely-moving conscious animals was employed in the second set of experiments.

In order to further elucidate the interactions between the opioids and aminergic neurotransmitters in the regulation of the LH surge, μ-, κ- and δ-opioid agonists and antagonists were administered and co-administered intracerebroventricularly to ovariectomised and steroid-primed rats.

MATERIALS AND METHODS

Animals

Adult female Sprague-Dawley rats were purchased from Harlan UK Ltd. (Oxon, England). Upon their arrival in the Animal Unit, they were numbered by ear marking under light halothane anaesthesia and maintained in groups of three in transparent polycarbonate cages. The animals were housed under controlled temperature (21±1 °C) and light conditions (lights on from 07.00h to 19.00h). Food (standard pellet diet) and water were supplied *ad libitum*.

Two different types of animal models were employed during the course of this study. In Experiments I and II, intact female rats and, in Experiment III, ovx and steroid-primed rats were used.

1. Intact Female Rats

Vaginal smearing was performed between 08.30h-10.30h each morning and the smears examined under the X10 objective of a light microscope. The morphology of the cells sloughed from the vagina were used to identify the four different stages of the oestrous cycle of each rat.

Stage of cycle	Vaginal Smear
Dioestrus I	Some nucleated epithelial cells and many poly-morphonuclear leucocytes with some keratinised squamous epithelial cells.
Dioestrus II	Nucleated epithelial cells and many leucocytes.
Pro-oestrus	Predominantly nucleated epithelial cells.
Oestrus	Keratinised (cornified) squamous cells only.

Table 2.1. Assessment of oestrous cyclicity by vaginal cytology.

Only those animals (weighing 220-300g) which had exhibited at least three consecutive four-day oestrous cycles were selected for experimentation on the morning of pro-oestrus.

2. Ovariectomised and Steroid-Primed Rats

Ovariectomy:

Adult female Sprague-Dawley rats were arbitrarily selected from the colony and bilaterally ovx under halothane anaesthesia.

While under deep anaesthesia, the rats were secured to a dissecting board in a dorso-ventral position. The upper abdominal skin was neatly trimmed and swabbed with absolute alcohol. A small incision was made in the skin, and the exposed subcutaneous fat was deflected to reveal the abdominal muscle coat. The muscle layer was then cut. The ovaries were very gently withdrawn, and a haemostatic ligature (silk thread) was made around the uteri before the removal. The tied ends were returned to the abdominal cavity and the muscle layer was sutured with surgical silk. The skin was closed using Michel clips. Polybactrin spray (The Wellcome Foundation Ltd., London, UK) was applied to the area to prevent any post-operative infection.

After the surgery, the animals were allowed to recover in individual cages for a period of ten days to three weeks before use. They were subcutaneously injected with β-oestradiol 3-benzoate (5μg/0.2ml olive oil, Sigma Chemicals Co., St. Louis, USA) 48h before and P (0.5mg/0.2ml olive oil, Sigma Chemicals Co., St. Louis, USA) 4h prior to experimentation.

On the day of the anticipated LH surge, the animals were anaesthetised with Vetalar (Ketamine hydrochloride 100mg/ml; Parke-Davis Veterinary Com. Pontypool, Gwent, UK). The surgical anaesthesia (complete cessation of the hind limb flexor withdrawal reflex) was maintained by periodic intramuscular (i.m.) injections of Vetalar (0.5-0.7ml) until the end of the experiment i.e. at 19.00h. A heparinised (sodium heparin, 10 units/ml; Leo Laboratories Ltd., Bucks, UK) cannula was inserted into the right femoral artery and the animals mounted on a stereotaxic frame to perform icv infusions. These procedures will be explained in detail later in this chapter.

Drugs

The properties of the drugs used as tools in this study are summarised in Table 2.2. Their chemical names are given in the 'List of Abbreviations'.

Morphine is a highly specific μ-agonist and displays very little activity at other opioid receptor types. In this study a rapid-acting preparation of morphine (diamorphine hydrochloride) was used which exhibits potent analgesic effects (Kruk and Pycock, 1993).

Drug	Receptor Properties
Naloxone	opioid antagonist
Diamorphine	μ-agonist
U-50488H	κ-agonist
U-69593	κ-agonist
MR2266	κ-antagonist
MR1452	κ-antagonist
DPDPE	δ-agonist
ICI 174,864	δ-antagonist
ICI 154,129	δ-antagonist

Table 2.2. Drugs used in the present study and their receptor properties.

Naloxone is a specific opioid antagonist which has greatest affinity for the μ-receptor but also possesses substantial affinity for both κ- and δ-receptors (Kruk and Pycock, 1993).

U-50488H and U-69593 are both highly selective κ-opioid agonists, being more potent at the κ-receptor than at any other opioid receptor (Vonvoigtlander *et al,* 1983; Lahti *et al,* 1985). These two κ-agonists do not show any affinity towards μ-opioid subtypes.

MR2266 and MR1452 are two selective κ-opioid receptor antagonists (Vonvoigtlander *et al,* 1983; Cicero *et al,* 1988). MR2266 shows some affinity

towards μ-opioid receptor subtypes (Vonvoigtlander *et al,* 1983). Affinities of MR1452 towards the other opioid receptor subtypes are at present unknown.

DPDPE, an enkephalin analogue (peptide) is a highly selective δ-opioid receptor agonist (Mosberg *et al,* 1983; Crook *et al,* 1992).

ICI 174,864 and ICI 154,129 are two selective δ-opioid receptor antagonists (Cowan and Gmerek, 1982; Crook *et al,* 1992). These peptide ligands prevent actions of DPDPE at δ-receptors, and do not have affinities towards μ-opioid receptors (Cowan and Gmerek, 1982; Crook *et al,* 1992).

I would like to thank to the following institutions for the gifts of the drugs employed in the experiments: Boehringer Ingelheim Ltd. (Ingelheim am Rhein, Germany) for the gifts of MR2266 and MR1452, the Upjohn Company (Kalamazoo, Michigan, USA) for U-50488H and U-69593, the National Institute on Drug Abuse (Rockville, Maryland, USA) for DPDPE.

Naloxone was purchased from Sigma Chemicals Corporation (Poole, Dorset, UK), diamorphine from Napp Laboratories Ltd. (Cambridge UK), ICI 174,864 and ICI 154,129 from Cambridge Research Biochemicals Ltd. (Cheshire, UK).

Femoral Artery Cannulation

Surgical anaesthesia was induced under halothane anaesthesia as described in Experiments II and III. A plastic cannula (Portex, outside diameter 0.63mm) combined with vinyl tubing (internal diameter 0.55mm) was inserted into the right femoral artery. With the aid of a stainless steel guide cannula, the vinyl tubing was fed under the dorsal skin to emerge at the back of the neck where it was loosely stitched to the skin. This procedure took no longer than 20-25 minutes.

The animals were allowed to recover for around two hours prior to the blood sampling commencing at 13.00h. Blood samples (200μl) were withdrawn through the heparinised cannula at hourly intervals from freely-moving conscious animals throughout the afternoon and early evening of pro-oestrus. A small volume (20-30μl) of warmed, heparinised physiological saline was injected after

each blood sample collection to maintain the cannula open. The animals were kept warm throughout the experiment by the use of a heating lamp.

Only one or two rats were operated upon on the same day. All drug administrations were carried out under light halothane anaesthesia.

The animals were killed by decapitation just after the final blood collection at 19.00h. The blood samples were centrifuged at 4°C for 5 mins at 3000 rpm. The plasma was transferred into fresh tubes and stored at -20°C until assayed for LH determination by radioimmunassay (RIA) at St. George's Hospital Medical School in London.

Carotid Artery Cannulation

On the day of pro-oestrus at around 12 noon, the right carotid artery was cannulated (Portex, outside diameter 0.63mm) under urethane anaesthesia (ethyl carbamate, 1g/kg; Sigma Chemical Co., St. Louis, USA) as described in Experiment I. The surgical anaesthesia was maintained by periodic intraperitoneal (IP) injections of urethane. Trunk blood (200µl) was collected via the heparinised cannula at hourly intervals commencing at 13.00h. After centrifugation and separation of the plasma, the red blood cells were reconstituted with 100µl of warmed, heparinised saline (10 units/ml) and reinjected through the cannula to maintain a constant plasma volume.

The animals were kept warm throughout the experiment by the use of a heating lamp. Decapitation of the rats and centrifugation of the blood samples procedure were carried out as explained above.

Removal of the Brain

Following the decapitation, the rat's head was placed on crushed dry ice. The skin lying over the skull was removed and the occipital muscles cut to reveal the foramen magnum. One blade of the scissors was cautiously introduced into the foramen. Cuts were made through the bone by keeping the blade parallel to the inner wall of the skull and as close to it as possible to avoid damaging the brain. These cuts were extended as rostrally as far as bregma. After two parallel cuts had been made, the skull was lifted forward and its rostral point of attachment separated

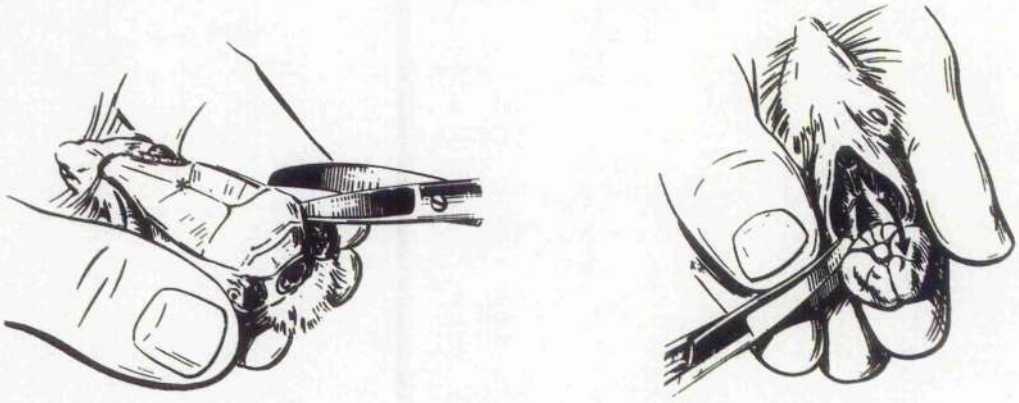


Figure 2.1. Removal of the brain. *Bregma.

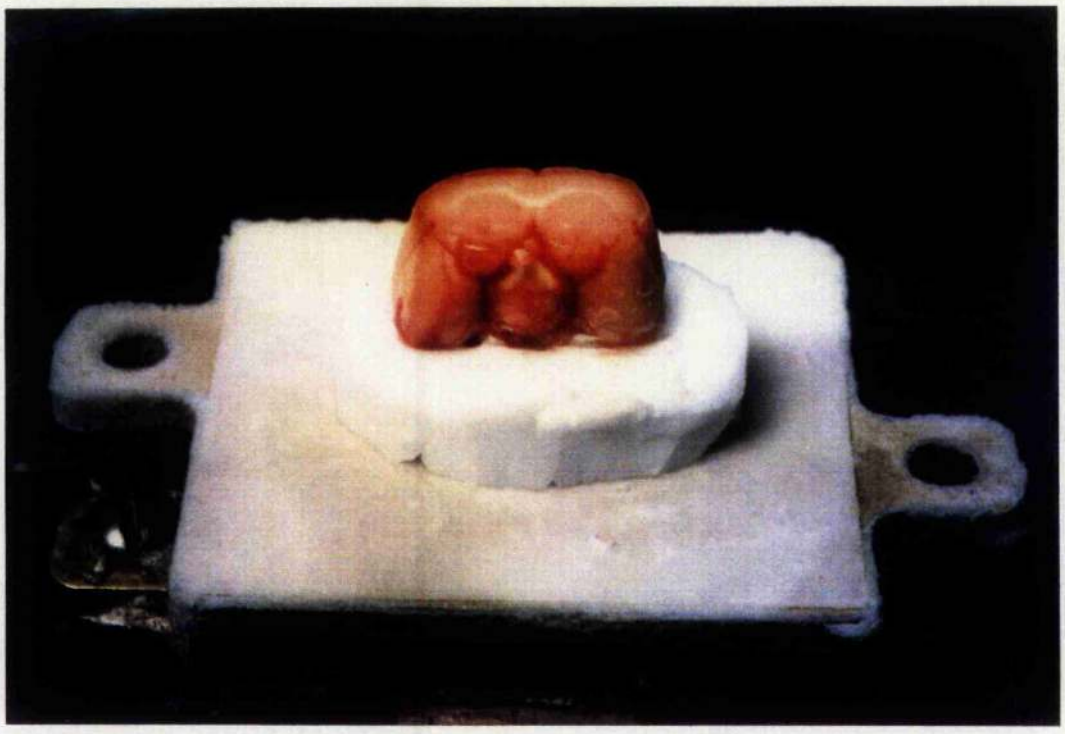


Figure 2.2. Placement of the brain on the mounting stage of the microtome.

(Figure 2.1.). 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. The entire procedure was carried out within 60 seconds and the brains were frozen on dry ice to prevent *post mortem* degradation of the brain aminergic neurotransmitters prior to their analysis.

Brain Microdissection and Micropunch

Brain microdissection and micropunch procedures were always carried out on the same day that the experiments were undertaken. The MPOA, SCN, ME and ARN of the hypothalamus were surgically isolated by a modification of the Palkovits Technique (Palkovits and Brownstein, 1983). The frozen brains were allowed to partially thaw to promote adherence to the mounting platform of a freezing microtome. The hind brain and the anterior portion of the forebrain were removed by coronal cuts. The dorsal cut surface of the brain was placed on the mounting stage of the microtome (-80°C; Leitz-Wetzler, Germany) and the tissue was held firmly in place by encasement of the base in Cryo-Gel (BDH Lab. Supplies, Poole, England) (Figure 2.2.).

There are several landmarks in various parts of the brain which are of guidance during microdissection. 100µm coronal brain slices were cut until the fusion of the corpus callosum and thereafter the anterior commissure could be seen. Subsequently, serial 500µm sections were taken in the frontal plane, numbered 0-10 and put on cooled microscope slides resting upon dry ice. There were no punches made from the first section (0); it was merely cut to ensure the correct plane of the sectioning. The remainder of the brain following removal of the 11 sections was discarded.

The slides, section numbers 1-8 (excepting number 4) which contained the hypothalamic areas of interest were then placed on a petri dish containing dry ice and viewed under a binocular dissection microscope (X16 magnification). Bilateral homologous tissue samples of the MPOA, SCN, ME and ARN were micropunched from the sections using modified stainless steel hypodermic needles (0.7 and 0.3mm internal diameter). The locations of discrete brain regions were identified by reference to the Stereotaxic Atlas of the Rat Brain (Paxinos and Watson, 1986) in conjunction with section number and standard neuroanatomical landmarks such as the third ventricle and midbrain (Figure 2.3.). A different

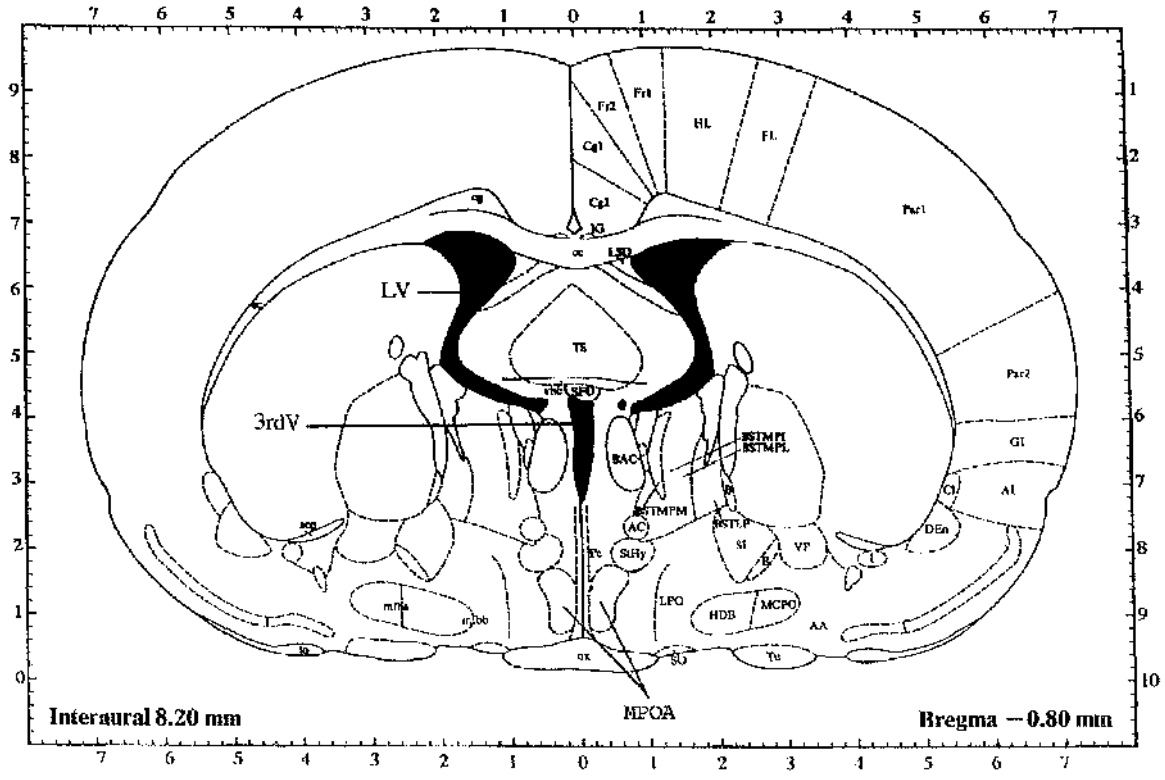


Figure 2.3. Coronal section (number 3) through the rat brain showing the location of the medial preoptic area (MPOA) to be isolated by micropunch. (Adapted from Paxinos and Watson, 1986).

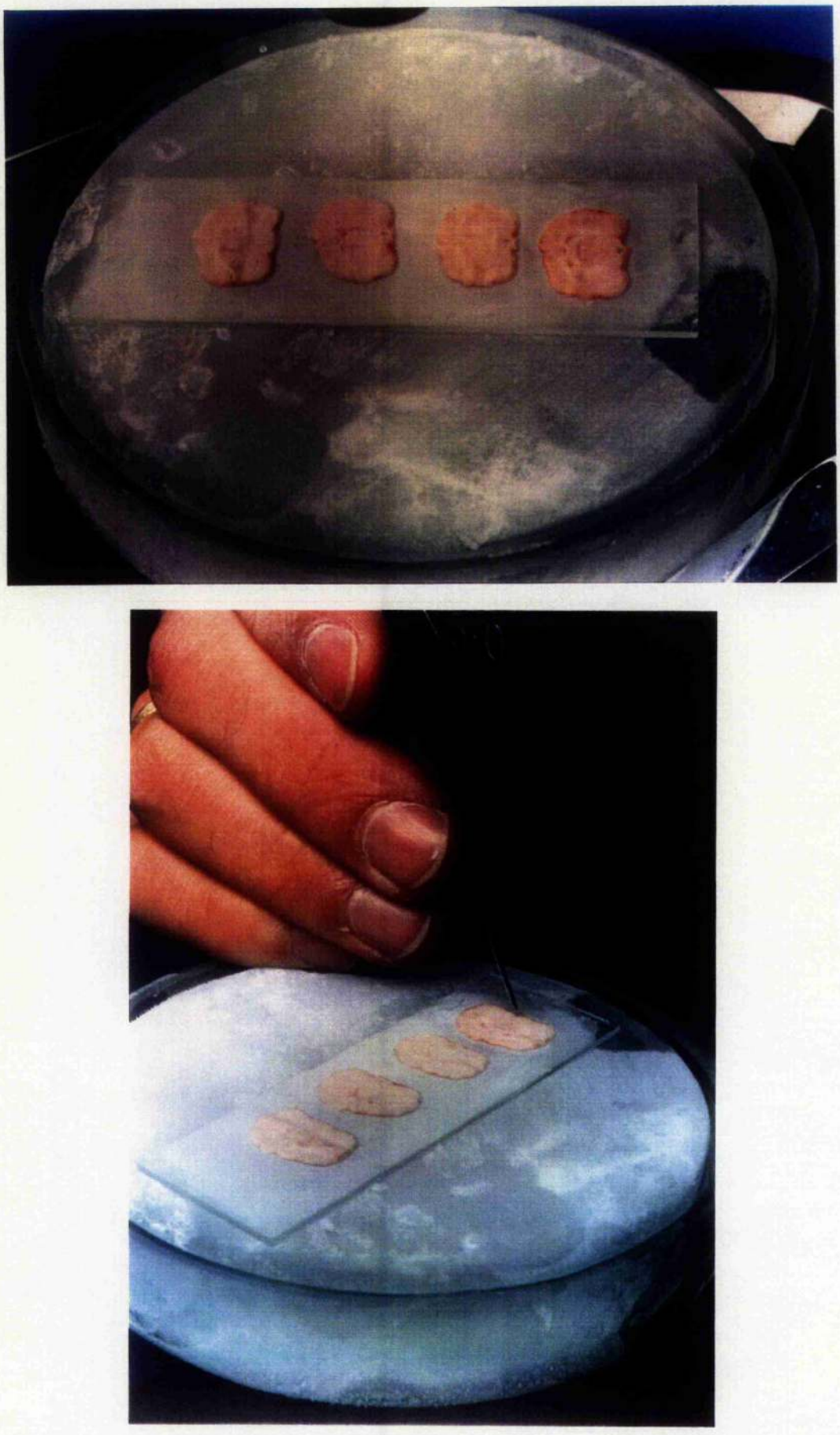


Figure 2.4. Isolation of the specific hypothalamic regions by micropunch technique.

needle was used for each area and the needles were rinsed in absolute alcohol between samples and always kept cold on dry ice before use.

The punch needle was held at an angle of 45° and brought to the surface of the section. When the tip of the needle was in the appropriate location, it was placed in a vertical position and gently pressed on to the slice. The punch was then rotated to free the surrounding tissue and withdrawn (Figure 2.4.). After a tissue sample was successfully withdrawn a sharp edged hole remained in the section, and the tissue disc is contained within in the needle. The ME was isolated by punches of half the needle cross-sectional area. Finally, the tissue discs were pushed out of the dissecting needle by its stylet. The isolated hypothalamic regions were immediately transferred into separate, cooled Eppendorf tubes and frozen in liquid nitrogen. They were stored at -80°C and processed (on the following day) for the measurement of their biogenic amine content.

As the hypothalamic areas were isolated by multiple micropunches, it was possible that a fraction of each area (particularly in the case of the SCN) may have been missed. A record of the success of the punches was therefore kept.

Intracerebroventricular Infusion

Icv infusion of the drugs to the ovx and steroid-primed rat model took place only in Experiment III.

Stereotaxic Apparatus

All infusions were performed using a 10µl graduated Hamilton Syringe (Hamilton Company, Reno Nevada, USA) mounted upon David Kopf stereotaxic equipment (Tujunga, California, USA). Three dimensional construction of the frame of this instrument allows the fixation of the rat's head at three different points. The ear bars were fixed in the external auditory meatus on either side and the nose was adjusted to a position in which the upper incisor bar firmly supported the upper jaw at the gingival margin behind the incisor teeth and a clamp was lowered on to the nasal region. The interaural rostro-caudal fixation maintained a recognised position of the head during infusion. Thus the infusion apparatus could be moved anteriorly and posteriorly along the long axis of the skull and the underlying brain.

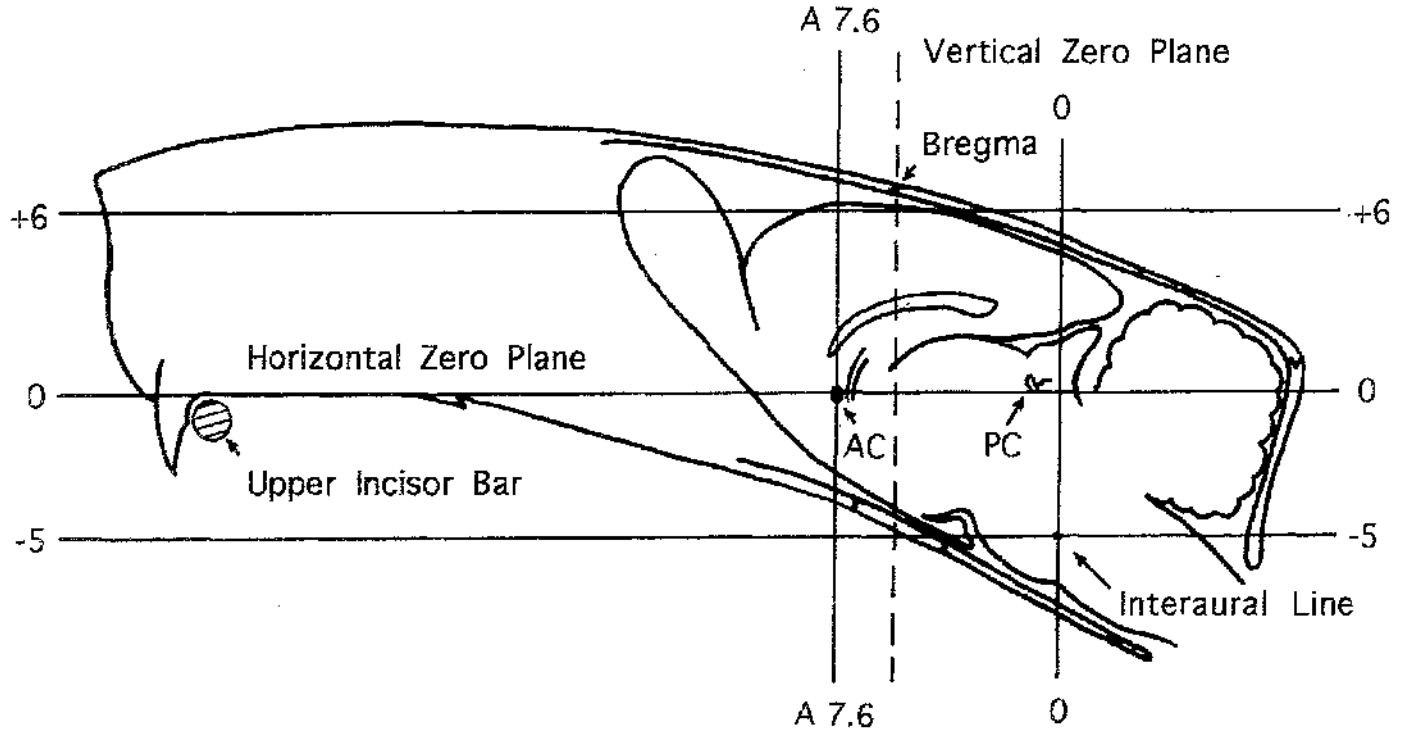


Figure 2.5. Mid-sagittal section through the rat's head, illustrating the construction of the horizon and vertical reference planes as positioned in the stereotaxic apparatus. AC, Anterior Commissure; PC, Posterior Commissure. (Adapted from De Groot's Rat Brain Atlas, 1972).

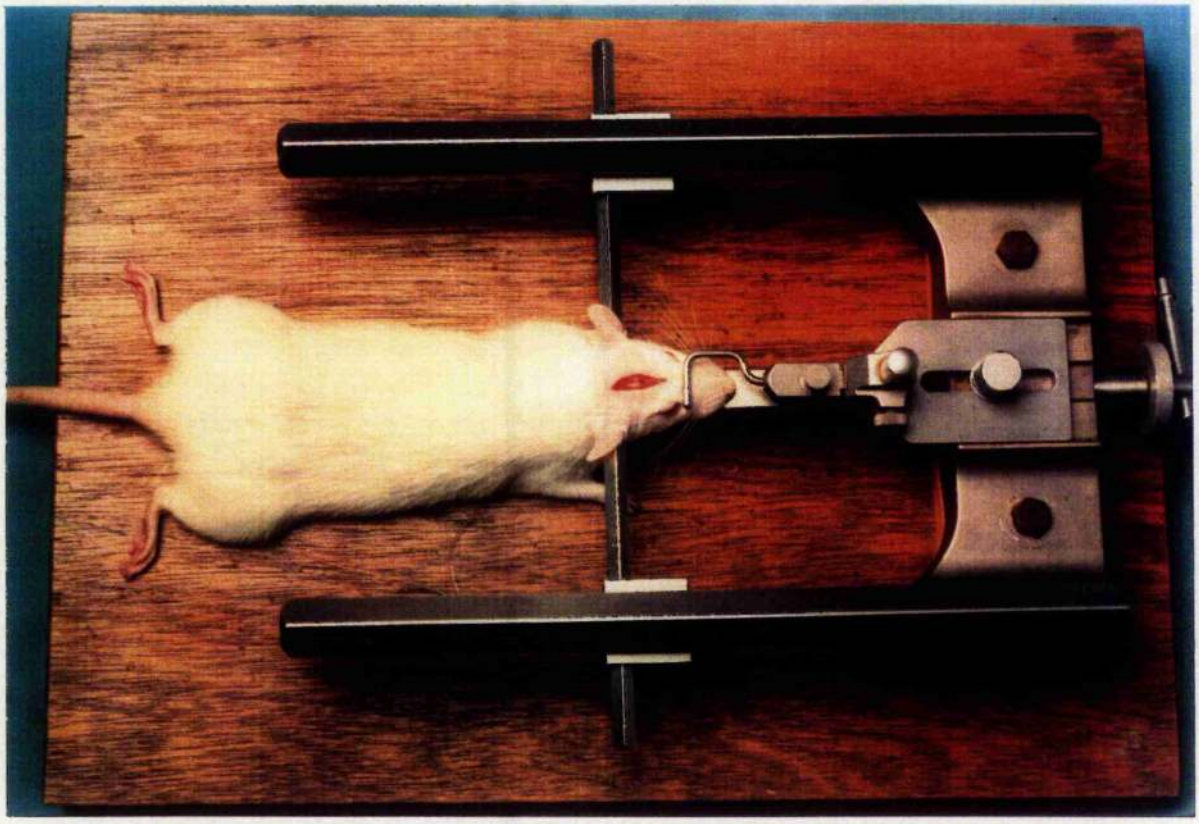


Figure 2.6.a. Fixation of rat in the stereotaxic apparatus for intracerebroventricular infusion of sterile saline, opioid agonists and antagonists.

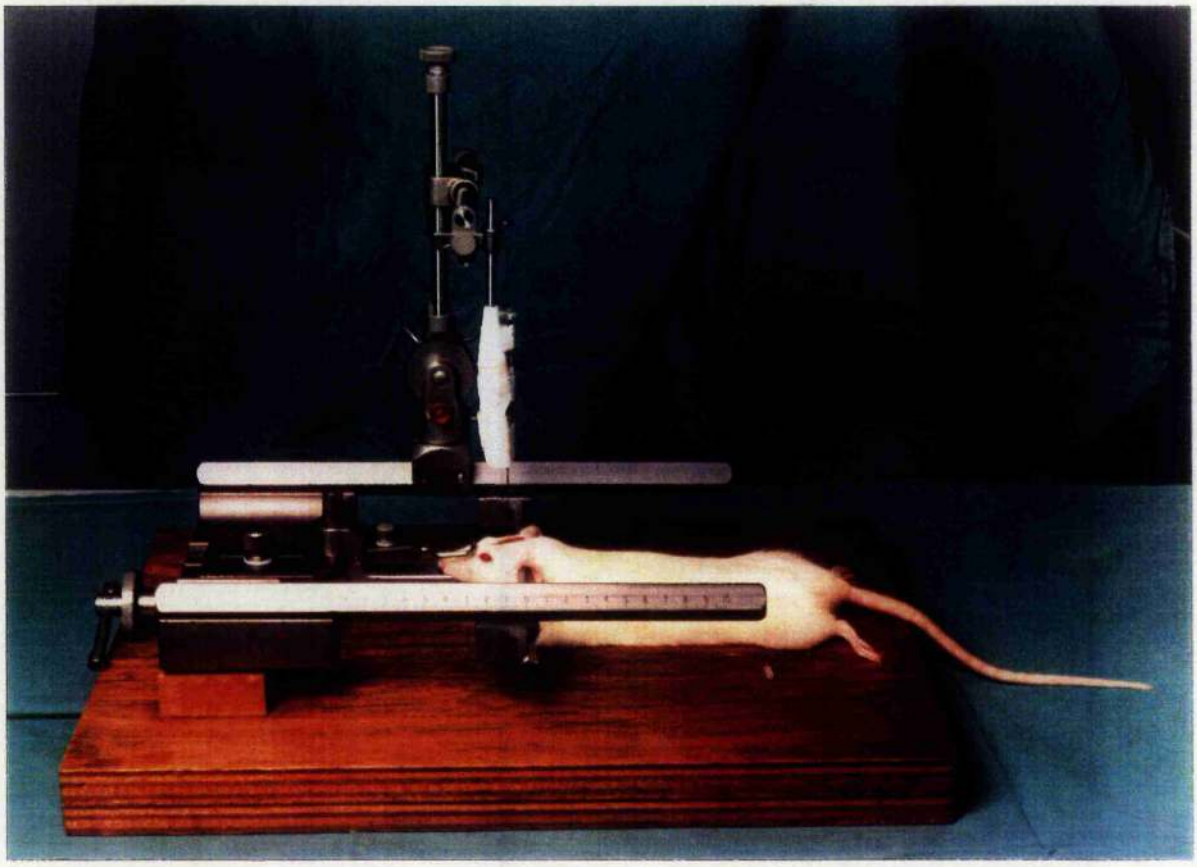


Figure 2.6.b. Stereotaxic apparatus showing the intracerebroventricular infusion technique.

The placement of the Hamilton syringe needle into the lateral ventricle was assessed with the aid of the De Groot Rat Brain Atlas (1972) 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 2.5.).

On the afternoon of the anticipated LH surge, following the femoral artery cannulation, the Vetalar-anaesthetised rat was mounted on the stereotaxic apparatus. The dorsal skin of the skull was trimmed and swabbed with alcohol. A midline incision was made in the scalp, the skin deflected and the membraneous connective tissue scraped from the superior aspect of the skull (Figure 2.6.a). This revealed the bregma. The position of the bregma was marked and the vertical, lateral and anterio-posterior co-ordinates noted. A hole was drilled in the appropriate location (determined by reference to the De Groot Atlas) of the skull, using a 5mm dental burr, to expose the superior sagittal sinus. Care was taken not to injure the surface of the brain. The Hamilton syringe was filled with 10µl of a freshly-made solution containing the drug under investigation, or the vehicle (sterile saline) 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 the pre-determined position (Figure 2.6.b). The solution was infused gently over a period of 60 seconds into the right lateral ventricle. The syringe was then raised and the animal carefully removed from the stereotaxic apparatus.

Once the icv infusion had been completed, the anaesthetised animal was kept warm by using a heating lamp. Blood samples (200µl) were collected from the indwelling cannula at hourly intervals throughout the afternoon and early evening as explained earlier.

Histological Verification

In order to assess the effectiveness of icv infusion, the position of the syringe needle was histologically verified in two separate rats. The Hamilton syringe was filled with 5µl of a marker, indian ink, which was subsequently infused into the right lateral ventricle. No hypothalamic samples were collected from these marker-infused rat brains. The brains were fixed and kept in formol saline. The frontal lobes and the hind brain were cuts, and the dorsal portion of the brain was mounted

on the specimen holder of a cryostat (Jung Frigocut 2800E, -25°C). 50µm sections were cut and arranged on a slide in series. They were then examined under a dissection microscope (2X10 magnification).

Because of the fact that the brains had to be frozen following their removal and the hypothalamic regions had to be micropunched from the microdissected brain slices, a slightly different method was adapted to verify the position of the syringe. During the brain microdissection and micropunch, the location of the syringe needle was examined under a dissection microscope (X16 magnification). The infusions were considered to be intraventricular if the tract of the needle was observed in the lateral ventricle or if drops of blood were seen within its swollen cavity. Only those brain samples, which fulfilled one of these criteria, were included in the results.

Chromatography

To identify and quantify monoamines such as NA, ADR, DA, 5-HT and aminergic metabolites in the brain samples, this study employed reversed-phase high performance liquid chromatography with electrochemical detection (HPLC-ECD), because of its high sensitivity and specificity.

HPLC involves the physical separation of components in a mixture which are distributed between a mobile phase and a stationary phase under high pressure. The separation occurs by differential migration and depends on the relative affinities of the various solute molecules for the mobile and stationary phases. The separated compounds are then quantitatively measured by an electrochemical detector (Jussofie *et al*, 1993). A schematic diagram of the main constituents which comprise the HPLC-ECD set-up utilised for this study is shown in Figure 2.7.

i) Analytical column: The column (S5ODS2-250A, 4.6mm i.d.x25cm, HICHROM) was packed with octadecyl silane (ODS) hypersil (5µm) non-polar packing material comprised of silica particles bonded to a C_{18} moiety. This forms a column environment suitable for reversed-phase chromatography. The solid phase thus enables separation according to the size, structure and polarity of the analyte, and determines the time taken for elution from the column. The more

polar a compound, the faster it is eluted, as there is little attraction between it and the non-polar packing gel.

ii) Mobile phase (solvent reservoir): HPLC grade reagents were used in the preparation of the solvent. The aqueous medium used consisted of 6.74g citric acid, 4.81g sodium citrate, 47mg ethylenediamine tetra-acetic acid (EDTA), 200mg heptasulphonic acid, 1.15ml glacial acetic acid, 3ml tetrahydrofuran and 25ml methanol. It was made up to 1 litre with deionised water and then brought to pH 4.9 using 10M NaOH to ensure complete ionisation of the biogenic amine molecules. The solvent was then degassed with helium for 10 minutes in order to eliminate the presence of oxygen, which increase baseline noise. The mobile phase was renewed every two to three weeks depending on the degree of use.

iii) Pump: A computer-controlled HPLC pump (Gilson 302) fitted with a manometric module (Gilson 802) capable of producing high pressure (up to 6000 psi) was used. The mobile phase was drawn into and delivered from the pump head (Gilson 10.WSC) through the inflow and outflow check valves. The flow rate was set at 1ml/min during analysis. The system was set to a low flow rate of 0.2ml/min when not in use.

iv) Sample loading: The supernatant sample or composite mixture was introduced into the column through an injection valve (Rheodyne 7125). The valve was turned from 'load' position to 'inject' position simultaneously with 'start command' on the computer. The mobile phase was then delivered through the loop to carry the sample on to the analytical column. The injection port was always flushed with HPLC grade methanol prior to and after each sample injection.

v) Detector: An electrochemical detector (Model 141, Gilson) was used to quantitatively measure the separated compounds. The ECD consisted of a carbon working electrode (BAS MF-1000), a platinum wire auxiliary electrode (BAS MF-2020) and a silver/silver chloride reference electrode (BAS RE-1 Ag/AgCl), which was immersed in a 3M solution of NaCl.

The cell consists of a Plexiglass block divided in half by a 0.2mm polytetra fluoroethylene (teflon) gasket. A flow cell with a volume of <1ml, containing the glassy carbon working electrode, is formed by a slit (1.6X0.5cm) cut in the gasket.

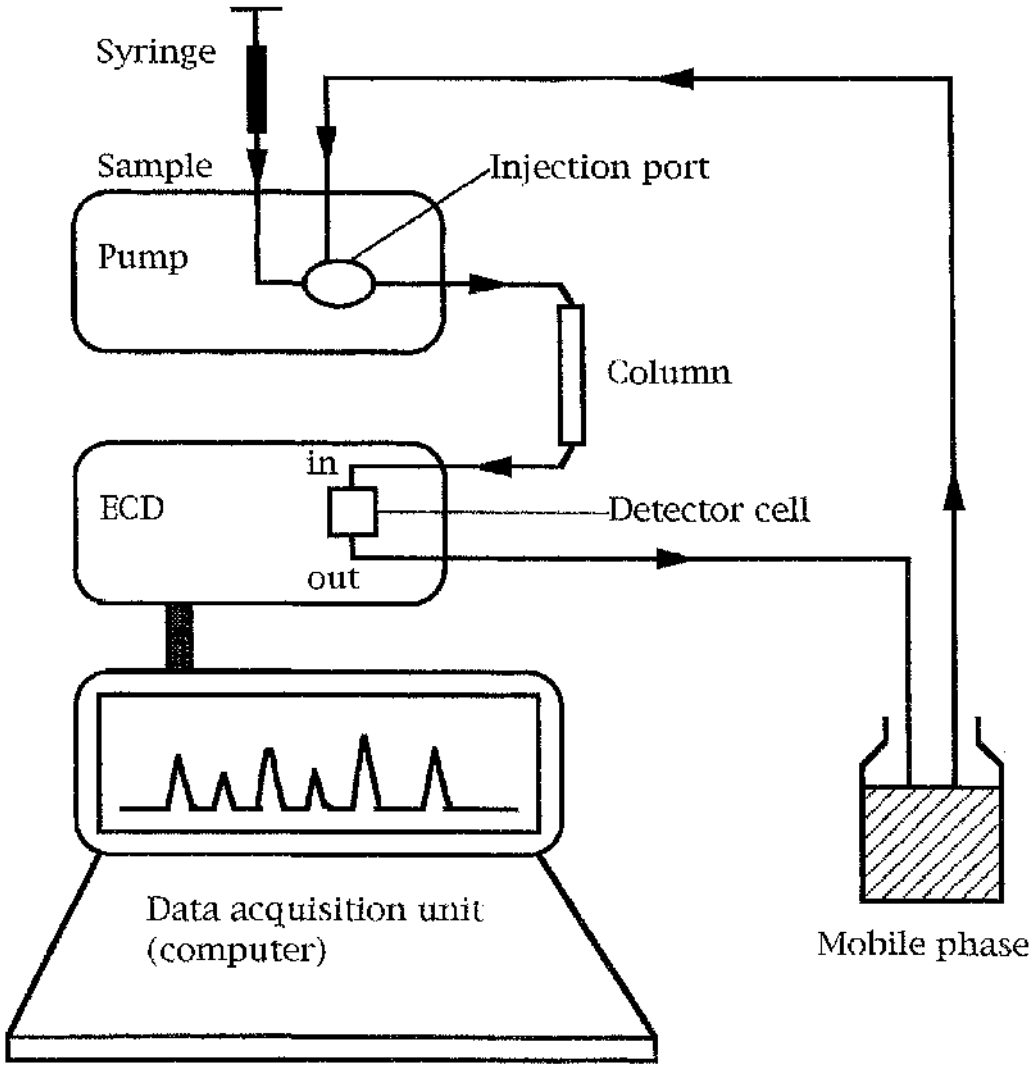
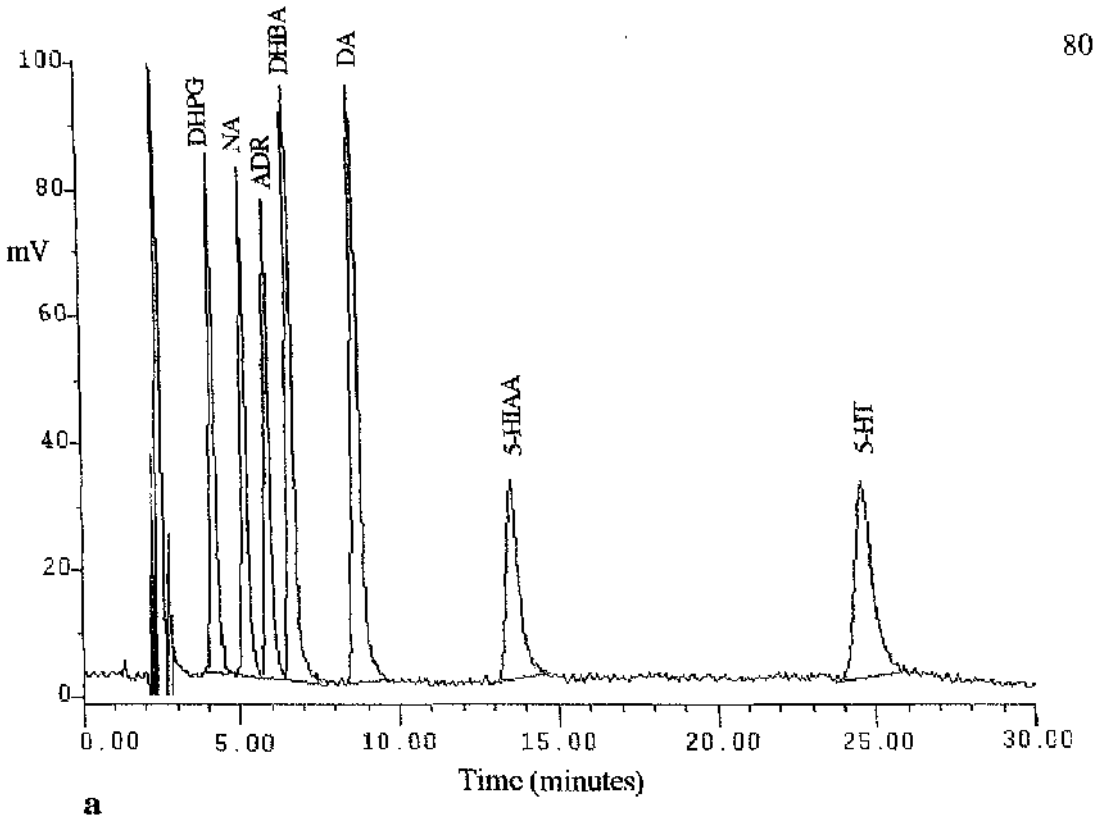


Figure 2.7. A schematic representation of the HPLC-ECD experimental set-up. The flow of the mobile phase is depicted by arrows. The supernatant sample is introduced to the system through the injection port. The mobile phase is delivered by the pump to carry the sample injected on to the analytical column. The separated compounds are then quantitatively detected by the ECD. Chromatograms are analysed and displayed by an integrating computer.



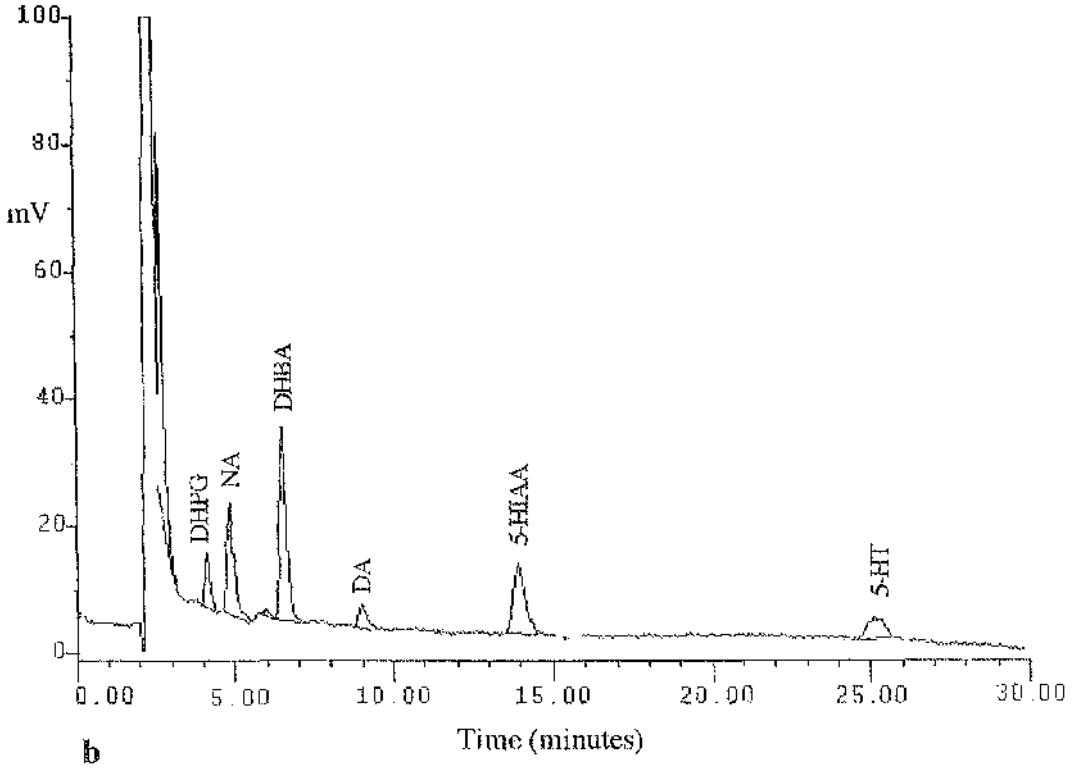


Figure 2.8. a. Chromatogram of a composite mixture containing some of the major neurotransmitters and their metabolites (1ng/10µl). **b.** A typical chromatogram of a sample obtained from the ARN of the rat hypothalamus.

Electrochemical detection is based on the principle that compounds capable of oxidation or reduction in an electrical field result in the passage of current. 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 determined. This introduces some measure of selectivity into the system. For the catechol and indoleamines under investigation, a potential of +0.70V was used.

vi) Chromatogram: As oxidisable bands of each compound pass through the detector, the current (and resultant voltage) rises and falls, as a function of time, to yield a liquid-chromatography, electrochemical chromatogram (Figure 2.8. a, b). The first peak on the chromatogram is called the solvent front which has the shortest retention time and leaves the analytical column first. This is followed by DHPG, NA, ADR, DHBA, DA, 5-HIAA and finally 5-HT. As 5-HT remains in the column longest, it is susceptible to band broadening and the peak is short and broad compared to the rest. Therefore, compounds are separated in order of decreasing polarity and increasing time. Chromatograms were analysed by an integrating computer (Hyundai HCM-428 Super VGA, with Gilson 712 HPLC application software) and quantified using the peak area ratio of DHBA to analyte method (Table 2.3.).

Specific hypothalamic regions were collected and kept at -80°C until assayed. At a later date (maximum two days later), 100µl of 0.1M HCl was added to each sample, along with 50µl of 3,4-dihydroxybenzylamine (DHBA: 1ng). DHBA was used as an internal standard because it does not react with any of the biogenic amines in the samples and is very stable. Since it is added in known concentrations, peak area of DHBA can be used to calculate the concentrations of unknown amines. Samples were centrifuged at 3000rpm at 4°C for 10 mins in a Denley BR401 refrigerated centrifuge to ensure all the tissue was sedimented at the bottom of the tube. The samples were homogenised using a vortex mixer for 30 seconds and then re-centrifuged (as above). The supernatants were transferred into fresh tubes and the remaining protein pellets were stored at -80°C until the protein assay was carried out.

10µl aliquots of supernatant were drawn off and injected on to the HPLC column. The concentrations of DHPG, NA, ADR, DA, 5-HIAA and 5-HT were simultaneously measured by the ECD. All samples were measured in duplicate.

The response of the HPLC system was checked by running composite mixtures of the amines under investigation in known concentrations each morning. Stock solutions of NA, DHPG, ADR, DA, 5-HIAA and 5-HT were made up in 0.1M HCl to a final concentration of 1ng/10µl. They were kept at 4°C and renewed every two weeks. The amines in the samples were identified by the coincidence of their retention times with the known amines in the composite mixture. The ratio of the amine peak areas to the peak area of DHBA in the standard mixture indicates the percentage recovery at the detector. This ratio is known as the response factor. Addition of internal standard (1ng) to the samples thus allows the integrating computer to calculate the content of the amines in the samples by taking the ratio of the peak areas with the internal standard and incorporating the response factor. The mean amine concentration of each sample was calculated and is shown in Table 2.3. The minimum detectable quantity of each of the biogenic amines was 4-5pg/10µl of supernatant, dependent on the noise level of the system.

