Opiate actions on central and systemic oxytocin release

Julia.E.Coombes

Doctor of Philosophy
University of Edinburgh
1991

The studies described in this thesis were performed in the Department of Physiology, University Medical School, Teviot Place, Edinburgh; AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge and National Institute for Medical Research, The Ridgeway, Mill Hill, London.

All of the work in the thesis was performed by the author unless credited otherwise in the text and was compiled by her alone.

March 1991 J.E.Coombes

ABSTRACT

The experiments in this thesis were designed to investigate the modification of oxytocin secretion by mechanisms acting at the posterior pituitary gland and on centrally-projecting oxytocin neurones.

Electrical stimulation of the neurohypophysial stalk to release oxytocin combined with autobioassay of oxytocin secretion was used in anaesthetized, lactating rats to compare actions of opioid-receptor subtype specific agonists on oxytocin neurone terminals in the posterior pituitary. The kappa-receptor was identified as the opioid-receptor subtype responsible for inhibition of oxytocin secretion at the posterior pituitary by the use of the selective opioid-receptor agonists U50,488H and morphine. It was determined that in rats with oxytocin neurones made tolerant to morphine there was no cross tolerance to the actions of kappa-opioid agonists at the posterior pituitary.

Electrically-stimulated isolated posterior pituitaries **in vitro** were used to study effects on oxytocin of opioid-antagonists with different receptor affinities: the non-selective antagonist naloxone was more effective than the relative μ-selective RX8008M and κ-selective MR2266.

Cerebrospinal fluid (CSF) samples were collected from the cisterna magna, for oxytocin radioimmunoassay from anaesthetized rats, which were also blood sampled to monitor opioid actions on centrally-projecting oxytocin neurones. It was found that oxytocin neurones projecting centrally and to the posterior pituitary could not be made dependent upon the kappa-agonist U50,488H, as assessed by lack of withdrawal excitation of oxytocin secretion after naloxone. Dependence upon the μ-opioid receptor agonist, morphine, could be

acheived for oxytocin neurones releasing into CSF as well as oxytocin neurones secreting into blood; it was concluded that both centrally-releasing and magnocellular neurones can be inhibited by opioids. Studies on morphine dependent rats with ablated paraventricular nuclei, indicated that magnocellular supraoptic neurones could release oxytocin into CSF during morphine withdrawal.

The effects of a synthetic, μ-selective opiate, pethidine, upon the progress of parturition was determined by monitoring the timecourse of parturition and ensuing maternal behaviour. Plasma samples taken during parturition shows oxytocin secretion was decreased following administration of the opiate, indicating how pethidine slows parturition.

Finally, the opioid receptors involved in the control of oxytocin secretion were examined more closely. Changes in receptor density of μ and κ receptors in the posterior pituitary during the development of morphine tolerance were sought by specific in situ ^{3}H-receptor ligand binding autoradiography and computer assisted image analysis. Animals were also treated with i.c.v. pertussis toxin, known to inactivate $G_{i/o}$ proteins in order to investigate the role of these proteins in post-receptor mechanisms involved in the inhibitory action of opiates on oxytocin secretion.

ACKNOWLEDGEMENTS

Firstly, I would like to thank Professor W.E. Watson for allowing me to use the facilities of the Dept. of Physiology for the duration of my PhD and the Faculty of Medicine, who awarded me the Gordon-Lennie Bursary for the three years of my studies.

I would also like to thank my supervisor Dr. J.A. Russell for his unfailing enthusiasm and sense of humour during my three years in his laboratory.

Dr. B.E.H. Sumner (Department of Physiology, Edinburgh), Dr. W.J. Sheward (MRC Brain Metabolism Unit, Edinburgh, Dr. R.J. Bicknell, (AFRC Institute, Cambridge) and Dr. I.C.A.F. Robinson (NIMR, London) all kindly gave me the opportunity to collaborate with them for some of the experiments described in this thesis and their help is greatly appreciated.

Mr. B. McGrory, Mrs. K. Grant, Mrs. G. Grant (Department of Physiology, Edinburgh) and Mr. C. Chapman (AFRC Institute, Cambridge) gave their invaluable technical assistance with assays and histology.

Finally, I would like to thank my husband Stephen and my parents for their support and encouragement during my PhD 'experimental' and 'writing-up' periods.

ABBREVIATIONS

AV3V	region anterior and ventral to the third ventricle.
CSF	cerebrospinal fluid
i.c.v.	intracerebroventricular
i.v.	intravenous
MPA	medial preoptic area.
MR2266	5,9 α-diethyl-2-(3-furylmethyl)-2-hydroxy-6,7-benzomorphan.
PVN	paraventricular nucleus
RX8008M	16-Me cyprenorphine
SON	supraoptic nucleus
U50,488H	trans(±)-3,4-dichloro-N-Me-N-[2-(1-pyrrolidinylcyclohexyl)] benzene acetamide methane sulphonate.

CONTENTS

FIGURES & PLATES

page

PLATES

GENERAL INTRODUCTION

GENERAL INTRODUCTION

THE DISCOVERY OF OXYTOCIN.

Understanding of the pituitary gland and physiology of the hormones it secretes has progressed far since the days when it was described as a filter for the excretion of 'pituita' or 'brain waste' into the nasopharynx.

The pressor function of a pituitary body extract administered intravenously into a dog was noted by **Oliver & Schäfer** in 1895. The rise in blood pressure produced was rapid, the fall slow and the maximum pressure maintained for many minutes if the dose given was sufficient. The rise was attributed to contraction of arterioles and augmentation of heart beats. The mechanism of action was concluded to be peripheral, since when added to Ringers circulating fluid and perfused through a frog with the nervous system destroyed, the flow of fluid was greatly diminished.

In 1901 this study was extended by **Schäfer & Magnus** to demonstrate that the pituitary extract caused a marked 'expansion' of the kidney and a prolonged diuresis. The 'active' substance responsible for these described actions was documented to be present in the infundibular or nervous portion of the pituitary gland.

In 1906, **Sir Henry Dale** was investigating the physiological actions of ergot in the cat. He had noted that the actions of adrenaline on blood pressure and uterine activity was reversed by ergot so that a fall in arterial blood pressure and relaxation of the uterus was seen. The pressor activity of posterior pituitary lobe extract was now familiar and he decided to see if this action was reversible by ergot. The cat being recorded from happened to be in the early stages of pregnancy and injection of the extract not only showed that it retained its pressor activity in the

presence of ergot but that the extract could also produce a strong contractile response of the uterus in the pregnant animal not hitherto described. This fortunate experimental 'accident' led to the systematic studies that were then carried out on neurohypophyseal extracts, and the hormone oxytocin was characterized. Oxytocin is now known to play a major role in parturition, lactation, osmoregulation and certain stress mechanisms.

BIOSYNTHESIS OF OXYTOCIN.

(i) The Gene

Oxytocin is made as part of a large precursor molecule together with the neurophysin polypeptide which is associated with the hormone in the neurosecretory granules found in oxytocin neurones. The precursor is processed en-route from its site of synthesis in the cell bodies of the hypothalamic magnocellular neurones in its passage along the axons through the median eminence and neurohypohysial stalk, to the granule sites in the pituitary nerve endings (**Ivell & Richter, 1984**).

In the rat, oxytocin and vasopressin are encoded by separated genes. The two genes do not appear to be expressed together - thus a cell produces either oxytocin or vasopressin but not both. This has been confirmed immunocytochemically (**Dierickx, 1980**) and by in-situ hybridization studies (**Coghlan, Aldred et al, 1984**). The biosynthesis of oxytocin and neurophysin I are shown in **Figure (i)**.

(ii) Co-localization

One third of oxytocin neurones projecting to the posterior pituitary produce both oxytocin and corticotrophin releasing factor (CRF) (**Swanson, Sawchenko et al, 1983**). CRF is also located in parvocellular vasopressin neurones (**Whitnall, Mezey et al, 1985**). The hormones co-exist within neurosecretory

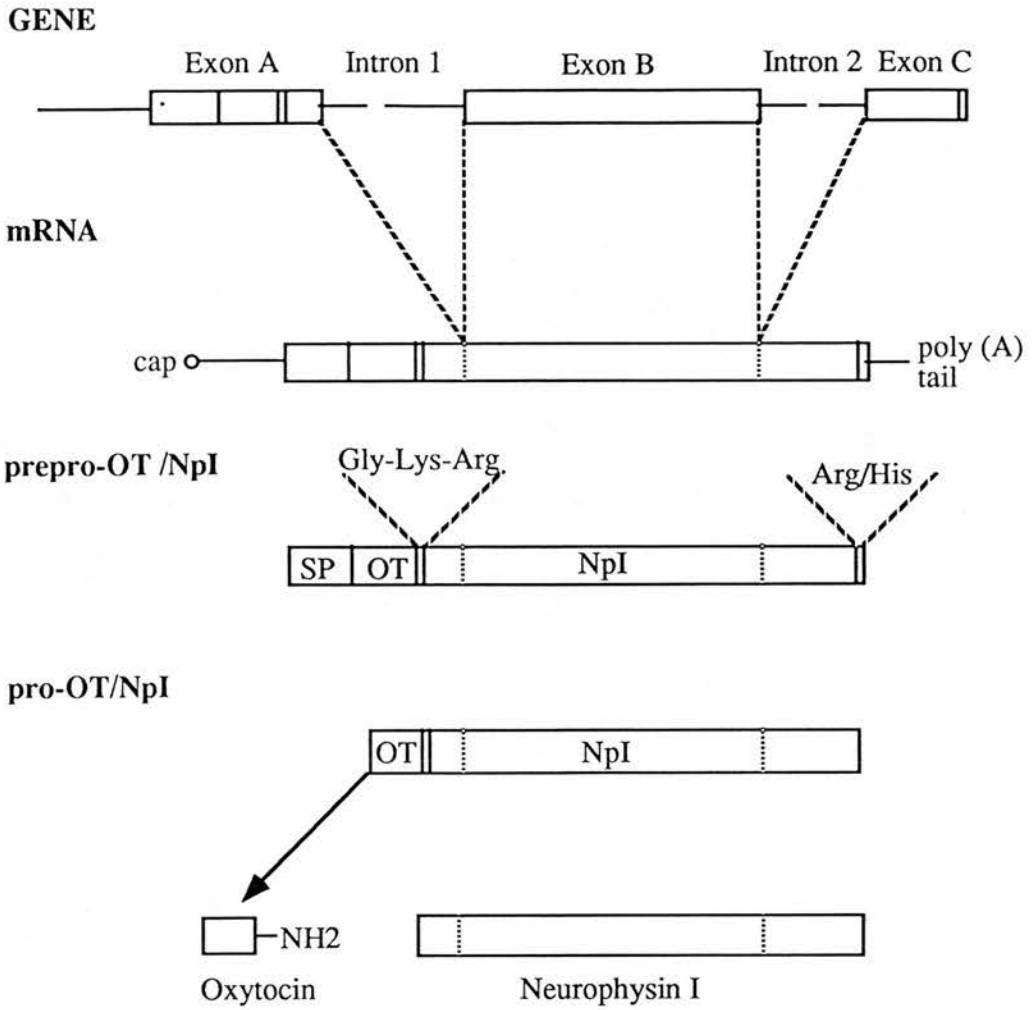


Fig (i)

Biosynthesis of oxytocin and neurophysin I based on data obtained from the cow and rat.
From 'Oxytocin' : Clinical and Laboratory studies,Excerpta Medica International Congress Series.Editors : J.A.Amico and A.G.Robinson (1985)

granules.

Cholecystokinin-8 (CCK) is an abundant regulatory peptide found in the brain and posterior pituitary of the rat (**Deschapper,Lotstra et al,1983**).

Immunocytochemical studies on CCK have shown that it is co-localized with oxytocin (**Kiss,Williams et al,1984**).Oxytocin and CCK appear to be co-localized in the same neurosecretory granules (**Martin,Geis et al,1983**).Since one third of magnocellular oxytocin neurones contain CRF there may be a population of oxytocin/CRF/CCK containing cells.CCK is not co-produced with vasopressin.

Opioid peptides have also been isolated within oxytocin and vasopressin neurones.Met-enkephalin has been shown to be present in oxytocin containing neurosecretory granules (**Martin,Geis et al,1983**) but is undetectable by immunocytochemical methods in the SON/PVN (**Weber,Roth et al,1982**).

Pro-enkephalin A has been isolated in bovine SON and PVN (**Vanderhaegen,Lotstra et al,1983**) and it has been suggested that pro-enkephalin A could be processed to met-enkephalin in the rat magnocellular system.

Leu-enkephalin is found in the posterior pituitary in much greater amounts than met-enkephalin (**Giraud,Castanas et al,1983**).It is likely that at least some of this is derived from pro-enkephalin B and possibly some from pro-enkephalin A.The product of pro-enkephalin B,dynorphin co-exists and is co-secreted with vasopressin in magnocellular neurones (**Watson,Akil et al,1982**).The significance of these endogenous opioid peptides in the regulation of oxytocin secretion will be discussed later.

ELECTROPHYSIOLOGY.

Oxytocin and vasopressin neurones can be distinguished by their contrasting patterns of electrical activity.The characteristic firing patterns of the cells in part reflect their secretory activity and

also their afferent synaptic inputs.

The first determination of magnocellular oxytocin neurone firing came from an experiment that initially set out to investigate oxytocin release in the anaesthetized lactating rat following dilatation of the uterine cervix. Oxytocin secretion was to be quantified by milk let-down in response to oxytocin secretion and subsequent weight gain of the litter of pups suckling at the nipples of the anaesthetized dam. It was noticed that in the absence of any stimulus from the experimenters that the pups showed a stretching reflex and increased vigorous suckling for about 10 seconds every 5-10 minutes (**Wakerley & Lincoln, 1971**). This phenomenon was the milk-ejection reflex, and coincided with a sharp increase in intramammary pressure.

Following these observations, electrophysiological recordings were made from a population of neurones in the paraventricular nucleus (PVN) that were continuously firing but showed explosive firing bursts for 2-4 seconds prior to milk-ejection. These were classified as putative oxytocinergic neurones (**Lincoln & Wakerley, 1971; Wakerley & Lincoln, 1973**). Neurones with a similar firing pattern were subsequently recorded in the supraoptic nucleus (SON) (**Lincoln & Wakerley, 1974**).

All these early experiments were performed on anaesthetized animals but identical electrical recordings were eventually made in unanaesthetized rats (**Summerlee & Lincoln, 1981**).

Initially the mammary gland autobioassay was used as a measure of oxytocin secretion during electrophysiological recording experiments. Plasma oxytocin measurements have now been achieved by RIA on blood samples withdrawn during suckling in conscious rats (**Higuchi, Tadokoro et al, 1986**) and has confirmed the release of a 'bolus' of oxytocin after each burst of electrical activity by the cell bodies of putative

oxytocin neurones.

Therefore, putative oxytocin neurones were identified as those cells in the SON/PVN which had a continuous background firing rate on which were superimposed synchronous, short duration bursts of high frequency (10-80Hz) electrical activity. Conversley, neurones which displayed an asynchronous phasic firing-pattern were classified as putative vasopressinergic cells.

These patterns of firing are also seen during recording from hypothalamic slices in-vitro (**Hatton, Ho & Mason, 1983**). Dye injection and immunohistochemistry following intracellular recording, demonstrated that the phasically firing neurones contained vasopressin (**Yamashita, Inenaga et al, 1983**). By exclusion, the continuously firing cells, in vitro, are classified as oxytocinergic.

Oxytocin neurones only display their bursting pattern of firing in response to certain physiological stimuli. The suckling stimulus, as described previously, leads to high frequency cell firing synchronized within all four magnocellular nuclei (**Lincoln & Russell, 1985**). There is summation of the afferent input in that 5-7 pups must be attached to the nipples for expression of the milk ejection reflex in the anaesthetized rat (less in the conscious animal).

These synchronous bursts of oxytocin neurones are also seen upon dilatation of the cervix, for example during labour (**Summerlee, 1981**)

Vasopressin cells are not seen to be activated under these conditions-the stimuli are oxytocin specific.

Changes in osmotic pressure stimulate oxytocin cell body firing and hence oxytocin secretion but by increasing the rate of continuous background firing rather than by induction of high frequency bursting (**Poulain, Wakerley & Dyball , 1977**).

The phasic firing of vasopressin neurones is believed to promote a continuous profile of vasopressin

secretion. The amount of vasopressin secreted is related to the ratio of the mean 'on phase' (during firing bursts) to the mean 'off phase' (during silent periods) and also to the firing rate of the neurones during the 'on phase'.

The continuous background activity of oxytocin neurones reflects the nature of the background secretion of the hormone whilst the high frequency bursts of electrical activity provide the pulses of oxytocin which stimulate uterine contractility during parturition and the mammary gland for the milk-ejection reflex during lactation. There is a non-linear relationship between firing-rate and oxytocin secretion, such that at higher frequencies there is a disproportionate increase in oxytocin secretion (**Lincoln & Russell, 1985**).

There is evidence that oxytocin released centrally in the vicinity of the magnocellular nuclei may act as an autoregulator upon oxytocinergic neurones and synchronize bursting between the four magnocellular nuclei (**Moos, Freund-Mercier et al, 1984; Mason, Hatton et al, 1986**).

There is extensive neuronal linkage between the four nuclei (**Silverman, Hoffman & Zimmerman, 1981**) and oxytocin-containing terminals have been reported in contact with oxytocin neurone cell bodies (**Theodosis, 1985**). Oxytocin is found to excite continuously firing but not phasically active neurones in the SON in vitro (**Yamashita, Okuya et al, 1983**).

Administration of oxytocin into the third ventricle enhances the neurosecretory bursts associated with milk-ejection and reduces the interval between them. An oxytocin antagonist administered to the same site has the opposite effect (**Freund-Mercier & Richard, 1984**).

A background of oxytocin secretion is, therefore, maintained by the continuous firing of magnocellular oxytocin neurones in the SON/PVN. High frequency, synchronized bursts of electrical activity

of these neurones leads to pulses of oxytocin seen during parturition and lactation. This bursting phenomenon is a gated response with centrally released oxytocin acting to augment the high frequency bursting patterns seen.

PROJECTIONS & PATHWAYS.

The cell bodies of oxytocin and vasopressin-containing neurones are mainly located in two discrete groups, the supraoptic (SON) and paraventricular (PVN) nuclei. The neurones in the PVN comprise two types, magnocellular neurones with cell body diameter 15-35μm and parvocellular neurones with cell body diameter 10-15 μm.

There is also a topographic segregation of oxytocin and vasopressin neurones which has been extensively described (**Sokol, Zimmerman et al, 1976; McNeill & Sladek, 1980; Swanson, Sawchenko et al, 1981; Rhodes, Morrell & Pfaff, 1981; Hou-Yu, Lemme et al, 1986**).

In general, the magnocellular neurones of the hypothalamus are located in the SON, PVN and anterior commissural nuclei (ACN), a number of accessory nuclei and also as individual cells within this region.

The SON is divided by the optic tract into the principal and retrochiasmatic SON regions. The former is found to contain oxytocin cells rostrally and dorsally whilst vasopressin neurones are located caudally and ventrally. The latter region contains almost exclusively vasopressin cells.

The PVN is divided into three main regions, the medial, lateral and posterior subnuclei on the basis of cellular morphology and peptide content (**Swanson & Kuypers, 1980; Armstrong, Warach et al, 1980**).

The medial PVN contains mainly oxytocin-secreting cells. The lateral PVN has a core of vasopressin-producing cells surrounded by a rim of oxytocin-containing cells. The posterior PVN contains mainly

oxytocin-producing cells. The accessory nuclei are found to contain both oxytocin and vasopressin producing cells.

Magnocellular oxytocin neurones of the principal portion of the SON project to the posterior pituitary gland (**Scherlock, Field & Raisman, 1975**)

The projections of oxytocin-containing neurones in the PVN are more complex. In essence, a core of magnocellular neurones project to the posterior pituitary gland whilst in the parvocellular regions oxytocin neurones project to the brainstem, spinal cord and the external lamina of the median eminence.

The magnocellular portion consists of three relatively dense cell groups projecting to the posterior pituitary. The posterior group contains both oxytocin and vasopressin cells whilst the medial and anterior groups contain oxytocin only.

Cells from the anterior, medial and lateral parvocellular PVN project to the dorsal-vagal complex; 10-15% of these cells project to the medulla and spinal cord (**Swanson & Kuypers, 1980**). Some oxytocin fibres from the PVN pass through the dorso-vagal complex to the central gray of the spinal cord (**Swanson & M^{c}Keller, 1979**). The dorsal parvocellular region has oxytocin cells which project to the spinal cord whereas the other regions of the PVN contain approximately equal numbers of cells which project to the spinal cord and/or the pituitary gland.

A medial parvocellular group projects to the median eminence, where the nerve terminals abut onto capillaries located in the external lamina and they release their neurohumoural content into the portal capillary system which irrigates the anterior pituitary (**Wiegand & Prise, 1980**).

Oxytocin projections from the PVN have also been traced to innervate the locus coeruleus and parabrachial nucleus in the pons (**Swanson, 1977**). Oxytocin-stained fibres also form a

THE UNIVERSITY *of* EDINBURGH

plexus in the marginal zone of the spinal nucleus of the trigeminal nerve in the medulla (**Swanson & Hartman, 1980**).

In a study by **Taniguchi, Yoshida et al, 1988** using retrograde horseradish peroxidase and immunohistochemical tracing techniques, 'CSF-contacting neurones' which have been reported in the subependymal layer with their cytoplasmic processes projecting to the ventricular lumen were observed (**Vich & Vich-Teichman, 1973**). This study however located neuronal perikarya not only beneath the ependymal layer but also within the ependymal layer. The existence of these CSF-contacting, oxytocin-containing neurones implies that some of the neurones which project to the posterior pituitary have a direct connection with the CSF and may be a source of the detectable CSF oxytocin.

The anterior commissural nucleus contains many magnocellular oxytocin perikarya which project to the posterior pituitary but not to the brainstem or spinal cord and have one or two dendrites which project to the third ventricle (**Armstrong, Warach et al, 1980**). These too may contribute to the oxytocin measured in CSF.

STIMULI FOR INCREASED OXYTOCIN SECRETION.

(i) Parturition

Oxytocin is a potent uterotonic agent and has therefore been recognized as a possible trigger for the activation of uterine contractions during parturition. The action of oxytocin on the myometrium is dependent upon the presence of extracellular Ca^{2+} and is inhibited by Ca^{2+} channel blocking agents (**Forman, Gandrup et al, 1982**). Ca^{2+} is probably the second messenger involved.

Oxytocin binds to specific binding sites on the muscle sarcolemmal membrane (**Crankshaw, Branda & Matlieb, 1978**). These receptor sites may be linked to

Ca^{2+} channels or gates, thus controlling the influx of Ca^{2+} into the muscle cells (**Mironneau, 1976**).
Oxytocin is also found to inhibit the activity of a Ca^{2+} extrusion pump which consequently leads to an increase in intracellular Ca^{2+} (**Soloff & Sweet, 1982**).
In the rat, uterine responsiveness to oxytocin is rapidly increased in late gestation so that it is maximal for the impending parturition (**Fuchs, 1985**).
In this species, oxytocin receptor density on myometrial cells is increased with oestrogen (**Fuchs, Periyasami et al, 1983**) an effect which is inhibited by progesterone. In rats, this steroid-mediated increase in oxytocin receptors is a major factor in uterine responsiveness to oxytocin at term.
The electrical activity of antidromically identified magnocellular oxytocin neurones has been recorded during parturition in unanaesthetized rats (**Summerlee, 1981**). Preceding any signs of delivery such as abdominal contractions, the oxytocin neurones show a gradual increase in their basal continuous firing pattern upon which is superimposed rapid bursts of high frequency activity. These bursts tend to occur just prior to delivery of individual pups. Vasopressin cell firing was also increased.
The stimulation of neurosecretory cells in the posterior pituitary occurs well before delivery of the pups and is not therefore due to reflexes from the birth canal during foetal-expulsion.
Measurements of plasma oxytocin and uterine activity by intra-uterine pressure recordings have shown that plasma oxytocin does not change in the few hours before expulsion of the pups (**Higuchi, Honda et al, 1985; Higuchi, Tadokoro et al, 1986**). This would imply that increased oxytocin secretion is not the critical element for triggering parturition. Frequent blood samples taken around the time of foetal expulsion show a small, gradual increase in plasma oxytocin which decreases approximately one minute after the foetal

expulsion reflex has occurred. However, electrophysiological recordings show high frequency bursting of oxytocin neurones during the abdominal contractions prior to the foetus-expulsion.

The apparently different findings with electrophysiology and RIA could be due to several reasons; the foetus-expulsion reflex may not be the trigger for the burst like release of oxytocin or maybe the frequency of sampling was not high enough to detect the rapid, sharp increase in plasma oxytocin.

There is some speculation that the bursting pattern of oxytocin neurones seen at this time is not synchronous unlike milk ejection and that not all of the oxytocin neurones are activated under these conditions.

Disruption of oxytocin secretion during parturition and its effect on the time course of pup delivery will be discussed later.

(ii) The milk-ejection reflex

Suckling is a major stimulus for the increased secretion of oxytocin. The mechanism of this is as follows. Pressure sensitive receptors located in the nipple are activated by the pups suckling (**Findlay, 1966**). Nerve impulses travel via segmental nerves, via the spinal cord and brainstem to the supraoptic and paraventricular nuclei. The magnocellular oxytocin neurones in these nuclei fire intermittently as described above and oxytocin is released in pulses from their axon terminals in the pituitary into blood to bind to oxytocin receptors on myoepithelial cells in the mammary gland (**Soloff & Schwarz, 1973**). Binding of oxytocin to these receptors leads to contraction of the myoepithelial cells and milk from the mammary glands is forcibly ejected to the suckling pups. This is called the milk ejection reflex.

Concomitant with the observed extensor reflex of the

suckling pups, there is a pulsatile release of oxytocin which decreases rapidly once into the circulation due to its short half life (ca. 100 sec) (**Higuchi, Honda et al, 1985; Higuchi, Tadokoro et al, 1986**)

The frequency of milk ejections or the time spent suckling by the pups seems not to affect the amount of oxytocin released at milk-ejection. This suggests that the amount of oxytocin released is strictly controlled. The effect of endogenous opioid peptides on the release of oxytocin during the milk-ejection reflex will be discussed later.

(iii) Maintenance of hydromineral balance

For oxytocin to have a role in salt/water balance as a putative natriuretic hormone, it would be expected to be released in response to increased plasma osmolality, Na^+ concentration and/or decreased blood volume.

In experiments that involved measurements of intra-uterine pressure and urine flow in dogs, hypertonic saline gave rise to the release of 10-20 times as much oxytocin as vasopressin (**Abrahams & Pickford, 1954**).

In the rat, it was shown that dehydration by means of water deprivation or saline intake causes similar depletion of neurohypophysial oxytocin and vasopressin content (**Jones & Pickering, 1969**).

To investigate changes in plasma osmolality upon the firing rate of neurones in the SON and PVN, hypertonic saline was injected intraperitoneally in order to elevate plasma osmolality without changes in blood volume (**Brimble & Dyball, 1977**).

An elevation in firing rate of the oxytocin and vasopressin neurones was seen but it was low and sustained compared to that observed during milk-ejection. Oxytocin neurones responded with an increase in their continuous firing rate rather than high frequency burst patterns. Plasma oxytocin was elevated in parallel with the firing rate-a rapid response

immediately following the hypertonic stimulus, which was maintained 35-40 minutes after the stimulus (**Brimble, Dyball & Forsling, 1978**).

As well as a peripheral release of oxytocin following osmotic stimulation, central release of oxytocin, presumably from centrally projecting processes of hypothalamic and extrahypothalamic neurones has been shown (**Landgraf, Neumann and Schwarzberg, 1988**).

Following hyperosmotic stimulation, an elevation of oxytocin was seen in plasma and CSF. Using push-pull perfusion techniques, an elevation of oxytocin in the septum and dorsal hippocampus was detected simultaneous with the changes seen in CSF and plasma.

This shows that osmotic stimulation leads to the same secretory response from two separate oxytocin pathways, one inside, and one outside the blood-brain barrier. As mentioned in the anatomical description of oxytocinergic pathways some neurones appear to project to the region close to the third ventricle (" CSF-contacting neurones"). These neurones may well be responsible for the similar response of central and peripherally projecting neurones.

The question must be raised - how is oxytocin secretion elevated following hypertonic saline ? What stimulates the neurones ?

It is possible that, like vasopressin neurones, oxytocin neurones are osmosensitive (**Mason, 1980**) but there is now evidence to suggest that hyperosmotic or hypernatremic effects are relayed to oxytocin neurones from elsewhere and angiotensin II is a likely candidate as a neuroregulator in this pathway.

Angiotensin II receptors are found in the subfornical organ (SFO) and the organ vasculosum of the lamina terminalis (OVLT), which are part of a circumventricular system (**Simpson, 1981**). Receptor sites are also found in the SON and PVN (**Mendelsohn, Quirion et al, 1984**).

Angiotensin II i.c.v. stimulates vasopressin release, a response mediated via the region anterior and ventral to the third ventricle (AV3V region) (**Sladek, 1983**).

There is now evidence that angiotensin II stimulates oxytocin secretion. Plasma oxytocin is elevated rapidly (within one minute) of injecting angiotensin II i.c.v.-this response is blocked by saralasin, an angiotensin II antagonist (**Lang, Rascher et al, 1981**).

Lesions of the AV3V region inhibit both oxytocin and vasopressin release following water deprivation (**Russell, Hatton & Robinson, 1984**).

The OVLT and SFO project to the SON and PVN as well as having interconnections between themselves indicating that the stimulation of oxytocin could involve all of these structures.

Interruption of the pathway between the SFO and the SON/PVN reduces the increased electrical activity of oxytocinergic neurones following i.p. hypertonic saline or angiotensin II i.c.v. (**Dyball & Prilusky, 1981**).

This lesion may remove the angiotensin II influence on the AV3V oxytocin pathway thus removing any input from the SFO. This results in a decreased secretion of oxytocin following water deprivation, for example. In addition, the AV3V region may also contain osmoreceptors projecting to the magnocellular nuclei, although magnocellular neurones in the SON and PVN may themselves be osmosensitive, they apparently require the AV3V input to express this (**Leng, Blackburn et al, 1989**).

(iv) Stress

Stress can be defined as activation of the hypothalamo-hypophysial-adrenal axis (**Gibb, 1984**).

CRF-41 is believed to be the hypothalamic hormone which mediates the ACTH response to 'stress'. CRF-41 is present in hypophysial-portal blood in sufficient amounts to stimulate ACTH secretion. There is increased

secretion of CRF into portal blood following haemorrhagic stress and, following passive immunization against CRF-41 the stress response is nearly abolished.

Oxytocin and vasopressin are now thought to be involved in the modulation of ACTH secretion probably by a facilitatory effect on the action of CRF-41. Oxytocin is present in portal blood along with CRF-41 and CRF-41 is also found co-localized in magnocellular oxytocin neurones and in parvocellular vasopressin neurones.

The pathways for stress-evoked oxytocin secretion are not yet known. Oxytocin secretion from the posterior pituitary appears to be affected differently by different kinds of stress.

Disturbance of parturition is found to disrupt the progress of pup delivery due to an inhibition of oxytocin secretion (**Leng, Mansfield et al, 1987**) and release of oxytocin during suckling is also blocked by emotional stress (**Lincoln, Hill et al, 1973**).

It has been shown that stress-evoked increases in oxytocin secretion are diminished in lactating rats (Schlein, Zarrow et al, 1974) which may be due to high levels of circulating prolactin. Hyperprolactinaemia (produced by the administration of either dopamine agonists or ovine prolactin) reduces the oxytocin response to stress. This effect is not seen in ovariectomized rats but is restored upon replacement of oestradiol and progesterone. The effect of hyperprolactinaemia on oxytocin secretion is not mediated by steroids however, since oestradiol and progesterone have no modulatory effect on oxytocin secretion (**Carter & Lightman, 1987**). The mechanisms by which hyperprolactinaemia modifies oxytocin secretion is not known.

OPIOID INTERACTION WITH OXYTOCIN SECRETION.

(i) Opioid receptors

There are three main opioid receptor subtypes - a 'classic' morphine receptor called the mu receptor (μ) and two 'non-classic' receptors termed kappa (κ) and delta (δ) (**Martin, Eades et al, 1976**).

Peripheral organ bioassays have shown that the potency of opioid agonist and antagonists varies according to which tissue is used in the assay, demonstrating that opioid receptors represent a heterogeneous population (**Chang, Hazum & Cuatrecasas 1980**).

There are three endogenous opioid precursor molecules, pro-opiomelanocortin (**Nakanishi, Inoue et al, 1979**), pro-enkephalin (**Gubler, Seeberg et al, 1982**) and pro-dynorphin (**Fischli, Goldstein et al, 1982; Kilpatrick, Wahlström et al, 1982**).

Pro-opiomelanocortin gives rise solely to the β-endorphin fragment, whereas pro-enkephalin A gives rise five peptide fragments; [Met] and [Leu] enkephalin, [Met] enkephalyl-Arg-Phe, [Met] enkephalyl-Arg-Gly-Leu and [Met] enkephalyl-Arg-Arg-Val-NH_2. Pro-dynorphin gives rise to pro-dynorphin A and B, α and β-neoendorphin and dynorphin A (1-8). These fragments of the precursor molecules have different affinities for the different opioid receptor subtypes.

β-endorphin binds with approximately equal affinity at both μ and δ sites. Leu and Met-enkephalin bind with highest affinity at the δ site but the other pro enkephalin derived fragments bind at both μ and δ sites. Fragments of the pro-dynorphin precursor molecule bind at the κ-receptor, dynorphin A and B having the greatest affinity at this site (**Kosterlitz, 1985**).

The distribution of opioid receptors has been determined by specific ligand-binding autoradiographic techniques.

A dense distribution of μ-receptors has been located in the neocortex, caudate putamen, nucleus

accumbens, thalamus, hippocampus, amygdala, inferior and superior colliculi, nucleus tractus solitarius, spinal trigeminal nucleus and the dorsal horn. There is a moderate distribution of μ-receptors observed in the periaqueductal gray and raphé nucleus whilst a sparser distribution is observed in the hypothalamus, preoptic area and globus pallidus. This distribution of μ-receptors corresponds well with their putative function in pain regulation and sensorimotor integration (**Mansour, Khachaturian et al, 1988**).

There is a less widespread distribution of δ-receptors in the CNS. They are most densely located in olfactory-related areas, neocortex, caudate putamen, nucleus accumbens and amygdala with a very sparse distribution in the thalamus, hypothalamus and brainstem. the function of the δ-receptor is not yet known but from its distribution it is thought that it may play a role in motor integration and olfaction (**Mansour, Khachaturian et al, 1988**).

There is a dense distribution of κ-receptors in the caudate-putamen, nucleus accumbens-amygdala, hypothalamus, neural lobe of the pituitary, median eminence and the nucleus tractus solitarius. A less dense but significant population of κ-receptors are found in the periaqueductal gray, raphé nuclei, spinal trigeminal nucleus and the dorsal horn. This distribution is consistent with the probable role of κ-receptors in water balance and food-intake control, pain perception and neuroendocrine functions (**Mansour, Khachaturian et al, 1988**).

Activation of μ or δ opioid-receptor subtypes causes an increase in potassium conductance. This effect has been characterized for μ-receptors on rat locus coeruleus neurones (**Williams & North, 1984**) and for the δ-receptor in cell of the submucosal plexus (**Mihara & North, 1986**). It has not yet been demonstrated if μ and δ- receptors co-exist on the same neurone (**North, 1986**).

Activation of κ-receptors leads to a reduction of voltage dependent calcium conductance. This is believed to be through the N but not the L-type calcium channels (**Xiang, Adamson et al, 1990**).
The results of all these channel events is a reduction in the rate of neuronal discharge and a reduction in the amount of transmitter or hormone released by each action potential.

(ii) Opioid-mediated modulation of oxytocin secretion.
The secretion of oxytocin is inhibited by the action of endogenous and exogenous opiates.
Autoradiographic studies on the rat brain have shown that there is a distribution of opioid receptors in close association with oxytocin neurones. There is a high density of κ-opioid receptors located in the neural lobe with a few μ-receptors but virtually no δ-receptors present (**Herkenham, Rice et al, 1986**). These opioid receptors may be located on the oxytocin nerve terminals or on surrounding pituicytes (**Lightman, Ninkovic et al, 1983**). In the SON, both κ and μ receptors are found (**Sumner, Coombes et al, 1990**).
As described previously, both oxytocin and vasopressin are co-localized with opioid peptides. Vasopressin with dynorphin, derived from pro-dynorphin (**Watson, Akil et al, 1982**) and oxytocin with Met (and possibly Leu) enkephalin (**Martin, Geis et al, 1983**).
Endogenous opioids have a modulatory interaction with oxytocin neurones which acts at the level of the cell bodies in the hypothalamus and at the nerve terminals in the pituitary.
Opioids inhibit the milk-ejection reflex by decreasing oxytocin secretion. This was first observed in mice (**Haldar & Sawyer, 1978**) when morphine (a μ-opioid receptor agonist) and its analogues butorphanol and oxilorphan inhibited the suckling-induced release of oxytocin. Evidence was also provided that opiates could inhibit carbachol induced, as well as electrically or

osmotically stimulated oxytocin release (**Clarke, Wood et al, 1979; Wright, Pill et al, 1983**).

It was believed that the opiates were acting on or close to the nerve terminals in the posterior pituitary although now, the actions of morphine on oxytocin neurones is believed to be a centrally mediated effect due to the lack of μ-receptors at the posterior pituitary (**Coombes & Russell, 1988**)

Environmental disturbance is found to prolong parturition in the rat and this is partly due to a decrease in plasma oxytocin (**Leng, Mansfield et al, 1985**). Naloxone (a general opioid-receptor antagonist) administration was found to prevent the disruption of parturition, presumably by reversal of endogenous opioid actions, since it was observed to elevate plasma oxytocin content.

It was shown that naloxone did not potentiate oxytocin secretion in non-pregnant rats or on Day 1 post-partum but did potentiate oxytocin secretion on Day 22 of pregnancy, even before the onset of parturition. This suggests a tonic opioid-inhibition of oxytocin on Day 22 immediately prior to parturition which is absent in non-pregnant or immediately post-parturient rats (**Leng, Mansfield et al, 1988**).

Which opioid system may be responsible for maintaining a regulatory opioid tone on oxytocin neurones during pregnancy is not known. Release of oxytocin from terminals in the posterior pituitary is postulated to be inhibited by κ-opioid agonists, possibly dynorphin co-released from neighbouring vasopressin neurones (**Bicknell, Chapman & Leng, 1985; Summy-Long, Denlinger et al, 1986**). Elevated vasopressin (and hence dynorphin) is found during pregnancy in the rat (**Lindheimer, Barron et al, 1985**) but compared to oxytocin secretion, the release of vasopressin during pregnancy is not significant (**Hartman, Rosella-Dampman and Summy-Long, 1987**).

Oxytocin secretion is also under the control of

opioids acting at the cell bodies of magnocellular oxytocin neurones (**Bicknell,Leng et al,1988**);these receive β-endorphin projections from the arcuate nucleus (**Sawchenko,Swanson et al,1982**). Interestingly,central β-endorphin levels are elevated during late pregnancy until 1-2 days post-partum (**Wardlaw & Franz,1983**).

Relaxin concentrations are also elevated during late pregnancy and relaxin administration to rats apparently inhibits oxytocin secretion by an endogenous opioid mechanism (**Summerlee,O'Byrne et al,1984;O'Byrne & Summerlee,1985;O'Byrne,Eltringham et al,1986**).

Endogenous opioid control of oxytocin secretion may be mediated both centrally and at the neural lobe,and which is important in various circumstances is not presently clear.

Oxytocin secretion can also be inhibited during parturition by the acute administration of exogenous opiates such as morphine,resulting in a prolongation of litter delivery (**Russell,Gosden et al,1989**).Morphine is effective when administered subcutaneously (s.c.) or intracerebroventricularly (i.c.v.).The i.c.v. dose was however,ineffective when administered peripherally,favouring a central site of action for the opiate.Morphine was found to inhibit foetal ejection-the movement of pups through the uterus to the cervix-by decreasing circulating oxytocin.Infusion of oxytocin in physiological concentrations (5mU for one minute and then 2mU/min until delivery was complete,reversed the interruption of parturition by i.c.v. morphine,showing that morphine was inhibiting oxytocin release from the neural lobe of the mother rat.It also showed that physiological concentrations of oxytocin are necessary for the normal progress of parturition.

(iii) Tolerance and dependence.

Oxytocin neurones can be made tolerant to and dependent upon morphine; this has been demonstrated by infusing rats i.c.v. with morphine, in increasing doses, over a period of five days (**Rayner, Robinson & Russell, 1988**).

Tolerance is demonstrated by the fact that subsequent acute doses of morphine have less effect on the electrical or secretory activity of oxytocin neurones. Dependence is revealed by a characterized withdrawal response in which removal of the opiate with the opioid antagonist naloxone leads to a dramatic increase in oxytocin neurone firing rate with a coincident hypersecretion of oxytocin into plasma (**Rayner, Robinson & Russell, 1988; Bicknell, Leng et al, 1988; Leng, Russell & Grossman , 1989**).

Chronic morphine treatment initially blocks the suckling-induced milk-ejection reflex in lactating rats but this returns to normal within three days, whilst still receiving the morphine infusion (**Russell, 1984**). These results demonstrate the development of tolerance; evidently, during infusion of the opiate, the oxytocin neurones modify their activity to restore a normal state of electrical and neurosecretory activity. When the opiate action is acutely reversed by naloxone administration, the oxytocin cells are left in a hyperexcitable state, revealing dependence.

This overview of oxytocin biosynthesis, the pathways regulating secretion and its regulation by opioids demonstrate the complexity of the magnocellular and parvocellular oxytocin system. This thesis now attempts to elucidate the acute and chronic actions of opioids on the regulation of oxytocin from certain pathway elements under various physiological conditions.

CHAPTER ONE

GENERAL METHODS

CHAPTER 1 : General methods.

1.1 ANIMALS

Unless otherwise stated, all the animals used in the following experiments were supplied by **Bantin & Kingman (Hull, U.K.)** and were Sprague-Dawley strain. Pregnant and lactating animals were always primiparous and housed under controlled temperature (22-23°C) and light cycle conditions (13hrs light/11hrs dark, lights on 8a.m GMT). Non-pregnant animals were virgin females which were kept under the same temperature but a slightly different lighting schedule (12hrs light/12hrs dark, lights on 8.50a.m. GMT). Rat breeder diet and tap water were freely available to all the animals.

1.2 ANAESTHESIA AND ROUTINE CANNULATION

Surgical procedures to be followed by recovery were performed under ether anaesthesia, maintained throughout surgery by means of a gauze mask for inhalation of the solvent. For all terminal surgical procedures, the rats were anaesthetized with urethane (ethyl carbamate), administered intraperitoneally as a 25% weight/volume solution, 0.5ml/100g bodyweight.

For experiments in which blood sampling was necessary and/or drugs were given intravenously, rats were fitted with polythene cannulae (**Portex Ltd., Hythe, Kent. OD 0.75mm.**). Vessels routinely cannulated were the femoral artery, femoral vein and a side branch of the external jugular vein. Three abdominal mammary gland main milk ducts were cannulated when the experiment necessitated recording of intramammary pressure. The cannulae were composed of a syringe needle (**Becton-Dickinson microlance 25 gauge**) connected to ≃10cm length of polythene tubing (OD **0.7mm**). In experiments where it was possible that surgery could interrupt respiration, the rats were fitted with a tracheal

cannula made from polythene tubing bent to a 90° angle (*OD 2.0mm/ID 1.7mm*) to keep the trachea patent.

1.3 INFUSION OF MORPHINE SULPHATE SOLUTION INTO A LATERAL CEREBRAL VENTRICLE.

To investigate morphine tolerance and dependence in relation to oxytocin secretion in the rat, an intracerebroventricular (i.c.v.) infusion of morphine sulphate in increasing doses was given over 5 days from a subcutaneous osmotic minipump via a stainless steel i.c.v. cannula.

The animals were anaesthetized with ether and placed in a stereotactic frame to keep the head immobilized. A skin incision was made in the midline to expose the sagittal suture and the dorsal surface of the skull exposed.

Using bregma, a well known anterior intersection of the sagittal and coronal skull sutures as a reference point, a hole (to fit a 21 gauge needle) was drilled through the parietal bone 2mm lateral and 3mm posterior to bregma to a depth of 1mm (the skull thickness).

A stainless steel cannula (**Becton-Dickinson 21** gauge, 1cm length bent at 90°, 4.5mm from the tip) was lowered vertically through this hole into the right lateral cerebral ventricle. Before insertion, the cannula was attached to an 18cm coil of sterile polythene tubing (**ID 0.7mm/OD 1.2mm**) and the whole assembly filled with 40µl of 10µg/µl morphine sulphate solution closest to the steel cannula separated by a 1µl air bubble from 40µl of 20µg/µl morphine sulphate solution.

All morphine solutions were made up in sterile pyrogen free water and sterilized by passage through a **Millipore filter (0.22µm)**.

The other end of the polythene coil was attached to a Hamilton microsyringe (100µl) which allowed the small volumes of morphine solutions to be drawn up into the

tubing coil. Once the cannula was secured in position, the tubing was severed from the microsyringe and connected to a sterile osmotic minipump (**Alzet 2001**) filled with 50μg/μl morphine sulphate solution.

The minipump was filled a few hours previously and allowed to equilibrate in sterile isotonic saline at room temperature. The minipump pumping rate was 1μl/hr and the rats were left for 120hrs (5 days) to develop dependence, finally receiving 40μl 10μg/μl, 40μl 20μg/μl and 40μl 50μg/μl morphine sulphate solution.

Two other holes were drilled in the skull antero-lateral and postero-lateral to the cannula. Into each of these was inserted a stainless steel screw (**3.2mm x 10 BA**) which provided anchor points for the dental acrylic which was finally applied to the skull surface in order to hold the cannula in place.

The minipump and polythene coil were placed subcutaneously in the subscapular space and the incision closed with skin sutures. The animals were immediately returned to their cages, the whole procedure lasting approximately 20 minutes.

For vehicle infused control animals, the minipump and polythene tubing were filled with sterile water.

In an experiment where animals were infused with the κ-opioid receptor agonist U50,488H, the polythene tubing length was increased to 32cm and was filled with 40μl 10μg/μl U50,488H, 40μl 20μg/μl U50,488H and 80μl 50μg/μl separated by 1μl air. The minipump was filled with sterile pyrogen free water so that it would pump the contents of the polythene coil into the lateral cerebral ventricle. This alternative method was used to avoid using large volumes of U50,488H solution to fill the minipump which would be wasted. All U50,488H solutions were made up in sterile water and filtered through a Millipore filter.

Russell (1984); Rayner, Robinson & Russell (1988)

1.4 CENTRAL RELEASE OF OXYTOCIN: COLLECTION OF CEREBROSPINAL FLUID (CSF) SAMPLES UNDER URETHANE ANAESTHESIA.

The release of oxytocin into CSF was measured in some experiments as well as plasma oxytocin content. In order to do this the rats were fitted with a cannula which allowed CSF samples to be obtained via the cisterna magna. The CSF samples collected were free flowing and care was taken not to accumulate samples that were too large and would disrupt the dynamics of CSF production (normal rate of production 150µl/hr). Cserr, 1965

The rats were anaesthetized with urethane. The atlanto-occipital membrane covering the cisterna magna was cannulated and the fluid allowed to collect under gravity.

The animals were held in a head frame to keep the head immobilized with the neck flexed for access to the region of the cisterna magna. A midline dorsal skin incision was made starting between the ears and extending ≈ 1.5cm caudally. The posterior dorsal surface of the skull was exposed by clearing away the overlying muscle (cranial portion of the levator auris longus) with fine curved forceps. The external occipital crest was located and traced along its length to the rostral attachment of the occipital foramen. Using curved forceps the first cervical vertebra was located and the overlying muscle gently cleared away; this bone marks the caudal attachment of the membrane. The muscle layers overlying the membrane were retracted and any connective tissue remaining gently freed.

The cannula was constructed from a 4mm length of stainless steel tubing (**Becton-Dickinson microlance, 23 gauge**) which had been ground to a 45° bevel at one end; the other end was attached to a length of silicon rubber tubing through which the CSF could be collected.

To secure the cannula once it had pierced the membrane

and to render it immobile, especially during respiratory movement, a silicon rubber flange was placed at the joint of the cannula and tubing. This was trimmed to the size and shape of the atlanto-occipital membrane and was secured to the surface using a cyanoacrylic adhesive (**Perma Bond C2: Permabond Adhesives Ltd., Eastleigh, Hants.**). As well as securing the cannula this method sealed the membrane and prevented loss of CSF from leakage. Robinson & Jones, 1982

1.5 EXPOSURE OF THE NEURAL STALK IN THE ANAESTHETIZED RAT AND SUBSEQUENT STIMULATION OF DESCENDING OXYTOCIN NEURONES.

For investigation of opioid-mediated regulation of oxytocin release from nerve terminals in the neurohypophysis any opioid manipulation of oxytocin secretion must take place solely at the terminals and be isolated from any facilitatory or inhibitory actions at the cell bodies in the hypothalamus.

This is achieved in an **in-vitro** system where an isolated, perifused, neural lobe preparation can be induced to secrete oxytocin by means of electrical stimulation, any changes in secretion being attributed to effects at the nerve terminals.

In-vivo, by stimulating descending oxytocinergic nerve axons in the neural stalk, the cell bodies are for the mostpart, effectively bypassed and any changes seen in secretion are likely be mediated via receptors located on or in the vicinity of the nerve terminals. The following method was used in such studies.

1.5 (i) SURGERY demonstrated by G. Leng

For this procedure the animal was anaesthetized with urethane and transferred to a stereotactic headframe (**Narishige**) where it was immobilized in a supine position for ventral exposure of the neural stalk. The lower jaw was split with scissors in the midline between the lower incisors and both the jaw and tongue

retracted laterally to expose the soft palate **(Fig 1(a))**.

Overlying soft tissue was removed by cautery, with care not to disrupt blood vessels lateral to the midline and near the junction of the hard and soft palate, thus avoiding unnecessary blood loss.

Underlying bone was gently drilled away with a dental burr , repeated applications of bonewax **(Ethicon, Edinburgh, Scotland.)** preventing bleeding from the sinus which lies ventral to the pituitary stalk. The bone was drilled away until the final layer could gently be lifted off with fine forceps revealing the neural stalk within its dural membrane beneath. Once this had been exposed the animals were left for an equilibration period of at least 1 hr.

1.5(ii) STIMULUS

In order to stimulate oxytocinergic (and vasopressinergic) axons descending from the hypothalamus through the neural stalk to terminate in the neural lobe, a concentric bipolar stimulating electrode was lowered into the pituitary stalk with a micromanipulator under visual control with a dissecting microscope.

The stimulus delivered to the neural stalk was generated from a **Neurolog pulse generator system**. The number of pulses in individual trains and their frequency were controlled by a **Digitimer D4030**.

The final stimulus was a train of matched biphasic pulses (usually between 60-240 pulses in a train), 0.5mA peak to peak, 1ms duration and at a frequency of 50Hz.

These parameters were chosen since they represented activity similar to that seen during the synchronous bursting of oxytocin neurones during the milk-ejection reflex and would result in a near maximal stimulus evoked oxytocin release **(Lincoln & Wakerley, 1975; Summerlee & Lincoln, 1981)**. The

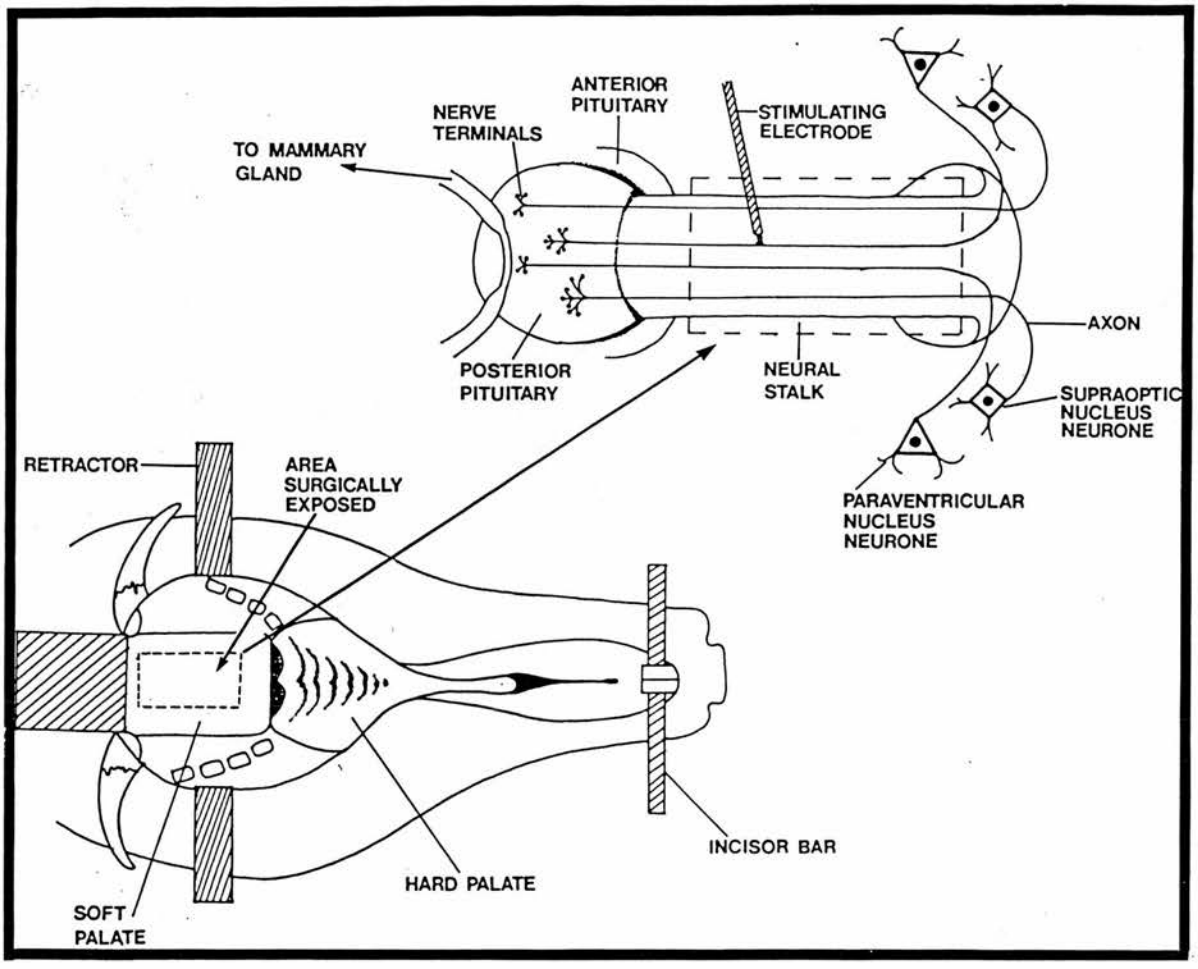


Figure 1(a)

The bottom left-hand picture is a schematic representation of the hard and soft palate of the rat. The dotted oblong on the soft palate demarkates the area surgically exposed and is enlarged in the top right-hand picture. The stimulating electrode was positioned on the stalk, as shown, to deliver trains of pulses (parameters described in the text) to descending oxytocin neurones.

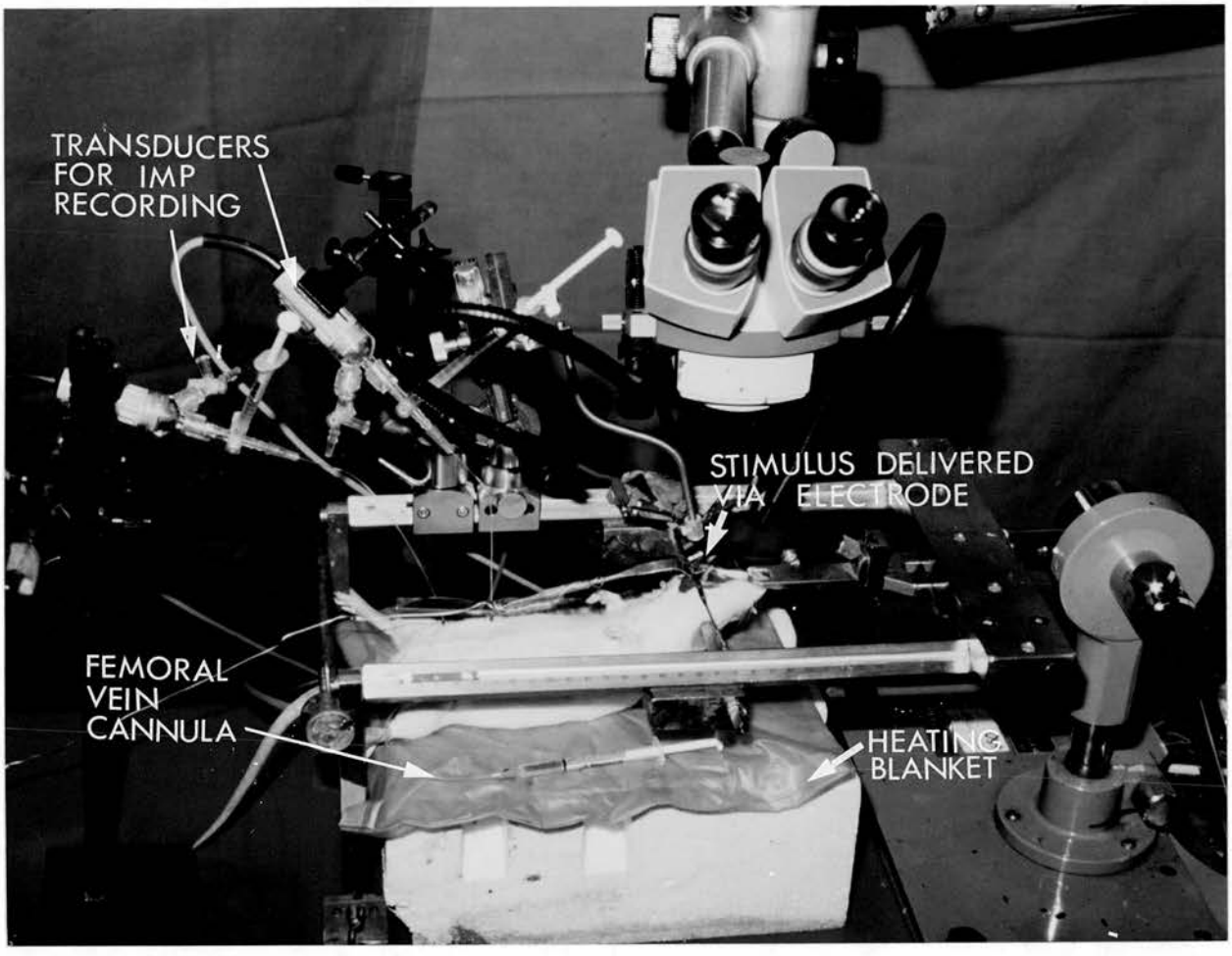


Plate 1

Set up for stimulation of the neural stalk. Two abdominal mammary glands are cannulated for the recording of intramammary pressure; the femoral and jugular veins cannulated for injection of oxytocin and opiates and the electrode positioned as labelled.

experimental set-up for neural stalk stimulation of oxytocin neurones is shown in **Plate 1.**

1.6 MAMMARY GLAND AUTOBIOASSAY

For all experiments involving stimulation of the neural stalk, oxytocin release was quantified using a mammary gland autobioassay which utilizes the response of myoepithelium to oxytocin.

Cannulation of three abdominal gland milk ducts allowed measurement of intramammary pressure changes, in response to oxytocin secretion, by connecting the cannulae to force transducers (**Gould P231D**) which converted pressure variations (mmHg) to a deflection on a chart recorder (**J&J Instruments CR652S Recorder**).

Recordings were taken from two mammary glands simultaneously so that pressure changes arising from spontaneous contractile activity rather than a defined stimulus could be detected.

Bolus doses of synthetic oxytocin 0.25.0.50, 0.75 and 1.0mU from a 12.5mU/ml stock solution, were injected via the femoral vein with a **Hamilton** 1ml glass syringe attached to a **Hamilton PB600** dispenser, set to deliver 20µl per strike, allowing construction of an oxytocin-dose intramammary-pressure response curve.

Stimulation of the neural stalk via a stimulating electrode results in a bolus release of oxytocin, the amount of which can be determined by extrapolation from the dose-response curve.

In experiments where the effect of opioid receptor agonists and antagonists on oxytocin release from nerve terminals in the neurohypophysis were to be measured, the number of pulses within a stimulus train was set for an optimal release of oxytocin into the circulation; i.e. at the top or bottom of the steep slope on the dose-response curve so that a small change in oxytocin secretion would be represented by a larger change in intramammary pressure.

Dose response curves were obtained before and after each drug injection so that any changes in mammary gland sensitivity could be taken into account. Once the amount of oxytocin evoked upon neural stalk stimulation had been determined for a control period and after each drug administration, the results were expressed as the percent of the initial amount of oxytocin released on stimulation of the neural stalk during the control period. All final results are expressed as the mean ± standard error of the mean for these values.

1.7 RADIOIMMUNOASSAY TECHNIQUE FOR OXYTOCIN DETERMINATION IN PLASMA AND CEREBROSPINAL FLUID SAMPLES.

1.7.1 The method developed by I.C.A.F. Robinson (1980) was used for oxytocin determination in arterial blood samples and cerebrospinal fluid samples taken during experiments described in this thesis

1.7.1 (i) PLASMA SAMPLES

Blood samples were drawn up into heparinized syringes and cooled over ice before being centrifuged (3000rpm for 3 mins) to obtain plasma. The resulting plasma aliquots were immediately frozen and stored at -20°C until assayed.

1.7.1 (ii) ASSAY PROCEDURE

The buffer used in the following assay procedure was a 100mM tris buffer (**tris-(hydroxymethyl)-methylamine, BDH chemicals**) with 3mg/ml bovine serum albumin added (**Fraction V, Sigma**) and adjusted to pH 7.4 with 5M hydrochloric acid.

The antibody was $RIII_S$ which was used at a final dilution of 1:230,000 in the assay volume.

Oxytocin was iodinated by the iodogen method (**Salacinsky, Hope, McLean et al, 1979**) and purified by

HPLC. ^{125}I-oxytocin stock (3,000 000 cpm/100µl) was diluted in assay buffer to give ≃ 7000 cpm/100µl i.e. 7000 cpm per tube.

For the plasma assay, the standard curve was made up using human plasma which did not cross react with the assay. The standard curve was set up in the following way:-

Tc tubes: 100µl tracer

Ab tubes: 100µl tracer , 100µl buffer, 100µl 50% plasma

Bo tubes: 100µl tracer, 100µl 50% plasma, 100µl $RIII_s$

The stock oxytocin standard was 5ng/ml. 100µl of this was added to 100µl 100% plasma in tube S_1. This was vortexed thoroughly and 100µl removed and placed in tube S_2 with 100µl 50% plasma. After vortexing, 100µl was taken from S_2 and placed in S_3 with 100µl 50% plasma. This procedure was repeated so that there were ten doubling dilutions (S_1-S_{11}) giving a range of standards from 250-0.25 pg/ml.

Antibody (100µl at a final dilution of 1:230,000) and tracer (7000cpm/100µl) were also added to these tubes. The standard curve was pipetted in triplicate.

Experimental plasma samples (50µl) were set up in duplicate. Added to this was 50µl buffer (so that there was a total of 50% plasma present in the tube plus 100µl $RIII_s$ (1:230,000) and 100µl tracer (7000cpm/100µl)

All tubes had a total volume of 300µl and were vortexed thoroughly before incubation. Two incubation procedures were possible,

a) simultaneous addition

Antibody and tracer were added at the same time and the tubes incubated overnight at room temperature.

b) delayed addition

Antibody was added on Day 1 and the tubes incubated overnight at 4°C. On day 2, the tracer was added, vortexed to mix well and incubated overnight at 4°C before separation on Day 3.

The latter method gives a more sensitive assay and is

often used if low levels of oxytocin are expected in the samples.

The method of separation of bound oxytocin from free oxytocin for this assay was with polyethylene glycol 6000 (**BDH Chemicals**). An 18% polyethylene glycol (PEG) solution is made up in tris buffer from a 27% PEG stock solution with bovine γ globulin (**Sigma, Cohn fraction II**). 600µl PEG was added to all the tubes except the Tc tubes. The tube contents were vortexed to enure thorough mixing and left for 30 minutes. After this they were centrifuged at 3000rpm for 10 minutes at 4°C. The resulting supernatant was aspirated carefully and the the remaining pellet counted in a gamma counter (**LKB Wallac 1260 Multigamma II**).

1.7.2 CEREBROSPINAL FLUID SAMPLES

The basic assay procedure for oxytocin determination in CSF was the same as for plasma. Only 50µl CSF was collected per sample so, the standard curve was set up using 50µl of standard, the top standard being 125 pg/ml with ten doubling dilutions to 0.125 pg/ml. The total volume of the assay was 150µl.

Antibody dilution was 1:230,000 but calculated for a final volume of 150µl and the tracer was diluted to give no more than 4000cpm/50µl (per tube).

To dispense 50µl accurately a **Hamilton dispense 200** was used to add antibody and tracer to the relevant tubes.

This time, assay buffer (as previously described) was added in place of 50% plasma. Levels of basal oxytocin in CSF samples are sometimes much lower than that found in plasma and the volume assayed much smaller so a delayed addition protocol was **always** followed to give the maximum sensitivity.

1.7.3 Modified Higuchi radioimmunoassay (1984) used at AFRC, Babraham for detection of oxytocin in in vitro perfusates and plasma samples.

Prior to aliquoting into assay tubes, the samples were centrifuged briefly in a bench centrifuge to spin down any precipitate. The assay buffer used in this procedure was a phosphate buffer :- 0.01M PO_4^{--}, 0.5% bovine serum albumin (**Sigma - No A-8022**), 1mg/ml bovine γ globulin (**Sigma - No G5009**) and 1mg/ml thiomerosol.
The antibody used was Higuchi THF-3 used at a final assay dilution of 1:300,000.
Oxytocin was iodinated using the chloramine-T method, the ^{125}I-oxytocin being stored in 0.2% acetic acid/0.25% BSA at -20°C until used. The tracer was diluted in assay buffer to give 8000-10000 cpm/50µl.
A standard curve was set up with the top standard 10000 pg/ml and ten doubling dilutions to 9 pg/ml.
On day 1 antibody (50µl) was added to sample (50µl) or standard (50µl) tubes. These were incubated for 24hrs at 4°C. On day 2, 50µl tracer was added to all the tubes which were incubated for a further 48hrs at 4°C.
Separation was achieved with polyethylene glycol 6000 (PEG) 30% weight/volume. The tubes were vortexed thoroughly and then centrifuged at 3000rpm for 30 minutes at 4°C. The supernatant was aspirated and the remaining immunoglobulin precipitate counted in a gamma counter.

MATERIALS

The following list of materials and drugs were routinely used in the experiments described.

CHEMICALS AND DRUGS

Isotonic sterile saline: "Steriflex", sodium chloride intravenous infusion BP (0.9% w/v), The Boots Company plc, Nottingham, U.K.

Sodium chloride: BDH Chemicals Ltd., Poole, Dorset, U.K.

U50,488H, trans-(±)-3,4,dichloro-N-methyl-N-(2-[1-pyrroldinyl]-cyclohexyl] benzene acetamide (methane sulphonate salt) :

(i)A gift from the Upjohn Company, Crawley, West Sussex, U.K.

(ii)Product no. D0908, Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Morphine sulphate: Supplied by the Pharmacy, Edinburgh Royal Infirmary.

Naloxone hydrochloride:

(i)A gift from Endo Laboratories Inc., Garden City, New York, USA.

(ii)Product no. N7758, Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

MR2266, 5,9-α-diethyl-2-(3-furylmethyl)-2'-hydroxy-6,7,benzomorphan: A gift from Boehringer Ingelheim.

RX8008M, 16-Me-cyprenorphine: A gift from Reckitt & Colman Pharmaceuticals Division, Hull, U.K.

Propranolol: "Inderal" propranolol injection BP (1mg/ml), Imperial Chemical Industries plc, Pharmaceuticals Division, Macclesfield, Cheshire, UK

Oxytocin: "Syntocinon" oxytocin injection BP (10units/ml), Sandoz Pharmaceuticals, Feltham, Middlesex, UK.

Pethidine: pethidine (meperidine) injection BP (100mg/2ml) Roche Pharmaceuticals.

Heparin: "Monoparin" heparin injection BP (5000units/ml), CP Pharmaceuticals, Wrexham, UK.

SURGERY

Urethane, ethyl carbamate: Fisons Laboratory Reagents, Loughborough, UK.

Anaesthetic ether: Fisons Laboratory Reagents, Loughborough, UK.

Osmotic minipumps: ALZET 2001, ALZA, Palo Alto, California. (European Distributors, Scientific Marketing, 67. Mildmay Grove, London, UK.)

Bone wax: Ethicon, Edinburgh, UK.

Surgicel: sterile oxidized cellulose, Ethicon, Edinburgh, UK.

CHAPTER TWO

INVESTIGATION OF A KAPPA-OPIOID RECEPTOR AGONIST, U50,488H AND A MU-OPIOID RECEPTOR AGONIST MORPHINE, ON ELECTRICALLY-EVOKED OXYTOCIN RELEASE FROM THE NEURAL LOBE OF THE ANAESTHETIZED LACTATING RAT.

CHAPTER 2 : Investigation of a κ-opioid receptor agonist, U50,488H and a μ-opioid receptor agonist, morphine, on electrically evoked oxytocin release from the neural lobe of the anaesthetized lactating rat.

INTRODUCTION

Oxytocin is secreted from nerve terminals situated in the neural lobe of the rat, as a result of electrical activity arising in the cell bodies of magnocellular neurones located in the supraoptic nuclei of the hypothalamus (**Lincoln & Wakerley, 1974; Poulain & Wakerley, 1982; Bourque & Renaud, 1985**). Oxytocin is released into the circulation where it is transported to its target organs.

Regulation of oxytocin by endogenous opioids can be effected at several sites; the oxytocin neurone cell bodies, nerve terminals and their afferent synaptic input. At the cell bodies, oxytocin secretion may be inhibited by enkephalins co-produced and released by oxytocin neurones (**Martin, Geis et al, 1983; Vanderhagen, Lotstra et al 1983**), dynorphin co-produced and released by vasopressin neurones (**Watson, Akil et al, 1982**) and β-endorphin from projections of the arcuate nucleus (**Sawchenko, Swanson & Joseph, 1982; Finley, Maderdrut & Petrusz, 1983**). These peptides may act directly on the cell bodies or on their afferent synaptic input.

At the neural lobe, inhibition of oxytocin secretion may be inhibited by enkephalins and dynorphin co-produced and released from oxytocin and vasopressin nerve terminals respectively (**Van Leeuwen, Pool & Sluiter, 1983; Summy-Long, Denlinger, 1984**). β-endorphin from the intermediate or anterior pituitary lobes may also be responsible for modification of oxytocin secretion.

Autoradiographic studies have shown that, in the

rat, the predominant opioid receptor subtype located at the neural lobe are κ-receptors with a few μ-receptors at this site; δ-receptors are virtually undetectable (**Herkenham, Rice et al, 1986; Brady & Herkenham, 1987; Bunn, Hanley & Wilkins, 1985; Zukin, Eghbali et al, 1988; Sumner, Coombes et al, 1988**). The κ-opioid receptors are at their densest on the rim of the neural lobe indicating close proximity to or location on oxytocin nerve terminals. There is however, evidence to suggest that opioid receptors may be located on the specialized astrocytic glial cells, pituicytes, which have a close positional relationship with oxytocin nerve terminals in the neural lobe. Transection of the neural stalk and subsequent nerve degeneration does not lead to a loss of ligand binding to opioid receptor subtypes indicating this alternative site (**Lightman, Ninkovic et al, 1983; Bunn, Hanley & Wilkin, 1985**).

Pituicytes have been suggested as one possible mediator for regulating oxytocin secretion. They are in close proximity to the nerve terminals, some encapsulating the terminals entirely and changes in their morphological arrangement have been noticed during times of high oxytocin and vasopressin secretion i.e. dehydration and lactation (**Hatton, Perlmutter, , 1984; Perlmutter, Tweedle & Hatton, 1985**). These changes may lead to variation in the immediate extracellular enviroment of the oxytocin nerve terminals which lead to a change in oxytocin release.

In-vitro experiments using the isolated neural lobe preparation have indicated through use of selective opioid receptor subtype antagonists MR2266 and naloxone that it is mainly opioids acting via κ or μ receptors respectively that suppress electrically evoked oxytocin secretion, although inclusion of specific opioid receptor agonists β-endorphin (μ), dynorphin 1-13 and bremazocine (κ) was

unsuccessful at inducing significant inhibition of oxytocin secretion (**Bicknell, Chapman & Leng, 1985**). Vasopressin secretion was not affected by naloxone or MR2266 indicating that its release is not controlled by endogenous opioids. Later experiments showed significant inhibition of oxytocin and vasopressin by the selective κ-agonist U50,488H in a naloxone reversible manner indicating the effect to be opiate receptor mediated.

Since the predominant type of opioid receptor at the neural lobe is kappa whose endogenous ligand is found to be dynorphin (**Corbett, Paterson et al, 1982; Kosterlitz, 1985**)) it seems likely that dynorphin-binding to opiate receptors on or around oxytocin nerve terminals could play a role in the modulation of oxytocin secretion.

In the following experiments, modulatory effects of the κ and μ-opioid system on oxytocin secretion at the neural lobe in the anaesthetized lactating rat in vivo was investigated. The neural stalk was stimulated electrically in order to elicit oxytocin release from nerve terminals in the neural lobe. This ensured that any inhibitory effects of opioids seen would be isolated to the neural lobe since the cell bodies were no longer involved in generation of action potentials normally associated with oxytocin release and the neural lobe was in effect isolated.

The κ- opioid receptor ligand used to try and modify oxytocin release was U50,488H (trans-3,4-dichloro-N-methyl-N-[2-(1-pyrroldinyl)-cyclohexyl benzene-acetamide) which has been shown to be a relatively selective κ-ligand with poor affinity for μ_1, μ_2 and δ sites (**Pasternak & Wood, 1986; Clarke & Pasternak, 1988**). Evidence has been put forward that intrathecal administration of U50,488H, in the rat, acts through a β-adrenergic mechanism originating in the adrenal medulla (**Clarke & Wright, 1987**). An intact, fully functioning adrenal medulla has been shown to be

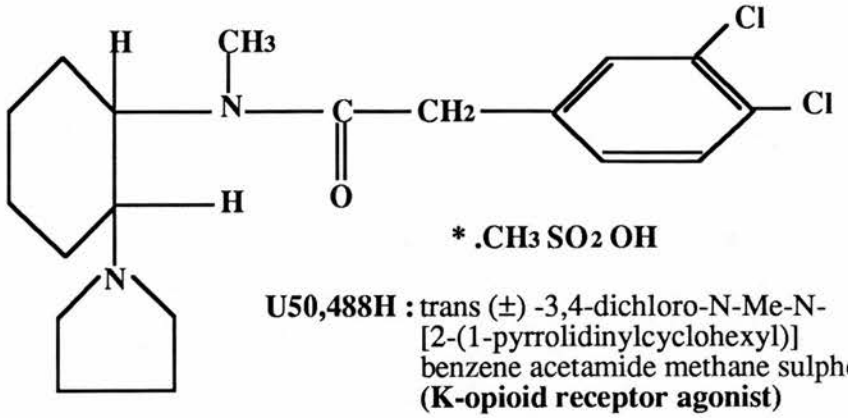
necessary for κ-opioid agonist induced diuresis in the rat (**Blackburn, Boronski, 1986; Borkowski, 1989**). A β-adrenergic antagonist propranolol was used as pretreatment prior to U50,488H intravenous injection to determine whether a β-adrenergic system has any contribution to opioid modification of oxytocin secretion at the level of the neural lobe.

Initial studies on opiate actions on oxytocin secretion indicated that morphine acts on the neural lobe although there are few μ-receptors at this site. However, μ-opioid receptors, located in the supraoptic nucleus respond to opiates and influence oxytocin secretion by changes in electrical activity at the neurone cell bodies (**Wakerley, Noble & Clarke (1983)**

To distinguish these two sites of action, the effects of a kappa agonist on oxytocin secretion was investigated in rats which were morphine dependent. Dependence on morphine in relation to oxytocin secretion is centrally mediated, in the hypothalamus, since peripheral infusion of morphine at the same dose is ineffective in producing dependence. Naloxone injection leads to a withdrawal response involving excitation of oxytocin neurone cell bodies and subsequent hypersecretion of oxytocin into the blood. This response denotes dependence on the opiate (**Rayner, Robinson & Russell, 1988**).

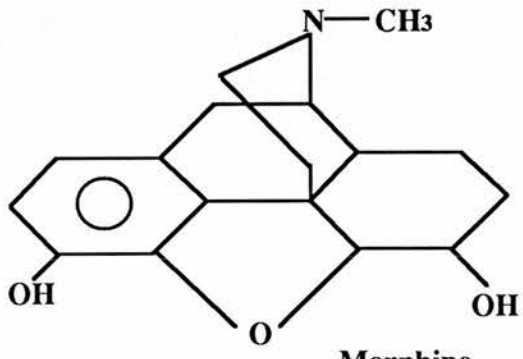
In addition morphine tolerance develops, since the milk-ejection reflex returns and oxytocin neurone activity and secretion are within normal limits. Intravenous injection of U50,488H was used in conjunction with measurement of electrically-stimulated oxytocin secretion to establish whether there is any cross tolerance to opiates acting via kappa receptors conferred at the neural lobe when the cell bodies are tolerant to and dependent on morphine.

The structures of U50,488H, morphine , naloxone and propranolol are shown in **Fig 2.1(a)**

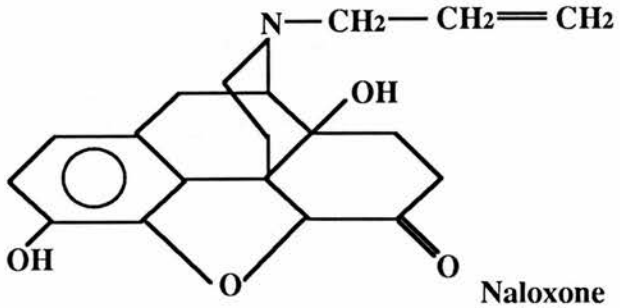


* .CH_3SO_2OH

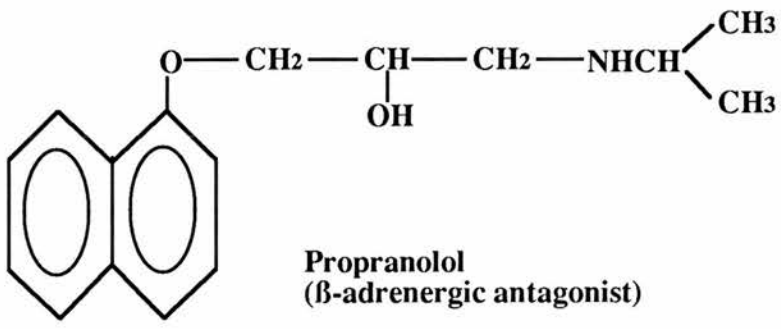
U50,488H : trans (±) -3,4-dichloro-N-Me-N-[2-(1-pyrrolidinylcyclohexyl)] benzene acetamide methane sulphonate.
(K-opioid receptor agonist)



Morphine
(μ-opioid receptor agonist)



Naloxone
(opioid receptor antagonist)



Propranolol
(ß-adrenergic antagonist)

Fig 2.1(a)

2.1 METHODS

2.1(i) ANIMALS

Adult female Sprague-Dawley rats 7-14 days post-partum in their first lactation, with litters standardized to 10 pups 2 days post-partum were used. The lactating rats weighed ca. 400g and were housed as described in **1.1**.

2.1(ii) SURGERY

The dam was separated from all but one of her litter overnight in order to encourage milk accumulation into the mammary glands which would subsequently be used in an oxytocin autobioassay (**Lincoln, Hill & Wakerley, 1973**). On the morning of the experiment, under urethane anaesthesia a tracheal cannula was fitted. Insertion of a femoral arterial cannula allowed blood sampling; a femoral venous cannula for injection of graded doses of oxytocin, a jugular venous cannula for injection of opiates and three abdominal mammary gland cannulae for the measurement of intramammary pressure and hence a method of quantifying oxytocin release were fitted. With the animals' head immobilized in a stereotactic frame, the neural stalk was exposed by ventral surgery as described in detail in **1.5** and the animal left for at least one hour so that circulating oxytocin which may have been elevated due to surgical 'stress' could equilibrate to a steady basal secretion.

For the morphine dependent animals, five days prior to the experiment they were anaesthetized with ether and fitted with an intracerebroventricular infusion device to deliver graded doses of morphine into a lateral cerebral ventricle as described fully in **1.3**

2.1(iii) STIMULUS

Trains of pulses were delivered to the exposed neural stalk via a concentric bipolar stimulating

electrode. The stimulus consisted of matched biphasic pulses (0.5mA, 1ms duration at 50Hz) as described in detail in **1.5(ii)**.

2.1(iv) OXYTOCIN AUTOBIOASSAY

The amount of oxytocin released on stimulation was extrapolated from a dose response curve constructed from the rises in intramammary pressure resulting from intravenous injection of graded doses of synthetic oxytocin (**see 1.6** for details). A dose-response curve was constructed before and after each drug addition so that changes in mammary gland sensitivity due to direct actions of opiates on the mammary gland or other systems affecting sensitivity (e.g. the adrenal medulla) could be accounted for.

