HYPERNATRAEMIC STIMULATION OF OXYTOCIN SECRETION : EFFECTS OF OPIOIDS AND PREGNANCY

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Ph.D.

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1993

DECLARATION

The studies reported here were performed solely by the author unless indicated otherwise and the thesis was composed by himself alone in the Physiology Department of the University of Edinburgh .

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March 1993

ACKNOWLEDGEMENTS

I am greatly indebted to Dr John Russell for his instruction and help during my postgraduate training and invaluable advice in compiling this thesis . I would also like to thank Dr Barbara Sumner who provided me with training in quantitative opioid-receptor autoradiography (Chapter 1) and Dr Alison Douglas with whom I collaborated in the study undertaken in Chapter 2 . In addition the microdialysis studies described in Chapter 3 were performed in collaboration with Dr Thomas Horn of Leipzig University .

I am grateful for all the advice and assistance of the Histology Laboratory under the expert supervision of Mrs Kay Grant, and to Mr Brendan McGrory for the measurement of all the plasma sample osmolalities and sodium concentrations reported in Chapter 4 .

I received financial support from the Agricultural and Food Research Council who provided me with a postgraduate research studentship .

I would finally like to thank all my family and friends who provided me with encouragement and additional financial support without which I would not have been able to complete my studies . I especially thank my fiancé Gaynor Barnes for her patience and understanding during the completion of my research and compilation of this thesis .

In memory of my Father

"Each neuron of the cortex is interwoven into a highly complex maze of the finest nerve fibers, some coming from distant parts . It may be safely assumed that most cortical neurons are directly or indirectly connected with every cortical zone . Herein lies the anatomical basis of the cortical association complexes ..... If one million cortical nerve cells were connected in all possible combinations in groups of only two neurons each, then the number of different interneural connections thus formed would total $10^{2783000}$."

C. Judson Herrick, 1928 .

The Brains of Rats and Man

University of Chicago Press

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Chapter 3 Where are the osmoreceptors regulating oxytocin neurones?

ABSTRACT

The thesis describes investigations into the responsiveness of the neuroendocrine oxytocin system to hypernatraemic stimulation during pregnancy in the rat . The effects of acute intraperitoneal and intravenous hyperosmotic saline were investigated in virgin rats and in pregnant rats after 16 and 21 days of gestation . Plasma oxytocin concentration was measured by radioimmunoassay, and the inhibitory effects of endogenous opioids and exogenous opiates on the osmotic stimulation of oxytocin secretion were also investigated .

The response of the oxytocin system to intraperitoneal hyperosmotic saline was strikingly attenuated at day 21 of pregnancy . These results reveal a reduced influence of the osmoregulatory input to oxytocin neurones in pregnancy . Experiments to test whether endogenous opioids, angiotensin II or acute ovarian hormone effects were involved in this reduced oxytocin activity during pregnancy indicated that it was independent of these factors .

However, the oxytocin response of 21 day pregnant rats to the intravenous administration of hyperosmotic saline after the opioid antagonist naloxone was significantly elevated . This may be due to an increased sensitivity of oxytocin neurones to changes in plasma volume produced by the intravenous infusion of hyperosmotic saline that is absent when administered by the intraperitoneal route .

The sites of the osmoreceptors regulating the response of oxytocin neurones to changes in plasma osmolality were investigated in virgin rats . This involved the discrete application of hyperosmotic saline into the brain using infusion and microdialysis techniques . The results of these experiments indicate that the osmoreceptors are partly in the lamina terminalis but through their direct osmosensitivity the magnocellular oxytocin neurones themselves function as osmoreceptors .

Evidence was sought for tolerance to exogenous opiate actions on the oxytocin system as a result of increased inhibitory endogenous opioid activity on oxytocin neurone secretion at the end of pregnancy . However, the effects of mu- and kappa-opiate agonists against hyperosmotic stimulation and oxytocin release evoked by electrical stimulation of the lamina terminalis were found to be similar in pregnant and virgin rats . In contrast, after naloxone there was a clear decrease in the excitatory effect of such electrical stimulation on oxytocin secretion at day 21 of pregnancy, indicating partial uncoupling of this input to oxytocin neurones in pregnancy . In virgin rats part of this excitatory input was found to be modulated by the excitatory amino acid NMDA receptor, but this pathway was not activated during hyperosmotic stimulation of oxytocin release . In rats made morphine-tolerant, responses to hyperosmotic saline were also reduced, as in pregnancy, but sensitivity to administered morphine was less than in morphine-naive rats, in contrast with pregnancy .

Plasticity of opioid actions on the oxytocin system was further investigated by measuring the time course of kappa-receptor down-regulation in the posterior pituitary during salt loading with *in situ* quantitative autoradiography . Within 2-5 days the kappa-receptor binding decreased significantly in virgin rats . Facilitated release of oxytocin, as a result of the down-regulation of inhibitory pituitary kappa-opioid receptors, would promote renal excretion of the excess salt load because of the synergistic natriuretic activity of oxytocin with vasopressin .

GENERAL INTRODUCTION

GI.1 The pituitary gland

The pituitary gland was described in aged medical literature as an anatomical entity assumed to act as a filter for the slime, pituita, or waste material excreted by the brain into the nasopharynx . A. von Haller (1766) appears to have been the first to report a distinction between the anterior and posterior parts of the gland . Then in 1778 Soemmering introduced the term hypophysis as an alternative to pituitary gland due to the nondescript appearance of the posterior lobe when studied with ordinary fixation and staining techniques . There was little progress in research on the neurohypophysis during the nineteenth century and as late as 1889 Macalister described the pituitary as "probably the rudiments of an archaic sense organ" (Heller, 1970) .

Ramon y Cajal (1894) originally described bundles of non-myelinated nerve fibres entering the neural lobe through the pituitary stalk in two day old mice . Subsequent studies revealed that most of these fibres were derived from the hypothalamic supraoptic and paraventricular nuclei in the higher vertebrates (Heller, 1970) . Besides nerve fibres and their terminals the posterior pituitary also contains glial elements (Berkeley, 1894) . Bucy in 1930 reported that these glial cells differed from astrocytes and oligodendrocytes of the adjacent brain and introduced the term pituicyte, and proposed that the pituicytes were the site of neurohypophysial hormone secretion . This view was supported by Gersh (1939) who claimed that there was a depletion of neurosecretory material from the pituicytes in water deprived rats but these findings were not confirmed by other workers (Hickey *et al.*, 1941) . Subsequent electron microscope investigations have revealed that as the axons enter the neural lobe they form large swellings filled with a variety of organelles including lysosomes, vacuoles, mitochondria and

secretory vesicles . Eventually the axons branch to form endings that lie near or actually apposed to portions of the basal lamina separating the neural lobe from the perivascular space (Palay, 1957) . Nordmann (1977) used the two criteria of contact with the basal lamina and presence of microvesicles to define neurosecretory terminals in the neural lobe of the rat . Estimates of such terminals in the rat pituitary number 40,000,000 and since there are only approximately 18,000 hypothalamic neurosecretory axons projecting to the neural lobe this implies each neurone branches into about 200 endings (Nordmann, 1977 ; Maddrell and Nordmann, 1979) . Pituicyte processes often extend to the immediate vicinity of blood vessels in the perivascular space (Lederis, 1965) and are additionally in intimate contact with axonal terminals as if wrapped around the potential release site (Baumgarten *et al.*, 1972 ; Tweedle, 1983), consequently neurosecretory endings often terminate within pituicytes (Tweedle and Hatton, 1980b) . This probably explains the early misconception that the pituicytes were the site of hormone secretion based on relatively low resolution light microscope investigations .

Tweedle and Hatton have also shown that the degree of intimacy depends upon the stimulatory state of the system (Tweedle and Hatton, 1987) . Stimuli which increase hormone release, such as dehydration via water deprivation (1980a) or saline drinking (1987), parturition (1982) and suckling (1987), lead to a decrease in the extent to which axon terminals are covered by the pituicytes, and thereby exposing the neurosecretory endings to a larger expanse of the basal lamina, as if to increase the surface area for release .

These results have been confirmed by numerous reports of changes in pituicyte morphology in response to increased neurohypophysial secretion

(Morris *et al.*, 1978) . Conversely castration in the male rat which decreases neurohypophysial hormone release (Crofton *et al.*, 1985) results in a greater than normal enclosure of axon terminals by pituicytes accompanied by a decrease in the proportion of the basal lamina occupied by neural membrane (Tweedle *et al.*, 1988a) . The physiological significance of this increased pituicyte enclosure may involve the formation of a physical barrier to hormone transport through the basal lamina, inhibit the conductance of the descending action potential into the secretory terminal and/or the release of inhibitory substances onto the axons or their terminals (Hatton, 1988a and b ; Hatton *et al.*, 1984 ; Tweedle, 1983 and 1987) .

Additionally tritiated opioid binding has been demonstrated in cultured rat neural lobes (Bicknell *et al.*, 1989) . Opioids are known to inhibit hormone release at the level of the neural lobe (Zhao *et al.*, 1988) . It therefore appears that the pituicytes may also play an important part in facilitating hormone secretion by binding inhibitory opioid agonists in combination with the morphological adaptations linked with increased demand on hypothalamo-neurohypophysial activity .

GI.2 The establishment of neuroendocrinology

The first indications that neurones might possess secretory activity were reported in 1919 by Speidel who described giant nerve cells in the spinal cord of the skate that also apparently possessed many cytological characteristics of glandular cells . Subsequently Scharrer (1928), whilst conducting a histological investigation of the hypothalamus in the minnow, described cells with all the attributes of typical neurones that also contained secretory material normally associated with an endocrine function . He proposed the idea that some cells could have both neuronal and glandular

properties and consequently the hypothesis of neurosecretion was initiated . But it was not until the elegant series of studies both of the Scharrers and by Bargmann that the concept of neurosecretion was established (Bargmann and Scharrer, 1951 ; Scharrer and Scharrer, 1940) . These workers reported that sectioning the pituitary stalk led to a proximal accumulation and a distal depletion of neurosecretory material . This provided evidence that the hypothalamic nuclei are the site of hormone synthesis the products of which are transported by axon flow to the nerve terminals in the posterior pituitary . Electron microscope studies by Palay (1955, 1957) showed that the neural lobe was composed of axons containing dilatations filled with dense cored vesicles . These vesicles, of approximately 150nm in diameter, were subsequently referred to as neurosecretory granules and were thought to be the storage containers for the hormones . Differential centrifugation studies soon confirmed this hypothesis (Lederis and Heller, 1960 ; Barer *et al.*, 1963) and the neurosecretory granules were also reported to contain hormone associated neurophysins (Ginsburg and Ireland, 1966) .

In conjunction with the histological evidence of neurosecretory activity were investigations undertaken to determine whether or not neurones containing secretory material could display both typical neurone and secretory cell activity . Cross (1958a and b) initially reported that electrical stimulation of the supraoptic nucleus, paraventricular nucleus and hypophysial tract produced effects upon the uterus and mammary gland that were unaffected by spinal transsection and could be mimicked by the injection of oxytocin . It was subsequently reported that representative neurosecretory neurones could propagate and conduct action potentials (Cross and Green, 1959 ; Kandel, 1964) . Kandel (1964) also introduced antidromic identification to the study of preoptic neurones in his experiments

on the goldfish . Dyball (1969) used this method in the rat to selectively identify hypothalamic neuroendocrine neurones that project to the neurohypophysis . Lincoln and Wakerley (1971), working at the same university, were the first to report electrophysiological recordings from antidromically identified neurones in the paraventricular nucleus of the lactating rat which were activated during OT release evoked by suckling of the pups . A similar relationship was found in cells of the supraoptic nucleus (Lincoln and Wakerley, 1974) which also demonstrated this synchronous high frequency discharge or burst of activity preceding a rise in intramammary pressure . It was therefore assumed that the burst of neuronal activity facilitates the release of OT from the nerve endings in the neurohypophysis into the circulation, resulting in a milk ejection response in the lactating rat . This assumption was confirmed a year later by Cross *et al.* (1975) who also demonstrated a relationship between the electrical and secretory activity of these neurones .

GI. 3 Structure and plasticity of the hypothalamo-neurohypophysial system

From lesion studies it was first believed that the synthesis of OT was confined to the paraventricular nuclei and that of vasopressin to the supraoptic nuclei (Olivercrona, 1957 ; Nibbelinck, 1961) . It now seems certain however that there is considerable overlap between the two nuclei (Sokol, 1970 ; Burford *et al.*, 1974 ; Dierickx *et al.*, 1978) . Differential control of the release of the two neuroendocrine hormones oxytocin and vasopressin was first explained by Heller (1966) in terms of the one neurone-one hormone hypothesis . Subsequent reports lead to the overwhelming conclusion in favour of Heller's proposal of a separate

neurone for each hormone (Aspeslagh *et al.*, 1976 ; Morris *et al.*, 1977 ; Dierickx *et al.*, 1978) .

Half the cell bodies of the neuroendocrine axons that terminate in the posterior pituitary lie mainly in the hypothalamic supraoptic and paraventricular nuclei (Pines, 1925 ; Greving, 1926 ; Fisher *et al.*, 1979) . The latter nuclei were found not only to contain neuroendocrine neurones of about 20-35μm in diameter, referred to as magnocellular, but also smaller parvocellular neurones (Sawchenko and Swanson, 1982 ; Silverman and Zimmerman, 1983) . The supraoptic nucleus (SON) in the rat during periods of stimulated activity has demonstrated a great degree of plasticity . These changes not only involve alterations in the numbers of cellular organelles and types of mRNA expressed but also dramatic changes in interneural membrane distances and the formation of new synapses . The time scale for these changes was first thought to take hours even days but further investigation has shown that for some of these modifications it is more in the order of minutes (Hatton, 1990) .

Morris (1978) reported that various stimuli for either vasopressin or OT release produced a number of subcellular modifications including increases in nuclear and Golgi apparatus volume . However his work was preceded by Hatton and Walters (1973) who reported that dehydration by 24 hours of water deprivation produced a 20% increase in the number of magnocellular neurones possessing more than one nucleolus per cell in the SON . An enlargement of OT neurones in the paraventricular nucleus (PVN) during lactation has also been reported (Russell, 1983) . It is possible that the creation of new and larger nucleoli in response to stimulation may be involved in the related increases in hormone, opioid and ribosomal mRNA expression observed (Sherman *et al.*, 1986 a and b ; Kawata *et al.*, 1988) .

Structural modifications also occur during dehydration and lactation involving both glial cell activity and axon-axon interactions . The SON is normally composed of mainly neurosecretory cell bodies which although in places lie very close together are usually separated by intervening astrocytic processes (Theodosis and Poulain, 1984) . However, several earlier investigators reported that occasionally neurosecretory somata and dendrites were directly apposed without an intervening glial layer (Enestrom, 1967 ; Rechardt, 1969 ; Lafarga *et al.*, 1975) . These appositions were found to vary by up to 10% in number as a direct function of the hydration state of the animal (Tweedle and Hatton, 1976 and 1977) . They subsequently reported that the chronic stimulus of suckling also induced similar increases in direct somatic appositions in SON cells (Hatton and Tweedle, 1980 and 1982) . Quantitative analysis has revealed that between 33-40% of the neurosecretory cell bodies in the lactating rat are of this type (Tweedle and Hatton, 1982 ; Theodosis and Poulain, 1984) . Theodosis and Poulain (1989) have similarly reported changes in the OT cells in the PVN during lactation . One popular hypothesis describing a functional consequence of these changes is postulated to result in an increased potassium concentration between the neuronal elements (Hatton and Tweedle, 1982 ; Theodosis and Poulain, 1987) . The astrocytic glial processes may act as potassium sponges and their removal therefore, in conjunction with the facilitated firing rate of neuronal elements due to increased excitatory influences, could result in elevations of interneuronal cleft potassium concentration in the order of several millimolar (Hatton, 1988a and b) . This may facilitate further neuronal activity as such elevations in potassium concentration have been shown to increase the excitability of neurons (Poolos *et al.*, 1987)

Despite the apparent loss of glial interaction the intracellular space between apposed neurone elements is maintained at normal levels (Theodosis and Poulain, 1984) . However Peters *et al.* (1970) reported the existence of attachment plaques between the exposed membranes which may serve a variety of functions including action electrotonic conduction (Spray and Bennet, 1985) . At the same time there is also an increase in the number of synapses coupling cell bodies and dendrites that lie directly apposed . This is often the result of single synaptic boutons making contact with more than one post-synaptic element referred to as "double" or "multiple" synapses (Lafarga *et al.*, 1975) . The number of these synapses dramatically increases in the SON (Theodosis *et al.*, 1981 ; Hatton and Tweedle, 1982) and PVN (Theodosis and Poulain, 1989) of lactating rats . These morphological changes have been found to appear during pregnancy reaching significance at parturition . They are maintained during lactation but disappear within two months of weaning and therefore seem to be transitory (Theodosis *et al.*, 1985) .

Multiple synapses have also been reported in male and female during dehydration and in the Brattleboro rat (Chapman *et al.*, 1986 ; Modney and Hatton, 1989 ; Tweedle and Hatton, 1984a) . Upon rehydration, only 14 days was required to re-establish control levels of multiple synapses (Tweedle and Hatton, 1984a), a substantially shorter period than in lactating rats after weaning . The rate of formation of these multiple synapses has been estimated in one report as early as 10 minutes after the transcardial infusion of hyperosmotic medium (Tweedle *et al.*, 1989) . Multiple synapses in the SON have been found to contain a variety of excitatory and inhibitory neurotransmitter substances including noradrenaline (Tweedle and Hatton, 1984b), dopamine (Buijs *et al.*, 1984) and GABA (Theodosis *et al*, 1986) . It

therefore appears that multiple synapses may be involved in both the facilitation of hypothalamic nuclei activity during osmotic challenge, and the modulation of the bursting activity of OT neurones recorded during lactation .

GI.4 Discovery and early clinical use of oxytocin

The first decisive report of the physiological importance of the posterior pituitary gland was made by Oliver and Schafer in 1895 who showed that the intravenous injection of an extract of the whole pituitary produced a rise in blood pressure . This was followed by the report of Howell (1898) that the active pressor substance was present in the posterior portion of the hypophysis . Shortly afterwards Dale (1906) whilst investigating the effects of ergot and adrenaline also reported a powerful contractile response to posterior pituitary extracts of the uterus in the pregnant cat . Further physiological effects of posterior pituitary extracts were reported by Ott and Scott (1910) who demonstrated milk ejecting activity . The uterine contracting activity of Dale's pituitary extracts were almost immediately applied to the human by Blair Bell (1909) . He injected this extract into two women during caesarean delivery and observed that "the uterus contracted into a blanched ball" . These observations quickly stimulated the use of pituitary extracts to treat the clinical conditions of post partum haemorrhage and delayed labour . These results were so widely known that by 1913 the Canadian obstetrician B.P. Watson commented "it seldom happens that a new drug or remedy comes into universal usage in such a short space of time as has been the case with pituitary extract in obstetrical practice" . In addition it was also at this time that the antidiuretic effect of pituitary extract was first reported to be of therapeutic benefit in treating the excess urine output of diabetes insipidus (Heller, 1970) .

G.I.5 Extraction and identification of oxytocin

Dudley (1919) found that the pressor and oxytocic activities of posterior lobe extracts could be separated by extraction with butanol and concluded in 1923 that "the uterine stimulant and pressor principles are two distinct chemical substances" . In collaboration with Dale in 1921, Dudley also reported the effects of proteolytic enzymes on the active principles of pituitary extracts and concluded that they were polypeptides . Research continued into isolating the active oxytocic and pressor principles from pituitary extract . In 1928 Kamm and his colleagues successfully isolated highly active oxytocic and pressor fractions and were the first to institute the universally accepted nomenclature of oxytocin and vasopressin . The structure of oxytocin (OT) was simultaneously determined by du Vigneaud *et al.* (1953a) and by Tuppy and Michl (1953) . Also that same year the structure of vasopressin was established by du Vingneaud *et al.* (1953b) and the synthesis of both hormones was successfully performed by 1954 (du Vigneaud *et al.*, 1953b and 1954) .

GI.6 Synthesis, release and fate of oxytocin

Investigating the *de novo* hypothalamic synthesis of OT and vasopressin Sachs and Takabatake (1964) demonstrated that initial production is of a high molecular weight precursor molecule which is subjected to proteolysis to yield the active hormones . Specific neurophysins are associated with each hormone (Legros and Loui, 1974) and it has been suggested that these peptide entities represent the inactive end of the cleaved molecule (Brownstein, 1983) . Evidence for this conclusion is also provided by the finding that the DNA sequences coding for OT and

vasopressin also code for their associated neurophysin (Land *et al.*, 1982 and 1983) . The primary secretory product of active hormone and neurophysin is reported to be produced on the richly endowed rough endoplasmic reticulum of the cytoplasm, from which it is transferred to the Golgi apparatus for packaging into the neurosecretory granules (Zambrano and de Robertis, 1966) . After their formation the vesicles are carried by axonal transport towards the nerve terminals in the posterior pituitary at a rate of 50-100mm/day (Jones and Pickering, 1970 ; Burford and Pickering, 1973) . Whilst in transit to the nerve ending there is good evidence that the hormones are processed (Pickering *et al.*, 1971 and 1975 ; Brownstein *et al.*, 1980) . Stimulus secretion coupling in the nerve terminal initially involves an increase in calcium permeability followed by exocytosis of the vesicle contents (Douglas, 1973 and 1974) . After release into the blood stream OT and vasopressin circulate as free peptides (Brook and Share, 1966) . The main site of clearance is the liver (Chaudhury and Walker, 1959) with only small amounts of intact hormone excreted in the urine (Frandsen and Jensen, 1971) . The half life of OT has been estimated at 100 seconds in the rat (Higuchi *et al.*, 1985) and to range from 3-17 minutes in other animals (Fabian *et al.*, 1969 ; Chard *et al.*, 1970 ; Gibbens *et al.*, 1972 ; Vankrieken *et al.*, 1983) . Additionally the clearance rate does not seem to vary during pregnancy (Amico *et al.*, 1984) .

GI.7 Established stimuli for OT release

The classical established stimuli for OT release involves two reflex mechanisms involved in reproduction . The first is associated with the stimulation of stretch receptors in the lower genital tract during the expulsive phase of parturition and is known as the Ferguson reflex (Ferguson, 1941) .

This spurt of OT release is associated with the direct, via OT receptors, and indirect, by increasing prostaglandin release, stimulation of myometrial contractions (Fuchs, 1985) . The second reflex involves tactile stimulation of the nipple which promotes the release of OT and results in the milk ejection reflex (Ely and Peterson, 1941 ; Findlay, 1966 and Pacheco *et al.*, 1979) . The milk ejection reflex is the mechanism by which pre-formed milk is ejected from the breast under pressure during suckling . Oxytocin released in response to tactile stimulation of the teat produces a contraction of the myoepithelial cells which surround the alveoli expelling milk into the larger ducts from which it escapes to the exterior through the nipple of the mammary gland (Mena *et al.*, 1985) .

GI.8 A role for oxytocin in sodium excretion ?

The high concentration of sodium ion in the extracellular fluid accounts for approximately half of the osmotic pressure exerted by this fluid . If we take into account the negatively charged ions associated with sodium then in effect the concentration of sodium ions of the extracellular fluid determines over 90% of the osmotic pressure of this fluid . Rarely does the extracellular fluid sodium concentration fluctuate more than 3% illustrating the effectiveness of the control systems regulating it (Young *et al.*, 1977 ; Guyton, 1986) .

In an attempt to excrete as much urea from the blood as possible the kidneys form large amounts of glomerular filtrate . In terms of sodium excretion in man this creates a serious problem for this filtrate contains approximately 26,000mEq of sodium each day compared to an intake of 150mEq . Consequently the principle role of the kidney tubular system in sodium excretion is to reabsorb sodium, about 99.3% of the total filtered by

the kidney glomerulus. Careful adjustment of the residual sodium concentration in the urine enables the body to balance the daily intake of sodium . 65% of sodium re-absorption occurs in the proximal tubular epithelial cells due to active transport and by the time the tubular fluid reaches the distal tubules approximately 92% of the sodium load has been removed (Guyton, 1986) . Sodium re-absorption from the distal tubules and collecting ducts is highly variable . Its rate is stimulated by the adrenal cortex hormone aldosterone, such that either virtually all or none of the sodium entering the distal tubules is reabsorbed (Young *et al.*, 1976 ; Anderson, 1977) . Experiments in dogs however have revealed that fluctuations in aldosterone concentration are not important in the control of extracellular sodium concentration . This is demonstrated in dogs that have received an infusion of aldosterone at a constant rate after the endogenous system was blocked by removal of the adrenal glands . If such animals are then subjected to in excess of a 600% increase in sodium intake their plasma sodium concentration fluctuates by only 2% (Young *et al.*, 1976) . This apparent lack of importance of aldosterone in plasma sodium modulation is explained by its associated water retaining effects . Such that a reduction in plasma aldosterone leading to a decrease in the re-absorption of sodium from the distal tubules, produces a concomitant decrease in the re-absorption of water which completely nullifies the osmotic effects produced by the loss of sodium (Guyton, 1986) . The predominant role of aldosterone is therefore restricted to the maintenance of blood volume and pressure (Young *et al.*, 1976) . Low blood pressure stimulates the release of renin from the juxtaglomerular apparatus of the kidney (Tobian, 1960 ; Vander and Miller, 1964 ; Tagawa *et al.*, 1974 ; Davis and Freeman, 1976) . Renin increases the production of angiotensin II which causes a vasoconstriction of

the arterioles resulting in a direct increase in blood pressure (Tagawa *et al.*, 1974 ; Gibbons *et al.*, 1984) and an indirect effect via an increased release of aldosterone from the adrenal cortex (Coghlan *et al.*, 1971) . The renal actions of aldosterone stimulate the re-absorption of sodium and its associated water of hydration leading to an increase in blood volume and pressure . In addition angiotensin II also has a direct effect on the kidney in the regulation of renal haemodynamics and electrolyte excretion (Hall *et al.*, 1977 and 1979 ; Baer and McGiff, 1980) .

More recently another hormone, atrial natriuretic factor (ANF), has been implicated in blood pressure control via salt regulation . Cardiac muscle fibres of the atria secrete this peptide hormone in response to agonists, during exercise and when stretched as might occur in response to increased blood volume (Verees *et al.*, 1988 ; Walsh *et al.*, 1988 ; Garcia *et al.*, 1987 ; Sonnenberg and Verees, 1984) . ANF appears to act as a potent natriuretic and diuretic agent via an action on the proximal tubules (Sonnenberg *et al.*, 1982 ; Flynn *et al.*, 1983 ; Burnett *et al.*, 1984 ; Hammond *et al.*, 1985 ; Baum and Toto, 1986 ; Zimmerman *et al.*, 1987 ; Itabashi *et al.*, 1985 ; Harris *et al.*, 1988 and 1989) . ANF receptors have been localised in both the kidney and adrenal gland (Ballermann *et al.*, 1985 ; Chai *et al.*, 1986 ; Koseki *et al.*, 1986 ; Suzuki *et al.*, 1987) . ANF has also been found to inhibit aldosterone production by suppression of adrenal glomerulosa cell activity (Kudo and Baird, 1984 ; Atarashi *et al.*, 1984 ; Goodfriend *et al.*, 1984) and to antagonise angiotensin-stimulated sodium and water re-absorption within the kidney (Harris *et al.*, 1987) . ANF immunoreactivity has additionally been reported in the brain (Kawata *et al.*, 1985) with a high concentration in areas innervating the hypothalamus as well as in the hypothalamus itself implying a central role for ANF in

cardiovascular control and fluid homeostasis (Kawata *et al.*, 1985b ; Jacobowitz *et al.*, 1985 ; Skofisch *et al.*, 1985 ; Saper *et al.*, 1985 ; Zamir *et al.*, 1986 ; Palkovits *et al.*, 1987 ; Geiger *et al.*, 1989) . Peripheral administration of ANF produces an increase in the urine flow rate and urine sodium excretion whilst central administration also produces an increase in the rate of urine production, an inhibitory effect on angiotensin II stimulation of water intake and a suppression of vasopressin release (Samson, 1985 ; Lee *et al.*, 1987 ; Al-Barazanji and Balment, 1989 ; Rohmeiss *et al.*, 1989) .

Mechanisms thought to be involved in restoring normal fluid dynamics during hypernatraemia are attributed to the renal actions of vasopressin, in conjunction with an increase in thirst and a decrease in the appetite for salt associated with an action of the angiotensin in the hypothalamus (Young *et al.*, 1977 ; Fregley and Rowland, 1985) . Hyperosmotic stimulation of vasopressin release produces an increase in fluid, but not solute, re-absorption by the kidneys (Robertson, 1974 ; Smith *et al.*, 1979). The combination of thirst, appetite and vasopressin effects results in a dilution of extracellular electrolytes and a reduction in osmolality .

However in the rat at least OT is also involved in fluid homeostasis (see also the introduction to Chapter 4) . Early investigators (Magnus and Schafer, 1901 ; Schafer and Herring, 1908) described a natriuretic effect of posterior pituitary extract in anaesthetised animals . By 1913 two independent researchers reported the effects of pituitary extract in human subjects (Farini, 1913 ; von den Velden, 1913) . Farini's report appeared first and it described the treatment of patients with diabetes insipidus using injections of Pituitrin (Parke, Davis and Co., 200mg posterior lobe/ml) . One such patient had a pre treatment daily urine output of 7.5-8.0 l which was

decreased to 2.0 l/day by Pituitrin . Von den Velden (1913) also reported similar results to Farini but in addition he also demonstrated an inhibitory effect on water diuresis in healthy men thus providing the first indication of the physiological importance of the neurohypophysis in normal fluid balance (Pickford, 1966) .

It was work by Frank (1910) that first drew attention to the evidence that it was dysfunction of the posterior pituitary that led to the production of diabetes insipidus . A few years later Camus and Roussy (1913) reported that hypothalamic lesions also lead to the appearance of diabetes insipidus . Cajal (1894) had already reported that the posterior pituitary was innervated by neurones arising in the hypothalamus and Greving (1923) localised these fibres to the supraoptic and paraventricular nucleus . Verney summarised his view by the statement that normal water antidiuresis depends upon the integrity of the pars nervosa (Verney, 1936) .

Early work with whole pituitary extract revealed an antidiuretic action accompanied by increased urinary excretion of phosphorous salts in dogs (Stehle and Bourne, 1925) . Nelson and Woods (1934) also found that small doses of pituitary extract in mice inhibited water diuresis whilst at the same time promoting salt excretion . Melville (1936), from a study of the effect of salt saturation on the urinary response to pituitary extract , concluded that the natriuretic and antidiuretic responses were unrelated .

After methods had been devised for the separation of oxytocin and vasopressin from pituitary extracts (Kamm *et al.*, 1928) studies were undertaken to determine which hormone was responsible for the various actions of the whole extract . Fraser (1937 and 1942) was the first to point out that in the rat oxytocin had a chloriuretic and diuretic action . His observations were later confirmed by other workers using both synthetic and

OT extracted from natural sources, and in addition to the above actions a natriuretic response was also noted (Kuschinsky and Bundshuh, 1939 ; Dicker and Heller, 1946 ; Brunner *et al.*, 1957 ; Horster, 1960) . Berde (1959) also reported that in rats loaded with saline solution there was a clear dose-response relationship between OT and sodium excretion . Abrahams and Pickford (1954) concluded that in the dog administration of hypertonic saline caused the release of 10-20 times as much OT as vasopressin . Jones and Pickering (1969) found that in rats, dehydration by water deprivation or salt loading depleted the vasopressin and OT content of the neurohypophysis to a similar extent . An increase in the firing rate of OT neurones in the supraoptic and paraventricular nucleus in response to changes in plasma osmolality has also been reported (Brimble and Dyball, 1977 ; Brimble *et al.*, 1978) . Jewell and Verney (1957) attempted to localise the osmosensitive site responsible for this increase in neuronal activity and concluded that the supraoptic neurones themselves contained osmoreceptive elements but did not discount the involvement of other areas within the hypothalamus that synapse with the supraoptic cells . Leng (1980) reported that the application of minute volumes of hypertonic saline to the immediate vicinity of antidromically identified neurones in the rat supraoptic nucleus increased the activity of virtually all cells tested . Local application of hypertonic solutions into regions that project to the paraventricular nuclei have also been reported to excite magnocellular nuclei (Honda *et al.*, 1987) . *In vitro* studies in which the osmotic pressure of the bathing medium has been manipulated by as little as 7-15mosmol/l has produced significant increases in the firing rate of neurons in the SON (Mason, 1980 ; Bourque and Renaud, 1984) . In contrast nearby hypothalamic cells and pyramidal

neurons from the hippocampus require an osmotic stimulus in excess of 100mosmol/l to evoke appreciable increases in firing rate (Bourque, 1989) .

Combining these studies it appears that both the magnocellular neurons of the SON and other hypothalamic neurons are osmosensitive . It would therefore seem probable that a central osmoreceptor complex exists capable of detecting fluctuations in plasma osmolality and sodium concentration and stimulating OT and vasopressin release (see chapter 3) .

Elevated plasma OT in response to 24 hours of water deprivation and salt loading associated with depleted pituitary stores of OT has also been reported in the male rat (George, 1976 ; Dogterom *et al.*, 1977 ; Balment *et al.*, 1980) . Forsling *et al.* (1982) reported that OT administered to rats infused with hypotonic saline was associated with saliuresis and altered renal water excretion . Recently Verbalis *et al.* (1991) infused graded doses of OT subcutaneously in conscious male rats maintained on a sodium deficient diet . There results demonstrated that OT caused a dose related increase in urinary sodium excretion at levels well within the physiological range of the rat . This may therefore provide an explanation for the presence of a functional OT system in the male aside from its normal roles associated with female reproduction .

Despite the reports in support of a natriuretic effect of OT in the rat Balment *et al.* (1980 and 1982) provided evidence that OT plays no major role in the day to day regulation of salt or water balance in the water replete rat . However it was proposed to be of physiological significance during dehydration produced by water deprivation or salt loading .

A role for OT has also been suggested in the control of sodium appetite in the rat . Stricker and Verbalis (1987) reported that sodium appetite was inhibited by increased activity in the supraoptic and

paraventricular nuclei . In a subsequent report Stricker *et al.* (1987) provided additional evidence that sodium appetite was inversely related to the activity of hypothalamic oxytocinergic neurones . It therefore seems probable that under certain conditions of hyperosmolality in the rat OT produces the dual effects of increasing natriuresis from the kidney and decreasing salt intake via a reduced sodium appetite . These two effects would obviously be synergistic in promoting the re-establishment of isotonic fluid composition from a hypertonic condition .

GI.9 Oxytocin in other animals

Osmotic stress has also been demonstrated to significantly decrease the amount of OT like neurosecretory material in the neural lobe of the water snake Natrix maura (Mancera, 1990) . A more familiar species of animal in which a lot of work has been performed is the dog . Early reports indicated OT had no effect on sodium excretion (Heller and Stephenson, 1950 ; Anslow and Wesson, 1955) . However if a large enough dose is given an increased electrolyte excretion can be shown even in hydrated dogs (Brooks and Pickford, 1956 ; Ali *et al.*, 1958 ; Chan and Sawyer, 1961 ; Bartter and Mills, 1969) . Weitzman *et al.* (1976) also reported that the infusion of hypertonic saline caused a similar increase of both plasma OT and vasopressin in the dog .

Disappointingly in man few reports to date have demonstrated that OT has a facilitatory effect on the natriuretic activity of the kidney . Most early reports concluded that OT had no effect on salt excretion in man (Chalmers *et al.*, 1951 ; Brunner *et al.*, 1957 ; Thomson, 1959 and 1960 ; Cross *et al.,* 1960) . Goodwin *et al.* (1970) reported that prolonged OT administration for 5-10 days had no effect under normal conditions on

sodium excretion . Nussey *et al.* (1986) could also find no evidence that OT was involved in osmoregulation in man . Also in 1986 Williams *et al.* concluded that vasopressin was selectively secreted in response to both dehydration and high sodium intake . Even after rapidly increasing plasma osmolality via the intravenous (i.v.) infusion of 0.85M NaCl (0.05mol/kg/min) they did not obtain a significant increase in plasma OT concentration . However there is evidence to the contrary, which is in favour of a role for OT in human osmoregulation . Mertz (1960) reported an increased excretion of electrolytes in man following administration of OT . Ezrin *et al.* (1962) also reported an interesting clinical use of OT in a patient with reversed diurnal rhythm of water excretion . The patient's condition was thought to have arisen due to an injury to the head and resulted in fluid retention during the day accompanied by increased excretion during the night . When the patient was treated three times a day with intramuscular injections of OT the rhythm returned to normal . More recently Ekstrom *et al.* (1991) have reported that the infusion of hypertonic saline increases menstrual pain by stimulating uterine contractions, thereby indicating a release of OT . They have subsequently reported that plasma OT concentration was indeed increased in these experiments (Ekstrom *et al.*, in press) . Despite earlier reports to the contrary it does therefore appear that OT is released in response to osmotic stimulation in the human at least during menstruation in women . This response of OT may produce a natriuretic effect via a direct action on the kidneys and involve an interaction with vasopressin (as discussed more fully in Chapter 4) and/or ANF .

GI.10 Control of oxytocin secretion

Oxytocin secretion stimulated by suckling, angiotensin II, cholinergic drugs and hyperosmotic saline is inhibited by the administration of exogenous opiates such as morphine (Haldar and Sawyer, 1978 ; Russell and Spears, 1984 ; Evans and Olley, 1988 ; see also Chapter 4) . Endogenous opioids appear to inhibit OT (Coombes and Russell, 1988) and to a lesser extent vasopressin secretion at the level of the neurohypophysis (Bicknell and Leng, 1982 ; Zhao *et al.*, 1988a and b) . Opioid receptors in the posterior pituitary are predominantly of the kappa-receptor subtype (Bunn *et al.*, 1985 ; Herkenham *et al.* 1986 ; Sumner *et al.*, 1990) . Possible sources of endogenous opioids acting at this site are the adjacent vasopressin terminals and the OT neurons themselves . Vasopressin neurones are found to contain and release dynorphins which are kappa opioid receptor agonists (Corbett *et al.*, 1982 ; Whitnall *et al.*, 1983 ; Lorenz *et al.*, 1985) . Enkephalin opioids have also been reported in the neural lobe (Martin *et al.*, 1983) and were subsequently reported to be co-localised in OT neurones (Gayman and Martin, 1987) .

Opiates mediate their effects at the level of the cell bodies of OT neurones via mu- and kappa-receptor subtypes (Clark *et al.*, 1986 ; Mansour *et al.*, 1988 ; Sumner *et al.*, 1990 and 1992) . Opiate receptors have also been reported at sites that provide excitatory innervation to the supraoptic and paraventricular neurones (Mansour *et al.*, 1987 ; Russell *et al.*, 1988 ; Sumner *et al.*, 1990) . Similar to the neuronal plasticity displayed during lactation and dehydration opioid receptor density shows a significant amount of change during dehydration (Brady and Herkenham, 1987 ; see also Chapter 1) and pregnancy (Sumner *et al.*, 1992) .

A catecholamine presence in the neural lobe has also been reported and both dopamine and noradrenaline activity are stimulated by dehydration (Alper *et al.*, 1980 and 1982 ; Holzbauer *et al.*, 1980) . Dopamine concentrations in neural lobe have been estimated at between 4-10 times the concentration of noradrenaline (Holzbauer *et al.*, 1980) . Tyrosine hydroxylase activity in SON and PVN magnocellular neurons has been reported to increase during osmotic stimulation of the rat (Kiss and Mezey, 1986) and it therefore appears that dopamine may possibly be another neurosecretory product of magnocellular neurons . Co-release of dopamine may serve a negative feedback function as dopamine has been reported to inhibit both vasopressin and OT secretion (Barnes and Dyball, 1982 ; Lightman *et al.*, 1982) .

Zhao *et al.* (1988b) reported that naloxone, the non selective opioid antagonist, increased noradrenaline release from isolated neural lobes . These results were interpreted as evidence for endogenous opioid suppression of noradrenaline release . In a subsequent report Zhao *et al.* (1988c) found evidence for alpha- and beta- adrenergic facilitation of OT release which may therefore contribute to the selective inhibition of OT release by kappa opioids . Opioid-noradrenaline interactions may involve the neural lobe pituicytes which display both kappa-opioid and beta-adrenergic receptors (Bicknell *et al.*, 1989 ; Bunn *et al.*, 1986) . Beta-adrenergic agonists have been reported to cause a retraction of pituicyte processes similar to that reported in chronic stimulatory states discussed above (Smithson *et al.*, 1988) which may have a facilitatory effect on hormone release .

GENERAL METHODS

GM1 OXYTOCIN RADIOIMMUNOASSAY

GM1.1 Introduction

The radioimmunoassay (ria) employed was a method from that of Higuchi *et al.* 1984 . Higuchi et al. raised and purified antibodies to a synthetic oxytocin-b.s.a. conjugate in the rabbit and showed that this antibody could be used to measure rat blood plasma OT without extraction . Specificity of the anti-OT antibody was measured by comparing the inhibition of ^{125}I-labelled OT binding in the presence of various substances including arginine-vasopressin, arginine-vasotocin, angiotensin II, rat follicle stimulating hormone and rat luteinizing hormone . Cross reactivity of these hormones was found to be 0.0005% or less . Recovery of OT, in the range of 6.3 - 400 pg/ml, from incubations in male and pregnant female rat plasma by this assay was found to be between 94-107% .

We added a limited concentration of this rabbit anti-OT antibody to mixtures of a constant ^{125}I-OT concentration and rat plasma aliquots, containing unknown concentrations of rat OT, in a phosphate buffer . Mixtures were left for 48 hours to reach equilibrium after which the proportion of ^{125}I-OT to rat OT bound to the rabbit antibody reflects the concentration of OT in the plasma sample . In order to measure the radioactivity of the rabbit anti-OT-^{125}I-OT aggregate it was precipitated using a second antibody method . Goat anti-rabbit gammaglobulin was added to the mixture to bind to the rabbit anti-OT first antibody and the resulting complex precipitates out of solution . The precipitate (ppt) was separated by centrifugation and the resulting supernatant aspirated . A suspension of white pansorbin cells added prior to centrifugation was used to aid precipitation and visualisation of the ppt . Radioactivity of the ppt was measured with a gamma counter .

To measure the unknown concentration of OT in rat plasma samples, radioactivity of sample precipitates was compared to a standard curve constructed from precipitate radioactivity of mixtures containing known OT concentrations instead of an aliquot of rat plasma .

GM1.2 MATERIALS

PHOSPHATE BUFFER : The phosphate buffer (pH 7.0) used was prepared in de ionised double distilled water containing :-

0.025% (w/vol) sodium dihydrogen orthophosphate $NaH_2PO_4.2H_2O$ (BDH)

0.119% (w/vol) di sodium hydrogen orthophosphate Na_2HPO_4 (BDH)

0.1% (w/vol) sodium azide NaN_3 (BDH)

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

(BDH LTD, Broom Road, Poole, Dorset, BH12 4NN . Sigma Chemical Company LTD, Fancy Road, Poole, Dorset, BH17 7NH) .

FIRST ANTIBODY BUFFER : - phosphate buffer containing normal rabbit serum, to aid the binding of the second antibody, at a dilution tested for maximum ^{125}I-OT binding and economical use, typically 1:100 dilution . Testing involved the preparation of trial assays containing standards only and no rat plasma samples . The lowest concentration of normal rabbit serum that produced the highest % binding of ^{125}I-OT in the precipitates was chosen .

FIRST ANTIBODY : rabbit anti-OT antibody THF-3 was kindly donated by Dr T. Higuchi, stored diluted 1:600 in phosphate buffer at -20^0C and used at a final dilution of 1:120,000 in antibody buffer .

SECOND ANTIBODY : goat anti-rabbit gammaglobulin, obtained from the AFRC IAPGR, Babraham, Cambridge, was stored at –20°C and used at a dilution of 1:100 in phosphate buffer .

^{125}I-OT : also obtained from the AFRC IAPGR, Babraham, was stored at –20°C and used at a concentration calculated to give between 6-8000 cpm/50 µl aliquot . OT was iodinated by the chloramine T method and purified on a Sephadex G-25 column (as Higuchi *et al.*, 1985) .

STANDARDISED PANSORBIN CELLS :supplied as a 10% (w/vol) solution in phosphate buffered saline containing 0.1% (w/vol) sodium azide was stored at 4°C, used at a dilution of 1:20 in ria buffer and obtained from Novabiochem (UK) LTD, 3 Heathcoat Building, Highfields Science Park, University Boulevard, Nottingham NG7 2QJ .

GM1.3 METHODS

In order to quantify the unknown OT concentration of experimental rat plasma samples a standard curve of ppt radioactivity versus OT concentration was constructed . Stock OT aliquots containing 10µg OT (Sigma) were previously lyophilised and stored at –70°C . On the day of assay serial dilutions of a stock lyophilised OT aliquot were made in phosphate buffer, to yield a range of eleven concentrations from 2.44-2500 pg/ml, which were then used to construct the standard curve . Three more standards were also prepared for construction of the curve :-

TC, contained an aliquot of ^{125}I-OT only, to determine Total Count of radioactivity added ;

NSB, contained ^{125}I-OT and second antibody but no first antibody or unlabelled OT, to determine Non Specific Binding of radioactivity ;

B0, contained ^{125}I-OT trace, first and second antibody but no unlabelled OT, to determine the maximum % binding of radioactivity .

Also on the day of assay experimental plasma samples were removed from the freezer, thawed to 4^{0}C as required, and centrifuged for 30 secs at 15,000g (Haemofuge, Heraeus-Christ, GMBH, Osterode, Germany) before being aliquoted into the appropriate tube . The assay was performed in pre numbered 0.75ml polystyrene tubes (LP2 tubes, Luckham LTD, Victoria Gardens, Burgess Hill, Sussex) . Standards were done in triplicate and rat plasma samples in duplicate . If we expected to get an OT concentration in excess of 1250pg/ml in the rat plasma sample, e.g. morphine dependant rats given the opioid antagonist naloxone, then a 1 in 3 dilution of the plasma sample in phosphate buffer was made .

The experimental procedure for the addition of reagents to the tubes was as follows .

Day 1

TC : nothing

NSB : 50μl ria buffer, 50μl antibody buffer

BO : 50μl ria buffer, 50μl first antibody solution

Standards/samples : 50μl first antibody solution, 50μl of standard/plasma sample .

Tubes were then vortex mixed, covered in aluminium foil and stored overnight at 4^{0}C .

Day 2

50μl of ^{125}I-OT was added to all tubes which were then vortex mixed re-covered in foil and left for 2-4 days at 4^{0}C .

Day 4

50μl of second antibody was added to all tubes, except TC, which were then vortex mixed, re-covered in foil and left for 2 days at 4°C .

Day 6

All tubes except TC received 50μl of diluted pansorbin cells, were then vortex mixed and centrifuged for 30mins at 3,000rpm and 4°C (Minifuge 2, Heraeus-Christ, GMBH, Osterode, Germany) . The resulting supernatant was aspirated and the radioactivity of each ppt then measured for 3mins in a gamma scintillation counter (LKB-Wallac 1272 Clinigamma with a single detector consisting of a NaI crystal in a 3" aluminium tube of wall thickness 0.25mm . Wallac Oy, 20101 Turku 10, Finland) .

Wallac Ultroterm 2 software was used . This automatically produces a curve from the standards using a spline-function best fitted curve . This construction provides a smooth continuous line through the points on the curve . Curve calculation also incorporates a weighting and smoothing function ; this automatically rejects bad points which can then be edited . Weighting the points provides information on the difference between the standards and the fitted spline function .

In order to assess inter-assay variation stock solutions of 20, 100 and 500 pg/ml OT were prepared in phosphate buffer , aliquoted and stored frozen at −20°C . Duplicates of each inter-assay standard were incorporated at the beginning and end of the plasma samples being assayed . This also allowed any intra-assay variation to be detected, but this was never found to be greater than 15% . The program we used also provided estimated OT concentrations for 80, 50 and 20% of the reference (B0) standard value (ED80, ED50, ED20 see page 35) . These could also be used to calculate inter-assay variation .

Raw counts were corrected for background radiation and, except for TC and NSB, non-specific radioactive binding . The corrected counts were automatically used to calculate inter-assay and plasma sample OT concentration from the standard curve .