Response Factor (RF):

$$\frac{\text{Peak area of DHBA in composite mixture}}{\text{Peak area of amine in composite mixture}}$$

Peak Concentration:

$$\frac{\text{Peak area of unknown in sample}}{\text{Peak area of DHBA in sample}} \times RF \times *DF$$

Final Amine Calculation:

$$\frac{\text{Peak concentration}}{\text{Weight of protein}} \text{ (pg amine/µg protein)}$$

*DF: Dilution Factor i.e. 150/10 = 15

Table 2.3. HPLC amine concentration calculations.

Protein Estimation

The amount of brain tissue in the homogenate was estimated by its protein content. The protein content of the brain is fairly stable, and therefore the amount of protein in a sample is proportional to its brain tissue content. The tissue pellets remaining in the Eppendorf tubes after centrifugation were stored at -80°C for protein determination by a modified method of Lowry *et al.* (1951).

A standard curve of protein concentrations versus absorbance at 595nm after reaction with Coomassie Blue G250 (Pierce, Life Science Laboratories Ltd., Luton, U.K.) was first obtained (Figure 2.9.). Known weights of bovine serum albumin (BSA) were prepared in a range of 0-200µg by using 0.1M sodium hydroxide (NaOH) and incubated overnight at 4°C. On the following morning, the BSA (100µl) was diluted to 0.9ml with distilled water. Then, 1ml of Coomassie Blue G250 was added and the absorbance at 595nm read immediately on an LKB Biochrom Ultrospec 4050 spectrophotometer. The blank solution consisted of 200µl of 0.1M NaOH plus 1800µl of distilled water. The absorbance of each tube was read in duplicate and the mean used for the calculation.

The protein pellet remaining after the preparation of the supernatant for injection on to the HPLC column was dispersed in 210µl 0.1M NaOH by a vortex mixer (Hook & Tucker Instruments Ltd.) for 30-50 seconds and incubated overnight at 4°C. On the following day, duplicate 100µl samples were transferred to two separate tubes and each treated as described above for the standard BSA curve. The protein concentration in each tube was calculated by substitution of the absorbance into the regression equation of the standard curve. The protein content of the duplicate tubes were added together to give the total protein content of the original sample.

If the sample tissue content lay below two standard deviations of the mean and no tissue had been lost in the micropunch procedure itself, then it was assumed that the protein had been lost during the course of storage/assay/transfer of the samples and was replaced by the mean protein content of the area for the calculation of the amine concentrations.

Amine Concentration Calculation

The total content of the individual amines in each sample was divided by the total protein content of the sample, or the mean protein concentration for the particular area, to give the concentration of each amine in the original tissue in pg amine/µg protein.

LH Radioimmunassay

The immunoreactivity of LH was used to determine its relative abundance in each of the serum samples using a homologous double antibody. The samples were assayed in duplicate and the standard curve in triplicate. The latter involved an eight point curve representing concentrations which ranged from 6.25pg/10µl to 800pg/10µl which was provided by a stock solution of LH (100ng/ml). Three further sets of tubes were set up as (1) total counts, (2) blank and (3) zero reference (Table 2.4.).

Reagents:

1) Merthiolated phosphate buffer; 8.8g NaCl (0.15M) and 100mg Thiomersal were mixed well in 1 litre of distilled water on a stirrer, and the pH was adjusted to 7.0 using 1M sodium hydroxide (NaOH). Then, 20ml was discarded and 7.8ml NaH_2PO_4 (0.5M) and 12.2ml Na_2HPO_4 (0.5M) were added. Once made up, the solution was stored at 4°C.

2) 1% Egg white; this was prepared by dissolving 1g of egg albumin in 100ml merthiolated phosphate buffer (this had to be stirred very fast for a long period) and then filtering through acetone-washed glass wool. The pH was adjusted to 7.0 using 1M NaOH when necessary. This solution was stored at 4°C and made up freshly each week as required.

3) Serum diluent; 0.05M EDTA (disodium salt) was prepared with merthiolated phosphate buffer i.e. 1.86g EDTA in 60-80ml of merthiolated phosphate buffer. The volume was made up to 100ml with merthiolated phosphate buffer and pH was ensured to be at 7.0. This solution too was stored at 4°C. On the day of assay, 2% normal rabbit serum was added.

4) LH standard; NIH - rLH - RP3 was used. 984ml of egg white-merthiolated phosphate buffer was added to 16µl aliquot of standard (100ng/ml) which then gave a 160ng/ml (1600pg/10µl) solution. This was then double diluted in egg white/merthiolated phosphate buffer to give the curve.

5) Antibody; NIH - rLH S10 was diluted to 1:60,000 with serum diluent so that on adding 50µl to each tube, a final dilucnt 1:180,000 was achieved.

6) Precipitating antibody; Donkey-anti-rabbit antibody (Guldhay Antisera Ltd.) was diluted to 1:24 with egg white-EDTA.

Protocol:

Day 1.

A 20µl sample was taken from each of the collected plasma which were then maintained in the tubes at similar volumes with egg white/merthiolated phosphate buffer. Serum diluent was added to the blank (tube 2). This was composed of normal rabbit serum at a dilution of 1:400, and 0.5M EDTA in merthiolated phosphate buffer a pH of 7.0. 50µl of the anti-serum (NIH-rLH-S10) was added to all the tubes (except tube 1 and 2). The tubes were incubated at 4°C for 24 hours.

Day 2.

Radiolabelled LH with total counts/min. of between 15-20,000/50µl was then added. Egg white/merthiolated phosphate buffer was used to achieve the correct dilution and again the tubes were incubated at 4°C for a further 24 hours.

Day 3.

The precipitating serum, donkey-anti-rabbit immunoglobulin G, was used with 0.1% $NaNH_3$ at a dilution of 1:24 in merthiolated phosphate buffer/EDTA.

Day 4.

500µl of egg white/merthiolated phosphate buffer was added to all the tubes (except tube1) which were then centrifuged at 4000rpm for one hour at 4°C. The supernatant was then drained and the sedimented pellets allowed to dry overnight. The totals were neither spun nor counted.

Day 5.

The pellets were measured for LH radioactivity using an LKB rack gamma counter. The sensitivity of the assay was 10 pg/tube (1ng/ml) and the inter- and intra-assay co-efficients of variation were 8.0% and 9.5% respectively.

Tube No.	No	Sample (µl)	EW/MPS (µl)	SD (µl)	A/S (µl)	*LH (µl)	PPS (µl)	EW/MPS (µl)
1. Total	3	-	-	-	-	50	-	-
2. Blank	3	-	110	50	-	50	50	500
3. Zero	3	-	110	-	50	50	50	500
4-11. Curve I	3	10	100	-	50	50	50	500
12-n. Sample	2	20	100	-	50	50	50	500
Curve II	2	10	100	-	50	50	50	500

Table 2.4. The schedule and reagents used for the assay. EW/MPS = egg white/merthiolated phosphate buffer; SD = serum diluent; A/S = anti-LH antibody in serum diluent; *LH = ^{125}I-LH; PPS = precipitating serum.

LH radioimmunassay reagents were obtained from the National Hormone and Pituitary Program (Baltimore, Maryland, USA).

Statistics

Tukey's one-way analysis of variance (MINITAB for Windows, 10) was performed on the hypothalamic monoamine results. When the F-test was significant, it was followed by the Kruskal-Wallis non-parametric test in groups where standard deviations were large between means. The Mann-Whitney U test was utilised to analyse the 5-HT and 5-HIAA results obtained from the urethane-anaesthetised animals (Experiment I), because in some cases the levels of these amines were undetectable; these were entered into the data sheet as zero.

Turnover estimation

Concentrations of each neurotransmitter and metabolite were first worked out in pg amine/µg protein as explained previously. The ratios of DHPG to NA and

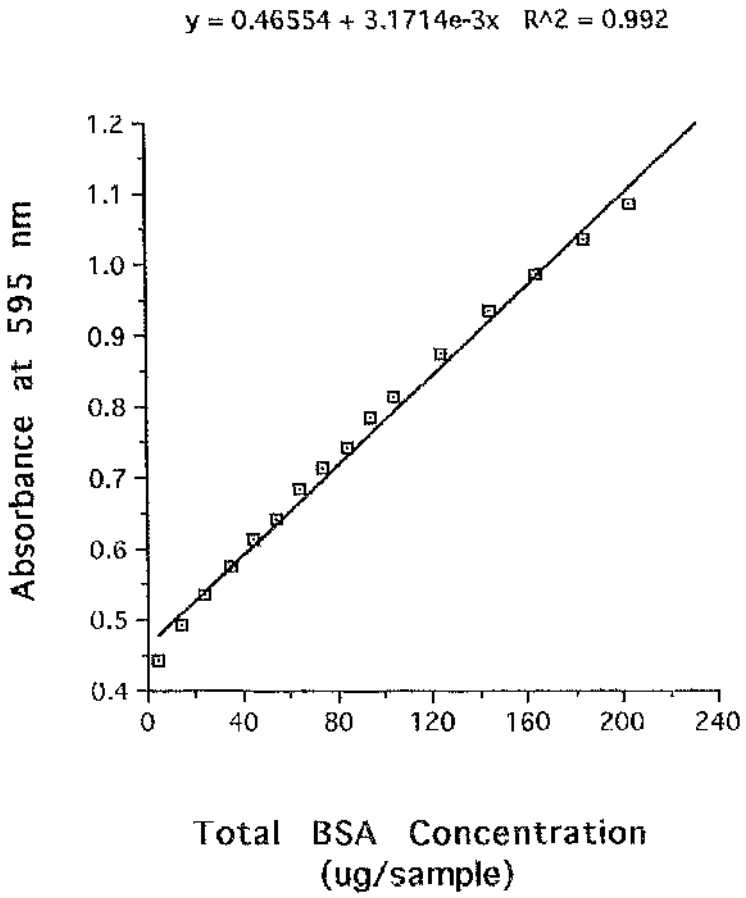


Figure 2.9. Standard curve of absorbance at 595nm after reaction with Coomassie Blue G250 versus concentration of bovine serum albumin (BSA).

5-HIAA to 5-HT were then calculated from the individual values obtained. The statistical analysis carried out on the results was as explained above.

Analysis of hormone results

In most cases, statistical analysis could not be performed on the LH results because of the lack of variables in the groups. In Experiment I, plasma LH levels were always below the limit of detection. In Experiment II, plasma LH levels were mostly undetectable in the drug-treated groups. Also, at some sampling intervals blood samples could not be obtained due to the spontaneous blockage of the cannula (see Tables 4.5. to 4.11.). ANOVA and Kruskal-Wallis non-parametric test were utilised to analyse the changes in plasma LH levels between the saline and diamorphine-treated groups in Experiment II. In these cases, individual values at all sampling intervals (between 15.00h and 19.00h) were used for comparisons. In Experiment III, plasma LH levels were mostly undetectable even in some of the control group animals (Tables 5.5 to 5.13).

EXPERIMENT I

Effects of various opioid agonists and antagonists on the hypothalamic monoamine concentrations during the pre-ovulatory LH surge in the urethane-anaesthetised rat

The GnRH pulse generator involves a complex neural circuitry and feedback effects of the gonadal steroids. EOPs have been reported to exert a profound inhibitory influence on LH release. Although direct connections have been demonstrated between the opioid peptidergic and GnRH neurons in the hypothalamus, effects of opioids on LH secretion appears to be indirect. It has been suggested that opioid inhibition of the pre-ovulatory LH surge involves alterations in the central monoaminergic neurotransmission.

This experiment was designed to investigate the effects of various selective opioid receptor agonists and antagonists, administered just before the critical period on the day of pro-oestrus, on hypothalamic monoamine concentrations and plasma LH levels in the intact female rat.

Any relationship between the opioid peptidergic system and the aminergic neurotransmitters in the MPOA, SCN, ME and ARN in the central regulation of LH secretion was also investigated. These specific hypothalamic regions were studied to establish the effective site of action of the opioids on the amine content and LH release.

This method was chosen such that it would allow us to serially collect blood samples throughout the afternoon of pro-oestrus for LH determination by RIA and to obtain specific regions of the hypothalamus by micropunch at the end of the LH surge.

Materials and Methods

Pro-oestrous rats were selected for experimentation in the morning. Following anaesthesia with urethane at around 12 noon, a heparinised cannula was inserted into the right carotid artery. The animals were injected IP with either diamorphine (3mg/kg), U-50488H (10mg/kg), DPDPE (1mg/kg), U-50488H plus MR2266 (5mg/kg) or DPDPE plus ICI 174,864 (1mg/kg) at 13.00h. Controls received physiological saline (1ml/kg) IP alone.

Subsequently, trunk blood (200µl) was collected via the indwelling cannula at hourly intervals throughout the afternoon of pro-oestrus. The red blood cells were reconstituted with 100µl of warmed, heparinised saline (10 units/ml) and re-injected through the cannula to maintain a constant plasma volume. The surgical

anaesthesia was maintained by periodic IP injections of urethane. The animals were kept warm throughout the experiment by the use of a heating lamp and were killed by decapitation just after the final blood collection at 19.00h. The experimental design is explained in details in the Materials and Methods. The number of animals in each group is shown in the Tables and Figures.

Results

The monoamine results are summarised in Tables 3.1 to 3.4. and Figures 3.1 to 3.14.

a) Noradrenaline

Control values found for NA concentrations (Mean ± SEM) in the MPOA, SCN, ME and ARN of the urethane-anaesthetised rats at 19.00h on pro-oestrus were 13.1 ± 1.2, 12.1 ±2.1, 13.6 ± 2.4 and 16.7 ± 1.5 pg/μg protein respectively.

(i) Diamorphine

Diamorphine did not significantly alter NA concentrations in the MPOA, SCN and ME, however, it significantly reduced the amine levels in the ARN compared to those seen in the control animals.

(ii) U-50488H

Similarly, this κ-opioid agonist also significantly lowered NA concentrations in the ARN compared to the control group values, but it did not have any significant effect in the MPOA, SCN and ME.

(iii) U-50488H + MR2266

This treatment significantly increased NA levels in the SCN and ARN compared to the group which received U-50488H alone. The NA levels were not significantly changed in the MPOA and ME.

(iv) DPDPE

Administration of this δ-opioid agonist resulted in significant decreases in NA concentrations in the MPOA, SCN and ARN compared to the saline-treated animals, but it had no significant effect in the ME.

(v) DPDPE + ICI 174,864

When DPDPE was co-administered with ICI 174,864, NA concentrations were significantly raised only in the SCN compared to the group receiving DPDPE alone. This treatment did not significantly alter the amine levels in the other hypothalamic regions examined.

b) 3,4-Dihydroxyphenylglycol

DHPG concentrations (Mean ± SEM) in the MPOA, SCN, ME and ARN of the urethane-anaesthetised control animals at 19.00h on pro-oestrus were 13.2 ± 2.3, 11.1 ± 2.1, 15.0 ± 3.1 and 11.0 ± 1.4 pg/μg protein respectively.

(i) Diamorphine

DHPG concentrations were significantly reduced by the μ-opioid agonist in the MPOA and ARN in comparison to the control values. Diamorphine had no significant effect in the SCN and ME.

(ii) U-50488H

Administration of U-50488H brought about significant decreases in DHPG concentrations in the MPOA, ME and ARN compared to the saline-treated animals, but not in the SCN.

(iii) U-50488H + MR2266

Treatment with the κ-opioid agonist and antagonist significantly increased DHPG levels only in the MPOA compared to the group which received U-50488H alone. The levels of this NA metabolite were not significantly altered in the SCN, ME and ARN.

(iv) DPDPE

DPDPE significantly lowered DHPG concentrations in all the hypothalamic regions examined compared to the values seen in the control animals.

(v) DPDPE + ICI 174,864

When DPDPE was co-administered with the δ-opioid antagonist, DHPG levels were significantly elevated in the SCN and ARN compared to the group receiving DPDPE alone. However, this treatment had no significant effect in the MPOA and ME.

c) Dopamine

Control values for DA concentrations (Mean ± SEM) in the MPOA, SCN, ME and ARN of the urethane-anaesthetised animals at 19.00h on pro-oestrus were 3.4 ± 0.7, 4.2 ± 1.2, 9.3 ± 3.3 and 5.0 ± 0.9 pg/μg protein respectively.

(i) Diamorphine

DA levels were significantly decreased by diamorphine only in the ARN compared to the saline-treated animals. The amine levels were unaffected in the MPOA, SCN and ME in this group.

(ii) U-50488H

The κ-opioid agonist significantly reduced DA concentrations only in the MPOA, but not in the SCN, ME and ARN, in comparison to the control values.

(iii) U-50488H + MR2266

Co-administration of U-50488H with MR2266 significantly reversed the inhibitory effects of U-50488H on DA concentrations in the MPOA. However, the amine levels were not significantly altered in the SCN, ME and ARN of this group.

(iv) DPDPE

Administration of DPDPE produced significantly lower concentrations of DA in the MPOA, SCN and ARN compared to the control group values. This δ-opioid agonist had no significant effect in the ME.

(v) DPDPE + ICI 174,864

This treatment did not significantly alter DA levels in any of the hypothalamic regions examined.

d) 5-Hydroxyindoleacetic Acid

Concentrations (Mean ± SEM) of 5-HIAA in the MPOA, SCN, ME and ARN of the urethane-anaesthetised control animals at 19.00h on pro-oestrus were 1.1 ± 0.8, 4.2 ± 3.5, 1.6 ± 0.6, 1.5 ± 0.7 pg/μg protein respectively.

(i) Diamorphine

(ii) U-50488H

(iii) U-50488H + MR2266

(iv) DPDPE

(v) DPDPE + ICI 174,864

None of the above drug regimes did significantly affect 5-HIAA concentrations in any of the four hypothalamic areas studied.

e) Serotonin

Control values obtained for 5-HT in the MPOA, SCN, ME and ARN of the urethane-anaesthetised rats at 19.00h on pro-oestrus were 2.3 ± 1.0, 1.8 ± 0.8, 2.6 ± 0.9 and 4.3 ± 1.3 pg/μg protein ± SEM respectively.

(i) Diamorphine

5-HT concentrations were not significantly changed by diamorphine in any of the hypothalamic regions examined.

(ii) U-50488H

The κ-opioid agonist significantly reduced 5-HT levels in the MPOA and ARN compared to the saline-treated animals, but had no significant effect in the SCN and ME.

(iii) U-50488H + MR2266

When U-50488H was co-administered with MR2266, 5-HT concentrations were significantly raised in the MPOA and ME compared to the group receiving U-50488H alone. This treatment did not significantly change the indoleamine levels in the SCN and ARN.

(iv) DPDPE

Administration of the δ-opioid agonist caused significant reductions of 5-HT concentrations compared to those seen in the control animals in the ME and ARN, but had no significant effect in the MPOA and SCN.

(v) DPDPE + ICI 174,864

This treatment produced significantly higher concentrations of 5-HT only in the ME compared to the DPDPE-treated animals. However, the 5-HT levels were not significantly changed in the MPOA, SCN and ARN.

f) Ratio of DHPG/NA

Control group values for the ratio of DHPG/NA in the MPOA, SCN, ME and ARN of the urethane-anaesthetised animals at 19.00h on pro-oestrus were 1.001 ±

0.119, 0.969 ± 0.171, 1.094 ± 0.100 and 0.678 ± 0.091 respectively (results expressed as Mean ± SEM).

(i) Diamorphine
The μ-opioid agonist did not significantly alter the ratio of DHPG to NA in any of the hypothalamic region examined.

(ii) U-50488H
U-50488H significantly lowered the ration of DHPG/NA in the MPOA and ME compared to the control group values, but had no significant effect in the SCN and ARN.

(iii) U-50488H + MR2266
Co-administration of the κ-agonist with the κ-antagonist significantly increased the ratio of DHPG/NA only in the SCN compared to the group which received U-50488H alone. The ratio was unaffected in the MPOA, ME and ARN.

(iv) DPDPE
DPDPE significantly reduced the ratio of DHPG/NA compared to those seen in the saline-treated animals in the ME and ARN, but not in the MPOA and SCN.

(v) DPDPE + ICI 174,864
This treatment did not produce significant alterations in the ratio of DHPG/NA in any of the hypothalamic regions examined.

g) Ratio of 5-HIAA/5-HT

The ratio of 5-HIAA/5-HT in the MPOA, SCN, ME and ARN of the urethane-anaesthetised control group at 19.00h on pro-oestrus were 0.323 ± 0.144, 1.217 ± 0.918, 0.712 ± 0.211 and 0.310 ± 0.129 respectively (results expressed as Mean ± SEM).

(i) Diamorphine
(ii) U-50488H
(iii) U-50488H + MR2266
(iv) DPDPE
(v) DPDPE + ICI 174,864

None of these drug regimes significantly changed the ratio of 5-HIAA/5-HT in any of the four hypothalamic areas studied.

h) Luteinising Hormone

In none of the groups plasma LH levels were detectable (including the controls). When the opioid agonists were administered alone or co-adminstered with their respective antagonists, again no detectable changes in LH levels were found.

Discussion

Luteinising Hormone

In the present study, the expected pre-ovulatory LH surge was completely abolished for seven hours on the afternoon of pro-oestrus in the urethane-anaesthetised and saline-treated rats.

It has been reported that activation of both μ- and κ-opioid subtypes by specific agonists brings about an inhibition of LH surge (Dyer *et al*, 1988). However, administration of μ-opioid agonists at low doses have been found to be stimulatory to LH release (Pang *et al*, 1977; Brown *et al*, 1994). Compared to work carried out μ- and κ-opioid subtypes, there have been less attempts to study the involvement of δ-opioid subtypes in the central control of LH secretion. Only moderate doses of opioid agonists were employed in the present study. Plasma LH levels were found to be undetectable at all sampling intervals following the administration of diamorphine, U-50488H or DPDPE on the afternoon of pro-oestrus. Moreover, co-administration of U-50488H with the specific κ-opioid antagonist or of DPDPE with a selective δ-opioid antagonist did not bring about any detectable changes in LH levels either. Therefore, it is difficult to interpret these findings and draw any conclusions regarding which opioid receptor subtypes may be involved in opioid inhibition of the LH surge.

These results have led us to hypothesise that it was the urethane anaesthesia which inhibited the pre-ovulatory LH surge in even the control animals. Previously reported doses of urethane were used to induce surgical anaesthesia. Our attempt in employing urethane as an anaesthetic in neuroendocrine experiments was based on the reports of de Greef *et al* (1987), who claimed that the urethane

administration did not prevent the LH surge. The clear discrepancies between these studies may be a result of the use of different strains of rats; the de Greef group used a hybrid strain, bred at their own institution. In addition, the rats were ovx and steroid-primed. It is possible that the ovx hybrid strain may be more susceptible to anaesthesia, or their neural circuitry involved in the LH surge more resistant to pharmacological suppression, than the intact pro-oestrous Sprague-Dawley rats used in the present study.

However, the mechanisms by which urethane inhibits the LH surge are unknown. It could be either by reducing the responsiveness of the gonadotrophs to the hypothalamic GnRH or by altering the GnRH neuronal activity. Since urethane is a centrally-acting anaesthetic, it seems likely that its site of action would be within the brain. Indeed, it has recently been reported that urethane anaesthesia decreases neuronal activity by promoting expression of Fos protein in the rat CNS (Takayama *et al,* 1994). It appears that urethane anaesthesia may suppress the neural stimulus necessary for LH release. This therefore suggests that 13.00h, the time around which the animals were anaesthetised, is still within the critical period for the initiation of the pre-ovulatory LH surge on the early afternoon of pro-oestrus. It has previously been shown that drugs may abolish the LH surge and ovulation if administered during a particular time period on pro-oestrus (Everett *et al,* 1949). After this time, the LH surge has been found to be resistant to pharmacological blockade.

There have been many reports in the literature regarding the use of anaesthetics in neuroendocrine experiments and several anaesthetics are suspected to influence LH release in the female rat (Sherwood *et al,* 1980; Dyer and Mansfield, 1984; de Greef *et al,* 1987; Hartman *et al,* 1989; Strutton and Coen, 1996). However, the results are conflicting. Kimura and Sano (1995) have suggested that separate GnRH pulse and surge generators exist in the brain. They demonstrated that pentobarbitone administered in the afternoon to ovx-steroid-primed rats inhibited the ensuing LH surge, while it had no effect on pulsatile LH release when given in the morning of the same day. Urethane may be similar since we have shown that it abolishes the LH surge, while others have found it did not prevent the occurrence of LH pulses although it reduced their amplitude (de Greef *et al,* 1987; Matzen *et al,* 1987). It is possible that urethane arrested the GnRH surge generator by decreasing the stimulatory noradrenergic input, since it significantly reduced NA levels in all areas investigated. In a similar study, Hartman *et al* (1989) showed

that amplified LH release occurred in urethane-anaesthetised rats following icv infusion of exogenous NA. Even greater increases were found in plasma LH levels after electrochemical stimulation of the MPOA in conjunction with icv NA administration to these rats (Hartman *et al,* 1989). It seems therefore that the exogenous NA infusion may have replaced the loss in NA concentrations brought about by the urethane anaesthesia. Pentobarbitone and chloral hydrate have also been found to reduce NA turnover and release respectively, concomitant with their inhibition of the LH surge (Rance and Barraclough, 1981).

Recently, it has been suggested that primary cause of LH pulse suppression in the anaesthetised animals may be hypothermia (Strutton and Coen, 1996). Administration of pentobarbitone at the standard laboratory temperature of 21°C induced hypothermia and a concurrent inhibition of plasma LH levels in the female Wistar rats. However, when the same experiment was performed at an ambient temperature of 35°C, which precludes the induction of hyperthermia, the suppressive effects of pentobarbitone were no longer observed. This suggests that hypothermia may disrupt LH pulses. These findings would therefore point to the importance of ambient temperature during such experiments. In this study, the surgical anaesthesia and the experiments were performed at standard ambient temperature (21±1°C). The animals were kept warm by the use of a heating lamb throughout the experiment, however, the body temperature was not monitored. In a different study, pentobarbital has been shown to inhibit LH release by a non-specific mechanism, inducing hypothermia in ovx rats (Simpkins and Katovich, 1987). Since pentobarbitone decreases the hypothalamic NA activity (Rance and Barraclough, 1981), it is possible that a reduction in the core temperature may reduce the noradrenergic input to the GnRH pulse generator. Interestingly, the same treatment had no inhibitory effects on LH secretion when the ovx rats were pre-treated with E_2 and P. These observations thus demonstrate that the response of the GnRH neurons is dependent on the steroidal state of the animal (Simpkins and Katovich, 1987). It is thought that occurrence of a hypothermia in the present experiment was unlikely. Unrecognised hypothermic effects of the treatments may also have been the cause of the pulse suppression.

It is concluded from the present experiment that care should always be taken when using general anaesthetics in neuroendocrine studies. Ambient temperature may have crucial effects on the drug treatments.

Hypothalamic Aminergic Activity

Monoamine content of the MPOA, SCN, ME and ARN was measured by the HPLC-ECD at 19.00h on the afternoon of pro-oestrus. Of the amines investigated, DHPG and 5-HIAA were the only metabolites which appeared within the detectable range of the HPLC-ECD system employed. When increased concentrations of NA were detected, raised levels of DHPG were almost always observed. This indicates that when the release of the neurotransmitter is elevated, its synthesis is also stimulated by an autoregulation mechanism (For review see Kruk and Pycock, 1993). Surprisingly, such a close relationship was not seen between the concentrations of 5-HT and 5-HIAA in most instances. Their levels were generally low and in some of the hypothalamic samples below the limit of detection. None of the DA metabolites could be detected in the present study. Therefore, the conclusions regarding the central dopaminergic transmission, if any, to be made from this experiment will solely be based on the neurotransmitter levels found. These premises will be reviewed in more detail in the General Discussion.

Opioid-induced decrease in the hypothalamic NA content and turnover has been reported (Dyer *et al,* 1988). It has also been demonstrated that morphine inhibits electrically stimulated NA release from slices of MPOA *in vitro* (Diez-Guerra *et al,* 1987). In the present study, DPDPE was the only drug which significantly reduced NA concentrations compared to control group values in the MPOA. However, DHPG levels were significantly lowered by all three opioid receptor agonists investigated in this hypothalamic area. Moreover, the falls in the NA metabolite's concentrations were prevented by the selective κ- and δ-opioid receptor antagonists following their co-administration with U-50488H and DPDPE, respectively. It has been shown that the noradrenergic nerve terminals contain a mixed population of the opioid receptors in the MPOA as well as in the several other hypothalamic areas. It is possible that although limited to some extent, the opioid peptides may have modulatory effects on the noradrenergic neurotransmission in this hypothalamic area. Since no detectable changes were observed in plasma LH levels in these animals, no correlation can be made between the changes in the hypothalamic NA or DHPG concentrations and the GnRH secretory system.

Neither diamorphine nor U-50488H caused any significant changes in the NA levels in the SCN and ME. However, they both reduced NA levels in the ARN.

It seems that both the opioid agonists have area-dependent inhibitory effects on the NA release. Surprisingly, the δ-opioid agonist reduced the NA concentrations in the SCN and ARN, but was ineffective in the ME. Its effects were reversed by ICI 174,864 only in the SCN, indicating a specific δ-opioid modulation in this area. Indeed, the highest density of δ-opioid binding has been visualised in the SCN (Desjardins *et al,* 1990). DHPG concentrations were selectively reduced by DPDPE in the SCN, ME and ARN, by U-50488H in the ME and ARN, by diamorphine only in the ARN. Administration of morphine lowers the NA turnover in the hypothalamus (Kalra *et al,* 1989), but dual effects of κ-opioid agonists have been reported (Brown *et al,* 1994). These results seem to favour a modulatory role for the μ- and κ-opioid receptors on the noradrenergic neurotransmission in the ARN, however, it is difficult to compromise the findings from the other hypothalamic areas examined. Prevention of the decreases in the DHPG levels by the selective δ-antagonist would point to a specific δ-opioid action in the SCN and ARN.

It has been postulated that the opioid peptides have both stimulatory and inhibitory effects on the dopaminergic system. Administration of morphine reduces DA synthesis and turnover in the ME by inhibiting activity of the TIDA neurons (Alper *et al,* 1980; Akabori and Barraclough, 1986). *In vitro*, the stimulation of both κ- and μ-opioid receptors with their respective agonists decreases DA release from the MBH (Heijna *et al,* 1991). On the contrary, morphine has been shown to activate the incertohypothalamic dopaminergic neurons and increase the DA turnover (Gopalan *et al,* 1989a). In the present study, none of the drug treatments had any significant effect on the dopaminergic activity in the SCN and ME. However, DA levels were selectively lowered by DPDPE in the MPOA and ARN and by diamorphine only in the ARN. The only significant effect of the κ-opioid agonist and antagonist on the DA levels was observed in the MPOA. This suggests a specific modulatory action of κ-opioid receptors in this area. However, these results seem to be inconclusive regarding inhibition of the dopaminergic activity in discrete hypothalamic regions via specific opioid receptor subtypes.

An inhibitory action of opioid peptides on the serotonergic system in the brain has been suggested. Activation of κ-, but not μ-opioid receptors has been shown to decrease the response of 5-HT-sensitive neurons to electrical stimulation in the raphe nucleus (Pinnock, 1992). The presence of high numbers of various opioid receptors has been shown in this brain region (Mansour *et al,* 1988). Increased

turnover of 5-HT has been observed in the SCN and AHA following administration of κ-and σ-, but not μ-opioid agonists (Gopalan *et al,* 1989b). However, the same treatments did not have any effect in the MPOA, ME and ARN. In the present study, none of the drug regimes employed significantly altered 5-HIAA concentrations in any of the hypothalamic areas examined. The μ-agonist, diamorphine, was ineffective in causing any significant changes in hypothalamic 5-HT levels. There were isolated area-dependent effects in the rats treated with the κ- and δ-agonists. U-50488H reduced 5-HT levels in the MPOA and ARN; however, its effects were reversed by the κ-antagonist in only the MPOA. DPDPE also lowered the 5-HT content in the ME and ARN and its inhibitory effects were prevented by the δ-opioid antagonist in only the ME. It is suggested that the serotonergic transmission may be selectively mediated by opioid receptors other than those of the μ-subtype. The nature of intermodulatory action between the opioidergic and serotonergic systems still remains to be investigated.

In general, the monoamine levels obtained from the urethane-anaesthetised animals have been found to be lower than those values seen in the conscious rats as reported in the next chapter. Since urethane anaesthesia has been observed to reduce neuronal activity in the CNS (Takayama *et al,* 1994), it is possible that urethane itself may have interfered with the monoaminergic systems. However, the mechanisms underlying these postulated effects are at present unknown.

Neurotransmitter Concentrations in the MPOA							
Treatment	**NA**	**DHPG**	**DA**	**5-HIAA**	**5-HT**	**Ratio of DHPG/NA**	**Ratio of 5-HIAA/5-HT**
Saline (5)	13.1±1.2	13.2±2.3	3.4±0.7	1.1±0.8	2.3±1.0	1.001±0.119	0.323±0.144
Diamorphine (5)	11.2±1.8	6.0±1.6 **d**	2.0±0.4	1.7±0.6	1.2±0.5	0.557±0.171	0.982±0.379
U-50488H (7)	9.4±1.4	4.8±0.3 **a**	1.2±0.1 **b**	0.5±0.2	0.2±0.1 **b**	0.608±0.113 **d**	0.560±0.560
U-50488H+MR2266 (5)	13.5±1.7	8.3±1.0 **e**	1.8±0.2 **f**	0.9±0.2	2.9±0.3 **e**	0.682±0.144	0.326±0.090
DPDPE (6)	7.4±0.6 **b**	5.7±0.8 **c**	1.5±0.4 **d**	1.2±0.3	0.8±0.3	0.773±0.100	1.090±0.325
DPDPE+ICI174,864 (4)	10.9±1.8	6.1±0.2	1.4±0.2	1.4±0.4	2.0±0.8	0.609±0.112	0.662±0.084

Table 3.1. Monoamine concentrations (pg amine/μg protein ± SEM) and ratios of DHPG/NA and 5-HIAA/5-HT in the MPOA of the urethane-anaesthetised rats at 19.00h on pro-oestrus following administration of various opioid agonists and antagonists at 13.00h. **a:** $p<0.001$; **b**: $p<0.005$; **c**: $p<0.01$; **d**: $p<0.05$ compared to the saline-treated animals, **e:** $p<0.005$; **f**: $p<0.05$ compared to the U-50488H-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

Neurotransmitter Concentrations in the SCN							
Treatment	**NA**	**DHPG**	**DA**	**5-HIAA**	**5-HT**	**Ratio of DHPG/NA**	**Ratio of 5-HIAA/5-HT**
Saline (5)	12.1±2.1	11.1±2.1	4.2±1.2	4.2±3.5	1.8±0.8	0.969±0.171	1.217±0.918
Diamorphine (5)	7.4±2.1	8.2±1.7	3.0±0.7	3.1±2.1	0.9±0.5	1.538±0.640	3.356±2.160
U-50488H (7)	9±0.8	9.1±1.3	2.6±0.9	0.3±0.2	0.3±0.2	1.000±0.094	0.333±0.333
U-50488H+MR2266 (5)	13.5±1.2 **c**	8.2±0.7	4.2±3.1	0.6±0.5	2.0±0.9	0.634±0.088 **d**	0.226±0.226
DPDPE (6)	6.1±1.0 **b**	5.6±0.4 **a**	1.4±0.6	1.4±0.5	0.4±0.2	1.025±0.152	1.000±0.577
DPDPE+ICI174,864 (4)	10.3±1.4 **e**	7.4±0.7 **e**	1.6±0.3	1.3±0.1	1.0±0.6	0.747±0.106	1.487±0.519

Table 3.2. Monoamine concentrations (pg amine/μg protein ± SEM) and ratios of DHPG/NA and 5-HIAA/5-HT in the SCN of the urethane-anaesthetised rats at 19.00h on pro-oestrus following administration of various opioid agonists and antagonists at 13.00h. **a:** $p<0.02$; **b:** $p<0.05$ compared to the saline-treated animals, **c:** $p<0.01$; **d:** $p<0.05$ compared to the U-50488H-treated animals, **e:** $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

Neurotransmitter Concentrations in the ME							
Treatment	**NA**	**DHPG**	**DA**	**5-HIAA**	**5-HT**	**Ratio of DHPG/NA**	**Ratio of 5-HIAA/5-HT**
Saline (5)	13.6±2.4	15.0±3.1	9.3±3.3	1.6±0.6	2.6±0.9	1.094±0.100	0.712±0.211
Diamorphine (5)	11.5±3.4	8.3±1.7	4.5±2.6	0.6±0.3	1.2±0.5	0.778±0.101	0.557±0.255
U-50488H (7)	12.4±1.6	7.5±1.1 **b**	4.6±1.0	0.5±0.3	0.8±0.4	0.636±0.090 **a**	0.250±0.194
U-50488H+MR2266 (5)	15.2±1.6	10.5±1.2	5.4±1.0	1.1±0.5	3.5±0.4 **c**	0.731±0.125	0.368±0.173
DPDPE (6)	9.3±1.1	5.3±0.6 **a**	2.7±0.4	0.9±0.4	0.3±0.1 **b**	0.611±0.089 **a**	1.500±0.806
DPDPE+ICI174,864 (4)	13.4±1.4	8.9±2.2	5.7±1.8	1.9±0.4	1.8±0.5 **d**	0.739±0.278	1.209±0.257

Table 3.3. Monoamine concentrations (pg amine/μg protein ± SEM) and ratios of DHPG/NA and 5-HIAA/5-HT in the ME of the urethane-anaesthetised rats at 19.00h on pro-oestrus following administration of various opioid agonists and antagonists at 13.00h. **a:** $p<0.01$; b: $p<0.05$ compared to the saline-treated animals, **c**: $p<0.01$ compared to the U-50488H-treated animals, **d:** $p<0.01$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

Neurotransmitter Concentrations in the ARN							
Treatment	**NA**	**DHPG**	**DA**	**5-HIAA**	**5-HT**	**Ratio of DHPG/NA**	**Ratio of 5-HIAA/5-HT**
Saline (5)	16.7±1.5	11.0±1.4	5.0±0.9	1.5±0.7	4.3±1.3	0.678±0.091	0.310±0.129
Diamorphine (5)	9.1±1.8 **c**	6.2±1.0 **c**	1.5±0.2 **b**	0.7±0.5	1.1±0.5	0.771±0.156	0.208±0.208
U-50488H (7)	9.9±1.0 **b**	5.9±0.5 **b**	2.4±0.8	0.5±0.2	0.5±0.3 **c**	0.637±0.100	0.640±0.546
U-50488H+MR2266 (5)	17.3±3.5 **d**	7.7±0.7	2.8±0.9	1.7±0.8	2.7±0.9	0.536±0.124	0.439±0.203
DPDPE (6)	9.6±1.0 **b**	3.8±0.2 **a**	1.1±0.03 **a**	1.1±0.4	1.0±0.4 **c**	0.427±0.062 **c**	1.128±0.634
DPDPE+ICI174,864 (4)	13.2±3.1	6.7±0.5 **e**	1.6±0.3	2.2±1.0	3.2±1.6	0.588±0.136	0.663±0.226

Table 3.4. Monoamine concentrations (pg amine/μg protein ± SEM) and ratios of DHPG/NA and 5-HIAA/5-HT in the ARN of the urethane-anaesthetised rats at 19.00h on pro-oestrus following administration of various opioid agonists and antagonists at 13.00h. **a**: $p<0.001$; **b**: $p<0.005$; **c**: $p<0.05$ compared to the saline-treated animals, **d**: $p<0.05$ compared to the U-50488H-treated animals, **e**: $p<0.001$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

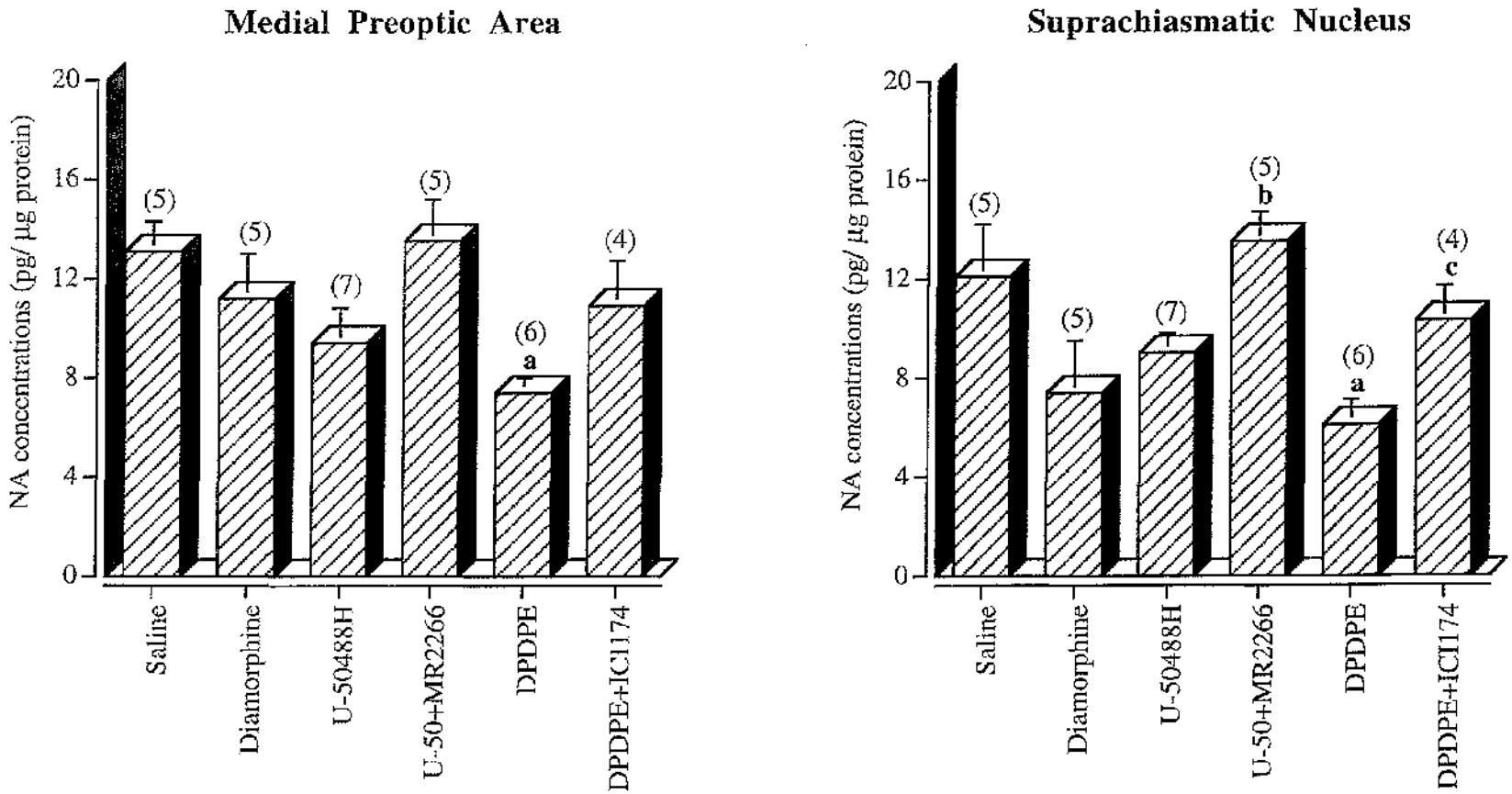


Figure 3.1. NA concentrations (pg/µg protein ± SEM) in the MPOA and SCN of urethane-anaesthetised female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.005$ compared to the saline-treated animals, **b:** $p<0.01$ compared to the U-50488H-treated animals, **c:** $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

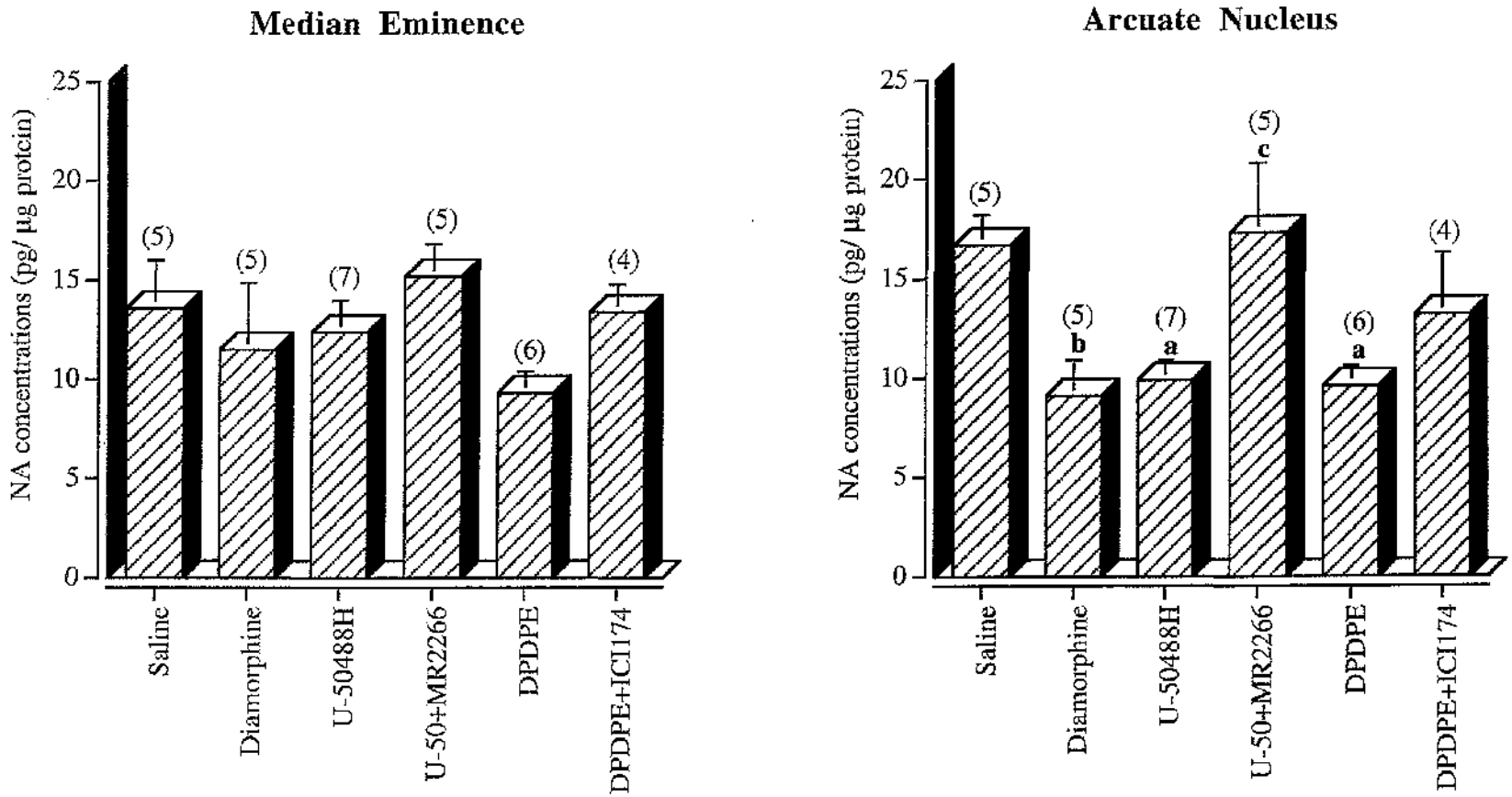


Figure 3.2. NA concentrations (pg/µg protein ± SEM) in the ME and ARN of urethane-anaesthetised female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.005$; **b:** $p<0.01$ compared to the saline-treated animals, **c:** $p<0.05$ compared to the U-50488H-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

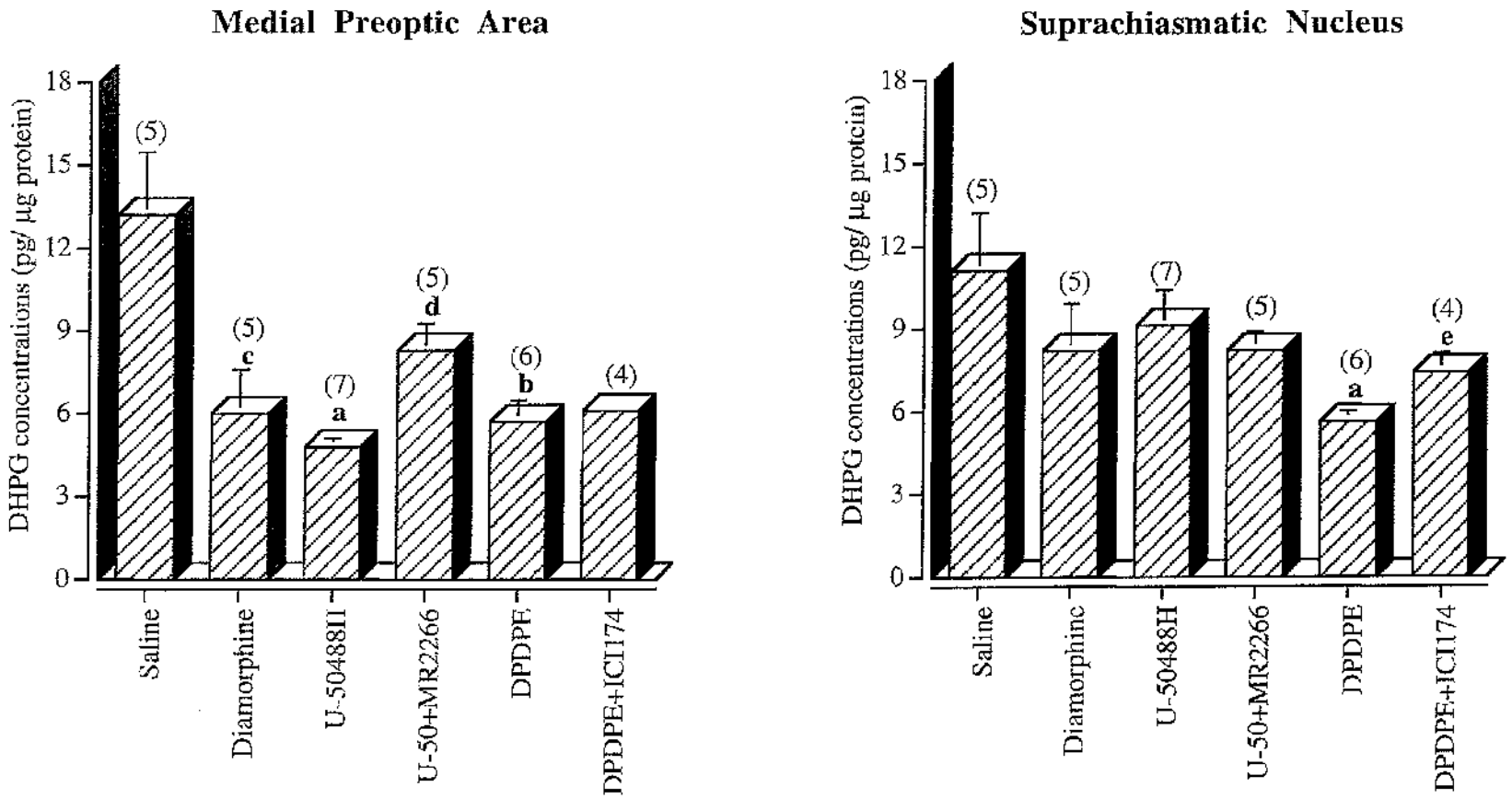


Figure 3.3. DHPG concentrations (pg/µg protein ± SEM) in the MPOA and SCN of urethane-anaesthetised female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.001$; **b:** $p<0.01$; **c:** $p<0.05$ compared to the saline-treated animals, **d:** $p<0.005$ compared to the U-50488H-treated animals, **e:** $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

