OXYTOCIN NEURONE ADAPTATIONS TO OPIOIDS.

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Declaration

The studies outlined in this thesis were undertaken in the Department of Physiology, University Medical School, Edinburgh under the supervision of Dr. J. A. Russell. All of the work described was performed by the author unless indicated otherwise.

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Acknowledgements

First and foremost I would like to thank Dr. John Russell primarily for his expert guidance and assistance on the subject matter described throughout the course of this thesis. However John has also managed to show that basic scientific research can be fun and that those partaking in this adventure, contrary to public opinion do possess a sense of humour.

The fruits of this work could not have been gathered without the help of Dr. Kathryn Liddington and Dr. Alison Douglas who were always more than willing to provide help as required (quite often in the good old early days!). A number of other people including Dr. Gareth Leng, Dr. Inga Neumann, Dr. Colin Brown, Ms. Louise Johnstone, Dr. Phillip Bull, Ms. Hanneke Meeren, Dr. Irina Antonijevic, Mr. Niall Murphy and Dr. Ruth Blackburn have also helped in making the lab a sunny place to work in, even on the darkest days.

In my final year I spent a period of time at the University of Leipzig supported by the British Council and was well looked after (apart from a near fatal rodent attack) by Dr. Mike Ludwigs' entourage in the lab of Professor Rainer Landgraf.

Expert technical service was provided by the Faculty Animal Unit headed by Mr. Don Henderson and by the Departmental Histology Lab fronted by Mrs. Kay Grant.

Finally but by no means least I would like to reserve my love and most sincere gratitude for a very dear friend over the past three years, Ms. Yvonne McLaren. She more than anybody else has been only too well aware of the strain involved and put up with more than any mere mortal could be expected to do. I would also like to attribute any spelling mistakes which may be incurred upon your wanderings through this text solely to her.

Supported by the AFRC and more often than not by my mother and father.
"I also fancied myself as a scientist...

I found out that if you gave a duck a piece of fatty pork, something in its intestinal make-up caused the bird to pass the pork within a minute or two. From beak to exit it was a spectacle you could observe very swiftly.

We had plenty of ducks in our back yard. I pondered a night over this.

It occurred to me that it would be interesting to tie a string about ten feet long to the pork.

Out came the pork, which I then gave to another duck with the same result, holding onto the string that entered the first duck's mouth. In a few minutes I had a half-dozen ducks tied together beak to rectum on this greased string.

I was in a stroke, and at the age of eight or nine, inventor of the first living bracelet. No scientist discoverer of an antibiotic could have been more enchanted than I.

At once I commercialised. I sold tickets to my friends. The ducks dragged one another around in all directions.

Father came home and witnessed my venture into the world of science.

'You cruel little devil!' He broke his unopened umbrella across my back. I scampered away with a cry of fright and a burst of tears.

'Dad' I said, 'you cut open animals all day long in your laboratory. What did I do wrong?'

He looked at his broken umbrella. He saw I had a point. Tears came to his eyes.

It was the only violence I ever experienced at his hands.

So I was destined never to become a laboratory technician-unless my experience with women can be called such."

(Taken from Errol Flynn's autobiography entitled My Wicked, Wicked Ways.)
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Abstract

In the rat, magnocellular oxytocin neurones within the supraoptic nucleus (SON) become both tolerant to and dependent upon the central inhibitory actions of morphine (Pumford et al., 1991 J. Physiol. 440: 437). Previous studies have shown that tolerance can be demonstrated at the level of the single neurone and may be a result of μ-opioid receptor downregulation. However dependence as shown by withdrawal excitation of neurones upon administration of the opioid antagonist naloxone would appear to be a property of an intact neuronal network. Withdrawal excitation of oxytocin neurones is mediated by a variety of excitatory inputs which converge upon the neurones to share a limited number of post-effector mechanisms leading to transduction of the electrical event. During morphine treatment normal excitability returns to the oxytocin neurone probably by increasing the contribution of excitatory input. Removal of morphine inhibition by naloxone may leave an upregulated system to respond in typical hyperexcitatable fashion. This study has focussed on the inputs responsible for driving oxytocin neurones during the withdrawal process.

Systemic administration of sulphated cholecystokinin octapeptide (CCK8S) activates central noradrenergic pathways (including possibly the A6 cell group of the locus coeruleus which projects to the SON) and this stimulus to oxytocin secretion into the blood measured by radioimmunoassay was dose-dependently inhibited by the α2-adrenergic agonist clonidine and by morphine; α2- and μ-receptor mediated effects may converge on the same post-receptor mechanism. Cells of the locus coeruleus also show tolerance and dependence to opioids. Withdrawal hypersecretion of oxytocin was significantly attenuated by clonidine, possibly by pre-synaptically blocking the
noradrenergic input from the A6 cell group although activation of this pathway by CCK8S alone did not initiate withdrawal.

Magnocellular oxytocin neurone action potentials exhibit a Ca$^{2+}$ dependent shoulder on the repolarisation phase. Withdrawal excitation of oxytocin neurone firing rate resulting in secretion of oxytocin into blood from the neurohypophysis, and release of oxytocin within the SON measured by microdialysis was attenuated by prior intracerebroventricular (ICV) administration of the L-type Ca$^{2+}$ channel blocker verapamil, indicating involvement of L-type Ca$^{2+}$ channel activation in this excitation. Oxytocin is excitatory to its own release within the SON. ICV injection and infusion of a specific oxytocin antagonist reduced withdrawal hypersecretion of oxytocin into blood implicating a requirement for autofacilitation of oxytocin release for the withdrawal process to be sustained. CCK is co-released with oxytocin from magnocellular neurones and has been proposed to act as an endogenous opioid antagonist. CCK8S opposed the inhibitory actions of morphine on firing rate of putative oxytocin neurones in the hypothalamic slice whilst CCK8S binding site density measured by receptor autoradiography was increased within the SON of morphine dependent rats suggesting that endogenous CCK mechanisms may be upregulated during morphine dependence.

Thus withdrawal excitation of supraoptic oxytocin neurones may involve an excitatory noradrenergic input from the A6 cell group which may become more active during dependence. Tolerance to opioids on excitatory inputs to the SON and within the SON itself may increase the expression of several components excitatory to oxytocin neurones such as endogenous CCK function. An amplification in release of noradrenaline within the SON after removal of central morphine inhibition by naloxone, may then excite oxytocin
neurones directly by increasing a voltage dependent Ca$^{2+}$ conductance. Co-release of oxytocin and CCK would feed back onto the oxytocin neurones and sustain the withdrawal process, whilst the increase in synaptic drive from the A6 cell group and locally within the SON would continue until release of oxytocin and CCK or a pool of readily available Ca$^{2+}$ had become depleted thereby bringing the withdrawal process to an end.
INTRODUCTION
1.1. Morphine; a historical perspective

The opium poppy *Papaver Somniferum* ('the poppy that brings sleep') through its chemical products which have potent analgesic actions, has eased human suffering for thousands of years, but has also caused considerable misery throughout history, through widespread abuse of the euphoric properties of the same products. A great deal of governmental effort has gone, unsuccessfully, into controlling its cultivation and distribution, and so a greater understanding of the physical and chemical properties of the constituents is obviously required.

The opium poppy has provided one of the greatest of all pain killers. Morphine, in the form of opium has been employed in medicine in the Eastern Mediterranean, the Middle East and Western Asia since the earliest times. Extraction of the opium, or latex, is achieved by cutting the green capsule with a small sharp implement and scraping off the soft material which exudes over the next twenty-four hours. The poppy remains the prime source of morphine and the capsules contain at least twenty-five alkaloids, four of which have been exploited medicinally- the phenanthrene derivatives morphine and codeine, and the benzylisoquinoline derivatives, papaverine and noscapine (Jaffe and Martin, 1990).

Archaeological evidence suggests that over 5000 years ago in Mesopotamia the Sumerians treated various illnesses with medicines obtained from the poppy. Such skills were to be inherited and practised by the Assyrians and Babylonians whilst the ancient Egyptians considered the poppy so important that pharaohs' were entombed with it at their sides (Zackon, 1988). Egyptian traders were to sail across the Mediterranean to Greece and Rome where opium was often regarded as a gift from the Gods.
as referred in Homers' Odyssey- "Into the wine they were drinking, Helen, of the line of Zeus cast an Egyptian drug which melted sorrow and sweetened gall, which made men forgetful of their pains." The Greek physician Hippocrates, often regarded as the father of modern medicine prescribed opium frequently for relief from medical ailments. Another Greek physician, Galen, who practised in the Roman Empire in the 2nd century, created a recipe called mithridate, by standardising the preparation of opium.

By the 7th century the Turks had succeeded in cultivating large crops of the poppy and Arab merchants, who traded with peoples and new markets in India and China provided an economic incentive for its cultivation.

The drinking or eating of mild opium potions to ease the pain of minor ailments continued for centuries although by the 14th century Arab scientists had noted that continued use of the drug led to a deterioration of the users' mind, with common knowledge that a single overdose could lead to death (Zackon, 1988).

In Europe, the strict medical doctrine of the Catholic church saw a decline in use of the opium poppy, until the 16th century when Paracelsus, a Swiss physician and chemist with remarkable insight, created a potion called laudanum, whose principal ingredient was opium (Sigerist, 1941). His fantastic claims for the rejuvenating powers of laudanum were to attract a widespread following and throughout the Western world laudanum was eventually to become one of the most popular and abused medications of all time. However until the 18th century the availability of strong opium in Europe was still limited until a new era was to dawn with increases in trade.

Under the auspices of the British Crown the British East India Company in its endeavours to maximise English trade in India and the far East was to
saturate the markets of Southeastern Asia at this time, and although the use and frequent abuse of opium contravened many strongly held religious beliefs and cultural values in these areas they were no match for the profit-seeking traders. Indeed, the British were also to acquire an opium habit of their own where chemists and pharmacists were only too willing to praise the virtues of the drug to a naive market. Although doctors were aware of the contraindications for the drug, it was difficult to document social health problems at the time and many were frequent consumers themselves, such that even educated opinion (along with valuable revenue for the Government) would render a cheap and plentiful availability of the drug (Berridge, 1977).

In 1803, Frederick Sertürner, a German scientist, derived from opium a crystal alkaloid so potent that extremely small amounts could produce pronounced effects. Sertürner was to call this chemical morphine after Morpheus (the Greek god of dreams and sleep), (Jaffe and Martin, 1990). Morphine, even more so than opium, would transform the practice of medicine over the following 20 years and with it the experience of drug addiction.

The nature of morphine abuse was to be further aggravated by the development of the hypodermic syringe by Dr. Alexander Wood of Edinburgh and its refinement in the 1840's and 1850's. The hypodermic syringe made possible the administration of morphine by injection, which was much more quickly absorbed into the bloodstream, than the enteral route, giving maximum intensity of effect extremely rapidly. This method of morphine administration was initially practised only by doctors, since intravenous injection required a degree of experience and skill, but such was their
affirmed confidence in this mode of administration that soon patients were able to obtain hypodermic syringes with appropriate instruction. The rapidity and degree of euphoria experienced with injection led to a new populus who were to become addicted to opiates. In the United States, this method of administration was to render the over-use of morphine in the American Civil War as the "army-disease" (it was taken medicinally as a painkiller to combat dysentery).

Not everyone however, was so eager to celebrate the drug-induced euphoria of opiate addiction. The British writer Thomas de Quincey published in 1822 a mesmerising account of opiate addiction called "Confessions of an English Opium Eater." This is his personal confession of how he was to acquire a need for laudanum that transgressed the bounds of mere medicinal use and eventually led him to living a nightmare. His intimate description documented the requirement for increasing quantity of laudanum to achieve a given state of euphoria with repeated administration, and the mental and physical trauma associated with cessation of use (Schiller, 1976).

The smoking of raw opium in China at this time heralded a massive increase in the trade of opium from the British traders to the Chinese. So huge was the market and its profits and such was the Emperors' contempt for the trade that in 1839 the Opium War began. The Chinese effort was futile and in 1852 a second war began and again, failure. At this point in history imperial exploitation was at its very peak (Zackon, 1988).

The incidence of opiate dependence grew dramatically in the United States along with the rapid expansion and dispersion of its population. Legislation came in the form of the Narcotic Act of 1914 but the problem was never fully eradicated. Similarly in the United Kingdom the Dangerous Drugs
Introduction

Act of 1920 was imposed to try and curb the problem of dependence.

Earlier in 1874, a British chemist, C.R. Wright synthesised diaceytlmorphine or diamorphine from morphine. This compound aceylated at both -OH groups was to receive a wider audience 25 years later when in 1898 Heinrich Dreser synthesised diamorphine and gave it its common name, heroin.

Heroin is catabolized to morphine in the body and because of its greater lipid solubility it crosses the blood-brain barrier quicker than morphine and so gives a more intense euphoria or "rush" than morphine. Heroin is reputed to be less emetic than morphine, another added attraction to abusers of the drug. However there is no evidence that heroin differs from morphine in either its respiratory depressant effect or in its liability to cause dependence (Jaffe and Martin, 1990).

1.2. Chemical Aspects of Opioids

![Chemical structure of morphine](image)

Figure (1.2.1.)- Chemical structure of morphine.

Morphine is a substituted phenanthrene molecule, with two planar rings and two aliphatic ring structures which occupy a plane roughly at right angles to the planar rings (Fig. 1.2.1.).
The other opiate used throughout the studies in this thesis is the general antagonist naloxone, which preferentially blocks $\mu$-receptors, but also the $\delta$ and $\kappa$ types (Kosterlitz, 1985). Naloxone differs from morphine at position 6 where the alcoholic OH is substituted by an oxygen atom, lacks a double bond between carbon atoms 7 and 8 and at the nitrogen atom at position 17 where the methyl group is replaced with a -CH$_2$CH=CH$_2$ extension.

1.3. Pharmacology of Opioids

It was postulated that morphine and its derivatives produce their effects by binding to specific receptor sites in the central nervous system (CNS) and elsewhere. Morphine is an extremely effective analgesic acting within the CNS on pain pathways to reduce the sensation of pain and the associated distress. As mentioned previously morphine induces a strong feeling of happiness and relaxation (euphoria) which is an important component of its analgesic effect, and this euphoria is particularly intense when morphine is injected intravenously, leading in part to the use of opiates solely for recreational purposes. For several minutes a pleasant surge, reportedly felt initially in the abdomen, spreading throughout the body occurs, before these feelings fade and are followed by several hours of gradually decreasing sensation. Intravenous injection can often result in vomiting in the non-habitual user (Jaffe and Martin, 1990).

Morphine is a powerful depressant slowing the pulse and acting on respiratory centres in the brain stem to depress breathing rate. Lethal effects of morphine are almost always a result of respiratory depression. Thus naloxone is used clinically to antagonise such agonist effects in the case of opiate overdose, until the patient resumes a normal respiratory pattern (Jaffe and Martin, 1990).
Propulsive contractions of the small intestine are markedly reduced by morphine, and in the colon peristalsis is abolished by direct actions on local opioid receptors. Consequently constipation is a common feature of morphine administration (Jaffe and Martin, 1990).

1.4. Opioid receptors and endogenous opioid peptides

A family of opioid receptors was first postulated by Martin in 1967 to explain the dual action of nalorphine in humans, where although analgesic, it was also capable of antagonising morphine analgesia. It was concluded that this effect was mediated by the $\kappa$-receptor (Martin, 1967). Differing pharmacological profiles and binding site characteristics of opiates in neurophysiological and behavioural tests in the spinal dog (Martin, 1976), and in the guinea-pig ileum and mouse vas deferens preparations (Lord et al., 1977), have indicated the existence of three classically defined opioid receptors, termed $\mu$-, $\delta$- and $\kappa$ (Goldstein and James, 1984; Paterson et al., 1983; Zukin and Zukin, 1984). Morphine binds preferentially to the $\mu$-receptor with little relative affinity for the $\kappa$- or $\delta$-subtype of opioid receptor (Kosterlitz, 1985). Recently the $\delta$-opioid receptor has been cloned by functional expression (Evans et al., 1992) and appears to show closer homology with the somatostatin receptor, than with a putative $\kappa$-receptor which has also been cloned (Xie et al., 1992; Meng et al., 1993); although this receptor is also a member of the G protein-coupled receptor family.

It is now firmly established that there are three major classes of endogenous opioid peptides. The enkephalins (Hughes et al., 1975), endorphins (Bradbury et al., 1976) and dynorphin-related peptides (Goldstein et al., 1981) arise from three separate precursor molecules encoded by different genes (Weber et al., 1983) which have some differential receptor
specificities (see Table 1.4.1.).

<table>
<thead>
<tr>
<th>Polypeptide precursor</th>
<th>Principal endogenous ligands</th>
<th>Exogenous ligand</th>
<th>Receptor specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proenkephalin</td>
<td>[Met$^5$]-enkephalin</td>
<td>DADLE</td>
<td>$\delta &gt; \mu$</td>
</tr>
<tr>
<td></td>
<td>[Leu$^5$]-enkephalin</td>
<td></td>
<td>$\delta &gt; \mu$</td>
</tr>
<tr>
<td></td>
<td>[Met$^5$]-Arg-Gly-Leu-enkephalin</td>
<td></td>
<td>$\mu, \delta &gt; \kappa$</td>
</tr>
<tr>
<td>Proopiomelanocortin</td>
<td>$\beta$-endorphin</td>
<td>Morphine</td>
<td>$\mu, \delta$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DAGO</td>
<td></td>
</tr>
<tr>
<td>Prodynorphin</td>
<td>Dynorphin $(1-17)$</td>
<td>U50, 488H</td>
<td>$\kappa &gt; \mu, \delta$</td>
</tr>
<tr>
<td></td>
<td>Dynorphin $(1-13)$</td>
<td></td>
<td>$\kappa &gt; \mu, \delta$</td>
</tr>
<tr>
<td></td>
<td>Dynorphin $(1-8)$</td>
<td></td>
<td>$\kappa &gt; \mu, \delta$</td>
</tr>
<tr>
<td></td>
<td>[Leu$^5$]-enkephalin</td>
<td></td>
<td>$\delta &gt; \mu$</td>
</tr>
</tbody>
</table>

Table 1.4.1.- Pharmacological profile of opioid peptides.

The longer dynorphin-related peptides are more stable and probably act as long-acting diffusible neurotransmitters, whereas the shorter forms and the enkephalins are subject to rapid hydrolysis by various peptidases, indicative of a typical neurotransmitter function (McKnight et al., 1983).

1.5. Morphine Tolerance and Dependence

Tolerance is defined as a requirement for an increase in dose of a particular drug to elicit a pre-determined pharmacological effect or, after repeated administration a given dose of the drug produces a decreased effect (Jaffe, 1990). Physical dependence develops with repeated administration of a drug, whereupon the neuronal system in question
undergoes a form of adaptation to the drug, thereby resulting in the manifestation of an associated characteristic withdrawal response upon removal of the drug (Jaffe, 1990).

With the realisation that different physiological responses are effected by the binding of an opiate to a proposed particular receptor subtype it has been deemed plausible that the analgesic component of an opiate such as morphine could be differentiated from the component which renders a subject dependent upon it (Woods et al., 1992). This however requires a full understanding of the cellular mechanisms underlying these distinct phenomena. As far as dependence is concerned this has been well characterised in a number of neuronal systems such as rat locus coeruleus cells and the guinea pig myenteric plexus.

Tolerance and dependence to the actions of morphine and opiates in general at the cellular level can potentially involve receptor down-regulation and/or internalisation, altered ionic conductances (particularly Ca\(^{2+}\)), modulation of receptor coupling with intracellular second messenger systems and increased activity of proposed endogenous antagonists. It should be noted however that although dependence has a cellular component it is extremely difficult to demonstrate in neuronal cell lines and isolated preparations since an important aspect of dependence appears to be derived from excitatory synaptic inputs which converge upon the neurone to share a common pool of second messenger systems leading to neuronal activation. It has been speculated that changes in the balance of these inputs modulate these second messenger systems to initiate dependence so that removal of the inputs renders its demonstration impossible.

Opiates have been shown to inhibit acutely adenyl cyclase, the enzyme
Introduction

responsible for the synthesis of cAMP (Sharma et al., 1975), and an accumulation of evidence suggests that this and other acute opiate actions are mediated by G proteins (Aghajanian and Wang, 1986). The ability of opiates to regulate neuronal activity can be attenuated by treatment with pertussis toxin (Nestler et al., 1989). This selectively inactivates two types of G protein designated G_i and G_o, by catalysing the ADP-ribosylation of their α subunits and it has been proposed that neuronal μ-receptors may be linked to these G proteins (Ueda et al., 1990). In these same neurones binding to μ- and δ-opioid receptors activates a K⁺ conductance responsible for prolonged after hyperpolarisation (North and Williams, 1985). This in turn contributes to a shortened action potential and may attenuate an associated Ca²⁺ conductance thereby slowing down gene transcription and turnover of essential neurotransmitters (North, 1989). Activation of κ-receptors reduces Ca²⁺ conductance but is without effect on the K⁺ conductance suggesting a direct mode of action (North, 1986).

Several studies have shown that voltage-dependent Ca²⁺ channel blockers such as the phenylalkylamines and modulators (typified by the dihydropyridines) diminish morphine tolerance (Contreras et al., 1988) and attenuate the behavioural signs associated with morphine withdrawal in rats (Bongianni et al., 1986; Baeyens et al., 1987) and mice (Barrios and Baeyens, 1991). In vitro they reduce naloxone-precipitated [³H]-noradrenaline release from cortical slices of dependent rats (Pellegrini-Giampietro et al., 1988). This is accompanied by an increase in dihydropyridine binding after prolonged morphine treatment (Ramkumar and El-Fakahany, 1988).

In the locus coeruleus opiate withdrawal dramatically increases firing rate
of the neuronal population and this is defined as a physical demonstration of dependence (Rasmussen et al., 1990). Another aspect to this response, is the expression of c-fos mRNA and subsequently of Fos protein within the locus coeruleus (Hayward et al., 1990). The proto-oncogene c-fos is an immediate early gene which serves to transmit information within and between cells by encoding the general transcription factor protein product Fos, which is induced by various enviromental signals (Morgan and Curran, 1991). Thus immunohistochemical staining for Fos within the nuclei of cells, can be used as an indicator of neuronal activity in response to a variety of stimuli at the level of the single cell (Morgan and Curran, 1991).

1.6. Oxytocin; a historical perspective

After it had been shown that intravenous injection of an extract of whole hypophysis into a dog displayed vasopressor activity (Oliver and Schafer, 1895), Sir Henry Dale whilst investigating the physiological actions of ergot in the cat accidentally discovered the uterotonic action of neural lobe extract (Dale, 1906). Dale was attempting to show that the pressor activity of the extract could be reversed by ergot. However, the cat from which the recordings were being taken happened to be in the early stages of pregnancy with the result that the extract not only retained its pressor activity in the presence of ergot, but also produced a strong contractile response of the uterus. Interest in the active components contained within the neural lobe extracts was stimulated further by Ott and Scott (1910) who demonstrated the milk-ejection activity of such extracts.

It was questionable at this point in time as to whether the same agent within the neural lobe extract accounted for both its pressor and oxytocic activities. Dudley (1919) was able to separate these activities by extraction
with butanol and to show that they were subject to degradation by proteolytic enzymes, concluding therefore that they were peptidergic in nature (Dale and Dudley, 1921). The progression towards isolating the two active principles of neural lobe extract was continued by Kamn and colleagues in 1928 when they succeeded in isolating highly active pressor and oxytocic fractions (Kamn et al., 1928). The amino acid sequence of oxytocin was elucidated in 1950 (Pierce and Du Vigneaud, 1950) and the structure (Fig 1.5.1.) simultaneously determined by Du Vigneaud and by Tuppy (Du Vigneaud et al., 1953a; Tuppy, 1953).

Figure (1.5.1.)- Structure of the neurohypophysial hormones oxytocin and vasopressin.

The structure of vasopressin was determined shortly afterwards (Fig 1.5.1.), (Du Vigneaud et al., 1953b) and the synthesis of both hormones completed by 1954 (Du Vigneaud et al., 1953a and 1954) laying to rest the controversy as to the number of physiologically active components contained within the neural lobe also referred to as the neurohypophysis. Interestingly,
all the jawed vertebrates examined thus far possess neurohypophysial hormones similar to those of mammals, differing only in amino acid constituents at positions 3, 4 and 8, suggesting that they may have evolved from a common ancestral form which is presumed to be arginine vasotocin.

As understanding of neuronal structure and function improved with clearer morphological and biochemical evidence, the concept of neurosecretion and the hypothalamic origin of oxytocin and vasopressin became firmly established as shown by Bargmann and Scharrer (1951) and Scharrer and Scharrer (1954). By transecting the neural stalk and using the "Gomori stain" for identification purposes they were able to demonstrate a proximal accumulation and a distal depletion of neurosecretory material. They were to conclude that the supraoptic and paraventricular nuclei (SON and PVN) were the site of hormone synthesis and that the neurosecretory material was transported from here by axonal flow to the nerve terminals in the neurohypophysis.

Oxytocin is synthesised as part of a large precursor molecule together with neurophysin I (Schmale and Richter, 1980) and post-translationally modified en route along the axons from its site of synthesis to the neurohypophysial nerve endings (Ivell and Richter, 1984) where they are released separately.

1.7. Electrical properties of magnocellular neurones

Studies concerning the neural control of the neural lobe advanced significantly with work completed by Barry Cross in the late 1950's. Cross made the first electrical recordings from putative magnocellular neurones (Cross and Green, 1959) whilst applying osmotic and other stimuli known to liberate neurohypophysial hormone release. Such recordings were later to be characterised by antidromically activating neurones projecting from the
SON and PVN to the neurohypophysis (Dyball and Koizumi, 1969). Further characterisation, enabling distinction between oxytocin and vasopressin neurones, followed with the discovery by Jonathan Wakerley in the lactating rat that suckling pups showed a stretch response every 5-10 min followed by voracious suckling for approximately 10 s; the first description of the milk-ejection reflex in the rat (Wakerley and Lincoln, 1971a). Thus, antidromically-activated continuously active neurones within the PVN and SON responded to a continuous suckling stimulus with pulsatile secretion of oxytocin (reflecting increased electrical activity in the range of 50-80 spikes/s lasting 2-4 s from a background firing rate of 1-5 spikes/s some 10 s before the rise in intramammary pressure indicative of milk-ejection), (Lincoln and Wakerley, 1971, 1974; Wakerley and Lincoln, 1973).

By exclusion, previous recordings of cells from the PVN in the rat, which fired in phasic bursts, with 20-40 s periods of activity alternated with similar durations of silence and firing rate in the range of 5-15 spikes/s were identified as vasopressinergic since activity did not correlate with the release of oxytocin at milk-ejection (Wakerley and Lincoln, 1971b). More recently this correlation of phasic activity with vasopressin neurones has been demonstrated with staining techniques combined with electrophysiology (Yamashita et al., 1983; Cobbett et al., 1984; Cobbett et al., 1986).

Such a wealth of evidence suggests that magnocellular neurosecretory cells are extremely dynamic as regards firing pattern and release of hormone. Indeed, stimulation of the isolated neural lobe preparation has been used to demonstrate that release of oxytocin and vasopressin increases with stimulation intensity, and can be further augmented by clustering the stimuli into bursts rather than by applying the same number of
stimuli at a fixed frequency (Bicknell, 1988). This optimisation of neurohormone release is more readily appreciated when one looks at the various stimuli which cause release of oxytocin and vasopressin from the neurohypophysis into the bloodstream.

1.8. Established stimuli for oxytocin release

Milk-ejection reflex

The synchronised bursting of oxytocin neurones which occurs during the milk-ejection reflex results in a bolus release of 0.5-1.5 mU of oxytocin from the neurohypophysis. This causes contraction of the myoepithelial cells surrounding the alveoli and ducts of the mammary gland some 10-20 s later, with a corresponding increase in intramammary pressure and subsequent milk let-down.

Injection of intracerebroventricular (i.c.v.) oxytocin increases the frequency and amplitude of these bursts unlike intravenous injection (Freund-Mercier and Richard, 1981, 1984) and can initiate bursts in the anaesthetised lactating rat previously unable to display milk-ejections (Freund-Mercier and Richard, 1984). Furthermore, centrally administered oxytocin promotes rapid recruitment of oxytocin neurones at the beginning of suckling (Moos and Richard, 1988) with evidence that oxytocin is released within the SON during the milk-ejection reflex (Moos et al., 1989; Neumann et al., 1993). Since i.c.v. injection of an oxytocin antagonist can interrupt the milk-ejection reflex (Freund-Mercier and Richard, 1984), the role of endogenous oxytocin in autofacilitating its own release is paramount in promoting the regular bursting activity of oxytocin neurones stimulated by suckling. Oxytocin is probably released from dendritic and axonal processes within the SON (Pow and Morris, 1989) where it can bind to oxytocin binding sites located on the
dendritic processes (Freund-Mercier and Stoeckel, 1993). This autofacilitation may be partly mediated via the bed nucleus of the stria terminalis which contains specific oxytocin binding sites that increase in density during lactation (Insel, 1990) and injection of oxytocin into this region facilitates bursting of magnocellular oxytocin neurones within the SON during milk-ejection (Moos et al., 1991).

Parturition

Oxytocin plays an important role in regulating the progress of parturition in the rat. Uterine responsiveness to oxytocin increases rapidly in late gestation up to the period of parturition (Fuchs, 1984). At parturition oxytocin is released in response to distension of the vagina and cervix (Higuchi et al., 1986b), stimuli associated with the Ferguson reflex and in response to the forceful abdominal contractions which propel the foetus along the birth canal; the foetal-expulsion reflex (Higuchi et al., 1986b; Higuchi et al., 1987). Measurement of plasma oxytocin concentrations following the birth of the first three pups has shown that there is an increase in oxytocin release once parturition has started and that levels continue to increase throughout parturition (Higuchi et al., 1986b; Leng et al., 1987). The electrical activity of antidromically-identified magnocellular oxytocin neurones is similar to that seen during milk-ejection, with an increase in background firing interspersed with periods of bursting activity; 10-32 Hz lasting 6-14 s after each forceful abdominal contraction and short 32-80 Hz bursts lasting 5-12 s some 15-28 s before the delivery of each pup (Summerlee, 1981). Such bursts tend to occur just before delivery of individual pups. Although this bursting activity is associated with pulsatile release of oxytocin in the milk-ejection reflex (Higuchi et al., 1986c) it has been extremely difficult to show using plasma
sampling such a manner of release during parturition, probably due to the short half-life (approximately 90 s) of oxytocin in the plasma (Higuchi et al., 1986c).

Oxytocin and osmotic balance

Considering the homology in structure which is conserved between oxytocin and vasopressin and that approximately one half of the magnocellular cells in the SON and PVN synthesise oxytocin (Rhodes et al., 1981), it is hardly surprising that oxytocin has overlapping actions with vasopressin in addition to its involvement in lactation and parturition. Magnocellular neurosecretory neurones within the SON respond to systemic hyperosmotic stimulation by increasing their electrical activity (Brimble and Dyball, 1977) which leads to release of oxytocin and vasopressin from the neurohypophysis (Brimble et al., 1978, Wakerley et al., 1978). Interestingly, this enhancement in release of oxytocin is achieved by a gradual increase in spontaneous activity rather than intermittent high frequency bursting (Brimble and Dyball, 1977). Hyperosmotic stimulation also leads to increased Fos-like immunoreactivity (Giovannelli et al., 1990) and induces c-fos-mRNA (Hamamura et al., 1992) and eventually oxytocin-mRNA expression within the hypothalamic magnocellular nuclei (Lightman and Young, 1987).