Total binding (TB) of an assay was calculated as the % of the TC radioactivity bound by the B0 standards, which contained no unlabelled OT . This was calculated using the following equation :-

$$TB = \frac{B0 - NSB}{TC}$$

Assay sensitivity was calculated from the % assay error which was estimated using the standard deviation (std dev) of the ^{125}I-OT binding in the three B0 tubes . In order to convert this to an OT concentration first a graph was produced of the % B0 radioactivity bound by each standard Vs the standard concentration . The % of B0 radioactivity bound by each standard was calculated using the following equation :-

$$\frac{\text{mean corrected standard cpm - mean NSB}}{\text{mean B0 - mean NSB}}$$

A graph of log standard [OT] Vs % B0 radioactivity bound was then constructed . Assay sensitivity, expressed as a % of B0, was then calculated using the following equation : -

$$\frac{[BO - (2 \times \text{std dev B0})] - NSB}{B0 - NSB}$$

For example :-

CODE (pg/ml)	COUNTS (counts per 3min)	CORRECTED CPM	%TB
TC	22263	7311.0	
TC	21778	7149.3	
TC	21908	7192.7	
MEAN		7217.7	100

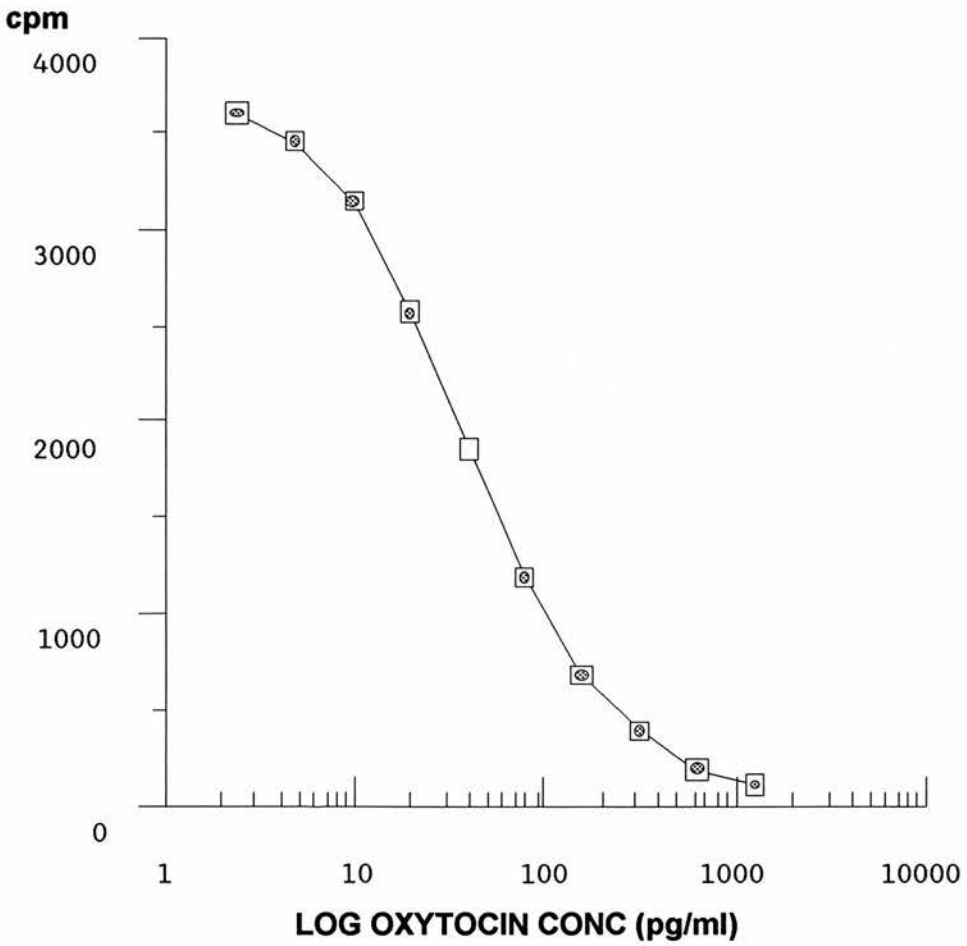
NSB	1082	250.7	
NSB	940	203.3	
NSB	1066	245.3	
MEAN		233.1	
B0	12541	3837.2	
B0	12312	3760.9	
BO	12883	3951.2	
MEAN		3849.8	53.3
2.4	11866	3612.2	% OF TB
2.4	11765	3578.6	
2.4	11895	3621.9	
MEAN		3604.2	93.6
4.8	11410	3460.2	
4.8	11509	3493.2	
4.8	11248	3406.2	
MEAN		3453.2	89.7
9.7	10591	3187.2	
9.7	10404	3124.9	
9.7	10385	3118.6	
MEAN		3143.6	81.0
19.5	8695	2555.2	
19.5	8935	2635.2	
19.5	8646	2538.9	
MEAN		2576.4	66.9
39.1	6392	1787.6	
39.1	6805	1925.2	
39.1	6611	1860.6	
MEAN		1857.8	48.3
78.1	4341	1103.9	
78.1	4559	1176.9	
78.1	4934	1301.6	
MEAN		1194.0	31.0
156	3063	677.9	
156	3126	698.9	
156	3090	686.9	
MEAN		687.9	17.9

312	2227	399.2	
312	2210	393.6	
312	2194	388.2	
MEAN		393.7	10.2
625	1577	182.6	
625	1593	187.9	
625	1609	193.2	
MEAN		187.9	4.9
1250	1344	104.9	
1250	1319	96.6	
1250	1435	135.2	
MEAN		112.2	2.9
2500	1181	50.6	
2500	1203	57.9	
2500	1277	82.6	
MEAN		63.7	1.7

STAND	CONC (pg/ml)	WEIGHT (%)
1	2.4	1.33
2	4.8	1.37
3	9.7	1.47
4	19.5	2.28
5	39.1	2.23
6	78.1	9.00
7	156.0	5.29
8	312.0	8.88
9	625.0	18.09
10	2500.0	29.96

	CPM	CONC
ED80	3079.8	10.686
ED50	1924.9	36.656
ED20	770.0	136.098

FIGURE GM.1 Example standard curve

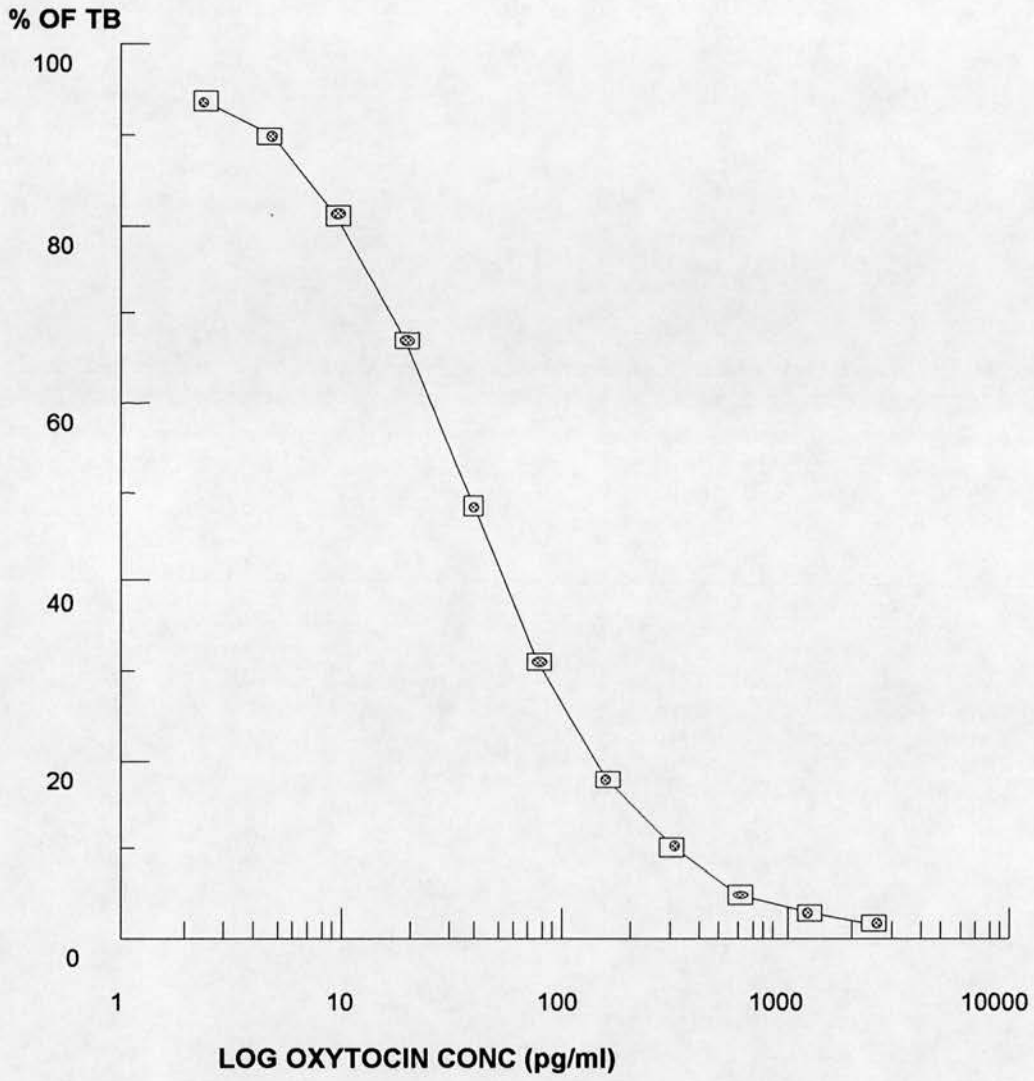


Sensitivity of this example assay was calculated as :-

$$\frac{[\,3849.8 - (2 \times 95.8)\,] - 233.1}{3849.8 - 233.1} = \underline{94.7\% \text{ of TB}}$$

This was converted into an OT concentration using Fig GM.2

FIGURE GM.2 % Total binding Vs Log oxytocin concentration



From Fig GM.2 the sensitivity of the assay was found to be at a lower OT concentration than the first standard of 2.4 pg/ml, the sensitivity of the assay was therefore set at this value .

GM1.4 SUMMARY ASSAY DETAILS

Chapter 1

Sensitivity of the oxytocin radioimmunoassay in Chapter 1 was calculated as 2.4pg/ml and the mean (± s.e.m.) intraassay variation was 12.2 ± 1.5% (n = 6) .

Chapter 2

Sensitivity of the oxytocin radioimmunoassays in Chapter 2 ranged from 2.4-13.4 pg/ml . Mean (± s.e.m.) intraassay variation was 10.1 ± 2.75% (n = 36) and interassay variation was 23.8 ± 13.9% (n = 6) .

Chapter 3

Sensitivity of the oxytocin radioimmunoassays in Chapter 3 ranged from 2.4 to 10 pg/ml . Mean (± s.e.m.) intraassay variation was 9.2 ± 1.1% (n = 24) and interassay variation was 27.1 ± 5.7% (n = 4) .

Chapter 4

Sensitivity of the oxytocin radioimmunoassays in Chapter 4 ranged from 2.4 to 10pg/ml . Mean (± s.e.m.) intraassay variation was 12.6 ± 4.3% (n = 60) and interassay variation was 24.5 ± 11.5% (n = 10) .

GM2 RATS

Adult female Sprague-Dawley rats, obtained from Bantin & Kingman, were used throughout this series of experiments . They were housed under controlled conditions at 21-23^{0}C with a 12 hour light/dark cycle, and allowed food (standard breeder diet) and water ad libitum, and usually acclimatized for 2-3 weeks upon arrival before use in experiments or mating .

In order to obtain pregnant rats adult females were housed individually in mesh floored breeder cages with a stud male . Day 0 of pregnancy was determined by the appearence of a vaginal plug of semen on the underlying tray . After this the females were caged separately until the day of experimentation . The number of pups was recorded post mortem in pregnant rats and data excluded from final analysis if less than four pups were found .

In studies on lactating rats, the day of the litter birth was recorded as day 0 of lactation .

GM3 NON-RECOVERY CANNULATION OF THE LEFT FEMORAL ARTERY AND VEIN .

Experiments involving the removal of serial blood samples to determine plasma osmolality and sodium and oxytocin concentration required the cannulation of the left femoral artery and vein . Prior to surgery a sufficient number of cannulae (approx. 5-15cm in length, OD 0.75mm, size 3FG, Portex Ltd., Hythe, Kent CT21 6JL) were filled with heparinised saline and connected to 1ml plastic syringes (Steriseal poly-vu syringes, Thornhill Road, Redditch, Worcestershire B98 9NL) also containing 0.75ml of heparinised saline . Heparinised saline was prepared by a 1:100 dilution of a 5000 units/ml heparin solution (Multiparin, Weddel Pharmaceuticals Ltd., London) in isotonic saline (Steriflex No.1, FL (manufacturing) Ltd., Fresnius Health Care Group, Basingstoke, England) . On the day of experimentation rats were anaesthetised with urethane (1.25g/kg i.p., product number U-2500, Sigma Chemical Company) and after a sufficient level of anaesthesia had been reached a 10-20mm incision beginning at the second inguinal nipple and proceeding towards the base of the tail was made . The surface musculature and underlying membranes were then prised apart using forceps to expose the sheath which encapsulates the femoral artery, vein and nerve . The sheath was then gently opened and a 20mm length of artery and vein separated from each other and accompanying nerve . The exposed vessels were then ligated with EP 1 suture silk (Davis and Geck, Cyanamid, Gosport, Hampshire, UK) at a site as distal from the inguinal ligament as possible and clamped as close to the ligament as possible . A small incision was then made through the vessel wall and a cannula inserted into the vessel in the direction of the ligament . This was held in place with a ligature

of EP 1 suture silk, placed between the incision and the clamp, at a tension that would prevent blood loss but still allow movement of the cannula along the vessel . The clamp was then removed and the cannula fed along the vessel for approximately 2-3cm . The effectiveness of the cannulation was then tested by attempting to withdraw blood along the cannula . If the blood flowed easily then the cannula was secured in place by tightening the present ligature and applying two further ligatures . The skin and muscle incision was then closed with EP 3 suture silk .

GM3.1 Non-recovery cannulation of right femoral vein

In experiments involving the intravenous infusion of hyperosmotic saline and angiotensin II the right femoral vein was cannulated . The procedure was exactly the same as that for the left femoral vessel except the cannulae were 5cm lengths of polythene tubing (external diameter 1.6mm, internal diameter 1mm) the leading edges of which were trimmed to 45^0 to ease insertion into the vein .

GM4 BLOOD SAMPLING

In order to asses the OT concentration of rat blood 0.3ml samples were taken, via a femoral arterial cannula into heparinised, 1ml syringes (Steriseal poly-vu) and then transferred into 1.5ml eppendorf tubes and chilled on ice for a few minutes . The samples were then spun at 15,000g for approximately 2min (Haemofuge, Heraeus-Christ, GMBH, Osterode, Germany) and the plasma aliquoted, normally 0.15ml, into separate pre numbered eppendorf tubes . The plasma samples were then transferred to the -20^{0}C freezer and stored there till assay . If plasma osmolality and sodium concentration measurements were required at the same time then a 0.6ml blood sample was taken . These were treated in the same manner as the samples for OT ria except after centrifugation an extra 0.15ml plasma aliquot was taken and stored frozen in a separate tube for osmolality and sodium concentration measurements .The remaining blood cells were re-suspended in a sufficient amount of isotonic saline (Steriflex No.1) to provide an equivalent amount of fluid as that which was originally removed . This solution was returned to the rat as quickly as possible via the i.v. cannula .

It should be noted that the freezing and thawing of plasma samples prior to osmolality measurements significantly affects the results (Bohnen *et al.*, 1992) . Therefore the absolute osmolality values as reported in this thesis are unreliable . However it is the intention of the author to demonstrate mean changes in osmolality between and more importantly within similarly treated sample groups .

GM5 HISTOLOGY

GM5.1 Verification of electrode sites

In order to locate electrode positions in experiments involving electrical stimulation of the brain, at the end of the experimental protocol a continuous train of pulses was passed (2.5mA, 0.5msec duration, monophasic pulses at 100Hz for 1min) to deposit Fe^{2+} ions from the electrode tip . The brains were then removed and fixed in 10% formal saline (0.9% w/vol NaCl) for at least two days . The addition of 3% (w/vol) potassium ferro- and ferri- cyanide to the fixation solution enabled the position of the ferrous ions to be visualised using the Prussian Blue reaction . This produces a blue spot within the brain at the position of the electrode tip .

After fixation 50μm coronal brain sections were cut at -20^{0}C on a freezing microtome (Mod 1206, Reichert - Jung LTD, 820 Yoevil Road, Slough SL1 43B) and collected in sequence into section trays containing distilled water . Sections were then floated on to microscope slides previously subbed with a 0.5% (w/vol) gelatine, 0.05% (w/vol) chromic potassium sulphate solution .

The sections were then left to dry before being counterstained in 1% neutral red for 2min . Excess stain was removed in running water and then the sections were dehydrated via 2min washes in 70% then 90% and finally absolute alcohol . Sections were then dipped in xylene before being mounted in DPX (BDH Chemicals LTD, Poole, Dorset) and covered with a glass slip .

Positions of blue spots were mapped onto a series of drawings of coronal brain sections produced according to a rat brain atlas (Koning & Klippel, 1963) .

GM5.2 Verification of infusion sites

At the end of experiments involving infusions into brain tissue the cannula tip position was located by the injection of 1μl of 1% Evans blue solution . The brains were then removed and fixed in 10% formal saline for at least 2 days . After this the brains were processed and the locations of the blue spots caused by the dye were mapped as in the previous section .

Intracerebroventricular infusion sites were verified post mortem by coronally sectioning the cortex by hand in the region of the infusion site . The cortex was then inverted and examined for penetration of the infusion cannula tip into the lateral ventricle .

At the end of experiments involving the use of microdialysis first the probes were removed from the skull and then the brains which were promptly immersion fixed in 10% formal saline . After at least two days the brains were cut, mounted, counterstained and then the probe positions mapped as above .

GM5.3 Preparation of tissue for receptor autoradiography studies

Prior to removal of tissue cryostat chucks were coded and chilled on dry ice . Rats were killed by decapitation with a guillotine and the brain with or without pituitary removed as quickly as possible . Brains were then trimmed to the region required, mounted separately for coronal sectioning onto the pre-chilled chucks using Tissue-Tek OCT compound (Miles Inc., Diagnostics Division, Elkhart, IN 461515 U.S.A.) and frozen in pelleted dry

ice . When solid the tissue was covered in Nesco film (Nippon Shoji Kaisha LTD, Osaka, Japan) , placed in a sealed plastic pot and stored frozen at -70^{0}C .

Prior to sectioning the tissue, microscope slides were cleaned by soaking them in 10% (w/vol) potassium dichromate overnight and then subsequently washed in running water for 1day . Slides were then rinsed twice in distilled water, dunked twice in 80% alcohol and then dried with paper tissue . Slides were then subbed with chrome alum gelatine solution, as before, and left to dry . Vinyl examination gloves were worn when handling the slides after they had been washed to prevent contamination .

On the day of sectioning brains or pituitaries were removed from the -70^{0}C freezer and left to "warm" to -15^{0}C in the cryostat (5030 Microtome, Bright Instrument Company LTD, Huntingdon, UK) for approximately 2 hours, then sectioned at 15μm and thaw mounted onto the prepared slides . Cut sections were stored in sealed plastic boxes containing a sachet of silica gel at -70^{0}C until processed .

Sections prepared for receptor autoradiography were stored at -70^{0}C for not more than four weeks before the commencement of biding studies .

GM6 STATISTICS AND PRESENTATION

Statistical analysis was performed using the Number Cruncher Statistical System software package . Parametric statistics were used when results were normally distributed about their mean value . Normal distribution of the data was tested using the Martinez and Iglewicz test . If this test produced values greater than the rejection values, indicating non-normality of the data, then nonparametric statistics were used . When comparing data within a group the parametric paired t test and nonparametric Wilcoxon signed ranks test were used where appropriate . To test the difference between the means of two independent groups then the parametric two sample t test and nonparametric Mann-Whitney U test were used as appropriate . When comparing the differences among more than two groups then the analysis of variance procedure was performed followed by Duncan's range test when a difference was indicated .

Results were considered significantly different at $P \leq 0.05$. It is possible however, in a small number of experiments where repeated tests have been used on the same data at this level of significance to generate false positive results .

The thesis was compiled on a Viglen Genie 3SX25 IBM compatible computer using Word for Windows, SigmaPlot and Cricketgraph software and was printed on a Hewlett Packard Laserjet (ii) printer .

CHAPTER 1

Opioid inhibition of oxytocin secretion

1.1 INTRODUCTION

Early reports of opioid interactions with neurohypophysial hormone release were associated with a modulatory effect on vasopressin release (de Bono, 1944 ; Duke *et al.*, 1951 ; Scheneiden and Blackmore, 1955) . An interaction with OT was not described until 1978 when Haldar and Sawyer reported that subcutaneous morphine in lactating mice inhibited the weight gain of the suckling young, used as an index of milk ejection activity . They concluded that morphine inhibited OT release as exogenous OT was still effective at initiating a reflex milk ejection . These results were subsequently extended to the rat (Clarke *et al.*, 1979 ; Russell *et al.*, 1989) . Clarke *et al.* postulated, that since oxytocin cell bursts still occurred and milk ejections could be evoked with exogenous OT even in the presence of morphine, that the opiate was active at the neural lobe terminals . In addition they reported that the opiate antagonist naloxone, administered without morphine, enhanced the rise in intramammary pressure associated with suckling induced milk ejection . They therefore suggested that endogenous opioids were active by producing a tonic suppression of OT secretion at this time .

1.1.1 Site of opioid inhibition

Work by other investigators generally supports the conclusions of Clarke *et al.* (1978) . Naloxone has been found to greatly facilitate OT release from isolated, perfused, electrically stimulated neural lobes (Bicknell and Leng, 1982 ; Wammack and Racke, 1988), reinforcing the idea that endogenous opioids attenuate OT release at the level of the neural lobe . However in contrast to the findings of Clarke *et al.* (1979) morphine was reported by Coombes and Russell (1989) not to act at the posterior pituitary

but on the cell bodies of magnocellular neurones to inhibit electrical activity . Subsequently both *in vitro* and *in vivo* experiments have revealed that opioid peptides inhibit the background electrical activity of supraoptic OT neurones (Wakerley *et al.*, 1983 ; Pumford *et al.*, 1987 ; Inenaga *et al.*, 1990 ; Pumford *et al.*, 1991) .

It is now well established that both the cell bodies and terminals of neurosecretory neurones are the targets for opiate actions . The cell bodies of supraoptic and paraventricular magnocellular neurones appear to receive immunocytochemically identified opioid peptide innervation from the arcuate nucleus (Sawchenko *et al.*, 1982) and / or dorsal medulla (Cuello, 1983 ; Sawchenko *et al.*, 1989) . Opiate actions have also been demonstrated at sites that provide an excitatory input on to OT neurones . Subfornical organ neuronal projections innervate supraoptic magnocellular neurones (Sgro *et al.* 1984 ; Ferguson *et al.*, 1984 ; Ferguson and Renaud., 1986 ; Okuya *et al.*, 1987) via both direct and indirect connections (Lind *et al.*, 1982 ; Tanaka *et al.*, 1988 ; Gutman *et al.*, 1986 and 1989) . Indirect innervation appears to synapse in the region anterior and ventral to the third ventricle (AV3V region) which consists of the organum vasculosum of the lamina terminalis (OVLT) and the median preoptic nucleus (MPN or nucleus medianus) . In addition to innervation arising from the subfornical organ (SFO) cells within the AV3V region have also been demonstrated to provide a tonic excitatory drive to OT neurones (Leng *et al.*, 1988 ; Russell *et al.*, 1988) . Buranarugsa and Hubbard (1979) demonstrated morphine sensitive cells in the AV3V region and a number of reports have demonstrated opioid peptide binding in this area (Mansour *et al.*, 1987 ; Sumner *et al.*, 1990) . It is apparent therefore that opioids not only mediate their effects at the level of the cell body but may also inhibit excitatory inputs to the magnocellular neurones .

Specific opioid receptor binding sites in neuronal tissue have been reported to be both saturable and competitive in their opioid ligand binding (Goldstein *et al.*, 1971 ; Pert and Snyder, 1973 ; Simon *et al.*, 1973) . Subtypes of opioid receptors were first postulated by Martin (1967) to account for the morphine antagonism and analgesic action of nalorphine . Multiple opioid receptors were subsequently described and classified as mu, delta, kappa, sigma and epsilon (Martin *et al.*, 1976 ; Lord *et al.*, 1977 ; Schulz *et al.*, 1979 ; Chang and Cuatrecasas, 1981 ; Pfeiffer and Herz, 1981 ; Zukin and Zukin, 1981) . Using selective radiolabelled opioid ligands and *in vitro* autoradiography both mu- and kappa- receptor types have been reported in the SON (Leng and Russell, 1989 ; Mansour *et al.*, 1988 ; Sumner *et al.*, 1990) whilst in the pituitary gland there is a predominance of the kappa type with only a small population of mu-receptors present (Herkenham *et al.*, 1986 ; Mansour *et al.*, 1988) . In addition to an action of opioids at the level of the cell body opioids may therefore also inhibit neurohypophysial hormone release by an action on the neurone terminals or associated pituicytes .

1.1.2 Endogenous opioid activity

Morphine is a selective ligand for the mu-opioid receptor with little relative affinity for the kappa-receptor type (Kosterlitz, 1985) . Wright and Clarke (1984) have reported that in urethane anaesthetised lactating rats both mu- and delta- opioid receptor agonists inhibit the rise in intramammary pressure following intracerebroventricular administration of hyperosmotic saline which is associated with OT release . Application of morphine therefore may have an action at receptive sites innervating the OT neurones but at least part of its action is at the level of the cell bodies (Leng and

Russell, 1989) . However endogenous opioids do not appear to be tonically-active at this level since the opiate antagonist naloxone does not produce a change in the firing rate of these neurones even after hyperosmotic stimulation (Shibuki *et al.*, 1988 ; Bicknell *et al.*, 1988a and b) and an action at the terminals is therefore proposed . There is evidence that towards the end of pregnancy however, that the firing rate of OT neurones may be modulated by endogenous opioids at the same time as mu-opioid receptor binding is reduced (Bicknell *et al.*, 1988a and b ; Sumner *et al.*, 1992) .

Endogenous opioids are produced by the hypothalamic magnocellular neurosecretory neurones . Vasopressin containing neurones co-synthesise, localise and release the kappa-receptor agonist dynorphin $A_{(1-8)}$ (Watson *et al.*, 1982 ; Whitnall *et al.*, 1983 ; Sherman *et al.*, 1986) and dynorphin $A_{(1-8)}$ kappa-opioid receptor binding has been reported in the neural lobe (Gertsberger and Barden, 1986) . Also [met]-enkephalin peptides have been reported to be co-localised with OT in neurohypophysial nerve terminals (Martin and Voigt, 1981 ; Adachi *et al.*, 1985) . Martin and Voigt proposed that secreted enkephalins may act on autoreceptors located on their own nerve terminals or adjacent fibres to produce an inhibition of OT release . This finding is supported by other workers who have reported that extended [met]-enkephalin peptides present in the posterior pituitary have kappa-agonist activity (Castanas *et al.*, 1985 ; Panula and Lindberg, 1987) .

Falke and Martin (1985) reported the specific binding of opiate receptor ligands by separated OT and vasopressin nerve endings (neurosecretosomes) . Zhao *et al.* (1988a) subsequently reported that kappa-agonists were effective at attenuating potassium depolarisation evoked hormone release from neural lobe neurosecretosome preparations . Despite these findings Pitzel and Konig (1984) and Nordman *et al.* (1986)

reported that [met]-enkephalin and [leu]-enkephalin do not influence either spontaneous secretion or electrical, ouabain and potassium induced release from the isolated neural lobe .

1.1.3 Effects of endogenous opioids on oxytocin and vasopressin secretion

A cross inhibitory function of dynorphins released from vasopressin neurones resulting in the selective inhibition of OT was first suggested by Clarke *et al.* (1979) . Bicknell and Leng (1982) supported this suggestion using electrically stimulated neural lobes *in vitro* . Summy-Long *et al.* (1984) subsequently reported that the opioid antagonist naltrexone amplified the OT but not the vasopressin response to dehydration and haemorrhage . The site of action of endogenous opioid tone was later proposed to be on the terminals of the magnocellular neurones and/or the adjacent pituicytes (Summy-Long *et al.*, 1986) . Evidence for an action on both the cell bodies and terminals comes from the finding that intracerebroventricular administration of dynorphin attenuates the release of OT but not vasopressin in osmotically stimulated rats (Summy-Long *et al.*, 1987) . A similar result is obtained when dynorphin is added to the perfusion fluid of electrically stimulated neural lobes in vitro (Bondy *et al.*, 1988 ; Falke, 1988) . It has been proposed that at the level of the terminals opioid inhibition may involve an interaction with the pituitary noradrenergic innervation together with some direct activity on OT neurosecretory terminals (Bicknell *et al.*, 1988 ; Zhao *et al.*, 1988b and c) .

Summy-Long (1989) proposed that two physiological consequences for the inhibition of OT release by endogenous opioids in the rat are ; 1, to allow the preferential release of vasopressin when it is of physiological importance, and 2, to conserve OT stores in the pituitary and reduce fatigue

of OT neurons during prolonged periods of dehydration . This may be demonstrated by the proposed role of OT in electrolyte regulation . Balment *et al.* (1986) demonstrated a synergistic renal action of OT and vasopressin in the neurohypophysectomised rat . Water deprivation in the rat produces a vasopressin associated antidiuresis accompanied by a natriuresis which in the rat at least may involve OT release in response to increased osmotic pressure of the blood . If water deprivation is maintained then the resultant fall in blood pressure and volume may selectively further stimulate vasopressin release and its associated dynorphins . Increased vasopressin and dynorphin secretion would enhance the vasopressin stimulated antidiuretic activity of the kidney facilitating the maintenance of blood volume whilst at the same time dynorphin would suppress OT induced natriuresis . Such suppression may be important when the conservation of blood volume becomes the critical factor as natriuresis is ultimately associated with a concomitant fluid loss (see Chapter 4) .

1.1.4 Auto-inhibition or cross-inhibition of OT release by endogenous opioids ?

Structures in, or fibres that pass through the AV3V region send extensive projections to the magnocellular nuclei of the hypothalamus (Carithers *et al.*, 1981 ; Sgro *et al.*, 1984) . Evidence suggests that these circuits are crucially involved in thirst (Johnson and Buggy, 1978), blood pressure control (Hartle and Brody, 1984 ; Haywood *et al.*, 1987), and the maintenance of salt and fluid homeostasis (Johnson, 1985) . Electrolytic destruction of neuronal tissue in the AV3V region results in a persistent hypernatraemia (Johnson, 1985) . This is associated with a severe reduction in osmotically stimulated OT and vasopressin release (Johnson, 1985 ;

Blackburn *et al.*, 1986) . Leng *et al.* (1989) reported that immediately after electrolytic lesion of the AV3V region supraoptic neurons showed little spontaneous activity, and their responses to increased plasma osmolality were severely impaired . However milk ejection during lactation continues to occur and the course of parturition is essentially unaffected by acute AV3V lesion (Russell *et al.*, 1988) . It therefore appears that a tonic excitation of magnocellular neurones by the AV3V region is required for the generation of spontaneous activity and normal osmoregulation . In contrast even in the absence of an excitatory input from the AV3V region magnocellular OT neurons can still be excited by suckling and parturition stimuli which involve projections through other areas .

We have used AV3V lesioned rats to further investigate the contribution of co-localised opioid peptides in the OT neurons to the auto-regulation of OT release . As already mentioned vasopressin neurones are silenced by an AV3V lesion resulting in decreased vasopressin release and presumably a decrease in co-localised dynorphin release . Dynorphins as discussed above may have a cross inhibitory function on the release of OT . By lesioning the AV3V region we therefore attempted to create an OT system free of opioid inhibition as a result of dynorphin release from vasopressin neurones, allowing the selective investigation of OT neurone opioid activity .

Systemic administration of cholecystokinin octapeptide (CCK8) has been shown to produce an abrupt increase in both the firing rate of OT neurones and circulating levels of OT but not vasopressin (Renaud *et al.*, 1987 ; Blackburn and Leng 1990) . It has been suggested that the physiological importance of this response may be important in eliciting feelings of satiety or nausea (Verbalis *et al.*, 1986) . Blackburn and Leng

(1990) reported that ablation of the AV3V region did not significantly impair CCK8 stimulated OT release and suggested that the path by which CCK stimulates OT release might involve vagal projections to the nucleus tractus solitarius (NTS) . Luckman (1992) has provided further evidence for this by staining the NTS and area postrema for nuclear *fos*-like protein following peripheral administration of CCK8 . Results suggested that cells in both the NTS and area postrema were excited but since the two areas intercommunicate it was not possible to determine whether one region activated the other . The NTS however is the principle recipient of sensory inputs conveyed by the vagus, it also projects to the magnocellular nuclei (Sawchenko and Swanson, 1983) and would therefore appear to be the likely route .

Flanagan *et al.* (1988) demonstrated that stimulation of OT by systemic CCK was potentiated by the opioid antagonist naloxone . This potentiation was reported by Leng *et al.* (1992) not to involve an increase in neuronal firing rate, indicating an action at the terminals in the neural lobe . As CCK does not stimulate vasopressin activity they suggested that this result provides further evidence of endogenous opioid regulation of OT secretion . We have attempted to demonstrate using AV3V lesioned rats that this endogenous opioid action is at least in part due to opioid peptide co-released from OT neurones themselves . This involved measuring OT release into the plasma following the administration of CCK both before and after naloxone in lesioned compared to control rats .

1.1.5 Opioid-receptor density is modulated by endogenous opioid activity

The density of the receptors by which opioids mediate their effects on the neurohypophysial system has been shown to respond to the stimulatory state of the animal . The opioid antagonist naloxone given to conscious late pregnant and parturient rats increases oxytocin secretion but in contrast has no effect in non-pregnant rats (Hartman *et al.*, 1986 ; Bicknell *et al.*, 1988 ; Leng and Russell, 1989) . This shows that at these times OT release is under tonic opioid inhibition . Sumner *et al.* (1992) have reported that mu-receptor binding in the SON was reduced on the last day of pregnancy and kappa-selective binding of the posterior pituitary was less on day 16 . This may therefore reflect opioid receptor down-regulation in response to increased endogenous opioid ligand release .

Increased plasma osmolality is a potent stimulus of OT and vasopressin release (see Chapter 4) . Water deprivation or ingestion of 2% saline increases the release of vasopressin and OT from the neural lobe (Jones and Pickering, 1969) and simultaneously depletes the levels of immunoreactive dynorphin (Mata *et al.*, 1977 ; Milan *et al.*, 1984) . Brady and Herkenham (1987) reported that 5 days of such dehydration produced a 35% reduction in kappa-receptor binding in the posterior pituitary of the rat . They suggested that chronic dehydration produces a down-regulation of kappa-opiate receptors in the neural lobe of water deprived and saline treated rats, and that chronically elevated levels of dynorphin are a major part of this mechanism . We have used the kappa-selective assay protocol of Sumner *et al.* (1990) to conduct a preliminary investigation into the time course of this reduction in kappa receptor binding initially after two days of 2% saline inbibition and subsequently also after five days .

1.2 MATERIALS

1.2.1 Kappa-opioid receptor autoradiography

Rats were maintained according to the conditions in the General Methods section except that saline drinking rats had their normal drinking water replaced by 2% (w/v) NaCl . Rats ingested 2% saline for either two or five days at the end of which their pituitaries and those of contemporaneous control tap water drinking rats were harvested, mounted onto cryostat chucks and stored at -70^{0}C until sectioning (see General Methods, GM5.3) .

Quantitative receptor autoradiography was performed on the slide mounted sections using an incubation medium containing a tritiated form of the selective opioid receptor ligand bremazocine (specific activity 18.02 and 29.7 Ci/mmol, NEN Research Products) in the absence and presence of competitive ligands . These ligands were the non-selective opioid antagonist naloxone (naloxone hydrochloride, product No. N-7758, Sigma Chemical Company Ltd), the highly selective mu-opioid receptor agonist Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH (DAGO, lot No. 034/315, Cambridge Research Biochemicals) and the highly selective delta-opioid receptor agonist [D-Pen^2, D-Pen^5]-enkephalin, (DPDPE, product No. E2260, Sigma Chemical Company Ltd) .

Ligands were diluted in an incubation medium consisting of 0.05M phosphate buffer (pH 7.4) containing 0.4M NaCl (BDH Laboratory Supplies) and 0.1% (w/v) bovine serum albumin (BSA, Sigma Chemical Company Ltd) . The recipe for the pre-incubation medium was similar except that 0.015M phosphate buffer (pH 7.4) was used containing 0.15M NaCl and 0.1% (w/v) BSA . Following the incubation procedure slides were apposed to single coated autoradiography film (Hyperfilm-^{3}H, code No. RPN.12, Amersham) for approximately 16 weeks . The films were processed in Kodak D19 developer

diluted according to the manufacturers instructions (800g in 5 litres of water at 38^{0}C) and Ilford Hypam fixer diluted 1 in 5 with tap water .

1.3 METHODS

1.3.1 Receptor autoradiography

The receptor binding method we employed was a modified method of Brady and Herkenham (1987) which has been previously reported by Sumner *et al.* (1990) . Two experiments were performed, an initial study attempted to assess kappa-opioid receptor binding in the posterior pituitary two days after the commencement of 2% saline drinking and a subsequent experiment after five days .

Kappa-opioid receptor density was measured by autoradiography of ^{3}H-bremazocine binding in the presence of displacers . The two day binding study was restricted to three incubation groups :- a, 2nM tritiated bremazocine only ; b, 2nM tritiated bremazocine + 2μM naloxone and c, an incubation medium only control . The subsequent five day experiment involved the use of a fourth incubation group containing :- 2nM tritiated bremazocine + 2μM DAGO and 2μM DPDPE . These protocols enabled us to investigate specific kappa-receptor binding in the posterior pituitary . Naloxone controls were used to test for non-opioid receptor binding by the tissue which could later be corrected for . The use of DAGO and DPDPE ensured that the opioid binding observed was specific for the kappa-receptor subtype . Buffer only controls were used to test for any chemographic effects created by the tissue on the film producing a false positive result, but this was never found to be significantly above background .

The previously prepared and mounted 15μm, coronal frozen sections were removed from the freezer and allowed to warm to room temperature in

their sealed storage boxes . After approximately one hour the slides were transferred to plastic sandwich boxes lined with Benchkote . 20μl of pre-incubating medium was then dispensed onto individual sections forming a discrete puddle which covered the whole surface area of each section being tested . After 30 minutes at 0°C the pre-incubating medium was tapped off and replaced with incubating medium, containing the tritiated ligand with or without inhibitors as appropriate, and further incubated for 3 hours at 0°C . After this incubation period the incubating medium was tapped off and the slides gently immersed in four successive baths of ice-cooled 0.05M potassium phosphate buffer for one minute each . Following this the slides were quickly dipped in ice-cold distilled water to remove any excess salts and then allowed to dry . Once dry the slides were placed in sealed plastic boxes (containing a sachet of silica gel as a desiccant) and stored overnight at 0°C .

The following day the sections were transferred from the refrigerator to the darkroom and allowed to warm to room temperature . After approximately one hour the slides were removed from the storage boxes and placed face down in a predetermined sequence onto autoradiography film under safelight conditions . In addition to the sections each film was loaded with two sets of tritium standards calibrated from 21.1 - 0.06 nCi/mg rat brain tissue equivalent (5μm sections mounted onto microscope slides, ^{3}H-microscales, Amersham). All slides were affixed to the film with autoclave tape to prevent shifting during exposure . The slides and film were sandwiched between two mirror tiles (30 x 30 cm mirror coated glass wall tiles, Texas Homecare Ltd) with their silvered surfaces facing outwards . The tiles were taped together and then wrapped in a sheet of brown packing paper followed by a sheet of aluminium foil and finally sealed in a black

plastic bag . These wrappings were used to prevent light, damp and stray radiation affecting the film during the exposure period . The films were exposed in a cold room at 4^{0}C for approximately 16 weeks . After which the exposed film was allowed to return to room temperature before it was unwrapped under safelight conditions . The slides were then removed and the film developed for 5 minutes in Kodak D19 developer at room temperature (approximately 20^{0}C) . After developing, the film was quickly rinsed in tap water before fixation in two successive baths of Ilford Hypam fixer for five minutes each . After fixation the film was washed in running tap water for 30 minutes and then hung up to dry .

The dry film was stored in a plastic bag until the images were analysed and the sections processed as follows . First the sections were fixed for 10 minutes in a mixture of acetic acid, absolute ethanol and commercial formalin (1:17:2 by volume) before being rehydrated in successive baths of 90% then 70% followed by 50% alcohol and then finally allowed to stand in running tap water for a few minutes . The rehydrated sections were stained for 15 minutes in 1% aqueous Cresyl Fast Violet before being dehydrated in 50% then 70% followed by 90% and finally absolute alcohol . The dehydrated, stained sections were finally immersed in xylene, mounted in DPX and covered with a glass slip .

1.3.2 Image analysis

The portions of film exposed to the sections were cut out and attached to microscope slides with tape . The boundaries of the posterior pituitaries were marked on the film with a fine felt tip pen under a dissecting microscope using the appropriate stained sections as reference guides . Silver grain densities were measured using Joyce-Loebl μMagiscan image

analyzer software receiving a video input from a black and white video camera (Philips Video 40) mounted with a Newvicon tube on a Vickers M17 microscope . The microscope magnification was set at X 10 objective and X 1.6 intermediate lens . The video:monitor scale factor was 1.1878 μm per pixel and counting was restricted to within a rectangular frame of dimensions 201.63 x 133.04 μm . This frame size enabled a number of non overlapping measurements to be taken from each neural lobe section . Silver grain density was calculated using the following equation :-

$$\frac{\text{total area of silver deposited}}{\text{total area of counting frame .}}$$

Grain densities were corrected by the subtraction of background counts measured over nearby film that was not exposed to tissue . For each neural lobe three measurements were taken from film exposed to tissue and two measurements of background were made . The two means from each type of measurement were then calculated after which the background mean was subtracted from the tissue mean to give the corrected measurement . This measurement was further corrected for non-opioid receptor binding by the subtraction of the similarly measured naloxone control values . A statistical comparison of mean grain densities between groups was then carried out using a two sample t test .

Films were also exposed to radioactive standards calibrated in terms of brain grey matter tissue equivalent (Sumner *et al.*, 1990) . Grain density was measured in triplicate over each step of the standard curve and the background density subtracted as before . This enabled the construction of a straight line calibration graph of silver grain density vs. log radioactivity . The equation for the line of best fit was calculated by linear regression which enabled us to transform the experimental data (grain density) into tissue radioactivity values . Taking into consideration the specific radioactivity of

the probe used we were then able to calculate fmol of bremazocine bound/mg of posterior pituitary tissue .

1.3.3 Technical considerations

Hyperfilm-^{3}H is designed to detect beta-radiation primarily from tritium sources but can also suffer fogging from natural background radiation and light . In view of this, precautions were designed to limit background effects by wrapping exposing films in paper, aluminium foil and polythene and the use of darkroom facilities and safelight conditions when handling unprocessed films . Despite these precautions all films when developed showed background fogging although this background effect was not measurably different between films .

We have expressed the results in terms of ligand bound per mg of brain tissue in an attempt to compare results generated from different experiments in this laboratory with each other and with those previously reported by other workers . In order to do this films were also exposed to sets of standards covering a range of known activity . This enabled the production of a standard curve from which the unknown experimental grain densities could be transformed into a bound radioactivity form . However this type of analysis not only introduces the possibility of an error in the mathematical procedure but may also fail to detect a non-linear response of the film to radiation fogging at the extremes not covered by the standards .

Interpretation of radioligand binding studies rests on the following assumptions :- 1, all receptors are accessible to all ligands ; 2, the binding properties of the receptors do not change ; 3, the number of receptors remains constant ; 4, the ligand structure does not change ; 5, the concentration of receptor binding modulators do not change and 6, the

reaction reaches equilibrium (Motulsky *et al.*, 1985) . It is possible that incubation of receptors with agonist and or antagonist ligands may lead to a decrease in receptor density by a number of factors including down-regulation . It is conceded that these factors may affect the total binding of the tissue during the incubation period . However with the low temperature and short duration of pre-incubation and incubation used one would assume only minimal down-regulation could occur .

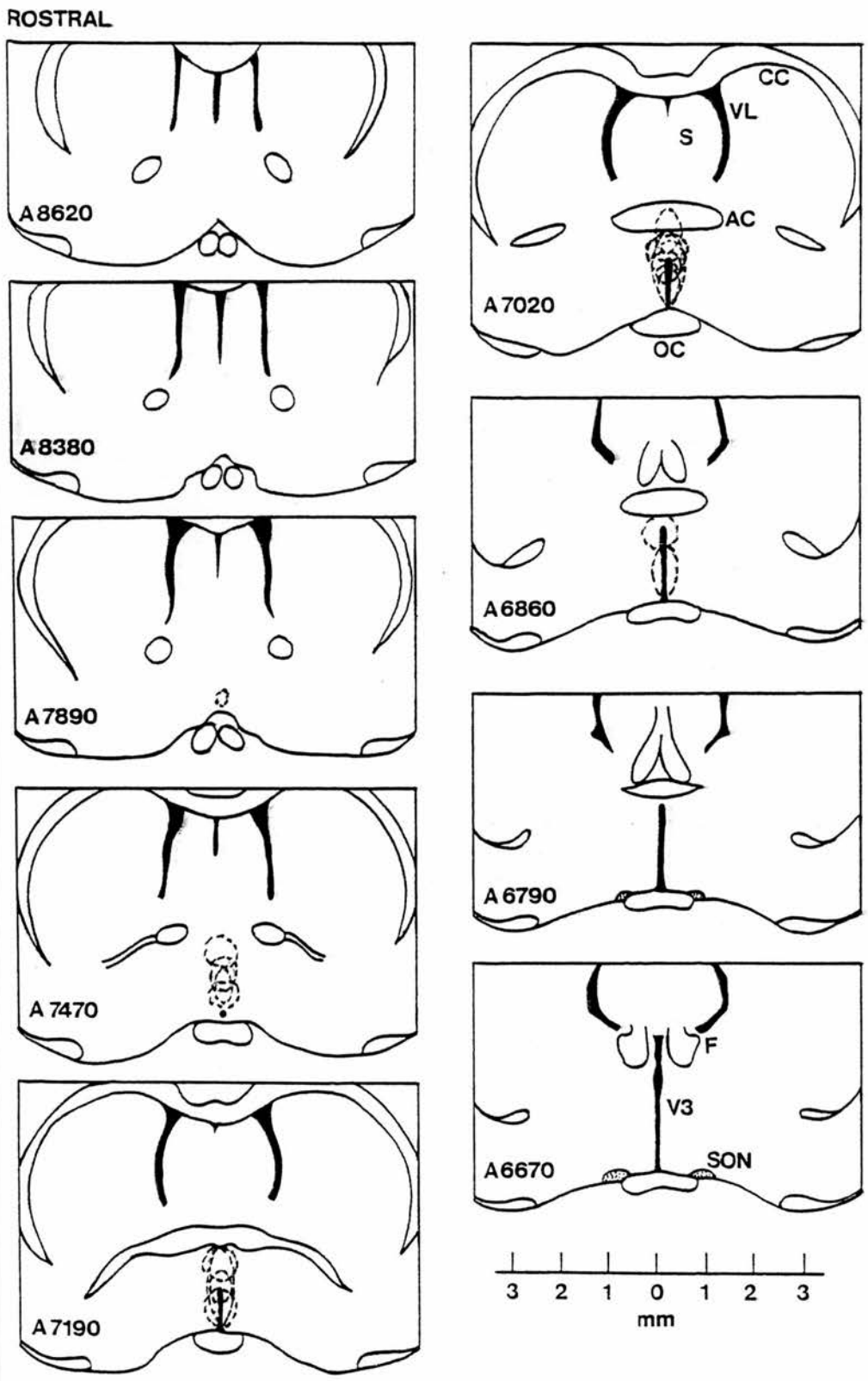
1.3.4 AV3V lesion - CCK experiment

A second experiment was designed and executed to investigate whether endogenous opioids co-localised with oxytocin produce an auto-inhibitory effect on oxytocin secretion . Adult female Sprague-Dawley rats housed in standard laboratory conditions were anaesthetised with urethane and a femoral artery and vein cannulated (see General Methods, GM2 and GM3) . Rats were then placed in a stereotaxic frame (Stoelting Co., Chicago, IL 60623, U.S.A.) and a skin incision made to expose the dorsal surface of the skull . The skull was then levelled between bregma and lambda after which a nichrome monopolar lesioning electrode was lowered through a burr hole in the skull into the AV3V region (0.3mm posterior to bregma, 8.3mm below the skull surface in the midline) . The electrode was connected to the anode of a lesion producing device (cat. No. 58041V, Stoelting Co.) and the circuit completed with a cathode ear clip . An electrolytic lesion was produced by passing 3mA of direct current for 15 seconds after which the rats were left for 2 hours to recover . A control series of rats were subjected to an identical preparation procedure except the lesioning electrode was

lowered to a position outside the AV3V region (0.3mm posterior to bregma, 5.3mm below the skull surface in the midline) and no current passed .

The experimental protocol involved blood sampling for plasma oxytocin radioimmunoassay (see General Methods, GM1 and GM4) before and after the i.v. administration of CCK8S (20μg/kg, Bachem UK LTD, Saffron Waldon, Essex CB10 1AA), repeated after i.v. naloxone (5mg/kg in 0.5ml/kg, naloxone hydrochloride, Sigma Chemical Company LTD) . At the end of the experiment rats were sacrificed and their brains removed and fixed for at least 2 days in 2% formal saline . The fixed brains were coronally sectioned at 50μm on a freezing microtome, mounted and then stained as for brains from AV3V stimulated rats (see General Methods, GM5.1) . The processed sections were then examined and the extent of the AV3V lesion or electrode tract mapped onto serial brain section drawings illustrated in Fig 1.A . A typical lesion damaged the periventricular tissue between the anterior commissure and the optic chiasm but did not include the supraoptic and paraventricular nuclei .

Plasma vasopressin was measured by radioimmunoassay at the I.A.P.G.R. Cambridge Research Station by Chris Chapman . Blood sampling for vasopressin concentration measurements was similar to that for OT except 1ml blood samples were removed, the plasma aliquoted after centrifugation and acidified with acetic acid prior to storage in the freezer .



Locations of electrolytic lesions of the AV3V region mapped on to a series of drawings after the atlas of Konig and Klippel (1963) . The broken lines represent the extent of destroyed tissue in all rats (n = 7) and included the OVLT together with most of the median preoptic nucleus . CC corpus callosum, F fornix, OC optic chiasma, S septum, SON supraoptic nucleus, VL lateral ventricle and V3 third ventricle .

FIGURE 1.A Extent of hypothalamic tissue destroyed by the electrolytic lesioning of the AV3V region .

1.4 RESULTS

1.4.1 Plasma oxytocin concentration in response to i.v. CCK and naloxone in AV3V lesioned rats (Fig 1.1) .

Basal plasma OT concentration was non-significantly greater in the intact control animals compared to the AV3V lesioned group ($P > 0.05$ two sample t test) . Five minutes after the initial CCK8 injection there was a significant increase of 41 ± 19 pg/ml in the plasma OT concentration of lesioned rats ($P = 0.02$ paired t test) and a significantly greater increase in control compared to lesioned rats ($P < 0.02$ two sample t test) of 79.1 ± 14.6 pg/ml ($P < 0.002$ paired t test Vs basal) . 50 minutes after the initial CCK8 injection plasma OT concentration had returned to basal in both the lesioned and control group .

Naloxone (5mg/kg) at 65min significantly increased the plasma OT concentration of lesioned rats by 82.1 ± 13.2 pg/ml ($P < 0.002$ paired t test Vs zero) and by 227.1 ± 75.5 pg/ml in control rats ($P < 0.03$ paired t test Vs zero) . The increase after naloxone was not significantly greater in the control rats compared to lesioned rats . CCK8 at 70min produced a further significant increase in the plasma OT concentration compared to the previous sample in both the lesion and control group ($P < 0.02$ paired t test) . The increase in plasma OT concentration with CCK8 after naloxone (sample 6-5) was not significantly different to that attained before naloxone in the control group ($P= 0.09$ paired t test) and in the lesioned animal group ($P = 0.3$ paired t test) .

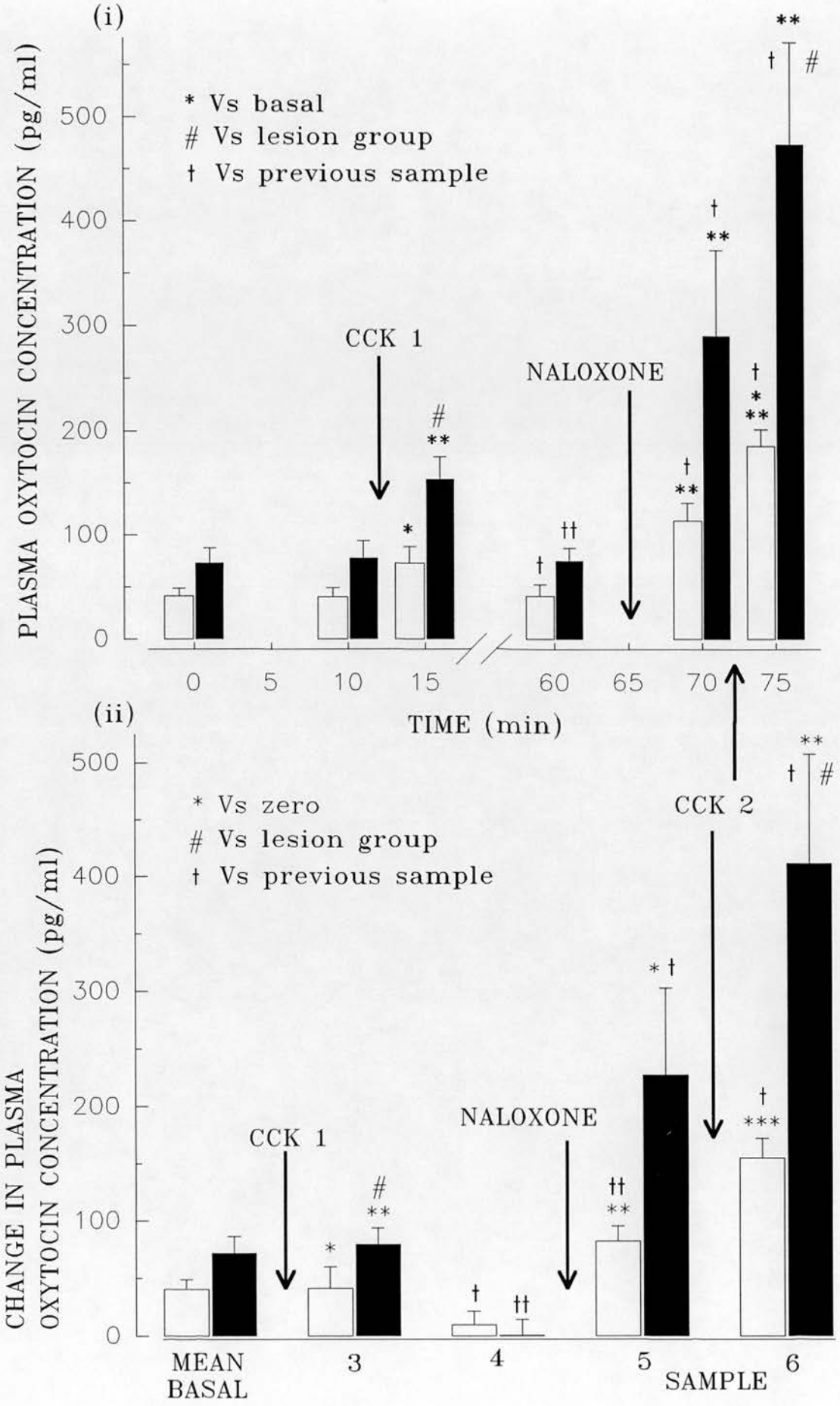


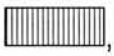
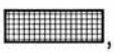
FIGURE 1.1 Plasma oxytocin concentration in response to i.v. CCK and naloxone in AV3V lesioned rats

1.4.2 Opioid ligand binding of the posterior pituitary in rats after two and five days of 2% saline drinking (Fig 1.2) .

After 2 days of saline ingestion there was a non significant 15% decrease in bremazocine only opioid receptor binding ($P > 0.05$ two sample t test) from 9.44 ± 3.72 to 7 ± 3.6 fmol/mg by the posterior pituitary .