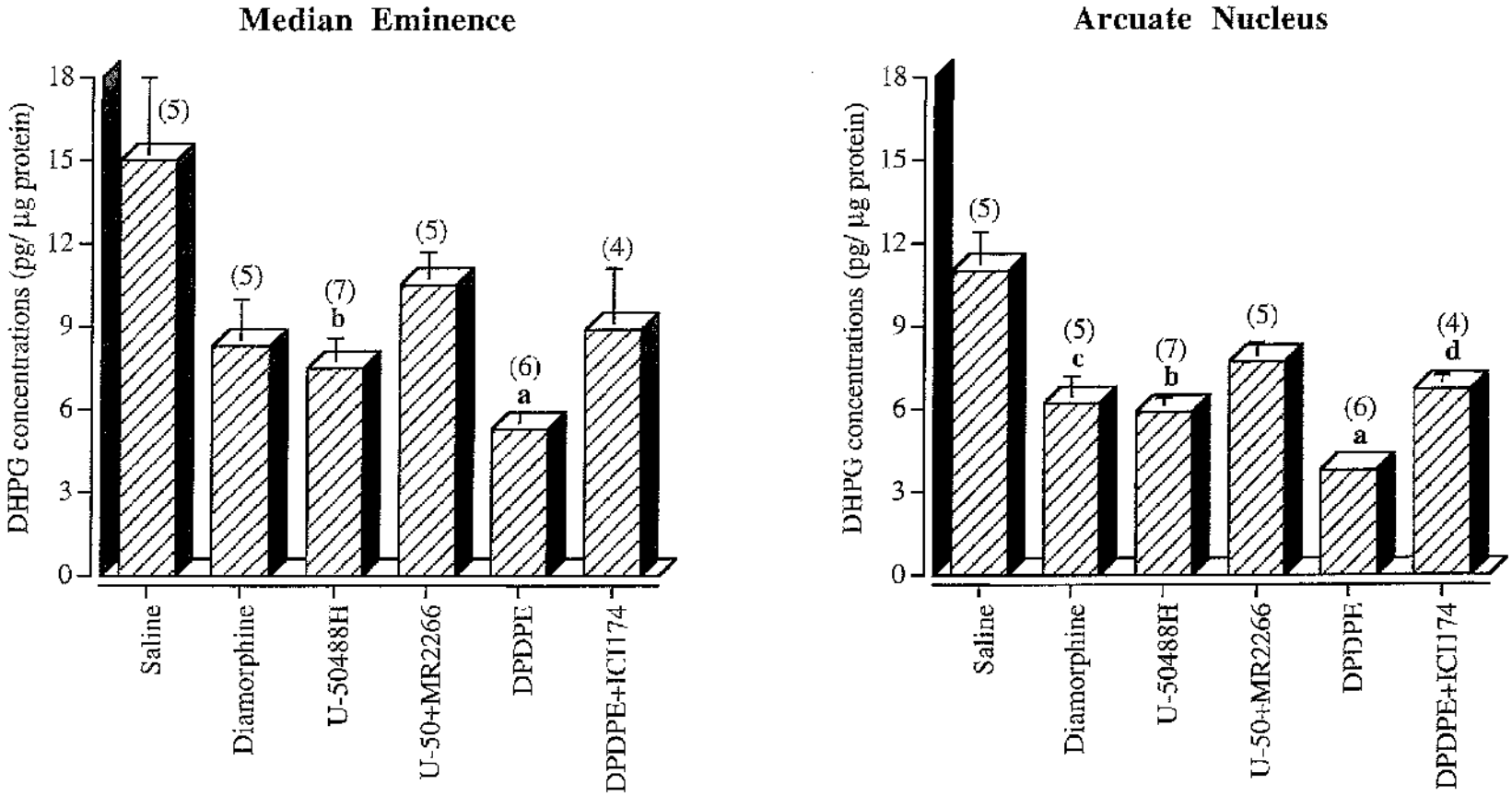


Figure 3.4. DHPG concentrations (pg/µg protein ± SEM) in the ME and ARN of urethane-anaesthetised female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.001$; **b:** $p<0.005$; **c:** $p<0.01$ compared to the saline-treated animals, **d:** $p<0.001$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

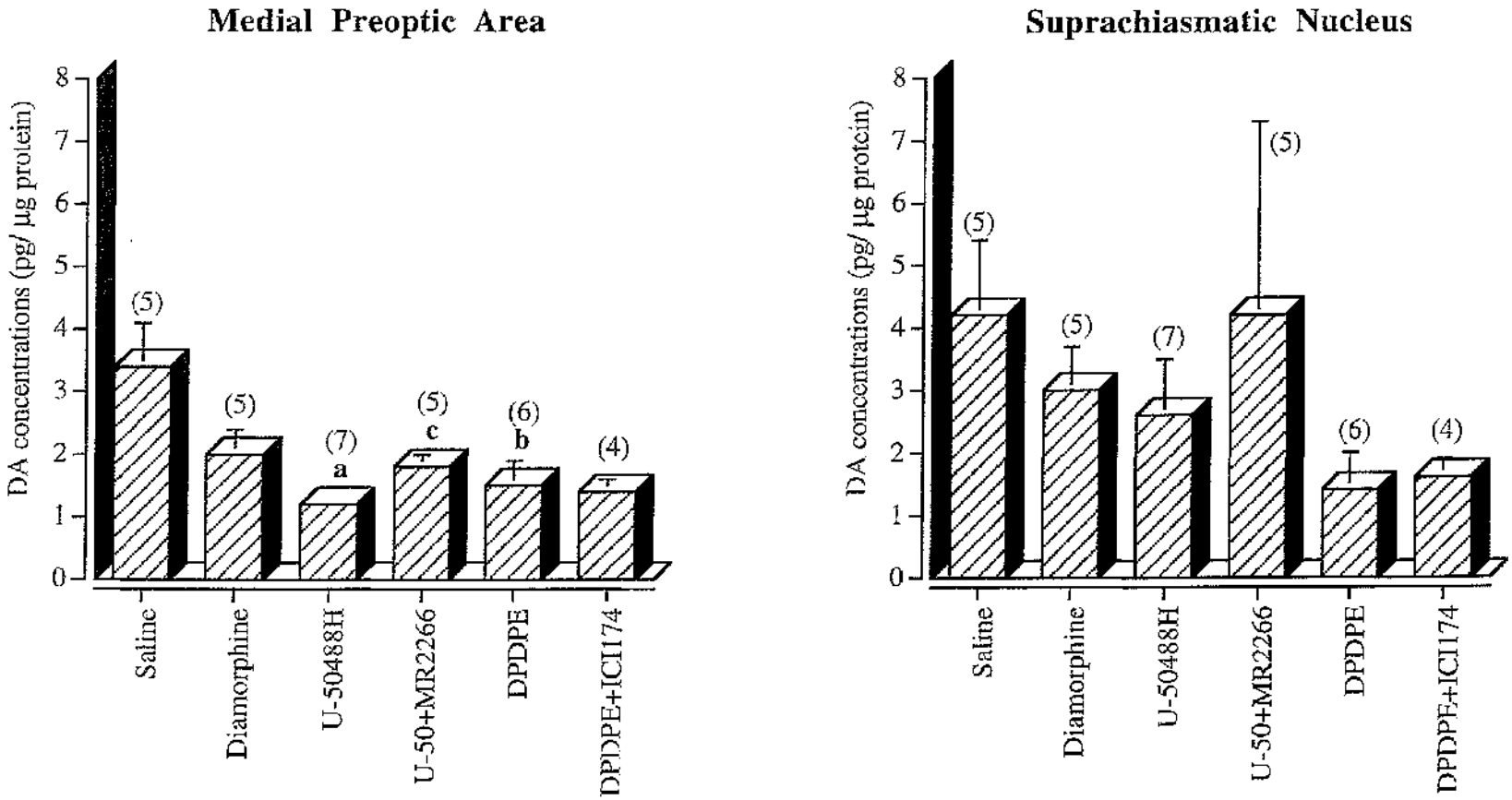


Figure 3.5. DA concentrations (pg/µg protein ± SEM) in the MPOA and SCN of urethane-anaesthetised female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.005$; **b:** $p<0.05$ compared to the saline-treated animals, **c:** $p<0.05$ compared to the U-50488H-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

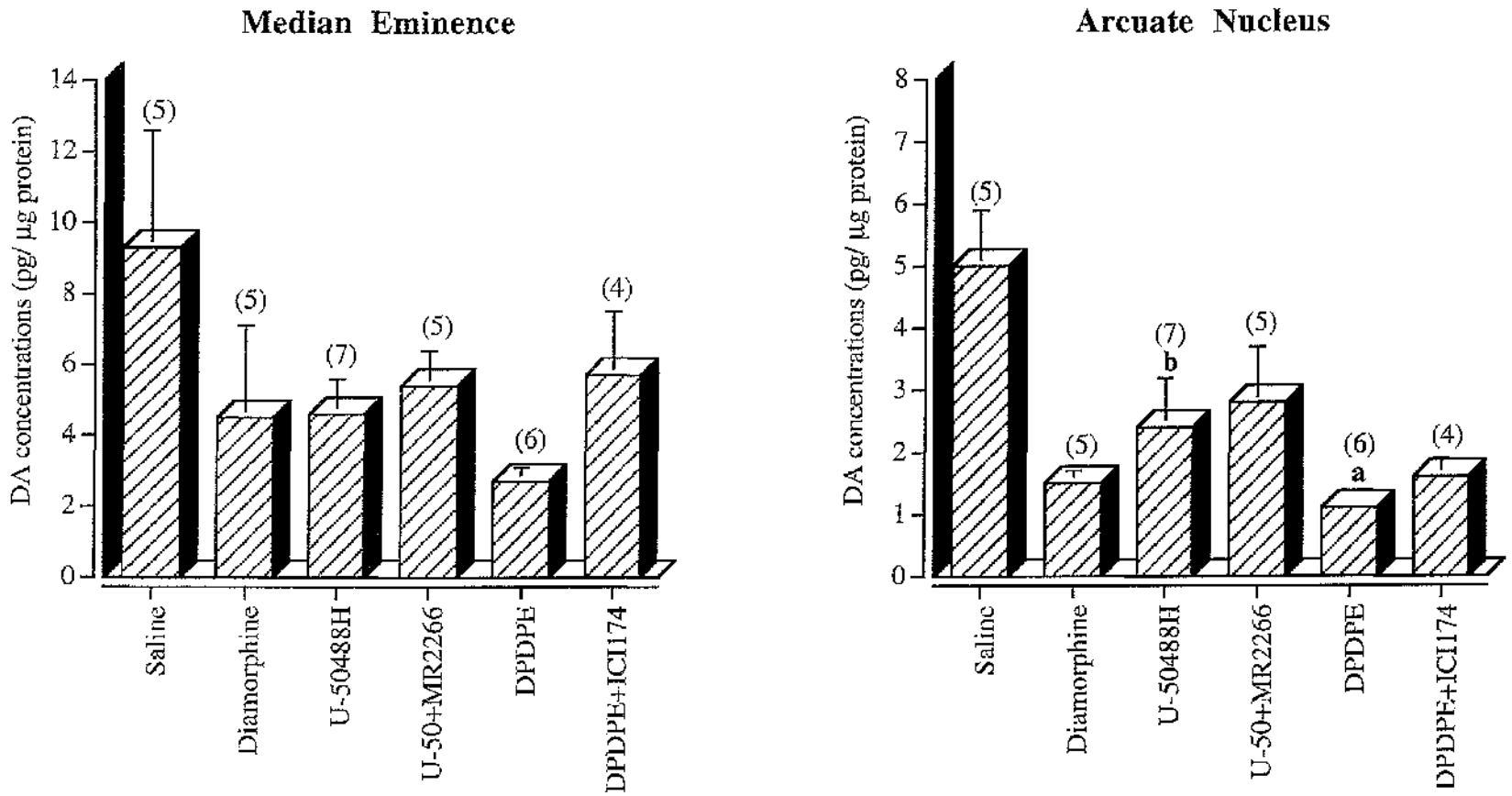


Figure 3.6. DA concentrations (pg/µg protein ± SEM) in the ME and ARN of urethane-anaesthetised female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.001$; **b:** $p<0.005$ compared to the saline-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

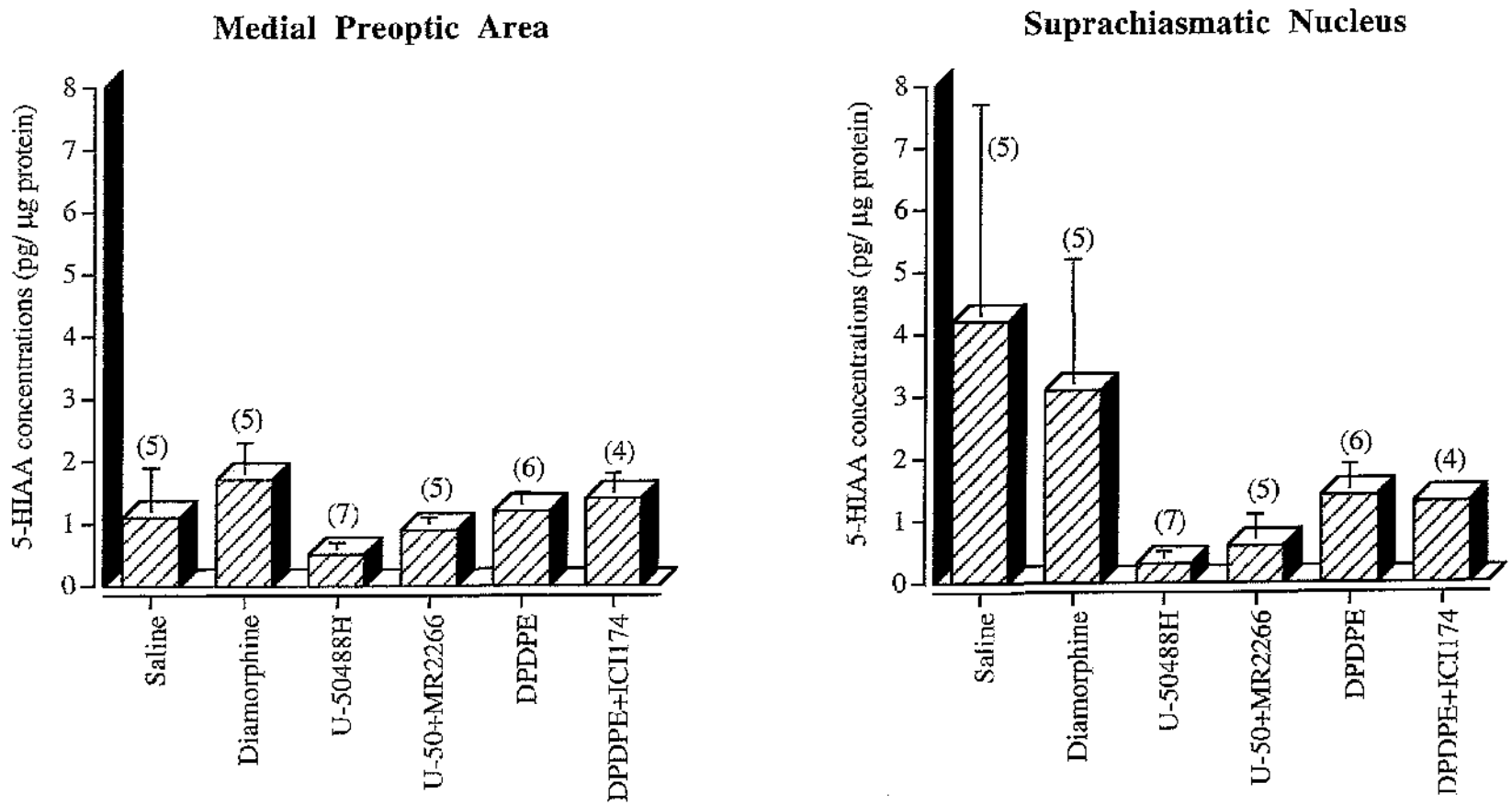


Figure 3.7. 5-HIAA concentrations (pg/µg protein ± SEM) in the MPOA and SCN of urethane-anaesthetised female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. The number of observations in each group is given in brackets.

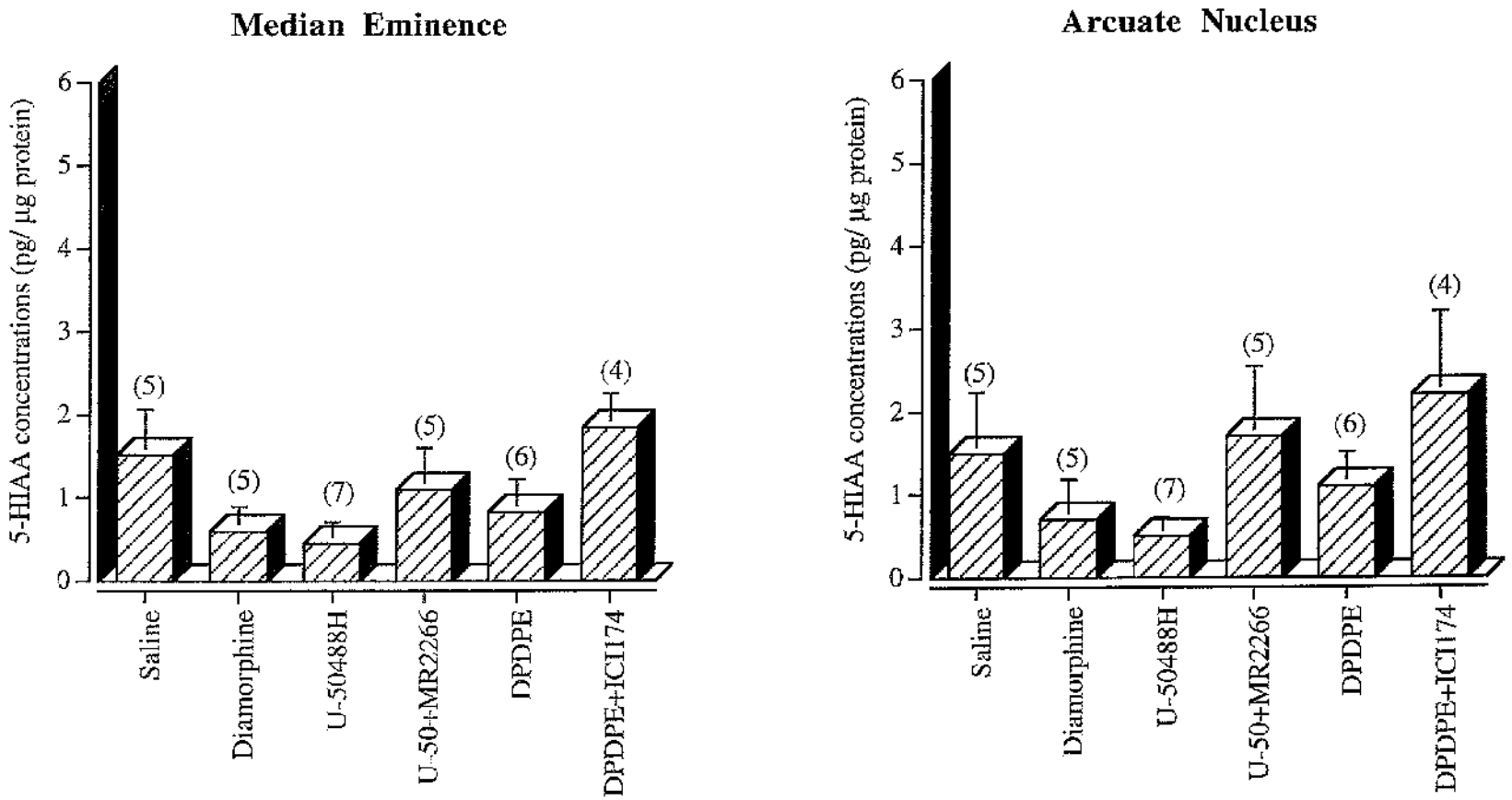


Figure 3.8. 5-HIAA concentrations (pg/µg protein ± SEM) in the ME and ARN of urethane-anaesthetised female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. The number of observations in each group is given in brackets.

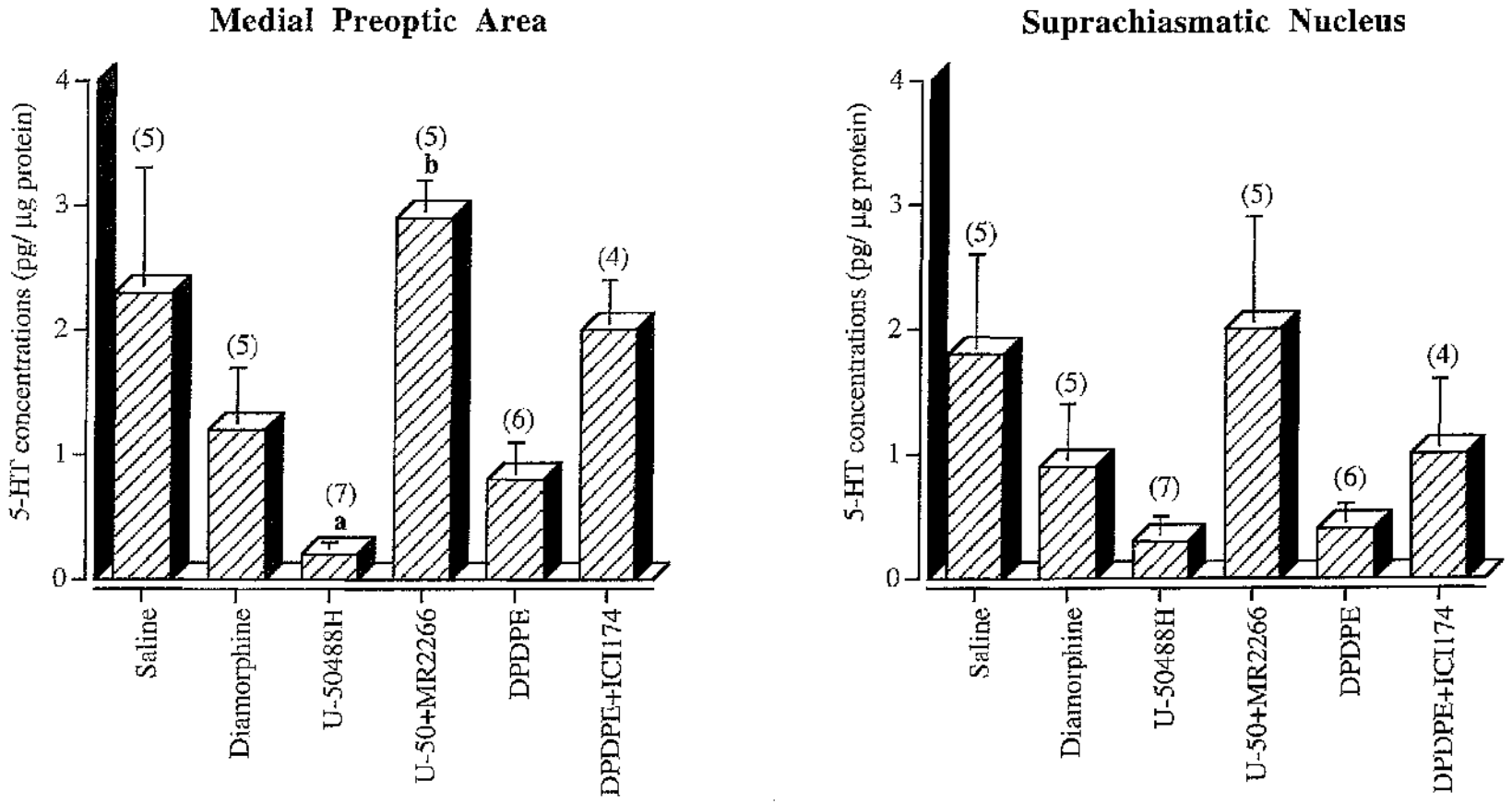


Figure 3.9. 5-HT concentrations (pg/µg protein ± SEM) in the MPOA and SCN of urethane-anaesthetised female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.005$ compared to the saline-treated animals, **b:** $p<0.005$ compared to the U-50488H-treated animals. One Way Analysis of Variance and the Mann-Whitney U test were utilised to examine the data. The number of observations in each group is given in brackets.

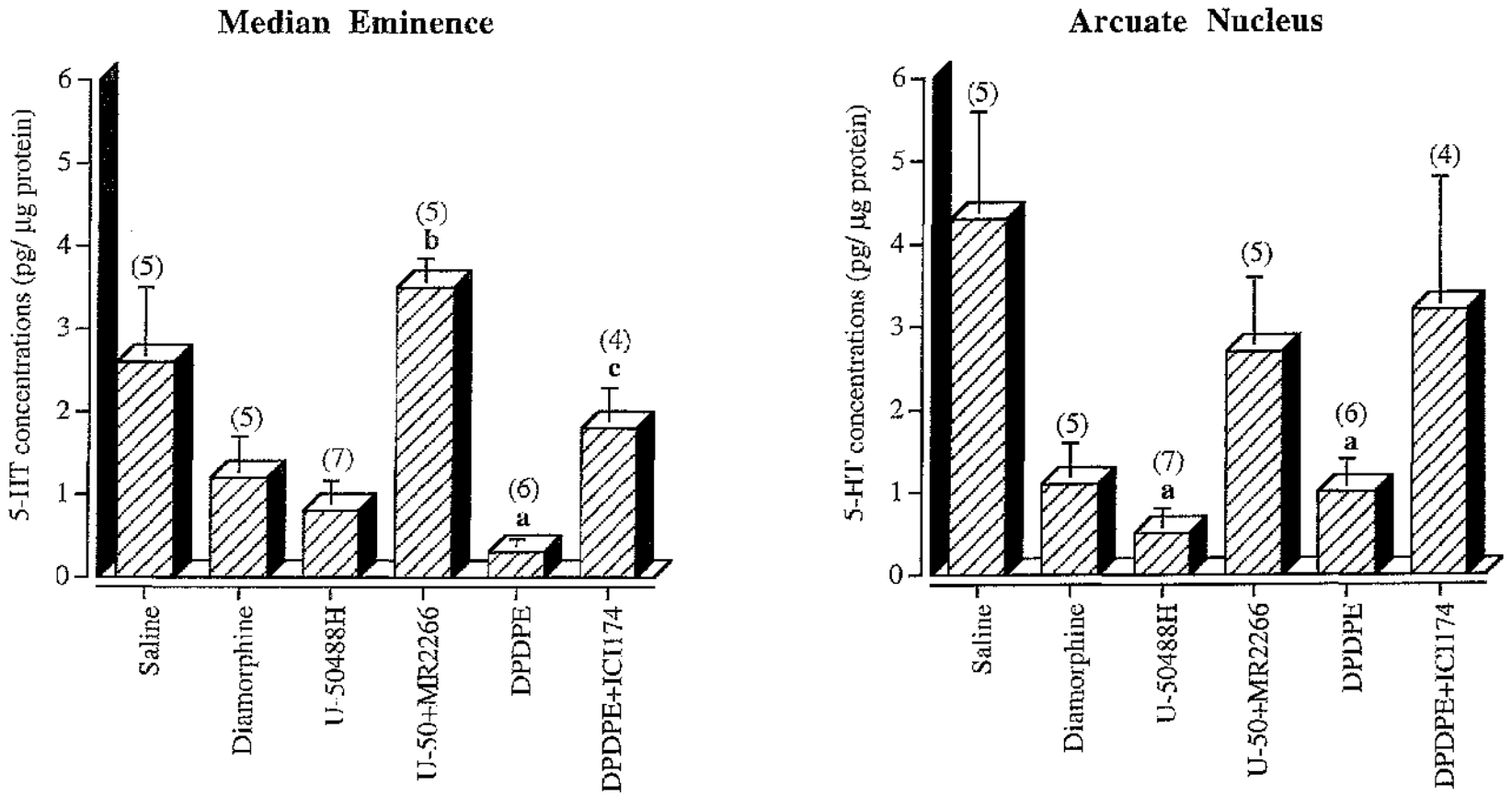


Figure 3.10. 5-HT concentrations (pg/µg protein ± SEM) in the ME and ARN of urethane-anaesthetised female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.05$ compared to the saline-treated animals, **b:** $p<0.01$ compared to the U-50488H-treated animals, **c:** $p<0.01$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Mann-Whitney U test were utilised to examine the data. The number of observations in each group is given in brackets.

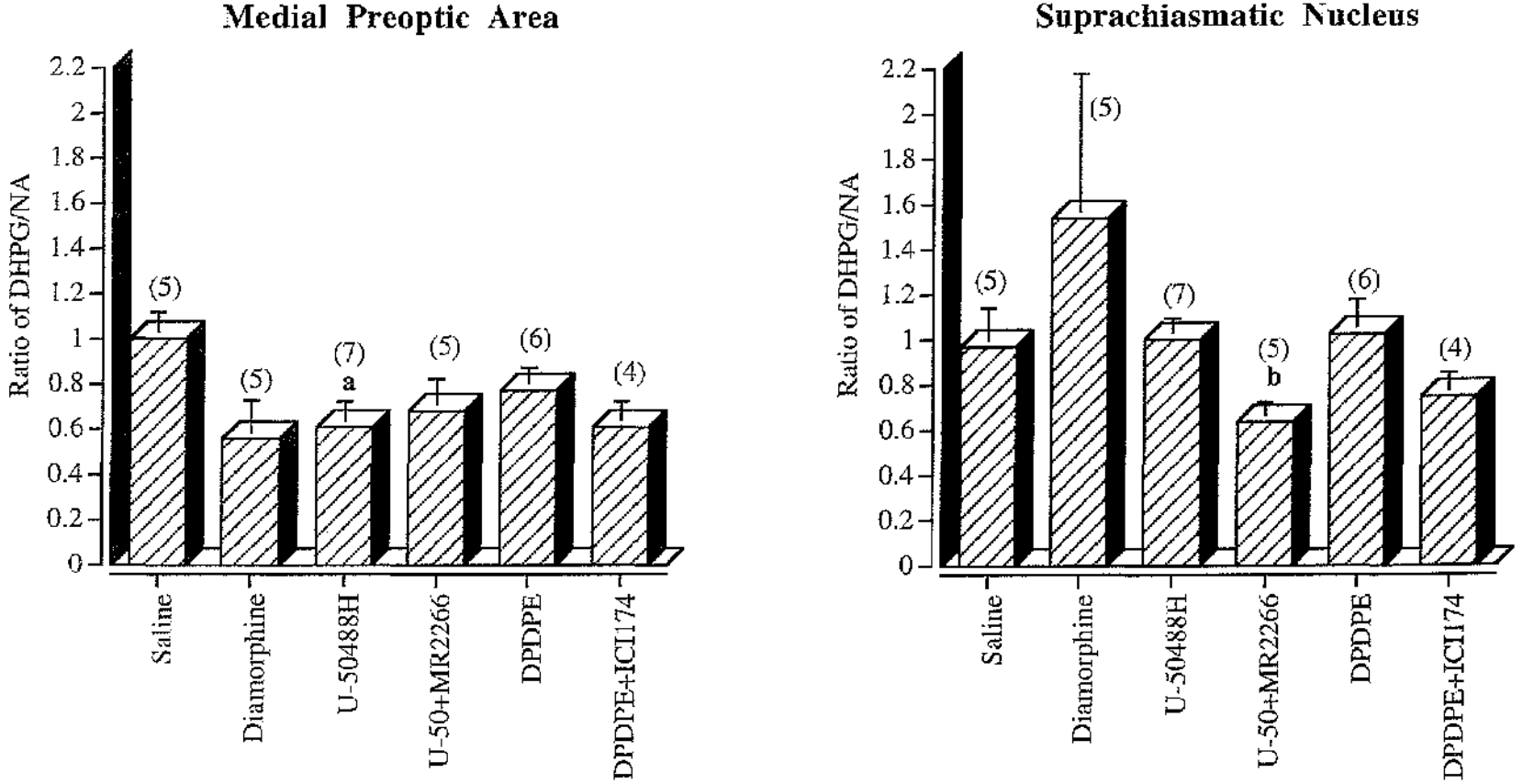


Figure 3.11. Ratio of DHPG/NA (Mean ± SEM) in the MPOA and SCN of urethane-anaesthetised female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.05$ compared to the saline-treated animals, **b**: $p<0.05$ compared to the U-50488H-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

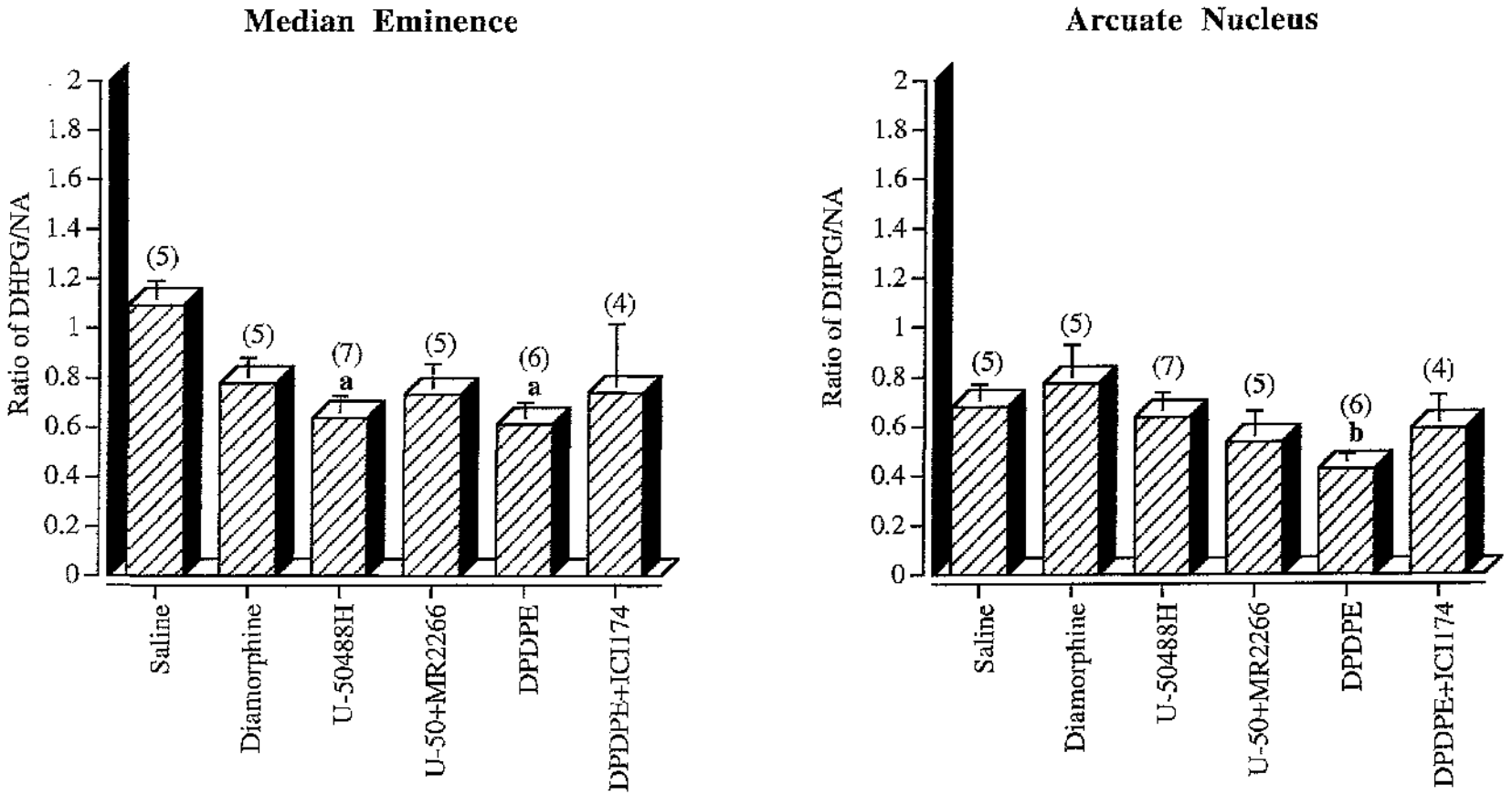


Figure 3.12. Ratio of DHPG/NA (Mean ± SEM) in the ME and ARN of urethane-anaesthetised female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.01$; b: $p<0.05$ compared to the saline-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

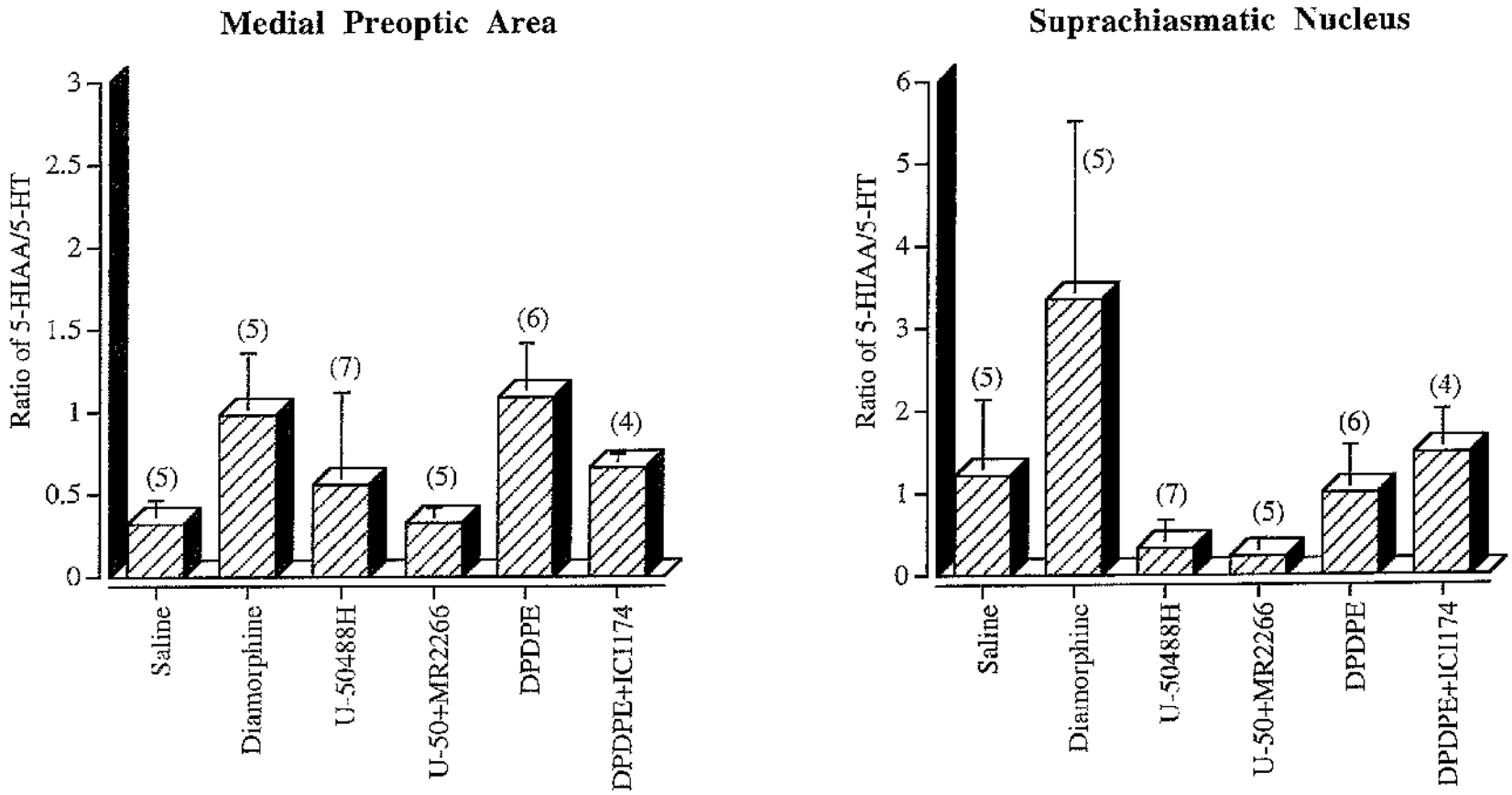


Figure 3.13. Ratio of 5-HIAA/5-HT (Mean ± SEM) in the MPOA and SCN of urethane-anaesthetised female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. The number of observations in each group is given in brackets.

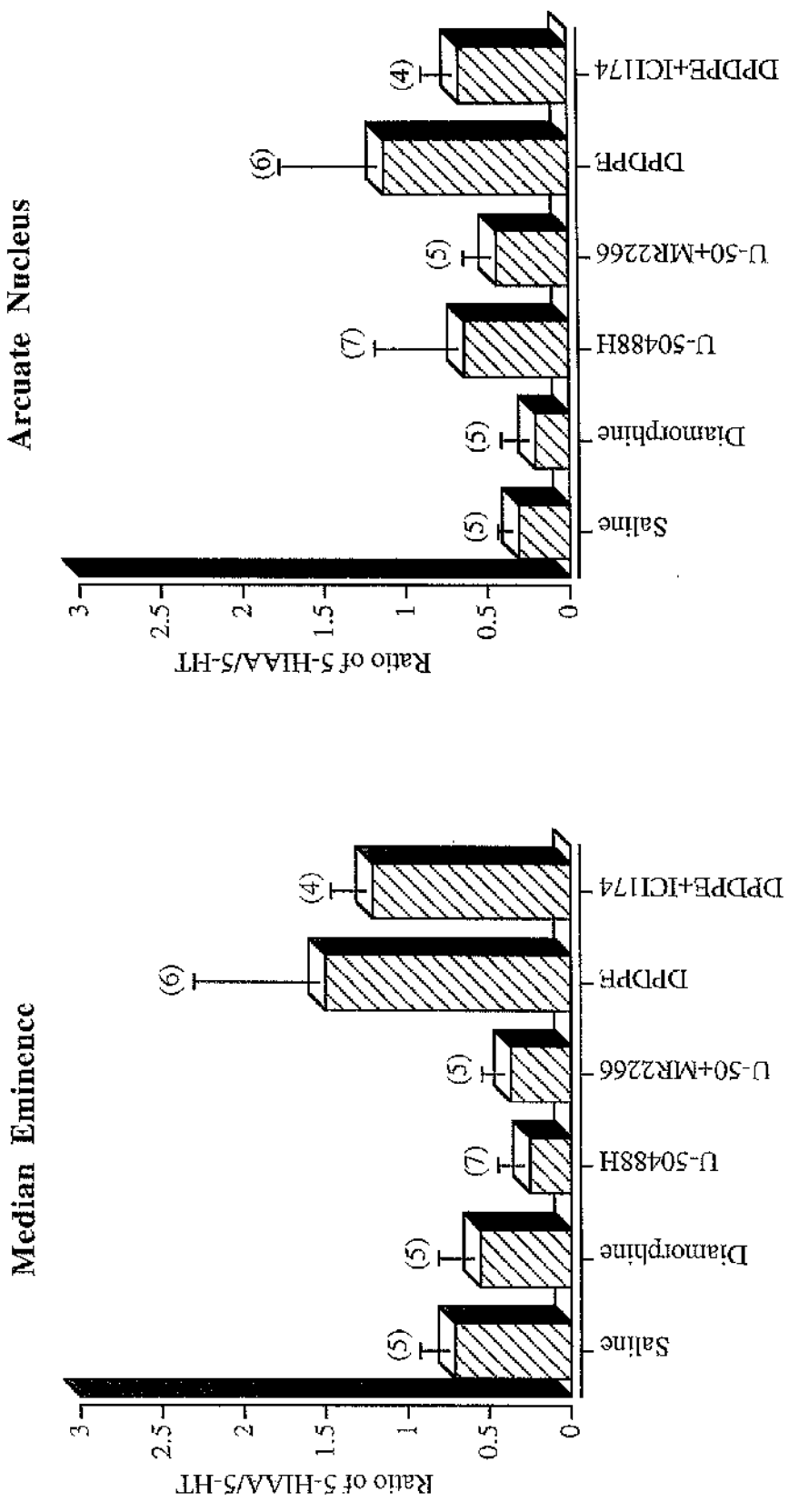


Figure 3.14. Ratio of 5-HIAA/5-HT (Mean ± SEM) in the ME and ARN of urethane-anaesthetised female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. The number of observations in each group is given in brackets.

EXPERIMENT II

Effects of various opioid agonists and antagonists on the hypothalamic monoamine concentrations during the pre-ovulatory LH surge in the freely moving conscious rat

As explained in detail in the Introduction, release of the pre-ovulatory LH surge is regulated by a series of complex neuroendocrine mechanisms. It has been reported that monoaminergic neurotransmitters, particularly NA, play an important role in the generation of the LH surge. These neurotransmitters may mediate the inhibitory effects of the EOPs on LH secretion. Indeed, morphine-induced inhibition of the LH surge has been attributed to a reduction in NA levels in the preoptic-tuberal pathway of the hypothalamus. Furthermore, naloxone-induced LH stimulation can be prevented by prior administration of α-adrenergic blockers.

In the previous experiment, while we were attempting to study the effects of various opioid drugs on the hypothalamic monoamine concentrations and plasma LH levels in the anaesthetised rats on the afternoon of pro-oestrus, it became clear that urethane anaesthesia totally inhibited the expected pre-ovulatory LH surge. These initial findings then led us to use a modified method for blood sampling employing conscious animals.

The same objectives in the Experiment I were adopted in this experiment. Because of the unexpected interference by the urethane in Experiment 1, no conclusions could be made regarding the opioid modulation of the hypothalamic monoamines in the control of the pre-ovulatory LH surge.

In the present experiment, the inter-relationship between the opioid and aminergic systems in the control of GnRH activity has been further investigated and the modulating effects of specific μ-, κ- and δ-opioid receptor agonists and antagonists on hypothalamic monoaminergic content and LH release have been noted simultaneously. The investigation has been confined to the modulation of the pre-ovulatory LH surge in conscious female rats on the day of pro-oestrus.

It was hoped that this modified method would allow us to avoid interference by the urethane-anaesthesia to the hypothalamo-pituitary axis while serially collecting blood samples throughout the afternoon of pro-oestrus for LH determination.

Materials and Methods

On the morning of pro-oestrus, rats were selected for experimentation. The right femoral artery was cannulated under halothane anaesthesia. The animals were allowed to recover and then early in the afternoon, just before the onset of the pre-

ovulatory LH surge, were IP injected with either DPDPE (1mg/kg), diamorphine (3mg/kg), U-50488H (10mg/kg), DPDPE plus naloxone (10mg/kg), diamorphine plus naloxone (15mg/kg) or U-50488H plus MR2266 (10mg/kg). Controls received physiological saline (1ml/kg) IP alone. The number of observation in each group is given in the Tables and Figures.

Blood samples (200µl) were withdrawn through the indwelling cannula at hourly intervals from freely-moving conscious animals throughout the afternoon and early evening of pro-oestrus. Only a small volume (20-30µl) of warmed, heparinised physiological saline was injected after each blood sample collection to maintain the cannula open. The animals were kept warm throughout the experiment by the use of a heating lamp and were killed by decapitation just after the final blood collection at 19.00h to obtain specific regions of the hypothalamus by micropunch. Only one or two rats were operated upon on the same day. All drug administrations were carried out under light halothane anaesthesia. More details on the methodology of this experiment are given in the Materials and Methods.

Results

The monoamine results of this experiment are summarised in the Tables 4.1 to 4.4 and Figures 4.1 to 4.14.

a) Noradrenaline

Control group values for NA concentrations (Mean ± SEM) in the MPOA, SCN, ME and ARN of the conscious rats at 19.00h were 34.5 ± 1.5, 35.1 ± 2.5, 33.2 ± 1.7 and 32.5 ± 1.6 pg/µg protein respectively.

(i) Diamorphine

This µ-opioid agonist significantly decreased NA concentrations in all the hypothalamic regions examined compared to the control group values.

(ii) Diamorphine + Naloxone

Co-administration of diamorphine with naloxone significantly increased NA levels in all the hypothalamic regions examined compared to the diamorphine-treated animals. In this group, the NA concentrations were even higher than the control group values.

(iii) U-50488H

NA concentrations were significantly lowered by U-50488H in the four hypothalamic regions examined compared to the saline-treated animals.

(iv) U-50488H + MR2266

When the κ-opioid agonist was co-administered with MR2266, NA concentrations were significantly elevated in the SCN, ME and ARN compared to the group which received U-50488H alone. This treatment had no significant effect on the NA levels in the MPOA.

(v) DPDPE

DPDPE significantly reduced NA concentrations in all the hypothalamic regions examined compared to the control animals.

(vi) DPDPE + Naloxone

Treatment with DPDPE and naloxone caused significant increases of NA levels in the four hypothalamic areas studied in comparison to the DPDPE-treated group.

b) 3,4-Dihydroxyphenylglycol

DHPG concentrations (Mean ± SEM) in the MPOA, SCN, ME and ARN of the conscious control animals at 19.00h were 15.8 ± 1.9, 20.0 ± 1.5, 14.4 ± 1.6 and 16.0 ± 2.0 pg/μg protein respectively.

(i) Diamorphine

Diamorphine administration brought about significant decreases of DHPG concentrations in all the hypothalamic regions examined compared to those seen in the control animals.

(ii) Diamorphine + Naloxone

Naloxone significantly reversed the inhibitory effects of the μ-opioid agonist on the DHPG concentrations in the MPOA, SCN, ME and ARN following its co-administration with diamorphine.

(iii) U-50488H

The κ-agonist significantly reduced concentrations of DHPG in all the hypothalamic regions examined compared to the saline-treated animals.

(iv) U-50488H + MR2266

This treatment significantly raised DHPG levels in all four hypothalamic areas compared to the group which received U-50488H alone.

(v) DPDPE

The δ-opioid agonist significantly decreased DHPG concentrations in all the hypothalamic regions studied compared to those seen in the saline-treated animals.

(vi) DPDPE + Naloxone

DHPG levels were significantly elevated following co-administration of DPDPE with naloxone in the hypothalamus compared to those receiving DPDPE alone.

c) Dopamine

Control values for DA concentrations (Mean ± SEM) in the MPOA, SCN, ME and ARN of the conscious animals at 19.00h were 6.1 ± 0.8, 6.2 ± 1.1, 19.1 ± 1.6 and 6.2 ± 1.0 pg/μg protein respectively.

(i) Diamorphine

DA levels were significantly reduced by the μ-opioid agonist only in the MPOA compared to the control group values. They were unaffected in the SCN, ME and ARN of this group.

(ii) Diamorphine + Naloxone

When naloxone was co-administered with diamorphine, DA levels were significantly increased in all the hypothalamic regions examined compared to the group which received diamorphine alone.

(iii) U-50488H

The κ-opioid agonist had no significant modulatory effects on DA concentrations in any of the hypothalamic areas examined.