Magnocellular neurosecretory neurones are known to be directly osmosensitive (Leng et al., 1982) and direct osmotic stimulation of the SON using the microdialysis technique has been shown to induce release of vasopressin (Landgraf and Ludwig, 1991; Ludwig and Landgraf, 1992) and oxytocin (Neumann et al., 1993) within the SON as well as into the periphery. Firing rate of supraoptic neurones within isolated slices or explants is increased following perfusion with hypertonic saline (Wakerley et al., 1983)
Introduction

and the associated depolarisation persists in Ca\(^{2+}\)-free media suggesting a post-synaptic location on the neurones themselves (Bourque and Renaud, 1984). Recently isolated magnocellular neurosecretory cells from the SON, devoid of long neuritic processes, have been shown to be directly osmosensitive (Oliet and Bourque, 1993). In addition, the persistence of a reduced osmosensitivity among supraoptic neurones in the absence of synaptic transmission indicates that their osmosensitivity is enhanced through synaptic input from adjacent neurones such as those located in the region anterior and ventral to the third ventricle (AV3V region). Indeed it is necessary for the functional integrity of afferent pathways from regions such as the subfornical organ (SFO) and the AV3V region to be maintained in order for magnocellular neurosecretory neurones to express full osmoreponsiveness (Leng et al., 1989). The SFO has efferent connections with the AV3V preoptic area and the hypothalamus (Miselis, 1981) and a predominantly excitatory influence on the excitability of both oxytocinergic and vasopressinergic neurosecretory cells (Sgro et al., 1984). Ablation of structures in the AV3V region results in reduced secretion of both oxytocin and vasopressin in response to a systemic hyperosmotic stimulus (Leng et al., 1989).

1.9. Modulation of magnocellular neurosecretory activity

It is apparent that magnocellular oxytocin and vasopressin neurones are subject to well defined forms of excitation and inhibition in terms of their expression of firing pattern to a given physiological stimulus. Such regulation is likely to be derived from modulation by local neurotransmitters, of ionic currents responsible for the generation of the firing patterns described.
Introduction

Cholecystokinin

The presence of cholecystokinin (CCK) as immunoreactive 'gastrin-like' material was first demonstrated by Vanderhaeghen and colleagues (Vanderhaeghen et al., 1975) and the majority of this 'gastrin-like' material is present as the C-terminal octapeptide, (CCK (26-33) or CCK8S). This peptide is widely distributed throughout the CNS (Hökfelt et al., 1988; Hökfelt et al., 1991) and has been shown to be immunocytochemically co-localised within magnocellular neurosecretory neurones located within the SON of the hypothalamus (Hökfelt et al., 1988). Two types of receptor are known to exist within rat brain (CCK$_A$ or 'peripheral' receptors and CCK$_B$ or 'central' receptors) as demonstrated by varying sensitivities of a number of preparations to a variety of agonists and antagonists (Innis and Snyder, 1980; Moran et al., 1986), and further verified by their recent cloning (Wank et al., 1992).

The proposed role for CCK acting as a neurotransmitter is now well supported since its exogenous application into the CNS is associated with specific behavioural changes (Crawley, 1985; Moran et al., 1986) and its co-localisation and synthesis within central neurones is associated with functionally identified receptors (Boden and Hill, 1988a; Boden and Hill, 1988b; Jarvis et al., 1992).

CCK is able to selectively evoke release from magnocellular oxytocin neurones via separate peripheral and central sites of action. Systemic injections of CCK8S selectively activate oxytocin-secreting neurones and not vasopressin neurones in a dose-dependent manner via a vagally mediated mode of action (Verbalis et al., 1986; Verbalis et al., 1991; Hamamura et al., 1991; Leng et al., 1991). Lesions in the vicinity of the area postrema
attenuate this response (Carter and Lightman, 1987) and there is evidence to suggest that the principal action of systemic CCK8S is mediated via the nucleus tractus solitarius (NTS), which is adjacent to the area postrema where it has an inhibitory effect on NTS spike discharges (Morin et al., 1983). Consequently the strong noradrenergic components of the NTS which project to the SON release noradrenaline when stimulated by systemic CCK8S, and the noradrenaline is principally excitatory to oxytocin neurones (Yamashita et al., 1987; Kendrick et al., 1991). In vitro electrophysiological studies clearly demonstrate that CCK8S and associated fragmented peptides can directly influence the excitability of magnocellular neurosecretory cells (Jarvis et al., 1992) whilst in vitro autoradiographic studies have shown high-affinity binding for [125I]-CCK8S in the SON which can be modulated by osmotic activation of the hypothalamo-neurohypophysial axis (Day et al., 1989; Hinks et al., 1993). CCK has been proposed to act as an endogenous opioid antagonist in some systems since central administration blocks opioid analgesia (Faris et al., 1983) whilst specific CCK receptor antagonists can enhance morphine analgesia and attenuate development of morphine tolerance (Dourish et al., 1990a).

**Opioid peptides**

Vasopressin synthesising neurones express prodynorphin mRNA (Sherman et al., 1986) and contain prodynorphin products, including [Leu5]-enkephalin (Seizinger et al., 1984). Osmotic activation of vasopressin neurones results in enhanced expression of both provasopressin and prodynorphin mRNA (Lightman and Young, 1987).

Oxytocin synthesising neurones contain [Met5]-enkephalin (an enkephalin not contained in the prodynorphin precursor), (Martin and Voigt, 1981; Martin
et al., 1983a). Although neurohypophysial oxytocin is depleted in response to chronic osmotic stimuli, such stimuli have been shown to be without effect on [Met5]-enkephalin content (Zamir et al., 1985; Nordmann et al., 1986a) whereas content of prodynorphin derived peptides is depleted. However [Met5]-enkephalin neurohypophysial content has been shown to be depleted along with a simultaneous decline in oxytocin content in response to naloxone-precipitated morphine withdrawal whilst having no effect on either vasopressin or dynorphin content within the neurohypophysis (unpublished observations). Furthermore immunocytochemical studies indicate that these opioid peptides are colocalised within neurosecretory granules with their respective neurohypophysial peptide (Martin et al., 1983a; Adachi et al., 1985; Nordmann et al., 1986a).

Early on, opioid binding sites were observed in membranes prepared from bovine hypophysis (Simantov and Snyder, 1977) and comparative studies using rat brain have demonstrated that the high concentration of opioid binding sites is located in the hypothalamus and hypophysis (Castanas et al., 1986). Within the neurohypophysis these opioid binding sites have been characterised as being predominantly or exclusively κ-sites by various groups (Bunn et al., 1985; Herkenham et al., 1986; Falke and Martin, 1986). Within the hypothalamus itself both μ- and κ-binding sites have been shown to be present (Mansour et al., 1986; Morris and Herz, 1986) and although initially only κ-type receptors were found to be present in the SON (Mansour et al., 1988; Tempel and Zukin, 1987), it has since been demonstrated that both μ- and κ-types are present in this nucleus (Sumner et al., 1990).

1.10. Oxytocin and opioid interactions

In magnocellular neurosecretory cells the action potentials display a
Introduction

frequency dependent Ca$^{2+}$ shoulder on the repolarisation phase both in vitro (Bourque and Renaud, 1985b; Bourque et al., 1993) and in vivo (Renaud and Bourque, 1989) which is most probably a primary target of action for neurotransmitters and neuropeptides. Magnocellular oxytocin and vasopressin secreting neurones are subject to regulation by exogenously administered opioids and speculatively by endogenous opioids. Opioids act both at the level of the cell bodies to reduce electrical activity (Arnauld et al., 1983; Wakerley et al., 1983a; Inenaga et al., 1990; Pumford et al., 1991; Bourque et al., 1993) and at the terminals in the neurohypophysis to attenuate release into blood (Iversen et al., 1980; Bicknell and Leng, 1982; Zhao et al., 1988; Russell et al., 1993).

In urethane-anaethetised rats, magnocellular neurosecretory neurones do not appear to be subject to a tonically active input from endogenous opioids since systemic administration of naloxone has very little effect on neuronal firing rate (Shibuki et al., 1988; Bicknell et al., 1988), although naloxone does increase release of oxytocin; indicative of it acting at the neurohypophysis. Both acute and chronic intracerebroventricular (i.c.v.) infusion of morphine initiate a profound inhibition of oxytocin release (Russell et al., 1989a; Pumford et al., 1991) and in lactating rats to failure of milk let-down upon induction of a suckling stimulus from the pups. During chronic morphine treatment, within two days milk let-down returns and after 5 days oxytocin release is normal (Rayner et al., 1988).

In the magnocellular oxytocin system dependence is demonstrated when a morphine tolerant rat is administered naloxone producing a withdrawal excitation of the neurones. This involves a large increase in neuronal firing rate and consequently a hypersecretion of oxytocin from both the dendritic
processes within the SON (Neumann et al., 1993) and from terminals in the neurohypophysis (Bicknell et al., 1988). Since this action of naloxone at the neurohypophysis does not appear to differ between morphine treated and morphine naive animals (Russell et al., 1993) it would appear that tolerance to and dependence upon morphine as regards release of oxytocin develops centrally. Previous reports of i.c.v. morphine having no effect on bursting activity of oxytocin neurones in the lactating rat (Clarke et al., 1979) can probably be explained by a reduced sensitivity to inhibition by opioids as the intensity of stimulation increases (Pumford et al., 1991; Pumford et al., 1993).

Earlier reports that the AV3V region provides a tonic excitatory input to oxytocin neurones necessary for their full expression of the effects of withdrawal have since been modified to suggest that it is at the level of the magnocellular nuclei (both SON and PVN) themselves, or upon afferents other than those from the AV3V region that are the sites of morphine withdrawal with respect to hypersecretion of oxytocin (Russell et al., 1992).

By now much has been mentioned about the extensive interactions between oxytocin, opioids and the mechanisms of tolerance and dependence both in vitro and in vivo. The inhibitory actions of opioids on magnocellular neurosecretory neurones and oxytocin neurones in particular certainly provides an excellent paradigm to both study and understand these phenomena, but what of their physiological relevance in the intact animal?

At the end of gestation in the rat, neurohypophysial content of oxytocin is increased by approximately 30% and is depleted by the same amount thereafter (Fuchs and Saito, 1971) whilst the pulsatile release of oxytocin into the periphery is essential for the normal progress of parturition (Luckman et al., 1993). Since exogenous administration of morphine or U50,488H which
are selective for \(\mu\)- and \(\kappa\)-opioid receptors respectively, can interrupt parturition (Gosden et al., 1985; Douglas et al., 1993) one could speculate on numerous roles for endogenous opioids within the neurohypophysial oxytocin system at this time. Dynorphin\(_{\text{(1-8)}}\) which is selective for the \(\kappa\)-receptor (Corbett et al., 1982) and co-released with vasopressin binds to \(\kappa\)-receptors located on neurosecretory terminals isolated from rat neurohypophyses (Falke et al., 1989) and inhibits stimulated release of oxytocin (Falke, 1988). Similarly in the isolated neural lobe preparation U50,488H inhibits stimulated release of oxytocin (Zhao et al., 1988a) although this action is less pronounced at the end of pregnancy (Douglas et al., 1993). Consequently it would appear that another opioid mechanism (probably related to the \(\mu\)-receptor) is responsible for the central inhibition of oxytocin release during gestation.

**Aim of thesis**

This study has focussed on the relative contributions of excitatory inputs and adaptive cellular changes, both locally within the SON and those located perhaps within the brainstem, postulated to drive magnocellular oxytocin neurones during the withdrawal process, in the rat treated chronically with morphine.
2.1. Animals

Female Sprague-Dawley rats obtained from Bantin & Kingman were used throughout this work, except where specified. They were housed under controlled conditions, ambient temperature 21-23°C with a 12 h light/12 h dark cycle and had free access to food (standard breeder diet) and water. Rats were usually acclimatised for 2-3 weeks after arrival before use in experiments.

2.2. Anaesthesia

1. Urethane

For non-recovery experiments rats were anaesthetised with urethane (ethyl carbamate; 25% weight/volume solution, Sigma, UK). A single i.p. (intraperitoneal) dose of 1.25 g/kg is known to produce a level of anaesthesia which can be maintained for up to 12 h. In experiments involving the milk-ejection reflex a lower dose of 1.1 g/kg was used since this is less likely to impair the occurrence of the milk-ejection reflex.

2. Xylocaine

To supplement the low dose of urethane (1.1 g/kg) in experiments involving the milk-ejection reflex animals were given the local anaesthetic 'Xylocaine 2%' (Lignocaine hydrochloride BP; Astra Pharmaceuticals Ltd, England) sub-cutaneously at sites of surgery.

3. Ether

For recovery experiments involving the induction of morphine tolerance and dependence rats were anaesthetised by ether inhalation (diethyl ether, peroxide free; May & Baker, UK). The rats were anaesthetised in a glass
bowl containing cotton wool soaked in ether and the level of anaesthesia maintained throughout the period of surgery with a cotton wool plug soaked in ether, contained in an open glass vial positioned over the rat's nose.

2.3. Surgical preparation

1. Cannulation of the femoral artery and vein

Portex cannulae (size 3FG; Portex Ltd, Kent) connected to 1 ml polypropylene syringes (Steriseal, Worcestershire) were filled with heparinised saline (50 units per ml isotonic, 0.9% saline). The rat was laid flat on its back and a small horizontal incision was then made just below the rostral left inguinal nipple. The exposed musculature and connective tissue was then gently but firmly teased apart with blunt forceps to reveal the femoral artery, femoral vein and nerve enclosed within a sheath of connective tissue. This sheath was carefully opened with sharp forceps and two silk ligatures (EP 1; Davis & Geck, Lancashire) were placed around the vein. The distal ligature was tightened to prevent venous return. A small incision was made proximal to this ligature and the cannula inserted approximately 1 cm into the length of the vein at which point the proximal ligature was tightened to prevent blood loss. The cannula was then pushed further into the vein until it had passed under the inguinal ligament and if blood could be drawn back the second ligature was fully tightened. The same procedure was followed for cannulation of the femoral artery except that the vessel was clamped rostral to the second ligature to prevent rapid arterial blood loss when the vessel was opened for insertion of the cannula. This clamp was removed enabling the cannula to be fully inserted into the artery. With both cannulae tied in place the wound was closed with 2-3
2. Cannulation of the mammary gland

On Day 7-13 of lactation, a day prior to experimentation, all pups but one were separated from the mother overnight enabling the mammary glands to engorge with milk. Depending on the experimental protocol 1-3 glands (1 abdominal and 2 inguinal) were cannulated. The teat of the mammary gland to be cannulated was stretched and the end secured. The milk duct was exposed and two ligatures (EP 1) placed around it. The tissue duct was incised for the insertion of a polythene cannula (Portex; o.d. 0.96 mm) filled with isotonic saline. The cannula was pushed in until saline passed easily into and out of the gland and milk was seen in the cannula, at which point the ligatures were tightened to secure the cannula. The cannula was connected to a pressure transducer (Model P231d; Gould Stratham Instruments Inc.) relayed to a chart recorder (Model BS 314; Kontron Electronik, GmbH or CR 652S Recorder; J.J. Instruments Ltd, Southampton) for measurement of intramammary pressure changes. Two hours after surgery was complete four doses of 0.1, 0.25, 0.5 and 1.0 or 0.25, 0.5, 0.75 and 1.0 mU (milliunits) synthetic oxytocin (Syntocinon, 10 units/ml diluted in saline to 12.5 milliunits/ml; Sandoz Pharmaceuticals, Middlesex) were each intravenously injected as a bolus in random order 3 min apart to construct dose-response curves for the mammary glands.

3. Induction of morphine tolerance and dependence

Female Sprague-Dawley rats (virgin or lactating) were anaesthetised with ether and placed in a stereotaxic frame. The dorsal surface of the skull was exposed by a skin incision along the midline and the underlying tissue
retracted to expose bregma. Two 1.3 mm diameter holes were drilled through the bone lateral to the cannula placement to hold two stainless steel screws (3.2 mm x 10 BA). A 1.0 mm diameter guide hole was drilled 2 mm right lateral and 3 mm posterior to bregma through which to introduce the infusion cannula. The infusion assembly consisted of a stainless steel cannula made from a 1 cm length of 21 gauge tubing bent at 90° 4.5 mm from its tip and bevelled at 45°. This was attached to an 18 cm length of coiled polythene tubing (1.2 mm o.d., 0.76 mm i.d.; Portex Ltd, Kent) which was attached temporarily at the other end to a 100 μl microsyringe. The whole assembly was then positioned close to the skull and the infusion cannula inserted vertically through the guide hole so that its tip lay in the right lateral cerebral ventricle, 4.0 mm below the skull surface. The cannula was held firmly in place by a mound of dental acrylic (Simplex Rapid; Associated Dental Products Ltd, Swindon) bonded to the two stainless steel screws which were anchored to the skull. An osmotic mini-pump (1 μl/h, Alzet 2001; Alza Corp.) was placed subcutaneously in the subscapular region, the microsyringe detached and the cannula connected to the mini-pump. Three reverse sutures were used to close the wound.

Morphine sulphate B.P. was dissolved in sterile pyrogen-free water and filtered (Milllex - GV, 0.22 μm; Millipore S.A., Molsheim, France) to give a stock solution of 50 mg/ml. All dilutions were made from this stock solution which was kept at 4°C. The osmotic mini-pump was filled with the stock solution of morphine sulphate and left to prime in 0.9% saline overnight at room temperature. Prior to insertion the infusion cannula was filled with 40 μl of a 20 mg/ml morphine solution, a 1 μl air bubble and then 40 μl of a 10 mg/ml morphine solution. Thus the infusion system was filled with morphine
General Methods

sulphate solution in increasing concentration (10 μg/h for 40 h, 20 μg/h for 40 h and 50 μg/h for 40 h) in order to increase the degree of tolerance achieved over 5 days (Bicknell et al., 1988).

4. Blood sampling

In all experiments each blood sample taken was of 0.3 ml volume. The sample was placed into a pre-numbered 1.5 ml polypropylene tube (Eppendorf; Hamburg, Germany), chilled and spun for 2-3 min at 13,000 g (MSE Micro Centaur). A 150 μl aliquot of oxytocin-containing plasma could then be pipetted off and stored immediately on ice. The remaining red blood cells were then resuspended in 150 μl warmed isotonic saline prior to reinjection after the next blood sample had been obtained. In all experiments 0.3 ml isotonic saline was injected straight after the first blood sample had been taken in order to maintain blood volume. At the end of the experiment samples were stored at -20°C prior to radioimmunoassay for oxytocin content.

2.4. Radioimmunoassay for oxytocin

1. Introduction

The radioimmunoassay used to determine oxytocin content (pg/ml) of unextracted rat plasma samples was a modified method from that of Higuchi et al., (1985a). The specificity of the assay was determined by comparing the inhibition of binding of 125I-labelled oxytocin to the first antibody with that of various peptides including arginine-vasopressin and arginine-vasotocin. Melanocyte stimulating hormone-release inhibiting factor had the highest cross-reactivity with the raised antibody at 0.0017%. Recovery of oxytocin after the antibody had been incubated with plasma from non-pregnant or
pregnant rats was 94-107%.

First antibody was added to solutions containing a fixed amount of $^{125}$I-oxytocin and either rat plasma aliquots of unknown concentration or aliquoted oxytocin of known concentration (for preparation of the standard curve) in phosphate buffer. Mixtures were left to equilibrate after which the proportion of $^{125}$I-oxytocin to oxytocin bound to first antibody reflects the concentration of oxytocin present. This conjugate was then precipitated from solution using a second antibody which binds to the first antibody. To further aid precipitation a suspension of white Pansorbin cells was added prior to centrifugation. The supernatant was then aspirated and the radioactivity of the precipitate measured. Radioactivity of the tubes to which known amounts of oxytocin had been added was then used to compile a standard curve against which concentration of the unknown samples could be determined.

2. Materials

Phosphate buffer

Phosphate buffer (pH 7.0) was prepared in double distilled de-ionised water containing;

0.025% (w/vol) sodium dihydrogen orthophosphate NaH$_2$PO$_4$·2H$_2$O (BDH)

0.119% (w/vol) disodium hydrogen orthophosphate Na$_2$HPO$_4$ (BDH)

0.1% (w/vol) sodium azide NaN$_3$ (BDH)

0.5% (w/vol) bovine serum albumin BSA (RIA grade) (Sigma)

(BDH Ltd, Dorset. Sigma Chemical Company Ltd, Dorset).

First antibody buffer

Frozen-stored normal rabbit serum was thawed and diluted (usually 1:400) in phosphate buffer to aid the binding of the second antibody. This
concentration was the lowest concentration of normal rabbit serum that produced the highest % binding of $^{125}$I-oxytocin in the precipitates and was determined from trial assays containing standards only.

First antibody

Rabbit anti-oxytocin antibody THF-3 was kindly donated by Dr. T. Higuchi, stored diluted at 1:200 in phosphate buffer at -20°C and used at a final concentration of 1:120,000 in phosphate buffer.

Second antibody

Donkey anti-rabbit serum was obtained from IDS, Tyne and Wear, stored at 4°C and diluted to 1:100 in phosphate buffer.

$^{125}$I-oxytocin

Iodinated oxytocin was obtained from the AFRC Babraham Institute, Cambridge, stored at -20°C and diluted in phosphate buffer to a concentration calculated to give 6-8000 cpm/50 µl aliquot.

Standardised Pansorbin cells

These were supplied as a 10% (w/vol) solution in phosphate buffered saline containing 0.1% (w/vol) sodium azide, stored at 4°C, diluted 1:25 in phosphate buffer and obtained from Novabiochem (UK) Ltd, Nottingham.

3. Methods

In order to determine the unknown oxytocin concentration of rat plasma samples a standard curve of precipitated radioactivity versus oxytocin concentration was compiled. Previously lyophilised stock oxytocin aliquots containing 10 µg oxytocin (Sigma) stored at -70°C were used. On the morning of assay phosphate buffer was added to the lyophilised oxytocin to
obtain a top standard dilution of 2500 pg/ml. A further ten serial doubling dilutions of this solution yielded a range of concentrations from 2.44-2500 pg/ml, which were then used to compile the standard curve. Three further standards were also required for preparation of the curve:

TC, contained an aliquot of $^{125}$I-oxytocin alone to determine Total Count of radioactivity added.

NSB, contained aliquots of $^{125}$I-oxytocin and second antibody to determine Non-Specific Binding of radioactivity.

B$_0$, contained aliquots of $^{125}$I-oxytocin, first and second antibody to determine the maximum percentage binding of radioactivity.

Next, experimental plasma samples were removed from the freezer as required, thawed to 4°C and centrifuged for 1-2 min at 15,000 g (Haemofuge, Heraeus-Christ, Osterode, Germany) before being aliquoted into the appropriate tube. The assay was performed in pre-numbered 0.75 ml plastic tubes (LP2 tubes, Luckham Ltd, Sussex). Standards were assayed in triplicate and intraassay, interassay standards and rat plasma samples in duplicate. Any samples which were expected to contain a concentration of oxytocin in excess of 500 pg/ml were assayed also, diluted appropriately (usually 1: 3) in phosphate buffer. The protocol for the addition of reagents to the tubes is shown in Table 2.4.3.1. overleaf:
General Methods

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC: nothing</td>
<td>50 μl ¹²⁵I-oxytocin was</td>
<td>100 μl antibody buffer and 100 μl antibody buffer were added to all tubes except TC.</td>
<td>50 μl pansorbin cells were added to all tubes except TC.</td>
</tr>
<tr>
<td>NSB: 100 μl RIA buffer</td>
<td>added to all tubes.</td>
<td>second antibody were added to all tubes except TC.</td>
<td></td>
</tr>
<tr>
<td>B₀: 50 μl first antibody solution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standards/samples: 50 μl first antibody solution, 50 μl standard/plasma sample.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4.3.1.

After addition of reagents on the appropriate day, tubes were vortex mixed, covered in aluminium foil and left for the specified time at 4°C. On the final day after all tubes had been vortex mixed (except TC) they were then centrifuged for 30 min at 3,000 rpm and 4°C (Minifuge 2, Heraeus-Christ, Germany). The resulting supernatant was carefully aspirated and the radioactivity of each suspended pellet then measured for 3 min in a gamma scintillation counter (LKB-Wallac 1272 Clinigamma, Wallac Oy, 20101 Turku 10, Finland).

4. Calculation of radioimmunoassay results

Assay Sensitivity

The sensitivity of an assay, which provides information as to the minimum acceptable peptide content per sample was determined using the following equation;

\[
\frac{[B_0 - (2 \times SD) - NSB]}{B_0 - NSB}
\]

where SD is the standard deviation around the mean of \( B_0 \). The value
obtained from this equation was then compared against a series of values for
% $B_0$ radioactivity bound by each standard of known concentration. The %
$B_0$ radioactivity bound by each standard (2.4-2500 pg/ml) was calculated
from the equation;

\[
\frac{\text{mean standard cpm} - (2 \times \text{SD } B_0) - \text{NSB}}{B_0 - \text{NSB}}
\]

Assay sensitivity was taken at the point where the value obtained using
the first equation was greater than those values obtained for the standards
using the second equation and converted to a concentration in pg/ml.

**Inter- and intraassay variation**

Variability between samples of comparable experiments from different
assays could be determined from known concentrations of hormone which
were stored in aliquots at -20°C; and for each assay six duplicates of each
concentration (three at the beginning of the assay and three at the end) were
included. This also allowed any intraassay variation to be determined. In
practise, all samples from any experiment were measured in a single assay.

**2.5. Microdialysis**

1. **Introduction**

The microdialysis sampling technique can be used for the *in vivo*
measurement of release of specific neurotransmitters during particular
behavioural and neuroendocrine events. In the context of this work
microdialysis has been used to sample oxytocin release within the supraoptic
nucleus upon a particular challenge to the magnocellular oxytocin system.
The microdialysis probe has a length of semi-permeable tubing connected to
separate inflow and outflow cannulae. Artificial cerebrospinal fluid (aCSF)
can then be pumped through the closed environment of the probe and as the membrane is semi-permeable and the concentrations of peptide in the tissue are greater than in the dialysate, a concentration gradient is established enabling small amounts of peptide to pass from the extracellular compartment across the membrane and into the aCSF pumped through the probe. The sample can then be collected at the outflow of the probe and peptide content determined by radioimmunoassay.

2. Probe Specifications

The dialysis probe consisted of a 1.3 mm U-shaped dialysis fibre (molecular weight cut-off 5 kDa, 3 mm total length and 0.2 mm outside diameter; probe diameter 1 mm), glued into the ends of two pieces of parallel attached 24-gauge stainless steel tubing connected to a syringe pump (flow rate 3 µl/min) via polythene tubing (PE 20; Beckton Dickinson, New Jersey, USA). The microdialysis fibre was threaded with a fine stainless steel wire to strengthen and guide the fibre through the brain tissue.

*In vitro* recovery of oxytocin has been tested and estimated previously (Neumann et al., 1993). Typical recovery of oxytocin for these microdialysis probes, which were similar to the those used in the studies outlined here were 1.99% for [³H]oxytocin and 1.62% for [¹²⁵I]oxytocin.

3. aCSF composition

The probe was perfused with either normal aCSF (pH 7.2, mM); NaCl 138, KCl 3.36, NaHCO₃ 9.52, Na₂HPO₄ 0.49, urea 2.16, CaCl₂ 1.26, MgCl₂ 1.18) or aCSF made hypertonic (0.5 M) by adding NaCl.

4. Surgery

Lactating or virgin female rats were anaesthetised with ether or urethane
depending on whether the experimental protocol was of a recoverable or non-recoverable nature respectively. Animals were placed in a stereotaxic frame, holes drilled through the skull and a small slit was made in the dura using a sharp needle. Microdialysis probes were positioned into the left or both left and right supraoptic nuclei 1.1 mm posterior to bregma, 1.8 mm lateral to midline and 9.1 mm below the surface of the skull. They were then anchored to the skull with two stainless steel screws and dental acrylic.

5. **Histological and morphological verification of probe location**

At the end of an experiment brains were removed, fixed in formal saline and serial frontal 60 μm Vibratome sections cut to locate the placement of the microdialysis probe histologically (see Fig 2.5.5.1.). In addition either during or at the end of an experiment direct hyperosmotic stimulation (0.5 M NaCl aCSF) of the perfused area via the probe was used in order to verify its placement in the region of the SON. It has previously been demonstrated that the 'rebound effect' (where oxytocin content increases in the dialysate containing isotonic aCSF following hyperosmotic stimulation) is a reliable indicator of probe placement within the SON (Neumann et al., 1993). Both methods were analysed independently and then taken in conjunction to positively verify probe placement within the SON.

6. **Radioimmunoassay**

Acidified (10 μl 0.1 M HCl) microdialysates were lyophilised and oxytocin content measured without extraction by specific radioimmunoassay as previously described (Neumann et al., 1993). Typical assay sensitivity was 0.1 pg at 95% binding (50% inhibition occurring at approximately 4.0 pg). The intraassay coefficient of variation was <10%. To eliminate interassay
variation, all dialysates to be compared were measured in the same assay. Oxytocin radioimmunoassays of microdialysates were expertly carried out by Frau Brigitte Wolff at the University of Leipzig, Germany.

2.6 Drugs

Morphine sulphate B.P. was obtained from Edinburgh Royal Infirmary.

CCK8S (26-33) was purchased from Palliard (Palliard Chemical Co, Sudbury, UK) and from Bachem (Bachem, Saffron Walden, UK).

± Verapamil hydrochloride was purchased from Sigma.

Naloxone hydrochloride was purchased from Sigma.

U50, 488H was purchased from Sigma.

2.7. Statistics

Unless otherwise stated, the non-parametric Wilcoxon Paired test was used to test for statistical differences within groups of animals and the Mann-Whitney U-test for differences between groups of animals. p<0.05 was considered statistically significant. Data are expressed throughout as mean ± SEM.
Figure (2.5.5.1.)- Representative 60 μm coronal brain section showing bilateral location of microdialysis probe tracts (long arrows) within or immediately adjacent to the SON (short arrows). Scale bar=1mm.
DETERMINING THE ROLE OF THE L-TYPE VOLTAGE-ACTIVATED Ca^{2+} CHANNEL BLOCKER VERAPAMIL ON SELECTED STIMULI TO OXYTOCIN RELEASE
3.1. INTRODUCTION

Calcium ions (Ca\(^{2+}\)) play a fundamental role in the regulation of synaptic processing of information which is primarily mediated by exocytotic release of various neurotransmitters (Perney et al., 1986). The role of Ca\(^{2+}\) in cellular functioning cannot be overemphasised, since it serves to regulate enzymatic activity such as kinases and phosphatases, ion channel function (Blatz and Magelby, 1987) and expression of immediate early genes such as c-fos (Morgan and Curran, 1991).

Ca\(^{2+}\) homeostasis

Intracellular Ca\(^{2+}\) acts as a second messenger and transduces electrical activity in neurones into biochemical events (McBurney and Neering, 1987; Scott et al., 1991). There are two mechanisms which when activated, produce an increase in intracellular Ca\(^{2+}\) concentration. The first involves Ca\(^{2+}\) entry from the extracellular environment via voltage-activated Ca\(^{2+}\) channels, ligand gated channels and second messenger operated channels; the result is membrane depolarisation and activation of Ca\(^{2+}\) dependent processes. The second involves release of Ca\(^{2+}\) from intracellular stores (mitochondria, endoplasmic reticulum) which may be activated directly by extracellular Ca\(^{2+}\) or indirectly via ligand-receptor binding and the associated post-effector signal transduction mechanism (G proteins), giving rise to intracellular messengers and further processing of information. The resting intracellular Ca\(^{2+}\) concentration may be 10^4 fold lower than the immediate extracellular concentration. Intracellular Ca\(^{2+}\) is kept low by Ca\(^{2+}\) binding buffering proteins (e.g. calmodulin, parvalbumin) which vary in concentration within different tissues, uptake into Ca\(^{2+}\) stores and export back into the
extracellular environment by cation exchange and active transport pumps.

It is hardly surprising therefore given this range of functions which Ca\(^{2+}\) has within a cell, that multiple types of Ca\(^{2+}\) channels can coexist within the same cell. Four types of Ca\(^{2+}\) channel have been identified according to their biophysical properties (Tsien et al., 1991). It is widely accepted that there are two classes of voltage-activated Ca\(^{2+}\) channels as defined by the pattern of channel activation: low voltage-activated, also termed T (transient) and high voltage-activated channels, which include the remaining three subclasses; L (long-lasting), N (neuronal) and P (Purkinje cell), (Bertolino and Llinás, 1992; Catterall and Striessnig, 1992). More recently a high voltage-activated Ca\(^{2+}\) channel with novel functional properties has been identified in rat cerebellar granule neurones (Ellinor et al., 1993).