2.1(v) DRUGS AND ADMINISTRATION PROTOCOL

Once a sub-maximal secretion of oxytocin had been achieved in response to stimulation of the neural stalk (0.7-0.8mU, see **1.5** for rationale), U50,488H or morphine were given intravenously at different doses, to see if they had any inhibitory action on oxytocin secretion from the neural lobe

All drugs were made up in sterile isotonic saline and were injected via the jugular vein. The dose range of U50,488H tested was 10-5000μg/kg (n=9). In animals that were morphine dependent the dose range of U50,488H tested was 10-1000μg/kg (n=6). Animals treated with acute injections of morphine were exposed to a dose range of 100-5000μg/kg (n=11).

Drugs were given in increasing doses, with time for recovery between doses, typically ≈ 20mins, as judged by recovery of stimulus evoked oxytocin release. Summaries of the drug administration protocols used in morphine naive and morphine dependent rats are shown in **Figs 2.1(b) & 2.1(c)**.

To investigate the involvement of β-adrenergic receptors in kappa mediated effects on oxytocin

Dose response curve 1

Exogenous oxytocin injection of 0.25.0.50.0.75 and 1.0mU via the femoral vein administered in random order.

↓

Neural stalk stimulation

Pulse train sufficient to elicit ~ 0.8mU oxytocin from the neural lobe delivered to the neural stalk.

↓

Drug administration

Intravenous injection of opioid receptor agonists U50,488H or morphine in appropriate dose.

↓

Dose response curve 2

Constructed from injection of 0.25,0.50,0.75 and 1mU oxytocin.

↓

Neural stalk stimulation

Same pulse train as previously used to determine any inhibition of oxytocin secretion due to the presence of the opiate agonist.

Repetition of the above sequence.

Fig 2.1 (b)

Protocol showing oxytocin does-response curve construction and administration of opioid agonists in neural stalk stimulation experiments.

t = -2 mins	Blood sample 1 (basal oxytocin secretion)
t = 0 mins	1mg/kg naloxone i.v.
t = 5 mins	Blood sample 2 (withdrawal oxytocin secretion)
t = 8 mins	5 or 10 mg/kg U50,488H i.v.
t = 14 mins	Blood sample 3 (post-agonist oxytocin secretion)
t = 17 mins	1 or 5 mg/kg naloxone i.v.
t = 25+ mins	Blood sample 4 (re-initiated withdrawal oxytocin-secretion)

Fig. 2.1(c)

Approximate timing protocol of opiate injections and blood samples from morphine dependent rats undergoing withdrawal from morphine with naloxone.

secretion, a group of animals were pretreated with the β-adrenergic antagonist propranolol (n=6). It was hoped to determine whether the antagonist would affect modification of electrically stimulated oxytocin release by the κ-opioid receptor agonist, at the level of the nerve terminals in the neural lobe. Propranolol was given as a dose of 1mg/kg (1mg/ml), approximately 20 mins prior to commencing the sequence of U50,488H administration.

For the experimental group which were morphine dependent naloxone (1 or 5mg/kg) was administered intravenously at the end of the experimental protocol in order to precipitate a withdrawal response from chronic morphine infusion. Withdrawal was characterized by hyperventilation, vibrissae and jaw twitching and a hypersecretion of oxytocin recorded as a large and sustained rise in intramammary pressure. In n=3 animals U50,488H (5 or 10mg/kg) was given during the withdrawal period and timed blood samples withdrawn (2.1(c)).

In n=3 animals, U50,488H was applied directly to the neural stalk at doses of 1μg, 5μg, 10μg and 20μg/5μl isotonic saline using a 50μl Hamilton syringe. Direct application of the opiate onto the tissue was intended to give a more precise application of the drug and any effects could be attributed solely to actions at the neural lobe and distinguish indirect from direct effects of the opiate. The dural membrane overlying the neural stalk was gently cut to allow distribution of the drug onto the neural stalk surface in these animals.

In n=4 animals, before the neural stalk was exposed, the rats were fitted with a stainless steel intracerebroventricular guide cannula into the right lateral cerebral ventricle (**Rayner, Robinson & Russell, 1988**) through which acute injections of morphine could be made. Morphine solutions were made up in sterile isotonic saline and administered as four

doses 1μg, 5μg, 10μg and 20μg/5μl saline. The drug was injected using a 25 μl Hamilton syringe connected to the i.c.v. cannula by means of a length of polythene tubing.

2.2 EXPRESSION OF RESULTS

Amounts of oxytocin released upon electrical stimulation of the neural stalk were extrapolated from the relevant dose response curve (in milliunits), see **1.6** for details. The effects of morphine (intravenous and intracerebroventricular), U50,488H (intravenous and direct stalk application) and U50,488H with propranolol pretreatment (intravenous) and U50,488H in morphine dependent animals were expressed as a percentage of the initial, pre-drug amount of oxytocin released upon stimulation of the neural stalk.

Statistical comparisons:

Comparisons of responses within groups of animals were made using a paired t-test and between groups of animals by an unpaired t-test.

The raw data from individual animals of log dose opiate vs. intramammary pressure response was used to obtain a linear regression line from which the ID_{50} was calculated.

$$X_{50} = \frac{50-a}{b}$$

a: y intercept

b: gradient

The standard error range was calculated as follows:-

$$s.e.\ X_{50} = \frac{Var\ (a)}{(50-a)^2} + \frac{Var\ (b)}{b^2} + \frac{2Cov\ (a,b)}{b(50-a)}$$

where Var (a) = variance a

Var (b) = variance b

Cov (a,b) = covariance (a,b)

which are derived from values obtained from the linear regression.

2.3 RESULTS

(i) EFFECT OF U50,488H ON ELECTRICALLLY-EVOKED OXYTOCIN SECRETION.

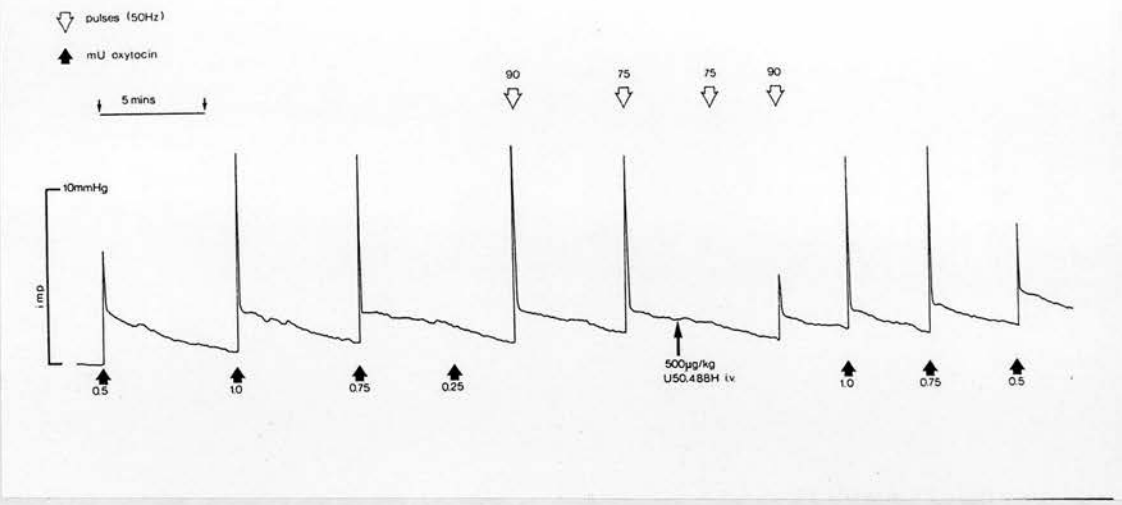
The κ-opioid receptor agonist U50,488H administered intravenously and tested over the dose range 10μg/kg-5mg/kg inhibited the release of stimulus evoked oxytocin in a dose dependent manner.

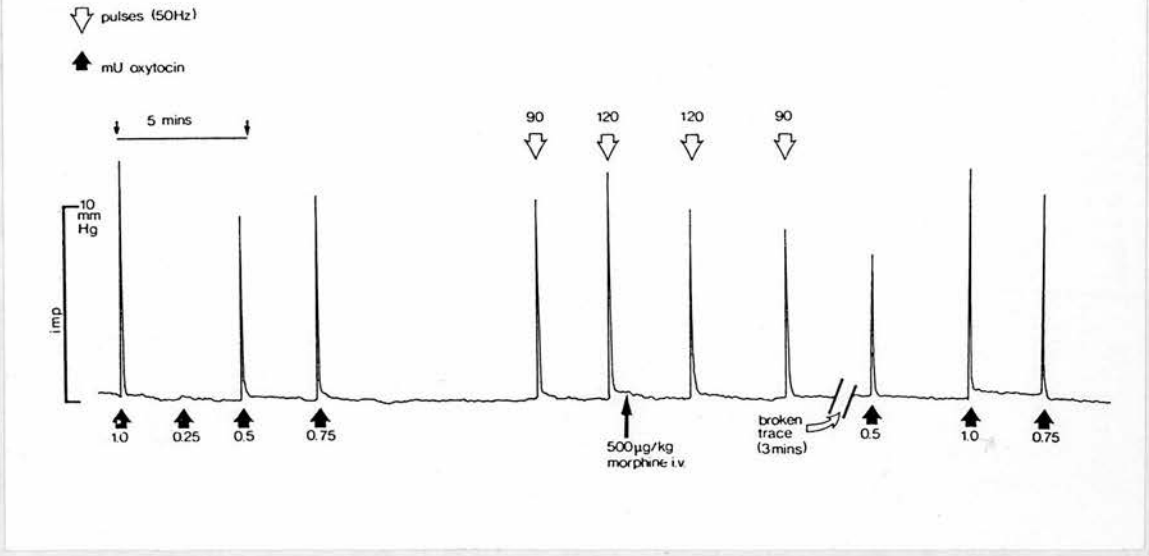
A typical intramammary pressure recording trace from an animal that had received an inhibitory dose of U50,488H is shown in **Fig 2.3(a)**.

U50,488H did not alter the sensitivity of the mammary gland to any great extent as shown in **Fig 2.3(b)** which depicts typical dose response curves and stimulus response to 60 pulses before and after 500μg/kg U50,488H. A slight shift in sensitivity of the mammary gland to oxytocin at the lower range of the dose-response curve was noted. The profound inhibitory effect of U50,488H can be seen by the response to 60 pulses) closed squares) which shift to the left on the curve after U50,488H.

Naloxone (1mg/kg i.v.)administered at the end of the experiment fully reversed the inhibition of stimulus evoked oxytocin secretion without potentiation of the response.

The dose response relation of electrically stimulated oxytocin release to U50,488H is shown in **Fig 2.3(c)**. At doses above 500μg/kg the inhibition by U50,488H was significant with respect to the initial amount of oxytocin elicited upon stimulation of the neural stalk ($p<0.05>0.01$, paired t-test). Using the raw data, a linear regression line was calculated from which the ID_{50}, the dose of U50,488H to inhibit the initial release of oxytocin by 50% was obtained. Using covariance values as described in 2.2 the standard errors for this value were calculated. The ID_{50} for U50,488H was found to be 441μg/kg (+ 194/- 136μg/kg).

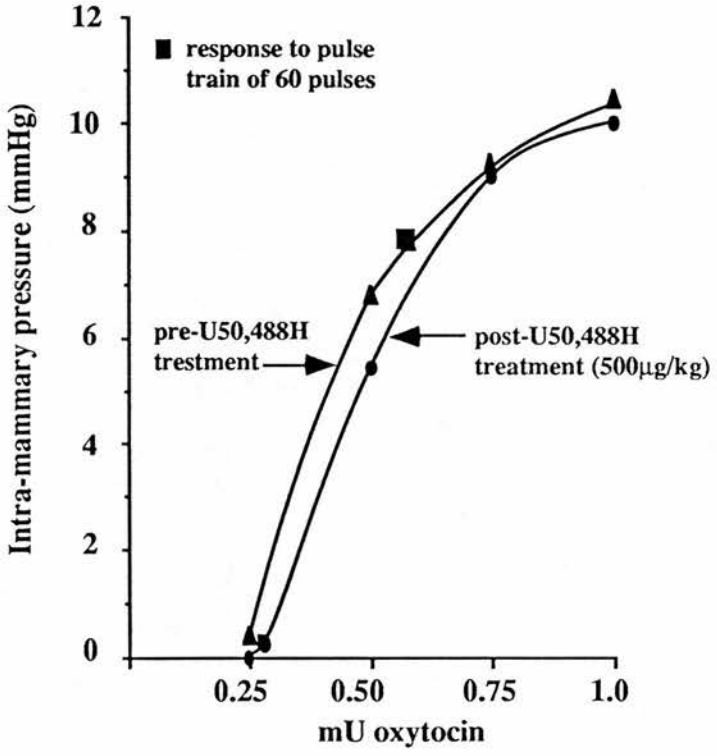




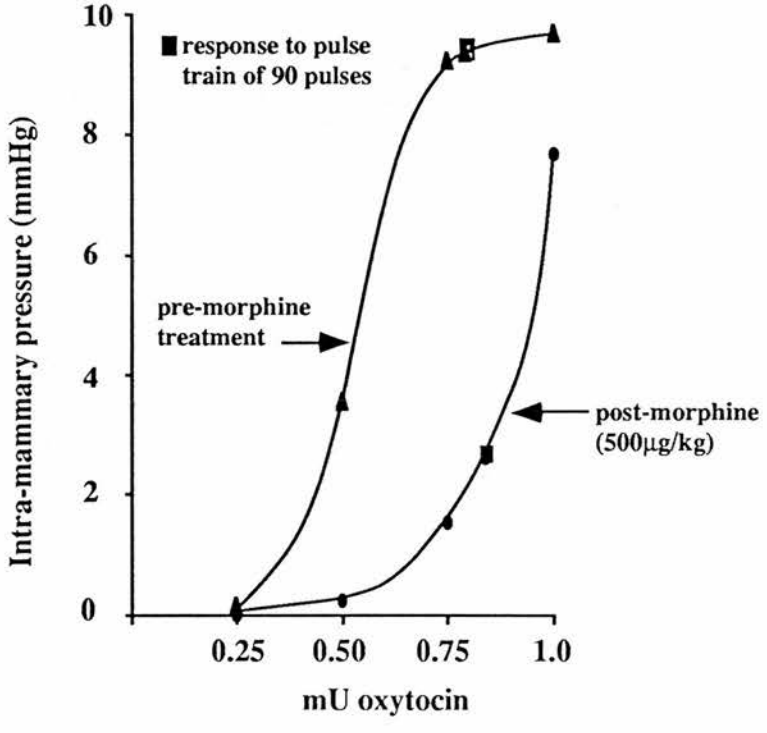
Figs. 2.3 (a) & (f)

Intramammary pressure recording traces from two rats that received 500µg/kg U50,488H (**2.3 a**) or morphine (**2.3**f) i.v..U50,488H can be seen to completely inhibit oxytocin released in response to a pulse train of 75 pulses and profoundly reduce the response to 90 pulses without a loss in sensitivity of the mammary gland to oxytocin.Morphine appears to slightly reduce the response to 90 and 120 pulses but this is only due to a loss in sensitivity of the mammary gland (see **Fig 2.3(g)**).

2.3 (b)



2.3 (g)



Dose-response curves to illustrate changes in sensitivity of the mammary gland that occur following i.v. injection of U50,488H (2.3 b) and morphine (2.3 g) at a dose of 500µg/kg.

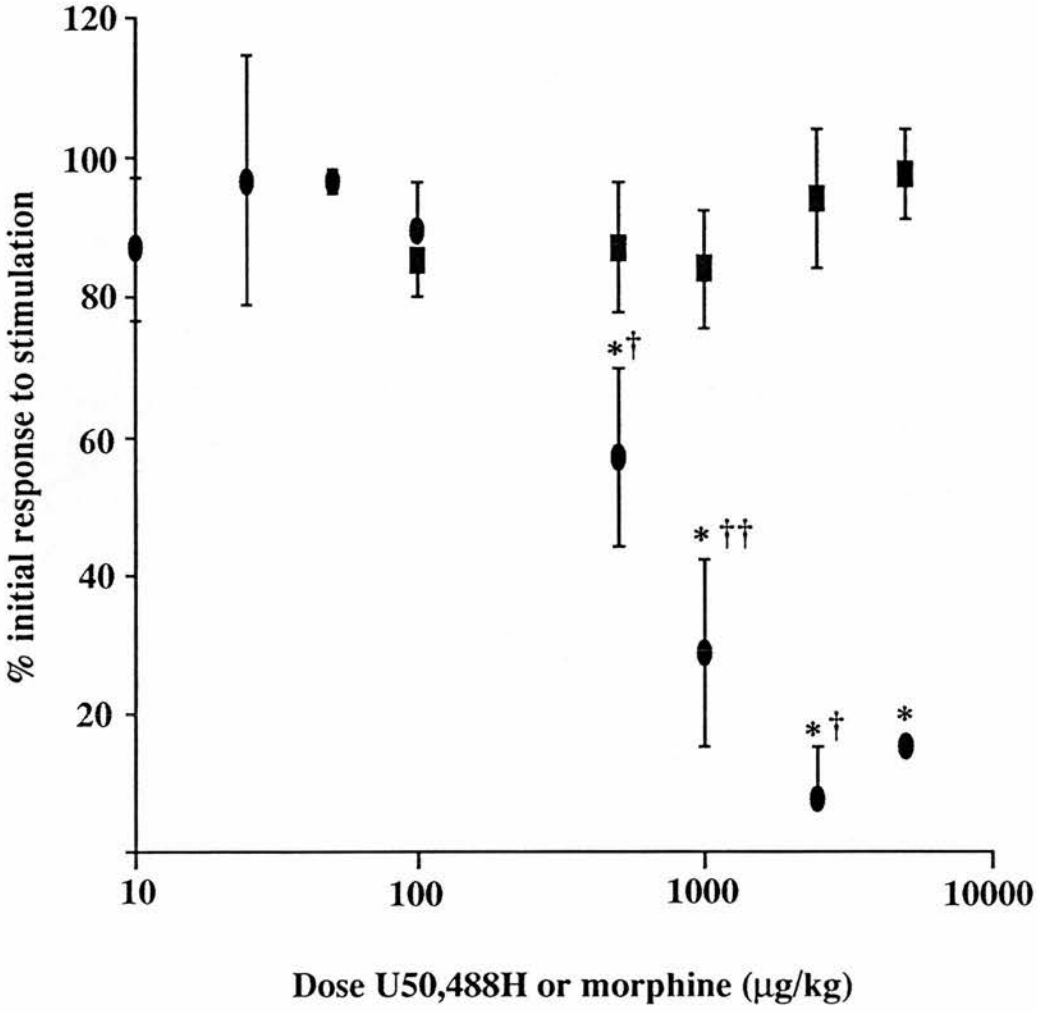


Fig 2.3 (c)

Graph to show the inhibition of electrically-evoked oxytocin release in urethane anaesthetized,lactating rats following i.v. administration of the opioid agonists U50,488H or morphine. Values shown are mean ± sem.Filled circles represent rats that received U50,488H (n=9) and filled squares the rats which received morphine (n=11).
Statistical comparisons:
* $p < 0.05 > 0.01$ with respect to initial response to stimulation (paired t-test).
† $p < 0.05$, †† $p < 0.01$ with respect to rats treated with the same dose of morphine (unpaired t-test).
ID_{50} U50,488H 441 (+194,-136) μg/kg.

(ii) EFFECT OF PROPRANOLOL ON ELECTRICALLY-STIMULATED OXYTOCIN SECRETION.

Pretreatment with propranolol approximately 20 minutes prior to the U50,488H administration protocol, firstly, resulted in an enhancement of mammary gland sensitivity to oxytocin as shown by the representative dose response curves constructed before and after propranolol injection in **Fig 2.3(d)**.

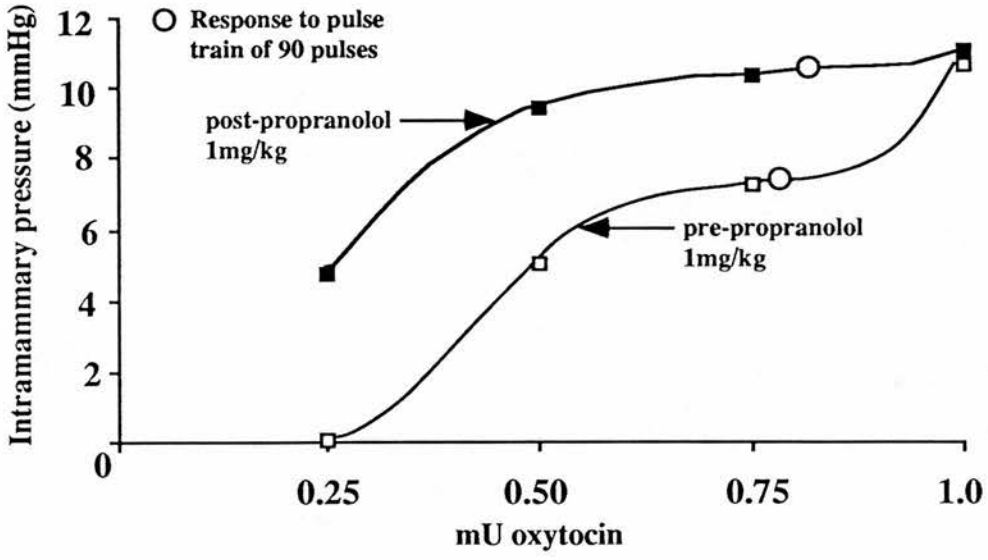
U50,488H still caused a dose dependent inhibition of electrically-stimulated oxytocin following propranolol pretreatment but a change in sensitivity to U50,488H was noted. The dose/response relation appears to have been shifted to the right, as seen in **Fig 2.3(e)** indicating propranolol to reduce the inhibitory effect of U50,488H on oxytocin secretion. Significant inhibition of electrically evoked oxytocin was achieved at doses of 500μg/kg and above ($p=0.01$, paired t-test). Comparison of the two experimental groups, propranolol pretreated and propranolol naive receiving i.v. U50,488H only showed significant differences at U50,488H doses of 100μg/kg and 1000μg/kg ($p<0.05$ unpaired t-test; $p=0.05$ unpaired t-test). Using regression analysis the ID_{50} for U50,488H in propranolol pretreated rats was found to be 1585 μg/kg (+ 603/- 437μg/kg) . This was significantly different from the ID_{50} obtained from rats that received only U50,488H ($p<0.05$, derived from regression statistics.

Naloxone restored the stimulus evoked oxytocin reponse in this experimental group to initial values without any potentiation.

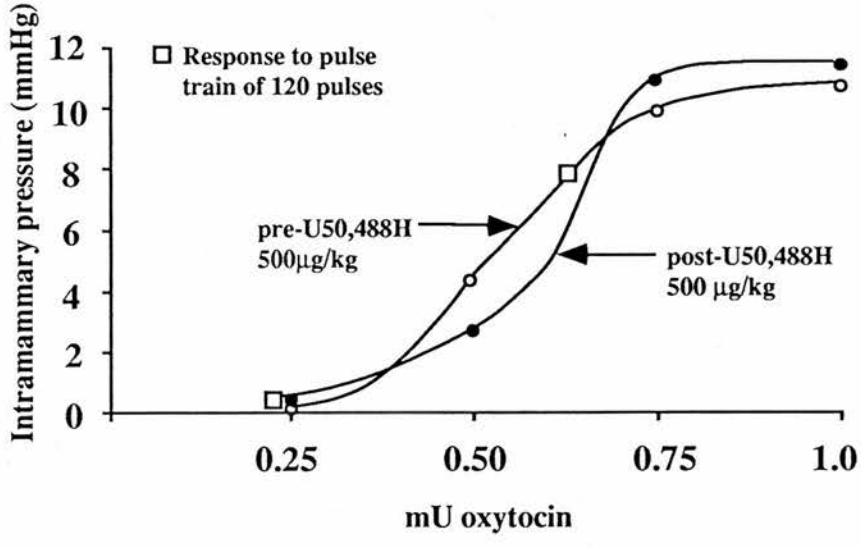
(iii) EFFECT OF MORPHINE ON ELECTRICALLY-STIMULATED OXYTOCIN SECRETION.

Intravenous morphine sulphate in the dose range 100μg/kg-5mg/kg had no significant effect on stimulus evoked oxytocin secretion when compared to initial values as seen in **Fig 2.3(c)** . Naloxone (1mg/kg i.v.)

2..3 (d) **propranolol pre-treated group**



2.3 (j) **morphine dependent group**



Dose-response curves to illustrate changes in sensitivity of the mamary gland that occur following i.v. injection of 1mg/kg propranolol (2.3 d) or 500µg/kg U50,488H in morphine dependent animals (2.3 j).

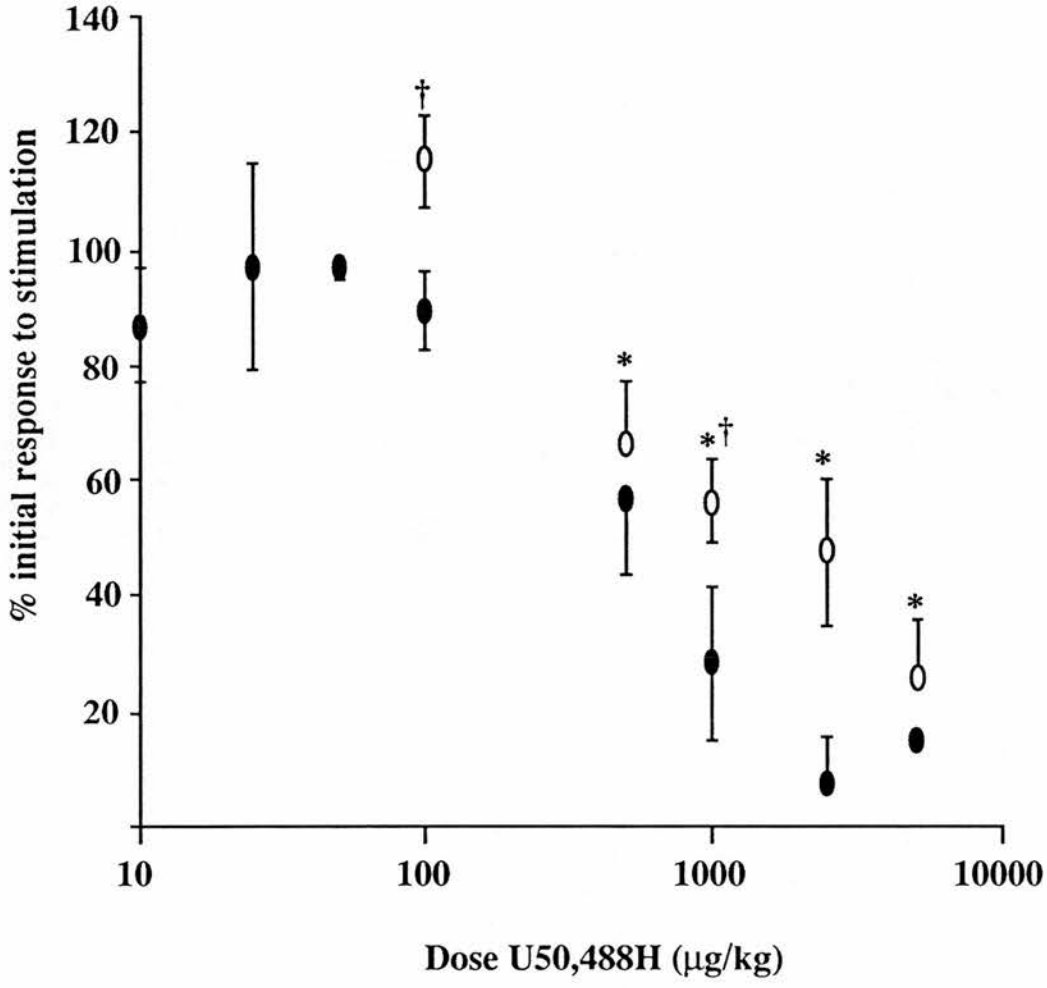


Fig 2.3 (e)

Graph to show the inhibition of electrically-stimulated oxytocin release in urethane-anaesthetized,lactating rats following i.v. administration of the opioid agonist U50,488H.Values shown are mean ± sem.Filled circles represent rats that received U50,488H (n=9),open circles represent rat that were pre-treated with propranolol prior to U50,488H injection (n=6). Statistical comparisons:
* $p < 0.01$ with respect to initial response to stimulation (paired t-test)
† $p < 0.05$ with respect to inhibition caused by U50,488H alone (unpaired t-test).
ID_{50} U50,488H + propranolol 1585 (+603,-437) µg/kg ($p < 0.05$ with respect to ID_{50} U50,488H alone.

elevated oxytocin secretion slightly but not in a significant manner.

A typical intramammary pressure recording trace during the morphine drug administration protocol is shown in **Fig 2.3(f)**.

Mammary gland sensitivity to oxytocin was depressed by i.v. morphine as demonstrated in **Fig 2.3(g)** where a shift in the dose response curve to the right after 500μg/kg can be seen.

(iv) EFFECT OF U50,488H ON ELECTRICALLY-STIMULATED OXYTOCIN SECRETION IN MORPHINE DEPENDENT RATS

In the morphine dependent group of animals U50,488H effectively inhibited stimulus evoked oxytocin secretion in a dose-dependent manner as seen in **Fig 2.3(h)**.

A typical intramammary pressure recording trace from a morphine dependent animal receiving i.v.U50,488H is shown in **Fig 2.3(i)**.

Dose response curves constructed before and after each drug administration in this group of animals showed that there was no change in mammary gland sensitivity.This is illustrated in **Fig 2.3(j)**.At a dose of 50μg/kg and above from a dose range of 10-1000μg/kg there was significant inhibition of oxytocin with respect to initial,pre-drug values ($p \leq 0.01$, paired t-test).The ID_{50} for U50,488H was found to be 170μg/kg (+ 78/- 54μg/kg).This was significantly different from the morphine naive group ($p \leq 0.05$,derived from the regression statistics).

In all morphine treated animals naloxone caused a sustained elevation of intramammary pressure response sufficient to satisfy that the animals were morphine dependent.A typical elevation of intramammary pressure seen upon naloxone induced morphine withdrawal is shown in **Fig.2.3(k)**.The sustained elevation of intramammary pressure is often maintained for 20-40 mins.

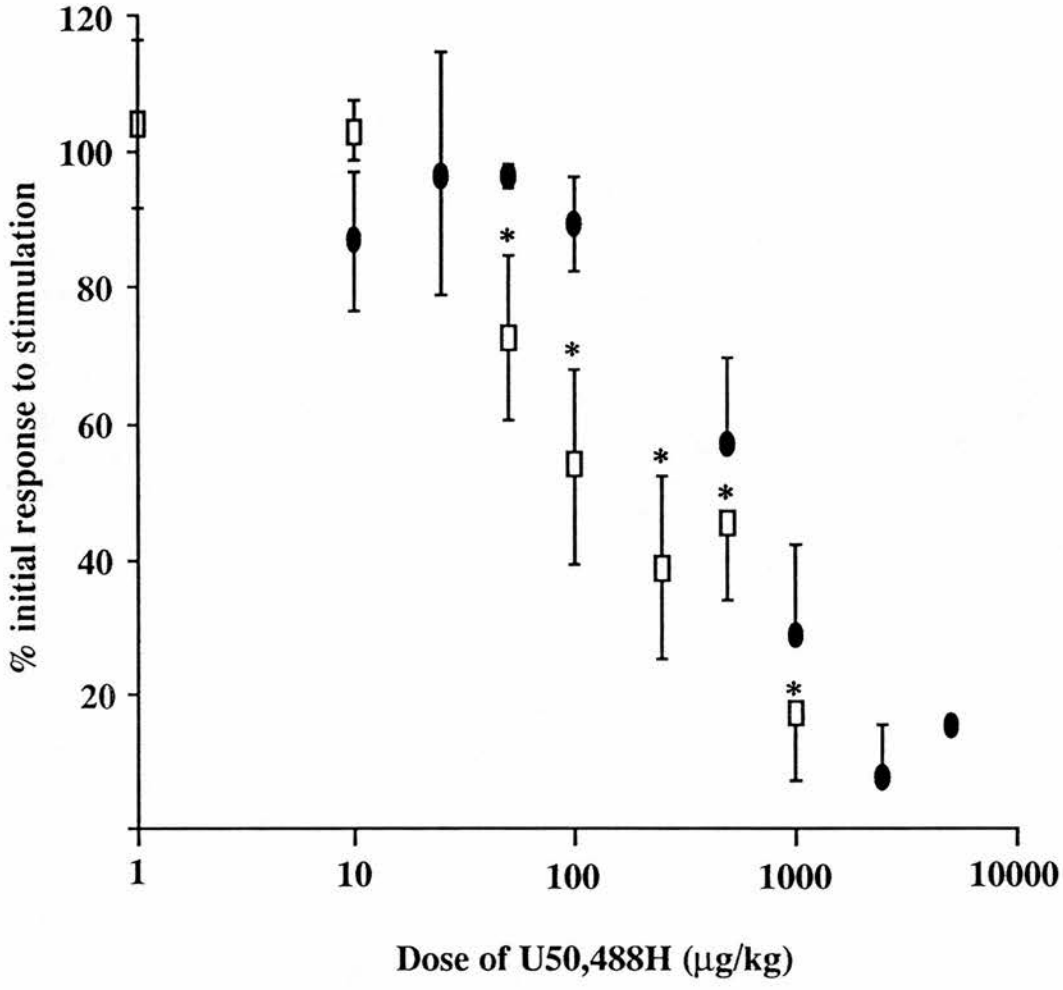
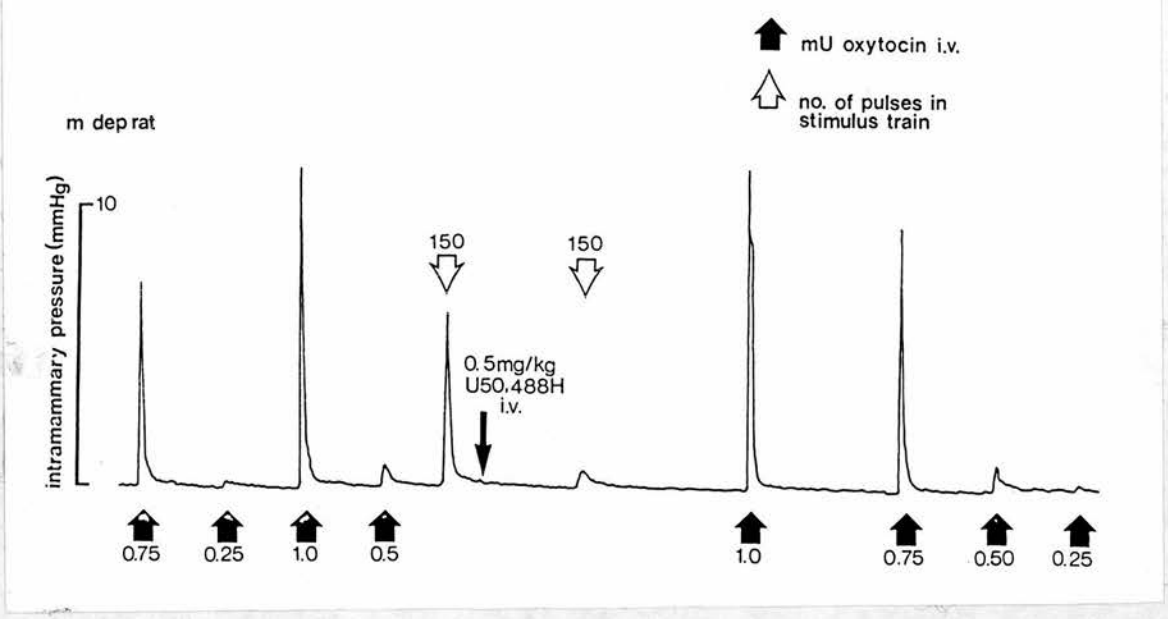
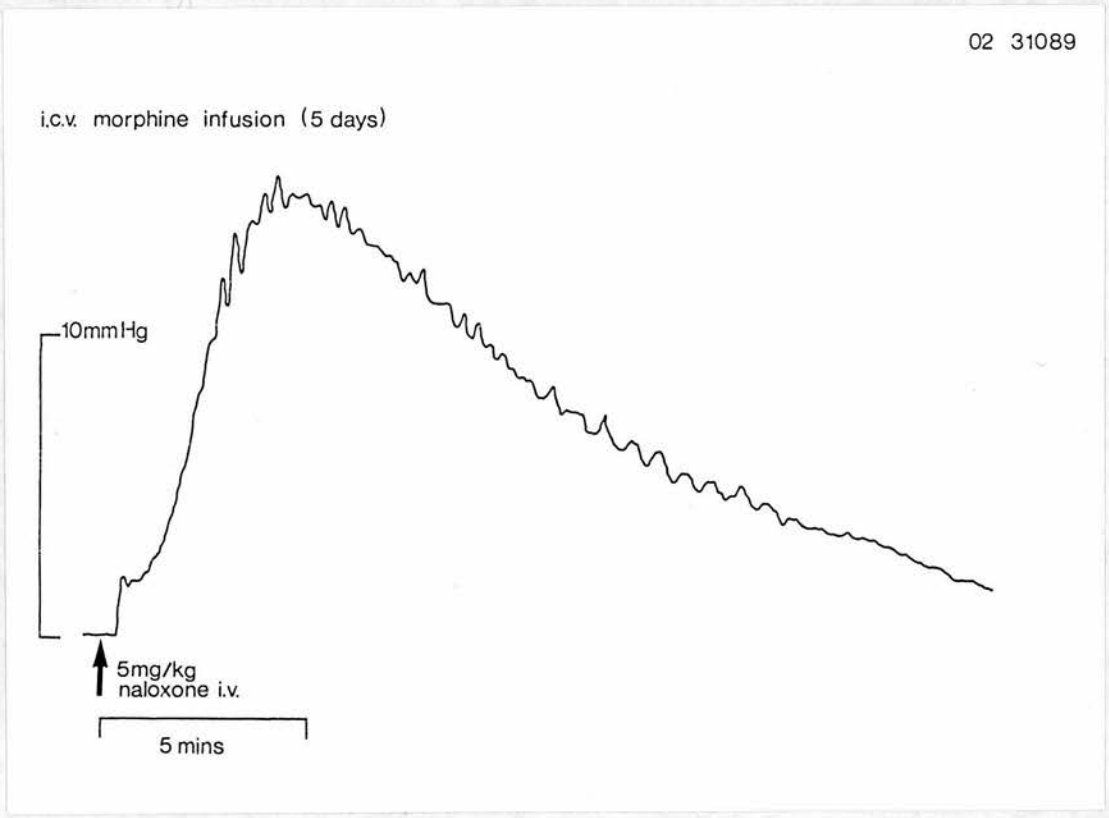


Fig 2.3 (h)

Graph to show the inhibition of electrically-evoked oxytocin release in urethane-anaesthetized,lactating rats following i.v. administration of the opioid agonist U50,488H.Values shown are mean ± sem.Filled circles represent morphine-naive rats that received U50,488H (n=9) and the open squares morphine-dependent rats that received U50,488H(n=6).
Statistical comparisons:
* $p < 0.01$ with respect to initial response to stimulation (paired t-test)
ID_{50} U50,488H (morphine dependent rats) 170 (+78,-54) μg/kg $p < 0.05$ with respect to ID_{50} U50,488H (morphine naive rats).





Figs 2.3(i) & 2.3(k)

Fig 2.3(i) shows an intramammary pressure recording trace from a morphine dependent rat which received 0.5mg/kg U50,488H i.v.This dose effectively inhibits the release of oxytocin evoked by a stimulus train of 150 pulses without loss of sensitivity of the mammary gland to oxytocin.

Fig 2.3 (k) shows a the typical sustained elevation of i.m.p. during naloxone-induced withdrawal of a morphine dependent rat

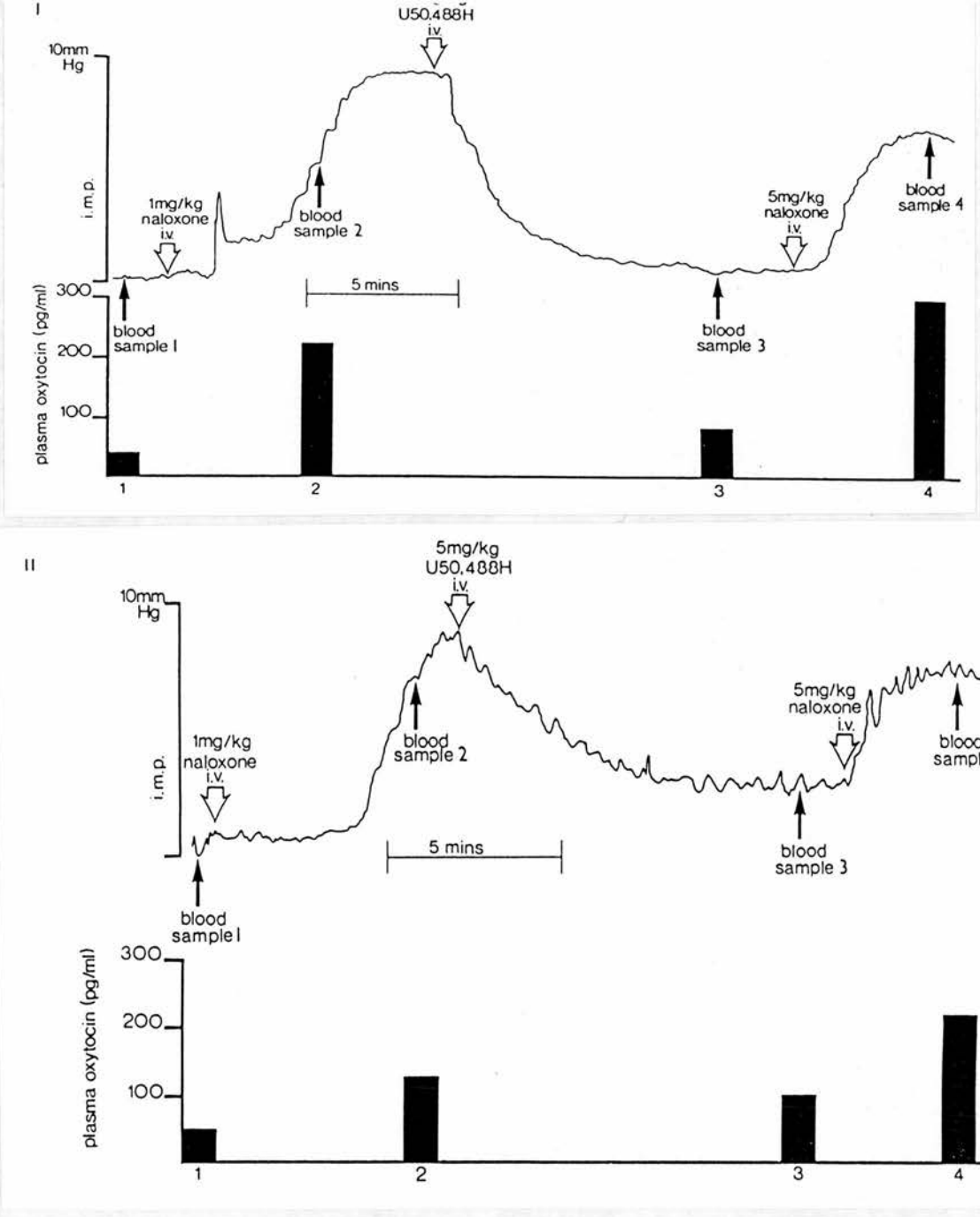


Fig 2.3 1 (i & ii)

Intramammary recording traces from two morphine dependent rats, withdrawn with naloxone (1mg/kg), and then subsequently challenged with the κ-opioid agonist U50,488H at 10mg/kg (2.31(i)) and 5mg/kg (2.31(ii)) i.v.. In both cases the withdrawal hypersecretion of oxytocin is re-initiated with 5mg/kg naloxone i.v.. Plasma oxytocin at the times indicated is shown by the solid bars.

In those animals that received U50,488H during withdrawal it would appear that the hypersecretion of oxytocin can be inhibited by a U50,488H mediated action as demonstrated in the traces shown in **Fig 2.3 1 (i & ii)**.

In trace (i) injection of 1mg/kg naloxone i.v. was able to precipitate withdrawal from morphine as seen from the elevation in intramammary pressure and elevation of plasma oxytocin.Once the response was maximal,10mg/kg U50,488H was injected i.v. and a rapid decrease in intramammary pressure was seen over the next 3-4 minutes after which it had reached basal,pre-naloxone values.Plasma oxytocin was also decreased.Subsequent injection of 5mg/kg naloxone was able to re-initiate the withdrawal response although to a lesser degree than the initial response. Circulating levels of oxytocin in peripheral plasma are also increased.

In trace (ii) the rat was given 1mg/kg naloxone i.v.which precipitated a sustained rise in intramammary pressure due to the hypersecretion of oxytocin into plasma following withdrawal from morphine.Once the response had reached a 'plateau' 5mg/kg U50,488H was injected i.v.Intramammary pressure decreased rapidly over a period of about 5 minutes,although it did not reach initial basal values.The animal was then given 5mg/kg naloxone i.v.There was a rapid elevation of intramammary pressure over about 2 minutes indicated withdrawal to have been re-initiated.

Blood samples were taken from the rat at the points indicated in **Fig 2.3(1).These showed naloxone to increase plasma oxytocin and U50,488H to decrease circulating oxytocin.**

(v) THE EFFECT OF STALK APPLICATION OF U50,488H ON THE ELECTRICALLY-STIMULATED RELEASE OF OXYTOCIN.

Application of a dose range of U50,488H (1-20µg/5µl

isotonic saline) directly onto the neural stalk gave no conclusive results. Due to the inconclusive results obtained this method was abandoned for further experiments into the effects of different opioid agonists and antagonists on oxytocin secretion from the neural lobe.

(vi) THE EFFECT OF INTRACEREBROVENTRICULAR MORPHINE ON ELECTRICALLY-STIMULATED RELEASE OF OXYTOCIN.

Acute injection of morphine sulphate (1-20μg/5μl isotonic saline) directly into the right lateral cerebral ventricle also presented inconsistent results and this method of drug delivery abandoned for further experiments.

2.4 DISCUSSION

Results from this series of **in-vivo** experiments complement autoradiographic ligand binding opiate receptor studies (**Bunn, Hanley & Wilkin, 1985; Herkenham, Rice et al, 1986; Zukin, Eghbali et al, 1988**) and **in vitro** experiments using the isolated neural lobe preparation (**Bicknell et al, 1985; Zhao, Chapman & Bicknell , 1988a; Zhao, Chapman & Bicknell, 1988b**) which have indicated the predominant opioid receptor present at the neural lobe to be of the κ-subtype.

When examining stimulus-evoked oxytocin release in the presence of a κ or μ opioid receptor agonist, only the κ-agonist U50,488H was able to show an inhibitory influence on oxytocin secretion. Morphine sulphate, the μ-opioid receptor agonist showed no influence on stimulus-evoked oxytocin release from the neural lobe although it is known to act at opioid receptors in the hypothalamus to modify oxytocin secretion as demonstrated by studies on oxytocin secretion, electrophysiologial and tolerance and dependence studies (**Bicknell, Leng et al, 1984; Russell & Spears, 1984; Rayner, Robinson &**

Russell, 1988; Leng, Russell & Grossman, 1989)
This would indicate that there are (a) virtually negligible numbers of functionally-coupled μ-receptors in the neural lobe of the rat either on or around oxytocin nerve terminals which contribute to the endogenous opioid tone influencing oxytocin release or (b) μ-receptors detected at the neural lobe are of a different subtype and binding affinity than those which predominate at the hypothalamus.
The μ-opioid receptor comprises of two subtypes μ_1 and μ_2, of which μ_2 is the highly selective morphine receptor (**Pasternak & Wood, 1986**). It may be the case that at the hypothalamus the population of μ receptors comprises of mainly the high affinity morphine μ_2 receptors whilst those at the neural lobe are mainly of the μ_1 subtype.
U50,488H is a selective ligand for the κ-type receptor and although ethylketocyclazocine is approximately five times more potent at the κ receptor than U50,488H it is much less selective and will compete at both μ_1 and μ_2 sites (**Vonvoigtlander, Lahti & Ludens, 1983**). In fact, it has been found to label μ_1 receptors ten times more effectively than κ-sites. U50,488H therefore, was a more suitable agonist for investigating opioid receptor mediated modification of oxytocin secretion since it is thirty-fold more potent at κ than μ_1 receptors and forty-fold more potent at κ than μ_2. Any inhibition of oxytocin secretion could be attributed more confidently to actions via kappa opioid receptors.
Autoradiographic evidence now suggests that there are two kinds of kappa receptors - κ_1 and κ_2 which bind endogenous κ-opioids and U50,488H (**Clark and Pasternak, 1988; Zukin, Eghbali et al, 1988**). In the latter study, homogenates from guinea pig brain and ratbrain were taken and a species difference in distribution of these receptors was noted; a single population of high affinity κ_1 being present in guinea

pig brain whilst in the rat brain homogenate two populations of receptors were characterized:-high affinity sites at low density (κ_1) and low affinity sites at high density (κ_2).

The two sites show different ligand selectivity profiles U50,488H being much more potent at the κ_1 than the κ_2 site. It is thought that the two subtypes of κ receptors may either couple to different membrane channels or perhaps the same channel but a different second messenger.

There is also evidence for a third type of κ-receptor, κ_3. This is insensitive to U50,488H binding and can therefore be distinguished from κ_1 and κ_2 receptors(**Clark,Lui et al,1989**).

When naloxone was given at the end of the experiments where the animals had undergone a U50,488H or morphine administration protocol,oxytocin secretion was only restored to its initial value,in the case of the κ-receptor agonist U50,488H which had caused an inhibitory response, or remained unchanged as in the case of morphine injected animals. This was an unexpected result as it was thought that as well as reversing any inhibition mediated by exogenously applied opiates that there would be reversal of endogenous opioid tone acting at oxytocin nerve terminals.

It isbelievedthat the endogenous ligand for the κ-receptor is dynorphin A(1-17) which is co-secreted and co-released with vasopressin from vasopressin nerve terminals also in the neural lobe (**Watson,Akil et al,1982**). Dynorphin has been shown to be potent at the κ_2 (low affinity site) whilst U50,488H is more potent at the κ1 (high affinity site) (**Zukin,Eghbali et al,1988**). Competitive binding studies have shown that U69,593,a κ-agonist can displace 85% of κ_1 receptor binding but only 15% of κ_2 binding. It is possible that under the conditions of the experiment that naloxone preferentially binds to the κ_1-type opioid receptor to

reverse the effects of U50,488H but not the endogenous κ-ligand binding at κ_2 receptors.

Oxytocin secretion is elevated by naloxone in both **in vivo** and **in vitro** studies demonstrating its ability to reverse endogenous opioid tone acting on oxytocin neurones. If U50,488H was inhibiting vasopressin secretion, as has been shown **(Forsling & Wells, 1989)** then dynorphin secretion would also be reduced so that there would be less endogenous opioid tone acting at these neurones and naloxone is just reversing the effect of U50,488H.

Another proposal is that endogenous opioid tone has been reversed and there is incomplete antagonism of U50,488H, which leads to restoration of oxytocin secretion to pre-drug values but without superceding them. Electrophysiological recordings from putative oxytocinergic neurones in the SON show that U50,488H will profoundly inhibit firing rate of these neurones but that naloxone only restores this to control values whereas morphine inhibition of oxytocin neurone cell body activity is reversed by more than 100% by naloxone. The inhibitory actions of U50,488H at the cell bodies in the SON are therefore, mirrored by its effects on oxytocin secretion at the neural lobe **(Leng, Pumford & Russell, 1990)**.

By pretreating a subsection of the experimental animals receiving U50,488H with the β-adrenergic antagonist propranolol it had been hoped to elucidate the contribution of a β-adrenergic system to κ-mediated inhibition of oxytocin secretion. In the suckled rat it has been shown that intraperitoneal or intrathecal U50,488H interupts the milk-ejection reflex. Following injection of propranolol, intrathecal U50,488H was ineffective whilst the action of intraperitoneal U50,488H was significantly reduced thus suggesting that there may be two sites of action for κ-agonists, one within the spinal cord acting through a β-adrenergic mechanism and one at a

supraspinal level (**Clarke & Wright, 1987**).

Using radioligands, opiate receptors have been localized at the bovine adrenal medullary membranes of which a high percentage are identified as κ-receptors (**Castanas, Giraud et al, 1983**).

In our experiments, pretreatment of the rats with propranolol did not prevent the inhibitory action of U50,488H on oxytocin secretion from the neural lobe but it did reduce its effects, as judged from comparison of the ID_{50} values obtained from propranolol pretreated rats and propranolol naive animals.

From this it is concluded that the adrenergic system may be partly involved in κ-opioid modification of oxytocin secretion from the neural lobe of the rat but that some of the inhibitory actions of U50,488H are not mediated by an adrenergic mechanism.

In the morphine dependent group of animals there was no cross tolerance to the effects of i.v. U50,488H. Conversely, morphine dependency led to a significant enhancement of κ- mediated inhibition of electrically evoked oxytocin secretion.

Previous experiments which have addressed this question fom another viewpoint i.e. does U50,488H administration affect the development of morphine tolerance and dependence in rats, have produced a mixture of results.

Yamamoto, Ohno & Ueki, 1988, reported that the development of tolerance to morphine analgesia was blocked by intraperitoneal U50,488H, which did not block the analgesic effect of morphine when administered with a single acute injection in the morphine naive rat. In morphine tolerant animals, U50,488H restored the analgesic effect of morphine. These results led to the suggestion that development of morphine tolerance can be blocked by a κ-mediated action but that once tolerance has developed, U50,488H, which has previously shown no

analgesic properties, expressed an analgesic effect through up-regulation of κ-receptors.

Fukagawa, Katz et al, 1989 made rats morphine dependent by food admixture which were subsequently challenged with naltrexone to precipitate withdrawal. U50,488H administered prior to withdrawal had no effect on the visible signs of withdrawal and concommitant administration during chronic morphine exposure also had no effect on withdrawal indicating tolerance and dependance to have developed normally. The analgesic effect of morphine was used as an index to demonstrate tolerance and dependence.

Rats chronically exposed to U50,488H and subsequently challenged with morphine were used by **Bhargarva, Hemendra et al, 1989** to examine the cross-tolerance of μ and κ receptor systems. They concluded that there was tolerance to some effects of U50,488H but not to the κ-agonist induced diuresis and found no cross-tolerance properties were conferred to the μ-system as demonstrated by morphine induced analgesia.

In the urethane anaesthetized rat model used in the present study there was no sign of cross tolerance between μ and κ-receptors. U50,488H appeared to be more effective at inhibiting stimulus evoked oxytocin secretion which would tend to comply with the idea of upregulation of the kappa system put forward by **Yamamoto, Ohno & Ueki**. This would have to be at the neural lobe since neural stalk stimulation isolates the inhibitory action of U50,488H to this site. However we have no other evidence to support this theory as autoradiographic studies on receptor changes at the neural lobe during chronic morphine exposure showed no change in the κ-opioid receptor density (**see chapter 7**).

There can be independent control of oxytocin by means of μ and κ-opioid receptors demonstrated by the ability of U50,488H to inhibit the withdrawal hypersecretion of oxytocin from the neural lobe

following naloxone administration.We cannot say from this study the site of the κ-receptors involved in this;they could be located at either the neural lobe or hypothalamus .It does however show that κ-opioid receptor modification of oxytocin secretion can take place when the μ-opioid receptors are inactivated

The unreliable results obtained from direct application of U50,488H onto the neural stalk arose from the problems in cutting the dural membrane overlying the stalk.Cutting the membrane led to release of CSF at the site of drug application.It was difficult to tell whether the drug reached the tissue or was washed away in the flow of CSF.It was also impossible to quantify the concentration of U50,488H reaching the tissue since it was immediately diluted in CSF.

Intracerebroventricular injection of morphine proved difficult due to the rats being held in a supine position.During injection,morphine solution tended to leak out of the i.c.v. cannula making it impossible to deliver a precise amount of drug to this compartment.

In conclusion, it seems that in the urethane anaesthetised rat,inhibition of stimulus evoked oxytocin at the site of the nerve terminals is mediated by κ rather than μ-receptors.The effect may partly but not entirely involve a β-adrenergic component at this site and there is no cross-tolerance between the morphine and U50,488H at the neural lobe.Speculatively,endogenous opioid tone which modifies oxytocin secretion under physiological conditions would appear to act at a different subset of κ-receptors (κ_2) than the κ-opiate receptor agonist U50,488H.Binding affinity studies have shown dynorphin to be a potent agonist at these sites and this would further emphasize its possible role as the endogenous ligand acting to modify oxytocin secretion at the level of the terminals under physiological conditions **in-vivo**.

CHAPTER THREE

INVESTIGATION OF THE ACTIONS OF MORPHINE AND OPIOID-RECEPTOR ANTAGONISTS ON OXYTOCIN SECRETION FROM THE POSTERIOR PITUITARY GLAND OF THE RAT IN VIVO AND IN VITRO.

CHAPTER 3 : Investigation of the actions of morphine and opioid-receptor antagonists on oxytocin secretion from the posterior pituitary gland of the rat in-vivo and in-vitro.

INTRODUCTION

In the rat, the magnocellular oxytocin secretory system can be influenced by both endogenous and exogenous opioids which exert their actions at the cell bodies in the hypothalamus (**Gosden, Humphreys et al, 1985**), demonstrated by changes in electrical activity (**Bicknell, Leng et al, 1984**) and at the neurosecretory terminals in the neural lobe (**Bicknell & Leng, 1982; Bicknell, Chapman & Leng, 1985; Coombes & Russell, 1988**).

Naloxone, an opioid receptor antagonist, active at all receptor subtypes (**Paterson, Robson & Kosterlitz, 1983**) is found to enhance oxytocin secretion in the urethane anaesthetized rat of either sex (**Hartman, Rosella-Dampman & Summy-Long, 1987**) but without alteration of recorded electrical activity from identified oxytocinergic SON neurones (**Clarke, Wood et al, 1979**). This action is assumed to be from reversal of endogenous opioid tone acting at oxytocin nerve terminals as a pre-synaptic mechanism in the posterior pituitary (**Bicknell, Chapman & Leng, 1985**).

As described in the introduction, immunoreactive dynorphin, the endogenous ligand for the κ-opioid receptor subtype (**Chavkin, James & Goldstein, 1982; Corbett, Paterson et al, 1982**) has been found in magnocellular vasopressin neurones and terminals (**Watson, Akil et al, 1982**) and is colocalized within neurosecretory granules (**Martin, Geis et al, 1984**).

The predominant opioid receptor subtype localized at the neural lobe is the κ-receptor although a small number of μ-receptors have been reported (**Bicknell, Chapman & Zhao, 1988[c]; Herkenham, Rice et**

al, 1986). From our own studies, the presence of μ-receptors in the posterior pituitary remains unresolved (**Sumner, Coombes et al, 1990**). In the SON both μ and κ-receptors are found (**Sumner, Coombes et al, 1990**).

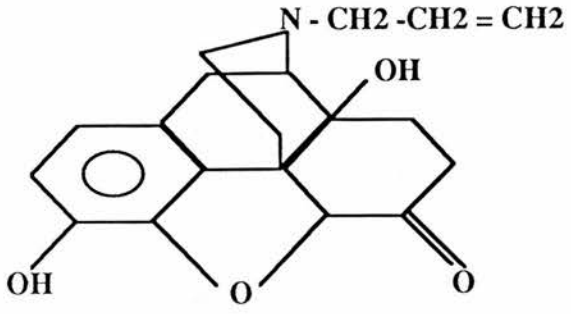
Inhibitory endogenous opioid effects have been attributed to dynorphin released from vasopressin nerve terminals acting via κ-receptors located on oxytocin nerve terminals or pituicytes in the posterior pituitary (**Lightman, Ninkovic et al, 1983; Bunn, Hanley & Wilkin, 1985**).

Acute intracerebroventricular (i.c.v.) injection of morphine (2-4μg), which is considered to be a relatively selective μ-opioid agonist (**Paterson, Robson et al, 1983**), inhibits oxytocin secretion and hence the milk-ejection reflex in suckled, anaesthetized, lactating rats (**Clarke, Wood et al, 1979**). The characteristic, high frequency synchronous bursting pattern associated with milk-ejection activity (**Poulain & Wakerley, 1982**), recorded from identified oxytocinergic SON cells, is unaffected.

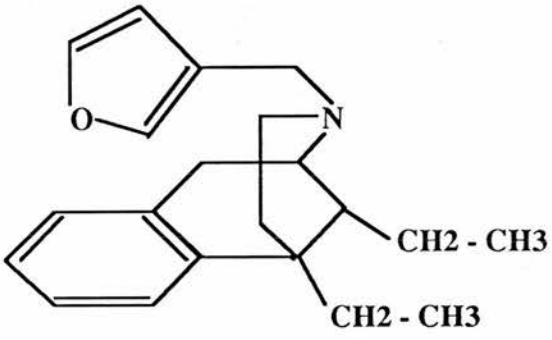
This indicates morphine to act by un-coupling electrical activity in the hypothalamus from events leading to hormone secretion in the neural lobe. The effect was found to be naloxone reversible and attributed to the actions of morphine on oxytocin nerve terminals in the neural lobe.

More recently, acute i.v. injection of morphine has been found to be inneffective at inhibiting electrically-evoked secretion from oxytocin nerve terminals (**Chapter 2 this thesis, Coombes & Russell, 1988**), although it inhibits the spontaneous firing activity from putative oxytocin neurones on the SON in hypothalamic slices, indicating its actions to be mediated at the cell bodies (**Pittman, Hatton & Bloom, 1980; Wakerley, Noble & Clarke, 1983**).

In the following experiment, morphine was given as an acute i.v. injection to occupy μ-receptors and to test

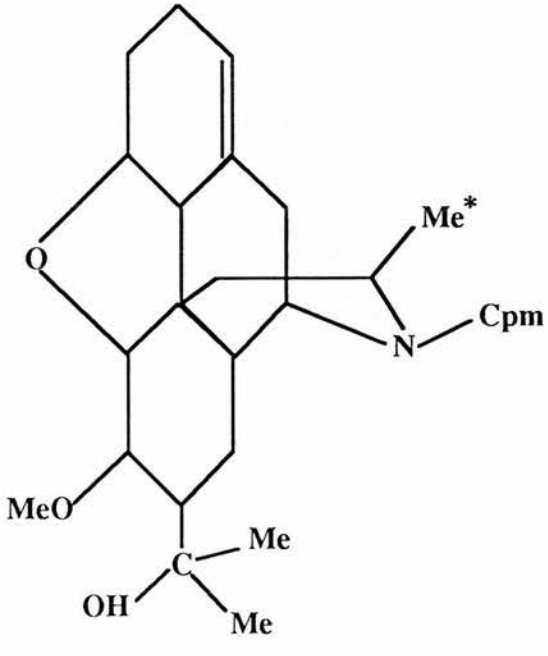


Naloxone



MR2266

5, 9 -Diethyl -2- (3-furylmethyl) -2- hydroxy - 6, 7 -benzomorphan



RX8008M

N-cyclopropylmethyl group

Fig 3.1 (a)

Structures of the opioid antagonists naloxone,MR2266 and RX8008M

whether it decreased plasma oxytocin. The animals were then given the opioid antagonists naloxone or MR2266 i.v.

Naloxone acts at all opiate receptor subypes but has a higher relative affinity for μ-receptors and therefore may be selective for μ-receptors at low concentrations.

MR2266 is a benzomorphan derivative with ~ 40 times greater affinity for κ than μ-receptors (derived from binding studies using guinea-pig brain membranes **(Kosterlitz, 1985)**

MR2266 and naloxone have similar affinity at μ-receptors and in the in-vitro neural lobe preparation are equipotent at reversing endogenous opioid peptide inhibition of oxytocin secretion. At low concentrations MR2266 and naloxone may have different effects on oxytocin secretion according to whether μ or κ-receptors are involved in its regulation.

In this case, using low concentrations of both antagonist, MR2266 would be expected to reverse endogenous opioid tone acting via κ-receptors at oxytocin nerve terminals in the neural lobe **(Coombes & Russell, 1988)** to increase oxytocin secretion and naloxone would be expected to reverse the effects of morphine, believed to be a μ-receptor agonist and hence acting centrally on oxcytocin neurone cell bodies. The structures of naloxone and MR2266 are shown in **Fig 3.1(a)**.

3.1.1 METHOD

3.1.1(i) ANIMALS

Virgin female Sprague-Dawley rats of mean bodyweight 286.5 ± 7.3g, as described in **1.1** were used.

3.1.1(ii) SURGERY

On the morning of the experiment the animals were anaesthetized with urethane (1.25g/kg i.p.) and fitted with a femoral arterial and femoral venous cannula for

blood sampling (**see 1.2 for details**).The animals were left for two hours to allow equilibration of oxytocin secretion after surgery.The animals body temperature was maintained at 37°C using a thermostatically controlled blanket connected to a rectal probe.

3.1.1(iii) SAMPLING/TREATMENT PROTOCOL

The sampling protocol is shown in **Fig 3.1(b)**. .Half the rats received 500µg/kg morphine i.v. after sample 2,the first two samples being a measure of basal plasma oxytocin.At this dose,morphine has profound inhibitory effects on the milk ejection reflex in the urethane anaesthetized rat and is analgesic (**Clarke & Wright,1984**).Control rats received isotonic sterile saline (0.5ml/kg) i.v.

After plasma sample 4,by which time any changes in plasma oxytocin would have been detected,both morphine treated and control rats received 50µg/kg of naloxone or MR2266.It was hoped that by using this low concentration of antagonist,that their relative abilities to antagonize a predicted µ-mediated inhibition of oxytocin secretion would be discriminated.

Blood samples (0.3ml) were taken and plasma separated as described in **1.7.1(i)** and oxytocin content determined by the Higuchi radioimmunoassay (for details see **1.7.3**).The first plasma sample was replaced with an equivalent volume of sterile isotonic saline,subsequent samples were replaced with resuspended red cells in an equivalent volume of isotonic saline.

3.1.1(iv) DRUGS

Morphine sulphate and naloxone hydrochloride were made up to the required concentration in an appropriate volume of isotonic sterile saline.MR2266,5,9 α-diethyl-2-(3-furylmethyl)-2-hydroxy-6,7-benzomorphan (originally a gift from **Boehringer-Ingelheim**),was

Surgery completed	t = 120 mins
Plasma sample 1	t = 0 mins
Plasma sample 2	t = 10 mins
Morphine (500μg/kg) i.v. or saline (0.5ml/kg) i.v.	t = 15 mins
Plasma sample 3	t = 20 mins
Plasma sample 4	t = 30 mins
MR2266/naloxone (50μg/kg i.v.)	t = 34 mins
Plasma sample 5	t = 40 mins
Plasma sample 6	t = 50 mins
Plasma sample 7	t = 60 mins

Fig 3.1(b)

Sampling protocol showing the schedule for timed arterial blood sampling for the measurement of oxytocin concentration in urethane anaesthetized rats.

initially dissolved in 0.1N HCl, 1mg MR2266:30µl 0.1N HCl. The resulting solution was then made up to the required concentration in isotonic sterile saline.

3.1.1(v) STATISTICS

Values of plasma oxytocin from radioimmunoassay are mean ± sem. Significant changes within a group were determined by a paired t-test and between groups by an un-paired t-test.

3.1.2 RESULTS

Graphs **3.1(c)** and **3.1(d)** show plasma oxytocin values in the four groups of animals used. For construction of these graphs, the plasma oxytocin concentrations for the two groups of morphine animals have been combined (samples 1-4), until the animals received either naloxone or MR2266 i.v., when the results have been plotted separately for each experimental group. Similarly, the plasma oxytocin concentrations in the control groups were combined until after sample 4 when the animals received one of the two opioid antagonists.

In graph **3.1(c)**, oxytocin content in plasma sample 4, taken at t=30 minutes, 15 minutes after morphine administration was significantly decreased compared to basal levels ($p < 0.05$, paired t-test). Both naloxone and MR2266 caused a significant elevation in oxytocin secretion. Plasma oxytocin was elevated from 11.5 ± 2.2 pg/ml post morphine injection to 52.7 ± 19.9 pg/ml by naloxone and to 78.1 ± 27.6 pg/ml by MR2266. Statistical comparisons using a paired t-test show that oxytocin content in samples 6 and 7 (t=16 and t=26 mins post antagonist) were significantly elevated with respect to oxytocin content pre-antagonist ($p < 0.05$ for both naloxone and MR2266). Although MR2266 would appear to have caused a greater elevation in plasma oxytocin content than naloxone, statistical tests show that there is no

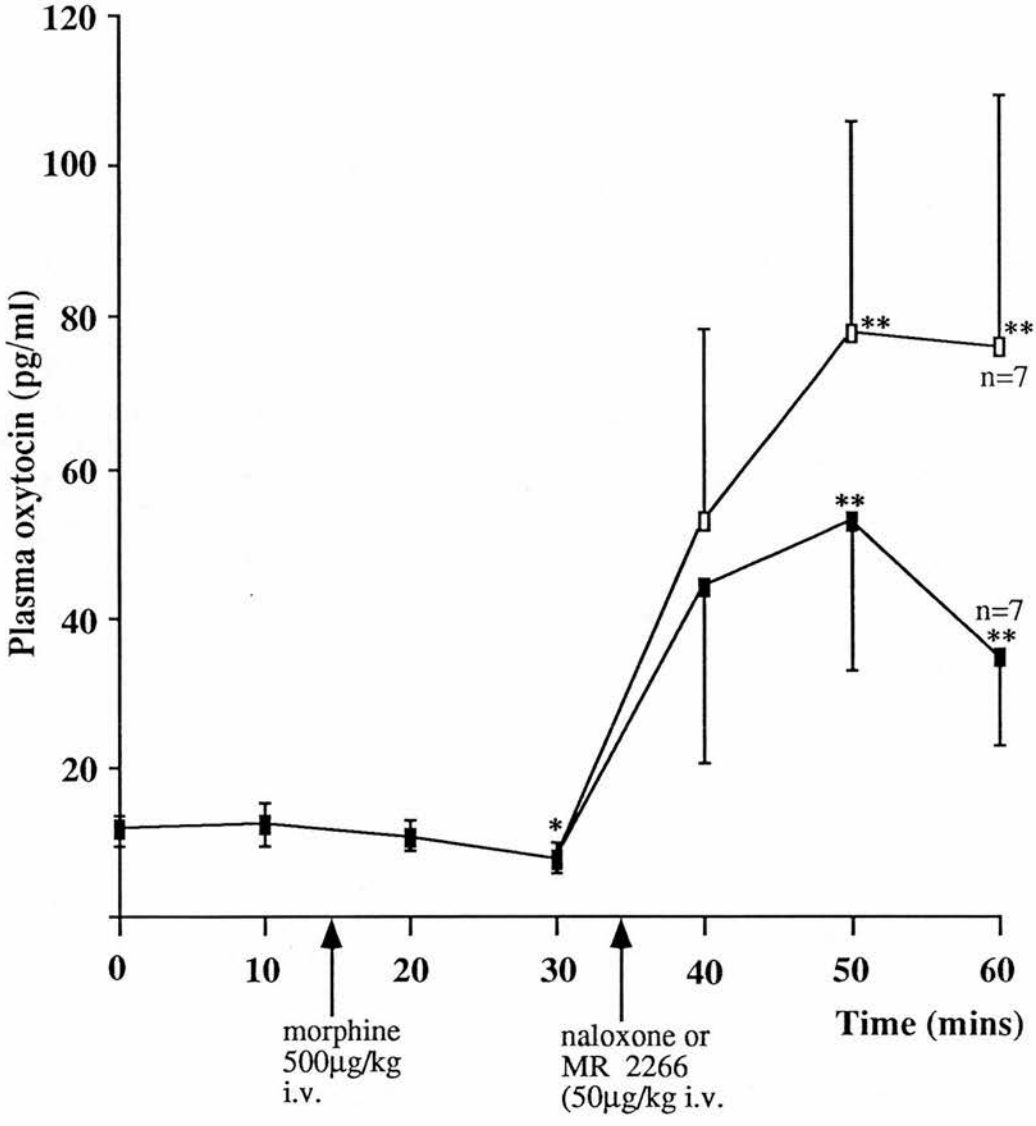


Fig.3.1 (c)

Plasma oxytocin in arterial blood samples taken from urethane anaesthetized rats.Morphine (500µg/kg) was administered i.v. at t=15 mins.At t=34 minutes the rats were given naloxone (50µg/kg) i.v. ,represented by the filled squares or MR2266 (50µg/kg) i.v.,shown by the open squares.
Statistical comparisons:
* $p < 0.05$ with respect to sample 1 (paired t-test)
** $p < 0.05$ with respect to sample 4 (paired t-test)

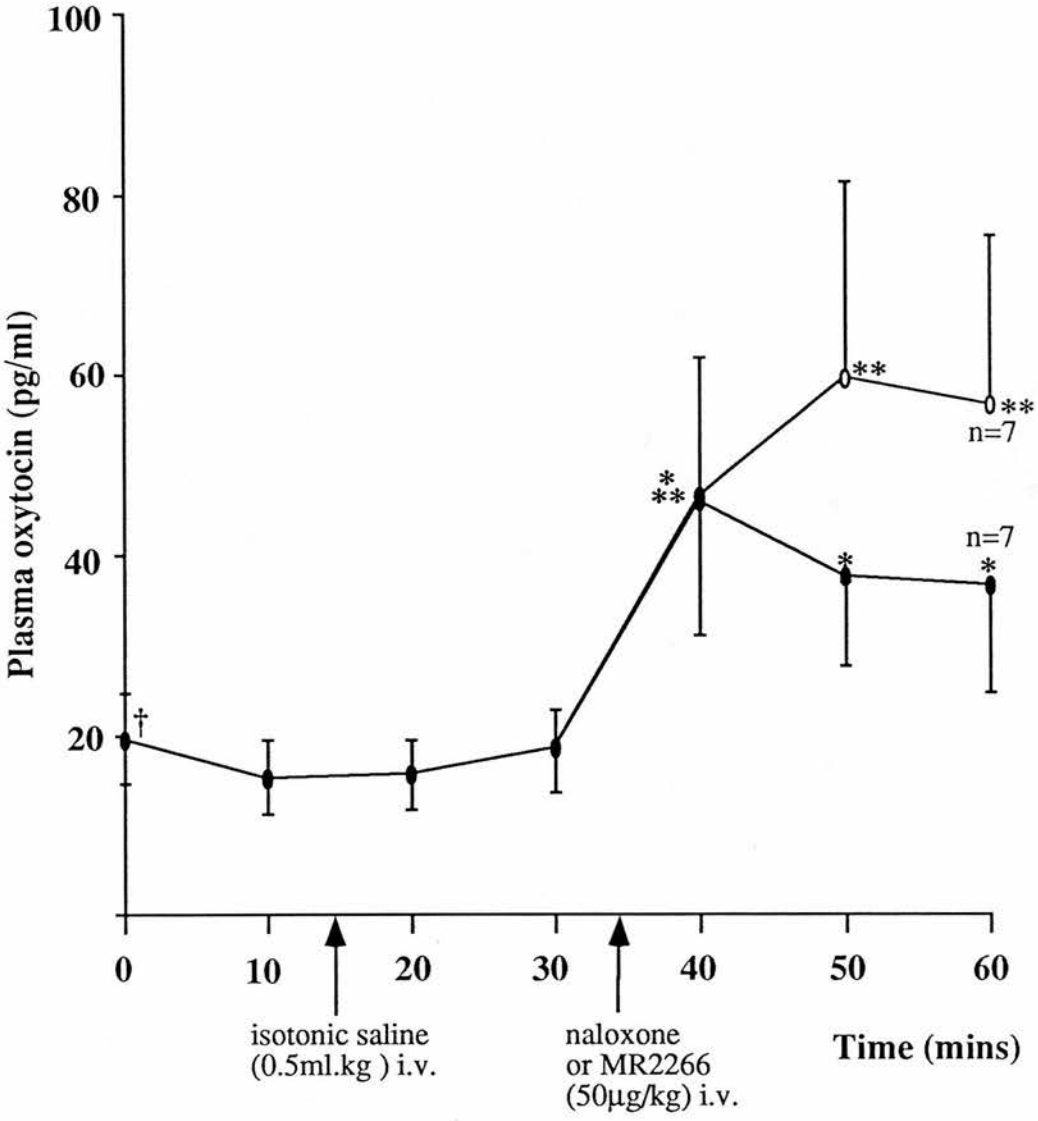


Fig 3.1 (d)

Plasma oxytocin in arterial blood samples taken from urethane anaesthetized rats.Saline (0.5ml/kg) was administered i.v. at t=15 mins.at t=34 minutes the rats were given naloxone (50µg/kg) i.v.,represented by the filled circles or MR2266 (50µg/kg) i.v.,shown by the open circles.
Statistical comparisons:
† $p < 0.05$ with respect to sample 1,morphine group (unpaired t-test).
* $0.005 > p < 0.05$ with respect to sample 4 (paired t-test)
** $p < 0.05$ with respect to sample 4 (paired t-test).

significant difference in the magnitude or time course of their antagonism against morphine using an unpaired t-test.

In control animals, shown in graph **Fig 3.1(d)**, the basal concentration of oxytocin in peripheral plasma started off higher than in the group of rats that received morphine ($p < 0.05$, unpaired t-test). Intravenous injection of isotonic saline (0.5ml/kg) had no significant effect on oxytocin secretion. Both naloxone and MR2266 caused significant elevation in oxytocin secretion, plasma oxytocin being elevated from 18.3 ± 4.8 pg/ml post saline injection to 45.8 ± 14.5 pg/ml by naloxone and 59.3 ± 22.0 pg/ml by MR2266. Statistical comparisons using a paired t-test show that oxytocin concentration in plasma samples 5, 6 and 7 (t=6, t=16 and t=26 minutes post antagonist) to be significantly elevated from pre-antagonist values. ($p > 0.005$ < 0.05 for naloxone and $p < 0.05$, for MR2266). Once again there was no significant difference between the magnitude of response to naloxone or MR2266 using an un-paired t-test.

3.1.3 DISCUSSION

In these experiments, i.v. morphine significantly decreased basal plasma oxytocin in urethane anaesthetized rats with respect to saline treated control animals. Since there are no μ-receptors present in the posterior pituitary (or possibly a very low distribution) this action is likely to be centrally mediated at oxytocin neurone cell bodies. Acute morphine may have an inhibitory effect on the electrical activity of oxytocin neurones such as that observed in hypothalamic slice preparations (**Wakerley et al, 1983; Pumford, Hunter & Russell, 1987**).

Acute dependence to morphine does not develop since there is no significant difference between elevated plasma oxytocin values in the control or morphine treated rats following naloxone injection.

In both the morphine and saline treated rats there was no significant difference between the effects of MR2266 or naloxone to elevate oxytocin secretion. The predominant effect seen therefore, is probably due to reversal of endogenous opioid tone at the posterior pituitary and not due to reversal of centrally mediated morphine actions. It is not possible to discriminate their effects with respect to the opioid receptor subtpes involved or their site of action.

Although naloxone is regarded primarily as a μ-opioid receptor agonist, it has been shown to effectively reverse opioid-mediated inhibition of oxytocin secretion known to be mediated by κ-receptors in the neural lobe (**Coombes and Russell, 1988, chapter 2 this thesis**).

In the morphine treated group, following injection of naloxone, plasma oxytocin is elevated in plasma sample 5 and continues to rise in sample 6 (t=6 and 16 minutes post-naloxone respectively). In control animals, plasma oxytocin content is elevated in sample 5 but is starting to decline in sample 6 (although there is no significant difference between plasma oxytocin in the two groups fromsample 6).

The initial elevation of plasma oxytocin may be due to reversal of endogenous opioids acting at the posterior pituitary and the subsequent continued elevation of plasma oxytocin in the morphine treated group from reversal of centrally acting morphine. Although the values of plasma oxytocin are not different between the groups, the timed secretion profile of oxytocin may be, an effect which could arise due to the time taken for diffusion access of the opioid antagonist, from the periphery, to central μ-receptors where morphine is acting.

In-vitro experiments using the isolated neural lobe preparation have shown that both naloxone and MR2266 elevate stimulus evoked oxytocin presumably by actions against κ-receptor mediated endogenous opioid tone

(**Bicknell, Chapman & Leng, 1985**). Detailed dose-response analysis showed that MR2266 displayed a biphasic dose response relationship with oxytocin secretion, displaying inhibitory actions at high doses in the perifusion medium (10^{-4}M). MR2266 was regarded as unsuitable for use to determine κ-receptor involvement in the regulation of oxytocin secretion in this system due to its partial agonist activity.

Other **in-vitro** experiments (**Kosterlitz & Paterson, 1985**) determined that MR2266 could not be used as a selective κ-opioid receptor antagonist except in isolated tissues where there are no μ or δ receptors - such as the rabbit vas deferens.

In an experiment where MR2266 was used to investigate the role of opioids in water and isotonic saline intake in mice (**Ukai, Nakayama and Kameyama, 1988**) MR2266 was found to reduce water intake (believed to be a κ-mediated effect) with an accompanying motor dysfunction. These locomotory effects were similar to those induced in mice by μ-opioid receptor antagonists naloxone and naltrexone (**Ukai & Kamayama, 1985**). However MR2266 did not produce opposing effects to those induced by specific μ (morphine) and κ (dynorphin 1-7 and ethlyketocyclazocine) agonists implying the effects may be due to non-specific opioid receptor blockade rather than selective μ and κ actions.

Acute naloxone excites oxytocin neurones in anaesthetized, morphine dependent rats, which at the lowest dose used (10^{-8}M) was considered to be a μ-opioid effect (**Leng, Russell & Grossman, 1989**).

MR2266 was ~ 100 times less potent than naloxone in exciting oxytocin neurones in morphine dependent rats (as estimated from the lowest doses needed to produce significant excitation).

The withdrawal excitation is mediated via receptors with different properties from the κ-receptor in the posterior pituitary, since MR2266 is equipotent, or more potent than naloxone in enhancing stimulated oxytocin

secretion from the neural lobe (**Carter & Lightman, 1987**).

These observations indicate that μ-receptors are involved in the central actions of morphine on oxytocin neurones, including the development of dpendence but they do not exlude the involvement of κ-receptors in centrally-mediated opioid modification of oxytocin secretion (since κ-receptors are also found in the SON).