5 days of 2% saline ingestion however produced a significant 76% decrease in kappa-receptor binding by the posterior pituitary compared to control rats from 25.4 ± 5.03 to 6.4 ± 6.4 fmol/mg in ^{3}H-bremazocine + DAGO + DPDPE labelled rats ($P = 0.002$ two sample t test) and from 23.6 ± 4.7 to 5.36 ± 4.7 fmol/mg in ^{3}H-bremazocine only labelled pituitaries ($P < 0.0001$ two sample t test) .

LEGEND : FIGURE 1.2

Kappa-opioid receptor binding was measured by receptor autoradiography followed by image analysis on posterior pituitary sections from rats after 2 and 5 days of 2% saline ingestion . Values are mean ± s.e.m. ;  , are 2 day ^{3}H bremazocine labelled posterior-pituitaries from control rats (n=7) ;  , are 2 day saline loaded rats (n=8) ;  , are ^{3}H bremazocine + DAGO + DPDPE labelled posterior-pituitaries from 5 day control rats ;  , are 5 day saline loaded rats (n=4) ;  , are ^{3}H bremazocine labelled posterior-pituitaries from 5 day control rats (n=4) and  , are from 5 day saline loaded rats (n=4) .

** = $P \leq 0.005$; *** = $P \leq 0.0005$

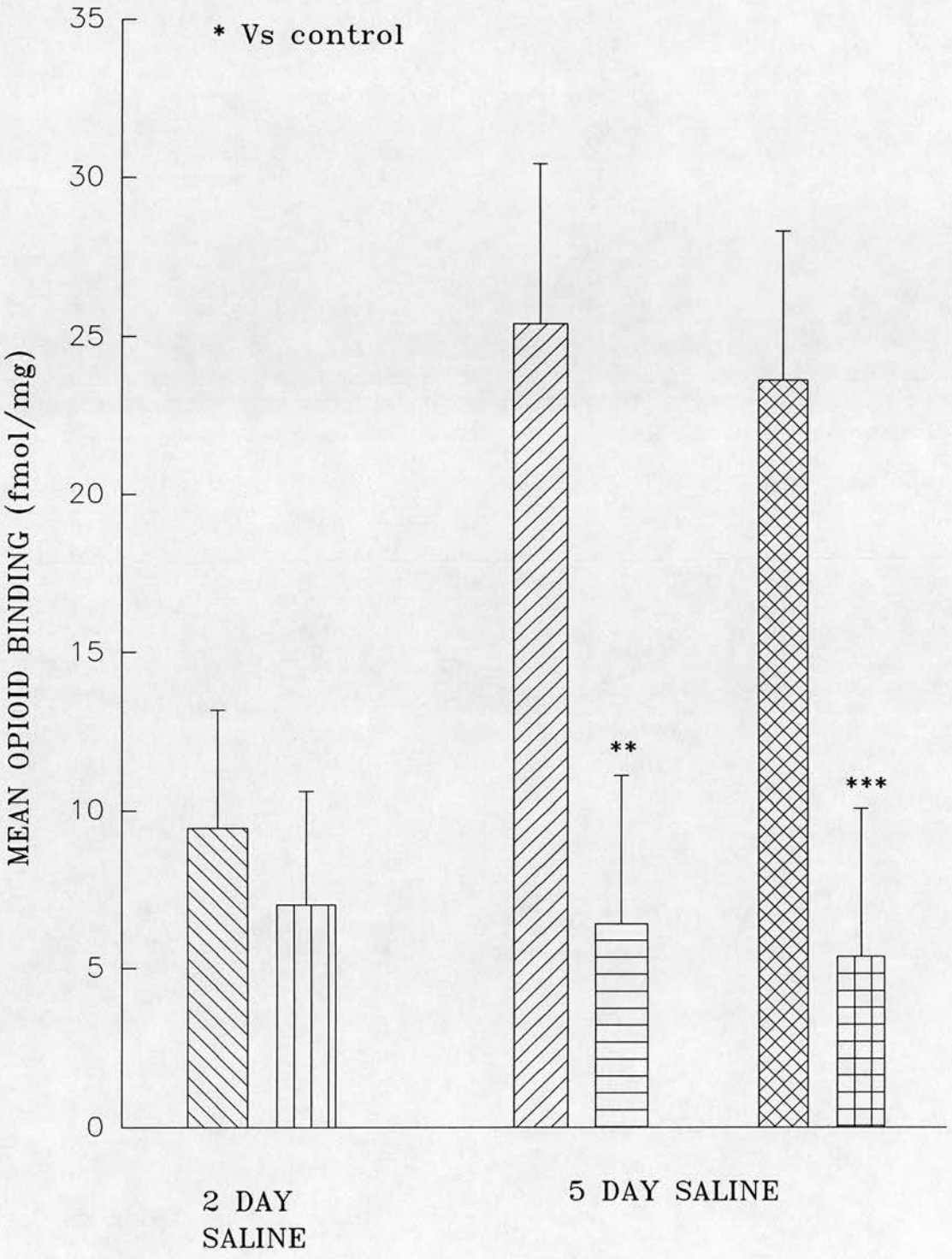


FIGURE 1.2 Opioid ligand binding of the posterior pituitary in rats after two and five days of 2% saline drinking

1.5 DISCUSSION

1.5.1 Effects of co-released opioids from oxytocin neurones

In agreement with other workers we have found that an acute AV3V lesion in the rat tends to decrease basal plasma OT concentration (Blackburn *et al.*, 1987 ; Leng *et al.*, 1989) . This may be accounted for by the loss of tonic excitatory tone on the magnocellular neurones arising from nuclei in and axons passing through this region resulting in a reduction in the spontaneous activity of supraoptic neurones (Leng *et al.*, 1989) . This loss of excitatory tone also severely attenuates the osmotic response of OT neurones (Russell *et al.*, 1988 ; Leng *et al.*, 1989) which would include a reduced responsiveness of these neurones to the hyperosmotic effect of urethane under basal conditions (Hartman *et al.*, 1987) .

Blackburn and Leng (1990) have previously reported that an acute AV3V lesion does not abolish the release of OT in response to systemic CCK . They did however demonstrate a reduction in response that we found to be significant in our rats after the initial administration of CCK8 ($P < 0.02$ two sample t test) . The reduced spontaneous activity of OT neurones following an AV3V lesion may reflect the loss of tonic excitatory input from this region which would render them less responsive to activation by other excitatory inputs - including that from the brainstem stimulated by systemic CCK (see Introduction of this chapter) .

Following administration of the opioid antagonist naloxone we obtained a significant increase in the plasma OT concentration of lesioned and intact rats ($P < 0.002$ and $P = 0.03$ respectively, paired t test) . Assuming that the vasopressin system is silenced at this time, as indicated by previous electrophysiological studies (Leng *et al.*, 1989), then this may provide evidence of an endogenous opioid tone on OT secretion from

another source possibly the OT neurones themselves . Naloxone administered to control rats produced a greater increase in the plasma OT concentration than it did in lesioned rats . This may reflect not only the increased activity of the OT opioid system but also an increased activity of the vasopressin opioid system . Following an identical administration of CCK8 to that before naloxone we again obtained a significant increase in the plasma OT concentration of lesioned and control rats versus the previous sample ($P < 0.02$ paired t test) . The increase in plasma OT concentration following CCK8 administration was approximately twice as large in the lesion and control groups compared to that produced before naloxone . These results were not significant in the control group ($P = 0.09$ paired t test) nor in the lesion group due to the relatively large standard errors involved with the calculations . However, the increased responsiveness in the lesioned rats of the OT system to CCK after naloxone compared to before is suggestive of an auto-inhibitory effect of opioids co-localised with OT .

Plasma vasopressin was also measured at the end of the experiment before and after an i.p. hyperosmotic stimulus (i.p. 1.5M NaCl, 4ml/kg) to test the responsiveness of the system . Plasma vasopressin was found to be non-significantly different between lesioned and control groups at 59 ± 20.7 and 56.5 ± 10.4 pg/ml respectively . This is a similar finding to that of Leng *et al.* (1989) who also reported similar plasma vasopressin levels in lesioned and intact rats . Our basal vasopressin values are greater than those reported by these workers and may reflect activation of the vasopressin system due to volume depletion as a result of the blood sampling protocol .

The increments in vasopressin release in response to hyperosmotic stimulation were similar in lesioned (19.2 ± 9.6 pg/ml) and control (16.8 ±

16.6) rats . This once again may reflect the conditions in which the experiment was conducted . Leng *et al.* (1989) demonstrated that two i.p. injections of 1.5M NaCl administered an hour apart produced a similar vasopressin response in lesioned and intact rats . The similar vasopressin responses obtained in this experiment may reflect the synergistic interaction of excitatory inputs on to the vasopressin system due to volume depletion, with the direct osmosensitivity of the neurons . The combination of these two stimuli being sufficient to produce near normal activity in the silent vasopressin neurones similar to that obtained by Leng *et al.* (1989) when they repeated their hyperosmotic insult .

The purpose of electrolytically lesioning the rats was an attempt to silence the vasopressin system which we have apparently failed to accomplish based on the assay results . Vasopressin release following hyperosmotic stimulation was however not significantly increased in the lesioned group . In contrast to numerous other workers (see General Introduction) we failed to demonstrate a significant vasopressin response to hyperosmotic stimulation in the intact group . It appears therefore that the results of the vasopressin assay should be interpreted with some caution .

As already discussed opioids can exert a central effect on OT neurone activity and a peripheral effect on OT release by an action on the terminals in the posterior pituitary . The first evidence that opioid peptides are present in the neural lobe was reported Rossier *et al.* (1977) . Extraction of dynorphin opioids was subsequently reported by Goldstein and Ghazarossian (1980) and dynorphin immunoreactive fibres were shown to be present in the neurohypophysis (Watson *et al.*, 1981) . Although the concentrations of enkephalins and dynorphins reported in the posterior pituitary are amongst the highest in the brain they are still 300-1000 fold less

abundant than OT and vasopressin (Bicknell and Zhao, 1989) . This implies that the opioids serve in an autocoid capacity rather than performing a systemic function .

Immunocytochemical studies report that in the rat at least the dynorphin peptides are localised in vasopressin neurones (Watson *et al.*, 1982 ; Bicknell and Zhao, 1989) . In addition [met]enkephalin peptides have been reported to be co-localised with OT in their secretory terminals and granules (Martin *et al.*, 1983 ; Adachi *et al.*, 1985) . However chronic osmotic stimulation which depletes neurohypophysial OT has been reported to have no effect on [met]enkephalin content (Morris and Livingston, 1983 ; Zamir *et al.*, 1985 ; Nordmann *et al.*, 1986) . In contrast the content of neural lobe dynorphin derived peptides declines (Hollt *et al.*, 1981) suggesting that dynorphin but not [met]enkephalin peptides are important in the modulation of neurohypophysial hormone release .

The lack of a decline in [met]enkephalin peptides may be explained by the report by Schriefer (1991) that magnocellular neurones can differentially regulate co-localised peptides . Thus a depletion of OT but not [met]enkephalin may not simply represent a difference in release but a difference in the ratio of release to synthesis for each peptide . In addition a recent communication by Bicknell *et al.* (1992) has reported that a proportion of dynorphin$_{(1\text{-}17)}$ (a highly kappa-selective peptide) is also co-secreted with oxytocin . This communication also described evidence of increased [met]enkephalin release from the rat neural lobe following electrical stimulation of the neural stalk for 90 minutes . In the light of these findings an autoinhibitory function of opioid peptides co-localised and co-released with OT appears more feasible and we have attempted to provide further evidence for this in the present experiment .

1.5.2 Site and mode of action of opioid peptides

In the model presented by Bicknell and Zhao (1989) a direct action of opioid peptides on kappa-receptors located directly on the terminals of OT neurones is proposed . Electrophysiological studies by North (1986) have suggested that kappa-receptors may be coupled to calcium channels and reduce Ca^{2+} entry across membranes . Stimulus-release coupling involves the entry of Ca^{2+} ions through voltage-sensitive channels during the depolarisation of the presynaptic membrane . Additionaly an opioid inhibition of noradrenaline release from noradrenergic innervation terminating in the posterior pituitary has also been reported . Noradrenaline itself has a facilitatory effect on OT release via activation of beta-adrenergic receptors which appear to be located on the pituicytes (Zhao *et al.*, 1988c) . Kappa-receptor agonists may therefore mediate their effects by reducing the influx of calcium ions during depolarisation of the neurone terminals and/or a reduction in facilitatory noradrenergic activity .

As discussed in the General Introduction, pituicytes also appear to possess opioid binding sites . These may be important in modulating the inhibitory effects of opioids on hormone release by binding co-secreted opioids and thereby preventing their action at opioid receptors on the neurone terminals . Alternatively activation of pituicyte opioid receptors may induce morphological changes similar to those reported during dehydration and parturition (see General Introduction) . Pituicyte processes normally ensheath neurosecretory terminals forming a barrier between terminal and perivascular space . During periods of intense neurohypophysial secretion however these processes are remarkably reduced exposing a greater surface area for potential hormone release . It is possible that these

morphological changes of the pituicyte result from chronic exposure to opioids co-released with OT and vasopressin in response to dehydration and during pregnancy and lactation .

Several sites and modes of action of opioids are therefore implicated in the acute and chronic control of neurohypophysial hormone release involving both the terminals and the pituicytes within the neural lobe .

1.5.3 Opioid-receptor density is dynamic

We have confirmed the findings of Brady and Herkenham (1987) in that 5 days of 2% saline ingestion produces a significant decrease of opiate-receptor binding in the posterior pituitary of the rat . We have additionally demonstrated that this involves a dramatic decrease in kappa-receptor binding since we included the use of delta- and mu-opioid displacers in the experimental protocol . Total kappa-receptor binding in our control assays was measured at 9.44 ± 3.72, 23.6 ± 4.7 and 25.4 ± 5.03 fmol/mg . The difference between the results may be due to the use of different stocks of ^{3}H-bremazocine and the fact that the two day experiment was performed approximately 18 months before the subsequent five day experiment . Nevertheless these results are in agreement with other studies which have reported total kappa-receptor binding in the neural lobe at between 11.6 ± 6.3 and 74.4 ± 16 .2 fmol/mg (Sumner *et al.*, 1990 and 1992) . However our results differ from those of Brady and Herkenham (1987) in the magnitude of the reduction after 5 days of saline drinking as they reported a 35% reduction and we have obtained a 76% reduction .

These results may represent a change in the affinity of the receptors due to masking by prebound endogenous ligand . This appears unlikely however as the high sodium concentrations used in the preincubation and

incubation mediums favours the dissociation of endogenous opioid peptides such as dynorphin (Simantov *et al.*, 1976 ; Garzon *et al.*, 1984) . Assuming therefore that this reduction in ^{3}H-bremazocine binding by the posterior pituitary is a real phenomenon then it may represent kappa-opioid receptor down-regulation as a result of prolonged opioid release in response to increased plasma osmolality, but apparently not before two days of such stimulation .

1.5.4 Mechanisms of receptor down-regulation

Down-regulation of opiate receptors in neural tissue has been reported in neuroblastoma cell lines and in aggregating foetal brain cells after exposure to enkephalin (Blanchard *et al.*, 1983 ; Lenoir *et al.*, 1984) . Down-regulation of delta-opioid receptors has also been reported in neuroblastoma hybrid cells after chronic opiate exposure accompanied by an increase in the lysosomal content of the ligand-receptor complex (Law *et al.*, 1984) .

Receptors can undergo internalisation by endocytosis into "coated pits" and are subsequently either broken down or preserved (Dickson, 1985) . It appears that the formation of a receptor-ligand complex stimulates endocytosis of the receptor although internalisation of unbound receptors also occurs . Cells can internalise the equivalent of 150% of the total cell surface area by endocytosis per hour (Raffa, 1985) and once internalised the coated pit contents can be metabolised, modified or reinserted into the membrane unchanged . Receptor density can also be regulated by phosphorylation of receptor proteins following ligand binding . Various receptors are phosphorylated on their serine, threonine and tyrosine residues after ligand binding . For example, insulin binding at its receptor

induces rapid phosphorylation of tyrosine residues initiating a cascade of further phosphorylations resulting in a reduced tyrosine kinase activity (Hanover and Dickson, 1985) .

1.6 CONCLUSIONS

We have attempted to provide further evidence of an autoinhibitory function for opioid peptides co-localised with OT . Our results are in agreement with this hypothesis but the evidence we have provided is not very strong .

We have also measured kappa-receptor density in the posterior pituitary by receptor autoradiography followed by image analysis . After 2 days of 2% saline ingestion we have found a non-significant 15% decrease in ^{3}H-bremazocine binding . After 5 days of 2% saline drinking we have found a further reduction in kappa-receptor binding to a significant 24% of control values measured at 25.4 ± 5.04 and 23.6 ± 4.7 fmol/mg ($P = 0.002$ and $P < 0.0001$ respectively, two sample t test) . Total receptor binding is in the same range as that reported by other workers but the reduction in receptor binding is approximately double that reported by Brady and Herkenham (1987) also after 5 days of 2% saline drinking in rats .

A decrease in SON mu-opioid receptor binding has been reported in the late pregnant rat at a time when endogenous opioid activity and OT secretion are increased (Sumner *et al.*, 1992) . It would therefore appear that chronic stimulation of neurohypophysial hormone and opioid release, via salt loading, produces a down-regulation of kappa-opioid receptors in the posterior pituitary which may facilitate the release of OT . OT has been shown to act synergistically with vasopressin in the promotion of sodium excretion (Balment *et al.*, 1986b) . A facilitated OT release during chronic saline ingestion would therefore be advantageous at increasing renal excretion of the excess plasma salt load .

CHAPTER 2

Electrical AV3V stimulation of oxytocin secretion : effects of pregnancy, opioids and MK801

2.1 INTRODUCTION

2.1.1 Role of the AV3V region

As mentioned in the previous chapter magnocellular OT neurones receive a major, predominantly excitatory, input from the region anterior and ventral to the third ventricle (AV3V region) (Blackburn *et al.*, 1987 ; Russell *et al.*, 1988) . Contained within this region is the organum vasculosum of the laminae terminalis which sends projections both directly and indirectly, via the median preoptic nucleus, to the supraoptic nuclei (Wilkin *et al.*, 1989) . In a similar manner projections from the subfornical organ (SFO) also pass through the AV3V region either synapsing in the median preoptic nucleus or advancing directly to the supraoptic nuclei (Renaud *et al.*, 1983 ; Sgro *et al.*, 1984) . Innervation to the SON from the AV3V region is crucial for the normal osmoresponsiveness of these neurones and in the maintenance of normal fluid homeostasis (Ramsay *et al.*, 1983 ; Russell *et al.*, 1988 ; Chaudhry *et al.*, 1989) . Similarly the input from the SFO has been shown to be involved in fluid homeostasis, in addition to the stimulation of OT release in response to angiotensin II and relaxin (Hosutt *et al.*, 1981 ; Miselis, 1982 ; Johnson, 1985 ; Ferguson and Kasting, 1988 ; Russell *et al.*, 1990 ; Way and Leng, 1991) .

An acute electrolytic lesion of tissue in the AV3V region silences the spontaneous continuous firing activity of OT neurones and the increase in firing rate of these neurones following hyperosmotic stimulation (Leng *et al.*, 1989) . Supraoptic neurones have been reported to be directly osmosensitive as demonstrated by their depolarisation on increased osmolality *in vitro* (Bourque, 1989) . In the presence of excitatory post synaptic potentials, this osmotically induced depolarisation is sufficient to significantly raise the firing rate of supraoptic neurones . However if all synaptic inputs are blocked the osmotically induced membrane

depolarisation is insufficient to generate an increase in action potential production (Leng *et al.*, 1988) .

In vivo, electrolytic ablation of tissue in the AV3V region results in adipsia and persistent hypernatraemia in the dog (Buggy and Johnson, 1977) . A reduced OT response to intraperitoneal administration of hyperosmotic saline has also been reported in AV3V lesioned rats (Blackburn *et al.*, 1987), in addition to a similar hypernatraemic state as reported in the dog (Johnson, 1985) . A synergistic effect of OT with vasopressin has been reported in the promotion of sodium excretion (Balment *et al.*, 1986b ; see also Chapter 4 of this thesis) . Thus the apparently reduced responsiveness of the OT neurones to hyperosmotic stimulation in AV3V lesioned rats may result in the loss of natriuretic ability due to an attenuated plasma OT concentration resulting in the persistent hypernatraemic condition reported .

At the ultrastructural level, destruction of AV3V region tissue in the rat results in the degeneration of fibres and terminals in the supraoptic nucleus and accumulation of neurosecretory vesicles in the posterior pituitary (Carithers *et al.*, 1981) . In addition, hyperosmotically stimulated lesioned rats also show a reduced uptake of ^{35}S-cysteine, a measure of biosynthetic activity , compared to sham operated controls (Gruber *et al.*, 1986) .

Elements within or projecting through the AV3V region therefore appear to provide a tonic excitatory drive to the OT neurones necessary for the expression of both spontaneous activity and normal osmoresponsiveness .

2.1.2 The effect of electrical stimulation in the AV3V region .

Electrical stimulation of the AV3V region influences the activity of most supraoptic neurones in a complex manner with the predominant response involving a short-latency, short-duration inhibition followed by long-latency, long-duration excitation resulting in an increase of plasma OT concentration in the rat (Leng *et al.*, 1989) . This increase in plasma OT concentration is dependent on both the frequency and intensity of the electrical stimulation with the greatest release at higher frequencies and currents (Blackburn *et al.*, 1988) . The inhibitory neurotransmitter GABA has been proposed to be involved in the pathway regulating these responses of the OT neurones to electrical stimulation (Randle and Renaud, 1987) . Excitatory neurotransmitters thought to be involved include excitatory amino acids (EAAs), acetyl choline and angiotensin II (Akaishi and Negoro, 1983 ; Gribkoff and Dudeck, 1988 ; Gribkoff *et al.*, 1988 ; Jhamandas *et al.*, 1989) . There is strong evidence that circulating angiotensin II concentrations are an important influence on neuronal activity in the SFO (Tanaka *et al.*, 1985 ; Ferguson and Renaud, 1986 ; Ferguson 1988) . However intracerebroventricular (i.c.v.) administration of the angiotensin II antagonist saralasin has been reported to have no effect on electrically stimulated OT release with the electrode placed either in the AV3V region or the SFO (Leng *et al.*, 1989 ; Russell *et al.*, 1990) . It would appear therefore that the action of angiotensin II is mainly at the level of the SFO in the stimulation of OT release .

Due to the availability of a variety of agonists and antagonist analogues it now appears that neurones contain at least two classes of excitatory amino acid receptors based on their affinity for the glutamate analog N-methyl D-aspartate (NMDA, Mayer and Westbrook, 1987) . Non-

NMDA receptors include the quisqualate and kainate receptor types . Activation of non-NMDA receptors results in a voltage insensitive, fast depolarising inward current of Na^+ and K^+ ions . Activation of NMDA receptors results in an inward depolarising current primarily carried by Na^+ and K^+ ions in addition to an increase in intracellular Ca^+ . However NMDA receptor activity is dependent upon membrane voltage and extracellular Mg^{2+} concentration . Such that, at resting membrane potentials and normal concentrations of extracellular Mg^{2+} most NMDA receptor ion channels are blocked (Fagg *et al.*, 1986 ; MacDermott and Dale, 1987) . The SON and PVN have some of the highest concentrations of glutamate in the hypothalamus (Palkovitis *et al.*, 1986), and bath or iontophoretic application of EAAs to magnocellular neurones produces a brisk increase in activity (Moss *et al.*, 1971 and 1972 ; Haller and Wakerley, 1980 ; Arnauld, *et al.*, 1983) . *In vitro* voltage clamp experiments have revealed that SON cells express functional post-synaptic EAA receptors for both NMDA and non-NMDA subtypes (Hu and Bourque, 1990) . Gribkoff and Dudek (1990) reported that EAAs mediate the fast excitatory synaptic responses of SON neurones to stimulation of cells and axons in the region dorsolateral to the SON . They also reported the blockade of all spontaneous excitatory post synaptic potentials by broad spectrum EAA antagonists indicating that EAAs are responsible for the majority of fast excitatory input involving kainate and/or quisqualate-type receptors .

We have investigated the effect of the NMDA receptor antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a.d] cyclo-hepten-5,-10-immine maleate (MK801) on electrical AV3V and hyperosmotic saline stimulated OT release in virgin female rats . MK801 has been reported to antagonise NMDA receptor activity by binding to a high affinity site on the receptor ion channel

complex distinct from the recognition site (Wong *et al.*, 1988) . We used an i.v. dose of 1mg/kg MK801 which has been reported to decrease the firing rate of spinal nerves in response to noxious stimulation in the hind paw for up to 50 minutes (Harley *et al.*, 1990) .

2.1.3 Opioids, pregnancy and AV3V stimulation .

The activity of OT neurones can be powerfully inhibited by opioid peptides . Given during parturition opioids attenuate OT secretion, which delays the delivery of pups and during lactation they block the milk ejection reflex (Clarke and Wright, 1984 ; Russell *et al.*, 1989 and 1991 ; Douglas *et al.*, 1990) . Opioids mediate their effects on OT neurone activity at various sites including sensory inputs in the spinal cord, subfornical organ and AV3V region, the cell bodies in the supraoptic nucleus and the OT neurone terminals in the posterior pituitary (Buranarugsa and Hubbard, 1979 ; Wright, 1985 ; Bicknell and Zhao, 1989 ; Leng and Russell, 1989 ; Shariff and Hughes, 1989 ; Leng *et al.*, 1990) . Intravenous administration of the mu-opioid agonist morphine and the kappa-opioid agonist U50,488 inhibits the firing rate of OT neurones (Leng *et al.*, 1990 ; Pumford *et al.*, 1991) . *In vivo* and *in vitro* electrophysiological studies have also revealed an inhibitory action of both morphine and U50,488 on the firing rate of OT neurones (Wakerley *et al.*, 1983 ; Leng *et al.*, 1990) . An effect of opioids directly on the OT neurones is implicated as they are still effective in conditions where synaptic transmission is blocked (Russell *et al.*, 1989 ; Inenega *et al.*, 1990)

Autoradiographic analysis of radioligand binding studies has revealed that the supraoptic nucleus contains both mu- and kappa- opioid receptors with a predominance of kappa receptors (Mansour *et al.*, 1988 ; Sumner *et al.*, 1990) . In the posterior pituitary there is a small population of mu-

receptors but most of the opioid-receptor binding is of the kappa-receptor type (Mansour *et al.*, 1988 ; Sumner *et al.*, 1990) .

As discussed in Chapter 1 and 4 the cell bodies of OT neurones in the supraoptic nucleus probably receive opioid innervation from the arcuate nucleus and dorsal medulla (Sawchenko *et al.*, 1982 and 1990 ; Cuello, 1983) . Whilst at the level of the neurone terminals in the neural lobe opioid activity probably results from release of co-localised opioid peptides from the magnocellular neurones themselves (Bicknell *et al.*, 1988 ; Zhao *et al.*, 1988a) .

Despite the identification of opioid receptors and evidence for opioid innervation at the level of the cell bodies, conditions have not yet been reported which demonstrate an inhibitory effect of endogenous opioid on the firing rate of OT neurones . For example, the administration of the opioid antagonist naloxone following either hyperosmotic or CCK8S stimulation does not further enhance OT neurone activity (Shibuki *et al.*, 1988 ; Leng *et al.*, 1992) . Consequently naloxone administered to conscious virgin rats or rats in early gestation produces no effect on oxytocin secretion . However after two weeks of pregnancy naloxone has a stimulatory action on OT secretion which increases to a maximum effect during parturition and rapidly diminishes thereafter (Hartman *et al.*, 1986b ; Leng *et al.*, 1988b) . This effect of naloxone may reveal an inhibitory role for endogenous opioids during the development of pregnancy in the rat and during the subsequent parturition process . The increased activity of endogenous opioids during parturition may have a function in the regulation of the birth process . The established role of OT during parturition involves stimulating the contractions of the uterus . Opioid peptides may therefore be useful in regulating the

release of OT allowing the rat to regulate the contractions of the uterus and space the delivery of successive pups .

If virgin rats are exposed to an i.c.v. infusion of morphine for 5 days they develop tolerance to the exogenously applied opiate (Rayner *et al.*, 1988 ; Russell, 1989) . Tolerance is displayed by the decreased sensitivity of OT neurones, chronically exposed to i.c.v. morphine, to the inhibitory effect of an acute i.v. dose of morphine (Pumford *et al.*, 1991) . Similarly one might expect that the proposed increase in opioid activity at the end of pregnancy might produce a state of opioid tolerance in the rat . Mechanisms involved in the development of tolerance to chronic i.c.v. morphine include a reduction in the density of opioid receptors in the AV3V region and more specifically of mu-receptors in the SON (Sumner *et al.*, 1990) . Likewise in the 21 day pregnant rat a decrease in mu-opioid receptor binding in the SON has also been reported (Sumner *et al.*, 1992) .

Opiates have been reported to inhibit the firing rate of OT neurones excited by electrical stimulation of the AV3V region (Leng and Russell, 1989) . We therefore investigated whether AV3V stimulated 21 day pregnant rats display tolerance to the effects of either mu- or kappa- opioids administered by i.v. injection . This involved the stereotacic placement of a stimulating electrode in the AV3V region of 21 day pregnant and virgin rats . Rats were then stimulated at either 25 or 10Hz and the effects of the mu-opioid agonist morphine, the kappa-opioid agonist U50,488 and the non-selective opioid antagonist naloxone on the subsequent rise in plasma OT concentration measured by radioimmunoassay . The effects of AV3V stimulation and drugs on blood pressure were also measured . Projections from the AV3V region to the nucleus tractus solitarius, an area associated with blood pressure regulation, are known to exist (Hartle and Brody, 1984 ; Haywood *et al.*,

1987 ; Hatton, 1990) . Monitoring the blood pressure of AV3V stimulated rats therefore provided us with a useful online bioassay of the electrical stimulation during the experiment . In a separate experiment the effect of the excitatory amino acid NMDA receptor antagonist MK801 on 10Hz AV3V and i.p. hyperosmotic saline stimulation of OT release was also investigated .

2.2 METHODS

Adult virgin female and age matched 21 day pregnant Sprague-Dawley rats were used . Rats were housed in standard conditions and the day of pregnancy determined according to the procedure outlined in the General Methods (GM2 Rats) section of this thesis .

2.2.1 Surgical procedures

On the day of experimentation, rats were anaesthetised with urethane and a femoral artery and vein cannulated (see General Methods, GM3) . Rats were then placed in a stereotaxic frame and the dorsal surface of the skull exposed by a midline skin incision . The skull was then levelled between bregma and lambda and a concentric bipolar stimulating electrode (SNEX 100, Clarke Electromedical Instruments, Berks, UK.) lowered through a burr hole into the AV3V region (8.3mm below the skull surface, 0.3mm posterior to bregma, in the midline) . Rats were left for 2 hours after completion of surgery before the start of the experimental protocol .

2.2.2 Experimental procedure

In order to assess the OT concentration of rat blood 0.3ml samples were taken, via a femoral arterial cannula, chilled on ice for a few minutes before centrifugation at 15,000g for approximately 2min and then the plasma aliquoted . The plasma samples were then transferred to the -20^{0}C freezer and stored there till assay . The radioimmunoassay employed was a modified double antibody method from that of Higuchi *et al.* (1984), further details of which can be obtained from the General Methods (GM1) section of this thesis .

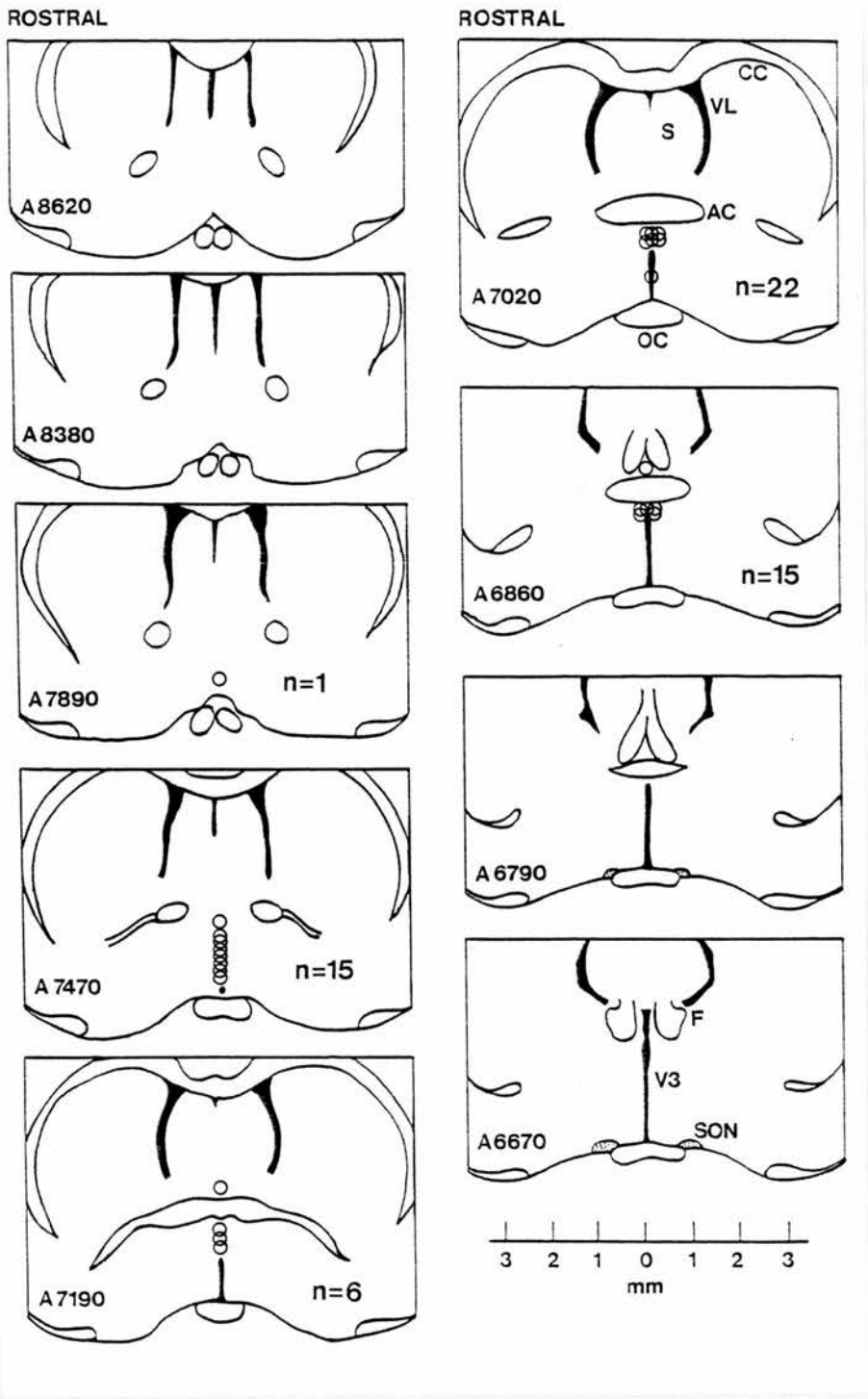
Verification of the electrode position was performed according to the description in the General Methods (GM5) section . Briefly at the end of the experimental protocol a continuous train of pulses was passed to deposit Fe^{2+} ions from the electrode tip . The brains were then removed and fixed in a solution of 10% formal saline containing 3% (w/vol) potassium ferro- and ferri- cyanide for at least two days . This solution not only fixed the tissue but produced the Prussian Blue reaction where Fe^{2+} ions had been deposited from the electrode tip . After fixation frozen 50μm coronal brain sections were cut, collected and mounted in sequence . Positions of blue spots were mapped onto a series of drawings of coronal brain sections produced according to the Rat Brain Atlas (Konig & Klippel 1963) . An example of the drawings produced is displayed as Fig 2.A . Electrodes were judged to be correctly positioned when in the midline and either in or immediately adjacent to the median preoptic nucleus and/or OVLT .

Electrical AV3V stimulation was performed at 25Hz for 2 min and 10Hz for 10 min (matched biphasic pulses, 0.5mA peak-peak, 1msec duration, 10sec on/off) in the presence of vehicle, opioid agonists and/or inhibitors and the excitatory amino acid antagonist MK801 . The pulse generator used was made by the Department of Physiology Electronics Workshop of this University and its power output was modulated by two stimulus isolators connected in parallel to produce a biphasic pulse (Neurolog NL800) . The output of the stimulation set up was calibrated on each day of use and the on/off switching of the stimulation periods monitored continuously throughout the experiment using an oscilloscope .

Femoral arterial blood pressure was continuously measured via a pressure transducer (P231D, Gould Stratham, USA) and chart recorder . The signal from the pressure transducers used was boosted by amplifiers

calibrated at 100mmHg (made by the Department of Physiology Electronics Workshop of this University) . Blood pressure changes were computed by hand, this involved measuring the height above the base line of the systolic and diastolic pressures at regular intervals along the trace . A mean height was then calculated of diastolic + 1/3 pulse pressure which was converted to mean arterial blood pressure using the calibration function . Mean arterial blood pressure was measured every 3 min over 10 sec segments of the trace in-between stimulation periods and during stimulation the maximum or minimum pressures were measured during each on/off phase of the stimulation cycle .

The effects of the mu-opioid agonist morphine (morphine sulphate B.P.), the kappa-opioid agonist U50,488 (trans-(±)-3,4-DICHLORO-N-METHYL-N-(2-[1-PYROLIDINYL]CYCLOHEXYL0BENZENE-ACETAMIDE, FW 465.4, cat. No. D-0908, Sigma Chemical Co.) and the non-selective opioid antagonist naloxone (naloxone hydrochloride, cat. No. n7758, Sigma Chemical Co.) on AV3V stimulation were investigated . In another experiment the effect of the excitatory amino acid NMDA receptor antagonist MK801 (MW 337.37, cat. No. M-107, Research Biochemicals Inc.) on electrical AV3V and i.p. hyperosmotic saline (4ml/kg, 1.5M NaCl) stimulated OT release were also investigated .



❍ = electrode positions (from FIG 2.1 - 2.4) visualised by use of the Prussian Blue reaction, which forms a blue spot in the brain tissue where Fe^{2+} ions were deposited from the electrode tip . Brains were then sectioned, mounted in sequence and counterstained before being examined under a dissecting microscope . Positions of the blue spots were then mapped on to a series of drawings produced in accordance with a rat brain atlas (Konig and Klippel, 1963) .

AC anterior commissure, CC corpus callosum, F fornix, OC optic chiasm, S septum, SON supraoptic nucleus, VL lateral ventrical and V3 third ventricle .

FIGURE 2.A Extent of electrode sites within the brains of AV3V stimulated rats that were considered to be correctly positioned .

2.3 RESULTS

2.3.1 Effect of U50,488 and naloxone on 25Hz AV3V stimulated oxytocin release in virgin and 21 day pregnant rats (Fig 2.1) .

The inaugural AV3V stimulation period produced a significant rise in the plasma OT concentration from basal in virgin rats of 33 ± 7.5 pg/ml ($P < 0.005$ paired t test) and a similar increase in pregnant rats of 36.7 ± 22.9 pg/ml ($P = 0.03$ Wilcoxon signed ranks test) .

The initial dose of the kappa-selective opioid agonist U50,488 (U50 1) was administered 5 minutes before the second period of AV3V stimulation . The ensuing period of AV3V stimulation produced a non-significant increase in plasma OT concentration from basal in virgin rats of 7.3 ± 4 pg/ml ($P = 0.1$ paired t test) and in pregnant rats of 7.6 ± 5.6 pg/ml ($P > 0.05$ Wilcoxon signed ranks test) . These responses in virgin and pregnant rats were significantly attenuated compared with those evoked in the absence of U50,488 ($P = 0.0002$ paired t test and $P < 0.03$ Wilcoxon signed ranks test respectively) .

Similarly the second dose of U50,488 (U50 2) abolished the response to the succeeding electrical stimulation in both virgin and pregnant rats ($P > 0.05$ paired t test and Wilcoxon signed ranks test versus respective basal values) .

Naloxone (NLX) produced a significant increase in plasma OT concentration from basal in virgin ($P < 0.05$ paired t test) and pregnant rats ($P < 0.03$ Wilcoxon signed ranks test) . The fourth AV3V stimulation period was commenced 15 minutes later and resulted in a further significant increase in the plasma OT concentration in virgin rats of 146.5 ± 45.2 pg/ml

(P = 0.009 paired t test versus zero) and also in the pregnant rats of 108.5 ± 72.7 pg/ml (P < 0.03 Wilcoxon signed ranks test versus zero). These values were significantly greater than the plasma OT concentration evoked by the first stimulation period in both virgin and pregnant rats (P < 0.03 paired t test and P < 0.05 Wilcoxon signed ranks test respectively) .

LEGEND : FIGURE 2.1

Plasma OT in femoral arterial blood samples from AV3V stimulated virgin and 21 day pregnant rats . Graph a) denotes plasma oxytocin concentration and graph b) change in plasma OT concentration from previous sample . Values are mean ± s.e.m. ; ❍ and ☐ are virgin rats (n = 11), ● and ▬ are 21 day pregnant rats (n = 6) .

Rats received four separate 25Hz AV3V stimulation periods of 2 min duration at 5, 25, 45 and 75 min (▬). Two doses of the kappa-opioid receptor antagonist U50,488 were administered, U50 1 (0.5mg/kg, i.v.) and U50 2 (2.5mg/kg, i.v.) . The opioid antagonist naloxone (NLX) was given 60 minutes into the experiment (5mg/kg, i.v.) .

*, #, † = $P \leq 0.05$; **, ##, †† = $P \leq 0.005$;

††† = $P \leq 0.0005$

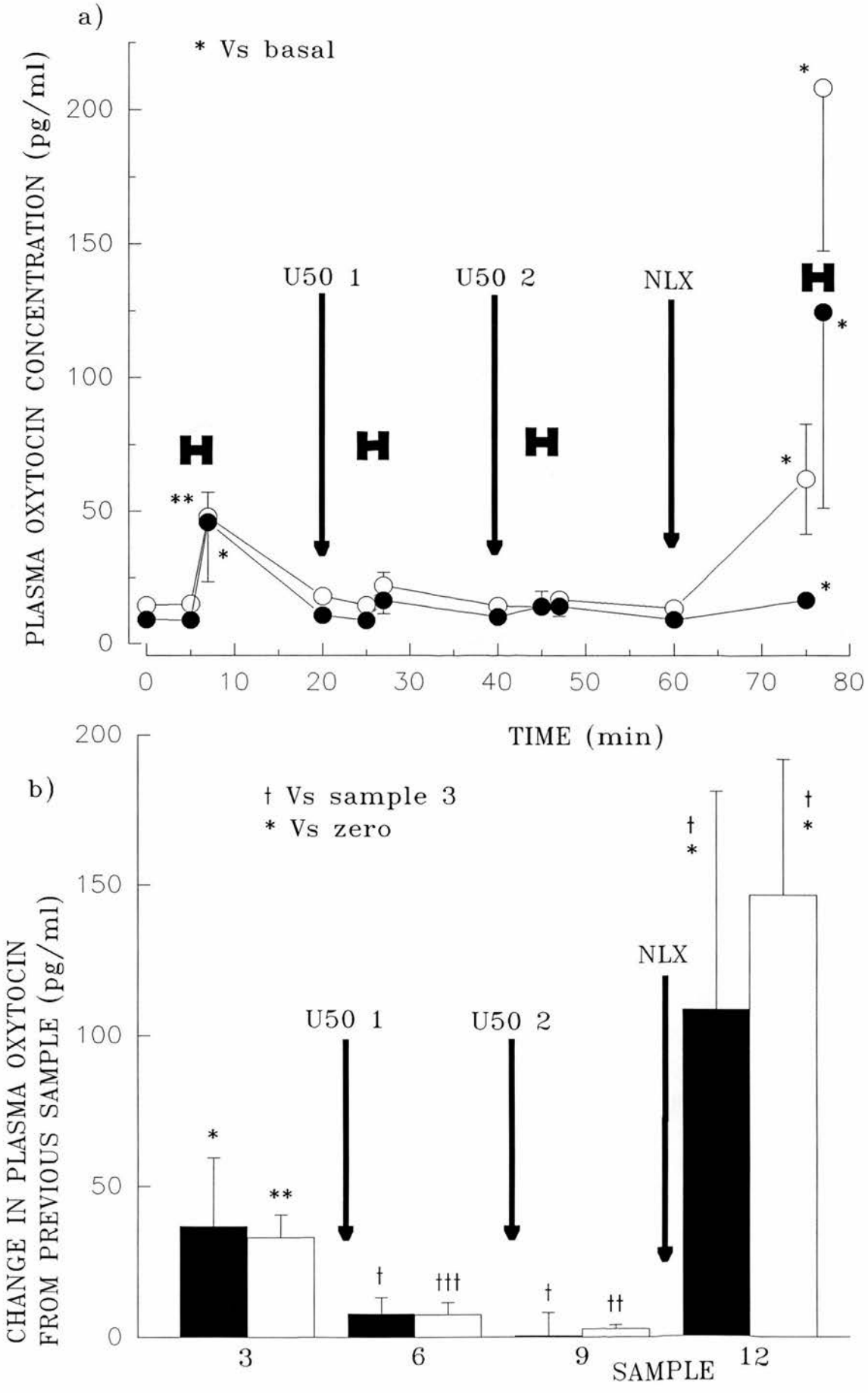


FIGURE 2.1 Effect of U50,488 and naloxone on 25Hz AV3V stimulated oxytocin release in virgin and 21 day pregnant rats

2.3.2 Effect of morphine and naloxone on 25Hz AV3V stimulated oxytocin release in virgin and 21 day pregnant rats (Fig 2.2) .

The inaugural AV3V stimulation period produced a significant rise in the plasma OT concentration from basal in virgin rats of 61.4 ± 15 pg/ml ($P < 0.05$ paired t test) and in pregnant rats of 66.1 ± 9.9 pg/ml ($P = 0.0005$ paired t test) .

The initial dose of the mu-opioid agonist morphine (M1) was administered 5 minutes before the second AV3V stimulation period . The OT response to electrical stimulation after the first dose of morphine was not significantly different from that produced by the previous AV3V stimulation period in both virgin and pregnant rats ($P > 0.6$ and $P > 0.9$ respectively, paired t test) .

The final dose of morphine (M2) was administered 5 minutes before the third AV3V stimulation period . The subsequent electrical stimulation produced a significant increase from basal in the plasma OT concentration in virgin rats of 50.7 ± 12.7 ($P < 0.02$ paired t test) and pregnant rats of 42.8 ± 15.7 ($P < 0.05$ paired t test) . The response of the pregnant rats was lower and that of the virgin rats significantly lower compared with that produced by the initial stimulation period ($P = 0.04$ paired t test versus sample 3) .

Naloxone (NLX) was administered 15 minutes before the final electrical stimulation period and significantly increased the response to 25Hz AV3V stimulated OT release in virgin and pregnant rats ($P < 0.05$ paired t test versus sample 3) . The response of virgin rats was significantly greater than that of pregnant rats to 25Hz AV3V stimulation in the presence of naloxone ($P < 0.05$ two sample t test) .

LEGEND : FIGURE 2.2

Plasma OT measured by ria. on femoral arterial blood samples from 25Hz AV3V stimulated virgin and 21 day pregnant rats . Graph a) denotes plasma oxytocin concentration and graph b) change in plasma OT concentration from previous sample . Values are mean ± s.e.m. ; ○ and □ are virgin rats (n = 7), ● and ■ are 21 day pregnant rats (n = 7) .

Rats received four separate 25Hz AV3V stimulation periods of 2 min duration at 5, 25, 45 and 77 min (▬) . Two doses of the mu-opioid agonist morphine were administered M1 (1mg/kg, i.v.) and M2 (5mg/kg, i.v.) . The opioid antagonist naloxone (NLX) was given 62 minutes into the experiment (5mg/kg, i.v.) .