**Characteristics of L-type channels**

L-type Ca\(^{2+}\) channels are not strongly inactivated by depolarisation to -40mV, have the largest single-channel conductance among the voltage-activated Ca\(^{2+}\) channels and have both a slow voltage-dependent, and a more prominent Ca\(^{2+}\) dependent mechanism of inactivation.

Studies with monoclonal antibodies specific for the L-type Ca\(^{2+}\) channel have revealed a wide distribution throughout the CNS. Microfluorometric imaging studies have reported their localisation on the cell bodies and dendrites of neurones (Thayer et al., 1987).

The properties of voltage-activated L-type Ca\(^{2+}\) channels are altered by a number of Ca\(^{2+}\) channel antagonist drugs. There are two main groups- the phenylalkylamines typified by verapamil and the dihydropyridines typified by nifedipine and nitrendipine (Catterall and Streissnig, 1992). The dihydropyridines are modulators of the L-type Ca\(^{2+}\) channel exhibiting
antagonistic and agonist modes of action. They bind with high affinity to inactivate Ca$^{2+}$ channels and bind to their receptor site from the extracellular surface of the Ca$^{2+}$ channel. The phenylalkylamines have a more profound inhibitory effect on Ca$^{2+}$ channel function as the channels become more activated by depolarising pulses, indicating that they gain more rapid access to the receptor site when the channel is open (Lee and Tsien, 1983). It has been suggested that the phenylalkylamines bind to an intracellular binding site to occlude the channel pore (Catterall and Striessnig, 1992).

The L-type Ca$^{2+}$ channel is present in high concentrations in the transverse tubules of vertebrate skeletal muscle, which has enabled their purification using high-affinity dihydropyridine Ca$^{2+}$ channel antagonists which isolate the protein constituents of the channel. These channels are composed of five protein subunits. The $\alpha_1$ subunit is the central functional component of the unit and contains the dihydropyridine and phenylalkylamine binding site (Tanabe et al., 1987). $\alpha_1$ subunits are present in a complex with four remaining subunits designated $\alpha_2$, $\beta$, $\gamma$ and $\delta$. There are in turn, five major classes of $\alpha_1$ subunits described in mammalian neurones (termed A, B, C, D and E), and functional studies are beginning to associate the biophysical properties of these cloned Ca$^{2+}$ subunits with the individual Ca$^{2+}$ channel types previously mentioned (Zhang et al., 1993).

**Opioid receptor interaction with Ca$^{2+}$ channels**

A number of studies have described the interaction of $\mu$-opioid agonists such as morphine with Ca$^{2+}$ channels in vitro (North, 1986; Pelligrini-Giampietro et al., 1988; Kennedy and Henderson, 1991; Kennedy and Henderson, 1992; Carter and Medzihradsky, 1993) and in vivo (Bongianni et al., 1986; Baeyens et al., 1987; Barrios and Baeyens, 1991). Acute exposure
to opioids in vivo has been shown to decrease the number of dihydropyridine binding sites within regions of rat brain (Gandhi and Ross, 1988) which is consistent with their ability to reduce Ca\(^{2+}\) conductance by direct (North, 1986) and indirect (North, 1989) modes of action; this latter mechanism involves direct activation of a K\(^+\) conductance with sustained hyperpolarisation of the neurone, and as a result the neurone may reduce the level of Ca\(^{2+}\) in the cell body thereby slowing gene transcription and reducing turnover of essential transmitters, receptors and channels. Chronic exposure to opiates in vivo in the morphine dependent rat on the other hand, increases the number of dihydropyridine binding sites in the brain (Ramkumar and El-Fakahany, 1988) which could reflect an increase in the number and/or affinity of L-type Ca\(^{2+}\) channels.

Numerous behavioural studies have demonstrated that dihydropyridine L-type Ca\(^{2+}\) channel inhibitors and the phenylalkylamine verapamil, can inhibit the expression of several naloxone precipitated withdrawal signs in morphine dependent rats (Bongianni et al., 1986; Baeyens et al., 1987) and mice (Barrios and Baeyens, 1991). Related in vitro work has corroborated these findings where Ca\(^{2+}\) channel inhibitors have been shown to inhibit naloxone-induced release of \(^{[3]}H\)noradrenaline from cortical slices of morphine dependent rats (Pelligrini-Giampietro et al., 1988). This Ca\(^{2+}\) sensitive component is absent in morphine naive rats. The ability of verapamil to inhibit the expression of withdrawal signs in morphine dependent rats would appear to depend on route of administration. I.c.v. administration of verapamil has been reported to reduce body weight loss and jumping behaviour whereas diarrhoeic weight loss was only prevented after intraperitoneal injection (Baeyens et al., 1987). Such actions are supported
by the autoradiographic localisation of centrally located Ca$^{2+}$ channels (Gould et al., 1985; Ramkumar and El-Fakahany, 1988). Thus the relative efficacy of verapamil in differentially inhibiting behavioural signs associated with withdrawal would appear to depend upon route of administration.

In cultured SH-SY5Y human neuroblastoma cells chronic exposure to morphine has been shown to induce tolerance to $\mu$-opioid receptor inhibition of an N-type voltage-dependent Ca$^{2+}$ channel current (Kennedy and Henderson, 1991), although in this cell line morphine does not induce dependence by changing either N- or L-type Ca$^{2+}$ channel activity (Kennedy and Henderson, 1992). The absence of morphine dependence in these cells may result from the inability of these cells to express the intracellular mechanisms associated with dependence or may be due to lack of functional synapses between the cells (Kennedy and Henderson, 1992). This suggests that tolerance and dependence to opioids can evolve as separate entities within neurones, and that the integration of a functional neuronal network would appear to be required for the expression of dependence in neuronal populations.

Effector responses mediated by ion channels can be directly regulated by G proteins. The bacterial exotoxin pertussis toxin blocks the receptor mediated activation of certain G proteins such as $G_\text{i}$ and $G_\text{o}$ through the ADP ribosylation of the $\alpha$-subunit. This toxin interferes with the inhibitory actions of the $\delta$-opioid agonist d-Ala-d-Leu enkephalin on Ca$^{2+}$ currents recorded from NG108-15 cells (Hescheler et al., 1987). Evidence from various preparations suggests that Ca$^{2+}$ current inhibition by activation of opioid receptors involves a pertussis toxin sensitive G protein (Surprenant et al., 1990; Seward et al., 1991). Tolerance to morphine in SH-SY5Y cells may be
due to an uncoupling of μ-receptors from associated G proteins enabling it to develop at the level of the single neurone (Kennedy and Henderson, 1991). The inhibitory actions of morphine on magnocellular oxytocin neurone activity would appear to involve mediation by $G_i$ and $G_o$ proteins (Pumford et al., 1993) which may affect Ca$^{2+}$ conductance within these cells.

**Ca$^{2+}$ channels in magnocellular neurosecretory neurones**

Magnocellular neurosecretory cells display a frequency dependent Ca$^{2+}$ shoulder on the repolarisation phase of the action potential both *in vivo* (Renaud and Bourque, 1989) and *in vitro* (Bourque and Renaud, 1985b; Bourque et al., 1993). A similar mechanism may occur at the terminal level in the neurohypophysis, accounting for the frequency and pattern dependent release of neuropeptide observed in the isolated neural lobe preparation (Bicknell, 1988). The cell bodies of magnocellular neurosecretory cells express T-, N-, and L-like Ca$^{2+}$ currents whereas isolated nerve terminals would appear not to express a T-type current suggesting that the cell bodies and terminals of these cells can differentially regulate the expression of Ca$^{2+}$ channels (Fisher et al., 1992). Other experiments have shown that a major part of the inward Ca$^{2+}$ current during sustained depolarisation of neurosecretosomes with K$^+$ (Kato et al., 1992) is carried by dihydropyridine sensitive L-type Ca$^{2+}$ channels (Tsien et al., 1988). N-type Ca$^{2+}$ channels appear to mediate the majority of Ca$^{2+}$ entry and secretion when depolarisation is evoked by Na$^+$ action potentials in the neural lobe preparation (Von Spreckelsen et al., 1990).

In view of the roles undertaken by Ca$^{2+}$ within a cell one might expect that i.c.v. injection of verapamil would have profound effects on neuronal activity, and on established oxytocin release in particular. This appears not
to have been reported thus far as regards such stimuli, especially withdrawal excitation of oxytocin neurones in morphine dependent rats. It may well be that the development of tolerance and dependence to opiates in SON oxytocin neurones is mediated primarily by changes in Ca\(^{2+}\) channels on these neurones. Therefore the actions of verapamil were studied in morphine dependent and morphine naive rats on 1) naloxone-induced release of oxytocin both within the SON and from the neurohypophysis into blood measured by microdialysis and blood sampling 2) on oxytocin release into blood stimulated by systemic CCK8S and 3) on suckling-induced oxytocin release measured by simultaneous microdialysis of the SON and intramammary pressure changes to examine the role played by L-type Ca\(^{2+}\) channels in the regulation of magnocellular oxytocin neurones.

Since magnocellular neurones appear to differentially regulate the expression of Ca\(^{2+}\) channels within the SON and the neurohypophysis, and the ability of verapamil to attenuate specific behavioural withdrawal signs appears to depend on route of administration, it was also decided to conduct a series of experiments which could localise the site of action of verapamil. Therefore neural stalk stimulation at a frequency and magnitude within the range elicited by bursting activity of oxytocin neurones during the milk-ejection reflex (Russell et al., 1993) was utilised and the corresponding intramammary pressure recordings monitored to determine the effect of centrally administered verapamil on this response. Finally, use of an in vivo electrophysiological approach (Leng, 1981) enabled extracellular recordings from supraoptic magnocellular putative oxytocin neurones exposed by ventral surgery to be made, giving a direct indication as to whether the attenuation of withdrawal, induced by verapamil is peripherally or centrally mediated.
3.2. MATERIALS AND METHODS

3.2.1. I.c.v. verapamil on withdrawal hypersecretion of oxytocin

1. Animals

Virgin female Sprague-Dawley rats (body weight mean ± SEM; 268 ± 4 g, n=14) were used, housed singly with access to food and water ad libitum on a 12 h light/dark cycle at an ambient temperature of 21-23°C.

2. Intracerebroventricular (i.c.v.) infusion

Under ether anaesthesia rats were implanted with an i.c.v. cannula into the right lateral cerebral ventricle, attached to an infusion assembly consisting of a polythene cannula and osmotic mini-pump (Alzet 2001, Alza Corp.), which was to deliver morphine over 5 days in increasing dose (10 μg/h for 40 h, 20 μg/h for 40 h and 50 μg/h for 40 h; see General Methods, section 2.3.3. for full details). A guide cannula (22ga, C313G Plastics One Inc, USA) was also inserted (1.6 mm lateral and 0.6 mm posterior to bregma for i.c.v. injection of verapamil (160 μg/5 μl 0.9% isotonic saline) or vehicle and sealed with a dummy cannula of wire until the day of the experiment.

3. Blood sampling/injection procedure

On the morning of the experiment, 5 days after implantation of i.c.v. cannulae, rats from both treatment groups were anaesthetised with urethane (1.25 g/kg; 25% w/vol, i.p.). The femoral artery and vein were then cannulated for withdrawal of blood samples and injection of drugs (see General Methods, section 2.3.1.). Upon completion of surgery a 2 h equilibration period enabled effects of operative stress to subside. Rats were randomly assigned to the control or experimental groups (n=7 for each group). After a basal sample had been obtained sample 2 was taken 10 min
later, and immediately followed with an i.c.v. injection of verapamil or vehicle and 10 min later the third sample was followed by an i.v. injection of naloxone. Finally two samples were taken 5 and 20 min after injection of naloxone. Blood samples were of 0.3 ml volume. A 150 μl aliquot of plasma was removed and stored immediately on ice. The remaining red blood cells were then resuspended in 150 μl warmed isotonic saline prior to reinjection after the next blood sample had been obtained. At the end of the experiment samples were removed and stored at -20°C ready for radioimmunoassay for oxytocin content (see General Methods, section 2.4.).

3.2.2. I.c.v. verapamil on naloxone-stimulated release of oxytocin in normal rats

1. Animals

Virgin female Sprague-Dawley rats (254 ± 3 g, n=16) housed under standard conditions were anaesthetised with urethane (ethyl carbamate, 1.25 g/kg i.p.) and the femoral artery and vein were cannulated for blood sampling and injection of drug. Animals were then placed in a stereotaxic frame (Stoelting) and a midline skin incision over the skull made and the skin retracted. The skull surface was levelled between lambda and bregma and a guide cannula inserted (1.6 mm lateral and 0.6 mm posterior to bregma) for i.c.v. injection of verapamil (160 μg) or vehicle (5 μl isotonic saline) and sealed with a dummy cannula of wire until the start of the experiment.

2. Blood sampling/injection procedure

The protocol used was the same as outlined previously in section 3.2.1.3. except that a second i.c.v. injection of either verapamil or vehicle was given 10 min immediately after the fifth blood sample and 10 min before an i.v.
injection of CCK8S (20 µg/kg). A final blood sample was taken 5 min after the injection of CCK8S.

3.2.3. Effect of i.c.v. verapamil on central and peripheral oxytocin release during naloxone-induced morphine withdrawal

1. Animals

Virgin female Sprague-Dawley rats (275 ± 3 g, n=13) under ether anaesthesia were implanted with i.c.v. cannulae for infusion of morphine sulphate over 5 days and acute injection of either verapamil (160 µg/5μl) or vehicle on the day of experiment (see section 3.2.2.). After 5 days rats were anaesthetised with urethane (1.25 g/kg) and the left femoral artery and vein were cannulated for withdrawal of blood and injection of drug respectively. Rats were then placed in a stereotaxic frame and a U-shaped microdialysis probe implanted into the right SON, 1.8 mm lateral, 1.0 mm posterior to bregma and 9.1 mm below the skull surface (see General Methods, section 2.5.4).

2. Dialysate/blood sampling

After a first 30 min dialysate had been discarded, six more consecutive 30 min dialysates were collected via the microdialysis probe. At the end of the first collection proper, verapamil (160 µg/5μl) or vehicle was injected i.c.v. and 5 min later naloxone (5 mg/kg) was injected i.v., immediately after which the second microdialysis collection was started. After the third perfusion with aCSF, the fourth perfusion was with aCSF made hypertonic by adding 0.5 M NaCl followed by the fifth and sixth perfusions with normal aCSF. Blood samples were taken 5 min before i.c.v. injection of verapamil or vehicle and 5 min after injection of i.v. naloxone. The separated plasma was stored on ice
and then at -20°C ready for radioimmunoassay for oxytocin content as previously described (General Methods, section 2.4.).

3.2.4. Effect of i.c.v. verapamil on CCK8S-stimulated oxytocin release

1. Animals

Virgin female Sprague-Dawley rats housed under standard conditions (261 ± 3 g, n=31) were anaesthetised with urethane (1.25 g/kg) and the left femoral artery and vein were cannulated for withdrawal of blood and injection of drug, and return of resuspended red blood cells. A guide cannula was stereotaxically implanted as described in section 3.2.2.1.

2. Blood sampling/injection procedure

CCK8S (20 μg/kg; i.v.) was given directly after the first blood sample. After second and third blood samples had been taken 5 and 25 min after CCK8S, the rats received an i.c.v. injection of either verapamil (160 μg/5μl) or vehicle and a fourth blood sample was taken 10 min later. A second injection of CCK8S was given at this point, 5 min before the next blood sample. Rats which had previously been treated with verapamil or vehicle were given a second similar i.c.v. injection immediately after the sixth blood sample had been obtained at t=60 min. A seventh blood sample was taken 10 min later before both groups of rats were given an i.p. injection of 1.5 M NaCl (4 ml/kg) and the time course of this response followed with three more blood samples taken at 10 min intervals.

3.2.5. Effect of i.c.v. verapamil on suckling-stimulated central oxytocin release in the lactating rat

1. Animals

Lactating Sprague-Dawley rats (302-373 g, n=12) were used. These
animals were day 7 to 13 post partum and had been housed with their litter, with free access to food (standard breeder diet) and water under standard conditions (see General Methods, section 2.1.). On the evening prior to experimentation all but one of the pups were removed from the mothers overnight, to ensure that the pups would be hungry enough to suckle and the mammary glands engorged the following day. The mother was anaesthetised with a low dose of urethane (ethyl carbamate, 1.1 g/kg i.p.) since level of anaesthesia can interfere with the milk-ejection reflex. This was supplemented with local anaesthetic (Xylocaine 2%; Lignocaine hydrochloride BP) at sites of surgery. The distal right inguinal mammary gland was cannulated for measurement of intramammary pressure via a pressure transducer relayed to a chart recorder (see General Methods, section 2.3.2.). The left jugular vein was cannulated for i.v. bolus injection of oxytocin 0.1, 0.25, 0.5 and 1.0 mU (12.5 mU/ml 0.9% saline; Syntocinon) each separated by at least 3 min to give mammary gland responses enabling a dose-response curve to be obtained.

A U-shaped microdialysis probe (5 kDa cut-off) was lowered stereotaxically into the right SON (1.8 mm lateral, 1.0 mm posterior to bregma and 9.1 mm below the skull surface) and a guide cannula was implanted (1.6 mm lateral, 0.6 mm posterior to bregma) for i.c.v. administration of verapamil (160 µg) or vehicle (5 µl 0.9% NaCl).

2. Microdialysis sampling/injection procedure

Eight consecutive 30 min dialysates were collected via the microdialysis probe after an initial 30 min dialysate had been discarded. Synthetic oxytocin was given i.v. during the second perfusion as described, before the third perfusion with normal aCSF. After the third microdialysis collection the
pups which had been separated overnight were attached to the nipples and allowed to suckle to initiate the milk-ejection reflex. When a first mammary gland milk-ejection response had been observed verapamil or vehicle was injected i.c.v. and 5 min later the fourth microdialysis perfusion was started. After the fifth dialysate had been collected, the pups were removed from the nipples prior to collection of a sixth post-suckling dialysate. With the seventh perfusion the aCSF contained 0.5 M NaCl before a final 30 min perfusion with normal aCSF.

As well as perfusing the SON in order to quantify central oxytocin release, a count of milk-ejections was also made during the 30 min periods of the fourth and fifth perfusions to give an on-line indication as to whether i.c.v. verapamil was capable of attenuating the occurrence of the milk-ejection reflex which is related to release of oxytocin within the SON (Moos et al., 1989).

Histological verification of the microdialysis probe placement was carried out at the end of the experiment as previously described (General Methods, section 2.5.5.)

3.2.6. Effect of i.c.v. verapamil on oxytocin secretion evoked by neural stalk stimulation

1. Animals

Lactating Sprague-Dawley rats (day 7 to 13 post partum; 391 ± 9 g, n=4) were housed with litter and free access to food (standard breeder diet) and water under standard conditions (see General Methods, Chapter 2.1.). On the day prior to experimentation all but one of the pups were removed from their respective mothers.
2. Surgical preparation

On the morning of experimentation the remaining pup was removed and the mother was anaesthetised with a reduced dose of urethane (1.1 g/kg) and supplemented with Xylocaine (2%) at sites of surgery. One abdominal mammary gland milk duct and two inguinal milk ducts were cannulated for measurement of intramammary pressure (see General Methods, Chapter 2.3.2.). The left femoral vein was cannulated for i.v. injection of oxytocin.

The rat was then placed in a stereotaxic frame and a midline skin incision made. The skull surface was levelled between lambda and bregma and a guide cannula inserted (1.6 mm lateral and 0.6 mm posterior to bregma) for i.c.v. injection of verapamil (160 μg/5μl). This was fixed in position with a mound of dental cement before drilling a second hole in the skull 4.0 mm rostral to lambda through which to introduce the stimulating electrode. The bipolar stimulating electrode (SNEX 200; Clarke Electrochemical Instruments, Reading) was initially lowered 10.5 mm below the skull surface and subsequently moved in 0.1 mm gradations to deliver from conventional equipment, matched biphasic square-wave D.C. pulses (0.5 mA peak-to-peak, 1.0 ms duration, 50 Hz) in trains of 60-150 pulses. The stimulating electrode was judged to be resting on the neural stalk when a depth was reached which provided an optimal intramammary pressure response to one of the lower stimulation parameters.

3. Injection procedure

The rat was left for 1 h and then a series of pulse trains alternated with bolus i.v. injection of 0.25, 0.5, 0.75 or 1.0 mU oxytocin (12.5 mU/ml 0.9% saline; Syntocinon), each separated by approximately 3 min, were given at least twice to elicit mammary gland responses. I.c.v. verapamil (160 μg/5μl)
was then injected and the cycles of stalk stimulation and i.v. bolus injection of oxytocin repeated using parameters which were most likely to be affected by verapamil. In two animals i.c.v. verapamil injection and the following cycle of stalk stimulations and oxytocin injections was repeated twice.

4. Histological verification of electrode placement

Location of the stimulating electrode was further confirmed at the end of the experiment by passing unipolar current as a continuous train of pulses (0.5 mA peak-to-peak, 1.0 ms duration, 100 Hz) for 60 s (reversing polarity after 30 s) to deposit ferrous ions from the electrode tip. The brains including the neural stalks were then removed and fixed in 10% formal saline containing potassium ferro- and ferri-cyanide (0.4 mM and 0.5 mM respectively) for 2-3 days to develop the Prussian Blue reaction. Using a binocular dissecting microscope the ferrous ions were visualised as a blue spot at the tip of the electrode and in all cases the electrode tips were in the neural stalk.

3.2.7. Effect of i.c.v. verapamil on electrical activity of oxytocin neurones stimulated by naloxone-induced morphine withdrawal

1. Animals

Under ether anaesthesia virgin female Sprague-Dawley rats (265-340 g, n=5) were implanted with an i.c.v. cannula into the right lateral cerebral ventricle, which was attached to an infusion assembly consisting of a polythene cannula and osmotic mini-pump (Alzet 2001, Alza Corp.), which was to deliver morphine over 5 days in increasing dose (10 µg/h for 40 h, 20 µg/h for 40 h and 50 µg/h for 40 h). See General Methods, section 2.3.3. for full details. A guide cannula was also inserted (1.6 mm lateral and 0.6 mm
posterior to bregma for i.c.v. injection of verapamil (160 μg/5 μl 0.9% isotonic saline) or vehicle and sealed with a dummy cannula of wire until the day of the experiment.

2. Electrophysiology

On the 6th day of infusion rats were anaesthetised with urethane (1.25 g/kg) and the trachea and the left femoral vein cannulated. The rat was mounted in a stereotaxic frame and the neurohypophysial stalk and right SON surgically exposed (ventral surgery kindly performed by Dr. G. Leng; Leng, 1981). A concentric bipolar stimulating electrode (SNEX 200; Clarke Electrochemical Instruments) was placed on the neural stalk and a glass microelectrode filled with 0.9% saline (20-50 MOhm) was placed into the SON to obtain extracellular recordings from antidromically-activated neurones. Neurones were antidromically activated by stimulation of the neural stalk (1 ms matched biphasic pulses <1 mA peak-to-peak, 0.3 Hz) and were tested for constant latency to confirm their projection from the SON to the neurohypophysis. This enabled exclusive identification of magnocellular neurosecretory neurones. To further identify oxytocin neurones i.v. CCK8S (20 μg/kg) was given as this has been shown to selectively activate putative oxytocin neurones while putative vasopressin neurones are unaffected or inhibited (Renaud et al., 1987). Recordings of oxytocin neurones were relayed from a spike processor (Digitimer D.130) to a digital to analogue convertor (CED 1401) and stored on a Spike 2 software programme (version 3.22 CED Ltd, Cambridge) for later analysis. Firing rate was recorded for at least 10 min prior to injection of i.v. naloxone in order to obtain a stable control period. Once withdrawal was established i.c.v. verapamil was injected. If at this point cell firing rate was abolished then the cell was
antidromically activated to make sure that the cell had not drifted away from
the recording electrode. In two animals a repeated injection of i.c.v.
verapamil was given.

3.2.8. Radioimmunoassay for oxytocin

Oxytocin content of all blood plasma samples from experiments described
in this chapter was determined using the antiserum and modified method of
Higuchi et al., (1985a). Assay sensitivity was <4.8 pg/ml and the interassay
coefficient of variation was 18%. Intraassay coefficients of variation were
never more than 17% at standard concentrations of 10 pg/ml and 100 pg/ml.
All samples from a single experiment were measured in the same assay to
eliminate any interassay variation.

Microdialysis samples were assayed with typical recovery values as
described previously (General Methods, section 2.5.6.).
Chapter 3

3.3. RESULTS

3.3.1. i.c.v. verapamil on withdrawal hypersecretion of oxytocin

I.c.v. verapamil had no effect on basal plasma oxytocin levels compared with vehicle alone (Fig. 3.3.1.1.). When naloxone was given i.v. 10 min after i.c.v. verapamil plasma oxytocin concentration increased in verapamil-treated rats by 275.1 ± 192.4 pg/ml and in vehicle-treated rats by 760.5 ± 152.7 pg/ml, and this augmentation from basal was significant 5 min and up to 20 min after the injection of naloxone (p<0.05, n=7; Paired Wilcoxon). The observed rise in plasma oxytocin 5 min after the injection of naloxone was significantly greater (p<0.05; Mann-Whitney U-test) in the vehicle group than in the verapamil-treated group but this was not the case 20 min later, when the level in the vehicle group tended to decline.

3.3.2. i.c.v. verapamil on naloxone-stimulated release of oxytocin in normal rats

After i.c.v. injection of verapamil or vehicle there was no significant difference in basal plasma oxytocin levels between the two groups. 10 min after the initial i.c.v. injection an i.v. injection of naloxone was given which significantly increased (p<0.05; Paired Wilcoxon) plasma oxytocin concentration in both vehicle-treated rats by 80.0 ± 14.2 pg/ml and verapamil-treated rats by 21.1 ± 10.6 pg/ml after 5 min respectively (Fig. 3.3.2.1.). The elevation in plasma oxytocin in the vehicle group was significantly greater than in the verapamil group (p<0.05; Mann-Whitney U-test) 5 min after naloxone injection. CCK8S given i.v. 10 min after a second injection of either i.c.v. vehicle or verapamil increased plasma oxytocin significantly in i.c.v. vehicle-treated rats by 298.4 ± 95.5 pg/ml, (p<0.005,
Mann-Whitney U-test) but had no effect on oxytocin levels in i.c.v. verapamil-treated rats.

3.3.3. Effect of i.c.v. verapamil on central and peripheral oxytocin release during naloxone-induced morphine withdrawal

There was no significant difference (Mann-Whitney U-test) in mean basal oxytocin release within the SON between rats which were to receive either i.c.v. verapamil (1.20 ± 0.27 pg/sample, n=8) or i.c.v. vehicle (0.60 ± 0.29 pg/sample, n=5). Injection of i.v. naloxone 5 min after i.c.v. vehicle precipitated withdrawal, increasing oxytocin release within the SON by 213.0 ± 50.8% in the second dialysate whereas this release was completely suppressed in animals injected with i.c.v. verapamil (p<0.01; Mann-Whitney U-test), (Fig. 3.3.3.1.a.). The increase in intranuclear release of oxytocin was sustained over the following 30 min perfusing period in the i.c.v. vehicle-treated group although release fell to 121.0 ± 119.9% (no sig. diff.; Paired Wilcoxon). Oxytocin release within the SON remained unchanged over this period in the i.c.v. verapamil-treated group.

I.v. naloxone injection significantly increased plasma oxytocin concentration in both i.c.v. vehicle-treated animals to 439.5 ± 186.4 pg/ml (from 49.0 ± 9.4 pg/ml) and i.c.v. verapamil-treated animals to 106.6 ± 15.7 pg/ml (from 52.0 ± 7.2 pg/ml), (p<0.05; Paired Wilcoxon, Fig 3.3.3.1.b.). Despite this however, prior i.c.v. verapamil injection reduced the increment in plasma oxytocin concentration by 86% (p<0.05; Mann-Whitney U-test).

3.3.4. Effect of i.c.v. verapamil on CCK8S-stimulated oxytocin release

Injection of i.v. CCK8S significantly increased plasma oxytocin from basal levels, from 24.0 ± 2.1 pg/ml to 37.7 ± 4.8 pg/ml in the vehicle-treated group

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(p<0.002, n=15; Paired Wilcoxon) and from 24.3 ± 1.9 pg/ml to 36.3 ± 2.1 pg/ml in the verapamil-treated group (p<0.0005, n=16; Paired Wilcoxon), (Fig. 3.3.4.1.). Injection of i.c.v. verapamil 10 min before a second injection of CCK8S completely eliminated (p<0.00001; Mann-Whitney U-test) the CCK8S-stimulated release of oxytocin observed in the i.c.v. vehicle-treated group, (p<0.001; Paired Wilcoxon). Rats previously given i.c.v. vehicle were then given an i.p. injection of hypertonic saline which increased oxytocin secretion markedly (p=0.0001; Paired Wilcoxon) over the following 10 min and it continued to increase over the following 20 min. I.c.v. injection of verapamil 10 min before injection of hypertonic saline significantly attenuated the oxytocin response for at least 20 min (p<0.0005; Mann-Whitney U-test), although 30 min after injection of hypertonic saline the increase was significant (p<0.01; Paired Wilcoxon), but still less than in i.c.v. vehicle-treated rats (p<0.05)

3.3.5. Effect of i.c.v. verapamil on suckling-stimulated central oxytocin release in the lactating rat

In rats treated subsequently with either i.c.v. vehicle or verapamil mean basal oxytocin release for the first 3 dialysates within the SON was 0.52 ± 0.26 pg/sample and 0.35 ± 0.11 pg/sample respectively (no significant difference; Paired Wilcoxon). After injection of i.c.v. vehicle in the first 30 min perfusion during suckling, oxytocin recovery increased significantly by 266 ± 46 % (p=0.01; Mann-Whitney U-test) while in rats which received i.c.v. verapamil there was no change in oxytocin release during either this perfusion or the second perfusion during the suckling period (Fig. 3.3.5.1.). However oxytocin release returned to its pre-suckling level in the i.c.v.
vehicle-treated group. There was no further change in either group of animals during the sixth perfusion period after the pups had been removed.

During the first 30 min perfusion period the total number of observed milk-ejections was significantly less (p<0.01; Mann-Whitney U-test) in the i.c.v. verapamil-treated group than in the group which received i.c.v. vehicle (Fig. 3.3.5.2. and Fig. 3.3.5.3.). There was no difference in number of milk-ejections between groups as suckling continued during the second 30 min perfusion.

### 3.3.6. Effect of i.c.v. verapamil on oxytocin secretion evoked by neural stalk stimulation

Pulse trains of 90-120 stimulus pulses at 50 Hz (typical parameters for an oxytocin neurone milk-ejection burst) applied to the neural stalk evoked the release of approximately 0.75 mU oxytocin (Fig. 3.3.6.1.a.). Injection of i.c.v. verapamil in all four animals appeared to initially diminish the mammary gland response to exogenous oxytocin (Fig. 3.3.6.1.a.) and to neural stalk stimulation (Fig. 3.3.6.1.a. and Fig. 3.3.6.1.b.), although this was not deemed to be significant (Paired Wilcoxon).

### 3.3.7. Effect of i.c.v. verapamil on electrical activity of oxytocin neurones stimulated by naloxone-induced morphine withdrawal

Injection of i.v. naloxone increased the firing-rate of antidromically-identified putative oxytocin neurones (excited by i.v. CCK8S, 20 μg/kg) recorded from morphine dependent rats by 4.7 ± 1.0 spikes/s from a mean basal level of 2.3 ± 1.4 spikes/s (p<0.05, n=5; Paired Wilcoxon). Once withdrawal was fully established an initial injection of i.c.v. verapamil abolished firing completely in 3 cells for approximately 12 min (Fig. 59).
3.3.7.1.a) and attenuated firing-rate in a further 2 cells by 6.3% and 8.5% (p<0.05 by confidence limits; Fig. 3.3.7.1.b.).