These experiments confirm the results obtained in this Chapter. MR2266 is an effective non-specific opioid-antagonist, its partial agonist activity and non-selectivity with regard to κ-opioid-receptors, however make it unsuitable (at the doses used) to discriminate between μ and κ-receptor mediated control of oxytocin when coupled with naloxone.

This experiment could not isolate the site or the receptor subtype for morphine action on oxytocin secretion using these opioid antagonists.

3.2 Investigation of actions on oxytocin secretion from the neural lobe of the rat of RX8008M (16-Me cyprenorphine, a relatively selective δ and μ-opioid receptor antagonist

INTRODUCTION

Research into the function of μ, δ and κ-opioid receptors subtypes is frustrated by the lack of selective antagonists for these receptors. This is effectively demonstrated by the experiment described in **3.1**.

RX8008M (16-Me cyprenorphine) is reported to be a selective, non-peptide, δ-receptor antagonist. Its structure is shown in **Fig 3.1(a)**.

Cyprenorphine, an oripavine, has a virtually identical antagonist profile to naloxone and naltrexone but RX8008M with its 16-methyl substituent group has very

different properties.

(i) It has no partial agonist activity demonstrated by cyprenorphine in the mouse vas deferens (MVD) and guinea pig ileum (GPI) preparation.

(ii) It has decreased affinity for the κ and μ-opioid receptors compared to cyprenorphine.

(iii) The affinity for the δ-opioid receptor is unchanged (**Smith, 1987**).

The Ke values for naloxone and RX8008M are as follows (data obtained from the MVD preparation)

	μKe(nM)	κKe(nM)	δKe(nM)
Naloxone	1.4 ± 0.1	15.9 ± 6.7	9.6 ± 2.3
RX8008M	1.7 ± 0.2	59.6 ± 9.8	0.73 ± 0.15

As well as being a selective δ-antagonist, RX8008M shows selectivity for μ-receptors over κ-receptors, which makes it a possible tool for discriminating between μ and κ-opioid receptor mediated events.

In-vivo studies using RX8008M (**Birch, Hayes et al, 1988**) tested its effects on the antinociceptive action of μ (morphine) and κ (U50,488H) selective agonists in the mouse abdominal constriction test.

RX8008M was co-administered subcutaneously with the agonist, preceded the agonist by 30 or 60 minutes, or was given 15 minutes after administration of the agonist.

RX8008M (0.33, 1 and 3mg/kg) was found to cause a dose dependent antagonism of the antinociceptive action of μ-selective agonist morphine but had no effect on antinociceptive effects mediated by the κ-selective agonist U50,488H. Pretreatment with RX8008M 30 and 60 minutes prior to agonist injection only produced a small shift in the dose-response curve of morphine indicating its actions to be short lived.

The advantages of this compound as a tool for differentiating between μ and κ opioid receptor mediated actions are twofold. Firstly, it is a

competitive antagonist unlike β-fulnaltrexamine (a non-competitive antagonist with relative selectivity for μ over k-opioid receptors) which has also been tested for its ability to distinguish between μ and κ opioid receptors (**Hayes, Skingle & Tyers, 1987**). Secondly it is effective by the subcutaneous route of administration unlike the selective μ-opioid antagonist CTP (D-Phe-Cys-Tyr-D-Try-Lys-Thr-Pen-Thr-NH_2, a cyclic somatostatin octapeptide) which is only effective **in vivo**, by intrathecal or intracerebroventricular administration (**Shook, Pelton et al, 1987**).

A species difference in antagonist activity of RX8008M has been found (**Hayes & Birch, 1988**). A shift to the right of dose-response curves for antinociceptive activity of μ-agonists morphine and fentanyl by RX8008M, were similar in the mouse and guinea pig but only the guinea pig showed a significant shift in its dose-response curve to κ-agonists U50,488H, U69,593 and tifluadom. However, this effect was found not to be dose dependent indicating RX8008M to be acting in a non-specific manner or U50,488H, U69,593 and trifluadom to exert some μ or δ receptor mediated antinociceptive effects in the guinea pig. In the rat RX8008M was unable to antagonize U50,488H induced diuresis at doses of up to 16mg/kg administered subcutaneously.

The following study set out to test the ability of naloxone and RX8008M, to antagonize endogenous and exogenous opiates acting at the neural lobe of the rat **in-vitro** and **in-vivo**. If successful, this would allow opioid effects on oxytocin secretion from nerve terminals in the neural lobe to be distinguished on the basis of whether they were mediated through μ or κ-opioid receptors.

3.2.1. IN VITRO METHODOLOGY.

These experiments were performed at the AFRC Institute

for Animal Physiology and Genetics Research, Babraham, Cambridge with the help of Dr. R. J. Bicknell.

3.2.1.(i) ANIMALS

Neural lobes were obtained from adult, virgin female Sprague-Dawley rats (250-300g bodyweight) which were housed under controlled photoperiod (14hrs light/10hrs dark) and temperature (22-23 °C). Standard rat diet and tap water were freely available.

3.2.1.(ii) TISSUE PREPARATION

On the morning of the experiment the animals were stunned and killed by cervical dislocation. A dorsal midline skin incision exposed the skull vault and the parietal bones were quickly removed with bone nibblers and scissors. A transverse cut across the brainstem just anterior to the cerebellum allowed the brain to be tilted forward in the cranial cavity. This enabled severance with scissors of the exposed optic nerves and facilitated removal of the whole brain leaving the pituitary gland and part of the neural stalk **in-situ**. The dural membrane covering the pituitary was removed using watchmakers forceps and the pituitary gently lifted out into a petri dish of pre-warmed medium at 37°C (see **3.2.1.(iv)**). The tissue remained in medium from this point onwards throughout the experiment.

The neurointermediate lobe was separated from the anterior lobe of the pituitary under a dissecting microscope by careful handling with watchmakers forceps.

In a fresh dish of medium the neurohypophysis was secured by remnants of the neural stalk and lobules of the intermediate lobe were carefully cleared away until they could be freed at the edges using a microscalpel with minimal disruption to the neural lobe tissue.

The neural lobe was impaled on one of a pair of platinum electrodes protruding from the upper part of the superfusion chamber (**Fig 3.2.(a)**).The electrode assembly was immersed in fresh medium forming a bubble of solution and was lowered into the perspex chamber,which was screwed onto the electrode assembly to seal the chamber.

The perifusion medium was pumped through the chamber at 150µl/min by a peristaltic pump (**Gilson minipuls 2**) and the perifusate collected from an outlet into an automatic fraction collector preset to collect fractions over time intervals of 5 minutes.The entire assembly was housed in a thermostatically controlled cabinet at 37°C.

3.2.1.(iii) TIMING SCHEDULE

The neural lobes were left for 45 minutes in the chamber filled with medium without collecting any fractions,to allow hypersecretion of hormones resulting from tissue trauma to subside.After this period the tissue was perifused for 5 minutes to "wash out" secretions of the gland during equilibration,the resulting perifusate being discarded.Subsequently,15 fractions each accumulated over 5 minutes were collected from the perifused tissues.

The first stimulus train S_1 was delivered at the start of fraction 2.At the end of fraction 6 the tissues were perifused with medium containing RX8008M or naloxone (10^{-5}M; 10^{-6}M; 3.3×10^{-7}M) or with medium containing no drug as time controls.

The second stimulus S_2 was delivered at the commencement of fraction 11.At the end of the experiment fractions 6-9 were discarded as these represented fractions collected during the changeover period of media and the amount of oxytocin secreted during this time was not required.Fractions 1-5 and 10-15 were frozen at -20°C until assayed for oxytocin and vasopressin content (see 1.7).

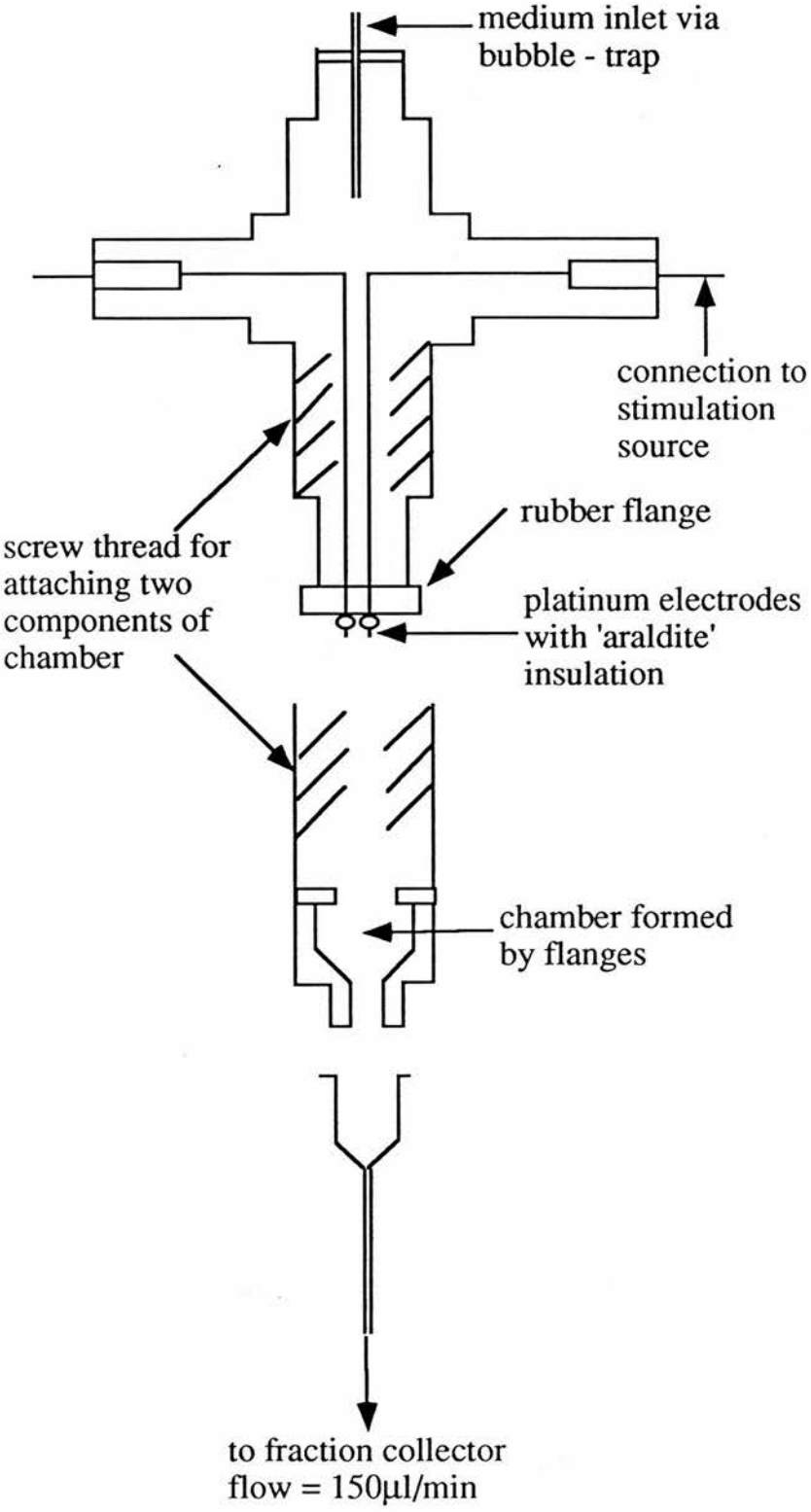


Fig 3.2 (a)

Chamber used for stimulation and perifusion of isolated neural lobe preparations

Previous studies have shown that secretion of AVP can be inhibited by the action of opioids at nerve terminals in the neural lobe (**Iversen, Iversen & Bloom, 1980; Lightman, Iversen & Forsling, 1982**). Naloxone however, has no potentiating effect on vasopressin release (**Bicknell & Leng, 1982**) which implies that the interaction of opioids with the vasopressin system may not involve naloxone-sensitive opioid receptors or that there is differential opioid innervation within the neural lobe to vasopressin neurones (**Bicknell, Chapman & Leng, 1985**).

3.2.1.(iv) MEDIA AND DRUGS

The buffered isotonic stock medium was made up as follows:- $MgCl_2.6H_2O$ (0.02mM/l); Hepes pH 4.3 (0.1mM/l); KCl (0.05mM/l); $CaCl_2.2H_2O$ (0.02mM/l); NaCl (1.45mM/l); $NaHCO_3$ (0.05mM/l).

On the day of use, glucose (1g/l) and bovine serum albumin (0.5g/l) were added to the medium at 37°C with stirring. The pH was buffered to 7.3-7.4 by adding 1.0M NaOH dropwise. All drug solutions were made up in the medium.

RX8008M, kindly provided by Dr. C. Smith (**Reckitt & Colman**), was initially dissolved in 0.1M HCl (≈30µl/mg) and made up to volume with distilled water, gently warmed, to give a stock solution of 2.45mM. Aliquots of 100µl were diluted in an appropriate volume of stock medium to give solutions of 1×10^{-5}M, 1×10^{-6}M and 3.3×10^{-7}M.

Naloxone was dissolved in distilled water to give a 5mM stock solution which was frozen as 100µl aliquots until ready for use. These were also diluted in an appropriate volume of stock solution to give doses of 1×10^{-5}M, 1×10^{-6}M and 3.3×10^{-7}M.

3.2.1.(v) ELECTRICAL STIMULUS

The stimulus parameters used to elicit oxytocin secretion from the isolated neural lobe were similar

to those associated with the firing of oxytocin neurones during milk-ejecting activity and could therefore be used in the parallel **in-vivo** study where intramammary pressure changes recorded in lactating rats were used as an index of oxytocin secretion. The stimulus consisted of matched 0.5mA biphasic pulses of 1ms pulse width administered as a train of 150 pulses at 50Hz.

3.2.2. IN VIVO METHODOLGY

3.2.2.(i) ANIMALS

The animals used were female Sprague-Dawley rats between day 7-14 of their first lactation of bodyweight 340-400g (as described in **1.1**)

3.2.2.(ii) SURGERY

On the morning of the experiment the rat which had been separated from all but one of its litter overnight (**Lincoln, Hill & Wakerley, 1973**), was anaesthetized by intraperitoneal injection of urethane (1.25g/kg).

The trachea, jugular vein, femoral vein and three abdominal mammary glands were cannulated (see **1.2**).

Drugs were administered via the jugular vein and oxytocin in bolus doses, through the femoral vein. The mammary gland cannulae were connected to pressure transducers which recorded changes in intra-mammary pressure for the bioassay (see **1.6**).

The neural stalk was exposed by the ventral approach using the surgical method described in **1.5.(i)**.

A concentric bipolar stimulating electrode lowered into the stalk enabled stimulation of descending magnocellular oxytocin neurone axons from the hypothalamus resulting in the release of oxytocin from the nerve terminals in the neural lobe.

3.2.2.(iii) STIMULUS

Trains of matched biphasic pulses (0.5mA peak to peak, 1ms duration at 50Hz were used. Individual stimulus trains consisted of between 60 and 240 pulses, designed to release approximately 0.75mU oxytocin (see **1.5.(ii)**)

3.2.2.(iv) OXYTOCIN BIOASSAY

Oxytocin released as a result of neural stalk stimulation resulted in an increase in intramammary pressure. By administering bolus doses of oxytocin 0.25, 0.5, 0.75. and 1mU, a pressure/response relationship was determined from which the amount of stimulus evoked oxytocin could be calculated. This is described in more detail in **1.6.**

3.2.2.(v) DRUG PROTOCOL

In the **in-vitro** experiment the concentrations of RX8008M and naloxone used in the perifusion medium were 10^{-5}M, 10^{-6}M and 3.3×10^{-7}M. **In-vivo**, it is more difficult to expose the neural lobe to such a precise drug dose due to the uncertain time course of tissue distribution.

An intravenous route of administration was chosen to permit rapid exposure to the antagonists but a decrease in initial effect was expected as the drugs distributed into various body compartments. For this reason the antagonists effect on stimulus evoked oxytocin was tested approximately 2 minutes after injection.

In order to obtain drug doses as similar as possible to those used on the isolated neural lobe **in-vitro**, the following procedure was carried out. The blood volume of each individual rat was estimated by assuming the total blood volume to be equivalent to 50ml/kg. The drug solutions were then made up so that intravenous injection of 0.1ml of solution would result in the required concentration of drug being present, initially, in the bloodstream of the rat. The

oxytocin terminals in the highly vascular posterior pituitary would be exposed to slightly less, allowing for tissue distribution.

Two lower doses of RX8008M and naloxone were used, 3.3×10^{-10} and 3.3×10^{-9}M, after preliminary experiments indicated RX8008M and naloxone to be equipotent at the higher doses used in the **in-vitro** study.

In the **in-vitro** experiment the drugs were compared by their ability to antagonize endogenous opioids acting at the neural lobe. **In-vivo** the animals were given a dose of the kappa opioid receptor antagonist U50,488H, sufficient to cause ≃ 50% inhibition of stimulus evoked oxytocin release (500μg/kg) and the effectiveness of RX8008M and naloxone at wholly or partly reversing this inhibition compared. This approach was chosen as subtle changes in endogenous opioid tone acting on oxytocin neurones would be difficult to detect in an autobioassay and naloxone has variable effects on electrically-evoked oxytocin secretion **in-vivo**.

The strategy of drug administration was as follows:-

(i) doses of exogenous oxytocin were given to construct a dose response curve followed by electrical stimulation of the neural stalk to elicit oxytocin release from the neural stalk as described in **1.5.(ii)**.

(ii) 500 or 1000μg/kg U50,488H was injected intravenously and 2½-3 minutes later the stimulus repeated to check that oxytocin secretion in response to stimulation had been inhibited by ≃ 50%.

(iii) Immediately after this the lowest dose of RX8008M was administered intraveously and 1½-2 minutes post injection another stimulus applied to see if the inhibition had been reversed.

(iv) The lowest dose of naloxone was then administered intravenously and 1½-2 minutes after this the stimulus was repeated so that any effects of naloxone on

U50,488H mediated inhibition of oxytocin release could be determined.

(v) Another dose response curve to exogenous oxytocin finished the sequence so that the drug effects could be calculated and changes in mammary gland sensitivity to oxytocin taken into account.

(vi) The neural lobe was allowed to recover, aided by further dilution of the antagonist in the extracellular volume and its metabolism, until its response to stimulation of the neural stalk resulted in approximately the same release of oxytocin as before any drug treatment. The regime (i)-(v) was repeated with the same dose of U50,488H and the next doses of RX8008M and naloxone.

To eliminate any influence that the order of drug addition may have had on the results, in a further 5 animals naloxone was given prior to RX8008M but otherwise the protocol (i)-(vi) was used.

To check that U50,488H mediated inhibition of stimulus evoked oxytocin outlasted the time taken to give RX8008M and naloxone in any cycle, some animals were given 500 or 1000µg/kg of U50,488H and the time course of its action monitored without giving the antagonists.

3.2.3. ANALYSIS OF RESULTS

Statistical comparisons were made within experimental groups using a paired t-test and between groups using an unpaired t-test.

3.2.3.(i) IN_VITRO EXPERIMENT

For the 15 perifusate fractions collected from each isolated neural lobe chamber, fraction 1 represented the basal secretion of oxytocin from the gland prior to the first stimulus train and was subtracted from each of the values of oxytocin obtained in fractions 2-5. The excess oxytocin secreted above this basal value from fractions 2-5 were combined and this taken

as the increase in oxytocin released in response to stimulus train S_1.

The same procedure was carried out for fractions 10-15 where fraction 10 represented the basal secretion of oxytocin prior to stimulus S_2 and the excess oxytocin release above this from each of samples 11-15 were combined and taken as the stimulated release due to the stimulus train S_2.

To overcome variability between glands, absolute amounts of oxytocin or vasopressin (detected by radioimmunoassay as described in **1.7**), released by the neural lobe **in vitro** were combined as described above and the $S_2:S_1$ ratio for each hormone calculated for each neural lobe.

Mean $S_2:S_1$ ratios were then calculated for each experimental group of isolated tissue preparations. Clearly a value greater than 1.0 would indicate a facilitatory effect on hormone release from the nerve terminals and a value less than 1.0 would indicate inhibition of hormone release.

$S_2:S_1$ ratios for oxytocin and vasopressin obtained for each dose of naloxone and RX8008M are shown in the histogram **3.2(b)** .

3.2.3.(ii) IN VIVO EXPERIMENT

The depression of electrically evoked oxytocin following administration of the κ-opioid receptor agonist U50,488H and any subsequent change following RX8008M and naloxone injection were expressed as percentages of the initial amount of oxytocin released on stimulation of the neural stalk before U50,488H or the antagonists were given.

The values were obtained from the dose-response curves constructed from the mammary gland autobioassay. The results from animals which received RX8008M before naloxone were combined with the animals that received naloxone before RX8008M as there was no difference in the antagonist action relating to the order in which

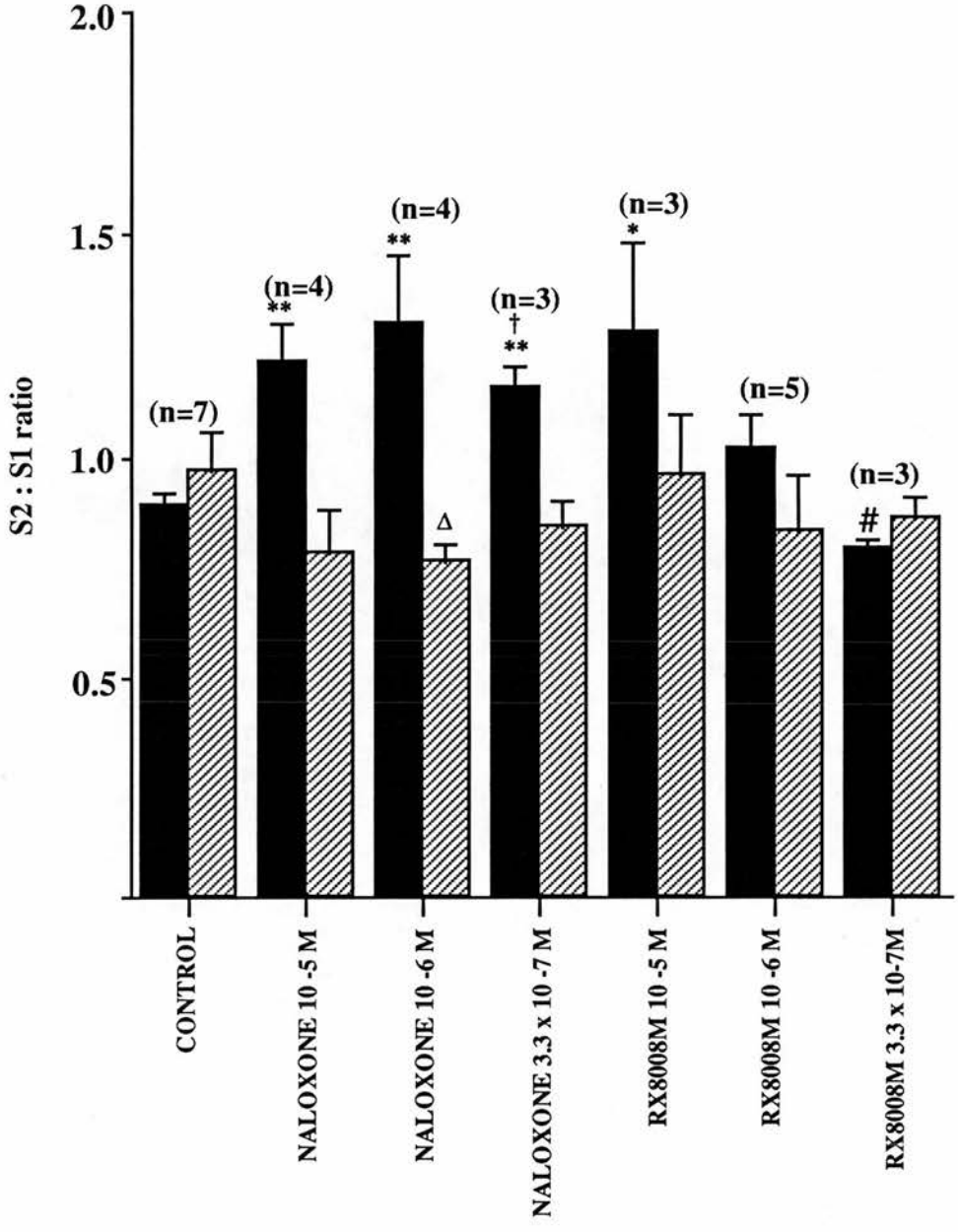


Fig 3.2 (b)

In vitro S2 : S1 ratios obtained from RIA values of oxytocin and vasopressin released from isolated neural lobes upon electrical stimulation.
Statistical comparisons:
* $p < 0.05$,** $p < 0.005$,# $p < 0.05$ (inhibition)with respect to control S2 :S1 oxytocin ratio (un-paired t-test).
† $p < 0.005$ with respect to S2 : S1 oxytocin ratio from 3.3 x 10 -7M RX8008M (un-paired t-test).
Δ $p = 0.05$ with respect to control vasopressin S1 : S2 ratio (un-paired t-test).
Solid bars represent stimulated oxytocin release,striped bars stimulated vasopressin release.

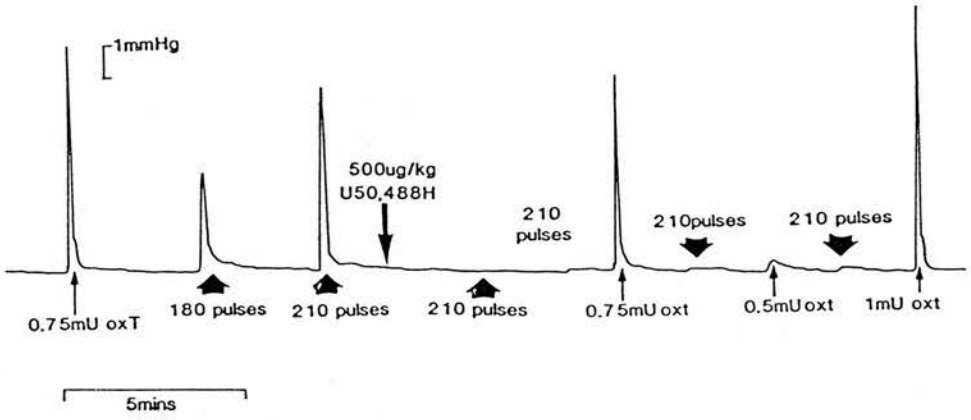
Legend for 3.2(c)

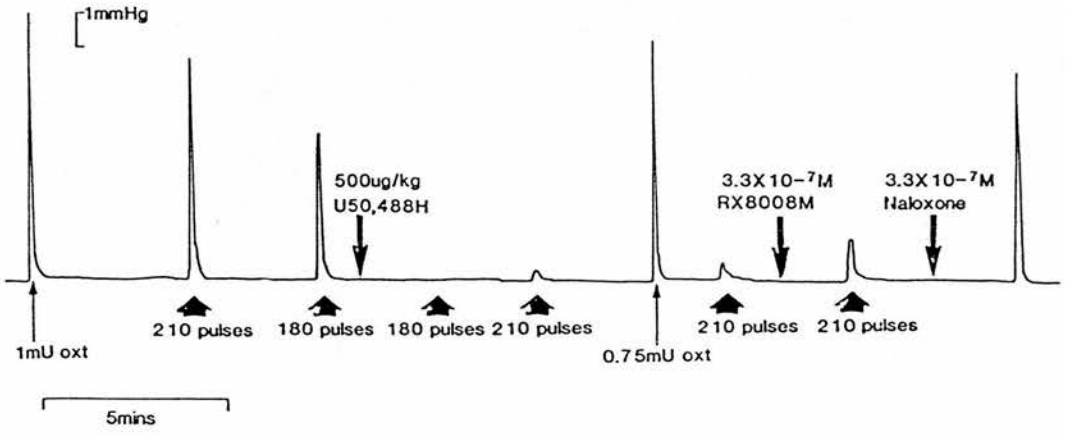
Intramammary pressure recording traces from animals that were treated with the opioid antagonists RX800M or naloxone.

The top trace shows how U50,488H (500μg/kg i.v.) profoundly inhibits electrically-evoked oxytocin release, from stimulation of the neural stalk (see **1.5**) whilst sensitivity of the mammary gland to oxytocin is unaltered (see response to 0.75 mU exogenous oxytocin).

The centre trace shows how RX800M and naloxone (3.3×10^{-7}M i.v.) reverse U50,488H mediated inhibition of oxytocin release, naloxone reversing the inhibition to the greatest extent.

The bottom trace show the effect of giving the same dose of opioid antagonists in reverse order, with naloxone still showing the greater anatagonism to U50,488H, indicating that the order than the drugs are given does not significantly affect the subsequent response (i.e. there is no appreciable summation effect).


1mmHg
500ug/kg
U50,488H
210
pulses
210pulses
210 pulses
0.75mU oxT
180 pulses
210 pulses
210 pulses
0.75mU oxt
0.5mU oxt
1mU oxt
5mins


1mmHg
500ug/kg
U50,488H
3.3X10-7M
RX8008M
3.3X10-7M
Naloxone
1mU oxt
210 pulses
180 pulses
180 pulses
210 pulses
0.75mU oxt
210 pulses
210 pulses
5mins

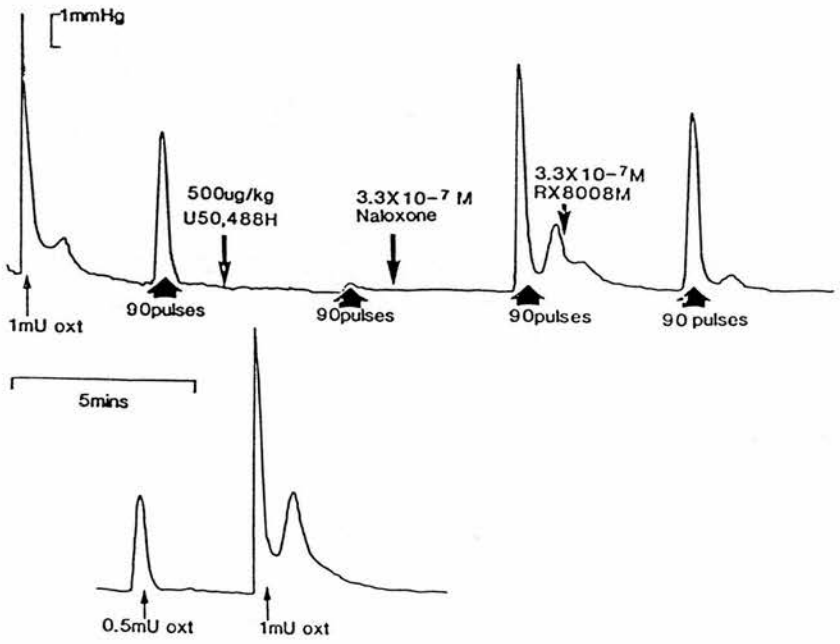

1mmHg
500ug/kg
U50,488H
3.3X10-7 M
Naloxone
3.3X10-7M
RX8008M
1mU oxt
90pulses
90pulses
90pulses
90 pulses
5mins
0.5mU oxt
1mU oxt

Fig 3.2c

they were given as shown in the traces in **Fig 3.2(c)**. The results have also been expressed in an alternative way. The difference was calculated between oxytocin release (expressed as a % initial amount of oxytocin released on neural stalk stimulation) after U50,488H but before RX8008M and naloxone (Bd) to that after antagonist administration (Ad) i.e by relating the effect of antagonist to oxytocin secretion during U50,488H mediated inhibition (Ad-Bd). This was to try and isolate effects of the antagonists and clarify the extent of their ability to antagonize U50,488H mediated inhibition of stimulus evoked oxytocin release from the neural lobe. It must be remembered that the doses of RX8008M and naloxone are those initially in the bloodstream of the rat and may change on tissue distribution. The doses injected are approximately equivalent to 30ng/kg, 0.3μg/kg, 3μg/kg, 10μg/kg and 0.1mg/kg naloxone and 40ng/kg, 0.4μg/kg, 4μg/kg, 12μg/kg and 0.12μg/kg RX8008M (corresponding to 3.3×10^{-9}M, 3.3×10^{-8}M, 3.3×10^{-7}M, 1×10^{-6}M and 1×10^{-5}M respectively) for the bodyweight of each rat.

3.2.4. RESULTS

IN VITRO RESULTS

Mean $S_2:S_1$ values obtained for each experimental group of **in vitro** neural lobe preparations, illustrated in **Fig 3.2(b)** show that naloxone enhanced oxytocin secretion above control values when included in the perifusion medium at all the concentrations tested ($p < 0.005$, unpaired t-test). RX8008M showed a dose-related effect on oxytocin secretion, significantly elevating it at 10^{-8}M ($p < 0.05$) but just causing an inhibition at 3.3×10^{-7}M ($p = 0.05$, unpaired t-test.) Naloxone was only more effective than RX8008M in its elevation of oxytocin secretion at a dose of 3.3×10^{-7}M ($p < 0.005$, unpaired t-test). Vasopressin secretion from these isolated neural lobe preparations was unaffected

with respect to the control values in the presence of RX8008M and naloxone with the exception of naloxone at a concentration of 1×10^{-6}M which caused a just significant depression in vasopressin secretion (p =0.05). **In vitro** it would appear that action of RX8008M against endogenous opioids acting at receptors on the neural lobe to modify oxytocin secretion is dose dependent, only having a significant effect at the highest dose tested.

IN VIVO RESULTS

U50,488H (500μg/kg) was an effective dose at achieving a 50% inhibition of oxytocin secretion elicited by electrical stimulation of the exposed neural stalk in the rat (p <0.0005, paired t-test). This dose was chosen on the basis of the results obtained in **Chapter 2**, where the ID_{50} of U50,488H against oxytocin secretion was found to be ≈500μg/kg (**Coombes & Russell, 1988.**)

The duration of U50,488H mediated inhibition outlasted the time course of the protocol for intravenous injection of RX8008M and naloxone.

Intramammary pressure recording traces showed naloxone to be more effective at antagonizing κ-opioid receptor mediated inhibition of oxytocin secretion in the anaesthetized rat. The order of injection of the antagonists proved to be unimportant responses to intravenous RX8008M being smaller than to naloxone even when it was administered after naloxone, demonstrating that summation of the antagonist effects was not occurring (see **Fig. 3.2(c)**).

Both RX8008M and naloxone in increasing doses were able to wholly or partly reverse U50,488H effects at the neural lobe, in what appeared to be a dose-dependent manner. The results, expressed as a % initial amount of electrically evoked oxytocin release before administration of either agonist or antagonists, are shown in the histogram in **Fig 3.2(d)**. RX8008M elevated

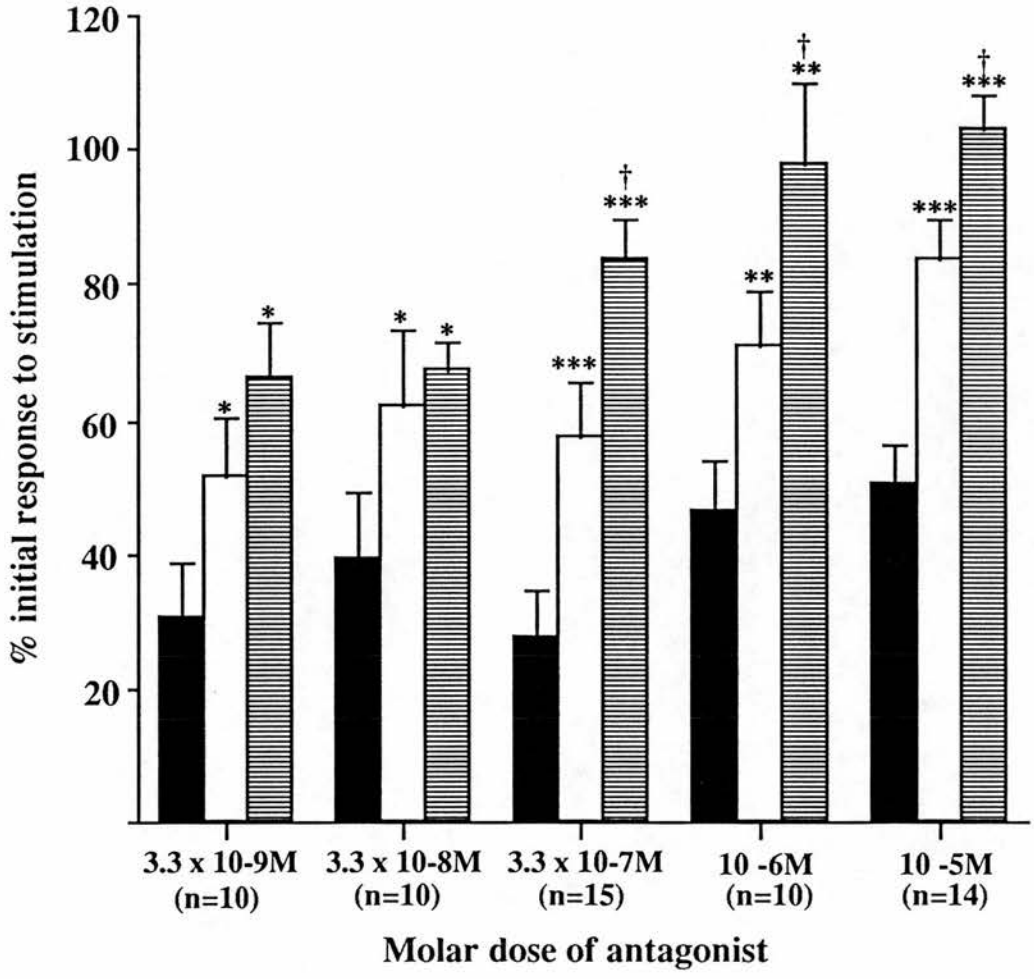


Fig 3.2 (d)

Effect of RX8008M (open bars) and naloxone (striped bars) on stimulated oxytocin secretion inhibited to < 50% by U50,488H (500μg/kg i.v.,solid bars) in urethane anaesthetized lactating rats.
Statistical comparisons :
* $p < 0.05$, **$p < 0.005$, *** $p < 0.0005$, for both RX8008M and naloxone with respect to U50,488H inhibited oxytocin response to neural stalk stimulation.
† $p < 0.05$ naloxone stimulatory effect on electrically-evoked oxytocin secretion with respect to the effect by RX8008M

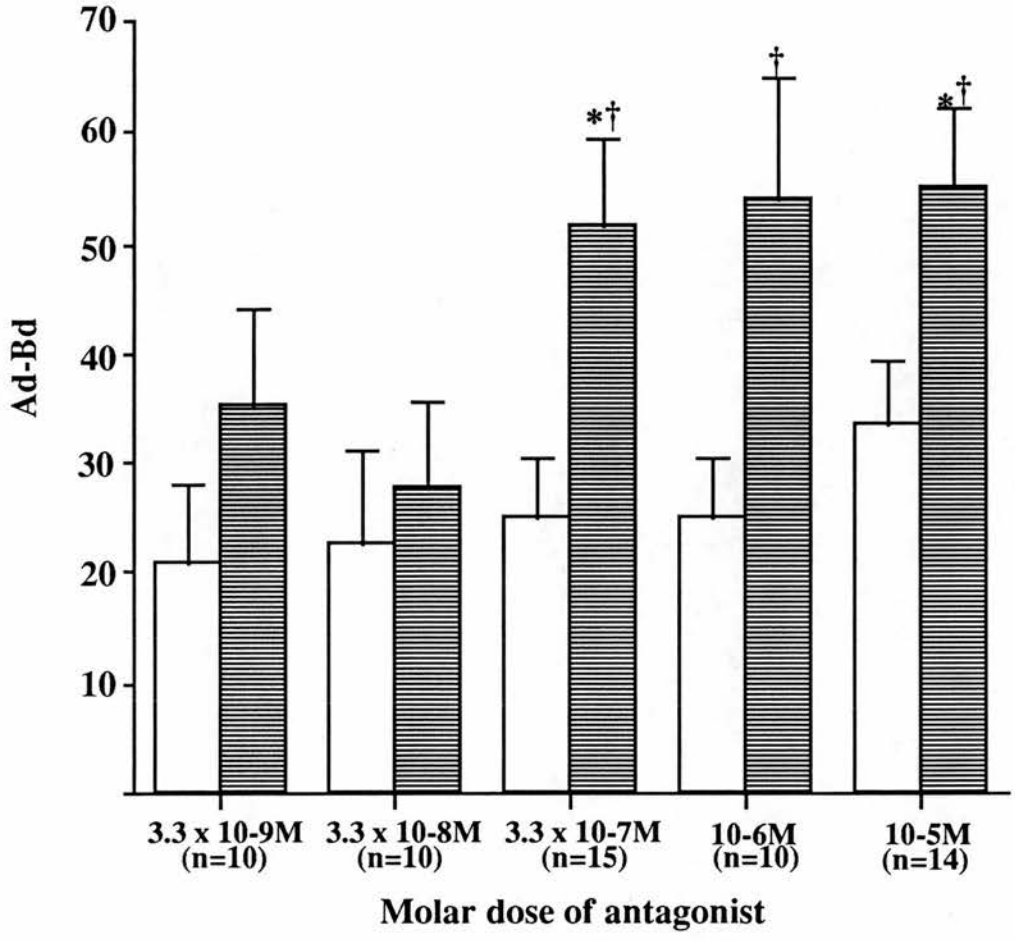


Fig.3.2 (e)

Elevation of electrically-evoked oxytocin secretion in urethane anaesthetized rats by increasing i.v. doses of RX8008M (open-bars and naloxone (striped bars).The initial oxytocin response to stimulation of the neural stalk had been inhibited by 500μg/kg U50,488H i.v. Values represent increases relative to oxytocin released during U50,488H mediated inhibition.. (For full description of Ad-Bd calculation see page 94).
Statistical comparisons :
* $p < 0.05$ with respect to initial effect of RX8008M or naloxone (paired t-test)
† $p < 0.05$ with respect to effect of RX8008M at these doses (un-paired t-test).

oxytocin secretion at 3.3×10^{-9}M, 3.3×10^{-8}M ($p<0.05$), 3.3×10^{-7}M, 10^{-5}M ($p\leq0.00005$) and 10^{-4}M ($p\leq0.0005$) using a paired t-test for statistical comparisons. Naloxone elevated oxytocin secretion at all doses, 3.3×10^{-9}M ($p<0.005$), 3.3×10^{-8}M ($p<0.05$), 3.3×10^{-7}M and 10^{-5}M ($p<0.00005$) and 10^{-4}M ($p\leq0.0005$) using paired t-tests. Naloxone was significantly more effective at antagonizing κ-opioid mediated effects at doses of 3.3×10^{-7}M and higher ($p<0.05$, unpaired t-tests).

When the results were expressed as Ad-Bd values, as shown in **Fig 3.2(e)**, which reflected the increment in facilitataion of oxytocin secretion during inhibition by U50,488H effected by the agonists, RX8008M does not appear to facilitate oxytocin secretion in a dose dependent manner. Statistical analysis, using a paired t-test shows that there is no significant difference between the increments of facilitation of oxytocin secretion at any of the doses tested. In contrast, naloxone is significantly more potent at doses above 3.3×10^{-7}M ($p<0.05$, paired t-test) although it is a large jump in response rather than a stepwise change. At doses above 3.3×10^{-7}M, naloxone is more effective than RX8008M at antagonizing the U50,488H modulation of oxytocin secretion ($p<0.05$, unpaired t-test).

3.2.5 DISCUSSION

Naloxone stimulated electrically-evoked oxytocin secretion from the isolated neural lobe preparations at all doses tested. This was probably due to its antagonism of endogenous opioid peptides acting via κ-opioid receptors to inhibit oxytocin secretion from nerve terminals in the neural lobe. Vasopressin secretion was not affected by naloxone at doses of 3.3×10^{-7}M and 1×10^{-5}M but was inhibited by a dose of 1×10^{-4}M naloxone. This indicates that vasopressin secretion is not modified by endogenous opioids (or at

least not via naloxone-sensitive receptors) although the reason for the inhibitory response seen is unclear.

RX8008M showed a biphasic effect on oxytocin release- it was stimulatory at a dose of 1×10^{-5}M, had no effect at 1×10^{-6}M and was inhibitory at 3.3×10^{-7}M.

At the high dose, the facilitatory effect may have been due to non-selective binding at κ-receptors, since the antagonist was at a high concentration and reversal of κ-mediated opioid tone acting at the oxytocin nerve terminals in the posterior pituitary.

The reason for the inhibitory effect of RX8008M at 3.3×10^{-7}M is not clear. It could be that at this low concentration, in addition to its high μ/δ selectivity over κ-recptors, that it shows partial κ-agonist activity. Previous **in-vivo** studies did not reveal any partial agonist activity in the mouse vas deferens or guinea pig ileum preparation (**Smith, 1987**).

There is now evidence for a multiplicity of κ-receptors which have been identified in guinea pig and rat brain.

There is a population of high affinity κ-sites found in guinea pig brain that is only found in low density in rat brain (κ_1) and a low affinity κ-site found in high density in rat brain but at very low density (or maybe absent) in guinea pig brain (κ_2).

The two sites display different ligand selectivity, U50,488H and U69,593 being more potent at the κ_1 site, the latter inactive at the κ_2 site **(Zukin, Eghbali et al, 1988)**.

There is now evidence for a κ_{1a} and κ_{1b} site following binding studies in which classical κ-opiates and dynorphin A have a high affinity for the two types of receptor located in guinea pig cerebellum whereas dynorphin B and α-neoendorphin bind to κ_{1b} fifty times more potently than κ_{1a} **(Clark, Liu et al, 1989)**.

A third type of κ-opioid receptor subtype has now been identified. This receptor (κ_3) is U50,488H insensitive

(as demonstrated by its inabilty to displace naloxone benzoylhydrazone from its κ_3 site.

In the rat brain there is predominantley a distribution of κ_2 receptors. It may be that RX8008M has some partial agonist activity at this receptor site but that this type of κ-receptor was not presentin the tissues in which the properties of RX8008M were characterized (**Smith, 1987**).

In-vivo, RX8008M elevated U50,488H inhibited oxytocin secretion in all doses tested but to a greater extent at higher doses. Once again this may be due to non-selective effects at κ-receptors at high concentrations or due to RX8008M being able to antagonize the κ-opioid receptor subtype involved despite having far higher affinity for the μand δ-opioid receptors. The effect is not strictly dose dependent indicating non-selective actions of RX8008M.

Naloxone was effective at reversing the U50,488H-mediated inhibition and was more effective than RX8008M at doses of 3.3×10^{-7}M and higher. **Chapter 2** has already shown that naloxone effectively reverses U50,488H mediated inhibition of electrically-evoked oxytocin secretion but without apparently having an effect on endogenous opioid tone, presumably due to the different types of receptors involved.

Once again, these experiments show that RX8008M is not a suitably selective opioid-receptor antagonist to discriminate between μ and κ-opioid receptor mediated action on oxytocin secretion.

CHAPTER FOUR

STUDY OF WHETHER CENTRALLY-PROJECTING OXYTOCIN NEURONES ARE SUBJECT TO OPIOID-MEDIATED MODULATION OF HORMONE SECRETION AND WHETHER THEY CAN BE MADE MORPHINE DEPENDENT FOLLOWING CHRONIC MORPHINE INFUSION.

CHAPTER 4 : Study of centrally-projecting oxytocin neurones - are they subject to opioid-mediated modulation of hormone secretion and can they be made morphine dependent following chronic morphine infusion.

INTRODUCTION

The distribution and projections of oxytocin and vasopressin-containing neurones has been described in the general introduction,

A measurable amount of oxytocin (and vasopressin) is found in cerebrospinal fluid (CSF) which, it was assumed, was secreted from the hypothalamo-neurohypophysial system perhaps by transport across the ependymal lining of the third ventricle (**Vorherr, Bradbury et al, 1968**).

Large amounts of exogenously administered peptides only poorly cross the blood-brain barrier (**Zaidu & Heller, 1974**) so it was later believed that oxytocin secreted into CSF came from centrally-projecting oxytocin neurones.

In the guinea pig, oxytocin release into peripheral plasma during suckling occurs without a similar elevation in central oxytocin, indicating that CSF oxytocin does not just reflect plasma oxytocin content (**Robinson & Jones, 1982**). It also demonstrated that the magnocellular oxytocin neurones which project to the posterior pituitary are not the primary source of CSF oxytocin.

Discrete brain areas which have been shown to contain parvocellular oxytocinergic projections (demonstrated immunohistochemically), show a local release of oxytocin during suckling, following hyperosmotic stimulation and after stimulation of the PVN (**Landgraf, Neumann & Schwarzberg, 1988; Neumann & Landgraf, 1989; Neumann, Schwarzberg & Landgraf, 1988**)

Oxytocin released into the extracellular fluid of

these brain areas can probably diffuse into CSF (**Robinson, 1983**).

Although stimulation of the PVN increases CSF oxytocin content, stimulation of the neurohypophysis does not, confirming that magnocellular neurones projecting to the posterior pituitary are not the main source of this peptide. However, oxytocin release from the dendrites or axons of these neurones cannot be ruled out.

The anterior commissural nuclei contain many magnocellular cell bodies (**Armstrong, Warach et al, 1980**) which project to the neural lobe but not to the brainstem or spinal cord, contain neurophysin and have one or two dendrites which project to the third ventricle. These cells are separated from the PVN by 300-400μm. Cells from the PVN (medial and lateral regions) are also seen to have dendrites projecting medially toward the ventricular wall. Due to presence of cilia extending from these processes , it has been proposed that these projections to the ventricular system are receptive in function. (**Vigh-Teichman, Vigh & Aros, 1976**)

Detailed ultrastructural studies have not been carried out but it is known that cilia have been observed from neurosecretory perikarya (**Gregory & Hatton, 1979**). As well as having a receptive function, these sub- and intra- ventricular peptidergic projections may be secretory in function. It has been observed that there is a large rise of vasopressin in CSF following hypophysectomy without a concomitant rise in plasma vasopressin indicating that AVP processes may secrete directly into the CSF (**Dogterom, Van Wimersma Greidanus & Swaab, 1977**)

Oxytocin and vasopressin secretion from nerve terminals in the neural lobe is regulated by endogenous opioid peptides co-secreted and co-released with oxytocin and vasopressin. Oxytocin is principally regulated by dynorphin acting at kappa opioid

receptors located on or near to the oxytocin nerve terminals. (**Clarke, Wood et al, 1979; Coombes & Russell, 1988**).

Magnocellular oxytocin neurones display both tolerance to and dependence on morphine when exposed to a chronic i.c.v. infusion over several days. (**Bicknell, Leng et al, 1988**). Withdrawal by an opioid antagonist such as naloxone administered i.v. results in a hypersecretion of oxytocin. This type of control over oxytocin secretion is thought to be mediated via μ-opioid receptors located centrally in the hypothalamus.

Mechanisms controlling oxytocin secretion from centrally projecting oxytocin neurones into the CSF are not known. To investigate whether opioids are involved in modification of centrally secreted oxytocin, both plasma and CSF oxytocin concentration was measured in morphine dependent and morphine naive rats before and after a withdrawal challenge with naloxone. In order to determine whether any oxytocin detected in the CSF came from oxytocin neurone projections originating from the PVN, in a separate group of animals this nucleus was ablated bilaterally prior to infusing the animals with morphine or vehicle.

Morphine is known to influence the secretion of vasopressin in an inhibitory fashion (**Aziz, Forsling & Woolf, 1981**) whereas growth hormone and ACTH are known to be increased by morphine (**Buckingham & Cooper, 1984; Wehrenberg, Bloch & Ling, 1985**). Withdrawal from morphine with naloxone also leads to an increase in plasma ACTH but this is probably due to the stressful nature of the withdrawal stimulus. Concentrations of these hormones were also measured in the plasma samples since their secretion is not only influenced by morphine but by a 'stressful' stimulus.

4.1 METHODS

The animals used in the following experiments were virgin female Sprague-Dawley rats (250-300g average bodyweight) housed under conditions described in **1.1**.

4.1.1 INTACT RATS

Five days prior to the experiment, under ether anaesthesia, one group of animals (n=7) was implanted with an intracerebroventricular (i.c.v.) infusion device connected to a subcutaneous osmotic minipump in order to continuously deliver graded doses of morphine sulphate into the left lateral cerebral ventricle. Control animals (n=6) were fitted with the same infusion system to deliver vehicle (sterile pyrogen free water) in the ventricle. Surgical details are described fully in **1.3**.

After five days the animals were anaesthetized with urethane (1.25g/kg i.p.) and were fitted with a tracheal cannula and femoral venous and arterial cannulae for terminal blood sampling.

The animals' heads were immobilized in a simple head frame with the neck flexed to facilitate exposure and cannulation of the atlanto-occipital membrane so that cerebrospinal fluid (CSF) from the cisterna magna could be collected. Details of this cannulation technique are fully described in **1.4**.

Terminal blood and CSF sampling were carried out according to the protocol described in **Fig. 4.1(a)**.

Seventy minutes was left after the completion of surgery to the collection of the first blood and CSF samples in order that any increase in basal oxytocin arising from surgical 'stress' would have subsided.

Blood samples (1ml) were withdrawn from the femoral artery, immediately cooled over ice, centrifuged at 0°C and plasma aliquots frozen at -20°C until assayed for oxytocin (OT), vasopressin (AVP), growth hormone (GH) and adrenocorticotrophic hormone (ACTH). The first sample was replaced with sterile isotonic saline (1ml)

Surgery completed	**t= -70 mins**
Plasma sample 1	**t= 0 mins**
CSF sample 1	**t= 5 mins**
5mg/kg naloxone or 0.9% saline	**t= 30 mins**
Plasma sample 2	**t= 35 mins**
CSF sample 2	**t= 40 mins**
Plasma sample 3	**t= 60 mins**
CSF sample 3	**t= 70 mins**
Plasma sample 4	**t= 120 mins**
CSF sample 4	

Fig 4.1 (a)

Terminal blood and CSF (from the cisterna magna) sampling protocol applied to all groups of rats used in the experiment.

via the femoral vein, subsequent samples were replaced with red cells resuspended in isotonic sterile saline to a volume of 1ml.

CSF samples were collected under gravity into preweighed polystyrene tubes so that over each collection period ≈ 50µl was collected (assumption that 1µl CSF = 1µg CSF). The CSF samples were cooled over ice and frozen at -20°C until assayed for oxytocin.

Naloxone (5mg/kg) was administered intravenously to all the animals via the femoral vein at t=30 mins.

4.1.2 TIME CONTROLS

Morphine infused (n=7) and vehicle infused (n=8) rats were set up as described in **4.1.1.** After five days, terminal blood and CSF samples were taken according to the schedule in **Fig. 4.1(a)**. In this group of rats (n=15 total) an intravenous injection of sterile isotonic saline was administered at t= 30 mins instead of naloxone to investigate whether changes take place in oxytocin content of CSF and plasma with repeated sampling. Plasma ACTH was also measured.

4.1.3 PVN LESIONED ANIMALS

4.1.3 (i) SURGERY

Nine days prior to starting an i.c.v. morphine or vehicle infusion, this group of experimental animals (n=27) underwent a bilateral knifecut lesion of the paraventricular nucleus under barbiturate anaesthesia **(lesions performed by Dr. F. Antoni, Dept. of Anatomy, Oxford.)** so that the contribution of magnocellular and parvocellular PVN oxytocin neurones to changes in oxytocin in both CSF and plasma could be measured.

After the experiment, histological preparation and examination of the brains confirmed the extent of the lesion in each animal.

The PVN lesioned experimental group underwent the same

regimen of i.c.v. morphine and vehicle infusion and terminal sampling protocol as described in **4.1.1.**

4.1.3 (ii) HISTOLOGY

(a) TISSUE FIXATION AND CUTTING

At the end of the experiment, brains from the PVN lesioned animals were removed and immediately placed in Bouins fixative. After 48hrs the brains were transferred to 70% alcohol and left overnight. The 70% alcohol was then replaced with two changes of 90% alcohol over two hours then three changes of 95% alcohol and finally three changes of absolute alcohol over 1½ hours. The fixed brains were left in cedarwood oil for two days. Following this, the brains were immersed in three changes of molten paraffin wax in an embedding oven over three hours and finally set in paraffin wax in a cubic mould.

The resulting wax block was trimmed to a parallel sided block prior to sectioning on a microtome (**Reichert-Jung, model 1130/Biocut**). 15μm frontal sections were cut through the hypothalamus and floated out in water at ≈ 40°C onto prewashed chrome-alum gelatine dip coated slides. The slides were dried in a 37°C oven.

(b) STAINING

The following protocol was used to stain the sections with cresyl fast violet (CFV).

The slides in slide racks were placed in xylene for 15 minutes to remove wax around the tissue sections. The sections were then hydrated through absolute alcohol (5 mins), 90% alcohol (3 mins) and 70% alcohol (3 mins).

The slides were immersed in 70% alcohol with $LiCO_3$ until the Bouins colouration had disappeared from the sections. The sections were then stained in cresyl fast violet for 3-5 mins and washed under running water for 10 mins to remove any excess stain.

The stained sections were dehydrated in successive alcohol baths: 70% alcohol (3 mins), 90% alcohol (3 mins) and absolute alcohol (5 mins). Finally, the slides were immersed in xylene until the sections cleared. The sections were mounted in DPX and cover slipped.

Examination under a low power microscope revealed the extent of the lesions which were visualised on diagrams of serial frontal sections taken from a stereotactic atlas (**König & Klippel**).

4.1.4 RADIOIMMUNOASSAY

Oxytocin was measured in unextracted plasma and CSF samples by a specific radioimmunoassay previously described by **I.C.A.F. Robinson (1980)**. Plasma samples were assayed twice on 10 and 50µl volumes, CSF samples were assayed once in 50µl samples. The sensitivity of the assay was 0.25-0.5 pg/tube. (Details of assay in 1.7)

Vasopressin was measured in unextracted plasma volumes of 10µl according to the method described by **Horn, Robinson & Fink (1985)**. The sensitivity of the assay was 0.5-1.0pg/tube.

Growth hormone was also measured in unextracted plasma samples of 10µl according to the method of **Clark & Robinson (1985)**. The sensitivity of the assay was 20-50pg/tube.

ACTH was measured in unextracted plasma volumes of 20µl by a specific second antibody radioimmunoassay. The sensitivity of the assay was 0.2fmol/tube. The primary rabbit antibody (AS8514), which was provided by **Dr. G. Makara, Budapest**, was raised against 1-32 ACTH coupled with carbodiimide to bovine serum albumin. This antibody specifically recognizes the mid portion of ACTH but will only react weakly with 1-24 ACTH and not at all with α-MSH or β-endorphin. Full details of this procedure are described by **Kovacs & Makara (1988)**.

All assays for oxytocin, AVP and GH were carried out with the help of Dr. Iain Robinson at NIMR, Mill Hill, London. ACTH assays were carried out by Dr. F. Antoni, Dept. of Anatomy, Oxford.

4.1.5 STATISTICS

Data points are represented by the group mean value ± s.e.m. Statistical comparisons were made within animals by paired t-tests and between groups with the Wilcoxon test where appropriate.

4.2 RESULTS

4.2.1 INTACT RATS

4.2.1 (i) OXYTOCIN

The results of changes in oxytocin secretion into peripheral plasma are shown in **Fig 4.2 (a)**. Basal plasma oxytocin content in sample 1 (t=5 mins) was 51.8 ± 20.8 pg/ml in vehicle infused rats (n=6) and 28.6 ± 15.2 pg/ml in morphine infused rats (n=7). There was no significant difference between these values.

Following i.v. injection of naloxone at t=30 mins, the vehicle group showed an approximate ten-fold increase in plasma oxytocin to 574.8 ± 135.1 pg/ml whereas the morphine treated animals showed oxytocin levels elevated approximately one hundred-fold to 2285.3 ± 264.5 pg/ml. Statistical comparison by a paired t-test showed these values to be significantly increased with respect to pre-naloxone values ($p = 0.01$ vehicle group, $p < 0.001$ morphine group). Oxytocin levels in the morphine treated animals were also significantly elevated with respect to the vehicle group, after naloxone ($p = 0.002$ Wilcoxon).

In the following two plasma samples oxytocin levels in the morphine treated animals fell to 785.4 ± 113.8 pg/ml and 322.4 ± 52.0 pg/ml but were still significantly elevated with respect to pre-naloxone values ($p < 0.001$, paired t-test) and also at t=60mins with respect to oxytocin levels in plasma samples from

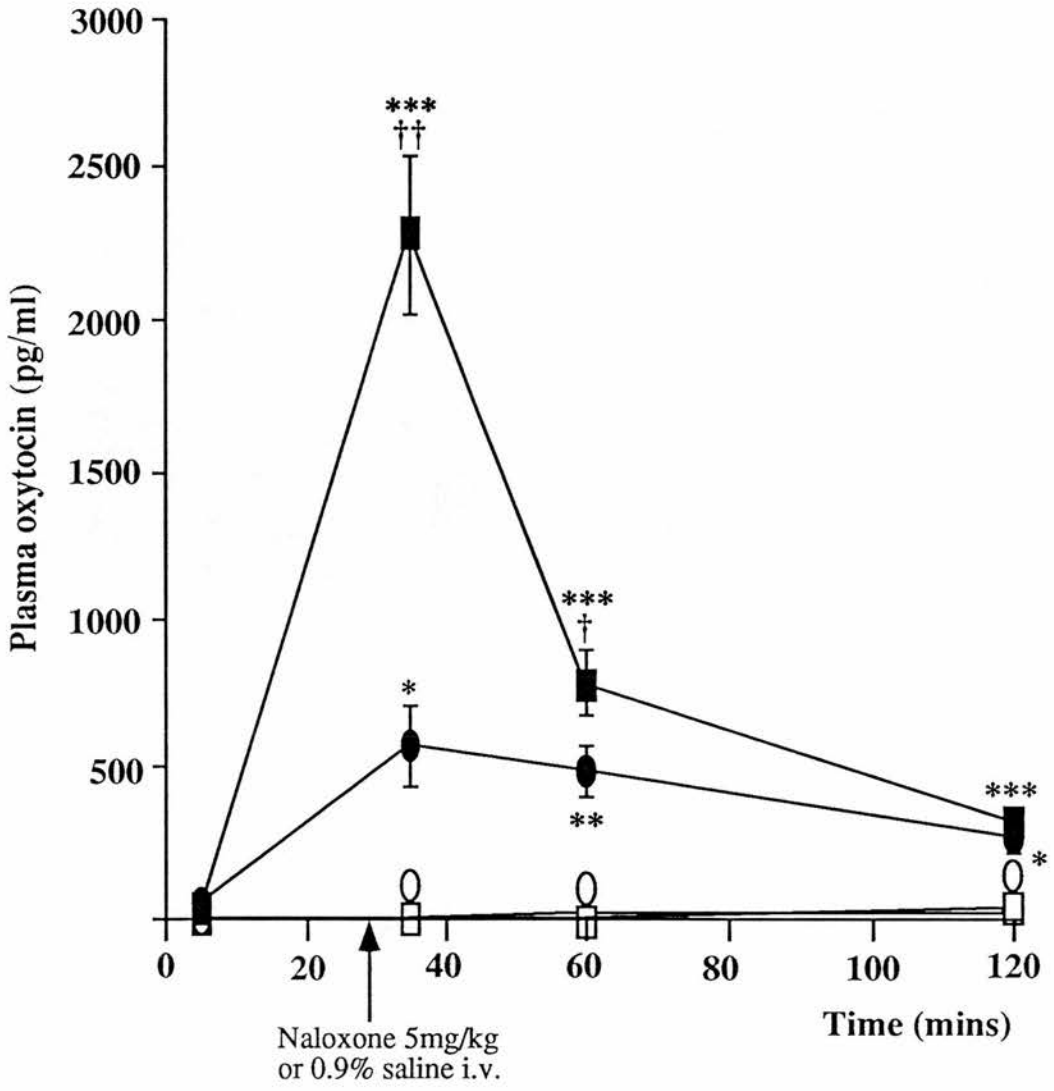


Fig 4.2(a)

Oxytocin in peripheral blood plasma of rats under urethane anaesthesia that received naloxone (5mg/kg,filled symbols) or 0.9% saline (open symbols) i.v. at t=30 minutes.Square symbols: i.c.v. morphine infusion (n=7 both groups);circular symbols: i.c.v. vehicle infusion (n=6,n=8 time controls)
* p=0.01,** p=0.002,***p<0.001 with respect to pre naloxone values (paired t-test).
† p<0.05,†† p=0.002 vehicle treated v. i.c.v. morphine infused rats at the same time points post-naloxone (Wilcoxon).

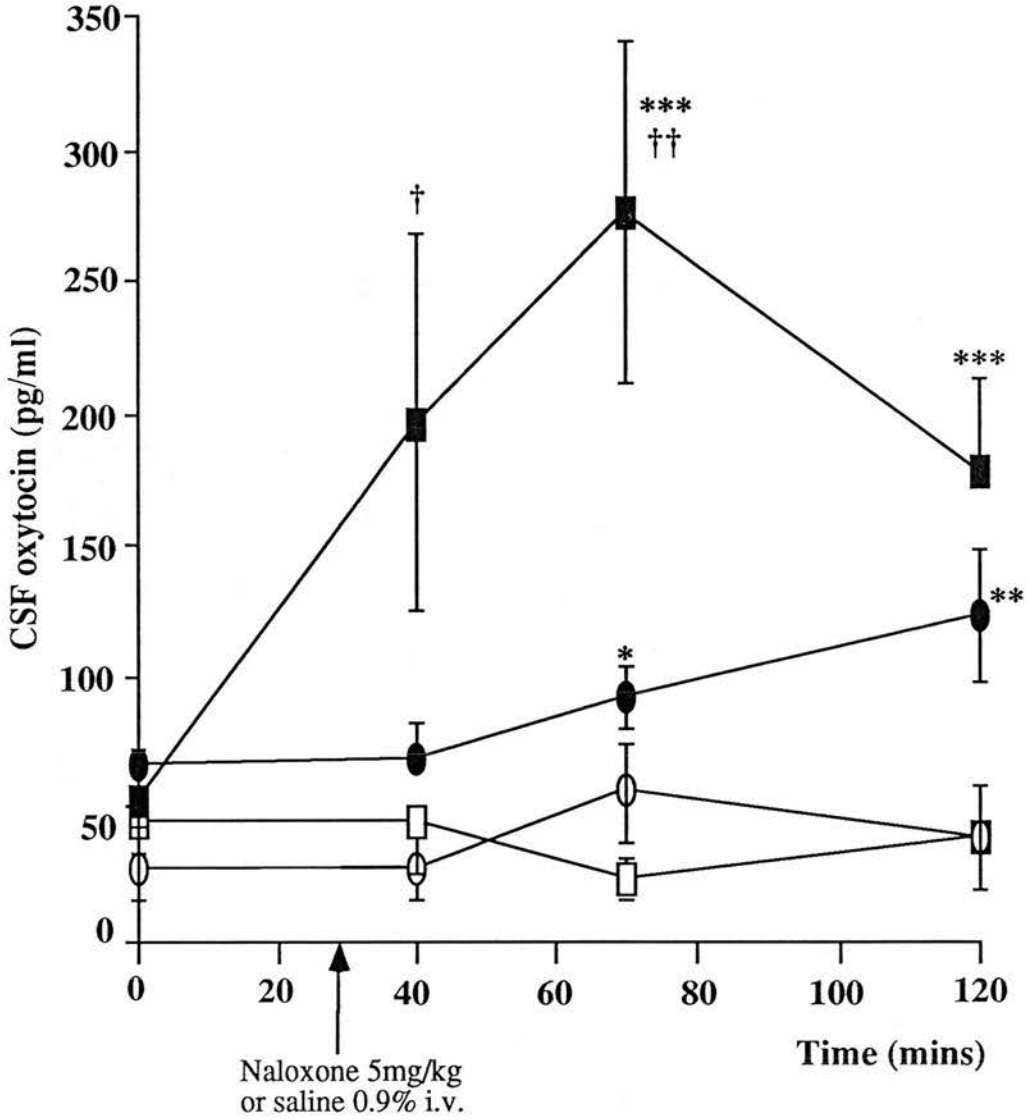


Fig 4.2(b)

Oxytocin in CSF of rats under urethane anaesthesia which received naloxone (5mg/kg ,filled symbols) or 0.9% saline (open symbols) i.v. at t=30 minutes.Square symbols:i.c.v. morphine infusion (n=7 both groups).Circular symbols:i.c.v. vehicle infusion (n=6, n=8 time controls).

* $p=0.05$,** $p<0.05$,*** $p<0.02$ with respect to pre-naloxone values (paired t-test)

† $p<0.05$,†† $p<0.02$ morphine infused v. vehicle infused animals at the same time points post-naloxone (Wilcoxon).

the vehicle treated animals (p <0.05, Wilcoxon).
Oxytocin in the vehicle infused group of rats also remained elevated with respect to pre-naloxone values in the following two samples at 490.3 ± 82.2 pg/ml and 264.8 ± 39.3 pg/ml (p = 0.002 and p = 0.01 respectively, paired t-test).
Oxytocin content in CSF samples from the two groups of experimental animals is shown in **Fig 4.2 (b)**
Initial basal oxytocin levels were 56.4 ± 6.4 pg/ml in vehicle treated animals and 53.7 ± 10.6 pg/ml in morphine treated animals. there was no significant difference between these values.
Following i.v. naloxone injection at t=30 mins, oxytocin levels in the CSF samples from morphine treated rats were elevated to 196.0 ± 71.0 pg/ml rising to 275.6 ± 65.1 pg/ml at t=60 mins and then falling to 178.3 ± 35.3 pg/ml. At t=60 and t=120 mins these values were significantly higher than pre-naloxone values (p < 0.02, paired t-test) and at t=35 and t=60 mins the values were significantly greater than those found in the morphine-naive animals (p < 0.05 and p < 0.02 respectively, Wilcoxon).
The vehicle-treated group of rats did not show an increased level of oxytocin in their CSF samples until t=60 mins when it rose to 92.2 ± 12.3 pg/ml (p = 0.05, paired t-test, with respect to pre-naloxone values). This elevation in oxytocin continued and at t=120 mins was 123.3 ± 25.1 pg/ml (p < 0.05, paired t-test with respect to pre-naloxone values.

4.2.1 (ii) VASOPRESSIN (AVP)

The results of vasopressin content measurement in plasma samples from the two groups of rats are shown in **Fig. 4.2 (c)**. Basal vasopressin content in the vehicle infused experimental group of rats was 72.6 ± 6.7 pg/ml and in the morphine treated group 87.0 ± 5.3 pg/ml. There was no significant difference between these initial values.

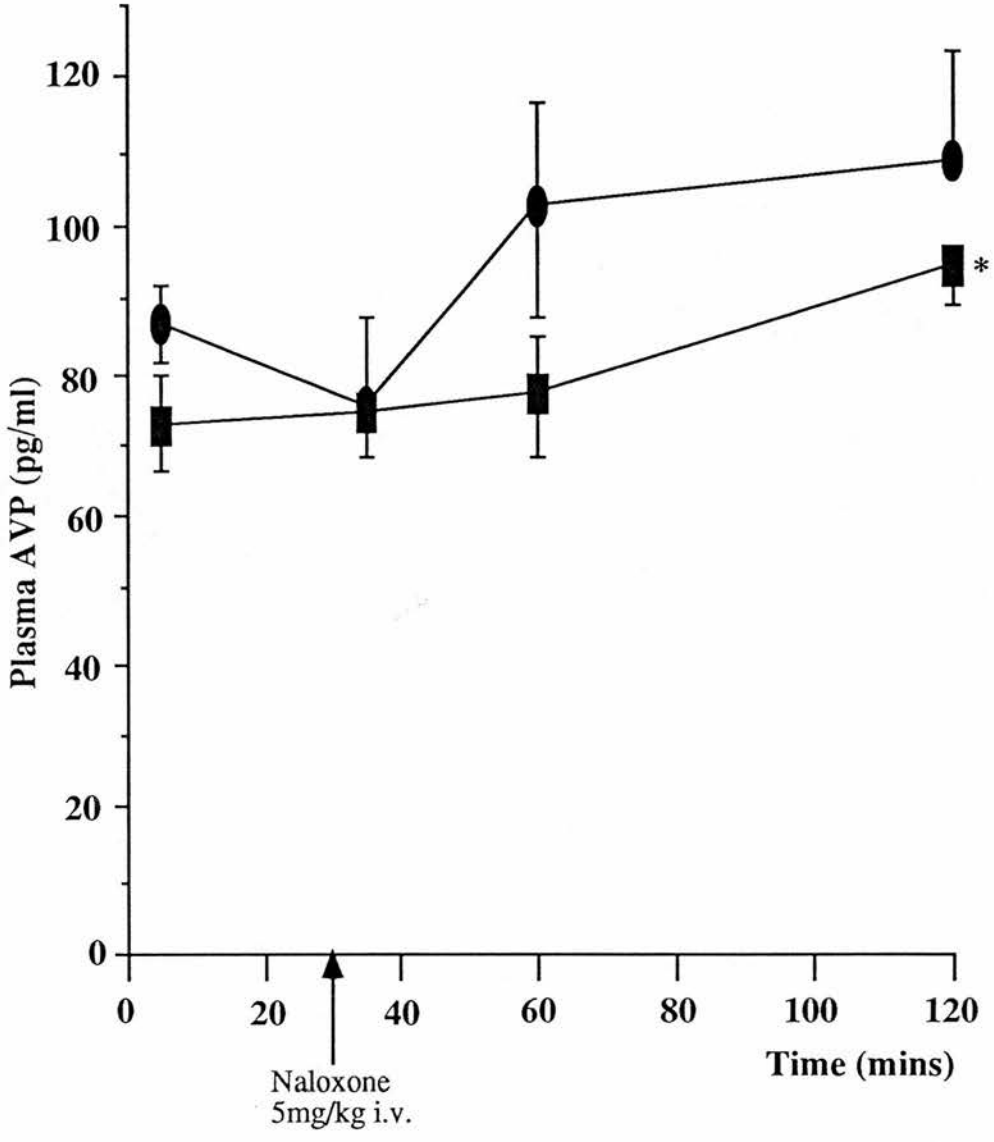


Fig 4.2(c)

Vasopressin (AVP) in peripheral blood plasma from two groups of experimental animals under urethane anaesthesia.Filled circles: i.c.v. morphine infused rats (n=7),filled squares:i.c.v vehicle infused (n=6).All animals received naloxone (5mg/kg i.v.) at t=30 minutes.
* $p<0.01$ with respect to pre-naloxone value (paired t-test)..

Following naloxone injection there appeared to be an increase in plasma vasopressin in the morphine treated animals but this is because the sample taken at t=35 mins had a lower AVP content than the basal sample (75.4 ± 12.3 pg/ml). Although this decrease was not statistically significant, it gave the impression that subsequent samples at t=60 and t=120 mins were elevated.

Vasopressin in plasma samples from vehicle treated animals showed a gradual elevation which was significantly above pre-naloxone values at t=120 mins (94.3 ± 5.3 pg/ml, $p < 0.01$, paired t-test).

4.2.1 (iii) GROWTH HORMONE (GH)

The results of GH measurements in plasma samples from the two groups of rats are shown in **Fig. 4.2 (d)**. Basal values of GH were 34.6 ± 4.0 pg/ml in the vehicle treated group and 36.2 ± 6.7 pg/ml in the morphine infused group. There was no significant difference between these.

Following naloxone injection neither group of animals showed any significant change from pre- naloxone values or from each other.

4.2.1 (iv) ADRENOCORTICOTROPHIC HORMONE (ACTH)

Basal plasma ACTH in the morphine treated group of rats was significantly higher than that in the morphine naive animals ($p<0.05$, Wilcoxon), as shown in **Fig 4.2e (i)**. It was also significantly higher at t=35 mins ($p<0.01$, Wilcoxon).

Neither group of animals showed a significant increase in ACTH immediately following naloxone administration at t=30 mins. During the course of the experiment ACTH in the morphine dependent group decreased until it was of the same magnitude as the morphine naive group. This decrease was significant with respect to basal values ($p<0.05$ at t=60 mins, $p<0.02$ at t=120 mins, paired t-test.)

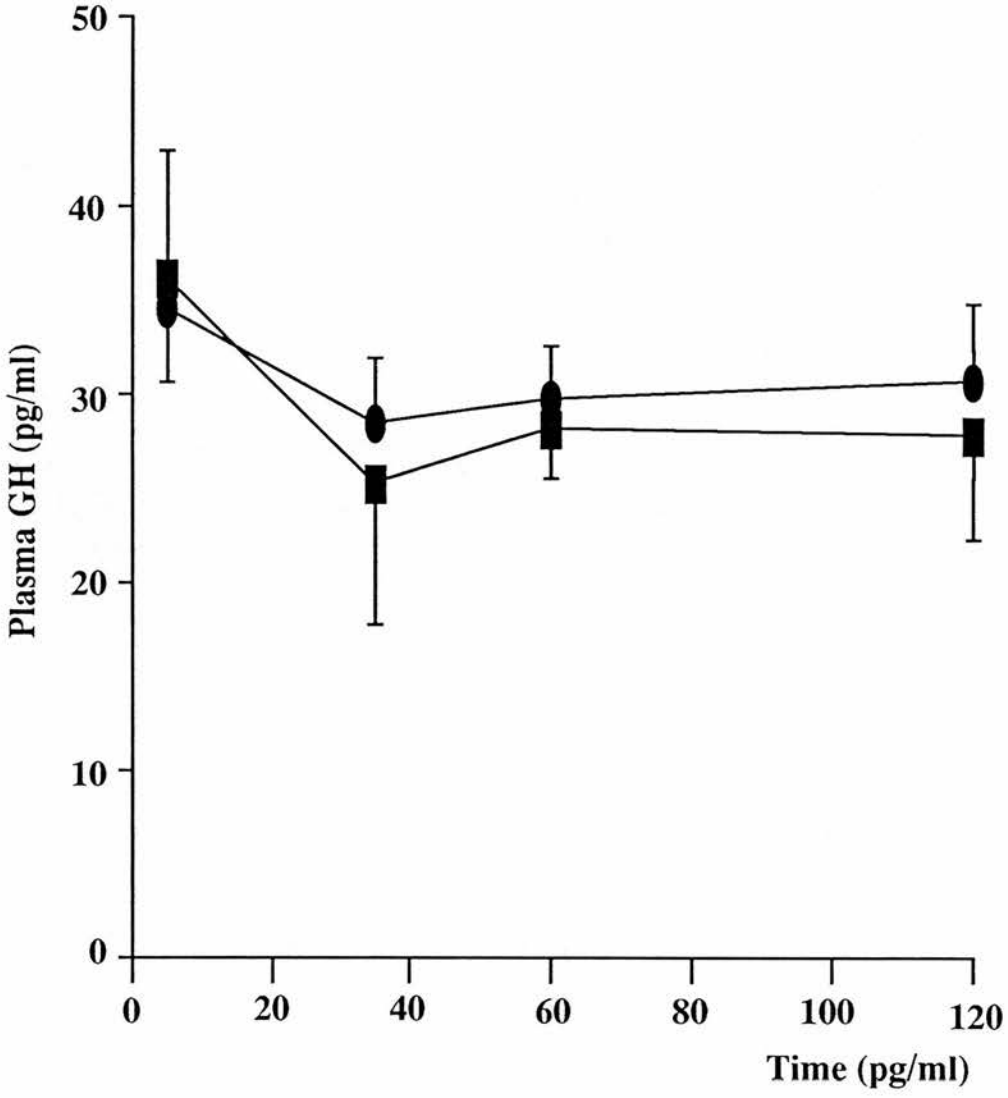


Fig 4.2 (d)

Growth hormone (GH) in peripheral blood plasma from two groups of experimental animals under urethane anaesthesia. Filled circles:i.c.v. morphine infused rats (n=7),filled squares: i.c.v. vehicle infused rats (n=6).All animals received 5mg/kg naloxone i.v.
There was no significant difference in plasma GH values within or between groups at any time.

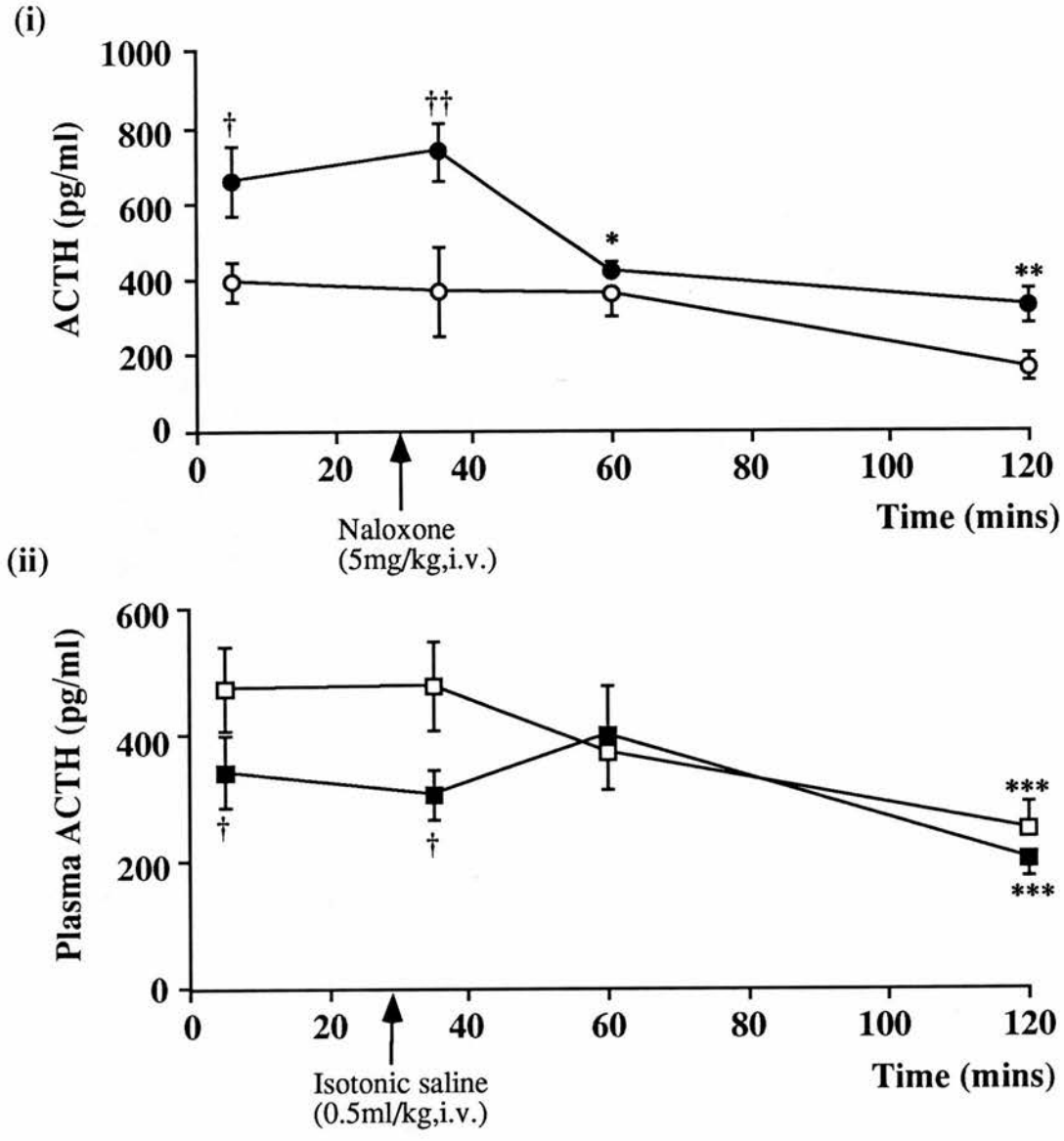


Fig 4.2 (e)

Concentrations of ACTH in peripheral blood plasma in two groups of urethane anaesthetized rats which had been treated with either i.c.v. morphine (filled symbols) or i.c.v. vehicle (open symbols) for 5 days..