*, #, † = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$

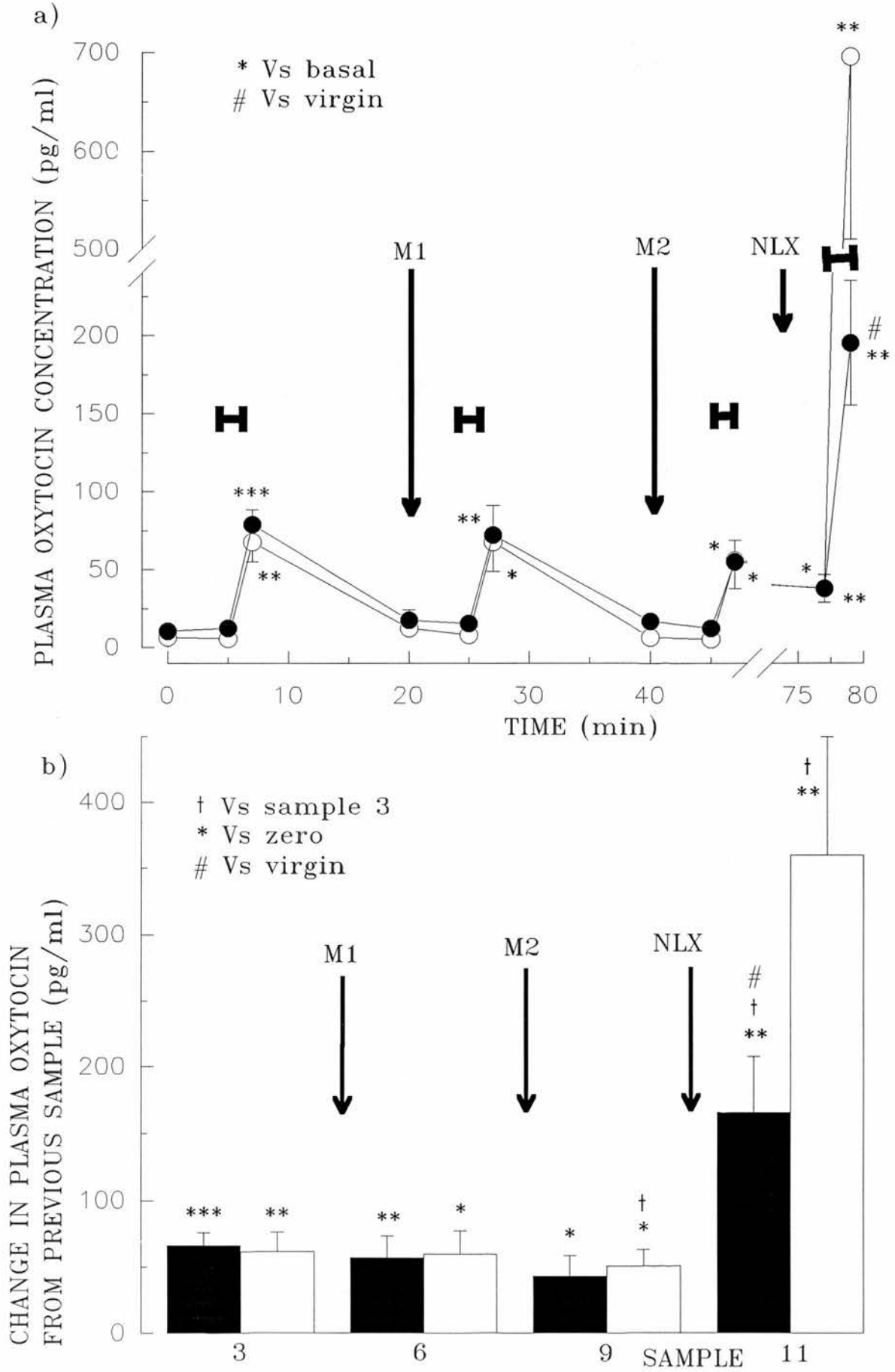


FIGURE 2.2 Effect of morphine and naloxone on 25Hz AV3V stimulated oxytocin release in virgin and 21 day pregnant rats

2.3.3 Effect of morphine and naloxone on 10Hz AV3V stimulated oxytocin release in virgin and 21 day pregnant rats (Fig 2.3) .

The inaugural stimulation period produced a significant rise in the plasma OT concentration from basal in virgin rats of 91.7± 19.6 pg/ml ($P < 0.005$ paired t test) and in pregnant rats of 83.1 ±12.2 pg/ml ($P = 0.0001$ paired t test) .

The initial dose of the mu-opioid agonist morphine (M1) was administered 5 minutes before the second AV3V stimulation period . The OT response to the subsequent electrical stimulation produced a significant increase in plasma OT concentration from basal, in virgin rats of 38.1 ± 14.4 pg/ml ($P < 0.005$ paired t test) and in pregnant rats of 43.1 ± 10.6 pg/ml ($P < 0.01$ paired t test) . These responses were significantly less than those produced by the first AV3V stimulation period in both virgin and pregnant rats ($P = 0.01$ and $P < 0.0005$ respectively, paired t test) .

The final dose of morphine (5mg/kg, M2) was administered 5 minutes before the third AV3V stimulation period . The subsequent electrical stimulation produced a significant increase from basal in the plasma OT concentration in virgin rats of 16.6 ± 4.7 ($P < 0.01$ paired t test) and in pregnant rats of 26.1 ± 9.5 ($P < 0.02$ paired t test) . These responses were lower than those produced in the presence of 1mg/kg morphine and significantly lower than those produced in the absence of morphine ($P = 0.002$ and $P = 0.0002$ respectively, paired t test versus increase in plasma OT with the initial electrical stimulation) .

Naloxone (NLX) was administered 10 minutes after the start of the final AV3V stimulation period and produced a greatly enhanced response in virgin and pregnant rats compared to that produced during the first stimulation period ($P = 0.0008$ and $P < 0.0001$ respectively, paired t test) .

LEGEND : FIGURE 2.3

Plasma OT measured by ria on femoral arterial blood samples from 10Hz AV3V stimulated virgin and 21 day pregnant rats . Graph a) denotes plasma oxytocin concentration and graph b) change in plasma OT concentration from previous sample . Values are mean ± s.e.m. ; ❍ and ▭ are virgin rats (n = 10), ● and ▬ are 21 day pregnant rats (n = 13) .

Rats received three separate 10Hz AV3V stimulation periods of 10 minute duration at 5, 35min and for 25 minutes at 65min (▬) .

Two doses of the mu-opioid agonist morphine were administered, M1 (1mg/kg, i.v.) and M2 (5mg/kg, i.v.) . The opioid antagonist naloxone (NLX) was given 75 minutes after the start of the experiment (5mg/kg, i.v.) .

$*, \dagger = P \leq 0.05$; $**, \dagger\dagger = P \leq 0.005$;

$***, \dagger\dagger\dagger = P \leq 0.0005$

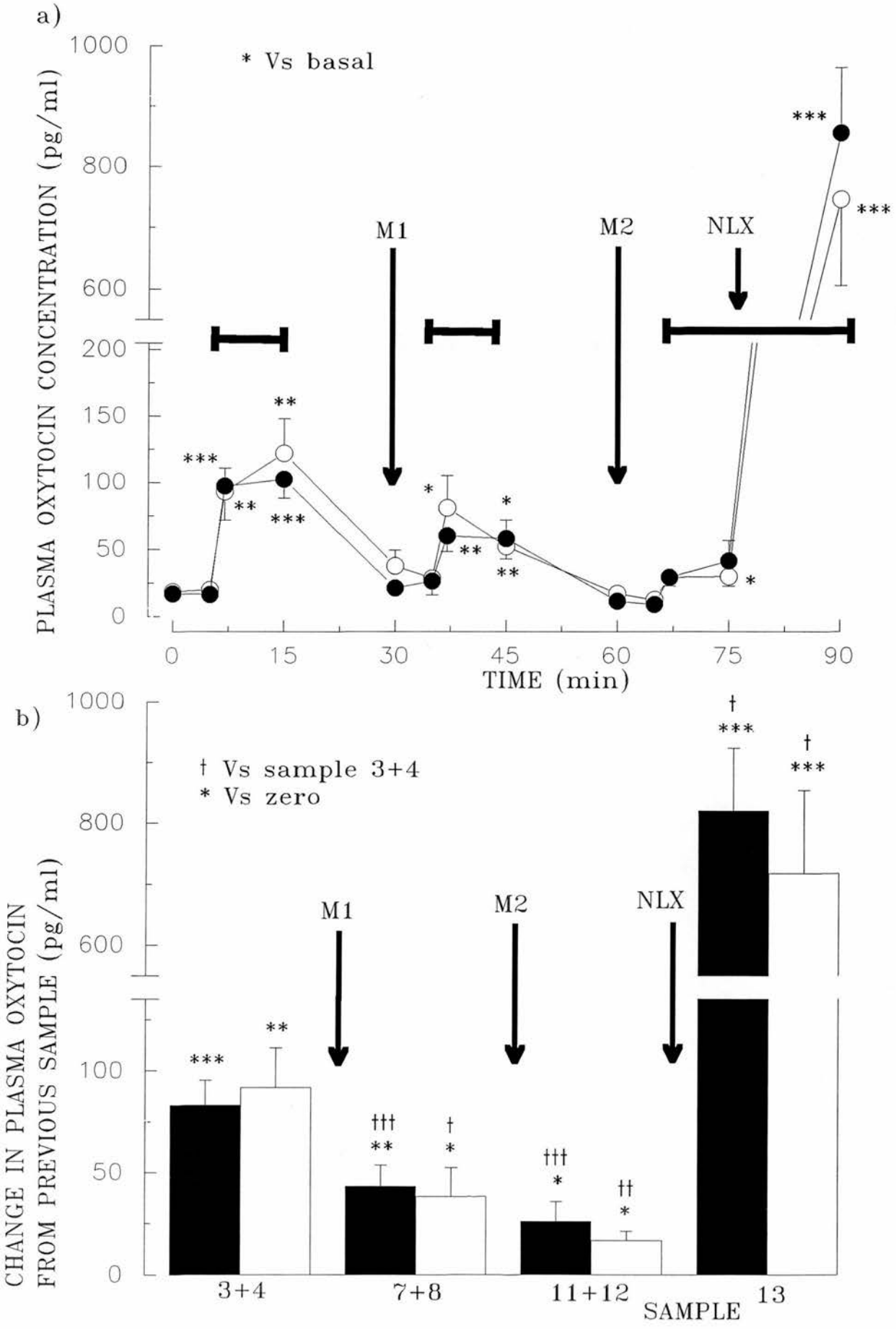


FIGURE 2.3 Effect of morphine and naloxone on 10 Hz AV3V stimulated oxytocin release in virgin and 21 day pregnant rats

2.3.4 Effect of time and naloxone on repeated 25Hz AV3V stimulated oxytocin release in virgin and 21 day pregnant rats (Fig 2.4) .

The inaugural stimulation period produced a significant rise in the plasma OT concentration from basal in virgin rats of 111.3 ± 22.7 pg/ml (P = 0.003 paired t test) and in pregnant rats of 121.1 ± 29.8 pg/ml (P = 0.002 paired t test) .

Five minutes before the second AV3V stimulation period a vehicle injection was administered (VEH) . The ensuing electrical AV3V stimulation produced a significant increase in the plasma OT concentration versus basal in virgin rats of 113.3 ± 25.9 (P < 0.02 paired t test) and in pregnant rats of 79.4 ± 24 (P = 0.01 paired t test) . The response of the virgin rats was slightly greater than during the previous stimulation period but in the pregnant rats it was significantly less (P = 0.02 paired t test) .

The third stimulation period once again produced a significant increase in the plasma OT concentration compared to basal in virgin rats of 103.3 ± 19.8 pg/ml (P = 0.0003 paired t test) and in pregnant rats of 70.3 ± 27.2 (P < 0.05 paired t test) five minutes after the second vehicle administration . The response of virgin rats was less and pregnant rats significantly less than that produced by the initial stimulation period (P = 0.007 paired t test) .

15 minutes before the fourth stimulation period naloxone was administered (NLX) and produced a significant increase, compared to basal, in the plasma OT concentration in virgin and pregnant rats (P = 0.01 paired t test) . The plasma OT concentration following naloxone in virgin rats was significantly greater than in pregnant rats (P = 0.01 two sample t test) . The subsequent AV3V stimulation elicited a further significant increase in the plasma OT concentration from basal in virgin rats of 549.9 ± 102.9 pg/ml (P

= 0.0001 paired t test) and in pregnant rats of 284.8 ± 92.2 pg/ml (P = 0.003 paired t test) . Once again the response of the virgin rats was significantly greater than that of the pregnant rats in response to electrical AV3V stimulation at 25Hz (P < 0.05 two sample t test) .

LEGEND : FIGURE 2.4

Plasma OT in femoral arterial blood samples from 25Hz AV3V stimulated virgin and 21 day pregnant rats . Graph a) denotes plasma oxytocin concentration and graph b) change in plasma OT concentration from previous sample . Values are mean ± s.e.m. ; ❍ and ▭ are virgin rats (n = 10), ● and ▬ are 21 day pregnant rats (n = 12) .

Rats received four separate 25Hz AV3V stimulation periods of 2 min duration at 5, 25, 45 and 75 min (▬) . Two doses of isotonic saline vehicle (VEH) were administered (0.5ml/kg, i.v.) . The opioid antagonist naloxone (NLX) was given 15 minutes before the fourth and final stimulation period (5mg/kg, i.v.) .

*, #, † = $P \leq 0.05$; **, ##, †† = $P \leq 0.005$; *** = $P \leq 0.0005$

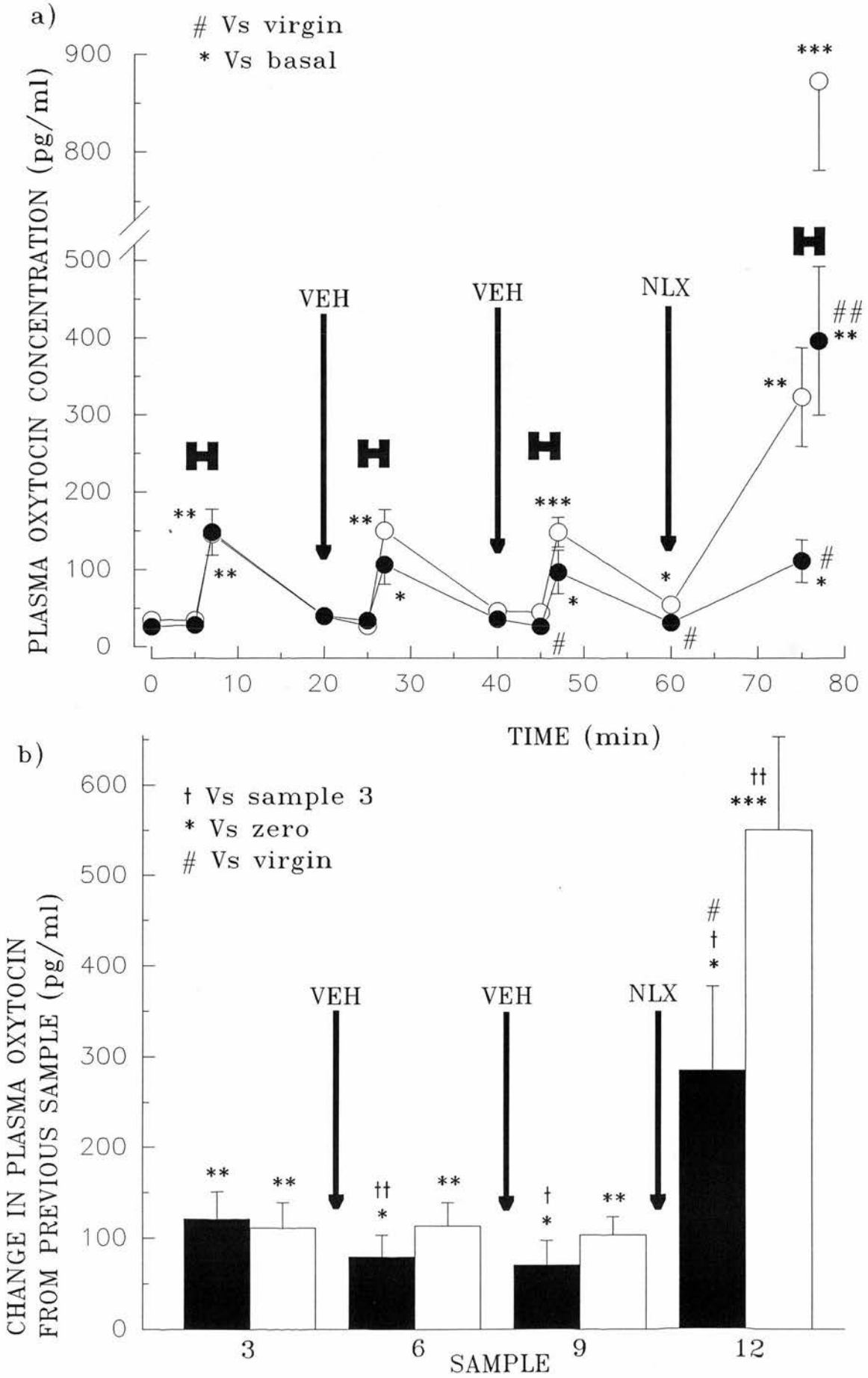


FIGURE 2.4 Effect of time and naloxone on repeated 25Hz AV3V stimulated oxytocin release in virgin and 21 day pregnant rats

2.3.5 Effect of time on naloxone facilitated 25Hz AV3V stimulated oxytocin release in virgin and 21 day pregnant rats (Fig 2.5) .

The inaugural stimulation period produced a significant rise in the plasma OT concentration from basal in virgin rats of 243.8 ± 87.2 pg/ml ($P = 0.03$ paired t test) and in pregnant rats of 123.1 ± 58 pg/ml ($P < 0.02$ Wilcoxon signed ranks test) .

Naloxone (5mg/kg) was administered 15 minutes before the second AV3V stimulation period and caused an increase in the plasma OT concentration in virgin and a significant increase compared to basal in pregnant rats ($P < 0.02$ paired t test) . Following the second AV3V stimulation period plasma OT concentration rose significantly in virgin rats by 803 ± 244.8 pg/ml ($P < 0.002$ paired t test) and in pregnant rats by 418.9 ± 109.7 ($P = 0.0004$ paired t test) . As in the previous experiment, electrical stimulation after the administration of naloxone was significantly more effective in virgin than pregnant rats ($P < 0.05$ two sample t test) .

LEGEND : FIGURE 2.5

Plasma OT was measured by ria on femoral arterial blood samples from 25Hz AV3V stimulated virgin and 21 day pregnant rats . Graph a) denotes plasma oxytocin concentration and the graph b) change in plasma OT concentration from previous sample . Values are mean ± s.e.m. ; ❍ and ▭ are virgin rats (n = 7), ● and ▬ are 21 day pregnant rats (n = 7) .

Rats received two separate 25Hz AV3V stimulation periods of 2 min duration at 5 and 35 min (▬) . Naloxone was administered 15 minutes before the second stimulation period (5mg/kg, i.v.) .

$*, \#, \dagger = P \leq 0.05$; $**, \dagger\dagger = P \leq 0.005$;

$***, \dagger\dagger\dagger = P \leq 0.0005$

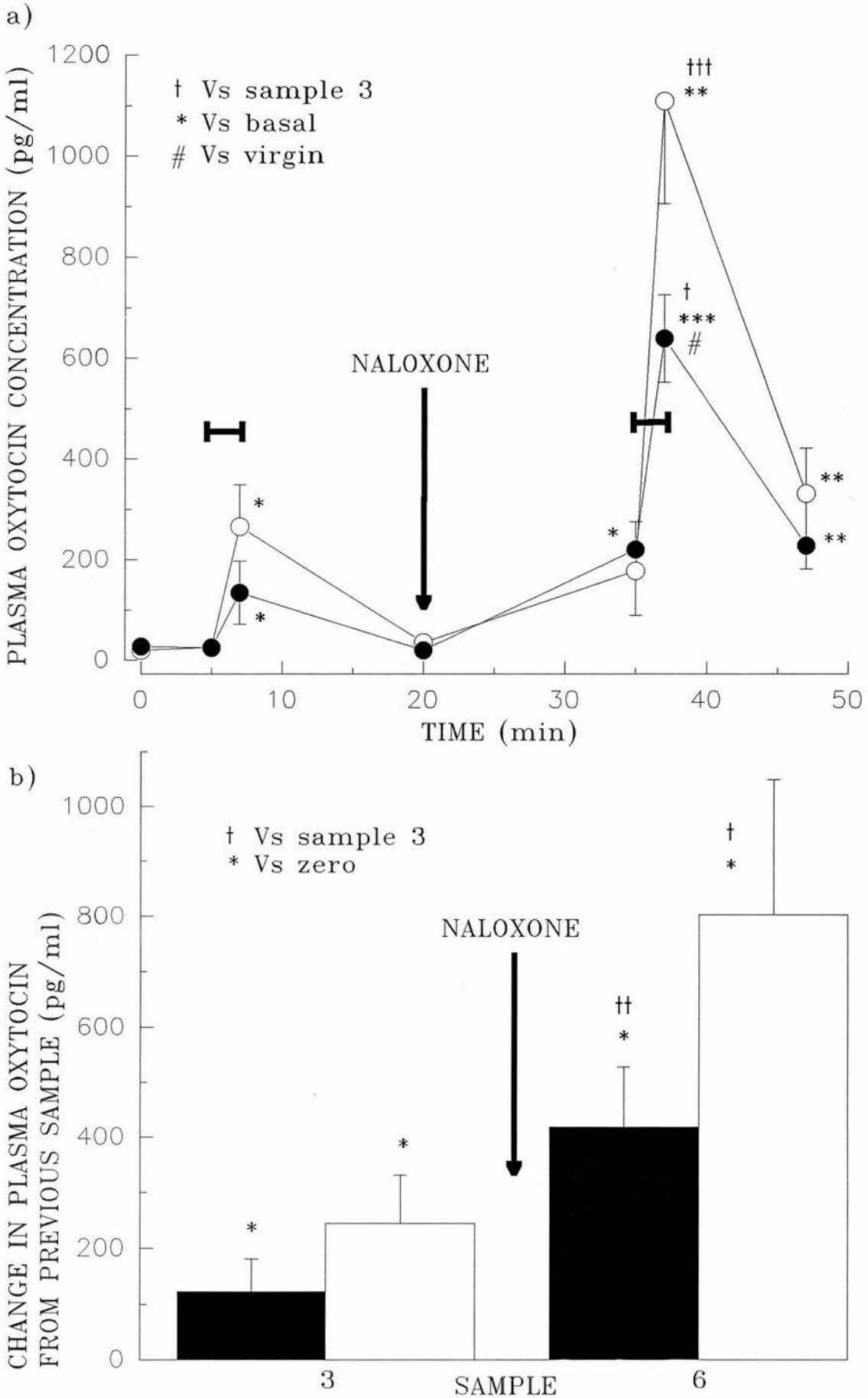


FIGURE 2.5 Effect of time on naloxone facilitated 25Hz AV3V stimulated oxytocin release in virgin and 21 day pregnant rats

2.3.6 Effect of MK801 on AV3V and hyperosmotic saline stimulated oxytocin release in virgin rats (Fig 2.6) .

The inaugural stimulation period produced a significant rise in the plasma OT concentration from basal in control rats of 140.5 ± 41.2 pg/ml (P = 0.01 paired t test) and in the MK801 group of 62.2 ± 29.9 pg/ml (P = 0.02 Wilcoxon signed ranks test) . The response of the control group was greater than that of the MK801 treatment group but the difference was not significantly different (P > 0.05 Mann-Whitney U test) .

MK801 or vehicle were administered 5 minutes before the second AV3V stimulation period . The subsequent electrical stimulation produced a significant rise in the plasma OT concentration of vehicle treated rats compared to basal of 101 ± 29.8 pg/ml (P < 0.02 paired t test) and a non significant increase in the MK801 treated rats of 28 ± 18.4 pg/ml (P > 0.05 Wilcoxon signed ranks test) . The responses were significantly lower than that of the previous AV3V stimulation period in both control (P < 0.05 paired t test) and MK801 treated (P = 0.03 Wilcoxon signed ranks test) rat groups . The response of the control group was also significantly greater than that of the MK801 treated group at this point (P = 0.03 Mann-Whitney U test) .

Hyperosmotic saline was administered at the 75min point and stimulated a significant increase in the plasma OT concentration of both vehicle and MK801 treated groups at 10 (P = 0.04 and P = 0.0007 paired t test respectively) and 30 (P = 0.002 and P = 0.003 respectively, paired t test) minutes after the i.p. injection . There were no significant differences between the release of OT in MK801 and vehicle treated rats in response to hyperosmotic saline stimulation .

LEGEND : FIGURE 2.6

Plasma OT in femoral arterial blood samples from 10Hz AV3V and i.p. 1.5M NaCl stimulated virgin rats . Graph a) denotes plasma oxytocin concentration and graph b) change in plasma OT concentration from previous sample . Values are mean ± s.e.m. ; ❍ and □ are vehicle treated control rats (n = 7), ● and ■ are MK801 treated rats (n = 6) .

Rats received two separate 10Hz AV3V stimulation periods of 15 minutes duration at 10 and 45min before and after the administration of MK801 . MK801 (1mg/kg, i.v.) or vehicle (0.5ml/kg of 0.15M NaCl, i.v.) was administered 5 minutes before the second AV3V stimulation period

*, #, † = $P \leq 0.05$; ** = $P \leq 0.005$; *** = $P \leq 0.0005$

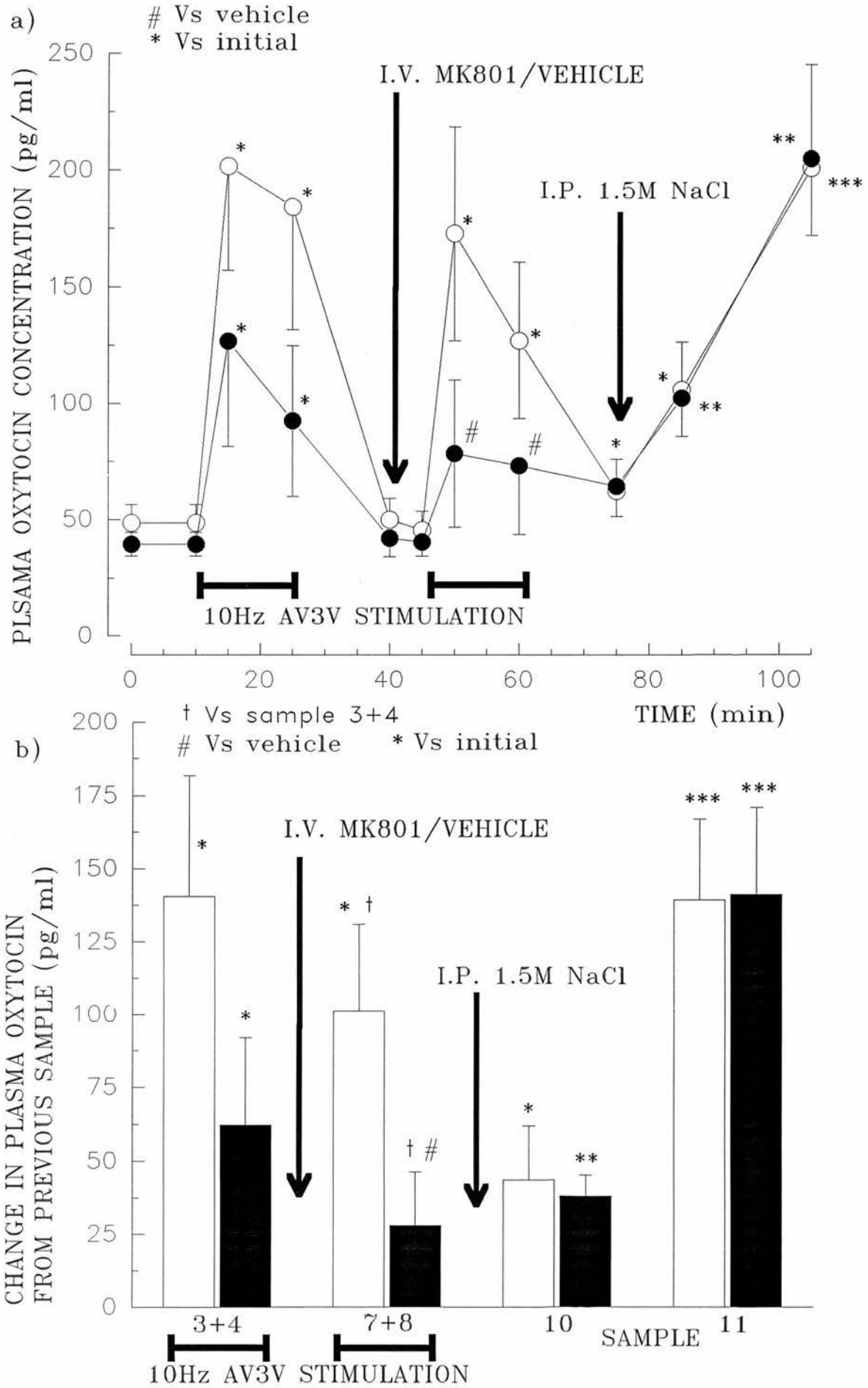


FIGURE 2.6 Effect of MK801 on electrical AV3V and hyperosmotic saline stimulated oxytocin release in virgin rats

2.3.7 Effect of 25Hz AV3V stimulation, morphine and U50,488H on mean arterial blood pressure (Fig 2.7) .

Graph a) depicts the mean change in blood pressure during 25Hz AV3V stimulation for the initial stimulation periods of all experiments involving 25Hz stimulation (figures 2.1, 2.2, 2.4 and 2.5) . Basal arterial blood pressure of virgin rats was measured at 83.6 ± 8.9 mmHg and in 21 day pregnant rats was significantly lower at 65.7 ± 2.9 mmHg ($P = 0.01$ two sample t test) . During AV3V stimulation the blood pressure of both pregnant and virgin animals fell during the off phases and rose again during the on phases of the stimulus train . Mean maximum arterial blood pressure of virgin rats during the on phases was 69.4 ± 3.1 mm Hg which was significantly lower than basal ($P = 0.007$ paired t test) but was significantly greater than the mean minimum pressure during the off phases, which fell to 56.8 ± 2.3 mm Hg ($P < 0.0001$ paired t test) . In the pregnant rats arterial blood pressure rose during the on phases to a mean of 62.6 ± 2.8 mmHg which was significantly less than basal ($P = 0.08$ paired t test, but $P = 0.02$ Wilcoxon signed ranks test) and fell to a mean of 52.7 ± 1.8 mmHg during the off phases which was significantly lower than during the on phases ($P < 0.0001$ paired t test) . Mean arterial blood pressure was not significantly different between virgin and pregnant rats during the AV3V stimulation period ($P > 0.1$ two sample t test) . Three minutes after the AV3V stimulation had been terminated basal arterial blood pressure in both pregnant and virgin rats had returned to levels not significantly different to basal ($P > 0.3$ paired t test) .

Graph b) depicts the mean fall in blood pressure following the administration of morphine at 1, 5 and 10 mg/kg in two separate experiments . Once again the mean arterial blood pressure of pregnant rats was found to

be significantly lower than virgin animals ($P = 0.03$ two sample t test) . Morphine produced a similar significant fall in the blood pressure of both pregnant and virgin rats 3 and 6 minutes after administration ($P < 0.0001$ and $P = 0.003$ respectively, paired t test) .

Graph c) represents the mean fall in blood pressure following the administration of U50,488 at 0.5 and 2.5 mg/kg in the same experiment (figure 2.1) . Three minutes after the injection of U50,488 there was a significant fall in the mean arterial blood pressure in pregnant rats of 8.9 ± 3.8 mmHg ($P = 0.04$ paired t test) . At the same time there was a significant decrease in the mean arterial blood pressure of virgin rats of 32.5 ± 4 mmHg ($P < 0.0001$ paired t test) which was significantly greater than the decrease in pregnant rats ($P = 0.0006$ two sample t test) .

LEGEND : FIGURE 2.7

Mean arterial blood pressure (MABP) was measured over a 10 second interval every 3 minutes between stimulation periods . During electrical AV3V stimulation the maximum or minimum pressure was measured during each 10 second on/off phase of the stimulus pulse train . Values are mean ± s.e.m. : ❍, ❑ and Δ are virgin rats (n = 44, 14 and 11) ; ●, ■ and ▲ are 21 day pregnant rats (n = 33, 12 and 6) .

Graph a) depicts the mean change in blood pressure during 25Hz AV3V stimulation for the initial stimulation periods of all experiments involving 25Hz AV3V stimulation (figures 2.1, 2.2, 2.4 and 2.5) . Graph b) depicts the mean transient fall in blood pressure following the i.v. administration of morphine at 1, 5 and 10 mg/kg in two separate experiments . Graph c) represents the mean fall in blood pressure following the administration of U50,488 at 0.5 and 2.5 mg/kg (i.v.) in the same experiment (Fig 2.1) .

$*, \#, \dagger = P \leq 0.05$; $**, \#\#, \dagger\dagger = P \leq 0.005$;

$***, \dagger\dagger\dagger = P \leq 0.0005$

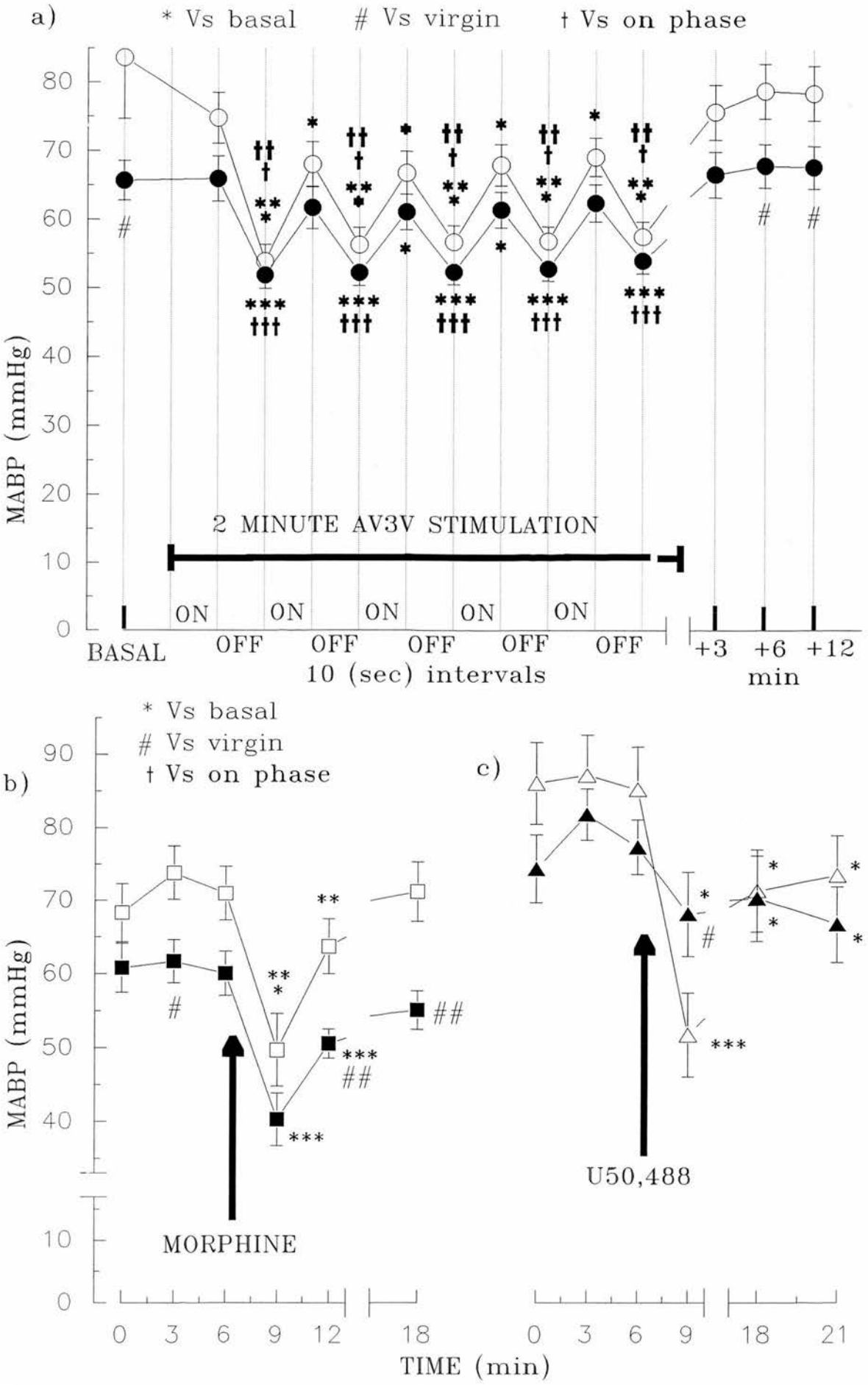


FIGURE 2.7 Effect of 25Hz AV3V stimulation, morphine and U50,488 on mean arterial blood pressure (MABP)

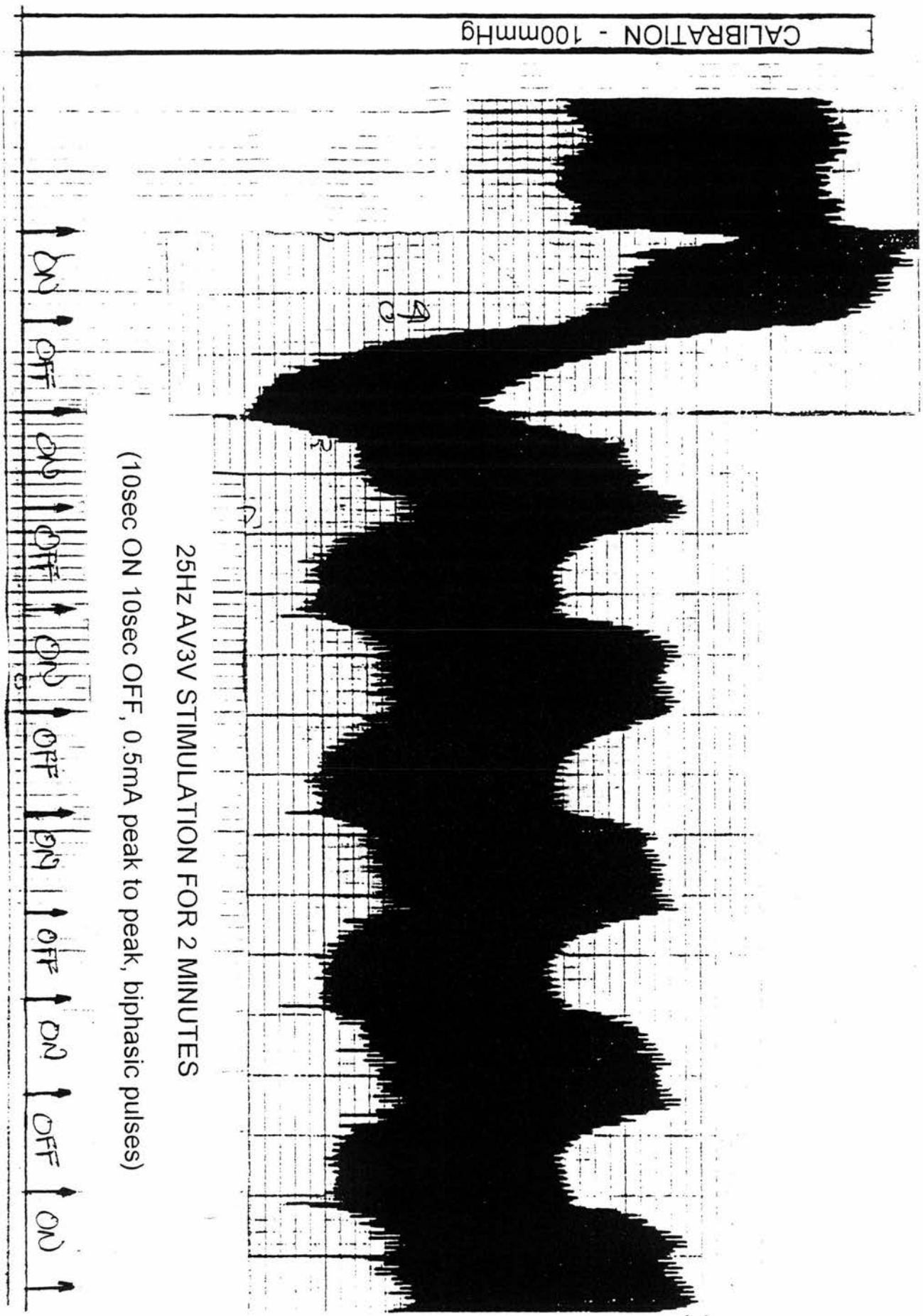


FIGURE 2.8 A typical blood pressure trace of a rat undergoing AV3V stimulation at 25Hz

2.4 DISCUSSION

2.4.1 The effect of U50,488 on AV3V stimulated oxytocin release

The increase in plasma OT concentration in response to electrical AV3V stimulation, at 10 and 25Hz, was similar in both 21 day pregnant and virgin animals . Intravenous administration of the kappa-opioid peptide U50,488, (U50) at 0.5 and 2.5 mg/kg, significantly reduced the response to 25Hz stimulation in a comparable manner in virgin and 21 day pregnant rats (Fig 2.1) . U50 has been demonstrated to inhibit OT secretion via an action on the posterior pituitary both *in vivo* and *in vitro* (Coombes and Russell, 1988 ; Douglas *et al.*, 1992) .

Towards the end of pregnancy there is a decrease in the kappa-receptor binding of the posterior pituitary in the rat (Sumner *et al.*, 1992) . In addition U50 has been demonstrated to be less effective at inhibiting OT release from the neural lobe of pregnant rats *in vitro* (Douglas *et al.*, 1990) . However U50 also acts centrally, potently inhibiting the basal activity of supraoptic neurones *in vivo* and *in vitro* by an action on both pre- and post-synaptic receptors (Russell *et al.*, 1989 ; Leng *et al.*, 1990) . Therefore any decreased kappa-mediated actions of U50 in the posterior pituitary of the pregnant rat was probably masked by an action of U50 on the cell bodies in pregnant rats, and it would appear that at this central site there is no loss of effectiveness of the kappa-agonist at the end of pregnancy .

2.4.2 The effect of morphine on AV3V stimulated oxytocin release

The effect of i.v. morphine on the response to 10 and 25Hz AV3V stimulation was also similar in both virgin and pregnant rats . Using 25Hz stimulation (Fig 2.2) there was a slight decrease in plasma OT release during the experiment which was significant in the virgin rats after 5mg/kg

morphine administration . However in the subsequent experiment using an identical protocol without morphine a similar decrease in release of OT was obtained with repeated AV3V stimulation (Fig 2.4) . The decrease in the morphine experiment using 25Hz stimulation therefore appears to be a result of repeated stimulation rather than an effect of morphine . A similar experiment using AV3V stimulation at 10Hz produced a more pronounced reduction in plasma OT released after the administration of morphine at 1 and 5 mg/kg i.v. (Fig 2.3) . Repeated 10Hz AV3V stimulation in virgin rats (n = 5) in the absence of morphine but using an identical protocol as Fig 2.3 produced similar mean increases in plasma OT concentration of 60 ± 25.5, 48.6 ± 16.7 and 60.7 ± 27.3 pg/ml . It appears therefore that the decreased release in plasma OT in response to 10Hz stimulation represents an effect of morphine rather than the protocol .

Morphine is known to inhibit OT secretion in response to several stimuli including hyperosmotic saline (see Chapter 4 of this thesis) . Morphine acts centrally to inhibit the release of OT via an action on the cell bodies of OT neurones in the supraoptic nucleus (Wakerley *et al.*, 1983 ; Coombes and Russell 1988 ; Pumford *et al.*, 1991) . The activity of morphine on AV3V stimulated neurone activity has been reported to be less effective as the stimulation frequency increases (Pumford *et al.*, 1992) . Morphine inhibits neurone activity by hyperpolarising OT neurones via post-synaptic mu-receptors (Inenega *et al.*, 1990), and it is possible that at higher stimulation frequencies enough excitatory post synaptic potentials are generated to depolarise the neurones from a morphine induced hyperpolarised state . This would explain the lack of an effect of morphine at 25Hz stimulation compared to the profound effect of morphine against 10Hz stimulation . This dependence of the inhibitory action of

morphine on the frequency of stimulation of the OT neurones may explain why earlier studies showed lack of inhibition of milk ejection bursts of oxytocin neurones (Clarke *et al.*, 1979) .

We were attempting however to demonstrate a reduced effect of mu-opioid agonist activity in view of the significantly reduced mu-receptor binding by the SON at the end of pregnancy . In this respect we have found no evidence for tolerance to the effects of the mu-opioid agonist morphine on electrical AV3V stimulated OT release in pregnant rats . However AV3V stimulation probably excites OT neurones projecting from the paraventricular nucleus (Takana *et al.*, 1987) . An increase in the sensitivity of OT neurones in the paraventricular nucleus to mu-opioid agonists such as morphine could therefore mask any decrease in the sensitivity of SON neurones . With this interpretation, the results indicate lack of tolerance to endogenous opioids acting on OT neurones in pregnancy .

2.4.3 The effect of AV3V stimulation and naloxone on oxytocin secretion

Naloxone was found to significantly increase the OT response of virgin and pregnant rats to AV3V stimulation and to completely abolish the inhibitory effects of U50 and morphine . In all 25Hz experiments this enhancement was significantly greater in virgin rats compared to pregnant rats . The effect of naloxone in virgin rats was apparently not dependent upon time as a similar effect was obtained in the shortened protocol using only two stimulation periods (Fig 2.5) . These results indicate a difference in the endogenous opioid activity between virgin and pregnant rats . In conscious unstimulated virgin rats naloxone has no effect on OT secretion and under urethane anaesthesia naloxone does not potentiate the firing rate of magnocellular neurones (Leng *et al.*, 1992) . In addition naloxone does

not further enhance the firing rate of OT neurones in virgin rats following AV3V stimulation (Leng and Russell, unpublished data) . Therefore in the virgin rat, endogenous opioid activity appears to be restricted to the level of the posterior pituitary . However in the conscious unstimulated 21 day pregnant rat , naloxone increases plasma OT concentration (Hartman *et al.*, 1986b ; Leng *et al.*, 1988b) . There is presently a lack of evidence concerning the central effects of naloxone on the firing rate of OT neurones in pregnancy but it would appear that endogenous opioid tone increases near term and during parturition . This is accompanied by a significant reduction in mu-opioid receptor binding in the SON of 21 day pregnant rats and reduction of kappa-opioid receptor binding in the posterior pituitary (Sumner *et al.*, 1992) . *In vitro* electrically stimulated neural lobes from 21 day pregnant rats release greater amounts of OT than those from virgin rats, and the facilitating effect of naloxone is less on pregnant rat pituitaries (Douglas *et al.*, in press) . The reduced effectiveness of naloxone on the pituitaries from pregnant rats possibly reflects the reduced kappa-receptor density expressed by this tissue and reduced enkephalin content, since extended enkephalins, co-produced in OT neurones, act via kappa-receptors and would auto-inhibit OT secretion (Douglas *et al.*, in press) . This may also account for the increased release of OT in the absence of naloxone as release of co-localised opioids from magnocellular neurones may be less effective at attenuating OT release . Also at this time the OT content of the pregnant rat pituitary is increased and this too may contribute to the increased release of OT in the absence of naloxone (Leng *et al.*, 1988b) .

We have applied an electrical stimulation to a site more central than in the above experiments in both virgin and pregnant rats . The dose of naloxone used in the present study would be expected to be supra-maximal

for antagonising endogenous opioids acting at all opioid receptor subtypes, so that the OT system was essentially opioid free (Kosterlitz, 1985) . The effects of naloxone in the experiments presented here were similar to those obtained by Douglas *et al.* (in press) in that naloxone was more effective in enhancing the release of OT in virgin compared to 21 day pregnant rats . This may reflect the reduced kappa receptor binding of 21 day pregnant rat pituitaries as discussed above .

In contrast we have obtained a similar release of OT in the absence of naloxone after 25Hz electrical stimulation applied to the AV3V region in both virgin and pregnant rats . As the posterior pituitary of pregnant rats releases more OT than virgin rats when stimulated directly these results indicate that AV3V stimulation in pregnant rats was less effective at exciting OT release . Electrical stimulation of the AV3V region produces both excitatory and inhibitory effects on the firing rate of supraoptic OT neurones (Leng *et al.*, 1989) . A reduction in the excitatory input or an increase in the inhibitory input would produce the effects reported here . Clearly increased inhibitory endogenous opioid tone cannot be involved since naloxone had less effect in pregnant rats than in virgins .

2.4.4 Possible mechanisms involved in the decreased excitatory effects of AV3V stimulation on oxytocin neurones

There are dramatic morphological changes of magnocellular neurones in the supraoptic nucleus at this time in pregnancy, involving increased synaptic contact (Montagnese *et al.*, 1988 ; see also General Introduction (GI.3) of this thesis) . GABA innervation on oxytocin neurones also appears to undergo a degree of structural plasticity in the build up to lactation (Theodosis *et al.*, 1987) . GABA is an inhibitory transmitter on OT neurones

from the AV3V pathway and pre treatment of rats with intracerebroventricular (i.c.v.) GABA or nipecotic acid (a GABA uptake blocker) reduces or blocks vasopressin release in response to i.v. hypertonic saline and i.c.v. angiotensin II (Iovino *et al.*, 1983a and b ; Brenan *et al.*, 1984 ; Brenan and Haywood, 1985) . In addition both the SON and the PVN have high affinity GABA binding sites (Palacios *et al.*, 1981), in particular SON cells contain numerous GABA-immunoreactive axosomatic and axodendritic appositions and synapses (Theodosis *et al.*, 1986 ; Jhamandas *et al.*, 1989) . It has been subsequently estimated that up to 50% of synapses on SON cells may be GABAergic (Theodosis, 1988) . Structural re-organisation of the GABA input to SON oxytocin neurones from the AV3V region may therefore mediate the reduced excitatory effectiveness of electrical AV3V stimulation .

The inhibitory effects of GABA are mediated by two classes of receptors, the $GABA_A$ and $GABA_B$ receptors . The $GABA_A$ receptors are coupled to chloride channels which when opened produce inhibition in the postsynaptic target cell (Olsen, 1991) . Progesterone and its metabolites, produced in large amounts during pregnancy, interact with the GABA system to enhance the activity of $GABA_A$ receptors (Majewska *et al.*, 1986) . It is therefore possible that increased progesterone activity during pregnancy mediates increased inhibitory $GABA_A$ activity in response to AV3V stimulation .

Excitatory input from the AV3V region is also essential for the normal osmoresponsiveness of oxytocin neurones (Leng *et al.*, 1989) . Reduced excitatory and/or increased inhibitory input from the AV3V region to the magnocellular system would therefore be expected to result in an attenuated OT response to hyperosmotic stimulation in pregnancy . We hyperosmotically stimulated 21 day pregnant rats using intraperitoneal 1.5M

saline and found 21 day pregnant rats to respond significantly less than virgin rats in terms of plasma OT secretion (see Chapter 4) . This result therefore corroborates the hypothesis that the input from the AV3V region onto the SON is less excitatory during pregnancy .

Magnocellular neurones also receive excitatory amino acid (EAA) input from the AV3V region (see Introduction to this chapter) . By using the excitatory amino acid NMDA-receptor antagonist MK801 we attempted to evaluate the importance of this receptor type in mediating this input in virgin rats (Fig 2.6) . We used both 10Hz AV3V and i.p. hyperosmotic stimulation as methods of stimulating OT secretion . The response of the experimental group to the initial AV3V stimulation period in the presence of vehicle was not significantly different to the control group although the levels of plasma OT attained were alot lower . Such a difference in response to electrical stimulation between experimental and control groups prior to drug treatment does however limit the conclusions that can be drawn from this experiment .

Following the administration of i.v. MK801 (1mg/kg) in the experimental group and vehicle in the control group rats were exposed to another AV3V stimulation period of 10 minutes in duration . The effect was significantly reduced in both groups . However the plasma OT concentration of the MK801 treated group following subsequent AV3V stimulation was significantly lower than the control group . This implies that a small portion of the excitatory input from the AV3V region onto magnocellular OT neurones is mediated by NMDA receptors . The effect of i.p. hyperosmotic stimulation was however not affected by the presence of MK801 suggesting that this input is not involved in the response of OT neurones to hyperosmolality . An effect of EAA via other receptor types is therefore implicated, and this requires further study .

2.4.5 The effect of AV3V stimulation, morphine, U50,488 and naloxone on mean arterial blood pressure

Fig 2.7 depicts the changes in mean arterial blood pressure (MABP) in virgin and 21 day pregnant rats following AV3V stimulation, morphine and U50,488 . The mean basal blood pressure of virgin rats was measured at 83.6 ± 8.9 mmHg compared to pregnant rats which was significantly lower at 65.7 ± 2.9 mmHg . This probably results from a decrease in peripheral resistance perhaps related to increased blood flow to the uterus . Also during pregnancy there is an increase in the blood flow supplying the liver and other major organs to compensate for an increased metabolic activity in the rat . This results in a decreased peripheral resistance of vessels supplying the skin in order to dissipate the increased heat produced by the accelerated metabolic rate .