In one cell once firing rate recovered to a level comparable with that observed during the initial control period after i.c.v. injection of verapamil (48 μg). A second injection of verapamil completely abolished the firing rate response (Fig. 3.3.7.1.a.).
Figure (3.3.1.1.)- Effect of i.c.v. verapamil on withdrawal hypersecretion of oxytocin release in the morphine-dependent rat. I.v. naloxone (5 mg/kg) given 10 min after i.c.v. verapamil (160 μg/5 μl) increased plasma oxytocin both in verapamil (▲; n=7) and in vehicle-treated (○; n=7) rats and this elevation was significant at both 5 min and 20 min after injection of naloxone (*p<0.05; Paired Wilcoxon). The elevation of plasma oxytocin in the vehicle group was significantly greater than that observed in the verapamil group (#p<0.05; Mann-Whitney U-test) 5 min after the injection of naloxone.
Figure (3.3.2.1.)- Effect of i.c.v. verapamil on naloxone-induced and CCK8S-stimulated release of oxytocin in the virgin female rat. I.v. injection of naloxone 10 min after i.c.v. verapamil significantly increased plasma oxytocin in both vehicle-treated (O; n=8) and verapamil-treated (▲; n=8) groups (*p<0.05; Paired Wilcoxon; ) after 5 min. The elevation of plasma oxytocin in the vehicle group was significantly greater than in the verapamil group (#p<0.05; Mann-Whitney U-test) 5 min after naloxone injection. CCK8S (i.v.; 20 μg/kg) given 10 min after either a second injection of i.c.v. vehicle or verapamil significantly increased plasma oxytocin in i.c.v. vehicle-treated rats (**)p<0.005, n=8), but had no effect in i.c.v. verapamil-treated rats.
Figure (3.3.3.1; overleaf)- Effect of i.c.v. verapamil on central and peripheral oxytocin release during naloxone-induced morphine withdrawal.

(a) I.v. injection of naloxone increased oxytocin content within the SON in the second dialysate in i.c.v. vehicle-treated animals (open bars), (**p<0.01; Mann-Whitney U-test). Prior injection with i.c.v. verapamil (hatched bars) completely suppressed this increase. Central release of oxytocin remained unchanged in the following dialysate for both groups relative to the previous respective dialysate.

(b) Injection of i.v. naloxone increased peripheral release of oxytocin in both i.c.v. vehicle-treated (open bars) and i.c.v. verapamil-treated animals (hatched bars), (*p<0.05, n=5 and n=8 respectively; Paired Wilcoxon) although prior administration of i.c.v. verapamil reduced the increment in release (#p<0.05; Mann-Whitney U-test).
(a) OXT content in SON dialysate (post/pre nalox % change)

(b) Plasma oxytocin (pg/ml)
Figure (3.3.4.1.)- Effect of i.c.v. verapamil on CCK8S-stimulated release of oxytocin secretion in virgin female rats. I.v. injection of CCK8S resulted in a significantly increased release of oxytocin in both vehicle-treated (○; *p<0.002, n=15; Paired Wilcoxon;) and verapamil-treated animals (▲; **p<0.0005, n=16). Injection of i.c.v. verapamil 10 min prior to a second injection of CCK8S completely abolished (#p<0.00001; Mann-Whitney U-test) the CCK8S-stimulated increase in oxytocin concentration seen in the i.c.v. vehicle-treated group, (Tp<0.001). In rats given hypertonic saline (4 ml/kg, i.p.) oxytocin secretion increased dramatically (TTp=0.0001) over the following 10 min and continued to increase steadily for the next 20 min. Prior injection of i.c.v. verapamil delayed this increase in plasma oxytocin secretion by at least 20 min.
Figure (3.3.5.1.)- Effect of i.c.v. verapamil on suckling-stimulated central oxytocin release in the lactating rat. Suckling-induced oxytocin release within the SON increased significantly from basal levels (**p=0.01; Mann-Whitney U-test) after injection of i.c.v. vehicle (open bars) 10 min before collection of the fourth dialysate whereas there was no change in central oxytocin release after i.c.v. verapamil injection (hatched bars), during this perfusion or the following perfusion during the suckling period. Central oxytocin release returned to pre-injection levels during the fifth perfusion. There was no further change in central oxytocin levels in either group during the sixth perfusion after removal of the pups.
Figure (3.3.5.2.)- Effect of i.c.v. verapamil on the milk-ejection reflex. Injection of i.c.v. verapamil (hatched bars) reduced the total number of observed milk-ejections (**p<0.01; Mann-Whitney U-test) during the first 30 min perfusion following injection compared with i.c.v. vehicle (open bars). As suckling continued through a second 30 min perfusion period there was no difference in number of observed milk-ejections in either group.
Figure (3.3.5.3.)- Typical intramammary pressure recordings obtained from urethane-anaesthetised rats (7-13 days post-partum). In the upper trace (a) injection of i.c.v. verapamil impaired milk-ejection (*) for at least 12 min in contrast to the lower trace (b) where i.c.v. vehicle had no effect on milk-ejections. Black arrow denotes 0.5 mU exogenous oxytocin (i.v.) and MD4 represents microdialysis dialysate number 4.
Figure (3.3.6.1.; overleaf)- Effect of i.c.v. verapamil on oxytocin secretion evoked by neural stalk stimulation.

(a) Stimulation of the neural stalk with 60-120 pulses at 50 Hz produced a graded increase in release of oxytocin into the periphery (measured from intramammary pressure) in lactating rats (n=4) which was unchanged after administration of i.c.v. verapamil.

(b) Typical recording obtained from a urethane-anaesthetised rat showing graded increases in intramammary pressure (which reflects oxytocin secretion) in response to electrical stimulation with 60-120 pulses (empty arrows) of the neural stalk and i.v. bolus injection of oxytocin (filled arrows). I.c.v. verapamil had an apparent though not significant transient depressing effect on the intramammary pressure response to 90 pulses and also to 0.75 mU oxytocin.
Chapter 3

pre-verapamil

mu oxytocin/stimulus

stimulus freq (Hz) 60 90 120

post-verapamil

60 90 120

b.

i.c.v. verapamil

5 min

10 mmHg

1.0 mU OXT

90 60 90 0.75 90 0.5 120 1.0 90 0.75 60 90 0.5

pulses 50 Hz
Figure (3.3.7.1.; overleaf)- Traces of electrical activity of supraoptic putative oxytocin neurones showing the two most extreme responses obtained after injection of i.c.v. verapamil. I.v. injection of CCK8S (20 μg/kg) caused a rapid, transient increase in firing rate in both cells tested.

(a) I.c.v. injection of verapamil (48 μg) completely abolished the naloxone-induced withdrawal increase in firing rate for 10-12 min before the cell slowly began to recover to a firing rate similar to that seen during the control period. A second injection of verapamil completely blocked the recovery in firing rate.

(b) I.c.v. injection of verapamil (160 μg) significantly attenuated the withdrawal-induced increase in firing rate (p<0.05) as measured by the 95% confidence limits during the post-injection period compared with the period immediately prior to injection.
3.4. DISCUSSION

Naloxone-induced release of oxytocin

The withdrawal excitation of magnocellular oxytocin neurones after intravenous injection of naloxone results in a pronounced hypersecretion of oxytocin into blood from the neurohypophysis as shown in this (Figs. 3.3.1.1. and 3.3.2.1.b.) and previous studies (Bicknell et al., 1988; Leng et al., 1989b).

The involvement of L-type Ca\(^{2+}\) channels would appear to be central to this response, since injection of verapamil into a lateral cerebral ventricle significantly attenuated the withdrawal-induced release of oxytocin into blood (Figs. 3.3.1.1. and 3.3.2.1.b.) and within the SON (Fig. 3.3.2.1.a.). In morphine naive animals anaesthetised with urethane, i.c.v. injection of verapamil although diminishing the oxytocin secretory response to systemic injection of naloxone, did not block it completely. This is consistent with an action of naloxone at the neurohypophysis, as shown by the unaffected firing rate response in untreated rats (Shibuki et al., 1988) and intimates that verapamil probably does not act at the neurosecretory terminals to attenuate release of oxytocin into blood. Prolonged exposure to morphine in vivo increases dihydropyridine binding site density within the brain (Ramkumar and El-Fakahany, 1988) and this could explain the effect of verapamil in attenuating naloxone-induced oxytocin release in morphine dependent rats. This may well infer that L-type Ca\(^{2+}\) channels play a profoundly important role in the withdrawal excitation of oxytocin neurones.

CCK8S- and osmotically-stimulated release

In morphine naive animals i.v. injection of naloxone would appear to have
potentiated the CCK8S-stimulated release of oxytocin in animals which had previously been treated with i.c.v. vehicle; in a separate study which was assayed together with the present study (Fig. 3.3.2.1.), the CCK8S-stimulated increase in plasma oxytocin although significant in itself was approximately 10 fold lower. This agrees with reports of naloxone potentiated release of oxytocin induced by systemic administration of CCK (Flanagan et al., 1988; Leng et al., 1992).

Systemic administration of CCK8S excites magnocellular oxytocin neurones as shown by their increased electrical activity and by the expression of Fos protein within the SON following application of this stimulus (Hamamura et al., 1991). Consequently there is an increase in plasma oxytocin as shown by this and previous studies (Blackburn and Leng, 1990). The pathway extends from the gastric vagus (Verbalis et al., 1986) and involves a central noradrenergic component projecting to the NTS, onwards by direct and indirect pathways to the hypothalamic magnocellular system. Since i.c.v. injection of verapamil completely abolished CCK8S-induced oxytocin release into blood from the neurohypophysis, this indicates that L-type Ca^{2+} channels may be an important factor in mediating the response. CCK8S also facilitates release of both vasopressin and oxytocin when applied to the isolated rat neural lobe (Bondy et al., 1989a) although this is apparently independent of external Ca^{2+}. Consequently verapamil when injected centrally, would appear not to act at the neurohypophysis to block release of oxytocin in response to systemic CCK8S.

Interestingly i.c.v. injection of verapamil also attenuated the osmotic activation of oxytocin neurones. The region anterior and ventral to the third ventricle (AV3V region) which receives projections from the organum
vasculosum of the lamina terminalis (OVLT) provides a major input to supraoptic magnocellular oxytocin neurones which is essential for maintaining their full osmoreposiveness (Leng et al., 1989), although this pathway does not seem to be essential for either withdrawal excitation of oxytocin secretion (Russell et al., 1992) or systemic CCK8S-stimulated oxytocin release (Blackburn and Leng, 1990). The subfornical organ (SFO) projects to the magnocellular nuclei and the AV3V region (Miselis et al., 1981), and may also be involved in the full maintenance of osmoreposiveness of magnocellular neurones (Leng et al., 1989).

Peripheral osmotic activation of oxytocin neurones involves at the cellular level a steady increase in continuous firing of oxytocin neurones which is reflected by a gradual increase in plasma oxytocin concentration (Fig. 3.3.4.1.). The delay in onset of activation of oxytocin secretion after i.c.v. injection of verapamil resembles that which takes place after lesioning of the SFO and AV3V region (Leng et al., 1989). Cells within the OVLT are themselves intrinsically osmosensitive, and it has been suggested that an osmotically activated depolarising response is relayed to magnocellular neurosecretory cells whereby it can be amplified and manifest itself as an electrical event in these cells, only if their intrinsic osmosensitivity is still intact (Bourque et al., 1993). An action of verapamil at either of these sites would effectively interrupt synaptic transmission and delay the magnocellular response to the hypertonic stimulus. The eventual appearance of the response was probably a result of verapamil ceasing to antagonise after a given period, the movement of extracellular Ca$^{2+}$ into the cells responsible for affecting either directly (within the SON) or indirectly (on afferents from the AV3V region) the increase in plasma oxytocin concentration.
Suckling stimulus

The suckling stimulus initiated by a hungry litter of rat pups has previously been shown to provide a reliable on-line bioassay of oxytocin release from the neurohypophysis (Wakerley and Lincoln, 1971a). The pathway involved in suckling-induced release of oxytocin has not been clearly described although a number of components essential for its normal functioning have been identified. One of the most important of these is the role of oxytocin itself in facilitating its own release within the magnocellular nuclei during the suckling process. Oxytocin release measured by push-pull perfusion and microdialysis within the SON increases during the milk-ejection reflex (Moos et al., 1989; Neumann et al., 1993) and this release is significantly reduced by replacing Ca$^{2+}$ in the perfusion medium with EGTA (Neumann et al., 1993). In these present experiments the rise in intramammary pressure associated with bolus release of oxytocin occurring over regular intervals (the milk-ejection reflex) was to provide a correlate as to the actions of verapamil on both peripheral and central oxytocin release. Injection of verapamil into a lateral cerebral ventricle significantly attenuated the frequency of milk-ejections and their magnitude (the magnitude of the intramammary pressure response being related to the amount of oxytocin released from the neurohypophysis). Recent reports have suggested that modulation of Ca$^{2+}$ channels can directly influence the occurrence of overlapping and functional after currents in magnocellular neurosecretory cells, and hence spike-induced Ca$^{2+}$ influx which may be important for the bursting of oxytocin neurones during milk-ejection (Bourque et al., 1993); such currents are likely to be modulated by verapamil.

In view of the facilitatory effect of oxytocin on its own release within the
magnocellular nuclei both *in vivo* and *in vitro* (Moos et al., 1984; Yamashita et al., 1987) it is likely that the disruption of central oxytocin release by verapamil prevented its further dendritic and axonal release within the SON (Pow and Morris, 1989; Ward and Morris, 1993) and subsequent release from the neurohypophysis. Release of oxytocin within limbic brain areas has also been shown to be Ca$^{2+}$ dependent (Landgraf et al., 1988) and it has been proposed that in the suckled lactating rat, septal neurones could modulate the bursting of oxytocin neurones within the PVN and SON via the bed nucleus of the stria terminalis (Ingram and Moos, 1992). From these data an action of verapamil at this site cannot be excluded although it is likely that Ca$^{2+}$ channel involvement occurs at both sites. However this problem could be resolved by injecting verapamil directly into the SON of the lactating rat using a modified triple probe system, and microdialysing for oxytocin upon initiation of a suckling stimulus.

**Verapamil acts centrally to attenuate activity of magnocellular oxytocin neurones**

Both the cell bodies and the terminals of magnocellular neurosecretory cells express N- and L-type Ca$^{2+}$ currents (Fisher et al., 1992). As a result verapamil could attenuate release of oxytocin into blood from the neurohypophysis by acting at either of these sites; modulation of Ca$^{2+}$-induced spike influx regulates firing pattern of magnocellular cells (Bourque et al., 1993), whilst depolarisation of the terminal membrane opens Ca$^{2+}$ channels allowing influx of extracellular Ca$^{2+}$ and subsequent neurohypophysial peptide secretion (Bicknell et al., 1993) which can be suppressed by Ca$^{2+}$ channel antagonists (Jørgensen et al., 1993). However it would appear that the inhibitory actions of verapamil on SON oxytocin
neurones are mediated centrally since i.c.v. verapamil a) attenuated oxytocin release within the SON during suckling and morphine withdrawal b) had no significant effect on oxytocin release from the neurohypophysis evoked by stimulation of the neural stalk (Fig. 3.3.6.1.) and c) completely abolished the withdrawal-induced firing rate in three magnocellular oxytocin cells and significantly attenuated firing rate in a further two cells of morphine dependent rats (Fig. 3.3.7.1.). Of course these central actions of verapamil on magnocellular oxytocin neurones may have been mediated pre-synaptically on afferents to these neurones and merit further investigation if a precise location of action is to be specified. Recently it has been reported that multiple L-type Ca\(^{2+}\) channels (which may be uniquely associated with dendritic and somatic Ca\(^{2+}\) dependent functions) can be co-expressed within the same cell (Tomlinson et al., 1993). Differential expression of such channels within magnocellular oxytocin neurones could explain the wide variation in firing rate response of these cells (Fig. 3.3.7.1.) to i.c.v. verapamil administration.

**Role of the Ca\(^{2+}\) channel in tolerance and dependence**

The dihydropyridine L-type Ca\(^{2+}\) channel antagonist nimodipine, attenuates naloxone precipitated noradrenaline release from various brain regions *in vivo* in morphine dependent rats (Bongianni *et al.*, 1986) and prevents release *in vitro* from cortical slices taken from morphine dependent rats (Pelligrini-Giampietro *et al.*, 1988).

The SON receives noradrenergic inputs from the A2 cell group, and to a lesser extent the A6 cell group located in the brainstem (Loizu, 1969; Sawchenko and Swanson, 1982). Systemic administration of CCK8S increases plasma oxytocin concentration and increases extracellular
noradrenaline concentration measured by microdialysis and high performance liquid chromatography in the dorsal region of the SON (Kendrick et al., 1991). However it is not known if noradrenaline concentration increases within the SON during withdrawal, although intuitively it might be expected to do so. It is possible that verapamil injected centrally attenuates a presynaptic Ca$^{2+}$ conductance thereby preventing release of oxytocin within the SON and perhaps ultimately into blood during morphine withdrawal, via pre-synaptic inhibition of Ca$^{2+}$-mediated noradrenaline release. Paradoxically, noradrenaline itself, acting upon $\alpha_2$-adrenoceptors has been shown to reduce the activity of high voltage-activated Ca$^{2+}$ channels (Lipscombe et al., 1989; for review see Scott et al., 1991). However noradrenaline is principally excitatory to magnocellular oxytocin neurones (Yamashita et al., 1987).

The part played by L-type Ca$^{2+}$ channels in the control of oxytocin within the SON and from the neurohypophysis in response to a wide range of stimuli has been readily demonstrated from these studies. Whether this is as a result of a direct action at the level of the channel itself (either pre- or post-synaptically) or due to modulation of other post effector mechanisms remains to be established. In relation to tolerance the reduction in Ca$^{2+}$ conductance which involves a pertussis toxin sensitive G protein (Seward et al., 1991) is well defined. It is doubtful however whether inactivation of such G proteins is important for the development of dependence in magnocellular oxytocin neurones (Pumford et al., 1993). The establishment of tolerance and dependence in this system to the inhibitory actions of morphine may result from the ongoing occupancy of the $\mu$-opioid receptor by morphine and adaptation of a Ca$^{2+}$ current associated with the K$^+$ conductance activated by
morphine (North, 1989). An upregulation of \( \text{Ca}^{2+} \) channel expression compensating for this decrease in \( \text{Ca}^{2+} \) conductance would mean that displacement of morphine from the \( \mu \)-receptor by naloxone would remove the inhibition exerted on these neurones and allow a massive flood of \( \text{Ca}^{2+} \) into the cell. This in turn would initiate neuronal activation due to increased intracellular \( \text{Ca}^{2+} \) concentration and associated turnover of second messenger events to initiate exocytotic release of oxytocin both centrally from dendritic processes and peripherally into the plasma (see General Discussion, Fig. 7.1.).

However, maintenance of other excitatory afferent inputs may also be required to mediate the full expression of withdrawal excitation in magnocellular oxytocin neurones, in view of the difficulty of its demonstration \textit{in vitro}. 
CAN ENDOGENOUS OXYTOCIN FACILITATE ITS OWN RELEASE DURING NALOXONE-PRECIPITATED MORPHINE WITHDRAWAL?
Chapter 4

4.1. INTRODUCTION

Oxytocin is released from the neurohypophysis into the periphery in response to a variety of stimuli including parturition, suckling, osmotic activation, stress, gastric distension and peripheral administration of CCK8S (Higuchi et al., 1985b; Lang et al., 1983; Brimble et al., 1978; Higuchi et al., 1986c; Renaud et al., 1989; Verbalis et al., 1986).

Oxytocin also has important actions when released within the brain being actively involved in both male and female sexual behaviour (Arletti et al., 1992) and facilitating maternal behaviour in the post-parturient rat (Pedersen et al., 1992). Injection of exogenous oxytocin into a lateral cerebral ventricle (unlike systemic oxytocin) can initiate the milk-ejection reflex in the anaesthetised suckled lactating rat (Freund-Mercier and Richard, 1984) whilst oxytocin has been shown to be stimulatory to oxytocin neuronal activity in vitro (Moos et al., 1984; Yamashita et al., 1987). Since central administration of an oxytocin antagonist not only prevents the facilitatory effect of oxytocin, but when given alone interrupts the milk-ejection reflex (Freund-Mercier and Richard, 1984) this indicates that endogenous brain oxytocin is crucial for mediating its own release in response to specific stimuli. This effect of the oxytocin antagonist in vivo combined with in vitro evidence where, in contrast with oxytocin, exogenous vasopressin has been reported to have no effect on oxytocin neurosecretion, suggests that these effects are mediated by specific oxytocin receptors. Using traditional autoradiographic techniques oxytocin binding sites have not been demonstrated within the SON, (Tribollet et al., 1988; Tribollet et al., 1992), until recently however, where oxytocin binding sites have been histoautoradiographically shown to be located on the dendritic processes of
the oxytocin neurones around the ventral rim of the SON (Freund-Mercier and Stoeckel, 1993).

Separate studies using the push-pull perfusion and microdialysis techniques have revealed that oxytocin release within the SON increases during the milk-ejection reflex (Moos et al., 1989; Landgraf et al., 1992). The rise in intranuclear release of oxytocin is both Ca\(^{2+}\) and Na\(^{+}\) dependent and is likely to be dendritic in origin (Pow and Morris, 1989; Ward and Morris, 1993) although the cell bodies of the oxytocin neurones themselves probably contribute (Pow and Morris, 1989). Release of oxytocin within the SON is also increased by both systemic and central administration of CCK8S (Neumann et al., 1993) which correlates with the increase in electrical activity of magnocellular oxytocin neurones in response to these stimuli (Leng et al., 1991; Jarvis et al., 1992).

In the rat prolonged administration of morphine results in dependence upon and tolerance to the inhibitory actions of morphine on oxytocin release, whilst administration of naloxone results in a withdrawal hypersecretion of oxytocin due to an increase in firing rate of the neurones (Bicknell et al., 1988). Naloxone-induced withdrawal is a robust stimulus to oxytocin release within the SON (Russell et al., 1992) and one may expect that this release of oxytocin within the SON may have a profound autofacilitatory effect, contributing to the hypersecretion of oxytocin and helping to sustain the elevated electrical activity of these neurones during withdrawal. Until now, the role of oxytocin within the SON in facilitating its own release during morphine withdrawal has remained undetermined. Preliminary blood sampling studies using a specific oxytocin antagonist (Manning et al., 1989) administered into a lateral cerebral ventricle were undertaken to ascertain a
role for central oxytocin during the withdrawal process in the morphine dependent rat.
4.2. MATERIALS AND METHODS

4.2.1. Effect of acute i.c.v. oxytocin antagonist injection on withdrawal hypersecretion of oxytocin

1. Animals

Virgin female Sprague-Dawley rats (294 ± 4 g, n=15) housed singly in a 12 h light/dark cycle with free access to food and water were used.

Under ether anaesthesia the rats were implanted with an i.c.v. infusion assembly connected to a sub-cutaneously implanted osmotic mini-pump (see General Methods, section 2.3.3.). The infusate delivered over 5 days into a lateral cerebral ventricle was morphine sulphate dissolved in sterile pyrogen-free distilled water in sequentially increasing concentrations (10 μg/μl, 20 μg/μl and 50 μg/μl), each delivered at a rate of 1 μl/h over a 40 h period. A guide cannula (1.6 mm lateral and 0.6 mm posterior to bregma) was also implanted for acute injection of i.c.v. vehicle (1% 0.01 M HCl/5 μl 0.9% NaCl) or oxytocin antagonist (1 μg) as required.

2. Properties of oxytocin antagonist

The oxytocin antagonist used was desGly\textsubscript{6}\text{d(CH\textsubscript{2})\textsubscript{5}[Tyr(Me)\textsubscript{2}}, Thr\textsuperscript{4}]OVT. From hereon it is referred to as Manning 16 (Manning et al., 1989). This peptide is an analogue of a peptide which was previously reported to have nonselective oxytocin/vasopressin antagonist effects. Manning 16 however exhibits substantially reduced antivasopressor potency compared with antioxytocic potency \textit{in vivo}. The \(pA_2\) values (negative log of the concentration required to reduce the agonist effect by half) for its antioxytocic activity \textit{in vitro} and \textit{in vivo} are 8.56 ± 0.09 and 7.61 ± 0.08 respectively. The \(pA_2\) value for its antivasopressor activity \textit{in vitro} is 6.98 ± 0.07. Finally, the
ED$_{50}$ (dose which reduces the response to an agonist by half) of Manning 16 required for an antivasopressor effect is 4.3 times greater than that required for an antioxytocic effect (Manning et al., 1989).

3. Blood sampling/injection procedure

On the morning of the sixth day of i.c.v. infusion the rats were anaesthetised with urethane (1.25 g/kg i.p.) and a femoral artery and vein cannulated for the withdrawal of blood samples and injection of drugs (see General Methods, section 2.3.1.). Rats were randomly assigned to the control or experimental group (n=7 and 8 respectively) and a 2 h equilibration period allowed before sampling commenced. One basal sample was obtained before the second blood sample, which was immediately followed with an i.c.v. injection of either vehicle or Manning 16 (1 µg). The second sample was followed 10 min later with an i.v. injection of naloxone (5 mg/kg). A fourth sample was taken 5 min later and followed by three more samples over the next 30 min to follow the time-course of the withdrawal response. Plasma separated from the red blood cells was stored immediately on ice and subsequently at -20°C prior to radioimmunoassay for oxytocin content (see General Methods, section 2.4.).

4.2.2. Effect of i.c.v. oxytocin antagonist infusion on withdrawal hypersecretion of oxytocin

1. Animals

Virgin female Sprague-Dawley rats (283 ± 9 g, n=15) housed singly under standard conditions (see General Methods, section 2.1.) were used throughout these experiments.

Surgery for induction of morphine tolerance and dependence and
placement of the guide cannula for subsequent injection of vehicle or oxytocin antagonist was as described in General Methods (section 2.1.) and section 4.2.2.1.

2. Blood sampling/infusion procedure

The protocol used was the same as that outlined previously in section 4.2.3. except that the acute i.c.v. injection of either vehicle or antagonist was followed immediately with a continued infusion over the following 15 min until the first post-naloxone blood sample was obtained. 2 μg of antagonist dissolved in 8 μl vehicle was infused at a rate of 0.5 μl/min from a slow perfusion pump (B. Braun, GmbH). Similarly blood plasma samples were taken and stored at -20°C prior to radioimmunoassay for oxytocin content.

4.2.3. Radioimmunoassay for oxytocin

Oxytocin content of all samples from experiments in this chapter were assayed using a modified method and the antiserum of Higuchi et al., (1985a). Assay sensitivity was <4.8 pg/ml and the interassay coefficient of variation between experiments was 0.3%. Intraassay coefficients of variation were 8.8, 21.8 and 19.5% for standard concentrations of 20, 100 and 500 pg/ml respectively. All samples from a single experiment were measured in the same assay to eliminate any interassay variation.
4.3. RESULTS

4.3.1. Effect of acute i.c.v. oxytocin antagonist injection on withdrawal hypersecretion of oxytocin

I.c.v. injection of oxytocin antagonist had no effect on basal plasma oxytocin levels compared with vehicle alone (Fig. 4.3.1.1.). I.v. injection of naloxone 10 min after the acute i.c.v. injection significantly increased plasma oxytocin concentration in the antagonist-treated rats 5 min later, by 339.7 ± 105.3 pg/ml from a basal level of 14.4 ± 1.9 pg/ml (p<0.05; n=8, Paired Wilcoxon). A similar increase in oxytocin release of 476.9 ± 98.7 pg/ml was seen in the vehicle-treated animals (p<0.05; n=7). The withdrawal-induced elevation in plasma oxytocin remained significant in both groups of animals up to 35 min after injection of naloxone (p<0.05).

4.3.2. Effect of i.c.v. oxytocin antagonist infusion on withdrawal hypersecretion of oxytocin

Injection of i.v. naloxone 10 min after acute i.c.v. injection of vehicle and 5 min before the i.c.v. infusion of vehicle was due to end produced a withdrawal-induced increase in plasma oxytocin release of 886.6 ± 182.8 pg/ml, significant at 5 min and up to 35 min after injection of naloxone (p<0.02 n=7; Paired Wilcoxon), (Fig. 4.3.2.1.). The rise in plasma oxytocin of 274.8 ± 51.6 pg/ml after i.v. naloxone, in rats administered an acute i.c.v. injection followed by infusion of oxytocin antagonist although significant (p<0.05; n=8), was significantly less than that in the i.c.v. vehicle-treated group (p=0.01; Mann-Whitney U-test). This difference in plasma oxytocin levels between groups was still significant 35 min after injection of naloxone (p<0.05).
Figure (4.3.1.1.)- Effect of acute i.c.v. oxytocin antagonist injection on withdrawal hypersecretion of oxytocin. I.v. naloxone (5 mg/kg) given 10 min after i.c.v. oxytocin antagonist (1 μg/5μl) increased plasma oxytocin in both vehicle-treated (○; n=7) and antagonist-treated animals ( ▲; n=8) and this elevation was significant at 5 min and up to 35 min after injection of naloxone (*p<0.05; Paired Wilcoxon).
Figure (4.3.2.1.)- Effect of i.c.v. oxytocin antagonist infusion on withdrawal hypersecretion of oxytocin. i.v. naloxone given 10 min after acute injection and 5 min before the end of i.c.v. infusion (1 μg; 2 μg/8 μl) of oxytocin antagonist increased plasma oxytocin in both vehicle-treated (O; n=7) and antagonist-treated (▲; n=8) animals (#p<0.02 and *p<0.05 respectively; Paired Wilcoxon). This remained significant in both groups up to 35 min after injection of naloxone (*p<0.05). The elevation in plasma oxytocin in the vehicle-treated group was significantly greater than that observed in the oxytocin antagonist-treated group (**p=0.01; Mann-Whitney U-test) 5 min after injection of naloxone.
4.4. DISCUSSION

Intravenous administration of naloxone to the morphine dependent rats produced an expected withdrawal-induced hypersecretion of oxytocin release in both blood sampling experiments in accordance with previously reported studies (Bicknell et al., 1988; Rayner et al., 1988; Leng et al., 1989); this is due to increased electrical excitation of the magnocellular oxytocin neurones in conjunction with removal of opioid tone at the nerve terminals in the neurohypophysis. Acute i.c.v. injection of the oxytocin antagonist Manning 16 alone, had no significant effect on the time course of the withdrawal response despite mean oxytocin release tending to be lower compared with vehicle at each post-naloxone sampling point in the antagonist-treated group. Since this particular oxytocin antagonist is derived from the peptide ornithine vasotocin (Manning et al., 1989) it is most probable that its half-life within the brain would be of a short duration since peptides are subject to rapid degradation by endogenous processing enzymes (McKelvy and Blumberg, 1986). Preliminary studies of its effects on the milk-ejection reflex (using a similar concentration of Manning 16 for i.c.v. injection) initiated by stimulation of the neural stalk as described for verapamil (see Chapter 3, section 3.2.6.), or after i.v. administration, suggested that it may have a duration of action in the range of 20-30 min (unpublished observations) since the established milk-ejection reflex could be blocked for this amount of time.

The autofacilitatory effect of oxytocin on the bursting of oxytocin neurones during the milk-ejection reflex is so pronounced (Freund-Mercier and Richard, 1984) however, that it was decided to infuse the oxytocin antagonist after the initial acute i.c.v. injection had been made. Since specific oxytocin binding sites have been demonstrated histoautoradiographically (Freund-
Mercier and Stoeckel, 1993) within the SON and these studies show that i.c.v. oxytocin antagonist can affect oxytocin receptors within the SON, the i.c.v. infusion of antagonist directly after the acute injection should have ensured that a steady state of drug concentration was maintained, into the early part of withdrawal, offsetting enzymatic hydrolysis of this peptide. In this experiment administration of oxytocin antagonist significantly attenuated the withdrawal process 5 min and up to 35 min after injection of naloxone which is indicative of a central role for oxytocin in sustaining this process. Whether acute injection followed by infusion of this antagonist could affect the naloxone-induced increase in firing rate in the morphine dependent rat remains to be established.