(i) Received 5mg/kg naloxone i.v. at t=30 mins (n=13,square symbols.)

(ii) Received isotonic saline (0.5mls/kg) i.v. at t=30 mins (time - controls (n=16,circular symbols)

Statistical comparisons:

† $p > 0.05$,†† $p > 0.02$ with respect to i.c.v. vehicle group (Wilcoxon).

* $p > 0.05$,** $p > 0.02$,*** $p > 0.01$ with respect to basal ACTH values (paired t-test).

4.2.2 TIME CONTROLS

4.2.2 (i) OXYTOCIN

Oxytocin in peripheral blood plasma samples of the animals which received 0.9% saline at t=30 mins remained at basal values throughout the experiment, as shown in **Fig 4.2(a)** .Neither the morphine infused group (n=7) nor those receiving i.c.v. vehicle showed any significant change in oxytocin levels.There was no significant difference in oxytocin content of the plasma samples between the two experimental groups.

Oxytocin in CSF samples of these animals showed a similar result to that seen in plasma.Neither the morphine infused or vehicle control animals showed any significant change in oxytocin in their CSF following intravenous injection of 0.9% saline, as shown in **Fig 4.2 (b)**.There was no statistical difference in response between the two groups of animals.

4.2.2 (ii) ADRENOCORTICOTROPHIC HORMONE (ACTH)

Basal plasma ACTH in the morphine dependent group of rats was significantly lower than that in the morphine naive group ($p<0.05$, Wilcoxon), the opposite of the finding in the above experiment, as shown in **Fig 4.2e (ii)** .

This difference was still apparent at t=35 mins, following i.v. 0.9% saline i.v. ($p<0.05$ Wilcoxon).After 120 minutes plasma ACTH levels were significantly decreased with respect to basal values in both the morphine and vehicle infused groups ($p<0.01$, paired t-test).

4.2.3 PVN LESIONED RATS

4.2.3 (i) OXYTOCIN

Oxytocin levels in peripheral blood plasma samples from rats with a bilateral ablation of the paraventricular nucleus are shown in **Fig 4.2(f)**.

Following naloxone injection (5mg/kg i.v.) at t=30mins the animals which had received an i.c.v. infusion of

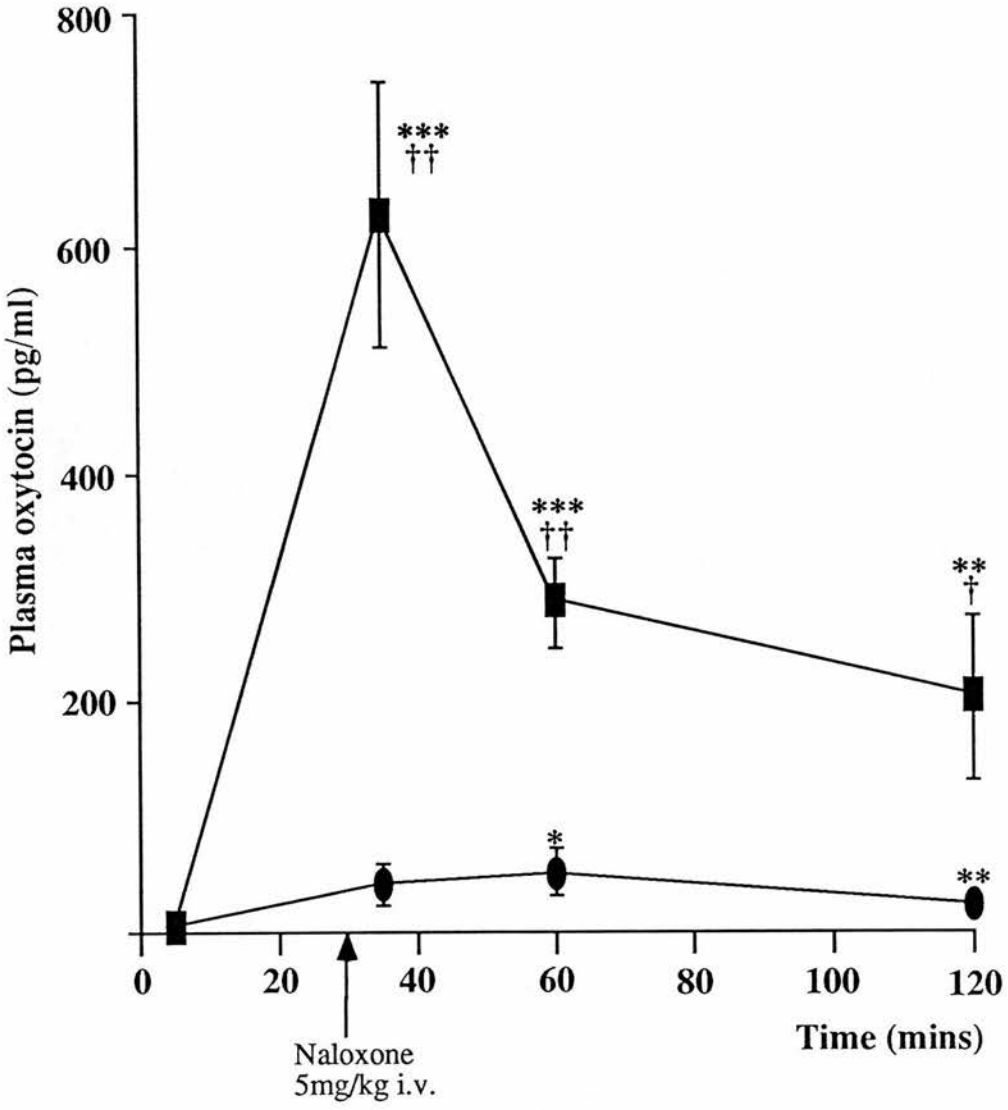


Fig 4.2(f)

Oxytocin in peripheral blood plasma of rats which had undergone a bilateral ablation of the PVN 14 days previously.Filled squares : i.c.v. morphine infused (n=9), filled circles : i.c.v. vehicle infused (n=11).
* $p<0.05$,** $p<0.02$,*** $p<0.001$ with respect to pre-naloxone values
† $p<0.05$,†† $p<0.001$, i.c.v. morphine treated rats v. i.c.v. vehicle treated rats at the same time points post-naloxone.

morphine for 5 days showed an approximate 120-fold increase in plasma oxytocin ($p < 0.001$, paired t-test with respect to pre-naloxone values). The peak concentration of plasma oxytocin from these animals (624.20 ± 114.54 pg/ml) was considerably less than that from the intact rats which had been made morphine dependent (2285.3 ± 264.5 pg/ml), although the magnitude of the increase was of the same order (≃ 100-fold). This was likely to be because basal plasma oxytocin levels in the PVN lesioned rats receiving an i.c.v. morphine infusion was considerably lower than that found in the intact rats; 5.0 pg/ml compared to 28.6 pg/ml. In the following two samples the level of oxytocin fell but was still significantly higher than pre-naloxone levels ($p < 0.001$ and $p < 0.02$ respectively, paired t-test).

In the control, vehicle-infused animals there was a more gradual increase in plasma oxytocin following naloxone injection. The peak level recorded at t= 60 minutes (52.82 ± 20.24 pg/ml) showed an approximate ten-fold increase from basal values. This rise was of the same magnitude as seen in the intact control animals following naloxone but basal levels in the PVN lesioned rats were considerably lower (5 pg/ml compared to 51.8 pg/ml in intact rats).

After naloxone injection the oxytocin response in plasma samples from morphine treated rats was significantly higher than that in samples from control rats at t= 35, 60 and 120 minutes ($p < 0.001$, $p < 0.001$ and $p < 0.05$ respectively).

Oxytocin levels in CSF from animals with a bilateral lesion of the PVN are shown in **Fig 4.2 (g)**. In contrast to basal oxytocin levels found in plasma samples from PVN lesioned rats, basal oxytocin levels in CSF from these rats was much higher than those measured in the CSF from intact rats. Following naloxone injection, the morphine treated animals showed a rapid elevation in oxytocin from 377.68 ± 65.40 pg/ml to 945.31 ± 426.67

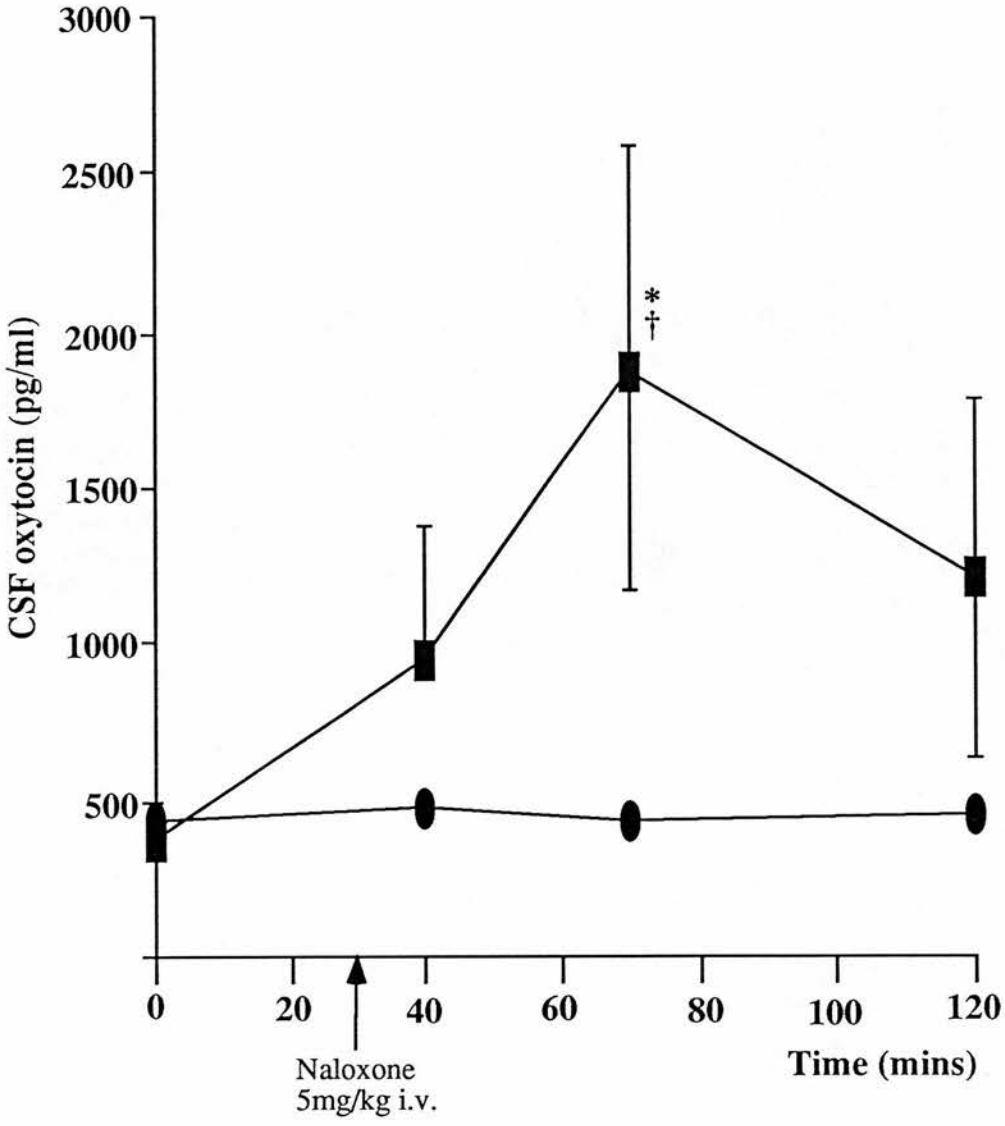


Fig 4.2(g)

Oxytocin in CSF of rats under urethane anaesthesia which had undergone a bilateral ablation of the PVN 14 days previously.Filled squares :i.c.v. morphine infused rats (n=6),filled circles : i.c.v. vehicle infused rats (n=9)
* $p<0.05$,with respect to pre-naloxone values (paired t-test)
† $p<0.02$, i.c.v. morphine treated rats with respect to i.c.v vehicle treated rats at the same time point post-naloxone (Wilcoxon)

pg/ml at t=40 minutes and 1876.41 ± 710.89 pg/ml at t=70 minutes. In the control group of animals basal oxytocin was measured as 435.94 ± 53.25 pg/ml and was not significantly changed following naloxone injection. Levels remained close to basal oxytocin at 471.27 ± 48.04, 436.11 ± 27.90 and 456.62 ± 39.18 pg/ml at t= 40, 70 and 120 minutes respectively. Due to the rather large errors in the morphine-infused group following naloxone it was only at t=70 mins that oxytocin in the morphine- treated group was greater than pre-naloxone values and also greater than levels in CSF sample from control animals. The magnitude of increase in oxytocin secretion from the morphine-treated PVN lesioned animals following naloxone treatment was the same as that in morphine treated PVN intact animals (≃ four-fold).

4.2.3 (ii) ADRENOCORTICOTROPHIC HORMONE (ACTH)

The ACTH concentrations measured in plasma samples from the two groups of PVN lesioned rats are shown in **Fig. 4.2 (h)**. Morphine-infused rats showed a significantly higher level of basal plasma ACTH at 595.4 ± 67.2 pg/ml compared to control animals which showed a basal ACTH content of 418.3 ± 89.1 pg/ml ($p < 0.05$, paired t-test).

Following naloxone, the morphine treated animals appeared to show an elevation in plasma ACTH but this was not significant, due to the large error arising from individual variation. The morphine animals then showed a steady decrease in ACTH in plasma until in the last sample at t=120 at 377.4 ± 50.8 pg/ml the level was significantly lower than the basal concentration measured ($p < 0.01$, paired t-test).

In the vehicle-treated group there was a steady decrease in measured ACTH in the plasma samples until in the last sample , when, at 299.3 ± 51.3 it was significantly decreased from the basal measured level ($p < 0.05$, paired t-test). The morphine-treated animals

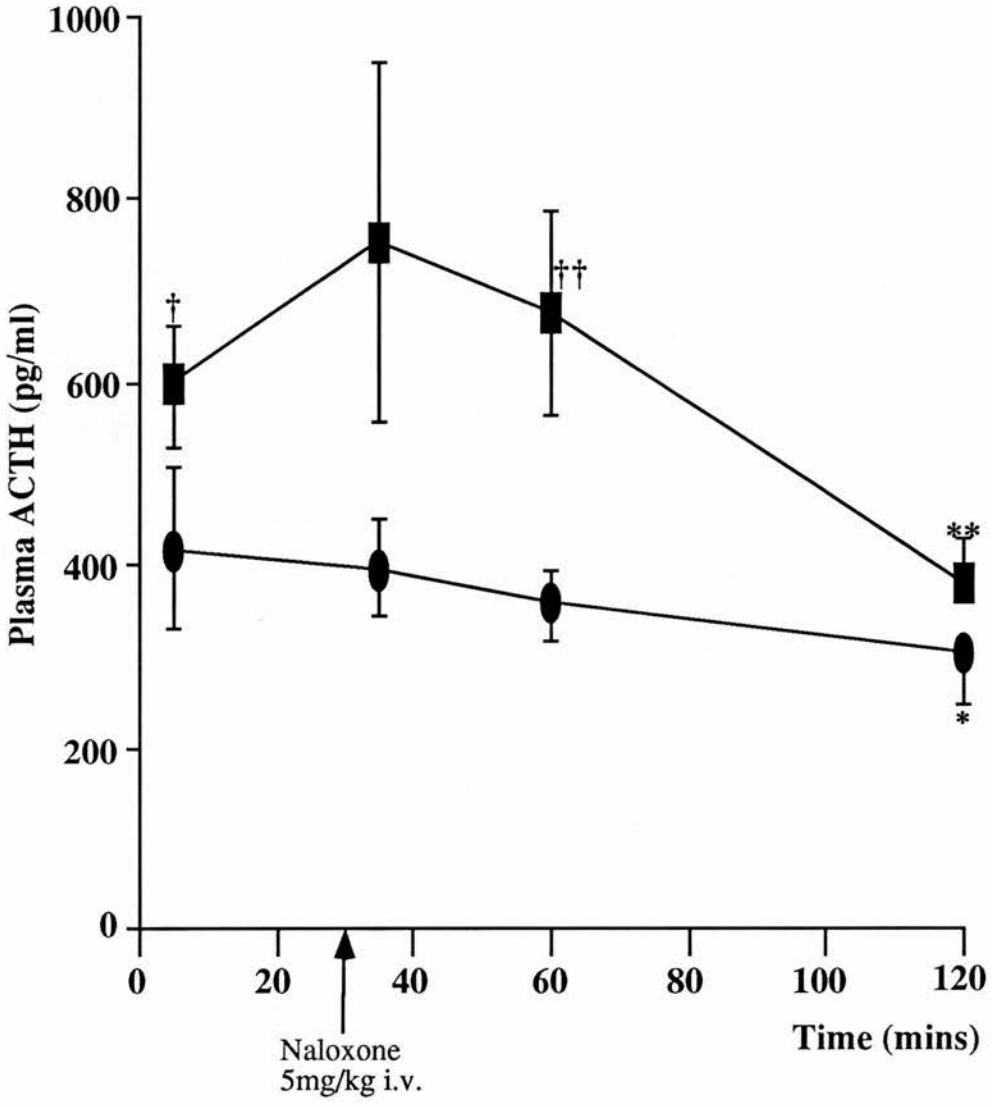


Fig 4.2 (h)

Adrenocorticotrophic hormone (ACTH) in peripheral blood plasma of rats under urethane anaesthesia which had undergone a bilateral ablation of the PVN 14 days previously.
Filled squares : i.c.v. morphine infused rats (n=8);filled circles i.c.v. vehicle infused rats (n=11)
* $p<0.05$,** $p<0.01$,with respect to pre-naloxone values (paired t-test).
† $p<0.05$,†† $p<0.02$,i.c.v. morphine v. i.c.v. vehicle treated rats at the same time points (Wilcoxon).

showed a much higher concentration of ACTH in peripheral plasma at t=60 mins,30 minutes post-naloxone injection (p < 0.02,Wilcoxon).

4.2.3 (iii) HISTOLOGICAL CONFORMATION OF LESIONS

Following histological examination of the frontal sections through the hypothalamus of the PVN- lesioned rats only the animals satisfying the following criteria were included in the data analysis.Bilateral ablation of the medial,lateral and posterior portions of the PVN had to be complete.The supraoptic nuclei were intact as were the fornical nuclei and the circularis nuclei.The anterior commissural nuclei were not routinely damaged in PVN ablation but were fully ablated in 3/5 i.c.v. morphine infused rats and 3/7 i.c.v. vehicle infused rats from which a full complement of CSF and plasma samples were obtained.

Illustrations of typical lesions are shown in **Fig. 4.2 (i-k)**.A lesion from a vehicle i.c.v. infused animal is pictured in **Fig 4.2.(i)**,from an i.c.v. morphine infused animal in **Fig. 4.2 (j)** and a lesion that was deemed to be incomplete and hence rejected from the study in **Fig. 4.2 (k)**.

4.3 DISCUSSION

4.3.1 INTACT RATS

4.3.1 (i) OXYTOCIN

In control rats,injection of 5mg/kg naloxone elicited an increase in plasma oxytocin consistent with antagonism of endogenous opioids which exert an inhibitory action on oxytocin secretion from the neural lobe.

Experiments described in **Chapter** 2 demonstrated that electrical stimulation of the neural stalk (and hence descending oxytocin axons from the hypothalamic nuclei) elicited oxytocin secretion into plasma,an effect which could be inhibited by the systemic introduction of a κ-opioid receptor agonist

Legend for 4.2 (i-k)

Illustrations of bilateral knife-cut ablation of the paraventricular nucleus. The outlines of three lesions identified in cresyl violet-stained frontal sections are shown superimposed as hatched areas onto drawings of serial forebrain sections (**from König, J.F.R. & Klippel, R.A., 1963**). The coordinate at the bottom left of each section gives the distance in µm anterior to the inter-aural line.

ACN : anterior commissural nucleus
F: fornix
NC : nucleus circularis
PVN : paraventricular nucleus
V3 : third ventricle

(i) shows a lesion from the brain of a vehicle infused rat

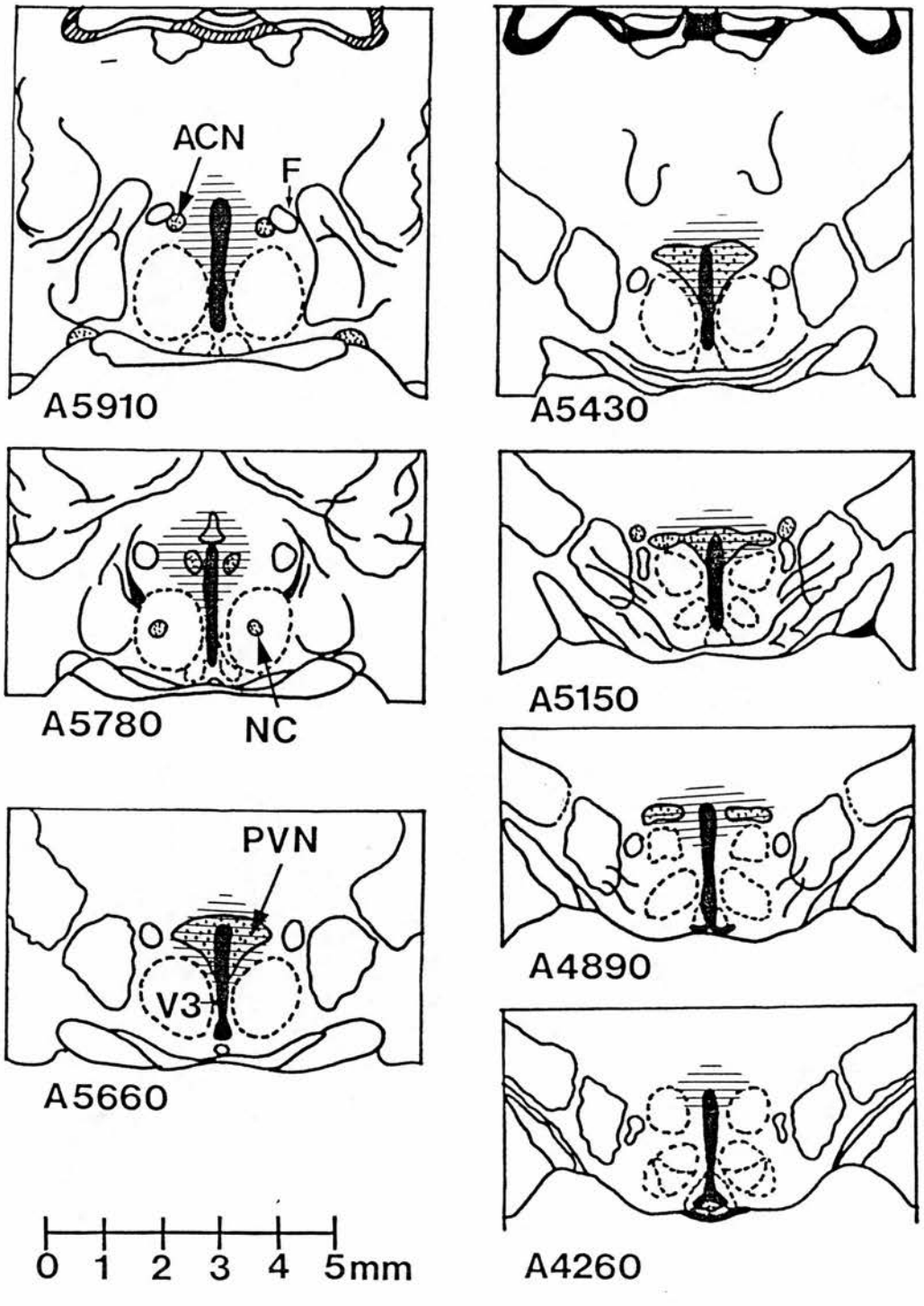
(j) shows a lesion from the brain of a morphine infused rat.

The plasma concentrations of oxytocin in the two rats illustrated were, before and 5, 30 and 90 minutes after naloxone, for (i) <5, 91, 100 and 41 pg/ml and for (j) <5, 448, 337 and 173 pg/ml. The values for CSF oxytocin before, and 10 and 40 minutes after naloxone were (i) 401, 358, and 451 pg/ml and for (j) 524, 2634 and 1493 pg/ml

(k) shows a lesion that was deemed to be incomplete with magnocellular neurone cell bodies clearly visible where marked by open arrows (A5660, A5430).

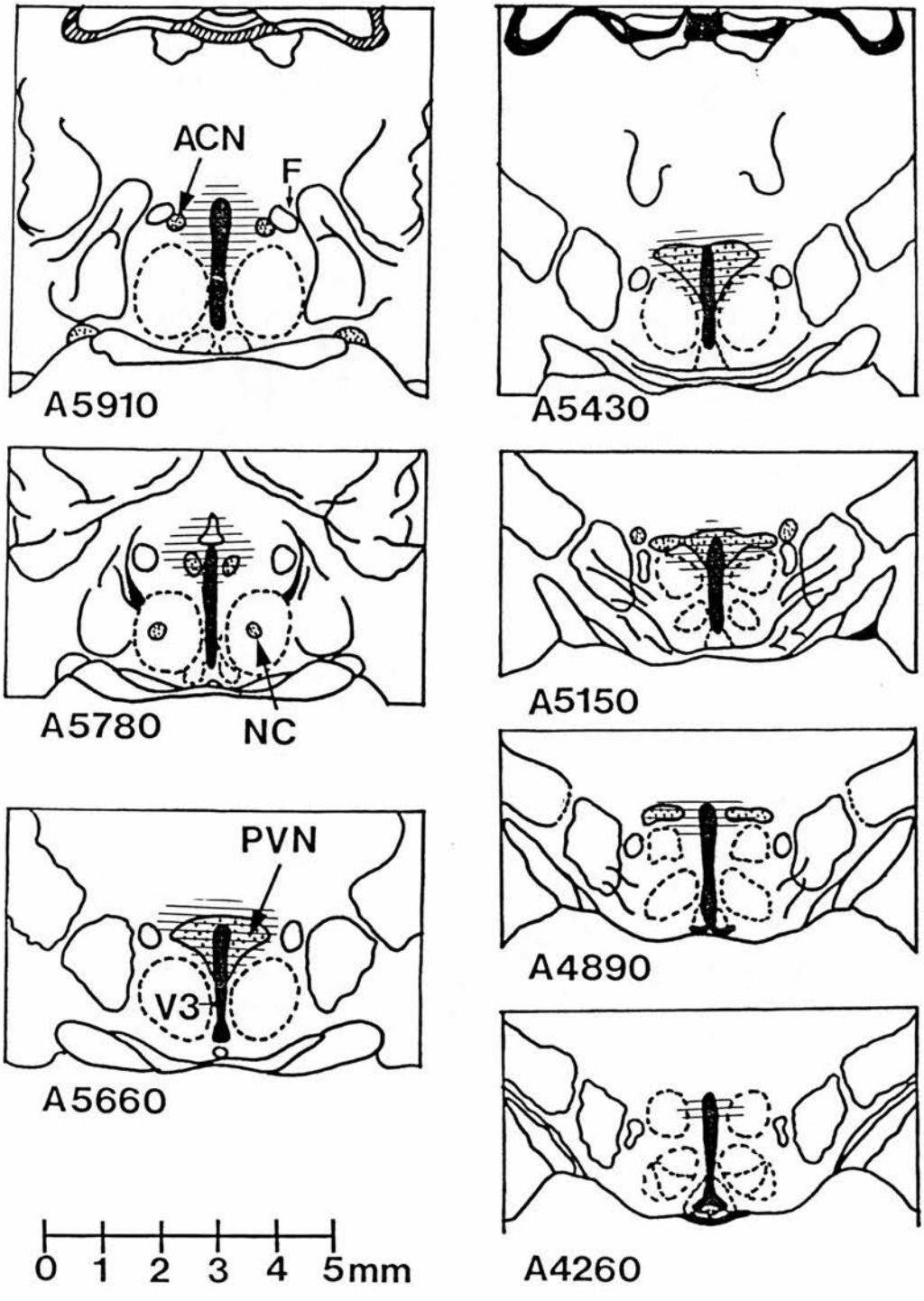
This animal was rejected from the study.

vehicle 26



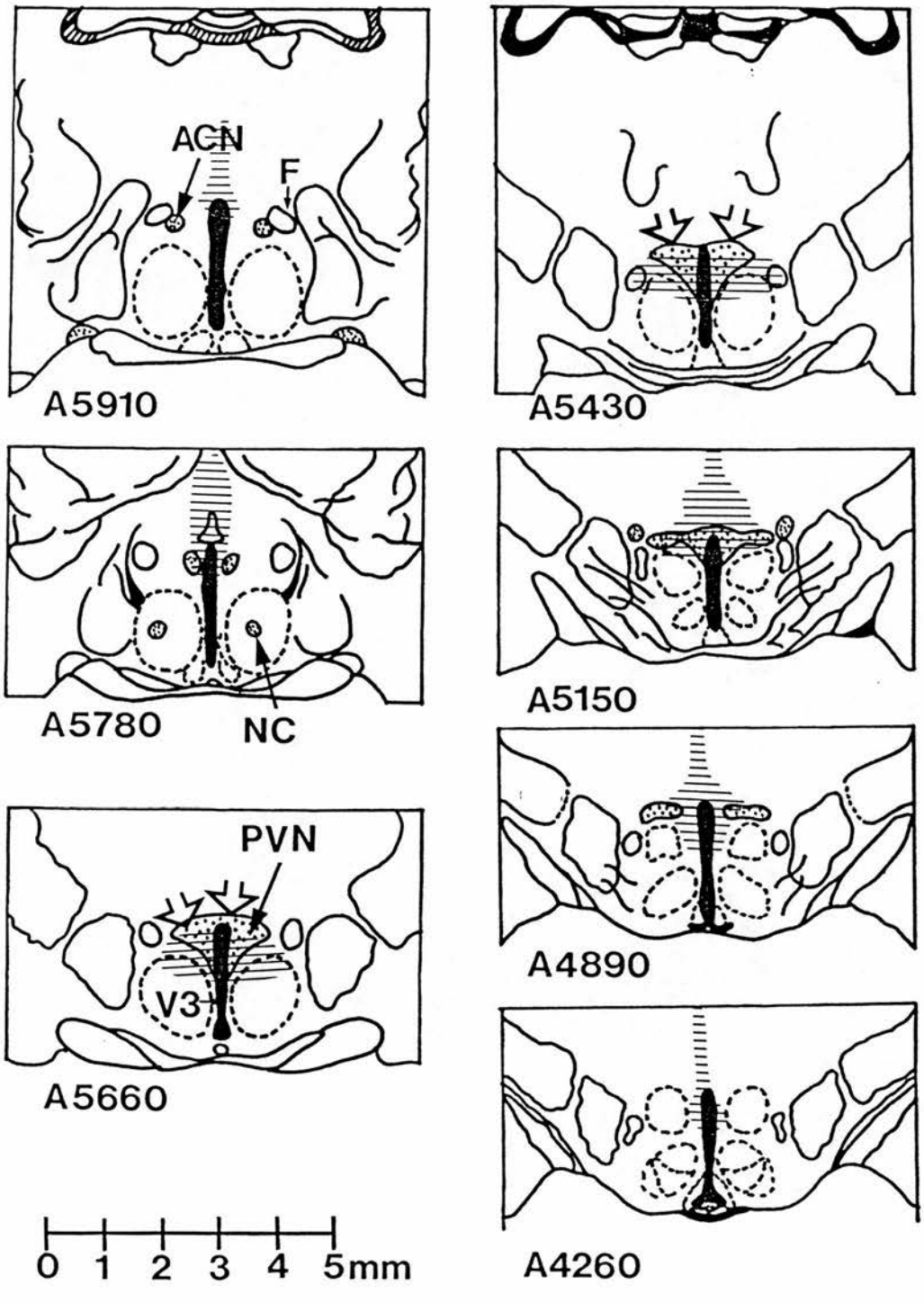
4.2i

morphine 21



4.2j

vehicle 5 (incomplete)



4.2k

U50,488H. This inhibition could be fully reversed by i.v. naloxone (**Coombes & Russell, 1988**). Thus, naloxone probably acts to antagonize endogenous opioid peptides acting at κ-receptors in the neural lobe.

In the morphine-treated rats, basal plasma oxytocin was not significantly different from control animals indicating the animals to be tolerant to morphine, since acute morphine inhibits oxytocin secretion in urethane-anaesthetized rats (**Chapter 2, this thesis**). Tolerance involves restoration of electrical activity of oxytocin neurones to normal and a down regulation of μ-opioid receptors in the hypothalamus (**Rayner, Robinson & Russell, 1988; Sumner, Coombes, Pumford & Russell, 1990**).

After naloxone administration the large increase in plasma oxytocin is the result of a withdrawal-excitation response of oxytocin neurones. The resulting hypersecretion of oxytocin stems from increased electrical activity arising in the oxytocin neurone cell bodies and is amplified by reversal of endogenous opioid tone at the neural lobe.

Oxytocin release into the CSF of control rats given naloxone showed a gradual increase with time, an effect not seen in the CSF samples from the time control rats which received 0.9% saline i.v. rather than naloxone. These results suggest that in urethane anaesthetized rats , oxytocin released centrally gradually reaches the cisterna magna, from neurones under endogenous opioid control.

In the morphine-treated rats the basal secretion of oxytocin into CSF was no different from rats in the control group-indicating that the centrally projecting oxytocin neurones are subject to tolerance to infused morphine.

After systemic naloxone, CSF oxytocin content was increased although the time course for this increase was much greater than the rise in oxytocin secretion seen in plasma. However, it is known that the appearance

and clearance of oxytocin is approximately ten times slower in CSF than in peripheral plasma (**Mens, Witter & Van Wimersma-Greidnaus, 1983**).

These results imply that centrally projecting oxytocin neurones undergo a withdrawal-excitation response and hypersecretion of oxytocin i.e. that they can be made dependent on morphine after a chronic i.c.v. morphine infusion.

Unlike the oxytocin neurones projecting from the hypothalamic nuclei to neural lobe there is no information as to whether this hypersecretion is due to increased electrical activity at the cell bodies or due to a change in excitability at the terminals. However, since morphine is a μ-opioid receptor agonist and naloxone is most potent at the μ-receptor it would appear that this opioid modification of oxytocin secretion from these central oxytocin neurones is mediated by the μ-opioid receptor subtype.

High levels of oxytocin in the circulation could cross the blood-brain barrier, possibly by influencing its permeability. The morphine infusion cannula, chronic morphine treatment or the action of naloxone-induced withdrawal may all disturb the integrity of the blood-brain barrier (**Ermishe, Rühle et al, 1985**). Physiological plasma concentrations of oxytocin do not transfer appreciable amounts into the CSF (**Mens, Witter & Van Wimersma Greidanus, 1983**).

Further experiments to investigate this possibility by measuring the transfer of ^{3}H-oxytocin into CSF are now being done in morphine naive animals with an artificially elevated plasma oxytocin concentration acheived by intravenous infusion and morphine dependent animals treated with naloxone.

4.3.1 (ii) AVP, ACTH & GH

Urethane anaesthesia was applied to all groups of rats in this experiment and can be considered to be a stressful stimulus as determined from the resulting

increase in basal circulating ACTH following its administration (**Hamstra, Doray & Dunn, 1987**).

Growth hormone in the rat is also affected by urethane anaesthesia, which interrupts its pulsatile secretory pattern (**Clark, Carlsson et al, 1988**) and a decrease in secretion occurs during stress which is believed to arise as a result of an increase in circulating somatostatin (**Arimura, Smith & Schally, 1976**).

Taking into account these effects on the secretion of ACTH and GH it was clear that the effect of naloxone was specific to increase oxytocin in peripheral plasma.

Basal levels of vasopressin in morphine-dependent and morphine-naive animals were not significantly different implying the system to be unaffected by morphine infusion or tolerant to its effects. Following i.v. naloxone there was no significant change in AVP secretion in either group indicating the secretion of this hormone is not under endogenous opioid control and that dependence does not develop for morphine.

Plasma ACTH in control rats was not affected by i.v. naloxone. This result was not unexpected since CRF released into hypothalamic-hypophysial portal blood in morphine naive animals remains unaltered following naloxone administration (**Sheward, Coombes et al, 1990**). In the morphine dependent rats basal plasma ACTH was much greater than in the morphine naive group.

Following naloxone there was no increase in ACTH which was once again consistent with results that show there is no increase in CRF secretion into hypothalamo-hypophysial portal blood after naloxone in urethane anaesthetized morphine dependent rats (**Sheward, Coombes, Bicknell, Fink & Russell, 1990**). ACTH levels gradually decreased throughout the sampling protocol until they were of the same magnitude as the control group of rats.

The combination of urethane anaesthesia (**Walker, Perrin**

et al,1986) and naloxone-induced morphine withdrawal could be regarded as a stressful stimulus which would keep ACTH levels elevated in the morphine-dependent group but in the time controls,both the morphine-dependent and morphine-naive rats had basal levels of the same magnitude as the naloxone treated control rats.In this group it was the morphine- naive rats that had the higher plasma ACTH concentration prior to saline injection.Repeated administration of morphine may lead to inhibition of the release of excitatory neurotransmitters for ACTH release (**Yaksh,Jessell et al,1980**).Naloxone may then lead to an overshoot in excitatory neurotransmitter release.Evidence suggests that neurotransmitters from primary afferent C fibres are involved in inducing ACTH release during naloxone induced morphine-withdrawal (**Donnerer & Lambeck,1988**). Basal plasma growth hormone secretion in the morphine-dependent and the morphine-naive groups of rats was not significantly different.Following naloxone,there was no change in secretion in either group-implying that there is no endogenous opioid influence on GH secretion (**Gabriel,Simpkins & Millard,1985;Russell,Antoni et al,1988**).

From the measurements of AVP,ACTH and GH it can be concluded that any increase in oxytocin secretion after naloxone induced morphine withdrawal results from the change in excitability of the oxytocin neurones and not from a generalized stressful stimulus arising from the withdrawal.Since the secretory profiles of these hormones are also sensitive to stressful stimuli,the lack of effect of morphine withdrawal on these hormones in urethane anaesthetized rats cannot be considered 'stressful'.

4.3.2 PVN LESIONED RATS

In rats with a bilateral ablation of the PVN,plasma oxytocin in morphine-dependent and morphine-naive rats follwed the same pattern as the intact rats.An

increase in oxytocin secretion reflecting removal of endogenous opioid tone was seen in the controls and a much larger increase indicating withdrawal from morphine dependence in the morphine infused group.
The actual concentrations of oxytocin in the plasma samples in both groups of PVN lesioned rats is much lower than in intact rats although the magnitude of the increase in secretion following naloxone remains the same.
Plasma oxytocin secretion was expected to be decreased by ablation of the PVN since the contribution of PVN oxytocin neurones projecting to the neural lobe and hence secreting into peripheral plasma had been removed. Oxytocin neurones in the neural lobe arising from the SON were undisturbed and reduced oxytocin levels seen reflected their secretion and withdrawal response. The results however, suggest that PVN magnocellular neurones contribute to the withdrawal hypersecretion of oxytocin into plasma.
Conversely, basal oxytocin levels in CSF in both morphine dependent and morphine naive groups with a bilateral ablation of the PVN, were higher than in intact rats. The increase in CSF oxytocin in the morphine dependent group was almost the same as that seen in intact rats although no change in oxytocin secretion was seen in the morphine naive rats unlike the slow increase detected in the intact group.
These results indicate that in normal rats, naloxone acts on oxytocin secreting PVN neurones which project centrally releasing oxytocin into CSF and that this effect is abolished by ablation of the PVN, which unlike the SON contains a significant proportion of centrally-projecting oxytocin neurones (**Swanson & Kuypers, 1980; Vich & Vich-Teichman, 1983**).
Since oxytocin secretion into the CSF continues in the morphine dependent PVN lesioned rats following naloxone, it could be concluded that centrally projecting oxytocin neurones are not confined to the

PVN.

However, in these PVN-lesioned rats there may be a new source of oxytocin. Following a knifecut bilateral ablation of the PVN, reorganization of oxytocin fibres occurs within the median eminence. After 2 weeks there is a large increase in the amount of oxytocin that can be released following stimulation of an **in-vitro** preparation, although there is a decrease in the amount of oxytocin released 1 week after ablation (**Antoni, Kovacs & Mazurek, 1988**). Magnocellular neurones release oxytocin from their dendrites/axons in the median eminence and some of these neurones have centrally projecting processes from which release could also take place. Following PVN lesion there could be reorganization of these processes to somehow facilitate the release of oxytocin as demonstrated (**Antoni, Kovacs & Mazurek, 1998**).

Anterior commissural oxytocin neurones have been shown to express centrally projecting dendrites (***Armstrong, Warach et al, 1980***), however, this nucleus was destroyed in some of the lesioned rats and intact in others without reflecting any difference in CSF oxytocin content implying that these projections could not account for the high levels of oxytocin seen.

Chronic morphine infusion has not been demonstrated to enhance oxytocin content of magnocellular neurones (**Rayner, Robinson & Russell, 1988**) although its influence on non magnocellular components has not been reported. The reduced plasma oxytocin levels in PVN lesioned animals demonstrates that bilateral destruction of the PVN suggests that there is not a compensatory increase in activity of oxytocin SON neurones.

The results of this study show that centrally projecting oxytocin neurones like those projecting to the neural lobe are subject to control from endogenous opioids as demonstrated from the response of CSF oxytocin in control animals to i.v. naloxone. These

infusion for 5 days but that they do not become dependent upon the opiate as they do with morphine. Althought there is no naloxone-induced withdrawal seen, naloxone still leads to an increase in plasma oxytocin (as seen in the vehicle treated animals) indicating that there is no cross tolerance from U50,488H to the endogenous κ-ligand.

Tolerance to i.c.v. infused U50,488H was inferred from the lack of change in the parameters measured in these and control animals i.e. weight, body temperature, water consumption, plasma osmolality, $[Na^+]$, $[K^+]$ and basal plasma and CSF oxytocin content. Another explanation could be that the infused U50,488H was ineffective due to

a) excessive dilution from the sterile water in the minipump in the modified i.c.v. infusion protocol (**see 1.3**).

b) degradation of the U50,488H during the infusion period.

The latter point could be resolved in future by testing the activity of the U50,488H remaining in the infusion device in another experimental system (e.g. stalk-stimulation preparation, **2.1(v)**.)

CHAPTER FIVE

INVESTIGATION OF WHETHER OXYTOCIN NEURONES DEVELOP TOLERANCE TO AND DEPENDENCE UPON A SELECTIVE KAPPA-OPIOID RECEPTOR AGONIST U50,488H.

CHAPTER 5 : Investigation of whether oxytocin neurones develop tolerance to and dependence upon a selective kappa-opioid receptor agonist, U50,488H.

INTRODUCTION

The principal opioid-receptor subtype located at the neural lobe of the rat is the kappa receptor. This has been demonstrated by **in-vitro** (**Zhao, Chapman & Bicknell, 1988**[c]), **in-vivo** (**Coombes & Russell, 1988** and **Chapter 2, this thesis**) and autoradiographic studies (**Herkenham, Rice et al, 1986**).

Oxytocin secretion from magnocellular nerve terminals in the neural lobe of the rat can be modified by intravenous (i.v.) administration of the kappa opioid receptor agonist U50,488H (**Coombes & Russell, 1988**). Since in these experiments oxytocin secretion arose from stimulating the neural stalk, the neural lobe was, in effect, isolated from the hypothalamus and we could conclude that the inhibition of oxytocin secretion was due to the action of the kappa agonist at the neural lobe. The effect was fully reversible by i.v. naloxone, a relatively non-selective opioid-receptor antagonist indicating it to be opioid-receptor mediated.

In-vitro, it has been shown that kappa opioid agonists U50,488H and dynorphin A_{1-13} inhibit oxytocin (and vasopressin) secretion evoked by K^+ induced depolarization of isolated neurosecretory terminals. U50,488H will also inhibit electrically-evoked release of oxytocin and vasopressin in the intact neural lobe preparation (**Zhao, Chapman & Bicknell, 1988**[c]).

Oxytocin neurones can be made tolerant to and dependent upon morphine following i.c.v. infusion of graded doses of morphine for five days (see **1.3**). Naloxone induced morphine withdrawal is characterized in the rat by an increase in cell body firing rate, hypersecretion of oxytocin into plasma and CSF and a sustained elevation

of intramammary pressure in the lactating rat (**Bicknell, Leng et al, 1988; Coombes, Robinson & Russell, 1988; Rayner, Robinson & Russell, 1988**).

Tolerance and dependence are believed to be mediated via μ-opioid receptors in the hypothalamus at the level of the cell bodies.

There are κ-opioid receptors located in the rat SON as well as at the posterior pituitary (**Mansour, Khatchaturian et al, 1988; Tempel & Zukin, 1987**).

Dependence studies upon κ-opioid agonists have been carried out in the rat (**Cowan, Zhu et al, 1988**). Following a 70 hour infusion of U50,488H into the Sylvian aqueduct, rats were given 3mg/kg naloxone s.c. and naloxone withdrawal (abstinence) scored. The dose of U50,488H infused was 2.9μmol (≃ 1.2mg/ml) at 1μl/hr. Rats treated with U50,488H showed negligible withdrawal syndrome scores like the animals treated with distilled water, the only condition to score being hypothermia). Rats infused with U50,488H for 2 weeks showed no signs of withdrawal when challenged with 3mg/kg naloxone (**Tang & Collins, 1985**).

Tolerance has been demonstrated to the antinociceptive and hypothermic effects of U50,488H, following twice daily i.p. injections but not to its diuretic response (**Bhargarva, Hemendra et al, 1989**).

In studies which investigated the advantages of κ over μ-receptor mediated analgesia, the rat has been shown to be a far from ideal model due to its low density of supraspinal κ-receptors compared to the guinea-pig (**Millan, 1990**). However, oxytocin neurones have been shown to be potently inhibited by κ-receptor agonists both at the cell bodies and at the level of the posterior pituitary.

The aim of this experiment was to see whether oxytocin neurones could be made tolerant to and dependent upon the κ-opioid receptor, U50,488H acting via kappa-receptors in the neural lobe and hypothalamus.

The rats were infused i.c.v. with U50,488H in the same

way morphine was infused to cause tolerance and dependence (see **1.3**). Tolerance to the κ- agonist was determined from the effect of U50,488H i.c.v. infusion on weight change, water consumption, body temperature and plasma $[Na^+]$, $[K^+]$, osmolality and initial oxytocin content of plasma and CSF.

Dependence of oxytocin neurones upon U50,488H was tested by giving the rats i.v. naloxone (5mg/kg) to try and precipitate a withdrawal response. Oxytocin was assayed for in plasma and CSF to see if the oxytocin neurones projecting to the neural lobe and central brain regions would respond with a hypersecretion of oxytocin.

Tolerance to and dependence upon a kappa-opioid receptor agonist by oxytocin neurones would imply that, in the rat, secretion of oxytocin can be modified by an endogenous kappa opioid agonist.

5.1 METHODS

5.1.1 ANIMALS.

The animals used were virgin female Sprague-Dawley rats (mean weight 262.53 ± 5.98 g) which were housed in individual cages under controlled light and temperature conditions described in **1.1**.

Following implantation of an intracerebroventricular (i.c.v.) infusion device (day 0) until terminal blood and cerebrospinal fluid (CSF) sampling (day 5), the animals were weighed daily, had their core body (rectal) temperature monitored and their daily water consumption determined gravimetrically.

These parameters were monitored due to the results obtained from previous studies which showed chronic U50,488H treatment to cause hypothermic effects (**Bhargarva, Hemendra et al, 1989**) and the diuretic effect seen upon acute administration of U50,488H (**Leander, 1983(a) & (b)**). Chronic U50,488H resulting in a diuresis would lead to disturbances of water and electrolyte metabolism and ultimately a loss in weight.

5.1.2 SURGERY.

Five days prior to the terminal blood and CSF sampling experiment, the rats were anaesthetized with ether and fitted with an intracerebroventricular infusion cannula driven by a subcutaneous osmotic minipump designed to continuously infuse sterile pyrogen free water ("vehicle" for the control animals) or graded doses of U50,488H at 1µl/hr for five days into a lateral cerebral ventricle. The surgery was carried out as described in **1.3** with the following modifications. The length of the polythene cannula was increased from 18cm to 32cm which, in the U50,488H treated animals was filled with 40µl 10µg/µl, 40µl 20µg/µl and 80µl 50µg/µl U50,488H solution, each separated by 1µl air. The minipump was filled with vehicle to drive the system thus reducing the total amount of U50,488H needed for the infusion. In the control animals, the whole system was filled with vehicle.

Five days after implantation of the infusion device, the animals were anaesthetised with urethane (1.25g/kg i.p.) and fitted with a tracheal cannula, a femoral venous cannula and a femoral arterial cannula. The animals were then placed in a simple headframe to immobilize the head with the neck flexed and the atlanto-occipital membrane cannulated to collect cerebrospinal fluid from the cisterna magna. Details of the surgery are given in **1.4**.

5.1.3 TERMINAL BLOOD AND CSF SAMPLING PROTOCOL.

The sampling protocol is shown in **Fig 5.1(a)** and is the same as that used in **Chapter 4**. Blood samples of 1ml were withdrawn from the femoral artery, cooled over ice, centrifuged at 0°C and the resulting plasma removed and frozen at -20°C until assayed for oxytocin.

The first plasma sample was divided into two aliquots, 200µl for Na^+, K^+ and osmolality measurements and the residue for oxytocin radioimmunoassay.

To maintain plasma volume, the first sample was replaced

Time (mins)	Sample No.
t = -70	Surgery completed
t = 0	CSF sample 1 (~50µl)
t = 5	Plasma sample 1 (1ml)
t = 30	Naloxone (5mg/kg i.v.)
t = 35	Plasma sample 2 (1ml)
t = 40	CSF sample 2 (~50µl)
t = 60	Plasma sample 3 (1ml)
t = 70	CSF sample 3 (~50µl)
t = 120	Plasma sample 4 (1ml) CSF sample 4 (~50µl)

Figure 5.1(a)

Protocol for plasma and cerebrospinal fluid (CSF) sampling in urethane anaesthetised female Sprague-Dawley rats. The rats had either received an intracerebroventricular (i.c.v.) infusion of U50,488H (n=8) or vehicle (n=7) for the previous five days. Blood samples were taken from a femoral artery; plasma from sample 1 was used for osmolality, $[K^+]$ and $[Na^+]$ analysis, as well as oxytocin determination. Oxytocin content of plasma aliqots and CSF samples was determined by radioimmunoassay.

with 1ml isotonic sterile saline via the femoral vein, subsequent samples were replaced with resuspended red blood cells made up to a volume of 1ml in isotonic sterile saline.

CSF samples of ≈ 50µl were collected into preweighed polystyrene tubes, immediately cooled over ice and then frozen at -20°C until assayed for oxytocin.

[Oxytocin radioimmunoassay of plasma and CSF samples was carried out at N.I.M.R., The Ridgeway, Mill Hill, London with the help of Dr. Iain Robinson.]

At the end of the experiment, the animals were killed by an overdose of barbiturate (0.6ml Sagatal) and the infusion devices removed and examined to check that the system remained patent and did not leak or become blocked. The parietal bones of the skull were removed to expose the brain surface in order to check that the cannula entry position into the brain was correct and thick sections cut with a scalpel blade to track the path of the cannula tip to the lateral ventricle .

5.2 RESULTS

Statistical comparisons were made within groups using a paired t-test and between group using an unpaired t-test.

5.2.1 WEIGHT CHANGE

The initial mean weight of the rats at the beginning of the experiment (Day 0) was 255.5 ± 10.2g (n=8, U50,488H infused group) and 254.5 ± 5.6g (n=9, vehicle infused group). There was no significant difference between these starting weights. The rats were weighed daily, at the same time each day (9-10 a.m.), for the duration of the 5 day i.c.v. infusion. The results are shown in **Fig.5.2(a)** as mean change in weight (± s.e.m.) from the mean initial weight (± s.e.m.) of each group of rats taken on Day 0. On Day 1 both groups of rats experienced a loss in weight although there was no significant difference between the two groups. On day 2

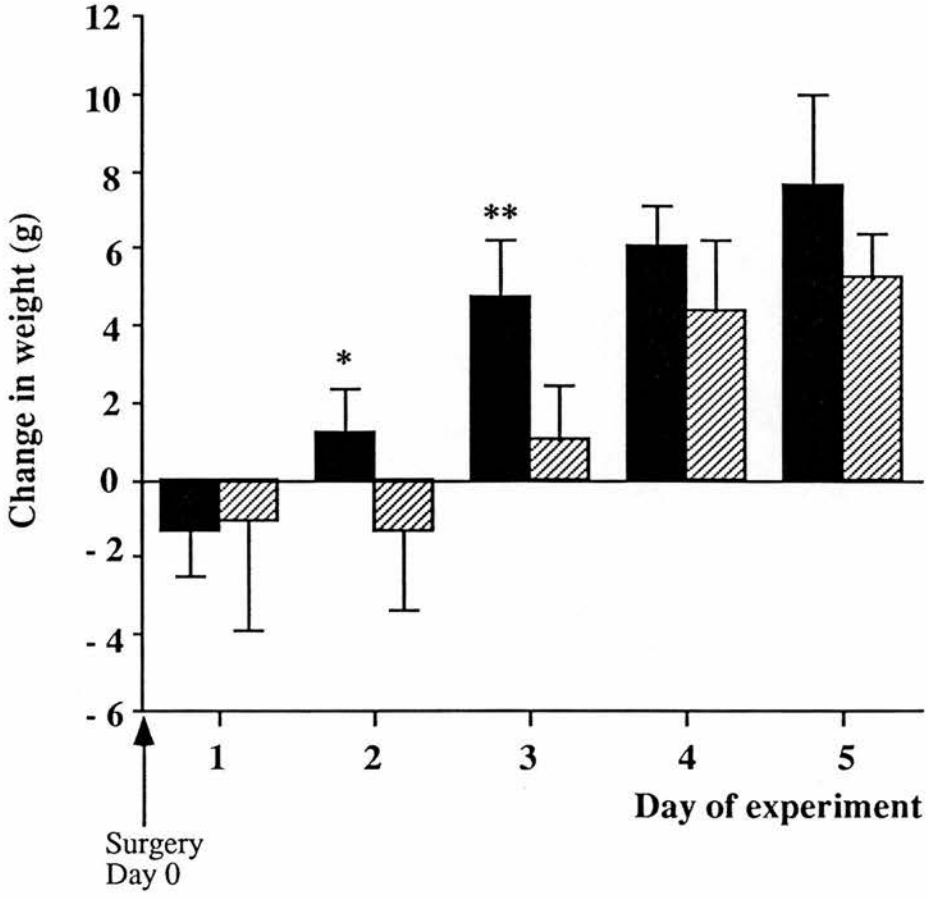


Fig 5.2 (a)

Daily weight change in rats during a five day infusion of vehicle (n=9,solid bars) or U50,488H (n=8,striped bars).
Statistical significance:
* $p<0.05$ with respect to U50,488H treated group (unpaired t-test)
** $p<0.01$ with respect to U50,488H treated group (unpaired t-test).

the vehicle infused animals gained weight with respect to their initial weight on Day 0, whereas the U50,488H infused group of animals did not gain weight and still remained below their initial starting weight ($p < 0.05$ with respect to vehicle infused group, unpaired t-test). On the subsequent days both groups of animals increased their weight above that recorded on Day 0 but there was no significant difference in weight change between the two groups of animals on these days.

5.2.2 CORE BODY TEMPERATURE

Core body temperature was monitored daily in the two groups of rats, the results are shown in **Fig 5.2b (i)**. There was no significant change in temperature recorded in either group over the 5 day infusion period and no difference between the U50,488H infused and vehicle infused groups of rats on any day.

5.2.3 WATER CONSUMPTION

Daily water consumption was measured in the two groups of experimental animals during the 5 day infusion period. The results are shown in **Fig 5.2b (ii)**. There was no significant change in daily water consumption over the 5 day infusion period in either the U50,488H treated or vehicle treated groups. Daily water consumption between the two groups of rats was not significantly different.

5.2.4 PLASMA OSMOLALITY, [Na^+] and [K^+]

Osmolality, [Na^+] and [K^+] were measured in plasma aliquots obtained from the first (basal) blood sample of each rat after induction of anaesthesia with urethane. The results are shown in **Fig 5.2 (c)**. Osmolality values were 280.6 ± 2.2 mOsm/kg in the vehicle infused rats and 280.6 ± 1.2 mOsm/kg in the U50,488H infused rats. Plasma [Na^+] values were 137.2 ± 0.8 mm/L in the vehicle treated animals and 137.3 ± 0.9

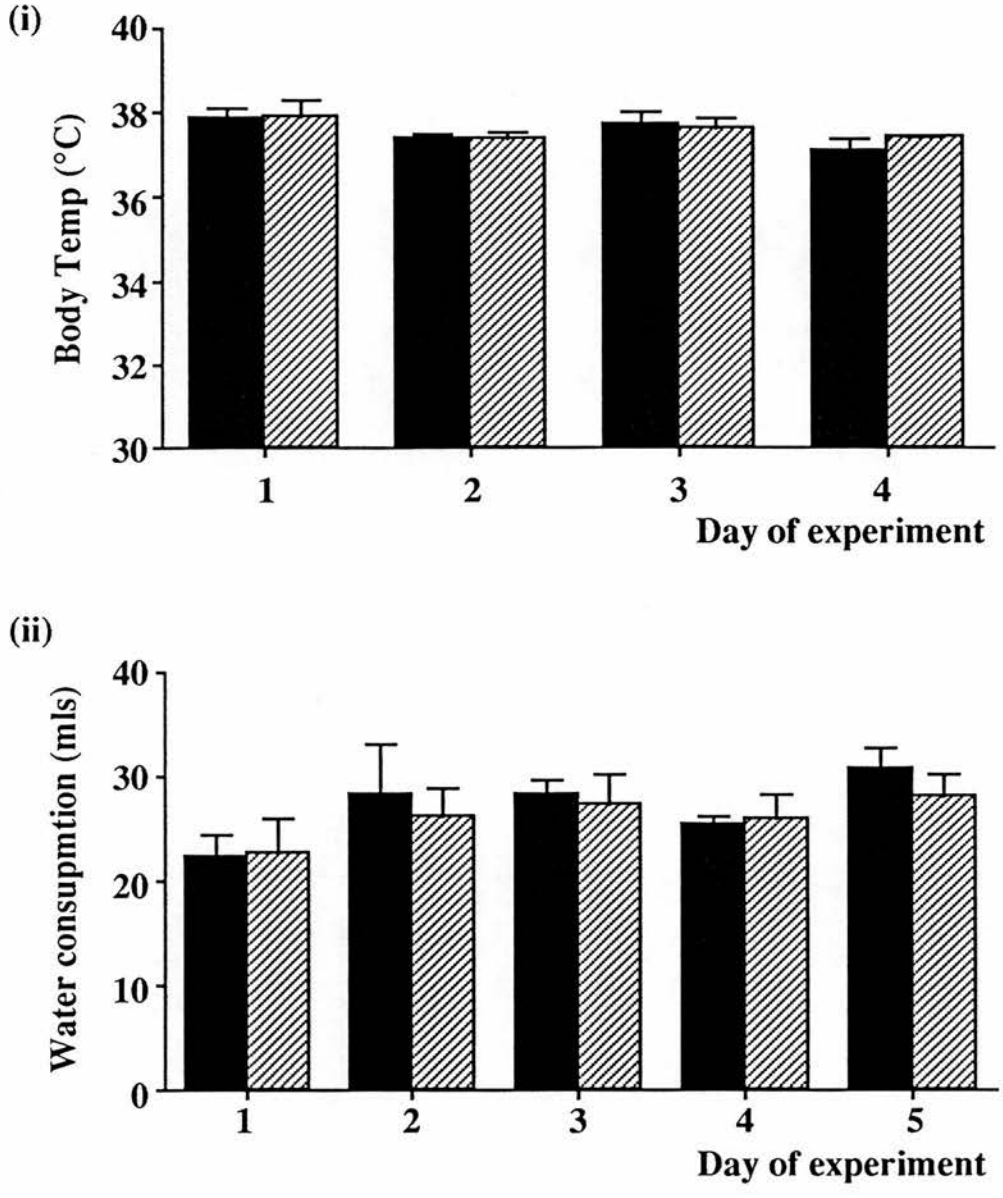


Fig 5.2 (b)

Graphs showing (i) core body temperature (°C) and (ii) daily water consuption in two groups of animals during a five day infusion of vehicle (solid bars,n=6) or U50,488H (striped bars,n=7).Values shown are mean ± sem.There was no significant difference between the two groups of rats in either temperature or water consumption on any day of the experiment.

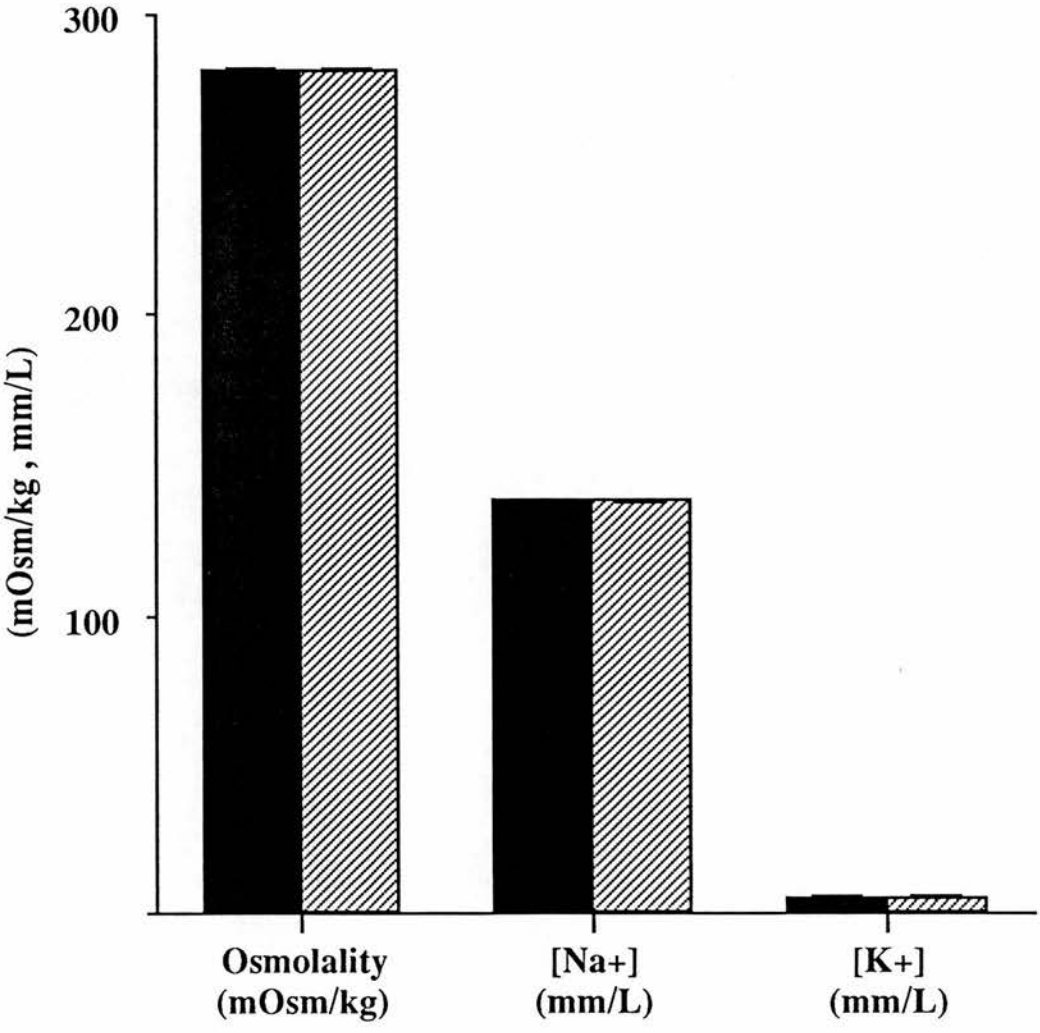


Fig 5.2 (c)

Osmolality,[Na+] and {K+] measurements taken from the first blood sample withdrawn from two groups of rats under urethane anaesthesia.The animals had received an i.c.v. infusion of vehicle (solid bars,n=9) or U50,488H (striped bars,n=8) for the previous five days.There was no significant difference in any of the parameters measured between the two groups of animals.

mm/L in the U50,488H infused group. Plasma [K^+] values were 5.0 ± 0.5 mm/L in the vehicle treated rats and 4.8 ± 0.5 mm/L in the U50,488H treated rats. There was no significant difference between these results in either treatment group of rats.

5.2.5 PLASMA OXYTOCIN

Mean plasma oxytocin values in both groups of animals during the blood sampling protocol are shown in **Fig 5.2(d)**.

Basaloxytocin levels were 35.5 ± 26.3 pg/ml in vehicle infused animals and 39.4 ± 18.7 pg/ml in U50,488H infused animals (n=7 in each group). The standard errors are large due to a few animals in each group showing high levels of oxytocin throughout the experiment but there is no significant difference in basal oxytocin levels between the two treatment groups.

Following i.v. naloxone at t=30mins plasma oxytocin was elevated in both groups of rats (382.0 ± 195.7 pg/ml in vehicle infused rats and 357.9 ± 120.6 pg/ml in U50,488H infused rats). This elevation in plasma oxytocin was significant with respect to pre-naloxone values in the vehicle infused group ($p < 0.03$, paired t-test) but not in the U50,488H infused group (this is due to the large variation in plasma oxytocin values for this group of animals and the resulting large errors). There was no difference in response to naloxone between the two groups.

Plasma oxytocin remained elevated in the following two plasma samples in both groups of rats (413.6 ± 152.5 and 323.4 ± 168.9 pg/ml in vehicle treated rats and 387.2 ± 105.6 and 330.7 ± 107.4 pg/ml in U50,488H infused rats). These values are significantly elevated from pre-naloxone plasma oxytocin levels, ($p = 0.008$, vehicle infused group, $p < 0.04$, U50,488H infused group, paired t-test). There was no significant difference in plasma oxytocin levels between the two

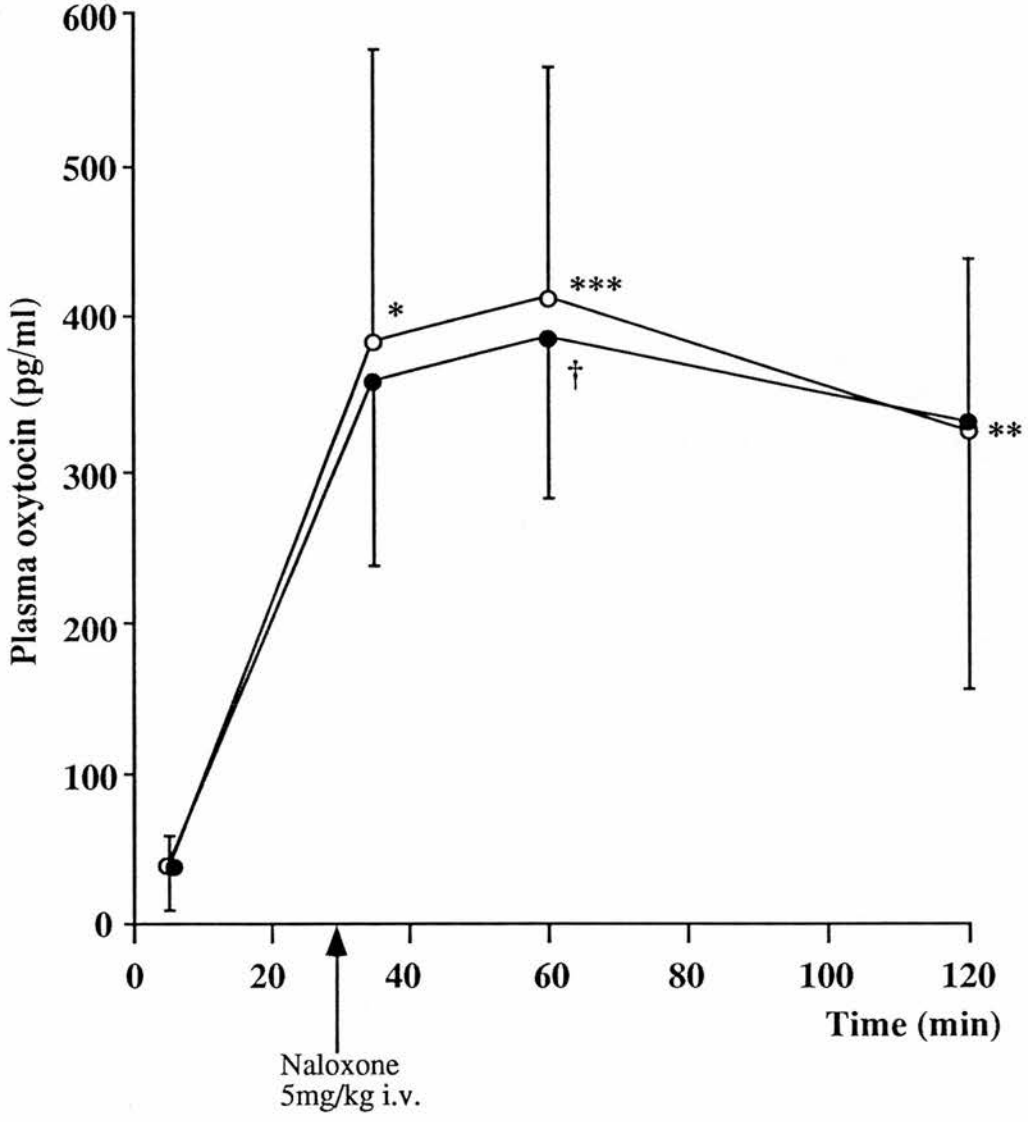


Fig. 5.2 (d)

Oxytocin measured in plasma of urethane-anaesthetized rats which had received an i.c.v. infusion of U50,488H (filled circles,n=7) or vehicle (open circles,n=7) for the previous 5 days.Naloxone was given at t=30 mins. Statistical comparisons :
* $p< 0.03$,** $p< 0.02$,*** $p< 0.008$,† $p<0.04$ with respect to pre-naloxone values (paired t-test).There was no significant difference in plasma oxytocin between the groups.

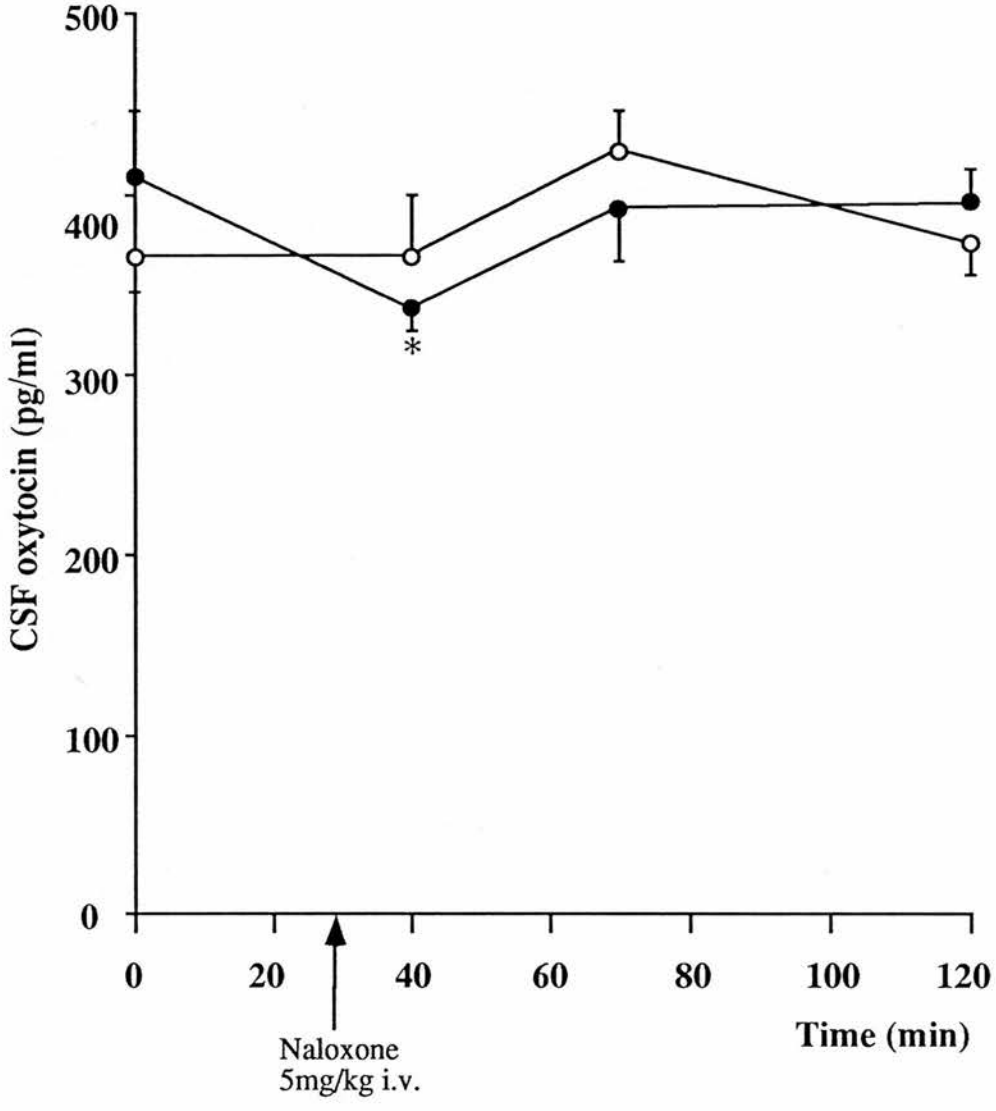


Fig 5.2 (e)

Oxytocin measured in CSF of urethane-anaesthetized rats which had received an i.c.v. infusion of U50,488H (filled circles,n=7) or vehicle (open circles,n=7) for the previous 5 days Naloxone was given at t=30 mins.
Statistical comparisons:
* $p < 0.05$ with respect to pre- naloxone value (paired t-test).
No significant differences in CSF oxytocin between the groups.

groups of rats at any time point during the blood sampling protocol.

5.2.6 CSF OXYTOCIN

Mean CSF oxytocin levels in both groups of animals are shown in **Fig 5.2(e)**. Basal CSF oxytocin concentration was 365.7 ± 19.9 pg/ml in vehicle infused rats and 410.9 ± 35.5 pg/ml in U50,488H infused rats. There was no significant difference between these values.

Following i.v. naloxone injection at t=30 minutes there was no change in CSF oxytocin in the vehicle treated rats (365.7 ± 34.8 pg/ml) and a decrease in CSF oxytocin in the U50,488H treated rats (337.4 ± 12.8 pg/ml, $p<0.05$ with respect to pre-naloxone values, paired t-test).

At t=70 minutes CSF oxytocin in the vehicle treated group had risen to 423.3 ± 22.4 pg/ml and to 391.6 ± 27.9 pg/ml in the U50,488H treated group. These changes in oxytocin were not significant and CSF oxytocin did not significantly differ between the two groups of experimental animals at any time.

5.3 DISCUSSION

Measurements of weight change, water consumption, body temperature, hydromineral balance and plasma oxytocin failed to reveal any significant differences between the U50,488H infused rats compared to the vehicle treated groups. This can be interpreted in two ways, (i) U50,488H infused for 5 days does not affect these parameters or, (ii) the animals have become tolerant to the effects of U50,488H.

Previous experiments have shown that acute administration of U50,488H has profound diuretic effects in rats (**Leander, 1983 (a & b)**). This is believed to be opioid-mediated and is dependent upon an intact and functional adrenal medulla, implicating a blood-borne diuretic factor of adreno-medullary origin (**Borkowski, 1989**). Although the animals were not

housed in metabolism cages and hence their urinary output accurately determined, there was no obvious sign of a diuretic response to the U50,488H infusion and measurement of plasma osmolality and electrolyte balance showed that there was no disturbance to hydromineral balance on Day 5.

The U50,488H infused group of rats maintained a weight loss following surgery until Day 3, when their weight was not significantly lower than the vehicle infused group. Animals treated with vehicle (and animals infused with morphine observed in other studies) show a weight loss on Day 1 after surgery but by Day 2 have gained weight to above their initial recorded mass on Day 0. This would indicate that the maintained weight loss was an effect of the U50,488H, possibly as a result of a diuresis but that the animals became tolerant to the U50,488H and that there was not a continued diuresis or weight loss.

Plasma and CSF oxytocin concentration in the first samples taken showed that there was no significant difference in these basal values between the U50,488H treated or vehicle treated animals. Inhibition of oxytocin release from the isolated neurohypophysis and isolated neurosecretory terminals has been effected by κ-opioid receptor agonists (**Bicknell, Chapman & Zhao, 1987 (a & b)**). Acute injection of the κ-opioid receptor agonist bremazocine (0.1mg/kg s.c.) decreases plasma oxytocin as the initial effect in a biphasic response to the opiate (**Grell, Fjalland & Christensen, 1989**). However, it is not possible to say whether the similar plasma oxytocin levels in the two groups of rats is due to lack of effect of U50,488H or tolerance to the opiate.

Administration of naloxone (5mg/kg i.v.) did not produce the hypersecretion of oxytocin into plasma and CSF associated with morphine withdrawal (**Chapter 4, this thesis; Coombes, Robinson & Russell, 1988**). This implies that the oxytocin system does not become dependent upon

κ-opioid agonists as it does upon the μ-opioid agonist morphine. There was a significant elevation in plasma oxytocin in both groups of rats following naloxone injection but this was probably due to antagonism of endogenous opioid tone acting at oxytocin nerve terminals in the neural lobe.

Dependence of oxytocin upon morphine and subsequent naloxone-induced withdrawal is believed to be mediated via μ-opioid receptors. In this experiment, dependence upon U50,488H following continuous infusion for 5 days would be via κ-opioid receptors. There are several reasons why dependence was not established or withdrawal from the opiate not seen.

(i) Morphine crosses the blood brain barrier relatively easily and therefore finds the μ-receptors within the SON easily accessible from its site of infusion in the lateral ventricle. It is possible that U50,488H does not cross the blood brain barrier so easily and therefore is not so easily distributed to central κ-opioid receptors. Previous studies where U50,488H has been infused into the Sylvian aqueduct have shown that the drug is distributed successfully (**Cowan, Zhu et al, 1988**) and therefore this cannot account for the lack of dependence of oxytocin neurones on U50,488H.

(ii) There is now evidence for at least three kinds of κ-opiate receptors, κ_1, κ_2 and κ_3 (**Zukin, Eghbali et al, 1988; Clark, Liu et al, 1989**). The density of these receptors is species specific and they bind different κ-opiate receptor agonists with differing affinity. It is possible that the subtype of κ-receptor within the SON is different to that found at the neural lobe where U50,488H has profound inhibitory effects (**Coombes & Russell, 1988**). U50,488H is found to inhibit magnocellular oxytocin cell body firing rate in the anaesthetized rat (**Leng, Pumford & Russell, 1990**) and it is therefore unlikely that the predominant type of κ-receptor found in the rat hypothalamus only binds U50,488H with low affinity.

It has been shown that there is down-regulation of κ-receptors in the neural lobe as a result of increased endogenous opioid release (**Brady, Rothman & Herkenham, 1988**). It is possible that exposure of central κ-opioid receptors to U50,488H over 5 days would also lead to down regulation of receptors, which is what is seen to happen to μ-receptors in the SON during the development of morphine tolerance (**Sumner, Coombes et al, 1990**). Tolerance to U50,488H may, therefore, have occurred

(iii) If down regulation is a sign of the development of tolerance then a withdrawal excitation such as that seen upon withdrawal from morphine is difficult to explain. There may be changes in post-receptor mechanisms regulating ion fluxes that account for the withdrawal excitation seen in morphine dependent oxytocin neurones challenged with naloxone.

Mu opioid receptors are coupled to a G protein ($G_{i/o}$) which has been demonstrated in the anaesthetized rat pretreated with pertussis toxin to inactivate the G protein by ADP-ribosylation. In these animals, the inhibitory action of morphine on oxytocin cell body firing is significantly reduced (Russell, Pumford & Leng, 1991).