(iv) U-50488H + MR2266

This treatment significantly raised DA concentrations only in the ME compared to the U-50488H-treated animals, but not in the MPOA, SCN and ARN of this group.

(v) DPDPE

DPDPE significantly lowered DA levels in the MPOA, ME and ARN compared to those seen in the control group. However, DA levels were not significantly altered in the SCN.

(vi) DPDPE + Naloxone

DA concentrations were significantly increased following co-administration of DPDPE with naloxone in the MPOA, ME and ARN compared to those receiving DPDPE alone. There was no significant alteration of the DA levels in the SCN of this group.

d) 5-Hydroxyindolcacetic Acid

Concentrations (Mean ± SEM) of 5-HIAA in the MPOA, SCN, ME and ARN of the saline-treated conscious animals at 19.00h were 7.2 ± 0.9, 7.3± 1.3, 7.8 ± 1.1, 6.7 ± 1.1 pg/μg protein respectively.

(i) Diamorphine

Diamorphine significantly decreased 5-HIAA levels in the MPOA and ME compared to the control group values. However, the 5-HIAA levels were unaffected in the SCN and ARN.

(ii) Diamorphine + Naloxone

Treatment with naloxone plus diamorphine significantly elevated concentrations of 5-HIAA in the respective hypothalamic regions (MPOA and ME) compared to the group which received diamorphine alone. Again, there was no alteration of the 5-HIAA concentrations in the SCN and ARN.

(iii) U-50488H

The κ-opioid agonist reduced 5-HIAA levels only in the ME compared to the saline-treated animals, but not in the MPOA, SCN and ARN.

(iv) U-50488H + MR2266

This treatment significantly increased 5-HIAA concentrations only in the ARN compared to the U-50488H-treated animals, but it did not significantly affect those of the MPOA, SCN and ME in this group.

(v) DPDPE

5-HIAA levels were significantly lowered following administration of DPDPE in the MPOA, ME and ARN compared to those values seen in the saline-treated animals. But they were not significantly altered in the SCN.

(vi) DPDPE + Naloxone

When DPDPE was co-administered with naloxone, 5-HIAA concentrations were significantly increased in all the hypothalamic regions examined compared to the group which received DPDPE alone.

e) Serotonin

Control values found for 5-HT in the MPOA, SCN, ME and ARN of the conscious rats at 19.00h were 8.7 ± 1.3, 6.2 ± 0.7, 7.7 ± 0.6 and 5.9 ± 0.6 pg/μg protein ± SEM respectively.

(i) Diamorphine

5-HT concentrations were not significantly changed by diamorphine in any of the hypothalamic areas examined compared to those values seen in the controls.

(ii) Diamorphine + Naloxone

Co-administration of diamorphine with naloxone had no significant effect of 5-HT levels in the MPOA, ME and ARN, but it significantly increased the indoleamine levels only in the SCN compared to those receiving diamorphine alone.

(iii) U-50488H

The κ-opioid agonist did not significantly alter 5-HT concentrations in any of the hypothalamic areas studied compared to the controls.

(iv) U-50488H + MR2266

5-HT levels were significantly elevated in the SCN and ARN, but significantly reduced in the MPOA following co-administration of the κ-agonist with MR2266 compared to the group which received U-50488H alone. This treatment had no significant effect in the ME of this group.

(v) DPDPE

DPDPE significantly reduced 5-HT levels in the four hypothalamic areas compared to the control group values.

(vi) DPDPE + Naloxone

When DPDPE was co-administered with naloxone, 5-HT concentrations were significantly raised in all the hypothalamic regions examined compared to the DPDPE-treated animals.

e) Ratio of DHPG/NA

Control group values for the ratio of DHPG/NA in the MPOA, SCN, ME and ARN of the conscious animals at 19.00h were 0.452 ± 0.047, 0.589 ± 0.041, 0.432 ± 0.039 and 0.498 ± 0.061 respectively (results expressed as Mean±SEM).

(i) Diamorphine

The μ-opioid agonist significantly lowered the ratio of DHPG/NA in the MPOA, SCN, ARN and ME compared to the control group.

(ii) Diamorphine + Naloxone

Co-administration of diamorphine with naloxone increased the ratio of DHPG to NA in the MPOA, SCN and ARN compared to the group which received diamorphine alone. However, the ratio was unaffected in the ME of this group.

(iii) U-50488H

Administration of U-50488H brought about significant decreases of the ratio of DHPG/NA in the MPOA, SCN and ARN compared to the saline-treated animals. The ratio was not significantly changed in the ME.

(iv) U-50488H + MR2266

Treatment with the κ-opioid agonist plus MR2266 significantly raised the ratio of DHPG/NA in the MPOA, SCN and ME compared to those receiving U-50488H alone, but it had no effect in the ARN.

(v) DPDPE

The ratio of DHPG/NA was significantly decreased by DPDPE in the MPOA, SCN and ARN compared to the control group values. However, there was no significant alteration of the DHPG/NA ratio in the ME.

(vi) DPDPE + Naloxone

This treatment caused no significant changes in the ratio of DHPG/NA in any of the four hypothalamic areas studied.

f) Ratio of 5-HIAA/5-HT

The ratio of 5-HIAA/5-HT in the MPOA, SCN, ME and ARN of the conscious control group at 19.00h were 0.962 ± 0.140, 1.311 ± 0.300, 1.172 ± 0.234 and 1.379 ± 0.329 respectively (results expressed as Mean ± SEM).

(i) Diamorphine

The ratio of 5-HIAA/5-HT was not significantly affected by diamorphine in the SCN, ME and ARN, however, it was significantly reduced only in the MPOA compared to the control group values.

(ii) Diamorphine + Naloxone

Treatment with diamorphine plus naloxone produced a significantly higher ratio of 5-HIAA/5-HT in the MPOA compared to the group which received diamorphine alone. However, the 5-HIAA/5-HT ratio was not significantly altered in the other hypothalamic areas.

(iii) U-50488H

(iv) U-50488H + MR2266

(v) DPDPE

(vi) DPDPE + Naloxone

None of these drug regimes significantly changed the ratio of 5-HIAA/5-HT in any of the hypothalamic regions examined.

g) Luteinising Hormone

LH results observed in individual animals are shown in the Tables 4.5 to 4.11. In the saline controls, plasma LH levels (ng/ml ± SEM) at the 15.00h, 16.00h, 17.00h, 18.00h and 19.00h sampling intervals on the afternoon of the pro-oestrus were 3.6 ± 1.5, 12.7 ± 4.4, 28.8 ± 8.6, 29.2 ± 8.7, 25.8 ± 6.5 respectively. Although the blood sampling commenced at 13.00h, we found no detectable changes in circulating LH levels before 15.00h. In some cases, although the indwelling cannula was heparinised, blood samples could not be obtained due to spontaneous blockage. In four out of 16 control animals no LH surge was seen.

Therefore, the mean values indicated above are taken from those in which the plasma LH levels were detectable.

(i) Diamorphine

The μ-opioid agonist tended to suppress plasma LH levels. Some of the diamorphine-injected animals showed detectable changes in LH levels over the 17.00h, 18.00h and 19.00h sampling periods. These changes were not found to be statistically significant compared to the saline-treated group.

(ii) Diamorphine + Naloxone

In some of the diamorphine with naloxone co-administered animals, plasma LH concentrations were elevated over the 16.00h, 17.00h, 18.00h, 19.00h sampling intervals.

(iii) U-50488H

Plasma LH levels were undetectable in all the κ-agonist-injected animals ($p<0.001$).

(iv) U-50488H + MR2266

In only one out of nine animals which received U-50488H with MR2266 LH surge was seen. In the remainder of the animals LH levels were all undetectable.

(v) DPDPE

The δ-opioid agonist either reduced or completely inhibited the pre-ovulatory LH surge ($p<0.05$). Plasma LH concentrations were below the limit of detection in eight out of 10 DPDPE-injected animals. Only in two animals, some rises in LH levels were observed over the 17.00h, 18.00h and 19.00h sampling periods, but these increases did not reach the levels seen in the controls.

(vi) DPDPE + Naloxone

When naloxone was co-administered with DPDPE, plasma LH levels were elevated at various sampling intervals on the afternoon of pro-oestrus. In three out of eight animals in this group no LH surge occurred.

Discussion

Luteinising Hormone

In the conscious group, the LH surge was seen in approximately 70% of the control animals (n:16). In these, it occurred late on the afternoon of pro-oestrus reaching a peak at 17.00-18.00h and then beginning to decline. This indicates that there may be an increase in the GnRH discharge and also an enhanced response of the pituitary gonadotrophs to the GnRH signal on the afternoon of pro-oestrus. Although the blood sampling commenced at 13.00h, there was no detectable changes in circulating LH levels before 15.00h. This slight delay in the pre-ovulatory LH surge could be a result of the mild stress caused by the surgery and by the halothane anaesthesia. The animals were operated upon on the late morning of pro-oestrus and the entire procedure did not take longer than 25 minutes. These LH results are consistent with those reported by Dow *et al.* (1994) using the same experimental technique. In their study, the LH surge was observed after 16.00h on the afternoon of pro-oestrus. Unexpectedly, in four (out of 16) control animals no rises in LH levels were seen over the hourly sampling periods. In some cases, the animals had to be handled for the collection of the blood samples. However, this was done very gently. Since vaginal smearing had been performed on these rats for at least three weeks, it is thought that they would have been accustomed to handling. Perhaps, the surgery might have caused a degree of stress to these animals, however, the underlying basis for this effect is unclear. As it will be detailed later in this chapter, NA levels in the specific regions of the hypothalamus in these rats were also high.

The ability of κ-, μ- and δ-opioid agonists to suppress the pre-ovulatory LH surge may result from the inhibition of the pituitary responsiveness to GnRH in the rats. The hypothalamus is densely populated by opioid receptors while the anterior pituitary contains only a very low density of these (Khachaturian *et al,* 1985). Since the opioids have been shown to have no direct action on LH release from the anterior pituitary (Bicknell, 1985), it is suggested that the opioid agonists under investigation in this study have exerted their inhibitory effects at the hypothalamic level. This is in line with the evidence that administration of morphine suppresses the release of GnRH (Mehmanesh *et al,* 1988) and naloxone infusion increases its secretion from the MBH-preoptic area *in vitro* (Leadem *et al,* 1985). Furthermore, stimulation of LH release by NA may be the consequence of

hypersecretion of GnRH into the hypophyseal portal system (Negro-Vilar *et al,* 1980).

It has been widely reported that administration of morphine inhibits the surge release of LH on the day of pro-oestrus (Leadem and Kalra, 1985; Pfeiffer *et al,* 1987). Work from this laboratory has also shown that high doses of a long acting μ-opioid agonist, duromorph, abolishes the LH surge when administered before the critical period on pro-oestrus (Brown *et al,* 1994). The present study confirms these findings that activation of the μ-opioid receptors with diamorphine brings about the suppression of the pre-ovulatory LH surge. However, four of the diamorphine-treated animals (n: 10) showed some rises in LH levels during the 17.00h, 18.00h and 19.00h sampling periods, but these increases did not reach the levels seen in the responsive controls. This could imply a recovery in plasma LH levels, since diamorphine is a rapid-acting drug. However, this supposition is unlikely because hypothalamic NA concentrations were still significantly lower at 19.00h than those in the controls. Moreover, diamorphine is known to be more lipid soluble than morphine and is slowly metabolised to morphine, thus its higher concentrations occur in the CNS (Kruk and Pycock, 1993). It could also be that since the drugs were administered systemically, the effective dose acting upon the central opioidergic system might, in these cases, have been low. It has been shown that low doses of morphine and duromorph stimulate the LH surge (Pang *et al,* 1977; Brown *et al,* 1994). Overall, these results also indicate that a decrease in the central opioidergic tone during the critical period on pro-oestrus is associated with the LH surge.

When diamorphine was co-administered with naloxone, the opioid inhibition of the LH release was prevented. This indicates that increased LH release results from the antagonism of EOP secretion by acting at post-synaptic μ-receptors. Also, the inhibitory opioidergic tone, although decreased, is not totally eliminated during the critical period. Administration of naloxone alone has been shown to enhance the amplitude of the pre-ovulatory LH surge (Crowley, 1988; Kalra *et al,* 1989).

There are conflicting reports on the involvement of κ-opioid receptors in the suppression of LH secretion. Inhibition of LH release occurs after administration of specific κ-opioid agonists (Leadem and Yagenova, 1987; Gopalan *et al,* 1989a). However, the specificity of the κ-opioid effect has been questioned since the μ-opioid antagonist, naloxone, reversed the κ-agonist's effects (Pfeiffer *et al,*

1987). In the present study, the LH surge was completely abolished by a more selective κ-agonist, U-50,488H, throughout the afternoon of pro-oestrus following its administration at 13.00h. These results thus provide further evidence for the involvement of the κ-opioid receptor subtypes in the inhibition of LH secretion. Furthermore, the effect is thought to be exerted via the central noradrenergic system. The κ-opioid action on LH release is also believed to be mediated at the level of the hypothalamus, since κ-agonists have been shown to inhibit GnRH release *in vitro* (Leposavic *et al,* 1991). Contrary to the expectation, MR2266, a κ-opioid receptor antagonist, failed to reverse the suppressive effects of U-50488H on plasma LH levels. Only one U-50488H plus MR2266-treated animal showed a LH surge late on the afternoon of pro-oestrus. It has previously been noted that MR2266 also failed to elevate LH levels in young rats (Cicero *et al,* 1988). It seems that this opioid antagonist either has no affinity for the κ-opioid receptors or that the relevant EOP ligands are absent. Although the present results appear to suggest the participation of κ-opioid receptors in the LH secretory systems, further studies, using more selective antagonists when they are available may reveal additional information.

The significance of δ-opioid receptors in the regulation of LH release is poorly understood. Activation of the δ-opioid receptors have been shown to suppress GnRH and LH secretion on the day of pro-oestrus (Leadem and Yagenova, 1987). However, their involvement in this process has been questioned since a specific δ-opioid antagonist has failed to elevate plasma LH levels (Cicero *et al,* 1988; Leposavic *et al,* 1991). We have shown that administration of DPDPE, a δ-opioid agonist, reduced or completely blocked the pre-ovulatory LH surge. Recently there have been reports of cross-communication between the δ- and μ-opioid receptor subtypes in the brain (Stadimis and Young, 1992; Traynor and Elliott, 1993). In the present experiment, the tonic inhibition of the LH surge by DPDPE was largely reversed by naloxone, the non-specific opioid antagonist. These results therefore indicate that both μ- and δ-opioid receptors may mediate the inhibitory effects of DPDPE. Alternatively, naloxone may have affinity towards δ-opioid receptors along with its well-known actions at μ-opioid subtypes. The δ-opioid receptors may thus be involved in the neuroendocrine control of the pre-ovulatory LH surge.

In conclusion, the results from the present experiment indicate that opioidergic influence on the LH release is inhibitory and multiple opioid receptors may be involved in mediating these effects.

Hypothalamic Aminergic Activity

As stated in the Experiment I, DHPG and 5-HIAA were the only metabolites of the biogenic amines under investigation which appeared within the detectable limits of the HPLC-ECD system employed. When raised concentrations of NA or 5-HT were detected, increased levels of DHPG or 5-HIAA were almost always observed in the four hypothalamic regions studied. This indicates that when the release of the neurotransmitter is elevated, its synthesis is also stimulated by an autoregulation mechanism. These premises will be discussed in detail in the General Discussion chapter.

The results of this experiment show that the aminergic activity in the MPOA, SCN, ME and ARN of the rat hypothalamus is high at 19.00h on the afternoon of pro-oestrus. Similarly, the opioid drugs administered before the critical period are able to extend their modulatory effects on the hypothalamic monoamine concentrations towards the end of the pre-ovulatory LH surge. It has already been demonstrated that the monoamine levels within the discrete hypothalamic areas are high at early stages of pro-oestrus and during the afternoon LH surge (Barraclough *et al,* 1984; Dyer *et al,* 1988). These results indicate that the neural circuitry upon which the opioids exert their actions is still active even at the time of the LH surge. However, previous work from this laboratory has also shown that opioid agonists are relatively ineffective at altering neurotransmitter levels at 18.00h following their administration at 12.30h on the same day. It was thought that the drugs might have been metabolised to their inactive forms by this time. However, this supposition is unlikely as duromorph is known to have potent analgesic effects up to 24h and can be detected in the circulation throughout this period (Johnston, 1990). Moreover, of all the opioid agents investigated, duromorph had the least effect on the biogenic amine concentrations (Brown *et al,* 1994). It thus appears that lack of effects of the opioids can not be attributed to breakdown during the course of experiment.

In the present experiment, plasma LH levels started to rise after 15.00h. It has been documented that in the rat the total pre-ovulatory LH surge occurs in about

270 minutes (Barraclough *et al,* 1984). Increased NA release/turnover within the specific regions of the hypothalamus has been correlated with the LH surge on the afternoon of pro-oestrus (Rance and Barraclough, 1981). Therefore, it is expected to see such an enhanced activity in the central aminergic system even at 19.00h on the late afternoon of pro-oestrus.

In this study, all three opioid receptor agonists tested significantly reduced the NA content in all the hypothalamic areas examined concomitantly with the abolition of the pre-ovulatory LH surge. These drug regimes also lowered DHPG levels, the NA metabolite, in the same hypothalamic regions. From this experiment, it is apparent that a close relationship exists between the increased NA release and turnover in the preoptico-MBH and the GnRH pulse generator which in turn induces the pulsatile secretion of the LH surge. It has previously been suggested that NA plays a crucial role in the generation of the pre-ovulatory LH surge (Barraclough *et al,* 1984; Ramirez *et al,* 1984). However, inhibitory effects of this catecholamine has also been reported in the absence of steroid milieu in the ovx rat (Dotti and Talcisnik, 1984; Bergen and Leung, 1987). It appears that the steroid hormones determine the direction of NA action on the pulsatile LH release through their receptors on the noradrenergic nerve terminals (Sar, 1984; Lehman and Karsch, 1993). Pharmacological work by Kalra and Simpkins (1981) has provided indirect evidence for this hypothesis by showing that opioids inhibit the positive feedback effects of E_2 and P on LH release via their action on the noradrenergic system.

A considerable amount of literature available appears to attribute a greater significance to the activity of NA in the MPOA on LH release (Honma and Wuttke, 1980; Grossman and Dyer, 1989). However, it has been suggested that the effective site of NA in evoking GnRH release is not solely the MPOA, but also involves other components of the preoptico-suprachiasmatic-tuberoinfundibular GnRH system (Barraclough, 1994). Noradrenergic nerve terminals have been observed to make synaptic contacts with the GnRH neurons in the MPOA and to a lesser extent with GnRH nerve terminals in the ARN and ME (Watanabe and Nakai, 1987). These results support the concept that NA concentrations were decreased by the opioid agonists and raised by their respective antagonists in the MPOA, SCN, ME and ARN. Changes in the NA content of the ME is also likely to affect GnRH release since this area is regarded as the final common pathway in regulating LH secretion. Indeed, increased NA activity within this hypothalamic

area has been considered to be important in initiating the pre-ovulatory LH surge (Rance *et al,* 1981).

The ability of morphine to suppress LH secretion has been shown in a variety of experimental conditions (see Kalra *et al,* 1989) and the effect has been attributed to an inhibition of release of NA from its nerve terminals (Barraclough, 1994). It has been demonstrated that naloxone is able to activate the brain catecholaminergic systems which in turn enhance the release of GnRH (Kalra and Kalra, 1985). The findings presented here indicate that naloxone negated the inhibitory influence of diamorphine on the concentrations of NA and its metabolite in all four areas studied. In some cases, in the diamorphine+naloxone-treated group, amine levels were found to be even higher than those seen in the controls. Naloxone has been shown to have its own stimulatory action on NA release and/or turnover within the hypothalamus during the afternoon of pro-oestrus (Akabori and Barraclough, 1986; Gopalan *et al,* 1989a). The presence of μ-opioid receptors has been visualised in the hypothalamus as well as in the other brain areas (Mansour *et al,* 1988). In addition, the noradrenergic terminals contain a mixed population of opioid receptors. This provides a functional relationship between the noradrenergic and opioid peptidergic systems in the brain. It is therefore suggested that modulatory effects of opioids on the central noradrenergic neurotransmission are exerted via the μ-opioid subtypes, which is inhibitory.

U-50488H brought about significant decreases in both NA and DHPG levels in all the hypothalamic regions examined. When MR2266 was co-administered with this κ-opioid agonist, the falls in both amine and metabolite concentrations were prevented in the SCN, ME and ARN, but not in the MPOA. The lack of effect of MR2266 was unexpected because the κ-opioid receptors are found in the MPOA as well as in the other hypothalamic sites (Mansour *et al,* 1988). It has been suggested that the EOPs alter NA neurotransmission by their action at pre-synaptic nerve terminals. Reduction of the amount of neurotransmitter released is a common feature of opioid action (Grossman and Dyer, 1989). It is thought that U-50488H lowers NA synthesis by inhibiting its release from the nerve terminals, as both NA and DHPG concentrations were reduced in parallel. Prevention of the falls in the amine levels by MR2266 would point to a specific κ-opioid action in discrete hypothalamic areas. It was also found that U-50488H lowered the ratio of DHPG/NA in the MPOA, SCN and ARN, but not in the ME. The κ-opioid antagonist negated these effects of U-50488H. It also raised the ratio of

DHPG/NA in the ME compared to those values seen in the κ-opioid agonist-treated animals. Previously, a highly selective κ-agonist has been reported to cause a significant decrease in NA turnover in the SCN and VMH (Burkard, 1984).

The δ-opioid receptor agonist reduced the secretion of LH on the afternoon of pro-oestrus and concomitantly decreased concentrations of both NA and its metabolite DHPG within the hypothalamus in a naloxone-reversible manner. In some cases, in the DPDPE+naloxone-treated group, amine levels were even higher than those values observed in the controls. DPDPE significantly lowered the ratio of DHPG/NA in the MPOA, SCN and ARN. The δ-opioid receptors have a more restricted distribution than those of μ- and κ-opioid receptors in the rat brain. Dense areas of δ-opioid receptors have been found in several other brain areas including the brain stem. In the hypothalamus, the highest concentration of δ-receptors have been located in the SCN and MPOA (Mansour *et al,* 1988; Desjardins *et al,* 1990). The δ-opioid receptors are known to have great affinity towards enkephalin neurons (Traynor and Elliott, 1993). The presence of enkephalin-containing neurons in most hypothalamic regions, including the preoptic area, and in other brain areas has been demonstrated (Schafer *et al,* 1991). In view of these reports and the present findings, it is suggested that the δ-opioid receptors have modulatory effects on NA release and turnover around the time of the pre-ovulatory LH surge.

The central dopaminergic system has been implicated in the regulation of LH release. However, its role in the intact female rat is less clear-cut, as it appears to have both stimulatory and inhibitory effects on LH release depending on the site of action (Ramirez *et al,* 1984; MacKenzie *et al,* 1988). Neurons of the TIDA dopaminergic system originate in the ARN and PeVN, and project to the ME (Lindvall and Bjorklund, 1982). A second group of DA neurons, the incertohypothalamic tract is of extrahypothalamic origin and projects principally to the MPOA. It has been suggested that DA is stimulatory in the MPOA (Kawakami *et al,* 1975) and ZI (MacKenzie *et al,* 1988) but inhibitory in the ARN and ME (Rose and Weick, 1986; He *et al,* 1994) to LH secretion. In this study, there were isolated area-dependent effects in the rats treated with diamorphine or DPDPE. We observed significant decreases in the DA levels in the MPOA following the administration of both diamorphine and DPDPE and this was accompanied by the abolition of the LH surge. These suppressive effects of both μ- and δ-opioid

agonists were reversed by naloxone in the same hypothalamic region. This suggests that both μ- and δ-opioid receptors have inhibitory effects on the dopaminergic activity in this area. Interestingly, U-50488H did not alter DA levels in any of the hypothalamic areas examined. The co-administration of MR2266 with U-50488H significantly elevated DA concentrations in the ME, however, this effect was not consistently seen in the other three areas. It might thus appear that the central dopaminergic system may not be involved in mediating the inhibitory effects of the κ-opioid receptors on LH release. However, it has been reported that activation of κ-opioid receptors reduces DA release from the MBH *in vitro* (Heijna *et al,* 1991) and inhibits the TIDA dopaminergic neurons (Manzanares *et al,* 1992).

Systemic administration of morphine has been shown to lower the rate of synthesis and turnover of DA in the ME by inhibiting the activity of the TIDA neurons (Alper *et al,* 1980). Similarly β-endorphin decreases the hypothalamic DA release from the TIDA dopaminergic neurons (van Loon *et al,* 1980; Wilkes and Yen, 1980). On the contrary, the activity of the TIDA neurons has been reported to be increased following the activation of δ-opioid receptors (Manzanares *et al,* 1993). Of the opioid agonists investigated in this study, only DPDPE significantly reduced the concentrations of DA in both the ME and ARN. Further, the opioid inhibition of dopaminergic activity was prevented by naloxone in these areas. It is therefore suggested that opioidergic influence on DA release via the δ-opioid receptors is inhibitory. Although DA levels were also detected in the SCN, no significant alterations were found in this hypothalamic area following the administration of DPDPE alone or together with naloxone. This is surprising because the SCN is known to contain DA projections (Bjorklund *et al,* 1975).

The studies on the involvement of the serotonergic system upon LH release have been controversial; both a stimulatory and an inhibitory role of 5-HT on LH secretion have been reported (Vitale and Chiocchio, 1993; Rodriguez *et al,* 1994). 5-HT may mediate, at least in part, the effects of the EOPs on the LH secretory systems. Morphine stimulates LH release in the presence of exogenous 5-HT, but is inhibitory in its absence (Lenahan *et al,* 1987). Synaptic contacts between the serotonergic terminals and immunoreactive GnRH perikarya have been visualised in the rat hypothalamus (Kiss and Halasz, 1985; King *et al,* 1986). The μ-opioid agonist, diamorphine reduced the LH surge in association but had no effect on 5-HT levels in any of the four areas studied. However, it selectively decreased the

concentrations of 5-HIAA in the MPOA and ME in a naloxone-reversible fashion. Moreover, the ratio of 5-HIAA/5-HT was also lowered in the MPOA, and the effect was negated by naloxone in this area. It has previously been suggested that naloxone enhancement of the pre-ovulatory LH surge is primarily mediated by 5-HT in the MPOA (Brown *et al,* 1994). These results would point to a modulatory action of the μ-opioid subtype on the 5-HT metabolism (or turnover) in the MPOA and ME rather than its release from the nerve terminals. However, it was expected that the opioids would reduce not only the metabolism of this indoleamine but also its release as well.

Although U-50488H was very successful in blocking the LH surge, it did not alter the concentrations of 5-HT or its metabolite in any of the hypothalamic areas examined except that 5-HIAA levels were lowered in the ME. Interestingly, 5-HT levels were elevated in the MPOA, SCN and ARN following the co-administration of U-50488H with MR2266. This κ-opioid antagonist also increased concentrations of 5-HIAA in only the ARN in comparison to those rats receiving U-50488H alone. Activation of κ-opioid receptors has been shown to pre-synaptically inhibit 5-HT release in the DRN (Pinnock, 1992). The results of the present study were not consistent and difficult to interpret. Previous attempts to reveal the putative nature of interaction between κ-opioid receptors and serotonergic neurotransmission have also been inconclusive (Gopalan *et al,* 1989b). Perhaps direct infusion of opioid drugs into specific hypothalamic areas where rich serotonergic projections have been demonstrated (Steinbusch, 1984) would pinpoint the site of action of the κ-opioid types on 5-HT release/turnover.

In this study, the most conclusive evidence for the serotonergic mediation of opioid inhibition of the pre-ovulatory LH surge resulted from experiments in which DPDPE was administered. This δ-opioid agonist significantly decreased concentrations of both 5-HT and its metabolite in parallel in all the hypothalamic regions examined (except 5-HIAA in the SCN) concomitant with the suppression of the LH surge. Furthermore, naloxone prevented these inhibitory actions of DPDPE in the MPOA, ME and ARN and SCN. However, the ratio of 5-HIAA/ 5-HT was unaffected in the hypothalamus following administration of DPDPE alone or together with naloxone. These results thus provide the first direct evidence for δ-opioid modulation of 5-HT release and metabolism in the specific regions of the hypothalamus at the time of the afternoon LH surge. It is thought that DPDPE exerts its inhibitory effects at pre-synaptic 5-HT terminals. It has

been suggested that in the MBH, 5-HT exerts its inhibitory influence on LH secretion whereas the MPOA represents the region where the stimulatory effect takes place on the afternoon of pro-oestrus (King *et al*, 1986; Johnson and Crowley, 1986). On the other hand, it has also been shown that the DRN exerts a stimulatory influence on the pre-ovulatory LH surge by means of its serotonergic projections to the ME (Kerdelhue *et al*, 1989). Accordingly, 5-HT stimulates GnRH release from the ME *in vitro* (Vitale *et al*, 1986). Pharmacological manipulation with 5-HT antagonists blocks the LH surge and hence ovulation (Dow *et al*, 1994). The present study, together with the above reports, appear to suggest a stimulatory role for 5-HT in the generation of the pre-ovulatory LH surge. It may also be concluded that site of action of 5-HT is not only the MPOA, but also likely to be the ME, ARN and SCN.

In summary, we conclude that the inhibitory effects of the EOPs on the surge release of LH may be mediated in large part by NA, and also by DA and 5-HT. It appears that an increase in the hypothalamic NA activity may be a critical event in triggering the surge release of GnRH and LH. Both the dopaminergic and serotonergic neurotransmitter systems may facilitate the pre-ovulatory LH surge, although their effects are thought to be minor in this process.

Neurotransmitter Concentrations in the MPOA							
Treatment	**NA**	**DHPG**	**DA**	**5-HIAA**	**5-HT**	**Ratio of DHPG/NA**	**Ratio of 5-HIAA/5-HT**
Saline (16)	34.5±1.5	15.8±1.9	6.1±0.8	7.2±0.9	8.7±1.3	0.452±0.047	0.962±0.140
Diamorphine (10)	24.9±1.8 **a**	5.4±0.7 **a**	3.5±0.3 **c**	4.0±0.9 **d**	9.4±0.9	0.213±0.022 **a**	0.430±0.106 **c**
Diamorphine+NAL (13)	70.9±6.6 **e**	23.1±2.5 **e**	8.1±0.9 **e**	10.0±1.6 **f**	12.4±2.6	0.330±0.026 **f**	1.048±0.165 **f**
U-50488H (8)	26.9±2.5 **c**	6.0±0.8 **b**	5.8±0.7	5.5±1.1	12.4±1.1	0.219±0.019 **b**	0.796±0.324
U-50488H+MR2266 (9)	31.7±2.0	12.5±2.1 **h**	5.2±0.6	5.7±1.4	6.2±0.9 **g**	0.388±0.047 **h**	0.783±0.169
DPDPE (10)	20.1±1.6 **a**	3.9±0.4 **a**	2.3±0.2 **b**	2.9±0.5 **b**	3.7±0.7 **b**	0.199±0.018 **a**	1.033±0.234
DPDPE+NAL (8)	46.3±3.6 **i**	11.5±2.7 **j**	5.7±0.5 **i**	14.8±1.3 **i**	17.8±1.1 **i**	0.265±0.063	0.839±0.057

Table 4.1. Monoamine concentrations (pg amine/μg protein ± SEM) and ratios of DHPG/NA and 5-HIAA/5-HT in the MPOA at 19.00h on pro-oestrus after administration or co-administration of various opioid agonists and antagonists at 13.00h on the same day. **a:** $p<0.001$; **b**: $p<0.005$; **c**: $p<0.01$; **d**: $p<0.05$ compared to the saline-treated animals, **e:** $p<0.001$; **f**: $p<0.01$ compared to the diamorphine-treated animals, **g:** $p<0.005$; **h**: $p<0.01$ compared to the U-50488H-treated animals, **i:** $p<0.001$; **j**: $p<0.005$; compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

Neurotransmitter Concentrations in the SCN							
Treatment	**NA**	**DHPG**	**DA**	**5-HIAA**	**5-HT**	**Ratio of DHPG/NA**	**Ratio of 5-HIAA/5-HT**
Saline (16)	35.1±2.5	20.0±1.5	6.2±1.1	7.3±1.3	6.2±0.7	0.589±0.041	1.311±0.300
Diamorphine (10)	23.8±1.9 **c**	6.9±0.4 **a**	4.7±0.5	6.1±1.6	6.0±1.2	0.301±0.023 **a**	1.365±0.440
Diamorphine+NAL (13)	57.9±4.0 **e**	22.8±1.7 **e**	7.9±0.6 **f**	11.1±2.4	10.7±2.1 **g**	0.419±0.044 **g**	0.968±0.156
U-50488H (8)	22.4±1.3 **b**	7.5±0.5 **a**	5.8±0.9	5.7±1.0	4.7±0.8	0.334±0.018 **a**	0.997±0.213
U-50488H+MR2266 (9)	40.2±3.0 **h**	17.4±1.9 **h**	6.9±0.6	7.5±1.3	8.9±1.5 **i**	0.427±0.029 **i**	0.736±0.116
DPDPE (10)	25.3±1.6 **c**	6.9±1.4 **a**	4.0±0.6	5.3±0.4	4.2±0.5 **d**	0.289±0.056 **a**	1.272±0.210
DPDPE+NAL (8)	51.9±5.8 **j**	14.7±1.9 **k**	7.0±0.8	12.2±2.5 **l**	9.4±2.6 **l**	0.316±0.052	1.212±0.371

Table 4.2. Monoamine concentrations (pg amine/μg protein ± SEM) and ratios of DHPG/NA and 5-HIAA/5-HT in the SCN at 19.00h on pro-oestrus after administration or co-administration of various opioid agonists and antagonists at 13.00h on the same day. **a:** $p<0.001$; **b:** $p<0.005$; **c:** $p<0.01$; **d:** $p<0.05$ compared to the saline-treated animals, **e:** $p<0.001$; **f:** $p<0.005$; **g:** $p<0.05$ compared to the diamorphine-treated animals, **h:** $p<0.001$; **i:** $p<0.05$ compared to the U-50488H-treated animals, **j:** $p<0.001$; **k:** $p<0.005$; **l:** $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

Neurotransmitter Concentrations in the ME							
Treatment	**NA**	**DHPG**	**DA**	**5-HIAA**	**5-HT**	**Ratio of DHPG/NA**	**Ratio of 5-HIAA/5-HT**
Saline (16)	33.2±1.7	14.4±1.6	19.1±1.6	7.8±1.1	7.7±0.6	0.432±0.039	1.172±0.234
Diamorphine (10)	21.6±2.3 **a**	6.0±0.7 **a**	17.5±2.0	4.5±1.1 **d**	5.7±1.1	0.290±0.029 **d**	1.153±0.281
Diamorphine+NAL (13)	71.1±7.8 **e**	27.8±3.9 **e**	30.2±3.1 **f**	14.5±3.0 **g**	10.2±2.1	0.406±0.050	1.078±0.112
U-50488H (8)	19.5±1.4 **a**	6.0±0.8 **b**	16.6±2.7	4.1±0.6 **d**	8.5±1.8	0.309±0.041	0.624±0.144
U-50488H+MR2266 (9)	33.9±2.1 **h**	15.8±1.9 **h**	26.0±2.9 **i**	5.6±1.2	8.0±1.5	0.478±0.064 **i**	0.643±0.149
DPDPE (10)	18.2±1.8 **a**	5.3±0.9 **a**	11.2±1.8 **c**	3.2±0.6 **b**	3.2±0.5 **a**	0.325±0.064	1.122±0.162
DPDPE+NAL (8)	46.1±4.2 **j**	14.3±3.2 **k**	35.9±4.8 **j**	17.8±3.1 **j**	13.6±2.7 **j**	0.335±0.074	1.207±0.161

Table 4.3. Monoamine concentrations (pg amine/μg protein ± SEM) and ratios of DHPG/NA and 5-HIAA/5-HT in the ME at 19.00h on pro-oestrus after administration or co-administration of various opioid agonists and antagonists at 13.00h on the same day. **a:** $p<0.001$; **b**: $p<0.005$; **c**: $p<0.01$; **d**: $p<0.05$ compared to the saline-treated animals, **e:** $p<0.001$; **f**: $p<0.005$; **g**: $p<0.01$ compared to the diamorphine-treated animals, **h:** $p<0.001$; **i**: $p<0.05$ compared to the U-50488H-treated animals, **j: p<0.001**; **k**: $p<0.01$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

Neurotransmitter Concentrations in the ARN							
Treatment	**NA**	**DHPG**	**DA**	**5-HIAA**	**5-HT**	**Ratio of DHPG/NA**	**Ratio of 5-HIAA/5-HT**
Saline (16)	32.5±1.6	16.0±2.0	6.2±1.0	6.7±1.1	5.9±0.6	0.498±0.061	1.379±0.329
Diamorphine (10)	21.4±2.1 **a**	4.4±0.8 **a**	4.2±0.5	6.0±1.5	6.2±1.0	0.207±0.024 **a**	1.070±0.246
Diamorphine+NAL (13)	69.3±5.2 **e**	23.8±2.1 **e**	8.3±0.9 **e**	13.8±5.0	8.4±2.0	0.363±0.042 **f**	1.133±0.255
U-50488H (8)	21.3±2.2 **b**	6.1±0.8 **b**	5.9±1.1	4.1±0.9	5.3±1.0	0.288±0.033 **d**	0.859±0.224
U-50488H+MR2266 (9)	31.9±4.4 **h**	14.8±2.6 **g**	7.0±0.7	7.4±1.2 **h**	8.5±0.9 **h**	0.511±0.096	0.836±0.123
DPDPE (10)	18.9±1.7 **a**	4.4±0.7 **a**	3.3±0.6 **c**	2.6±0.3 **c**	3.0±0.6 **b**	0.290±0.089 **d**	1.025±0.094
DPDPE+NAL (8)	41.3±3.9 **i**	11.1±3.1 **k**	5.2±0.4 **k**	15.6±2.4 **i**	12.1±2.6 **j**	0.267±0.062	1.544±0.279

Table 4.4. Monoamine concentrations (pg amine/μg protein ± SEM) and ratios of DHPG/NA and 5-HIAA/5-HT in the ARN at 19.00h on pro-oestrus after administration or co-administration of various opioid agonists and antagonists at 13.00h on the same day. **a:** $p<0.001$; **b:** $p<0.005$; **c:** $p<0.01$; **d:** $p<0.05$ compared to the saline-treated animals, **e:** $p<0.001$; **f:** $p<0.005$ compared to the diamorphine-treated animals, **g:** $p<0.01$; **h:** $p<0.05$ compared to the U-50488H-treated animals, **i:** $p<0.001$; **j:** $p<0.005$; **k:** $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

Serum LH levels (ng/ml)							
Animal ID	**13.00h**	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
C-8	LOW	LOW	LOW	NS	NS	LOW	LOW
C-9	LOW	LOW	LOW	1.96	10.8	16.6	15.9
C-15	LOW	LOW	LOW	LOW	LOW	LOW	LOW
C-16	NS	NS	NS	NS	NS	NS	10.2
C-23	LOW	LOW	LOW	LOW	LOW	3.3	2.8
C-27	LOW	1.66	NS	NS	NS	NS	NS
C-33	LOW	LOW	LOW	LOW	LOW	LOW	LOW
C-39	LOW	1.2	15.8	16.8	17.5	10.4	9.5
C-64	NS	NS	2.38	31.6	64.5	NS	38.0
C-65	NS	NS	3.26	36.4	65.0	50.7	38.5
C-67	NS	NS	2.54	1.80	1.25	1.53	1.96
C-70	NS	NS	1.84	2.5	34.78	45.84	46.14
C-73	NS	NS	2.18	2.63	24.61	62.41	63.45
C-77	NS	NS	1.4	8.0	1.6	NS	NS
C-78	NS	NS	1.5	NS	NS	NS	31.5
C-88	NS	NS	1.38	12.7	1.44	NS	1.64

Table 4.5. Plasma LH levels (ng/ml) recorded in individual conscious animals at hourly intervals between 13.00h and 19.00h on the afternoon of pro-oestrus following intraperitoneal saline administration at 13.00h. LOW: concentration below the limit of detection, NS: no sample obtained due to the spontaneous blockage of the indwelling cannula.

Serum LH levels (ng/ml)							
Animal ID	**13.00h**	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
C-3	NS	NS	LOW	LOW	LOW	LOW	LOW
C-5	LOW	LOW	LOW	LOW	LOW	LOW	LOW
C-34	LOW	LOW	LOW	LOW	LOW	10.7	9.2
C-35	LOW	LOW	LOW	5.06	16.5	11.5	9.8
C-38	NS	LOW	LOW	LOW	LOW	NS	18.5
C-41	LOW	LOW	LOW	LOW	28.8	24.4	16.8
C-47	LOW	LOW	LOW	LOW	11.9	20.2	14.7
C-51	NS	NS	LOW	LOW	LOW	LOW	1.20
C-58	NS	NS	2.65	3.08	1.96	2.61	1.49
C-89	NS	NS	1.69	2.89	2.21	2.47	1.53

Table 4.6. Plasma LH levels (ng/ml) seen in individual animals at hourly intervals between 13.00h and 19.00h on the afternoon of pro-oestrus following intraperitoneal diamorphine administration at 13.00h. LOW: concentration below the limit of detection, NS: no sample obtained due to the spontaneous blockage of the indwelling cannula.

Serum LH levels (ng/ml)							
Animal ID	**13.00h**	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
E-40	NS	LOW	LOW	LOW	LOW	LOW	LOW
E-41	NS	NS	NS	NS	NS	NS	5.2
E-50	NS	LOW	LOW	NS	NS	NS	LOW
E-53	NS	LOW	LOW	LOW	11.75	18.7	22.35
E-55	NS	33.45	NS	NS	NS	NS	7.10
E-56	NS	LOW	LOW	8.95	7.25	4.70	5.57

Table 4.7. Plasma LH levels (ng/ml) recorded in individual animals at hourly intervals between 13.00h and 19.00h on the afternoon of pro-oestrus following intraperitoneal diamorphine plus naloxone administration at 13.00h. LOW: concentration below the limit of detection, NS: no sample obtained due to the spontaneous blockage of the indwelling cannula.

Serum LH levels (ng/ml)							
Animal ID	**13.00h**	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
C-2	NS	NS	LOW	NS	NS	NS	LOW
C-40	LOW	LOW	LOW	LOW	LOW	0.34	LOW
C-42	0.4	LOW	LOW	LOW	LOW	LOW	LOW
C-44	LOW	LOW	LOW	LOW	LOW	LOW	LOW
C-49	LOW	NS	NS	NS	NS	NS	LOW
C-50	LOW	LOW	LOW	LOW	LOW	LOW	LOW
C-56	NS	NS	LOW	LOW	LOW	LOW	LOW
C-59	NS	NS	LOW	NS	NS	NS	LOW

Table 4.8. Plasma LH levels (ng/ml) observed in individual animals at hourly intervals between 13.00h and 19.00h on the afternoon of pro-oestrus following intraperitoneal U-50488H administration at 13.00h. LOW: concentration below the limit of detection, NS: no sample obtained due to the spontaneous blockage of the indwelling cannula.

Serum LH levels (ng/ml)							
Animal ID	**13.00h**	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
D-11	NS	LOW	LOW	LOW	LOW	NS	LOW
D-12	NS	LOW	LOW	LOW	LOW	5.2	19.5
D-13	NS	LOW	LOW	LOW	LOW	NS	LOW
D-15	NS	LOW	LOW	LOW	NS	LOW	LOW
D-17	NS	LOW	LOW	LOW	LOW	NS	LOW
D-19	NS	LOW	LOW	LOW	LOW	NS	LOW
D-21	NS	LOW	LOW	LOW	LOW	LOW	LOW
D-25	NS	LOW	NS	NS	NS	NS	LOW
D-28	NS	LOW	LOW	LOW	LOW	LOW	LOW

Table 4.9. Plasma LH levels (ng/ml) recorded in individual animals at hourly intervals between 13.00h and 19.00h on the afternoon of pro-oestrus following intraperitoneal U-50488H plus MR2266 administration at 13.00h. LOW: concentration below the limit of detection, NS: no sample obtained due to the spontaneous blockage of the indwelling cannula.

Serum LH levels (ng/ml)							
Animal ID	**13.00h**	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
C-45	NS	NS	5.9	18.7	18.8	NS	15.1
C-46	NS	NS	NS	LOW	NS	NS	LOW
C-52	NS	NS	LOW	LOW	LOW	LOW	LOW
C-53	NS	LOW	LOW	LOW	LOW	LOW	LOW
C-54	NS	NS	LOW	LOW	1.6	2.7	2.0
C-55	LOW	LOW	NS	NS	NS	NS	LOW
C-60	NS	LOW	LOW	LOW	LOW	NS	LOW
C-74	NS	NS	LOW	LOW	LOW	LOW	LOW
C-83	LOW	LOW	1.4	1.1	NS	NS	1.3
C-84	NS	NS	2.2	NS	NS	NS	19.0

Table 4.10. Plasma LH levels (ng/ml) observed in individual animals at hourly intervals between 13.00h and 19.00h on the afternoon of pro-oestrus following intraperitoneal DPDPE administration at 13.00h. LOW: concentration below the limit of detection, NS: no sample obtained due to the spontaneous blockage of the indwelling cannula.

Serum LH levels (ng/ml)							
Animal ID	**13.00h**	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
D-49	LOW	LOW	1.25	34.3	NS	NS	44.3
D-50	NS	LOW	1.0	0.5	LOW	LOW	LOW
D-54	LOW	0.5	2.6	NS	0.63	1.65	8.3
D-64	NS	NS	NS	NS	NS	NS	LOW
E-2	NS	LOW	0.22	0.25	NS	NS	12.0
E-19	NS	LOW	LOW	LOW	LOW	NS	11.0
E-23	LOW	0.75	12.2	61.5	21.2	NS	16.0
E-29	NS	LOW	LOW	NS	NS	NS	LOW

Table 4.11. Plasma LH levels (ng/ml) recorded in individual animals at hourly intervals between 13.00h and 19.00h on the afternoon of pro-oestrus following intraperitoneal DPDPE plus naloxone administration at 13.00h. LOW: concentration below the limit of detection, NS: no sample obtained due to the spontaneous blockage of the indwelling cannula.