There is substantial immunocytochemical and electrophysiological evidence in support of an oxytocin-containing pathway from the PVN to the bed nuclei of the stria terminalis (BNST) in the lactating rat (Ingram and Moos, 1992). The existence of specific oxytocin binding sites within the BNST has been demonstrated (Insel, 1990) and microinjection of oxytocin into this region facilitates the electrical activity of magnocellular oxytocin neurones, within both the SON and PVN of lactating rats (Moos et al., 1991). It has been suggested that afferents from the BNST may be involved in autoexcitation of oxytocin neurones during the milk-ejection reflex (Ingram and Moos, 1992). As yet this pathway, and a subsequent autoexcitatory effect of oxytocin on magnocellular oxytocin neurones has not been investigated in terms of withdrawal excitation of these neurones.

It is likely that this self-stimulating action of oxytocin during the withdrawal response is a property of the magnocellular neurosecretory system, although this system does share features with other neuronal systems such as the
locus coeruleus which displays the typical withdrawal characteristics of hyperexcitation of electrical activity and hypersecretion of noradrenaline release (North and Williams 1985). In addition both systems show increased expression of c-fos mRNA and Fos protein during withdrawal in the morphine dependent rat (Hayward et al., 1990; Russell et al., 1992; Johnstone et al., 1993). This then questions the functional significance of autofacilitatory release of oxytocin during withdrawal. Since release of oxytocin within the SON in vivo is Ca$^{2+}$ dependent (Landgraf et al., 1992) and in the hypothalamic slice preparation oxytocin can stimulate further dendritic release of oxytocin by a voltage-sensitive Ca$^{2+}$ channel mode of action (Yamashita et al., 1987) it would appear that the whole withdrawal process is primarily Ca$^{2+}$ dependent with central oxytocin acting as a messenger for its own release. Positive feedback would continue until the source of oxytocin had become depleted, or another self-limiting process had become activated, bringing the withdrawal process indicative of physical dependence to an end. An intrinsic relationship between oxytocin and Ca$^{2+}$ within the neurohypophysial magnocellular neurosecretory system such as this merits further consideration. Chronic exposure to morphine in vivo increases dihydropyridine sensitive Ca$^{2+}$ channel binding site density within the brain (Ramkumar and El-Fakahany, 1988), probably as a compensatory measure to ongoing occupancy of the $\mu$-opioid receptor by morphine, which is known to decrease a K$^+$ dependent conductance and may decrease an associated Ca$^{2+}$ dependent conductance in neuronal preparations (North, 1989). Since oxytocin neurones become tolerant to the inhibitory actions of morphine (Pumford et al., 1991) it is not entirely unreasonable to propose upregulation of oxytocin receptor expression during this treatment in a compensatory
fashion analogous to that achieved for the Ca\(^{2+}\) channel. Two adaptive mechanisms such as these acting in concert with each other could provide a partial explanation for withdrawal hypersecretion of oxytocin in the morphine dependent rat.
5.1. INTRODUCTION

Noradrenergic receptor classification

Two types of adrenergic receptor termed $\alpha$ and $\beta$ were originally proposed on the basis that adrenaline, isoprenaline and noradrenaline produce excitatory, inhibitory and mixed excitatory/inhibitory effects in smooth muscle respectively (Ahlquist, 1948). Later, $\beta$-receptors were subdivided into $\beta_1$ (those in the myocardium) and $\beta_2$ (smooth muscle and most other sites), whilst recently a third receptor designated $\beta_3$ has been identified in human adipose tissue. Likewise $\alpha$-receptors have been further characterised as $\alpha_1$ and $\alpha_2$. These receptor subtypes were first described on the basis that in general $\alpha_1$-agonists were post-synaptically located and excitatory to noradrenaline release from neurones, whereas $\alpha_2$-agonists were pre-synaptically located and inhibitory (Lefkowitz et al, 1990). However it is now clear that $\alpha_2$-adrenergic receptors are also present at post-synaptic sites in several tissues. Thus the anatomical concept of pre-synaptic $\alpha_2$ and post-synaptic $\alpha_1$ has been abandoned in favour of pharmacological and functional classification. In addition the aforementioned receptors have been isolated to different genes using molecular cloning techniques. Further subdivisions of the $\alpha$-receptor subtypes have been reported (for review see receptor nomenclature supplement accompanying Trends Pharmacol. Sci. 1993).

Noradrenergic innervation of magnocellular neurosecretory cells

A number of noradrenergic pathways project from the brainstem to magnocellular neurones in the SON and PVN (Sawchenko and Swanson, 1982; Wilkin et al., 1989). The most dense of these inputs projecting to the SON is from the noradrenergic A1 cell group which passes through the
ventrolateral medulla to almost exclusively innervate vasopressin neurones (Raby and Renaud, 1989). The A2 cell group of the nucleus tractus solitarius (NTS) and the A6 cell group originating in the locus coeruleus although providing a less extensive projection framework innervate amongst other regions oxytocin neurones within the SON. Oxytocin cells in the SON are directly excited by electrical stimulation of the A2 group, whilst this stimulation only affects vasopressin neurones indirectly via the ventrolateral medulla (Raby and Renaud, 1989). Much less is known regarding direct stimulation of the A6 group. However, unilateral lesions in the locus coeruleus decrease catecholamine fluorescence in the SON (Loizu, 1969) whilst direct injection of the fluorescent retrograde tracer doxorubicin into the SON labels catecholamine cells within the A6 cell group identified by histochemistry (Wilkin et al., 1989). Reports that lesions of efferents from the A6 region result in impairment of the vasopressin secretory response to haemorrhage (Everitt et al., 1983), may provide some physiological support for an A6-magnocellular pathway.

The NTS acts as an extremely important integrative relay structure, its afferents having been reported to convey a variety of information to the hypothalamic nuclei. This information would appear to be gastrointestinal rather than cardiovascular in origin, with gastric distension and peripheral administration of CCK8S being potent stimuli to NTS neurones in the rat (Raybould et al., 1985). These same stimuli are also able to selectively excite oxytocin neurones within the SON (Renaud et al., 1987). Systemically administered CCK8S induces expression of Fos protein within the NTS and SON (Luckman, 1992; Hamamura et al., 1991) and increases firing rate of SON oxytocin neurones (Hamamura et al., 1991) resulting in oxytocin release
from the neurohypophysis into the periphery (Blackburn and Leng, 1990). Despite the locus coeruleus having been reported to show no overall increase in Fos-like immunoreactive material following systemic injection of CCK8S, it has not been excluded that changes may take place within specific neuronal populations in this region (Luckman et al., 1993). Further evidence in support of direct NTS noradrenergic innervation of magnocellular oxytocin neurones has been shown using microdialysis sampling of the SON, where noradrenaline concentration within the dorsal, oxytocin-rich region of the SON increases in response to intravenous CCK8S injection (Kendrick et al., 1991). The CCK8S-mediated oxytocin response is attenuated by lesions of the area postrema, a circumventricular organ which provides a dense input to the caudal NTS (Carter and Lightman 1987). This organ lacks a blood-brain barrier and as such can conceivably receive blood borne signals. However it is difficult to lesion the area postrema without damaging the NTS and it is unlikely that CCK8S passes through this window to the brain to have direct effects on magnocellular neurosecretory cells.

**A proposed role for noradrenaline in withdrawal excitation of neurones**

In the rat the neurones of the locus coeruleus become both tolerant to and dependent upon the inhibitory actions of opiates in vivo (Aghajanian, 1978; Kosten, 1992), although such evidence for dependence has been much harder to obtain in related in vitro models (Christie et al., 1987). Locus coeruleus neurones show increased expression of c-fos mRNA and Fos protein 1-2 h after initiation of withdrawal (Hayward et al., 1990). These neurones exhibit a hyperexcitation of firing rate during precipitated opiate withdrawal which shows a similar time course to that of the behavioural signs of withdrawal (Rasmussen et al., 1990). As yet dependence has not been
demonstrated at the level of the single neurone (Christie et al., 1987) thereby reinforcing the requirement for maintenance of synaptic input in order for withdrawal excitation to be shown.

Locus coeruleus noradrenergic neurones are inhibited by noradrenergic agonists such as clonidine (Aghajanian, 1978) acting at $\alpha_2$-adrenergic receptors and by opiates which bind preferentially to the $\mu$-opioid receptor (Christie et al., 1987). At the level of the single neurone, $\mu$-opioid and $\alpha_2$-adrenoceptors appear to converge on the same post-receptor mechanisms, utilising the same pool of pertussis-sensitive G proteins to modify the K$^+$ conductance responsible for membrane hyperpolarisation and subsequent inhibition of neuronal activity (Aghajanian and Wang, 1987; Christie et al., 1987). Local infusion of the $\alpha_2$ agonist clonidine in the vicinity of the locus coeruleus attenuates several behavioural changes associated with naloxone-precipitated withdrawal as well as reducing hippocampal concentration of the noradrenergic metabolite 3-Methoxy-4-hydroxyphenylglycol, indicative of decreased noradrenaline turnover (Taylor et al., 1988). Systemic administration of a non-lipophilic $\alpha_2$ agonist (clonidine is extremely lipophilic and crosses the blood-brain barrier very easily) has no effect on hippocampal noradrenaline turnover suggesting that clonidine attenuation of naloxone-precipitated withdrawal in locus coeruleus neurones is site-specific.

**Noradrenergic control of magnocellular secretory function**

It has been mentioned previously that noradrenergic projections arising in the brainstem can modulate activity of supraoptic oxytocin neurones by both direct and indirect modes of action (Raby and Renaud, 1989).

In the rat magnocellular neurosecretory system, noradrenaline has been proposed to facilitate release of vasopressin and oxytocin from
neurohypophysial terminals via stimulatory actions on \( \alpha_1 \)- and \( \beta \)-adrenergic receptors within the neurohypophysis, although this only occurs once presynaptic opioid or \( \alpha_2 \)-regulated restraint of noradrenaline release has been removed (Zhao et al., 1988b). Within the SON noradrenaline activates magnocellular neurosecretory cells via actions on post-synaptically located \( \alpha_1 \) receptors (Yamashita et al., 1987). The co-release of opioid peptides with oxytocin and especially vasopressin can inhibit oxytocin release partly through inhibiting the facilitatory action of noradrenaline on oxytocin neurones, both at the terminal level (Zhao et al., 1988a) and potentially at the cell body in the SON (Douglas et al., 1993); increased endogenous opioid tone acting on magnocellular oxytocin neurones during pregnancy has been suggested to regulate oxytocin secretion at parturition (Douglas et al., 1993). However the potential involvement of noradrenergic mechanisms in opioid control of oxytocin neurones has remained relatively poorly understood.

Preliminary blood sampling studies were undertaken to investigate the acute effects of opioids on oxytocin release stimulated by peripheral administration of CCK8S which is known to involve noradrenergic projections which originate in the NTS. This study was then repeated in morphine dependent rats to ascertain whether supraoptic oxytocin neurones develop tolerance to the actions of morphine on this excitatory noradrenergic input. The relative contribution of this input to oxytocin neurones was then determined by measuring release of oxytocin into blood from the neurohypophysis after stimulation by systemic CCK8S in animals given an intravenous injection of the \( \alpha_2 \) agonist clonidine. This blood sampling study was also undertaken to give an indication as to the required dose of clonidine needed to have a supposed influence on the initiation of withdrawal whilst
having a minimal effect on blood pressure, since clonidine has well defined hypotensive effects; initially increasing blood pressure via stimulation of peripheral $\alpha_2$ receptors followed by a more lengthly fall as a result of its central actions (Hoffmann and Lefkowitz, 1990). The effects of clonidine on the withdrawal hypersecretion of oxytocin in the morphine dependent rat were determined using blood sampling and radioimmunoassay for oxytocin content in conjunction with measurement of arterial blood pressure. Finally the control by endogenous opioids of oxytocin release initiated by peripheral administration of CCK8S was investigated in the 21 day pregnant rat by comparing the effects of naloxone on CCK8S-stimulated oxytocin release from the neurohypophysis with oxytocin release in the virgin female.

It was hoped that this series of experiments would provide an insight into the noradrenergic control of oxytocin release in response to various stimuli and whether such control mechanisms could play a pivotal role in the characteristic withdrawal process of magnocellular oxytocin neurones within the SON.
5.2. MATERIALS AND METHODS

5.2.1. Effect of acute i.v. and central morphine administration on CCK8S-stimulated oxytocin release

1. Animals

Virgin female Sprague-Dawley rats (body weight 252-267 g) housed under standard conditions (see General Methods, section 2.1.) were anaesthetised with urethane (ethyl carbamate, 1.25 g/kg; i.p.). The femoral artery and vein were then cannulated for withdrawal of blood samples and injection of drugs respectively. I.c.v. injections were given via an implanted guide cannula (1.6 mm lateral, 0.6 mm posterior to bregma and 4.0 mm below the skull surface). Rats were allowed to recover for 2 h after completion of surgery before obtaining the first blood sample.

2. Blood sampling/injection procedure

In experiments in which rats were given acute morphine i.v. the first blood sample was followed by an i.v. injection of CCK8S (20 μg/kg). Three more i.v. injections of CCK8S, 35 min apart, followed the fourth, seventh and tenth blood samples respectively. Five min after the third blood sample, rats received an injection of i.v. morphine (0.5 mg/kg) or vehicle (0.9% NaCl; 0.5 ml/kg). A second, larger dose of i.v. morphine (5 mg/kg) or vehicle was given 5 min after the sixth blood sample. All rats were given naloxone (5 mg/kg) i.v. immediately after the ninth blood sample was taken at t=95 min. Three more blood samples were taken over the following 35 min.

Rats given an acute i.c.v. injection of morphine (10 μg) or vehicle (5 μl) 5 min after the third blood sample were given two injections of i.v. CCK8S separated by 35 min. Both groups of rats then received an i.p. injection of
1.5 M NaCl (4 ml/kg) after the sixth blood sample was taken and two more samples followed separated by 10 min intervals. Blood samples were centrifuged and the plasma separated and stored on ice before being frozen to -20°C prior to radioimmunoassay for oxytocin content.

5.2.2. Effects of i.v. CCK8S and acute i.v. morphine administration on oxytocin release in the morphine dependent rat

1. Animals

Virgin female Sprague-Dawley rats (274 ± 5 g, n=15) housed singly under standard conditions with free access to food and water were used.

2. I.c.v. infusion

Under ether anaesthesia rats were implanted with an i.c.v. cannula (2 mm lateral, 3 mm posterior to bregma, 4.5 mm below skull surface) for infusion of morphine sulphate (up to 50 µg/h; see General Methods, section 2.3.3.) delivered from a subcutaneously implanted osmotic mini-pump over 5 days. The i.c.v. vehicle-treated group were implanted with an i.c.v. infusion assembly as mentioned, but containing sterile pyrogen-free distilled water.

3. Blood sampling/injection procedure

On the morning of the sixth day the rats were anaesthetised with urethane (1.25 g/kg i.p.) and the femoral artery and vein cannulated for blood sampling and injection of drugs. The first blood sample was followed directly by an i.v. injection of CCK8S (20 µg/kg) and two more i.v. injections of CCK8S followed the fourth and seventh blood samples. Five min after the third blood sample the rats were given an i.v. injection of morphine (0.5 mg/kg) and a subsequent dose of i.v. morphine (5 mg/kg) was administered after the sixth blood sample. Naloxone (5 mg/kg) was injected i.v. after the ninth blood
sample. Two more blood samples were taken separated by 10 min intervals.

5.2.3. Effect of i.v. clonidine on the CCK8S-stimulated oxytocin neurosecretory response

1. Animals

Virgin female Sprague-Dawley rats (261 ± 2 g, n=12) housed in groups of six animals, with free access to food and water were used. They were maintained in a 12 h light/dark cycle at an ambient temperature of 21-23°C.

2. Blood sampling/injection procedure

Rats were anaesthetised with urethane (1.25 g/kg) and the femoral artery and vein cannulated for withdrawal of blood samples and injection of resuspended blood cells and drugs (see General Methods, section 2.3.4.). Blood sampling was started 2 h after completion of surgery. CCK8S (20 μg/kg) was given i.v. directly after the first blood sample had been taken. Two more i.v. injections of CCK8S followed the fourth and seventh blood samples in both groups of animals. Immediately after the third blood sample, rats received an i.v. injection of either clonidine (0.5 mg/kg) or vehicle (0.9% NaCl). A second injection of i.v. clonidine (2.5 mg/kg) or vehicle was given immediately after the sixth blood sample had been obtained. Animals in both groups were given an i.v. injection of naloxone (5 mg/kg) following the ninth blood sample and a final sample was taken 5 min later.

5.2.4. Effect of i.v. clonidine on withdrawal hypersecretion of oxytocin in the morphine dependent rat

1. Animals and i.c.v. morphine infusion

Virgin female Sprague Dawley rats (278 ± 3 g, n=15) were housed singly with access to food and water ad-libitum. Under ether anaesthesia the rats
were stereotaxically implanted with an i.c.v. cannula for infusion of morphine sulphate over 5 days to induce morphine tolerance and dependence (see General Methods, section 2.3.3.).

2. Blood sampling/injection procedure

On the morning of day 6 of i.c.v. infusion rats were anaesthetised with urethane (1.25 g/kg). The femoral artery and vein were cannulated for withdrawal of blood samples and injection of drugs as described previously (General Methods, section 2.3.4.). After a first blood sample had been taken, a single i.v. injection of either clonidine (2.5 mg/kg) or vehicle (0.9% NaCl) was given directly after a second basal blood sample had been obtained. In both groups of rats this was followed 10 min later with an i.v. injection of naloxone (5 mg/kg). Two more samples were taken over the next 10 min to monitor the withdrawal response. A further three samples were obtained 20, 40, and 70 min after injection of naloxone.

In a few rats the arterial cannula was connected to a pressure transducer (Model P231d; Gould Stratham Instruments) relayed to a chart recorder (J.J. Instruments Ltd, Southampton) for on-line measurement of arterial blood pressure and heart rate. Mean arterial blood pressure (MABP) was defined as- diastolic blood pressure + 0.33 (diastolic-systolic blood pressure).

5.2.5. Effect of naloxone on CCK8S stimulation of oxytocin release during pregnancy

1. Animals

Virgin female Sprague-Dawley rats (n=28) were housed under standard conditions and given food, water and pellets ad libitum. One group was mated overnight during oestrus with an experienced male and defined as
pregnant following production of a vaginal plug the following morning. This was designated day 0 of pregnancy and pregnant rats (404 ± 9 g, n=16) were taken subsequently on day 21 of pregnancy. Where possible virgin female rats (267 ± 4 g, n=12) from the same stock were used as control animals.

2. Blood sampling/injection procedure

All rats were anaesthetised with urethane (1.25 g/kg i.p.) and the femoral artery and vein cannulated for withdrawal of blood samples and injection of drugs respectively. CCK8S (20 μg/kg i.v.) was given directly after a third basal sample had been obtained. Two samples were taken 5 and 45 min later in order to follow the oxytocin response to CCK8S stimulation. At this point either naloxone (2 mg/kg) or vehicle (0.9% NaCl) was injected intravenously before three more blood samples were taken, each separated by 5 min intervals. A second injection of CCK8S was followed by two final blood samples taken at t=80 and t=90 min respectively.

5.2.6. Radioimmunoassay for oxytocin

Oxytocin content for all samples from experiments described in this chapter was determined using the antiserum and modified method of Higuchi et al., (1985a) as described previously (see General Methods, section 2.4.). Assay sensitivity was <2.4 pg/ml and the interassay coefficient of variation was 10.6%. Intraassay coefficients of variation were 10 and 11% at standard concentrations of 20 and 100 pg/ml respectively.
5.3. RESULTS

5.3.1. Effect of acute i.v. and central morphine administration on CCK8S-stimulated oxytocin release

i.v. morphine

The first injection of i.v. CCK8S (20 μg/kg) increased plasma oxytocin concentration by 123.7 ± 25.1 pg/ml (p<0.001, n=10; Paired Wilcoxon) and two further i.v. injections of CCK8S 5 min after i.v. vehicle produced similar significant increases (p<0.05, n=5), (Fig. 5.3.1.1.). Morphine was given i.v. 5 min before the second and third CCK8S injections and produced a dose-related inhibition of oxytocin release significant after 5 mg/kg morphine (p<0.05, vs the first CCK8S response and vs the third CCK8S response in the vehicle controls; Mann-Whitney U-test). When naloxone was given i.v. 30 min after i.v. morphine (5 mg/kg) plasma oxytocin concentration increased in both morphine-treated rats to 936.9 ± 401.4 pg/ml and in vehicle-treated rats to 1343.8 ± 563.0 pg/ml, (p<0.05; Paired Wilcoxon). The response to i.v. CCK8S given 10 min after i.v. naloxone was restored in the morphine-treated rats (p<0.05).

i.c.v. morphine

Prior to acute i.c.v. morphine injection, an i.v. injection of CCK8S increased plasma oxytocin concentration by 99.6 ± 19.5 pg/ml from a basal level of 33.3 ± 5.7 pg/ml, (p<0.001, n=12; Paired Wilcoxon) and a second i.v. injection of CCK8S given 35 min later, 5 min after injection of i.c.v. vehicle produced a similar increase (p<0.05, n=5), (Fig. 5.3.1.2.). Morphine (2 μg/μl) injected i.c.v. 5 min before the second injection of i.v. CCK8S abolished the oxytocin response seen in the vehicle-treated animals (p<0.001, n=7; Paired...
Hypertonic saline injected i.p. 25 min after the second CCK8S injection in i.c.v. vehicle-injected rats, increased plasma oxytocin concentration by $277.7 \pm 121.6$ pg/ml ($p<0.05$, n=5; Paired Wilcoxon) over the following 20 min but had no effect on plasma oxytocin in i.c.v. morphine-treated rats.

5.3.2. Effects of i.v. CCK8S and acute i.v. morphine administration on oxytocin release in the morphine dependent rat

After an initial injection of i.v. CCK8S plasma oxytocin concentration increased by $29.0 \pm 7.6$ pg/ml from a basal level of $16.4 \pm 2.3$ pg/ml in the i.c.v. vehicle-treated animals and by $27.3 \pm 3.1$ pg/ml from a basal level of $23.0 \pm 6.8$ pg/ml in the i.c.v. morphine-treated group, ($p<0.02$, n=8 and 7 respectively; Paired Wilcoxon). A second i.v. injection of CCK8S 5 min after an injection of i.v. morphine (0.5 mg/kg) produced a similar significant increase ($p<0.02$), (Fig. 5.3.2.1.). Two i.v. injections of morphine (0.5 and 5 mg/kg) were given 5 min before the second and third CCK8S injections respectively. These produced a dose-related inhibition of the oxytocin response to stimulation by CCK8S in the i.c.v. vehicle-treated animals ($p<0.02$, second CCK8S response vs the first CCK8S response; $p<0.05$ third CCK8S response vs the second CCK8S response) whilst in the morphine-treated animals, only the second larger dose of i.v. morphine attenuated the third CCK8S response ($p<0.05$, vs the first and second CCK8S responses). Injection of i.v. naloxone 25 min after the final injection of i.v. CCK8S, increased plasma oxytocin concentration 10 min after injection in both vehicle-treated (by $239.8 \pm 82.5$ pg/ml) and in morphine-treated animals (by $844.4 \pm 134.1$ pg/ml), ($p<0.05$; Paired Wilcoxon). However the increase was significantly greater in i.c.v. morphine-treated rats than i.c.v. vehicle-treated
rats (p<0.005; Mann-Whitney U-test).

5.3.3. Effects of i.v. clonidine on the CCK8S-stimulated oxytocin neurosecretory response

The first CCK8S injection increased plasma oxytocin concentration by 16.6 ± 6.5 pg/ml from a basal level of 10.7 ± 3.7 pg/ml (p<0.005, n=12; Paired Wilcoxon Fig. 5.3.3.1.). Two further injections of CCK8S after administration of i.v. vehicle produced similar significant increases of 15.4 ± 5.1 pg/ml and 15.0 ± 4.4 pg/ml respectively (p<0.05, n=5). Clonidine (0.5 and 2.5 mg/kg) was given i.v. before the second and third CCK8S injections and produced a complete inhibition of the oxytocin neurosecretory response to CCK8S (p<0.005 and p<0.05 vs the respective CCK8S-stimulated oxytocin responses in the vehicle controls; Mann-Whitney U-test). There was no significant difference in the oxytocin response to systemic injection of naloxone (5 mg/kg, i.v.) between vehicle-treated animals where plasma oxytocin concentration increased by 45.7 ± 16.6 pg/ml (p<0.05), and in clonidine-treated animals (n=7) where plasma oxytocin concentration increased by 12.3 ± 4.3 pg/ml (p<0.05).

5.3.4. Effects of i.v. clonidine on withdrawal hypersecretion of oxytocin in the morphine dependent rat

Administration of i.v. clonidine (2.5 mg/kg) had no effect on basal plasma oxytocin levels compared with vehicle alone (Fig. 5.3.4.1.). I.v. injection of naloxone (5 mg/kg) given 10 min after injection of either clonidine or vehicle, increased plasma oxytocin from a basal level of 21.3 ± 4.5 pg/ml, in clonidine-treated rats to 303.7 ± 113.3 pg/ml and in vehicle-treated rats to 702.4 ± 186.9 pg/ml 10 min after injection of naloxone and this elevation
remained significant 40 min later (p<0.05, n=8 and 7 respectively; Repeated measures ANOVA). The increase in plasma oxytocin in the vehicle-treated group was significantly greater than that observed in the clonidine group (p<0.05; lower tailed one-way t-test) 10 min after naloxone injection.

I.v. injection of vehicle had no effect on arterial blood pressure or heart rate (Fig. 5.3.4.2.a). I.v. injection of clonidine produced an initial increase in arterial blood pressure with a simultaneous decrease in heart rate from 420 bts/min (Fig. 5.3.4.2.b.). Naloxone given i.v. 10 min after injection of either vehicle or clonidine produced a small, transient increase in blood pressure lasting for approximately 10s in both groups, but had no effect on heart rate in morphine dependent rats.

5.3.5. Effect of naloxone on CCK8S stimulation of oxytocin release during pregnancy

Non-pregnant rats

The first CCK8S injection (20 μg/kg, i.v.) significantly increased plasma oxytocin concentration in the vehicle-treated rats by 14.3 ± 4.4 pg/ml (p<0.05, n=6; Paired Wilcoxon) from a basal level of 11.9 ± 3.3 pg/ml (Fig. 5.3.5.1.a.). Similarly in rats given a subsequent injection of naloxone (5 mg/kg) plasma oxytocin increased by 11.9 ± 3.3 pg/ml (p<0.05, n=6) from a basal level of 17.1 ± 2.1 pg/ml. 10 min after injection of i.v. naloxone plasma oxytocin concentration was significantly increased (p<0.05; Mann-Whitney U-test) compared with i.v. vehicle. A second injection of CCK8S once more significantly increased plasma oxytocin concentration by 13.7 ± 5.7 pg/ml in the vehicle-treated animals (p<0.05). The 300% elevation (p<0.05) in the oxytocin response stimulated by CCK8S in naloxone-treated animals was significantly greater than the corresponding CCK8S response in animals
injected with i.v. vehicle (p<0.05; Mann-Whitney U-test).

21 Day pregnant rats

As for the non-pregnant rats, the first CCK8S injection again increased plasma oxytocin concentration in both groups of animals from a basal level of 8.5 ± 1.0 pg/ml (p<0.05, n=8 each group; Paired Wilcoxon Fig. 5.3.5.1.b.). I.v. injection of naloxone significantly elevated plasma oxytocin concentration by 27.4 ± 6.8 pg/ml from basal (p<0.02) 10 min after injection. This steady increase in plasma oxytocin concentration was maintained over the following 10 min finally elevating plasma oxytocin by 61.2 ± 8.5 pg/ml (p<0.02) 20 min after injection of naloxone. Oxytocin levels in the naloxone-treated rats were significantly greater at each time point up to 20 min after naloxone injection compared with animals given i.v. vehicle (p<0.001 in each case; Mann-Whitney U-test). A second injection of CCK8S after naloxone, produced a potentiation in release of oxytocin compared with the corresponding CCK8S-stimulated oxytocin response in the vehicle-treated animals (p<0.001; Mann-Whitney U-test), resulting in a 250% increase in plasma oxytocin concentration (p<0.02) 5 min after injection.

The second CCK8S injection increased plasma oxytocin concentration to a significantly greater overall level in the 21 day pregnant rats compared with the non-pregnant rats (171.4 pg/ml compared with 80.4 pg/ml p<0.05; Mann-Whitney U-test).
Figure (5.3.1.1.; overleaf)- Effect of i.v. morphine on CCK8S-stimulated release of oxytocin in the virgin female rat. The first CCK8S injection increased plasma oxytocin (*** p<0.001, n=10; Paired Wilcoxon) and two further injections of CCK8S after i.v. vehicle produced similar significant increases (* p<0.05). Morphine (0.5 and 5 mg/kg) was given i.v. before the second and third CCK8S injections and produced a dose-dependent inhibition of the oxytocin response to stimulation by CCK8S, significant after 5 mg/kg morphine (# p<0.05, vs the first CCK8S response and vs the third CCK8S response in the vehicle controls, Mann-Whitney U-test). I.v. injection of naloxone (5 mg/kg) increased plasma oxytocin in both vehicle-treated (△) and morphine-treated animals (▲; #p<0.05). Naloxone restored the response to i.v. CCK8S in the morphine-treated rats (*p<0.05).
4000

/ A

i.v. morphine (0.5mg/kg) /vehicle

i.v. morphine (5mg/kg)

0 10 20 30 40 50 60 70 80 90 100 110 120 130 140

t CCK 1 (20ug/kg)

CCK 2 (20ug/kg)

CCK 3 (20ug/kg)

CCK 4 (20ug/kg)

i.v. Nal (5mg/kg)

plasma oxytocin (pg/ml)

time (minutes)
Figure (5.3.1.2) - Effect of i.c.v. morphine on CCK8S-stimulated release of oxytocin in the virgin female rat. The first CCK8S injection significantly increased plasma oxytocin concentration (*** p<0.001, n=12; Paired Wilcoxon) and a further injection of CCK8S after i.v. vehicle produced a similar increase (* p<0.05). Morphine (10 μg; 2 μg/μl) injected i.c.v. before the second CCK8S injection, abolished the oxytocin response, (# p<0.05, vs. CCK1 and p<0.001, vs. CCK2 in the vehicle group). Hypertonic saline (4 ml/kg) injected i.p. 25 min after the second CCK8S injection increased plasma oxytocin in i.c.v. vehicle rats (○) over the following 20 min (T p<0.05, n=5), but had no effect on plasma oxytocin in i.c.v. morphine rats (▲).
Figure (5.3.2.1.; overleaf)- Effects of i.v. CCK8S and acute i.v. morphine on release of oxytocin in the morphine dependent rat. I.v. injection of CCK8S increased plasma oxytocin from basal in both i.c.v. vehicle-treated and i.c.v. morphine-treated rats (* p<0.02, n=8 and 7 respectively; Paired Wilcoxon) and a second injection of CCK8S after i.v. morphine produced a similar significant increase (* p<0.02). Morphine (0.5 and 5 mg/kg) was given i.v. before the second and third CCK8S injections and produced a dose-related inhibition of the oxytocin response to stimulation by CCK8S in the vehicle-treated animals (TT p<0.02, second CCK8S response vs the first CCK8S response; T p<0.05, third CCK8S response vs the second CCK8S response). In the morphine-treated animals, morphine (5 mg/kg) reduced the third CCK8S response (@ p<0.05) relative to the first and second CCK8S responses. I.v. injection of naloxone (5 mg/kg) increased plasma oxytocin in both vehicle-treated (O) and morphine-treated animals ( ▲ ; # p<0.05), although the increase was significantly greater in the i.c.v. morphine-treated animals (**p<0.005; Mann-Whitney U-test).
i.v. morphine (0.5mg/kg)

i.v. morphine (5 mg/kg)

CCK 1 (20μg/kg)

CCK 2 (20μg/kg)

CCK 3 (20μg/kg)

i.v. Naloxone (5mg/kg)
Figure (5.3.3.1)- Effects of i.v. clonidine on the CCK8S-stimulated oxytocin neurosecretory response. The first injection of CCK8S increased plasma oxytocin from basal in rats later given vehicle (○) or clonidine (▲), (***p<0.005, n=12; Paired Wilcoxon). Two further injections of CCK8S produced similar significant increases in vehicle-treated animals (*p<0.05; n=7). I.v. injection of clonidine (0.5 and 2.5 mg/kg) before the second and third CCK8S injections completely suppressed the oxytocin responses to CCK8S seen in the vehicle-treated animals (***p<0.005 and *p<0.05; Mann-Whitney U-test). Naloxone increased plasma oxytocin concentration in both vehicle-treated and clonidine-treated animals (#p<0.05).
Figure (5.3.4.1)- Effects of i.v. clonidine on withdrawal hypersecretion of oxytocin in the morphine dependent rat. I.v. injection of naloxone (5 mg/kg) given 10 min after administration of i.v. clonidine (2.5 mg/kg) produced a significant withdrawal-induced increase in oxytocin secretion in both vehicle-treated (O) and clonidine-treated (▲) animals, (*p<0.05; Paired Wilcoxon) and this remained significant at each sampling point up to 40 min later (*p<0.05; Repeated measures ANOVA). The naloxone initiated withdrawal response was significantly greater in the vehicle group than in the clonidine group (#p<0.05; lower tailed one-way t-test) 10 min after injection of naloxone.
Figure (5.3.4.2.; overleaf)- Typical recordings obtained from urethane-anaesthetised morphine dependent rats showing changes in mean arterial blood pressure (MABP) and heart rate in response to i.v. clonidine administration.