U50,488H does not work via a G protein since it is still effective at inhibiting oxytocin cell body activity in pertussis toxin treated rats. U50,488H is known to decrease $[Ca^{2+}_i]$ by blocking the voltage-sensitive N-type calcium channel through a κ-opioid receptor mediated mechanism. This inhibits oxytocin secretion, since intracellular calcium is decreased thus preventing the release of oxytocin from its synaptic vesicles. Following infusion of U50,488H and possible development of tolerance it may be that the post-receptor mechanism leading to the reduction of $[Ca^{2+}_i]$ cannot be modified by naloxone and hence a withdrawal excitation of oxytocin neurones is not seen.

In conclusion, it would appear that oxytocin neurones

centrally projecting neurones can also be made morphine dependent by i.c.v. infusion of morphine and undergo withdrawal excitation following naloxone injection.

In control animals with a bilateral PVN lesion there was not a rise in CSF oxytocin following naloxone suggesting that endogenous opioids do act on centrally projecting neurones in the PVN. In morphine-dependent animals following naloxone administration, hypersecretion of oxytocin into CSF is seen in animals with a bilateral ablation of the PVN and it must therefore be concluded that it is not only oxytocin neurone projections from the PVN that are responsible for oxytocin seen in CSF, at least after PVN lesion.

The source and nature of the endogenous opioids concerned with regulation of centrally projecting oxytocin neurones and whether they act at the cell bodies or nerve terminals is not yet known.

The elevated basal CSF oxytocin content observed in PVN lesioned animals (**Fig 4.2g**) is similar to that seen in the i.c.v. vehicle- and i.c.v. U50,488H-infused animals in Chapter 5 (**Fig 5.2e**). No calculation errors can be detected from the assay results. It is possible that during storage of the samples some breakdown product was formed that cross-reacted with the assay but this is unlikely since the storage, transit and assay procedure was the same for all CSF samples generated. The similarity between the results from these two groups of animals remains difficult to resolve.

CHAPTER SIX

AUTORADIOGRAPHIC RECEPTOR BINDING STUDY TO DETERMINE CHANGES IN OPIOID RECEPTOR SUBTYPE POPULATIONS IN THE NEURAL LOBE OF THE RAT DURING THE DEVELOPMENT OF MORPHINE TOLERANCE.

CHAPTER 6 : Autoradiographic receptor binding study to determine changes in opioid receptor subtype populations in the neural lobe of the rat during the development of morphine tolerance .

INTRODUCTION

In the rat, both endogenous and exogenous opiates are known to inhibit oxytocin secretion from nerve terminals in the posterior pituitary (**Bicknell & Leng, 1982; Russell, Gosden et al, 1989; Russell & Spears, 1984**). Opiates acting on oxytocin neurones exert their influence at the perikarya (within the supraoptic (SON) and paraventricular nuclei (PVN) of the hypothalamus) and at the nerve terminals in the posterior pituitary.

There are three principle opioid receptor subtypes, identified pharmacologically by their binding selectivity for opiate agonists and antagonists (**Gillan & Kosterlitz, 1982**). In-vitro receptor autoradiography has been used to investigate the distribution of μ, δ and κ receptors in the rat brain (**Mansour, Lewis et al, 1986; Mansour, Khachaturian et al, 1987 & 1988; Tempel & Zukin, 1987**).

In the SON, κ but no μ or δ opioid-receptors were found. Both morphine, a μ-opioid receptor agonist and U50,488H, a κ-opioid receptor agonist have been shown to inhibit the electrical activity of oxytocin neurones in the SON (**Leng, Pumford and Russell, 1990**), so it is puzzling that no μ-receptors were located at this site. In the posterior pituitary, κ-opioid receptors were found to predominate with a few μ but no δ-receptors present (**Bunn, Hanley & Wilkin, 1985**). This correlates well with experiments previously described which demonstrated that in the anaesthetized rat, the κ-opioid receptor agonist U50,488H will inhibit oxytocin secretion evoked by electrical stimulation of the neurohypophysial stalk whilst morphine, a μ-opioid

receptor agonist is ineffective at this site (**Coombes & Russell, 1988; Chapter 2 this thesis**).

Infusion of morphine into a lateral cerebral ventricle of the rat for a period of 5 days, (as described in 1.3), results in oxytocin neurones becoming first tolerant and then dependent upon the opiate. Tolerance is described as lack of opiate effect or the need for a greater dose to acheive the initial effects seen on oxytocin neurones. Dependence is revealed upon administration of the opiate antagonist naloxone which results in excitation of oxytocin cell body activity and hypersecretion of oxytocin into plasma and CSF (**Bicknell, Leng et al, 1988; Rayner, Robinson & Russell, 1988**; Coombes, Robinson & Russell, 1988).

Chronic infusion of the κ-opioid agonist, U50, 488H appears to result in tolerance to the opiate (as determined by its lack of effect on basal plasma and CSF oxytocin, with respect to control animals) but there was no naloxone induced withdrawal response in terms of oxytocin neurone electrical activity or secretion into plasma or CSF (**Chapter 5, this thesis**).

Tolerance to opiates could be explained by a decrease in opioid receptors that modify oxytocin secretion. In the hypothalamus, including the SON, there are μ-opioid receptors (as judged by the actions of selective opioid-agonists on oxytocin neuronal activity) and it is thought that tolerance and dependence are mediated through μ-receptors at the cell bodies of oxytocin neurones and not at their terminals in the posterior pituitary (**Bicknell, Leng et al, 1988**). However, cross tolerance between receptor subtypes has been described in some systems (**Yamamoto, Ohno & Ueki, 1988; Bhargarva, Hemendra et al, 1989**) and this experiment was designed to look for changes in specific opioid-receptor populations in the neurohypophysis during the development of morphine tolerance.

6.1 METHODS

6.1 (i) ANIMALS

The animals used were female Sprague-Dawley rats which were caged individually under conditions of controlled photoperiod (12hrs light/12hrs dark, lights on 8am) and temperature (23°C). Food and water were freely available.

There were 6 rats in each experimental group and the mean weight (± sem) of the groups were 274.8 ± 5.1g in the morphine infused group and 288.8 ± 8.7g in the vehicle infused group.

6.1 (ii) SURGERY

The animals were implanted with a subcutaneous osmotic minipump and intracerebroventricular infusion cannula, under ether anaesthesia, five days prior to the terminal experiment. Full details of this procedure are described in 1.3.

Six animals were infused with vehicle and six with morphine in graded doses of 10, 20 and 50μg/μl at a rate of 1μl/hr. The rats were set up in pairs i.e. one morphine treated and one vehicle treated were set up on the same day so that one animal from each treatment group would be processed together.

6.1 (iii) TERMINAL EXPERIMENT : TISSUE COLLECTION

After 5 days of morphine infusion the rats were decapitated and trunk blood collected into heparinized tubes. The blood was centrifuged at 0°C and divided into aliquots for :-

a) Oxytocin determination : to see if morphine tolerance was reflected by basal plasma oxytocin levels (RIA as described in 1.7.3.

b) Osmolality and Na^+ : to determine whether chronic morphine treatment effected any change in hydromineral balance. Changes in Na^+ or osmolarity may stimulate the oxytocin/vasopressin system leading to changes in

opioid receptors in the posterior pituitary as described by **Brady & Herkenham, 1987.**

c) Glucose content : to see if chronic morphine treatment leads to any change in plasma glucose concentration, using a Boehringer kit: Glucos GOD-Perid. (Acute morphine is reported to elevate plasma glucose, **Gross & Pierce, 1935**).

The skull was opened and the brain quickly removed. Coronal frozen cryostat sections including the supraoptic nuclei (SON) and medial preoptic nucleus (MPN) were taken to determine changes in opioid receptor density in these regions during development of morphine tolerance and dependence (**Sumner, Coombes et al, 1990**).

The whole pituitary was removed from its protective meninges (see 3.1) and oriented for coronal sectioning on a layer of frozen Tissue-Tek II OCT compound (**Miles Laboratory**) on a chuck pre-cooled in solid CO_2. The gland was completely covered with more OCT compound and returned to the solid CO_2 until thoroughly frozen. 10μm pituitary sections were cut with a cryostat and thaw mounted on to pre-cleaned and pre-coated slides. (Cleaned in chromic acid, washed in running water followed by double distilled water and coated with chrome alum gelatine).

Four sections were taken for each incubation category per rat, each containing a coronal section of neural lobe surrounded by intermediate lobe and anterior lobe tissue. The slides with sections were kept in a slide tray, sealed in polythene bags and stored in a deep freeze at -20°C for up to three weeks before incubation with the appropriate ^{3}H-ligand.

6.1 (iv) INCUBATION MEDIA

The frozen sections of neural lobe were incubated with ^{3}-H ligands and displacers in order to identify and allow quantification of the opioid subtypes present. The

incubation medium used for these sections contained 2nM ^{3}H-(-) bremazocine. Bremazocine is a kappa opioid receptor ligand although it is not highly selective for kappa receptor binding (**Morris & Herz .1986**). This was preferred to using ^{3}H-etorphine (which was used to determine total opioid receptor binding in sections containing the SON and MPN taken from the same animals). ^{3}H etorphine was found to show negligible labelling of pituitary sections. [Pilot studies carried out by B.E.H.S].

Etorphine is a μ-opioid receptor ligand although it is regarded as being non-selective and has been used in autoradiographic studies to locate κ-opioid receptors (**Bunn, Hanley & Wilkin, 1985; Lightman, Ninkovic et al, 1983**). Since the predominant receptor subtype at the neural lobe is kappa, bremazocine would be expected to show greater binding at this site than etorphine, as found.

To expose the specific opioid receptor subtypes, unlabelled, highly selective agonists were used as competitive displacers.

i) **μ-receptor displacer**: DAGO (Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH.

ii) **δ-receptor displacer**: DPDPE [D-Pen^{2}, D-Pen^{5}] enkephalin.

iii) **κ-receptor displacer**: U50,488H (trans-(±)-3,4-dichloro-N-methyl-N-(2-[1-pyrroldinylcyclohexyl]) benzene methane sulfonate.

To detect individual receptor subtypes two unlabelled displacers were included with the ^{3}H-ligand in the medium so that only the third type of receptor would be selectively located by the ^{3}H-ligand.

2μM displacer (1000-fold excess) was used with 2nM ^{3}H-ligand. Pilot experiments (**B.E.H.Sumner**) showed that displacer in concentrations of up to 50μM did not further reduce ^{3}H-ligand binding. Naloxone (a non-selective opioid antagonist) was used in the medium on some sections to displace the probe and thus act as a

RECEPTOR TYPE ISOLATED	ISOLATING MEDIUM	CODE
All opiate receptors	2nM ^{3}H-bremazocine	B
Specificity controls	2nM ^{3}H-bremazocine + 2μM naloxone	B_N
μ-receptors	2nM ^{3}H-bremazocine + 2μM D-pen,D-pen + 2μM U50,488H	B_μ
δ-receptors	2nM ^{3}H-bremazocine + 2μM DAGO + 2μM U50,488H	B_δ
κ-receptors	2nM ^{3}H-bremazocine + 2μM DAGO + 2μM D-pen,D-pen	B_κ
Chemography controls	no ligand	B_c

Table 6.1 (I)

Summary table to show the specific labelled ligand and unlabelled displacers included in the incubation media for each receptor subtype isolated. Four sections of neural lobe were cut in each incubation category.

control for total opioid receptor-specific binding of the probe.

Other control sections were incubated without ^{3}H-ligand in the incubating medium to act as chemography controls - i.e. to check that the unlabelled sections did not produce images on the emulsion.

The combination of drugs in each incubation medium type is summarized in **Table 6.1 (I)**. All solutions were made up in double distilled water.

6.1 (v) INCUBATION PROCEDURE

The frozen sections were allowed to come to room temperature over one hour. They first underwent pre-incubation to detach and wash out endogenous opioid or exogenous opiate (arising from the morphine infusion) from the tissue. The pre-incubation medium (0.015M potassium phosphate buffer containing 0.15m NaCl + 0.1% BSA, pH 7.4) was applied to the sections with a Gilson pipette as a 20µl puddle. Pre-incubation was for 30 minutes at 0°C and took place in moist chambers, plastic boxes lined with filter paper saturated with ice cold pre-incubation medium.

After this, the puddles were drained off the slides and replaced with 20µl incubating medium containing ^{3}H-ligand and displacers as appropriate. The slides were incubated for 3hrs at 0°C in moist chambers. After this time, the medium was drained off the slides which were then loaded into slide racks. They were then immersed in successive baths of ice cold pre-incubation medium to remove excess unbound ligand displacer from the tissue, for one minute per bath. Finally, the slides were immersed in ice cold double distilled water to remove salts. The slides were propped vertically in drying racks and allowed to dry in air at room temperature, protected from dust by a canopy of paper towels. The dried slides were stored in sealed boxes with a gauze bag containing silica gel as a dessicant

for a maximum of 24hrs at 0°C before autoradiographic processing.

6.1 (vi) AUTORADIOGRAPHIC EXPOSURE

The slides from a pair of rats (one vehicle infused and one morphine infused) were allowed to come to room temperature slowly before opening the boxes, to avoid condensation forming. In a dark room under safe light conditions, the slides were apposed to Hyperfilm-^{3}H (**Amersham**), keeping slides from the same incubation category of the two rats together. A 5μM tritiated standard (^{3}H-microscales, **Amersham**) was also attached to each sheet of film for normalization purposes in grain density analysis. The slides were secured to the sheets of film with strips of adhesive masking tape. The sheets of film were then sandwiched between two mirror tiles held together with adhesive tape to maintain a good even contact between slides and film, wrapped in brown paper followed by aluminium foil and finally sealed in opaque plastic bags. The films were exposed at 0-4°C in a cold room for 16 weeks.

6.1 (vii) HYPERFILM PROCESSING

The following procedures were all carried out in the dark under safe light conditions. The sealed packages containing Hyperfilm and slides were allowed to come to room temperature. the packages were then unsealed and the slides removed from the Hyperfilm by slitting through the adhesive tape. The slides were loaded into labelled slide boxes for the sections to be stained later and the film marked by cutting notches in the corner to denote the orientation in which the slides had been exposed and which pair of rats it corresponded to.

The film was immersed, emulsion side uppermost, in developer (**Kodak D19 developer**) for five minutes. The film was then rinsed quickly in tap water and immersed in a tray of fixer, emulsion side upwards for five

minutes (**Ilford Hypam rapid fixer**, diluted 1:4 with tap water). The film was transferred to a second tray of fixer for five minutes and then washed in the sink under running tap water for 30 minutes and finally hung up to dry. When dry, the film was labelled with the slide serial numbers so that the images could be identified; films were stored in polythene bags to prevent scratching.

6.1 (viii) STAINING

The pituitary sections were fixed for 10 minutes in a 1:17:2 mixture by volume of acetic acid:absolute alcohol:formalin. Following this they were dehydrated, rehydrated, stained for 15 minutes in 1% cresyl fast violet, differentiated, dehydrated, cleared and mounted in DPX for neuroanatomical analysis (**Paxinos & Watson, 1982**).

6.2 IMAGE ANALYSIS

The sheets of film were cut into microscope slide sized strips and attached to microscope slides with adhesive tape for analysis. The autoradiographic images were assessed in two ways:-

6.2 (i) SUBJECTIVE QUALITATIVE ASSESSMENT

The autoradiographs were examined under a binocular microscope (**Wild M3**), magnification ×16 and the grain density assessed in the following way :- 0 (none), + (weak), ++ (moderate), +++ (moderately strong) and ++++ (strong).

6.2 (ii) QUANTITATIVE ASSESSMENT

The neurohypophysis boundary was scratched on the film with a needle under the microscope so that the area was clearly defined for analysis at high magnification. Analysing apparatus consisted of a Joyce-Loebl μMagiscan image analysing computer that received its input from a Phillips black and white video 40TV

camera mounted on a Vickers M17 microscope (**Sumner, Kawata & Russell, 1989**). Magnification in the microscope was ×10 (objective) with a further ×1.6 (intermediate lens), whilst on the TV monitor the scale factor was 1.1878μm per pixel.

A counting frame equivalent to 201.63μm × 133.04μm was placed over images on the TV monitor of both central and peripheral regions of the neurohypophysis for grain density measurements, since more opioid receptors have been reported in the external rim of the neurohypophysis (**Herkenham, Rice et al, 1986; Brady & Herkenham, 1987**). The "grey level" threshold which defined silver grains could be altered for each individual image so that only silver grains were detected by the computer. The grain density was calculated thus:

$$\frac{\text{Total area of silver deposit}}{\text{Total area of counting frame}}$$

(**Sumner et al, 1989**).

6.3 RESULTS

6.3 (i) EXPRESSION OF RESULTS

For each grain density measurement (**T**), a background measurement (**BT**) was subtracted. The background measurement was taken close to the tissue area counted but in a tissue free area. For each tissue incubation category, activity above background was determined by (**T-BT**). The mean (**T-BT**) value for each incubation category was calculated - as tissue from morphine and vehicle treated rats was processed in pairs, these values were compared by the paired t-test.

For conversion to absolute values, grain densities were measured in triplicate for each step of the standard scale exposed on each sheet of film and the background values subtracted. A mean standard curve of grain density against radioactivity in terms of its brain-gray matter-tissue equivalent (calibrated by **Geary &**

Wooten for Amersham) was computed for the SON and MPN analysis (9 weeks exposure) and for analysis of the posterior pituitary (16 weeks exposure). These were then fitted to a power function -

$y=0.34x^{0.14}$ and $r=0.96$ for posterior pituitary standard curve.

Tissue radioactivity values (nCi/mg of tissue) were then calculated from the mean grain densities and were converted to fmol of ^{3}H-ligand bound per mg of tissue by reference to the specific activity of the ^{3}H-ligand. Specific binding to opioid receptors was obtained by subtracting binding in the presence of naloxone. Comparisons between morphine and vehicle treated groups mean ^{3}H-ligand binding values were made using the Student t-test.

6.3 (ii) QUALITATIVE RESULTS

Autoradiographic images were obtained from the posterior pituitary but not from the anterior lobes. The subjective assessment results are shown in **Table 6.3 (I)**.

6.3 (iii) QUANTITATIVE RESULTS

There was no significant difference between the mean grain density or mean binding of central or peripheral regions of the posterior pituitary. Central and peripheral data, therefore, have been combined and the mean values used. The mean grain densities and mean specific binding values for each incubation category in both groups of rats are shown in **Tables 6.3 (II)** and **6.3 (III)** respectively.

The autoradiographic images obtained from the B_{κ}, B_{μ}, B_{ν} and the B_{N} incubation categories along with the corresponding CFV stained tissue section are shown in **Plate 6**.

6.3 (iii) BLOOD PLASMA ANALYSIS

The blood plasma analysis results are shown in **Table**

Incubation category and code	Assessment
[^{3}H](-)-Bremazocine alone (B)	+++
[^{3}H](-)-Bremazocine + DAGO + DPDPE (B_κ)	+++
[^{3}H](-)-Bremazocine + DPDPE + U50,488H (B_μ)	++
[^{3}H](-)-Bremazocine + U50,488H + DAGO (Bδ)	O-+
[^{3}H](-)-Bremazocine + naloxone (B_N)	O
Chemography controls	No image

Table 6.3 (I)

Subjective assessment results for the posterior pituitary for each incubation category. There was no difference in autoradiographic image intensity between morphine treated and control animals.

Incubation Category	Mean Binding μm^2 silver deposit/μm^2 field	
	Vehicle	Morphine
[^{3}H](-)-Bremazocine	0.18 ± 0.04	0.19 ± 0.05
[^{3}H](-)-Bremazocine + DAGO + DPDPE	0.13 ± 0.05	0.15 ± 0.03
[^{3}H](-)-Bremazocine + DPDPE + U50,488H	0.11 ± 0.05	0.10 ± 0.03

Table 6.3 (II)

Table of mean (± sem) binding results obtained for the B, B_κ and B_μ incubation categories in rat neural lobe tissue. (B_δ images were not significantly above background). The specificity control values have been subtracted from these results. There were no significant differences between morphine treated and control animals (n=6 animals per group).

Incubation Category	Mean Binding fmol/mg tissue	
	Vehicle	Morphine
[^{3}H](-)-Bremazocine	11.6 ± 6.30	18.0 ± 8.46
[^{3}H](-)-Bremazocine + DAGO + DPDPE	3.25 ± 2.09	2.72 ± 1.42
[^{3}H](-)-Bremazocine + DPDPE + U50,488H	2.00 ± 1.21	1.07 ± 0.73

Table 6.3 (III)

Table of mean binding results obtained for the B, B_κ and B_μ incubation categories in rat neural lobe, expressed as fmol/mg tissue. B_δ images were not significantly above background. There was no significant difference in binding between morphine treated or morphine naive animals. n=6 animals per experimental group.

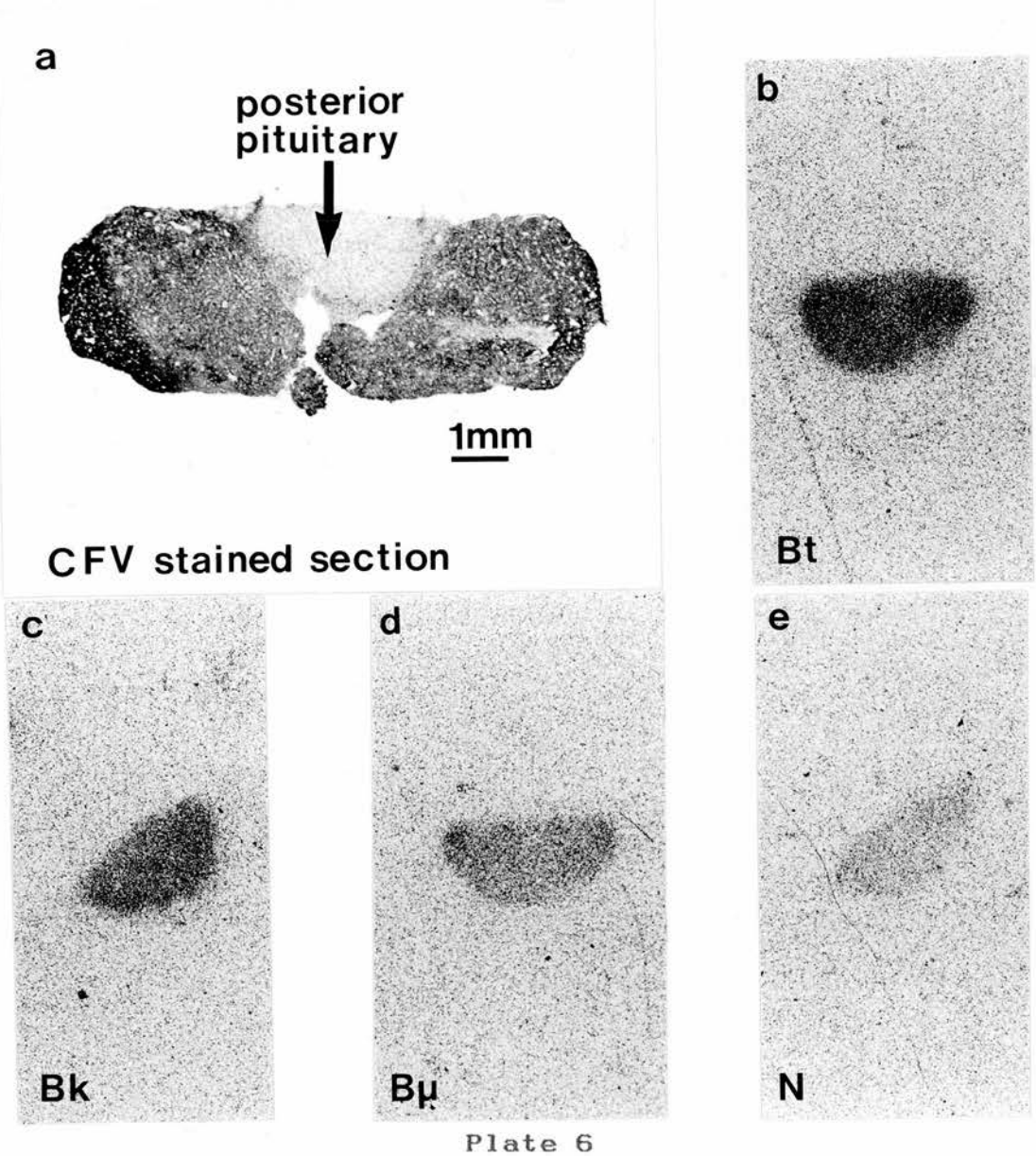


Plate 6

(a) shows a 10μm section of rat pituitary incubated with [^{3}H](-)-bremazocine, apposed to Hyperfilm-^{3}H for 16 weeks, then fixed in acetic acid-ethanol-formalin and stained with Cresyl Fast Violet. (b) is the autoradiographic image of (a), showing total binding to opioid receptors. Only the posterior pituitary produces an image. (c) & (d) show autoradiographic images selective for κ and μ-receptors respectively (by virtue of the displacers in the incubating media). (e) shows non-specific binding of probe in the posterior pituitary. Magnification ×8 throughout.

	Treatment Group	
	Vehicle infused (n=6)	Morphine infused (n=6)
Plasma oxytocin (pg/ml)	13.23 ± 5.06	8.67 ± 1.77
Plasma glucose (mM/l)	7.82 ± 0.43	7.31 ± 0.24
Plasma [Na^+] (mM/l)	138.29 ± 2.23	142.71 ± 1.41
Osmolality (mOsmol/kg)	270.00 ± 5.63	268.29 ± 5.99

Table 6.3 (IV)

Table to show the mean (± s.e.m.) values of data obtained from blood plasma analysis. There was no significant difference in values between the morphine naive and morphine tolerant rats.

6.3 (IV). There were no significant differences between morphine treated and control animals for any of the parameters measured.

6.4 DISCUSSION

In the control group of rats, highest levels of binding to opioid receptors was seen after incubating the posterior pituitary sections with [^{3}H](-) bremazocine alone, which was expected to bind to all types of opioid receptor present in the tissue. Strong binding was also seen in the tissue sections incubated with [^{3}H](-) bremazocine with DAGO and DPDPE included in the incubation medium which was intended to isolate kappa-opioid receptor binding. (DAGO which has a relatively high affinity for μ-receptors and DPDPE which has a relatively high affinity for δ-receptors were in 1000-fold excess-leaving predominantly κ-receptors available for binding) **(Brady & Herkenham, 1987; Bunn, Hanley & Wilkin, 1985; Herkenham, Rice et al, 1986).**

Addition of U50,488H to the medium containing DAGO or DPDPE significantly decreased grain density or specific binding values indicating that the κ-opioid receptor predominates in rat neural lobe. This correlates well with other autoradiographical, **in-vitro** and previously described **in-vivo** (see **Chapter 2**) studies, whose results indicate that it is principally κ-receptors that are found in the neural lobe **(Bunn, Hanley & Wilkin, 1985; Herkenham, Rice, Jacobson & Rothman, 1986; Zhao, Chapman & Bicknell[c], 1988; Coombes & Russell, 1988).**

The resolution of the autoradiographic method in this study was not sufficient to determine whether the receptors visualised were located on oxytocin nerve terminals or on surrounding pituicytes. Other studies have suggsted that κ-opioid receptors in the neurohyphophysis may be located on pituicytes **(Lightman, Ninkovic et al, 1983)** or neurosecretosomes.

There appeared to be some significant binding of

bremazocine when U50,488H and DPDPE were included in the incubation medium. These conditions should have been selective for μ-opioid receptor binding. In pilot studies (B.E.H.Sumner) on the posterior pituitary using tritiated etorphine as a probe, much less binding under conditions selective for isolating μ-opioid receptors was seen. These results are therefore ambiguous as to the presence of μ-receptors on the neural lobe. The density of this μ-type binding was not altered in chronic morphine-treated rats.

Although κ-opioid receptor binding was measured in central and peripheral regions of the neural lobe for each incubation category, no differences were found in binding between these regions.

It has been reported that κ-receptors are densest in the external rim of the posterior pituitary where oxytocin nerve terminals predominate (**Herkenham, Rice, Jacobson & Rothman, 1986**). No significant difference in binding between morphine treated rats and vehicle treated rats was detected in any incubation category.

In-vitro experiments have shown that endogenous opioids still inhibit oxytocin secretion from isolated neural lobe preparations taken from i.c.v. morphine infused rats (**Bicknell, Chapman, Leng & Russell, 1985**). Thus, chronic morphine treatment resulting in tolerance to and dependence upon morphine neither affects the number of available kappa receptors in the posterior pituitary nor induces cross tolerance to the endogeous ligand, which may be dynorphin, co-produced in and co-secreted from vasopressin neurones.

In-vivo experiments described in **Chapter 2** demonstrate that chronic morphine treatment confers no cross-tolerance to the inhibitory action of U50,488H on electrically stimulated oxytocin secretion but instead leads to increased sensitivity to the kappa-opioid receptor agonist; the mechanism of this is not clear. Binding affinity was not measured in the present

study.

The κ-receptor population in the rat posterior pituitary does display plasticity under certain conditions, for example during dehydration, when the osmotic stimulus leads to a decrease in κ-receptor numbers, although not to a change in receptor affinity (**Brady & Herkenham, 1987**). Therefore the system has the capacity for receptor number plasticity during some circumstances.

Measurements on plasma glucose, [Na^+] and osmolality showed that there was no significant difference in these parameters between morphine naive and morphine treated animals, demonstrating that there were no osmoreceptor mechanisms acting to alter opioid receptor density in the posterior pituitary.

So, morphine tolerance and dependence in the oxytocin system are not associated with detectable changes in opioid number in the posterior pituitary. The situation is different for the cell bodies of oxytocin neurones. In a parallel study to the present one, brain sections from the same rats were used to study opioid receptor changes in the SON and median preoptic nucleus (MPN), which is part of the region anterior and ventral to the third ventricle (AV3V region) that projects to the supraoptic nuclei and might contribute to tolerance of the oxytocin system. These data have been published (**Sumner, Coombes et al, 1990**) and are discussed here.

For the SON, brain sections were incubated with ^{3}H-etorphine (for non-selective binding to all opioid-receptor subtypes) and etorphine with displacers DPDPE and U50,488H for μ-receptor isolation. Kappa receptors were detected using [^{3}H](-) bremazocine as probe with DAGO and DPDPE as displacers since pilot studies had shown that this displayed stronger binding than did etorphine. MPN sections were incubated with etorphine alone for total opioid receptor binding.

In the control group of vehicle-infused rats, [^{3}H](-)

etorphine binding in the SON was found under conditions selective for μ-receptor binding i.e. when DPDPE and U50,488H were added to the incubation medium. Patches of intense binding under these conditions were also located in the caudate putamen which has been documented to contain μ-opioid receptors (**Tempel & Zukin, 1987**).

During chronic morphine treatment, μ-opioid receptor binding in the SON was reduced by over 80%, a significant change with respect to control animals. Morphine is known to be a selective μ-opioid agonist which has been shown to have a profound inhibitory effect on oxytocin neurone firing when applied in the vicinity of the cell bodies (**Wakerley, Noble & Clarke, 1983**) and tolerance to this action with respect to morphine but not U50,488H has been shown in chronic morphine-treated rats (**Leng, Pumford & Russell, 1990**). This decrease in receptor number may, at least in part, explain morphine tolerance of oxytocin neurones (**Bicknell, Leng et al, 1988; Rayner, Robinson & Russell, 1988**).

Binding to opioid receptors under conditions selective for κ-opioid receptor binding - (i.e. with [^{3}H](-) bremazocine as a probe and DPDPE and DAGO included in the incubating medium to exclude μ and δ-receptor binding) - was also detected in the SON and to a small extent in the caudate putamen. This binding was decreased to a very low level by addition of U50,488H to the incubating medium, indicating it to be mediated via κ-receptors. The presence of κ-receptors in the SON has been demonstrated by autoradiography and electrophysiological studies (**Mansour, Khachaturian et al, 1987; Russell, Pumford & Leng, 1989**). Chronic morphine exposure did not affect κ-selective [^{3}H](-) bremazocine binding in the SON, consistent with the sensitivity of firing-rate of oxytocin neurones in such rats (**Leng, Pumford & Russell, 1990**).

A significant reduction in [^{3}H](-) etorphine binding

was detected in the MPN following i.c.v. morphine infusion although this is only an indication of change in total number of receptors and not the opioid receptor subtype responsible since only total opioid receptor binding was measured. However, this could represent a decrease in opioid receptor number in an input pathway which contributes to oxytocin neurone regulation (**Russell, Blackburn & Leng, 1988**).

It is possible that morphine from the i.c.v. infusion, bound to μ-opioid receptors in the SON, competed with [^{3}H] etorphine binding and hence reduced the binding seen in this incubation category.

Another possibility is that morphine infusion alters endogenous opioid peptide secretion in the SON, thus preventing opioid receptor binding.

Hopefully, the preincubation procedure employed, with 5' guanylylimidodiphosphate included in the medium would have encouraged dissociation of any receptor-bound opiates and allowed binding of the labelled ligand (**Bunn, Hanley & Wilkin, 1985**).

A reduction in μ-receptors may allow normal functioning of SON oxytocin neurones (i.e. tolerance) which is indicated by the similar values for plasma oxytocin in both morphine naive and morphine treated rats. Partial loss of μ-receptors from the available pool will ultimately decrease the number of receptors binding morphine at any given concentration and thus decrease the efficacy of morphine. This partial loss of μ-opioid receptors may explain the phenomenon of tolerance to morphine in the oxytocin system of the female rat.

Conversely, the lack of change in κ-receptor numbers in the SON or posterior pituitary is consistent with the lack of tolerance to the κ-opioid receptor agonist, U50,488H in morphine tolerant rats, both with respect to electrical activity of the cell bodies (**Leng, Pumford & Russell, 1990**) and to actions in the

posterior pituitary (Coombes and Russell, 1991; Chapter 2, this thesis)

CHAPTER SEVEN

INVESTIGATION OF THE EFFECTS OF INTRACEREBROVENTRICULAR PERTUSSIS TOXIN ON OPIATE MEDIATED MODULATION OF OXYTOCIN SECRETION.

Chapter 7 : Investigation of the effects of intracerebroventricular pertussis toxin on opiate mediated modulation of oxytocin secretion.

INTRODUCTION

Experiments described in previous chapters have identified that in the rat, opiate receptors are involved in the regulation of oxytocin secretion from neurosecretory terminals located in the neural lobe. Manipulation of oxytocin release both **in vivo** (**Coombes & Russell, 1988**) and **in vitro** (**Bicknell, Chapman & Leng, 1985**) by the use of selective opioid receptor subtype agonists and antagonists implies that their binding to opiate receptors alters the activity of oxytocin neurones by opening or closing ionic membrane channels. The signal transduction system from binding of an effector molecule to opening an ion channel is not yet fully described.

THE SECRETORY EVENT.

During a depolarizing action potential, calcium enters the oxytocin nerve terminals, leading to an increase in $[Ca^{2+}_i]$ and acting as a primary signal for exocytosis of oxytocin from the terminals (**Bicknell, 1988**).

Studies using a suspension of isolated neurohypophyseal terminals (neurosecretosomes) loaded with the fluorescent Ca^{2+} indicator, fura-2, show that the rise in terminal $[Ca^{2+}_i]$ induced by electrical stimulation is enhanced by a phasic pattern of stimulation (**Brethes, Dayanithi et al, 1987**). During conditions of high oxytocin secretion, at parturition and lactation oxytocin neurones are seen to fire in a synchronized bursting pattern (**Lincoln & Wakerley, 1971; Lincoln & Wakerley, 1974; Summerlee, 1981**).

Isolated oxytocin nerve terminals from the rat show an increase in $[Ca^{2+}_i]$ during depolarization induced by elevation of extracellullar potassium ion concentration.

Kappa opioid receptors which are found on oxytocin nerve terminals inhibit

oxytocin secretion. Activation of these receptors by ligand binding does not affect basal intracellular calcium ion concentration but reduces the peak influx of calcium ions into the cell during depolarization **(Bicknell, 1988; Zhao, Chapman and Bicknell, 1988)**.

The κ-opioid receptor agonist U50,488H is found to decrease the entry of Ca^{2+} into rat cortical synaptosomes by inhibition of N but not L type calcium channels **(Xiang, Adamson et al, 1990)**.

OPIOID RECEPTORS

The three main opioid receptor subtypes can be viewed as initiators in a physiological signal transduction mechanism within the neuronal cell membrane and cytoplasm. In some cell culture systems and discrete brain areas opioid receptors have been linked with negative coupling to adenylate cyclase but it is often difficult to quantify a second messenger response.

In common with muscarinic and adrenergic receptor subtypes, opioid receptors are associated with the specific guanine nucleotide binding protein $G_{(i/o)}$ which accomplishes the first stage of signal transduction through hydrolysis of GTP **Haynes, 1988)**.

In the general model that has been proposed, ligands which interact with $G_{(i/o)}$ proteins can be negatively coupled to adenylate cyclase (vs. $G_{(s)}$ which stimulates adenylate cyclase). Although a decrease in intracellular cAMP may account for some of the physiological effects associated with opioid receptor binding some other mechanisms may contribute to the physiological responses seen via adeylate cyclase

independent mechanisms. Ionic conductances, particularly K^+ and Ca^{2+} have been shown in some neurones to play an important role in cell depolarization and may do so in oxytocin neuronal activity.
So, how do G proteins couple opioid receptor binding to ionic channels ?

G-PROTEINS

GTP binding proteins are heterotrimers consisting of an α-subunit and a βγ-dimer. There is conflicting evidence as to which subunit plays an active role in signal transduction **(Lutz Birnbaumer, 1987)**.
Initial experiments carried out on embryonic chick atrial cells showed that coupling of acetylcholine (ACh) binding to muscarinic receptors resulted in an increased potassium conductance from the atrial cells and hence slowing of pacemaker activity and that this required intracellular GTP. Changes in cAMP and cGMP were found not to affect the potassium channel or its response to muscarinic agonists and hence imply that cyclic nucleotide second messengers are not involved.
Pretreatment of GTP binding proteins with islet activating protein (IAP) from the bacterium **Bordetella Pertussis** leads to ADP-ribosylation of two GTP binding proteins and prevents the coupling of ACh binding to ionic channel activation **(Pfaffinger, Martin et al, 1985)**.
Following this finding, attempts were made to determine whether it was the α or the βγ subunits which activated ion channels. The effects of purified $G(_{i/o})$ subunits from bovine cerebral cortex on muscarinic K^+ channels in chick embryonic atrial cells was examined. The βγ-subunit was found to cause activation of single K^+ channels whilst the α-subunit did not. Additionally, α-subunits added in excess to the βγ fraction inhibited inactivation by the βγ dimer. This suggests that dissociation of the αβγ trimer is necessary for G-protein activation.

Magnesium ions are necessary for activation and dissociation of the G protein through binding and hydrolysis of GTP but is not required for βγ activation of the K^+ channel.

The role of the α-subunit was not discounted, it was noted that in this system its action could not be demonstrated (**Logothetis, Kurachi et al, 1987**). The α-subunit of the GTP binding protein G_k has been found to open K^+ channels in mammalian atrial myocytes (**Codina, Yatani et al, 1987**)

Somatostatin is found to reduce the voltage dependent Ca^{2+} current I_{Ca} and intracellular free Ca^{2+} in cells cultured from a pituitary cell line. Treatment of cells with pertussis toxin supressed the ability of $G_{i/o}$ to couple inhibitory receptors to adenylate cyclase and abolished the effect of somatostatin on calcium influx and intracellular calcium concentration.

However, intracellular application of cAMP did not alter the inhibitory effects of somatostatin implying that the GTP-binding protein is directly involved in the cAMP-independent receptor-mediated inhibition of voltage dependent Ca^{2+} channels (**Lewis, Weight & Luini, 1986; Koch & Schonbrunn, 1988**)

The active form of guanine nucleotide binding proteins is with GTP bound to the protein. Inactivation occurs by the slow hydrolysis of GTP to GDP which is mediated by the GTP-ase activity of the G protein itself. It is accepted that low Km GTP-ase stimulation is a post receptor event mechanism resulting from binding of the effector molecule. U50,488H, a kappa opioid receptor agonist was found to stimulate low Km GTP-ase activity which was antagonized by MR2266 (a μ/κ antagonist) but not by naloxone (a μ-antagonist) at 100 times the concentration of MR2266. DAGO (a μ-agonist) was found to stimulate low Km GTP-ase activity in a sodium dependent and fully naloxone reversible manner. Sodium is necessary for high-affinity agonist binding to the receptor (**Ueda, Misawa et al, 1987**).

It has been accepted that enhancement of low Km GTP-ase activity is followed by the automatic termination of signal transduction and hence opioid-receptor agonists would manifest an inhibitory response. The type of response following this, which ultimately leads to an inhibitory effect on the neurone has yet to be elucidated (Ueda, Misawa et al, 1987)

However, the key feature is the nature of the G-protein involved. GTP-ase stimulation indicates that the receptor-G-protein interaction is in progress, which in the case of opioid receptors will lead to inhibition of cell events. For other G-proteins, for example $G(_{s})$ binding of an agonist leads to stimulation of cell mechanisms by the stimulation of adenylate cyclase (**Sternweis & Pang, 1990**). By treating rats with i.c.v. pertussis toxin it was hoped to inactivate brain $G(_{i/o})$-proteins by ADP-ribosylation including those coupled to opioid-receptors. Following this we intended, to test the effectiveness of morphine on 1) basal oxytocin secretion and 2) on the stimulation of oxytocin secretion by hypertonic saline (**Shibuki, Leng & Way, 1988; Landgraf, Neumann & Schwarzberg, 1989**). In this way it was intended to demonstrate a role of $G_{(i/o)}$ in signal transduction from the μ-opioid receptor subtype to oxytocin secretion.

The evidence cited has demonstrated $G(_{i/o})$ proteins to be involved in the regulation of ion currents in that they can open K^{+} channels, modify I_{Ca} and hence alter $[Ca^{2+}{}_{i}]$.

These factors are all part of the neurosecretory event and it is possible that the actions of endogenous opioids on oxytocin neurone cell bodies and/or nerve terminals may involve $G(_{i/o})$-protein mediated mechanisms.

7.1 EXPERIMENT 1 : Effects of pertussis toxin given at a low or high dose 24hrs prior to terminal

experiment.

7.1.1. METHOD

7.1.1 (i) Animals

Virgin female rats housed under conditions described in 1.1 were used. Three groups of experimental animals were set up:-controls, low dose pertussis toxin treated and high dose pertussis toxin treated rats.

The rats were housed individually in metabolism cages for the duration of the experimental period.

The water bottle and food hopper were easily removed for daily weighing and hence measurement of food and water consumption. The rats were also weighed daily to monitor any weight changes.

The base of the cage comprised a wire mesh so that urine and faecal matter fell into a collecting funnel. The outlets of this funnel were arranged so that urine and faeces fell into separate bottles. Faeces were discarded but the urine was collected under a layer of liquid paraffin to prevent evaporation, the volume was measured gravimetrically and a sample of approximately 2mls withdrawn for Na^+, K^+ and osmolarity analysis.

Weighing and volume measurements were carried out at the same time each day (8.50-10.50 a.m.)

The day the rats were placed in the metabolism cages was designated day -3. Measurements were taken on days -2, -1 and 0, administration of toxin taking place on day 0. The rats were kept in metabolism cages for 24hrs after the toxin administration and measurements taken on day +1.

7.1.1 (ii) Surgery

On day 0, the animals were anaesthetized with ether and the head immobilized in a stereotactic frame. A midline skin incision exposed the skull surface and a hole drilled with a dental burr 1.6mm lateral and 0.6mm posterior to the skull sutures at bregma. The skull was levelled between lambda and bregma by

adjustment of the height of the incisor bar so that the hole drilled through the skull and the track of the drug delivery needle would be vertical. A 21 gauge needle attached to a 25µl through the needle Hamilton syringe was lowered vertically by a micromanipulator attached to the stereotactic frame to a depth of 4.5mm below the skull thickness via the drilled hole.
Vehicle (isotonic sterile saline, 5µl) or pertussis toxin (0.7µg/5µl or 7µg/5µl) was injected slowly into the lateral ventricle. After closing the skin incision with sutures, the animals were immediately returned to their metabolism cages - the whole surgical procedure lasted 10-15 minutes.

7.1.1 (iii) Terminal experiment (blood sampling)

Blood sampling took place 24hrs after the pertussis toxin or vehicle injections (day +1).
On the morning following i.c.v. injection the rats were anaesthetized by intraperitoneal injection of urethane (25% solution w/v, 1.25mg/kg). Each animal was fitted with a femoral arterial and femoral venous cannula for blood sampling into heparinized syringes and drug injection. The animals were left for 2hrs before sampling started, the sampling protocol being that shown in **Fig. 7.1.(a)**.
Blood samples 1 and 9 were 0.6ml, the plasma being divided into two aliquots, 200µl for oxytocin radioimmunoassay and the rest for sodium analysis. The rest of the samples (2-8) were 0.3ml, the plasma used for oxytocin determination only. Blood samples were withdrawn from the femoral artery; sample 1 being replaced with an equivalent volume of isotonic saline into the femoral vein. Subsequent samples were replaced with resuspended red blood cells made up to a volume of 0.3ml in isotonic saline. Plasma aliquots were immediately cooled over ice and then stored at -20°C until assayed.
Morphine sulphate or naloxone hydrochloride was

Plasma sample 1	**t= 0 mins**
Plasma sample 2	**t=10 mins**
Morphine (500μg/kg, 1mg/ml i.v.)	**t=15 mins**
Plasma sample 3	**t=20 mins**
Plasma sample 4	**t=30 mins**
Naloxone (50μg/kg, 0.1mg/ml i.v.)	**t=34 mins**
Plasma sample 5	**t=40 mins**
Plasma sample 6	**t=50 mins**
Plasma sample 7	**t=60 mins**
Hyperosmotic stimulus (1.5M NaCl, 4ml/kg)	**t=60 mins**
Plasma sample 8	**t=80 mins**
Plasma sample 9	**t=90 mins**

Fig 7.1.(a)

Summarized protocol of the blood sampling and drug administration procedure for rats treated 24hrs previously with i.c.v. pertussis toxin (0.7μg/5μl and 7μg/5μl saline) or control rats treated with 5μl sterile saline. Samples 1 and 9 were divided into two aliquots for oxytocin assay and sodium content analysis, all other samples were assayed for oxytocin only.

injected via the femoral vein (0.5mg/kg; 1mg/ml). Naloxone was given a) to antagonize any effects of morphine and b) to test for inhibitory endogenous opioid action acting on oxytocin neurones.
After blood sample 7 was taken, all the animals received a hyperosmotic stimulus by means of an intraperitoneal injection of hypertonic saline (4ml/kg, 1.5M NaCl); to compare the responsiveness of the oxytocin system in the three treatment groups.
Radioimmunoassay of oxytocin in the plasma samples were carried out by Mr.C.Chapman at A.F.R.C. Institute of Animal Physiology and genetics Research, Babraham.
Plasma osmolality measurements were carried out by Mr.B.McGrory, Dept. of Physiology, University of Edinburgh Medical School.
Once the blood sampling schedule was completed, the animals were killed by injection of 0.6ml Sagatal (60mg/ml) and the brains removed so that the position of the needle track to the lateral ventricle where the toxin or vehicle had been injected could be confirmed by dissection (see **Fig 7.1 (b)**).

7.1.2 RESULTS

7.1.2.(i) Rat Body Weight

The body weight data obtained is shown in **Fig 7.1 (c)**. The mean initial weights (± sem) of the treatment groups of rats were 219.63 ± 7.23g (controls), 225.81 ± 3.48g (low dose pertussis toxin) and 225.11 ± 5.10g (high dose pertussis toxin). There was no significant difference in the initial weight of the rats between groups.
All three groups gained weight up until day 0 when they underwent surgery for i.c.v. injection of toxin or vehicle. On day +1 both toxin treated groups lost weight but only those receiving 7μg/5μl dose showed a significant decrease with respect to the previous days weight and the mean weight of the other two groups ($p<0.05$ with respect to weight on day 0, paired t-

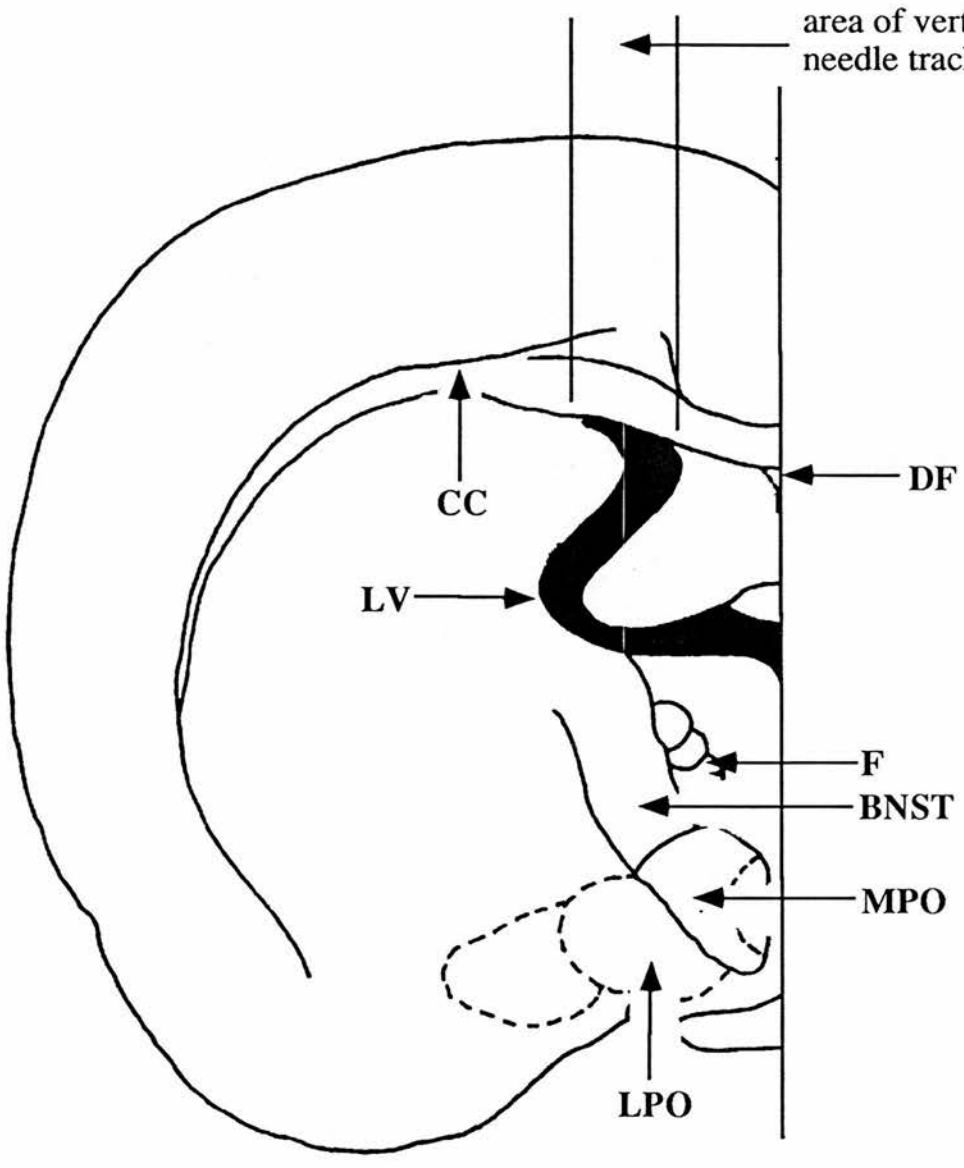


Fig. 7.1 (b)

Coronal section of the brain at 600μm posterior to the intersection of skull sutures at bregma,the area confined between the two parallel lines demarkates the acceptable area for needle tracks to the lateral ventricle.
[CC corpus callosum;DF dorsal fornix;LV lateral ventricle;
F fornix;BNST bed nucleurs of the stria terminalis;MPO medial preoptic nucleus;LPO lateral preoptic nucleus]

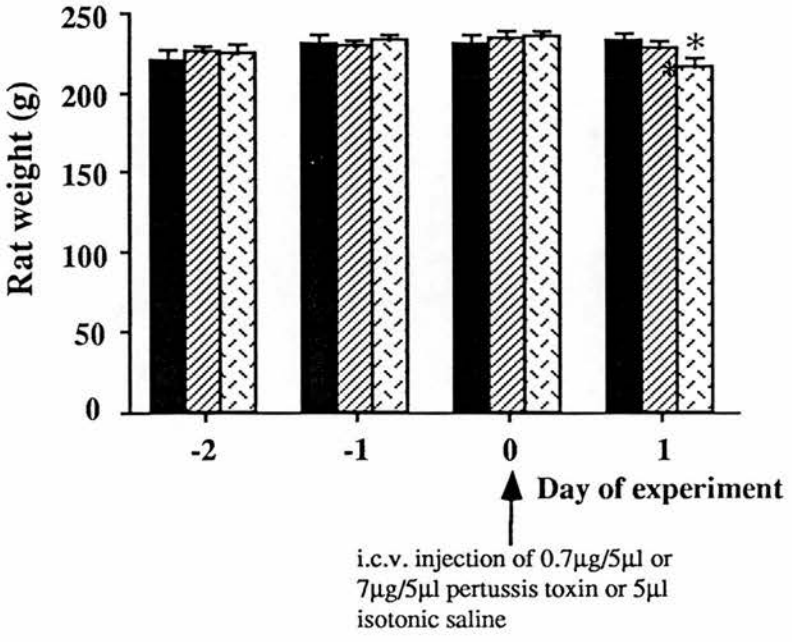


Fig. 7.1 (c)

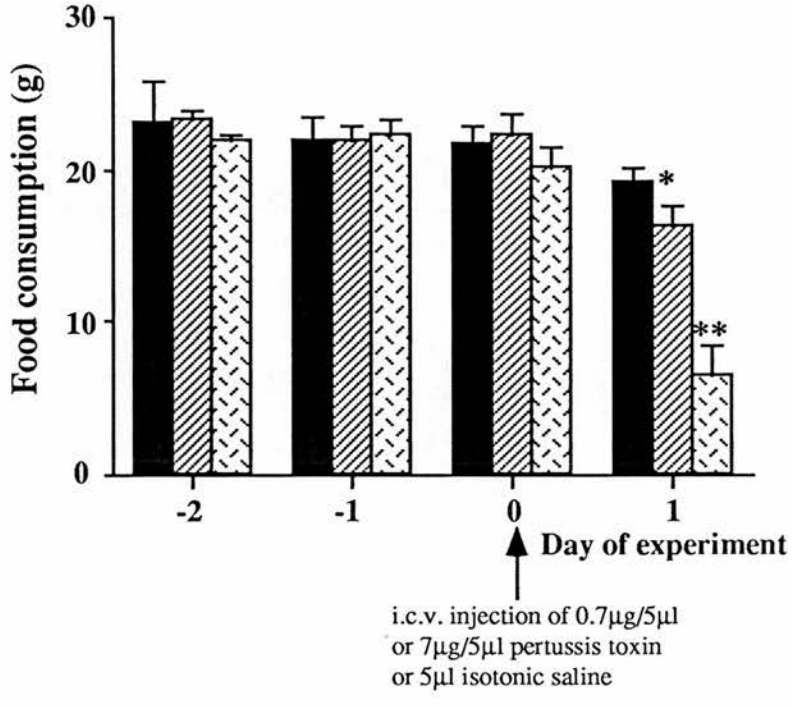


Fig. 7.1 (d)

Histograms representing the daily weight change (7.1c) and food consumption (7.1d) in three groups of rats (n=6 rats per group).Solid bars represent control animals, striped bars 0.7µg/5µl pertussis toxin treated and hatched bars 7µg/5µl pertussis toxin treated.
Statistical comparisons:
* $p<0.05$,** $p<0.02$,paired t-test with respect to previous days weight/food consumed.

test;p<0.05 with respect to weight of control group on day +1,unpaired t-test).

7.1.2.(ii) Food and water consumption.

Food and water consumption data are shown in **Figs. 7.1. (d)** and **7.1 (e)** respectively.There were no significant differences in food or water consumption until day +1 (24hrs post surgery).On this day food intake was decreased,compared to day 0 in the low dose toxin treated group (p<0.05,paired t-test) and in the high dose toxin treated group (p<0.02,paired t-test);water consumption was also significantly lower in the high dose toxin treated group p<0.05,paired t-test).

7.1.2.(iii) Urine output.

There was no significant change in urine output in any of the three experimental groups over the four days of sampling (Kruskal-Wallis).

Changes in urine electrolyte balance are shown in **Figures. 7.1 (f),7.1 (g).**

Urine osmolality,**Fig. 7.1(f)** in the high dose pertussis toxin treated group was significantly decreased 24hrs post toxin injection (p<0.0015,paired t-test).Urine sodium content was not significantly changed but there was a significant decrease in urine [K+] on day 1 (24hrs post i.c.v. toxin injection) p<0.001,paired t-test (**Figure 7.1 (g)**).

Urine osmolality in the low dose pertussis toxin treated group and control group was not significantly changed during the experimental period (paired t-test).Individually,neither sodium nor potassium content of urine samples was changed in either group before or after i.c.v. injection (paired t-test).

7.1.2.(iv) Plasma oxytocin.

Graphs representing plasma oxytocin profiles in each of the three experimental groups are shown in **Figs**

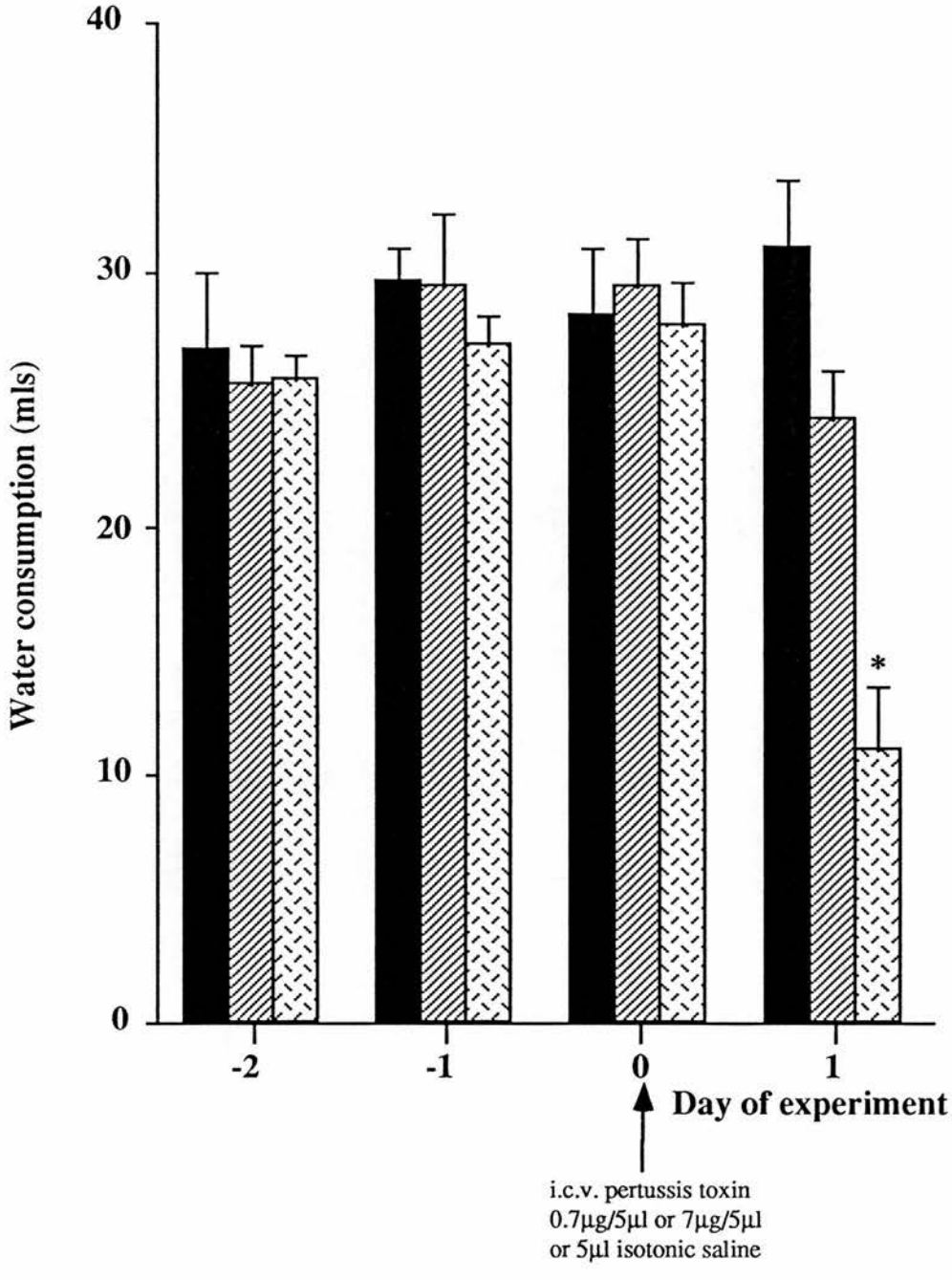


Fig. 7.1 (e)

Histogram representing daily water consumption in three experimental groups of animals,controls:solid bars,0.7µg/5µl pertussis toxin: striped bars, 7µg/5µl pertussis toxin: hatched bars (n=6 rats per group).
Statistical comparisons:
* $p < 0.05$,paired t-test ,with respect to previous days water consumption.

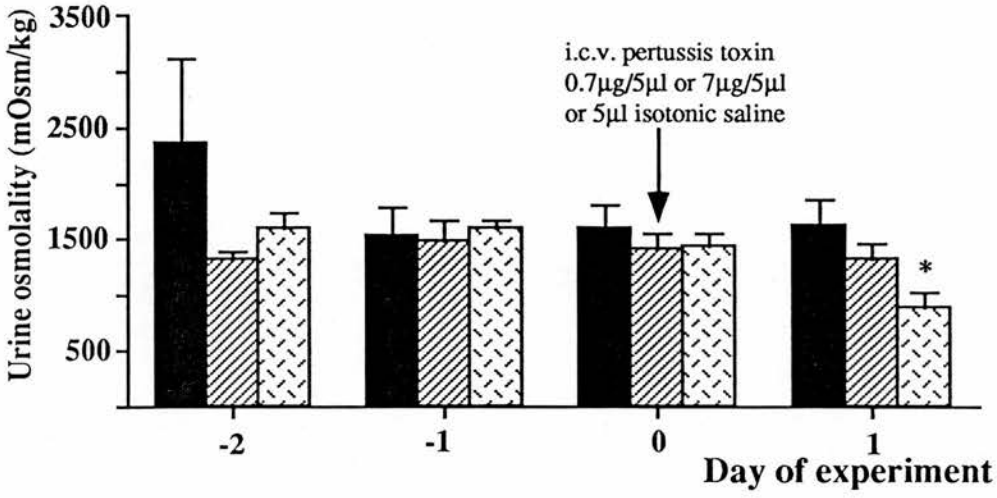


Fig 7.1 (f)

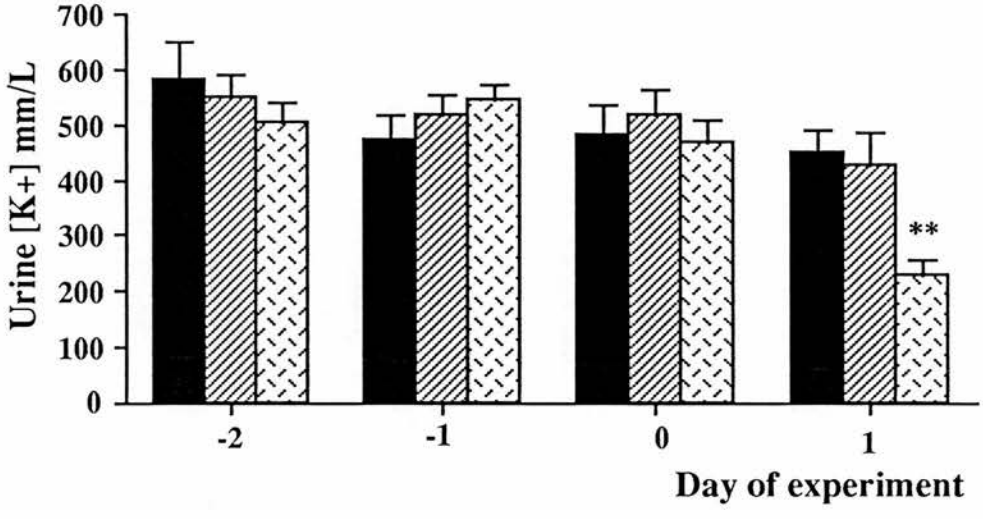


Fig 7.1 (g)

Histograms depicting urine osmolality (7.1(f) and urine [K+] in three groups of experimental rats.Solid bars:controls,striped bars: 0.7µg/5µl pertussis toxin,hatched bars 7µg/5µl pertussis toxin. (n=6 rats per group).
Statistical comparisons:
* $p<0.015$,** $p<0.001$,paired t-test, with respect to previous days value.

7.1.(h-j).

Basal oxytocin levels were not significantly different in the toxin treated groups 20.85 ± 3.52 pg/ml (low dose),27.48 ± 7.46 pg/ml (high dose) compared to the controls (17.95 ± 2.81 pg/ml).

Plasma oxytocin concentration in the control group,**Fig. 7.1(h)**, remained unchanged from basal values after injection of morphine (500µg/kg i.v.) and there was no statistically significant elevation of plasma oxytocin after naloxone injection (500µg/kg i.v.),although this was expected from several previous experiments (**Chapters 3,4,and 5**),and is likely to be due to large variation in this experiment.

After the hyperosmotic stimulus of an intraperitoneal injection of 1.5M NaCl plasma oxytocin was elevated to 217.38 ± 84.44 pg/ml 20 minutes after the stimulus in sample 8 ($p<0.05$,paired t-test) compared to basal oxytocin secretion (17.95 ± 2.81 pg/ml).Although oxytocin levels continued to rise in sample 9,30 minutes after the stimulus ,the large error makes this non-significant (paired t-test).

In the low dose pertussis toxin treated group of rats,**Figure 7.1(i)**, there was no change in plasma oxytocin after morphine administration but a significant elevation of oxytocin was measured in sample 6 (62.61 ± 12.24 pg/ml) and 7 (56.85 ± 11.21 pg/ml),16 and 26 minutes after intraveous injection of naloxone repectively compared to basal measurements of plasma oxytocin (20.85 ± 3.52pg/ml) ($p< 0.01$ and $p=0.01$,paired t-test).In sample 6 this elevation was also significant with respect to oxytocin measured in the previous sample ($p<0.05>0.01$,paired t-test).

A hyperosmotic stimulus led to elevation of plasma oxytocin in sample 8 (236.37 ± 65.85 pg/ml),20 minutes after i.p. injection of 1.5M NaCl ($p=0.01$ paired t-test).This rise was also significant with respect to oxytocin levels measured in the previous sample ($p=0.01$,paired t-test).Again,there was a continuing

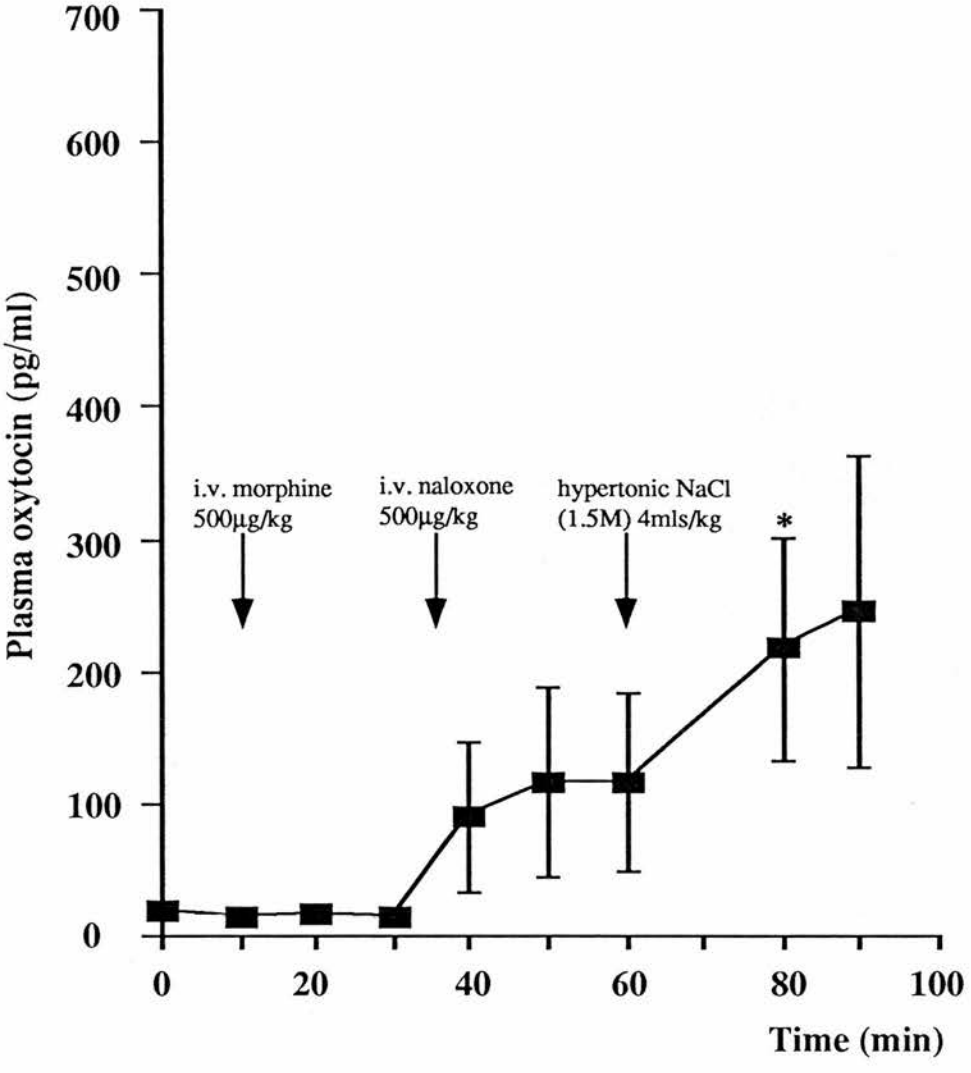


Fig. 7.1 (h)

Plasma oxytocin (pg/ml) in serial samples withdrawn from the femoral artery of rats given 5µl isotonic saline i.c.v. 24hrs previously.The values shown are mean ± sem.
Statistical comparisons:
* $p<0.05$,paired t-test with respect to basal values

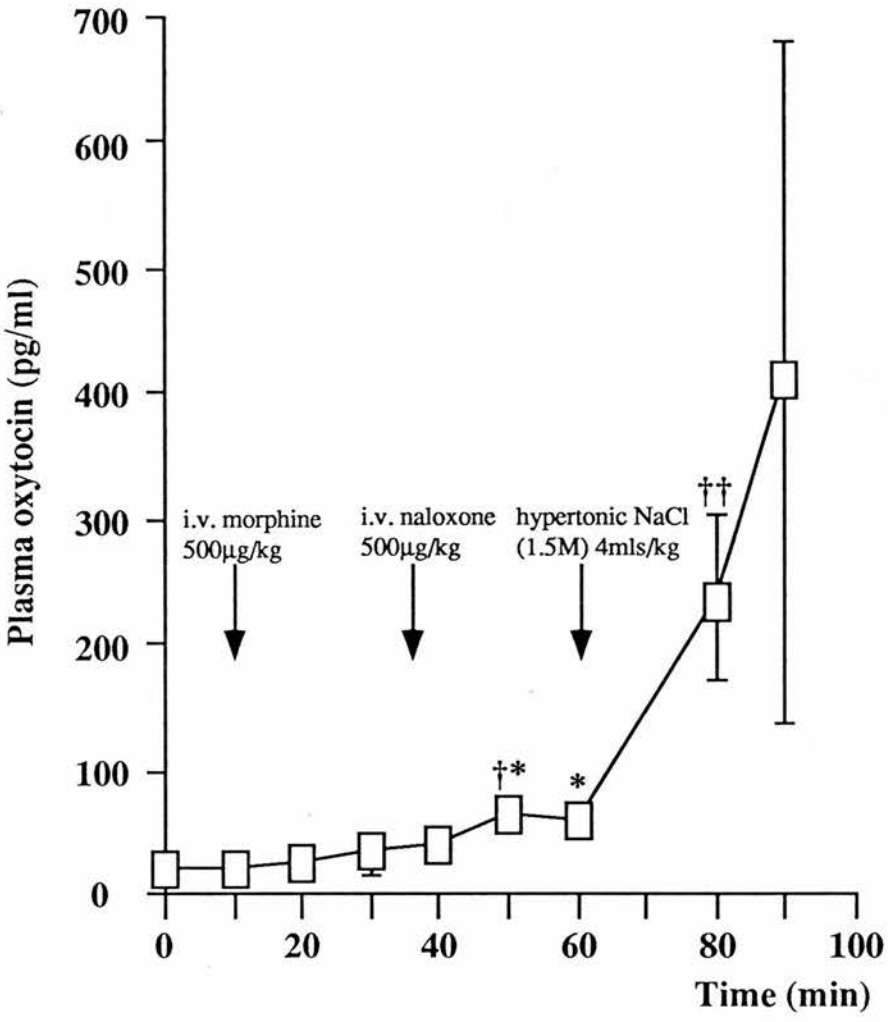


Fig. 7.1 (i)

Plasma oxytocin (pg.ml) in serial samples withdrawn from the femoral artery of rats given 0.7µg/5µl pertussis toxin i.c.v. 24hrs previously. Values shown are mean ± sem.
Statistical comparisons:
* $p<0.01$, paired t-test with respect to basal values
† $p<0.05>0.01$, paired t-test with respect to sample 5
†† $p=0.01$, paired t-test, with respect to sample 7.

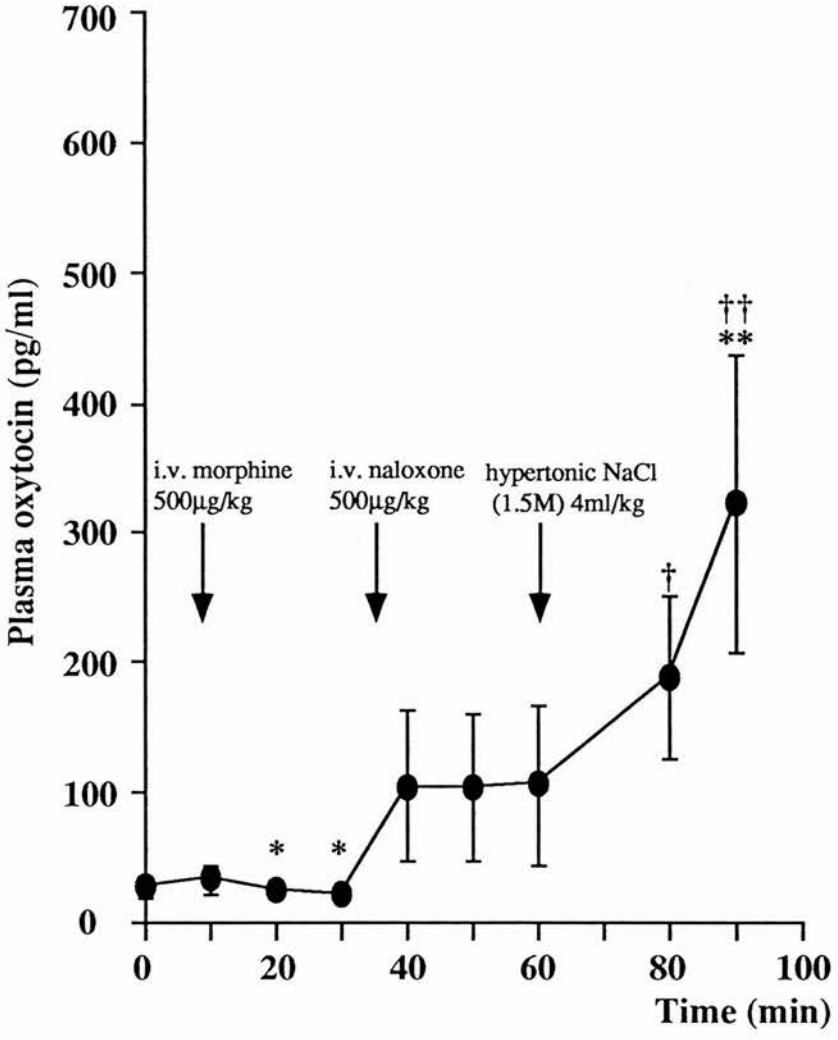


Fig. 7.1 (j)

Plasma oxytocin (pg/ml) in serial samples withdrawn from the femoral artery of rats given 7µg/5µl pertussis toxin i.c.v. 24hrs previously. Values shown are mean ± sem.
Statistical comparisons:
* $p<0.05>0.01$,† $p=0.05$, paired t-test with respect to basal values
** $p=0.05$,†† $p< 0.05$,paired t-test with respect to sample 7

rise in oxytocin measured in sample 9,30 minutes after the hyperosmotic stimulus but due to the large variation of responses within the experimental group and hence the large standard error this was not statistically significant. Plasma [Na^+] measured in samples 1 and 9 showed an increase in [Na^+] indicative of a successful hypertonic stimulus (ca.5-10mm/L).

Plasma oxytocin in the high dose pertussis toxin treated group of rats appeared to be decreased after i.v. injection of morphine and this was found to be statistically significant when comparing samples 3 (24.76 ± 5.09 pg/ml) and 4 (20.98 ± 4.14 pg/ml),10 and 20 minutes after morphine administration respectively to basal values (27.48 ± 7.46 pg/ml),($p<0.05>0.01$,paired t-test). After naloxone injection there appeared to be an elevation of oxytocin levels measured but this could not be demonstrated significantly.

After the hyperosmotic stimulus an elevation of plasma oxytocin was measured which was of similar magnitude to that seen in the control group of animals. With respect to the basal value (27.48 ± 7.46 pg/ml) the increase was significant in both samples 8 (187.84 ±63.68 pg/ml) and 9 (322.14 ± 114.63 pg/ml) ,20 and 30 minutes post osmotic stimulus respectively ($p=0.05$ and $p=0.02$,paired t-test). The rise in oxytocin was also significant when compared to the pre-stimulus levels recorded in sample 7 ($p<0.05$,paired t-test).

Changes in oxytocin in plasma samples 3 and 4 (post morphine injection),5,6,and 7 (post naloxone injection) and samples 8 and 9 in response to the hyperosmotic stimulus were compared between the control and low dose pertussis toxin group,control and high dose pertussis toxin group and between the two pertussis toxin treated groups. No significant difference in the measured oxytocin responses was detected at any stage of the experiment between any of the groups (Kruskal-Wallis).

7.2. Experiment 2 : The effects of pertussis toxin given at a high dose 72hrs before experiment.

7.2.1 METHOD

7.2.1 (i) Animals

The rats used were of the same sex and strain as in experiment 1.They were housed under the same conditions and had free access to food and tap water.There were two experimental groups,controls (i.c.v. saline) and pertussis toxin treated (i.c.v. 0.7µg/5µl saline),n=12 rats per group.

The rats were housed in individual cages (not metabolism cages) for the duration of this experiment but were weighed daily and had their water bottles weighed to determine water consumption gravimetrically.The experiment was carried out over 8 days - 4 days prior to surgery,day 0 when the animals underwent i.c.v. injection of saline or toxin and 72hrs post-surgery (day 1-3).

7.2.1 (ii) Surgery

The surgical procedure was the same as described in experiment 1.Only one dose of pertussis toxin was administered 0.7µg/5µl saline plus an equal number of saline control animals .The rats were returned to their cages and weighed and monitored for 72hrs.

7.2.1 (iii) Terminal experiment (blood sampling)

72hr post-surgery the rats were anaesthetized with urethane as before and fitted with femoral arterial and venous cannulae.The protocol was changed in this experiment so that morphine and the hyperosmotic stimulus were administered concommitantly at t=10 mins to study the effects of morphine on a stimulated,high secretion rate of oxytocin rather than on a basal secretion.The amended protocol is summarized in **Fig. 7.2 (a)**.

Blood samples 1 and 7 were 0.6ml and the plasma

Plasma sample 1	t=0 mins
Plasma sample 2	t=10 mins
Hyperosmotic stimulus (1.5M NaCl, 4ml/kg) + morphine 500µg/kg (1mg/ml) or 0.5ml/kg 0.9% NaCl i.v.	t=10 mins
Plasma sample 3	t=20 mins
Plasma sample 4	t=30 mins
Plasma sample 5	t=40 mins
Naloxone 5mg/kg (10mg/ml)	t=44 mins
Plasma sample 6	t=50 mins
Plasma sample 7	t=60 mins

Fig. 7.2 (a)

Summarized protocol of blood sampling schedule followed in experiment 2. Blood volumes withdrawn were the same as in experiment 1. Oxytocin content was detected by radioimmunoassay.

obtained divided into two aliquots for oxytocin radioimmunoassay and measurement of sodium content. All other samples were 0.3ml and were for oxytocin radioimmunoassay only. Blood volume was maintained by reinfusing resuspended red cells in an appropriate volume of isotonic sterile saline except the first sample which was replaced by 0.6ml isotonic saline. At the end of the protocol as in experiment 1, the position of the needle track made when delivering i.c.v. injections was checked by dissection.

7.2.2 RESULTS

7.2.2 (i) Rat Body Weight

Daily body weights are shown in **Fig 7.2 (b)**. The mean initial weight of the control animals was 253.6 ± 4.5g and the pertussis toxin treated group 249.9 ± 8.2g (n.s., paired t-test).

In the control group bodyweight increased daily throughout the experiment so that by day 0 it was significantly elevated above initial bodyweight ($p=0.006$, paired t test). In the pertussis toxin group body weight increased daily until day 1, the first day after pertussis toxin i.c.v. administration. On day 0 bodyweight was significantly above initial starting weight ($p<0.01$, paired t-test) but on day 1 it decreased from 255.4 ± 7.6g to 239.2 ± 6.3g ($p<0.0001$, paired t-test). On days 2 and 3 the rats gained weight as before and weighed 258.5 ± 5.3g compared to 266.0 ± 4.8g in the control group on day 3.