MABP remained stable during the first 10sec on phase of 25Hz AV3V stimulation but then fell significantly during the subsequent off phase to 53.9 ± 2.5 mmHg in the virgin rats and 51.9 ± 2 mmHg in the pregnant rats ($P < 0.0001$ paired t test) . MABP of both virgin and pregnant rats then followed a similar pattern for the rest of the stimulation period of rising during on phases and falling during off phases of the stimulation periods . A typical trace of changes in blood pressure during 25Hz AV3V stimulation is depicted in Fig 2.8 . In virgin rats MABP during the on phases was measured at 69.4 ± 3.1 mmHg which was significantly greater than the off phases at 56.8 ± 2.3 ($P < 0.0001$ paired t test) . The effect of 25Hz AV3V stimulation was the same in pregnant rats and during electrical stimulation was not significantly different to virgin MABP ($P > 0.1$ two sample t test) . MABP normalised within 3 minutes of the termination of the stimulation to levels not significantly different to basal in both virgin and pregnant rats .

The rapid increases and decreases (within 10 seconds) in blood pressure during electrical AV3V stimulation suggests that the effects are not mediated by vasopressin secretion . Magnocellular vasopressin neurones receive inputs from the A2 noradrenergic cell group in the caudal nucleus of the solitary tract (NTS) (Cunningham and Sawchenko, 1988) . A dense pathway extending from the caudal NTS and the adjacent reticular formation has also been described and verified as ending preferentially on OT neurones (Sawchenko *et al.*, 1988) . Neurones that convey information on blood pressure and volume are known to terminate in portions of the caudal NTS (Norgen, 1984) . It has therefore been proposed that the NTS could integrate information on blood pressure and volume and elicit the release of vasopressin and OT in a physiologically appropriate manner (Cunningham and Sawchenko, 1991) . Connections between the AV3V region and NTS have also been described (Hartle and Brody, 1984 ; Haywood *et al.*, 1987) . It is therefore likely that electrical stimulation of the AV3V region and subsequent magnocellular neurones produces fluctuations in blood pressure via these connections with the NTS .

Morphine at 1, 5 and 10 mg/kg produced a similar transitory decrease in the blood pressure of both virgin and pregnant rats . Morphine had no effect on the changes in blood pressure described during AV3V stimulation in both rat groups . Three minutes after the AV3V stimulation period (18 minutes from the administration of morphine) MABP of both pregnant and virgin animals had returned to levels not significantly different from basal . U50 had similar effects to morphine on the MABP of virgin and pregnant rats during AV3V stimulation . However MABP did not fully recover up to 21 minutes from the administration of U50 . Also in contrast to morphine, U50

was more effective in depressing the blood pressure of virgin than pregnant rats .

Naloxone tended to increase the blood pressure of rats that had received the exogenous opiates morphine and U50, had no effect in vehicle treated control groups and no effect on changes in blood pressure during AV3V stimulation . In addition opiate agonists had no effect on the changes in blood pressure obtained during electrical stimulation despite having profound effects on OT secretion . Therefore a central inhibitory action of exogenous opioids on the projections from the AV3V region and magnocellular neurones to the NTS seems unlikely . It therefore seems probable that the transitory effects of opiates on MABP are mediated by peripheral opioid receptors either directly on vasculature muscle or on innervation controlling their contractile state.

2.6 Conclusions

In conclusion the present experiments show that electrical stimulation of the AV3V region in virgin and 21 day pregnant urethane-anaesthetised rats produces similar increases in plasma OT concentration . Posterior pituitaries of 21 day pregnant rats *in vitro* however release more OT than equivalently stimulated pituitaries from virgin rats (Douglas *et al.*, in press) . In addition when endogenous opioid actions are antagonised with naloxone, the increase in plasma OT concentration at high frequency AV3V stimulation was greater in virgin than pregnant rats . These results indicate that in pregnant rats the AV3V input is less effectively coupled to OT neurones .

Morphine, a mu-opioid agonist, inhibition of OT secretion in response to electrical AV3V stimulation was frequency dependent and this probably results from its mode of action . U50,488, a kappa-opioid agonist, however was effective at inhibiting OT secretion in response to high frequency AV3V stimulation . Both morphine and U50,488 were equally effective in virgin and pregnant rats indicating there is no change in the sensitivity of central mu- and kappa-opioid mechanisms on OT neurones in pregnancy .

Following electrical stimulation of the AV3V region we have reported preliminary results which suggest that part of the input from this region on to OT neurones may be mediated via excitatory amino acid activity at NMDA receptors . However this requires further investigation before a conclusion can be drawn . In contrast we found no evidence for activation of this input following hyperosmotic saline stimulated OT release in virgin rats .

CHAPTER 3

Where are the osmoreceptors regulating oxytocin neurones ?

3.1 INTRODUCTION

Pioneering work by Verney (1947) identified the importance of the hypothalamus in body fluid osmoregulation . Jewell and Verney (1957) suggested that osmoreception in the mammalian brain occurs in the region of the anterior hypothalamus . Mouw and Vander (1970) some time later reported that perfusion of the cerebral ventricles in the rat with low-sodium fluids reduced sodium excretion without altering other renal haemodynamic parameters . Conversely, increasing the sodium concentration of the cerebrospinal fluid in the third ventricle potentiated sodium excretion in the rat (Dorn and Porter, 1970), dog (Dorn *et al.*, 1969) and sheep (Mckinley *et al.*, 1973) . It has also been shown that the natriuresis associated with infusions of hypertonic saline, dehydration or feeding could be attenuated by the concomitant lowering of cerebrospinal fluid sodium concentration (Olsson, 1973 ; Leksell *et al.*, 1981 and 1982 ; McKinley *et al.*, 1983 and 1987) . In addition Chodobski and McKinley (1989) reported that intravenous sodium loads within the physiological range in the sheep produced a five fold increase in the rate of natriuresis that was independent of associated increases in blood volume . They found that this facilitated sodium excretion could be blocked by lowering the CSF sodium concentration . The natriuresis reported may be due at least in part to an increase in both OT and vasopressin secretion found to occur following the icv administration of hypertonic solutions in the conscious rat (Hattori *et al.*, 1988 ; Wells *et al.*, 1990) .

Considerable evidence now implicates the brain in the renal regulation of sodium excretion . Both lesioning and electrophysiological studies have revealed a number of osmoreceptive sites within circumventricular organs and the concept of an osmoreceptor complex

involving the subfornical organ (SFO), the region anterior and ventral to the third ventricle (AV3V region), which consists of the median preoptic nucleus (MnPO or MPN) and organum vasculosum of the lamina terminalis (OVLT), and the magnocellular neurones themselves in the supraoptic and paraventricular nuclei (Leng *et al.*, 1982) .

3.1.1 The subfornical organ

The involvement of the subfornical organ in body fluid homeostasis is mainly reported in connection with angiotensin II stimulated drinking responses in the rat, cat, dog and Japanese quail (Felix, 1976, Hoffman and Phillips, 1976 ; Takei, 1977 ; Simpson *et al.*, 1978 ; Felix and Schlegel, 1978 ; Mangiapane and Simpson, 1979 and 1980a ;Thrasher *et al.*, 1982) . Studies have identified the perifornical zone of the lateral hypothalamus, the rostral zona incerta and the nucleus reuniens of the thalamus as a source of angiotensin II containing neuronal inputs to the SFO (Lind *et al.*, 1984a) . Scanning and transmission microscopy have distinguished three regions in the rat SFO, the rostral, central and caudal regions . The central region occupies the largest area and contains most of the neuronal perikarya and a dense capillary network with wide precapillary spaces permeates the tissue (Dellmann and Simpson, 1976) . These results combined with those of infusion studies have led to the proposal that the SFO contains dipsogenic receptors for circulating and centrally released angiotensin II (Simpson *et al.*, 1978) .

Specific angiotensin II receptive neurones have been reported in the cat (Phillips and Felix, 1976), and in the rat 52% of SFO neurones showed facilitated activity in the presence of electrophoretically applied angiotensin II (Ishibashi *et al.*, 1985) . Participation of beta-adrenergic and muscarinic-

cholinergic receptors in the thirst inducing effects of angiotensin II have also been demonstrated (Menai *et al.*, 1984 ; Saad *et al.*, 1985) . In addition the SFO has been proposed as a receptor site for atrial natriuretic peptide where it strongly depresses angiotensin II excitation of SFO neurones and may therefore act as an inhibitory neuromodulator of SFO activity (Hattori *et al.*, 1988 ; Yamashita *et al.*, 1989) .

Electrical stimulation of the SFO in the rat not only stimulates drinking behaviour but also increases femoral arterial blood pressure (Ishibashi and Nicolaidis, 1981 ; Robertson *et al.*, 1983) . The increase in blood pressure may be accounted for in part by the report of direct neural projections between the nucleus of the solitary tract (NTS) and the SFO in the rat (Zardetto-Smith and Gray, 1987) . Electrophysiological, degenerative and tracer studies have also revealed both direct projections of the SFO to the supraoptic and paraventricular neurosecretory neurones and indirect projections synapsing in the AV3V region (Carithers *et al.*, 1980 ;Lind and Johnson, 1982 ; Renaud *et al.*, 1983 ; Yamashita *et al.*, 1984 ; Ferguson *et al.*, 1985) . The importance of these connections for the dipsogenic and pressor effects associated with SFO stimulation was reported by Gutman *et al.* (1988a and b) . They found that bilateral chemical lesion of the paraventricular nuclei abolished the drinking response and attenuated the increase in blood pressure normally associated with the microinjection of angiotensin II into the SFO of the unrestrained rat .

Predictably both peripheral and central angiotensin II administration stimulates magnocellular neurone activity and increases plasma vasopressin and OT concentration (Lang *et al.*, 1981 ; Iovino and Steardo, 1984 ; Ferguson *et al.*, 1990) . Lesions or local anaesthesia of the SFO impair the osmotically stimulated release of vasopressin and OT in addition to

attenuating the drinking response to hyperosmotic stimulation (Saad *et al.*, 1979 ; Mangiapane and Simpson, 1980b ; Lind *et al.*, 1984b ; Leng *et al.*, 1989 ; Tanaka *et al.*, 1989) . There is evidence that some SFO neurones can respond directly to osmotic stimuli *in vitro,* and may be inhibited or excited by increased extracellular osmolality (Buranarugsa and Hubbard, 1988 ; Sibbald *et al.*, 1988) . In contrast the bolus intracarotid injection of 0.5M NaCl *in vivo* has been reported to only excite SFO neurone activity (Gutman *et al.*, 1988a) . This may reflect an SFO response to a change in blood volume and/or pressure, via neuronal NTS connections, as a result of the injection rather than a demonstration of the innate osmosensitivity of these neurones . Similarly glucose utilisation in the brain measured by autoradiographic assessment of the metabolic trapping of intravenously administered radioactive [1-^{14}C]glucose during two days of water deprivation, revealed a 74-103% increase in uptake of SON and PVN magnocellular neurones but no apparent change in the OVLT or SFO (Duncan *et al.*, 1989) . In addition Dyball and Leng (1989) reported that 6 out of 6 cells tested in the SFO failed to respond to the intraperitoneal injection of hypertonic saline which has no effect on blood volume but produces a prolonged increase in magnocellular neurone firing rate . The role of the SFO as an osmoreceptor is therefore controversial and we have attempted to provide further evidence for this role by the discrete infusion of hyperosmotic saline into the SFO of virgin female rats followed by measurements of the plasma OT response .

3.1.2 The AV3V region

Hypothalamic lesions in the rat have been reported to produce alterations in food, water and sodium chloride intake depending upon the

extent and location of the lesion (Smith and McCann, 1962 ; Covian and Antunes-Rodrigues, 1963) . The electrolytic lesion of tissue in the AV3V region of the dog results in adipsia and chronically increased plasma sodium concentration leading to severe dehydration (Buggy and Johnson, 1977 ; Brody *et al.*, 1978) . Consequently structures in this region, which includes the OVLT, have been proposed as osmoreceptors (Phillips, 1978) . Thrasher *et al.* (1982) also reported that dogs with discrete lesions of the OVLT have greatly diminished vasopressin responses to osmotic stimuli over a reasonably physiological range . Ramsay, Thrasher and Keil (1983) subsequently concluded that the OVLT was the anatomical home of the osmoreceptors . In addition Thrasher and Keil (1987) reported that lesions destroying greater than 90% of the OVLT dramatically increased the osmotic threshold at which drinking and vasopressin secretion are stimulated, and significantly reduced the magnitude of these responses to osmotic stimuli .

As mentioned in the previous section, the AV3V region contains many fibres of passage and synapses, within the median preoptic nucleus, relaying information from the SFO to the supraoptic and paraventricular nuclei . This has led to the suggestion that the AV3V region, and in particular the OVLT, function to integrate information derived from blood-borne angiotensin II and vascular pressure/volume receptor inputs from the nucleus tractus solitarius with innate osmoreceptive activity . The result of this integration may be important for the generation of thirst as a result of dehydration (Johnson, 1985) . In association with volume regulation, a functional connection has been reported between the AV3V region and renal nerves modulating renal excretion during changes in plasma volume (Huang *et al.*, 1988) .

Horseradish peroxidase tracer studies have demonstrated that the OVLT consists of two functionally different divisions . Both parts possess fenestrated capillaries which differ in their permeability for peroxidase with only one portion being labelled after i.v. injection (Krisch *et al.*, 1987) . Also the intravenous infusion of ^{125}I-labelled angiotensin II has been found to result in high ^{125}I-angiotensin II concentrations in the SFO and OVLT but not in the SON (Ramsay *et al.*, 1983) . The leaky capillary network of the OVLT therefore would imply that it is a prime candidate for an osmoreceptive site . *In vitro* electrophysiological studies have revealed osmosensitive neurones in the OVLT in brain slices taken from the rat . These neurones demonstrated increased activity in response to raised osmolality of the bathing medium even when synaptic transmission was prevented by a high magnesium concentration (Vivas *et al.*, 1990) . Also *in vivo* studies have reported dose related osmosensitive neurones in the AV3V region, and numerous projections from this region to both the supraoptic and paraventricular nuclei (Honda *et al.*, 1987 ; Chaudhry *et al.*, 1989) . However it appears that the neuronal interactions of the AV3V and magnocellular neurones are more complex than a simple osmosensitive excitatory drive . Chaudhry *et al.* (1989) reported that OVLT cells identified as projecting to the SON by antidromic activation were never themselves osmosensitive . Indeed Leng (1982) ascribed the osmoresponsiveness of the AV3V region to an indirect synaptically-mediated effect of activation of the magnocellular system . A projection from the SON to the AV3V which then extends back to the SON via the MPN has been reported, and the osmosensitive elements within the AV3V have been antidromically identified as projecting to the MPN (Honda *et al.*, 1990) . It may therefore be the case, that the important osmosensitive cells in the AV3V region are those that are involved in this

projection . We have attempted to further clarify the role of osmosensitive cells in the AV3V region in the regulation of OT secretion, by the hyperosmotic stimulation of this region with the discrete infusion and microdialysis application of hyperosmotic saline (Fig 3.1 and 3.2) .

3.1.3 The magnocellular oxytocin and vasopressin neurones

Electrophysiological studies using extracellular recordings have revealed that, in terms of spike frequency, the most osmosensitive cells yet recorded are those of the magnocellular SON (Poulain and Wakerley, 1982) . *In vitro* slice preparation reports indicate that cells lying in the SON respond to changes in osmotic stimuli that are within the physiological range (Mason, 1980 and 1983 ; Abe and Ogata, 1982 ; Bourque and Renaud, 1984 ; Bourque 1989) . *In vivo*, increased magnocellular neurone activity has been described in urethane anaesthetised rats in response to the intraperitoneal injection of hypertonic solutions of NaCl, LiCl and mannitol, to the intra-atrial injection of 1M NaCl, the substitution of normal drinking water with 2% saline and the direct application of hypertonic saline to the cell bodies (Brooks *et al.*, 1966 ; Dyball and Poutney, 1971 and 1973 ; Brimble *et al.*, 1978 ; Leng, 1980) . Correspondingly plasma OT concentration increases following peripheral and central administration of hyperosmotic saline (Brimble *et al.*, 1978 ; Hattori *et al.*, 1988 ; see also Chapter 4) . Interestingly Cheng and North (1986) concluded that OT release was greater than vasopressin release in response to acute increases in plasma osmolality when they measured plasma-associated neurophysin concentrations in acute salt loaded rats . The electrophysiological evidence therefore appears to indicate that the magnocellular OT and vasopressin secreting neurones are a major osmoreceptive site in the proposed osmoreceptor complex .

Immunohistochemical investigations using *in situ* hybridisation and immunoflourescence histochemistry have revealed an alteration in the OT and vasopressin mRNA content in the neurohypophysial system within 24 hours of the start of 2% saline ingestion (McCabe *et al.*, 1990) . In addition after 2 weeks of 2% saline loading there is a 200% increase in the hypothalamic magnocellular neuropeptide mRNA content (Van Tol *et al.*, 1987 ; Meiser *et al.*, 1990) . Associated with this is a decrease in the neuropeptide concentration of the supraoptic and paraventricular nuclei (Dellman *et al.*, 1988) . In addition increased levels of the nucleotide second messenger cAMP have been reported both in vivo and in vitro following 2% saline drinking for two days and in punched hypothalamic nuclei challenged with 290 to 310 mOsm/kg bathing medium (Carter and Murray, 1989) . Expression of Fos, the protein product of the protooncogene c-fos, has been shown to increase in neurones when they are stimulated by a number of procedures including electrical stimulation and neurotransmitter application (Dragunow and Faull, 1989 ; Morgan and Curran, 1989) . Fos expression has therefore been used as an indicator of neuronal activity and has been reported to increase in the supraoptic and paraventricular nuclei magnocellular neurones in response to dehydration or i.p. injection of hyperosmotic saline (Morgan *et al.*, 1987 ; Ceccatelli *et al.*, 1989 ; Giovannelli *et al.*, 1990 ; Hamamura *et al.*, 1992) . The consensus of molecular and electrophysiological information could therefore be interpreted as indicating that the magnocellular neurones of the supraoptic and paraventricular nuclei are key osmoreceptive elements, or simply that these neurones are driven by osmoreceptors elsewhere .

Fluorescent retrograde tracer studies have revealed that the SON receives projections from the SFO, median preoptic nucleus and OVLT

(Wilkin, 1989 ; Swanson *et al.*, 1980) . These findings are supported by electrophysiological investigations which provide evidence of a complex interaction between the SON and the AV3V region of the rat associated with osmotic regulation (Ingram, 1988 ; Chaudhry *et al.*, 1988 ; Honda *et al.*, 1990 ; Takano *et al.*, 1990)

Rats with electrolytic lesions of the AV3V region show little spontaneous SON activity and their responses to the i.p. injection of hypertonic saline are also impaired . Corresponding deficits are also reported in the secretion of OT and vasopressin in response to the hypertonic stimulus (Leng *et al.*, 1989) . The integrity of the AV3V region input is therefore crucial not only for the normal OT and vasopressin response to osmotic stimulation but also for the expression of any innate osmoreceptiveness of the magnocellular neurones .

Other data indicate that the medial nucleus tractus solitarius excites the supraoptic vasopressin cells via an adrenergic input (Day and Sibbald, 1988) . Afferent renal nerve stimulation also excites supraoptic vasopressin neurones (Day and Ciriello, 1985) . It would therefore appear that not only are the magnocellular neurones candidate osmoreceptors concerned with fluid balance, but may also be important in the co-ordination of neuronal information in the regulation of arterial pressure, both via vasopressin secretion and by the neuronal connections described above .

We have attempted to provide further evidence for the osmoreceptor sensitivity of supraoptic OT neurones to central osmotic stimulation by the direct microdialysis application of hypertonic saline to the SON (Fig 3.3) . In addition we have further investigated the role of the AV3V region input onto the OT neurones in this response by 'clamping' its activity with an icv infusion of the angiotensin II antagonist saralasin . Icv saralasin silences

supraoptic OT neurone activity and abolishes their response to i.p. 1.5M saline . However supraoptic neurones are still excited by electrical stimulation of the AV3V and SFO, so it appears that circulating angiotensin II acts on SFO and or AV3V region receptors to stimulate OT release (Leng *et al.*, 1989) . As OT neurones fail to respond to hyperosmotic stimulation in the absence of an excitatory drive from the AV3V region we have replaced the silenced input by electrically stimulating the SFO at a constant rate . By then administering an intraperitoneal hyperosmotic stimulus it was hoped to demonstrate facilitated OT neurone activity demonstrated as increased plasma OT release resulting from the direct osmoreceptiveness of the magnocellular neurones themselves (Fig 3.4) .

METHODS

3.2.1 Procedures

Virgin female Sprague-Dawley rats were used, and housed prior to experimentation under our standard laboratory conditions (see General Methods section, GM2 Rats) . On the day of experimentation rats were anaesthetised with intraperitoneal urethane (1.25g/kg body weight) and a femoral artery and vein cannulated for blood sampling and drug administration purposes . The blood sampling and OT radioimmunoassay procedure are described in the General Methods section (GM1, GM3, GM4)

.

The following experimental procedures were carried out :-

3.2.2 Experiment 3.1: SFO and MPN cannulation .

Experiments 3.1 involved the infusion of hypertonic and isotonic saline into discrete brain regions and required the stereotaxic implantation of a 28 gauge stainless steel cannula (C131I Plastic Products Co.) . Following vessel cannulation rats were positioned in a stereotaxic frame (Stoelting) and the dorsal surface of the skull exposed by a midline skin incision . The rat's skull was then levelled between bregma and lambda . The infusion cannulas used, were to be inserted in the midline which would have punctured the sagittal sinus which runs along the surface of the brain . Due to the relatively large bore of the cannula used this would have caused a significant amount of blood loss . To avoid this the sinus was displaced to one side whilst inserting the cannula and then the sinus was allowed to return to as near its normal position as possible . This procedure involved burring away the skull with a dental drill along the midline for about 2mm either side of where the cannula was to be inserted . Using a dissecting

microscope this produced a rectangular hole in the skull of the rat of approximately 2 x 4mm in size . The surface membranes of the skull surrounding the sagittal sinus were then separated from the sinus using the sharp end of a 26G hypodermic syringe needle . The sagittal sinus could then be carefully displaced to one side, with a 50mm length of wire shaped in a U at one end, without ripping the vessel open . The stainless steel guide cannula, cut so it's tip was 1mm above the target site when positioned, was then lowered into either the subfornical organ (target site, 0.92mm posterior to bregma, 5.8mm below the skull surface in the midline) or the median preoptic nucleus (target site, 0.8mm posterior to bregma, 7mm below the skull surface in the midline) through the burr hole in the skull . A stainless steel screw was also inserted into the skull near to the guide cannula as an anchorage point to secure the infusion cannula in place . Once in place the guide cannula was fixed in position with dental acrylic which also covered the exposed screw head . When the acrylic had set the rats were removed from the frame and left for approximately two hours before starting the experiment .

3.2.3 SFO and MPN infusion

The experimental protocol of Fig 3.1 involved the infusion of 0.2M hypertonic and 0.15M isotonic saline at 26nl/min for ten minutes each separated by a ten minute recovery period . The flow rate was accomplished using 1μl glass Hamilton syringes mounted on to a slow infusion pump (B.Braun perfusor mark 6, B.BraunMelsungen AG, Germany) which were then connected to the infusion cannula by equal lengths of polythene tubing . The order in which the rats received the hypertonic and isotonic infusions was randomised such that half the rats received the hypertonic infusion first

and half the isotonic infusion first . This protocol was used to try to account for changes in plasma OT produced by mechanical damage of neuronal tissue as a result of fluid infusion . After the infusion protocol an i.p. injection of 1.5M saline (4ml/kg) was given to test the integrity of the system and responsiveness to a known hyperosmotic challenge . At the end of the experiment the rats were injected with 1μl of 1% Evans blue solution through the brain infusion cannula to enable verification of the cannula position . Brains were then removed and fixed in 10% formal saline for at least 2 days . After fixation the brains were processed, by cutting frozen sections, and the locations of the blue spots mapped as in the General Methods (section GM5.2) (see Fig 3.A1 and 3.A2) .

3.2.4 Experiment 3.2: Microdialysis application of hyperosmotic saline to the AV3V region .

Microdialysis application of hypertonic saline required the insertion of microdialysis probes (made in the Biological Sciences Section of the University of Leipzig) consisting of a U shaped dialysis fibre (molecular weight cut-off 6,000 Da, 3mm in length) glued on to the ends of parallel lengths of 24 gauge stainless steel tubing . The microdialysis fibre was supported by a length of fine stainless steel wire threaded through it to strengthen it . For the experiments depicted in Fig 3.2, probes were stereotaxically inserted into the AV3V region (0.3mm posterior to bregma, 8.3mm below the skull surface in the midline) of the rat brain . The surface of the brain was exposed and the sagittal sinus displaced prior to the insertion of the probe, as in the previous experiment, before being secured in place with dental acrylic anchored to a stainless steel screw fixed in the skull .

Microdialysis probes were perfused with artificial cerebrospinal fluid (aCSF, pH 7.2, composition in mM : 138 NaCl, 3.36 KCl, 9.52 $NaHCO_3$, 0.49 Na_2HPO_4, 2.16 urea, 1.26 $CaCl_2$, 1.18 $MgCl_2$) at a flow rate of 3μl/min using a slow infusion pump fitted with 1ml glass Hamilton syringes . Hypertonic aCSF (as above except 1M NaCl) was perfused through the probes for 20 minutes in the experiments shown in Fig 3.2 and 30 minutes in the experiment in Fig 3.3 .

At the end of the experiment the microdialysis probes were removed from the skull and the brains fixed for 2 days in 2% formal saline . After fixation the brains were coronally sectioned, stained, mounted (see General Methods, GM5) and the position of the microdialysis probes mapped onto a series of drawings produced in accordance with a rat brain atlas (see FIG 3.B) .

3.2.5 Experiment 3.3: Electrical stimulation of the AV3V region followed by bilateral microdialysis application of hyperosmotic saline in the supraoptic nuclei .

For the experiments in Figure 3.3 two microdialysis probes were inserted into every rat : one in each supraoptic nucleus (1mm posterior to bregma, 1.7mm lateral and 9.1mm below the skull surface) through two burr holes in the dorsal surface of the skull . Also in this experiment a stainless steel stimulating electrode (SNEX 100, Clarke Electromedical Instruments) was implanted in the AV3V region (1mm posterior to bregma, 1.7mm lateral and 9.1mm below the skull surface, see Chapter 2) through a separate burr hole in the skull . The electrode and both microdialysis probes were then fixed in place with dental acrylic and secured with two stainless steel screws anchored in the skull . Electrical stimulation of the AV3V region (matched

biphasic pulses, 0.5mA peak to peak, 1msec duration, 10sec on/off at 10Hz for 30 minutes) was performed according to the method of and using the same stimulating set up as in Chapter 2 .

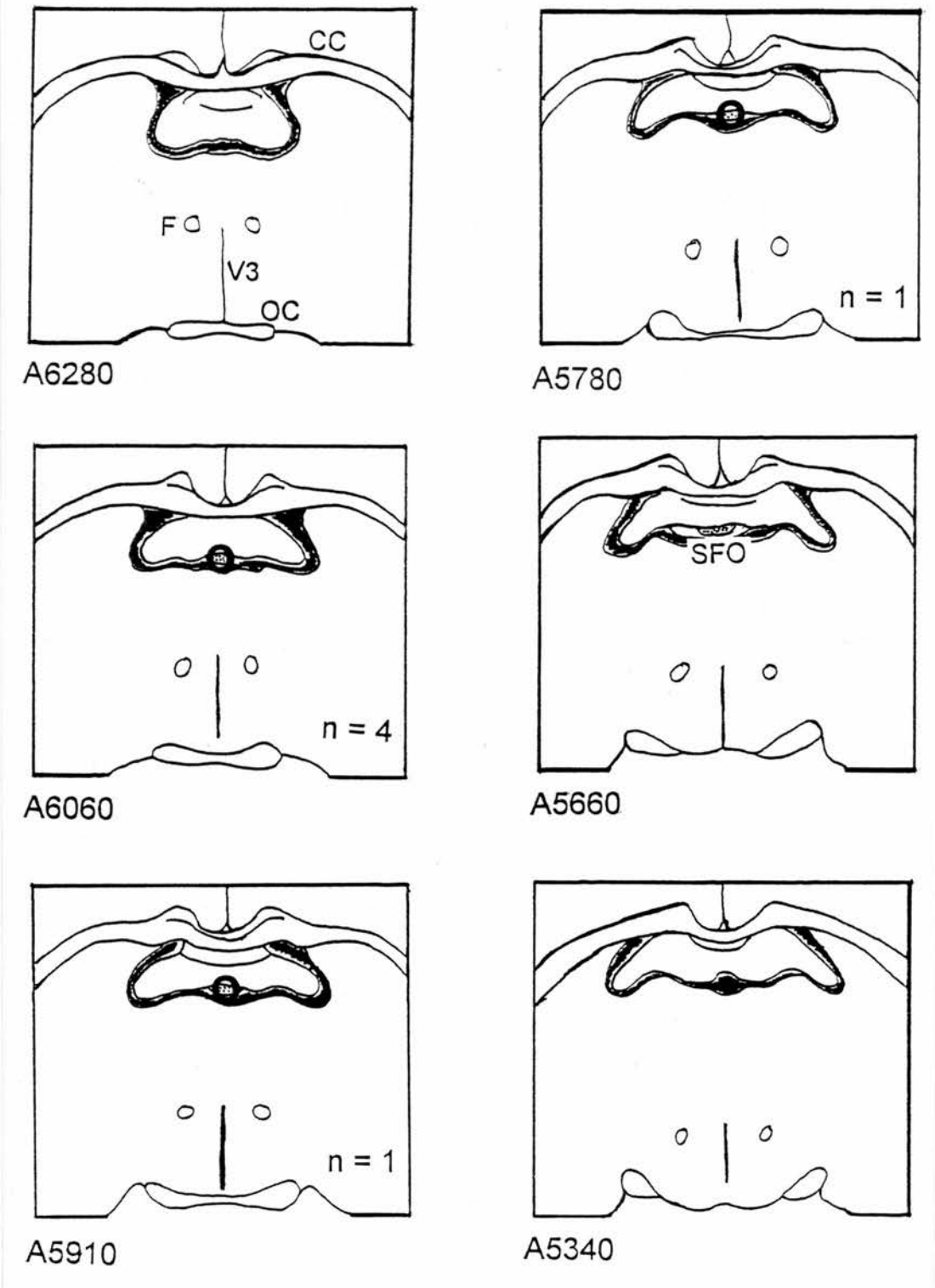
At the end of the experiment a continuous train of electrical pulses was passed (2.5mA, 0.5msec duration, monophasic pulses at 100Hz for 1 min) to deposit Fe^{2+} ions from the electrode tip which were later visualised using the Prussian Blue reaction . The microdialysis probes were then removed and the brain fixed in 10% formal saline containing 3% potassium ferro- and ferri-cyanide . After at least two days the brains were then sectioned, mounted and counterstained . The positions of the electrode tip (see Chapter 2) and microdialysis probes were then mapped on to a series of drawings produced in accordance with a rat brain atlas (see also General Methods, GM5) (Fig 3.C) .

3.2.6 Experiment 3.4: Electrical stimulation of the SFO and icv infusion of saralasin .

Electrical stimulation of the SFO was performed in the same way as electrical AV3V stimulation (experiment 3.3), except different co-ordinates were used for placement of the electrode (0.92mm posterior to bregma, 5.8mm below the skull surface in the midline) .

A similar method and cannulae were employed for intracerebroventricular (icv) cannulation as that for the infusion of brain tissue (experiment 3.1) . With the skull exposed and levelled in the stereotaxic frame a hole was drilled through the skull (1.6mm lateral, 0.6mm posterior to bregma) of a sufficiently large enough bore to allow the cannula to be lowered 4mm below the skull surface . This was then secured in place with dental acrylic anchored to a screw mounted in the skull .

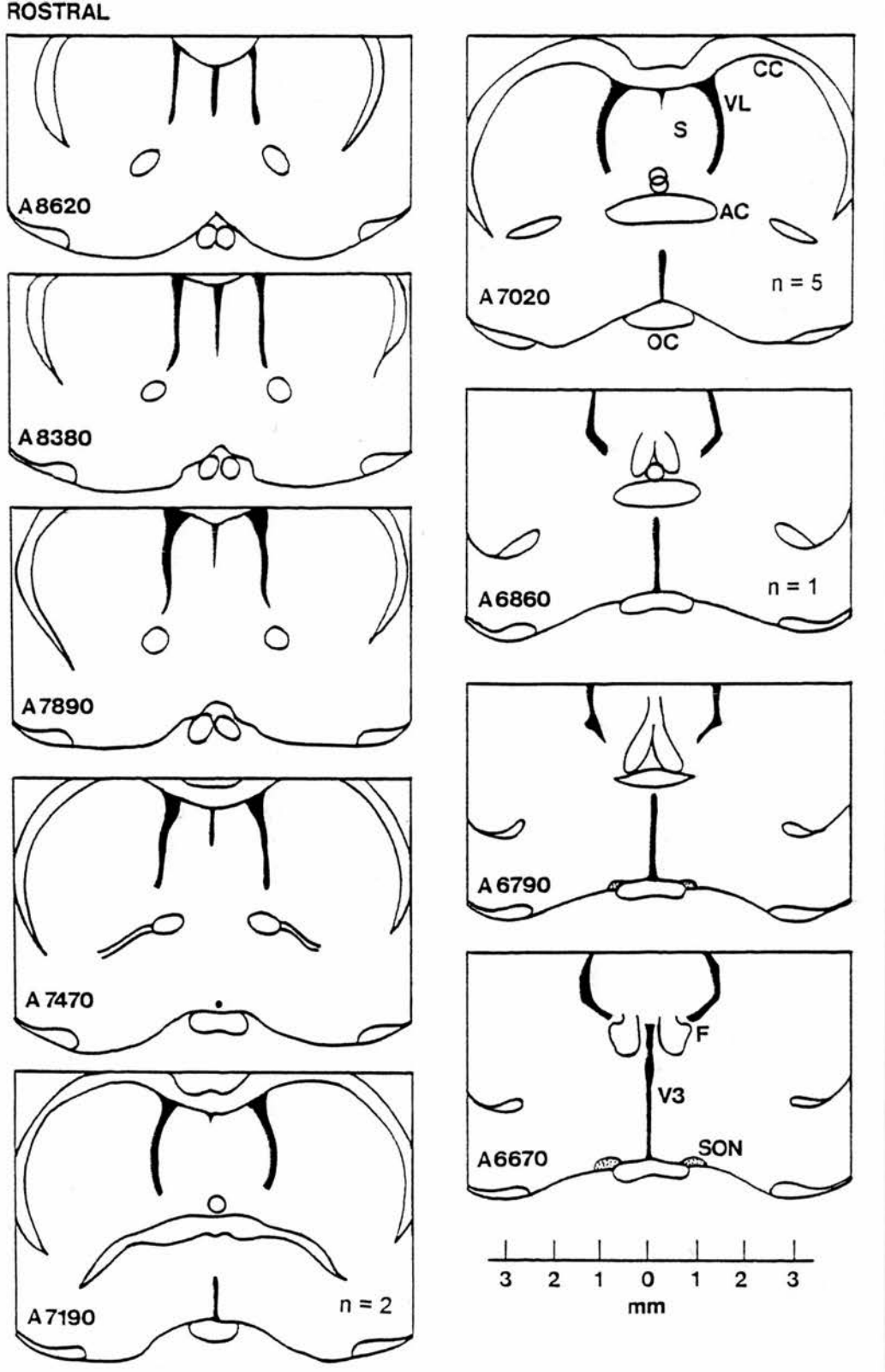
Icv infusion of the angiotensin II antagonist saralasin (2.5μg/min for 80 minutes at 0.5μl/min) was performed with the slow infusion pump (Braun Perfusor 6) fitted with 100μl glass Hamilton syringes connected to the cannula with equal lengths of polythene tubing . At the end of the experiment brains were removed post mortem and the cortex coronally sectioned by hand in the region of the infusion site . The cortex was then inverted and examined for the penetration of the infusion cannula tip into the lateral ventricle



O = location of blue spots produced by the introduction of Evans Blue into the rat brain through the infusion cannulae at the end of each experiment . Brains were then fixed for 2 days and subsequently sectioned, mounted in sequence and counterstained before the location of the blue spots were mapped on to a series of drawings produced in accordance with a rat brain atlas (Konig and Klippel, 1963) .

CC corpus callosum, F fornix, SFO subfornical organ, OC optic chiasm and V3 third ventricle .

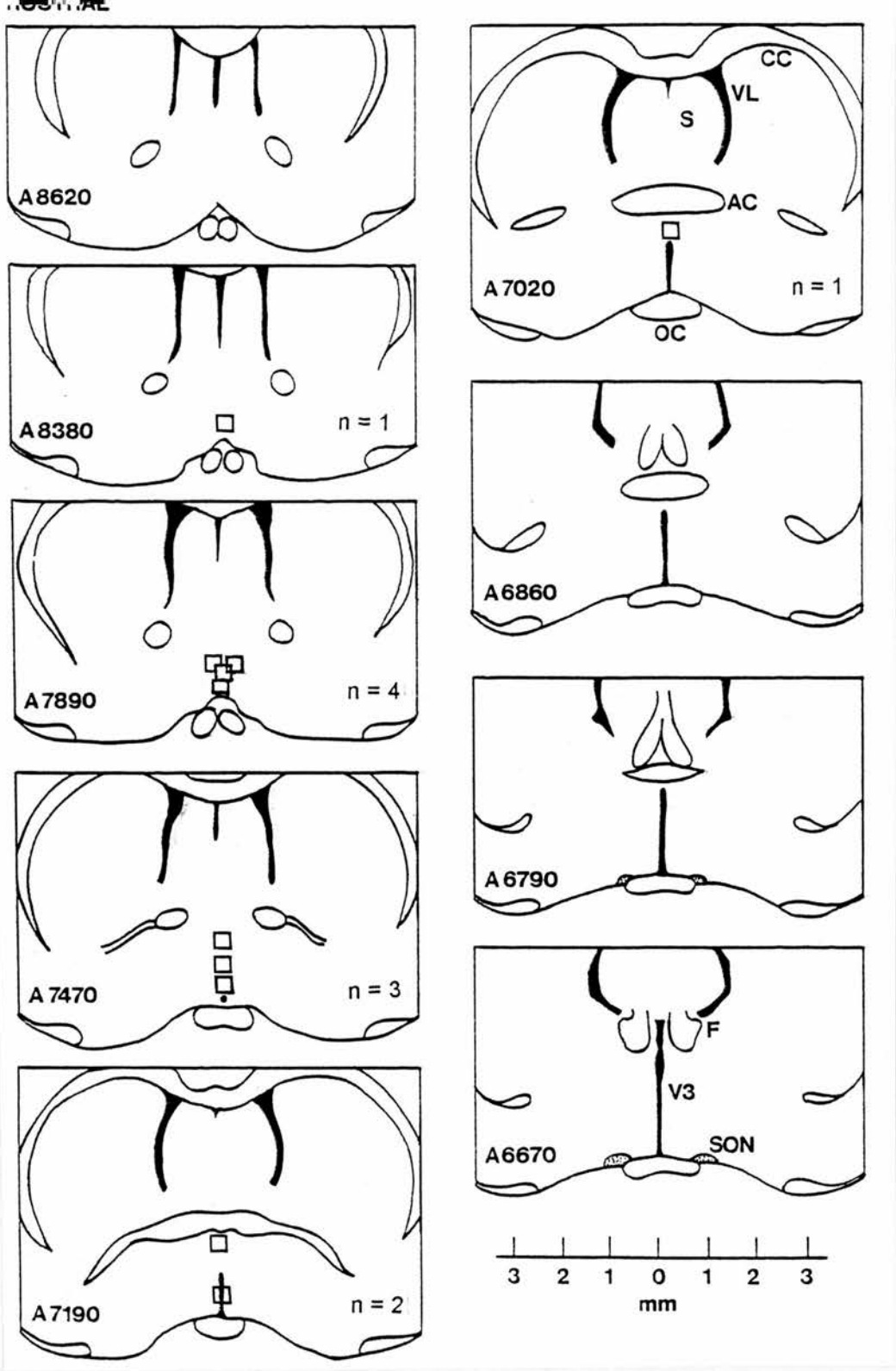
FIGURE 3.A1 Positions of infusion cannula within the subfornical organ



O = location of blue spots produced by the introduction of Evans Blue into the rat brain through the infusion cannulae at the end of each experiment . Brains were then fixed for at least 2 days and latter sectioned, mounted in sequence and counterstained before the location of the blue spots were mapped on to a series of drawings produced in accordance with a rat brain atlas (Konig and Klippel, 1963) .

AC anterior commissure, CC corpus callosum, F fornix, OC optic chiasm, S septum, SON supraoptic nucleus, VL lateral ventricle and V3 third ventricle .

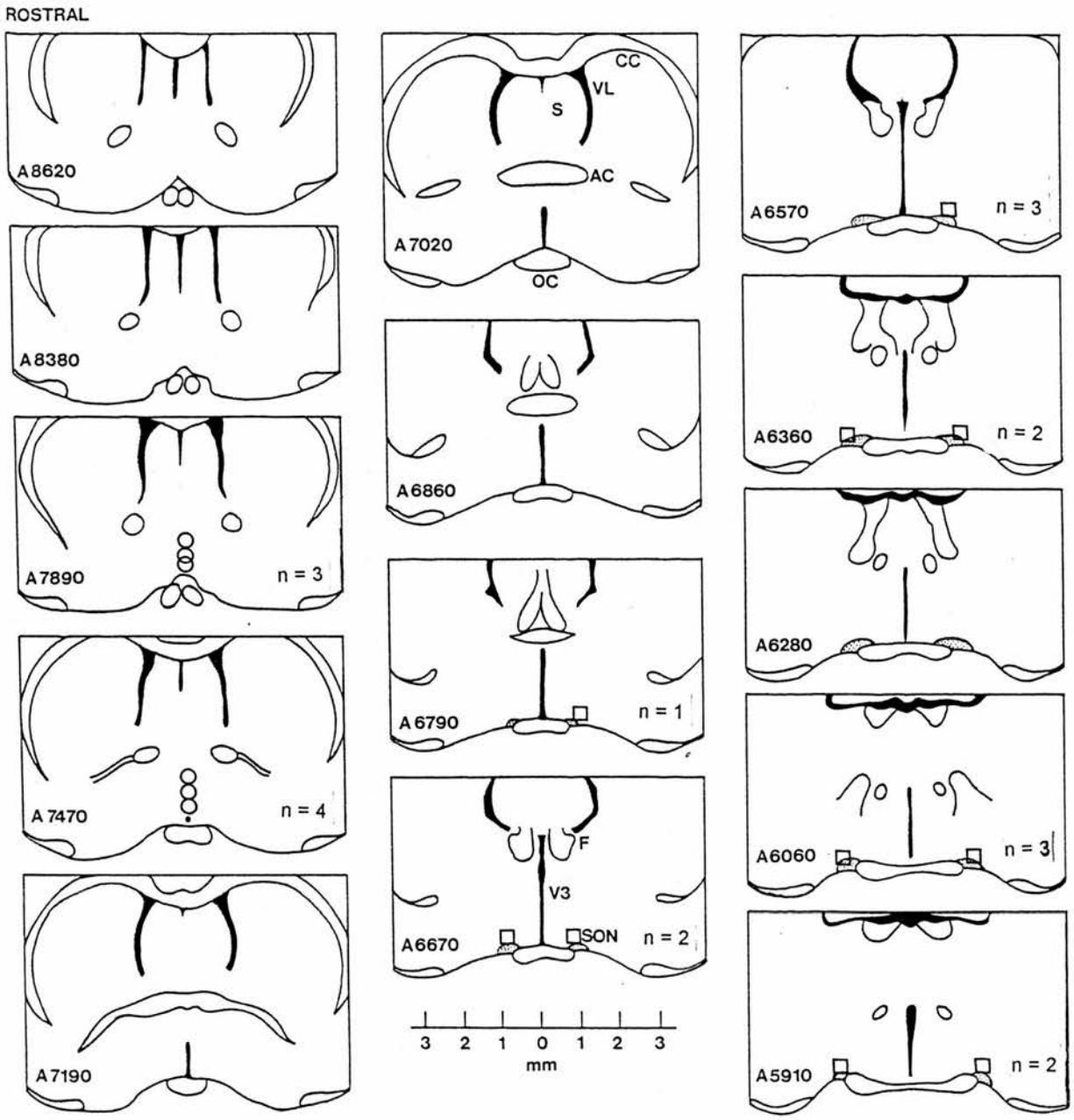
FIGURE 3.A2 Positions of infusion cannula within the median preoptic nucleus



□ = location of microdialysis probes within the AV3V region assessed from the tissue destruction caused by their insertion . At the end of each experiment the probes were removed and the brains fixed for at least 2 days and then sectioned, mounted in sequence and counterstained before the location of the probes were mapped on to a series of drawings produced in accordance with a rat brain atlas (Konig and Klippel, 1963) .

AC anterior commissure, CC corpus callosum, F fornix, OC optic chiasm, S septum, SON supraoptic nucleus, VL lateral ventricle and V3 third ventricle .

FIGURE 3.B Positions of microdialysis probes within the region anterior and ventral to the third ventricle (AV3V region) .



❑ = location of microdialysis probes within the SON assessed from the tissue destruction caused by their insertion (n = number of probes) . ❍ = electrode positions visualised by use of the Prussian Blue reaction . At the end of each experiment the probes and electrodes were removed and the brains fixed for 2 days before being sectioned, mounted in sequence and counterstained . The location of the probes and blue spots were then mapped on to a series of drawings produced in accordance with a rat brain atlas (Konig and Klippel, 1963) .

AC anterior commissure, CC corpus callosum, F fornix, OC optic chiasm, S septum, SON supraoptic nucleus, VL lateral ventricle and V3 third ventricle .

FIGURE 3.C Positions of microdialysis probes within the supraoptic nuclei and stimulating electrodes in the AV3V region .

3.3 RESULTS

3.3.1 Change in plasma oxytocin concentration from basal after hypertonic and isotonic infusions into discrete rat brain regions . (Fig 3.1)

Hypertonic saline infusion into the SFO and off target control areas produced no significant change in the plasma OT concentration of anaesthetised virgin female rats . When this same infusion was applied in the MPN however there was a small but non-significant increase in the plasma OT concentration . An equivalent volume of isotonic saline produced no significant changes of plasma OT concentration in any of the three groups . Ten minutes after the infusion protocol was terminated i.p. hyperosmotic saline produced a significant increase in the plasma OT concentration in the control and MPN infused groups ($P < 0.05$ paired t test) .

LEGEND : FIGURE 3.1

Change in mean femoral arterial plasma OT concentration, ± s.e.m., in urethane anaesthetised virgin female rats with ten minute infusions of 260nl of 0.2M hypertonic and 0.15M isotonic saline into either the SFO (❍, n = 6) or MPN (□, n = 8), with off target rats forming the control group (Δ, n = 6) . At 40 minutes into the experiments rats were given an i.p. hyperosmotic saline injection (1.5M saline, 4ml/kg) . Change in plasma OT is plotted taking the initial and recovery period samples (at 20 and 40min) as the mean basal

.

$* = P \leq 0.05$

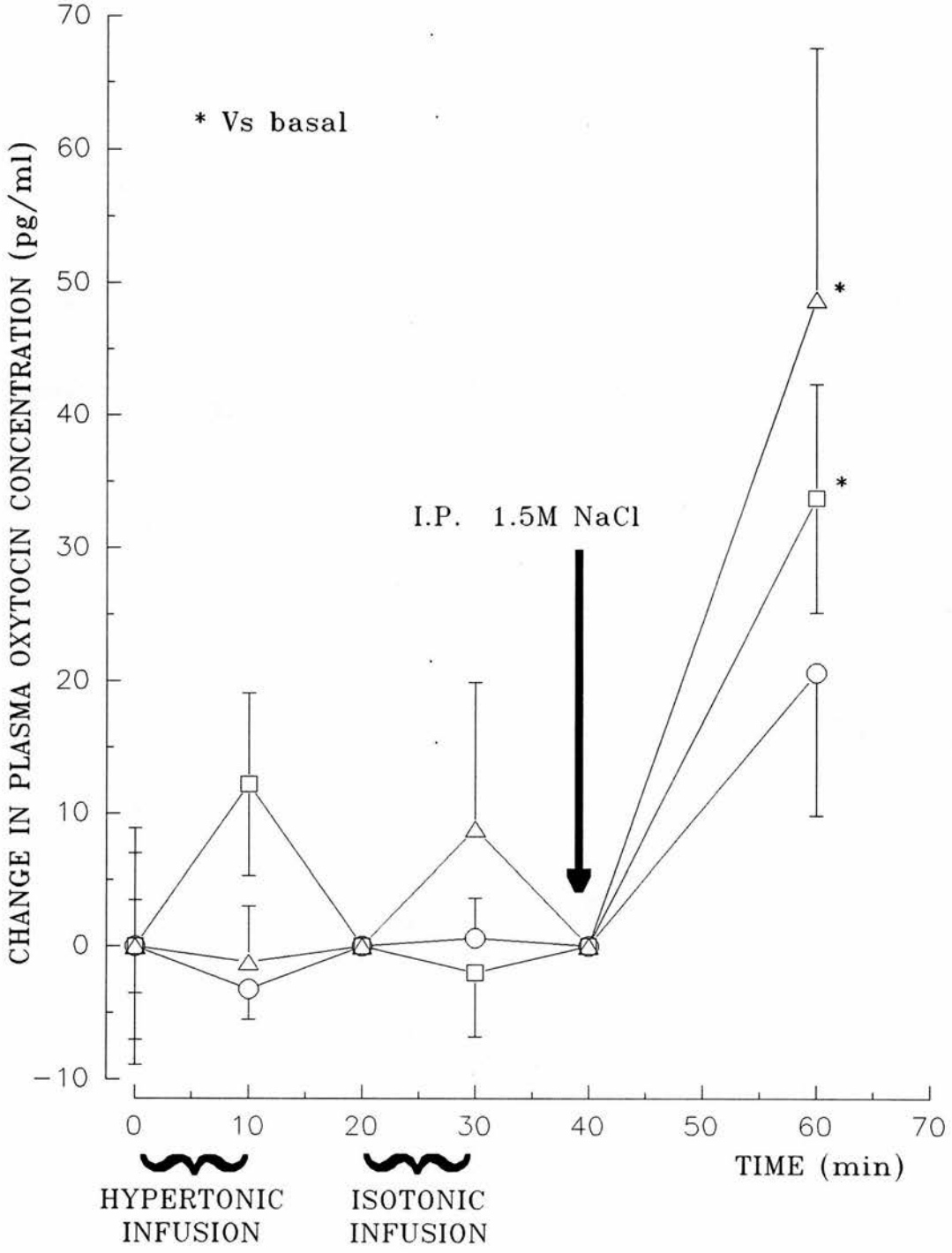


FIGURE 3.1 Change in plasma oxytocin concentration from basal after hypertonic and isotonic infusions into discrete rat brain regions

3.3.2 Plasma oxytocin concentration during microdialysis application of normal and 1M NaCl containing artificial cerebrospinal fluid followed by intraperitoneal hyperosmotic saline (Fig 3.2) .

1M aCSF perfused for 15 minutes via the microdialysis probe in the AV3V region produced a small but significant increase in plasma OT concentration ($P < 0.05$ paired t test) . 10 minutes after the return to the microdialysis application of normal aCSF, plasma OT had returned to levels not significantly different from basal . 95 minutes from the start of the experiment, perfusion of the microdialysis probes was terminated and the OT system challenged with an i.p. hyperosmotic stimulus . This produced a larger but highly variable and overall non significant increase in plasma OT concentration .