(a) Injection of i.v. vehicle had no effect on MABP or on heart rate. Naloxone (5 mg/kg i.v.) given 10 min after vehicle produced a small increase in MABP lasting approximately 10 s. Heart rate remained unchanged measured at 360 bts/min.

(b) Injection of i.v. clonidine (2.5 mg/kg) produced an initial rapid, short-lasting increase in MABP and simultaneous decrease in heart rate from 420 bts/min. Naloxone given i.v. had similar effects on MABP and heart rate to those observed for the control animal.

◆ recording stopped to allow arterial blood sample to be obtained.
a. 20 mm/sec 2 mm/sec

b. 20 mm/sec 2 mm/sec

i.v. vehicle

i.v. clonidine (2.5 mg/kg)

i.v. naloxone (2.5 mg/kg)

i.v. naloxone (5 mg/kg)

4 sec 100 mmHg
Figure (5.3.5.1.; overleaf): Effect of naloxone on CCK8S stimulation of oxytocin release during pregnancy.

(a) Non pregnant rats- plasma oxytocin concentration was significantly increased in both groups of rats after a first injection of CCK8S (*p<0.05, n=6 each case; Paired Wilcoxon). I.v. injection of naloxone (▲), (2 mg/kg) significantly increased (*p<0.05; Mann-Whitney U-test) plasma oxytocin 10 min after injection compared with i.v. vehicle (○). A second injection of CCK8S significantly increased plasma oxytocin concentration in vehicle-treated rats (*p<0.05) and naloxone-treated rats (*p<0.05). This latter increase was more marked in the naloxone-treated animals compared with vehicle-treated animals (#p<0.05).

(b) 21 Day pregnant rats- a first injection of CCK8S (20 μg/kg) increased plasma oxytocin levels in both treatment groups (*p<0.05, n=8 each case; Paired Wilcoxon). Injection of i.v. naloxone (▲) induced a gradual increase in plasma oxytocin concentration (#p<0.02) up to 20 min after injection, which was significantly different from each corresponding time point in the vehicle-treated animals (○), (***p<0.001; Mann-Whitney U-test). CCK8S potentiated release of oxytocin compared with the corresponding CCK8S-stimulated oxytocin response in the vehicle-treated rats (Tp<0.001) when injected for a second time.

The second CCK8S injection increased plasma oxytocin concentration to a significantly greater level in the 21 day pregnant rats compared with the non-pregnant rats (@p<0.05; Mann-Whitney U-test).
5.4. DISCUSSION

Opioid control of oxytocin release stimulated by systemic CCK8S

Systemic administration of CCK8S excites magnocellular oxytocin neurones within the SON via actions on the gastric vagus, which has been proposed to activate centrally ascending noradrenergic pathways originating from the brainstem. This proved to be a repeatable, short-lasting stimulus to release of oxytocin from the neurohypophysis in agreement with previous studies (Blackburn and Leng, 1990). The dose-dependent inhibition of this response by the µ-opioid agonist morphine, indicates that µ-receptors are present somewhere on the input to oxytocin neurones activated by intravenous CCK8S or on the oxytocin neurones themselves, although their location could not be verified from this single experiment alone. However acute i.c.v. administration of morphine was able to completely abolish the oxytocin response to stimulation by intravenous CCK8S, suggesting that morphine acts centrally to inhibit oxytocin secretion in response to this stimulus.

Opioid receptors are known to be widely distributed throughout the central nervous system (Mansour et al., 1988) and they are present within the magnocellular nuclei of the hypothalamus (Sumner et al., 1990), possibly on the afferent inputs to the magnocellular nuclei (Sumner et al., 1990) and at the terminal level in the neurohypophysis (Herkenham et al., 1986). Since only κ-agonists such as U50,488 inhibit oxytocin secretion at the level of the neurohypophysis (Zhao et al., 1988a) this further indicates the inhibitory actions of morphine on CCK8S-stimulated release as being centrally located. This also confirms that CCK8S enhances oxytocin secretion by a means other than at the neurohypophysis which is possible since CCK binding sites
have been localised in this region (Bondy et al., 1989b). However CCK-evoked neurohormone release from the isolated neural lobe occurs only after a long latency (Bondy et al., 1989a), whereas release in vivo is immediate and selective for oxytocin.

In rats treated chronically with morphine for 5 days an initial injection of systemic CCK8S was again able to stimulate oxytocin secretion and this response was similar to that observed in control rats. This is in agreement with in vivo electrophysiological studies where CCK8S is able to induce a rapid increase in neuronal firing rate of putative oxytocin secreting neurones in morphine dependent rats (Pumford et al., 1993). Therefore tolerance to the central inhibitory actions of morphine on CCK8S-stimulated oxytocin secretion must have developed in these morphine dependent rats, since acute i.v. and i.c.v. morphine can respectively dose-dependently and completely inhibit oxytocin secretion in response to systemic CCK8S (Figs. 5.3.1.1. and 5.3.1.2.). As with morphine naive animals, morphine (5 mg/kg) was able to significantly attenuate oxytocin secretion in response to stimulation by CCK8S in morphine dependent rats although seemingly not in a dose-dependent manner, indicating that tolerance to the inhibitory actions of i.c.v. morphine on magnocellular neurones is incomplete (Pumford et al., 1993); thus tolerance in these neurones to a systemic CCK8S stimulus is quantitative.

The inhibitory actions of morphine on oxytocin neuronal function would appear not to be stimulus-selective since the secretory response to stimulation with hypertonic NaCl was also abolished after the response to CCK8S had been suppressed by an acute i.c.v. injection of morphine.

Intravenous injection of naloxone increased basal oxytocin levels in
morphine naive rats confirming that endogenous opioids inhibit oxytocin secretion in urethane-anaesthetised rats, and this is at the neurohypophysis (Bicknell et al., 1988). The observed hypersecretion of oxytocin after i.v. injection of naloxone in chronic morphine-treated rats was indicative of physical dependence in these neurones as has been previously described (Bicknell et al., 1988). The potentiating effect of naloxone on oxytocin secretion stimulated by CCK8S (Flanagan et al., 1988; Leng et al., 1992) was confirmed in the first experiment where morphine naive rats had the CCK8S response inhibited by acute morphine restored by naloxone, supporting the interactive role postulated for noradrenaline and opioids in the control of oxytocin secretion.

Adrenergic control of oxytocin release in response to peripheral CCK8S

In conjunction with selectively stimulating release of oxytocin into blood and within the SON, intravenous CCK8S also increases noradrenaline and serotonin release measured by microdialysis within the dorsal region of the SON (Kendrick et al., 1991).

Using the isolated neural lobe preparation Zhao and colleagues have reported a requirement for removal of pre-synaptic α2-mediated and opioid mediated inhibition of noradrenaline release before noradrenaline can act on β and α1 receptors to facilitate oxytocin and vasopressin release (Zhao et al., 1988b). Such a mechanism of pre-synaptic control of noradrenaline from terminals in the SON and ultimately oxytocin release from dendritic and axonal processes of oxytocin neurones (Pow and Morris, 1989), may also occur within the SON.

Release of oxytocin from the neurohypophysis into blood in response to systemic CCK8S stimulation was completely suppressed by prior
administration of the $\alpha_2$ agonist clonidine, even at the lowest dose tested (0.5 mg/kg Fig. 5.3.3.1.). From this present study alone it is impossible to say whether clonidine suppressed CCK8S-stimulated oxytocin release via direct and/or indirect actions on oxytocin neurone firing rate within the SON or via pre-synaptically mediated inhibition of oxytocin release from oxytocin terminals within the neurohypophysis. However intracellular recordings from the SON in the rat hypothalamic slice preparation have shown that noradrenaline depolarises and increases the activity of magnocellular neurosecretory cells through an action on $\alpha$-adrenergic receptors (Yamashita et al., 1987) and this would appear to be mediated by $\alpha_1$- rather than $\alpha_2$- receptors, since clonidine is without affect on firing rate or firing pattern of SON neurones in vitro (Randle et al., 1984). However such reports concern only the direct actions of clonidine on basal firing rate and not on firing rate enhanced by a stimulus such as intravenous CCK8S. Despite CCK8S being able to evoke release of oxytocin from the isolated neurohypophysis (Bondy et al., 1989a), CCK8S when given systemically most probably increases release of oxytocin via actions on oxytocin neurone firing rate (Hamamura et al., 1991). It seems likely therefore that clonidine was able to completely suppress the oxytocin response associated with administration of CCK8S by a pre-synaptic inhibitory effect on firing rate, although an attenuation of secretory activity at the neurosecretory nerve terminals cannot be ruled out. Measurement of noradrenaline release by microdialysis of the SON (which would be attenuated if clonidine were acting to pre-synaptically inhibit oxytocin neurone activation in response to systemic CCK8S administration) would resolve this issue.

It should be noted however that in the lactating rat clonidine has both
inhibitory and facilitatory influences on the milk-ejection reflex depending on the exact dose given (Bailey et al., 1993). Such a finding could be a result of morphological reorganisation of magnocellular oxytocin neurones which takes place during lactation (Theodosis et al., 1986), which in turn may reflect the differential involvement of noradrenergic mechanisms in the control of oxytocin release in response to various stimuli.

Adrenergic and opioid interaction in control of oxytocin secretion from the neurohypophysis during tolerance/dependence

In morphine dependent anaesthetised rats, intravenous administration of naloxone greatly potentiates secretory activity of oxytocin neurones (Bicknell et al., 1988) as illustrated by the present data (Fig. 5.3.4.1.). This robust stimulus to both oxytocin release within the SON (Russell et al., 1992) and secretion from the neurohypophysis into blood (Bicknell et al., 1988) was significantly attenuated by prior administration of clonidine which is consistent with pre-synaptic inhibition of noradrenergic input, and suggests that this input is essential for expression of withdrawal excitation in magnocellular oxytocin neurones.

In vivo electrophysiological recording of SON oxytocin neurone firing rate in the morphine dependent rat has provisionally shown that clonidine can attenuate the naloxone-induced withdrawal increase in firing rate, this being a specific receptor mediated event, which can be reversed by the $\alpha_2$ antagonist idazoxan (unpublished data). Thus clonidine probably attenuates the withdrawal-induced secretory response by decreasing firing rate of magnocellular oxytocin neurones. This may be via the locus coeruleus where clonidine reduces withdrawal excitation of noradrenergic neurones which may project to the SON, or within the SON itself by inhibiting release of
noradrenaline from adrenergic inputs such as the A6 or A2 cell groups.

It could be argued that the dose of clonidine chosen (2.5 mg/kg) in an attempt to suppress the withdrawal response was too small since it did not completely suppress this robust stimulus to oxytocin secretion from the neurohypophysis. Even at this dose clonidine caused an initial rapid increase in blood pressure and decrease in heart rate compared with vehicle (Fig. 5.3.4.2.), presumably via stimulation of peripherally located post-synaptic $\alpha_2$ receptors which are thought to reside on smooth muscle cells of the blood vessel. The terminal half-life of clonidine in the human is approximately 9 h when taken orally, and the reported transient rise in blood pressure is normally followed by a more prolonged fall as a result of its central actions (Hoffmann and Lefkowitz, 1990), although this was not followed since measurement of blood pressure was discontinued shortly after administration of naloxone, which itself appeared to have little effect on blood pressure and heart rate. Such fluctuations in cardiovascular function could potentially modulate activity of magnocellular neurosecretory neurones; more so vasopressin than oxytocin neurones (Harris, 1979).

Treatment with clonidine completely removed the CCK8S-stimulated oxytocin response in control rats, but only partially removed the withdrawal-induced oxytocin response in morphine dependent rats. Adrenergic $\alpha_2$ receptors perhaps located within the SON, and certainly upon inputs to SON oxytocin neurones may be saturated during withdrawal explaining why this robust stimulus to oxytocin secretion is only partially attenuated by prior administration with clonidine. On this basis, such a saturation effect does not appear to have been in evidence during CCK8S stimulation of oxytocin secretion which was completely abolished by clonidine. It is also possible
that a facilitatory noradrenergic input is not essential for the full expression of withdrawal excitation, but is required for systemic CCK8S-stimulation of oxytocin neurones.

Cells of the locus coeruleus have been extensively studied in terms of opiate tolerance and dependence. Withdrawal excitation of their firing rate is also attenuated by administration of clonidine (Aghajanian, 1978) and tolerance to opioids, but not dependence is readily demonstrated in vitro (Christie et al., 1987). Supraoptic magnocellular oxytocin neurones can become tolerant to the inhibitory actions of morphine in vivo as previously described (Bicknell et al., 1988), but in vitro inconsistently display hyperexcitable characteristics typical of dependence during perfusion with naloxone. As such this infers that an intact neuronal framework is a prerequisite for initiation of the complete withdrawal response in these neurones, possibly requiring inputs from cells of the locus coeruleus. Consequently any noradrenergic input from this region which could potentially drive magnocellular oxytocin neurones in the hyperexcitable fashion associated with withdrawal, could only be a representative feature of dependence in the supraoptic cells to which the input projects.

Adrenergic and opioid interaction in control of oxytocin secretion from the neurohypophysis during pregnancy

Endogenous opioids can restrain release of oxytocin from the neurohypophysis (Zhao et al., 1988a) and potentially within the SON (Douglas et al., 1993). Dynorphin which is co-localised with vasopressin (Meister et al., 1990) is an endogenous ligand for the \( \kappa \)-opioid receptor (Corbett et al., 1982), the principal opioid receptor found in the neurohypophysis of the rat (Herkenham et al., 1986). These receptors may
be located post-synaptically on oxytocin neurosecretory terminals or on pituicytes which surround the terminals (Zhao et al., 1988a). Both μ- and κ-opioid receptors are located within the SON (Sumner et al., 1990) where the appropriate endogenous ligand can act to suppress oxytocin neurone firing rate by direct actions (Wakerley et al., 1983a; Inenaga et al., 1990) and possibly indirectly by inhibiting excitatory afferent pathways to the oxytocin neurones.

It has been demonstrated both here (Fig. 5.3.5.1.) and previously in the female virgin rat that naloxone enhances secretion of oxytocin in response to systemic CCK8S (Leng et al., 1992), whilst the firing rate response to CCK8S is unaffected by naloxone (Leng et al., 1992). In 21 day pregnant rats where endogenous opioid restraint of oxytocin release is increased, the CCK8S-stimulated oxytocin response in terms of firing rate is significantly greater after intravenous injection of naloxone (Douglas et al., 1993). On this basis it had been expected that the naloxone potentiation of CCK8S-stimulated oxytocin release from the neurohypophysis observed in virgin rats (Leng et al., 1992) would have been further enhanced in 21 day pregnant rats. The potentiation observed in virgin animals results from removal of endogenous opioid tone at the terminals in the neurohypophysis. This restraining action of opioid peptides is decreased towards the end of pregnancy within the neurohypophysis (Douglas et al., 1993) and increased within the SON (Douglas et al., 1993), although it would be expected that naloxone antagonism of these actions would occur at both locations, as is the case in rats treated chronically with morphine (Leng and Russell, 1989). As central opioid tone increases during pregnancy it may be expected that excitatory input activity to the magnocellular oxytocin system would increase tending
to offset the opioid inhibition on oxytocin neuronal function as has been proposed for morphine dependent rats. The markedly higher oxytocin levels in pregnant rats compared with virgin rats, in response to naloxone potentiation of CCK8S-stimulated oxytocin release supports the electrophysiological data where naloxone potentiation of CCK8S stimulation of oxytocin neurone firing rate is significantly greater in pregnant than in virgin rats (Douglas et al., 1993).

A possible ionic mechanism for noradrenaline actions on magnocellular oxytocin neurones

As yet the exact ionic mechanism by which noradrenaline exerts its influence upon neurosecretory cells remains to be fully defined. Noradrenaline and the $\alpha_1$ agonist methoxamine, depolarise SON neurosecretory cells and this can be diminished by membrane hyperpolarisation and raising extracellular K+ concentration (Bourque and Renaud, 1991). Yamashita and colleagues have speculated that the depolarising actions of noradrenaline on SON cells could be due to the opening of voltage dependent channels (Yamashita et al., 1987). Central administration of the L-type voltage-activated Ca\(^{2+}\) channel antagonist verapamil abolishes intravenous CCK8S stimulation of oxytocin release and significantly attenuates morphine withdrawal induced release of oxytocin from the neurohypophysis (Munro et al., 1993; see also Chapter 3). This action of verapamil has been shown to occur centrally since it has no effect on oxytocin release evoked by stimulation of the neural stalk. As a result of this, it can be speculated that noradrenaline may actually depolarise SON neurosecretory cells principally by opening of Ca\(^{2+}\) channels in conjunction with suppression of a steady-state K+ conductance as previously suggested.
Conversely verapamil may attenuate exocytotic release of noradrenaline within the SON. However the precise location of the effects mediated by verapamil require further investigation. Pre-synaptic release of noradrenaline would appear to have facilitatory actions on the ionic currents mentioned, perhaps within the noradrenergic terminals which in turn can directly enhance the activity of magnocellular oxytocin neurones.

At the molecular level it would be of interest to know whether clonidine and \( \alpha_2 \) agonists in general can inhibit the expression of \( c-fos \) mRNA and the associated protein product Fos in withdrawn animals, not only in magnocellular oxytocin neurones, but also in neurones which show opiate tolerance and dependence distributed throughout the CNS. These markers of neuronal activation undergo increased expression within oxytocin neurones of the SON after naloxone-induced morphine withdrawal (Russell et al., 1992; Johnstone et al., 1993). Such a study would provide a clearer indication as to where exactly clonidine acts to attenuate secretory activity of magnocellular oxytocin neurones during morphine withdrawal.
CCK WITHIN THE SON AS A PUTATIVE ENDOGENOUS OPIOID ANTAGONIST TO OXYTOCIN RELEASE FROM MAGNOCELLULAR NEURONES: A QUANTITATIVE AUTORADIOGRAPHIC STUDY
Chapter 6

6.1. INTRODUCTION

Two types of cholecystokinin (CCK) receptor have been identified using agonist response profiles in various tissue preparations (Innis and Snyder, 1980; Moran et al., 1986), which have subsequently been designated CCK\textsubscript{A} and CCK\textsubscript{B}. Recently these distinct receptor subtypes have been further characterised by molecular cloning techniques (Wank et al., 1992). CCK\textsubscript{A} receptors are located in the periphery and some distinct brain regions whereas CCK\textsubscript{B} receptors are widely distributed throughout the brain (Dietl and Palacios, 1989). Although several forms of CCK with varying lengths of amino acid chains have been isolated from the mammalian central nervous system, it is the sulphated octapeptide (CCK\textsubscript{8S}) fragment which appears to predominate (Larssen and Rehfeld, 1979). CCK interactions with dopamine, and specifically neuromodulation of dopamine release, have been extensively studied in the mesolimbic and nigrostriatal dopamine systems which have been implicated in the development of schizophrenia and Parkinson's disease respectively (Crawley, 1992). CCK has also been colocalised with opioid peptides in nociceptive neurones of the thalamus and brainstem (Gall, 1987).

CCK and opioid interactions

The antagonistic role of CCK to the actions of opioids has previously been demonstrated. Itoh et al., (1982) observed a blockade of i.c.v. \(\beta\)-endorphin-induced analgesia after i.c.v. administration of the CCK agonist caerulein and this finding was further refined to incorporate administration of physiological doses of CCK and blockade of opioid analgesia (Faris et al., 1983). In this second study CCK was also shown to modulate analgesia mediated by
endogenous as well as exogenous opioids as seen by its attenuating effect on classically conditioned front paw foot shock.

Neurones of the intralaminar nuclei of the thalamus respond with either excitation or inhibition to noxious stimuli (Han, 1992). Systemically administered morphine can suppress the excitation and enhance the inhibition, and both effects are blocked by i.c.v. CCK8S. In addition prolonged morphine treatment results in tolerance to the neurophysiological responses of its acute effects. These interactions between opioids and CCK at the neurophysiological level have been replicated using electro-acupuncture instead of systemic morphine (Han, 1992); electro-acupuncture stimulates the release of endogenous opioids and induces analgesia.

Recently it has been shown that CCK receptor antagonists can, depending on receptor subtype (CCK$_A$ or CCK$_B$), influence morphine place conditioning in the rat (Higgins et al., 1992) as well as enhancing morphine analgesia and preventing morphine tolerance (Dourish et al., 1988; Dourish et al., 1990a). CCK does not cross the blood-brain barrier very readily (Verbalis et al., 1986; Hamamura et al., 1991) and this could explain why some studies have failed to show CCK precipitated withdrawal in morphine-treated animals (Pournagash and Riley, 1991).

CCK and opioid interactions within the SON

Morphine inhibits secretion of oxytocin from the neurohypophysis as shown by its effects on the milk-ejection reflex in the lactating rat and on parturition. When administered intraventricularly morphine inhibits the milk-ejection reflex, whilst after chronic administration milk let-down returns after 2 days and within 4-5 days oxytocin release is normal indicating tolerance to the central actions of morphine (Rayner et al., 1988). Morphine given either
subcutaneously or by i.c.v. injection interrupts parturition for up to several hours (Gosden et al., 1985; Luckman et al., 1993) despite the uterus remaining responsive to oxytocin. Supraoptic neurosecretory cell activity is inhibited by morphine in the hypothalamic slice (Wakerley et al., 1983a) while low doses of morphine inhibit the firing-rate of putative oxytocin neurones in the supraoptic nucleus of virgin female rats indicating that μ-receptor inhibition of oxytocin release can occur centrally (Pumford et al., 1991). This is supported further by μ-receptor autoradiographic localisation within the SON (Sumner et al., 1990).

CCK is colocalised within supraoptic magnocellular oxytocin neurones as shown by immunocytochemistry (Vanderhaeghen et al., 1981; Hökfelt et al., 1988) and in situ hybridisation histochemistry (Ingram et al., 1989), whilst CCK levels within the neurohypophysis are depleted upon activation of the oxytocinergic system (Deschepper et al., 1983). Exogenously applied CCK8S can directly influence the excitability of rat supraoptic neurones within the hypothalamic explant maintained in vitro and these receptors would appear to be located on the oxytocin neurones (Jarvis et al., 1992).

Binding for $[^{125}\text{I}]-\text{CCK8S}$ within the SON is enhanced in salt-loaded and homozygous Brattleboro rats (Day et al., 1989). Salt-loading is known to activate the magnocellular neurohypophysial neurosecretory system as shown by electrophysiology (Brimble and Dyball, 1977) and localised expression of Fos immunoreactivity (Giovannelli et al., 1990). Furthermore, following lesions anterior and ventral to the third ventricle, an identified central site involved in the regulation of fluid balance, $[^{125}\text{I}]-\text{CCK8S}$ binding in the SON still increases in response to salt-loading (Blackburn et al., 1990). More recently salt-loading has been shown to increase both CCK$_A$ and CCK$_B$.
receptor content within the hypothalamus, indicating that the reported changes in binding site density probably reflect functional CCK receptors (Hinks et al., 1993). Food deprivation and dehydration in rats decreases and increases CCK receptor density within the SON respectively, and this is thought to be regulated in line with neurochemical activity of predominantly oxytocin neurones in this nucleus (O'Shea and Gundlach, 1993).

In view of the antagonistic actions between CCK and opioids (both endogenous and exogenous) and the well defined changes in CCK8S binding site density within the SON upon activation of the magnocellular oxytocin system, we have employed an in vitro autoradiographic method to try to assess if increased CCK8S binding within the rat SON may be responsible for the development of tolerance to and be partially involved in dependence upon morphine in magnocellular oxytocin neurones during prolonged morphine treatment.
6.2. METHODS

1. Animals

Under ether anaesthesia the rats (body weight; 241-282 g, n=6) were implanted with an i.c.v. infusion assembly connected to a subcutaneously-implanted osmotic mini-pump (Alzet 2001) containing morphine sulphate solution in three sequentially increasing concentrations (10 µg/µl, 20 µg/µl and 50 µg/µl) with each solution separated by a 1 µl air bubble and delivered at a rate of 1 µl/h over a 40 h period (see General Methods, section 2.3.3.). The infusate was delivered for 5 days into a lateral cerebral ventricle (2 mm right lateral to bregma, 3 mm posterior to bregma and 4.5 mm below the skull surface).

Rats in the i.c.v. vehicle-treated group (280-308 g, n=6) were implanted with an i.c.v. infusion assembly as outlined above, but containing sterile pyrogen-free distilled water.

The saline-treated group (249-291 g, n=4) had their normal drinking water replaced with a solution of 2% w/v sodium chloride (NaCl) for 48 h.

The control group (252-302 g, n=4) received no experimental treatments.

2. Tissue

Preparation of tissue obtained from rats took place over a 6 day period. On the first and second days, a number of rats prepared as above, were taken from each group and decapitated. The brains were removed and blocked coronally, including tissue 1 mm anterior to the optic chiasm through to the posterior limit of the mammillary bodies. These blocks were immediately frozen onto a pre-cooled layer of Tissue-Tek OCT compound on a cold cryostat chuck standing on dry ice. Blocks were stored in the freezer
at -70°C until sectioning was carried out at -18°C using a cryostat (Bright Instrument Co. Ltd, Cambridge, model FS/FAS/M) over the remaining four days. 10 µm transverse sections were thaw-mounted onto acid-cleaned, chrome alum-gelatine-subbed slides. 10 sections were taken from each rat, containing anteriorly located SON for the 3 incubation categories. Mounted sections were stored at -70°C in sealed Kartell boxes, containing silica gel for up to 11 days prior to the incubation stage.

3. Radioligand

The radioligand was CCK8 (sulphated), ¹²⁵I-labelled with Bolton and Hunter reagent, [¹²⁵I]-CCK8S, (specific activity ~2000 Ci/mmol, ~74 TBq/mmol, Amersham International plc, Amersham, UK). Stock [¹²⁵I]-CCK8S was added to 10 mM HEPES buffer containing NaCl [130 mM], KCl [4.7 mM], MgCl₂ [5 mM], EGTA [1 mM], bacitracin [0.25 mg/ml], and bovine serum albumin [5 mg/ml] to achieve a final concentration of 0.25 nM.

4. Unlabelled ligand

The unlabelled ligand CCK8 (sulphated), (Bachem, Saffron Walden, UK) was made up to a final concentration of 1 µM in 10 mM HEPES buffer containing [¹²⁵I]-CCK8S (0.25 nM). The excess was required in order to competitively displace the radioligand from the CCK binding sites in control studies.

5. Preincubation with [¹²⁵I]-CCK8S

[¹²⁵I]-CCK8S was used as the radioligand to probe for CCK binding sites in the SON and the following protocol was used (Day et al., 1989). Sections were preincubated for 15 min at room temperature in 50 mM Tris-HCl buffer, pH 7.4, which was applied as a 40 µl puddle in order to completely immerse
the section. Slides were kept in chambers moistened with the preincubation medium for this period, after which the puddles of preincubation medium were drained off the slides and replaced with 40 μl puddles of incubation medium made up in 10 mM HEPES buffer, pH 7.4 at room temperature. In this particular study we looked only for changes in specific binding to CCK binding sites and so there were 3 incubation categories (see table 6.2.5.1.).

1. \([^{125}\text{I}]\)-CCK8S alone (total CCK binding) 2. \([^{125}\text{I}]\)-CCK8S plus unlabelled CCK8S (non-specific CCK binding) 3. no \([^{125}\text{I}]\)-CCK8S or unlabelled CCK8S (chemographic control). The sections were then incubated for 2 h at room temperature in chambers moistened with HEPES buffer, after which the slides were removed and the incubation medium drained off. The slides were then placed in racks and gently immersed in 3 consecutive ice-cold buffer washes (50 mM Tris-HCl, pH 7.4 at 0°C) for 5 min each wash. Finally, sections were rinsed briefly in ice-cold double-distilled-deionised (DDD) water to remove salts and dried in air. Sections were then ready for autoradiography which was started on the same day as incubation.

Table (6.2.5.1.)- Incubation categories

<table>
<thead>
<tr>
<th>Incubation Code</th>
<th>Total Binding</th>
<th>Non-Specific Binding</th>
<th>Chemographic Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>KT</td>
<td>([^{125}\text{I}])-CCK8S (0.25 nM)</td>
<td>([^{125}\text{I}])-CCK8S (0.25 nM) + CCK8S (1 μM)</td>
<td>No radioligand or unlabelled ligand</td>
</tr>
<tr>
<td>KK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KC</td>
<td></td>
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</tr>
</tbody>
</table>
6. Autoradiography

Sections were at room temperature and apposed to Hyperfilm-$^3$H (Amersham) keeping slides which had been incubated in $[^{125}\text{I}]$-CCK8S only and $[^{125}\text{I}]$-CCK8S plus unlabelled CCK8S adjacent. Slides from a total of 5 rats were apposed to each sheet of film so that the sheet contained tissue from at least 1 rat per treatment group. A pre-cut 20 μm thick section of a $[^{125}\text{I}]$ standard (Autoradiographic $[^{125}\text{I}]$ microscales, Amersham) attached to a glass slide was placed in the centre of each sheet of Hyperfilm. The sheets of film with attached slides were then sandwiched between mirror-coated glass tiles, held in place with masking tape. They were then enclosed in aluminium foil, wrapped in brown paper and a black plastic bag to prevent moisture and light penetration before being left to expose at 0-4°C in the dark.

An initial exposure period of 2 days was employed to produce measurable grain densities over tissue sections which were below saturation in all positive incubations, but after trial this was adjusted to 4 days to obtain an optimum image.

After exposure, the films and slides were brought to room temperature and the slides removed for later histological preparation. The films were developed at room temperature in Kodak D19 developer for 5 min, rinsed briefly in tap water, fixed in Ilford Hypam rapid fixer for 5 min (1: 4 fixer: tap water), rinsed for 30 min in running tap water and dried in air.

The sections were fixed for 10 min in acetic acid: alcohol: formaldehyde (1: 17: 2 by volume), washed in running water for 5 min, dehydrated through a series of alcohol baths (70, 90 and 100%; 2-3 min in each) and transferred to a xylene bath for 10 min before rehydrating through alcohol baths in
reverse. After a 5-10 min wash in running water sections were stained for 10-15 min in 1% Cresyl Fast Violet, washed briefly in running water and dried. They were then dehydrated in absolute alcohol, transferred to an alcohol/xylene bath (50/50), cleared in xylene, mounted in DPX and coverslipped.