There was no significant difference between the daily mean weights of the two groups of animals except on day 1, the day after surgery when only the toxin treated group lost weight ($p=0.02$, unpaired t test.

7.2.2 (ii) Water consumption.

Water consumption was also measured daily, the results are presented in **Fig 7.2 (c)**. In the control group

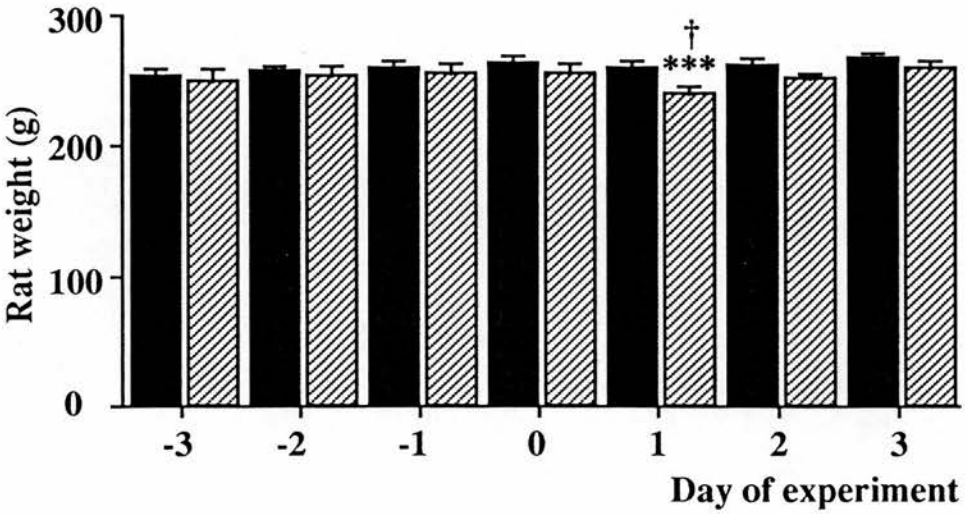


Fig 7.2 (b)

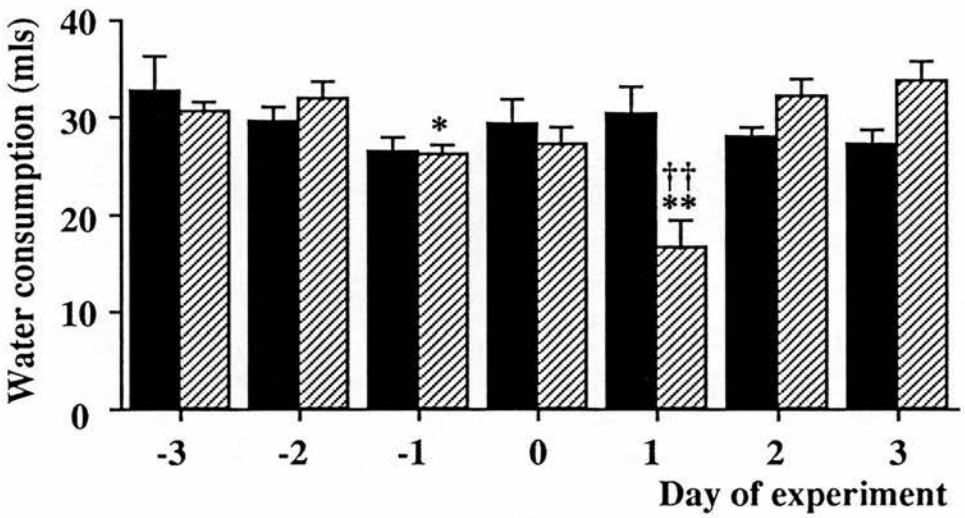


Fig 7.2 (c)

Histograms depicting rat bodyweight (7.2b) and water consumption (7.2c) in two groups of rats (n=12 per group).Control animals are shown by solid bars and pertussis toxin treated animals (0.7µg/5µl i.c.v.) by the striped bars.
Statistical comparisons:
* $p<0.005$,** $p<0.001$,*** $p<0.0001$,paired t-test with respect to previous days values.
† $p=0.02$,†† $p<0.005$,unpaired t-test,with respect to control group.

daily water consumption did not change significantly. In the pertussis toxin group water consumption decreased between day -2 and -1 ($p<0.005$, paired t-test) when it fell from 32.0 ± 1.7mls to 26.2 ± 1.0mls and between day 0 and day 1 ($p<0.001$, paired t-test) when it fell from 27.3 ± 1.6mls to 16.4 ± 2.9mls.

The second decrease in water intake corresponded to that in the initial 24hrs after pertussis toxin administration. Water consumption subsequently returned to pre- toxin administration values on days 2 and 3. There was only a significant difference in water intake between the two groups on day 1 after surgery ($p<0.005$, unpaired t-test).

7.2.2 (iii) Plasma oxytocin.

The plasma profiles of oxytocin in control animals and pertussis toxin treated animals are shown in **Fig. 7.2 (d)**. & (e)

Mean basal oxytocin in the plasma of control animals was 8.93 ± 1.80 pg/ml and in the pertussis toxin treated group 12.34 ± 2.82 pg/ml (n.s. unpaired t-test).

At t=10 minutes both groups of animals received an intraperitoneal injection of 1.5M NaCl (4mls/kg) to provide a hyperosmotic stimulus. $[Na^+]$ determination in plasma samples 1 and 7 showed a change in $[Na^+]$ (ca 5-10mm/L) sufficient to indicate a successful hyperosmotic stimulus. Immediately after this, half the animals in each group (n=6) were given i.v. morphine (500µg/kg), the other half an i.v. injection of isotonic saline (0.5ml/kg).

In the control rats (i.c.v. NaCl) treated with saline (i.v.), plasma oxytocin rose slightly from 8.93 ± 1.80 to 24.52 ± 8.24 pg/ml (sample 4, t=30 mins) but this rise was not significant with respect to basal values (paired t-test).

In the i.v. morphine injected control animals plasma

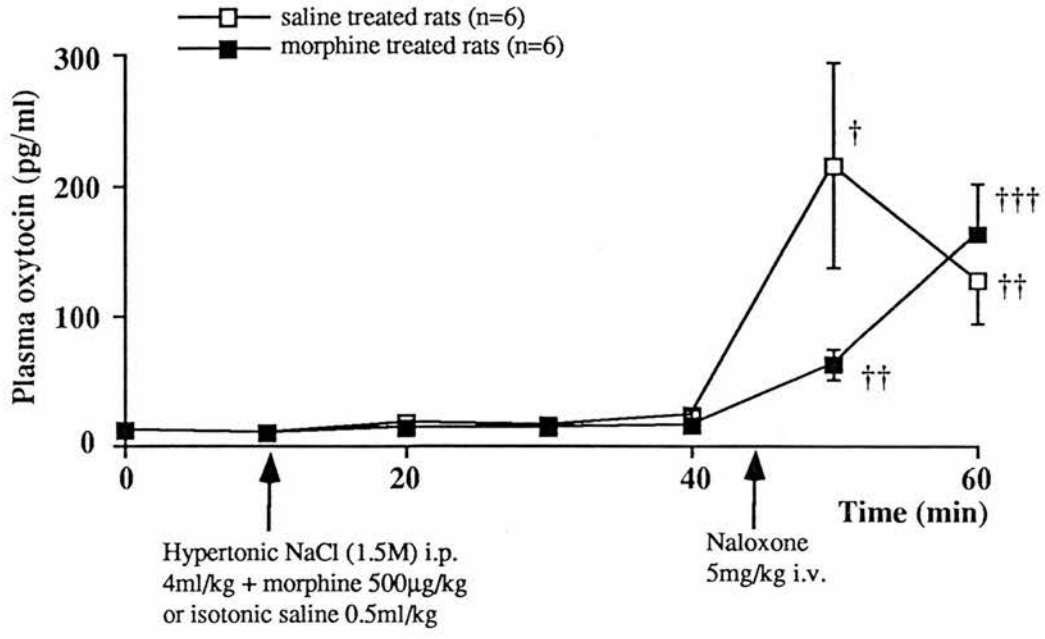


Fig 7.2 (d)

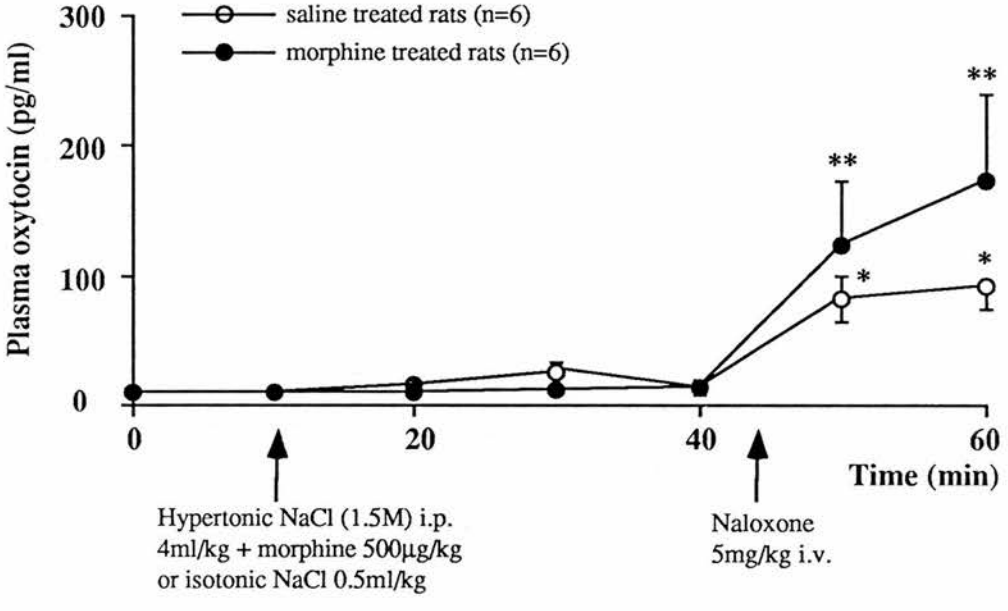


Fig 7.2 (e)

Plasma oxytocin in two groups of rats (n=12 per group),treated with 0.7µg/5µl i.c.v pertussis toxin (7.2 d) or 5µl i.c.v. isotonic NaCl (7.2 e) 72hrs previously.Six animals in each group were given either isotonic NaCl or morphine i.v. with hypertonic NaCl i.p. at t=10 mins.All animals received naloxone at t=44 mins.

Statistical comparisons:

† $p<0.05$,†† $p<0.02$,††† $p=0.009$,paired t-test with respect to pre-naloxone values.

*$p<0.05$,** $p< 0.01$,paired t-test with respect to pre-naloxone values.

oxytocin remained almost constant with respect to basal values (8.93 ± 1.81 pg/ml),rising to 13.72 ± 5.95 pg/ml in sample 5 (t=40 minutes).

There was no significant difference in response to the hyperosmotic stimulus in terms of oxytocin secretion between the i.c.v. vehicle treated rats subsequently injected with morphine or isotonic saline i.v. (unpaired t-test).

In the pertussis toxin treated groups, the i.v.saline injected animals showed a small,non-significant elevation of plasma oxytocin to 16.05 ± 4.39 pg/ml from basal values (12.34 ± 2.82 pg/ml).

The morphine injected i.c.v. pertussis toxin treated animals followed almost exactly the same trend resulting in a non-significant rise in plasma oxytocin to 16.43 ± 3.38 pg/ml from basal values of 12.34 ± 2.82 pg/ml at sample 5.

There was no difference in the response to a hyperosmotic stimulus between the two pertussis toxin treated groups.

At t=44 minutes all animals in the experiment were given an i.v. injection of naloxone.

In the i.v. saline treated control animals plasma oxytocin was elevated following naloxone.Samples 6 and 7 showed oxytocin concentrations of 82.27 ± 17.74 pg/ml and 92.82 ± 19.17 pg/ml,which were found to be significant with respect to the pre-naloxone value of 24.52 ± 8.24 pg/ml ($p < 0.05$,paired t-test).

In the morphine treated control animals, a large elevation in plasma oxytocin was seen following injection of the opioid antagonist.Samples 6 and 7 taken 6 and 16 minutes after naloxone showed a significant rise with respect to pre-naloxone values of 13.72 ± 5.95 pg/ml to 123.35 ± 48.6 pg/ml and 172.75 ± 66.02 pg/ml ($p = 0.009$ and $p = 0.01$,paired t-test).

There was no significant difference between the response to naloxone in the i.v. morphine and saline

treated control groups (unpaired t-test).

In the i.c.v. pertussis toxin treated group, the i.v. saline injected animals showed the greater response to naloxone in terms of oxytocin secretion. There was an immediate rise in sample 6 from 16.05 ± 4.39 pg/ml to 216.25 ± 78.4 pg/ml falling to 127.40 ± 34.19 in sample 7. These values were significantly elevated with respect to pre-naloxone levels ($p < 0.05, p < 0.02$, paired t-test).

Morphine injected animals showed an initially lesser response, an elevation from 16.43 ± 3.38 to 62.02 ± 11.67 pg/ml in sample 6 but this steadily increased to 163.63 ± 37.51 pg/ml in sample 7. Both these values were significantly higher than pre-naloxone oxytocin levels ($p < 0.02, p = 0.009$, paired t-test).

Once again there was no significant difference in oxytocin secreted as a response to naloxone between morphine and saline treated pertussis toxin animals.

Overall there was no difference between pertussis toxin treated or saline treated animals in magnitude of the oxytocin secretory response to a hyperosmotic stimulus, morphine or saline injection and naloxone administration.

7.3 DISCUSSION

7.3.1: Experiment 1

Intracerebroventricular (i.c.v.) injection of pertussis toxin caused a significant weight loss in the group of rats treated with the highest dose of toxin (7μg/5μl). These animals decreased both their food and water consumption significantly in the 24hrs following toxin administration compared to the vehicle trated control animals. The animals in this group looked unwell from their external appearance following surgery-tending to sit in a hunched position with their fur standing on end rather than exploring their cages like the rats in the other two treatment

groups. Urine output volume was not significantly changed in any of the three treatment groups but in the high dose pertussis toxin treated group, urine electrolyte balance was disrupted with urine [K^+] falling significantly in the 24hrs post injection. This effect is probably as a result of the decreased food intake seen in this group of rats.

Intravenous morphine sulphate has been shown to decrease basal oxytocin (**Chapter 3**) and silence continuously firing putative oxytocin neurones recorded in the supraoptic nucleus (SON) (**Pittman, Hatton & Bloom, 1980; Wakerley, Noble & Clarke, 1983**). Morphine binding to hypothalamic μ-opioid receptors may lead to alteration of potassium and calcium ionic conductances to favour the unresponsive state of oxytocin neurones, inhibiting cell body electrical activity and firing rate and decreasing oxytocin neurosecretion. Pretreatment of the rats with pertussis toxin was expected to inactivate the $G(_{i/o})$ proteins involved in ionic conductance regulation and hence negate the inhibiting effect of morphine.

The results obtained from this experiment are inconclusive since it was in the control and low dose pertussis toxin experimental groups that morphine injection was ineffective and the high dose toxin treated group where morphine effectively lowered basal plasma oxytocin. The controls behaved unexpectedly as morphine was expected to lower basal plasma oxytocin (see **chapter 3**).

The opioid receptor antagonist naloxone is most potent at the μ-opioid receptor subtype. However, it also antagonizes, at the dose used here, κ-opioid inhibition of oxytocin secretion at the level of the posterior pituitary, in i.v. morphine treated rats it would thus have dual actions. If morphine inhibition of oxytocin secretion is interrupted by the action of pertussis toxin on the receptor $G(_{i/o})$ proteins, then, naloxone binding which would normally

elevate plasma oxytocin and restore putative cell body firing ,may also act through modification of ion fluxes via G protein action.

In this experiment there was no statistically significant difference in response to naloxone between the three treatment groups due to the large standard errors from individual variations in response.Both the high and the low dose pertussis toxin treated groups displayed a significant elevation in basal oxytoxin following naloxone injection implying that the integrity of the $G(_{i/o})$ proteins is not required for naloxone mediated action endogenous opioid inhibition of oxytocin secretion or,as the response to morphine implies that the pertussis toxin had not affected G protein interactions.

There are several reasons that could account for the lack of results.

i) The dose of pertussis toxin may have been insufficient for inactivation of central $G(_{i/o})$ proteins.

ii) The time delay of 24hrs before the terminal experiment may have been insufficient for inactivation of central $G(_{i/o})$ proteins.

iii) The i.c.v. route of administration of the toxin into a lateral cerebral ventricle may have only given poor access to sensitive sites modifying oxytocin secretion.

iv) The $G(_{i/o})$ protein may not be involved in morphine actions on oxytocin neurones.

Administration of an intraperitoneal injection of hypertonic saline increases plasma osmotic pressure and produces a sustained activation of both oxytocin and vasopressin neurones (**Brimble & Dyball**) resulting in elevated plasma oxytocin levels (**Shibuki,Leng & Way,1988 ;Landgraf,Neumann & Schwarzberg,1988; Russell,Blackburn & Leng,1988**).

This stimulus to oxytocin secretion was given to check the responsiveness of the oxytocin system in animals

treated with pertussis toxin.
In all three groups an elevation of plasma oxytocin was noted after hypertonic saline but a difference in magnitude of this response between the three groups was not seen.

7.3.2: Experiment 2

The aim of this experiment was to see whether leaving the animals for 72hrs post i.c.v. toxin injection would increase $G_{i/o}$ protein disruption in the light of the results from Experiment 1 and give more clear cut results.
The pertussis toxin treated rats experienced weight loss in the 24hrs following surgery and i.c.v. toxin injection whereas the control rats did not. This could be attributed to non-opioid receptor linked actions of the toxin disrupting food and water intake - effects which only lasted for 24hrs after toxin treatment.
In experiment 1 the hyperosmotic stimulus was given towards the end of the blood sampling protocol (**Fig 7.1.(a)**). In experiment 2 this was amended so that the hyperosmotic stimulus could be given concommitantly with either a morphine or a saline injection. It was hoped that by giving these treatments close together that it could be determined whether the inhibition by morphine of the usually marked oxytocin response to a hyperosmotic stimulus could be mediated by receptor $G_{i/o}$ proteins. Once again the plasma oxytocin data proved disappointing with control animals unable to provide a significant oxytocin secretion in response to hypertonic saline, thus making it difficult to demonstrate any inhibitory effects of morphine or show that pertussis toxin pretreatment had any effect.
In the vehicle treated animals given saline i.v. followed by a hyperosmotic stimulus of 1.5M NaCl i.p. did not consistently show the rise in plasma oxytocin expected (**Shibuki, Leng & Way, 1988**) and it was therefore difficult to say if morphine injection had

suppressed a response since there was no significant difference in plasma oxytocin between the two groups.
Following i.v. naloxone injection at t=44 mins both the morphine and saline treated control animals experienced a significant rise in plasma oxytocin with respect to pre-naloxone values.
The morphine treated group appeared to experience a greater elevation implying that antagonism of endogenous opioids regulating oxytocin secretion as well as morphine binding to μ-receptors had been achieved but this difference between the groups was not significant.
In the animals that had received i.c.v. pertussis toxin (0.7μg/5μl) 72hrs previously neither animals injected with saline or with morphine showed a significant increase in plasma oxytocin following i.p hypertonic saline therefore confounding comparison between the two treatment groups with respect to morphine effects..Following naloxone,both the morphine and saline treated animals experienced a significant elevation in plasma oxytocin levels.It appears that the saline treated animals experienced an initially greater elevation than the morphine treated group.However there was no statistical significant difference between oxytocin secretion in morphine injected or saline injected animals and therefore no definite conclusions can be drawn from this.
Since the morphine treated i.c.v. pertussis toxin treated rats showed a similar elevation of plasma oxytocin as the morphine treated i.c.v. vehicle treated rats following naloxone suggests that the actions of both exogenous and endogenous opioid antagonists have been reversed i.e. both morphine and endogenous opioids are normally effective.
Another possibility for the lack of effect of the **pertussis toxin** was that the toxin was no longer active although changes in only the toxin treated animals following i.c.v. injection would imply that it

was active. These experiments gave inconclusive results as to whether a pertussis toxin sensitive G- protein ($G_{i}/_{o}$) is the signal transduction mechanism for μ-opioid receptor subtype binding.

In contrast, i.c.v. pertussis toxin (7μg/5μl) pretreatment attenuates morphine inhibition of electrical activity of putative oxytocin neurones in the SON. Oxytocin neurone electrical activity in i.c.v. pertussis toxin treated rats was found to be fully inhibited by i.v. U50,488H, at similar doses to control animals. Morphine however, showed a significantly decreased ability to inhibit oxytocin neurone cell firing in these pertussis toxin treated rats compared to control i.c.v. vehicle infused animals.

Other results indicate that morphine but not U50,488H inhibit oxytocin neurones by a mechanism involving a pertussis toxin sensitive $G_{i/o}$ protein and that it is μ-opioid receptors not κ-opioid receptors that involve a $G_{i/o}$ protein in their modification of oxytocin secretion (**Russell, Pumford & Leng, 1991**).

CHAPTER EIGHT

THE EFFECT OF PETHIDINE (MEPERIDINE), A SYNTHETIC OPIATE, ON OXYTOCIN RELEASE AND UTERINE CONTRACTILITY IN THE RAT.

Chapter 8 : The effect of pethidine (meperidine), a synthetic opiate, on oxytocin release and uterine contractility during parturition in the rat.

INTRODUCTION

A pregnant female rat will give birth to between 6 and 14 pups on the afternoon of day 21 or 22 following detection of a vaginal plug (day 0), taken as an indication of fertile mating. The pups are normally born over a period of 60-90 minutes provided the mother rat remains undisturbed (**Leng, Mansfield et al, 1988**).

In the rat, oxytocin is known to have a regulatory role in the progress of parturition. Oxytocin secretion steadily increases once parturition has been initiated and reaches a plateau some time after the 4th or 5th pup (**Higuchi, Honda et al, 1985; Higuchi, Takadoro et al, 1986**).

Superimposed upon this slightly elevated basal secretion are bolus releases of oxytocin associated with periodic high frequency bursting of supraoptic neurones similar to milk-ejection activity (**Summerlee, 1981**).

Oxytocin is a potent uterotonic agent but is unable to initiate contraction of the pre-parturient uterus and hence precipitate parturition earlier than 4-6 hours prior to expected delivery. Since plasma oxytocin does not increase dramatically during parturition and the uterus is unresponsive until just beforehand it would appear that it is a change in sensitivity of the uterine receptors to oxytocin rather than the modification of oxytocin secretion that initiates parturition (**Fuchs, Periyasami et al, 1983; Fuchs, 1985**).

Morphine administered intracerebroventricularly (i.c.v.) or intravenously (i.v.) interrupts

established parturition for up to several hours with a depression in circulating oxytocin .This effect is fully reversible by naloxone and can also be overcome by i.v. infusion of oxytocin (**Cutting,Fitzsimons et al,1985**).

The bolus release of oxytocin just prior to the appearance of a pup is thought to be responsible for the uterine contractions which propel the foetuses from their implantation site along the uterus - this is known as the foetus-ejection reflex.The foetal-expulsion reflex which finally moves the pup through the birth canal is dependent upon abdominal and respiratory movement and compliance of the cervix (**Higuchi,Uchide et al,1987**).

In rats where parturition has been held up by morphine administration,the foetuses remian adjacent to their implantation sites implying that depression of oxytocin has interrupted the foetus-ejection reflex.

Pethidine (meperidine) is a synthetic opiate used as an obstetric analgesic in humans.Pethidine is believed to act as a μ-opioid agonist (**Clarke & Wright,1984**) and also to have atropine-like properties.

In the following experiments,the effect of pethidine on parturition in the rat and upon uterine contractility of the post-partum uterus **in-vitro** were examined to establish whether it had central or peripheral actions.Co-administration of naloxone was used in an experimental group of animals to see whether pethidine was acting via opioid receptors.In these experiments blood samples were taken at timed intervals during delivery of the litter to determine the effect of pethidine or pethidine and naloxone on plasma oxytocin.

Observations were also on maternal behaviour in which oxytocin may play an important role (**Pederson & Prange,1979;Pederson,Caldwell et al,1985;Fahrbach,Morrell & Pfaff,1985**).

The effect of pethidine on osmotically stimulated plasma oxytocin levels was also investigated. This was to determine whether pethidine was able to depress oxytocin secretion by a central action, since the hyperosmotic stimulus acts both directly on magnocellular neurones and on central osmoreceptors in the AV3V region or subfornical organ (SFO) (**Leng, Blackburn et al, 1989**).

8.1. METHODS

8.1.(ii) ANIMALS

Female Sprague Dawley rats were placed in individual mating cages with wire mesh floors, with males, during late afternoon (~ 4pm) and left overnight. The following morning, the removable tray beneath each cage floor was checked for the presence of a vaginal plug - an indication that a fertile mating had taken place. Following detection of a plug, the female was removed and caged individually. The day of vaginal plugging was designated day 0 and parturition estimated for the afternoon of day 21. All pregnant females were kept under conditions of controlled photoperiod (13hrs light: 11hrs dark, lights on at 8a.m.) and constant temperature. Food and water were freely available.

From day 16 of pregnancy onwards, the animals were weighed daily at the same time each morning (8.30-9.30 a.m.) to

a) check that they were pregnant from their daily weight gain,

b) accustom the animals to being handled and removed from their cages.

8.1 (ii) Experiment 1 : The effect of subcutaneous pethidine injection on the timecourse of parturition in the undisturbed Sprague-Dawley rat.

On the morning of expected delivery the rats were

observed from the time of lights on.All signs of impending delivery were noted :-

i) nest building

ii) appearance of blood on bedding

iii) straining and muscular contraction of the abdomen

iv) excessive body grooming and licking of perineum.

The time of delivery of each pup was noted and whether they were born alive or dead.After the birth of the second pup pethidine or 0.9% isotonic saline was injected subcutaneously into the hind flank of the mother rat and the timecourse of continuing parturition noted.The time delay between the first and second pup is generally longer than subsequent inter-birth intervals and extremely variable between animals hence the drug/saline injection was not given until after pup 2 so that any effects on established paturition could be more reliably interpreted.

Pethidine was administered subcutaneously in one of three doses 60,30 and 10 mg/kg (taken from 120,60 and 20 mg/ml stock solutions).The control animals were injected subcutaneously with isotonic sterile saline (vehicle,0.5ml/kg).

All injections were performed by the same person so that variation in restraining the animal and the initial site of drug entry was minimized.

8.1 (iii) Experiment 2 : effect of pethidine,pethidine and naloxone,or vehicle on the timecourse of established parturition and levels of plasma oxytocin during parturition in the undisturbed Sprague-Dawley rat.

Early in the morning of expected delivery under ether anaesthesia a cannula was implanted via the jugular vein into the right atrium to allow withdrawal of timed blood samples in an unrestrained conscious rat during parturition (**Leng,Mansfield et al,1988**).

The cannula tubing was silicon and thus

extremely flexible which allowed it to be taken up over the shoulder under the skin to exit through a small incision at the base of the neck. The cannula was fed through a tightly coiled spring which was attached to the rat via two loose skin sutures at the base of the neck and tied to the cage lid at the other end. The cannula was thus protected from the animal chewing it by being encased in the spring whilst still allowing the rat free access to all parts of its cage since the spring allowed it complete freedom of movement. The cannula was filled with heparinized saline (Heparin 50U/ml saline) and was periodically flushed through to prevent blood clots.

An initial blood sample for basal oxytocin was taken 2hrs after completion of surgery and the other samples according to the protocol shown in **8.1(a)**.

Signs of impending delivery as previously described were noted and the times of each pup delivery. After the birth of the second pup, the rats were given a subcutaneous injection of pethidine (10mg/kg, 20mg/ml) or vehicle (isotonic saline 0.5ml/kg). Blood samples were withdrawn as described in **8.1(a)** and the time course of continuing parturition noted. In n=10 rats, an intravenous injection of 5mg/kg naloxone preceded pethidine administration to determine whether the effects of pethidine are naloxone reversible and hence opioid receptor mediated.

Blood samples (0.3ml) were immediately placed over ice and then centrifuged to obtain plasma. Plasma aliquots were stored frozen at -20°C until assayed. The method of radioimmunoassay used to measure oxytocin was that described by Dr. T. Higuchi **(T. Higuchi, K. Honda et al, 1985)**.

All oxytocin assays in this experiment were carried out at AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT. The detection limit of the assay was 5pg/ml plasma. The

Sample 1.....2hrs post surgery (basal oxytocin)

Sample 2.....After delivery of pup no.2

Subcutaneous injection of pethidine (10mg/kg) or saline.For method II(ii) naloxone (5mg/kg i.v.) preceded pethidine injection.

Sample 3.....10 minutes post injection

Sample 4.....20 minutes post injection

Sample 5.....30 minutes post injection

Sample 6.....60 minutes post injection

Sample 7.....90 minutes post injection

Sample 8.....120 minutes post injection

Sample 9.....After birth of pup 3 in animals delayed for >120 minutes.

Figure 8.1(a)

Protocol for the blood sampling procedure in cannulated rats receiving either subcutaneous vehicle, pethidine or pethidine preceded by intravenous naloxone.

intra- and inter- assay coefficients of variation were 9.5% and 3.3% respectively.

8.1 (iv) Experiment 3: Does pethidine affect maternal behaviour ?

During the course of parturition in experiments 1 and 2, maternal behaviour was monitored before and after drug administration.

(a) <u>During parturition</u>

Maternal behaviour comprised of (i) cleaning the pups immediately they were born (ii) gathering the pups together into a nest and (iii) eating newly delivered placentae (placentophagy) (iv) not eating the newly delivered pups.

(b) <u>Post-partum</u>

Maternal behaviour involved (i) construction of a nest area (ii) keeping the pups together as a litter in the nest (iii) keeping the pups warm and (iv) nursing the pups.

Since the mother usually moved from the nest if the cage was disturbed these observations were usually made early in the morning before moving the cage from the rack. The presence of milk-spots and litter weight gain were taken as an index of whether the pups were suckling.

8.1 (v) Experiment 4 : Does pethidine affect spontaneous contractions in the immediate (or <24hr) post-parturient isolated uterus muscle bath preparation ?

Animals from experiment 1 which had littered overnight without receiving any drug treatment and a few mated up at a later date and allowed to deliver without any disturbance were used in this experiment.

The rats were killed by stunning followed by cervical dislocation. The uterine horns were ligated and removed from the abdominal cavity . The horns were suspended in

a 40ml muscle bath containing de Jalon's solution gassed with 95% O_2/5% CO_2 maintained at 37°C by an external water bath. Each horn was suspended from a strain gauge and secured to a metal hook at its lower end (**see Fig 8.1(b)**). The muscle was initially tensed to 10g and contractile activity measured isometrically.

Once spontaneous contractile activity had attained a steady state, a control period of nine minutes was observed prior to introduction of drugs into the bathing medium. All drug solutions were made up in de Jalon's medium. Effects of the drugs were determined by measuring the mean amplitude of the last three contractions in the control period and the first three contractions in the series immediately post drug administration (excluding those occuring in the first 30 seconds except in the case of acetylcholine (**see later**).

Drug effects were expressed as the ratio of the mean amplitude of contractions pre- and post- drug additions. When the drug caused complete cessation of spontaneous contractions the trace was assigned a minimum scale value so that all ratios would be finite. Since the water bath accommodated two muscle baths, simultaneous recordings of contractile activity could be made from two uterine horns at the same time. This allowed time controls to be carried out, when one horn was not exposed to any drug, at the same time another horn was undergoing a drug addition protocol.

8.1v (a) Effect of pethidine on spontaeous uterine contractile activity.

A nine minute control period of spontaneous contractile activity was recorded after which pethidine was added to the bath. The doses tested were 5.16×10^{-6}M, 0.3×10^{-5}M, 2.06×10^{-5}M, 6.19×10^{-5}M and

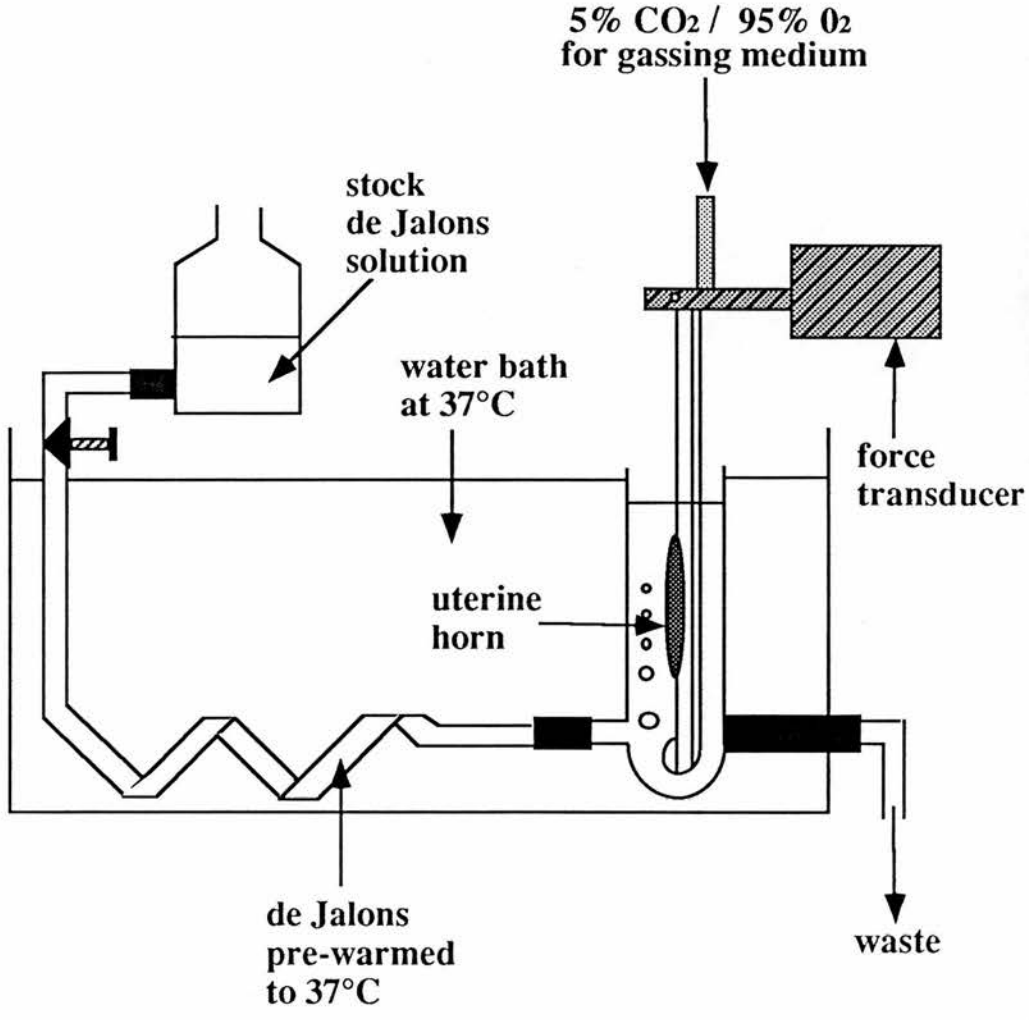


Fig 8.1 (b)

In- vitro muscle bath preparation set up.The uterine muscle was kept at 37°C in gassed de Jalons medium.Drugs were added directly to the medium bathing the muscle in the bath (volume of bath ca. 40ml).The medium in the bath was changed by a system of draining and filling the bath with pre-warmed fresh de Jalons medium.

2.47×10^{-4}M which were administered singly in random order. These doses were calculated to be of the same order of magnitude as the range of pethidine used in **Experiment 1** once distributed in the rats extracellular fluid volume. After four minutes the fluid in the bath was drained twice and replaced with fresh stock solution; the test cycle was then repeated with another dose of pethidine.

8.1v (b) Effect of pethidine on oxytocin stimulated contractions of uterine muscle.

Nine minutes of control spontaneous activity was measured as before after which oxytocin was added to the bath. The doses of oxytocin used were 0.875 mU/ml (1.75×10^{-6}M), 0.218mU/ml (4.375×10^{-7}M) and 0.109mU/ml (2.9×10^{-7}M).

After three minutes the bath was drained and refilled with fresh medium twice and a further nine minutes of spontaneous activity recorded. Pethidine was then added to the bath (6.19×10^{-5}M) and four minutes after this the same dose of oxytocin as previously administered was added and uterine contractions measured over a further three minutes. The bath was then drained and refilled twice with fresh medium and the whole cycle repeated using another dose of oxytocin.

Thus, the effects of different doses of oxytocin on uterine contractility before and after a fixed dose of pethidine compared to the control period of activity were examined.

8.1v (c) Interaction of naloxone with pethidine

After a nine minute control period oxytocin (middle dose: 4.375×10^{-7}M) was added to the bath and after three minutes was washed out. After a further nine minutes control period, pethidine (6.19×10^{-5}M) was added to the bath and its effect recorded for three minutes before being washed out with fresh stock de

Jalon's. This established the effect of oxytocin or pethidine alone on uterine contractions as compared to the control period.

Next, after another nine minute control period these doses of oxytocin and pethidine were added simultaneously and their combined effects recorded for three minutes before replacing the bathing medium with fresh solution.

Finally, after nine minutes of control activity, naloxone at 10^{-4}M or 10^{-5}M was added to the bath to determine its effect on uterine contractions alone then two minutes later pethidine (6.19×10^{-5}M) was added and lastly oxytocin (4.375×10^{-7}M) after three minutes. This allowed comparison of naloxone alone and naloxone effects on pethidine or pethidine and oxytocin combined effects as compared to control spontaneous activity.

8.1v (d) Pethidine effects on acetylcholine (ACh) stimulated contractions.

After a nine minute control period, ACh was added to the bath in doses of 10^{-6}M, 5×10^{-7}M, 10^{-7}M and 5×10^{-8}M administered singly in random order. After three minutes the medium was replaced twice and the cycle repeated with the next dose. From the dose response curve constructed 10^{-6}M was chosen to test against pethidine and after nine minutes of control activity this dose was added to the bath and the contraction in the next two minutes recorded. In the previous experiments, the first thirty seconds of activity after a drug addition was disregarded but since ACh acts and is degraded so quickly, the immediately following contractions were studied. After a further nine minutes of control activity, pethidine was added to the muscle bath in one of the doses 5.16×10^{-6}M, 1.03×10^{-6}M, 2.06×10^{-5}M and 6.19×10^{-5}M and three minutes later 10^{-6}M ACh added. After two minutes the bath was drained

and refilled twice with fresh stock solution and the cycle repeated with a different dose of pethidine but the same dose of ACh.

All drug concentrations given are those in the baths in which the uterine horns were suspended. Tests were carried out only once on each horn but each horn experienced several different tests but always received a thorough wash-out and adequate recovery time.

8.1 (vi) Experiment 5 : Does pethidine affect elevated plasma oxytocin concentration in osmotically stimulated virgin female Sprague-Dawley rats ?

Virgin female Sprague-Dawley rats in this experiment were caged separately under the same conditions as the rats in **8.1 (ii)** and **8.1.(iii)**.

On the evening of day 1 the rat and its water bottle were weighed at 8pm. On day 2 the rat and water bottle were weighed at 8am (to monitor normal overnight water consumption) and the rats implanted with a jugular cannula under ether anaethesia. At 12 noon the water was replaced with 2% saline and the bottles weighed at 8pm.

On day 3 the rats and water bottles were weighed at 8am to monitor overnight saline consumption. [If the rats continued to drink as normal they would be osmotically stimulated from the 2% saline solution but if they chose not to drink for the 24hrs exposure to saline they would be osmotically stimulated from water deprivation.]

Blood samples were taken according to the protocol shown in **Fig 8.1(c)**. Samples of 0.3ml were withdrawn for oxytocin radioimmunoassay, those of 0.6ml for radioimmunoassay and plasma osmolality and $[Na^+]$ measurements.

After 24hrs exposure to 2% saline the rats were

DAY 1:

Weigh rat and water bottle.....4pm

Weigh water bottle.....8pm

DAY 2:

Weigh rat and water bottle.....8am

Implant venous cannula

Sample 1 (0.6ml).....2hrs post surgery

Replace drinking water with 2% saline

Sample 2 (0.3ml).....8pm
Weigh water bottles

DAY 3:

Sample 3 (0.3ml).....8am
Weigh water bottles

Sample 4 (0.6ml).....12 noon

Subcutaneous pethidine (10mg/kg) or saline

Sample 5 (0.3ml).....10 mins post injection
Sample 6 (0.3ml).....20 mins post injection
Sample 7 (0.3ml).....30 mins post injection
Sample 8 (0.6ml).....60 mins post injection

Figure 8.1(c)

Blood sampling protocol for cannulated virgin female rats during exposure to 2% saline drinking water as an osmotic stimulus and subsequently injected subcutaneously with pethidine or vehicle

injected subcutaneously with either pethidine (10mg/kg, 20mg/ml) or vehicle and serial blood samples withdrawn.

8.2 RESULTS

8.2 (i) Experiment 1 : The effect of subcutaneous injection of pethidine on the timecourse of parturition.

The first rat to deliver in this experimental group of animals was given 60mg/kg pethidine s.c. following the birth of its second pup. Acute respiratiory depression ensued resulting in death of the animal. This dose was subsequently rejected for use in this study.

The timecourse of parturition following s.c. pethidine (30 & 10mg/kg) and vehicle is shown in **Figure 8.2(a)**. Cumulative birth-time against pup number has been plotted. Both doses of pethidine (10mg/kg & 30mg/kg) significantly increased the cumulative time of delivery with respect to vehicle treated animals ($p<0.05, 0.002$ and 0.005, Kruskal-Wallis)

For more detailed statistical comparisons, the time to deliver pups 2-6 and 6-10 were compared between the three experimental groups (see **table 8.2(I)**)

For the rats that received pethidine (30mg/kg) inhibition of the progress of parturition was severe enough to prevent reinitiation of parturition before lights out and therefore the data for these animals into the later stages of parturition has not been collected.

Injection of pethidine slowed parturition significantly compared to vehicle treated rats. The action had rapid onset, lengthening the interbirth intervals between pups 2-5 (10mg/kg pethidine s.c.) and between pups 2-3 (30mg/kg pethidine s.c.). Once parturition had been re-established it continued at a similar rate to that in the vehicle injected control rats.

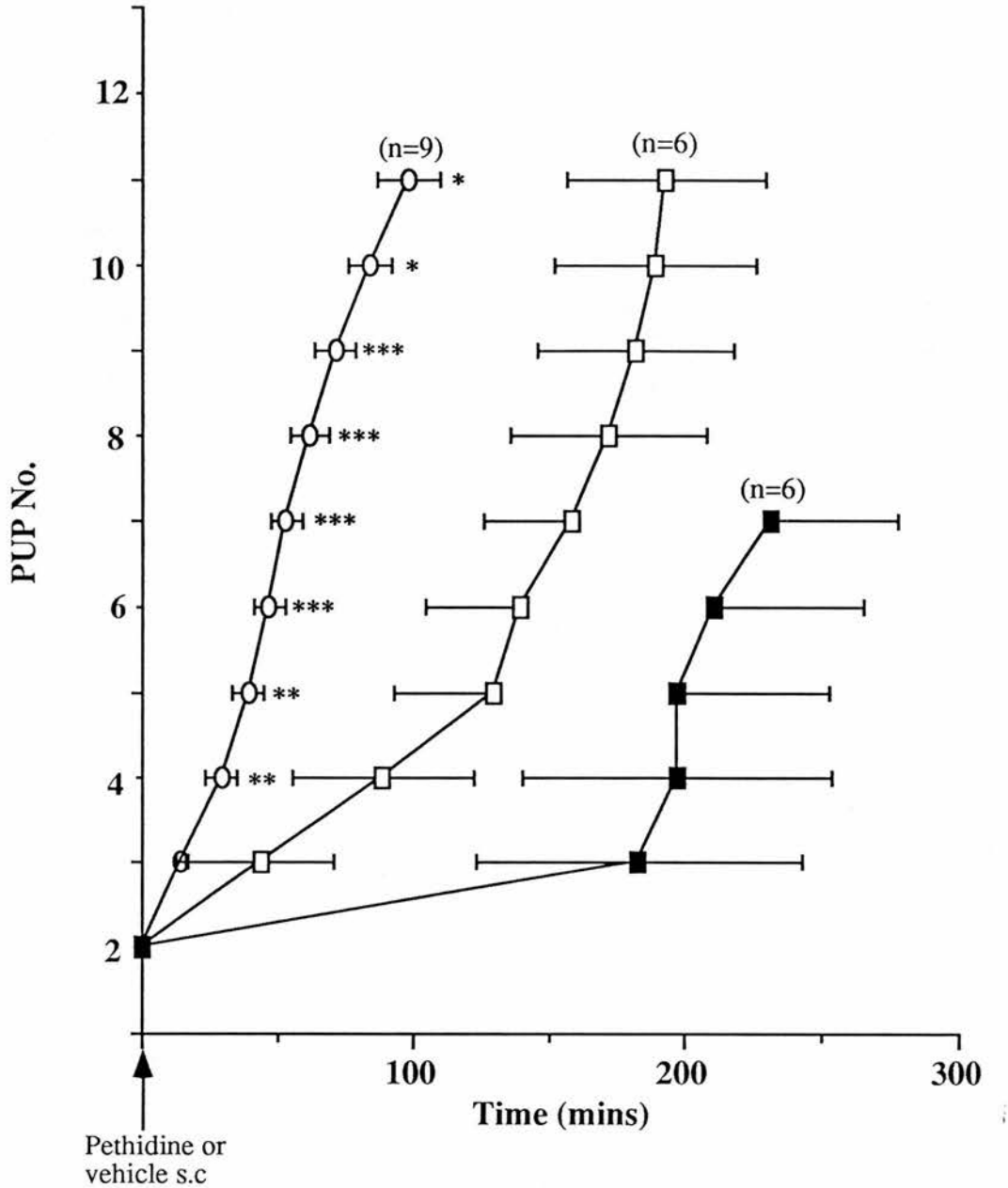


Figure 8.2 (a)

Graph depicting cumulative time of parturition against pup no. for three experimental groups of rats (vehicle,open circles; pethidine (10mg/kg),open squares;pethidine (30mg/kg),filled-squares.Subcutaneous administration of vehicle or pethidine was after pup no.2.
Statistical comparisons:
* $p<0.05$,** $p<0.02$,*** $p<0.005$ with respect to both groups of pethidine treated rats (Kruskal-Wallis),
The cumulative time to the delivery of pup 8 in the 30mg/kg pethidine treated group ranged from 62-1126 mins,therefore the mean values are not shown past pup no.7.

EXPERIMENTAL GROUP		INTERBIRTH INTERVAL (min) PUP 2-6	PUP 6-10
Vehicle	$\bar{x}$	46.8	39.9
	s.e.	5.9	5.5
	n	9	8
Pethidine 10mg/kg	$\bar{x}$	139.3 **	49.3 †
	s.e.	34.5	12.2
	n	6	6
Pethidine 30mg/kg	$\bar{x}$	210.5 **	-----
	s.e.	54.2	-----
	n	6	-----

Table 8.2(I)

Summary of statistical comparisons on the progress of parturition in uncannulated rats treated with pethidine (10/30mg/kg) or vehicle s.c.
** p<0.005 (Kruskal-Wallis w.r.t. vehicle)
† p<0.03 (Wilcoxon w.r.t. pup 2-6 interval).

EXPERIMENTAL GROUP		INTERBIRTH INTERVAL (min) PUP 2-6	PUP 6-10
Vehicle	$\bar{x}$	53.0	29.1 ≈
	s.e.	8.8	4.9
	n	8	8
Pethidine 10mg/kg	$\bar{x}$	184.3 **	52.8 †† '
	s.e.	28.4	7.3
	n	15	9
Pethidine 10mg/kg + naloxone 5mg/kg	$\bar{x}$	83.5 *	40.1 † ≈
	s.e.	22.2	7.7
	n	10	8

Table 8.2 (II)

Summary of statistical comparisons on the progress of parturition in cannulated rats treated with vehicle or pethidine (10mg/kg) s.c. or pethidine (10mg/kg) s.c. preceded by naloxone (5mg/kg i.v)
** p<0.001 w.r.t pethidine, * p<0.005 w.r.t vehicle (Wilcoxon)
† p<0.05 w.r.t pethidine, †† p<0.02 w.r.t vehicle (Wilcoxon)
' p<0.01, ≈ p<0.02 w.r.t interval 2-6 (Wilcoxon signed rank test)

8.2 (ii) Experiment 2 : Effect of pethidine (10mg/kg) s.c. on plasma oxytocin during parturition.

The timecourse of parturition in rats that received s.c. vehicle, s.c. pethidine (10mg/kg) or s.c. pethidine preceded by 5mg/kg naloxone i.v. is shown in **Figure 8.2 (b)**. The same statistical comparisons were made as in the uncannulated rats and are summarized in **Table 8.2 (II)**. The cannulation procedure and blood sampling protocol did not affect the progress of parturition in these animals. The cumulative time to delivery of pup 10 was not different to that in the uncannulated group (unpaired t-test).

Pethidine (10mg/kg) s.c. was once again followed by a slower progress of parturition. The interbirth intervals between pups 2-6 were significantly lengthened compared to the vehicle injected group ($p<0.001$ Wilcoxon). In both groups the interval between pups 6-10 was significantly less than that between pups 2-6 ($p<0.02$ vehicle, $p<0.01$ pethidine Wilcoxon) indicating that the interbirth intervals between pups decreased as parturition progressed.

Naloxone (5mg/kg i.v.) given prior to pethidine increased the rate of delivery of the four pups immediately after treatment (pups 3-6) compared to the group treated with pethidine alone ($p<0.005$, Wilcoxon rank sum test). There were no significant differences between the rate of delivery of the pups in the naloxone/pethidine treated group compared to those rats treated with vehicle.

Basal plasma oxytocin concentration in the pregnant and parturient rats was significantly elevated compared to the virgin rats used in **8.1 (vi)** (33.95 ± 3.82 vs 17.37 ± 2.40 pg/ml, $p<0.05$ Wilcoxon). The plasma oxytocin profile obtained from the serial blood samples taken from each group of rats is shown in **Figure 8.2(c)**. This graph represents the change in plasma oxytocin with respect to that measured in

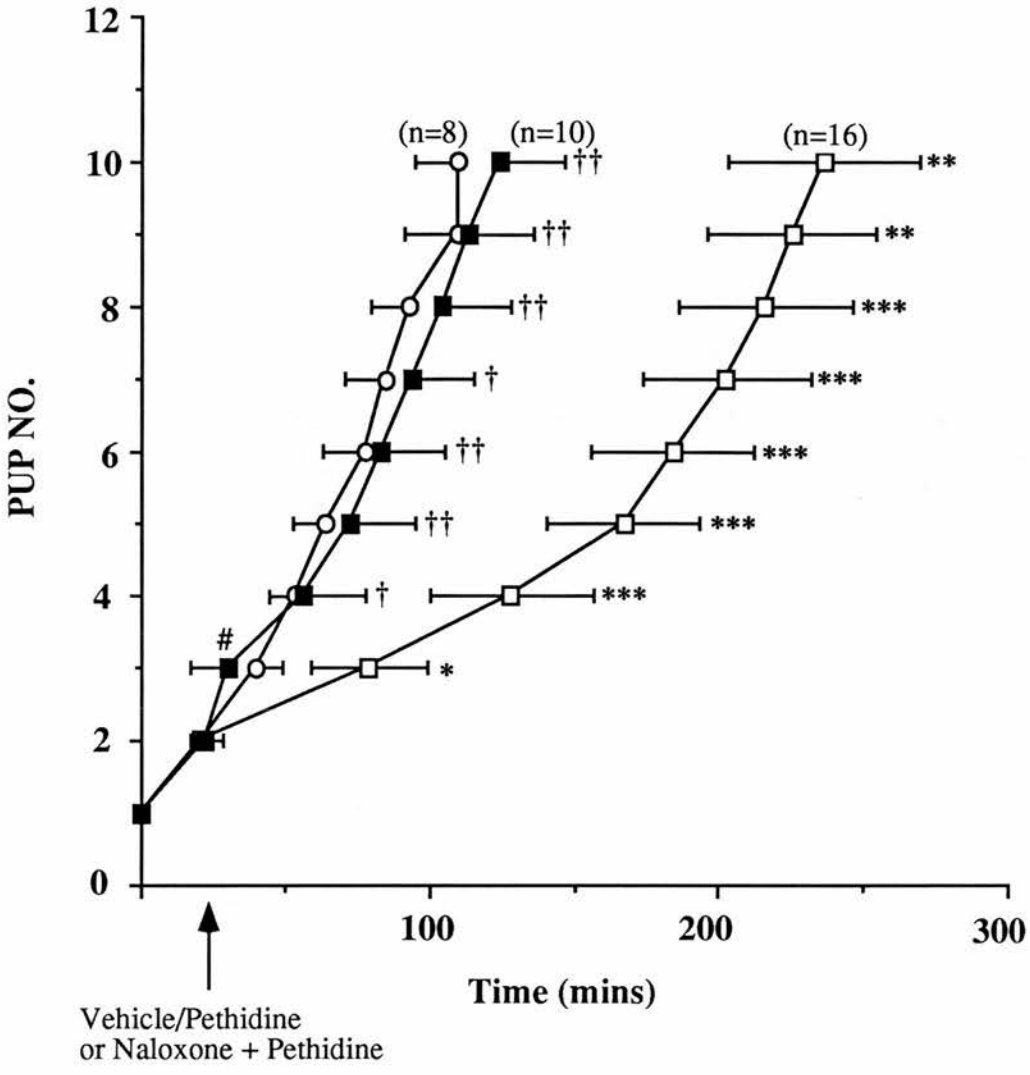


Figure 8.2 (b)

Graph depicting cumulative time of parturition against pup no. for three groups of experimental animals (vehicle,open circles; pethidine (10mg/kg),open squares;pethidine (10mg/kg) + naloxone (5mg/kg i.v.),filled squares.Subcutaneous administration of vehicle or pethidine or naloxone/pethidine took place after delivery of pup 2.
Statistical comparisons:
* $p<0.02$,** $p<0.005$,*** $p<0.001$ (with respect to vehicle treated group,Wilcoxon).
# $p<0.05$,† $p<0.01$,†† $p<0.005$,(with respect to pethidine treated group,Wilcoxon).

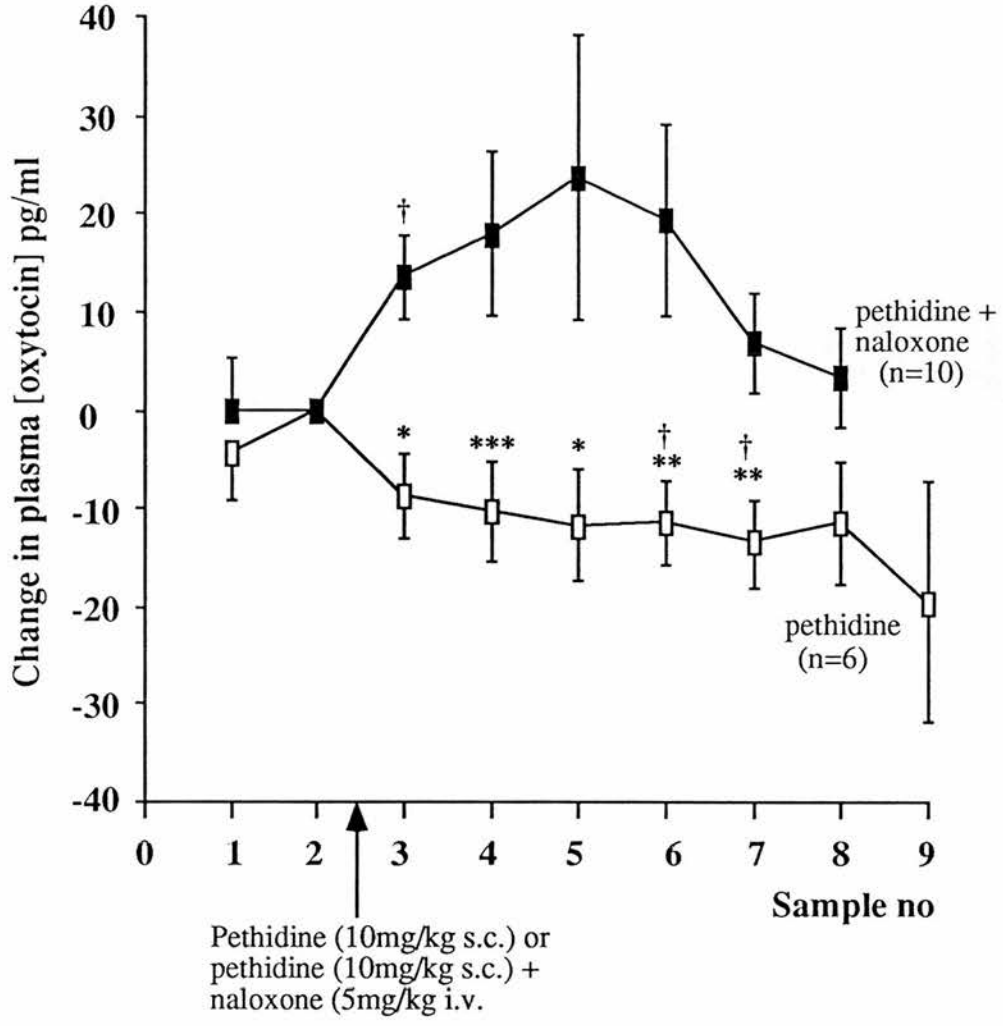


Figure 8.2 (c)

Change in plasma oxytocin in serial blood samples taken from rats during the course of parturition which were treated with pethidine (10mg/kg s.c.) or pethidine (10mg/kg s.c.) + naloxone (5mg/kg i.v.).The drugs were administered after the birth of pup no.2,immediately after sample 2. Sample 9 was taken from rats that resumed delivery after 120 mins and only occured in the pethidine treated group. Values shown are mean ± sem.

Statistical comparisons:

* $p<0.03$,** $p<0.006$,*** $p<0.007$ unpaired t-test,with respect to pethidine + naloxone group.

† $p<0.01$,paired t-test,with respect to value at sample 2.

sample 2, immediately after pup no. 2 and preceding any drug administration.

Following pethidine injection, plasma oxytocin in the timed samples decreased with respect to its initial value to a maximum change of -13.68 ± 6.97 pg/ml (sample 7). When pethidine was co-administered with naloxone, plasma oxytocin was elevated during the course of parturition to a maximum increase of 23.6 ± 14.4 pg/ml (sample 5). Statistical comparisons at sample times where significant, are shown in **Figure 8.2(c)**.

8.2 (iii) The effect of pethidine injection upon maternal behaviour.

During the course of parturition, female rats will clean their pups as they are born, gather the newborn into a nest and will eat the newly delivered placentae. Although the pups will gather beneath the mother, milk let down does not usually occur until about 2hrs after the completion of parturition. Oxytocin is believed to play an important role in prolactin secretion and milk-ejection **(Bisset, Clark et al, 1967)**.

Following injection of pethidine, the female rats appeared much quieter than those injected with saline or pethidine preceded by naloxone. In general they were disinterested in their increasing litter and made little or no attempt to clean or gather the pups together. However, this effect was short-lived and generally within two hours of completing parturition their interest in the litters had returned. No signs of dystocia were noted amongst the pethidine treated rats and despite the long delays apparent in producing the next pup after pethidine injection, there was a high pup survival rate. Details of litter size and perinatal pup viability with relevant statistical comparisons are summarized in tables 8.2 (III) & 8.2 (IV).

GROUP		LITTER SIZE	PUP VIABILITY 1hr POSTPARTUM (%)
Vehicle	$\bar{x}$	12.6	100
	s.e.	0.7	0.0
	n	9	9
Pethidine 10mg/kg	$\bar{x}$	13.8	86.7 *
	s.e.	0.5	6.0
	n	6	6
Pethidine 30mg/kg	$\bar{x}$	11.2	70.9
	s.e.	1.0	15.9
	n	6	4

Table 8.2 (III)

Litter size and pup viability data from uncannulated rats that received vehicle or pethidine (10 or 30mg/kg s.c.).

* $p<0.05$ (unpaired t-test, w.r.t. vehicle group).

GROUP		LITTER SIZE	PUP VIABILITY 1hr POSTPARTUM (%)
Vehicle	$\bar{x}$	11.6	100
	s.e.	0.7	0.0
	n	8	6
Pethidine 10mg/kg	$\bar{x}$	11.8	96.2
	s.e.	0.7	1.9
	n	17	19
Pethidine 10mg/kg + naloxone 5mg/kg	$\bar{x}$	12.5	90.6 *
	s.e.	0.1	3.2
	n	10	10

Table 8.2 (IV)

Litter size and pup viability data in cannulated rats that received either vehicle or pethidine (10mg/kg) s.c. or pethidine (10mg/kg) preceded by naloxone (5mg/kg i.v.)

* $p<0.05$ (unpaired t test, w.r.t vehicle group).

8.2 (iv) Effect of pethidine on uterine contractility in vitro

Pethidine inhibited spontaneous contraction of the post-partum uterus in a dose-dependent manner; the ID_{50} was approximately 2.6×10^{-5}M (**Fig 8.2(d)**). Pethidine also inhibited uterine contractions stimulated by oxytocin. This inhibition was not reversed by addition of oxytocin up to 1.8×10^{-6}M.

The stimulatory effect of acetylcholine on spontaneous uterine contractions is shown in **Figure 8.2(e)**.

Acetylcholine (10^{-6}M) had no effect on pethidine mediated inhibition of uterine contractions as shown in **Figure 8.2(f)**.

Naloxone alone had no effect on spontaneous uterine contractility and was not able to reverse pethidine mediated inhibition of spontaneous or oxytocin stimulated contractility. These results are summarized in **Figure 8.2(g)**.

8.2 (v) Experiment 5 : Effect of pethidine on plasma oxytocin elevated by an osmotic stimulus.

The results of fluid intake, plasma [Na^+] and plasma osmolality measurements are shown in **Table 8.2 (V)**. Fluid intake was reduced significantly once water had been replaced with 2% saline. Subsequently, plasma [Na^+] and osmolality were elevated by 24hrs either from the rats drinking the saline or by not drinking and elevating these parameters by water deprivation.

Following pethidine injection, plasma oxytocin was decreased in the serial blood samples withdrawn between 10 and 60 minutes post pethidine. Following injection of saline (vehicle), plasma oxytocin continued to rise from its post-osmotic stimulation values throughout the blood sampling protocol. The plasma oxytocin profiles from both groups of rats is shown in **Figure 8.2(h)**.

Plasma oxytocin was significantly elevated with

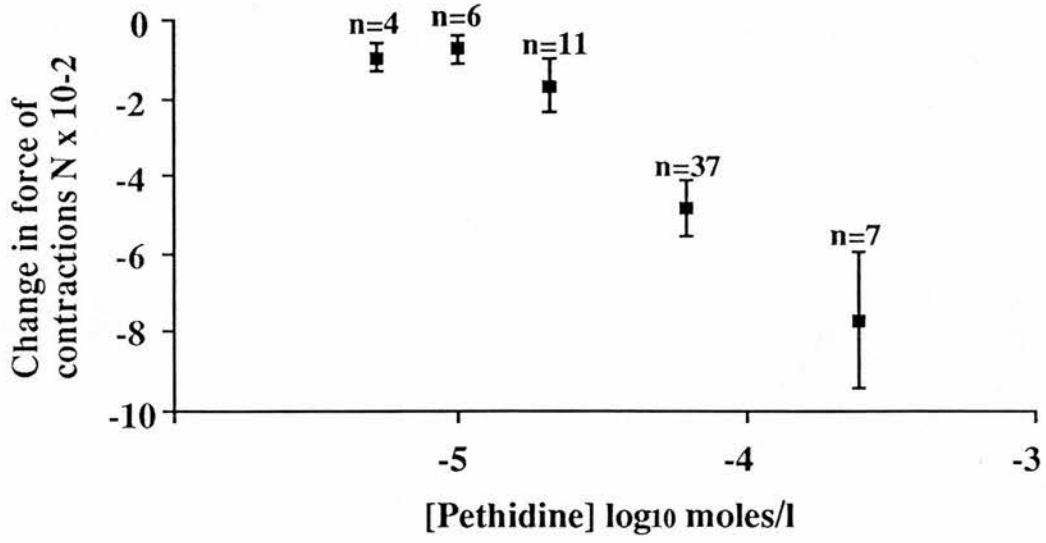


Fig 8.2 (d)

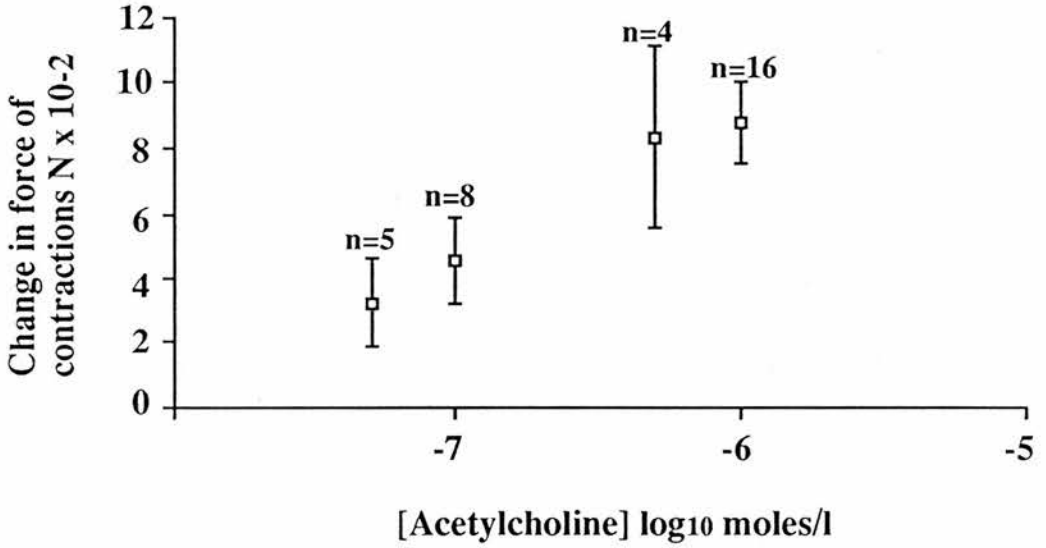


Fig 8.2 (e)

Dose response curves for pethidine (8.2 (d)) and acetylcholine (8.2 (e)) to demonstrate the inhibitory and facilitatory effects they show on spontaneous contractile activity of the immediate post-parturient uterus in vitro.Points shown are mean ± s.e.m.

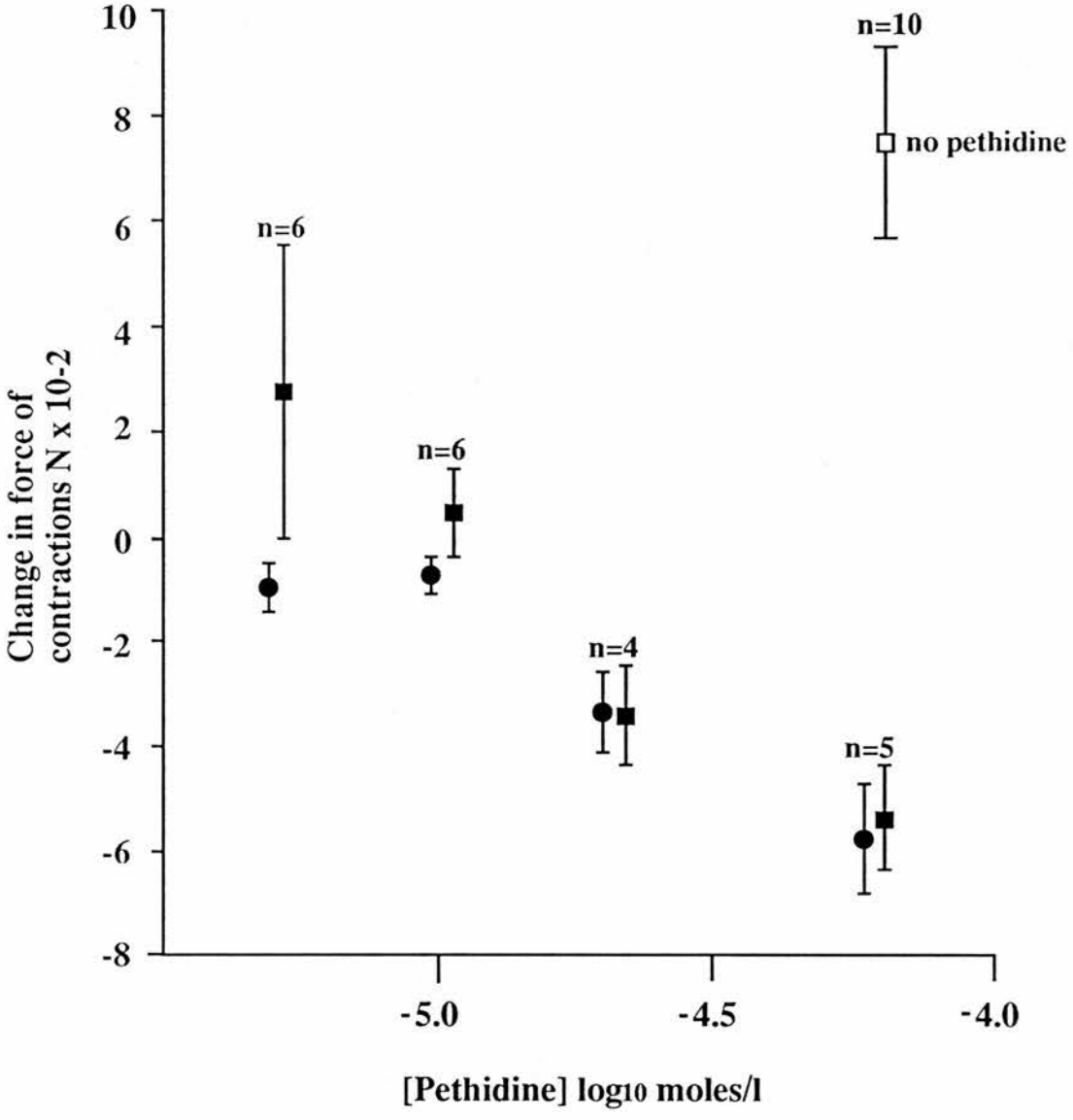


Fig 8.2 (f)

Change in contractile activity of the post-parturient uterus in vitro in the presence of pethidine (filled circles) , at various doses , and pethidine in the presence of acetylcholine at 10-6M (filled squares).Values are mean ± s.e.m.The open square symbol represents the change in force of contraction of the uterus in the presence of 10-6M acetylcholine.

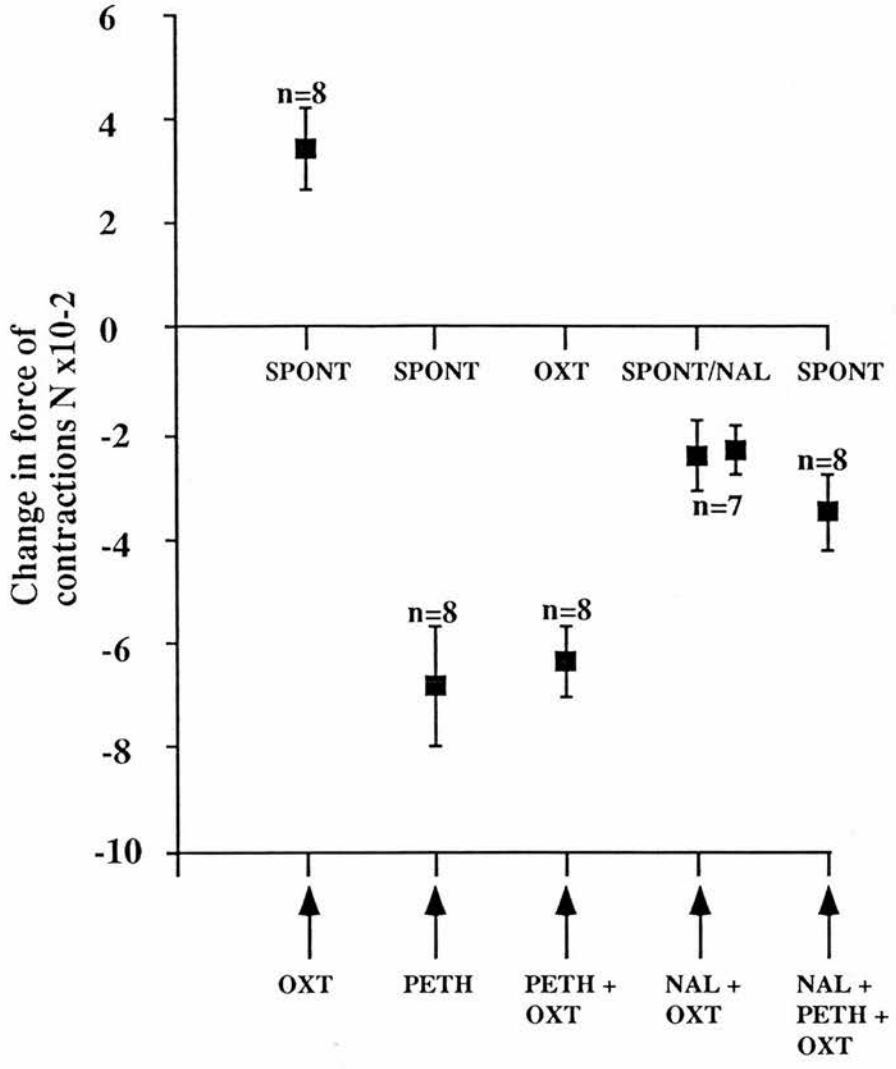


Fig 8.2 (g)

The effects of pethidine,oxytocin and naloxone on the force of contractions recorded from the post-parturient uterus in vitro.The drugs were given alone or in combination at the following doses;pethidine 6.2 x10-5M, oxytocin 4.4 x 10-7M and naloxone at 10-5M.The effects were recorded from the spontaneously contracting uterus, oxytocin stimulated contracting uterus or naloxone stimulated contracting uterus.All points represent mean ± s.e.m. values.

	Day 1	Day 2	Day 3
Mean body weight (g)	287 ± 9.1 (n=23)	287 ± 8.6 (n=23)	97▲ % Day 2 ± 0.5 (n=12)

	Day 1-2 (water)	Day 2-3 (2% NaCl)
Mean fluid intake (ml) 8pm-8am	20.6 ± 1.3 (n=22)	12.2 ± 1.6△ (n=25)

Mean Plasma	Day 2 (8pm)	Day 3 (12am)	Post injection veh	Post injection peth
$[Na^+]$ mm/L	143.7 ± 0.5 (n=23)	146.2● ± 0.5 (n=23)	145.7 ± 0.8 (n=12)	145.8 ± 0.6 (n=12)
Mean Plasma osmolality mOsm/kg	265.0 ± 5.3 (n=12)	283.5* ± 3.7 (n=12)	285.7** ± 1.9 (n=6)	288.0** ± 3.9 (n=6)

Table 8.2 (V)

Summary of the effects of substitution of 2% saline for drinking water for 24hr on body weight, fluid intake, plasma $[Na^+]$ and osmolality in virgin female rats fitted with jugular venous cannulae for blood sampling. Values shown are mean ± sem.

Statisical comparisons:

▲ $p<0.001$ with respect to Day 1 value (paired t-test)
△ $p<0.001$ with respect to Day 1 value (paired t-test)
● $p<0.001$ with respect to Day 2 value (paired t-test)
* $p<0.05$ with respect to Day 2 value (paired t-test)
** $p<0.01$ with respect to Day 2 value (paired t-test)

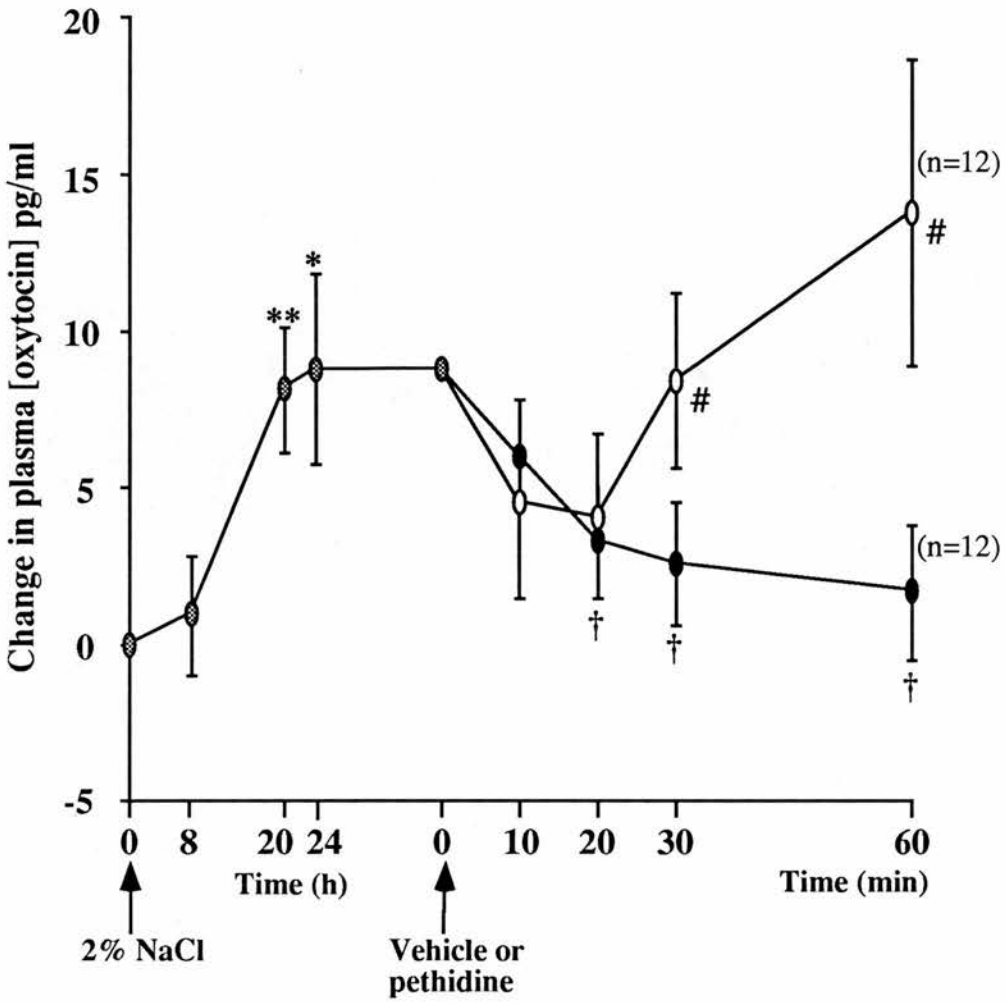


Fig 8.2 (h)

Change in plasma oxytocin concentration in blood samples withdrawn from the jugular vein of conscious rats which had been osmotically stimulated by replacing their drinking water with 2% NaCl for 24hrs (stippled symbols).The rats were then divided into two treatment groups,pethidine (filled circles) or vehicle (open circles) injected.The blood sampling protocol is shown in Figure 8.1(c).

Statistical comparisons:

* $p<0.01$, ** $p<0.001$ paired t-test with respect to basal value (t=0 h).

† $p<0.05$,paired t-test with respect to value at (t=0 min)

# $p<0.05$,unpaired t-test with respect to vehicle treated group at t=30 and t=60 min.

Mean plasma oxytocin at t=0h , 18.8 ± 2.1 pg/ml and at t=24h/0min , 26.5 ± 3.7 pg/ml.

respect to basal values at t=20hrs and t=24hrs. Following pethidine injection, plasma oxytocin concentration was decreased in samples withdrawn from the jugular vein at t=20, 30 and 60 minutes after drug injection with respect to plasma oxytocin concentration at t=0 mins. At t= 30 and 60 minutes plasma oxytocin concentration in the pethidine treated group was significantly lower than that of the saline injected controls. Statistical comparisons are shown in **Figure 8.2(h)**.

8.3 DISCUSSION

The reported effects of pethidine, when used as an obstetric analgesic, on the progress of labour in women are conflicting. A significant prolongation (**Gallen & Prescott, 1944**) and an acceleration (**Filler, Hall & Filler, 1967**) of labour have been documented.

In the experiments described here, pethidine acted to substantially prolong parturition at the two doses tested (30 & 10 mg/kg). Examination of delivery times of the rats show that this was through a lengthening of interbirth intervals. The effect of pethidine was rapid in onset and generally apparent within one to two pups after injection. For the low dose of pethidine (10mg/kg) there appeared to be an increase in the first four interbirth intervals following injection of the drug. Parturition then resumed to the rate observed in control animals. In the rats treated with the higher dose of pethidine (30 mg/kg) there appeared to be an increase in one interbirth interval, usually immediately after pethidine injection but this was for a considerable length of time and parturition continued at the normal rate on appearance of the next pup. This indicates a dose related effect dependent upon the half life of pethidine in the circulation.

There was little or no increase in perinatal mortality despite the interruption, often for several hours, to

the progress of parturition. This implies that the foetuses remained at their implantation sites on the uterus with foetal transport along the uterine horns rather than the foetal-expulsion reflex from the birth canal being affected (**Higuchi, Uchide et al, 1987**). Foetal-ejection relies in secretion of oxytocin and its uterotonic action, therefore pethidine may have an inhibitory action on oxytocin action at this stage of labour (**Higuchi, Uchide et al, 1986**).

Morphine acts centrally to inhibit oxytocin secretion (by its actions as an agonist at μ-opioid receptors) and hence prolong parturition. This effect is reversed by naloxone which is a potent μ-opioid receptor antagonist or by infusion of oxytocin (**Russell, Gosden et al, 1989**).

Pethidine has considerably less affinity for μ-opioid receptors than morphine so naloxone would be expected to completely reverse its effects and that of any endogenous opioids acting to regulate oxytocin secretion. Subcutaneous injection of pethidine reduced plasma oxytocin during the course of parturition, as seen in 8.2(c).