LEGEND : FIGURE 3.2

Mean plasma OT concentration, ± s.e.m., in femoral arterial blood samples from urethane anaesthetised virgin female rats (n = 11) during microdialysis application (15 minutes at 3μl/min) in the AV3V region of normal and 1M NaCl-containing artificial cerebrospinal fluid (aCSF), followed by the i.p. administration of 1.5M saline (4ml/kg) .

$* = P \leq 0.05$

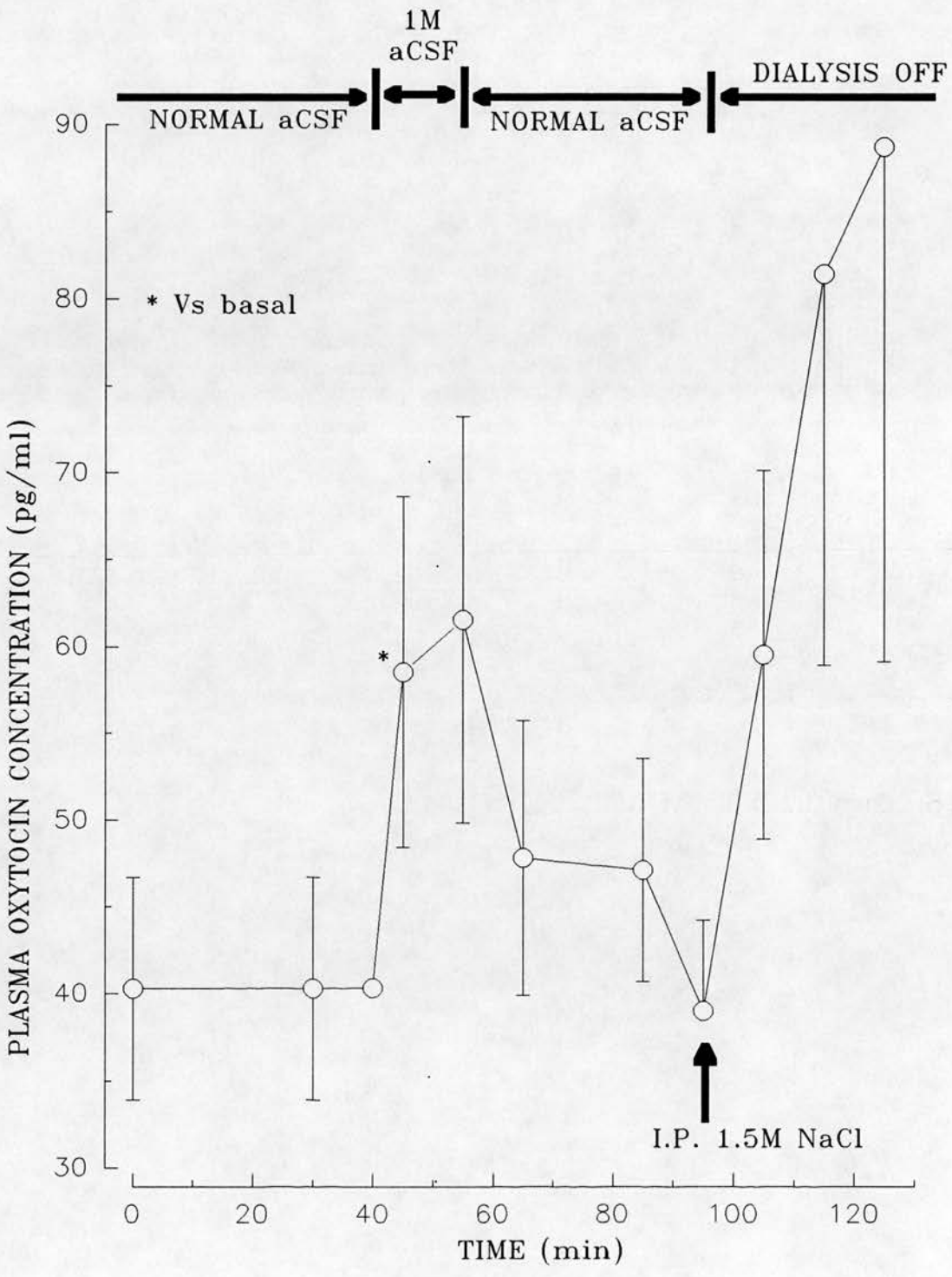


FIGURE 3.2 Plasma oxytocin concentration during microdialysis application of normal and 1M artificial cerebrospinal fluid followed by intraperitonial hyperosmotic saline

3.3.3 Effect of electrical AV3V and osmotic SON stimulation on plasma oxytocin concentration (Fig 3.3) .

Electrical stimulation of the AV3V region produced a large reversible significant increase in the plasma OT concentration of virgin female rats ($P < 0.001$ paired t test) . 60 minutes after the termination of the electrical stimulation of the AV3V region, 15 minutes of 1M hyperosmotic stimulation of both supraoptic nuclei via microdialysis probes also produced a significant increase in plasma OT concentration ($P < 0.005$ paired t test) .The OT response of rats to the SON hyperosmotic stimulation was not significantly different from that to the electrical AV3V stimulation .

LEGEND : FIGURE 3.3

Mean plasma OT concentration, ± s.e.m., of femoral arterial blood samples from urethane anaesthetised virgin female rats (n = 7) . Rats initially received 30 minutes of electrical AV3V stimulation (10Hz) followed 60 minutes later by 30 minutes of bilateral microdialysis with 1M hyperosmotic artificial cerebrospinal fluid of both supraoptic nuclei .

** = $P \leq 0.005$

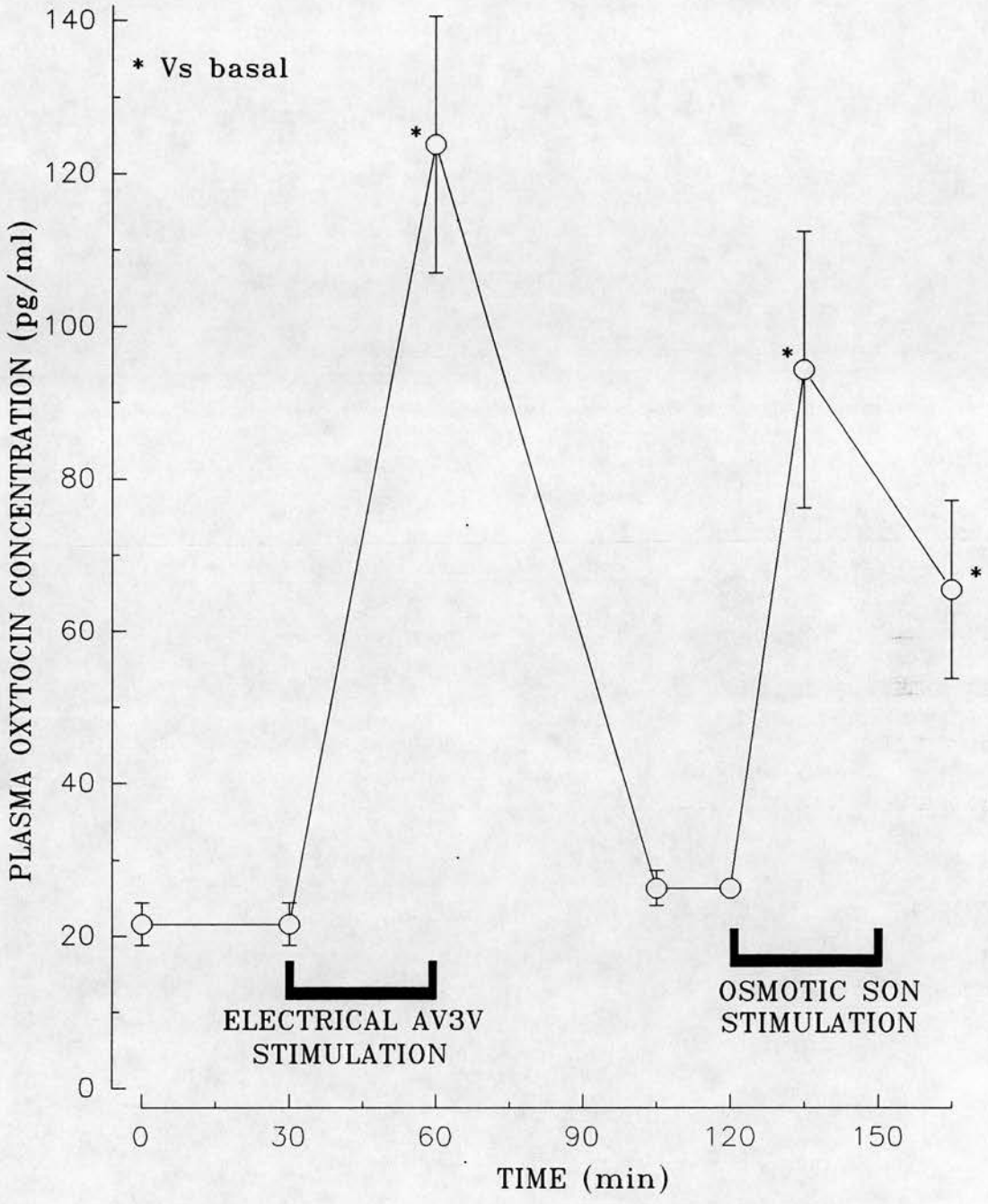


FIGURE 3.3 Effect of electrical AV3V and osmotic SON stimulation on plasma oxytocin concentration

3.3.4 Effect of icv saralasin and 10Hz SFO stimulation on the oxytocin response to i.p. hyperosmotic saline (Fig 3.4) .

In the icv vehicle, sham SFO stimulated group i.p. hyperosmotic stimulation produced a sustained significant increase in plasma OT concentration ($P < 0.05$ paired t test) . Icv saralasin completely abolished this response, rendering values non-significantly different from basal ($P > 0.05$ two sample t test) . However electrical SFO stimulation (10Hz) was effective, during icv saralasin infusion, in producing a significant increase in plasma OT concentration ($P < 0.05$ paired t test) . Nevertheless OT release was not further excited by subsequent i.p. hyperosmotic saline and plasma OT concentrations were significantly lower than in the control, icv vehicle, group ($P < 0.05$ two sample t test) .

LEGEND : FIGURE 3.4

Mean plasma OT concentration, ± s.e.m., in femoral arterial blood samples of urethane-anaesthetised virgin female rats . Δ, received an icv vehicle infusion and sham SFO stimulation (n = 5) ; ❍, received an icv saralasin infusion and 10Hz SFO stimulation (n = 4) and □, received an icv saralasin infusion and sham SFO stimulation (n = 6) . All groups were administered an i.p. hyperosmotic stimulus (1.5M NaCl, 4ml/kg) 40 minutes from the start of the experiment .

$*, \# = P \leq 0.05$

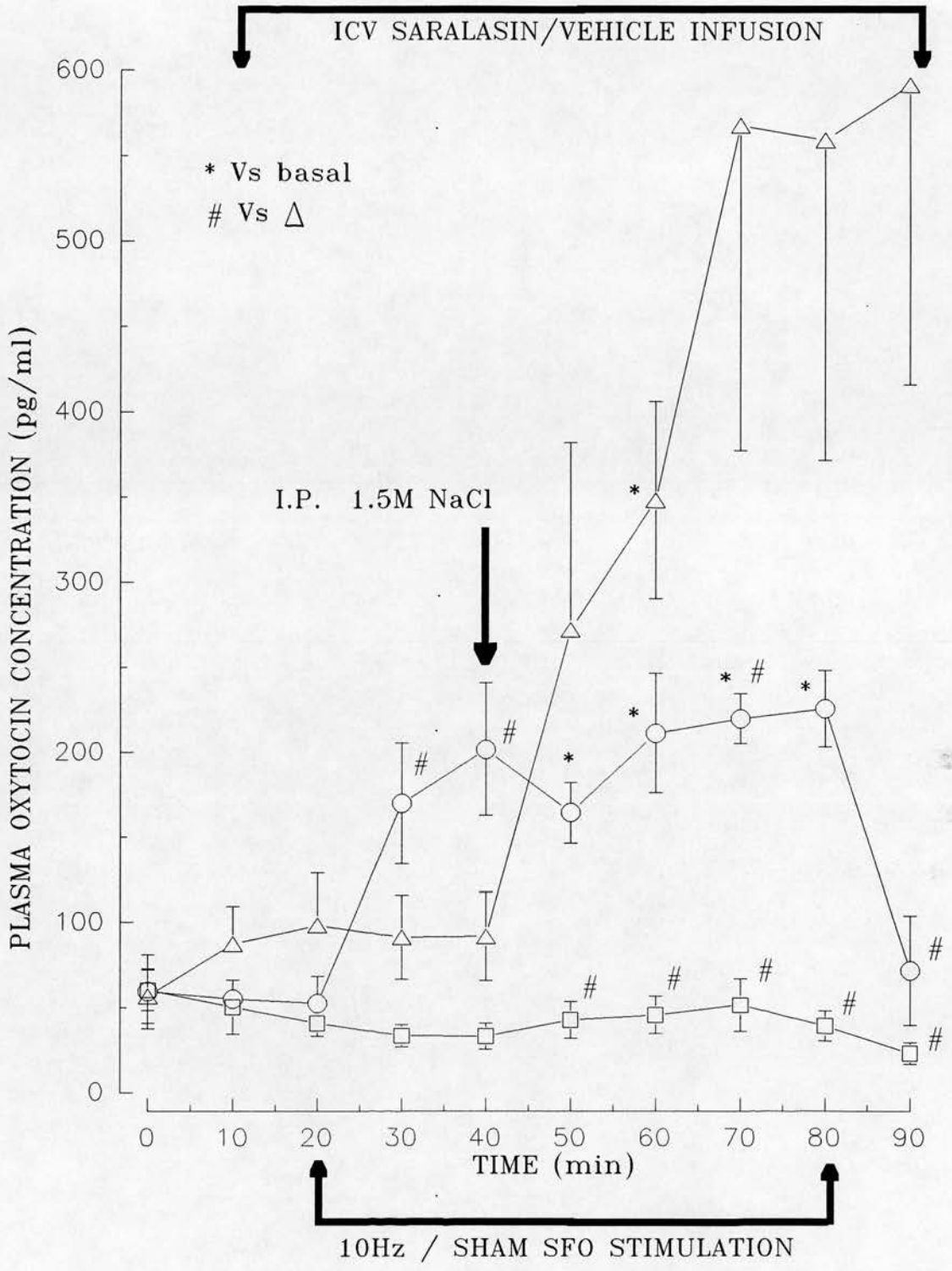


FIGURE 3.4 Effect of icv saralasin and 10Hz SFO stimulation on the oxytocin response to i.p. hypertonic saline

3.4 DISCUSSION

3.4.1 SFO and AV3V region osmoreceptor activity

Our results from the discrete infusion of hyperosmotic saline into the SFO and MPN region (Fig 3.1) would imply that the SFO is not an osmoreceptive site but the MPN may possess some osmoreceptor capability . This hypothesis is supported by the second experiment (Fig 3.2) in which hypertonic saline was administered in to the AV3V region, which includes the MPN and OVLT, via a microdialysis probe . A small but significant increase in plasma OT concentration was produced within 5 minutes of initiating the osmotic stimulus . *In vivo* electrophysiological studies performed by other workers have also confirmed the presence of osmosensitive neurones in the AV3V region with the discrete application of hyperosmotic saline (Honda *et al.*, 1987) . *In vitro* Bourque and Richard (1992) have recently reported that local hypertonic stimulation excited OVLT neurones . This was associated with a dramatic increase in the frequency of spontaneous excitatory post synaptic potentials and spike discharges recorded in supraoptic magnocellular neuroendocrine cells . In our experiments the subsequent response to the administration of systemic i.p. hyperosmotic saline in both the AV3V infusion and microdialysis experiments produced larger increases in OT concentration than central hyperosmotic stimulation, thus suggesting that the AV3V is not the only or most sensitive osmoreceptive region .

There are difficulties in the interpretation of these results which involve the determination of the spread and dilution of the hyperosmotic stimulus . It is impossible to determine the dilution effect of the blood and extracellular fluid surrounding the tissue under test with any great accuracy . Likewise one assumes that the hyperosmotic stimulus is only effective in a

relatively local area . A lack of reproducible effect may therefore be due to inconsistent failure of the osmotic stimulus to reach the appropriate osmoreceptive site in the nucleus . Osmotic stimulation may also produce localised vasoconstriction leading to reduced blood flow and a temporary depolarisation of neurones resulting in a false positive result (Dyball and Leng, 1989) . In view of the above problems in interpreting the results it is likewise open to discussion whether the infusion and microdialysis application of hyperosmotic saline produces a localised change in osmolality within the physiological range . False positive results may therefore also be obtained due to supraphysiological stimulation of neurones not normally stimulated by day to day fluctuations in plasma osmolality .

However both of these experiments demonstrate a reduced effectiveness of intraperitoneal hyperosmotic stimulation compared to other experiments involving virgin female rats performed by us (Fig 4.1, Chapter 4) . This may be the result of damage to elements of the proposed osmoreceptor complex due to the surgery performed in executing these experiments . This is comparable with lesion studies which demonstrate a lack of OT response to hyperosmotic stimulation when the integrity of the SFO and AV3V are compromised (Leng *et al.*, 1989) .

3.4.2 Magnocellular neurone osmoreceptor activity

Electrophysiological studies have revealed that in terms of spike frequency the most osmosensitive cells yet recorded are those of the magnocellular SON (Poulain and Wakerley, 1982) . This is commensurate with our results involving the microdialysis application of hypertonic saline to the SON (Fig 3.3) in which we obtained a large significant increase in plasma OT concentration . This increase was approximately 3 fold greater

than that produced by a similar stimulus applied to the AV3V region, and was not significantly different from that produced by 10Hz electrical AV3V stimulation . It would therefore appear that the magnocellular neurones themselves are the most osmosensitive region not only in terms of neuronal activity but also, as would be expected to follow, in the osmotic control of hormone release .

Electrophysiological evidence suggests that it is the magnocellular OT neurones that are most responsive to changes in osmolality and that cells of the SON are more responsive than those in the PVN (Brimble *et al.*, 1978 : see also the introduction to this chapter) . This is supported by immunocytochemical investigations of cAMP and neuropeptide concentration in addition to metabolic activity which all suggest that the SON is functionally more active than the PVN in responding to osmotic disturbances (Bandaranayake, 1976 ; Dellmann *et al.*, 1988 ; Carter and Murphy, 1989) .

3.4.3 Nature of the osmoreceptor.

Bourque (1989) voltage clamped magnocellular neurones in hypertonic medium, and found that standard voltage steps were associated with increased current flow across the membrane that was unaffected by doubling the external potassium concentration . It was suggested that the depolarisation may be due to activation of a non-voltage dependent, non-selective cation conductance . More recently Oliet and Bourque (1992) reported that the amplitude of the cation current was proportional to changes in external osmolality .

The precise nature of the osmoreceptor remains the subject of continual debate . Olsson (1969) observed that in the goat the infusion of icv hypertonic sodium solutions stimulated drinking and antidiuresis, whereas

similarly hypertonic infusions of sucrose were without effect . Olsson (1972, 1973) subsequently reported that the icv infusion of various saccharide solutions that lowered CSF sodium concentration also blocked the drinking and antidiuresis produced by intracarotid infusion of hyperosmotic saline . Andersson (1978) consequently postulated, based on these and other experiments, that the osmoreceptors are sensitive to sodium concentration rather than to osmolality . Using a similar *in vivo* microdialysis technique as in the present study, but with a probe inserted into the PVN, Hattori et al (1990) also concluded that sodium sensitive cells were responsible for the osmotically stimulated release of OT .

In contrast, Brimble, Dyball and Forsling (1978) concluded that in terms of both neuronal activity and neuropeptide release, OT and vasopressin cells were responsive to the osmotic pressure of the blood rather than to Na^+ or Cl^- concentration . The icv infusion of sucrose and sodium chloride were equally effective in the stimulation of drinking and vasopressin release in the dog indicating osmotic pressure sensitive cells (Thrasher *et al.*, 1980) . This view was shared by Negoro *et al.* (1988) who measured plasma OT concentration during the peripheral infusion of various osmotic solutions into unanaesthetised rats . Alternatively it has been proposed that both sodium receptors and osmoreceptors may be responsible for osmotically induced neuropeptide secretion (Wells *et al.*, 1990) .

Part of the discrepancy in these results may reflect the use of several animal species . In addition the different routes of administration of the various hyperosmotic solutions produce different results . For example, an 18% increase of the local osmolality in the third ventricle is required to elicit an increases in vasopressin secretion (McKinley *et al.*, 1980) . This is in sharp contrast with the 1-2% changes in plasma osmolality known to cause

vasopressin secretion, and indicates that the osmoreceptors are more likely to be located in an area of the hypothalamus influenced by the plasma rather than by CSF osmolality (McKinley *et al.*, 1978) . Both the SFO and OVLT are densely vascularised structures with capillaries that possess fenestrated endothelium and are thereby outside the blood-brain barrier . The SON and PVN are also highly vascularised structures (Ambach and Palkovits, 1979) but the capillary endothelium is complete, however penetration of small molecular weight solutes should still occur .

Conclusions drawn from the use of various hypertonic solutions of sodium chloride, glucose and urea have led to the proposal that to be effective a solute has to be excluded by cell membranes and cause cell shrinkage (Ramsay *et al.*, 1983) . This has been confirmed by Oliet and Bourque (1992) who concluded, it is volume-sensitive cationic channels that transduce osmosensitivity in rat supraoptic neurons .

During hyperosmotic stress the brain shrinks less than expected due to the accumulation of electrolytes within the tissue, while during hypo-osmolar conditions it swells less than predicted due to the net loss of electrolytes (Cserr *et al.*, 1991) . Acute (90 minute) hyperosmotic stress of neuronal tissue in the cerebral cortex of urethane anaesthetised rats via i.p. 1.5M NaCl, results in the maintenance of intracellular neuronal water content and volume by gaining Na^+, Cl^- and K^+ electrolytes from the extracellular compartment (Cserr *et al.*, 1987b and 1991) . The brain consequently loses water during hypernatraemia and stabilises at a new reduced volume within 15-30 minutes of elevating plasma osmolality (Cserr *et al.*, 1987) . In contrast intracellular water content remains approximately the same as control animals, maintaining cell volume at the cost of water loss from the extracellular compartment (Cserr *et al.*, 1991) .

Osmoreceptors are suggested to detect fluctuations in plasma osmolality due to changes in membrane conductances regulated by cell volume . One might therefore expect that these volume regulating mechanisms described in the cortex would be attenuated or totally absent in osmoreceptive cells . An attenuated volume regulating capability is implicated, resulting in delayed cell volume adjustment, as osmoreceptors show adaptation to chronic hypo-osmolar conditions . This is demonstrated by the normal OT and vasopressin response to graded intravenous infusions of hypertonic saline in chronically hypo-osmolar rats (Verbalis and Dohanics, 1991) . In addition the pregnant rat, which also has a chronically reduced plasma osmolality and sodium concentration (see Chapter 4), also displays normal osmotic vasopressin activity (Durr *et al.*, 1981 ; Atherton *et al.*, 1982) . Therefore it is possible that during prolonged disturbances in plasma osmolality the delayed volume regulation of osmoreceptors eventually adapts such that the altered conditions are perceived as normal . However, an exception appears to be the osmotic regulation of oxytocin secretion in pregnancy (see Chapter 4) .

3.4.4 OT neurone osmosensitivity and the AV3V region input

Electrical stimulation of the AV3V region in the rat influences the activity of most supraoptic neurones in a complex manner with the predominant response involving a short-latency, short-duration inhibition followed by long-latency, long-duration excitation resulting in an increase in plasma OT concentration (Leng *et al.*, 1989) . Neurotransmitters proposed to be involved in the pathways regulating these responses of the OT neurones to electrical stimulation include, GABA, excitatory amino acids, acetyl choline and possibly angiotensin II (Akaishi and Negoro, 1983 ; Randle and

Renaud, 1987 ; Gribkoff and Dudeck, 1988 ; Gribkoff *et al.*, 1988 ; Jhamandas *et al.*, 1989) .

Electrolytic ablation of tissue in the AV3V region results in the loss of OT release in response to increased plasma osmolality . OT neurones themselves are osmosensitive as demonstrated by depolarisations on increased osmotic pressure of the extracellular environment resulting in increased OT secretion (Leng, 1980 ; Mason, 1980 and 1983 ; Bourque and Renaud, 1984 ; Fig 3.3) . However such depolarisations do not lead to facilitated action potential production unless the osmotic stimulus is supraphysiological or the cells are receiving an excitatory synaptic input (Leng *et al.*, 1988) .

We have presented evidence that this excitatory input from the AV3V region results in part from the activation of osmoreceptive elements probably in the OVLT or MPN . We attempted to silence the osmoreceptive input from the AV3V region with the infusion of the angiotensin II antagonist saralasin, and subsequently replace the excitatory drive to the OT neurones with tonic exogenous electrical stimulation of the SFO (Fig 3.4) . The icv saralasin infusion used, completely abolished the hyperosmotic stimulation of OT secretion . Nevertheless 10Hz electrical SFO stimulation was still effective at producing OT release . It would appear therefore that the action of angiotensin II antagonist is mainly at the level of the SFO in the stimulation of OT secretion . However, the subsequent i.p. osmotic challenge during icv saralasin and electrical SFO stimulation was ineffective at further facilitating OT release .

As discussed above OT neurones are able to respond directly to fluctuations in plasma osmolality . It is therefore possible that the lack of an osmotic effect in the present experiment reflects the type of AV3V input onto

these neurones produced by the electrical SFO stimulation . Multiple neurotransmitters have been implicated in the neuronal connections of the AV3V region and SON . Therefore during osmotic stimulation the OT neurones may require a chemically specific input from the AV3V region in order to display their innate osmoresponsiveness . This may involve angiotensin II in some way which would have been antagonised by the icv administration of saralasin

3.5 Conclusions

We have attempted to provide further evidence for the roles of the SFO, AV3V region and supraoptic magnocellular OT neurones in the osmoreceptor complex which functions to maintain body fluid homeostasis .

Our results demonstrate that the osmoreceptors regulating OT neurone activity are partly in the lamina terminalis but mainly involve the OT neurones themselves .

In addition OT neurones may require a chemically specific synaptic input from the AV3V region in order to respond directly to systemic hyperosmotic stimulation .

No evidence was found for the existence of osmoreceptive elements within the SFO involved in OT regulation . However it was concluded that for normal osmotically stimulated release of OT, the integrity of the SFO and AV3V region were vital .

CHAPTER 4

REDUCED OXYTOCIN SECRETION IN RESPONSE TO HYPERNATRAEMIC STIMULATION DURING PREGNANCY

4.1 INTRODUCTION

4.1.1 Natriuretic activity of oxytocin

Interest in the oxytocin portion of neurohypophysial hormone secretion was stimulated in 1910 when Ott and Scott demonstrated the milk ejecting activity of posterior pituitary extracts (Ott and Scott, 1910) . A few years later, Gaines (1915) described the milk ejection response to the hormone in the lactating goat . It was in this animal model that the first demonstration of the specific release of OT in response to hyperosmotic stimulation was made by Andersson in 1951 . Andersson combined the findings of Ely and Peterson (1941) in which it was reported that the mechanical stimulation of the teats produced increased secretion of oxytocin from the posterior pituitary into the blood stream of lactating goats producing milk ejection, with the findings of Verney (1947) which described the release of neurohypophysial hormones in response to intra carotid infusion of hypertonic saline . In Andersson's experiments he injected 0.684-1.368M NaCl into the right common carotid artery of four lactating goats . Milk ejections were obtained with the rapid injection (2cc/sec or faster) of 20-60cc of solution . Rapid injection of 20cc of 1.368M NaCl was found to produce a certain amount of emotional stress so the experiments were repeated under nembutal anaesthesia but the same milk let down occurred . Similar injections of 40cc of Ringer solution had no effect so Andersson concluded that the increased osmotic pressure of the arterial blood supply to the brain, by way of the osmoreceptors in the hypothalamus, "stimulated the posterior lobe of the pituitary to secrete the oxytocic principle into the blood stream" . The difficulty was and still is today, whether oxytocin is of physiological importance in the control of electrolyte excretion and urine flow rate .

A considerable amount of evidence has been accumulated to date . Pioneering work by Fraser (1937) demonstrated that an extract of pituitary rich in oxytocin when injected subcutaneously increased the urine output in hydrated and non hydrated rats . Many subsequent studies have confirmed this early finding . For instance Dicker and Heller (1946) found that subcutaneous administration of 3mu/100g OT significantly increased glomerular filtration rate and renal plasma flow and was accompanied by a significant decrease in chloride re-absorption in rats . Fraser (1942) confirmed this result, also in the rat, with the finding that OT was much more active than vasopressin in increasing chloride and water excretion . Fraser also found oxytocin to be extremely active in decreasing phosphate excretion . Sawyer (1952) also observed an increase in sodium, potassium and chloride excretion in rats administered oxytocin rich pituitary extracts with essentially equal sensitivity to vasopressin . Subsequently Dicker (1957) reported that intravenous injection of oxytocin at 400μu/100g/min markedly increased the osmolar clearance, a measure of the rate at which osmotically active substances are cleared from the plasma, in the rat .

4.1.2 Hyperosmotic stimulation of oxytocin secretion

Hyperosmotic stimulation can be accomplished in several ways including removal of drinking water, substitution of drinking water with 2% saline, infusion of hyperosmotic saline as well as intraperitoneal injection of hyperosmotic saline . Abrahams and Pickford (1954) reported that in the dog intracarotid and intravenous infusion of 1.7M NaCl, 15-20ml, produced antidiuresis and a change in uterine motility . They concluded that hypertonic saline liberated 15-20 times as much OT as vasopressin . Cheng and North

(1986), also using iv. infusion of hyperosmotic saline, obtained a significant increase of 682 fmol/l in OT associated neurophysin in rat plasma .

Using i.p. 1.5M saline (1ml) Brimble *et al.* (1978) produced an increase in plasma osmotic pressure of 12 mosmol/kg resulting in an increase in the spontaneous firing rate of putative OT neurones in the paraventricular and supraoptic nuclei, which was associated with an increase in plasma OT concentration of 8.8 μu/ml . Using a similar stimulus Hartman *et al.* (1986a) found that an increase in plasma OT concentration in response to hyperosmotic stimulation could be elicited from rats as early as 2 days old . Naloxone was found to augment this response at all ages tested and enhance OT depletion in adult rats . Shibuki *et al.* (1988) reported that this augmentation of response by naloxone was not associated with an increase in the firing rate of putative OT neurones in the SON . This leads to the conclusion that following i.p. hyperosmotic stimulation, endogenous opioids act at the neurosecretory terminals to partially inhibit OT release .

Water deprivation has also been shown to be a potent stimulus of OT release, resulting in depletion of pituitary hormone stores estimated at 97 mu/day with a repletion rate of 41mu/day following rehydration (Young and van Dyke, 1968) . However this treatment has the disadvantage that experiments must be relatively short (7 days) . Another method of dehydration involves replacing normal drinking water with hyperosmotic saline (2-3% w/v) (Gilman and Goodman, 1937) . This stimulus has the advantage that animals can be kept in excess of 90 days (Delmann *et al.* 1988) . Using this stimulus Duchen (1962) found that mitotic divisions within the pars intermedia of the pituitary stopped compared to numerous divisions in the nueral lobe greatest after 4 days of exposure to 2% saline drinking water . Friesen and Astwood (1967) found that rats would drink up to

80ml/day of 2-2.5% saline and excrete similar volumes of urine . They also found that "within three to seven days the posterior pituitary lobe, which usually was pale and ill defined and rested above the anterior lobe, changed to a reddish, glistening, bulging structure that stretched the overlying capsule" . This result was confirmed by Dellman *et al.* (1988) who reported that the neural lobes of rats after 90 days of exposure to 2% saline were approximately 80% larger than in controls . In addition Van Tol *et al.* (1987) found that 14 days of exposure to 2% saline drinking produced a doubling in the OT and vasopressin mRNA levels within the supraoptic and paraventricular nuclei as well as a 70% depletion of both OT and vasopressin from the pituitary gland and a marked increase in the OT and vasopressin plasma levels .

4.1.3 Renal activity of oxytocin

Fraser (1937 and 1942) reported that subcutaneous administration of OT was much more active than vasopressin in increasing water and chlorine excretion whilst decreasing phosphorous excretion in hydrated and non hydrated rats at doses smaller than those required to produce an oxytocic effect . These results were confirmed by numerous workers who found that administration of OT increased the renal excretion of sodium, potassium, chlorine and water in hydrated and dehydrated rats (Dicker and Heller, 1946; Sawyer, 1952 and Jacobson and Kellogg, 1956) . More recently Verbalis *et al.* (1991) have reported the effects of infusing physiological plasma concentrations of OT in rats maintained on a sodium deficient diet . They found that OT in such rats caused a dose related increase in urinary sodium excretion during the initial day of infusion . This effect could be abolished

using an OT receptor antagonist but was only partially affected by a combined vasopressin 1 and 2 receptor antagonist .

These results have also been validated by the infusion of synthetic OT which produced an increase in urinary sodium excretion in the presence and absence of vasopressin in euvolemic Long Evans rats (Conrad *et al.*, 1986) . Experiments have also been performed using analogues of OT which have been found to have up to three times the natriuretic and diuretic activity of OT . The natriuretic effect appeared to be due to an inhibitory action of the peptides on tubular sodium re-absorption (Chan and Du Vigneaud, 1970 ; Hrbas *et al.*, 1984) . Chan and Hruby (1988) found that antagonists to the vasopressin 1 and 2 receptors in the kidney had no effect on the natriuretic and diuretic actions of OT and therefore implicated an OT receptor . Tribollet (1988) provided further evidence for this by demonstrating specific tritiated OT binding in glomerular structures of the rat kidney . The action of OT on the kidney epithelial LLC-PK1 cells has been characterised by Leitman *et al.* (1988) and found to be 10 times more potent than vasopressin at increasing cyclic GMP content . Isii *et al.* (1991) obtained the same result in cultured porcine kidney epithelial cells and attributed it to an activation of soluble guanylate cyclase .

4.1.4 A synergistic action with vasopressin in fluid balance

Evidence for the synergistic involvement of OT with vasopressin in fluid balance of the rat comes from experiments involving removal of the pituitary (hypophysectomy) . Demunbrun *et al.* (1954) using dogs found that hypophysectomy produced a striking and predictable decrease in renal glomerular filtration rate and renal plasma flow . Renal heamodynamics in such preparations were effectively restored only by the administration of the

total extract of the neurohypophysis . Administration of the vasopressin fraction alone had no effect, therefore demonstrating the importance of OT as well as vasopressin at promoting kidney function . Licardus and Ponec (1973) reported that acutely hypophysectomized rats avidly retained sodium . They were able to reverse this sodium retention with the i.p. injection of posterior but not anterior pituitary lobe homogenates . They therefore concluded that sodium retention resulted from the loss of posterior pituitary hormones in this preparation . These results were further ratified by Balment *et al.* (1986b) who concluded that in the neurohypophysectomized rat (removal of the posterior pituitary alone) "replacement of both neurohypophysial hormones , at plasma levels within the physiological range, largely reverses the renal sodium retention of neurohypophysectomised rats, oxytocin considerably potentiating the natriuretic action of vasopressin" . These results therefore provide evidence of a potentially important synergism involved in the management of sodium balance .

4.1.5 Evidence from Brattleboro rats

Further evidence for OT involvement in sodium balance is demonstrated in the Brattleboro rat which contains no effective means of vasopressin secretion and therefore displays the condition of diabetes insipidus . Balment *et al.* (1982) reported that an increase in plasma osmolality was an effective stimulus for oxytocin release in such rats . Subsequently Sherman *et al.* (1988) reported that replacement of normal drinking water with 2% saline for 18 hours a day effectively increased the plasma level of OT, maintained the posterior pituitary in a state of depleted stores of OT and increased the level of OT mRNA expression in Brattleboro rats .

Edwards and La Rochelle (1984) added further evidence when they found that OT was released during the early hours of dehydration in the Brattleboro rat . OT was also found to cause a natriuresis, maintain renal haemodynamics and glomerular filtration rate during volume contraction and elicit a weak antidiuretic response . Brimble *et al.* (1991) examined the effects of infusing Brattleboro rats with hypertonic saline . They also found that hypernatraemia and hyperosmolality were associated with greatly increased plasma concentration of OT in these rats . Following neurohypophysectomy they obtained a greatly reduced sodium excretion which was reversed by the infusion of OT at plasma levels equivalent to those in intact animals . They therefore concluded that "OT secretion evoked in response to saline infusion would thus appear to be effective in promoting renal sodium excretion in the absence of vasopressin in the Brattleboro rat "

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4.1.6 Effect of opioids

Release of OT from the neurohypophysis in response to hyperosmotic stimulation can be severely attenuated by the use of mu and kappa opioid agonists such as morphine (Evans *et al.*, 1989) . A functional role for opioid peptides in the differential secretion of vasopressin and OT was reported by Summy-Long (1984) . Using the opioid antagonist naltrexone she found that endogenous kappa opioid agonists preferentially inhibit the release of OT during dehydration in the conscious rat, thereby promoting the advantageous release of vasopressin . Bicknell *et al.* (1985) reported a similar result when they electrically stimulated the neural stalk as did Cheng *et al.* (1990) during the infusion of 18% saline with the non selective opioid antagonist naloxone . Gaymann and Martin (1987) confirmed that there was

co storage of immunoreactive dynorphin$_{(1-8)}$-like material with vasopressin and [met]enkephalin-like material with oxytocin . Possible sources of opioid inhibition therefore appear to be from the neighbouring vasopressin terminals and the OT terminals themselves .

Evidence for the preferential release of vasopressin by cross inhibition of OT secretion from the vasopressin terminals was put forward by Bondy *et al.* (1988) who reported that dynorphin$_{(1-8)}$ was effective in inhibiting OT secretion from electrically stimulated isolated neurointermediate lobes but had no effect on vasopressin secretion . Evidence in support of the regulation of OT secretion by co-localised opioids comes from the finding that systemic CCK stimulated OT release is enhanced by naloxone (Flanagan *et al.*, 1988) . CCK selectively stimulates OT release and is therefore assumed not to promote cross inhibition by dynorphin from the vasopressin terminals . Naloxone does not enhance CCK stimulated OT release at the level of the cell body (Leng *et al.*, 1991) so it must therefore be exerting its effect at the neural lobe . It thus appears that OT neurones contain a negative feedback mechanism involving the co-secretion of autoinhibitory opioids (see also Chapter 1) .

Endogenous opioid peptides have also been found to be active and have an inhibitory action on OT secretion during pregnancy . Fletcher *et al.* (1983) reported an increase in plasma beta endorphin-like immunoreactivity on day 18 of pregnancy in the rat . Leng *et al.* (1985) implanted subcutaneous minipumps to deliver naloxone to pregnant rats . They found that the time course of subsequent parturition was different and the interval between successive births was shorter . Hartman *et al.* (1986) administered subcutaneous naloxone to 20 and 21 day pregnant rats . Naloxone caused an elevation of plasma OT concentration in pregnant but not virgin rats .

Leng *et al.* (1988) subsequently found an increasing stimulatory action of naloxone from the second week of pregnancy . Endogenous opioids therefore appear to exert an inhibitory action on OT secretion during pregnancy and may modulate OT release during parturition in respect by spacing the delivery of successive pups .

4.1.7 Pregnancy and fluid homeostasis

Pregnancy in the rat also produces changes in kidney function and fluid homeostasis . Lindheimer *et al.* (1971) reported that the volume of distribution of inulin was increased at day 20 of pregnancy . Accompanying this there was an increase in the filtration and reabsorption rate of sodium associated with quantitatively comparable increments in renal Na^+-K^+-ATPase activity . Churchill *et al.* (1980) investigated the time course of salt retention and distribution during pregnancy . They found that sodium retention began during the second week with the bulk of retention occurring during the third week of pregnancy when the rat only excretes 67% of the sodium ingested . 59% of the retained sodium was found in the products of conception . These findings were confirmed by Atherton *et al.* (1982) who reported that electrolyte intake (food consumption) increased from as early as day three of pregnancy but was accompanied by an increase in renal ionic excretion . A net retention of sodium, potassium and chloride did not occur until the final week of pregnancy when the urinary output of these ions was reduced . There was also a significant increase in plasma volume from day 6 but an increase in fluid intake did not occur till day 13 of pregnancy . This indicated either an altered extrarenal output (the rat only excretes 1/6 of its daily water loss as urine) or a shift of fluid between body fluid compartments .

Maternal plasma sodium and total osmolality were also found to be reduced during the last week of pregnancy (Atherton *et al.*, 1982) . Despite this decrease in plasma sodium concentration and osmolality vasopressin responsiveness to decreases in plasma volume are not significantly different to virgin (Barron *et al.*, 1984) . The vasopressin response to increases in plasma sodium and osmolality are also similar to virgin rats with the apparent threshold for vasopressin secretion 11-14 mosmol/kg lower in the pregnant rat (Durr *et al.*, 1981) . Osmoregulation of OT secretion in pregnancy does not appear to have been studied previously . So we have undertaken a series of experiments investigating the responsiveness of the OT system to hyperosmotic stimulation via i.p. injection and i.v. infusion of hyperosmotic saline solutions in rats at 16 and 21 days of pregnancy and after 6-9 days of lactation .

As mentioned above endogenous opioid tone on the OT system increases during pregnancy . We have also investigated whether this increase in endogenous opioid activity produces tolerance in the OT system to the exogenous mu opioid agonist effects of morphine when stimulated by hyperosmolality during pregnancy .

We have further investigated the effects of long term exposure to opioid inhibition using chronic morphine treated virgin rats . These animals received an increasing dosage of icv morphine from a subcutaneous osmotic mini pump over 5 days . Such rats display tolerance to and dependence upon the exogenously applied morphine (Rayner *et al.*, 1988) . Tolerance is demonstrated by the return to near normal OT neurone activity and secretion (Russell, 1989) which involves a reduction in mu opioid receptor density in the SON (Sumner *et al.*, 1990) . By then applying a similar hyperosmotic challenge to these animals as that received by pregnant rats, and then

comparing the results from each group, we have attempted to provide evidence of tolerance to opioid inhibition in the pregnant rat .

Changes in fluid balance are known to occur during the human menstrual cycle (Baylis *et al.*, 1985) and changes in plasma vasopressin concentration have also been observed (Forsling *et al.*, 1981) . There are similar variations during the rat oestrous cycle (Forsling and Peysner, 1988b) and this group also observed a reduction in the circulating vasopressin concentration of ovariectomised rats (Peysner and Forsling, 1990) suggesting therefore that ovarian hormones influence neurohypophysial hormone release . Forsling *et al.* (1991) investigated the effect of ovariectomy of virgin rats on the vasopressin response to hypertonicity and hypovolaemia . It was found that after ovariectomy or treatment with the antioestrogen tamoxifen the response to hypertonicity was unaffected but that to hypovolaemia was attenuated . We have also investigated whether short term removal of ovarian hormones, via ovariectomy, produces an alteration in the response of OT neurones to hyperosmotic stimulation in 21 day pregnant rats .

Angiotensin II is known to produce an increase in plasma OT concentration (Lang *et al.*, 1981) and this is thought to stimulate OT neurone activity via an action on the subfornical organ (Gutman *et al.*, 1988a ; Leng *et al.*, 1989) . Ray *et al.* (1990) reported a down regulation of angiotensin II receptors in the SFO of young male rats by chronic dietary sodium depletion . A potentially similar condition to pregnant rats which express a decrease in plasma sodium concentration .

Chen and Printz (1983) reported that chronic oestrogen treatment reduced angiotensin II receptor binding in the anterior pituitary . In addition Jonklaas and Buggy (1985) and Fregley *et al.* (1985) reported that chronic

administration of oestradiol benzoate in ovariectomised rats decreased the response of angiotensin II stimulated drinking associated with reduced binding in the median preoptic nucleus , organum vasculosum of the lamina terminalis and the subfornical organ . A blunted pressor response to angiotensin II has been reported in pregnant rats mediated mainly by progesterone (Nakamura *et al.*, 1988) . A similar result was obtained during human pregnancy (Brown *et al.*, 1988) in which there was also a reduced effectiveness of angiotensin II at reducing sodium excretion . We have therefore investigated the effect of an iv angiotensin II infusion on OT secretion in the pregnant rat before and after naloxone which has been found to potentiate its effects (Coiro and Chlordera, 1991) .

4.2 METHODS

Rats were housed and the day of pregnancy or lactation, as required, was determined according to the procedures in the General Methods section (GM2) .

4.2.1 Non recovery preparation of animals .

On the day of experimentation rats were anaesthetised with i.p. urethane (product No. U-2500, Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset BH17 7NH, 1.25g/kg), taking care to avoid the gravid uteri in pregnant rats, and the left femoral artery and vein cannulated (GM3) . Rats were normally left at least 2 hours from the end of surgery before the start of the experiment . Blood sampling and radioimmunoassay determination of plasma OT concentration procedures are described in the General Methods section (GM1 and GM4) .

4.2.2 Determination of plasma sample osmolality and sodium concentration

Samples for the determination of plasma osmolality and sodium concentration were stored frozen until the day of assay . All samples were assayed by Mr B. McGrory, plasma osmolality was measured in duplicate (vapour pressure osmometer model 5100B, Wescor Inc., 459 South Main Street, Logan, Utah 84321, U.S.A.) and sodium concentration in one go (Corning 455 flame photometer, Corning Ltd., Halstead, Essex CO9 2DX) .

4.2.3 Drugs, chemicals, etc

1.5 molar hyperosmotic saline was prepared by dissolving 90g of sodium chloride (AnalaR, BDH Laboratory Supplies, Poole, BH15 1TD) in 1l of distilled water . This was administered by intraperitoneal injection using a

2ml plastic syringe and 23GX1" hypodermic needle (Steriseal, Thornhill Road, Redditch, Worcestershire B98 9NL) .

Morphine (morphine sulphate B.P.) and naloxone (naloxone hydrochloride, product No. N-7758, Sigma Chemical Company Ltd) were dissolved in isotonic saline (Steriflex No.1, FL (manufacturing) Ltd., Fresnius Health Care Group, Basingstoke, England) to the appropriate dilutions to administer 0.5ml of solution/kg body weight and were injected slowly (approximately 1-2 minutes duration) via the left intravenous cannula .

4.2.4 Recovery preparation of morphine tolerant animals

In experiments involving morphine tolerant and their control rats (Fig 4.6) virgin female rats were anaesthetised with ether and placed in a stereotaxic frame . The dorsal surface of the skull was then exposed by a midline skin incision and the skull levelled between bregma and lambda . A 1mm deep guide hole was then drilled through the skull (2mm lateral right of and 3mm posterior to bregma) for the insertion of the infusion cannula into the right lateral ventricle . The infusion cannula consisted of a 10mm length of 21G stainless steel tubing bent at 90^0 4.5mm from the end which was ground to 45^0 . The cannula was connected to an 18cm length of polythene tubing (1.2mm external diameter by 0.76mm internal diameter) and both cannula and tubing were sterilised in boiling water for 30mins prior to use . In the control group this infusion assembly was connected to a microsyringe filled with sterile pyrogen-free water (Phoenix Pharmaceuticals Ltd., Gloucester, UK) . The assembly was then flushed with water and positioned above the guide hole in the skull surface of the rat . Two 1.3mm holes were then drilled either side of the cannula hole and into each a stainless steel screw (3.2mm X 10BA) was inserted to provide anchorage points . The

infusion cannula was then introduced into the right lateral ventricle and both the cannula and screws covered with dental acrylic which was left for a few minutes to harden . The microsyringe was then replaced with an osmotic minipump calibrated to deliver 1μl/h (Alzet 2001, Alza Corporation, Palo Alto, CA 94303, USA) filled with sterile pyrogen-free water . The minipump was then placed subcutaneously in the subscapular region and the incision closed with sterile EP 3 suture silk (Davis and Geck, Cyanamid, Gosport, Hampshire, UK) . In rats receiving an i.c.v. morphine infusion the infusion tubing and pump were filled with increasing concentrations of morphine dissolved in sterile pyrogen-free water which was then filtered (Millex-GS, 0.22μm, Millipore S.A., 67 Molsheim, France) . The minipump was filled with 50mg/ml morphine and primed in 0.15M saline at approximately 37°C for 5 hours . The infusion cannula tubing was filled with 40μl aliquots of 10mg/ml then 20mg/ml followed by 50mg/ml morphine solution separated from each other by a 1μl air bubble . This procedure produces morphine tolerance and dependance over 5 days . All surgery for chronic infusion experiments was performed with the assistance of Dr J.A. Russell .

4.2.5 Non recovery preparation of ovariectomised rats .

In experiments involving sham operated and ovariectomised 21 day pregnant rats (Fig 4.7), the rats were anaesthetised with urethane and the left femoral artery and vein cannulated . Two 1cm incisions were then made through the skin and muscle layer on the back of the rat at the level of the last rib approximately 1cm either side of the spine . In ovariectomised rats the fallopian tubes and blood vessels supplying the ovaries were ligated with EP 1 suture silk . The ovaries were then carefully removed and the surrounding tissue inspected for any remnants . The incision was then

closed with EP 3 suture silk stitches . Sham operated animals had their ovaries exposed but not ligated or removed and the incision then closed with EP 3 suture silk . All surgery involved in the ovariectomy or sham operation of rats was performed with the assistance of Dr J.A.Russell . 4 hours was left from the end of surgery before the start of the experiment .

4.2.6 Angiotensin II infusion

In experiments involving angiotensin II infusion the left femoral artery and vein were cannulated as above for blood sampling and the right femoral vein was cannulated for the infusion of angiotensin II (product No. A9525, Sigma Chemical Company Ltd.) at 0.5 μg/kg body weight/min . This was achieved at a flow rate of 278.6μl/20min using a B.Braun perfusor mark 6 (B.BraunMelsungen AG, Germany) set at speed 6 and fitted with 1ml glass Hamilton syringes . The infusion syringes were connected to the right infusion cannula via a 45cm length of polythene tubing (1.6mm external diameter by 1mm internal diameter) .

4.2.7 Hyperosmotic saline infusion

Experiments involving the i.v. infusion of hyperosmotic saline required the dilution of weighed amounts of sodium chloride in isotonic saline administered at a low dose of 262.5mg NaCl/kg body weight and a high dose of 525mg NaCl/kg body weight . These solutions were infused via a right femoral venous cannula at 0.028ml/min using a B.Braun perfusor mark 6 at setting 7, connected to the cannula as above, fitted with 1ml plastic syringes (Steriseal poly-vu syringes) for 30 minutes to give a total infusion volume for all rats of 0.84ml .

4.3 RESULTS

4.3.1 Effect of morphine on hyperosmotic stimulation (I.P.) of OT release in adult virgin female rats (Fig 4.1) .

Hyperosmotic stimulation via I.P. 1.5M NaCl (first arrow) of control rats produced a significant increase in plasma OT concentration ($P < 0.05$ paired t test) from a mean basal of 14.6 ± 2.6 to a maximum of 93 ± 25.1 pg/ml . This response was unaffected by morphine at 0.25mg/kg compared to controls ($P > 0.05$ ANOVA) although a non significant increase in plasma OT concentration was obtained in the low dose morphine treated rats ($P > 0.05$ paired t test) from a mean basal of 11 ± 0.9 to a maximum of 61.9 ± 24.8 pg/ml . However a subsequent dose of 2.5mg/kg completely abolished this response of the low dose morphine treated rats compared to controls ($P < 0.05$ ANOVA then Duncan's range test) and plasma OT was not significantly different from basal ($P > 0.05$ paired t test) .