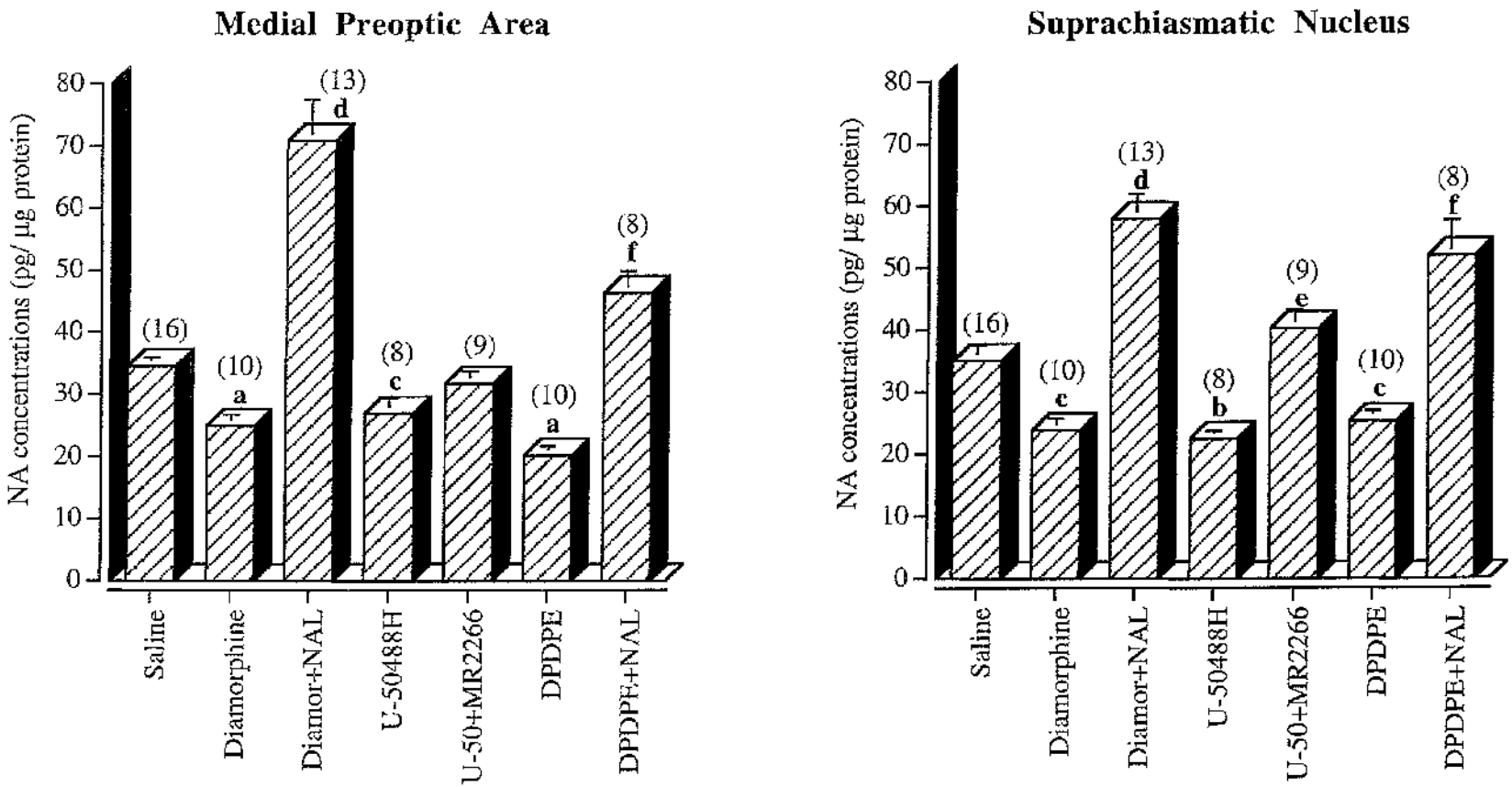


Figure 4.1. NA concentrations (pg/µg protein ± SEM) in the MPOA and SCN of conscious female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.001$; **b**: $p<0.005$; c: $p<0.01$ compared to the saline-treated animals, **d:** $p<0.001$ compared to the diamorphine-treated animals, **e:** $p<0.001$ compared to the U-50488H-treated animals, **f:** $p<0.001$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

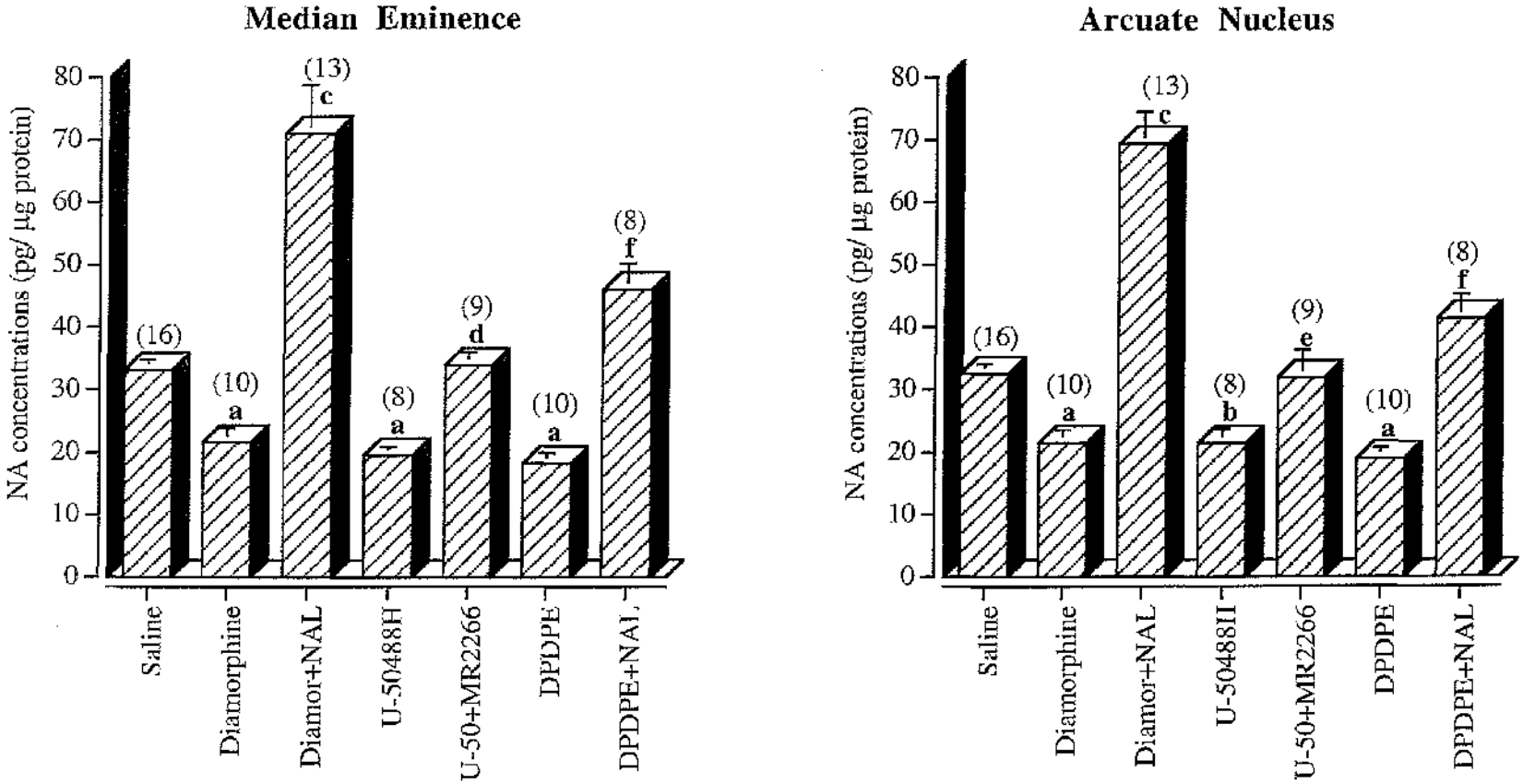


Figure 4.2. NA concentrations (pg/µg protein ± SEM) in the ME and ARN of conscious female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.001$; **b**: $p<0.005$ compared to the saline-treated animals, **c:** $p<0.001$ compared to the diamorphine-treated animals, **d:** $p<0.001$; **e**: $p<0.05$ compared to the U-50488H-treated animals, **f:** $p<0.001$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

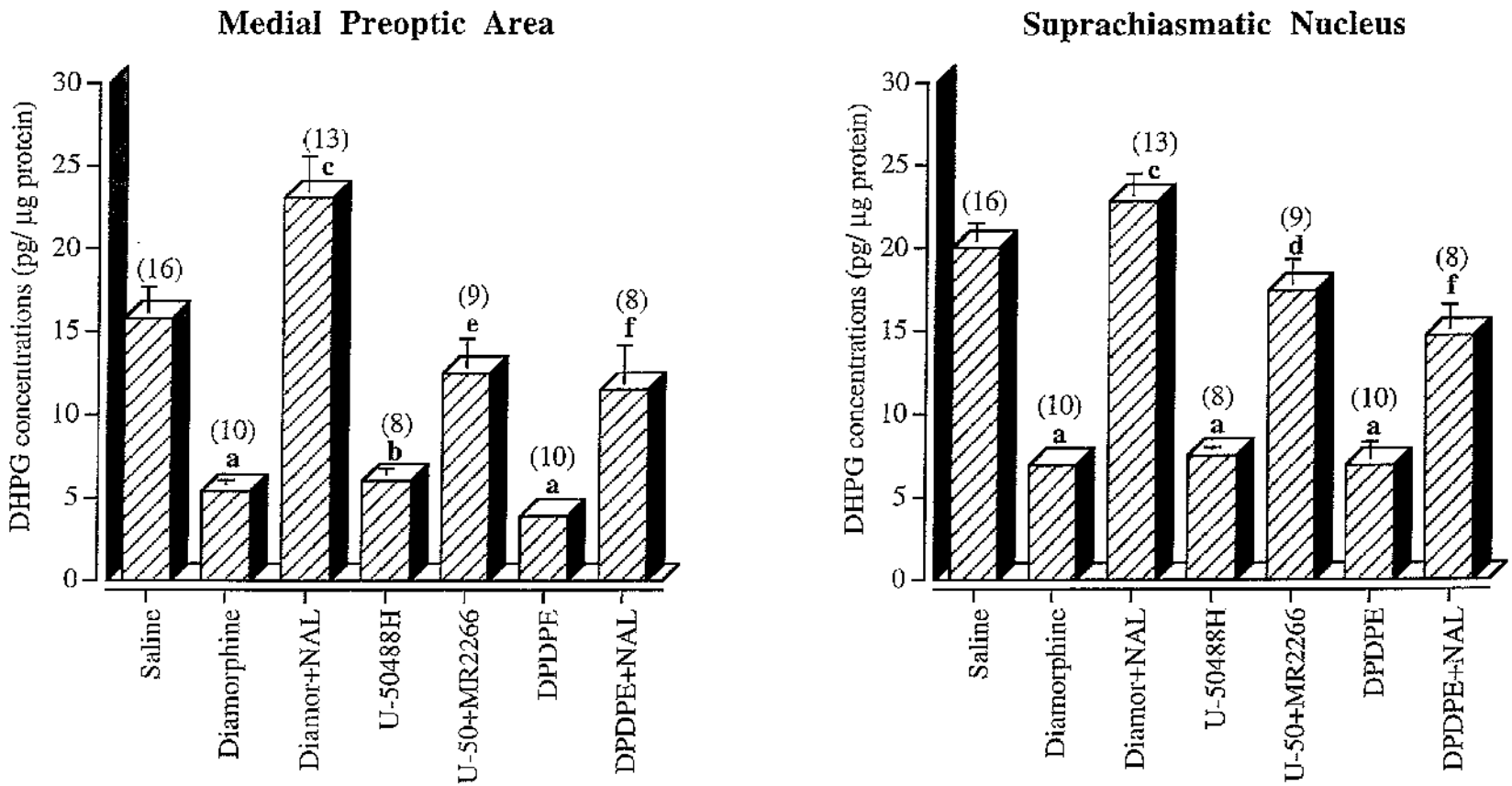


Figure 4.3. DHPG concentrations (pg/µg protein ± SEM) in the MPOA and SCN of conscious female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.001$; **b**: $p<0.005$ compared to the saline-treated animals, **c:** $p<0.001$ compared to the diamorphine-treated animals, **d:** $p<0.001$; **e**: $p<0.01$ compared to the U-50488H-treated animals, **f:** $p<0.005$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

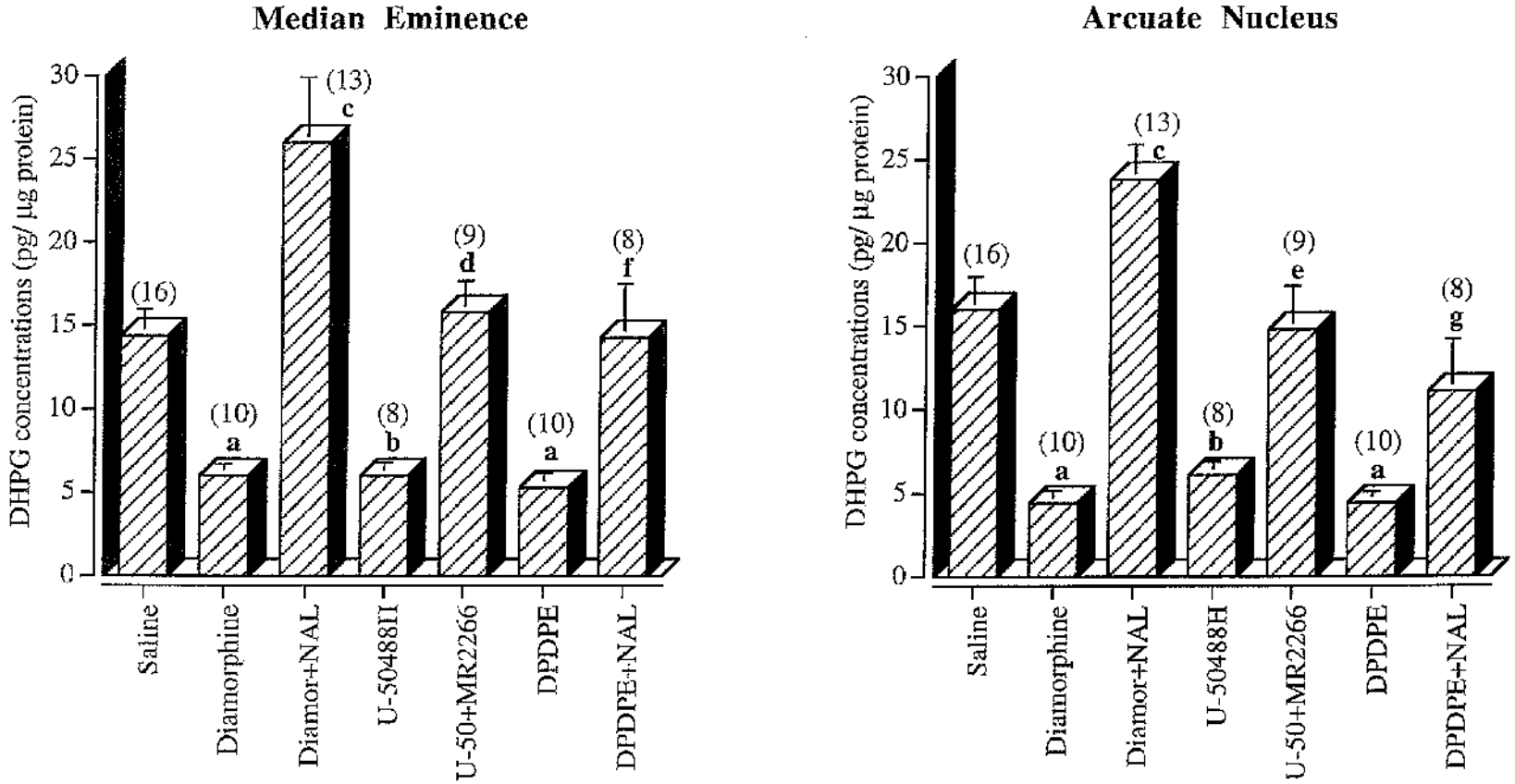


Figure 4.4. DHPG concentrations (pg/µg protein ± SEM) in the ME and ARN of conscious female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.001$; **b**: $p<0.005$ compared to the saline-treated animals, **c:** $p<0.001$ compared to the diamorphine-treated animals, **d:** $p<0.001$; **e**: $p<0.01$ compared to the U-50488H-treated animals, **f**: $p<0.01$; **g:** $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

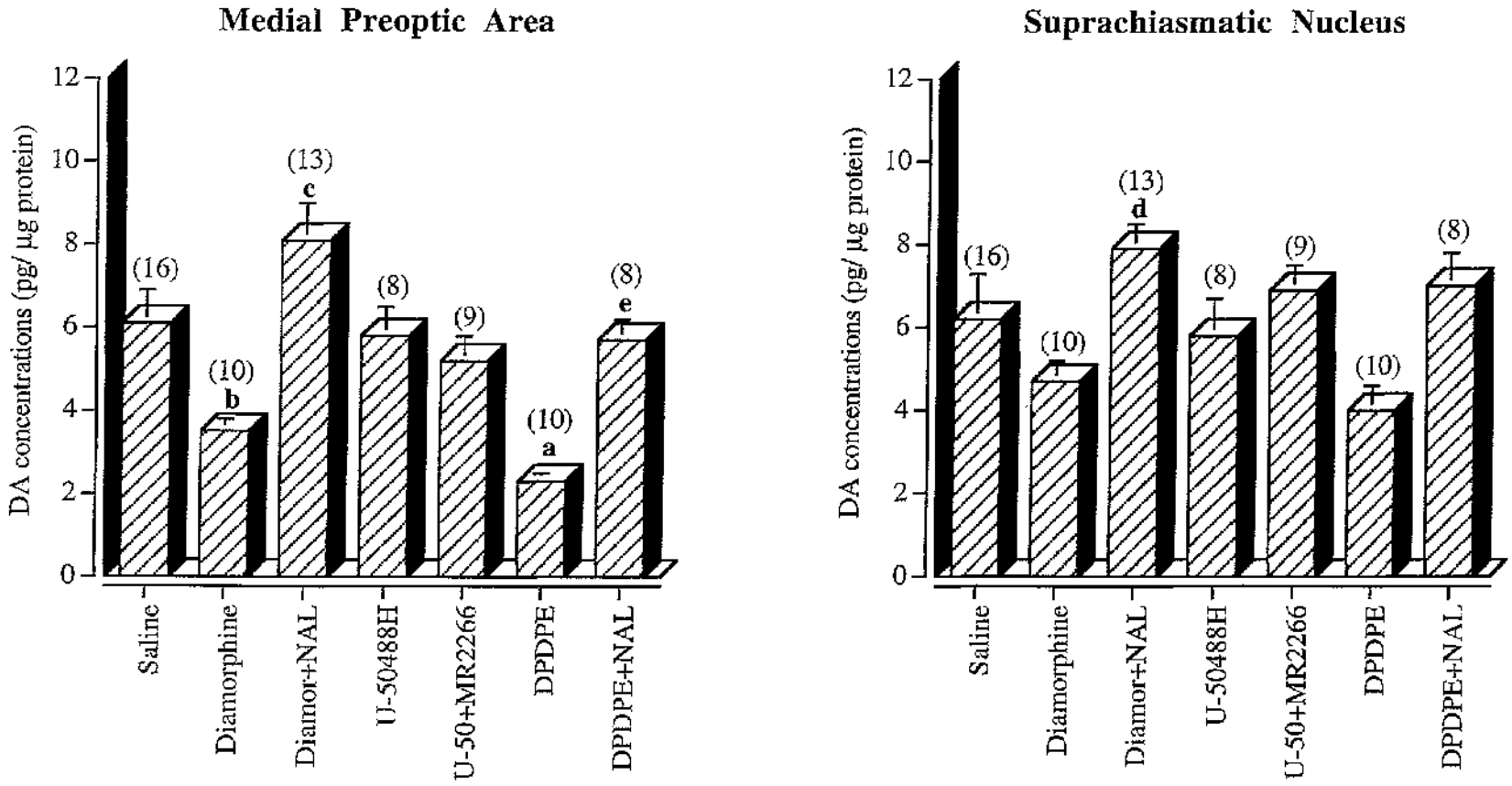


Figure 4.5. DA concentrations (pg/µg protein ± SEM) in the MPOA and SCN of conscious female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.005$; **b:** $p<0.01$ compared to the saline-treated animals, **c:** $p<0.001$; **d:** $p<0.005$ compared to the diamorphine-treated animals, **e:** $p<0.001$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

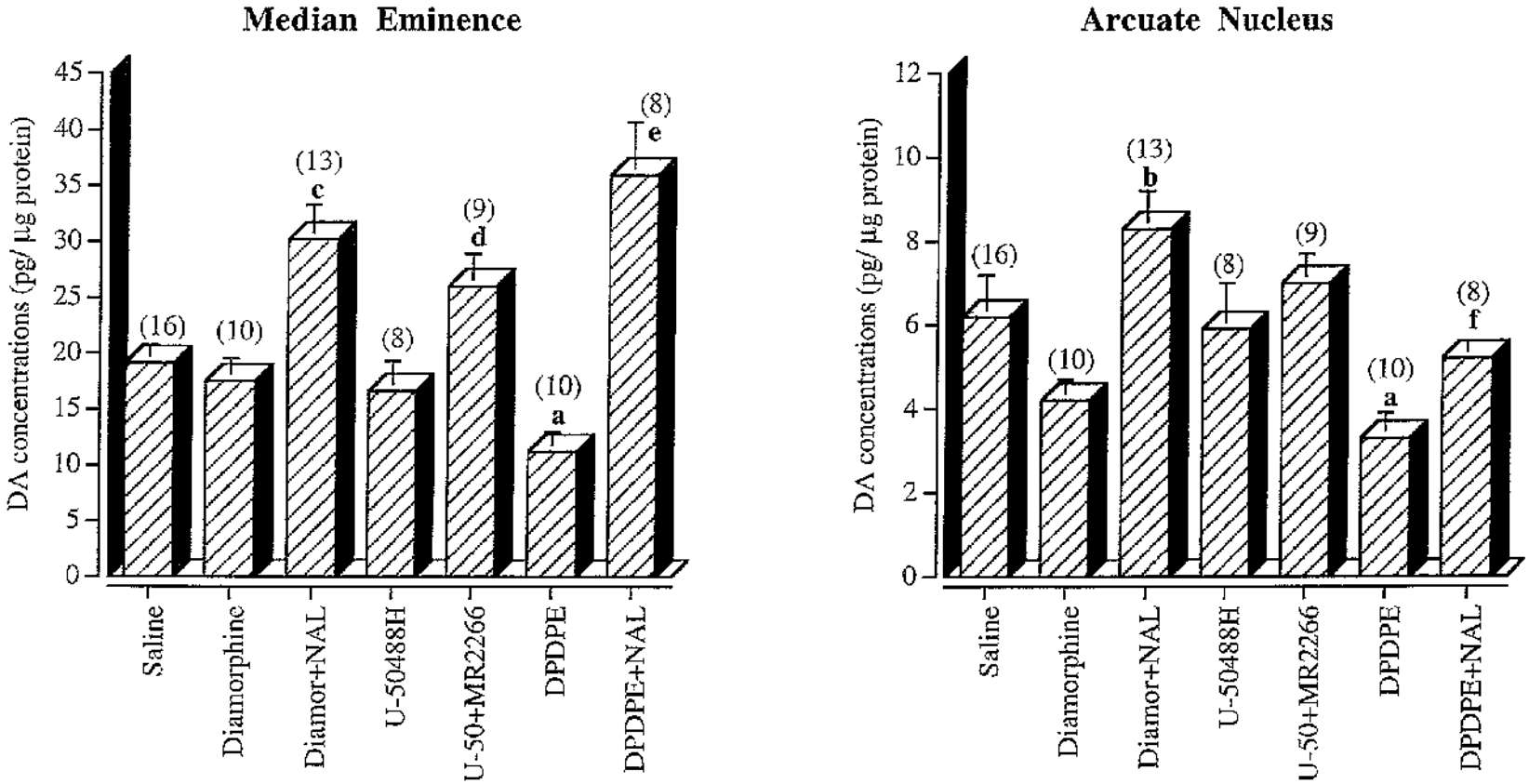


Figure 4.6. DA concentrations (pg/µg protein ± SEM) in the ME and ARN of conscious female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.01$ compared to the saline-treated animals, **b:** $p<0.001$; **c:** $p<0.005$ compared to the diamorphine-treated animals, **d:** $p<0.05$ compared to the U-50488H-treated animals, **e:** $p<0.001$; **f:** $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

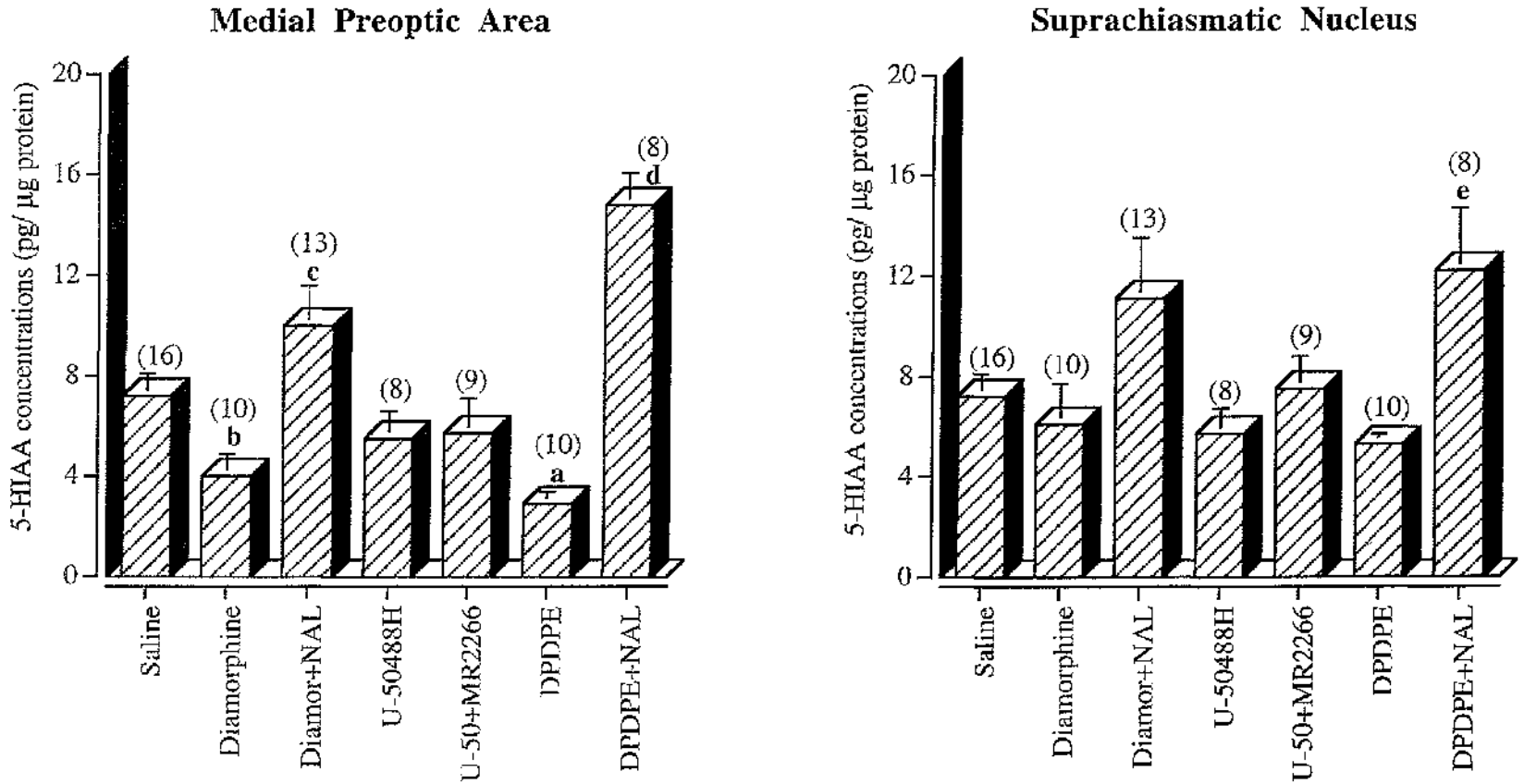


Figure 4.7. 5-HIAA concentrations (pg/µg protein ± SEM) in the MPOA and SCN of conscious female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.005$; **b:** $p<0.05$ compared to the saline-treated animals, **c:** $p<0.01$ compared to the diamorphine-treated animals, **d:** $p<0.001$; **e:** $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

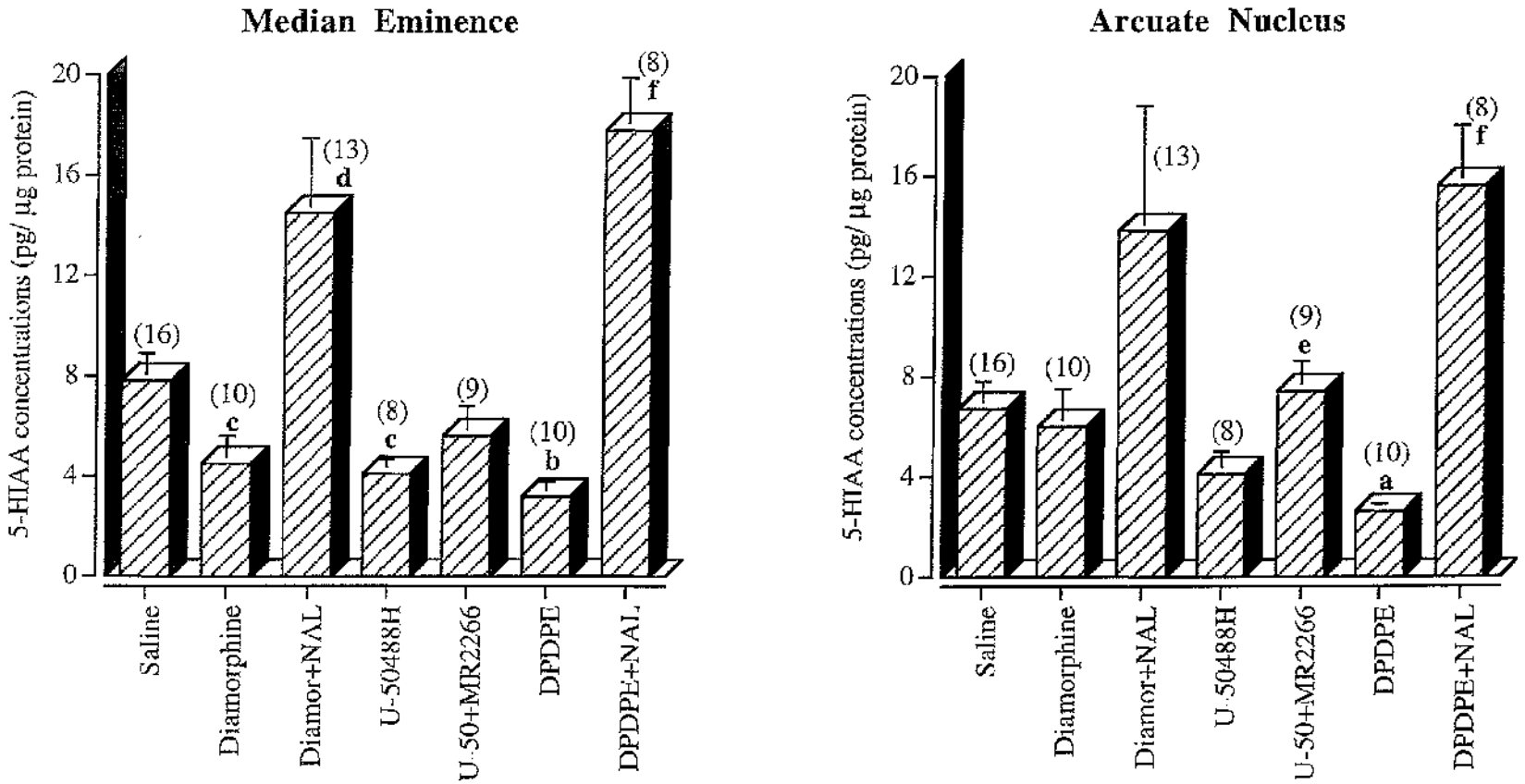


Figure 4.8. 5-HIAA concentrations (pg/µg protein ± SEM) in the ME and ARN of conscious female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.005$; **b**: $p<0.01$; **c**: $p<0.05$ compared to the saline-treated animals, **d:** $p<0.01$ compared to the diamorphine-treated animals, **e:** $p<0.05$ compared to the U-50488H-treated animals, **f:** $p<0.001$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

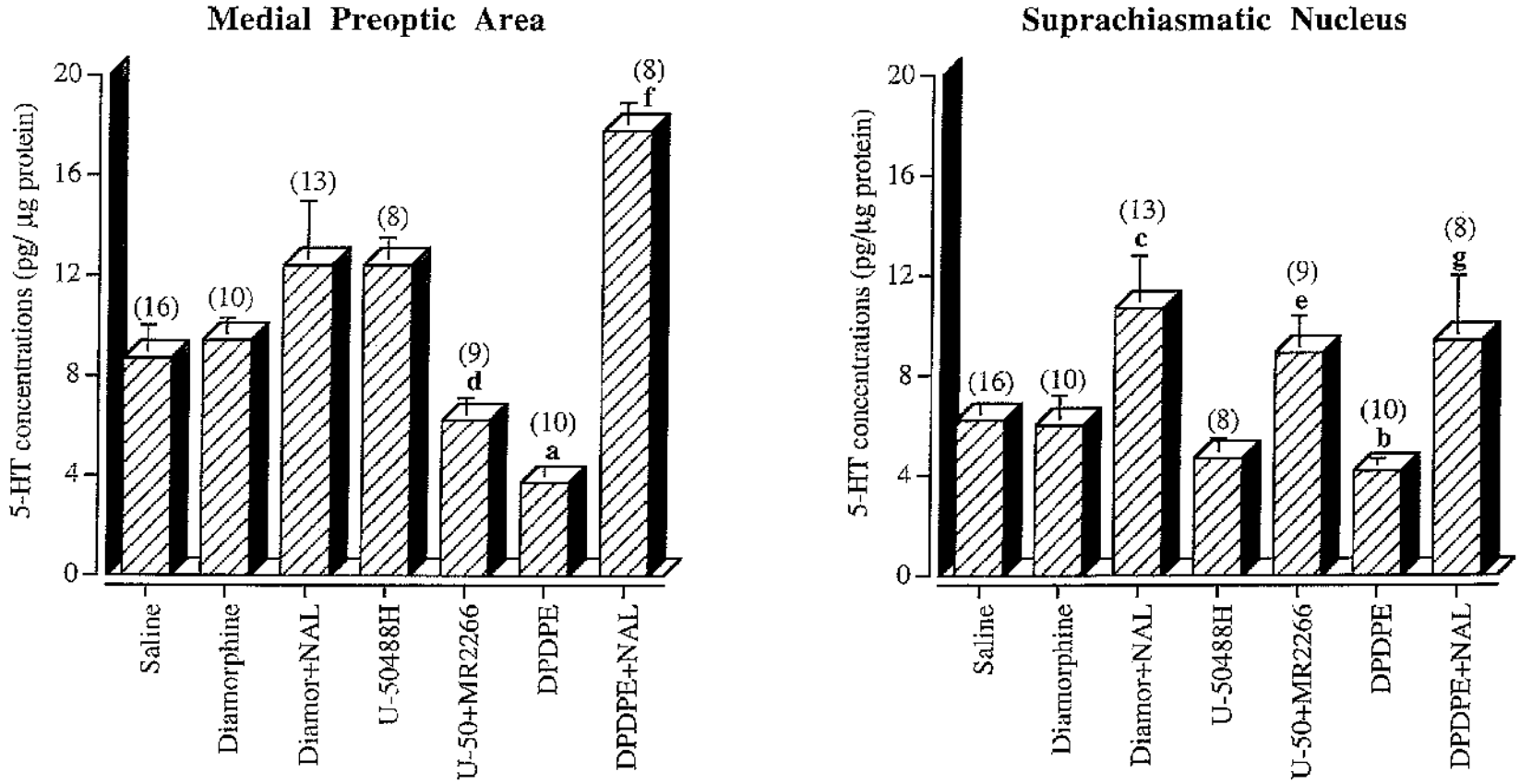


Figure 4.9. 5-HT concentrations (pg/µg protein ± SEM) in the MPOA and SCN of conscious female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.005$; **b**: $p<0.05$ compared to the saline-treated animals, **c:** $p<0.05$ compared to the diamorphine-treated animals, **d:** $p<0.005$; **e**: $p<0.05$ compared to the U-50488H-treated animals, **f:** $p<0.001$; **g**: $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

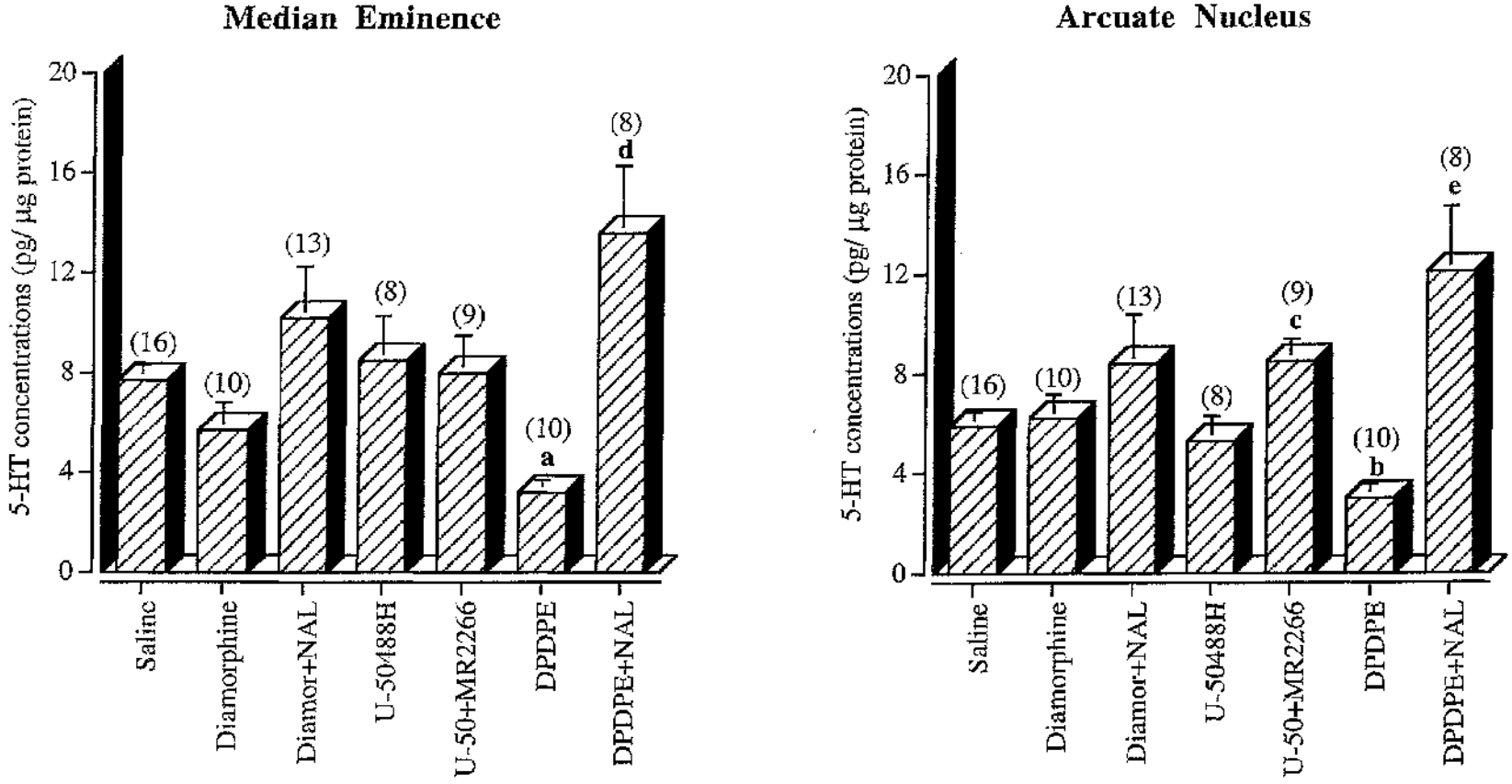


Figure 4.10. 5-HT concentrations (pg/µg protein ± SEM) in the ME and ARN of conscious female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.001$; **b**: $p<0.005$ compared to the saline-treated animals, **c:** $p<0.05$ compared to the U-50488H-treated animals, **d:** $p<0.001$; **e**: $p<0.005$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

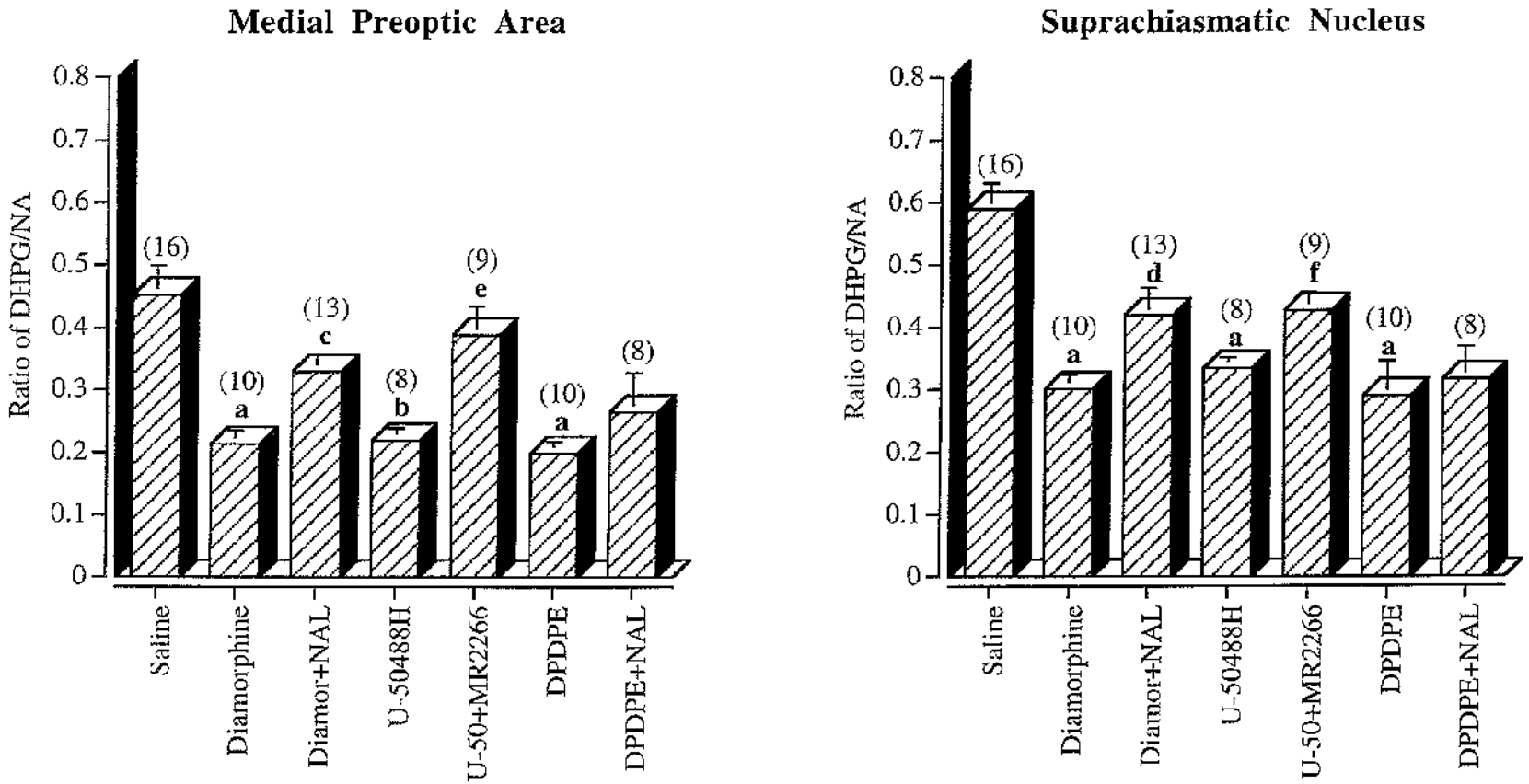


Figure 4.11. Ratio of DHPG/NA (Mean ± SEM) in the MPOA and SCN of conscious female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.001$; **b**: $p<0.005$ compared to the saline-treated animals, **c:** $p<0.01$; **d**: $p<0.05$ compared to the diamorphine-treated animals, **e:** $p<0.01$; **f**: $p<0.05$ compared to the U-50488H-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

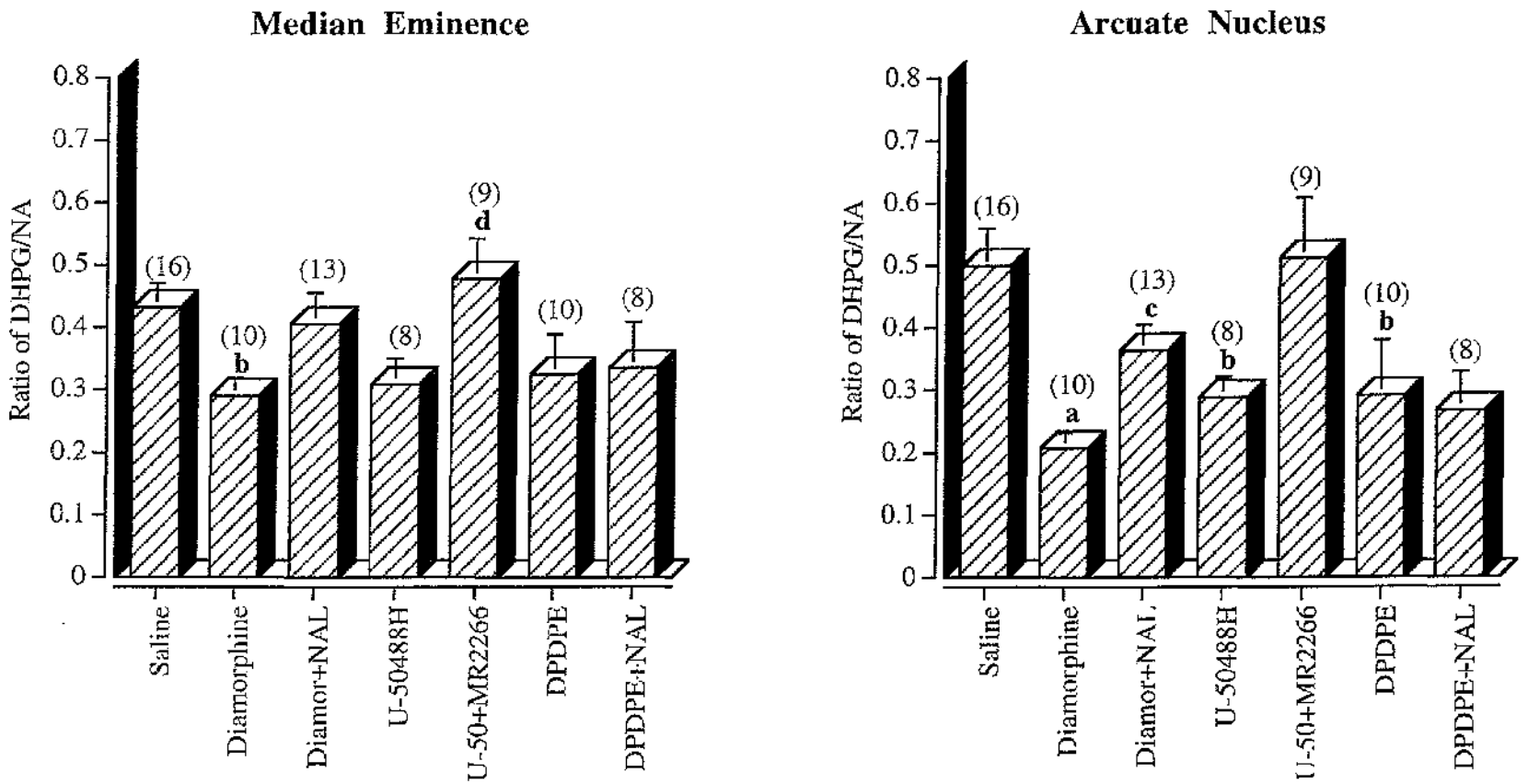


Figure 4.12. Ratio of DHPG/NA (Mean ± SEM) in the ME and ARN of conscious female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.001$; **b**: $p<0.05$ compared to the saline-treated animals, **c:** $p<0.005$ compared to the diamorphine-treated animals, **d:** $p<0.05$ compared to the U-50488H-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

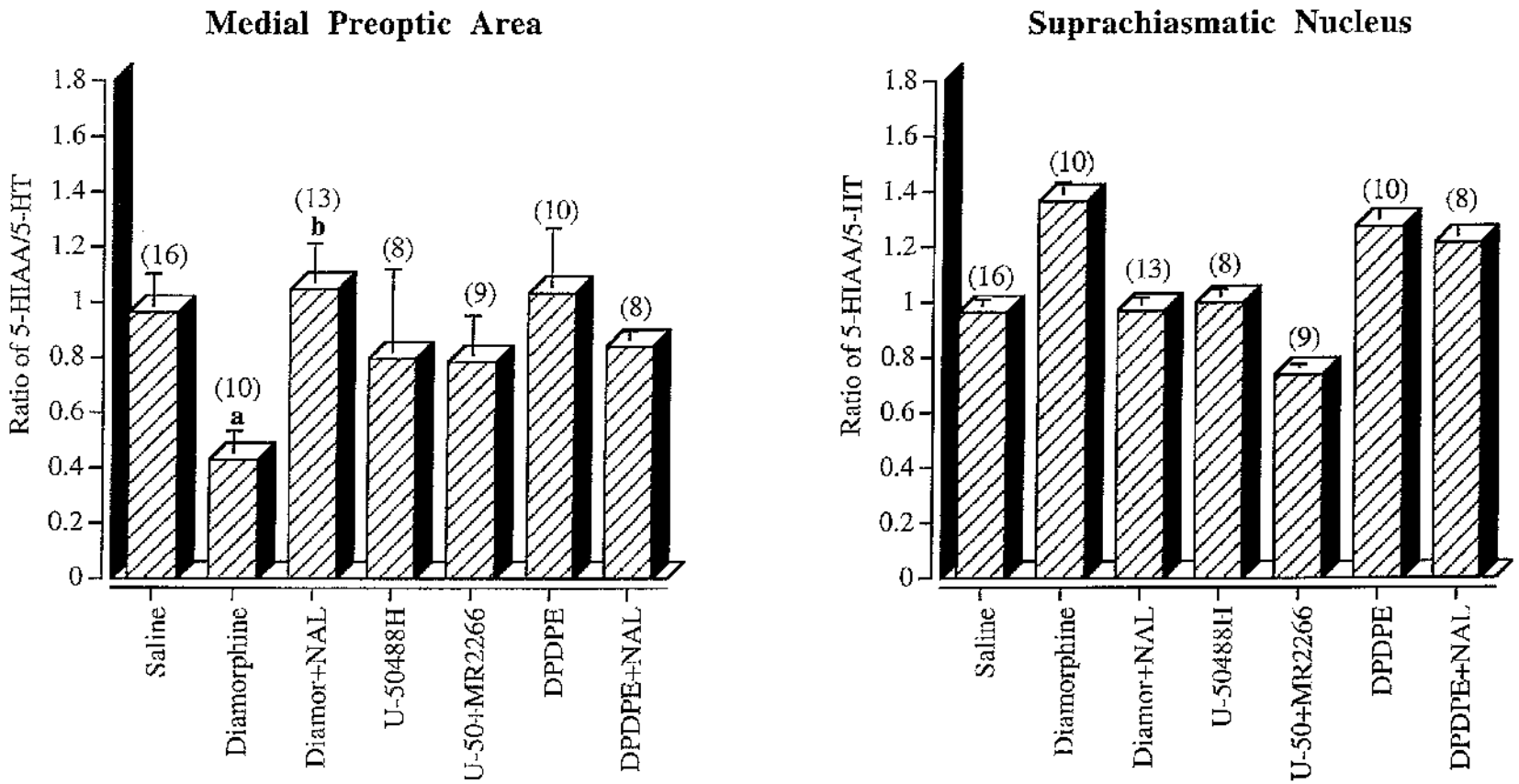


Figure 4.13. Ratio of 5-HIAA/5-HT (Mean ± SEM) in the MPOA and SCN of conscious female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. **a:** $p<0.01$ compared to the saline-treated animals, **b:** $p<0.01$ compared to the diamorphine-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

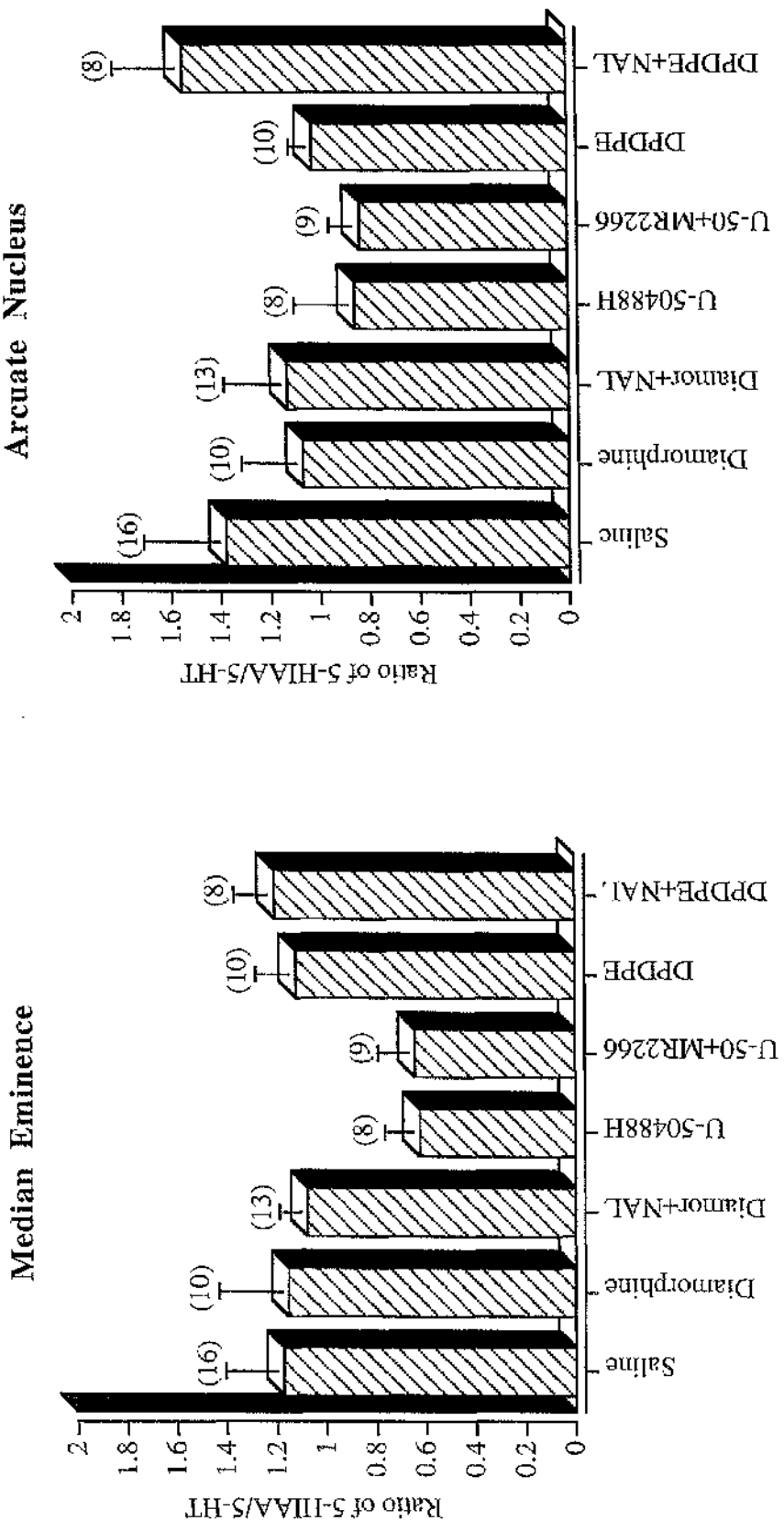


Figure 4.14. Ratio of 5-HIAA/5-HT (*Mean* ± SEM) in the ME and ARN of conscious female rats at 19.00h on the afternoon of pro-oestrus following the intraperitoneal administration of opioid agonists and antagonists or saline at 13.00h. The number of observations in each group is given in brackets.