Naloxone, when co-administered with pethidine elevated plasma oxytocin considerably, presumably through antagonism of pethidine and endogenous opioids (**Hartman, Rosella-Dampman et al, 1986; Leng, Mansfield etal, 1988**). Parturition was not accelerated in these rats, although when naloxone is given to rats in the early stages of labour it increases plasma oxytocin and shortens the duration of parturition (**Leng, Mansfield et al, 1987**). Pethidine is known also to have atropine like properties and it may be partly due to these non-opioid mechanisms that parturition is delayed.

In-vitro, pethidine profoundly inhibited spontaneous and oxytocin-stimulated uterine contractions as well as rendering the muscle insensitive to

aceylcholine. These results contrast with the results of previous studies on uteri from late pregnant rats in which pethidine increased both the frequency and amplitude of uterine contractions (**Fazackerley & Pleuvry, 1987; Sivalingham & Pleuvry, 1985**) Naloxone was not able to reverse the inhibition, implying that it was not mediated by opioid-receptors. Since the effects of acetylcholine are negated by pethidine it would appear that its actions at the uterus are due to its atropine-like properties. A consequence of this direct action on the uterus would be to prevent entry of the foetuses into the birth canal via the cervix and hence remove the Ferguson reflex stimulation of oxytocin secretion. Thus, pethidine probably interrupts parturition by this inhibitory action on the uterus, peripherally and indirectly, as well as reducing oxytocin secretion possibly by a central route.

It is difficult to monitor the effects of pethidine on uterine contractility in women since administration of other drugs such as oxytocin and prostaglandins during labour makes interpretation of any effects seen difficult. Pethidine has been reported to both stimulate (**Sica-Blanco, Rozada & Remedio, 1967**) and inhibit (**Petrie, Wu, Miller, Sacks et al, 1976**) uterine contractility.

Immediate post-partum maternal behaviour was disrupted by pethidine injection, in that the mother rats did not clean or gather their pups together in a nest. However, the litter sizes in the pethidine and pethidine/naloxone treated animals were not significantly different from the vehicle treated groups. There was a small significant decrease ($p<0.05$ with respect to the vehicle group) in pup viability 1 hour post-partum following 10mg/kg pethidine (experiment 1) or 10mg/kg pethidine + 5mg/kg naloxone (experiment 2).

It has been postulated that the hormonal changes at

the termination of pregnancy induce the rapid onset of maternal behaviour. Central oxytocin is also thought to have a role in the induction of maternal behaviour (**Pederson & Prange, 1979**) but that oestrogen priming is necessary for oxytocin receptor sensitization (**Pederson & Prange, 1985**). Central oxytocin is known to be elevated in the SON and PVN at the end of pregnancy (**Russell, 1980; Fuchs, 1985**).

Central injection of an oxytocin antagonist, d(CH_2) 5-8-ornithine vasotocin inhibits post-partum maternal behaviour (**van Leengoed, Kerker & Swanson, 1987**) and the partial inhibition of this central oxytocin by pethidine may account for the slight disruption of maternal behaviour seen.

Rats with raised plasma oxytocin secretion from drinking 2% saline for 24hrs experienced a decrease in plasma oxytocin following pethidine injection (10mg/kg). Oxytocin secretion is elevated in response to an osmotic stimulus by central mechanisms, implying that pethidine acts centrally to depress oxytocin secretion (**Leng, Mason & Dyer, 1982**). Hyperosmolality stimulates oxytocin secretion by actions at osmo-receptors located in the AV3V (region anterior and ventral to the third ventricle) (**Leng, Blackburn et al, 1989**) and this may be the site for the central effects of pethidine on oxytocin since there are opioid receptors in this region and the sub-fornical organ (SFO) (**Sharif & Hughes, 1989 ; Sumner, Coombes et al, 1990**).

However, parturition is not prolonged by lesion of the AV3V region which would be expected to resemble opioid inhibitory action, so pethidine may not impede parturition by acting at this site (**Russell, Blackburn & Leng, 1989**). Pethidine may, like morphine, also act on the cell bodies of magnocellular oxytocin neurones, but this has not been investigated.

In the rat, pethidine profoundly prolongs parturition

by depression of oxytocin secretion possibly by μ-opioid agonist activity and by abolision of uterine contractility. Since the effect on the uterus is not reversible by naloxone and renders acetylcholine ineffective at the isolated uterus, this may be by atropine like actions. These effects delay the movement of pups down the uterus and consequently prevent positive feedback secretion of oxytocin from the Ferguson-reflex. Pethidine severely affects both the secretion and action of oxytocin during parturition resulting in lengthening of labour.

It is not yet known, whether, when used in women as an obstetric analgesic, pethidine compromises the physiological role of oxytocin to such an extent.

REFERENCES

Abrahams,V.C. & Pickford,M. (1954) Simultaneous observations on the rate of urine flow and spontaneous uterine movements in the dog and their relationship to posterior lobe activity. J.Physiol.(Lond) **126** : 329-346

Antoni,F.A.,Kovacs,K.J.,Dohanits,J.,Makara,G.B., Holmes,M.C. & Mazurek,M.F. (1988) Hypophysiotropic function of vasopressin and oxytocin. Brain Res.Bull **20**: 729-736

Arimura,A.,Smith,W. & Schally,A.(1976) Blockade of the stress-induced decrease in blood GH by antisomatostatin serum in rats. Endocrinology **98** : 540-543

Armstrong,W.E.,Warach,S.,Hatton,G.I. & M=Neill,T.M. (1980) Subnuclei in the rat hypothalamic paraventricular nucleus : a cytoarchitectural , horseradish peroxidase and immunocytochemical analysis. Neuroscience **5** : 1931-1958

Aziz,L.A.,Forsling,M.L. & Woolf,C.J. (1981) The effect of intracerebroventricular injections of morphine on vasopressin release in the rat. J.Physiol.(Lond) **311** : 401-409

Bhargarva ,Hemendra,N.,Ramarao,P. & Gulati,A. (1989) Effects of morphine in rats treated chronically with U50,488H , a kappa opioid receptor agonist. Eur.J.Pharmacol. **162 (2)** :257-264

Bicknell,R.J. & Leng,G. (1982) Endogenous opiates regulate oxytocin but not vasopressin from the neurohypophysis. Nature (Lond). **298** :161-162

Bicknell,R.J.,Leng,G.,Lincoln,D.W. & Russell,J.A. (1984) Activation of putative oxytocin neurones following naloxone administration to rats chronically treated with intracerebroventricular morphine.J.Physiol.(Lond) **357** : 97P

Bicknell,R.J.,Chapman,C. & Leng,G. (1985) Effects of opioid agonists and antagonists on oxytocin and vasopressin release in vitro.Neuroendocrinology **41** : 142-148

Bicknell,R.J.,Chapman,C. & Zhao,B-G. (1987) Functional kappa-opioid receptors on oxytocin and vasopressin secretory terminals isolated from rat neurohypophysis. J.Physiol.(Lond) **394** : 123P

Bicknell,R.J.,Chapman,C. & Zhao,B-G. (1987) Opioid receptor subtypes mediating inhbition of oxytocin,vasopressin and noradrenaline release from the rat neurohypophysis in vitro. J.Physiol.(Lond) **388** : 9P

Bicknell,R.J. (1988) Optimizing release from peptide hormone secretory nerve terminals.J.Exp.Biol **139** : 51-65

Bicknell,R.J.,Leng,G.,Lincoln,D.W. & Russell,J.A. (1988) Naloxone excites oxytocin neurones in the supraoptic nucleus of lactating rats after chronic morphine treatment.J.Physiol.(Lond) **396** : 297-317

Birch,R.J.,Hayes,A.G.,Sheehan,M.J. & Tyers,M.B. (1988) In vivi studies on the opioid antagonist,16-methylcyprenorphine. J.Pharm.Pharmacol. **40**(3) : 213-214

Birnbaumer,L. (1987) Which G protein subunits are the active mediators in signal transduction ? Trends Pharmacol. **8** : 209-211

Bissett,G.W.,Clark,B.J.,Haldar,J.,Harris,M.C.,Lewis,G. P. & Roche E Silva,M.Jr (1967) Assay of milk ejecting activity in the lactating rat. Br.J.Pharmacol. **31** : 537-549

Blackburn,T.P.,Borkowski,K.R.,Friend,J. & Rance,M.J. (1986) Mechanisms of κ-induced diuresis. Br.J.Pharmacol. **89** : 593-598

Borkowski,K.R. (1989) Studies on the adrenomedullary dependence of kappa-opioid agonist-induced diuresis in conscious rats. Br.J.Pharmacol **98** :1151-1156

Bourque,C.W. & Renaud,L (1985) Calcium-dependent action potentials in rat supraoptic neurosecretory neurones recorded in vitro. J.Physiol.(Lond) **363** : 429-39

Brady,L.S. & Herkenham,M. (1987) Dehydration reduces κ-opiate receptor binding in the neurohypophysis in the rat. Brain Res. **425** : 212-217

Brady,L.S.,Rothman,R,B. & Herkenham,M (1988) Physiological regulation of neurohypophysial kappa-opiate receptors. Brain Res. **443** : 398-402

Brethes,D.,Dayanithi,G.,Lettelier,L. & Nordmann,J.J. (1987) Depolarization-induced Ca^{2+} increase in isolated neurosecretory nerve terminals measured with fura-2. Proc.Natl.Acad.Sci. **84** : 1439-1443

Brimble,M.J. & Dyball,R.E.J. (1977) Characterization of the responses of oxytocin and vasopressin-secreting neurones in the supraoptic nucleus to osmotic stimulation. J.Physiol. (Lond) **271** : 253-271

Brimble,M.J.,Dyball,R.E.J. & Forsling,M.L. (1978) Oxytocin release following osmotic activation of oxytocin neurones in the paraventricular and supraoptic nuclei.J.Physiol.(Lond) **278** :69-78

Buckingham,J.C. & Cooper,T.A. (1984) Differences in hypothalamo-pituitary-adrenocortical activity in the rat after acute and prolonged treatment with morphine.Neuroendocrinology **38** : 411-417

Bunn,S.J.,Hanley,M.R. & Wilkin,G.P. (1985) Evidence for a kappa-opioid receptor on pituitary astrocytes : an autoradiographic study.Neurosci.Letts **55** : 317-323

Buijs,R.M.,Swaab,D.F.,Dogterom,J. & Van Leeuwen,F.W. (1978) Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat. Cell Tiss.Res **186** : 423-433

Carter,D.A. & Lightman,S.L. (1987) Oxytocin responses to stressin lactating and hyperprolactinaemic rats. Neuroendocrinology **46** : 532-537

Castanas,E.,Giraud,P.,Audigier,Y.,Drissi,R., Boudouresque,F.,Conte-Devolx,B. & Oliver,C. (1984) Pharmacological characterization of kappa receptors in bovine adrenal medulla and human pheochromocytoma. Molec. Pharmacol. **25** : 38-45

Chang,K.J.,Hazum,E. & Cuatrecasas,P. (1980) Multiple opiate receptors. Trends Neurosci. 3(7) : 160-162

Chavkin,C.,James,I.F. & Goldstein,A. (1982) Dynorphin is a specific endogenous ligand of the κ-receptor. Science **215** : 413-415

Clarke,G.,Wood,P.,Merrick,L. & Lincoln,D.W. (1979) Opiate inhibition of peptide release from the neurohumoral terminals of hypothalamic neurones Nature (Lond) **282** : 746-748

Clarke,G. & Wright,D.M. (1984) A comparison of analgesia and suppression of oxytocin release by opiates. Br.J.Pharmacol. **83** :799-806

Clarke,G. & Wright,D.M. (1987) Opioids , catecholamines and the secretion of oxytocin. Neuroscience Letts. Supplement **29** : S40

Clark,J.A. & Pasternak,G.W. (1988) U50,488H: a kappa selective agent with poor affinity for mu, opiate binding sites. Neuropharmacology **27**(3) : 331-332

Clark,J.A.,Liu,L.,Price,M,Hersch,B.,Edelson,M. & Pasternak,G. (1989) Kappa opiate receptor multiplicity: evidence for two U50,488H-sensitive kappa$_1$ subtypes and a novel kappa$_3$ subtype. J.Pharmacol.Exp.Ther. **251**(2) : 461-468

Clark,R.G. & Robinson,I.C.A.F. (1985) Growth hormone responses to multiple injections of a fragment of growth hormone-releasing factor in conscious male and female rats. J.Endocrinol. **106** : 281-289

Clark,R.G.,Carlsson,L.M.S.,Raffery,B & Robinson,I.C.A.F (1988) The rebound release of growth hormone (GH) following somatostatin infusion in rats involves hypothalamic GH-releasing factor release. Endocrinology **119** : 397-404

Coghlan,J.P.,Aldred,P.,Barkus,A.,Crawford,R.J. Derby,I.A.,Fernley,R.T.,Haralambis,J.,Hudson,P.J., Mitri,R.,Niell,H.D.,Penshaw,J.D.,Roche,P.J.,Scanlon,D. B. & Treycar,G.W. (1984) Hybridization Histochemistry : Excerpta Medica (Int. Congress Series) **655** : 18 Elsevier (Amsterdam)

Coombes,J.E. & Russell,J.A. (1988) Inhibition by a kappa-opioid receptor agonist (U50,488H) of electrically stimulated oxytocin secretion from the neurohypophysis in anaesthetized lactating rats. J.Physiol. **401** :34P

Corbett,A.D.,Paterson,S.J.,McKnight,A.T.,Magnan,J. & Kosterlitz,H.W. (1982) Dynorphin (1-8) and dynorphin (1-9) are ligands for the κ-subtype of opiate receptor. Nature **299** : 79-81

Crankshaw,D.J.,Branda,L.A.,Matlieb,M.A. & Daniel,E.E. (1978) Localization of oxytocin receptors in plasma membrane of rat myometrium. Eur.J.Biochem. **86** : 481-486

Cserr,H. (1965) Potassium exchange between cerebrospinal fluid,plasma and brain. Am.J.Physiol. **209** : 1219-1226

Dale,H.H. (1906) On some physiological aspects of ergot. J.Physiol. **34** : 163-206

Deschepper,C.,Lotstra,F.,Vandesande,F. & Vanderhaegen,J.J. (1983) Cholecystokinin varies in the posterior pituitary and external median eminence of the rat according to factors affecting vasopressin and oxytocin. Life Sci. **32** : 2571-2577

Dierickx,K. (1980) Immunocytochemical localization of the vertebrate cyclic nonapeptide neurohypophyseal hormones and neurophysins Int.Rev.Cytol **62** : 119-185

Dogterom,J.,Van Wimersma Greidanus,T.B. & Swaab,D.F. (1977) Evidence for the release of vasopressin and oxytocin into cerebrospinal fluid : measurements in plasma and CSF of intact and hypophysectomized rats. Neuroendocrinology **24** : 108-118

Donnerer,J. & Lembeck,F. (1988) Neonatal capsaicin treatment of rats reduces ACTH secretion in response to peripheral neuronal stimuli but not to centrally acting stressors. Br.J.Pharmacol. **94** : 647-652

Dyball,R.E.J. & Prilusky,J. (1981) Responses of supraoptic neurones in the intact and deafferented rat hypothalamus to injection of hypertonic sodium chloride. J.Physiol. **311** : 443-452

Ermische,A.,Rühle-H.J.,Landgraf,R. & Hess,J. (1985) Blood-brain barrier and peptides. J.Cereb.Blood Flow Metab. **5** : 350-357

Fahrbach,S.E.,Morrell,J.I. & Pfaff,D.W. (1985) "Role of oxytocin in the onset of oestrogen facilitated maternal behaviour in rats" In Oxytocin:Clinical and Laboratory Studies. J.A.Amico,A.G.Robinson eds. Elsevier,New York.

Fazackerley,J. & Pleuvry,B.J. (1987) Stimulant action of pethidine on the pregnant rat uterus in vitro. J.Pharm.Pharmacol. **39** : 491-492

Filler,W.W.Jr.,Hell,W.C. & Filler,N.W. (1944) Analgesia in obstetrics.The effect of analgesia on uterine contractility and foetal heart rate. Am.J.Obstet.Gynaecol. **98** : 832-846

Findlay,A.L.R. (1966) Sensory discharges from lactating mammary glands. Nature(Lond) **211** : 1183-1184

Finley,J.C.W.,Manderdrut,J.L. & Petrusz,P. (1983) The immunocytochemical localization of enkephalin in the central nervous system of the rat. J.Comp.Neurol. **198** : 541-565

Fischli,W.,Goldstein,A.,Hunkapillar,M.V. & Hood,L.E. (1982) Isolation and amino acid sequence analysis of a 4000 dalton dynorphine from porcine pituitary. Proc.Natl.Acad.Sci.(USA) **79** : 5435-5437

Forman,A.,Gandrup,P.,Anderson,K-E. & Ulmsten,U. (1982) Effects of nifedipine on oxytocin-induced and prostaglandin-$F_{2\alpha}$-alpha-induced actinvity in the postpartum uterus. Am.J.Obstet.Gynaecol. **144** : 655-670

Forsling,M. & Wells,T. (1989) The κ-opioid influence on basal and osmotically-stimulated AVP secretion in the conscious rat. J.Physiol.(Lond.) **415** : 86P

Freund-Mercier,M.J. & Richard,Ph. (1984) Electrophysiological evidence for facilitatory control of oxytocin neurones by oxytocin during suckling in the rat. J.Physiol.(Lond) **352** : 447-466

Fuchs,A.R.,Periyasami,S.,Alexandrova,M. & Soloff,M.S. (1983) Correlation between oxytocin receptor concentration and responsiveness to oxytocin in pregnant rat myometrium-effects of ovarian steroids. Endocrinology **113** :742-749

Fuchs,A.R. (1985) "Oxytocin in animal parturition" In Oxytocin:Clinical and Laboratory Studies. J.A.Amico,A.G.Robinson eds. Elsevier,New York.

Fukagawa,Y.,Katz,J.L. & Suzuki,T. (1989) Effects of a selective κ-opioid agonist,U-50,488H on morphine dependence in rats. Eur.J.Pharmacol. **170** : 47-51

Gabriel,S.M.,Simpkin,J.W. & Millard,W.J. (1985) Changes in anterior pituitary hormone secretion and hypothalamic catecholamine metabolism during morphine withdrawal in the female rat. Brain Res. **346** : 15-21

Gallen,B. & Prescott,F. (1944) Pethidine as an obstetric analgesic.A report on 150 cases. Br.Med J. **1** : 176-179

Gibbs,D.M. (1984) Dissociation of oxytocin, vasopressin and corticotrophin secretion during different kinds of stress. Life Sci. **35** : 487-491

Giraud,P.,Castanas,E.,Patey,G.,Oliver,C. & Rossier,J. (1983) Regional distribution of Methionine-Enkephalin-Arg^6-Phe^7 in the rat brain-comparitive study with other opioid peptides. J.Neurochem. **41(i)** : 154-160

Goldstein,A.,NestorJ.J.Jr,Naidu,A. & Newman,S.R. (1988) DAKLI-a multipurpose ligand with a high affinity and selectivity for dynorphin (kappa-opioid) binding sites. Proc.Natl.Acad.Sci (USA) **85**(19) :7375-7379

Gosden, R.G., Humphreys, E.M., Johnston, V., Liddle, S. & Russell, J.A. (1985) Morphine acts centrally to interrupt established parturition in the rat. J. Physiol. **364** :59P

Gregory, W.A., Tweedle, C.D. & Hatton, G.I. (1980) Ultrastructure of neurons in the paraventricular nucleus of normal dehydrated and rehydrated rats. Brain Res. Bull. **5**(3) : 301-306

Grell, S., Fjalland, B. & Christensen, J.D. (1989) Biphasic effect of a kappa-opioid receptor agonist on plasma oxytocin levels in rats. Eur. J. Pharmacol. **166** : 41-48

Gross, E.G. & Pierce, I.H. (1935) Effect of morphine on the oxygen consumption of brain tissue in rat. J. Pharm. Exp. Ther. **53** : 156-168

Gubler, U., Seeberg, P., Hoffman, B.J., Gage, L.P. & Undenfriend, S. (1982) Molecular cloning establishes pro-enkephalin as precursor of enkephalin containing peptides. Nature **295** : 206-208

Haldar, J. & Sawyer, S.M. (1978) Inhibition of oxytocin release by morphine and its analogs. Proc. Soc. Exp. Biol. Med. **157** : 476-480

Hamstra, W.N., Doray, D. & Dunn, J.D. (1984) The effect of urethane on pituitary adrenal function in female rats. Acta Endocrinol. (Copenhagen) **106** : 362-367

Hartman, R.D., Rosella-Dampman, L.M. & Summy-Long, J.Y. (1987) Endogenous opioid-peptides inhibit oxytocin release in the lactating rat after dehydration and urethane. Endocrinology **121**(2) : 536-543

Hatton, G.I., Perlmutter, L.S., Salm, A.K. & Tweedle, C.D. (1984) Dynamic neuronal-glial interactions in hypothalamus and pituitary : implications for control of hormone synthesis and release. Peptides (Suppl.1) : 121-138

Hatton, G.I., Ho, Y.W. & Mason, W.T. (1983) Synaptic activation of phasic bursting in rat supraoptic nucleus neurones recorded in hypothalamic slices. J. Physiol(Lond) **345** : 297-317

Hayes, A.G., Skingle, M. & Tyers, M.B. (1986) Reversal by β-funaltrexamine of the antinociceptive effect of opioid agonists in the rat. Br. J. Pharmacol. **88** : 867-872

Hayes.A.G. & Birch,P.J. (1988) Reversal by β-funaltrexamine and 16-methyl cyprenorphine of the antinociceptive effects of opioid agonists in the mouse and guinea pig. Neuropharmacology **27**(8) : 813-816

Haynes.L. (1988) Opioid receptors and signal transduction. Trends Pharmacol. **9** : 309-311

Herkenham,M.,Rice,K.C.,Jacobson,A.E. & Rothman,R.B. (1986) Opiate receptors in rat pituitary are confined to the neural lobe and are exclusively kappa Brain Res. **382** : 365-371

Higuchi,T.,Tadokoro,Y,Honda,K. & Negoro,H. (1986) Detailed analysis of blood oxytocin levels during suckling and parturition in the rat. J.Endocrinol. **110** :251-256

Higuchi,T.,Honda,K.,Fukuoka,T.,Negoro,H. & Wakabayashi,K, (1985) Release of oxytocin during suckling and parturition in the rat. J.Endocrinol. **105** : 339-346

Higuchi,T.,Uchide,K.,Honda,K. & Negoro,H. (1986) Oxytocin release during parturition in the pelvic-neurectomized rat J.Endocrinol. **109** : 149-154

Higuchi,T.,Uchide,K.,Honda,K. & Negoro,H. (1987) Pelvic neurectomy abolishes the fetus-expulsion reflex and induces dystocia in the rat. Exp.Neurol. **96** : 443-455

Horn,A.M.,Robinson,I.C.A.F. & Fink,G (1985) Oxytocin and vasopressin in rat hypophysial portal blood : experimental studies in normal and Brattleboro rats. J.Endocrinol. **104** :211-224

Hou-You,A.,Lamme,A.T.,Zimmerman,E.A. & Silverman,A.J. (1986) Comparitive distribution of vasopressin and oxytocin neurons in the rat brain using a double-label procedure Neuroendocrinology **44** : 235-246

Ivell,R. & Richter,D. (1984) Structure and comparison of the oxytocin and vasopressin genes from rat. Proc.Natl.Acad.Sci.(USA) **81** : 2006-2010

Jones,C.W. & Pickering,B.J. (1969) Comparison of the effects of water deprivation and sodium chloride inhibition on the hormone content of the neurohypophysis of the rat. J.Physiol.(Lond) **203** : 449-458

Jones,P.M.,Robinson,I.C.A.F. & Harris,M.C. (1983) Release of oxytocin into blood and cerebrospinal fluid by electrical stimulation of the hypothalamus or neural lobe in the rat. Neuroendocrinology **37** : 454-458

Kilpatrick,D.L.,Wahlström,A.,Lahm,H.W.,Blacher,R. & Undenfriend,S. (1982) Rimorphine,a unique,naturally occurring [Leu] enkephalin-containing peptide found in association with dynorphin and alpha-neoendorphin. Proc.Natl.Acad.Sci.(USA) **79** :6480-6483

Kiss,J.Z.,Williams,T.H. & Palkovits,M. (1984) Distribution and projections of cholecystokinin-immunoreactive neurons in the hypothalamic paraventricular nucleus of the rat. J.Comp.Neurol.**227** : 173-181

König,J.F.R. & Klippel,R.A. (1963). The rat brain.A stereotaxic atlas of the forebrain and lower parts of the brainstem.Baltimore : Williams & Wilkins.

Kosterlitz,H.W. (1985) The Wellcome Foundation Lecture,1982.Opioid peptides and their receptors. Proc.R.Soc.Lond.B **225** : 27-40

Kovacs,K.J. & Makara,G.B. (1988) Corticosterone and dexamethasone act at different sites to inhibit adrenalectomy-induced adrenocorticotrophin hypersecretion. Brain Res. **474** : 205-210

Lang,R.E.,Rascher,W.,Heil,J.,Unger,Th.,Weidemann,G. & Ganten,D. (1981) Angiotensin stimulates oxytocin release. Life Sci. **29** : 1425-1428

Landgraf,R.,Neumann,I. & Schwarzberg,H. (1989) Central and peripheral release of vasopressin and oxytocin in the conscious rat after osmotic stimulation. Brain Res. **457 (2)** : 219-225

Leander,J.D. (1983 a) A kappa-opioid effect -increased urination in the rat J.Pharmacol.Exp.Ther **224** : 89-94

Leander,J.D. (1983 b) Further study of κ-opioids on increased urination. J.Pharmacol.Exp.Ther **227** : 35-41

Leng,G.,Mason,W.T. & Dyer,R.G. (1982) The supraoptic nucleus as an osmoreceptor Neuroendocrinology **34** : 75-82

Leng,G.,Mansfield,S.,Bicknell,R.J.,Brown,D,Chapman,CHollingsworth,S.,Ingram,C.D.,Marsh,M.I.C.,Yates,J.O. & R.G.Dyer (1987) Stress-induced disruption of parturition in the rat may be mediated by endogenous opioids. J.Endocrinol. **114** :247-252

Leng,G.,Mansfield,S.,Bicknell,R.J.,Blackburn,R.E., Brown,D.,Chapman,C.,Dyer,R.G.,Hollingsworth,S., Shibuki,K.,Yates,J.O. & Way,S. (1988) Endogenous opioid actions and effects of enviromental disturbance on parturition and oxytocin secretion in the rat. J.Reprod.Fert. **84** : 345-56

Leng,G.,Blackburn,R.E.J.,Dyball & Russell,J.A. (1989) Role of anterior peri-third ventricular structures in the regulation of supraoptic neuronal activity and neurohypophyseal hormone secretion in the rat. J.Neuroendocrinol. **1** : 35-46

Leng,G.,Russell,J.A. & Grossman,R. (1989) Sensitivity of magnocellular oxytocin neurones to opioid antagonists in rats treated chronically with intracerebroventricular (i.c.v.) morphine. Brain Res. **484** : 290-296

Leng,G.,Pumford,K.M. & Russell,J.A. (1990) Actions of the x-type opioid receptor agonist U50,488H on oxytocin neurones in the supraoptic nucleus (SON) in normal and morphine-tolerant urethane anaesthetized rats. J.Physiol.(Lond) **420** : 71P

Lightman,S.L.,Ninkovic,M.,Hunt,S.P., & Iversen,L.L. (1983) Evidence for opiate receptors on pituicytes. Nature (Lond.) **305** : 235-237

Lincoln,D.W. & Wakerley,J.B. (1971) Accelerated discharge of paraventricular neurosecretory cells correlated with reflex release of oxytocin during suckling. J.Physiol. (Lond.) **222** : 23P

Lincoln,D.W.,Hill,A & Wakerley,J.B. (1973) The milk-ejection reflex of the rat : an intermittent function not abolished by surgical levels of anaesthesia. J.Endocrinol. **57** : 459-476

Lincoln,D.W., & Wakerley,J.B. (1974) Electrophysiological evidence for the activation of supraoptic neurones during the release of oxytocin. J.Physiol. (Lond.) **242** : 533-554

Lincoln,D.W. & Wakerley,J.B. (1975) Factors governing the periodic activation of supraoptic and paraventricular neurosecretory cells during suckling in the rat. J.Physiol. (Lond.) **250** :443-461

Lincoln,D.W. & Russell,J.A. (1985) "The electrophysiology of magnocellular oxytocin neurones" In : Oxytocin:Clinical & Laboratory Studies 53-76. J.A.Amico & A.G.Robinson eds,Elsevier,New York.

Lindheimer, M.D., Barron, W.M. & Davison, J.M. (1985) "Water metabolism and vasopressin secretion in pregnancy" In : Vasopressin 229-240. R.W.Schrier ed Raven Press, New York.

Mansour, A., Khachaturian, H., Lewis, M.E., Akil, H.A. & Watson, S.J. (1988) Anatomy of CNS opioid receptors. Trends Neurisci. 11(7) : 308-314

Martin, R., Geis, R., Holl, R., Schafer, M. & Voigt, K.H. (1983) Co-existence of unrelated peptides in oxytocin and vasopressin terminals of rat neurohypophysis : immunoreactive methionine^{5}-enkephalin-, leucine^{5}-enkephalin and cholecystokinin-like substances. Neuroscience **8**(2) : 213-227

Martin, W.R., Eades, C.G., Thompson, G.A., Huppler, R.E. & Gilbert, P.E. (1976) The effects of morphine and nalorphine like drugs in the non-dependent and morphine dependent chronic spinal dog. J.Phamacol.Exp.Ther. **197** : 517-532

Mason, W.T. (1980) Supraoptic neurones of the rat hypothalamus are osmosensitive. Nature (Lond) **287** : 154-157

Mason, W.T., Hatton, G.I., Ho, Y.W., Chapman, C. & Robinson, I.C.A.F. (1986) Central release of oxytocin, vasopressin and neurophysin by magnocellular neurone depolarization: evidence in slices of guinea-pig and rat hypothalamus. Neuroendocrinology **42** : 311-322

McNeill, T.H. & Sladek, J.R. Jr (1980) Simultaneous monoamine histofluorescence and neuropeptide immunocytochemistry 2. Correlative distribution of catecholamine varicosities and magnocellular neurosecretory neurones in the rat supraoptic and paraventricular nuclei. J.Comp.Neurol. **193** : 1023-1033

Mendelsohn, F.A.O., Quirion, R., Saavedra, J.M., Aguillera, G. & Catt, K.J. (1984) Autoradiographic localization of angiotensin II receptors in rat brain. Proc.Natl.Acad.Sci. USA **81** : 1575-1579

Mens, W.B.J., Witter, A., & Van Wimersma Greidanus, Tj.B (1983) Penetration of neurohypophyseal hormones from plasma int cerebrospinal fluid (CSF) - half times of dissappearance of these neuropeptides from CSF. Brain Res. **262** : 143-149

Mihara, S. & North, R.A. (1986) Opioids increase potassium conductance in submucous neurones of guinea pig caecum by activating delta receptors. Brit.J.Pharmacol. **88** : 315-322

Millan,M.J. (1990) κ-opioid receptors and analgesia. Trends Neurosci. **11(7)** : 70-76

Mironneau,J. (1976) Effects of oxytocin on ionic currents underlying rhythmic activity and contractions of uterine smooth muscle. Pflügers Arch. **363** : 113-118

Moos,F.,Freund-Mercier,M.J.,Guerne,J.M.-Guerne,Y.,Stoekel,M.E. & Richard Ph. (1984) Release of oxytocin and vasopressin by magnocellular nuclei in vitro : specific facilitatory effect of oxytocin on its own release. J.Endocrinol. **102** : 63-72

Morris,M.,Barnard,R.R. & Sain,L.E. (1984) Osmotic mechanisms regulating cerebrospinal fluid vasopressin and oxytocin in the conscious rat. Neuroendocrinology **39** : 377-383

Nakanishi,S.,Inoue,A.,Kuta,T.,Nakamura,M.,Chang,A.C.Y,Cohen,S.H. & Numa,S **(1979) Nucleotide sequence of cloned cDNA for bovine corticotrophin-β-lipotropin precursor.** Nature (Lond) **278** : 423-427

Neumann,I.,Schwarzberg,H. & Landgraf,R. (1988) Measurement of septal release of vasopressin and oxytocin by the push-pull technique following electric stimulation of the paraventricular nucleus of rats. Brain Res. **462** : 181-184

Neumann,I. & Landgraf,R. (1989) Septal and hippocampal release of oxytocin but not vasopressin,in the conscious lactating rat during suckling. J.Neuroendocrinol. **1** : 305-308

North,R.A. (1986) Opioid receptor types and membrane ion channels. Trends Neurosci. **9(3)** : 114-117

Oliver,G. & Schäfer,E.A. (1895) On the physiological action of pituitary and certain other glandular organs J.Physiol. **18** : 277-279

O'Byrne,K.J. & Summerlee,A.J.S. (1985) Naloxone reverses relaxin inhibition of oxytocin release in the rat. Neurosci.Letts **22** : S573

O'Byrne,K.J.,Eltringham,I.,Clarke,G. & Summerlee,A.J.S. (1986) Effects of porcine relaxin on oxytocin release from the neurohypophysis in the anaesthetized lactating rat. J.Endocrinol. **109** : 393-397

Pasternak,G.W. & Wood,P.L. (1986) Multiple mu opiate receptors. Life Sci. **38** : 1889-1898

Paterson,S.J.,Robson,L.E. & Kosterlitz,H.W. (1983) Classification of opiate receptors. Br.Med.Bull. **39** : 31-39

Paxinos,G. & Watson,C. (1982) The Rat Brain in Stereotactic Co-ordinates.Academic Press,Sydney.

Pederson,C.A. & Prange,A.J.Jr (1979)Induction of maternal behaviour in virgin rats after intracerebroventricular administration of naloxone. Proc.Natl.Acad Sci.USA **76** : 6661-6665

Pederson,C.A.,Caldwell,J.D.,Johnson,M.F.,Fort,S.A. & Prange,A.J.Jr. (1985) Oxytocin antiserum delays onset of ovarian steroid induced maternal behaviour. Neuropeptides **6** : 175-182

Perlmutter,L.S.,Tweedle,C.D. & Hatton,G.I. (1985) Neuronal/glial plasticity in the supraoptic dendritic zone in response to acute and chronic dehydration. Brain Res. **361** : 225-232

Petrie,R.H.,Wu,R.,Miller,F.C.,Sacks,D.A.,Sugarman,R,Paul,R.H. & Hon,E.H. (1976) Effects of drugs on uterine activity. Am.J.Obstet.Gynaecol. **48** :431-435

Pfaffinger,P.J.,Martin,J.M.,Hunter Dale,D.,Nathanson,N.M. & Hille,B. (1985) GTP-binding proteins couple cardiac muscarinic receptors to a K-channel. Nature (Lond) **317** : 536-538

Pittman,Q.J.,Hatton,J.D. & Bloom,F.E. (1980) Morphine and opioid peptides reduce paraventricular neuronal activity : studies on the rat hypothalamic slice preparation. Proc.Natl.Acad.Sci. USA **77** : 5527-5531

Poulain,D.A.,Wakerley,J.B. & Dyball,R.E.J. (1977) Electrophysiological differentiation of oxytocin and vasopressin-secreting neurones. Proc.R.Soc.Lond.B. **196** : 367-384

Poulain,D.A. & Wakerley,J.B. (1982) Electrophysiology of hypothalamic magnocellular neurones secreting oxytocin and vasopressin. Neuroscience **7** : 773-808

Pumford,K.M.,Hunter,M.J. & Russell,J.A. (1987) Paradoxical effects of naloxone and morphine on the electrical activity of magnocellular supraoptic neurones in rat hypothalamic slices Neurosci.Letts Supplement **29** : S44

Rayner,V.,Robinson,I.C.A.F. & Russell,J.A. (1988) Chronic intracerebroventricular morphine and lactation in rats : dependence and tolerance in relation to oxytocin neurones. J.Physiol.(Lond) **396** : 319-347

Rhodes,C.H.,Morrell,J.I. & Pfaff,D.W. (1981) Immunohistochemical analysis of magnocellular elements in rat hypothalamus : distribution and number of cells containing neurophysin,oxytocin and vasopressin. J.Comp.Neurology **198** : 45-64

Robinson,I.C.A.F. (1980) The development and evaluation of a sensitive and specific radioimmunoassay for oxytocin in unextracted plasma J.Immunoassay **1(3)** : 323-347

Robinson,I.C.A.F. & Jones,P.M. (1982) Oxytocin and neurophysin in plasma and CSF during suckling in the guinea pig. Neuroendocrinology **34** : 59-63

Robinson,I.C.A.F. (1983) "Neurohypophysial peptides in CSF" In The Neurohypophysis Structure,Function and Control. Prog.in Brain Res. **60** : 129-145 B.A.Cross,G.Leng eds. Elsevier,Amsterdam.

Russell,J.A. (1980) Changes in the nucleolar dry mass of neurones in the paraventricular and supraoptic nuclei in the rat during pregnancy and lactation. Cell & Tissue Res. **208** : 313-312

Russell,J.A. & Spears,N. (1984) Morphine inhibits suckling-induced oxytocin secretion in conscious lactating rats but also disrupts maternal behaviour. J.Physiol.(Lond) **346** : 133P

Russell,J.A.,Hatton,G.I. & Robinson,I.C.A.F. (1984) Antero-ventral peri-third ventricle (AV3V lesions in rats impairs secretion of both oxytocin (OXT) and vasopressin (AVP) during water deprivation. J.Steroid Biochem. **20** : 1503

Russell,J.A. (1984) Naloxone provokes protracted secretion of oxytocin in morphine-dependent lactating rats anaesthetized with urethane. J.Physiol.(Lond) **355** : 34P

Russell,J.A.,Antoni,F.A.,Bicknell,R.J.,Coombes,J.E.,Fisher,P.,Mansfield,S. & Robinson,I.C.A.F. (1988) Naloxone selectively stimulates oxytocin secretion from the pituitary gland in urethane-anaesthetized control or morphine treated rats.8th International Congress Endocrinology,abstract 10-22 633

Russell,J.A.,Blackburn,R.E. & Leng,G. (1989) Ablation of the region anterior and ventral to the third ventricle (AV3V region) does not impede parturition in rats. J.Endocrinol. **121** : 109-115

Russell,J.A.,Pumford,K.M. & Leng,G. (1991) J.Physiol (in press).Communication accepted at Mill Hill meeting 9-10th November 1990.

Salacinsky,P.,Hope,J.,McLean,C.,Clement-Jones,V.,Sykes,J.,Price,J. & Lowry,P.J. (1979) A new simple methodm which allows theoretical incorporation of radioiodine into proteins and peptides without damage. J.Endocrinol. **81** : 131P

Sawchenko,P.E.,Swanson,L.W. & Joseph,S.A. (1982) The distribution and cells of origin of ACTH (1-39) - stained varicosities in the paraventricular and supraoptic nuclei. Brain Res. **232** : 365-374

Schäfer,E.A. & Magnus,R. (1901) The action of pituitary extracts upon the kidney. J.Physiol. **27** : ix

Schlein,P.A.,Zarrow,M.X. & Denenberg,V.H. (1974)Role of prolactin in depressed or buffered adrenocorticosteroid response of the rat. J.Endocrinol. **62** : 93-99

Sharif,N.A. & Hughes,J. (1989) Discrete mapping of mu and delta opioid receptors using selective peptides : quantitative autoradiography,species differences and comparison with kappa receptors. Peptides **10** : 499-522

Sherlock,D.A.,Field,P.M. & Raisman,G. (1975) Retrograde transport of horseradish peroxidase in the magnocellular neurosecretory system in the rat. Brain Res. **88** : 403-414

Kramer,T.H.,Shoock,J.E.,Kazmierski,W.,Ayres,E.A.,Wire, W.S.,Hruby,V.J. & Burks,T.F. (1989) Novel peptidic mu-opioid antagonists : pharmacologic characterization in vitro and in vivo. J.Pharm.Exp.Ther. **249(2)** : 544-555

Shibuki,K.,Leng,G. & Way,S. (1988) Effects of naloxone and of intraperitoneal hypertonic saline upon oxytocin release and upon supraoptic neuronal activity. Neuroscience Letts **88** : 75-80

Sheward,W.J.,Coombes,J.E.,Bicknell,R.J.,Fink,G & Russell,J.A. (1990) Release of oxytocin but not corticotrophin-releasing factor-41 into rat hypophysial portal vessel blood can be made morphine dependent. J.Endocrinology **124** : 141-150

Sica-Blanco,Y.,Rozado,H & Remedio,M.R. (1967) Effect of meperidine on uterine contractility during

pregnancy and prelabour. Am.J.Obstet.Gynaecol. **97** : 1096-1100

Simpson,J.B. (1981) The circumventricular organs and the central actions of angiotensin. Neuroendocrinology **32** :248-256

Sivalingham,T & Pleuvry,B.J. (1985) Actions of morphine,pethidine and pentazocine on the oestrus and pregnant rat uterus in vitro. Br.J.Anaesth. **57** : 430-433

Silverman,A.J.,Hoffman,D.L. & Zimmerman,E.A. (1981) The descending afferent connections of the paraventricular nucleus of the hypothalamus (PVN). Brain Res.Bull. **6** : 47-61

Sladek,C.D. (1983) Regulation of vasopressin release by neurotransmitters,neuropeptides and osmotic stimuli. Prog.Brain.Res **60** : 71-90

Smith,C.F.C. (1987) 16-Me-cyprnorphine (RX8008M) : A potent opioid antagonist with some δ-selectivity. Life Sciences **40** : 267-274

Sokol,H.W.,Zimmerman,E.A.,Sawyer,W.H. & Robinson,A.G. (1976) The hypothalamic-neurohypophyseal system of the rat : localization and quantitation of neurophysin by light microscopic immunohistochemistry in normal rats and Brattleboro rats deficient in vasopressin and neurophysin. Endocrinology **98** : 1176-1188

Soloff,M.S. & Schwarz,T.L. (1973) Characterization of a proposed oxytocin receptor in rat mammary gland. J.Biol.Chem. **248** : 6471-6478

Soloff,M.S. & Sweet,P (1982) Oxytocin inhibition of (Ca^{2+} + Mg^{2+})-ATPase activity in rat myometrial plasma membranes. J.Biol.Chem **257** : 10687-10693

Sternweis,P.C. & Pang,I.H. (1990) The G protein-channel connection. Trends Neurosci. **13(4)** : 122-125

Summerlee,A.J.S. & Lincoln,D.W. (1981) Electrophysiological recordings from oxytocinergic neurones during suckling in the unanaesthetized lactating rat. J.Endocrinol. **90** : 225-265

Summerlee,A.J.S. (1981) Extracellular recordings from oxytocin neurones during the expulsive phase of birth in unanaesthetized rats. J.Physiol.(Lond) **321** : 1-9

Summerlee,A.J.S.,O'Byrne,K.T.,Paisley,A.C.,Breize,M.F. & Porter,D.G. (1984) Relaxin affects the central control of oxytocin release. Nature (Lond) **309** : 372-374

Summy-Long, J.Y., Denlinger, C., Palm, D., Hartman, R.D. & Rosella-Dampman, L.M. (1986) Naloxone effects on plasma vasopressin and oxytocin concentrations elevated by histamine, nicotine, isoproteronol and an acute increase in [NaCl] in cerebrospinal fluid. Neuroendocrinology **44** : 157-162

Summy-Long, J.Y., Miller, D.S., Rosella-Dampman, L.M., Hartman, R.D. & Emmert, S.E. (1985) Brain.Res. **302** : 362-366

Sumner, B.E.H., Coombes, J.E., Pumford, K.M. & Russell, J.A. (1990) Opioid receptor subtypes in the supraoptic nucleus and posterior pituitary gland of morphine-tolerant rats. Neuroscience **37** :635-645

Sumner, B.E.H., Kawata, M. & Russell, J.A. (1989) Does acute, intense stimulation of oxytocin neurones in the supraoptic nucleus increase their content of mRNA ? Brain Res. **489** : 283-290

Swanson, L.W. (1977) Immunohistochemical evidence for a neurophysin-containing autonomic pathway arising in the paraventricular nucleus of the hypothalamus. Brain.Res **128** : 356-363

Swanson, L.W. & McKeller, S. (1979) The distribution of oxytocin- and neurophysin-stained fibres in the spinal cord of the rat and monkey. J.Comp.Neurol. **188** : 87-106

Swanson, L.W. & Hartman, B.K. (1980) Biochemical specificity in central pathways related to peripheral and intracerebral homeostatic functions. Neuroscience Letts **16** : 55-60

Swanson, L.W. & Kuypers, H.G.J.M. (1980) The paraventriculr nucleus of the hypothalamus : a cytoarchitectoral subdivisions and organization of projections to the pituitary, dorsal vagal complex and spinal cord as demonstrated by retrograde fluorescence double-labelling methods. J.Comp.Neurol. **194** : 555-570

Swanson, L.W., Sawchenko, P.E., Berod, A., Hartman, B.K., Helle, K.B. & van Orden, D.E. (1981) An immunohistochemical study of the organization of catecholaminergic cells and terminals in the paraventricular and supraoptic nuclei of the hypothalamus. J.Comp.Neurol **196** : 271-285

Swanson, L.W., Sawchenko, P.E., Rivier, J. & Vale, W.W. (1983) Organization of ovine-corticotrophin-releasing factor immunoreactive cells and fibres in the rat brain : An immunohistochemical study. Neuroendocrinology **36** :165-186

Tang,A.H. & Collins,R.J. (1985) Behavioural effects of a novel kappa-opioid analgesic U-50,488H in rats and rhesus maonkeys. Psychopharmacol. **85** : 309-314

Taniguchi,Y.,Yoshida,M.,Ishikawa,K.,Suzuki,M & Kurosumi K (1988) The distribution of vasopressin-or oxytocin-neurones projecting to the posterior pituitary as revealed by a combination of retrograde transport of horseradish peroxidase and immunohistochemistry. Arch.Histol,Cytol **51(1)** 83-89

Theodosis,D.T. (1985) Oxytocin-immunoreactive terminals synapse on oxytocin neurones in the supraoptic nucleus. Nature(Lond) **313** : 682-684

Ukai,M,Nakayama,S. & Kameyama,T. (1988) The opioid antagonist,MR2266,specifically decreases saline intake in the mouse. Neuropharmacology **27** : 1027-1031

Ueda,H.,Misawa,H.,Fukushima,N. & Takagi,H. (1987) The specific opioid kappa-agonist U-50,488H inhibits low Km GTPase. Eur.J.Pharmacol. **12** : 138(i) 129-132

Ukai,M. & Kameyama,T. (1985) Naloxone specifically blocks the linear locomotion in mice. Brain Res. **328** : 378-380

Vanderhaegen,J.J.,Lotstra,F.,Liston,D.R. & Rossier,J. (1983) Proenkephalin,[Met]enkephalin and oxytocin immunoreactivities are colocalized in bovine hypothalamic magnocellular cells. Proc.Natl.Acad.Sci. USA **80** : 5139-5143

Vandesande,F. & Dierikx,K. (1975) Identification of the vasopressin-producing and of the oxytocin-producing neurones in the hypothalamic magnocellular system of the rat. Cell.Tiss.Res **164** : 153-162

Van Leengoed,E.,Kerker,E. & Swanson.H.H. (1987) Inhibition of post partum maternal behaviour in the rat by injecting an oxytocin antagonist into the cerebral ventricles. J.Endocrinol. **112** : 275-282

Van Leeuwen,F.W.,Pool,C.W. & Sluiter,A.A. (1983) Enkephalin immunoreactivity in synaptoid elements on glial cells in the rat neural lobe. Neuroscience **8(2)** : 229-241

Vigh-Teichman, I., Vigh, B. & Aros, B. (1976) Cerebrospinal fluid-contacting neurones, ciliated perikarya and 'peptidergic' synapses in the magnocellular preoptic nucleus of teleost fishes. Cell Tiss. Res. **174**: 139-160

Vigh, B. & Vigh-Teichman, I. (1973) Comparitive ultra-structure of the cerebrospinal fluid-contacting neurones. Int. Rev. Cytol. **35** : 189-251

Vonvoightlander, P.F., Lahti, R.A. & Ludens, J.H. (1983) U50,488H : a selective and structurally novel non-mu (kappa) opioid agonist. J. Pharmacol. Exp. Ther. (1983) **224(1)** : 7-12

Vorherr, H., Bradbury, M.W.B., Hoghouchi, M. & Kleeman, C.R. (1968) Antidiuretic hormone in cerebrospinal fluid during endogenous and exogenous changes in its blood level. Endocrinology **83** : 246-250

Wakerley, J.B. & Lincoln, D.W. (1971) Intermittent release of oxytocin during suckling in the rat. Nature New Biol. **233** : 180-181

Wakerley, J.B. & Lincoln, D.W. (1973) The milk-ejection reflex in the rat : a 20- to 40-fold acceleration in the firing of paraventricular neurones during oxytocin release. J. Endocrinol. **57** : 477-493

Wakerley, J.B., Noble, R. & Clarke, G. (1983) Effects of morphine and D-Ala, D-Leu enkephalin on the electrical activity of supraoptic neurosecretory cells in vitro. Neuroscience **10** : 73-81

Walker, C.D., Perrin, M., Vale, W. & Rivier, C. (1986) Ontogeny of the stress response in the rat - role of the pituitary and the hypothalamus. Endocrinology **118** : 1445-1451

Wardlaw, S.L. & Franz, A.G. (1983) Brain beta-endorphin during pregnancy, parturition and the post-partum period. Endocrinology **113** : 1664-1668

Watson, S.J., Akil, H., Fischli, W., Goldstein, A., Zimmerman, E., Nilaver, G. & van Wimersma-Greidanus, T.B. (1982) Dynorphin and vasopressin : common localization in magnocellular cells. Science **216** : 85-87

Weber, E., Roth, K.A., Evans, C.J., Chang, J-K & Barchas, J.D. (1982) Immunohistochemical localization of dynorphin (1-8) in hypothalamic magnocellular neurones-evidence for absence of proenkephalin. Life Sci. **31** : 1761-1764

Wehrenberg, W.B., Bloch, B. & Ling, N. (1985) Pituitary secretion of growth hormone in response to opioid peptides and opiates is mediated through growth

hormone-releasing factor. Neuroendocrinology **41** : 13-16

Whitnall,M.,Gainer,H.,Cox,B.M. & Molineaux,C.J. (1983) Dynorphin-A-(1-8) is contained within vasopressin neurosecretory vesicles in rat pituitary. Science **222** : 1137-1139

Whitnall,M.,Mezey,E. & Gainer,H. (1985) Co-localization of corticotrophin releasing factor and vasopressin in the median eminence neurosecretory vesicles. Nature(Lond) **317** : 248-250

Wiegand,S.J. & Prise,J.L (1980) The cells of the afferent fibres to the median eminence in the rat. J.Comp.Neurol. **192** : 1-29

Williams,J.T. & North,R.A. (1984) Opiate-receptor interactions on single locus coeruleus neurones. Mol.Pharmacol. **26** : 489-497

Wright,D.M.,Pill,C.E.J. & Clarke,G (1983) Effect of ACTH on opiate inhibition of oxytocin release. Life Sci. **33** (supplement 1) : 495-498

Xiang,J.Z.,Adamson,P.,Bremner,M.J. & Cambell,I.C. (1990) The κ-opiate agonist U50,488H decreases the entry of ^{45}Ca into rat cortical synaptosomes by inhibiting N- but not L-type calcium channels. Neuropharmacol. **29(5)** 439-444

Yaksh T.L.,Jessell,T.M.,Gamse,R.,Mudge,A.W. & Leeman,S.E. (1980) Intrathecal morphine inhibits substance P release from mammalian spinal-cord in vivo. Nature(Lond) **286** : 155-157

Yamamoto,T.,Ohno,M. & Ueki,S. (1988) A selective κ-opioid agonist U-50,488H blocks the development of tolerance to morphine analgesia in rats. Eur.J.Pharmacol **156** : 173-176

Yamashita,H.,Inenaga,K.,Kawata,M. & Sano,Y. (1985) Neurosci.Letts **37** : 87

Yamashita,H.,Okuya,H.,Inenaga,K.,Kasai,M.,Uesugi,S.,Kannan,H. & Kaneuko,T. (1987) Oxytocin predominantly excites putative oxytocin neurones in the rat supraoptic nucleus **in vitro**. Brain Res. **416** : 364-368

Zaidi,S.M.A. & Heller,H. (1974) Can neurohypophyseal hormones cross the blood cerebrospinal fluid barrier? J.Endocrinol. **60** : 195-196

Zhao,B-G.,Chapman,C. & Bicknell,R.J. (1988a) Opioid-noradrenergic in the neurohypophyisis.I. Differential opioid-receptor regulation of vasopressin and

noradrenaline release. Neuroendocrinology **48(1)** : 16-24

Zhao,B-G,Chapman,C. & Bicknell,R.J. (1988b) Opioid-noradrenergic in the neurohypophysis.II. Does noradrenaline mediate the actions of endogenous opioids on oxytocin and vasopressin release. Neuroendocrinology **48(1)** : 25-31

Zhao,B-G,Chapman,C & Bicknell,R.J. (1988c) Functional κ-opioid receptors on oxytocin and vasopressin nerve terminals isolated from the rat neurohypophysis. Brain Res. **462** : 62-66

Zukin,R.S.,Eghbali,M.,Olive,D.,Unterwald,E.M. & Tempel,A (1988) Characterization and visualization of rat and guinea pig brain kappa opioid receptors : evidence for $kappa_1$ and $kappa_2$ opioid receptors. Proc.Natl.Acad.Sci USA **85(ii)** : 4061-4065

APPENDIX

PUBLICATIONS

Sheward, W.J., Coombes, J.E., Bicknell, R.J., Fink, G & Russell, J.A. (1990). Release of oxytocin but not corticotrophin-releasing factor-41 into rat hypophysial portal vessel blood can be made opiate dependent. J.Endocrinol. **124** : 141-150

Sumner, B.E.H., Coombes, J.E., Pumford, K.M. & Russell, J.A. (1990). Opioid receptor subtypes in the supraoptic nucleus and posterior pituitary gland of morphine-tolerant rats. Neuroscience 37(3) : 634-645

ABSTRACTS

Coombes, J.E., Robinson, I.C.A.F. & Russell.J.A. (1988) Release of oxytocin (OXT) into both cerebrospinal fluid (CSF) and blood by naloxone in anaesthetized morphine dependent rats. J.Physiol. **398** : 79P

Coombes, J.E. & Russell, J.A. (1988). Inhibition by a kappa-opioid receptor agonist (U50,488H) of electrically-stimulated oxytocin secretion from the neurohypohysis in anaesthetized lactating rats. J.Physiol **401** : 34P

Russell, J.A., Bicknell, R.J. Coombes, J.E., Fink, G., Robinson, I.C.A.F. & Sheward, W.J. (1988) Morphine dependence in oxytocin neurones projecting centrally and to hypophysial portal vessels. Satellite symposium on posterior pituitary hormones. (Yoshida ed.)

Bicknell, R.J., Coombes, J.E., Fink, G., Russell, J.A. & Sheward, W.J. (1988) Effects of naloxone and long term morphine administration on oxytocin release in hypophysial blood in urethane anaesthetized rats. J.Physiol. **401** : 35P

Russell, J.A., Antoni, F.C., Coombes, J.E. & Russell, J.A. (1988). Naloxone provokes secretion of oxytocin and not other pituitary hormones altered by stress in urethane anaesthetized rats. J.Endocrinol. **117**[Suppl] : 95

Russell, J.A., Antoni, F.C., Bicknell, R.J., Coombes, J.E., Fisher, P., Mansfield, S. & Robinson, I.C.A.F. (1988) Naloxone selectively stimulates oxytocin secretion from the pituitary gland in urethane anaesthetzed control or morphine treated rats. 8th International Congress of Endocrinology, Kyoto Abstract 10-22/033

Coombes,J.E. & Russell,J.A. (1990). U50,488H,a selective κ-opioid agonist,inhibits stimulated oxytocin secretion in morphine-tolerant rats and reduces hypersecretion provoked by naloxone. 13th Annual Meeting of the European Neuroscience Association,Stockholm. Supplement 3 to Eur.J.Neuroscience : Abstract 1182

Coombes,J.E. & Russell,J.A. (1991) Lack of cross tolerance in the posterior pituitary to the inhibitory actions of U50,488H on oxytocin secretion in morphine dependent anaesthetized lactating rats.(In press-accepted for publication Mill Hill Meeting of the Physiological Society (C82) 9-10th Nov.1990.

Release of oxytocin but not corticotrophin-releasing factor-41 into rat hypophysial portal vessel blood can be made opiate dependent

W. J. Sheward, J. E. Coombes*, R. J. Bicknell†, G. Fink and **J. A. Russell***

MRC Brain Metabolism Unit, University Department of Pharmacology, 1 George Square, Edinburgh EH8 9JZ
*Department of Physiology, University Medical School, Teviot Place, Edinburgh EH8 9AG
†AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT

RECEIVED 1 June 1989

ABSTRACT

The effects of morphine dependence and abrupt opiate withdrawal on the release of oxytocin and corticotrophin-releasing factor-41 (CRF-41) into hypophysial portal vessel blood in rats anaesthetized with urethane were investigated. Adult female Sprague–Dawley rats were made dependent upon morphine by intracerebroventricular infusion of morphine for 5 days; abrupt opiate withdrawal was induced by injection of the opiate antagonist naloxone. The basal concentrations of oxytocin in portal or peripheral plasma from morphine-dependent rats did not differ significantly from those in control, vehicle-infused rats. In rats in which the pituitary gland was not removed after stalk section, the i.v. injection of naloxone hydrochloride (5 mg/kg) resulted in a large and sustained increase in the concentration of oxytocin in both portal and peripheral plasma in control and morphine-dependent rats. The i.v. injection of naloxone resulted in a threefold increase in the secretion of oxytocin into portal blood in acutely hypophysectomized rats infused with morphine, but did not alter oxytocin secretion in vehicle-infused hypophysectomized rats. The concentration of oxytocin in peripheral plasma in both vehicle- and morphine-infused hypophysectomized rats was at the limit of detection of the assay and was unchanged by the administration of naloxone. There were no significant differences in the secretion of CRF-41 into portal blood in vehicle- or morphine-infused hypophysectomized rats either before or after the administration of naloxone. These data show that, as for oxytocin release from the neurohypophysis into the systemic circulation, the mechanisms which regulate oxytocin release into the portal vessel blood can also be made morphine dependent. The lack of effect of morphine or naloxone on the release of CRF-41 or other stress neurohormones suggests that the effect of opiate dependence and withdrawal is selective for oxytocin and is not simply a non-specific response to 'stress'.
Journal of Endocrinology (1990) **124,** 141–150

INTRODUCTION

Oxytocin is secreted in high concentrations into hypophysial portal blood (Gibbs, 1984; Sarkar & Gibbs, 1984; Horn, Robinson & Fink, 1985). The role of oxytocin released into portal blood has not been established, but it has been suggested that under some conditions it may act synergistically with corticotrophin-releasing factor-41 (CRF-41) and vasopressin in the regulation of adrenocorticotrophin (ACTH) secretion (Antoni, 1986) and may be involved in prolactin secretion (Sarkar & Gibbs, 1984).

Many studies have shown that opioids inhibit the secretion of oxytocin into the systemic circulation from the neurohypophysis under several physiological and experimental situations including suckling (Clarke, Wood, Merrick & Lincoln, 1979; Russell & Spears, 1984), parturition (Leng & Russell, 1989) and electrical stimulation of the neurohypophysis (Bicknell & Leng, 1982) or neuropharmacological manipulation (Haldar, Hoffman & Zimmerman, 1982; Keil, Rosella-Dampman, Emmert *et al.* 1984). The milk ejection reflex is initially blocked by long-term continuous infusion of morphine into the cerebroventricular system, but this effect is rapidly lost as tolerance develops to morphine (Russell, 1984; Rayner, Robinson & Russell, 1988). Continuous or repeated administration of opiates results in opiate dependence, as shown by a withdrawal response to the i.v. injection of the opiate antagonist naloxone. In urethane-anaesthetized lactating rats in

  Printed in Great Britain
0022–0795/90/0124–0141 $02.00/0

which morphine had been infused into the lateral ventricles continuously for 5 days, injection of naloxone resulted in a sustained increase in intramammary pressure, an increased firing rate of continuously firing cells (presumed oxytocinergic) in the supraoptic nucleus and a greater than 25-fold increase in the concentration of oxytocin in systemic plasma (Russell, 1984; Bicknell, Leng, Lincoln & Russell, 1988; Rayner *et al.* 1988). Morphine withdrawal by naloxone administration also results in increased firing of oxytocinergic neurones and hypersection of oxytocin from the posterior pituitary gland in non-lactating rats (Bicknell, Grossmann, Leng & Russell, 1987).

The aim of the present study was to determine the effects of long-term morphine administration and of naloxone-induced withdrawal on the secretion of oxytocin from the median eminence into hypophysial portal blood. Because opiate withdrawal is considered, in conscious rats, to constitute a marked stress, the release of CRF-41 into portal blood in the same animals was also measured in order to determine whether any effect on oxytocin release could simply be due to non-specific stress. Some aspects of this study have been reported previously in abstract form (Bicknell, Coombes, Fink *et al.* 1988).

MATERIALS AND METHODS

Adult female Sprague–Dawley rats (200–300 g body weight) supplied by Bantin and Kingman, Hull, Humberside, U.K. were maintained under controlled conditions of lighting (12 h light : 12 h darkness) and temperature (22 °C) and allowed free access to food (pelleted breeder diet) and tap water. Rats were anaesthetized with ether and implanted stereotaxically with a cannula positioned with its tip in the lateral cerebral ventricle. The cannula was connected by way of a coiled length of polythene tubing to an Alzet 2001 osmotic minipump (Alza Corp., San Diego, CA, U.S.A.) implanted s.c. By this means the animal received a continuous intracerebroventricular (i.c.v.) infusion of either morphine sulphate (supplied by the Pharmacy, Royal Infirmary of Edinburgh, Edinburgh, U.K.; 10 μg/h for 40 h, 20 μg/h for the next 40 h and 50 μg/h for the remainder of the experiment, all infused at 1 μl/h; Rayner *et al.* 1988) or vehicle (distilled water). Five days after the start of infusion of morphine or vehicle the rats were anaesthetized with urethane (ethyl carbamate; Sigma, Poole, Dorset, U.K.; 1·2 g/kg body weight administered i.p. as a 20% (w/v) solution in 0·9% (w/v) NaCl) and the hypophysial portal vessels exposed by the transpharyngeal approach (Worthington, 1966; Fink & Sheward, 1989). Once the dura overlying the pituitary gland and median eminence was exposed, each rat was left undisturbed for 3–4 h. The purpose of this delay period was to enable the establishment of stable baseline concentrations of oxytocin and CRF-41 in portal blood after surgery. Throughout the experiment the body temperature was monitored continuously and maintained at 37 ± 1 °C with the aid of an electric warming blanket. At the end of the delay period, the dura mater was cut and the exposure of the hypophysial portal vessels completed. The pituitary stalk was cut as close as possible to the pituitary gland which was removed in its entirety by aspiration. After a brief delay (less than 5 min) to achieve haemostasis, heparin (Multiparin; C.P. Pharmaceuticals, Wrexham, Clwyd, U.K.; 2500 IU) was injected by way of the external jugular vein, and the hypophysial portal vessels recut for the collection of portal blood (Fink & Sheward, 1989). In some animals the pituitary stalk was cut immediately after injection of heparin, and the pituitary gland was left *in situ* throughout the period of portal blood collection. The blood which issued from the cut pituitary stalk in the first 5 min was discarded to prevent the contamination of blood with tissue fragments from the cut stalk; thereafter, hypophysial portal blood was collected into ice-cold plastic tubes for a total of six consecutive 15-min collection periods. A proteinase inhibitor (Trasylol; Bayer UK, Newbury, Berks, U.K.; 500 Kallikrein inactivator units/ml blood) was added to prevent peptide degradation (Fink, Robinson & Tannahill, 1988). At the end of the third collection period either 0·9% NaCl (0·5 ml/kg) or naloxone hydrochloride (Sigma; 5 mg/kg in 0·9% NaCl; 0·5 ml/kg) was injected i.v. by way of the external jugular vein. Samples of peripheral blood (0·3 ml) were also withdrawn periodically from the external jugular vein as follows: (*a*) after removal of the pituitary gland and immediately before the injection of heparin, (*b*) at the end of the third period of portal blood collection (50 min after the first sample), (*c*) 5 min after the i.v. injection of naloxone or saline and (*d*) at the end of the final period of portal blood collection (45 min after i.v. injection of naloxone or saline). In some animals which were infused with morphine or vehicle, the pituitary gland was left in position throughout the experiment and only 0·9% NaCl (0·5 ml/kg) was injected i.v. after 45 min of blood collection. At the end of each experiment, animals were killed by decapitation and the brain was removed rapidly, placed on an ice-cold slide and the pituitary stalk-median eminence (SME) dissected out (Sheward, Watts, Fink & Smith, 1985). For animals in which the pituitary had remained in position throughout the portal blood collection, the pituitary gland was removed from its dural capsule and the neurointermediate lobe (NIL) dissected away from the anteror pituitary gland. Samples of SME and NIL tissue were homogenized in 200 μl ice-cold 0·1 mol HCl/l. All plasma and tissue samples were stored frozen at −40 °C until radioimmunoassay.

TABLE 1. Mean ± S.E.M. concentrations of oxytocin (pmol/l) in the peripheral plasma of female rats anaesthetized and prepared for the collection of hypophysial portal blood after long-term administration of morphine sulphate or vehicle into the lateral ventricle of the brain (i.c.v.). The numbers of animals in each treatment group are shown in parentheses

	Concentration of oxytocin (pmol/l)			
	P_1	P_2	P_3	P_4
Treatment				
Pituitary intact				
Vehicle i.c.v. + naloxone i.v. ($n=13$)	258 ± 69	224 ± 113	1168 ± 582*††	896 ± 254†
Morphine i.c.v. + naloxone i.v. ($n=13$)	172 ± 39	183 ± 82	1149 ± 192*††	808 ± 299†
Vehicle i.c.v. + saline i.v. ($n=7$)	71 ± 20	28 ± 13	32 ± 13	134 ± 89
Morphine i.c.v. + saline i.v. ($n=7$)	110 ± 48	101 ± 56	108 ± 56	79 ± 21
Pituitary removed[a]				
Vehicle i.c.v. + naloxone i.v. ($n=11$)	12 ± 2	5 ± 1	5 ± 1	4 ± 1
Morphine i.c.v. + naloxone i.v. ($n=11$)	12 ± 2	6 ± 1	5 ± 1	5 ± 1

*$P<0.05$ compared with saline-injected controls (Mann-Whitney U test); †$P<0.05$, ††$P<0.01$ compared with preinjection concentration (P_2) (Wilcoxen signed matched pairs test).
[a]The assay limit for these samples was 3 pmol/l.
The sample P_1 was withdrawn from the external jugular vein at the start of the first portal blood collection period; 45 min after P_1 a further sample (P_2) was taken (immediately before the i.v. injection of naloxone hydrochloride, 5 mg/kg, or saline) and sample P_3 was taken 5 min later. The final peripheral blood sample (P_4) was taken at the end of the experiment, 45 min after i.v. injection.

Plasma concentrations of oxytocin were determined by radioimmunoassay of unextracted plasma samples as described previously (Higuchi, Honda, Fukuoda *et al.* 1985). The lower limit of detection of the assay was 3·0 pmol/l and inter- and intra-assay coefficients of variation were 7 and 11% respectively. For determination of CRF-41, plasma samples were first extracted with ice-cold methanol and subsequently assayed by double-antibody radioimmunoassay using anti-rat-CRF-41 (IgG Corporation, Nashville, TN, U.S.A.) as described previously (Fink *et al.* 1988). All plasma concentrations of CRF-41 were determined in a single assay. The lower limit of detection of the assay was 7·4 pmol/l and the intra-assay coefficient of variation was 7·2%. The significance of differences between groups was determined by the Mann–Whitney U test and Wilcoxon matched pairs signed rank test.

RESULTS

Oxytocin was present in hypophysial portal blood in higher concentrations than in peripheral blood (Table 1 and Fig. 1) and there was no significant difference between the concentration or the content (concentration × plasma volume collected/15 min) of oxytocin in portal blood collected from intact rats or rats in which the pituitary gland was removed at the time of blood collection (Fig. 1). In contrast, the concentration of oxytocin in peripheral plasma was reduced by hypophysectomy to concentrations close to the detection limit of the radioimmunoassay (Table 1).

In animals in which the pituitary gland was not removed before portal blood collection, there was no significant difference in the baseline secretion of oxytocin (i.e. during collection periods 1–3) in portal

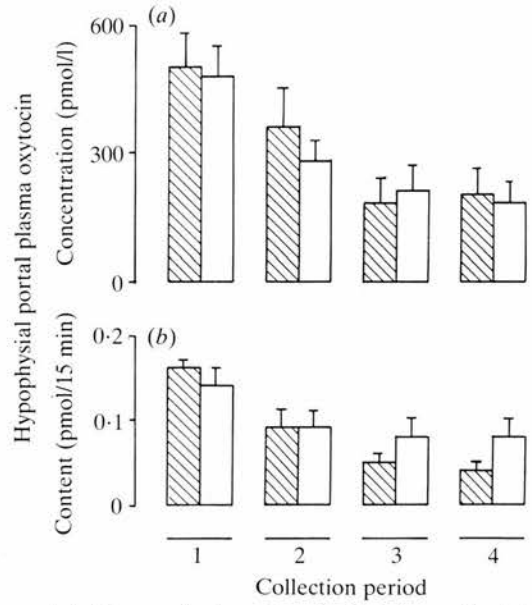


FIGURE 1. (*a*) Concentration of oxytocin in hypophysial portal vessel plasma and (*b*) the total amount released from intact female rats (hatched bars) or rats in which the pituitary gland had been removed immediately before the collection of portal blood (open bars). Hypophysial portal blood was collected under urethane anaesthesia for four consecutive 15-min periods. Values are means ± S.E.M. for six animals in each treatment group.

plasma from rats infused with vehicle compared with rats infused with morphine (Fig. 2). In both morphine- and vehicle-infused rats the injection of naloxone resulted in a prompt and sustained increase in the portal plasma concentration of oxytocin and in the total amount of oxytocin secreted during each 15-min

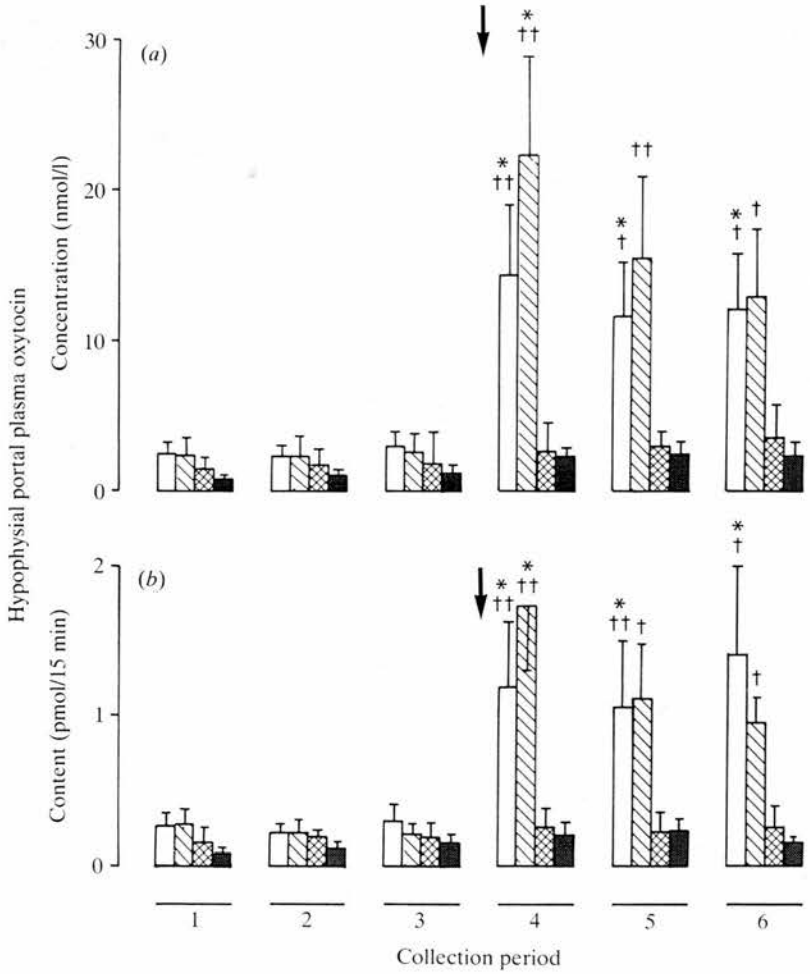


FIGURE 2. (*a*) Mean ± S.E.M. concentration of oxytocin in hypophysial portal blood and (*b*) the amount of oxytocin secreted during each of six consecutive 15-min periods of portal blood collection under urethane anaesthesia from female rats after 5 days of continuous intracerebroventricular infusion with either morphine sulphate or distilled water (vehicle). The pituitary gland was not removed during blood collection. At the end of the third collection period (arrows) animals were injected i.v. with 5 mg naloxone hydrochloride/kg or with 0·9% (v/v) NaCl (control). Rats were infused with vehicle and injected with naloxone (open bars; $n = 13$), infused with morphine and injected with naloxone (hatched bars; $n = 13$), infused with vehicle and injected with saline (cross-hatched bars; $n = 7$) or infused with morphine and injected with saline (shaded bars; $n = 7$). $^*P < 0{\cdot}05$ compared with control rats (Mann–Whitney U test); $\dagger P < 0{\cdot}01$, $\dagger\dagger P < 0{\cdot}005$ compared with preinjection values (Wilcoxon matched pair signed rank test).

collection period, with no significant differences between the i.c.v. treatment groups (Fig. 2). Similarly, there was a significant increase in the concentration of oxytocin in peripheral plasma withdrawn from the external jugular vein 5 and 45 min after the injection of naloxone in both vehicle- and morphine-infused rats, with no significant difference between the two i.c.v. treatment groups (Table 1). Injection of saline, instead of naloxone, did not result in any significant alteration in the output of oxytocin into portal blood or the concentration of oxytocin in peripheral blood in vehicle- or morphine-infused rats (Fig. 2 and Table 1).

In vehicle- and morphine-infused rats in which the pituitary gland was removed immediately before the

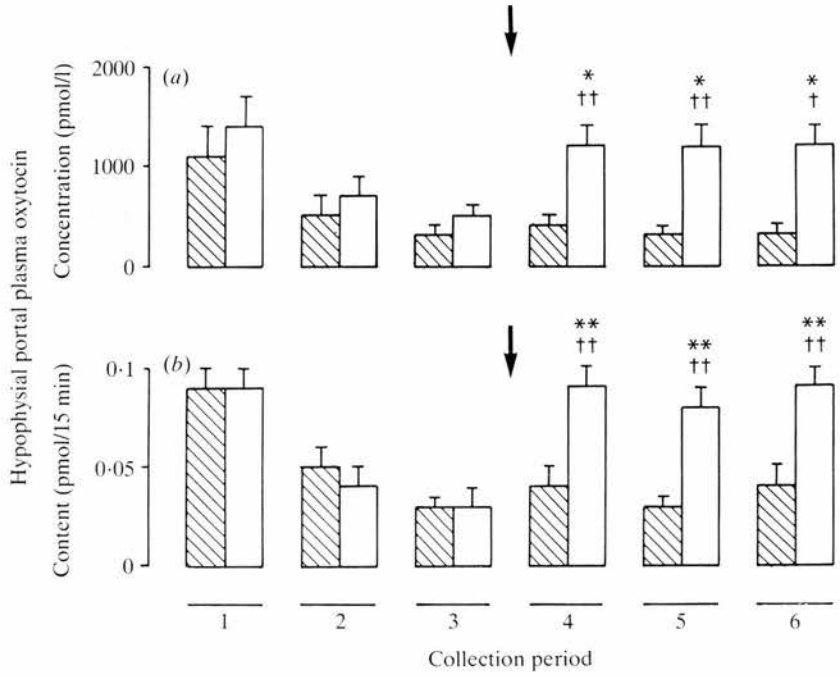


FIGURE 3. (*a*) Mean ± S.E.M. concentration of oxytocin and (*b*) oxytocin content in hypophysial portal vessel blood during each of six consecutive 15-min periods of blood collection from urethane-anaesthetized female rats after 5 days of continuous intracerebroventricular infusion of morphine sulphate (open bars) or vehicle (hatched bars). The entire pituitary gland was removed by aspiration immediately before the start of the first period of blood collection. Naloxone hydrochloride (5 mg/kg) was injected i.v. at the end of the third period of collection (arrows). There were 13 animals in each treatment group. $*P<0{\cdot}005$, $**P<0{\cdot}001$ compared with vehicle-infused rats (Mann–Whitney U test); $\dagger P<0{\cdot}01$, $\dagger\dagger P<0{\cdot}005$ compared with preinjection value (Wilcoxon matched pairs signed rank test).

start of hypophysial blood collection, there was no significant difference in the secretion of oxytocin into either portal or peripheral blood before the injection of naloxone (Table 1). However, in morphine- but not vehicle-treated rats, the injection of naloxone resulted in a significant increase in both the concentration of oxytocin and the total amount of oxytocin in portal plasma during each successive 15-min collection period (Fig. 3). In both vehicle- and morphine-infused rats, the concentration of oxytocin in peripheral plasma after hypophysectomy was close to the lower limit of detection of the assay (3·0 pmol/l) and was not affected by the i.v. injection of naloxone (Table 1).

Table 2 shows the oxytocin content of the median eminence and neurointermediate lobe of morphine- and vehicle-infused rats. Although in morphine-dependent rats in which the pituitary gland remained *in situ* throughout the blood collection period the content of oxytocin in the median eminence was apparently depleted by administration of naloxone; this finding was not confirmed in hypophysectomized rats. The content of oxytocin in the neurointermediate lobe was not altered significantly by administration of morphine or naloxone.

Figure 4 shows that CRF-41 was present in hypophysial portal plasma collected from acutely hypophysectomized rats; concentrations were high relative to those reported in peripheral plasma, and with the assay system used the concentrations of CRF-41 in peripheral plasma from the rats anaesthetized with urethane were below the limit of detection (< 7·4 pmol/l). The secretion of CRF-41 into hypophysial portal plasma did not differ significantly between morphine- and vehicle-infused rats and was not affected by administration of naloxone.

There were no significant differences in the volumes of portal blood collected from rats with intact pituitaries or hypophysectomized rats infused with either vehicle or morphine and the portal blood flow was not appreciably altered by the i.v. injection of naloxone or

TABLE 2. Mean ± S.E.M. content of oxytocin in the median eminence (pmol/median eminence) or neurointermediate lobe (nmol/neurointermediate lobe) of female rats after long-term infusion of morphine sulphate or vehicle into the lateral cerebral ventricle (i.c.v.) and i.v. injection of naloxone hydrochloride or saline. The numbers of animals in each treatment group are shown in parentheses

	Content of oxytocin	
	Median eminence (pmol)	Neurointermediate lobe (nmol)
Treatment		
Pituitary intact		
Vehicle i.c.v. + naloxone i.v.	12·0 ± 1·8*‡ (*n* = 11)	1·8 ± 0·2† (*n* = 11)
Morphine i.c.v. + naloxone i.v.	5·9 ± 0·4* (*n* = 11)	1·6 ± 0·3 (*n* = 11)
Vehicle i.c.v. + saline i.v.	7·0 ± 1·0 (*n* = 6)	0·9 ± 0·1 (*n* = 7)
Morphine i.c.v. + saline i.v.	8·5 ± 0·9 (*n* = 7)	1·1 ± 0·1 (*n* = 7)
Pituitary removed		
Vehicle i.c.v. + naloxone i.v.	9·7 ± 0·8 (*n* = 13)	—
Morphine i.c.v. + naloxone i.v.	9·9 ± 0·9 (*n* = 13)	—

*$P < 0.05$ compared with vehicle-saline group; †$P < 0.01$ compared with morphine-saline group; ‡$P < 0.001$ compared with morphine-naloxone group (Mann–Whitney U test).

saline. The mean (± S.E.M.) volume of portal blood collected from the various treatment groups ranged between 123 ± 14 and 267 ± 64 μl/15 min.

DISCUSSION

Our major finding was that the release of oxytocin into hypophysial portal blood, like the release of oxytocin into the systemic circulation, can be made morphine dependent. We have also confirmed that hypophysial portal plasma contains high concentrations of oxytocin (Gibbs, 1984; Sarkar & Gibbs, 1984; Horn *et al.* 1985) and CRF-41 (Plotsky, Otto & Sapolsky, 1986; Fink *et al.* 1988) relative to peripheral plasma, and that the output of oxytocin is unaffected by the removal of the pituitary gland immediately before portal blood collection (Sarkar & Gibbs, 1984; Horn *et al.* 1985). The source of the oxytocin in portal blood is most likely to be a projection from the paraventricular nuclei of the hypothalamus to the portal vessels, but the possibility cannot be excluded that, as suggested for vasopressin (Holmes, Antoni, Aguilera & Catt, 1986), oxytocin may be released from oxytocinergic fibres passing through the internal layer of the median eminence. Ultrastructural studies suggest that oxytocin may be released collaterally from axons or axon swellings of magnocellular neurones (Buma & Nieuwenhuys, 1988; Morris & Pow, 1988).