In the high dose morphine treated rats the first dose of 1mg/kg morphine was effective at suppressing any increase in plasma OT concentration after hyperosmotic stimulation, initially reducing values significantly lower than basal ($P = 0.02$ paired t test) before a non significant rise from a mean basal of 15.3 ± 3.4 to a maximum of 22.7 ± 5.5 pg/ml . The second dose of 5mg/kg morphine at 50min once again reduced plasma OT concentration to significantly less than basal ($P < 0.03$ paired t test) . Plasma OT concentration in the high dose morphine treated rats remained significantly less than that of control vehicle treated animals at all points between the hyperosmotic stimulation and the administration of naloxone ($P < 0.05$ ANOVA then Duncan's range test) .

After i.v. administration of naloxone there was a large significant rise in plasma OT concentration compared to sample 8 at 70min ($P < 0.01$ paired t test) in the control group from 93 ± 25.1 to 518.7 ± 92.1 pg/ml . There was also a significant rise in the plasma OT of the low dose morphine treated rats ($P < 0.001$ paired t test) from 13.2 ± 4.1 to 745.9 ± 86.1 pg/ml . In addition there was a highly significant increase in the plasma OT concentration of the high dose morphine treated rats ($P = 0.0002$ paired t test) from 6.5 ± 1.6 to 580.7 ± 33.6 pg/ml . These effects of naloxone indicate a complete reversal from the morphine inhibition of increases in plasma OT concentration . Naloxone also produced an "overshoot" from morphine inhibition in both low (at 5min) and high (at 15min) dose treated groups compared to the vehicle controls ($P < 0.05$ ANOVA then Duncan's range test) .

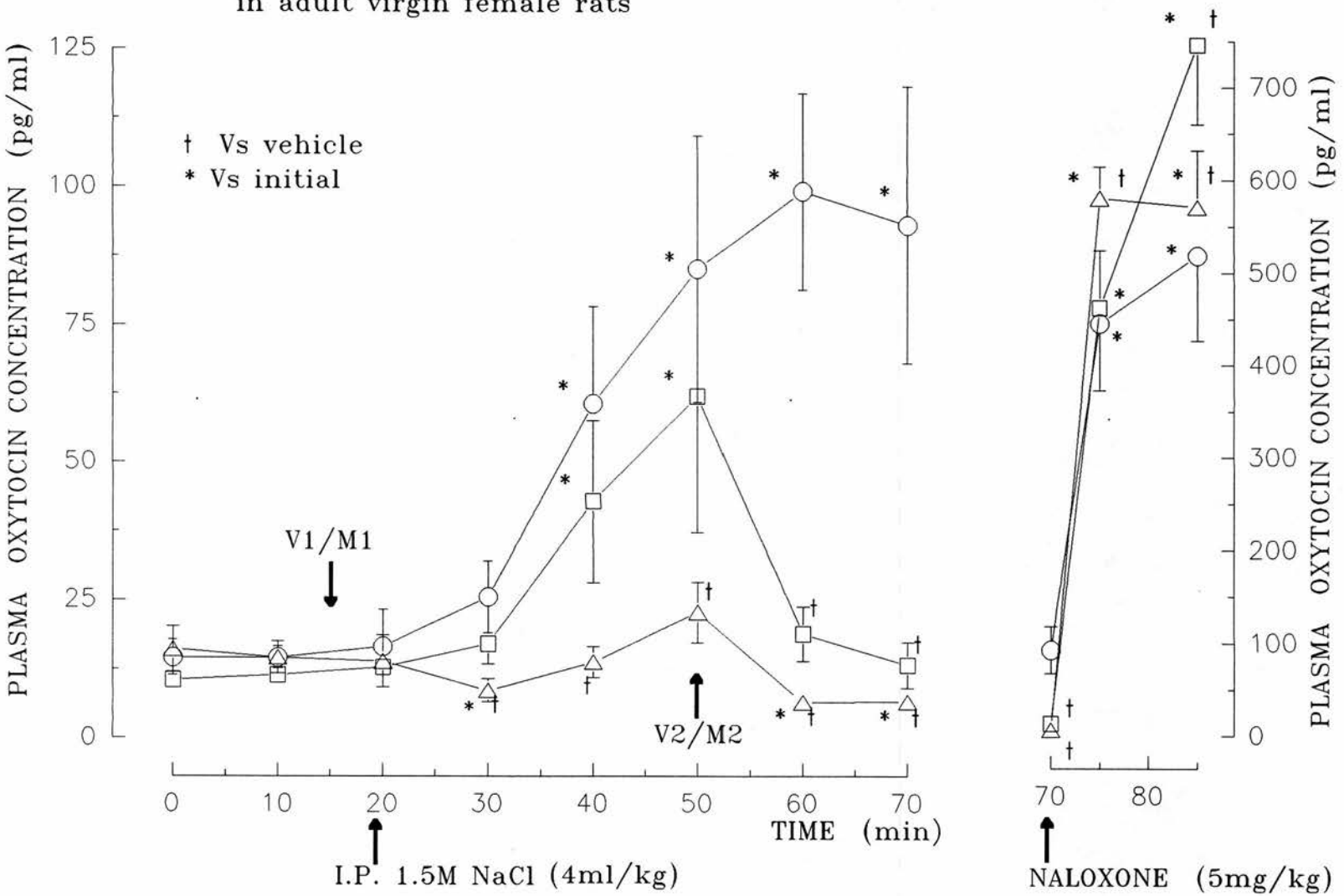
LEGEND : FIGURE 4.1

Plasma OT in femoral arterial blood samples, measured by r.i.a., from urethane anaesthetised adult virgin female rats . Values are mean ± s.e.m. . ❍, are control animals (n = 5) ; ❑, received an acute low dose course of i.v. morphine (n = 5) and Δ, received an acute high dose course of i.v. morphine (n = 6) . Control rats received i.v. vehicle injections at 15 and 50min (V1, V2) . Low dose morphine rats received two i.v. morphine injections, an initial dose of 0.25mg/kg at 15min and a final dose of 2.5mg/kg at 50min (M1, M2) . High dose morphine rats were also given two i.v. injections of morphine, an initial dose of 1mg/kg at 15min and a later dose of 5mg/kg at 50min (M1, M2) .

All groups received an i.p. hyperosmotic stimulus at 20min (4ml/kg, 1.5M NaCl) and naloxone at 70min (i.v., 5mg/kg) .

*, † = $P \leq 0.05$; **, = $P \leq 0.005$; ***, = $P \leq 0.0005$

FIGURE 4.1 Effect of morphine on hyperosmotic stimulation (I.P.) of OT release in adult virgin female rats



4.3.2 Effect of morphine on hyperosmotic stimulation (I.P.) of OT release in 21 day pregnant rats (Fig 4.2) .

In contrast to virgin control rats hyperosmotic stimulation via I.P. 1.5M saline in 21 day pregnant control rats produced a non significant rise in plasma oxytocin ($P > 0.05$ paired t test), from a mean basal of 9.8 ± 3.2 to a maximum of 22.1 ± 7 pg/ml . Sensitivity to morphine however is apparently similar to virgin with no effect of 0.25mg/kg whilst 1, 2.5 and 5mg/kg morphine produced values significantly less than vehicle treated pregnant rats ($P < 0.05$ ANOVA then Duncan's range test) . Morphine at 2.5 and 5mg/kg also significantly reduced stimulated plasma OT concentration compared to the respective mean basal value ($P < 0.05$ paired t test).

After i.v. naloxone administration at 70min there was a significant increase in plasma OT concentration of control rats compared to sample 8 at 70min ($P < 0.02$ paired t test) from 22.1 ± 7 to 120 ± 30.5 pg/ml . There was also a significant increase in the plasma OT concentration of low dose morphine treated rats compared to sample 8 ($P < 0.02$ paired t test) from 4.1 ± 0.6 to 318.9 ± 151.6 pg/ml . In addition there was a non significant increase in the plasma OT concentration of high dose morphine treated rats compared to sample 8 ($P > 0.05$ paired t test) from 4.3 ± 0.3 to 426 ± 167.4 pg/ml . Once again this indicates a complete reversal of morphine inhibition and a non significant "overshoot" of OT secretion .

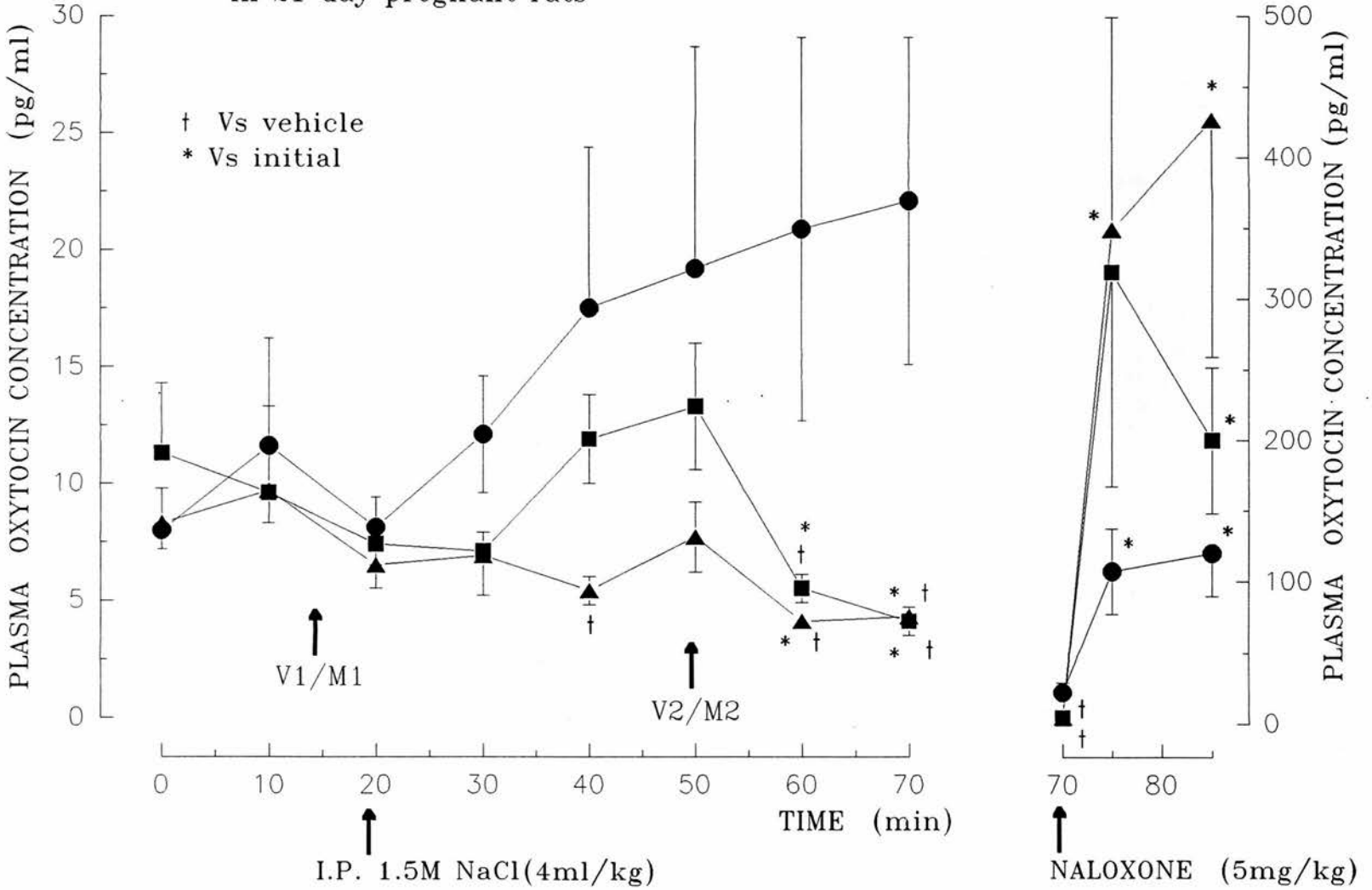
LEGEND : FIGURE 4.2

Plasma OT of femoral arterial blood samples, measured by r.i.a., from urethane anaesthetised 21 day pregnant rats . Values are mean ± s.e.m. . ●, are control animals (n = 6) ; ■, received an acute low dose course of i.v. morphine (n = 6) and Δ received an acute high dose course of i.v. morphine (n = 6) . Control rats received i.v. vehicle at 15 and 50min (V1, V2) . Low dose morphine rats received two doses of morphine , an initial dose of 0.25mg/kg at 15min and a final dose of 2.5mg/kg at 50min (M1, M2) . High dose morphine rats also received two doses of morphine, an initial dose of 1mg/kg at 15min and a subsequent dose of 5mg/kg at 50 min (M1, M2) .

All groups received an i.p. hyperosmotic stimulus at 20min (4ml/kg, 1.5M NaCl) and naloxone at 70min (i.v., 5mg/kg) .

$*, \dagger = P \leq 0.05$

FIGURE 4.2 Effect of morphine on hyperosmotic stimulation (I.P.) of OT release in 21 day pregnant rats



4.3.3 Plasma sodium concentration of virgin and 21 day pregnant rats after hyperosmotic (I.P.) stimulation (Fig 4.2B) .

Basal plasma sodium concentration of virgin rats was significantly greater than that of 21 day pregnant rats ($P < 0.01$ two sample t test) . Hyperosmotic stimulation at 20min significantly increased plasma sodium concentration of virgin rats ($P < 0.0001$ paired t test) from a mean basal of 138.8 ± 0.9 to a maximum of 148.9 ± 1.2 mmol/l . Hyperosmotic stimulation also significantly increased the plasma concentration of sodium in the 21 day pregnant rats ($P < 0.005$ paired t test) from a mean basal of 135.3 ± 0.8 to a maximum of 143.8 ± 1.7 mmol/l . However stimulated plasma sodium concentration of 21 day pregnant rats was significantly lower than that of virgin rats at 50min ($P < 0.0001$ two sample t test) .

LEGEND : FIGURE 4.2B

Plasma sodium concentrations were measured on three (0, 50 and 85min) femoral arterial blood samples from urethane anaesthetised virgin and 21 day pregnant rats that had received an I.P. injection of 1.5M saline (4ml/kg) at 20min (Figs 4.1 and 4.2) . Values are mean ± s.e.m. .

Graph (i) represents the changes in plasma sodium concentration for the individual groups ; ❍, virgin controls (n = 6) ; ❑, low dose morphine virgin (n = 5) ; Δ, high dose morphine virgin (n = 5) ; ●, 21 day pregnant controls (n = 5) ; ■, 21 day pregnant low dose morphine (n = 5) and ▲, 21 day pregnant high dose morphine (n = 6) . There were no significant differences in plasma sodium concentration within virgin and pregnant treatment groups ($P > 0.05$ ANOVA) . Therefore a mean plasma sodium concentration was taken for control, low dose morphine and high dose morphine treatments for virgin and 21 day pregnant rats .

Graph (ii) represents the mean plasma sodium concentrations for control, low dose morphine and high dose morphine treatments of virgin (❍, n = 16) and 21 day pregnant (●, n = 17) rats .

*, # = $P \leq 0.05$; **, = $P \leq 0.005$; ***, ### = $P \leq 0.0005$

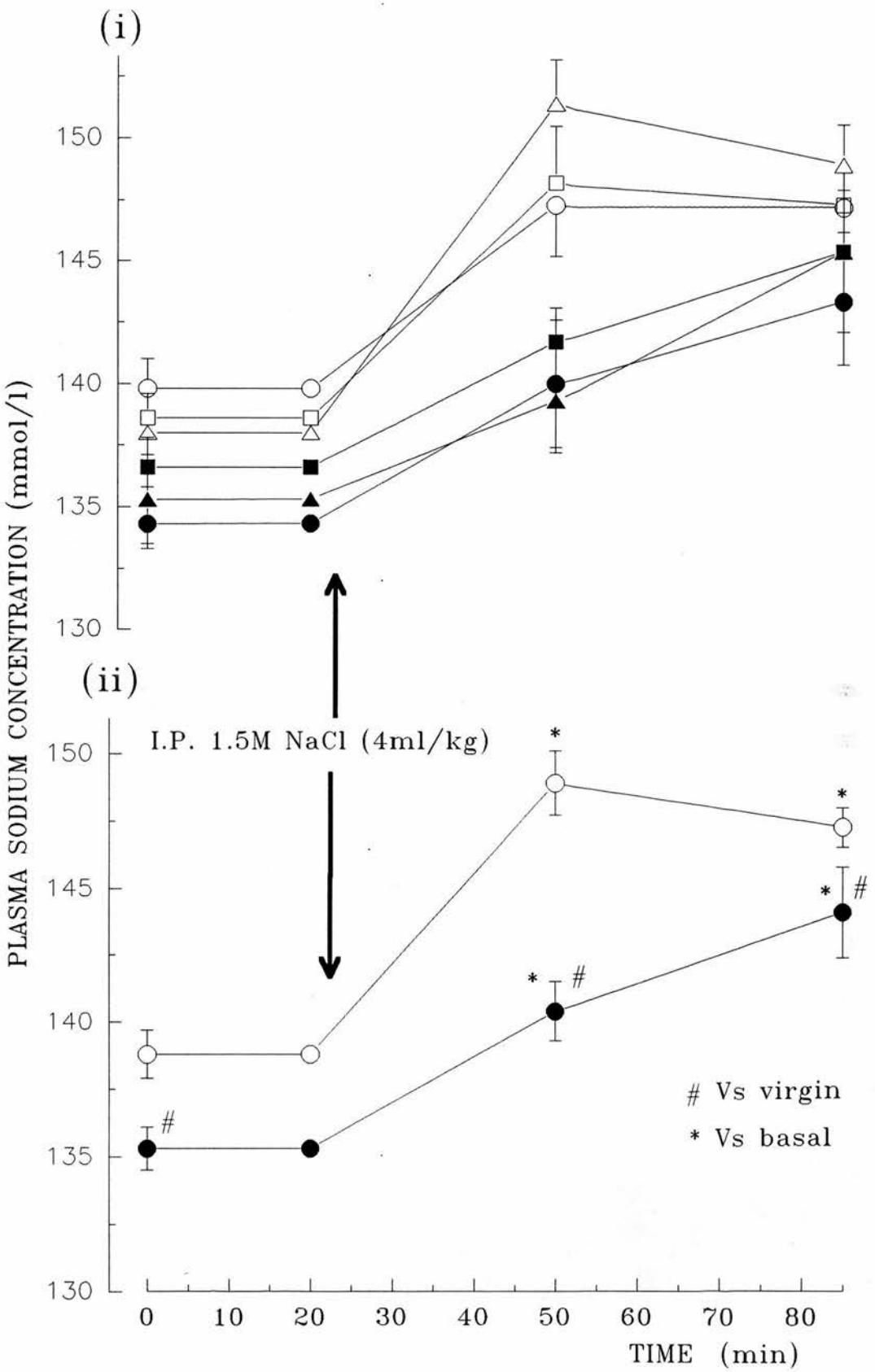


FIGURE 4.2B Plasma sodium concentration of virgin and 21 day pregnant rats after hyperosmotic (I.P.) stimulation

4.3.4 Plasma osmolality of virgin and 21 day pregnant rats after hyperosmotic (I.P.) stimulation (Fig 4.2C) .

Basal plasma osmolality of virgin rats was significantly greater than that of 21 day pregnant rats ($P = 0.0001$ two sample t test) . Hyperosmotic stimulation at 20min (I.P., 1.5M NaCl) significantly increased plasma osmolality of virgin rats ($P < 0.0001$ paired t test) from a mean basal of 299.2 ± 1.8 to a maximum of 320.4 ± 2.8 mosmol/kg . Hyperosmotic stimulation also significantly increased the plasma osmolality of the 21 day pregnant rats ($P < 0.003$ paired t test) from a mean basal of 275.1 ± 2.8 to a maximum of 293.4 ± 4.2 mosmol/kg . However stimulated plasma osmolality of 21 day pregnant rats remained significantly lower than that of virgin rats at both samples ($P = 0.0001$ two sample t test) .

LEGEND : FIGURE 4.2C

Plasma osmolality was measured on three (0, 50 and 85min) femoral arterial blood samples from urethane anaesthetised virgin and 21 day pregnant rats that had received an I.P. injection of 1.5M saline (4ml/kg) at 20min (Figs 4.1 and 4.2) . Values are mean ± s.e.m. of samples stored frozen at –20^{0}C until assay .

Graph (i) represents the change in plasma osmolality of the individual groups ; ❍, virgin controls (n = 5) ; ❑, low dose morphine virgin (n = 5) ; Δ, high dose morphine virgin (n = 5) ; ●, 21 day pregnant controls (n = 5) ; ■, 21 day pregnant low dose morphine (n = 5) and ▲, 21 day pregnant high dose morphine (n = 6) . There were no significant differences in plasma osmolality within virgin and pregnant treatment groups at any time point ($P > 0.05$ ANOVA) . Therefore a mean plasma osmolality was taken of control, low dose morphine and high dose morphine treatments for virgin and 21 day pregnant rats at each time point .

Graph (ii) represents the mean plasma osmolality of control, low dose morphine and high dose morphine treatments of virgin (❍, n = 15) and 21 day pregnant (●, n = 16) rats .

, = $P \leq 0.005$; *, ### = $P \leq 0.0005$

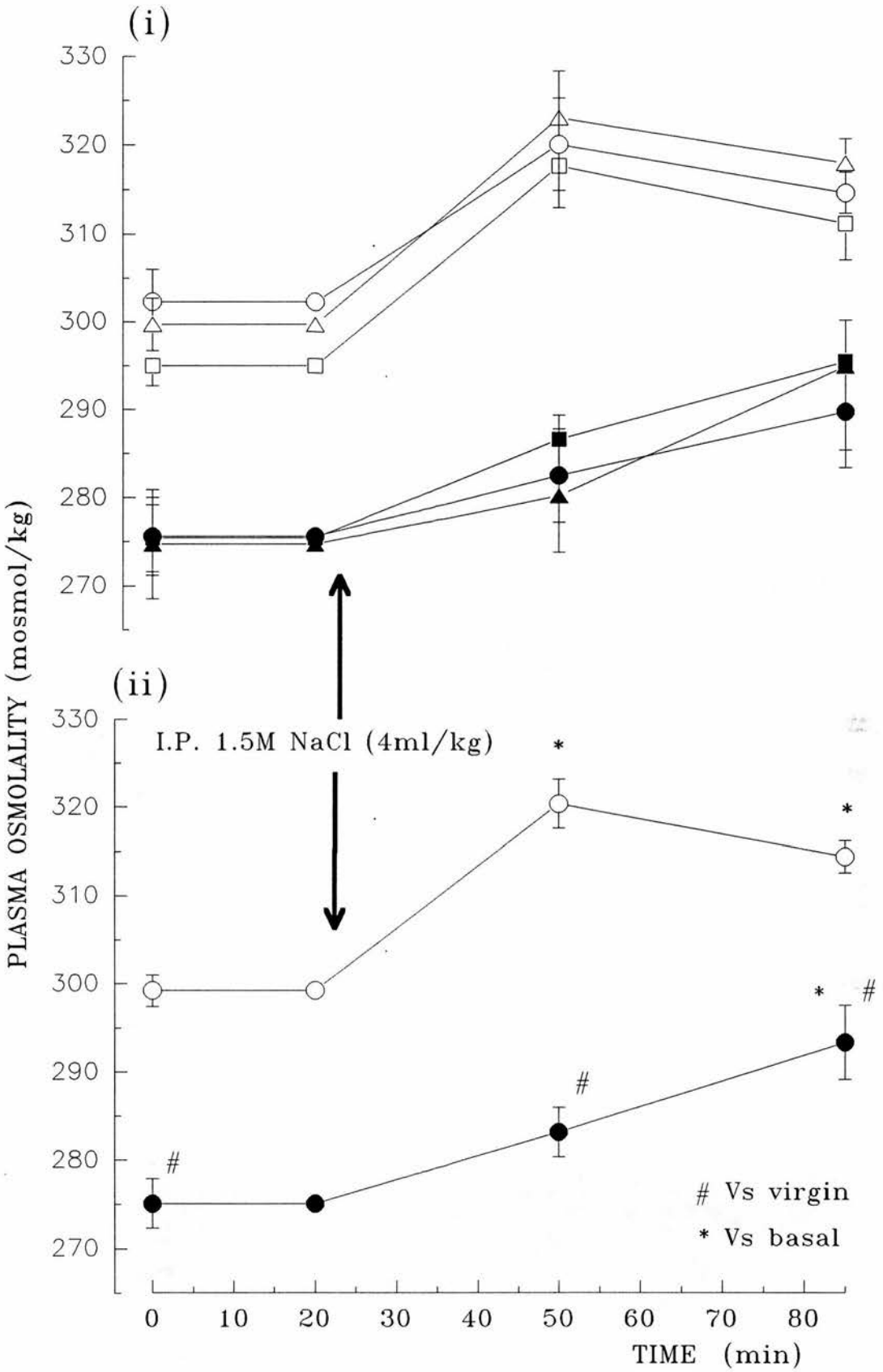


FIGURE 4.2C Plasma osmolality of virgin and 21 day pregnant rats after hyperosmotic (I.P.) stimulation

4.3.5 Effect of morphine on hyperosmotic stimulation (I.P.) of OT release in 16 day pregnant rats (Fig 4.3) .

I.P. injection of 1.5M saline in the virgin rats produced a sustained significant rise in plasma OT concentration ($P < 0.05$ paired t test) from a mean basal of 28.5 ± 8.9 to a maximum of 212.1 ± 74 pg/ml . An identical stimulus in 16 day pregnant rats also produced a significant increase in plasma OT concentration ($P < 0.02$ paired t test) from a mean basal of 23.7 ± 3.8 to a maximum of 109.6 ± 17.8 pg/ml . However this increase was not sustained and plasma OT concentration of 16 day pregnant rats was significantly less than virgin from 50min ($P < 0.05$ ANOVA then Duncans range test) . Morphine at 0.25mg/kg completely abolished I.P. hyperosmotic stimulation of OT release in 16 day pregnant rats ($P < 0.05$ ANOVA then Duncan's range test) and at 2.5mg/kg lowered plasma OT concentration below that of basal ($P < 0.05$ paired t test) .

Naloxone in virgin rats produced a large significant increase in plasma OT concentration compared to sample 8 at 70min ($P < 0.05$ paired t test) from 212.1 ± 74 to 836.4 ± 158.6 pg/ml . In 16 day pregnant control rats naloxone also produced a significant increase in plasma OT concentration ($P < 0.02$ paired t test) . In morphine treated 16 day pregnant rats there was also a significant increase in plasma OT concentration after naloxone, compared to sample 8, ($P < 0.005$ paired t test) from 9.1 ± 1.5 to 511.5 ± 110.8 pg/ml, naloxone producing a complete reversal of morphine inhibition in 16 day pregnant rats and a non significant "overshoot" ($P > 0.05$ ANOVA then Duncan's range test) . Nevertheless plasma OT concentration of 16 day pregnant rats remained significantly lower than in virgin control animals ($P < 0.05$ ANOVA then Duncan's range test) .

LEGEND : FIGURE 4.3

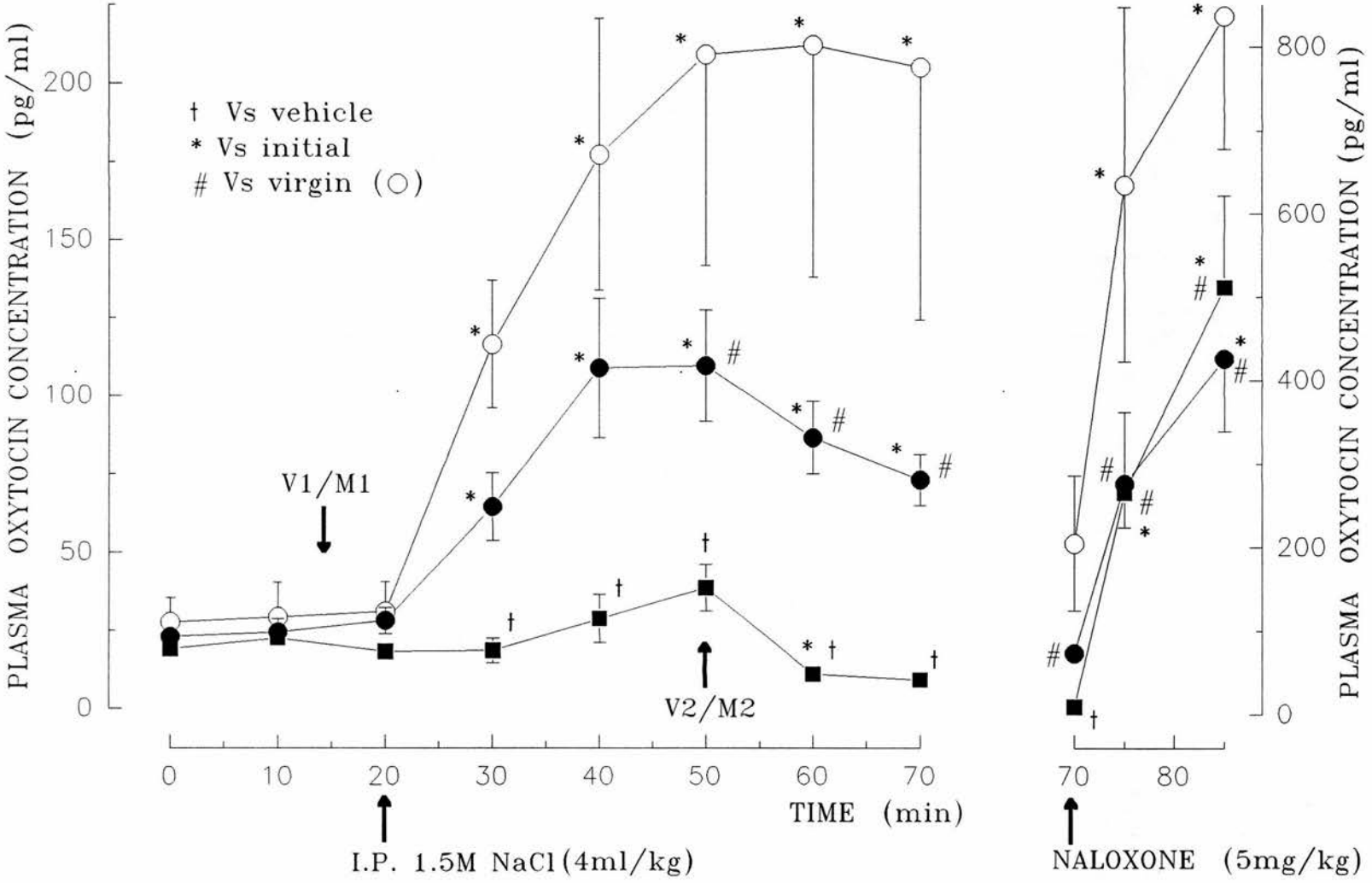
Plasma OT from urethane anaesthetised virgin and 16 day pregnant rats measured by r.i.a. on femoral arterial blood samples . Values are mean ± s.e.m. .

❍, are control virgin rats (n = 4) ; ●, are control 16 day pregnant rats (n = 6) and ■, are i.v. morphine treated 16 day pregnant rats (n = 7) . Control rats received i.v. vehicle at 15 and 50min (V1, V2) and morphine treated rats received an initial dose of 0.25mg/kg at 15min and a later dose of 2.5mg/kg at 50min (M1, M2) .

All groups received an i.p. hyperosmotic stimulus at 20min (4ml/kg, 1.5M NaCl) and naloxone at 70min (i.v., 5mg/kg) .

$*, \dagger, \# = P \leq 0.05$; $**, \#\# = P \leq 0.005$

FIGURE 4.3 Effect of morphine on hyperosmotic stimulation (I.P.) of OT release in 16 day pregnant rats



4.3.6 Effect of morphine on hyperosmotic stimulation (I.P.) of OT release in 7-9 day lactating rats (Fig 4.4) .

Basal plasma OT was significantly lower in lactating rats compared to virgin controls ($P < 0.05$ two sample t test) . Hyperosmotic stimulation by I.P. injection of 1.5M saline in virgin rats (the same virgin controls as in Fig 4.3) produced a significant increase in plasma OT ($P < 0.05$ two sample t test) from basal to a maximum of 212.1 ± 74 pg/ml . An identical stimulus in lactating rats produced a very small but significant increase in plasma OT ($P < 0.05$ paired t test) from a mean basal of 10.2 ± 1.1 to a maximum of 32 ± 10.7 pg/ml . Plasma OT of stimulated control lactating rats remained significantly lower than in stimulated virgin rats ($P < 0.05$ ANOVA then Duncan's range test) .

Morphine at 0.25mg/kg abolished this increase of plasma OT concentration in lactating rats compared to vehicle treated animals ($P < 0.05$ ANOVA then Duncan's range test) and a subsequent dose of 2.5mg/kg significantly reduced values below basal ($P = 0.02$ paired t test) .

In virgin rats after naloxone there was a large significant increase in plasma OT compared to sample 8 at 70min ($P < 0.05$ paired t test) from 212.1 ± 74 to 836.4 ± 158.6 pg/ml . In lactating controls there was also a significant increase compared to sample 8 ($P < 0.02$ paired t test) from 32 ± 10.7 to 138.2 ± 44 pg/ml . Similarly in morphine treated lactating rats there was a significant increase in plasma OT concentration compared to sample 8 ($P < 0.05$ paired t test) from 6.6 ± 0.61 to 84.6 ± 23.3 pg/ml . Plasma OT concentration in morphine treated lactating rats was also not significantly different from controls after naloxone indicating a complete reversal of morphine inhibition by naloxone ($P > 0.05$ ANOVA) . However plasma OT

concentration in lactating rats remained significantly different from that in virgin animals ($P < 0.05$ ANOVA then Duncan's range test) .

LEGEND : FIGURE 4.4

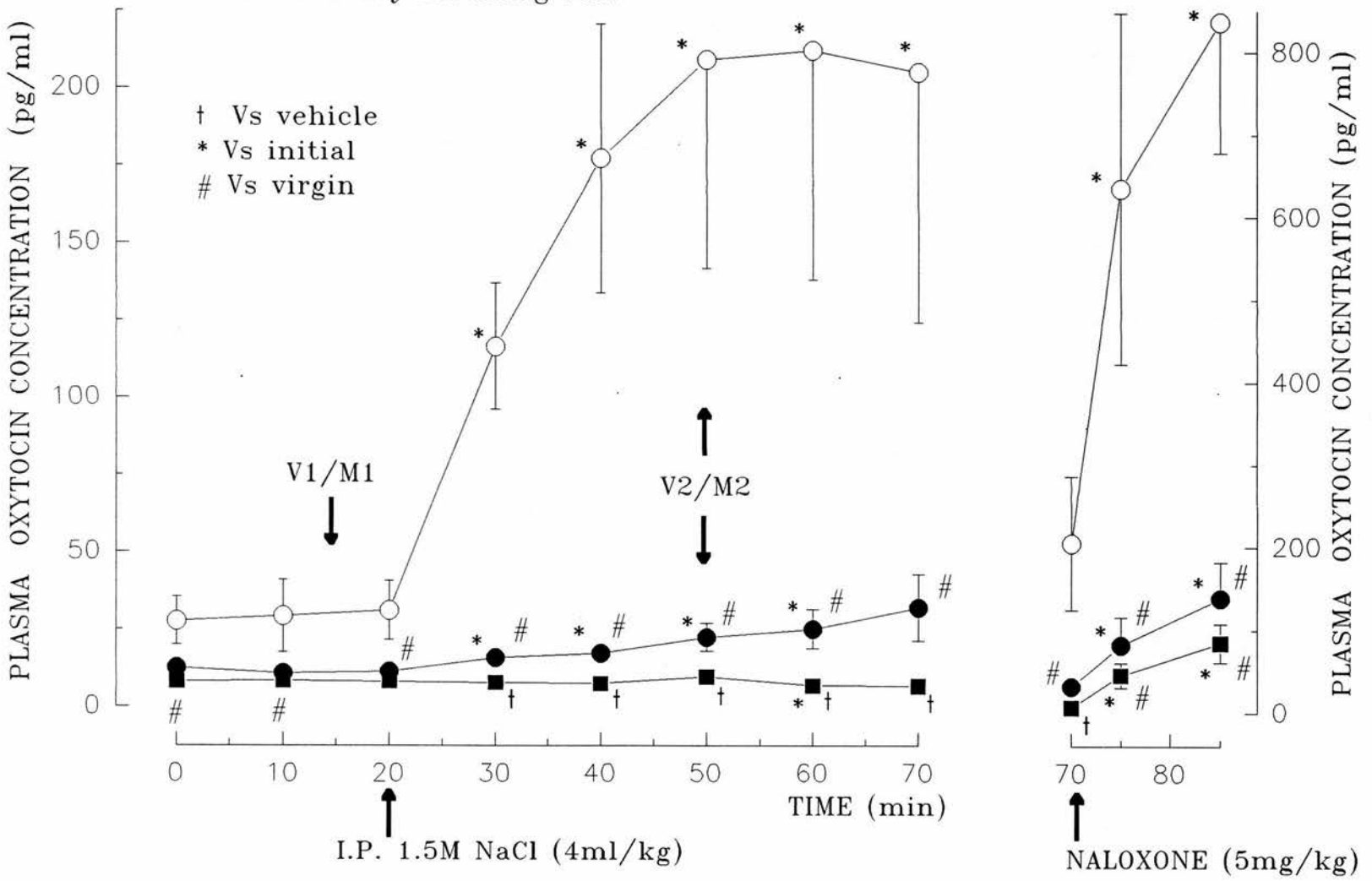
Plasma OT of femoral arterial blood samples, measured by r.i.a., from urethane anaesthetised virgin rats and rats after 7-9 days of lactation . Values are mean ± s.e.m. .

❍, are control virgin rats (as in Fig 4.3, n = 4) ; ●, are control lactating rats (n = 7) and ■, are lactating rats that received i.v. morphine (n = 8) . Control rats received i.v. vehicle at 15 and 50min (V1, V2) whilst rats administered i.v. morphine received two doses , an initial dose of 0.25mg/kg at 15min and a subsequent dose of 2.5mg/kg at 50min (M1, M2).

All groups received an i.p. hyperosmotic stimulus at 20min (4ml/kg, 1.5M NaCl) and naloxone at 70min (i.v., 5mg/kg) .

$*, \dagger, \# = P \leq 0.05$

FIGURE 4.4 Effect of morphine on hyperosmotic stimulation (I.P.) of OT release in 7–9 day lactating rats



4.3.7 Plasma sodium concentration of virgin, 16 day pregnant and lactating rats after hyperosmotic (I.P.) stimulation (Fig 4.4B) .

Basal plasma sodium concentration in lactating rats was significantly greater than in 16 day pregnant and virgin controls ($P < 0.05$ ANOVA then Duncans range test) . Hyperosmotic stimulation significantly increased plasma sodium concentration in virgin rats ($P < 0.02$ paired t test) from a mean basal of 133 ± 1.1 to a maximum of 143.6 ± 3.1 mmol/l ; in pregnant rats ($P < 0.0001$ paired t test) from a mean basal of 132.3 ± 0.45 to a maximum of 141 ± 0.8 mmol/l and in lactating rats ($P < 0.001$ paired t test) from a mean basal of 137.4 ± 0.6 to a maximum of 143.5 ± 0.3 mmol/l .

LEGEND : FIGURE 4.4B

Plasma sodium concentration measurements were made on three (0 , 50 and 85min) femoral arterial blood samples from urethane anaesthetised virgin, 16 day pregnant and 7-9 day lactating rats which had received an I.P. injection of 1.5M saline (4ml/kg) at 20min (Figs 4.3 and 4.4) . Values are mean ± s.e.m. .

Graph (i) represents the change in plasma sodium concentration of the individual groups ; ❍, virgin controls (n = 5) ; ●, 16 day pregnant controls (n = 7) ; ■, 16 day pregnant i.v. morphine group (n = 7) ; ∇, 7-9 day lactating controls (n = 6) and Δ, 7-9 day lactating i.v. morphine (n = 8) . There were no significant differences in plasma sodium concentrations within the pregnant and lactating rat groups at any point so a mean of each group was taken (two sample t test) .

Graph (ii) represents changes in plasma sodium concentration for virgin controls (❍, n = 5), pregnant rats with and without i.v. morphine (■, n = 14) and lactating rats with and without morphine (Δ, n = 14) .

*, # = $P \leq 0.05$; **, = $P \leq 0.005$; *** = $P \leq 0.0005$

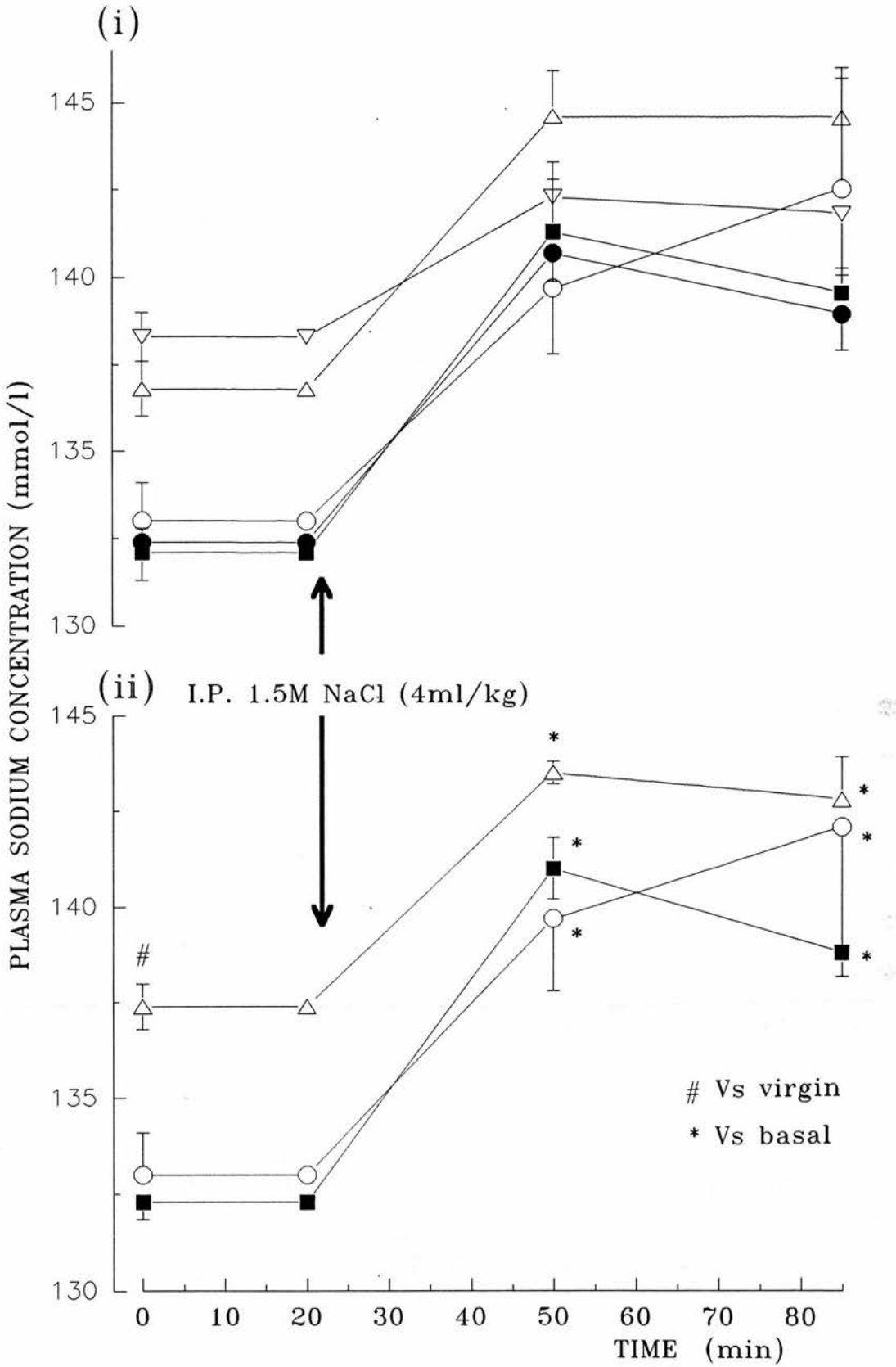


FIGURE 4.4B Plasma sodium concentration of virgin, pregnant and lactating rats after hyperosmotic (I.P.) stimulation

4.3.8 Plasma osmolality of virgin, 16 day pregnant and lactating rats after hyperosmotic (I.P.) stimulation (Fig 4.4C) .

Hyperosmotic stimulation significantly increased plasma osmolality in virgin rats ($P < 0.05$ paired t test) from a mean basal of 295.2 ± 4.4 to a maximum of 309.8 ± 10.5 mmol/l ; in pregnant rats ($P = 0.0002$ paired t test) from a mean basal of 277.2 ± 3.4 to a maximum of 292 ± 2.6 mmol/l and in lactating rats ($P = 0.0002$ paired t test) from a mean basal of 280 ± 1.9 to a maximum of 291 ± 1.9 mmol/l .

Basal and stimulated plasma osmolality of pregnant and lactating rats were significantly less than virgin controls ($P < 0.05$ ANOVA then Duncan's range test) .

LEGEND : FIGURE 4.4C

Plasma osmolality measurements were made on three (0, 50 and 85 min) femoral arterial blood samples from urethane anaesthetised virgin, 16 day pregnant and 7-9 day lactating rats which had received an I.P. injection of 1.5M saline (4ml/kg) at 20min (Figs 4.3 and 4.4) . Values are the mean ± s.e.m. of samples which were stored frozen until assay .

Graph (i) represents the change in plasma osmolality in the individual groups ; ❍, virgin controls (n = 6) ; ●, 16 day pregnant controls (n = 7) ; ■, 16 day pregnant i.v. morphine group (n = 7) ; ∇, 7-9 day lactating controls (n = 6) and Δ, 7-9 day lactating i.v. morphine (n = 8) . There were no significant differences in plasma osmolality between vehicle and morphine treated groups of pregnant and lactating rats at any time point so a mean of each group was taken (two sample t test) .

Graph (ii) represents changes of plasma osmolality in virgin controls (❍, n = 6), pregnant rats with and without i.v. morphine (■, n = 14) and lactating rats with and without morphine (Δ, n = 14) .

$*, \# = P \leq 0.05$; $*** = P \leq 0.0005$

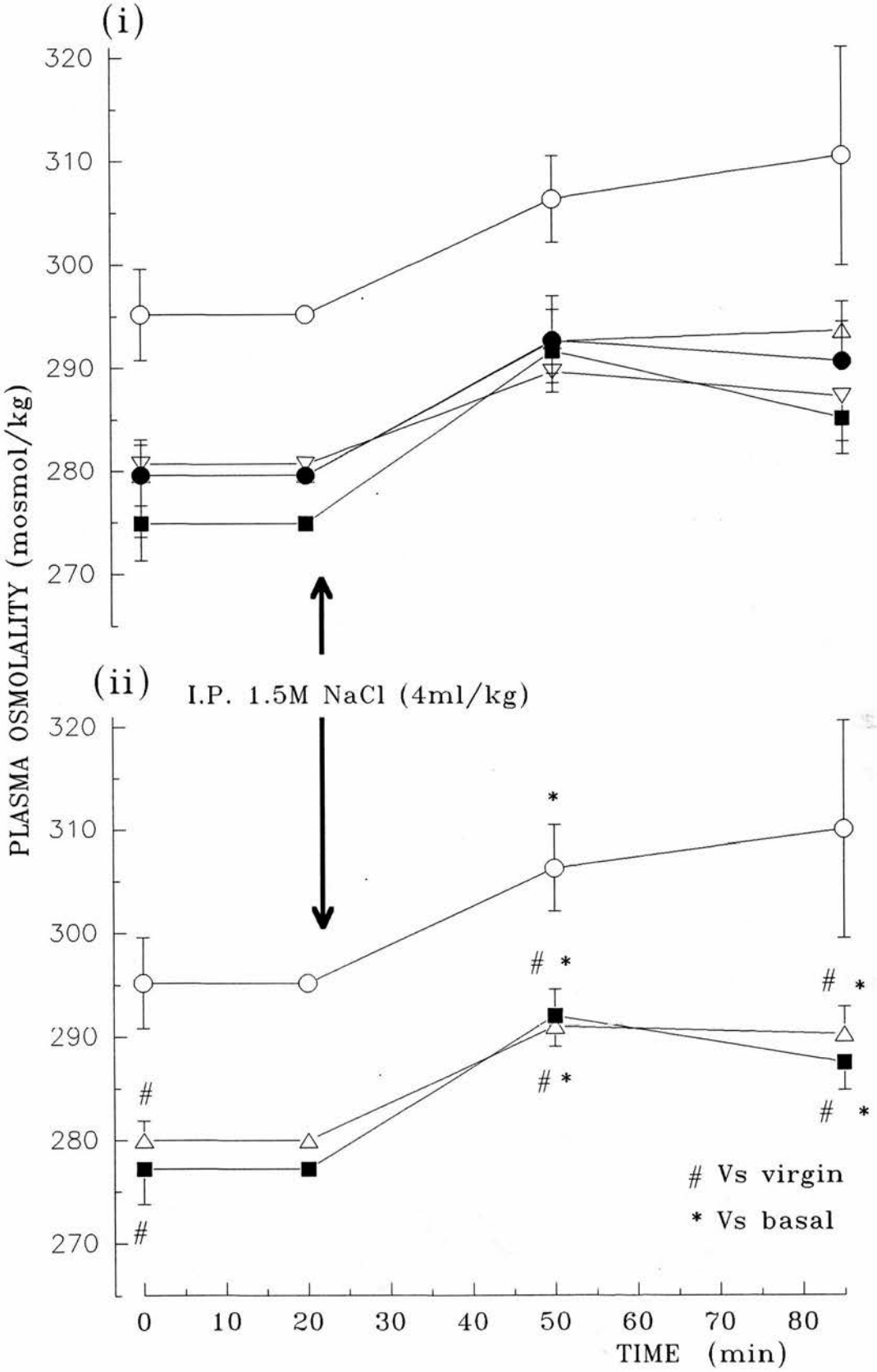


FIGURE 4.4C Plasma osmolality of virgin, pregnant and lactating rats after hyperosmotic (I.P.) stimulation

4.3.9 Changes in plasma sodium and oxytocin concentration of virgin , pregnant and lactating rats after I.P. 1.5M saline (Fig 4.5) .

Despite similar changes in plasma sodium 30 minutes after administration of the hyperosmotic stimulus 16 day pregnant and 7-9 day lactating rat plasma OT concentration changed significantly less than in virgin controls ($P < 0.05$ two sample t test) . Similarly the plasma OT concentration in 21 day pregnant rats was significantly lower compared to virgin controls 30 minutes after the administration of I.P. hypertonic saline ($P < 0.05$ two sample t test) .

15min after i.v. naloxone and 65min after I.P. 1.5M saline there were no significant differences for the changes in plasma sodium concentration between virgin, pregnant and lactating rats . However the increase in plasma OT concentration was still significantly less in 16 day pregnant ($P < 0.05$ two sample t test), 21 day pregnant ($P < 0.01$ two sample t test) and 7-9 day lactating rats ($P < 0.02$ two sample t test) than their virgin controls .

LEGEND : FIGURE 4.5

A comparison of the changes in plasma oxytocin with sodium concentration at equivalent time points after hyperosmotic stimulation with I.P. 1.5M NaCl (4ml/kg) in the two experiments involving virgin, 7-9 day lactating, 16 and 21 day pregnant rats (Figs 4.1, 4.2, 4.3 and 4.4) . Values are mean changes from basal ± s.e.m. . □, virgin (n = 4/5) ; ▦, 7-9 day lactating rats (n = 7) ; ▨, 16 day pregnant (n = 6) and ■, 21 day pregnant (n = 6) rats.