EXPERIMENT III

Opioid modulation of the hypothalamic monoamine content during the anticipated LH surge in the ovx and steroid-primed rat

As detailed in the Introduction, opioid modulation of the central noradrenergic, dopaminergic and serotonergic systems has been reported. There is a bulk of evidence suggesting that activation of different opioid receptor subtypes brings about a reduction in the hypothalamic NA concentrations and opioid antagonists, such as naloxone, reverses these effects. It is well documented that NA may play a crucial role in the generation of the LH surge in the rat. Therefore, opioid-induced decreases in the hypothalamic NA content may account for the opioid suppression of the LH surge. However, the nature of interaction(s) between opioids, DA and 5-HT have been controversial. Both stimulatory and inhibitory actions of opioids on dopaminergic and serotonergic release and/or turnover in the MPOA, SCN, ME and ARN have been suggested. The results from Experiment II have shown that direction of opioid action on hypothalamic NA, DA and 5-HT release and/or turnover may be inhibitory at the time of the LH surge.

This experiment was undertaken to further investigate the effects of various selective opioid receptor agonists and antagonists on hypothalamic monoamine concentrations and plasma LH levels in the ovx and steroid-primed rat. Drugs or saline were centrally administered at 14.00h on the afternoon of the anticipated LH surge. It was expected that plasma LH levels would be elevated by prior administration of E_2 plus P and the anticipated LH surge would be timed more precisely.

Some of the drugs (diamorphine, naloxone, DPDPE) selected for this experiment had already been used in Experiments I and/or II; in which they were injected IP at 13.00h on the afternoon of pro-oestrus. In the present experiment, it was intended that icv infusion of these opioid agonists and their respective antagonists would demonstrate more clearly the mechanism(s) by which the neural control of LH secretion is exercised. It was also thought that the peptidergic ligands under investigation would act directly at the hypothalamic sites without, if any, a blood brain barrier following icv infusion.

Materials and Methods

The design of this experiment has already been explained in details in the Materials and Methods.

Adult female Sprague/Dawley rats were ovx and allowed to recover for a period ranging between 10 to 21 days before use. They were subcutaneously injected with E_2 48h before and P 4h prior to experimentation. On the afternoon of the anticipated LH surge, the animals were anaesthetised with Vetalar. A heparinised cannula (Portex, outside diameter 0.63mm) was inserted into the right femoral artery. The animals were then mounted on a stereotaxic frame and icv infused with either diamorphine (25µg/10µl), U-69593 (50µg/10µl), DPDPE (50µg/10µl), diamorphine plus naloxone (60µg/10µl), U-69593 plus MR1452 (100µg/10µl), DPDPE plus ICI 154,129 (60µg/10µl) or DPDPE plus naloxone (60µg/10µl) at 14.00h on the afternoon of the anticipated LH surge. Controls received sterile saline (10µl) alone.

Trunk blood (200µl) was collected via the indwelling cannula at hourly intervals throughout the afternoon. Anaesthesia was maintained by periodic intramuscular injections of Vetalar. The animals were kept warm throughout the experiment by the use of a heating lamp and were killed by decapitation just after the final blood collection at 19.00h. The number of animals in each group is shown in the Tables and Figures.

Results

The monoamine results are summarised in Tables 5.1 to 5.5 and Figures 5.1 to 5.16.

a) Noradrenaline

Control group values for the NA concentrations in the MPOA, SCN, ME and ARN at 19.00h were 59.7 ± 5.1, 58.2 ± 3.9, 54.6 ± 5.6 and 54.6 ± 4.1 pg/µg protein ± SEM respectively.

(i) Diamorphine

This µ-opioid agonist significantly reduced NA concentrations in all the hypothalamic regions examined in comparison to the saline controls.

(ii) Diamorphine + Naloxone

When diamorphine was co-administered with naloxone, NA concentrations were significantly elevated in all the hypothalamic regions examined compared to the group which received diamorphine alone.

(iii) U-69593

This κ-opioid agonist also significantly reduced NA levels in all four areas examined compared to the controls.

(iv) U-69593 + MR1452

The κ-opioid receptor antagonist significantly increased NA concentrations compared to the group receiving U-69593 alone in all four hypothalamic areas following its co-administration with U-69593.

(v) DPDPE

This δ-opioid agonist significantly lowered NA concentrations in all the hypothalamic regions examined compared to the saline-treated animals.

(vi) DPDPE + ICI 154,129

NA concentrations in all hypothalamic regions examined were significantly raised following the co-administration of DPDPE with the δ-opioid antagonist compared to the DPDPE-treated animals.

(vii) DPDPE + naloxone

NA levels in the four hypothalamic areas were significantly increased following the co-administration of DPDPE with naloxone compared to the DPDPE-treated group.

b) 3,4-Dihydroxyphenylglycol

Control values for DHPG concentrations in the MPOA, SCN, ME and ARN at 19.00h were 22.9 ± 2.9, 20.6 ± 2.6, 25.8 ± 4.5 and 23.1 ± 3.2 pg/μg protein ± SEM respectively.

(i) Diamorphine

Diamorphine significantly decreased DHPG concentrations in the hypothalamus compared to those seen in control animals.

(ii) Diamorphine + Naloxone

Treatment with diamorphine plus naloxone caused no significant changes in DHPG levels in any of the hypothalamic areas examined compared those animals which received only diamorphine.

(iii) U-69593

DHPG concentrations were significantly reduced in the hypothalamus by the κ-opioid agonist compared to the control values.

(iv) U-69593 + MR1452

The co-administration of the κ-opioid agonist and antagonist resulted in significant increases in DHPG levels in the hypothalamus in comparison to the animals in (iii) above.

(v) DPDPE

DPDPE significantly decreased DHPG concentrations in all the hypothalamic regions examined compared to the saline-treated animals.

(vi) DPDPE + ICI 154,129

When DPDPE was co-administered with ICI 154,129, DHPG levels were significantly elevated in the hypothalamus compared to those rats which received DPDPE alone.

(vii) DPDPE + Naloxone

The treatment with DPDPE plus naloxone had no significant effects on DHPG levels in the MPOA, SCN or ARN, but levels in the ME were significantly raised in comparison to the group receiving DPDPE alone.

c) Dopamine

Dopamine levels (Mean ± SEM) in the MPOA, SCN, ME and ARN of the control animals at 19.00h were 9.9 ± 1.1, 9.9 ± 1.0, 38.2 ± 3.8 and 10.1 ± 1.1 pg/μg protein respectively.

(i) Diamorphine

Diamorphine significantly reduced DA levels in the MPOA, SCN and ME in comparison to saline-treated animals. However, there was no statistically significant alteration of DA levels in the ARN.

(ii) Diamorphine + Naloxone

DA concentrations were significantly increased in the MPOA, SCN and ME compared to diamorphine-treated animals following the co-administration of

diamorphine with naloxone. Again, the DA levels were not significantly altered in the ARN of this group.

(iii) U-69593
The κ-opioid agonist caused significant decreases in the DA concentrations in all hypothalamic areas examined in comparison to control group values.

(iv) U-69593 + MR1452
When U-69593 was co-administered with MR1452, DA levels were significantly elevated in the hypothalamus compared to values seen in the U-69593-treated animals.

(v) DPDPE
The δ-opioid receptor agonist significantly decreased DA concentrations in the hypothalamus compared to the controls.

(vi) DPDPE + ICI 154,129
Treatment with the δ-opioid agonist plus ICI 154,129 had no significant effects on DA levels in any of the hypothalamic regions examined.

(vii) DPDPE + naloxone
DA concentrations were significantly elevated following the co-administration of DPDPE plus naloxone in the MPOA, SCN and ME compared to the group which received DPDPE alone, but levels in the ARN were not significantly altered.

d) 5-Hydroxyindoleacetic Acid
Concentrations of 5-HIAA (Mean ± SEM) in the MPOA, SCN, ME and ARN of the control animals at 19.00h were 16.1 ± 2.7, 13.3 ± 1.8, 13.2 ± 1.8, 12.1 ± 2.1 pg/μg protein respectively.

(i) Diamorphine
Diamorphine brought about significant decreases of 5-HIAA concentrations only in the MPOA compared to the control group values.

(ii) Diamorphine + Naloxone

5-HIAA levels were significantly elevated following the co-administration of diamorphine plus naloxone in all hypothalamic regions examined in comparison to the diamorphine-treated animals.

(iii) U-69593

This κ-opioid agonist significantly reduced concentrations of 5-HIAA in the MPOA, SCN and ME compared to values seen in the saline-treated animals. However, 5-HIAA levels were not significantly altered in the ARN.

(iv) U-69593 + MR1452

When U-69593 was co-administered with MR1452, 5-HIAA concentrations were significantly increased in all hypothalamic areas examined compared to the group which received U-69593 alone.

(v) DPDPE

Administration of the δ-opioid agonist resulted in significant decreases in the 5-HIAA levels in the MPOA and ARN compared to the control group. However, DPDPE had no significant effect on the 5-HIAA concentrations in the SCN and ME.

(vi) DPDPE + ICI 154,129

The co-administration of DPDPE and ICI 154,129 caused no significant changes of 5-HIAA concentrations in any of the hypothalamic regions examined.

(vii) DPDPE + Naloxone

5-HIAA levels were significantly increased following the co-administration of DPDPE and naloxone in all hypothalamic regions examined compared to the DPDPE-treated animals.

e) Serotonin

Control values (Mean ± SEM) found for 5-HT in the MPOA, SCN, ME and ARN at 19.00h were 16.0 ± 1.9, 15.3 ± 1.7, 14.5 ± 1.4 and 14.5 ± 2.0 pg/μg protein respectively.

(i) Diamorphine

This μ-opioid receptor agonist significantly reduced concentrations of 5-HT in the hypothalamus compared to values recorded in the saline-treated animals.

(ii) Diamorphine + Naloxone

When diamorphine was co-administered with naloxone, 5-HT levels were significantly elevated in the MPOA, ME and ARN in comparison to the diamorphine-treated group, but they were not significantly affected in the SCN.

(iii) U-69593

U-69593 caused significant decreases of the 5-HT concentrations in the hypothalamus compared to the control group values.

(iv) U-69593 + MR1452

5-HT levels were significantly raised in the hypothalamus following the co-administration of the κ-opioid agonist with MR1452 compared to the group which received U-69593 alone.

(v) DPDPE

DPDPE significantly reduced 5-HT concentrations in the SCN, ME and ARN compared to levels observed in the saline-treated animals. However, there was no significant effect in the MPOA.

(vi) DPDPE + ICI 154,129

When DPDPE was co-administered with the δ-opioid receptor antagonist, 5-HT concentrations remained unaffacted in the SCN, ME and ARN, but were significantly decreased in the MPOA compared to the DPDPE-treated animals.

(vii) DPDPE + Naloxone

Co-administration of DPDPE with naloxone significantly increased the concentrations of 5-HT in all hypothalamic areas examined in comparison to DPDPE-treated animals.

e) Ratio of 5-HIAA/5-HT

The ratio of 5-HIAA/5-HT in the MPOA, SCN, ME and ARN of the control group at 19.00h were 1.209 ± 0.262, 0.811 ± 0.093, 0.919 ± 0.103 and 0.881 ± 0.157 respectively (results expressed as Mean ± SEM).

(i) Diamorphine

The ratio of 5-HIAA/5-HT was not significantly affected by diamorphine in any of the hypothalamic regions examined.

(ii) Diamorphine + Naloxone

Naloxone significantly increased the 5-HIAA/5-HT ratio after its co-administration with diamorphine, in the MPOA, ME and ARN compared to the group which received diamorphine alone. However, this treatment had no significant effect on the ratio of 5-HIAA/5-HT in the SCN.

(iii) U-69593

The κ-opioid agonist caused no significant changes in the ratio of 5-HIAA/5-HT in any of the hypothalamic areas studied.

(iv) U-69593 + MR1452

The co-administration of U-69593 with MR1452 likewise had no significant effect on the ratio of 5-HIAA/5-HT in any of the four areas examined.

(v) DPDPE

DPDPE significantly increased the ratio of 5-HIAA to 5-HT in only the ME compared to control group values.

(vi) DPDPE + ICI 154,129

This treatment resulted in a significant increase in the ratio of 5-HIAA/5-HT in the MPOA and ARN compared to the DPDPE-treated animals, but not in the SCN or ME.

(vii) DPDPE + Naloxone

When DPDPE was co-administered with naloxone, the ratio of 5-HIAA/5-HT was significantly increased only in the MPOA and ARN, but not in the SCN and ME, compared to the group which received DPDPE alone.

f) Ratio of DHPG/NA

The control group values for the ratio of DHPG/NA in the MPOA, SCN, ME and ARN at 19.00h were 0.388 ± 0.034, 0.367 ± 0.039, 0.467 ± 0.063 and 0.424 ± 0.046 respectively (results expressed as Mean ± SEM).

(i) Diamorphine (ii) Diamorphine + Naloxone
Neither the μ-opioid agonist alone nor its co-administration with naloxone significantly altered the ratio of DHPG/NA in any of the hypothalamic regions examined.

(iii) U-69593
U-69593 significantly lowered the ratio of DHPG/NA in the hypothalamus compared to control group values.

(iv) U-69593 + MR1452
When the κ-opioid agonist was co-administered with MR1452, the ratio of DHPG/NA was significantly increased in the MPOA and SCN, but not in the ME and ARN, compared to values seen in the U-69593-treated group.

(v) DPDPE (vi) DPDPE + ICI 154,129 (vii) DPDPE + Naloxone
None of these treatments significantly altered the ratio of DHPG/NA in any of the hypothalamic areas studied.

g) Luteinising Hormone
Results are shown in Tables 5.5 to 5.14.

In some of the control animals (eight out of 15) there were rises in plasma LH levels at various times on the afternoon of the anticipated LH surge. However, in the remainder of the saline-treated animals LH levels were undetectable.

(i) Diamorphine
Although some rises in plasma LH levels were seen in the diamorphine-treated rats, these were not different from those seen in the controls.

(ii) Diamorphine + Naloxone, (iii) U-69593, (iv) U-69593 + MR1452, (v) DPDPE, (vi) DPDPE + ICI 154,129, (vii) DPDPE + Naloxone
None of these treatments had any significant effect on plasma LH levels. In most instances LH levels were undetectable.

Discussion

Luteinising Hormone

In the present experiment, the animals were anaesthetised with Vetalar (ketamine) in order to perform icv infusion of the opioid agents under investigation. Since it appeared from the work described in Experiment I that urethane disrupted the expected pre-ovulatory LH surge, a careful selection of anaesthetics for this experiment had to be made. Following a detailed literature search, saffan and ketamine were chosen so that they would have the least effect on hormone secretion patterns. However, preliminary studies using both anaesthetics showed that plasma LH levels in the saffan-anaesthetised rats were very low while they seemed less affected by ketamine anaesthesia. Previous work from this laboratory had produced inconsistent results using saffan anaesthesia. Vetalar was therefore selected to anaesthetise the animals throughout Experiment III.

There were some rises in plasma LH levels in seven out of 15 control animals. However, in the remainder and also in the various opioid agonist and antagonist-treated groups LH levels were consistently either low or below the limit of detection. Interestingly, two of the diamorphine-treated animals showed some rises in LH levels at the 17.00h, 18.00h and 19.00h sampling intervals.

It has been documented that activation of both μ- and κ-opioid subtypes by specific agonists brings about an inhibition of the LH surge (Grossman and Dyer, 1989). These effects were confirmed in Experiment II. It was also observed that the selective δ-opioid receptor agonist, DPDPE, is as effective as the μ- and κ-agonists in suppressing the LH surge. In this experiment, only moderate doses of diamorphine, U-69593 and DPDPE were administered icv to the ovx and steroid-primed rats to further elaborate the participation of multiple opioid subtypes in the central regulation of LH release. Plasma LH levels were found to be either low or undetectable at all sampling intervals following the administration of diamorphine, U-69593 or DPDPE on the afternoon of the anticipated LH surge. These findings were expected and confirmed the inhibitory effects of the EOPs on pulsatile LH secretion. However, when all three opioid agonists were co-infused into the lateral ventricle with their respective antagonists, the anticipated rises in plasma LH concentrations were not observed. Naloxone is able to overcome morphine-induced suppression of the LH surge (Piva *et al*, 1985; Allen *et al*, 1987). It also

reversed the inhibitory effects of DPDPE in the conscious animal model as reported in Experiment II. Here, naloxone and two other opioid receptor antagonists studied, MR1452 and ICI 154,129, failed to elevate plasma LH levels in the ketamine-anaesthetised animals. It is therefore difficult to interpret these results and draw any conclusions regarding specific actions of different opioid receptor subtypes on the anticipated LH surge.

There have been previous reports in the literature on the use of ketamine and saffan as anaesthetic agents in neuroendocrine experiments (Sherwood *et al,* 1980; Ching, 1982; Matzen *et al,* 1987; Hartman *et al,* 1989). It has been claimed that in ketamine-anaesthetised rats, peak LH responses are not significantly different from those in unanaesthetised control animals (Hartman *et al,* 1989). Furthermore, in the latter study ketamine appeared to have no effect upon the NA-induced LH surge. It has also been reported that ketamine and saffan do not significantly influence plasma LH levels in the male rat (Matzen *et al,* 1987). Previously, the GnRH rise measured in the hypothalamo-hypophyseal portal blood under ketamine and/or saffan anaesthesia has been found to be of sufficient magnitude to initiate the spontaneous LH surge in the pro-oestrous rat (Sherwood *et al,* 1980; Ching, 1982). In contrast to earlier findings, the results described here have indicated that both saffan and ketamine administration either abolished or greatly reduced the anticipated LH surge, but did not alter hypothalamic NA release and/or turnover. This is surprising, because a reduction in LH release is usually associated with a fall in hypothalamic NA activity and a decrease in GnRH secretion into the hypophyseal portal blood vessels (for review see Ramirez *et al,* 1984; Barraclough, 1994; the current Introduction). However, it would appear that under the ketamine and saffan anaesthesia utilised in this experiment, the GnRH pulse generator and its essential component, the central noradrenergic system, were unaffected. Instead of acting at the hypothalamic level, perhaps these anaesthetics inhibited the LH surge by reducing the pituitary responsiveness to the GnRH discharge. Nevertheless, the mechanisms by which saffan and ketamine might produce such effects are currently unknown.

As mentioned earlier in Experiment I, hypothermia has been postulated as the primary cause of LH pulse suppression in anaesthetised animals. It is unlikely that hypothermia occurred under ketamine anaesthesia since the animals were kept warm by the use of a heating lamb throughout this experiment. Also, amongst a number of anaesthetics, ketamine has been shown to have the least effects on the

core temperature (Wixson *et al*, 1987). Furthermore, this anaesthetic causes minimal respiratory depression and therefore minimal hypoxia. Indeed, no anaesthetic-related mortality was observed during the entire course of the experiment. Unrecognised hypothermic effects of drug treatments may also suppress LH pulses (Strutton and Coen, 1996). However, those drugs employed in the present study are unlikely to have caused hypothermia since they were infused icv. Previously, opioids have been shown to inhibit LH secretion without having hypothermic effects following icv administration (Leadem and Kalra, 1985; Jansky, 1990).

In conclusion, it is thought that the ketamine anaesthesia may abolish the anticipated LH surge by reducing the pituitary responsiveness to GnRH release. Although ketamine has been suggested as a safe anaesthetic with the least effects on the LH secretory systems compared to other anaesthetics (Hartman *et al*, 1989), care should always be taken when using general anaesthetics in neuroendocrine studies.

Hypothalamic Aminergic Activity

It has been confirmed in this experiment that following icv infusion of opioid agonists and antagonists at the time of the anticipated LH surge, the release and/or turnover of biogenic amines in the specific hypothalamic regions is inhibited. Furthermore, it appears that these inhibitory effects on aminergic neurotransmission are exerted via multiple opioid receptor subtypes.

Administration of diamorphine significantly decreased concentrations of both NA and DHPG in all four areas studied. Naloxone antagonised the effects of the μ-opioid agonist on NA content of the hypothalamus, but had no effect on its metabolite (DHPG) levels. However, the ratio of DHPG/NA was significantly elevated in all the hypothalamic regions examined after the co-administration of naloxone with diamorphine. These results are consistent with previous reports suggesting that opioids inhibit NA release and/or turnover via opioidergic receptors located at the noradrenergic nerve terminals (Grossman and Dyer, 1989). Morphine decreases noradrenergic activity in the MPOA and ME of ovx and steroid-primed rats (Akabori and Barraclough, 1986). *In vitro*, activation of μ- and δ-opioid receptors inhibits electrically-stimulated release of NA from slices of the MPOA (Diez-Guerra *et al*, 1987). Naloxone has been shown to stimulate

NA release within the hypothalamus (Nishihara *et al*, 1991). It is thus confirmed that opioid modulation of the noradrenergic neurotransmission via μ-opioid subtypes is inhibitory.

There have been several attempts to demonstrate the involvement of the κ-opioid subtypes in the modulation of NA neuronal activity within the hypothalamus. Activation of κ-opioid receptors has been shown to cause a significant decrease in NA turnover in the SCN and VMH (Burkard, 1984). It has also been reported that κ-opioid agonists increase NA turnover in the whole hypothalamus (Gopalan *et al*, 1989a). However, it should be noted that by measuring amine levels in the entire hypothalamus, it is not possible to localise the effects of a particular drug, the actions of which may differ in specific regions. The results of the present experiment have indicated that icv infusion of a selective κ-agonist, U-69593, reduces NA release and turnover in the MPOA, SCN, ME and ARN. Therefore, it is suggested that, consistent with previous findings (Experiment II), κ-opioid influence on the noradrenergic system is inhibitory. Prevention of the decreases in the concentrations of both NA and DHPG by the κ-antagonist, MR1452 would point to a specific κ-opioid action.

The δ-agonist, DPDPE, reduced levels of NA and DHPG in parallel within all the hypothalamic regions examined. These findings appear to support the concept that δ-opioid receptors may also have inhibitory effects on NA release and turnover at the time of the LH surge. Naloxone, the non-selective opioid antagonist, prevented these suppressive effects of DPDPE. Due to the putative cross-communication between μ- and δ-opioid subtypes (Stadimis and Young, 1992; Traynor and Elliott, 1993), it was suggested that both receptor types could be involved in mediating the effects of DPDPE on NA neuronal activity (Experiment II). The results of the present experiment revealed that a selective δ-opioid antagonist, ICI 154,129 was as effective as naloxone in negating the inhibitory effects of DPDPE on the hypothalamic NA release and turnover. Thus it appears that the δ-opioid action on the central noradrenergic neurotransmission may operate independent of the μ-opioid receptors. Activation of δ-opioid receptors has been shown to inhibit NA release from slices of the MPOA *in vitro* (Diez-Guerra *et al*, 1987).

The EOPs differentially affect activity of various DA-containing neurons and pathways in the brain, particularly in the hypothalamus at the time of the LH surge

(reviewed in the Introduction). The DA results obtained from icv administration of various opioid agonists and antagonists to ovx and steroid-primed rats appear to confirm the observations made in Experiment II i.e. that activation of multiple opioid receptors inhibits DA release in discrete hypothalamic areas. Diamorphine reduced DA concentrations in the MPOA, SCN and ME in a naloxone-reversible manner. Paradoxically neither diamorphine alone nor, its administration together with naloxone, significantly altered DA levels in the ARN where short dopaminergic neurons are found (Lindvall and Bjorklund, 1982). It has been reported that the DA content of the ME is significantly reduced at the time of the LH surge compared to the values seen on the morning of the pro-oestrus (Akabori and Barraclough, 1986). Pharmacological stimulation of the μ-opioid receptors has resulted in an inhibition of evoked DA release from the MBH *in vitro* (Heijna *et al*, 1991). However, μ-opioid agonists have also been suggested to have no effects on the TIDA dopaminergic neurons (Manzanares *et al*, 1992). The present results further demonstrate that activation of μ-opioid receptors inhibits the central dopaminergic system.

DPDPE significantly reduced the DA content in all the hypothalamic regions examined. Naloxone prevented the negative effects of DPDPE on DA release in the MPOA, SCN and ME. As mentioned earlier, a cross-communication between the μ- and δ-opioid receptor subtypes in the brain has been postulated. It has been suggested that this δ-agonist may exert its effects on DA release by acting at both δ- and μ-opioid receptors (Experiment II). The specificity of δ-opioid modulation of the dopaminergic neuronal activity was tested by icv co-administration of DPDPE with ICI 154,129. Surprisingly, this selective δ-opioid antagonist did not significantly alter DA levels in any of the hypothalamic areas examined. Therefore, it is suggested that the modulatory actions of DPDPE on DA release may be exerted via μ-opioid receptors rather than those of δ-subtypes. It has been shown that in the rat brain μ- and δ-receptors can exist on the same neuron and are physically associated (Schoffelmeer *et al*, 1987). It is not inconceivable that the activation of the δ-receptors would allosterically influence μ-receptor activity (Passarelli and Costa, 1989). Naloxone can apparently therefore directly antagonise the inhibitory actions of DPDPE on dopaminergic neurotransmission by preventing its binding to the μ-opioid subtypes. However, this hypothesis remains to be further investigated.

Activation of the κ-opioid receptors results in an inhibition of the TIDA dopaminergic neuronal activity (Manzanares *et al,* 1992). Previous work from this laboratory has shown that a κ-agonist, tifluadom, increases DA turnover in the MPOA of pro-oestrous rats (Gopalan *et al,* 1989a). However, DA turnover has also been found to be elevated following the administration of a non-specific opioid antagonist, naloxone, in the same hypothalamic area. These contradictory results have been explained by a possible increase in the number of μ-opioid receptors on the afternoon of pro-oestrus as reported by Casulari *et al* (1987). However, it has been suggested that a decrease in the inhibitory opioid peptidergic tone occurs at the time of the LH surge (Kalra *et al,* 1989). It has recently been shown that the density of μ-opioid receptors declines as a result of elevated circulating gonadal steroids on the afternoon of pro-oestrus when plasma LH levels begin to rise (Maggi *et al,* 1993). Thus, it is possible that numbers of κ-opioid receptors may also fluctuate during the oestrous cycle depending on the steroid milieu. In the present study, icv infusion of the specific κ-opioid agonist (U-69593) reduced the concentrations of DA in all the hypothalamic regions studied. Prevention of the falls in DA levels by a selective κ-antagonist, MR1452, in the same hypothalamic regions clearly indicates that these effects are specifically exerted via the κ-opioid subtypes. In contrast to the earlier reports (outlined above) these results show that opioidergic influence on DA release via the κ-opioid receptors is also inhibitory.

As mentioned in the Introduction, it has been suggested that the EOPs alter the central serotonergic neurotransmission by their action at pre-synaptic nerve terminals. In the experiment described in this chapter, 5-HT concentrations in all four hypothalamic regions examined were significantly reduced following icv administration of diamorphine, DPDPE and U-69593 (except for the action of DPDPE in the MPOA). All three opioid agonists also decreased 5-HIAA levels in the MPOA, but had selective effects in the other three hypothalamic regions. Furthermore, the suppressive effects of these opioid agonists on the content of both 5-HT and its metabolite were prevented by their respective receptor antagonists. These results thus indicate that opioid inhibition of the serotonergic system within the hypothalamus may be exerted through multiple opioid receptor subtypes. It is thought that the exogenous opioids studied lower 5-HT synthesis by inhibiting its release from the nerve terminals as, in most cases, both 5-HT and 5-HIAA concentrations were reduced in parallel (this will be reviewed in the General Discussion). Unexpectedly, the ratio of 5-HIAA/5-HT was not significantly

changed by diamorphine and U-69593 in any of the hypothalamic areas. Surprisingly, DPDPE raised the ratio in only the ME compared to the control group values. Of the opioid antagonists investigated, only naloxone produced consistent effects. It significantly increased the ratio between 5-HIAA and 5-HT in the MPOA, ME and ARN compared to the animals receiving diamorphine or DPDPE alone. The relative concentrations of 5-HT and 5-HIAA were used to estimate the ratio of 5-HIAA/5-HT as described by Shannon *et al* (1986). However, it appears that this method may not necessarily reflect the turnover rate of 5-HT. Therefore, the conclusions regarding 5-HT neuronal activity (above) were based directly on the indoleamine levels detected in the discrete hypothalamic regions and not solely on the ratio of 5-HIAA/5-HT.

Interestingly, as in the case of DA release (explained earlier), the δ-opioid antagonist, ICI 154,129 failed to prevent the suppressive actions of DPDPE on the hypothalamic 5-HT and 5-HIAA levels. An enhanced serotonergic activity was observed after icv infusion of the δ-agonist concomitantly with naloxone. Therefore, it is postulated that DPDPE may influence 5-HT release and turnover primarily by acting at μ-opioid receptors. However, the nature of this interaction awaits further investigation.

It has been postulated that opioids may abolish the surge release of LH by inhibiting the serotonergic neurotransmission on the afternoon of pro-oestrus (Brown *et al*, 1994). All the opioid agonists investigated appeared to affect hypothalamic 5-HT release and turnover around the time of the anticipated LH surge in the ovx and steroid-primed rats. However, the present experiment failed to demonstrate such interactions between the EOPs and the serotonergic system in the control of LH release due to interference by the ketamine anaesthesia.

5-HT metabolism may also influence other neurotransmitter systems. Serotonergic innervation of the TIDA dopaminergic neurons has been demonstrated in the ZI and ARN (Bosler *et al*, 1984; Willoughby and Blessing, 1987). Therefore, suppression of serotonergic activity by the opioids may indirectly account for the reduced DA release within discrete hypothalamic areas.

Neurotransmitter Concentrations in the MPOA							
Treatment	**NA**	**DHPG**	**DA**	**5-HIAA**	**5-HT**	**Ratio of DHPG/NA**	**Ratio of 5-HIAA/5-HT**
Saline (16)	59.7±5.1	22.9±2.9	9.9±1.1	16.1±2.7	16.0±1.9	0.388±0.034	1.209±0.262
Diamorphine (9)	29.6±2.7 **a**	9.6±3.4 **c**	3.4±0.3 **a**	7.9±2.0 **d**	6.9±1.5 **b**	0.309±0.088	0.951±0.086
Diamorphine+NAL (7)	53.2±6.4 **f**	9.0±0.7	8.6±1.4 **e**	28.2±1.7 **e**	16.9±2.2 **f**	0.193±0.021	1.801±0.200 **e**
U-69593 (13)	37.9±3.2 **b**	7.9±1.1 **a**	5.3±0.8 **b**	8.1±1.2 **d**	7.6±1.4 **b**	0.200±0.017 **a**	1.489±0.350
U-69593+MR1452 (7)	59.0±6.5 **h**	18.0±2.6 **g**	9.0±1.0 **j**	19.5±5.1 **i**	21.3±4.4 **h**	0.315±0.038 **h**	1.304±0.400
DPDPE (8)	22.2±2.3 **a**	11.7±3.5 **d**	4.9±0.3 **b**	5.6±1.0 **d**	10.7±1.6	0.512±0.136	0.535±0.106
DPDPE+ICI 154129 (7)	65.2±5.5 **k**	31.0±1.7 **k**	7.5±1.3	9.0±2.1	4.8±0.8 **m**	0.489±0.029	1.98±0.424 **l**
DPDPE+NAL (7)	47.4±2.0 **k**	14.6±1.6	8.4±0.7 **k**	20.6±3.7 **k**	20.7±2.9 **m**	0.310±0.032	1.066±0.184 **n**

Table 5.1. Monoamine concentrations (pg amine/μg protein ± SEM) and ratios of DHPG/NA and 5-HIAA/5-HT in the MPOA at 19.00h on pro-oestrus after administration or co-administration of various opioid agonists and antagonists at 14.00h on the same day. **a:** $p<0.001$; **b**: $p<0.005$; **c**: $p<0.01$; **d**: $p<0.05$ compared to saline-treated animals, **e:** $p<0.001$; **f**: $p<0.005$ compared to diamorphine-treated animals, **g:** $p<0.001$; **h**: $p<0.005$; **i**: $p<0.01$; **j**: $p<0.05$ compared to U-69593-treated animals, **k:** $p<0.001$; **l**: $p<0.005$; **m**: $p<0.01$; **n**: $p<0.05$ compared to DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were used. Number of observations in each group is given in brackets.

Neurotransmitter Concentrations in the SCN							
Treatment	**NA**	**DHPG**	**DA**	**5-HIAA**	**5-HT**	**Ratio of DHPG/NA**	**Ratio of 5-HIAA/5-HT**
Saline (16)	58.2±3.9	20.6±2.6	9.9±1.0	13.3±1.8	15.3±1.7	0.367±0.039	0.811±0.093
Diamorphine (9)	29.7±1.9 **a**	10.7±3.6 **d**	3.8±0.3 **a**	8.5±2.3	6.6±1.6 **b**	0.349±0.104	1.047±0.282
Diamorphine+NAL (7)	53.4±5.3 **e**	14.7±2.6	8.4±1.0 **e**	22.0±3.4 **f**	12.0±4.6	0.297±0.061	1.258±0.096
U-69593 (13)	36.4±2.4 **a**	8.1±0.8 **a**	6.3±1.1 **d**	6.1±1.3 **b**	6.5±1.6 **a**	0.227±0.018 **c**	0.573±0.143
U-69593+MR1452 (7)	54.3±3.8 **g**	18.9±3.5 **g**	10.8±1.2 **h**	19.3±3.6 **g**	15.8±3.5 **h**	0.362±0.077 **h**	0.965±0.156
DPDPE (8)	22.7±2.6 **a**	10.6±2.5 **d**	5.2±0.9 **c**	8.6±1.9	5.3±1.4 **a**	0.439±0.069	1.153±0.306
DPDPE+ICI 154129 (7)	52.9±4.7 **i**	21.7±3.3 **k**	6.8±0.9	8.2±0.7	4.8±0.8	0.449±0.112	1.961±0.357
DPDPE+NAL (7)	52.2±2.0 **i**	13.9±2.8	11.7±1.5 **j**	21.8±4.2 **k**	17.6±1.8 **i**	0.264±0.045	1.330±0.275

Table 5.2. Monoamine concentrations (pg amine/μg protein ± SEM) and ratios of DHPG/NA and 5-HIAA/5-HT in the SCN at 19.00h on pro-oestrus after administration or co-administration of various opioid agonists and antagonists at 14.00h on the same day. **a:** $p<0.001$; **b**: $p<0.005$; **c**: $p<0.01$; **d**: $p<0.05$ compared to saline-treated animals, **e:** $p<0.001$; **f**: $p<0.005$ compared to diamorphine-treated animals, **g:** $p<0.001$; **h**: $p<0.05$ compared to U-69593-treated animals, **i:** $p<0.001$; **j**: $p<0.005$; **k**: $p<0.05$ compared to DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were used. Number of observations in each group is given in brackets.

Neurotransmitter Concentrations in the ME							
Treatment	**NA**	**DHPG**	**DA**	**5-HIAA**	**5-HT**	**Ratio of DHPG/NA**	**Ratio of 5-HIAA/5-HT**
Saline (16)	54.6±5.6	25.8±4.5	38.2±3.8	13.2±1.8	14.5±1.4	0.467±0.063	0.919±0.103
Diamorphine (9)	33.9±3.4 **d**	10.9±2.9 **d**	22.9±3.9 **d**	8.3±2.2	5.4±1.5 **a**	0.331±0.082	1.176±0.199
Diamorphine+NAL (7)	56.6±5.6 **f**	11.9±1.6	45.3±6.7 **g**	32.5±3 **e**	17.0±2.9 **f**	0.226±0.033	2.369±0.372 **h**
U-69593 (13)	33.5±3.0 **b**	8.5±1.1 **b**	25.0±2.3 **c**	6.8±0.8 **c**	5.2±1.3 **a**	0.268±0.026 **d**	1.090±0.222
U-69593+MR1452 (7)	57.5±4.3 **i**	18.4±3.1 **j**	39.5±6.7 **k**	22.4±6.0 **j**	18.4±3.7 **i**	0.318±0.047	1.940±0.840
DPDPE (8)	16.5±0.5 **a**	7.8±1.4 **d**	21.6±3.9 **d**	9.3±1.6	4.7±1.2 **a**	0.469±0.082	1.812±0.409 **c**
DPDPE+ICI 154129 (7)	65.4±4.1 **l**	31.9±2.9 **l**	32.3±3.5	10.5±2.5	8.2±1.9	0.509±0.070	1.549±0.444
DPDPE+NAL (7)	49.9±3.4 **l**	13.0±1.9 **m**	36.4±5.5 **m**	24.8±3.5 **l**	14.0±1.1 **l**	0.279±0.052	1.731±0.184

Table 5.3. Monoamine concentrations (pg amine/μg protein ± SEM) and ratios of DHPG/NA and 5-HIAA/5-HT in the ME at 19.00h on pro-oestrus after administration or co-administration of various opioid agonists and antagonists at 14.00h on the same day. **a:** $p<0.001$; **b:** $p<0.005$; **c:** $p<0.01$; **d:** $p<0.05$ compared to saline-treated animals, **e:** $p<0.001$; **f:** $p<0.005$; **g:** $p<0.01$; **h:** $p<0.05$ compared to diamorphine-treated animals, **i:** $p<0.001$; **j:** $p<0.005$; **k:** $p<0.05$ compared to U-69593-treated animals, **l:** $p<0.001$; **m:** $p<0.05$ compared to DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were used. Number of observations in each group is given in brackets.

Neurotransmitter Concentrations in the ARN							
Treatment	**NA**	**DHPG**	**DA**	**5-HIAA**	**5-HT**	**Ratio of DHPG/NA**	**Ratio of 5-HIAA/5-HT**
Saline (16)	54.6±4.1	23.1±3.2	10.1±1.1	12.1±2.1	14.5±2.0	0.424±0.046	0.881±0.157
Diamorphine (9)	26.1±1.8 **a**	10.6±3.4 **d**	7.3±1.1	9.6±2.4	6.1±1.5 **c**	0.380±0.107	1.209±0.309
Diamorphine+NAL (7)	46.5±6.1 **f**	8.9±0.7	8.1±0.8	29.7±2.6 **e**	13.9±1.4 **f**	0.217±0.028	2.229±0.242 **g**
U-69593 (13)	30.0±2.6 **a**	8.0±1.5 **a**	5.1±0.6 **a**	7.3±1.1	6.4±1.4 **b**	0.272±0.034 **d**	0.820±0.177
U-69593+MR1452 (7)	57.6±4.2 **h**	13.6±1.9 **i**	11.2±1.8 **h**	18.3±5.5 **i**	16.3±1.5 **h**	0.236±0.027	1.079±0.288
DPDPE (8)	17.0±1.1 **a**	8.5±2.6 **c**	6.0±1.3 **d**	5.2±1.3 **d**	3.7±1.1 **b**	0.538±0.177	0.781±0.220
DPDPE+ICI 154129 (7)	52.8±2.3 **j**	25.0±3.2 **k**	7.5±0.9	5.9±1.0	3.7±0.6	0.474±0.060	1.627±0.164 **l**
DPDPE+NAL (7)	41.5±4.9 **j**	12.3±2.1	8.0±1.1	19.9±3.8 **k**	11.8±3.0 **m**	0.303±0.031	1.533±0.230 **m**

Table 5.4. Monoamine concentrations (pg amine/μg protein ± SEM) and ratios of DHPG/NA and 5-HIAA/5-HT in the ARN at 19.00h on pro-oestrus after administration or co-administration of various opioid agonists and antagonists at 14.00h on the same day. **a:** $p<0.001$; **b**: $p<0.005$; **c**: $p<0.01$; **d**: $p<0.05$ compared to saline-treated animals, **e:** $p<0.001$; **f**: $p<0.005$; **g**: $p<0.05$ compared to diamorphine-treated animals, **h:** $p<0.001$; **i**: $p<0.05$ compared to U-69593-treated animals, **j:** $p<0.001$; **k**: $p<0.005$; **l**: $p<0.01$; **m**: $p<0.05$ compared to DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were used. Number of observations in each group is given in brackets.

Neurotransmitter Concentrations in the Controls (Saffan)							
Hypothalamic area	**NA**	**DHPG**	**DA**	**5-HIAA**	**5-HT**	**Ratio of DHPG/NA**	**Ratio of 5-HIAA/5-HT**
MPOA (7)	38.5±2.3	14.8±1.9	4.9±0.5	5.0±1.2	4.3±0.8	0.401±0.073	1.097±0.087
SCN (7)	37.6±6.4	16.2±2.7	6.7±1.5	7.2±1.7	4.9±0.8	0.471±0.102	1.163±0.080
ME (7)	38.5±1.6	16.5±0.9	25.9±6.5	5.3±0.5	5.9±0.8	0.431±0.03	0.963±0.137
ARN (7)	33.2±3.2	11.4±1.2	3.9±0.5	5.1±1.0	3.6±0.5	0.364±0.061	1.160±0.092

Table 5.5. Monoamine concentrations (pg amine/μg protein ± SEM) and ratios of DHPG/NA and 5-HIAA/5-HT in the MPOA, SCN, ME and ARN at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of sterile saline (10μl) to the saffan-anaesthetised rats at 14.00h on the same day. Number of observations is given in brackets.

Plasma LH levels (ng/ml)						
Animal ID	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
C-85	2.45	1.25	2.9	17.3	1.1	1.5
C-86	2.35	2.8	4.4	7.25	NS	NS
D-2	NS	3.1	1.75	5.5	5.1	4.2
D-3	NS	1.1	1.0	0.75	4.1	6.5
D-6	NS	0.5	0.65	0.8	NS	2.5
D-9	NS	LOW	LOW	LOW	LOW	2.0
D-14	NS	2.7	1.4	6.2	4.85	4.3
D-16	NS	LOW	LOW	LOW	LOW	LOW
D-61	1.65	1.1	2.3	1.35	1.3	2.8
E-9	LOW	LOW	LOW	LOW	LOW	LOW
E-10	LOW	LOW	LOW	LOW	LOW	LOW
E-12	LOW	LOW	LOW	LOW	LOW	LOW
E-15	LOW	LOW	LOW	LOW	LOW	LOW
E-24	LOW	LOW	LOW	LOW	LOW	LOW
E-46	LOW	LOW	LOW	LOW	LOW	LOW

Table 5.6. Plasma LH levels (ng/ml) recorded in individual animals at hourly intervals between 14.00h and 19.00h on the afternoon of the anticipated LH surge in the ketamine-anaesthetised rats following icv sterile saline infusion at 14.00h. LOW: concentration below the limit of detection, NS: no sample obtained.

Plasma LH levels (ng/ml)						
Animal ID	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
D-48	1.50	0.43	0.40	0.38	1.35	1.10
D-59	0.70	1.45	0.70	1.10	1.00	1.65
D-67	1.75	1.10	1.20	1.45	1.08	1.09
D-68	1.10	1.50	0.80	1.15	2.60	14.0
D-69	3.30	0.75	2.35	2.75	6.30	1.11
D-75	1.50	1.35	0.95	0.93	1.50	1.80
E-42	LOW	LOW	LOW	LOW	LOW	LOW

Table 5.7. Plasma LH levels (ng/ml) recorded in individual animals at hourly intervals between 14.00h and 19.00h on the afternoon of the anticipated LH surge in the ketamine-anaesthetised rats following icv diamorphine infusion at 14.00h. LOW: concentration below the limit of detection, NS: no sample obtained.

Plasma LH levels (ng/ml)						
Animal ID	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
E-13	LOW	LOW	LOW	LOW	LOW	LOW
E-14	LOW	LOW	LOW	LOW	LOW	LOW
E-22	LOW	LOW	LOW	LOW	LOW	LOW
E-25	LOW	LOW	LOW	NS	NS	NS
E-26	LOW	LOW	LOW	LOW	LOW	LOW
E-27	LOW	LOW	LOW	NS	LOW	LOW
E-28	0.45	1.6	LOW	LOW	LOW	LOW
E-30	LOW	LOW	LOW	LOW	1.45	2.0

Table 5.8. Plasma LH levels (ng/ml) recorded in individual animals at hourly intervals between 14.00h and 19.00h on the afternoon of the anticipated LH surge in the ketamine-anaesthetised rats following icv diamorphine plus naloxone infusion at 14.00h. LOW: concentration below the limit of detection, NS: no sample obtained.

Plasma LH levels (ng/ml)						
Animal ID	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
E-34	LOW	LOW	LOW	NS	NS	LOW
E-36	LOW	LOW	LOW	LOW	LOW	LOW
E-37	LOW	LOW	LOW	LOW	LOW	LOW
E-38	LOW	LOW	LOW	LOW	LOW	LOW
E-39	LOW	LOW	LOW	LOW	LOW	LOW
E-47	LOW	LOW	LOW	LOW	LOW	LOW
D-56	0.85	0.80	0.35	LOW	0.85	LOW
D-66	LOW	0.55	1.20	0.65	0.84	1.60

Table 5.9. Plasma LH levels (ng/ml) recorded in individual animals at hourly intervals between 14.00h and 19.00h on the afternoon of the anticipated LH surge in the ketamine-anaesthetised rats following icv U-69593 infusion at 14.00h. LOW: concentration below the limit of detection, NS: no sample obtained.

Plasma LH levels (ng/ml)						
Animal ID	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
E-5	LOW	LOW	LOW	LOW	LOW	LOW
E-7	LOW	LOW	LOW	LOW	LOW	LOW
E-8	LOW	LOW	LOW	LOW	NS	NS
E-11	LOW	LOW	LOW	LOW	LOW	LOW
E-16	LOW	LOW	LOW	LOW	LOW	LOW
E-17	LOW	LOW	LOW	LOW	LOW	0.85
E-18	LOW	LOW	LOW	LOW	NS	LOW
E-35	LOW	LOW	LOW	LOW	LOW	LOW

Table 5.10. Plasma LH levels (ng/ml) observed in individual animals at hourly intervals between 14.00h and 19.00h on the afternoon of the anticipated LH surge in the ketamine-anaesthetised rats following icv U-69593 plus MR1452 infusion at 14.00h. LOW: concentration below the limit of detection, NS: no sample obtained.

Plasma LH levels (ng/ml)						
Animal ID	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
D-45	LOW	LOW	LOW	LOW	LOW	LOW
D-47	LOW	LOW	LOW	LOW	LOW	LOW
D-53	LOW	LOW	LOW	LOW	LOW	LOW
D-55	LOW	LOW	LOW	LOW	LOW	LOW
D-63	LOW	LOW	LOW	NS	NS	LOW
D-65	LOW	LOW	LOW	LOW	LOW	LOW
E-31	LOW	LOW	LOW	LOW	0.30	LOW
E-32	LOW	LOW	LOW	LOW	LOW	LOW

Table 5.11. Plasma LH levels (ng/ml) recorded in individual animals at hourly intervals between 14.00h and 19.00h on the afternoon of the anticipated LH surge in the ketamine-anaesthetised rats following icv DPDPE infusion at 14.00h. LOW: concentration below the limit of detection, NS: no sample obtained.

Plasma LH levels (ng/ml)						
Animal ID	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
D-51	LOW	LOW	LOW	LOW	LOW	LOW
D-52	LOW	LOW	LOW	LOW	NS	LOW
D-57	0.25	LOW	0.35	LOW	LOW	0.43
D-58	0.42	0.33	LOW	LOW	0.12	LOW
E-1	LOW	LOW	LOW	LOW	LOW	LOW
E-3	LOW	LOW	LOW	LOW	LOW	LOW
E-20	LOW	LOW	LOW	LOW	LOW	LOW
E-21	LOW	LOW	LOW	LOW	LOW	LOW

Table 5.12. Plasma LH levels (ng/ml) seen in individual animals at hourly intervals between 14.00h and 19.00h on the afternoon of the anticipated LH surge in the ketamine-anaesthetised rats following icv DPDPE plus naloxone infusion at 14.00h. LOW: concentration below the limit of detection, NS: no sample obtained.

Plasma LH levels (ng/ml)						
Animal ID	**14.00h**	**15.00h**	**16.00h**	**17.00h**	**18.00h**	**19.00h**
E-43	LOW	LOW	1.35	1.30	0.40	1.0
E-45	2.15	LOW	1.0	1.8	2.15	1.45
E-48	0.45	0.70	0.60	0.40	1.25	4.35
E-49	LOW	LOW	LOW	LOW	LOW	LOW
E-52	LOW	LOW	LOW	LOW	LOW	LOW
E-54	LOW	LOW	LOW	LOW	LOW	LOW
E-57	LOW	LOW	LOW	LOW	LOW	LOW

Table 5.13. Plasma LH levels (ng/ml) recorded in individual animals at hourly intervals between 14.00h and 19.00h on the afternoon of the anticipated LH surge in the ketamine-anaesthetised rats following icv DPDPE plus ICI 154,129 infusion at 14.00h. LOW: concentration below the limit of detection, NS: no sample obtained.

Plasma LH Leves (ng/ml)							
Sampling Intervals	**Individual animals**						
	C-66	**C-69**	**C-71**	**C-72**	**C-75**	**C-80**	**C-82**
13.00 h	0.80	LOW	0.65	0.95	0.70	1.30	1.10
14.00 h	0.80	0.65	0.85	0.80	0.85	0.90	1.05
15.00 h	0.70	0.80	0.75	0.95	0.85	1.05	2.50
16.00 h	0.72	0.75	0.65	0.80	0.65	0.95	0.90
17.00 h	0.70	0.75	0.65	0.80	0.85	0.90	1.00
18.00 h	0.72	0.90	0.80	0.90	0.65	1.05	1.00
19.00 h	0.63	0.80	0.70	0.75	1.05	1.15	0.90

Table 5.14. Plasma LH concentrations (ng/ml) of individual animals at various times after icv administration of sterile saline (10µl) at 14.00h on the afternoon of the anticipated LH surge in the saffan-anaesthetised rats. LOW: Plasma hormone levels below the limit of detection.