7. Measurement of autoradiographs

Pieces of film which corresponded to the apposed slides were cut out and attached to clean microscope slides with clear adhesive tape. Autoradiographs and stained sections were paired and examined under a binocular dissecting microscope (Carl Zeiss, magnification x 1.6) to identify the SON. The boundaries of the SON were outlined by scratching the emulsion coated side with a fine needle under the dissecting microscope. Silver grain density was then measured using a Joyce-Loebl μMagiscan image analysing computer with video input from a COHU High Performance CCD black and white camera (Brian Reece Scientific Ltd.) mounted on a Vickers M17 microscope. Microscope magnification was x 10 (objective) and x 1.6 (intermediate lens). The video monitor scale factor was 1.1878 μm per pixel. A rectangular counting frame (201.63 x 133.04 μm) was outlined which was smaller than the area of any SON so that measurements could be made selectively over the dorsal region of the SON.

8. Expression of results as mean grain density values

Silver grain density was calculated as the total area of silver deposit/total area of counting frame. A non-tissue background count was made after each SON tissue count over an adjacent region of film and subtracted from the corresponding tissue count. These subtracted counts were then averaged.
for each rat, and then for each group to obtain a mean grain density value for each incubation category. To obtain a mean grain density value for specific binding sites for CCK for each rat, the mean grain density value for non-specific binding was subtracted from the mean count achieved for total binding. To account for variability between the sheets of film the 3 treatment groups on a single sheet of film were indexed to the control (untreated) group on that film. Mean grain density values for the 4 control sub-groups from the 4 sheets of film thus had an index value of 1. The mean grain density indexed values for rats in a given treatment group were then grouped and a mean value ± SEM obtained. All measurements of mean grain density were made 'blind'.

9. \([^{125}\text{I}]\) polymer standards

Expression of mean grain density values as absolute values requires reference to either \([^{125}\text{I}]\) brain paste standards or \([^{125}\text{I}]\) polymer standards. Both enable the optical density values obtained for the tissue autoradiographs to be expressed in radioactive units. Pre-cut strips of polymer standards (Amersham) consist of 10 layers of radioactive polymer arranged in order of increasing specific activity separated by non-radioactive layers. A standard strip was attached to each sheet of Hyperfilm enabling a standard curve of mean grain density vs radioactivity to be constructed. This is necessary since Hyperfilm does not respond in an absolutely linear fashion in terms of silver grain deposition upon exposure to increasing activity of radioactive sources.

Ideally the conversion from mean grain density values obtained for each treatment group should have been made to radioactive units to enable expression of results in terms of fmole ligand bound/mg tissue. In order to
obtain optimal images for measurement of silver grain deposition an exposure period of four days was required. However the specific activity of the standard strip (even after the removal of the top two steps) was so high, that after this time considerable background fogging had occurred around the top two steps. Consequently when the background counts were subtracted from the counts obtained for the respective steps, values markedly lower than the previous three bottom steps were obtained. On this basis the standard curve of mean grain density vs radioactivity would have had to have been plotted using just three points. Furthermore mean grain density values obtained for the chemography controls and vehicle-treated animals did not lie on this line, so that extrapolation of the line was required. However since the highest mean grain density values obtained for the remaining groups fell upon this line which in essence was linear, it can be assumed with a degree of confidence that the emulsion had responded in a linear fashion over the uppermost observed range of receptor binding. Although an extrapolation of the standard curve would have been required in order to estimate those values at the bottom end of the range it would have been of a small enough magnitude such that it would have been highly unlikely to be non-linear in nature. It was decided therefore to express results solely as mean grain density values since the aim was to seek changes between groups of animals.

10. CCK antagonists

The specific CCK receptor antagonists MK-329 (L-364, 718; 1-methyl-3-(2-indoloyl)amino-5-phenyl-3H-1,4-benzodiazepin-2-one) and L-365, 260 (3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N'-(3-methyl-phenyl)urea) which bind preferentially to CCK$_A$ and CCK$_B$
receptors in rat brain respectively (Hill and Woodruff, 1990) were used in an initial study to discriminate between CCK binding sites. These compounds were solubilised initially in vehicle (20% dimethyl sulphoxide/80% propylene glycol v/v) to a concentration of 10 mM (Dourish et al., 1988). They were further diluted in DDD water and HEPES buffer to a concentration of 10 μM.

During the wash procedure in ice-cold HEPES buffer as described in section 6.2.5., it became apparent that the antagonist vehicle did not rinse completely away from the sections, and upon subsequent autoradiographic exposure, sections which had been incubated with either antagonist or antagonist vehicle were markedly fogged. Indeed it was still possible to identify the outline of the incubation puddle around the majority of these sections. Although no mention is made of the antagonist vehicle used by Hill and Woodruff (1990), the concentrations of antagonist used were 100 fold lower for L-365, 260 (100 nM) and 1000 fold lower for L-364, 718 (10 nM). It is possible that the aforementioned problem could have been avoided if both antagonists had been further dissolved in buffer to concentrations similar to that used by Hill and Woodruff, (1990).

11. Statistics

Comparison of indexed mean grain density values between groups was made by performing a one-way ANOVA on the 3 treatment groups to obtain a pooled variance which was used to calculate t-values by Student’ s t-test.
6.3. RESULTS

6.3.1. Qualitative distribution of CCK8S binding sites within rat midbrain

Localisation of $[^{125}\text{I}]-\text{CCK8S}$ binding sites within discrete brain areas was determined using the rat atlas of Paxinos and Watson, (1982). The abbreviations used in the labelling of the autoradiographs and stained marker sections were obtained from the same source.

1. i.c.v. morphine-treated and i.c.v. vehicle-treated groups

$[^{125}\text{I}]-\text{CCK8S}$ binding sites, visualised as darker areas of silver grain density were clearly localised in the SON of the hypothalamus in both groups of animals (Figs. 6.3.1.1.A.a. and 6.3.1.1.B.a.), although less intensively in the vehicle group and this was statistically significant on quantification (section 6.3.2.). $[^{125}\text{I}]-\text{CCK8S}$ binding sites were also visually apparent within the anterior cingulate cortex, frontoparietal cortex and the caudate putamen in both groups. The thalamus which lies above the third ventricle demonstrated specific $[^{125}\text{I}]-\text{CCK8S}$ binding and this would appear to be have been specific to the i.c.v. vehicle group (Fig. 6.3.1.1.B.a.). Similarly $[^{125}\text{I}]-\text{CCK8S}$ binding within the reticular thalamic nuclei was specific to morphine-treated animals.

Non-specific $[^{125}\text{I}]-\text{CCK8S}$ binding (Figs. 6.3.1.1.A.b. and 6.3.1.1.B.b.) in the sections adjacent to those in Fig. 6.3.1.1.A.a. and Fig. 6.3.1.1.B.a. respectively, as defined with an excess of unlabelled CCK8S (1 μM), demonstrates that the majority of the $[^{125}\text{I}]-\text{CCK8S}$ binding seen in the total binding images corresponds to specific $[^{125}\text{I}]-\text{CCK8S}$ binding. The stained marker sections (Figs. 6.3.1.1.A.c. and 6.3.1.1.B.c.) clearly contain both SON and so the lack of $[^{125}\text{I}]-\text{CCK8S}$ binding (Figs. 6.3.1.1.A.a. and 6.3.1.1.B.a.)
within the SON can not be attributed to damaged or missing SON.

2. Salt loaded group and untreated controls

\(^{[125I]}\)-CCK8S binding sites were very densely labelled within the SON of salt-loaded animals (Fig. 6.3.1.2.A.a.) and specific \(^{[125I]}\)-CCK8S binding in this region was clearly increased in these animals compared with the untreated controls (Fig. 6.3.1.2.B.a.). \(^{[125I]}\)-CCK8S binding appeared to be homogeneous throughout the dorsal oxytocin neurone-rich region of the nucleus. \(^{[125I]}\)-CCK8S binding sites were also labelled within the anterior cingulate cortex, frontoparietal cortex and the caudate putamen in both groups of animals. \(^{[125I]}\)-CCK8S binding was also present in the region adjacent to the third ventricle in both groups and may have been specifically localised within the anterior paraventricular thalamic nucleus (which lies beside the third ventricle).

Non-specific \(^{[125I]}\)-CCK8S binding (Figs. 6.3.1.2.A.b. and 6.3.1.2.B.b.) in the sections adjacent to those described above demonstrates that total \(^{[125I]}\)-CCK8S binding seen in Figs. 6.3.1.2.A.a. and 6.3.1.2.B.a. corresponds to specific \(^{[125I]}\)-CCK8S binding.

6.3.2. Quantitative analysis of \(^{[125I]}\)-CCK8S binding within the supraoptic nucleus

Specific \(^{[125I]}\)-CCK8S binding in the SON increased significantly by 2.4 fold (p<0.01) after i.c.v. morphine treatment compared with the i.c.v. vehicle-treated group and by 1.4 fold (p<0.05) compared with the untreated control group (Fig. 6.3.2.1.). There was no difference in \(^{[125I]}\)-CCK8S binding between the morphine-treated group and the salt-loaded group. Salt-loading significantly increased \(^{[125I]}\)-CCK8S binding site density by 1.5 fold (p<0.05)
compared with the untreated controls. Specific $[^{125}\text{I}]-\text{CCK8S}$ binding was 1.76 fold (p<0.02) lower in i.c.v. vehicle-treated animals than in untreated controls.
Figure (6.3.1.1.A.; overleaf)- i.c.v. morphine-treated group

a) and b) are representative autoradiographs showing total $[^{125}\text{I}]-\text{CCK8S}$ binding and non-specific $[^{125}\text{I}]-\text{CCK8S}$ binding respectively, demonstrated by silver grain deposition obtained from 10 µm transverse sections. The difference in $[^{125}\text{I}]-\text{CCK8S}$ binding between a) and b) demonstrates that the majority of $[^{125}\text{I}]-\text{CCK8S}$ binding in a) clearly localised within the SON, ACg, FrPaM, CPu and Rt corresponds to specific $[^{125}\text{I}]-\text{CCK8S}$ binding.

c) and d) stained sections showing rostrally located SON adjacent to optic chiasm. Scale bar=1 mm.

Abbreviations used: SON-supraoptic nucleus, ACg-anterior cingulate cortex, FrPaM-frontoparietal cortex, motor area, CPu-caudate putamen, Rt-reticular thalamic nucleus.
Figure (6.3.1.1.B.; overleaf)- i.c.v. vehicle-treated group

a) and b) are representative autoradiographs showing total $[^{125}\text{I}]-\text{CCK8S}$ binding and non-specific $[^{125}\text{I}]-\text{CCK8S}$ binding respectively, demonstrated by silver grain deposition obtained from 10 μm transverse sections. As with Fig. 6.3.1.1.A, the difference in $[^{125}\text{I}]-\text{CCK8S}$ binding between a) and b) demonstrates that the majority of $[^{125}\text{I}]-\text{CCK8S}$ binding in a) clearly localised within the SON, ACg, FrPaM, CPu and Th (lying above the third ventricle) corresponds to specific $[^{125}\text{I}]-\text{CCK8S}$ binding.

c) and d) stained sections showing rostrally located SON adjacent to optic chiasm. Scale bar=1 mm.

Abbreviations used: SON-supraoptic nucleus, ACg-anterior cingulate cortex, FrPaM-frontoparietal cortex, motor area, CPu-caudate putamen, Th-thalamic structure (poorly defined).
Figure (6.3.1.2.A; overleaf)- saline-treated group

a) and b) are representative autoradiographs showing total $^{125}$I-CCK8S binding and non-specific $^{125}$I-CCK8S binding respectively, demonstrated by silver grain deposition obtained from 10 μm transverse sections. The difference in $^{125}$I-CCK8S binding between a) and b) demonstrates that the majority of $^{125}$I-CCK8S binding in a) clearly localised within the ACg, FrPaM, CPu and PVA corresponds to specific $^{125}$I-CCK8S binding. In this group binding within the SON was very dense compared with the other labelled areas.

c) and d) stained sections showing rostrally located SON adjacent to optic chiasm. Scale bar=1 mm.

Abbreviations used: SON-supraoptic nucleus, ACg-anterior cingulate cortex, FrPaM-frontoparietal cortex, motor area, CPu-caudate putamen, PVA-paraventricular thalamic nucleus, anterior.
a) and b) are representative autoradiographs showing total $^{125}\text{I}$-CCK8S binding and non-specific $^{125}\text{I}$-CCK8S binding respectively, demonstrated by silver grain deposition obtained from 10 \( \mu \text{m} \) transverse sections. The difference in $^{125}\text{I}$-CCK8S binding between a) and b) demonstrates that the majority of $^{125}\text{I}$-CCK8S binding in a) clearly localised within the SON, ACg, FrPaM, CPu and PVA corresponds to specific $^{125}\text{I}$-CCK8S binding.

c) and d) stained sections showing rostrally located SON adjacent to optic chiasm. Scale bar=1 mm.

Abbreviations used: SON-supraoptic nucleus, ACg-anterior cingulate cortex, FrPaM-frontoparietal cortex, motor area, CPu-caudate putamen, PVA-paraventricular thalamic nucleus, anterior.
Figure (6.3.2.1.)- Silver grain density (mm² silver deposit/mm² of field); corrected for non-specific binding and expressed as a ratio to the unoperated control group. * p<0.05, # p<0.02, ** p<0.01; calculated using Student's t-test after ANOVA, p<0.05. The number of rats in each group is given in parentheses.
6.4. DISCUSSION

CCK8S binding in rat brain with specific reference to the SON

The autoradiographic technique employed in this study used a peptide labelled with $^{125}\text{I}$. Different brain regions have been reported to show no differences from each other in the way they absorb emitted particles from this radionuclide (Davenport and Hall, 1988). As a result detection of binding throughout the brain regions mentioned should have been homogeneous.

CCK is extremely abundant in the mammalian cerebral cortex (layers II-III, V-VI) and has been ascribed a putative role as a neurotransmitter in this region (Vanderhaeghen and Schiffman, 1992). Little is known of its physiological function although a possible neurotransmitter interaction with $\gamma$-amino butyric acid (GABA) has been intimated. CCK8S binding sites were clearly localised in the anterior cingulate cortex and frontoparietal cortex in all four treatment groups. Similarly CCK8S binding sites were localised within the caudate putamen in each group and the intensity of labelling did not appear to differ significantly between groups in agreement with the study of Day et al., (1989).

CCK8S binding increases in the SON of salt-loaded rats and homozygous Brattleboro rats (Day et al., 1989). Salt-loading has subsequently been successfully used as a positive control for CCK8S binding within the SON in this present study. Other studies using different radioactively labelled ligands to probe for CCK binding sites have also reported the presence of CCK receptors within the SON (Carlberg et al., 1992). It has been suggested that the increase in CCK8S binding in the SON of salt-loaded rats occurs in the rostrally located dorsal oxytocin rich half of the nucleus (Day et al., 1989), primarily at CCK$_A$ receptors (Hinks et al., 1993), and autoradiographical
competition studies have shown that the change reflects an increase in binding site density \((B_{\text{max}})\) rather than affinity \((K_d)\), (Day et al., 1989).

The present results demonstrate that there is an increase in specific CCK8S binding site density within the SON in morphine dependent rats compared with i.c.v. vehicle-treated and untreated control rats (Fig. 6.3.2.1.). Recently it has been shown that salt-loading induces a small increase in \(\text{CCK}_B\) and a much larger increase in \(\text{CCK}_A\) receptor content within the hypothalamus (Hinks et al., 1993) and that this is associated with increased levels of preproCCK mRNA and oxytocin mRNA. Thus in rats treated chronically with morphine the increase in CCK8S binding site density within the SON (Fig. 6.3.2.1.) may be a result of upregulated synthesis of CCK reflecting increased activity of endogenous CCK control of magnocellular oxytocin neurone activation. The implications of this increase in CCK binding site density (which may reflect an increase in functional CCK receptor content) within the SON, in terms of the development of tolerance and dependence in SON oxytocin neurones is discussed later.

Thus far we have looked at the situation where CCK8S binding site density increases within the SON. However in animals which received an i.c.v. infusion of vehicle for 5 days, CCK8S binding was significantly attenuated compared with the unoperated controls (Fig. 6.3.2.1.). The vehicle infusate consisted of sterile, pyrogen-free distilled water which would tend to decrease tonicity of cerebrospinal fluid. Since a hypertonic stimulus upregulates CCK8S binding within the SON as shown in this present study and previously (Day et al., 1989; Blackburn et al., 1990), simplistically one might expect a hypotonic stimulus to have the converse effect, causing downregulation of CCK8S binding. Although this is a reasonable assumption
since magnocellular neurones are directly osmosensitive (Leng et al., 1982; Oliet and Bourque, 1993) a problem arises from the fact that the infusion rate of vehicle (1 μl/h) into a much larger ventricular space would have been unlikely to change local osmotic conditions in the SON region due to diffusion throughout the ventricle. The region anterior and ventral to the third ventricle (AV3V region) is itself intrinsically osmosensitive and includes the nucleus medianus which has been reported to contain CCK8-immunoreactive cell bodies (Kawano et al., 1989). Ablation of structures in the AV3V region markedly impairs the neuronal response of supraoptic neurones to systemic hyperosmotic stimulation (Leng et al., 1989), indicative of neuronal projections from the AV3V region to the SON. Recently it has been reported that brief osmotic stimulation of the organum vasculosum of the lamina terminalis (OVLT) which projects to the AV3V region and to the SON, produces a sustained enhancement of magnocellular neurosecretory cell firing rate because the osmotically-induced increase in firing in OVLT neurones is itself long-lasting (Bourque et al., 1993). One explanation for the observed decrease in CCK8S binding in i.c.v. vehicle-treated rats therefore may be that infusion of vehicle directly into the vicinity of the AV3V region could change local conditions in osmolality thereby affecting the neuronal population which would subsequently modulate activity of the magnocellular neurones in the SON.

Since the autoradiographic resolution obtained was of a fairly low order in the present study there was no means by which the components within the SON to which the silver grains were localised could be identified. One way in which this could be achieved however, would be to combine tissue receptor autoradiography with immunocytochemistry allowing visualisation of
CCK8S binding sites with identified components, be they neuronal, presynaptically located or on glial cells. Interestingly, within the SON, the glial cell bodies and the magnocellular neuronal dendrites which receive the majority of synaptic contacts are located in the ventral glial lamina (Armstrong et al., 1982) and as already mentioned oxytocin cells tend to be located more dorsally than vasopressin cells (Sofroniew et al., 1981). The glial cell processes which normally pervade the whole nucleus, retract following saline drinking especially from the dorsal region of the nucleus (Theodosis et al., 1984). Thus, the increase in specific CCK binding within the SON after saline drinking may be localised to the cell bodies of the magnocellular neurosecretory cells.

CCK involvement in development of tolerance and dependence

It was quickly realised from early studies that CCK could antagonise opiate-induced analgesia in the rat (Itoh et al., 1982), and since central administration of CCK8S has been shown to be a potent antagonist of opiate analgesia produced by foot shock and morphine it has been suggested that endogenous CCK could act physiologically as a specific functional opiate antagonist in pain-mediating pathways (Faris et al., 1983). Against these early studies have come reports suggesting that CCK is actually analgesic in its own right (Hill et al., 1987b; Hong and Takemori, 1989), although these results are probably best explained by the high administration doses of CCK employed throughout these experiments.

Magnocellular neurones within the SON respond to chronic morphine treatment further with a reduction in density of available μ-opioid binding sites and this is thought to account at least in part to morphine tolerance in relation to oxytocin secretion (Sumner et al., 1990). CCK receptors and μ-
opioid receptors are located on the cell bodies of supraoptic oxytocin neurones (Inenaga et al., 1990; Jarvis et al., 1992; Oliet and Bourque, 1992). CCK8S has been reported to inhibit the binding of \( \mu \)-ligands to their respective receptors in rat brain homogenates and this suppressive effect is almost completely blocked by the CCK antagonist proglumide implying that the inhibition is mediated by CCK receptors (Wang and Han, 1992). Thus the receptors responsible for mediating the effects of morphine and CCK8S on oxytocin neurone electrical activity may converge on the same effector component at the cellular level to mediate a neurosecretory response. Binding to \( \mu \)-receptors in the rat brain activates a pertussis-toxin-sensitive G-protein (Pumford et al., 1993) which has been postulated to act via a protein phosphatase regulated pathway to reduce dihydropyridine Ca\(^{2+}\) channel sensitivity (Armstrong and White, 1992), and blocking of these channels attenuates morphine withdrawal excitation of oxytocin neurones in the SON (Munro et al., 1993). The role of central CCK receptor activation on Ca\(^{2+}\) channel function remains to be fully defined. Although morphine and CCK mediated effects on magnocellular oxytocin neurones do not appear to involve a Ca\(^{2+}\) component acutely, the development of tolerance and dependence in these neurones does involve such a component. Consequently binding to CCK receptors may alter the balance of this effector component displacing it away from opioid receptor availability with subsequent development of tolerance to the inhibitory actions of morphine. Oxytocin neurones would then be able to function normally in morphine-treated rats as has been indicated by similar plasma oxytocin concentrations in control and morphine-treated rats (Bicknell et al., 1988; Rayner et al., 1988).
Chapter 6

This scenario has a physiological relevance since endogenous opioid restraint is thought to be an important factor in the control of oxytocin secretion throughout gestation leading into parturition. In late pregnant rats this endogenous opioid inhibition of oxytocin release is centrally mediated (Douglas et al., 1993). Pregnancy has been shown to alter the binding of opioids within the SON (Sumner et al., 1992) with a down-regulation of μ-receptors. Thus a tolerance-like state of oxytocin neurones to the inhibitory actions of endogenous opioids during pregnancy could be partly attributed to a manifestation of increased CCK binding within the SON, similar to that seen in the morphine treated animals of the present study. Removal of increased opioid tone either within the SON or on excitatory inputs to oxytocin neurones within the SON at the end of pregnancy would enable dendritically released oxytocin and CCK to facilitate oxytocin neurone activation and drive the process of parturition until it reached its natural conclusion.
ACUTE EFFECTS OF CCK AND OPIOIDS ON MAGNOCELLULAR NEURONAL ELECTRICAL ACTIVITY
7.1. Introduction

Cholecystokinin (CCK) is a 33 amino acid peptide originally isolated in the gastrointestinal tract before being shown to be widely distributed throughout the CNS. The sulphated octapeptide (CCK8S) is the predominant form present in the CNS and has been attributed functions as a neurotransmitter throughout the brain (Crawley, 1992). A number of studies have focussed on the potential for CCK to act as an endogenous opioid antagonist within neuronal systems (Itoh et al., 1982; Faris et al., 1983; Han, 1992; Zhou et al., 1993), based in part on evidence such as CCK colocalisation with opioid peptides in thalamus and brainstem nociceptive neurones (Gall, 1987).

CCK has been immunocytochemically colocalised within supraoptic oxytocin neurones (Vanderhaeghen et al., 1981; Martin et al., 1983a; Hökfelt et al., 1988), whilst CCK8S excites both supraoptic putative oxytocin and vasopressin neurones in vitro via activation of a non-selective cationic conductance (Jarvis et al., 1992). A similar response is seen for magnocellular cells isolated from their dendritic field and afferent input by an enzymatic neuronal separation procedure (Oliet and Bourque, 1992). The depolarising response is tetrodotoxin resistant, persists in Ca\(^{2+}\)-free media, and is reversibly attenuated by the high affinity antagonist L-365,260 indicating that the receptors are CCK\(_B\) receptors post-synaptically located on the oxytocin neurones (Jarvis et al., 1992). In addition direct administration of CCK8S into the SON stimulates oxytocin release measured by microdialysis into blood and within the SON itself (Neumann et al., 1993).

Both \(\mu\)- and \(\kappa\)-opioid receptors are present within the SON (Sumner et al., 1990). Application of the \(\mu\)-opioid agonist morphine to the SON in the perfused hypothalamic slice inhibits putative oxytocin neurone electrical
activity (Wakerley et al., 1983a; Inenaga et al., 1990). Dynorphin which is selective for the \( \kappa \)-type receptor inhibits both vasopressin and oxytocin neurone electrical activity (Inenaga et al., 1990; Bourque et al., 1993). Recently it has been reported using conventional intracellular recording techniques (which permit the relative involvement of separate ionic currents involved in cell function to be identified) that \( \kappa \)-receptor agonists suppress excitatory synaptic inputs to the SON by a probable pre-synaptic mode of action although post-synaptic suppression has not been excluded (Inenaga et al., 1994). In addition \( \kappa \)-receptor agonists were also shown to attenuate the duration of the \( \text{Ca}^{2+} \) component of the action potential in SON neurones whilst a \( \mu \)-receptor agonist was largely without effect on this \( \text{Ca}^{2+} \) component (Inenaga et al., 1994).

In the previous chapter CCK8S binding was shown to increase within the SON of morphine dependent rats and it was proposed that this may alter the balance of a shared post-effector component away from the inhibitory effects of \( \mu \)-opioid receptor occupancy towards that of the excitatory effects mediated by the CCK receptor. As yet the cellular mode of action by which CCK exerts a facilitatory influence on magnocellular electrical activity has not been described, whilst morphine tolerance in these neurones may be associated with activation of a pertussis-toxin-sensitive G protein (Pumford et al., 1993).

In a preliminary series of studies the direct actions of CCK8S and opiates (particularly morphine) on magnocellular neurosecretory cells were investigated \textit{in vitro} using the hypothalamic slice preparation to try and establish if a two way functional relationship on the electrical activity of magnocellular neurosecretory cells occurs between CCK and opiates;
implicative perhaps of a shared second messenger component mediating effects on neuronal electrical activity.
7.2. MATERIALS AND METHODS

7.2.1. Actions of CCK and opioids on supraoptic magnocellular neuronal firing rate

1. Animals

Virgin female Sprague-Dawley rats (body weight 200-300 g) housed under standard conditions (see General Methods, section 2.1.) were used throughout these experiments.

2. Electrophysiology

Animals were killed by decapitation using a guillotine (Harvard Apparatus Ltd, Kent). A midline skin incision was then made over the dorsal surface of the skull and the skin retracted. Scissors were then inserted into the foramen magnum and the bone lateral to this on either side was carefully sheared towards the rostral end of the cerebellum. The dorsal surface of the skull was then removed with a pair of bone nibblers to expose the brain. Using a scalpel incisions were made at the most rostral and caudally accessible regions of the cerebral hemispheres. This loosened the dura which was then completely removed to prevent damaging the soft brain tissue. The brain was then lifted gently to expose the optic nerves which were cut with either a small pair of scissors or a scalpel. Once cut, the scalpel was inserted underneath the ventral surface of the brain to aid its removal from the cranial vault. The brain was then bathed in oxygenated (95% O₂/5% CO₂), ice-cold medium before being blocked at the optic nerves and mammillary bodies. This block of tissue was fixed with cyanocrylate adhesive to the stage of a Vibraslice microtome (Campden Instruments Ltd, UK) rostral end uppermost and immediately immersed in the pre-cooled bathing medium in the
Vibraslice chamber. One or two 400 μm coronal sections containing SON were cut and transferred from the Vibraslice chamber to the perfused well of the recording chamber (Model QL-2; Fine Science Tools) and covered with a single piece of gauze swab (Vernon-Carus Ltd, Lancashire) to prevent the slices from moving in the well and to aid perfusion of the oxygenated bathing medium over the slice surface. Flow rate of the bathing medium was set to between 1-2 ml/min and the temperature of the bathing medium at the slice interface was maintained at 34°C. The slices were then left for 2 h to equilibrate before recording.

Extracellular recordings from magnocellular supraoptic neurones were made from a glass microelectrode (20-50 MOhm; filled with 0.9% NaCl) pulled on a Narishige electrode puller (Narishige, Japan) and then via an M707A probe and associated amplifier (World Precision Instruments, Inc, USA). Neuronal spike activity was discriminated from background using a spike processor (Digitimer D.130) which was connected to a digital to analogue converter (CED 1401) enabling data to be stored on a Spike 2 software programme (version 3.22 CED Ltd, Cambridge) for later analysis. The microelectrode was positioned in the dorsal region of the anteriorly located SON to enhance the likelihood of finding magnocellular oxytocin-like (continuously firing) cells.

When an apparently suitable cell was located, a 10-20 min control period was taken to enable the cell to establish a stable firing pattern. Mean firing rate was measured in 10 s bins and averaged over 5-10 min. Drugs were dissolved in bathing medium and superfused from a modified gravity feed system consisting of separate reservoirs (50 ml polypropylene syringes; B. Braun, GmbH) maintained at 37°C, which allowed direct switch over between
drugs. A drug flow through time of 2-3 min was estimated by superfusing 5% Alcian Blue dye from the reservoirs incorporated into the gravity feed system and measuring the time taken for the dye to appear in the recording chamber at the point where the hypothalamic slice would normally be placed.

The frequency and pattern of firing were not enough to identify a cell as exclusively magnocellular and so it was important to monitor the shape of the associated action potential, since magnocellular neurone action potentials have a characteristic Ca\(^{2+}\) dependent shoulder on the repolarisation phase both in vivo (Renaud and Bourque, 1989) and in vitro (Bourque and Renaud, 1985b) in the hypothalamic explant where magnocellular cells can be positively identified by antidromic activation.

3. Experimental protocol

Six out of a total of seven continously firing (oxytocin-like) magnocellular neurosecretory cells were perfused initially with CCK8S (10\(^{-6}\)M) given by bath application for 10-15 minutes. This was then followed by co-perfusion with CCK8S and morphine (10\(^{-6}\)M) until the cell adopted a stable firing pattern, or in the case of cells (a)-(c) until firing rate was completely abolished. In cells where firing rate was not completely inhibited [eg cells (e) and (f)] the perfusion was switched to morphine alone (10\(^{-6}\)M) to enable the full inhibitory effect of morphine on cell electrical activity to be determined. To verify if these inhibitory effects on cell activity were opioid mediated, naloxone (10\(^{-6}\) M) was co-perfused with morphine [cell (f)] in an attempt to antagonise the morphine mediated effects on cell firing rate. Finally, cell (g) was perfused initially with morphine for 10 minutes before being co-perfused with morphine and CCK8S.

In the case of one cell which appeared to fire in a phasic fashion typical of
vasopressin cells the k-selective agonist U50, 488H (trans-(±)-3,4-dichloro-
N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl) benzeneacetamide, methane sulphi
te salt) was used to antagonise the CCK8S-mediated excitation since 
μ-opioid agonists such as morphine are largely without effect on vasopressin 
neurones (Inenaga et al., 1990).

4. Bathing medium

The slice was cut and left to equilibrate for 2 h in standard medium (mM); 
NaCl 124, KCl 5, NaPO₄.2H₂O 1.2, NaHCO₃ 26, glucose 10, MgSO₄ 1.3, 
CaCl₂ 2.4.

For recording purposes and to aid spontaneous activity of magnocellular 
neurosecretory cells Ca²⁺ was lowered to 0.75mM.

5. Statistics

Data are expressed as mean ± SEM. Comparisons were made within 
groups of cells with Student's t-test (Paired). For individual cells, significant 
effects of treatment were sought by calculating the mean ± 95% confidence 
limits over a 5-10 minute period during a given treatment. These were then 
compared for the test periods and if the 95% confidence limits of the two 
mean values did not overlap, then p<0.05 was taken as being the 
significance of the difference.
7.3. RESULTS

7.3.1. Actions of CCK8S and opioids on supraoptic magnocellular neuronal firing rate

In six out of seven magnocellular continuously firing oxytocin-like cells tested, spontaneous activity was increased by more than 3-fold (p<0.02; Paired t-test) from a mean basal firing rate of 1.28 ± 0.33 spikes/s after addition to the bathing media of CCK8S (10^{-6} M), (see Table 7.3.1.1 and Fig. 7.3.1.1.a.-f.). 10 min after an initial effect on firing rate was observed the cells were perfused concomitantly with CCK8S and morphine (10^{-6} M) which resulted in an attenuation of firing rate to 0.96 ± 0.34 spikes/s (p<0.005; Paired t-test); a 4 fold reduction in firing rate below that seen during perfusion with CCK8S alone. Firing rate during perfusion with CCK8S and morphine returned to a level seen during the control period (no sig. diff.; Paired t-test). The inhibitory effect of morphine on this CCK8S-induced excitation was variable between the six individual cells with cells (a)-(c) exhibiting a complete suppression of firing rate, whilst in cells (e) and (f) co-perfusion of CCK8S and morphine although significantly attenuating the CCK8S excitation (p<0.05 by 95% confidence limits) did not abolish it completely. However further perfusion with morphine alone (10^{-6} M) did completely abolish cell activity (p<0.05) and in the case of cell (f) this action was significantly antagonised by naloxone (10^{-6} M), (p<0.05). In one cell, (g), morphine was perfused first and significantly reduced firing rate to 1.7 ± 0.1 spikes/s from a basal level of 4.4 ± 0.1 spikes/s (p<0.05) and this inhibition was then partially reversed by CCK8S (p<0.05).