The present results show that there was no significant difference between the secretion of oxytocin into portal blood of morphine- and vehicle-treated rats, suggesting the rapid development of tolerance to the effects of morphine on oxytocin release. Such tolerance to the effects of continuous administration of morphine develops rapidly (within the first 2–3 days) in the neurohypophysial oxytocin system (Russell, 1984; Bicknell *et al.* 1987). The immediate increased release of oxytocin into portal blood following administration of the opiate antagonist, naloxone, shows that the oxytocin neurones which project to the median eminence have been made morphine-dependent by long-term administration of opiates. In morphine-infused rats in which the pituitary gland was left *in situ* during the portal blood collection, concentrations of oxytocin in peripheral plasma were increased markedly following administration of naloxone. Antagonism of endogenous opioids released within the neurohypophysis is probably responsible for this effect, since neurohypophysial opioid receptors are of the κ-type and insensitive to μ-opioid agonists such as morphine (Coombes & Russell, 1988; Zhao, Chapman & Bicknell, 1988). This interpretation is supported by the finding of a similar rise in peripheral oxytocin seen following administration of naloxone in the pituitary-intact rats infused with vehicle. Previous in-vitro studies have also demonstrated a lack of opiate dependence at the level of the neurohypophysis in morphine-treated animals (Bicknell, Chapman, Leng & Russell, 1985). The increase in oxytocin concentration in portal blood, in response to naloxone in vehicle-treated animals from which the pituitary gland was not removed, may be

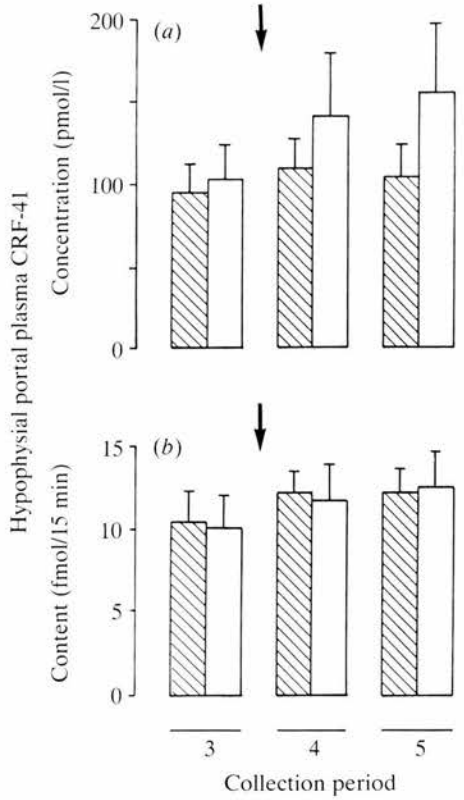


FIGURE 4. (*a*) Mean ± S.E.M. concentration and (*b*) content of corticotrophin-releasing factor-41 (CRF-41) in hypophysial portal blood from female rats under urethane anaesthesia after intracerebroventricular infusion of either vehicle (hatched bars) or morphine sulphate (open bars) for 5 days. Data shown are concentration and content of CRF-41 for the third, fourth and fifth of six consecutive 15-min periods of blood collection; after the third collection period (arrows) animals were injected i.v. with 5 mg naloxone hydrochloride/kg. The entire pituitary gland was removed immediately before starting the blood collection. There were 13 animals in each treatment group.

due to oxytocin in backflow blood from the pituitary. Alternatively, endogenous opioids from the pituitary gland could conceivably inhibit oxytocin release, an effect blocked by naloxone. In contrast, in hypophysectomized rats infused with vehicle, administration of naloxone did not stimulate any increase in oxytocin output into portal blood, suggesting that whilst the oxytocinergic neurones which project to the neurohypophysis and those which project to the median eminence are sensitive to the actions of morphine these neurones differ from one another with respect to their sensitivity to endogenous opioids. In the case of the oxytocinergic neurones which project to the neurohypophysis, it is evident that, under urethane anaesthesia, it is their terminals in the posterior pituitary that are under endogenous opioid tone and not their cell bodies (Bicknell *et al.* 1988). Clearly, with respect to the neurones which project to the median eminence, neither their cell bodies nor their terminals in the median eminence are under endogenous opioid inhibition, at least during urethane anaesthesia.

Although the present results demonstrate that the secretion of oxytocin into portal blood can be modified by naloxone after pretreatment with morphine, the site of action and nature of the opioid receptor involved has yet to be elucidated. Naloxone may act directly on receptors on the cell bodies of oxytocinergic neurones in the paraventricular (or supraoptic) nucleus, on the afferent input to these neurones or, alternatively, on the oxytocinergic terminals in the median eminence. In lactating rats, opiate withdrawal following long-term administration of morphine resulted in a 25-fold increase in plasma oxytocin concentration together with a three- to fourfold increase in the firing rate of oxytocin neurones in the supraoptic nucleus, whereas in vehicle-infused rats injection with naloxone also resulted in increased secretion of oxytocin, but no change in the firing rate. Such an effect on the electrical activity of the oxytocinergic neurones which project to the median eminence could explain the increased secretion of oxytocin into portal blood after naloxone-induced withdrawal in morphine-dependent rats. It is the effect on electrical activity, together with the reversal of endogenous opioid inhibition in the posterior pituitary, which accounts for the increased secretion of oxytocin from the posterior pituitary into peripheral blood in morphine-dependent rats. Although the lack of effect of naloxone on oxytocin secretion into portal blood in i.c.v. vehicle-treated rats indicates lack of endogenous opioid influence on these neurones under urethane anaesthesia, the enhanced secretion of oxytocin induced by naloxone in morphine-dependent rats indicates nonetheless that opioid receptors are available to influence these neurones, and that endogenous opioids could inhibit their activity. In particular, these receptors are likely to be μ-type opioid receptors (Leng, Russell & Grossman, 1989). As discussed above, there may be actions of opioids on the inputs to these neurones, or on their terminals in the median eminence. β-Endorphin, dynorphin(1–17) and the enkephalins are all present in portal blood (Wardlaw, Wehrenberg, Ferin *et al.* 1980; Castanas, Giraud, Drissi *et al.* 1984; Sarkar & Yen, 1985). Dynorphin(1–17) could be co-released from vasopressinergic neurones which synthesize this opioid (Anhut & Knepel, 1982; Watson, Akil, Fischli *et al.* 1982; Liston & Rossier, 1984); there is some evidence that oxytocinergic neurones themselves produce [Met]-enkephalin (Martin, Geis, Holl *et al.* 1983; Vanderhaeghen, Lotstra, Liston & Rossier, 1983); β-endorphin in portal blood presumably is secreted by arcuate nucleus neurones. Similarly, this range of

opioids could act after central release at the level of the cell bodies. Further studies are necessary to identify the endogenous opioids which regulate the secretion of oxytocin into portal blood.

Despite the greater release of oxytocin from the median eminence after injection with naloxone in morphine-dependent rats in comparison with vehicle-infused rats, there was no consistent evidence of greater depletion of median eminence oxytocin in the former (Table 2). In morphine-dependent hypophysectomized rats the injection of naloxone resulted in an increase of 150 fmol oxytocin released in 45 min, which represents approximately 1·5% of the total median eminence content of oxytocin. Similarly, naloxone did not significantly deplete the neurohypophysis of oxytocin (Table 2), despite the large and sustained increase in peripheral blood concentration of oxytocin (Table 1). It can be concluded that the stores of oxytocin in both the neurohypophysis and median eminence are adequate to meet major acute demands for increased secretion. Long-term i.c.v. infusion of morphine had no detectable effect on oxytocin content in the neurohypophysis or median eminence (Table 2); similarly, the oxytocin mRNA content of neurones in the supraoptic nuclei is not affected (Sumner, Kawata & Russell, 1989).

In the present experiments, insufficient plasma remained after assay of oxytocin and CRF-41 to enable the assay of arginine vasopressin (AVP). However, Bicknell *et al.* (1985) showed, that in contrast to oxytocin, there were only small and transient increases in the peripheral plasma concentration of AVP in morphine-treated rats after i.v. injection of naloxone.

One possible interpretation of the present and previous findings is that the oxytocin reponse to naloxone in morphine-dependent rats is a non-specific stress response. However, several measures of so-called stress hormones argue against this. First, naloxone did not stimulate ACTH release in morphine-dependent rats anaesthetized with urethane (Russell, Antoni, Coombes & Robinson, 1988*b*). Secondly, we found no significant difference between the baseline secretion of CRF-41 into portal blood of morphine- and vehicle-treated rats, and CRF-41 output was unaffected by the injection of naloxone. Thirdly, although administration of opiates increases the secretion of prolactin and growth hormone (Deyo, Swift, Miller & Fang, 1980; Wehrenberg, Bloch & Ling, 1985), and stressful stimuli increase prolactin secretion (Samson, McDonald & Lumpkin, 1985) and decrease growth hormone secretion (Terry, Willoughby, Brazeau *et al.* 1976), the plasma concentrations of these hormones are not altered by naloxone-induced opiate withdrawal in the model used in the present experiments (Russell, Antoni, Bicknell *et al.* 1988*a*; Russell *et al.* 1988*b*). Thus it appears that the hypersecretion of oxytocin after administration of naloxone is a specific effect on this system and not a general 'stress response' caused by abrupt withdrawal of opiates.

The present experiments and those of Russell *et al.* (1988*b*) show that the in-vivo secretion of CRF-41 and ACTH in morphine-dependent rats anaesthetized with urethane was not affected by administration of naloxone. This would appear to conflict with the finding of other groups, who reported that ACTH release was increased by naloxone treatment (Buckingham & Cooper, 1987; Donnerer & Lembeck, 1988). However, it must be stressed that changes in ACTH release may be mediated by factors other than CRF-41, such as AVP and/or adrenaline (Antoni, 1986). Plotsky (1986) reported that naloxone increased CRF-41 release into portal blood, but in contrast to our i.v. administration, Plotsky infused naloxone by the i.c.v. route. Administration of naloxone to morphine-dependent rats also increased the content of CRF-41 mRNA in the parvocellular division of hypothalamic paraventricular nuclei (Lightman & Young, 1988); however, there is not necessarily a close relationship between neuropeptide mRNA concentration and neuropeptide release.

It is possible that the secretion of CRF-41 in animals prepared for portal blood collection might be maximal after surgery in both morphine- and vehicle-infused rats, and this could explain the lack of a response by CRF-41 to naloxone. However, this is an unlikely explanation since it is possible to use the portal blood collection technique to demonstrate increases in the secretion of CRF-41 in response to long-term adrenalectomy or hypophysectomy (Fink *et al.* 1988; W. J. Sheward & G. Fink, unpublished data). Fink *et al.* (1988) have shown that rats anaesthetized with urethane have increased plasma concentrations of ACTH and corticosterone in comparison with animals anaesthetized with pentobarbitone. However, in the present experiments, in rats anaesthetized with urethane, and with a 3–4 h delay period before collecting portal blood, the portal plasma concentration and content of CRF-41 did not differ from the values reported in animals anaesthetized with sodium pentobarbitone (Fink *et al.* 1988). Our findings on CRF-41 secretion into hypophysial portal blood, therefore, provide an important positive control which shows that the effect of naloxone on oxytocin release is specific and not simply a general stress effect on neuroendocrine systems.

ACKNOWLEDGEMENTS

We wish to thank C. Leitch for preparing the manuscript and A. Wright for the illustrations.

REFERENCES

Anhut, H. & Knepel, W. (1982). Release of dynorphin-like immunoreactivity of rat neurohypophysis in comparison to vasopressin after various stimuli *in vitro* and *in vivo*. *Neuroscience Letters* **31,** 159–164.

Antoni, F. A. (1986). Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor. *Endocrine Reviews* **7,** 351–378.

Bicknell, R. J., Chapman, C., Leng, G. & Russell, J. A. (1985). Chronic morphine exposure and oxytocin neurones in rats: lack of both morphine dependence and cross-tolerance to endogenous opioids in the neurohypophysis. *Journal of Physiology* **361,** 32P.

Bicknell, R. J., Coombes, J. E., Fink, G., Russell, J. A. & Sheward, W. J. (1988). Effects of naloxone and long-term morphine administration on oxytocin release into hypophysial portal blood in urethane-anaesthetized rats. *Journal of Physiology* **401,** 35P.

Bicknell, R. J., Grossmann, R., Leng, G. & Russell, J. A. (1987). Hypersensitivity to naloxone in magnocellular neurones of virgin female rats treated chronically with intracerebroventricular (i.c.v.) morphine. *Journal of Physiology* **382,** 151P.

Bicknell, R. J. & Leng, G. (1982). Endogenous opiates regulate oxytocin but not vasopressin secretion from the neurohypophysis. *Nature* **298,** 161–162.

Bicknell, R. J., Leng, G., Lincoln, D. W. & Russell, J. A. (1988). Naloxone excites oxytocin neurones in the supraoptic nucleus of lactating rats after chronic morphine treatment. *Journal of Physiology* **396,** 297–317.

Buckingham, J. C. & Cooper, T. A. (1987). Interrelationships of opioidergic and adrenergic mechanisms controlling the secretion of corticotrophin releasing factor in the rat. *Neuroendocrinology* **46,** 199–206.

Buma, P. & Nieuwenhuys, R. (1988). Ultrastructural characterization of exocytotic release sites in different layers of the median eminence of the rat. *Cell and Tissue Research* **252,** 107–114.

Castanas, E., Giraud, P., Drissi, R., Chabrier, P. E., Conte-Devolx, B., Boudouresque, F., Cantau, P., Cesselin, F., Cupo, A., Eiden, L. E. & Oliver, C. (1984). Characterization of enkephalins and related peptides in rat hypophysial portal blood. *Brain Research* **310,** 1–6.

Clarke, G., Wood, P., Merrick, L. & Lincoln, D. W. (1979). Opiate inhibition of peptide release from the neurohumoral terminals of hypothalamic neurones. *Nature* **282,** 746–748.

Coombes, J. E. & Russell, J. A. (1988). Inhibition by a kappa opioid receptor agonist (U50, 488H) of electrically-stimulated oxytocin secretion from the neurohypophysis in anaesthetized lactating rats. *Journal of Physiology* **401,** 34P.

Deyo, S. N., Swift, R. M., Miller, R. J. & Fang, V. S. (1980). Development of tolerance to the prolactin-releasing action of morphine and its modulation by hypothalamic dopamine. *Endocrinology* **106,** 1469–1474.

Donnerer, J. & Lembeck, F. (1988). Neonatal capsaicin treatment of rats reduces ACTH secretion in response to peripheral neuronal stimuli but not to centrally acting stressors. *British Journal of Pharmacology* **94,** 647–652.

Fink, G., Robinson, I. C. A. F. & Tannahill, L. A. (1988). Effects of adrenalectomy and glucocorticoids on the peptides CRF-41, AVP and oxytocin in rat hypophysial portal blood. *Journal of Physiology* **401,** 329–345.

Fink, G. & Sheward, W. J. (1989). Neuropeptide release *in vivo*: measurement in hypophysial portal blood. In *Neuropeptides: A Methodology* pp. 157–188. Eds A. J. Harmar & G. Fink. New York: John Wiley and Sons.

Gibbs, D. M. (1984). High concentrations of oxytocin in hypophysial portal plasma. *Endocrinology* **114,** 1216–1218.

Haldar, J., Hoffman, D. L. & Zimmerman, E. A. (1982). Morphine, beta-endorphin and [D-Ala^2]met-enkephalin inhibit oxytocin release by acetylcholine and suckling. *Peptides* **3,** 663–668.

Higuchi, T., Honda, K., Fukuoda, T., Negoro, H. & Wakabayashi, K. (1985). Release of oxytocin during suckling and parturition in the rat. *Journal of Endocrinology* **105,** 339–346.

Holmes, M. C., Antoni, F. A., Aguilera, G. & Catt, K. J. (1986). Magnocellular axons in passage through the median eminence release vasopressin. *Nature* **319,** 326–329.

Horn, A. M., Robinson, I. C. A. F. & Fink, G. (1985). Oxytocin and vasopressin in rat hypophysial portal blood: experimental studies in normal and Brattleboro rats. *Journal of Endocrinology* **104,** 211–224.

Keil, L. C., Rosella-Dampman, L. M., Emmert, S., Chee, O. & Summy-Long, J. Y. (1984). Enkephalin inhibition of angiotensin-stimulated release of oxytocin and vasopressin. *Brain Research* **297,** 329–336.

Leng, G. & Russell, J. A. (1989). Opioids, oxytocin and parturition. In *Brain Opioid Systems in Reproduction* pp. 231–256. Eds R. G. Dyer & R. J. Bicknell. Oxford University Press.

Leng, G., Russell, J. A. & Grossman, R. (1989). Sensitivity of magnocellular oxytocin neurones to opioid antagonists in rats treated chronically with intracerebroventricular (i.c.v.) morphine. *Brain Research* **484,** 290–296.

Lightman, S. L. & Young III, W. S. (1988). Corticotrophin-releasing factor, vasopressin and pro-opiomelanocortin mRNA responses to stress and opiates in the rat. *Journal of Physiology* **403,** 511–523.

Liston, D. & Rossier, J. (1984). Synenkephelin is coreleased with met-enkephalin from neuronal terminals *in vitro*. *Neuroscience Letters* **48,** 211–216.

Martin, R., Geis, R., Holl, R., Schafer, M. & Voigt, K. H. (1983). Co-existence of unrelated peptides in oxytocin and vasopressin terminals of rat neurohypophyses: Immunoreactive methionine^5-enkephalin-, leucine^5-enkephalin-, and cholycystokinin-like substances. *Neuroscience* **8,** 213–227.

Morris, J. F. & Pow, D. V. (1988). Capturing and quantifying the exocytotic event. *Journal of Experimental Biology* **139,** 81–103.

Plotsky, P. M. (1986). Opioid inhibition of immunoreactive corticotropin-releasing factor secretion into the hypophysial-portal circulation of rats. *Regulatory Peptides* **16,** 235–242.

Plotsky, P. M., Otto, S. & Sapolsky, R. M. (1986). Inhibition of immunoreactive corticotropin-releasing factor secretion into the hypophysial-portal circulation by delayed glucocorticoid feedback. *Endocrinology* **119,** 1126–1130.

Rayner, V. C., Robinson, I. C. A. F. & Russell, J. A. (1988). Chronic intracerebroventricular morphine and lactation in rats: dependence and tolerance in relation to oxytocin neurones. *Journal of Physiology* **396,** 319–347.

Russell, J. A. (1984). Naloxone provokes protracted secretion of oxytocin in morphine-dependent lactating rats anaesthetized with urethane. *Journal of Physiology* **355,** 34P.

Russell, J. A., Antoni, F. A., Bicknell, R. J., Coombes, J. E., Fisher, P., Mansfield, S. & Robinson, I. C. A. F. (1988*a*). Naloxone selectively stimulates oxytocin secretion from the pituitary gland in urethane-anaesthetized control or morphine treated rats. *8th International Congress of Endocrinology*, Abstract No. 10-12-033.

Russell, J. A., Antoni, F. A., Coombes, J. E. & Robinson, I. C. A. F. (1988*b*). Naloxone provokes secretion of oxytocin and not other pituitary hormones altered by stress in urethane-anaesthetized morphine-treated rats. *Journal of Endocrinology* **117** (Suppl.), Abstract No. 95.

Russell, J. A. & Spears, N. (1984). Morphine inhibits suckling-induced oxytocin secretion in conscious lactating rats but also disrupts maternal behaviour. *Journal of Physiology* **346,** 133P.

Samson, W. K., McDonald, J. K. & Lumpkin, M. D. (1985). Naloxone-induced dissociation of oxytocin and prolactin releases. *Neuroendocrinology* **40,** 68–71.

Sarkar, D. K. & Gibbs, D. M. (1984). Cyclic variation of oxytocin in the blood of pituitary portal vessels of rats. *Neuroendocrinology* **39,** 481–483.

Sarkar, D. K. & Yen, S. S. C. (1985). Changes in β-endorphin-like immunoreactivity in pituitary portal blood during the estrous cycle and after ovariectomy in rats. *Endocrinology* **116,** 2075–2079.

Sheward, W. J., Watts, A. G., Fink, G. & Smith, G. C. (1985). Effects of intravenously administered 6-hydroxydopamine on the content of monoamines in the median eminence and neurointermediate lobe of the rat. *Neuroscience Letters* **55,** 141–144.

Sumner, B. E. H., Kawata, M. & Russell, J. A. (1989). Does acute, intense stimulation of oxytocin neurones in the supraoptic nucleus increase their content of oxytocin mRNA? *Brain Research* **489,** 283–290.

Terry, L. C., Willoughby, J. O., Brazeau, P., Martin, J. B. & Patel, Y. (1976). Antiserum to somatostatin prevents stress-induced inhibition of growth hormone secretion in the rat. *Science* **192,** 565–567.

Vanderhaeghen, J. J., Lotstra, F., Liston, D. R. & Rossier, J. (1983). Proenkephalin, [Met]enkephalin, and oxytocin immunoreactivities are colocalized in bovine hypothalamic magnocellular neurons. *Proceedings of the National Academy of Sciences of the U.S.A.* **80,** 5139–5143.

Wardlaw, S. L., Wehrenberg, W. B., Ferin, M., Carmel, P. W & Frantz, A. G. (1980). High levels of β-endorphin in hypophyseal portal blood. *Endocrinology* **106,** 1323–1326.

Watson, S. J., Akil, H., Fischli, W., Goldstein, A., Zimmerman, E., Nilaver, G. & van Wimersma Greidanus, T. B. (1982). Dynorphin and vasopressin: common localization in magnocellular neurons. *Science* **216,** 85–87.

Wehrenberg, W. B., Bloch, B. & Ling, N. (1985). Pituitary secretion of growth hormone in response to opioid peptides and opiates is mediated through growth hormone-releasing factor. *Neuroendocrinology* **41,** 13–16.

Worthington, W. C., Jr. (1966). Blood samples from the pituitary stalk of the rat: method of collection and factors determining volume. *Nature* **210,** 710–712.

Zhao, B.-G., Chapman, C. & Bicknell, R. J. (1988). Opioid-noradrenergic interactions in the neurohypophysis. I. Differential opioid receptor regulation of oxytocin, vasopressin and noradrenaline release. *Neuroendocrinology* **48,** 16–24.

Neuroscience Vol. 37, No. 3, pp. 635–645, 1990
Printed in Great Britain

0306-4522/90 $3.00 + 0.00
Pergamon Press plc

OPIOID RECEPTOR SUBTYPES IN THE SUPRAOPTIC NUCLEUS AND POSTERIOR PITUITARY GLAND OF MORPHINE-TOLERANT RATS

B. E. H. SUMNER,* J. E. COOMBES, K. M. PUMFORD and J. A. RUSSELL
Department of Physiology, University Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

Abstract—Morphine, given acutely, inhibits oxytocin secretion in adult female rats, but chronic intracerebroventricular infusion for five to six days induces tolerance and dependence in the mechanisms regulating oxytocin secretion. One explanation for tolerance could be that there is a loss of opioid receptors.

To test this hypothesis cryostat sections of selected brain regions and the pituitary, from six control and six intracerebroventricular morphine-infused rats, were processed for quantitative *in vitro* receptor autoradiography. [^{3}H]Etorphine or [^{3}H](−)-bremazocine were used as ligands, and DAGO, DPDPE and U50,488H as selective displacers from μ-, δ-, and κ-receptors, respectively. Control incubations had naloxone determined specificity. The supraoptic nucleus (site of oxytocin-secreting magnocellular perikarya) contained both μ- and κ-receptors in control rats (mean ± S.E.M. binding of μ-selective [^{3}H]etorphine was 91.8 ± 25.4 fmol/mg of tissue, and of κ-selective [^{3}H](−)-bremazocine was 130.4 ± 25.6 fmol/mg). Chronic morphine treatment caused a 83.9% decrease in binding in μ-selective conditions ($P < 0.05$), but no significant change in κ-selective binding. In the median preoptic nucleus (which projects to the supraoptic nucleus) mean ± S.E.M. binding of [^{3}H]etorphine decreased by 77.0% ($P < 0.01$) in chronic morphine-treated rats, from the control value of 76.2 ± 9.8 fmol/mg of tissue. In the posterior pituitary gland (site of the terminals of the oxytocin-secreting magnocellular perikarya) binding with [^{3}H](−)-bremazocine in controls was over 90% lower than in the supraoptic nucleus. No changes followed chronic morphine treatment.

Thus chronic morphine exposure reduces the numbers of available μ-receptors in the supraoptic nucleus, and of opioid receptors in the median preoptic nucleus, perhaps accounting for morphine-tolerance in relation to oxytocin secretion.

The inhibitory effects of opiates on oxytocin secretion were first demonstrated only a decade ago.[18] Since then inhibitory actions of both exogenous opiates and endogenous opioids have been shown in a range of studies on the oxytocin system, mainly in the rat (e.g. see Refs 20, 48, 50). Morphine acts centrally by inhibiting the firing rate of continuously firing (putatively oxytocin-secreting) neurons in the supraoptic nucleus (SON).[6,58] Endogenous opioids act also on oxytocin-releasing nerve terminals in the posterior pituitary (PP) gland.[3,5,6] Opioids can also inhibit vasopressin secretion *in vivo*,[2] but their effects on perikaryal electrical activity are inconsistent,[58] and their action on vasopressin release from the PP is less striking,[3–5,17,58] except for a dose-dependent inhibition by ethylketocyclazocine,[55] and U50,488H.[60,61]

The actions of opioids in brain are mediated by three principal opioid receptor subtypes, classified by agonist selectivity.[1,22,63] Mansour *et al.*[34–36] and Tempel and Zukin[57] have surveyed the distribution of μ-, δ- and κ-type opioid receptors in the rat brain, using *in vitro* receptor autoradiography. In the SON, κ-type receptors but not μ- or δ-receptors were found, yet both μ- and κ-opioid receptor agonists inhibit the electrical activity of SON neurons.[49] In the PP, κ-type receptors predominate, with few μ-receptors and no δ-receptors;[12,25] κ-selective, but not μ- or δ-selective, agonists inhibit oxytocin secretion at this site.[7,19]

Chronic morphine exposure leads to tolerance (loss of opiate effect, or a requirement for a larger dose to achieve the initial effect) and dependence (revealed as hyperexcitation on withdrawal). Both changes are seen in the mechanisms regulating oxytocin secretion in the rat after i.c.v. infusion of morphine for five days.[6,43,54] In contrast, dependence does not develop in the vasopressin system.[6]

One possible explanation for opioid tolerance is that the number of opioid receptors on tolerant neural elements diminishes. Previous *in vitro* autoradiographic studies on cryostat sections of morphine-tolerant rat brain found either no change in total opioid receptor numbers,[21] or an up-regulation of μ-receptors,[10] but with no observations on the SON

*To whom correspondence should be addressed.

Abbreviations: AV3V region, region anterior and ventral to the third ventricle; DAGO, Tyr-D-Ala-Gly-MePhe-NH(Ch$_2$)$_2$OH, a μ-type opioid receptor agonist; DPDPE, [D-Pen^2, D-Pen^5]-enkephalin, a δ-type opioid receptor agonist; G_i, inhibitory guanine nucleotide-binding protein; MPN, median preoptic nucleus; PP, posterior pituitary; SON, supraoptic nucleus; U50, 488H *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]-cyclohexyl) benzeneacetamide, methane sulphonate salt, a κ-type opioid receptor agonist.

or PP. Studies on ^{3}H-ligand-binding to other brain preparations have also yielded conflicting results in morphine-tolerance.[8,27,28,42,45,46,52,56,59] However, most of these studies did not distinguish between opioid receptor subtypes, and none focused on the SON and PP. The aims of the present study were to measure the densities of μ- and κ-opioid receptor subtypes in the SON and PP in normal and morphine-tolerant female rats, to try to explain morphine-tolerance in relation to oxytocin neurons.

We also measured opioid receptor density in the median preoptic nucleus (MPN), which is part of the region anterior and ventral to the third ventricle (AV3V region) that projects to the supraoptic nuclei,[47] and might contribute to tolerance of the oxytocin system.

EXPERIMENTAL PROCEDURES

Materials

The following were used as probes for opioid receptors *in vitro*. (i) [^{3}H]Etorphine (50 Ci/mmol ≡ 1.85 TBq/mmol Amersham);[12,33] (ii) [^{3}H](−)-bremazocine (21.3 Ci/mmol ≡ 0.79 TBq/mmol, NEN).[9,38]

The following were used as competitive displacers of the ^{3}H-ligands from individual opioid receptor subtypes. (i) Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$ OH, or DAGO (cat. No. PB2000A, Cambridge Research Biochemicals), for displacement from μ-receptors;[34] (ii) [D-Pen^2, D-Pen^5]-enkephalin, or DPDPE (cat. No. E-0260, Sigma), for displacement from δ-receptors;[34,40] (iii) *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl) benzeneacetamide, methane sulphonate salt, or U50,488H, (cat No. D-0908, Sigma) for displacement from κ-receptors.[16,38] To test specificity, naloxone hydrochloride (cat. No. N-7758, Sigma) was used.

Intracerebroventricular infusion

Adult virgin female albino Sprague–Dawley rats were implanted stereotaxically under ether anaesthesia with a cannula in a lateral cerebral ventricle, for continuous infusion from a subcutaneous osmotic minipump (Alzet, 2001) of either morphine sulphate (10 μg/h for 40 h increasing to 20 μg/h, and then 50 μg/h, each for 40 h), or vehicle (sterile, pyrogen-free distilled water, 1 μl/h).[43] There were six rats in each group, and the mean ± S.E.M. body weights were 274.8 ± 5.1 g in the morphine group and 288.8 ± 8.7 g in the vehicle group.

Tissue preparation

After five days of i.c.v. infusion, the rats were decapitated, their brains removed, and from each a thick coronal slice was cut extending from approximately 1 mm anterior to the optic chiasma, to the posterior limit of the mammillary body. Each slice was frozen onto a pre-cooled layer of Tissue-Tek II OCT compound (Miles Laboratories) on a cryostat chuck, in crushed dry ice. The pituitary gland containing the PP was frozen similarly after orientation for coronal sectioning.

Cryostat sections were cut at 10 μm, and thaw-mounted onto acid-cleaned, chrome alum–gelatine-subbed slides. From each rat for each incubation category there were four sections containing two SON profiles each, four sections of the MPN, and four sections of the PP. Mounted sections were stored at −20°C for up to three weeks before incubation.

Detection of opioid receptors

An incubating medium containing a broad-spectrum ^{3}H-ligand (2 nM), without additional agonists, was used for total opioid receptors, but for receptor subtypes two unlabelled selective agonists were included (2 μM each)[57] with the ^{3}H-ligand, with the intention of competitively displacing the ^{3}H-ligand from all but the third receptor subtype. In pilot studies, higher concentrations of displacer (up to 50 μM) did not reduce ^{3}H-ligand-binding further. The autoradiographic images from different incubation categories (see Results) suggested that at 2 μM the displacers were effective and selective in the protocol for either ^{3}H-ligand.

[^{3}H]Etorphine (with displacers) was used for μ-receptors in brain, but [^{3}H](−)-bremazocine (with displacers) was used for κ-receptors, since pilot studies showed more binding by [^{3}H](−)-bremazocine than by [^{3}H]etorphine in brain sections when DAGO and DPDPE were present. Only [^{3}H](−)-bremazocine (with or without displacers) was used for the pituitary, since we obtained negligible specific labelling with [^{3}H]etorphine, even after long exposure. Neither ^{3}H-ligand gave measurable binding in the presence of DAGO and U50, 488H at either site, or elsewhere in brain sections, so quantitative analysis of this category was omitted. Control sections were incubated with 2 nM ^{3}H-ligand and 2 μM naloxone hydrochloride to antagonize all binding to opioid receptors. In pilot studies, higher concentrations of naloxone (up to 20 μM) did not further reduce binding. Further control sections were incubated with ^{3}H-ligand containing all three displacers to control for possible direct chemical interaction between naloxone and [^{3}H]etorphine, and this reduced binding like naloxone; the effects of naloxone were used to calculate specific binding (see below). Other control sections were incubated without ^{3}H-ligand to test for chemography. None of these sections produced autoradiographic images. The incubation categories and protocols are shown in Tables 1 and 2. Sections were brought to room temperature and then preincubated to detach and wash out endogenous opioid or exogenous opiate. We used procedures optimized for each ^{3}H-ligand by others;[9,12] Na^+ was not included in the incubating medium with [^{3}H]etorphine to seek to distinguish high/low affinity binding to μ-receptors.[13] The preincubating and incubating solutions, made up in deionized double-distilled water, were applied as a 40- or 20-μl puddle to each brain or pituitary section, respectively, on slides in moist chambers. After ice-cold immersion washes, the slides were air-dried, and stored desiccated at 0–4°C for less than 48 h before autoradiography. All the slides from a pair of control and morphine-treated rats were processed identically at the same time.

Autoradiography

Slides at room temperature were apposed to Hyperfilm-^{3}H (Amersham), keeping similarly incubated slides from each pair of rats adjacent. A 5-μm section of a tritium standard (^{3}H-microscales, Amersham) was applied to each film. The loaded films were clamped between mirror-coated glass plates with adhesive tape, sealed in light-tight wrappers, and exposed in the dark at 0–4°C.

The optimum exposure periods (determined empirically to produce grain densities over tissue that were below saturation for all positive incubations) were nine weeks for brain sections and 16 weeks for pituitary sections. The exposed films were separated from the slides, developed at 18°C in Kodak D19 developer (5 min), rinsed in tap water, fixed in Ilford Hypam rapid fixer (two changes, 5 min each, of 1:4 fixer:tap water), washed for 30 min in running water, then air-dried. The sections were fixed for 10 min in acetic acid:absolute ethanol:commercial formalin (1:17:2 by volume), dehydrated, rehydrated, stained for 15 min in 1% aqueous Cresyl Fast Violet, dehydrated, cleared, and mounted in DPX.

Image analysis

Pieces of film were first cut out and attached to slides.

Subjective assessment. Autoradiographs were matched with the stained sections in a Wild M3 binocular dissecting

Table 1. Incubation categories for the detection of opioid receptor subtypes in the different tissue regions

Incubation category	Intended selectivity for different opioid receptor subtypes	Tissue region(s) probed
[^{3}H]Etorphine alone (without displacers)	All subtypes (non-selective)	SON and MPN
[^{3}H]Etorphine + DPDPE + U50,488H	μ-receptor selective	SON
[^{3}H]Etorphine + naloxone	No naloxone-sensitive opioid receptors detected	SON and MPN
[^{3}H]Etorphine + DAGO + DPDPE + U50,488H	No μ-, δ-, or κ-receptors detected	SON and MPN
No ^{3}H-ligand, but [^{3}H]etorphine protocol	No receptors of any kind detected	SON and MPN
[^{3}H](−)-Bremazocine alone (without displacers)	All subtypes (non-selective)	SON and PP
[^{3}H](−)-Bremazocine + DPDPE + U50,488H	μ-receptor-selective	PP
[^{3}H]−)-Bremazocine + DAGO + DPDPE	κ-receptor-selective	SON and PP
[^{3}H](−)-Bremazocine + naloxone	No naloxone-sensitive opioid receptors detected	SON and PP
[^{3}H](−)-Bremazocine + DAGO + DPDPE + U50,488H	No μ, δ-, or κ-receptors detected	SON and PP
No ^{3}H-ligand, but [^{3}H](−)-bremazocine protocol	No receptors of any kind detected	PP

microscope (magnification: ×16), to identify appropriate brain regions.[41] Regional silver-grain density was graded on a subjective scale of 0, +, ++, +++, and ++++ (none, weak, moderate, moderately strong, strong).

Quantitative assessment of autoradiographs. The boundaries of the SON, MPN, and PP profiles were first scored with a needle-point on the film under the dissecting microscope. Silver-grain density in these regions was measured with a Joyce–Loebl μMagiscan image-analysing computer, receiving video input from a Philips black and white Video 40 camera (with a Newvicon tube) mounted on a Vickers M17 microscope.[54] Microscope magnification was ×10 (objective), and ×1.6 (intermediate lens); the video monitor scale factor was 1.1878 μm per pixel. The rectangular counting frame (201.63 × 133.04 μm) was set to be smaller than the average area of the SON, to make measurements in the dorsal SON, where oxytocin perikarya predominate.[44,54] The same counting frame was used for both MPN, and for the PP where separate central and peripheral measurements were made because more opioid receptors have been found in the oxytocin terminal-rich periphery.[9,25] Silver-grain density was calculated from the fraction:

$$\frac{\text{total area of silver deposit}}{\text{total area of counting frame}},$$ as justified previously.[54]

Grain densities over tissue were corrected by subtraction of a background count (measured over nearby tissue-free film). For each rat the mean grain density was determined for each tissue region (eight, four and four images of SON, MPN, or PP, respectively) in each incubation category. The mean grain density representing specific binding to opioid receptors was obtained by subtraction of the mean grain density over the corresponding naloxone control sections, if significantly above background (paired t-test). Then, since tissue from morphine- and vehicle-treated rats was co-processed in pairs, the mean grain densities in each incubation category were compared by the paired t-test.

For conversion to absolute values, grain densities were measured similarly, in triplicate, and backgrounds subtracted, for each step of the standard scale on each sheet of film. A mean standard curve of grain density against radioactivity in terms of its brain–gray matter–tissue equivalent (calibrated by Geary and Wooten for Amersham), was computed for the SON and MPN analysis (nine weeks exposure) and for the PP analysis (16 weeks exposure), and fitted to a power function ($y = 0.27\,x^{0.23}$ and $r = 0.97$, for the SON and MPN standard curve, and $y = 0.34\,x^{0.14}$ and $r = 0.96$, for the PP standard curve). Tissue radioactivity values (nCi/mg of tissue), were then calculated from the mean grain densities, and were converted to fmol of ^{3}H-ligand bound per mg of tissue, by reference to the specific activity of the ^{3}H-ligand. Specific binding to opioid receptors was obtained by subtracting binding in the presence of naloxone.

I.c.v. morphine and control group mean ^{3}H-ligand-binding values were compared by Student's t-test.

Preincubation tests

To test the efficiency of the preincubation wash procedure in detaching opioids or opiates from the tissue, brain sections were preincubated for 0, 1, 10, 15, 30, 45, 60, 75, or 90 min, incubated with [^{3}H]etorphine (μ-selective for SON; non-selective for MPN), and then processed and analysed as above.

Blood plasma analysis

Trunk blood was collected from each rat into heparinized tubes, plasma separated immediately by centrifugation, and stored at −20°C. Measurements were made of (i) oxytocin concentration by radioimmunoassay (Higuchi *et al.*[26]) to assess tolerance; (ii) glucose concentration (using a Boehringer Kit; Glucosa GOD-Perid) since morphine raises blood glucose concentration, but tolerance develops;[23] (iii) osmolality and sodium concentration[43] to assess hydromineral state. Student's t-test was used to compare treatment groups.

RESULTS

Supraoptic nucleus

Subjective assessment. Representative autoradiographs of the SON in a control rat are shown in Fig. 1. Using [^{3}H]etorphine alone the SON was moderately strongly labelled (+++), as was adja-

Table 2. Protocols used for the ^{3}H-ligands

^{3}H-ligand	Preincubation	Incubation	Washes	Reference
[^{3}H]Etorphine (±displacers)	15 min at room temperature in 0.05 M Tris–HCl buffer (pH 7.4) containing 0.1 M NaCl and 50 μM Gpp(NH)p	1 h at room temperature in 0.05 Tris–HCl buffer (pH 7.4) containing 0.19 M sucrose, and 2 nM [^{3}H]etorphine (± 2 μM displacers)	1 min in each of four successive baths of ice-cold 0.05 M Tris–HCl buffer (pH 7.4) containing 0.19 M sucrose; then a quick dip in ice-cold deionized double-distilled water to remove salts	After Bunn *et al.*[12] (but with modified washes and displacers)
[^{3}H](−)-Bremazocine (±displacers)	30 min at 0–4°C in 0.015 M potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.1% BSA	3 h at 0–4°C in 0.05 M potassium phosphate buffer (pH 7.4) containing 0.4 M NaCl, 0.1% BSA, and 2 nM [^{3}H](−)-bremazocine (± 2 μM displacers)	1 min in each of four successive baths of ice-cold 0.05 M potassium phosphate buffer (pH 7.4) without additives, before the final quick rinse in ice-cold deionized double-distilled water	After Brady and Herkenham[9] (but with the addition of displacers)

BSA, bovine serum albumin; Gpp(NH)p, 5′-guanylylimidodiphosphate.

cent gray matter in the hypothalamus and thalamus. White matter gave only a feeble image (0 to +). The strongest labelling was in patches in the caudate–putamen, and in the amygdala (++++). Elsewhere in the caudate–putamen, amygdala, and cerebral cortex, labelling was moderate (++). The pattern and intensity of labelling was similar in sections probed with [^{3}H]etorphine and DPDPE and U50,488H. Sections probed in the presence of DAGO and U50,488H gave only weak images (0 to +) in the SON and elsewhere.

Sections probed with [^{3}H](−)-bremazocine alone gave strong images in which the pattern of labelling resembled that shown by [^{3}H]etorphine, except that the cerebral cortex and caudate–putamen were more heavily labelled (++ to +++). The SON was moderately strongly labelled (+++). Sections probed with [^{3}H](−)-bremazocine and DAGO and DPDPE gave moderate images in the SON and elsewhere in the hypothalamic gray matter (++). The intense patches in the caudate–putamen and the amygdala, probed with either ^{3}H-ligand alone, or with [^{3}H]etorphine and DPDPE and U50,488H, were absent from sections probed with [^{3}H](−)-bremazocine and DAGO and DPDPE.

Specificity control sections, incubated with ^{3}H-ligand either with naloxone, or with all three unlabelled displacers, gave extremely faint images.

In morphine-treated rats, the pattern of labelling was similar to control rats, but there was a decrease in intensity in sections incubated with [^{3}H]etorphine and DPDPE and U50,488H.

Quantitative assessment. The results for the SON are shown in Table 3, and Fig. 2. Specific (naloxone-displaceable) binding was over 97% of the total binding in SON sections probed by ^{3}H-ligand alone.

The grain density over the SON incubated with [^{3}H]etorphine alone was reduced by 26.9% in the chronic morphine group ($P < 0.05$; Student's paired t-test). With [^{3}H]etorphine, DPDPE and U50,488H the decrease was 46.9% ($P < 0.02$). There was no significant difference between morphine- and vehicle-treated rats for any other incubation category. In the SON of control and morphine-treated rats the group mean (± S.E.M.) specific binding of [^{3}H]etorphine alone was 102.0 ± 10.5 and 51.7 ± 25.4 fmol per mg of tissue, respectively (not significant). Over SON profiles probed by [^{3}H]etorphine and DPDPE and U50,488H, mean binding was 91.8 ± 25.4 and 14.8 ± 7.5 fmol per mg of tissue in the control and morphine groups, respectively (83.9% decrease, $P < 0.05$, Student's t-test).

Specific binding of [^{3}H](−)-bremazocine alone in the SON was not significantly altered by i.c.v. morphine treatment. In the control and morphine groups the means were 125.7 ± 35.0 and 70.9 ± 24.8 fmol per mg of tissue, respectively. Similarly, treatment group means for SON probed with [^{3}H](−)-bremazocine and DAGO and DPDPE showed no significant difference between control and morphine groups

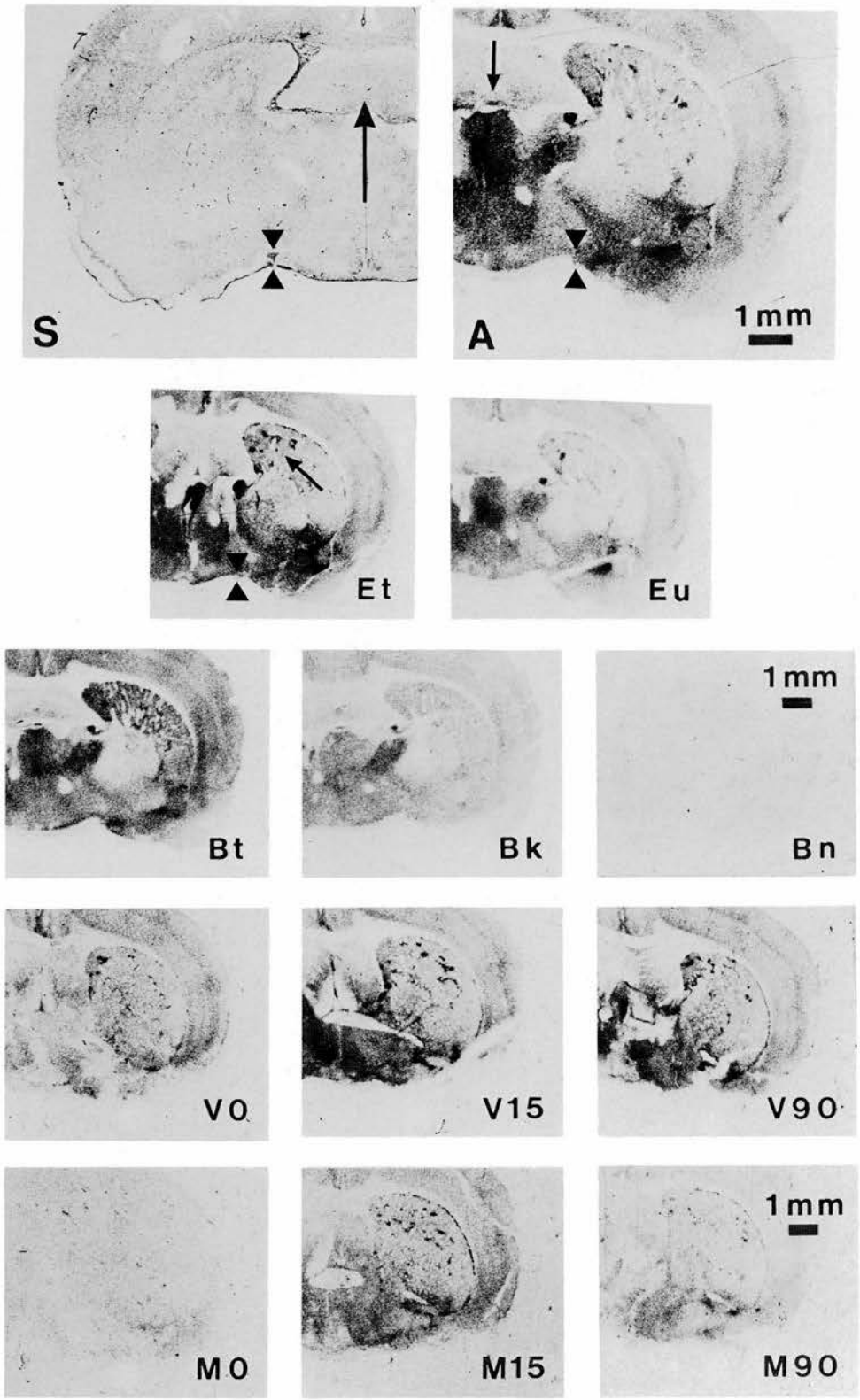


Fig. 1. (Top row) S is part of a 10-μm coronal section of rat brain, incubated with [^{3}H]etorphine, apposed to Hyperfilm-^{3}H for nine weeks, then fixed in acetic acid–ethanol–formalin, and stained with Cresyl Fast Violet. The SON is the small dark-staining area between the black triangles. The upward-pointing arrow is on the midline. A is the autoradiographic image corresponding to S, processed on the Hyperfilm-^{3}H after separation of S from the film. The position of the SON is indicated as in S, and the downward-pointing arrow is on the midline. Magnification × 6. (Second and third rows) Autoradiographic images of sections incubated with [^{3}H]etorphine alone (Et), or with [^{3}H]etorphine and DPDPE and U50,488H (Eu), or with [^{3}H](−)-bremazocine alone (Bt), or with [^{3}H](−)-bremazocine and DAGO and DPDPE (Bk), or with [^{3}H](−)-bremazocine and naloxone to antagonize all binding to opioid receptors (Bn). In [^{3}H]etorphine alone (Et), the SON is between the black triangles, and the arrow indicates intense patches of labelling in the caudate–putamen. For further neuroanatomical details, see Paxinos and Watson.[41] Magnification × 4. (Fourth and fifth rows) Autoradiographic images of sections from an i.c.v. vehicle-infused rat (V), and an i.c.v. morphine-infused rat (M), incubated with [^{3}H]etorphine and DPDPE and U50,488H following 0, 15, or 90 min preincubation as indicated. Magnification × 4.

Table 3. Treatment group means (±S.E.M.) of mean grain densities in autoradiographs of the supraoptic nucleus, median preoptic nucleus and posterior pituitary

Tissue region	Incubation category	Vehicle-treated animal group ($n = 6$)	Morphine-treated animal group ($n = 6$)	Morphine-treated versus vehicle-treated (Student's paired t-test)
SON	[^{3}H]Etorphine alone	0.38 ± 0.01	0.28 ± 0.04	$P < 0.05$
	[^{3}H]Etorphine + DPDPE + U50,488H	0.37 ± 0.02	0.20 ± 0.04	$P < 0.02$
	[^{3}H](−)-Bremazocine alone	0.32 ± 0.02	0.27 ± 0.03	n.s.
	[^{3}H](−)-Bremazocine + DAGO + DPDPE	0.32 ± 0.01	0.30 ± 0.02	n.s.
MPN	[^{3}H]Etorphine alone	0.36 ± 0.01	0.25 ± 0.02	$P < 0.05$
PP	[^{3}H](−)-Bremazocine alone	0.18 ± 0.04	0.19 ± 0.05	n.s.
	[^{3}H](−)-Bremazocine + DAGO + DPDPE	0.13 ± 0.05	0.15 ± 0.03	n.s.
	[^{3}H](−)-Bremazocine + DPDPE + U50,488H	0.11 ± 0.05	0.10 ± 0.03	n.s.

Data are expressed in μm^2 of silver deposit per μm^2 of field; specificity control values subtracted. n.s., not significant.

(means ± S.E.M. were 130.4 ± 25.6 and 95.5 ± 16.0 fmol per mg of tissue).

Without the preincubation step, the mean binding of [^{3}H]etorphine with DPDPE and U50,488H in chronic morphine-treated tissue was not significantly different from the binding after incubation with [^{3}H]etorphine and all three selective agonists. With only 1 min of preincubation, the mean binding in the presence of DPDPE and U50,488H in morphine-treated tissue had risen to 15.1% of the value in vehicle-treated tissue, and thereafter, up to the longest preincubation time tested (90 min), there was no further rise. Typical sections are illustrated in Fig. 1.

Median preoptic nucleus

Subjective assessment. Regional silver-grain distribution in sections containing the MPN, probed with [^{3}H]etorphine alone, was similar to sections containing the SON. Assessed subjectively, in control rats, the relative intensities were: white matter, 0 to +; MPN, +++; patches in the caudate–putamen, ++++; elsewhere in the caudate–putamen and in the cerebral cortex, ++. Specificity control sections, incubated with [^{3}H]etorphine and either naloxone or all three displacers, gave very weak images.

In i.c.v. morphine-treated rats the pattern of silver-grain distribution in [^{3}H]etorphine-probed sections

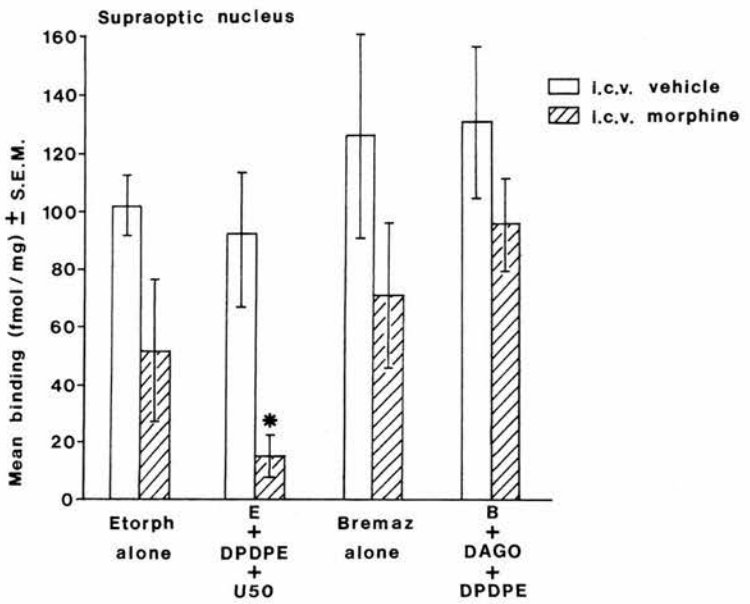


Fig. 2. Treatment group means (±S.E.M.) of ^{3}H-ligand binding to the SON of i.c.v. vehicle-infused rats (□) and i.c.v. morphine-infused rats (▨), in each incubation category. The values shown represent specific binding to opioid receptors since specificity control values have been subtracted. Values are expressed in fmol of ^{3}H-ligand per mg of tissue. Etorph alone, binding after incubation with [^{3}H]etorphine alone (without displacers). E + DPDPE + U50, binding after incubation with [^{3}H]etorphine and DPDPE and U50,488H. Bremaz alone, binding after incubation with [^{3}H](−)-bremazocine alone (without displacers). B + DAGO + DPDPE, binding after incubation with [^{3}H](−)-bremazocine and DAGO and DPDPE. *$P < 0.05$ (Student's t-test).

was similar to the control group, but the silver-grain density appeared lower in the MPN and elsewhere.

Quantitative assessment. Chronic morphine treatment significantly decreased grain density over MPN after incubation in [^{3}H]etorphine (by 29.7%, $P < 0.05$; Student's paired t-test, Table 3). Mean specific [^{3}H]etorphine-binding in the MPN was reduced by 77.0% in morphine-treated rats. In the control and morphine-treated groups the means ($\pm$S.E.M.) were 76.2 ± 9.8 and 17.5 ± 5.2 fmol per mg of tissue, respectively ($P < 0.01$, Student's t-test). Prolonged preincubation, for 90 min, before incubation in [^{3}H]etorphine did not affect the difference between control and morphine-treated rats.

Posterior pituitary

Subjective assessment. Whole pituitary sections were apposed to film, but only the PP gave an autoradiographic image. Typical autoradiographs are shown in Fig. 3. No consistent differences between central and peripheral parts of the posterior pituitary were found in any incubation category. Using [^{3}H](−)-bremazocine alone or with DAGO and DPDPE an intense image was obtained (+ + +). Inclusion of DPDPE and U50,488H in the incubating medium gave a less intense image (+ +). Incubation in the presence of DAGO and U50,488H resulted in a very weak image (0 to +). Specificity control sections, incubated with [^{3}H](−)-bremazocine and naloxone, also gave very weak images (0 to +), and chemography control sections, incubated without ^{3}H-ligand, gave no images at all.

Images from the posterior pituitaries of morphine-treated rats were similar to those from vehicle-treated controls.

Quantitative assessment. Whether the quantitative results from the posterior pituitary were expressed as mean grain densities, or as mean binding, no statistically significant differences were found between the centre, and the periphery, of the PP for any incubation category so central and peripheral data have been combined.

In control rats, the mean grain densities were highest after incubation with [^{3}H](−)-bremazocine alone (Table 3). Chronic morphine treatment had no significant effects (Table 3). Results from PP sections of control rats probed with [^{3}H](−)-bremazocine alone or with naloxone, showed that specific binding averaged 79% of the total. Mean ($\pm$S.E.M.) specific binding after incubation with [^{3}H](−)-bremazocine without displacers, was 11.6 ± 6.30 and 18.0 ± 8.46 fmol per mg of tissue in control and i.c.v.

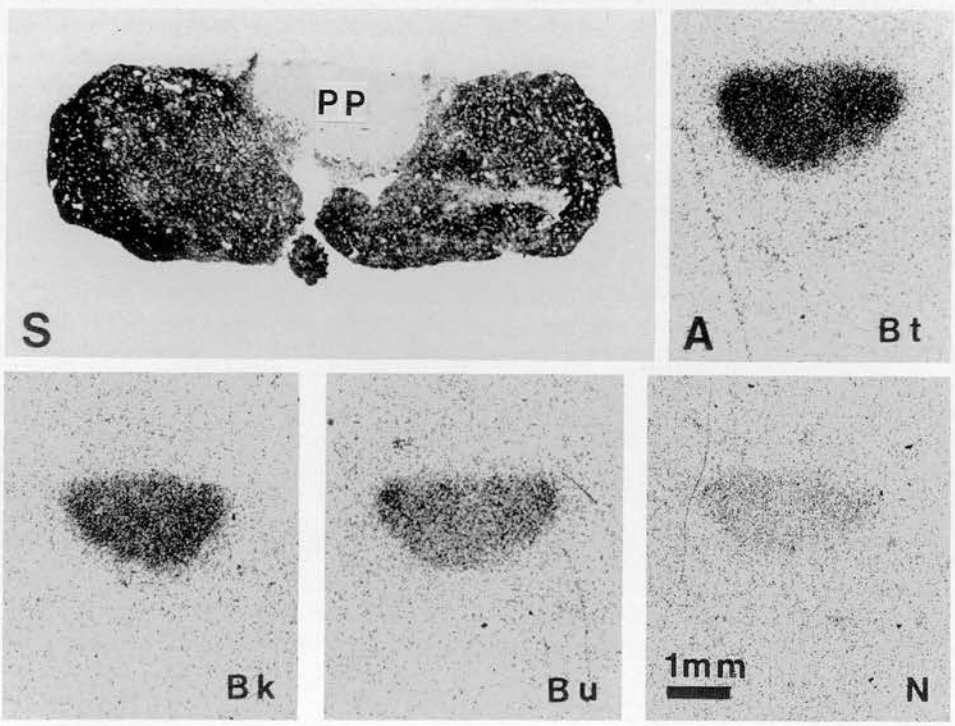


Fig. 3. (Top row) S is a 10-μm section of rat pituitary, incubated with [^{3}H](−)-bremazocine, apposed to Hyperfilm-^{3}H for 16 weeks, then fixed in acetic acid–ethanol–formalin, and stained with Cresyl Fast Violet. The PP is the pale-staining area. A is the autoradiographic image of S. Note that only the PP has produced an image. This image shows binding to opioid receptors after incubation in [^{3}H](−)-bremazocine alone (without displacers) (Bt). Magnification × 8. (Second row) Autoradiographic images of sections incubated with [^{3}H](−)-bremazocine and DAGO and DPDPE (Bk), or with [^{3}H](−)-bremazocine and DPDPE and U50,488H (Bu), or with [^{3}H](−)-bremazocine and naloxone to antagonize all binding to opioid receptors (N). Magnification × 8.

morphine-infused rats, respectively. In control rats binding was only 9.2% of that in the SON in the same incubation category. In the presence of DAGO and DPDPE the mean (±S.E.M.) values were 3.25 ± 2.09 and 2.72 ± 1.42 fmol of ^{3}H-ligand bound per mg of tissue in control and morphine groups, respectively. For control rats, this binding was only 2.5% of the level in the SON in similar conditions. Mean (±S.E.M.) specific binding after incubation with [^{3}H](−)-bremazocine and DPDPE and U50,488H was 2.00 ± 1.21 fmol (control group), and 1.07 ± 0.73 fmol (morphine group) per mg of tissue. There were no significant differences between control and morphine-treated rats for any incubation category.

Blood plasma analysis

There were no significant differences between the control and i.c.v. morphine groups for any of the parameters measured, i.e. trunk blood plasma oxytocin, glucose, or sodium concentration, or osmolality (Table 4).

DISCUSSION

Supraoptic nucleus

Control rats. Unlike some previous studies on male rats[35,36] we found ^{3}H-ligand-binding in the SON of female rats in conditions designed to be selective for μ-type opioid receptors. We suggest that this represents binding to μ-type opioid receptors, for the following reasons. Firstly, [^{3}H]etorphine is a broad spectrum ^{3}H-ligand for opioid receptors, but with a 1000-fold molar excess of the unlabelled agonists, DPDPE and U50,488H, known to have high relative affinity for δ- and κ-type receptors, respectively,[16,40] competition for binding sites would be expected to leave predominantly μ-receptors available for [^{3}H]-etorphine. Although U50,488H is a stronger displacer at κ-type opioid receptors of the guinea-pig (postulated type 1 κ-receptors) than at κ-receptors of the rat (postulated type 2 receptors),[62] it nonetheless blocks *in situ* [^{3}H](−)-bremazocine-binding to rat κ-receptors.[38,39] Secondly, incubation with DAGO (which has a high relative affinity for μ-receptors), in addition to DPDPE and U50,488H, reduced [^{3}H]etorphine-binding in the SON to a very low level. Thirdly, elsewhere in the sections containing SON profiles were intense patches of labelling, present after incubation in media designed to demonstrate μ-receptors, but absent after incubation in media designed to show δ- or κ-receptors. Other authors have claimed these intense clusters in the caudate–putamen to be μ-receptors.[57] Fourthly, the change in the SON during chronic morphine treatment was confined to μ-selective binding. This suggests that the subtype demonstrated by this selective incubation differs from the others, and that it is uniquely responsive to a known μ-selective agonist (morphine). Lastly, the demonstration of μ-type opioid receptors in the SON is consistent with the inhibitory action of morphine on the electrical activity of oxytocin neurons when applied close to their cell bodies.[49,58]

Specific binding to opioid receptors was also demonstrated in the SON after incubation with [^{3}H](−)-bremazocine in the presence of DAGO and DPDPE, a procedure designed to be selective for κ-receptors. In the caudate–putamen, this type of incubation did not show the intense patches of binding shown by ^{3}H-ligand alone, or by incubation in conditions intended to be μ-selective; it seems likely that DAGO and DPDPE prevented binding to the receptor subtype(s) at these clustered sites. Addition of U50,488H, a known κ-receptor-selective agonist,[16] to the other two unlabelled selective agonists reduced [^{3}H](−)-bremazocine-binding to a low level both in the SON and caudate–putamen, supporting the assumption that κ-receptors were predominantly involved. Demonstration of κ-receptor in the SON is in agreement with previous reports,[35,36] and with electrophysiological studies showing inhibitory actions of U50,488H on oxytocin (and vasopressin) neurons in the SON.[49] Indeed, suppression of electrical activity by U50,488H, and by morphine, has even been achieved on the same supraoptic neuron (Russell *et al.*, unpublished observations).

With the low resolution of the techniques used, we were unable to determine the distribution of μ- and κ-receptors on or between neurons,[24] or indeed whether they had been internalized.[29] It is possible, by analogy with the PP gland,[12,33] that some of the receptors were on glial cells, especially astrocytes.

Effects of intracerebroventricular morphine infusion. In the SON, binding of [^{3}H]etorphine in conditions designed to be μ-selective was reduced by over 80% by chronic morphine treatment compared with control rats. Morphine did not affect κ-selective [^{3}H](−)-bremazocine-binding.

One possible explanation for decreased binding of [^{3}H]etorphine to the SON after chronic morphine treatment could be that morphine still in the tissue

Table 4. Means (±S.E.M.) of data from blood plasma samples

Treatment group	Oxytocin concentration (pg/ml)	Glucose concentration (mM/l)	Sodium concentration (mM/l)	Osmolality (mOsmol/kg)
i.c.v. vehicle-infused ($n = 6$)	13.23 ± 5.06	7.82 ± 0.43	138.29 ± 2.23	270.00 ± 5.63
i.c.v. morphine-infused ($n = 6$)	8.67 ± 1.77	7.31 ± 0.24	142.71 ± 1.41	268.29 ± 5.99

competes with [^{3}H]etorphine. Morphine is remarkably persistent in brain after peripheral administration, [^{14}C]morphine still being detectable three weeks after a single injection,[37] however, morphine can be washed out *in vitro*.[59] Similarly, altered release of endogenous opioid in i.c.v. morphine-infused rats might compete with [^{3}H]etorphine. However, etorphine has a high affinity for opioid receptors,[22] and 5′-guanylylimidodiphosphate was included in the preincubating medium to encourage dissociation of receptor-bound opioids,[12] and we showed that preincubation for much longer than the 15 min used did not result in any increase in ^{3}H-ligand binding. It seems reasonable to conclude that *in vivo*, the number of μ-receptors in the SON is reduced by chronic i.c.v. infusion of morphine, and that this reduction explains the decreased binding of ^{3}H-ligand. However, a possible wash-resistant blockade by some of the i.c.v. infused morphine, cannot be ruled out. A significant reduction in [^{3}H]etorphine-binding was found also in the MPN, though not resolved to a particular opioid receptor subtype.

A reduction in number of available μ-receptors might allow SON oxytocin neurons, and their inputs, to function normally in morphine-treated rats, as indicated by similar concentrations of oxytocin in trunk blood plasma from the i.c.v. morphine- and vehicle-treated rats in the present study. Normal functioning has also been inferred in previous studies.[6,43] The consequence of a partial loss or blockade of μ-type receptors would be to decrease the pool of spare receptors.[14,32,53] Thus, even if the affinity of the receptors for morphine were unaffected, this would decrease the number of receptors which could be activated at a given concentration of morphine. This effect would contribute to tolerance, and the extent of the loss of binding we have measured might almost be great enough to explain morphine-tolerance[15] in the female rat oxytocin system. We found no evidence after i.c.v. morphine infusion for changes in trunk blood plasma glucose or sodium concentrations, or osmolality, that might stimulate the oxytocin system through osmoreceptor mechanisms,[47] and perhaps alter opioid receptor density in the SON.

Posterior pituitary

Control rats. In common with earlier work,[51] we found a much lower density of opioid receptors in the PP than in the brain. Longer exposures were required to produce autoradiographic images. Most binding was obtained after incubation with [^{3}H](−)-bremazocine alone, or in the presence of DAGO and DPDPE, which was intended to be selective for κ-receptors. Since addition of U50,488H to DAGO and DPDPE reduced [^{3}H](−)-bremazocine-binding to a low level, it is probable that κ-receptors were indeed responsible for most of this binding. Other authors have also suggested that opioid receptors in the PP are predominantly of the κ-receptor subtype.[9,12,25] In the presence of DPDPE and U50,488H, which was intended to be μ-selective, there was still some binding by [^{3}H](−)-bremazocine, but there was less binding in pilot studies with [^{3}H]etorphine so the presence of the μ-receptor subtype in the PP remains controversial.

We did not find any differences in opioid receptor density between the central and peripheral zones of the PP of female rats, unlike the studies of Herkenham *et al.*[25] and Brady and Herkenham[9] on male rats.

Effects of intracerebroventricular morphine infusion. Although i.c.v.-infused morphine reaches the PP,[43] we detected no change in [^{3}H](−)-bremazocine binding in any incubating conditions. The lack of effect of morphine on κ-receptors in the PP is consistent with the persistence of endogenous opioid- (probably dynorphin)-inhibition of oxytocin secretion in isolated PPs from i.c.v. morphine-infused rats *in vitro*.[3] Thus, chronic morphine treatment neither affects the number of κ-receptors, nor induces cross-tolerance to the endogenous ligand in the PP. However, the density of κ-receptors in the PP can show plasticity: during dehydration for five days the number of κ-receptors decreases by about 40% without a change in affinity.[11]

CONCLUSION

The data we have on the oxytocin and oxytocin mRNA content of SON neurons, their firing rate, and the responsiveness of their terminals in the PP to electrical stimulation, all indicate that the synthesis of oxytocin and the coupling of electrical activity to secretion are not altered in morphine-tolerance.[3,6,43,54] This leaves opioid–receptor interaction, and early post-receptor events regulating electrical excitability, as likely sites for tolerance. We have provided evidence in this study for a decrease in number of available μ-receptors in the SON, and of available opioid receptors in an input pathway, the MPN, which regulates oxytocin neurons, but we have not distinguished intracellular forms from surface forms of the receptor. There may also be reduced effectiveness of remaining receptors available for binding to ligand because of attenuated coupling of receptor to a $G_{i/o}$-like protein, as has been described for morphine-tolerant locus coeruleus neurons,[15] but this has not been investigated in SON neurons.

Morphine-dependence in the oxytocin system, revealed by injection of naloxone, is expressed as withdrawal excitation of oxytocin hypersecretion resulting from a large increase in the firing rate of oxytocin neurons.[6,30] This phenomenon is mediated through μ-receptors,[31] which the present study has shown to remain available in the SON at about 20% of the number in morphine-naive rats. It is difficult to explain withdrawal excitation of oxytocin neurons in terms of a reduced number of available μ-receptors. Adaptive changes in post-receptor mechanisms regu-

lating ionic fluxes seem more likely. There may also be compensatory changes in non-opioid pathways.

Acknowledgements—We thank Dr R. J. Bicknell for the measurements of oxytocin concentration in blood plasma by radioimmunoassay and Mr B. G. McGory for the remaining blood plasma analysis. We are also indebted to Dr R. J. Bicknell for helpful criticism of the manuscript. We are grateful to Mrs K. E. Grant and Mrs G. E. Grant for expert histological assistance, and to the M.R.C. for financial support.

REFERENCES

1. Atweh S. F. and Kuhar M. J. (1983) Distribution and physiological significance of opioid receptors in the brain. *Br. med. Bull.* **39,** 47–52.
2. Aziz L. A., Forsling M. L. and Woolf C. J. (1981) The effects of morphine on vasopressin release in the rat. *J. Physiol., Lond.* **311,** 401–409.
3. Bicknell R. J., Chapman C., Leng G. and Russell J. A. (1985) Chronic morphine exposure and oxytocin neurones in rats: lack of both morphine dependence and cross-tolerance to endogenous opioids in the neurohypophysis. *J. Physiol., Lond.* **382,** 151P.
4. Bicknell R. J. and Leng G. (1982) Enkephalin analogue inhibits oxytocin secretion from the rat neurohypophysis. *J. Physiol., Lond.* **332,** 87P.
5. Bicknell R. J. and Leng G. (1982) Endogenous opiates regulate oxytocin but not vasopressin secretion from the neurohypophysis. *Nature, Lond.* **298,** 161–162.
6. Bicknell R. J., Leng G., Lincoln D. W. and Russell J. A. (1988) Naloxone excites oxytocin neurons in the supraoptic nucleus of lactating rats after chronic morphine treatment. *J. Physiol, Lond.* **396,** 297–317.
7. Bicknell R. J., Leng G., Russell J. A., Dyer R. G., Mansfield S. and Zhao B.-G. (1988) Hypothalamic opioid mechanisms controlling oxytocin neurones during parturition. *Brain Res. Bull.* **20,** 743–749.
8. Bolger G. T., Skolnick P., Rice K. C. and Weissman B. A. (1988) Differential regulation of μ-opiate receptors in heroin- and morphine-dependent rats. *Fedn Eur. biochem. Socs Lett.* **234,** 22–26.
9. Brady L. S. and Herkenham M. (1987) Dehydration reduces κ-opiate receptor binding in the neurohypophysis of the rat. *Brain Res.* **425,** 212–217.
10. Brady L. S., Herkenham M., Long J. B. and Rothman R. B. (1989) Chronic morphine increases μ-opiate receptor binding in rat brain: a quantitative autoradiographic study. *Brain Res.* **477,** 382–386.
11. Brady L. S., Rothman R. B. and Herkenham M. (1988) Physiological regulation of neurohypophysial kappa-opiate receptors. *Brain Res.* **443,** 398–402.
12. Bunn S. J., Hanley M. R. and Wilkin G. P. (1985) Evidence for a kappa opioid receptor on pituitary astrocytes: an autoradiographic study. *Neurosci. Lett.* **55,** 317–323.
13. Carroll J. A., Shaw J. S. and Wickenden A. D. (1988) The physiological relevance of low agonist affinity binding at opioid mu-receptors. *Br. J. Pharmac.* **94,** 625–631.
14. Chavkin C. and Goldstein A. (1984) Opioid receptor reserve in normal and morphine-tolerant guinea pig ileum myenteric plexus. *Proc. natn. Acad. Sci. U.S.A.* **81,** 7253–7257.
15. Christie M. J., Williams J. T. and North R. A. (1987) Cellular mechanisms of opioid tolerance: studies in single brain neurons. *Molec. Pharmac.* **32,** 633–638.
16. Clark J. A. and Pasternak G. W. (1988) U50,488: a kappa-selective agent with poor affinity for mu_1 opiate binding sites. *Neuropharmacology* **27,** 331–332.
17. Clarke G. and Patrick G. (1983) Differential inhibitory action by morphine on the release of oxytocin and vasopressin from the isolated neural lobe. *Neurosci. Lett.* **39,** 175–180.
18. Clarke G., Wood P., Merrick L. P. and Lincoln D. W. (1979) Opiate inhibition of peptide release from the neurohumoral terminals of hypothalamic neurones. *Nature, Lond.* **182,** 746–748.
19. Coombes J. E. and Russell J. A. (1988) Inhibition by a kappa-opioid receptor agonist (U50,488H) of electrically-stimulated oxytocin secretion from the neurohypophysis in anaesthetized lactating rats. *J. Physiol., Lond.* **401,** 34P.
20. Cutting R., Fitzsimons N., Gosden R. G., Humphreys E. M., Russell J. A., Scott S. and Stirland J. A. (1986) Evidence that morphine interrupts parturition in rats by inhibiting oxytocin secretion. *J. Physiol., Lond.* **371,** 182P.
21. Geary W. A. and Wooten G. F. (1985) Regional saturation studies of [^{3}H]naloxone binding in the naive, dependent and withdrawal states. *Brain Res.* **360,** 214–223.
22. Gillan M. G. C. and Kosterlitz H. W. (1982) Spectrum of the μ-, δ-, and κ-binding sites in homogenates of rat brain. *Br. J. Pharmac.* **77,** 461–469.
23. Gross E. G. and Pierce I. H. (1935) Effect of morphine on the oxygen consumption of brain tissue in the rat. *J. Pharmac. exp. Ther.* **53,** 156–168.
24. Herkenham M. (1987) Mismatches between neurotransmitter and receptor localizations in brain: observations and implications. *Neuroscience* **23,** 1–38.
25. Herkenham M., Rice K. C., Jacobson A. E. and Rothman R. B. (1986) Opiate receptors in rat pituitary are confined to the neural lobe and are exclusively kappa (BRE 21754). *Brain Res.* **382,** 365–371.
26. Higuchi T., Honda K., Fukuoka T., Negoro H. and Wakabayashi K. (1988) Release of oxytocin during suckling and parturition in the rat. *J. Endocrin.* **105,** 339–346.
27. Holaday J. W., Hitzemann R. J., Curell J., Tortella F. C. and Belenky G. L. (1982) Repeated electroconvulsive shock or chronic morphine treatment increases the number of ^{3}H-D-Ala^2, D-Leu^5-enkephalin binding sites in rat brain membranes. *Life Sci.* **31,** 2359–2362.
28. Klee W. A. and Streaty R. A. (1974) Narcotic receptor sites in morphine-dependent rats. *Nature, Lond.* **248,** 61–63.
29. Law P.-Y., Griffin M. T. and Loh H. H. (1984) Mechanisms of multiple cellular adaptation processes in clonal cell lines during chronic opiate treatment. In *Mechanisms of Tolerance and Dependence* (ed. Sharp C. W.), pp. 119–135. National Institute on Drug Abuse Research Monograph, 54.
30. Leng G. and Russell J. A. (1988) Opioid control of oxytocin secretion. In *Recent Progress in Posterior Pituitary Hormones* (eds Yoshida S. and Share L.), pp. 89–96. Elsevier, Amsterdam.
31. Leng G., Russell J. A. and Grossman R. (1989) Sensitivity of magnocellular oxytocin neurones to opioid antagonists in rats treated chronically with intracerebroventricular (i.c.v.) morphine. *Brain Res.* **484,** 290–296.

32. Leslie F. M. (1987) Methods used for the study of opioid receptors. *Pharmac. Rev.* **39,** 197–249.
33. Lightman S. L., Ninkovic M., Hunt S. P. and Iversen L. L. (1983) Evidence for opiate receptors on pituicytes. *Nature, Lond.* **305,** 235–237.
34. Mansour A., Lewis M. E., Khachaturian H., Akil H. and Watson S. J. (1986) Pharmacological and anatomical evidence of selective μ, δ, and κ-opioid receptor binding in rat brain. *Brain Res.* **399,** 69–79.
35. Mansour A., Khachaturian H., Lewis M. E., Akil H. and Watson S. J. (1988) Anatomy of CNS opioid receptors. *Trends Neurosci.* **11,** 308–314.
36. Mansour A., Khachaturian H., Lewis M. E., Akil H. and Watson S. J. (1987) Autoradiographic differentiation of mu, delta and kappa opioid receptors in the rat forebrain and midbrain. *J. Neurosci.* **7,** 2445–2464.
37. Misra A. L., Mitchell C. L. and Woods L. A. (1971) Persistence of morphine in central nervous system of rats after a single injection and its bearing on tolerance. *Nature, Lond.* **232,** 48–50.
38. Morris B. J. and Herz A. (1986) Autoradiographic localization in rat brain of κ-opiate binding sites labelled by [^{3}H]bremazocine. *Neuroscience* **19,** 839–846.
39. Morris B. J. and Herz A. (1989) Control of opiate receptor number *in vivo*: simultaneous κ-receptor down-regulation and μ-receptor up-regulation following chronic agonist/antagonist treatment. *Neuroscience* **29,** 433–442.
40. Mosberg H. I., Hurst R., Hruby V. J., Gee K., Yamamura H. I., Galligan J. J. and Burks T. F. (1983) Bis-pencillamine enkephalins possess highly improved specificity towards opioid receptors. *Proc. natn. Acad. Sci. U.S.A.* **80,** 5871–5874.
41. Paxinos G. and Watson C. (1982) *The Rat Brain in Stereotaxic Co-ordinates.* Academic Press, Sydney.
42. Perry D. C., Rosenbaum J. S. and Sadee W. (1982) *In vivo* binding of [^{3}H]etorphine in morphine-dependent rats. *Life Sci.* **31,** 1405–1408.
43. Rayner V. C., Robinson I. C. A. F. and Russell J. A. (1988) Chronic intracerebroventricular morphine and lactation in rats: dependence and tolerance in relation to oxytocin neurones. *J. Physiol., Lond.* **396,** 319–347.
44. Rhodes C. H., Morrell J. I. and Pfaff D. W. (1981) Immunohistochemical analysis of magnocellular elements in rat hypothalamus: distribution and numbers of cells containing neurophysin, oxytocin and vasopressin. *J. comp. Neurol.* **198,** 45–64.
45. Rogers N. F. and El-Fakahany E. E. (1986) Morphine-induced receptor down-regulation detected in adult rat brain cells. *Eur. J. Pharmac.* **124,** 221–230.
46. Rothman R. B., McLean S., Bykov V., Lessor R. A., Jacobson A. E., Rice K. and Holaday J. W. (1987) Chronic morphine upregulates a mu-opiate binding site labeled by [^{3}H]cyclo FOXY: a novel opiate antagonist suitable for positron emission tomography. *Eur. J. Pharmac.* **142,** 73–81.
47. Russell J. A., Blackburn R. E. and Leng G. (1988) The role of the AV3V region in the control of magnocellular oxytocin neurons. *Brain Res. Bull.* **20,** 803–810.
48. Russell J. A., Gosden R. G., Humphreys E. M., Cutting R., Fitzsimons N., Johnston V., Liddle S., Scott S. and Stirland J. A. (1989) Interruption of parturition in rats by morphine: a result of inhibition of oxytocin secretion. *J. Endocr.* **121,** 521–536.
49. Russell J. A., Pumford K. M. and Leng G. (1989) Opiate dependence in a neuroendocrine neurone system. In *Advances in Biosciences Series 75: Progress in Opioid Research* (eds Cros J., Meunier J.-Cl. and Hamon M.), pp. 767–770. Pergamon Press, Oxford.
50. Russell J. A. and Spears N. (1984) Morphine inhibits suckling-induced oxytocin secretion in conscious lactating rats but also disrupts maternal behaviour. *J. Physiol., Lond.* **346,** 133P.
51. Simantov R. and Snyder S. H. (1977) Opiate receptor binding in the pituitary gland. *Brain Res.* **124,** 178–184.
52. Simon E. J. and Hiller J. M. (1978) *In vitro* studies on opiate receptors and their ligands. *Fedn Proc. Fedn Am. Socs exp. Biol.* **37,** 141–146.
53. Stephenson R. P. (1956) A modification of receptor theory. *Br. J. Pharmac.* **11,** 379–393.
54. Sumner B. E. H., Kawata M. and Russell J. A. (1989) Does acute, intense stimulation of oxytocin neurons in the supraoptic nucleus increase their content of oxytocin mRNA? *Brain Res.* **489,** 283–290.
55. Szligi G. R. and Ludens J. H. (1982) Studies on the nature and mechanisms of the diuretic action of the opioid analgesic ethylketocylazocine. *J. Pharmac. exp. Ther.* **220,** 585–591.
56. Tempel A., Habas J., Paredes W. and Barr G. A. (1988) Morphine-induced down regulation of μ-opioid receptors in neonatal rat brain. *Brain Res.* **469,** 129–133.
57. Tempel A. and Zukin R. S. (1987) Neuroanatomical patterns of the μ, δ, and κ-opioid receptors of rat brain as determined by quantitative *in vitro* autoradiography. *Proc. natn. Acad. Sci. U.S.A.* **84,** 4308–4312.
58. Wakerley J. B., Noble R. and Clarke G. (1983) Effects of morphine and D-Ala, D-Leu enkephalin on the electrical activity of supraoptic neurosecretory cells *in vitro*. *Neuroscience* **10,** 73–81.
59. Wilkinson M., Jacobson W. and Watson-Wright W. (1986) Tissue slices in radioligand binding assays: studies in brain, pineal and muscle. *Life Sci.* **39,** 2037–2048.
60. Zhao B.-G., Chapman C. and Bicknell R. J. (1988) Opioid–noradrenergic interactions in the neurohypophysis. I. Differential opioid receptor regulation of vasopressin, and noradrenaline release. *Neuroendocrinology* **48,** 16–24.
61. Zhao B.-G., Chapman C., Brown D. and Bicknell R. J. (1988) Opioid–noradrenergic interactions in the neurohypophysis. II. Does noradrenaline mediate the actions of endogenous opioids on oxytocin and vasopressin release? *Neuroendocrinology* **48,** 25–31.
62. Zukin R. S., Eghbali M., Olive D., Unterwald E. M. and Tempel A. (1988) Characterization and visualisation of rat and guinea pig brain kappa opioid receptors: evidence for kappa 1 and kappa 2 opioid receptors. *Proc. natn. Acad. Sci. U.S.A.* **85,** 4061–4065.
63. Zukin R. S. and Zukin S. R. (1984) The case for multiple opiate receptors. *Trends Neurosci.* **7,** 160–164.

(*Accepted* 23 *April* 1990)