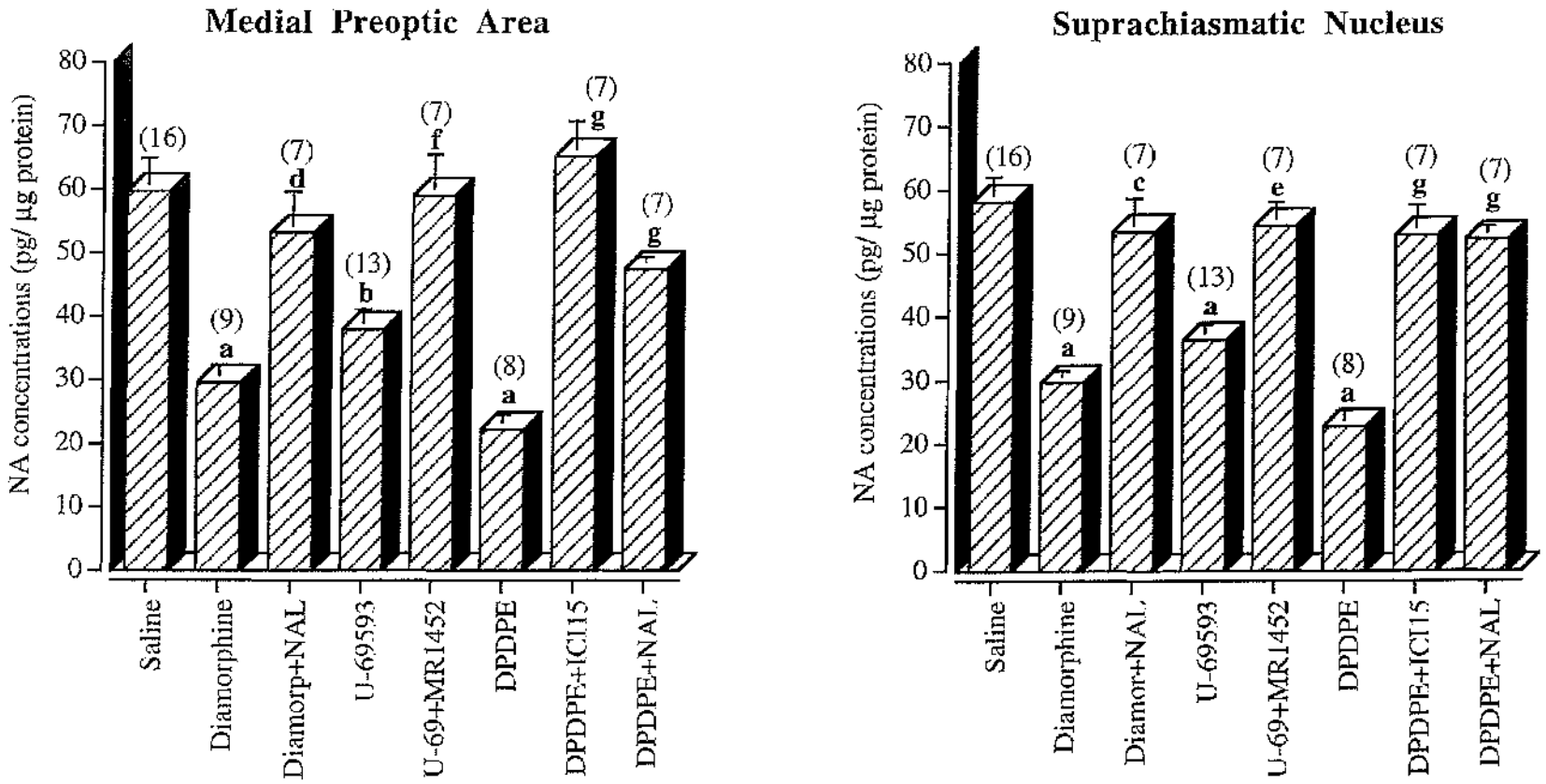


Figure 5.1. NA concentrations (pg/µg protein ± SEM) in the MPOA and SCN of ovariectomised and steroid-primed rats at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of drugs or saline at 14.00h. **a:** $p<0.001$; **b:** $p<0.005$ compared to the saline-treated animals, **c:** $p<0.001$; **d:** $p<0.005$ compared to the diamorphine-treated animals, **e:** $p<0.001$; f: $p<0.005$ compared to the U-69593-treated animals, **g:** $p<0.001$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

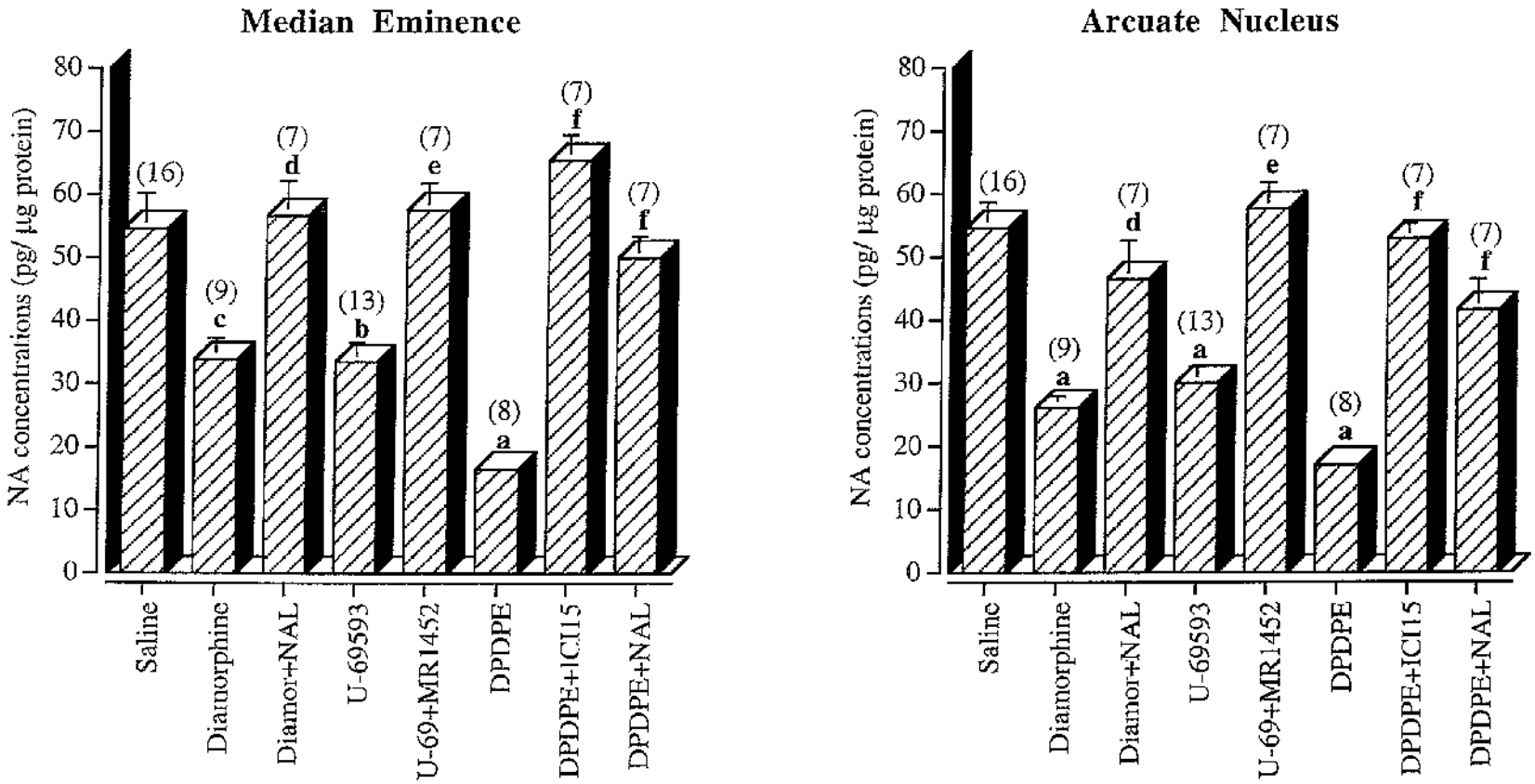


Figure 5.2. NA concentrations (pg/µg protein ± SEM) in the ME and ARN of ovariectomised and steroid-primed rats at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of drugs or saline at 14.00h. **a:** $p<0.001$; **b:** $p<0.005$; **c:** $p<0.05$ compared to the saline-treated animals, **d:** $p<0.005$ compared to the diamorphine-treated animals, **e:** $p<0.001$ compared to the U-69593-treated animals, **f:** $p<0.001$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

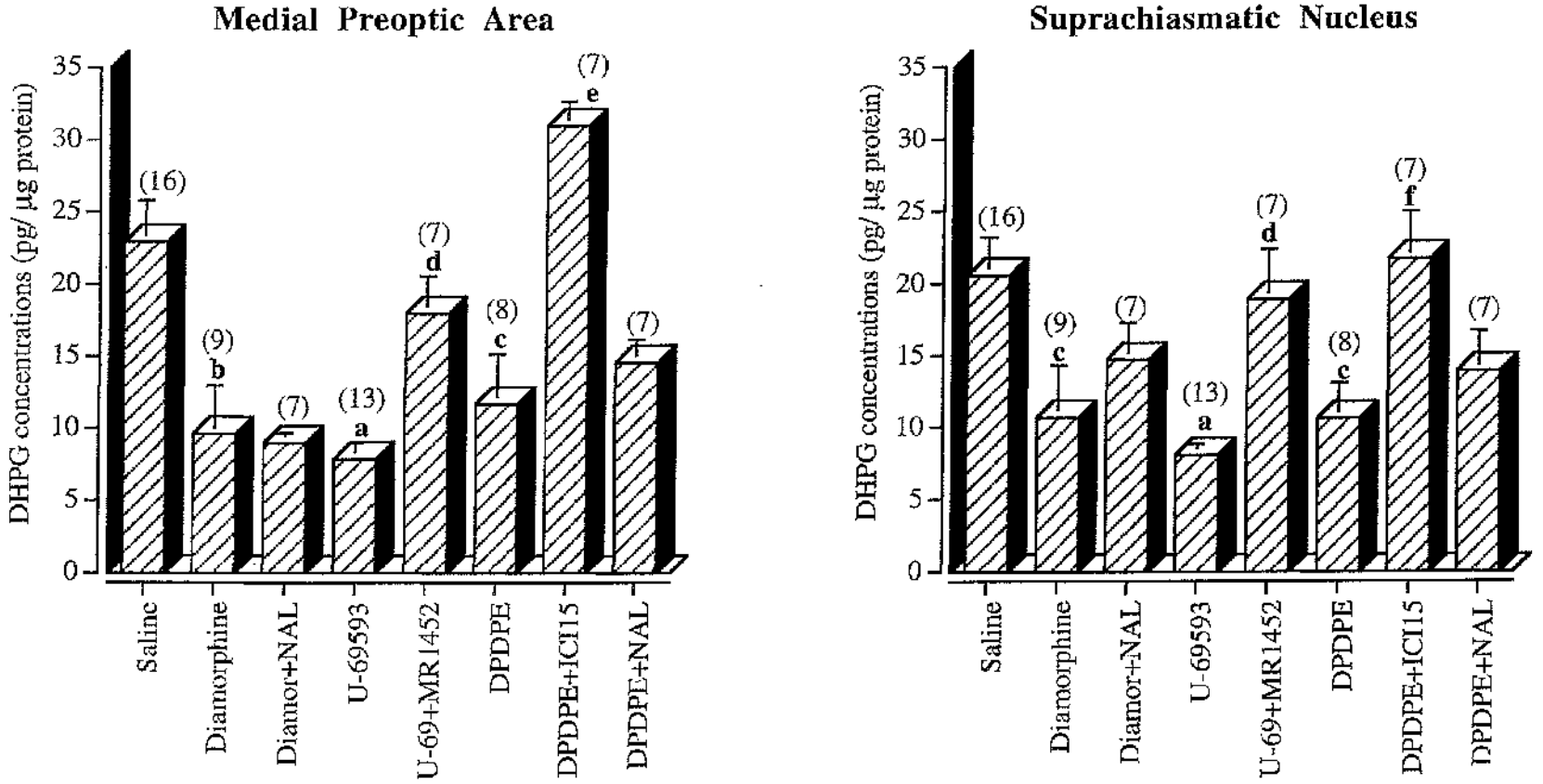


Figure 5.3. DHPG concentrations (pg/µg protein ± SEM) in the MPOA and SCN of ovariectomised and steroid-primed rats at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of drugs or saline at 14.00h. **a:** $p<0.001$; **b:** $p<0.01$; **c:** $p<0.05$ compared to the saline-treated animals, **d:** $p<0.001$ compared to the U-69593-treated animals, **e:** $p<0.001$; **f:** $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

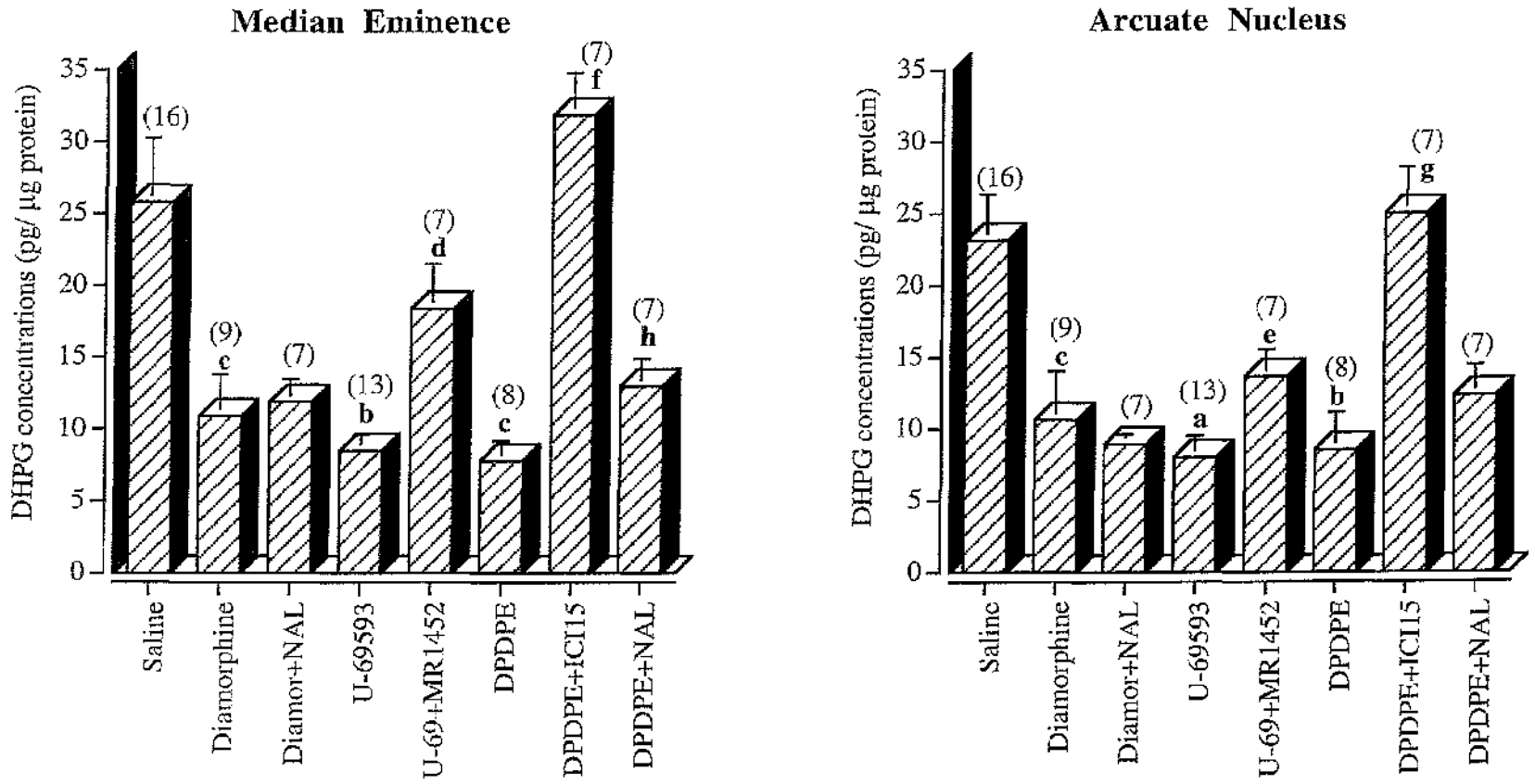


Figure 5.4. DHPG concentrations (pg/µg protein ± SEM) in the ME and ARN of ovariectomised and steroid-primed rats at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of drugs or saline at 14.00h. **a:** $p<0.001$; **b**: $p<0.005$; **c**: $p<0.05$ compared to the saline-treated animals, **d:** $p<0.005$; **e**: $p<0.05$ compared to the U-69593-treated animals, **f**: $p<0.001$; **g**: $p<0.005$; **h**: $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

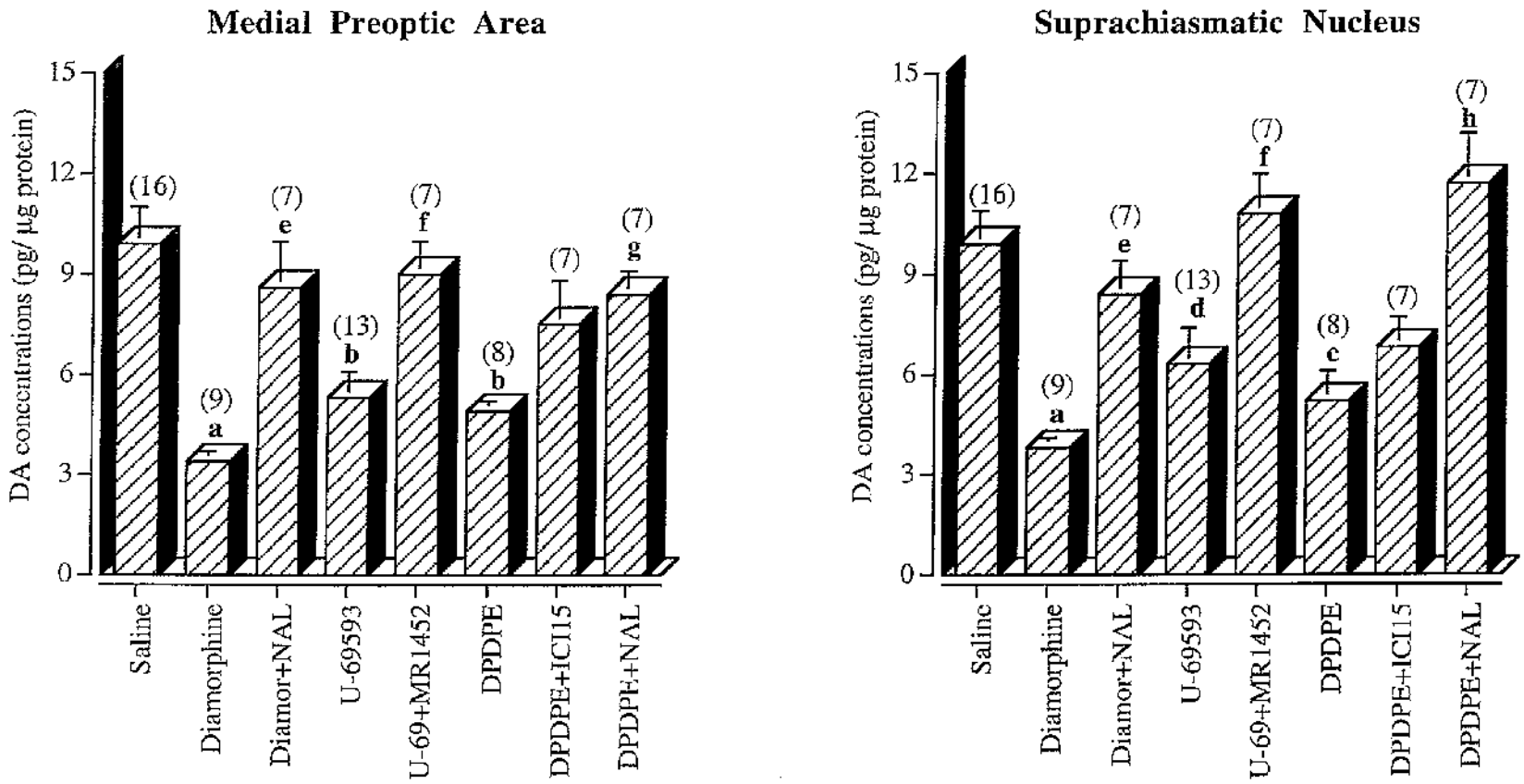


Figure 5.5. DA concentrations (pg/µg protein ± SEM) in the MPOA and SCN of ovariectomised and steroid-primed rats at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of drugs or saline at 14.00h. **a:** $p<0.001$; **b:** $p<0.005$; **c:** $p<0.01$; **d:** $p<0.05$ compared to the saline-treated animals, **e:** $p<0.001$ compared to the diamorphine-treated animals, **f:** $p<0.05$ compared to the U-69593-treated animals, **g:** $p<0.001$; **h:** $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

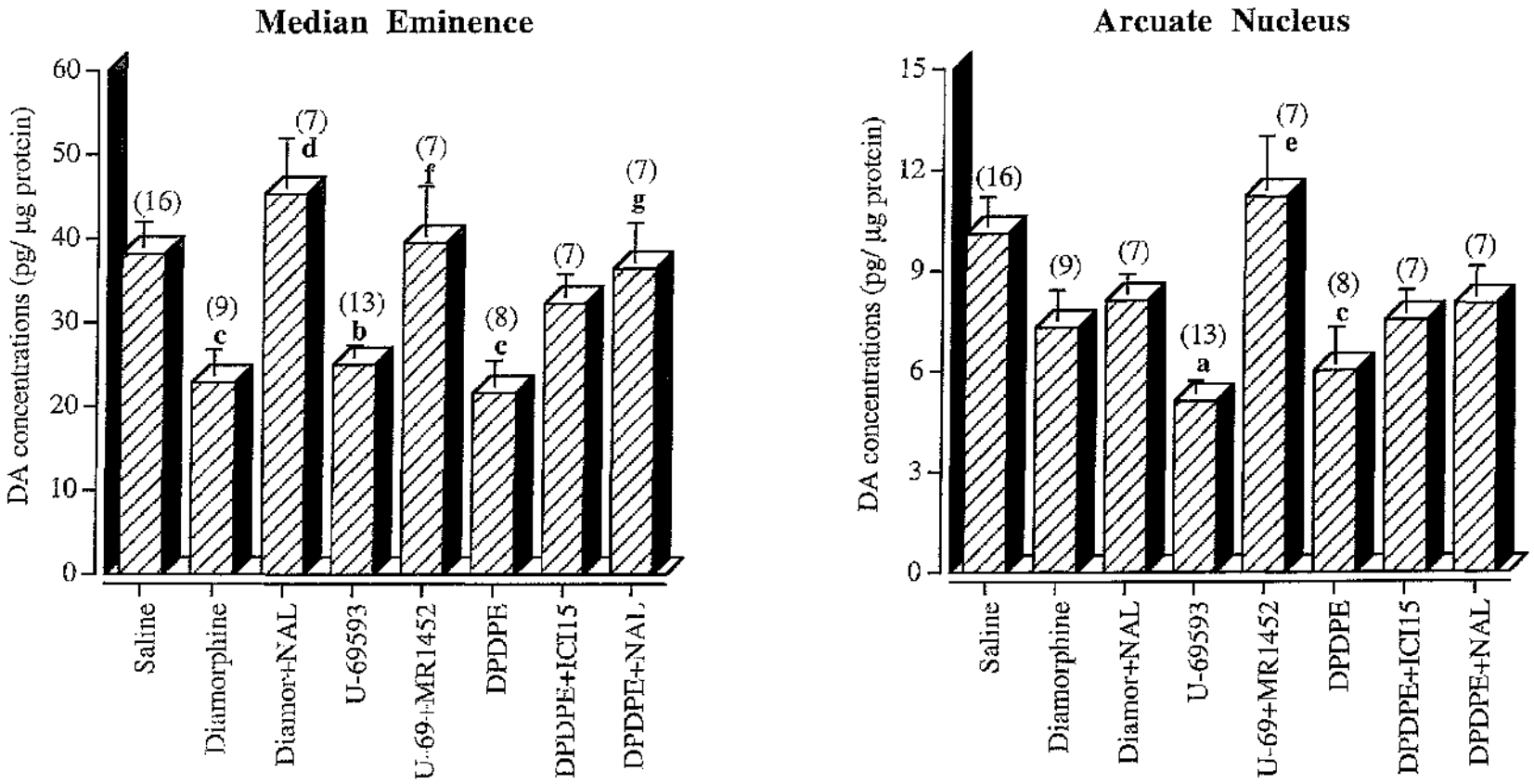


Figure 5.6. DA concentrations (pg/µg protein ± SEM) in the ME and ARN of ovariectomised and steroid-primed rats at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of drugs or saline at 14.00h. **a:** $p<0.001$; **b:** $p<0.01$; **c:** $p<0.05$ compared to the saline-treated animals, **d:** $p<0.01$ compared to the diamorphine-treated animals, **e:** $p<0.001$; **f:** $p<0.05$ compared to the U-69593-treated animals, **g:** $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

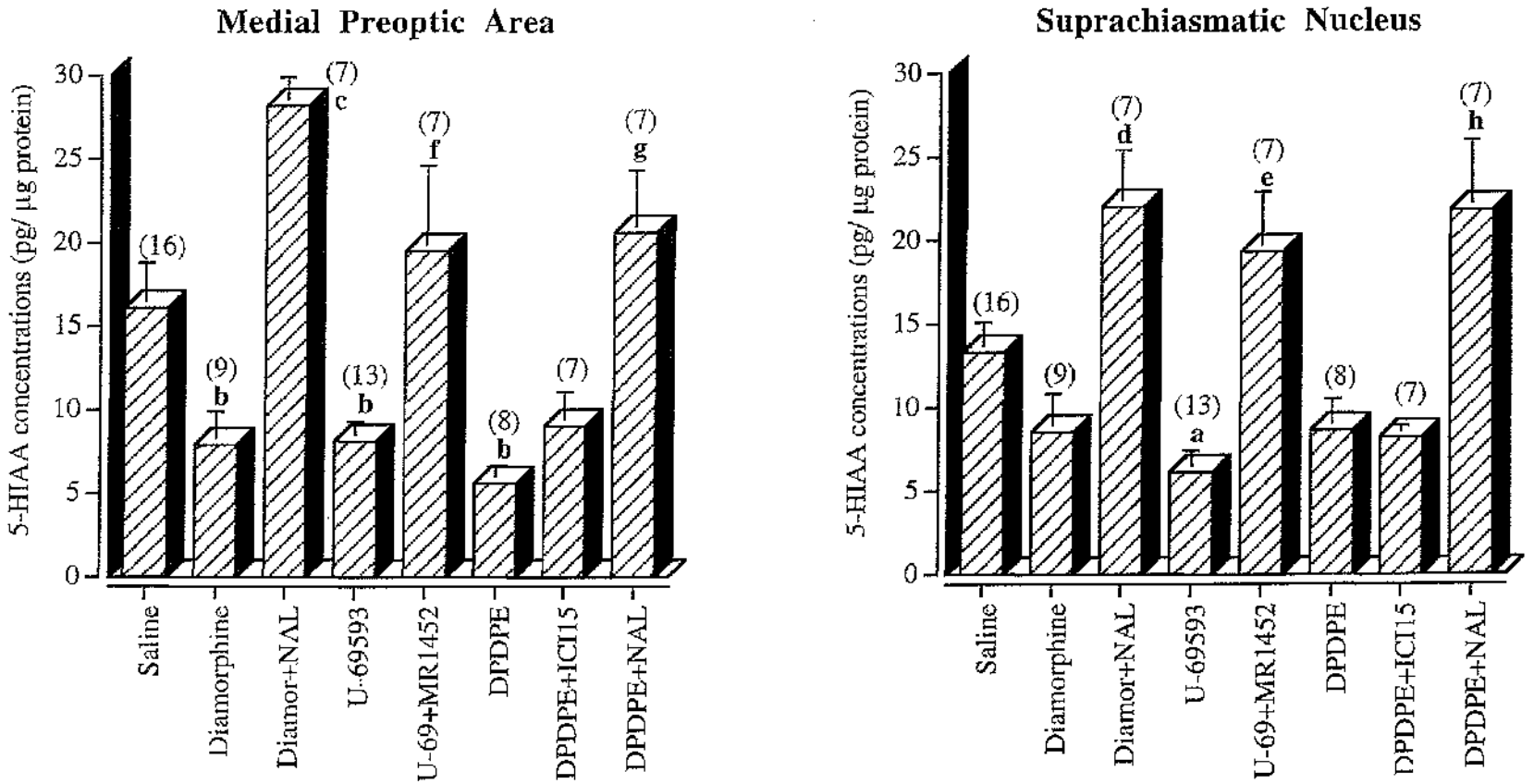


Figure 5.7. 5-HIAA concentrations (pg/µg protein ± SEM) in the MPOA and SCN of ovariectomised and steroid-primed rats at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of drugs or saline at 14.00h. **a:** $p<0.005$; **b:** $p<0.05$ compared to the saline-treated animals, **c:** $p<0.001$; **d:** $p<0.005$ compared to the diamorphine-treated animals, **e:** $p<0.001$; **f:** $p<0.01$ compared to the U-69593-treated animals, **g:** $p<0.001$; **h:** $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

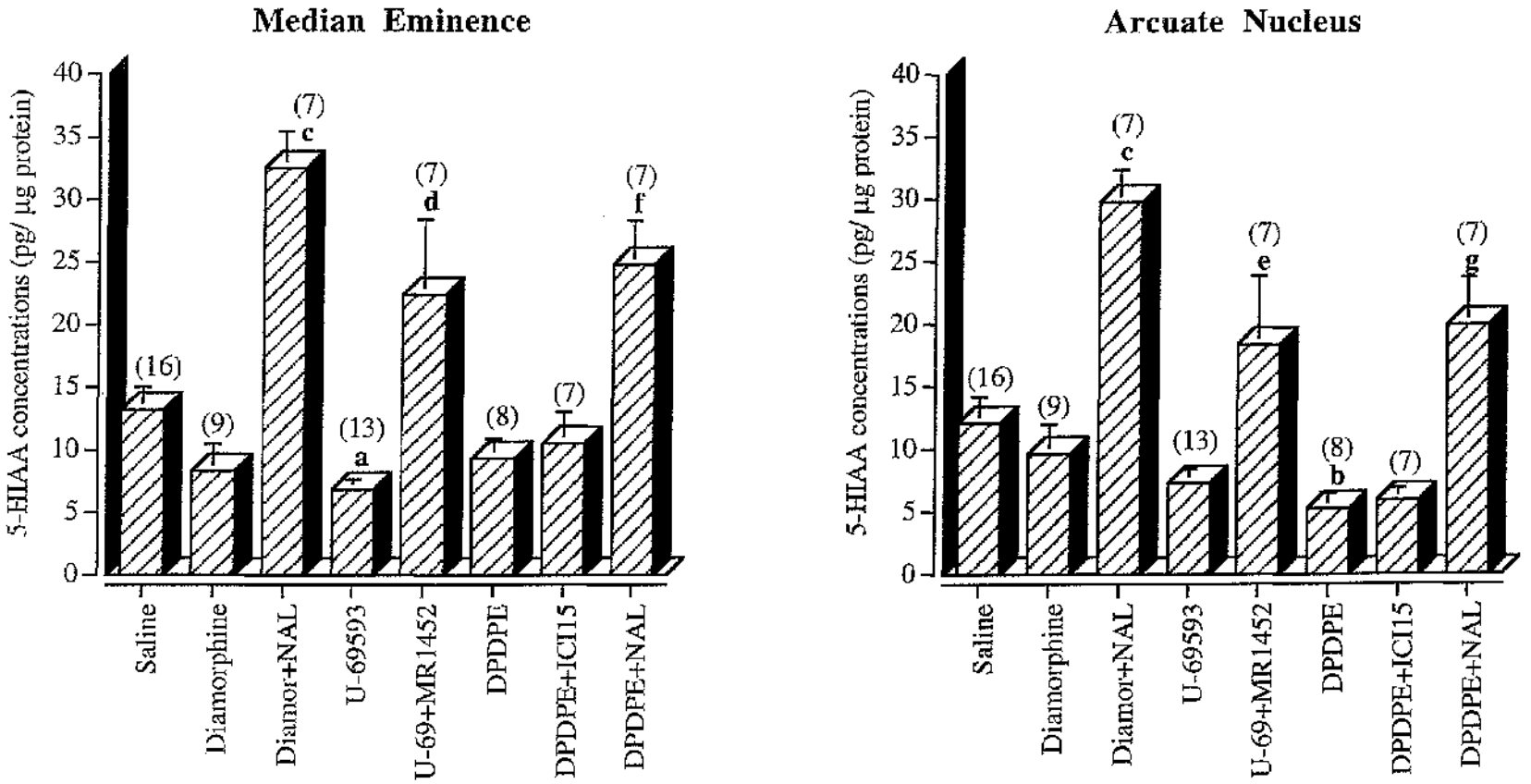


Figure 5.8. 5-HIAA concentrations (pg/µg protein ± SEM) in the ME and ARN of ovariectomised and steroid-primed rats at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of drugs or saline at 14.00h. **a:** $p<0.01$; **b:** $p<0.05$ compared to the saline-treated animals, **c**: $p<0.001$ compared to the diamorphine-treated animals, **d**: $p<0.005$; **e:** $p<0.05$ compared to the U-69593-treated animals, f: $p<0.001$; **g**: $p<0.005$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

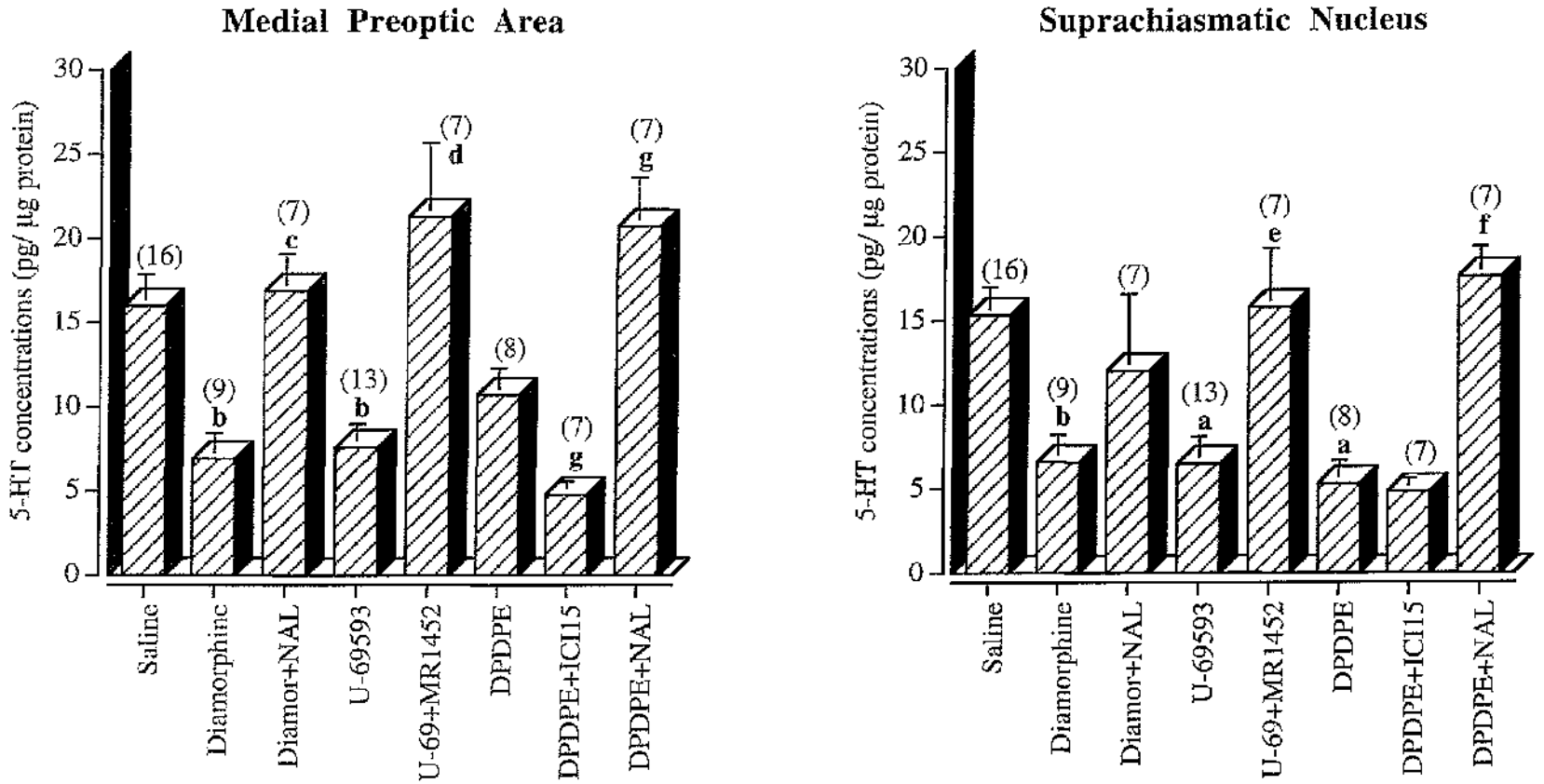


Figure 5.9. 5-HT concentrations (pg/µg protein ± SEM) in the MPOA and SCN of ovariectomised and steroid-primed rats at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of drugs or saline at 14.00h. **a:** $p<0.001$; **b:** $p<0.005$ compared to the saline-treated animals, **c:** $p<0.005$ compared to the diamorphine-treated animals, **d:** $p<0.005$; **e:** $p<0.05$ compared to the U-69593-treated animals, **f:** $p<0.001$; **g:** $p<0.01$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

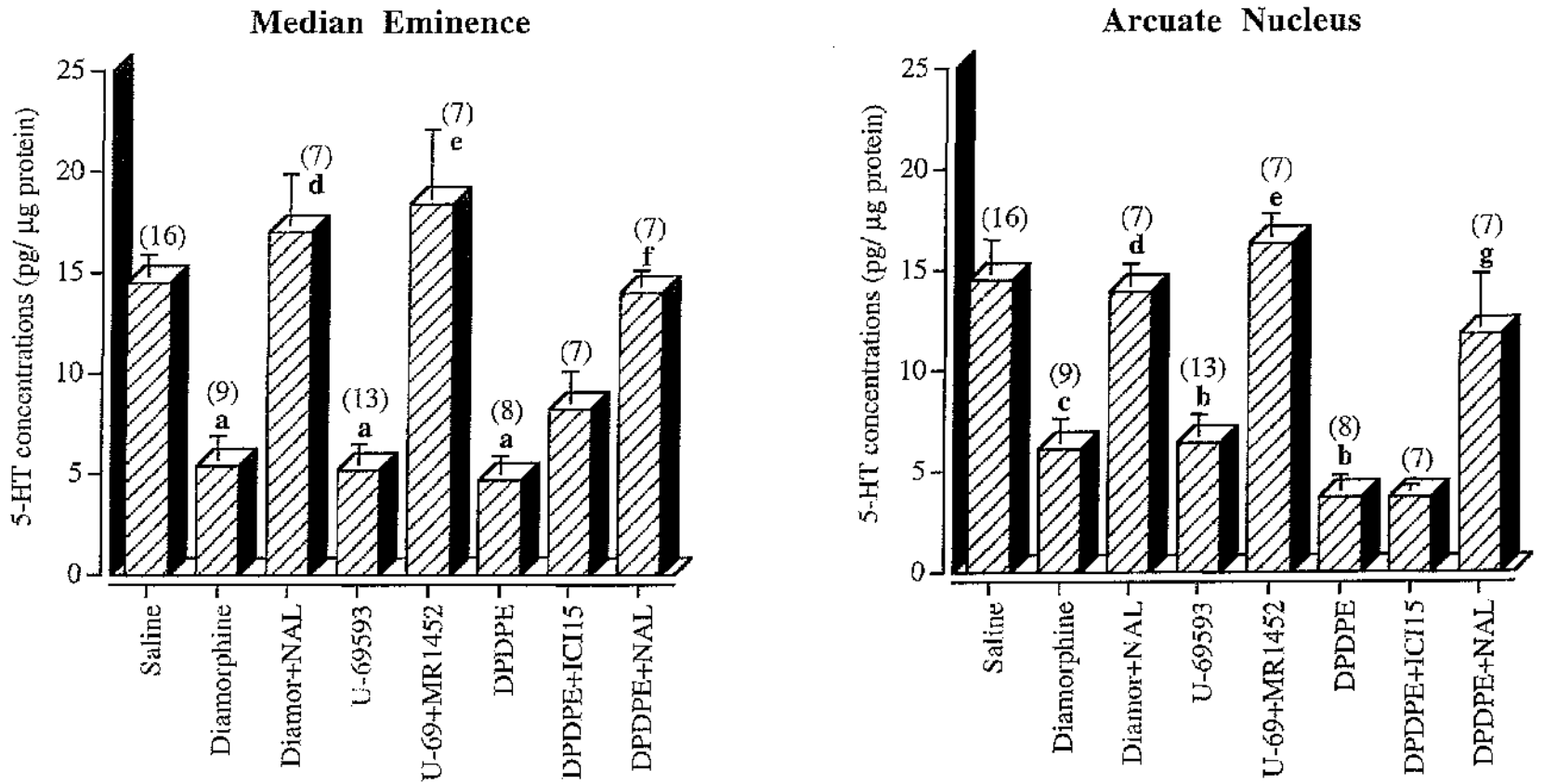


Figure 5.10. 5-HT concentrations (pg/µg protein ± SEM) in the ME and ARN of ovariectomised and steroid-primed rats at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of drugs or saline at 14.00h. **a:** $p<0.001$; **b:** $p<0.005$; **c:** $p<0.01$ compared to the saline-treated animals, **d:** $p<0.005$ compared to the diamorphine-treated animals, **e:** $p<0.001$ compared to the U-69593-treated animals, **f:** $p<0.001$; **g:** $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

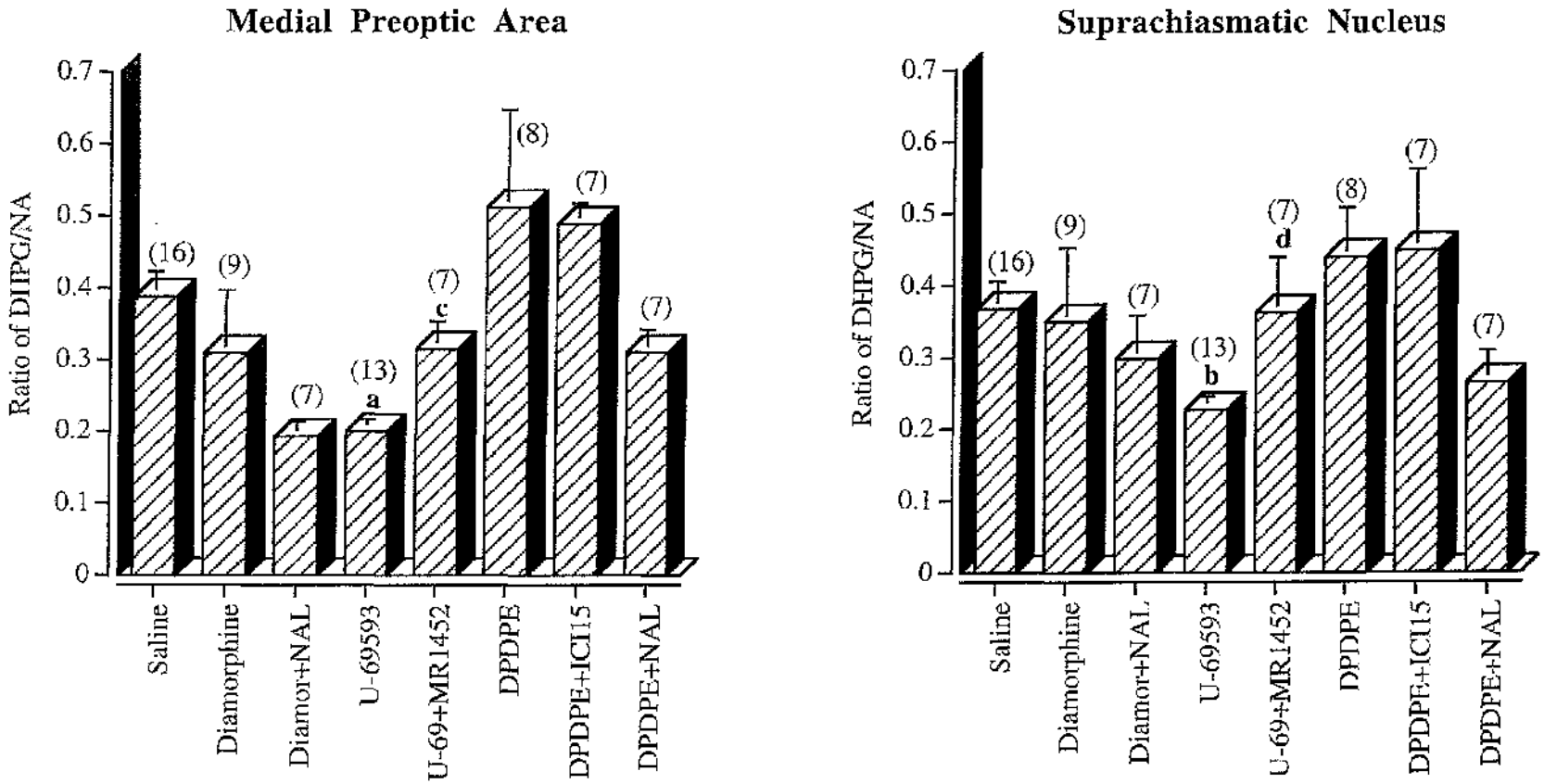


Figure 5.11. Ratio of DHPG/NA (Mean ± SEM) in the MPOA and SCN of ovariectomised and steroid-primed rats at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of drugs or saline at 14.00h. **a:** $p<0.001$; **b:** $p<0.01$ compared to the saline-treated animals, **c:** $p<0.005$; **d:** $p<0.05$ compared to the U-69593-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

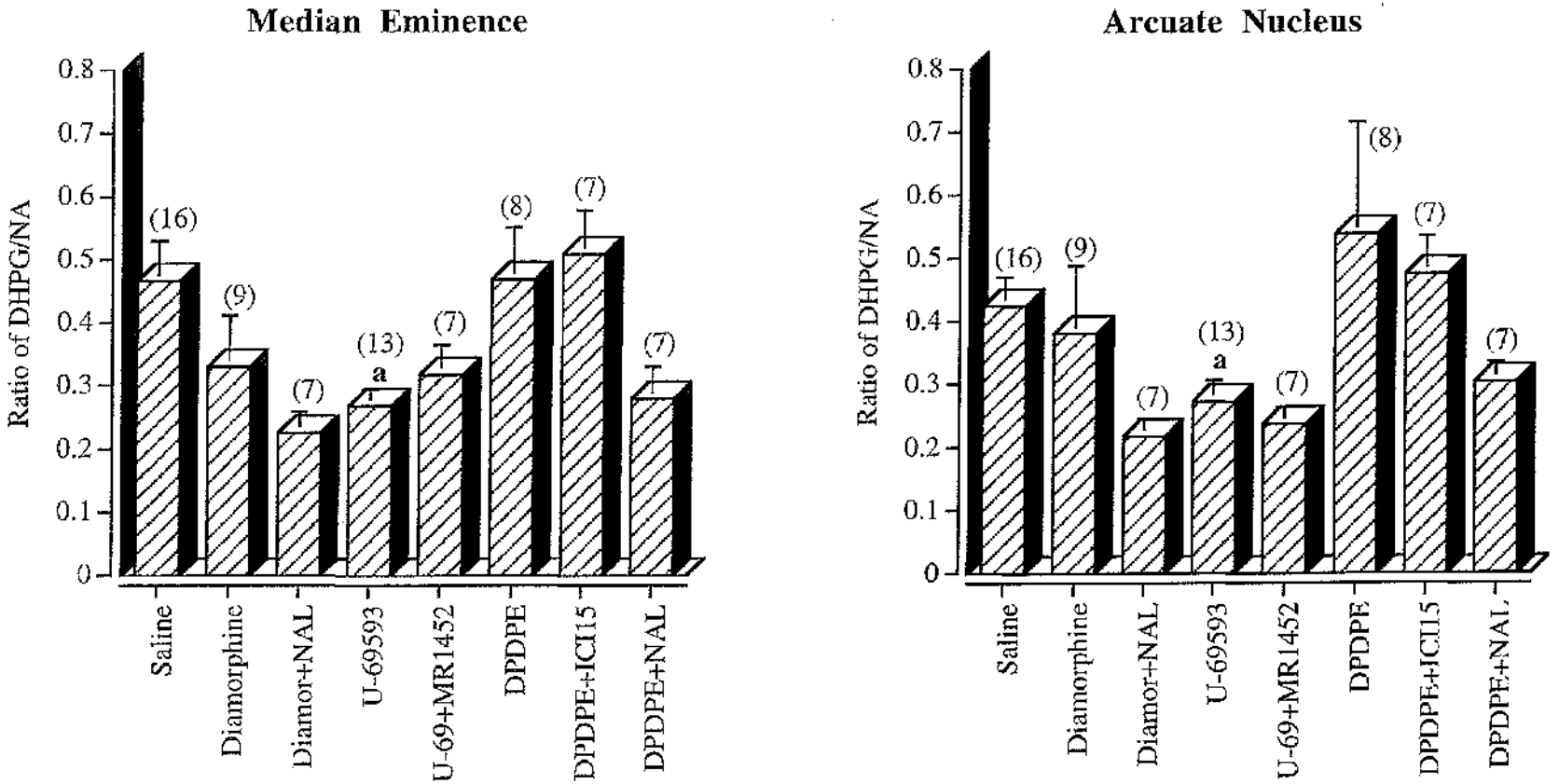


Figure 5.12. Ratio of DHPG/NA (Mean ± SEM) in the ME and ARN of ovariectomised and steroid-primed rats at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of drugs or saline at 14.00h. **a:** $p<0.05$ compared to the saline-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

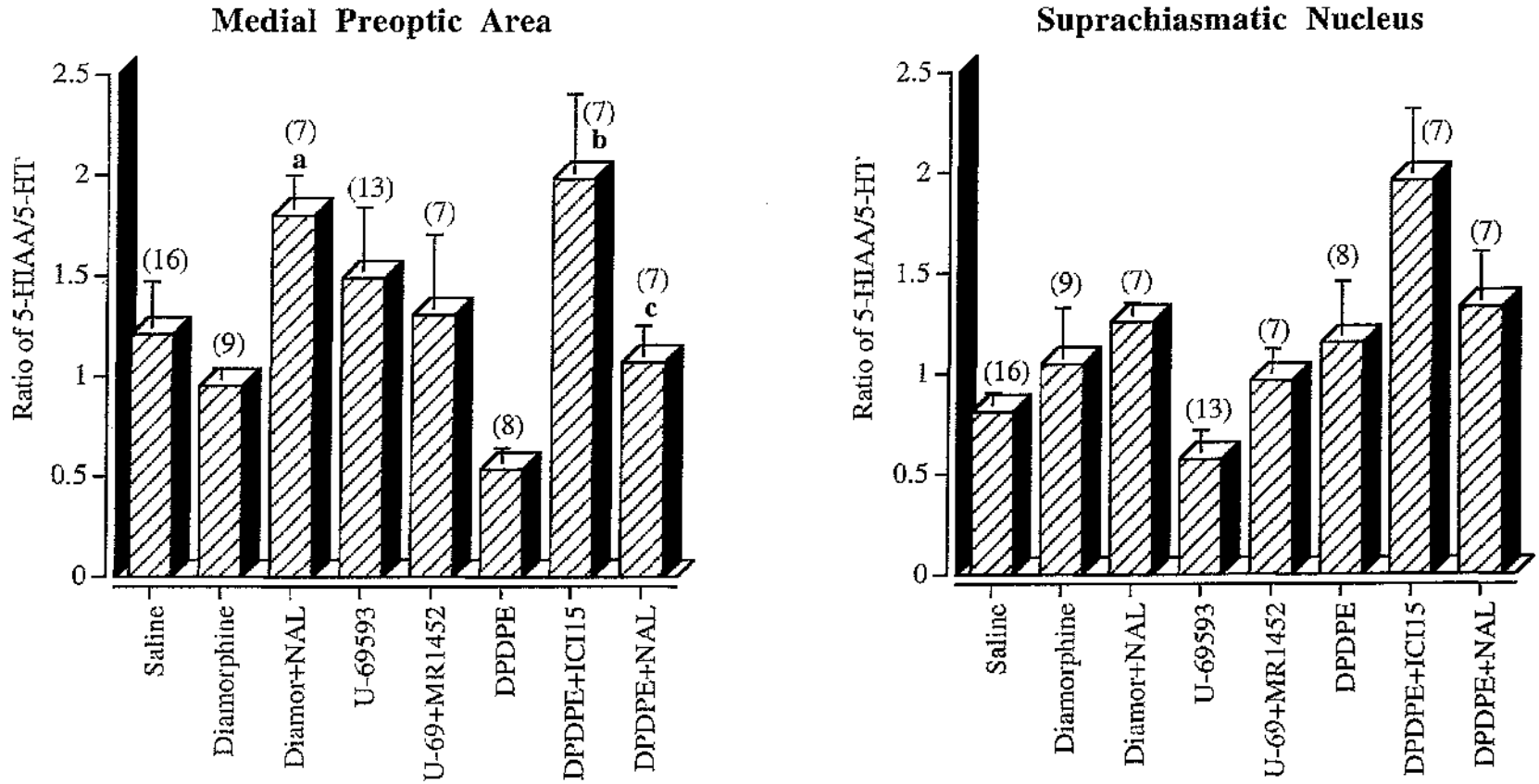


Figure 5.13. Ratio of 5-HIAA/5-HT (Mean ± SEM) in the MPOA and SCN of ovariectomised and steroid-primed rats at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of drugs or saline at 14.00h. **a:** $p<0.001$ compared to the diamorphine-treated animals, **b**: $p<0.005$; **c**: $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

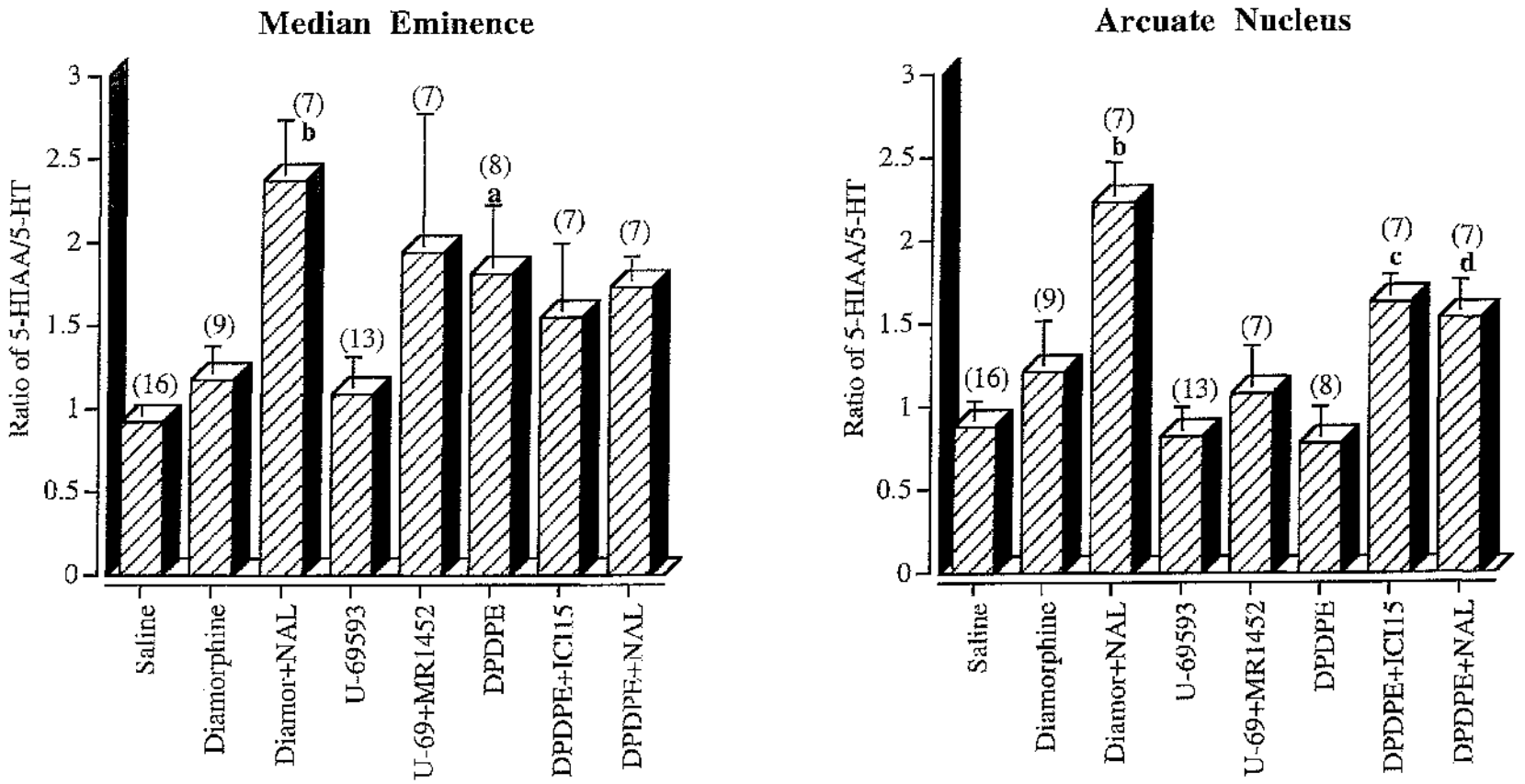


Figure 5.14. Ratio of 5-HIAA/5-HT (Mean ± SEM) in the ME and ARN of ovariectomised and steroid-primed rats at 19.00h on the afternoon of the anticipated LH surge following the intracerebroventricular administration of drugs or saline at 14.00h. **a:** $p<0.01$ compared to the saline-treated animals, **b:** $p<0.05$ compared to the diamorphine-treated animals, **c:** $p<0.01$; **d:** $p<0.05$ compared to the DPDPE-treated animals. One Way Analysis of Variance and the Kruskal-Wallis non-parametric test were utilised to examine the data. The number of observations in each group is given in brackets.