The remaining cell had a mean basal firing rate of 0.32 ± 0.13 spikes/s and was analysed separately as this cell appeared to fire in a phasic manner.
characteristic of a putative vasopressin cell (Fig. 7.3.1.2.). As with the other 6 cells, this cell was also excited when perfused with CCK8S; firing rate increasing to $5.41 \pm 0.55$ spikes/s ($p<0.05$ by confidence limits). Subsequent co-perfusion with the $\kappa$-selective agonist U50, 488H ($10^{-6}$ M) resulted in a marked attenuation in firing rate to $0.96 \pm 0.40$ spikes/s ($p<0.05$). This was then fully antagonised ($p<0.05$) by addition to the perfusion medium of naloxone ($10^{-5}$ M) which once more increased firing rate to $3.79 \pm 0.85$ spikes/s.

<table>
<thead>
<tr>
<th>Experimental Protocol</th>
<th>Control period</th>
<th>CCK8S (1 $\mu$M)</th>
<th>CCK8S (1 $\mu$M) + Morphine (1 $\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Firing Rate (spikes/s)</td>
<td>1.28 ± 0.33 (6)</td>
<td>3.97 ± 0.66 * (6)</td>
<td>0.96 ± 0.34 ** (6)</td>
</tr>
</tbody>
</table>

Table (7.3.1.1.)- Effect of CCK8S and morphine on magnocellular cellular activity in the hypothalamic slice preparation. After addition to the bathing medium of CCK8S ($10^{-6}$ M), there was an increase in magnocellular neurosecretory cell mean firing rate (* $p<0.02$; Paired t-test) followed by a marked reduction in mean firing rate after simultaneous perfusion with CCK8S ($10^{-6}$ M) and morphine ($10^{-6}$ M), (** $p<0.005$; Paired t-test). Numbers of cells are in parentheses.
Figure (7.3.1.1.; overleaf)- Electrical activity of continuously firing magnocellular neurosecretory cells recorded from the supraoptic nucleus in the hypothalamic slice preparation. Cells (a)-(f) represent those cells summarised in Table 7.3.1.1. Cells (a)-(d) were initially excited (p<0.02; Paired t-test) following bath application of CCK8S (10^{-6} \text{ M}). Simultaneous perfusion of CCK8S and morphine (10^{-6} \text{ M}) completely suppressed the CCK8S-induced excitation in cells (a)-(c), although cell (d) appeared to be unaffected in terms of firing rate. Cells (e) and (f) were also initially excited by perfusion with CCK8S although on this occasion co-perfusion of morphine with CCK8S although attenuating firing rate to a level comparable with that of the control period (p<0.05 by confidence limits), did not completely suppress firing rate as with cells (a)-(c). However subsequent perfusion with morphine (10^{-6} \text{ M}) alone did abolish firing rate (p<0.05) and this was antagonised by naloxone (10^{-5} \text{ M}) in cell (f), (p<0.05). Cell (g) was initially inhibited by morphine (10^{-6} \text{ M}), (p<0.05) and this inhibition was partially reversed by CCK8S (10^{-6} \text{ M}), (p<0.05).

Open and black bars indicate periods of drug perfusion.
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a.

b.

c.
Chapter 7

d.  

![Graph showing spikes per second (spikes/s) over time (minutes)]

- Time (minutes): 0, 10, 20, 30, 40, 50
- Spikes/s: 0, 2, 4, 6, 8, 10, 12

- 10^-6 M CCK
- 10^-6 M CCK + 10^-6 M Mor


e.  

![Graph showing spikes per second (spikes/s) over time (minutes)]

- Time (minutes): 0, 10, 20, 30, 40, 50
- Spikes/s: 0, 2, 4, 6

- 10^-6 M CCK
- 10^-6 M CCK + 10^-6 M Mor


f.  

![Graph showing spikes per second (spikes/s) over time (minutes)]

- Time (minutes): 0, 20, 40, 60, 80, 100, 120, 140
- Spikes/s: 0, 5, 10, 15, 20

- 10^-6 M CCK
- 10^-6 M CCK + 10^-6 M Mor
- 10^-6 M Mor + 10^-6 M NaCl
Chapter 7

g.

![Graph showing spikes per second over time with time in minutes on the x-axis and spikes per second on the y-axis. The graph illustrates changes in spikes per second with 10^{-6} M Mor, 10^{-6} M Mor + 10^{-6} M CCK.]
Figure (7.3.1.2.): Electrical activity of a phasically firing magnocellular neurosecretory cell recorded from the supraoptic nucleus in the hypothalamic slice preparation. The cell was excited following bath application of CCK8S (10^-6 M) as shown by the increase in firing rate (p<0.05 by confidence limits) and apparent decrease in interburst interval. Cell firing rate was then significantly attenuated (p<0.05) during simultaneous perfusion with U50, 488H (10^-6 M) and recovered (p<0.05) following further perfusion with naloxone (10^-5 M).
7.4. Discussion

A functional interaction between CCK8S and opioids (particularly morphine) on magnocellular neurosecretory activity within the SON was indicated from results obtained from the hypothalamic slice preparation. The limitations of this electrophysiological approach using conventional extracellular recording techniques make it difficult to positively identify a cell as being vasopressinergic or oxytocinergic in nature. Both cell types show a depolarising response in vitro in the hypothalamic explant to a bolus infusion of CCK8S (Jarvis et al., 1992); this preparation permits both antidromic identification of magnocellular neurones and a greater maintenance of synaptic input enabling the two separate cell types to fire in a recognisably characteristic fashion (Bourque and Renaud, 1991).

CCK receptors have been shown to be post-synaptically located on oxytocin neurones (Jarvis et al., 1992; Oliet and Bourque, 1992) although there is more dispute as to the location of μ-opioid receptors. It is likely that they are located on or close to the cell bodies of magnocellular oxytocin neurones, since morphine can inhibit the electrical activity of putative oxytocin neurones in the SON in vitro (Wakerley et al., 1983a; Inenaga et al., 1990). In the present study the excitatory effect of CCK8S on magnocellular neurones was completely suppressed by simultaneous perfusion with morphine (Table 7.3.1.1.), whilst morphine inhibition of SON putative oxytocin neurone firing rate was partially reversed by CCK8S (Fig. 7.3.1.1.g). In one vasopressin-like cell (Fig. 7.3.1.2.) the CCK8S mediated excitation was markedly reduced by the κ-opioid selective agonist U50, 488H, which confirms previous reports that vasopressin neurones are sensitive to κ- but not μ-opioid agonists (Inenaga et al., 1990). Thus this opioid inhibition of
CCK8S-induced excitation of firing rate would appear to be common to both cell types within the SON despite evidence that \( \kappa \)-opioid agonists seem to mediate inhibition in magnocellular neurones by a different mode of action from \( \mu \)-agonists (Inenaga et al., 1994), as in other neuronal preparations (North, 1986).

Although acute effects of CCK8S on magnocellular cell activity do not appear to involve a Ca\(^{2+} \) component, a mixed cationic conductance is involved (Jarvis et al., 1992), whilst the exact post-receptor mechanisms which mediate the excitation remain to be resolved. Morphine has been reported to inhibit neurones by activating a K\(^+ \) conductance thereby sustaining a more prolonged after hyperpolarisation, which may in turn with continued administration attenuate an associated Ca\(^{2+} \) conductance (North, 1989) and this may be G protein mediated. It has been speculated using a rat brain homogenate preparation that morphine and CCK may converge on the same post-receptor component to mediate their respective effects on cell activity (Wang and Han, 1992). Thus morphine may apparently antagonise the excitatory action of CCK8S on magnocellular putative oxytocin neurone function by displacing the balance of such an effector component (which for magnocellular neurones remains to be exclusively defined) and subsequent effects on cellular activity away from the CCK receptor to mediate its suppressive effect on electrical activity of the cell.

Ultimately it would be of considerable interest to determine whether such a relationship on magnocellular oxytocin cell electrical activity also exists between CCK and morphine when the cell is initially inhibited by morphine; that is could endogenous CCK8S antagonise the inhibitory action of morphine? As mentioned previously, preliminary studies have intimated that
this may be the case (Fig. 7.3.1.1.e. and f.), although further work is obviously required before such results can be verified. CCK has been proposed to act as an endogenous opioid antagonist (Faris et al., 1983; Higgins, 1992) and CCK8S binding site density within the SON is increased in morphine dependent rats (Munro et al., 1993). If synthesis of the CCK8S precursor preproCCK is also increased in these animals as is the case when the magnocellular oxytocin system is activated by salt-loading and dehydration (Hinks et al., 1993; O'Shea and Gundlach, 1993) then increased endogenous CCK function upon oxytocin cell electrical activity could be a partial candidate for the development of tolerance and dependence in these neurones; although the increased availability of CCK receptors may be sufficient for the excitatory actions of dendritically released CCK to antagonise the inhibitory actions of morphine and to help sustain withdrawal excitation of oxytocin neurones once initiated.

Although the increase in CCK8S binding within the SON during tolerance and dependence probably reflects an increase in binding site density rather than receptor affinity (Day et al., 1989) no studies have been completed concerning CCK-mediated excitation on magnocellular putative oxytocin neurone electrical activity from morphine tolerant hypothalamic slices. Such an approach is complicated by the requirement for tolerance to be maintained in vitro, and therefore the amount of morphine required to be added to the bathing medium during recording to maintain this state. Comparison of firing rate from supraoptic oxytocin cells between morphine tolerant and naive slices after perfusion with CCK8S would hopefully provide a more thorough insight into CCK receptor function during tolerance and dependence. The relative involvement of the two receptor subtypes could
also be elucidated from such a series of experiments. It has been proposed that CCK8S exerts its excitatory influence on magnocellular neurosecretory cells via a post-synaptically located CCK$_B$ receptor (Jarvis et al., 1992), yet osmotic activation of the magnocellular neurosecretory system has been reported to upregulate primarily CCK$_A$ receptor density within the SON (Hinks et al., 1993). This apparent conflict of findings may be due to magnocellular neurones being able to separately regulate CCK receptor subtypes consequent upon mode of activation. In the case of magnocellular neurones these two receptor subtypes may function via differing post-effector mechanisms to initiate the electrical event and if CCK and morphine do share a common pool of second messenger such as a specific G protein, then it is possible that only one of the CCK receptor subtypes has access to this pool, to offset the balance away from morphine to be subsequently upregulated during dependence.
GENERAL DISCUSSION
General Discussion

Recently it has become progressively clearer that the physical phenomena of tolerance and dependence can exist within a neuronal system as two separate entities. As such they are not necessarily co-expressed during prolonged opiate exposure especially at the level of the single neurone (Christie et al., 1987). The ongoing search for a site expressing a cellular component associated with dependence is far from resolved. Physical dependence as demonstrated by withdrawal excitation of neurones upon administration of the appropriate specific antagonist would appear to be a complex phenomenon requiring the maintenance of a functionally integrated neural network to synapse onto the neuronal population used to demonstrate withdrawal. The purpose of the following discussion therefore is to outline a scheme for withdrawal excitation of oxytocin neurones in the morphine dependent rat (see Fig. 7.1.).

The magnocellular oxytocin neurosecretory system located within the SON of the hypothalamus has been studied extensively in terms of cellular signalling leading to activation of neurones, as have the afferents which input to these neurones to modulate their activity upon application of an appropriate stimulus.

Acute administration of the μ-opioid agonist morphine inhibits electrical activity of supraoptic putative oxytocin neurones in vitro (Wakerley et al., 1983a; Inenaga et al., 1990). Continued administration of morphine results in the development of tolerance to and dependence upon the central inhibitory actions of morphine in vivo in these neurones. During this period normal electrical excitability returns to the cell. It is thought that this return to normal excitability occurs via upregulation of excitatory synaptic input to the
oxytocin neurones within the SON. Subsequent removal of this opioid inhibition by administration of naloxone enables the oxytocin neurone to respond in the appropriate hyperexcitable fashion; hypersecretion of oxytocin from the neurohypophysis into blood as demonstrated previously (Pumford et al., 1991) and throughout this thesis, occurring as a direct result of an increase in electrical activity of supraoptic oxytocin neurones in conjunction with removal of opioid tone by naloxone at the level of the neurohypophysis.

Systemic administration of sulphated cholecystokinin octapeptide (CCK8S) acts on the gastric vagus in the gastrointestinal tract to activate centrally ascending, probably noradrenergic, pathways which project to the SON to selectively activate magnocellular oxytocin neurones (Kendrick et al., 1991). These pathways which involve the A2 cell group which projects from the nucleus tractus solitarius (Sawchenko and Swanson, 1992) and possibly the A6 cell group of the locus coeruleus which also projects to the SON (Wilkin et al., 1989) mediate release of noradrenaline and serotonin within the SON as measured by microdialysis (Kendrick et al., 1991), whilst noradrenaline is principally excitatory to magnocellular oxytocin neurones (Yamashita et al., 1987). Initial blood sampling studies were able to show that prior intravenous administration of morphine could dose-dependently inhibit systemic CCK8S stimulation of oxytocin release into blood from the neurohypophysis in both morphine naive and morphine dependent rats by a central mode of action. However the oxytocin response to stimulation by systemic CCK8S was restored in morphine dependent rats; therefore tolerance to the central inhibitory actions of morphine on this excitatory input to oxytocin secretion must have developed. The development of tolerance would appear to have been quantifiable since the oxytocin response was
inhibited by morphine to a greater degree in the morphine naive rats. Similar blood sampling studies were also to demonstrate that prior intravenous administration of the $\alpha_2$ agonist clonidine could also suppress the oxytocin response to systemic stimulation by CCK8S as well as significantly attenuating naloxone-precipitated morphine withdrawal release of oxytocin from the neurohypophysis into blood. At the level of the locus coeruleus, $\alpha_2$- and $\mu$-mediated receptor effects have been shown to converge on the same post-receptor mechanism (Aghajanian and Wang, 1987) and this may also be the case for the noradrenergic afferents mentioned previously which project to the SON to modulate magnocellular neurosecretory cell activity, and even the SON oxytocin cells themselves, although clonidine has been reported to be without effect on basal firing rate of supraoptic neurones in vitro (Randle et al., 1984). Despite clonidine attenuation of the morphine withdrawal response, perhaps by a pre-synaptic mode of action at the level of the locus coeruleus or within the SON itself, the probable activation of this pathway by systemic CCK8S did not initiate withdrawal in magnocellular oxytocin neurones which reinforces the complex nature of this characterising dependence phenomenon.

Calcium ($\text{Ca}^{2+}$) is a ubiquitous ion involved in a variety of cellular processes within the CNS. Movement of $\text{Ca}^{2+}$ from the extracellular compartment into neurones can initiate exocytotic release of neurotransmitters from axon terminals, cell bodies and terminal processes (Pow and Morris, 1989; Scott et al., 1991). The L-type voltage-activated $\text{Ca}^{2+}$ channel blocker verapamil has been shown to inhibit the expression of several behavioural signs associated with naloxone-precipitated withdrawal in the morphine dependent rat depending upon route of administration
The present study was able to extend these findings to the magnocellular neurosecretory system and show that verapamil when given centrally attenuates withdrawal-induced secretion of oxytocin both from the neurohypophysis and within the SON and subsequently withdrawal excitation of oxytocin neurones by a central mode of action (Munro et al., 1993). Both the terminals in the neurohypophysis and the cell bodies within the SON of magnocellular oxytocin neurones express L- and N-type Ca\(^{2+}\) channels although the cell bodies do not express T-type Ca\(^{2+}\) channels suggesting that these neurones differentially regulate the expression of Ca\(^{2+}\) channels (Fisher et al., 1992). Differential regulation of magnocellular oxytocin neurone function and ultimately oxytocin release both within the SON and from the neurohypophysis has also been shown after application of an osmotic stimulus to both SON, whereupon neither intravenously administered naloxone nor morphine have any effect on osmotically stimulated intranuclear oxytocin release, whereas morphine does block oxytocin release from the neurohypophysis; perhaps via a central inhibitory action on the firing rate of oxytocin neurones (Munro et al., 1994).

Intracerebroventricular administration of a specific oxytocin antagonist can completely suppress the spontaneous occurrence of the milk-ejection reflex in the suckled lactating rat (Freund-Mercier and Richard, 1984) and oxytocin excites magnocellular oxytocin neurones \textit{in vitro} (Yamashita \textit{et al.}, 1987) probably by binding directly to oxytocin binding sites located upon dendritic processes within the SON (Freund-Mercier and Stoeckel, 1993). Oxytocin is also released within the SON during morphine withdrawal in a Ca\(^{2+}\) dependent manner (Neumann \textit{et al.}, 1993). The present results clearly demonstrate that endogenous central oxytocin is required for the full
expression of withdrawal in magnocellular oxytocin neurones since acute intracerebroventricular injection followed by infusion of a specific oxytocin antagonist referred to as Manning 16 (Manning et al., 1989) significantly attenuated withdrawal hypersecretion of oxytocin from the neurohypophysis into blood.

Oxytocin is colocalised within magnocellular oxytocin neurones with cholecystokinin (CCK) and this latter peptide has been widely attributed functions as a neurotransmitter throughout the brain (Crawley, 1992). The sulphated octapeptide fragment of CCK (CCK8S) predominates and CCK8S directly excites magnocellular neurosecretory cells *in vitro* (Jarvis et al., 1992; Oliet and Bourque, 1992) and this excitation can be suppressed by morphine as demonstrated here. Furthermore microinjection of CCK8S into the SON induces release of oxytocin measured by microdialysis (Neumann et al., 1993) probably from dendritic processes (Pow and Morris, 1989) and so endogenous CCK may function in an autocrine manner to enhance the electrical activity of magnocellular oxytocin neurones during morphine withdrawal. Activation of the magnocellular oxytocinergic system is associated with increased electrical activity of the neurones. Rats challenged with an ongoing osmotic stimulus achieved by salt-loading with 2% sodium chloride demonstrate both upregulated CCK8S binding and synthesis of preproCCK mRNA within the SON (Hinks et al., 1993) and this is also the case for rats which have been dehydrated previously (O’ Shea and Gundlach, 1993). Specific [125I]CCK8S binding was also increased in the SON of morphine dependent rats (Munro et al., 1993) and thus co-release of CCK with oxytocin during naloxone-precipitated morphine withdrawal may act at these sites to contribute towards maintaining the enhanced electrical
activity of these neurones during the withdrawal process. Preliminary electrophysiological studies were able to demonstrate that morphine can suppress CCK8S-induced excitation of supraoptic putative oxytocin neurone firing rate and it has been speculated that CCK and morphine may share a common pool of second messenger to mediate their effects on neuronal function (Wang and Han, 1992). These initial findings were extended to show morphine antagonism of CCK8S mediated excitation, and partial CCK8S antagonism of morphine inhibition of putative oxytocin neurone firing rate. Thus the development of tolerance to the inhibitory actions of morphine in these neurones may be the result of increased endogenous CCK activity offsetting morphine inhibition of oxytocin cell electrical activity.

These results suggest that withdrawal excitation of magnocellular oxytocin neurones within the SON involves a cellular component originating from the noradrenergic cell groups of the brainstem and such excitatory noradrenergic inputs may become more active during morphine withdrawal. However it may be that they are tonically upregulated during dependence as demonstrated by tolerance to the central inhibitory actions of morphine on systemic CCK8S stimulation of release from the neurohypophysis; such that increased release of noradrenaline within the SON per se need not necessarily be part of the withdrawal process in these neurones. This in itself may increase the expression of several components within the SON excitatory to oxytocin neurones such as the increase in CCK8S binding site density in morphine dependent rats. Such plasticity may manifest itself on other excitatory mechanisms by increasing oxytocin and Ca\(^{2+}\) channel binding site density for example, which could enhance the proposed autoexcitatory action of oxytocin during withdrawal excitation. Release of noradrenaline within the SON after
removal of central morphine inhibition by administration of naloxone may subsequently be amplified compared with the control situation. This enhanced release of noradrenaline which itself may be Ca\(^{2+}\) dependent could then excite oxytocin neurones directly (Yamashita et al., 1987) by increasing a voltage dependent Ca\(^{2+}\) conductance. Dendritic release of oxytocin and CCK in conjunction with the increase in synaptic drive from the noradrenergic cell groups could then continue until the availability of a readily accessible substrate such as Ca\(^{2+}\) which appears to be of paramount importance to the normal functioning of magnocellular neurones in response not only to withdrawal excitation, but to a variety of stimuli had become sufficiently depleted to bring the withdrawal process to its natural conclusion.
Figure 7.1. (overleaf)- Scheme based on data presented in this thesis to summarise sequence of events leading to excitation of oxytocin neurones following naloxone-precipitated morphine withdrawal in dependent rats.

Excitatory input activity (located both pre- and post-synaptically) to magnocellular oxytocin neurones within the SON may be upregulated due to ongoing \( \mu \)-receptor occupancy by morphine. Removal of this inhibition upon initiation of withdrawal has a number of cumulative effects to induce an increase in electrical activity of the cell (1). Pre-synaptic release of noradrenaline from brainstem noradrenergic afferents such as neurones of the nucleus tractus solitarius increases cell firing rate, in a \( \text{Ca}^{2+} \) dependent manner (2); such inputs may provide increased stimulation to the morphine-inhibited oxytocin neurone to enable resumption of normal electrical excitability. \( \text{Ca}^{2+} \) influx through L-type voltage-activated \( \text{Ca}^{2+} \) channels during cell depolarisation initiates exocytotic release of oxytocin and CCK from dendritic processes located in the ventral rim of the SON (3). Local release of these two peptides sustains the withdrawal process by further exciting the oxytocin neurones (4). Eventually depletion of \( \text{Ca}^{2+} \) or another readily available excitatory substrate results in the attenuation of the withdrawal process and its gradual drawing to an end. Other synaptic inputs (not shown) may be required for the full expression of withdrawal in these neurones.

Abbreviations used: SON-supraoptic nucleus, OT-oxytocin, CCK-cholecystokinin, M-morphine, Nx-naloxone, NA-noradrenaline, cNTS-caudal nucleus tractus solitarius.
REFERENCES


Blackburn, R.E. and Leng, G. (1990) Ablation of the region anterior and ventral to the third ventricle (AV3V region) in the rat does not abolish the


References


References


References


References


References

Nature **284**: 350-351.


Kato, M., Chapman, C. and Bicknell, R.J. (1992) Activation of \(\kappa\)-opioid receptors inhibits depolarisation-evoked exocytosis but not the rise in intracellular Ca$^{2+}$ in secretory nerve terminals of the neurohypophysis. *Brain Res.* **574**: 138-146.


Kendrick, K., Leng, G. and Higuchi, T. (1991) Noradrenaline, dopamine and serotonin release in the paraventricular and supraoptic nuclei of the rat in
response to intravenous cholecystokinin injections. J. Neuroendocrinol. 3: 139-144.


References


References


References


References


References


Nordmann, J.J., Cazalis, M., Dayanithi, G., Castanas, E., Giraud, P., Legros,
References


References


Raby, W.N. and Renaud, L.P. (1989) Dorsomedial medulla stimulation activates rat supraoptic oxytocin and vasopressin neurones through different


References

Comp. Neurol. 198: 45-64.


References


Theodosis, D.T., Chapman, D., Montagnese, C., Poulain, D.A. and Morris,


References

87-92.


Intracerebroventricular (i.c.v.) verapamil attenuates morphine withdrawal excitation of oxytocin neurones in anaesthetized rats

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i.c.v. infusion of morphine for 5 days in the rat leads to tolerance and dependence in magnocellular oxytocin neurones (Pumford et al. 1991). Naloxone then provokes a withdrawal excitation of oxytocin neurone firing rate and consequently a hypersecretion of peptide from the posterior pituitary. Magnocellular oxytocin neurone action potentials have a Ca⁺⁺-dependent shoulder and opening of centrally located voltage-activated Ca⁺⁺ channels has been implicated in withdrawal excitation of neurones. In behavioural studies the L-type Ca⁺⁺ channel antagonist verapamil inhibits withdrawal signs in morphine-dependent rats (Baeyens et al. 1987). We have given verapamil i.c.v. to see if it affects withdrawal excitation of magnocellular oxytocin neurones. Female Sprague-Dawley rats were anaesthetized with ether and the left lateral cerebral ventricle cannulated for subsequent injection whilst the right ventricle was fitted with a cannula connected to a subcutaneous osmotic mini-pump to deliver morphine sulphate (up to 50 μg ml⁻¹ h⁻¹). On day six of infusion, under urethane anaesthesia (1-25 g kg⁻¹), t.p., a femoral artery and vein were cannulated for blood sampling and injections. Plasma oxytocin was measured by specific radioimmunoassay. Verapamil (160 μg in 5 ml 0-9 % NaCl) or vehicle was injected i.c.v. 10 min after an initial blood sample. Naloxone (3 mg kg⁻¹) injected i.v. 10 min after i.c.v. vehicle raised plasma oxytocin by 761 ± 155 pg ml⁻¹ (mean ± s.e.m., n = 7) but after naloxone and i.c.v. verapamil the increase was only 276 ± 192 pg ml⁻¹ (n = 7, P < 0-05, Mann–Whitney U test). In a further experiment on morphine-dependent rats the supraoptic nucleus was exposed by ventral surgery to record via a microelectrode the extracellular activity of antidromically identified putative oxytocin neurones (continuously active, excited by t.v. CCK8S, 30 μg kg⁻¹) (Pumford et al. 1991). Naloxone increased the firing rate of continuous cells (mean baseline ± s.e.m., 23 ± 1-4 spikes s⁻¹, n = 5 cells in five rats) by 4-7 ± 1-9 spikes s⁻¹ (P < 0-05, Wilcoxon) and i.c.v. verapamil suppressed this withdrawal excitation, abolishing firing in three cells for ca 12 min and reducing firing in two cells by 93 and 95 % (P < 0-05 by confidence limits). These results show that verapamil, given centrally diminishes withdrawal excitation of oxytocin neurones, and indicate involvement of activation of L-type Ca⁺⁺ channels in this excitation.

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REFERENCES


From the Proceedings of the Physiological Society, September 1993.
Morphine Tolerance/Dependence Increases Cholecystokinin (CCK) Binding in the Rat Supraoptic Nucleus (SON)

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Magnocellular oxytocin neurones in the SON appear to co-produce CCK. These neurones are directly excited by CCK, this being reversed by morphine. Chronic i.c.v. infusion of morphine leads to tolerance and dependence in oxytocin neurones (Pumford et al. 1991). CCK may be an endogenous opioid antagonist involved in the development of tolerance. We have used autoradiography to quantify CCK binding within the SON in morphine-treated rats to seek evidence for a role of CCK in morphine tolerance of oxytocin neurones. Under ether anaesthesia female Sprague-Dawley rats were fitted with an i.c.v. cannula to infuse morphine sulphate (up to 50μg/h) or vehicle (1μl/h) from a subcutaneous osmotic mini-pump. After 5 days the rats were decapitated, their brains rapidly removed and frozen onto cryostat chucks on dry ice. A further group of rats was given 2% w/v NaCl to drink for 48h as a positive control, since it has previously been shown that this treatment increases CCK binding within the SON (Day et al. 1989); a control group was given water. 10μm transverse brain sections on gelatinised slides were incubated with [125I]CCK8 with or without excess unlabelled CCK8 to determine Total Binding (TB) and Non-Specific Binding (NSB) respectively, specific CCK binding was defined as (TB-NSB). The sections were then exposed to Hyperfilm [3H] for 4 days. Films were then processed, tissue sections fixed and the autoradiograms of SON profiles viewed under a microscope (objective ×10) for quantification using a Joyce-Loebl αMagiscan image analyser. Results were expressed as mean density of silver deposit over the measured area of dorsal SON (202 ± 135μm). Each film processed contained sections from at least one animal from each treatment group, the untreated group measurement on each film was used to calculate relative values from the measurements for each treatment group on the film, to overcome variability between films.

Specific [125I]CCK8 binding in the SON increased by 2.4 fold (P<0.01) after i.c.v. morphine treatment (n=6, mean relative value ± s.e. mean. 1.37 ± 0.18) compared with the vehicle treated group (n=6, 0.57 ± 0.1). There was no difference between the morphine-treated group and the salt loaded group (n=3, 1.48 ± 0.15); in both, binding was significantly increased (P<0.05) relative to the untreated controls. The increase in specific CCK8 binding within the SON, which may reflect an increase in receptor density, after chronic morphine treatment indicates that increased activity of endogenous CCK mechanisms within the SON could play a role in the development of morphine tolerance and dependence in oxytocin neurones in the rat.

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Morphine-induced withdrawal induces c-fos expression in magnocellular oxytocin neurones

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Magnocellular oxytocin (OXT) neurones are inhibited by acute morphine but develop tolerance and dependence during intracerebroventricular (i.c.v) morphine infusion over 5d (Bicknell et al. 1988). Withdrawal induced acutely by naloxone (NLX) increases OXT neurone firing rate 3.5 fold in dependent rats but has no effect in controls and as a result oxytocin secretion is greatly increased. Vasopressin neurones are unaffected. We have studied whether withdrawal activates the early onset gene, c-fos, and stimulates production of Fos in dependent OXT neurones. To establish morphine dependence, virgin female Sprague Dawley rats, n=8, were implanted with a lateral i.c.v cannula, under ether anaesthesia, to infuse morphine sulphate (up to 50ug/h) from a subcutaneous osmotic minipump; controls were given vehicle (1ul/h). In the first study after i.c.v. morphine infusion for 5d a femoral vein was cannulated, under urethane anaesthesia (1.25g/kg i.p.), for injection 2h later of NLX (5mg/kg) or vehicle. The rats were decapitated 0.5h after NLX, the brains removed, frozen on dry ice and stored at -70°C. 20um cryostat sections through the hypothalamus were hybridised with 35S-labelled oligonucleotide c-fos mRNA probe and exposed on film for 3 weeks (Hamamura et al 1991). Autoradiographs were viewed by microscope (x10 objective) and grain density above background over the supraoptic nucleus (SON) was measured with a Joyce Loebel image analyser. In the SON c-fos mRNA was barely detected in vehicle injected rats (n=4, mean grain density ± s.e.m. 0.8 ± 0.8% field area) whereas NLX induced c-fos mRNA expression (n=4, 12.2 ± 1.3%, p<0.001, t-test). In the second study after i.c.v. morphine or vehicle infusion for 5d conscious rats were given either s.c. NLX or vehicle(n=6 per group). The rats were decapitated 90min later and the brains removed, frozen on dry ice and stored at -70°C. 15um cryostat sections through hypothalamus were immunono-cytochemically processed using Fos antibody (Oncogene Sciences) and peroxidase labelled second antibody detected by the glucose oxidase-nickel-DAB method (Shu et al 1988). Sections were viewed at x10 mag and the density of Fos positive nuclei in the SON calculated by counting and by measuring the area of each SON profile with the image analyser. In the i.c.v. vehicle group after i.v. vehicle or NLX, and in the i.c.v. morphine group after i.v. vehicle, the density of Fos positive neurones in the SON was <1 neurone/100um² but in the morphine/OLX group the density was 22.6±3.30 (means ± s.e.m., p<0.001, ANOVA). Activation of the c-fos gene in SON neurones during morphine withdrawal may be confined to oxytocin neurones, and a consequence of their increased firing rate.

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