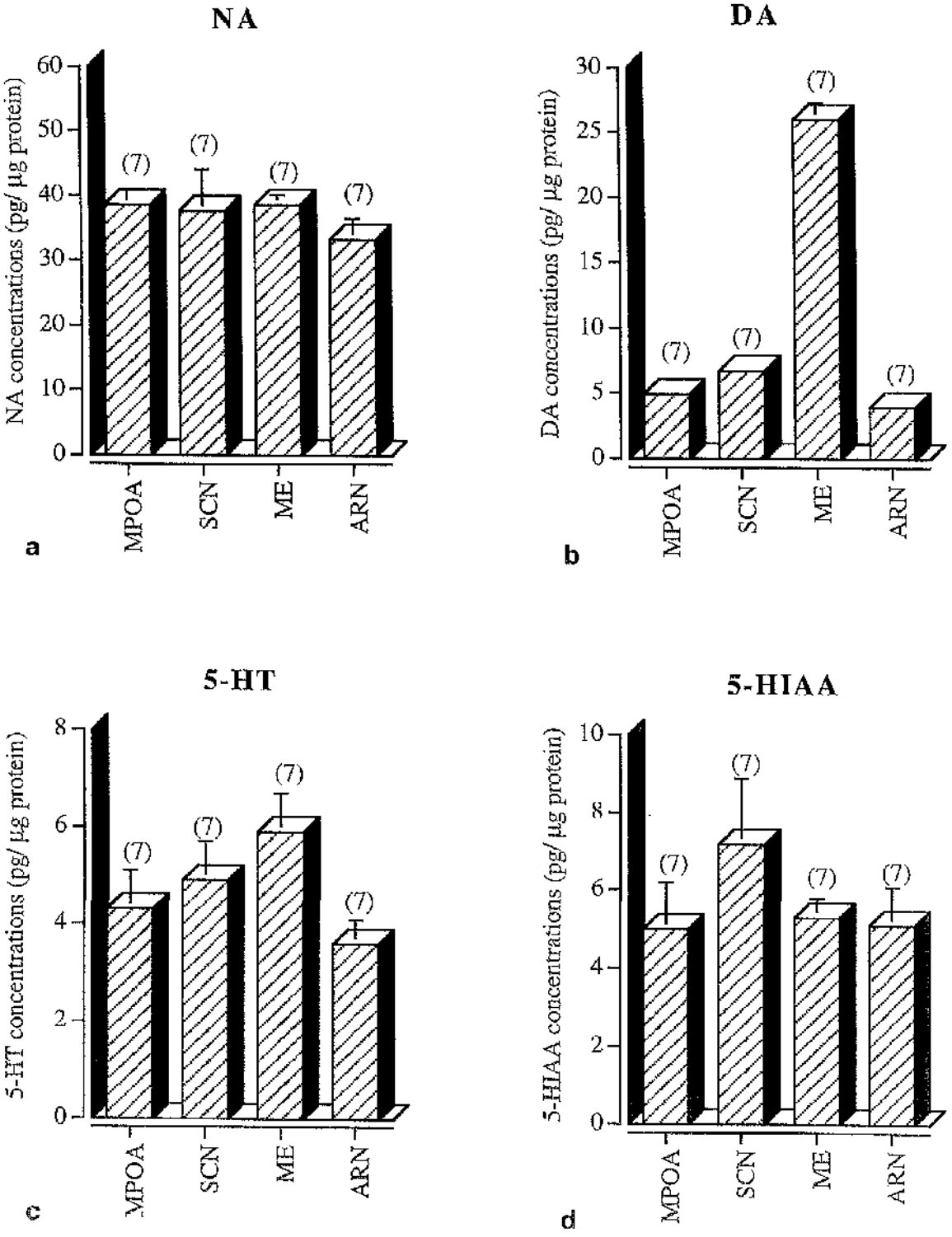


Figure 5.15. (a, b, c, d). Concentrations of NA, DA, 5-HT and 5-HIAA in the MPOA, SCN, ME and ARN of the saffan-anaesthetised control animals at 19.00h on the afternoon of the anticipated the LH surge.

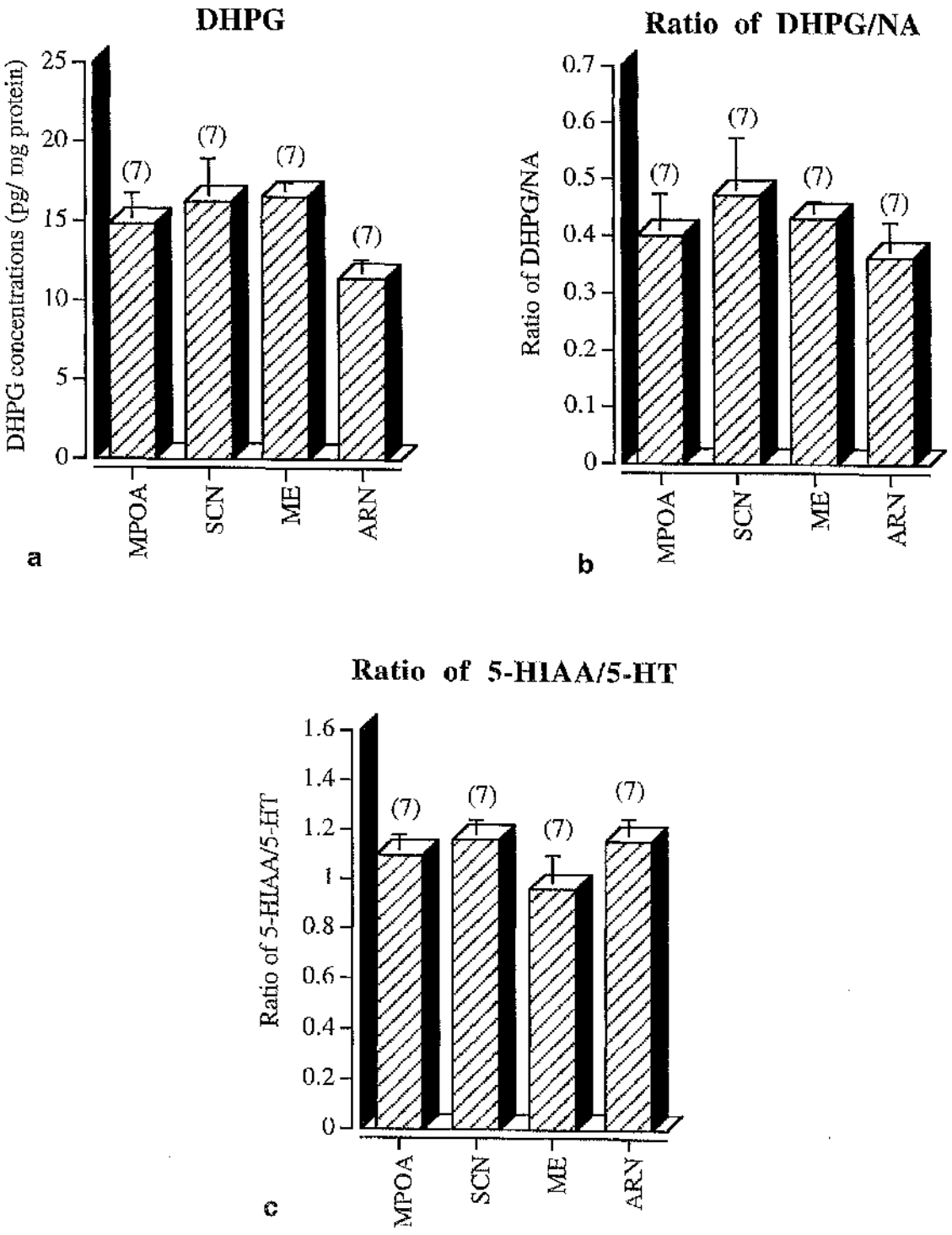


Figure 5.16. (a, b, c). DHPG concentrations and ratios of DHPG/NA and 5-HIAA/5-HT in the MPOA, SCN, ME and ARN of the saffan-anaesthetised control animals at 19.00h on the afternoon of the anticipated the LH surge.

GENERAL DISCUSSION

Site of Action of Opioids

Opioids have been shown to inhibit both basal and GnRH-induced LH release from cultured pituitary cells (Blank *et al,* 1986). However, they have also been reported to have no direct effects on LH release from the anterior pituitary (Bicknell, 1985). The anterior pituitary is relatively poor in opioid receptors (Khachaturian *et al,* 1985). It is therefore most likely that the endogenous opioid control of LH secretion is caused by actions and/or interactions within the brain; in particular, by modulating the secretion of GnRH into the hypophyseal portal system. β-endorphin inhibits GnRH secretion by preventing its release from the ME (Bonavera *et al,* 1993). Increased GnRH concentrations have been detected in the MPOA, ME and ARN at the time of the pre-ovulatory LH surge on the afternoon of pro-oestrus (Parnet *et al,* 1990). Furthermore, concentrations of GnRH in the hypophyseal portal blood have been well correlated with plasma LH levels in circulation following systemic administration of catecholaminergic drugs (Sarkar and Fink, 1981). These observations support the concept that drugs administered systemically exert their effects on LH release mainly through the hypothalamus.

In the rat, the response of the anterior pituitary to GnRH release increases steadily during the oestrous cycle and reaches maximum levels on the afternoon of pro-oestrus (Blake, 1976). It is known that the presence of steroid hormones in the circulation influences the way in which the gonadotrophs respond to the GnRH discharge (Everett, 1994). Elevated plasma levels of E_2 have been detected at the time of the pre-ovulatory LH surge, when a rapid increase in the GnRH content of the MBH occurs (Kalra, 1986). It has been suggested that opioid peptides might also alter the responsiveness of the pituitary to this neural signal from the hypothalamus (i.e. GnRH). However, this mechanism of opioid action is thought to be of least physiological importance since such an effect has not been shown *in vivo*.

In this study, exogenous opioid agonists and antagonists were administered systemically or icv to pro-oestrous or ovx and steroid-primed rats. The central effects of opioids on LH secretion may result from their action at one or several hypothalamic regions and perhaps also at extrahypothalamic loci. In the rat brain, the septal-preoptic-tuberal region is regarded as the final common pathway controlling LH secretion (Everett, 1994). The distribution of GnRH perikarya and neuronal processes mainly follow the discrete regional extension of this pathway in

the diencephalon (Silverman *et al,* 1994; for review see Introduction). Implants containing naloxone or icv infusion of naloxone into the MPOA and ME-ARN regions readily stimulated LH secretion (Kalra and Simpkins, 1981). However, implantation of naloxone into the regions outside the hypothalamus failed to elevate LH release in ovx and steroid-treated rats. Furthermore, naloxone also elicits the efflux of GnRH from the preoptic area-MBH zone *in vitro* (Leadem *et al,* 1985). Thus, the regional specificity of naloxone action indicates that the effective site of the opioids is confined to the preoptic-tuberal pathway. Here, the opioid drugs may directly affect the GnRH perikarya and nerve terminals. Alternatively, they may also alter the activity of the central aminergic system whose nerve terminals make synaptic contacts with the GnRH neurons (described in detail in the Introduction). Additional brain loci have also been implicated in the regulation of LH release. Changes in dopaminergic activity in the ZI and lesions of this brain region have been shown to alter LH release (MacKenzie *et al,* 1988). Microinjection of morphine into the DRN or MRN blocked the pre-ovulatory LH surge and ovulation (Johnson *et al,* 1986) and injection of opioids into the amygdaloid complex suppressed LH secretion (Lakoski and Gebhart, 1982). These findings indicate that the opioids may also exert their effects on the neuronal systems outwith the hypothalamus which project to the GnRH neurons in the preoptic-tuberal pathway.

Opioids and the Blood Brain Barrier

Capillaries in the brain, unlike those of most other organs, do not have pores between adjacent endothelial cells. Instead, these endothelial cells are fused together by tight junctions. Therefore, the brain cannot obtain molecules (such as drugs) from the blood plasma by a non-specific filtering process. Alternatively, the molecules within the brain capillaries are transported through the endothelial cells by either diffusion, active transport, endocytosis or exocytosis. This imposes a very selective blood brain barrier. However, the circumventricular organs such as the ME and OVLT do not have a functional blood brain barrier as these areas contain fenestrated capillaries.

Since the systemic (IP) route of administration was employed in Experiments I and II, it is necessary to consider the permeability of the blood brain barrier to the drugs investigated. Initially peptides were believed to be too large and unstable to cross the blood brain barrier to any significant extent. Although the precise pathways

(i.e. intracellular, extracellular, transmembrane) for passage of blood-borne peptides into the CNS seems equivocal, it has now been documented that the peptides (e.g. enkephalins, dynorphins) cross the blood brain barrier by saturable and nonsaturable mechanisms in amounts sufficient to affect CNS functions (Banks and Kastin, 1990). Recently a microdialysis study has provided even more direct evidence for the new concept by indicating that morphine is found in the dialysate of the striatum after systemic administration (Aasmundstad *et al,* 1995). It is therefore believed that the opioid peptides studied here passed through the blood brain barrier in amounts sufficient to exert effects at the hypothalamic and/or extrahypothalamic loci. Delivery of substances into the brain ventricular system however does not involve the blood brain barrier. The monoamine results from Experiment II, where the systemic route was used, have been found to be broadly in agreement with those obtained following icv administration of opioid agonists and antagonists to the ovx steroid-primed rats. These findings also appear to support the concept outlined above that opioids cross the blood brain barrier.

Opioids, Neuronal Activity and Monoamine Concentrations

It is generally accepted that opioid peptides depress spontaneous neuronal activity and hence neuronal transmission by hyperpolarising the membrane of their target cells (Henderson, 1983; Jiang and North, 1992). For ligands of μ-, κ- and δ-opioid receptors, the inhibitory hyperpolarisation results from activation of a calcium-sensitive potassium conductance (Williams *et al,* 1982; Lagrange *et al,* 1995). However, a second mechanism for action of the κ-opioid receptors has been suggested, i.e. that activation of these receptor subtypes decreases the voltage-dependent calcium conductance by reducing channel opening time (Macdonald and Werz, 1986). Thus it appears that EOPs decrease spontaneous electrical activity and the amount of neurotransmitter released from their target neurons in the brain.

As detailed in the Introduction, aminergic neurotransmitters are synthesised in the cell bodies and transported via the axon to the nerve terminals where they are stored in vesicles. Upon depolarisation, the stored transmitters are released into the synaptic cleft. Hence, they can be found both intra- and extra-cellularly. Subsequently, the released amines are rapidly metabolised and taken up into their respective neurons (for review see Rang and Dale, 1991). Perikarya of the

noradrenergic and dopaminergic neurons lie outwith the MPOA, SCN, ME and ARN in which the monoamine measurements in the present study were made using HPLC-ECD. The four hypothalamic regions are also densely innervated by 5-HT projections from the raphe nuclei (Steinbusch, 1984). It appears that the majority of the amines detected in this study were situated within the nerve terminals of their respective neurons.

Since the specific hypothalamic regions were isolated by the micropunch technique, biogenic amines in both intra- and extra-cellular compartments were collected. Hence, an increase in amine concentrations in the micropunched hypothalamic samples could be a result of increased release, synthesis and/or axonal transport. It is well established that secretion of neurotransmitters stimulates their own synthesis and axonal transport by an autoregulation mechanism (Kruk and Pycock, 1993). Therefore, the more amine released pre-synaptically the more produced by the neurons. Alternatively, raised concentrations of amines may also reflect a reduced release. However, results obtained in the present study do not support the latter assumption. Raised concentrations of NA or 5-HT were almost always accompanied by an increase in the levels of DHPG and 5-HIAA in the same hypothalamic samples, respectively. Production of DHPG or 5-HIAA directly reflects secretion of NA and 5-HT, respectively (Shannon *et al,* 1986). It thus appears that when release is elevated, so too is synthesis.

When a composite mixture was run through the reverse phase HPLC column, the retention time of ADR was between that of NA and the internal standard (DHBA) in the chromatogram (see Figure 2.9.a). However, it was observed that concentrations of this catecholamine were below the limit of detection in most of the hypothalamic samples examined. Therefore, those few results obtained have not been presented. The content of ADR in the rat brain is considered to be very low (Mefford, 1987). Indeed, previous work from this laboratory has also failed to detect ADR levels in the whole or in the specific regions of the hypothalamus (Gopalan *et al,* 1989a).

The metabolites of DA, DOPAC and HVA were consistently below the limit of detection in the supernatant sample of the hypothalamic areas. The conclusions made regarding hypothalamic DA release and neuronal activity were therefore based on only the concentrations of this neurotransmitter. As mentioned above, decreases in NA and 5-HT levels were accompanied by reduced concentrations of

their metabolites, DHPG and 5-HIAA, respectively. These findings thus indicate that the opioid agonists studied decrease the synthesis of NA and 5-HT by inhibiting their pre-synaptic release. In view of this, it is not inconceivable that when the DA release is reduced, the rate of its synthesis would also be lowered by the opioids.

Effects of Opioids on LH Release

It has been argued that a decrease in the EOP tone on the afternoon of pro-oestrus is an important event in the initiation of the pre-ovulatory LH surge (Allen and Kalra, 1986). Also, the number of opioid receptors fluctuate throughout the oestrous cycle (Limonta *et al,* 1989). The μ-opioid binding sites have been demonstrated to decline on the afternoon of pro-oestrus when plasma E_2 and P levels are elevated (Maggi *et al,* 1993). Naloxone antagonism of the inhibitory opioidergic actions on both GnRH and LH release has been widely reported (Van Vugt *et al,* 1981; Piva *et al,* 1985; Kim *et al,* 1991). Administration of morphine suppresses GnRH release (Mehmanesh *et al,* 1988) and naloxone infusion increases its secretion from the MBH-POA *in vitro* (Leadem *et al,* 1985). These observations imply that the opioidergic tone is not completely eliminated on the afternoon of pro-oestrus. In contrast to this hypothesis, Gabriel *et al* (1986) have reported that naloxone had no effect on LH release. The present results together with the previous work from this laboratory (Brown *et al,* 1994) appear to support the concept that the EOP tone persists to some degree during the LH surge.

The ability of morphine to inhibit both basal and surge release of LH has been demonstrated under various experimental conditions (Pfeiffer *et al,* 1987; Grossman and Dyer, 1989). It appears that suppression of the LH surge is a result of reduced GnRH release into the hypophyseal portal system since a μ-opioid agonist (DAMGO) has recently been shown to inhibit GnRH neuronal activity and thus its release *in vitro* (Lagrange *et al,* 1995). In the present study, activation of the μ-opioid receptors with diamorphine tended to lower the pre-ovulatory LH surge prior to 17.00h following its administration before the critical period on the afternoon of pro-oestrus. However, four of the 10 diamorphine-treated animals showed some rises in plasma LH levels, but only towards the end of the critical period. This observation is similar to previously reported work where low doses of morphine elevated surge release of LH (Pang *et al,* 1977; Barraclough, 1994). These results may provide qualitative evidence for a

stimulatory opioidergic input to the LH surge-generating system as suggested by others (Piva *et al*, 1986). However, the involvement of putative autoreceptors in this response is unknown as their presence has not been demonstrated on EOP neurons.

Although activation of κ-opioid receptors has been shown to inhibit LH and GnRH release (Leadem and Yagenova, 1987; Leposavic *et al*, 1991) the specificity of κ-opioid action has been questioned since naloxone, a predominantly μ-opioid antagonist, prevented the κ-agonist-induced LH inhibition (Pfeiffer *et al*, 1987). A highly specific κ-opioid agonist, U-50488H (Cicero *et al*, 1988) was used in the present study. The pre-ovulatory LH surge was completely abolished for seven hours by U-50488H on the afternoon of pro-oestrus. Unexpectedly, MR2266, a κ-opioid receptor antagonist, failed to reverse the suppressive effects of U-50488H on plasma LH levels. It could be that this opioid antagonist may not have affinity for the κ-opioid receptors or the dose used was insufficient to overcome the effects of U-50488H. However, these assumptions seem unlikely since the same treatment significantly increased the hypothalamic noradrenergic neuronal activity in the same animals (this is detailed later in this discussion). Possible modulatory effects of κ-opioid receptors were further investigated by using a different κ-agonist and an antagonist in the ovx and steroid-primed rat model. Icv infusion of U-69593 decreased, and its co-administration with MR1452 increased, NA and DHPG concentrations within the hypothalamus. However, neither U-69593 alone nor together with MR1452 caused significant changes in plasma LH levels due to the interference by the ketamine anaesthesia (as explained in the Experiment III). It is most likely that the complete cessation of the pre-ovulatory LH surge, evident after IP administration of U-50488H, results from a specific action at κ-opioid receptor subtypes.

To date, much of what is known regarding the effects of opioids on GnRH and LH release has been based upon studies using opioidergic compounds which act at either μ- or κ-opioid receptors. There have been relatively few attempts to study the significance of δ-opioid receptors in the regulation of LH release. Activation of δ-opioid receptors have been shown to suppress LH secretion on the day of pro-oestrus (Leadem and Yagenova, 1987). However, specificity of δ-opioid action appears to be controversial, since a specific δ-opioid receptor antagonist has failed to reverse the inhibition of LH release (Leposavic *et al*, 1991). In Experiment II, the use of a highly selective δ-agonist, DPDPE, profoundly inhibited the pre-

ovulatory LH surge, and its effects were reversed by naloxone. Naloxone exerts its antagonistic effects by acting predominantly at μ-receptor sites (Bicknell, 1985). δ-opioid receptors have a more restricted distribution than those of μ- and κ-opioid receptors in the brain (Mansour *et al,* 1988; Desjardins *et al,* 1991). It has recently been reported that there may be interaction between the δ- and μ-opioid receptor subtypes in the CNS (Stadimis and Young, 1992; Traynor and Elliott, 1993). The δ-opioid receptors are known to have great affinity towards enkephalin neurons (Traynor and Elliott, 1993). Furthermore, in the rat brain, μ- and δ-receptors can exist on the same neuron (Schoffelmeer *et al,* 1988). Therefore, it is possible that both receptor subtypes may mediate the inhibitory effects of DPDPE on the LH secretion. In order to reveal the specific site of δ-opioid action, DPDPE was co-administered (icv) with ICI 154,129, a selective δ-antagonist, and with naloxone to the ovx and steroid-primed rats on the afternoon of the anticipated LH surge. The LH results from that experiment have proved to be inconclusive since the ketamine anaesthesia again disrupted the expected surge release of LH. However, specific δ-opioid effects were observed on the hypothalamic amine (particularly NA) concentrations within the specific hypothalamic regions of the same animals. It is suggested that inhibitory effects of EOPs on LH secretion are also exerted via the δ-opioid subtypes. The nature of δ-opioid involvement in this process has to be further elucidated when more selective δ-opioid antagonist agents become available.

In conclusion, all three of the opioid receptor subtypes investigated appear to inhibit LH release when activated by their respective agonists on the afternoon of the pro-oestrus.

NA Mediation of Opioid Effects on LH Release

As detailed in the Introduction, it is generally believed that the noradrenergic system in the brain is stimulatory to GnRH and thus also LH release in the presence of circulating ovarian steroids but, inhibitory in their absence (See Kalra *et al,* 1989; Kordon *et al,* 1994). Blockade of the α-adrenoreceptors in the preoptic/AHA, ARN and ME as well as systemic application of the catecholamine synthesis inhibitor, diethyl dithiocarbamate, inhibits the LH surge and also basal LH pulses (Estes *et al,* 1982; Al-Hamood *et al,* 1985; Jarry *et al,* 1990). Noradrenergic nerve terminals make synaptic contacts with the GnRH neurons in the MPOA and to a lesser extent with GnRH nerve terminals in the ARN and ME

(Watanabe and Nakai, 1987). The noradrenergic terminals within the hypothalamus possess a mixed population of opioid receptors; which are inhibitory to GnRH release, and stimulation of opioidergic activity inhibits GnRH release. Morphine-induced suppression of LH secretion has been attributed to an inhibition of release of NA from its nerve terminals (Kalra *et al,* 1989; Barraclough, 1994).

In view of the reports outlined above, opioid suppression of LH release would be expected to involve a reduced NA activity in the hypothalamic areas containing GnRH neurons or terminals. In the present study, administration of specific κ- and δ-opioid agonists significantly decreased the concentrations of NA and its metabolite (DHPG) in the MPOA, SCN, ME and ARN concomitantly with the inhibition of the pre-ovulatory LH surge. These inhibitory effects of U-50488H and DPDPE were prevented following co-administration with their respective antagonists. Similar results were observed in all the hypothalamic regions examined after icv infusion of opioid agonists and/or antagonists to the ovx and steroid-primed rats under ketamine anaesthesia. However, the anticipated LH surge was blunted in all the drug treatment groups by ketamine itself. As activation of opioid receptors depresses spontaneous neuronal activity and hence neuronal transmission (Jiang and North, 1992), the present results suggest that opioid agonists studied suppress LH release. This action may involve a reduction in the hypothalamic noradrenergic neurotransmission.

It has previously been reported that administration of naloxone stimulates NA release and/or turnover within the hypothalamus during the pre-ovulatory LH surge (Akabori and Barraclough, 1986). Naloxone-induced increase in the noradrenergic activity enhances release of GnRH (Kalra *et al,* 1989). Consistent with these observations, the results presented here indicate that naloxone stimulation of the surge release of LH involves a greatly increased hypothalamic NA release and/or turnover within the preoptic-tuberal pathway. It thus appears that it is the changes in the hypothalamic NA content by which the opioid antagonist influences LH release. Indeed pharmacological studies have shown that naloxone-stimulated LH release is prevented by inhibitors of the noradrenergic synthesis (Van Vugt *et al,* 1981; Kalra and Simpkins, 1981).

During the pre-ovulatory LH surge, all GnRH neurons synchronise to initiate synthesis of this neuropeptide (Silverman and Witkin, 1994). This co-ordination of GnRH synthesis is achieved by intercellular communications through numerous

collateral synaptic contacts between GnRH-producing neurons and putative GnRH receptors (Wetsel *et al*, 1992). The simultaneous firing of many, if not all, GnRH neurons is required for the pulsatile discharge of GnRH. In the MPOA of the κ-opioid agonist and antagonist-treated group, there was no significant increase in NA concentrations. Perhaps the lack of an essential stimulatory noradrenergic component to the GnRH neurons in the MPOA accounts for the failure of MR2266 to elevate plasma LH levels. This suggestion is supported by the findings of Honma and Wuttke (1980) who concluded that the stimulatory effects of NA on GnRH and hence LH release are exerted within the MPOA.

There are claims that the central noradrenergic system also exerts an inhibitory effect on LH release (Dotti and Talcisnik, 1984; Leipheimer and Gallo, 1985; Bergen and Leung, 1987). However, the results described here have clearly indicated that a decrease in the hypothalamic NA content was associated with the inhibition of the pre-ovulatory LH surge in the pro-oestrous rats. These contradictory findings can be explained by the lack of a steroidal environment in the ovx animal models used in those studies reported above. Because the ovarian hormones have been reported to have a determining role on the action of NA which is considered to provide an essential stimulatory input to the LH secretory systems. It this context, it should be noted that there is usually an increase in the opioidergic neuronal activity in the absence of ovarian steroids (Genazzani *et al*, 1990). Opioids inhibit the stimulatory noradrenergic input to the LH secretory mechanisms. However, the EOP neurons contain and are themselves inhibited by circulating E_2 (Grossman and Dyer, 1989). Thus, increasing levels of E_2 on the afternoon of pro-oestrus frees the excitatory NA input from the tonic inhibition of the EOPs and leads to the pre-ovulatory LH surge. Likewise, enhanced noradrenergic content and turnover in the specific regions of the hypothalamus were detected following icv infusion of sterile saline or various opioid receptor antagonists to the ovx and steroid-primed rat model utilised in this study. However, these findings could not be correlated with the anticipated LH surge, again due to the unexpected interference by ketamine anaesthesia in the same animals (this was detailed in the Experiment III).

GABAergic neurons may function as inhibitory interneurons between the noradrenergic terminals and GnRH neurons. Activation of GABA neurons with muscimol and baclofen ($GABA_A$ and $GABA_B$ receptor agonist, respectively) reduces NA release in the MPOA and MBH in association with a sustained

decrease in serum LH levels (Demling *et al,* 1985; Adler and Crowley, 1986). On the other hand, stimulation of noradrenergic input projecting to the MPOA, results in GABA release in ovx rats when E_2 levels are relatively low, but not when they are high (Herbison *et al,* 1989). Furthermore, GABA may also modulate GnRH neuronal responsiveness to NA and its withdrawal at the time of the LH surge; this action would allow a greater NA release (Barraclough, 1994). These findings are taken to indicate that elevated E_2 levels on pro-oestrus also eliminate the inhibitory GABAergic influence on the excitatory NA input to the GnRH secretory system. Such a determining role of E_2 on this GABAergic action has indeed been reported (Flugge *et al,* 1986). Thus, it appears that expression of the triggering neural signal of NA for the LH surge may result from a reduction in the hypothalamic GABAergic activity (Jarry *et al,* 1991). Although the hypothalamic GABA release was not monitored in this study, the increased NA release and turnover in the MPOA, SCN, ME and ARN would indicate that the GABAergic neuronal activity was decreased or completely eliminated by the autoregulatory mechanisms at the time of the LH surge. These results thus support the hypothesis that a decrease in GABA release would also contribute to the GnRH pulse generating system.

DA Mediation of Opioid Effects on LH Release

There have been controversial reports about the involvement of the central dopaminergic system in pulsatile LH release; both stimulatory and inhibitory actions of DA have been suggested (Ramirez *et al,* 1984; MacKenzie *et al,* 1988). However, DA is not considered to play a primary role in the generation of the LH surge under physiological conditions.

Pharmacological manipulations of the dopaminergic systems projecting to the preoptic-tuberal pathway have been shown to alter LH secretion. Administration of DA antagonists inhibits the LH surge, and thus ovulation, when given to the pro-oestrous rat (MacKenzie *et al,* 1988). Conversely, infusion of DA or its agonists into the ZI elevates plasma LH levels and advances the LH surge (James *et al,* 1987). Lesions of the ZI have been demonstrated to disrupt the LH surge (Sanghera *et al,* 1991a). As mentioned in the Introduction, the dual effects of DA on LH release have been attributed to the pathway involved; the incertohypothalamic system appears to be stimulatory in the MPOA and SCN with the TIDA being inhibitory in the ARN-ME. Indeed, DA has been shown to be excitatory in the MPOA (Kawakami *et al,* 1975; MacKenzie *et al,* 1988) where

dopaminergic and GnRH neurons make synaptic contacts (Leranth *et al,* 1988a). Therefore, a decrease in DA levels of the MPOA would be expected following administration of opioid agonists which inhibit LH secretion. Both diamorphine and DPDPE reduced the DA concentrations in this hypothalamic area concomitantly with inhibition of the LH surge. Furthermore, naloxone-stimulation of LH release involved an increase in the DA activity both in the MPOA and SCN. It is suggested that EOPs may exert their effects, at least in part, on LH secretion also via a dopaminergic mechanism. This modulatory action of the opioids appears to be inhibitory in the MPOA and SCN.

DA has been suggested to be inhibitory to LH secretion in the ARN and ME (Rose and Weick, 1986). Morphine-induced LH inhibition involves an increase in the dopaminergic activity in these hypothalamic regions (He *et al,* 1994). In contrast, morphine has also been reported to lower the rate of synthesis and turnover of DA in the ME by inhibiting the activity of the TIDA neurons (Alper *et al,* 1980). Similarly β-endorphin decreases the hypothalamic DA release from the TIDA neurons (van Loon *et al,* 1980; Wilkes and Yen, 1980). In the present study, of the opioid agonists investigated, only DPDPE significantly reduced DA concentrations in the ME and ARN concomitantly with the inhibition of the pre-ovulatory LH surge. It may therefore be inferred that opioids decrease the TIDA dopaminergic activity too, during the pre-ovulatory LH surge.

It has very recently been reported that opioidergic suppression of LH release is accompanied by an inhibited dopaminergic input to the LH secretory systems (Chandolia *et al,* 1997). In the present study, DA concentrations were significantly increased in all the hypothalamic regions examined following the co-administration of naloxone with diamorphine or DPDPE. These results thus further indicate that naloxone-stimulation of the pre-ovulatory LH surge involves an increase in DA release in specific hypothalamic regions on the afternoon of pro-oestrus.

Administration of the κ-agonist, U-50488H, reduced the hypothalamic DA content in association with the abolition of the pre-ovulatory LH surge. However, the decreases in DA levels were not found to be statistically significant. Previous work from this laboratory using a different κ-opioid agonist, tifluadom, have also produced inconsistent results (Gopalan *et al,* 1989a; Brown *et al,* 1994). Activation of κ-opioid receptors has been shown to inhibit DA release from the

MBH slices *in vitro* (Heijna *et al,* 1991). Based on the findings here from administration of the μ- and δ-opioid agonists, it has been suggested that an increase in the hypothalamic DA release (particularly in the MPOA and SCN) may be stimulatory to LH release. If so, failure of the combination of MR2266 with U-50488H to elevate plasma LH levels in the pro-oestrous rats may be attributed to lack of effects of the κ-opioid antagonist on the DA content in the MPOA and SCN.

In the ovx and steroid-primed rats, central administration of μ-, κ- and δ-opioid receptor agonists brought about significant decreases in DA levels in all the hypothalamic regions examined (except for the action of diamorphine in the ARN). Thus these results further demonstrate that the effects of opioids on DA release is inhibitory at the time of the LH surge regardless of the dopaminergic pathway involved. Furthermore, prevention of opioid-induced falls in the hypothalamic DA content by naloxone, MR1452 and ICI 154,129 indicates that these inhibitory actions of the opioids are exerted via specific opioid receptor subtypes.

Direct neurohumoral effects of DA on prolactin release from the anterior pituitary have been well-documented (See Kordon *et al,* 1994). Dopaminergic projections to the external layer of the ME secrete this catecholamine into the hypophyseal portal system as a prolactin inhibiting factor (Ben-Jonathan, 1985). Naloxone inhibits prolactin secretion by increasing the outflow of DA from the ME and morphine produces the opposite effects (Pfeiffer *et al,* 1987). The possibility exists that the raised DA levels in the ARN-ME observed during the present study may be a reflection of naloxone's action on the regulation of prolactin secretion. Naloxone-enhancement of the dopaminergic activity in all the hypothalamic regions studied concomitant with the pre-ovulatory LH surge is therefore not surprising. It is known that dopaminergic drugs affect plasma LH levels, however, their action is exerted within the brain rather than at the anterior pituitary level. Indeed, a functional relationship between the dopaminergic and GnRH systems has been demonstrated in discrete hypothalamic areas. The action of DA neurotransmission is probably mediated by axo-axonic contacts between GnRH and DA fibres in the ME (Ramirez *et al,* 1984). The results of the present study also appear to support the concept that it is changes in the hypothalamic activity of this catecholamine which may influence LH discharge from the anterior pituitary.

In conclusion, although the dopaminergic system may be of minor importance in the mediation of opioid inhibition of LH release, an increase in its activity may facilitate the pre-ovulatory LH surge.

5-HT Mediation of Opioid Effects on LH Release

The physiological significance of the serotonergic system in the central regulation of LH secretion has not been well-established. Both stimulatory and inhibitory effects of 5-HT on LH release have been reported (See Vitale and Chiocchio, 1993; Kordon *et al,* 1994).

There is compelling evidence to suggest that 5-HT is excitatory to GnRH and hence LH release (Walker, 1983; James *et al,* 1989; Dow *et al,* 1994). Administration of a 5-HT agonist stimulates LH secretion. In addition, impairment of the serotonergic transmission abolishes the LH surge (Walker, 1980). *In vitro*, 5-HT stimulates GnRH release from ME fragments taken from the pro-oestrous rats (Vitale *et al,* 1987). The results of the present study have shown that there is usually an increase in the concentrations of 5-HT and its metabolite, 5-HIAA, in specific hypothalamic regions of the saline-treated animals at the time of the LH surge. Similar findings have previously been reported that an increase in serotonergic activity occurs in association with the pre-ovulatory LH surge on pro-oestrus (Kerdelhue *et al,* 1989). These observations appear to indicate a facilitatory role for 5-HT in the control of LH secretion.

There are reports of 5-HT mediation of opioid effects on LH release (Lenahan *et al,* 1987; Gopalan *et al,* 1989b). High numbers of the various opioid receptors have been detected in the raphe nucleus (Mansour *et al,* 1988). In the DRN, activation of κ-opioid receptors decreases the response of 5-HT-sensitive neurons to electrical stimulation suggesting that EOPs may pre-synaptically inhibit 5-HT release in this brain area (Pinnock, 1992). Thus, opioid agonist inhibition of the LH surge should involve a reduced 5-HT activity in the hypothalamus, if opioid effects on LH are mediated by 5-HT neurons. The hypothalamus receives dense serotonergic projections from the raphe nuclei (Steinbusch, 1984). Icv infusions of diamorphine, U-69593 and DPDPE have clearly indicated that all three (μ, κ, and δ) opioid agonists have modulatory effects on the hypothalamic serotonergic system at the time of the anticipated LH surge. The direction of the opioidergic action on the 5-HT neuronal activity appears to be inhibitory. Systemic

administration of diamorphine and U-50488H had area-dependent effects on the concentrations of 5-HT and 5-HIAA in the MPOA, SCN, ME and ARN. However, the δ-opioid agonist, DPDPE, significantly reduced the levels of both 5-HT and its metabolite in all the hypothalamic regions examined (except 5-HT in the SCN) concomitantly with the abolition of the pre-ovulatory LH surge. Thus, these results reaffirm the hypothesis that opioids may exert their inhibitory effects on LH secretory systems, at least in part, via a 5-HT mechanism.

It has previously been suggested that naloxone enhances LH secretion by increasing the hypothalamic 5-HT content and/or turnover (Brown *et al,* 1994). The results described here have clearly indicated that naloxone-stimulation of LH release involves an increase in the serotonergic neurotransmission in the MPOA, SCN, ME and ARN on the afternoon of pro-oestrus. Administration of naloxone antagonises the inhibitory effects of diamorphine on 5-HT release and/or turnover by acting principally at the μ-opioid receptor subtypes. These findings thus support the concept that the 5-HT system provides a stimulatory input to the LH surge generating system.

In conclusion, the serotonergic system may be involved in opioid inhibition of the LH surge. It is also believed that although 5-HT may facilitate the LH release, its role in this process appears to be of minor importance under physiological conditions.

Urethane and Ketamine Anaesthesia in Neuroendocrine Studies

There is increasing concern about the use of anaesthetics in neuroendocrine experiments. Several anaesthetics e.g. pentobarbitone, alphaxolone, ketamine, urethane, ether and chloral hydrate have been reported to influence LH release in the female rat, however, the results are often conflicting (Kimura and Sano, 1995; Hartman *et al,* 1989; de Greef *et al,* 1987; Dyer and Mansfield, 1984; Sherwood *et al,* 1980).

The employment of urethane as a useful anaesthetic in neuroendocrine studies has been recommended as it has been claimed that it does not prevent the occurrence of LH pulses, although it reduces their amplitude (de Greef *et al,* 1987; Matzen *et al,* 1987). Despite using the minimum effective dose in this study, urethane completely abolished the LH surge for seven hours on the afternoon of pro-oestrus

(see Experiment I). Furthermore, the overall hypothalamic monoamine concentrations, in particular those of NA and its metabolite (DHPG), in the urethane-anaesthetised animals were found to be significantly lower than those seen in the conscious, unrestrained rats. Pentobarbitone and chloral hydrate have also been reported to reduce NA turnover and release respectively, concomitant with their inhibition of the LH surge (Rance and Barraclough, 1981). An increase in the hypothalamic noradrenergic activity has been correlated to the pre-ovulatory LH surge, and the absence of this stimulatory neural signal may be responsible for the failure of ovulation (Ramirez *et al,* 1984; Akabori and Barraclough, 1986). The present results therefore indicate that urethane may have interfered with the GnRH surge secretory systems by decreasing the concentrations of NA and its metabolite (DHPG) in the specific hypothalamic regions.

Differential effects of anaesthetic agents on plasma LH levels have been explained by a different mechanism by Kimura and Sano (1995). These authors have reported that pentobarbitone anaesthesia arrested the anticipated LH surge in the afternoon, while it had no effect on pulsatile LH release when given in the morning of the same day. Furthermore, naloxone infusion increased the activity of GnRH neurons and plasma LH levels in the pentobarbitone-anaesthetised rats on the late morning of pro-oestrus (Funabashi *et al,* 1997). These findings were taken to suggest that separate GnRH pulse and surge generators exist in the hypothalamus and that these control basal and surge release of LH, respectively. In the present study, plasma LH levels were not monitored on the morning of pro-oestrus in the urethane-anaesthetised rats. However, it has previously been reported that this anaesthetic does not prevent basal release of LH in the male rat (Matzen *et al,* 1987). Therefore, it is suggested that urethane may exert its effects by a similar mechanism (see above) since it completely blocked the pre-ovulatory LH surge on the afternoon of pro-oestrus.

In contrast to the earlier reports (Sherwood *et al,* 1980; Ching, 1982; Hartman *et al,* 1989), it was found in the present study that ketamine anaesthesia also suppresses LH release from the anterior pituitary (see Experiment III). Interestingly, ketamine did not seem to disrupt central aminergic neurotransmission, as biogenic amine levels were largely in parallel with the values obtained from the conscious animals in which the LH surge took place. These findings suggest that the inhibition of the LH surge may be a result of reduced pituitary responsiveness to GnRH release in the ketamine-anaesthetised rats. It is

also suggested that the GnRH surge generator may still be active in these rats, since a GnRH surge has previously been detected in the portal blood of the ketamine-anaesthetised rats (Sherwood *et al,* 1980).

To date it would appear that there is no anaesthetic which does not interrupt the reproductive neuroendocrine axis in female rats. Therefore, it is recommended that use of general anaesthetics should be avoided if at all possible in such studies.

Conclusions

The results reported here provide strong evidence for the participation of κ- and δ-opioid receptors in the central regulation of LH release. Thus it appears that multiple opioid receptor subtypes are involved in the opioid inhibition of the pre-ovulatory LH surge.

Since all three opioid receptors agonists significantly reduced concentrations of NA and DHPG in all the hypothalamic regions examined, concomitantly with the abolition of the LH surge (except diamorphine), it is suggested that the inhibitory actions of EOPs on LH release are mediated in large part by NA. These findings suggest that an increase in hypothalamic noradrenergic activity may be a critical event in triggering the surge release of GnRH and LH. Both the dopaminergic and serotonergic neurotransmitter systems may also facilitate the pre-ovulatory LH surge, although their effects are thought to be of minor importance in this process.

Icv administration of diamorphine, U-69593 and DPDPE resulted in significant decreases in the hypothalamic biogenic amine levels. These results therefore indicate that modulatory actions of opioids on the hypothalamic noradrenergic, dopaminergic and serotonergic neurotransmission are inhibitory. The prevention of these effects by selective opioid antagonists would point to specific mediation via μ-, κ- and δ-opioid receptors.

DPDPE inhibited the pre-ovulatory LH surge in association with a decrease in the hypothalamic NA levels in a naloxone reversible-manner. Due to the postulated cross-communication between the μ- and δ-opioid subtypes, it is suggested that both μ- and δ-opioid receptors may mediate the inhibitory effects of DPDPE.

Recommendation for future work

In the experiments presented here, opioid suppression of the pre-ovulatory LH surge by a mixed population of receptors has been shown. Furthermore, the results have indicated that direction of opioidergic action on the central noradrenergic, dopaminergic and serotonergic neurotransmission via μ-, κ- and δ-opioid receptors is inhibitory. To further investigate the above findings, acute central administration of the opioids could be used. To this end, indwelling intracranial cannula could be placed (under halothane anaesthesia) to allow for icv administration of drugs to conscious, unrestrained animals. Blood samples would be collected from conscious animals at half hourly intervals for LH determination by RIA on the afternoon of pro-oestrus. Such an experimental design would provide for the deposition of drugs in predominantly hypothalamic loci and eliminate a possible interference caused by anaesthetic agents. At the end of the critical period, the animals would be decapitated, brains removed and MPOA and ME isolated as described earlier. The monoamine contents of these two hypothalamic regions would then be determined by HPLC-ECD.

To further investigate the opioid receptor subtypes involved, selective antagonists of each opioid receptor subtype could be co-administered with their respective agonists, and the effects on LH secretion, and monoamine activity in the hypothalamus, determined. To further elucidate the role of hypothalamic monoamines in the mediation of the effects of the opioids on LH secretion, microdialysis technique would be employed to deliver opioids and collect extracellular fluid for the measurement of monoamines in the MPOA and ME of conscious animals. Microdialysis would provide a clearer index for the release of monoamines in dialysates at the time of the LH surge.

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List of Communications

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