Graph (i) depicts changes in plasma oxytocin concentration and graph (ii) changes in plasma sodium concentration at equivalent time points .

#, = $P \leq 0.05$

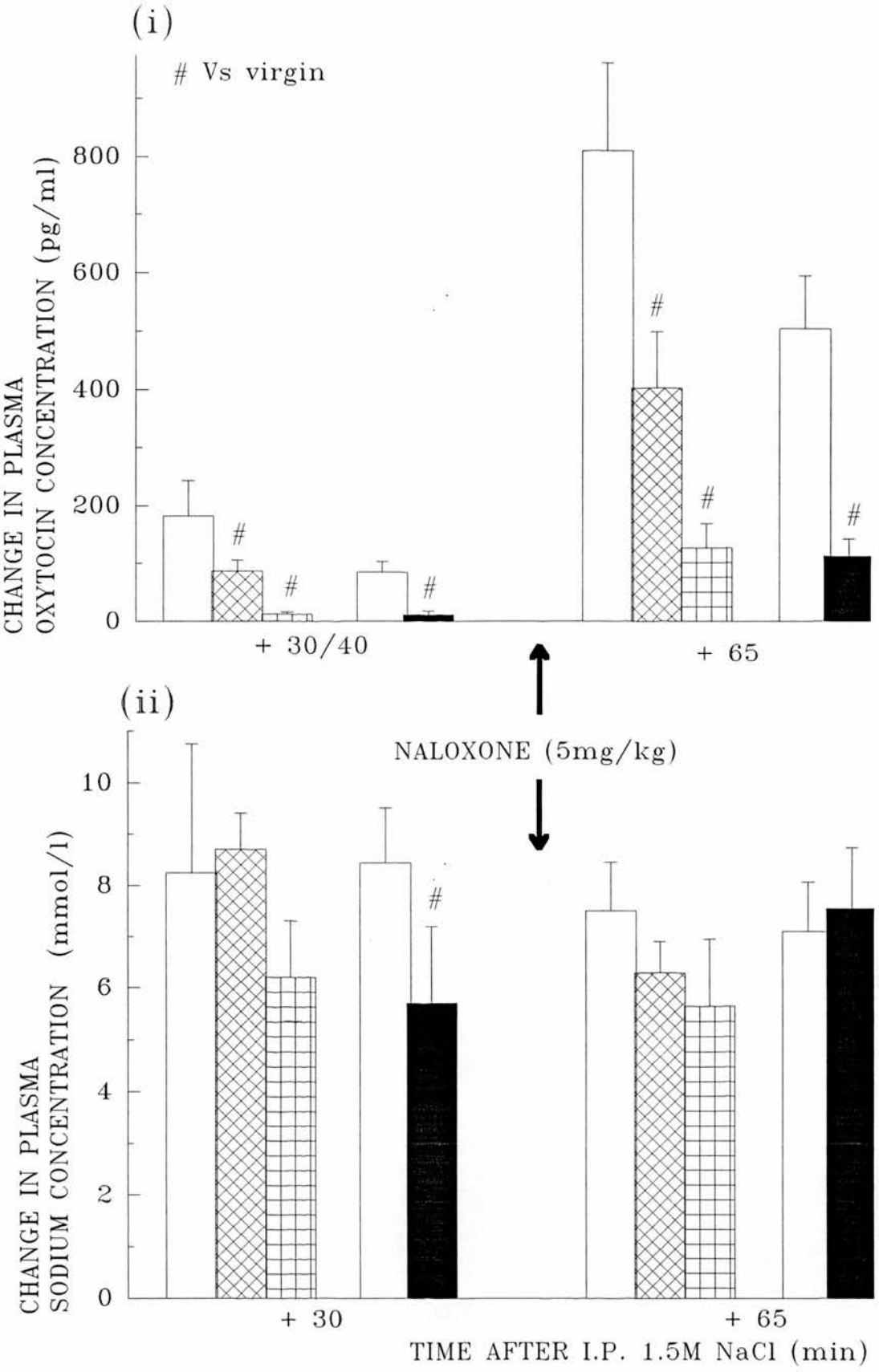


FIGURE 4.5 Change in plasma sodium and oxytocin concentration of virgin, pregnant and lactating rats after I.P. 1.5M saline

4.3.10 Effect of intravenous morphine on hyperosmotic (I.P.) stimulation of OT release in chronic intracerebroventricular morphine and vehicle infused rats (Fig 4.6) .

Hyperosmotic stimulation via I.P. 1.5M NaCl produced a significant increase in plasma OT concentration in the i.c.v. + i.v. vehicle group ($P < 0.05$ paired t test) from a mean basal value of 60.1 ± 14.8 to a maximum of 287.4 ± 77.6 pg/ml . Rats that received i.c.v. vehicle and i.v. morphine also showed a significant increase in plasma OT before administration of the second dose of morphine ($P < 0.05$ two sample t test) . However this rise was less than that of the i.c.v. + i.v. vehicle group and was significantly different at 30min ($P< 0.05$ two sample t test) . The response to hyperosmotic stimulation was completely abolished by the subsequent dose of 2.5mg/kg morphine in i.c.v. vehicle infused rats and was significantly less than basal at 70min ($P < 0.05$ two sample t test) .

Mean basal plasma OT concentration in i.c.v. morphine infused rats was significantly less than that of i.c.v. vehicle infused rats ($P < 0.05$ ANOVA then Duncans range test) . Hyperosmotic stimulation of i.c.v. morphine + i.v. vehicle infused rats produced a small but significant increase in plasma OT ($P < 0.03$ paired t test) from a basal value of 26.7 ± 4.6 to a maximum of 67.1 ± 4.6 pg/ml .

Like basal values, hyperosmotically stimulated values in i.c.v. + i.v. morphine rats were all significantly less than in i.c.v. + i.v. vehicle rats ($P < 0.05$ ANOVA then Duncan's range test) . Hyperosmotic stimulation of i.c.v. + i.v. morphine infused rats also produced a small but significant increase in plasma OT ($P < 0.05$ paired t test) from a mean basal of 29 ± 4.4 to a maximum of 97 ± 23.1 pg/ml . In contrast to i.c.v. vehicle rats, i.v. morphine

had no effect on hyperosmotically stimulated OT release, at the doses tested, in i.c.v. morphine infused rats ($P > 0.05$ ANOVA) .

Naloxone produced a significant increase in plasma OT concentration in i.c.v. vehicle infused rats and a complete reversal of i.v. morphine inhibition compared to sample 8 at 70min (❍ $P < 0.02$, ❑ $P < 0.03$ paired t test) . Naloxone also produced a large increase in plasma OT concentration in i.c.v. morphine infused rats (● $P < 0.003$, ■ $P < 0.0003$ paired t test) . In both i.c.v. morphine groups the responses to naloxone were significantly greater than in the i.c.v. vehicle groups ($P < 0.05$ ANOVA then Duncans range test) .

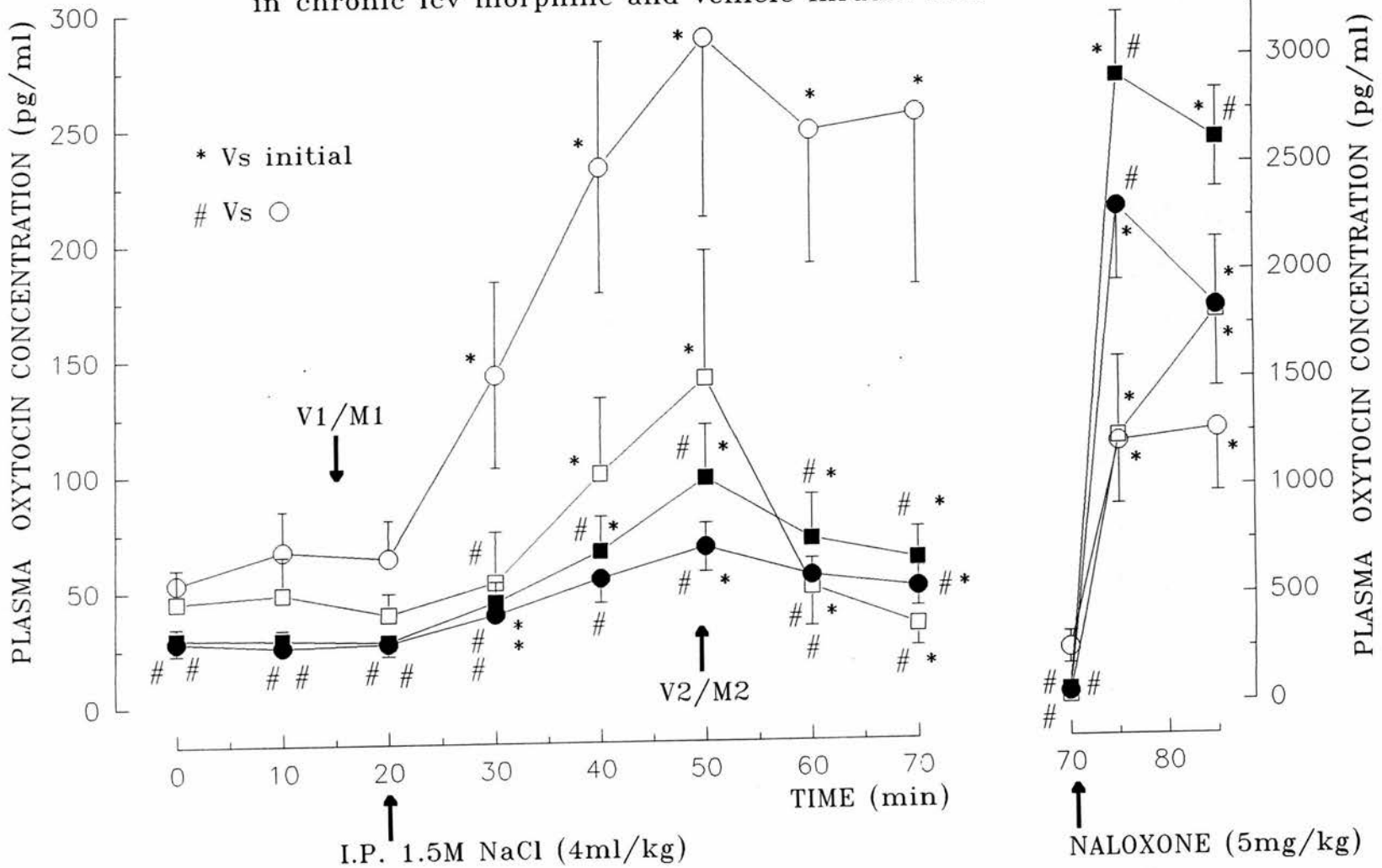
LEGEND : FIGURE 4.6

Plasma OT , measured by r.i.a. , in femoral arterial blood samples from urethane anaesthetised rats after 5 days of i.c.v. morphine or vehicle infusion . Values are mean ± s.e.m. ; ❍, received chronic i.c.v. vehicle and acute i.v. vehicle (n = 5) ; ❑, received chronic i.c.v. vehicle and acute i.v. morphine (n = 5) ; ●, received chronic i.c.v. morphine and acute vehicle (n = 6) and ■, received chronic i.c.v. morphine and acute i.v. morphine (n = 6) . Rats administered acute i.v. vehicle received two doses of 0.15M saline (0.4ml/kg) an initial dose at 15min and a second dose at 50min (V1, V2) . Acute i.v. morphine treated rats also received two doses, an initial dose of 0.25mg/kg at 15min and a second dose of 2.5mg/kg at 50min (M1, M2) .

All groups received an i.p. hyperosmotic stimulus at 20min (4ml/kg, 1.5M NaCl) and naloxone at 70min (i.v., 5mg/kg) .

$*, \# = P \leq 0.05$; $**, = P \leq 0.005$; $*** = P \leq 0.0005$

FIGURE 4.6 Effect of iv morphine on hyperosmotic (i.p.) stimulation of OT release in chronic icv morphine and vehicle infused rats



4.3.11 Plasma sodium concentration in chronic intracerebroventricular morphine and vehicle infused rats following hyperosmotic stimulation (I.P.) (Fig 4.6B) .

As can be seen after 5 days of i.c.v. morphine infusion basal plasma sodium concentration of virgin female rats was significantly greater than that of i.c.v. vehicle infused rats ($P < 0.002$ two sample t test) . Hyperosmotic stimulation significantly increased the plasma sodium concentration of i.c.v vehicle infused rats ($P = 0.0007$ paired t test) from a mean basal of 135.8 ± 1.0 to a maximum of 145.6 ± 1.1 mmol/l . An equivalent hyperosmotic stimulation in i.c.v. morphine infused rats also produced a significant increase in plasma sodium concentration ($P < 0.0001$ paired t test) from a mean basal of 140.1 ± 0.5 to a maximum of 149.1 ± 1.1 mmol/l .

As with basal values the stimulated values remained significantly different between i.c.v. treatments at both points ($P < 0.05$ two sample t test) .

LEGEND : FIGURE 4.6B

Measurements were made on three (0, 50 and 85min) femoral arterial plasma samples from urethane anaesthetised chronic i.c.v. morphine / vehicle infused rats which were stored frozen until assay . Values are mean ± s.e.m. .

Graph (i) represents changes in the individual groups ; ❍, i.c.v. vehicle + i.v. vehicle (n = 5) ; ❑, i.c.v. vehicle + i.v. morphine (n = 6) ; ●, i.c.v. morphine + i.v. vehicle (n = 6) and ■, i.c.v. morphine + i.v. morphine (n = 6) . There were no significant differences in sodium concentration within the i.c.v. treatment groups whether given acute i.v. morphine or not, so the data for each i.c.v. sub-group were meaned (two sample t test) .

Graph (ii) represents plasma sodium concentration in i.c.v. vehicle infused rats with and without i.v. morphine (❍, n = 11) and i.c.v. morphine infused rats with and without i.v. morphine (●, n = 12) .

= $P \leq 0.05$; **, ## = $P \leq 0.005$; *** = $P \leq 0.0005$

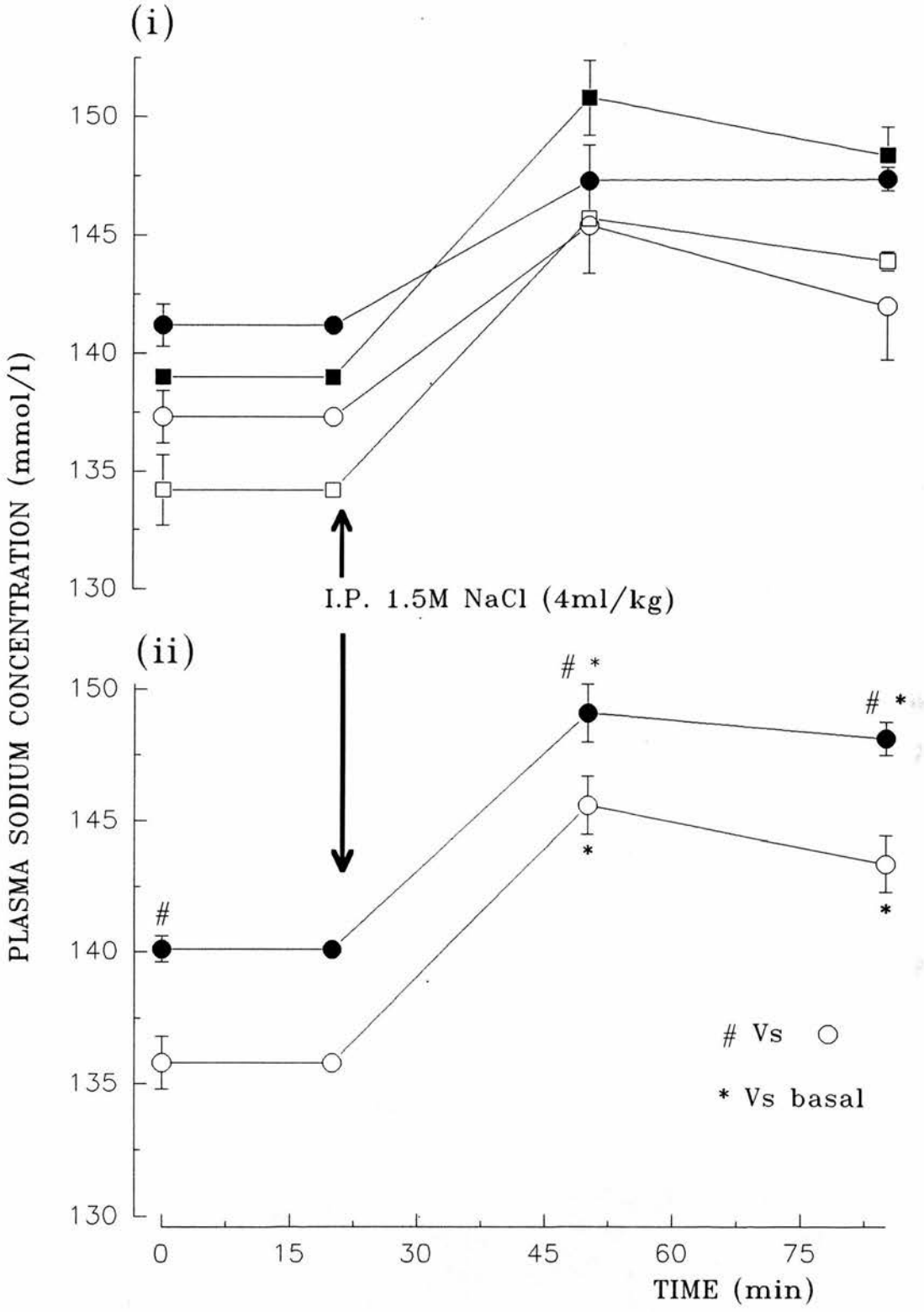


FIGURE 4.6B Plasma sodium concentration of chronic i.c.v. morphine and vehicle infused rats following hyperosmotic stimulation (I.P.)

4.3.12 Plasma osmolality in chronic intracerebroventricular morphine and vehicle infused rats following hyperosmotic stimulation (I.P.) (Fig 4.6C) .

In contrast to sodium concentration basal plasma osmolality was not significantly different (two sample t test) between chronic icv morphine and vehicle infused rats . Hyperosmotic stimulation significantly increased the plasma osmolality in i.c.v vehicle infused rats ($P < 0.002$ paired t test) from a mean basal of 293.2 ± 3.3 to a maximum of 314 ± 4.2 mosmol/kg . An equivalent hyperosmotic stimulation in i.c.v. morphine infused rats also produced a significant increase in plasma osmolality ($P < 0.0001$ paired t test) from a mean basal of 294.3 ± 1.8 to a maximum of 311.8 ± 2.1 mosmol/kg .

As with basal values the stimulated values remained non significantly different between i.c.v. treatments at both points (two sample t test) .

LEGEND : FIGURE 4.6C

Measurements were made on three (0, 50 and 85 min) femoral arterial plasma samples from urethane anaesthetised chronic i.c.v. morphine / vehicle infused rats which were stored frozen until assay . Values are mean ± s.e.m. .

Graph (i) represents changes in the individual groups ; ❍, i.c.v. vehicle + i.v. vehicle (n = 5) ; ❑, i.c.v. vehicle + i.v. morphine (n = 6) ; ●, i.c.v. morphine + i.v. vehicle (n = 6) and ■, i.c.v. morphine + i.v. morphine (n = 6) . There were no significant differences in plasma osmolality within the i.c.v. treatment groups whether given acute i.v. morphine or not so the data from each i.c.v. sub-groups were meaned (two sample t test) .

Graph (ii) represents the change in plasma osmolality in i.c.v. vehicle infused rats with and without i.v. morphine (❍, n = 11) and i.c.v. morphine infused rats with and without i.v. morphine (●, n = 12) .

** = $P \leq 0.005$; *** = $P \leq 0.0005$

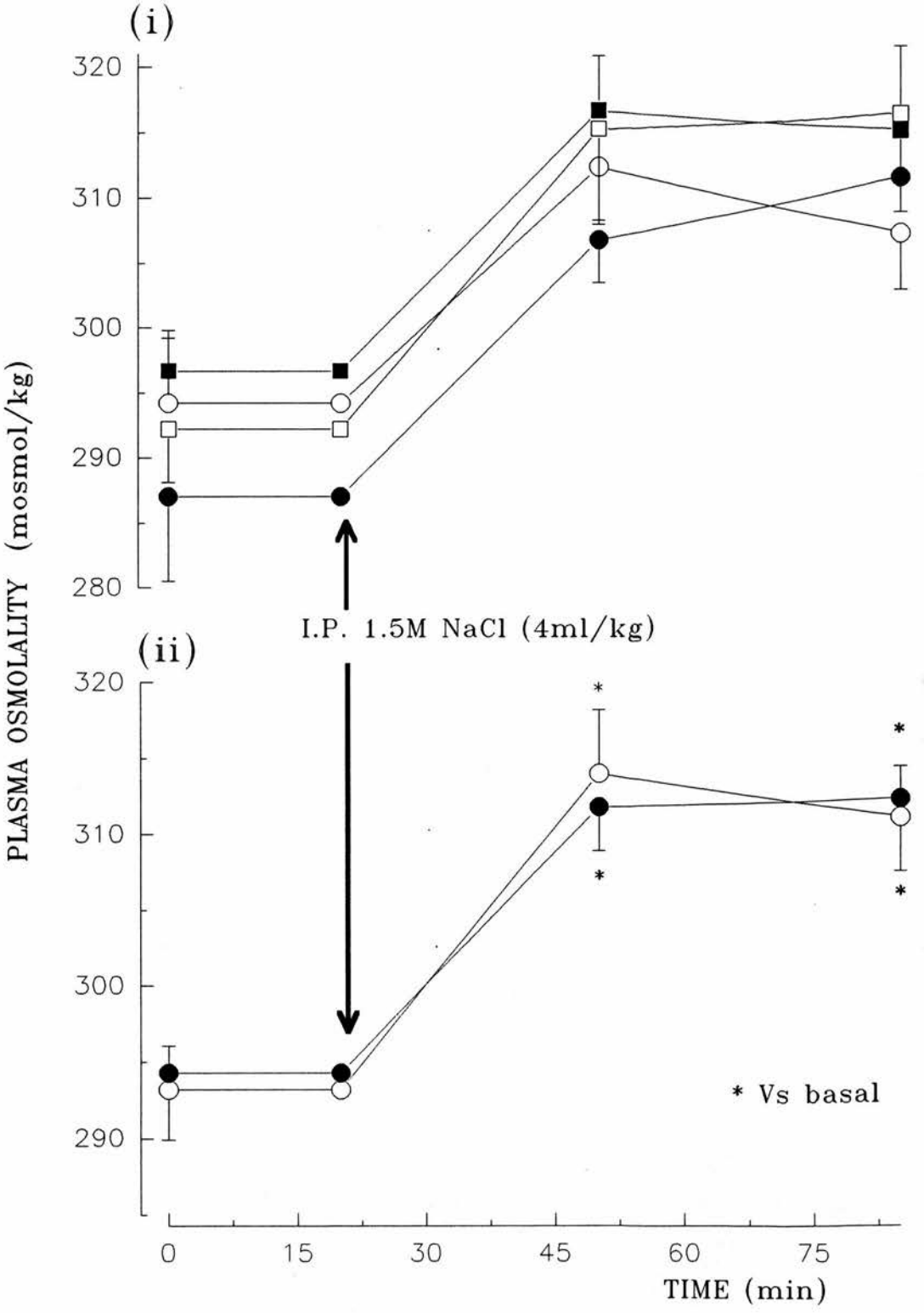


FIGURE 4.6C Plasma osmolality of chronic i.c.v. morphine and vehicle infused rats following hyperosmotic stimulation (I.P.)

4.3.13 Effect of ovariectomy on hyperosmotic (I.P.) stimulation of oxytocin release in 21 day pregnant rats (Fig 4.7) .

4 hours after surgery mean basal plasma OT concentration was found to be significantly greater in ovariectomised rats compared to sham operated controls ($P < 0.05$ two sample t test) .

Hyperosmotic stimulation via I.P. 1.5M saline produced a small but significant increase of plasma oxytocin concentration in sham operated animals ($P < 0.02$ paired t test) from a mean basal value of 13.2 ± 2.2 to a maximum of 48.9 ± 9.6 pg/ml 30 minutes after the I.P. injection . An equivalent hyperosmotic stimulus given to ovariectomised rats also produced a significant rise in plasma oxytocin concentration ($P < 0.02$ paired t test) from a mean basal of 22.8 ± 3.9 to a maximum of 45.4 ± 7.9 pg/ml at the same sample point as sham operated animals . Naloxone (5mg/kg , i.v.) produced a significant increase in plasma OT concentration compared to the previous blood sample in both ovariectomised ($P < 0.002$ paired t test) and sham operated rats ($P < 0.02$ paired t test) .

Ovariectomy however had no significant effect on either the rate of change or the absolute values of OT obtained from hyperosmotically stimulated 21 day pregnant rats both before and after naloxone (two sample t test).

LEGEND : FIGURE 4.7

Plasma oxytocin concentration in femoral arterial blood samples from urethane anaesthetised 21 day pregnant ovariectomised and sham operated rats . Values are mean ± s.e.m. , open circles (❍) are ovariectomised rats (n = 7) and closed circles (●) are sham operated rats (n = 7) .

Both groups received a hyperosmotic stimulus (i.p. 1.5M saline) at 10min and the opioid antagonist naloxone (iv) at 60min .

*, †, # = $P \leq 0.05$; **, †† = $P \leq 0.005$

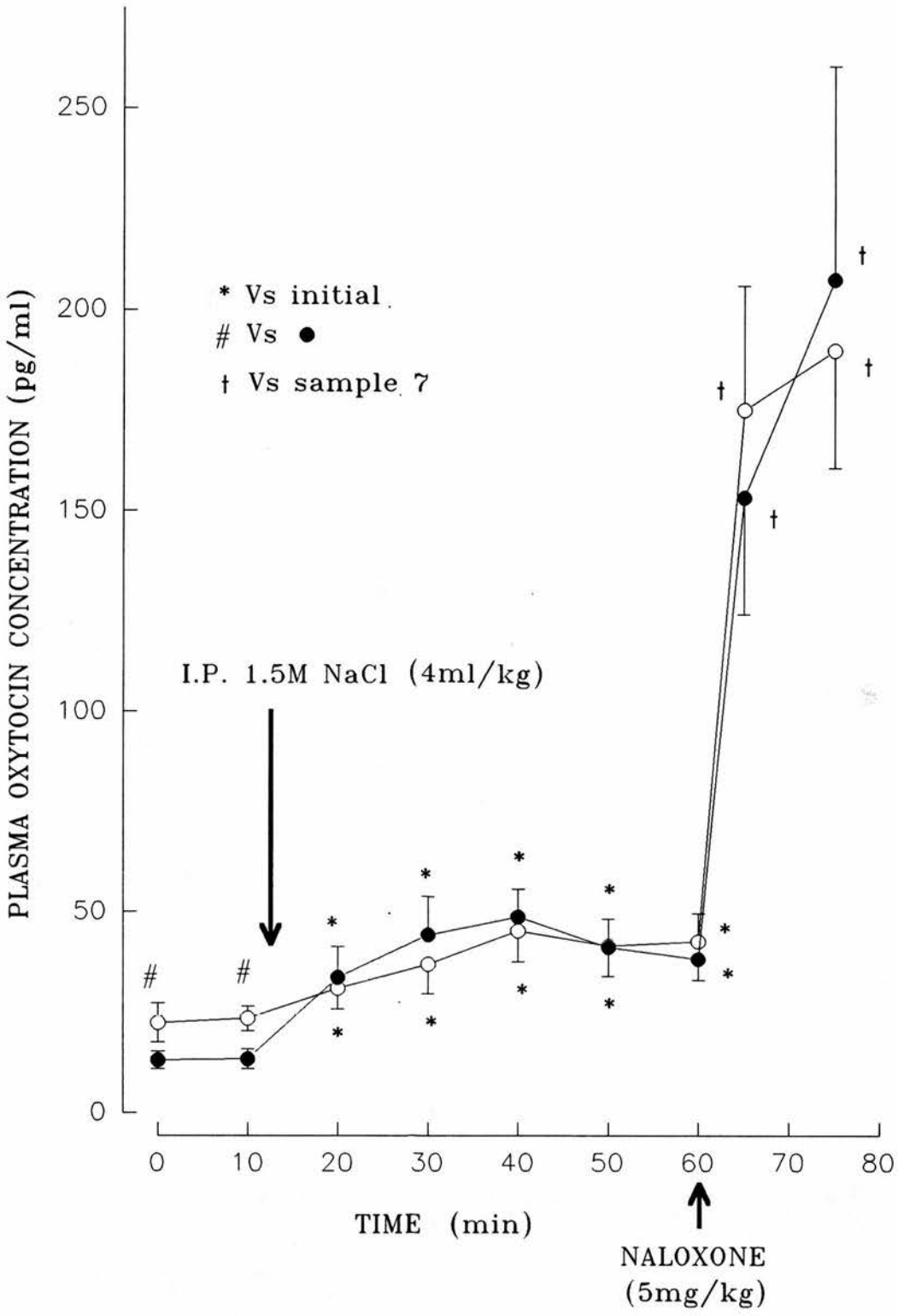


FIGURE 4.7 Effect of ovariectomy on hyperosmotic (i.p.) stimulation of oxytocin release in 21 day pregnant rats

4.3.14 Plasma osmolality and sodium concentration of ovariectomised and sham operated 21 day pregnant rats following hyperosmotic stimulation (I.P.) (Fig 4.7B) .

I.P. 1.5M saline significantly increased plasma sodium concentration (graph (ii)) of sham operated animals compared to basal ($P < 0.02$ paired t test) from 136 ± 2.5 to a maximum of 148.9 ± 3.3 mmol/l . An identical stimulus in rats ovariectomised 4 hours prior to the start of the experiment also produced a significant increase in plasma sodium concentration ($P < 0.05$ paired t test) from a basal level of 138 ± 1.4 to a maximum of 144.3 ± 1.0 mmol/l .

Plasma osmolality (graph (i)) followed similar changes to those of plasma sodium concentration . In the sham operated group osmolality rose significantly ($P < 0.02$ paired t test) from a basal value of 262.7 ± 5.3 to a maximum of 287.6 ± 7.4 mosmol/kg . In the ovariectomised group osmolality rose significantly ($P < 0.002$ paired t test) from a basal level of 272.9 ± 3.2 to a maximum of 284 ± 2.6 mosmol/kg .

There were no significant differences in the plasma osmolality and sodium concentration between intact and ovariectomised rat (two sample t test) .

LEGEND : FIGURE 4.7B

Osmolality (graph (i)) and sodium concentration (graph (ii)) measurements were made on five femoral arterial plasma samples (at 0, 20, 40, 60 and 75min) from urethane anaesthetised ovariectomised and sham operated 21 day pregnant rats which were stored frozen until assay (from Fig 4.7) . Values are mean ± s.e.m. ; ❍, are ovariectomised rats (n = 7) and ●, sham operated rats (n = 7) .

$* = P \leq 0.05$; $** = P \leq 0.005$; $*** = P \leq 0.0005$

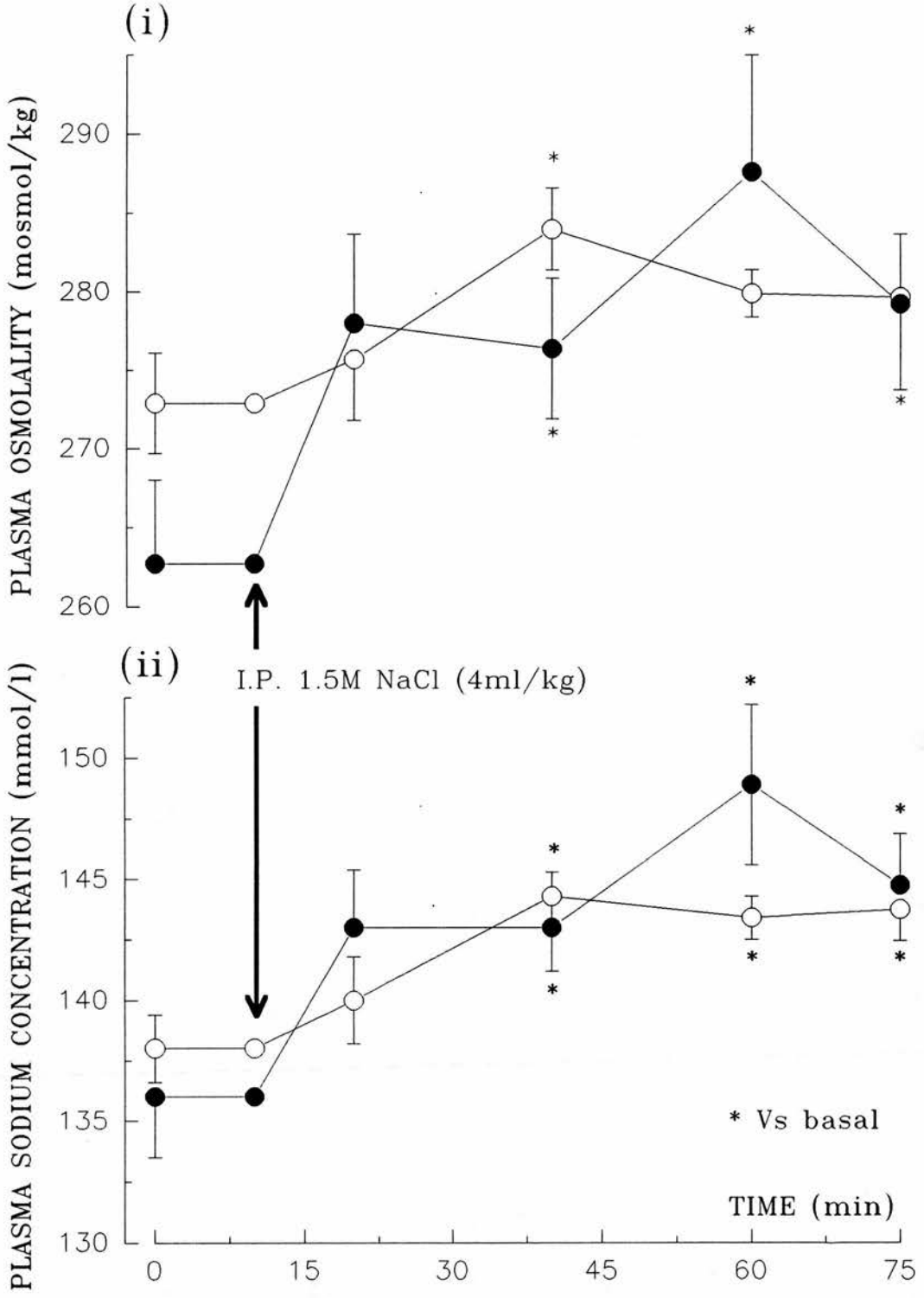


FIGURE 4.7B Plasma osmolality and sodium concentration of ovariectomised and sham operated 21 day pregnant rats following hyperosmotic stimulation (i.p.)

4.3.15 Effect of pregnancy on angiotensin II (i.v) stimulated OT release (Fig 4.8) .

Plasma OT concentration rose significantly during the first angiotensin II infusion in virgin rats ($P < 0.05$ paired t test) from a basal value of 96.9 ± 14.5 to 130.1 ± 23.7 pg/ml , and then returned to a basal level 20 minutes after the infusion ended . A matching stimulus in 21 day pregnant rats produced a similar but non significant rise in plasma OT concentration from 82.1 ± 9.5 to 104.6 ± 12.1 pg/ml before values returned to basal levels after the infusion was turned off .

Naloxone produced a significant increase in the plasma OT concentration of virgin rats ($P < 0.05$ paired t test) from 104.1 ± 22.6 to 261.4 ± 71.5 pg/ml and in 21 day pregnant rats ($P < 0.05$ paired t test) from 81.9 ± 7.6 to 186 ± 45.6 pg/ml . A subsequent angiotensin II infusion produced a further significant increase in plasma OT concentration of virgin rats ($P < 0.03$ paired t test) from 261.4 ± 71.5 to 364.3 ± 50.9 and in 21 day pregnant rats ($P < 0.02$ paired t test) from 186 ± 45.6 to 293.8 ± 60.1 pg/ml . OT concentration then returned to values non significantly different to pre infusion levels in both groups (paired t test) . There were no significant differences between the plasma OT concentration of virgin and 21 day pregnant rats throughout the experiment (paired t test) .

LEGEND : FIGURE 4.8

Plasma OT was measured by ria of femoral arterial blood samples in an experiment to investigate the effect of an angiotensin II infusion (i.v.) in virgin and 21 day pregnant rats before and after naloxone (i.v.) . Values are mean ± s.e.m. ; ❍, are virgin rats (n = 5) and ●, are 21 day pregnant rats (n = 6) .

Angiotensin II was infused (iv) for two periods of 20min at a rate of 0.5μg/min/kg , the first infusion period was from 0 to 20min and the second was from 75 to 95min . Naloxone (5mg/kg , i.v.) was administered at 60min .

$*, \#, \dagger = P \leq 0.05$; $**, \dagger\dagger = P \leq 0.005$

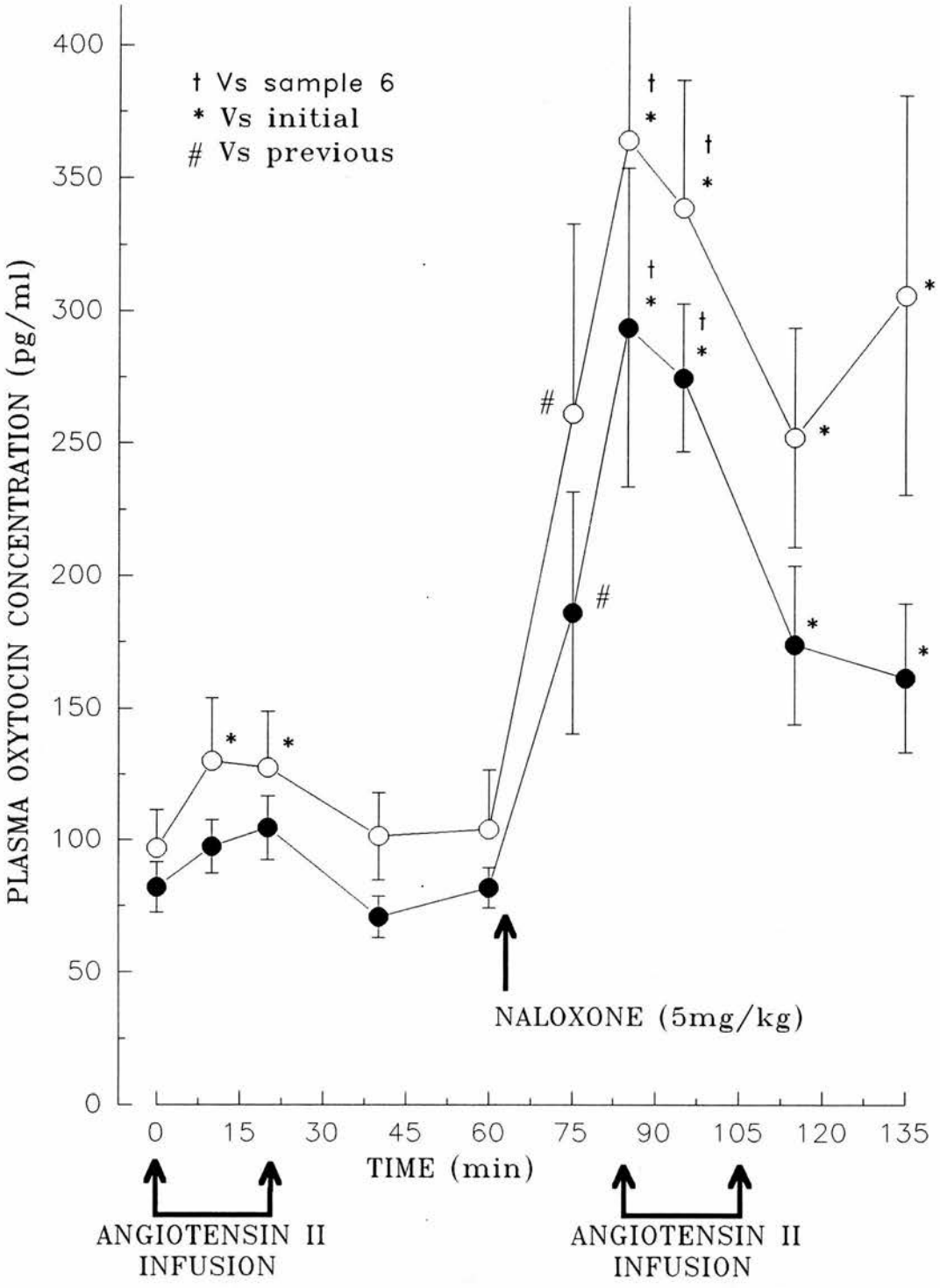


FIGURE 4.8 Effect of pregnancy on angiotensin II (i.v.) stimulated OT release

4.3.16 Change in plasma OT concentration of virgin and 21 day pregnant rats with an angiotensin II infusion (i.v.) (Fig 4.9) .

The initial angiotensin II infusion produced a significant increase in plasma OT concentration compared to zero in virgin ($P < 0.05$ paired t test) of 31.9 ± 10.2 pg/ml and in 21 day pregnant rats ($P < 0.01$ paired t test) of 29.6 ± 7.4 pg/ml . There was no significant change in plasma OT concentration from basal after the infusion was stopped in both virgin and pregnant rats (paired t test) .

Naloxone produced a significant change from basal in the plasma OT concentration of virgin rats ($P < 0.05$ paired t test) of 164.6 ± 62.7 pg/ml and in 21 day pregnant rats ($P < 0.05$ paired t test) of 133.3 ± 46.6 pg/ml . The subsequent angiotensin II infusion produced a further significant increase in plasma OT concentration compared to the increase of sample 6 in virgin ($P < 0.02$ paired t test) of 259.9 ± 33.4 pg/ml and in pregnant rats ($P = 0.005$ paired t test) of 230.8 ± 45 pg/ml . Values then returned to pre infusion levels when the angiotensin II infusion was turned off . There were no significant differences in the changes of plasma OT concentrations from basal between virgin and 21 day pregnant rats at any sample during the experiment .

LEGEND : FIGURE 4.9

The change in plasma OT concentration from basal was calculated for the rats that had received an intravenous infusion of angiotensin II in Fig 4.8 . Values are the mean changes from basal ± s.e.m. ; □, are virgin rats (n = 5) and ■, are 21 day pregnant rats (n = 6) . There were no significant differences between samples 2 and 3, 4 and 5, 7 and 8 or 9 and 10 so the mean changes were plotted .

Angiotensin II was infused (iv) for two periods of 20min at a rate of 0.5μg/min/kg , initially during samples 2 and 3 and subsequently during samples 7 and 8 . Naloxone (5mg/kg , i.v.) was administered after sample 5 .

$*, \dagger = P \leq 0.05$; $**, \dagger\dagger = P \leq 0.005$

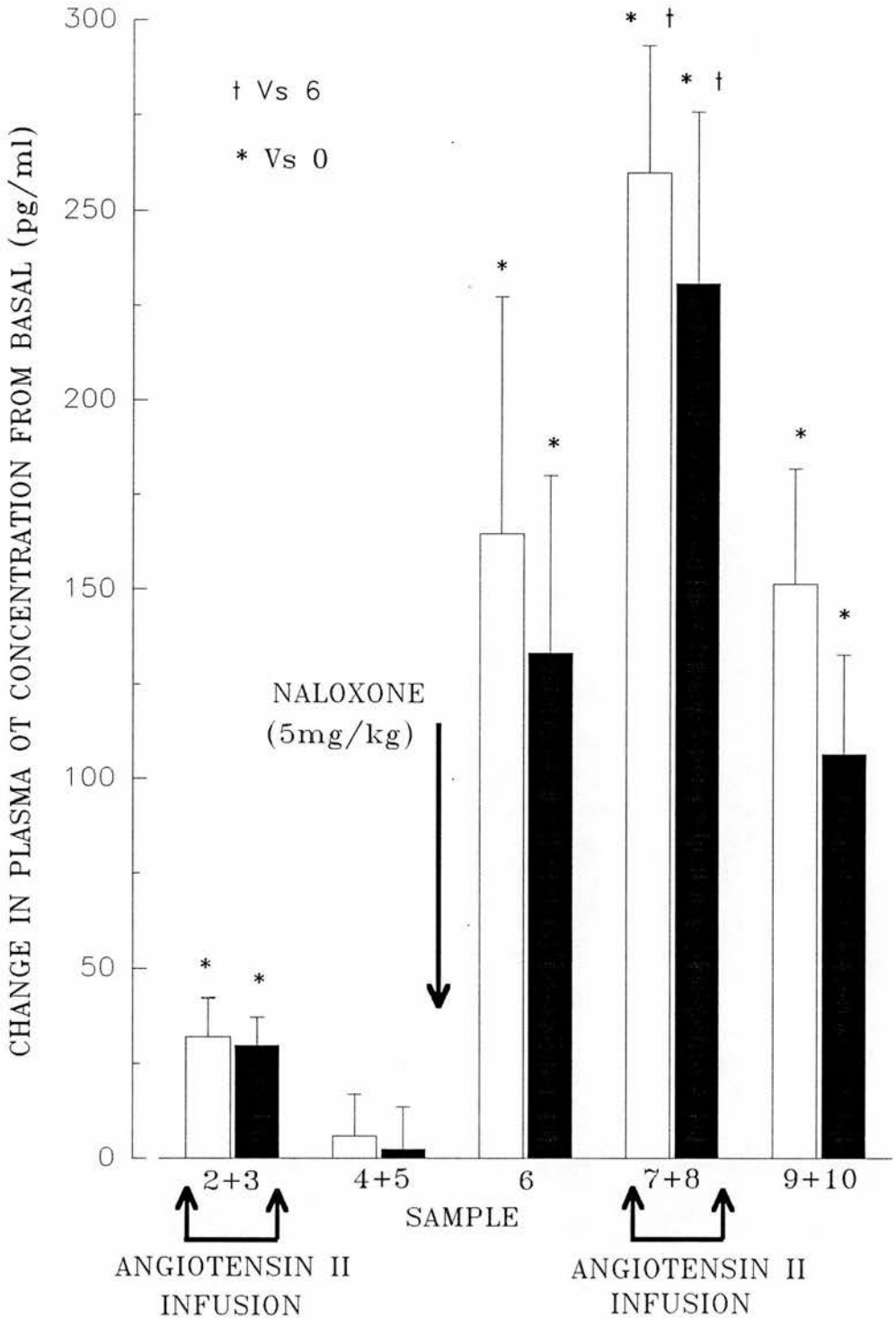


FIGURE 4.9 Change in plasma OT concentration of virgin and 21 day pregnant rats with an angiotensin II infusion (i.v.)

4.4 DISCUSSION

As expected hyperosmotic stimulation of urethane anaesthetised virgin female rats (Fig 4.1), via i.p. 1.5M NaCl, produced a significant increase in circulating plasma OT concentration . What was not expected was the apparent unresponsiveness of the OT system to an equivalent stimulus in the full term gravid rat (Fig 4.2) . The increase in plasma vasopressin concentration in response to 48 hours of water deprivation and administration of i.p. hyperosmotic saline were found to be similar in virgin and 20 day pregnant rats (Durr *et al.*, 1981) . This demonstrates that the apparent osmotic threshold for vasopressin secretion is reduced by 11-14mosmol/kg in association with the reduced plasma osmolality and sodium concentration of pregnant rats . In addition, Barron *et al.* (1984) reported that pregnant rats secrete vasopressin in response to fractional decreases in blood volume in a manner similar to virgin animals . These results suggest that the reduced plasma osmolality and increased plasma volume are perceived as normal by the vasopressin system in the pregnant rat and demonstrate a resetting of the receptor thresholds for these stimuli . A similar result has been demonstrated in rats maintained for 10-24 days under hyposmolar conditions (Verbalis and Dohanics, 1991) . In these rats detectable increases in plasma OT and vasopressin began at significantly lower plasma sodium concentrations compared to normosmolar control rats . They suggested that osmoreceptor activity is regulated to maintain extracellular fluid and plasma osmolality within narrow absolute ranges rather than responding to relative changes in osmolality . In contrast our results suggest that the response of the OT system to changes in plasma osmolality is attenuated during the hyposmolar and hypervolaemic conditions of pregnancy .

A reduced OT response to i.p. hyperosmotic saline was apparent from day 16 of pregnancy (Fig 4.3) when OT secretion was significantly different from virgin animals towards the end of the experiment . A similar unresponsiveness of the OT system was obtained in the 7-9 day lactating rat (Fig 4.4) . In the lactating rat a reduced OT response to hyperosmotic stimulation has already been noted (Hartman *et al.*, 1987 ; Evans and Olley, 1988) . This reduced response was postulated to be a mechanism by which the rat could conserve the pituitary stores of OT for essential milk ejection purposes . Higuchi *et al.* (1991) could find no conclusive evidence for a reduction in the excitability of OT neurones at the level of both the cell bodies and neural lobes of lactating rats . They attributed this reduction in OT release in response to a number of stimuli, including hyperosmotic saline, simply to a reduced pituitary content of oxytocin . This is not the case in the pregnant rat where pituitary content of OT is found to increase during pregnancy (Fuchs and Saito, 1971 ; Kumaresan *et al.*, 1979) . Indeed electrical stimulation of isolated neural lobes from 16 and 21 day pregnant rats releases more OT compared with those from virgin female controls (Douglas *et al.*, 1992 in press) .

4.4.1 Do opioid changes explain the loss of OT responses to increased plasma sodium concentration ?

As mentioned previously endogenous opioid tone apparently increases during the second week of pregnancy reaching a maximum effect during parturition (Hartman *et al.*, 1986b ; Leng et al., 1988b) . Opiates reduce OT secretion by an action on the cell bodies and terminals of the OT neurones . Both mu- and kappa-opioid receptor subtypes are found in the supraoptic nucleus of the rat (Mansour *et al.*, 1987 and 1988 ; Sumner *et al.*,

1990) and agonists for both these receptors inhibit the firing rate of OT neurones (Leng and Russell, 1989 ; Leng *et al.*, 1990) . In the rat posterior pituitary gland only the kappa-receptor subtype is present (Herkenam *et al.*, 1986) and kappa-receptor agonists are found to selectively inhibit OT release at this level (Zhao *et al.*, 1988a) . Sumner *et al.* (1992) reported that mu-selective binding in the SON was reduced on the last day of pregnancy and kappa-selective binding in the posterior pituitary was less on day 16 . Mu- and kappa-receptor binding in the SON were also reduced on day 16 of pregnancy although this result was not significant . These changes in endogenous opioid activity may be important in fine tuning the synergism between OT and vasopressin in promoting natriuresis during the fluid changes seen in pregnancy . Electrolyte intake is known to increase from day three of pregnancy and associated with this is an equivalent increase in renal excretion . Presumably if OT is involved in natriuresis this increase in excretion will involve an increase in the secretion of OT and its co-localised auto inhibitory [met]enkephalins . Sustained stimulation of the OT system during the first two weeks of pregnancy may therefore result in the reduced levels of neural lobe kappa-receptor binding due to desensitisation by co-released opioids . An increase in the plasma concentration of OT in unanaesthetized pregnant compared to virgin rats has been reported (Higuchi *et al.*, 1986) and associated with this is a decrease in the posterior pituitary content of [met]enkephalin (Douglas *et al.*, 1992 in press) .

Another explanation could involve the stimulation of vasopressin neurones and subsequent release of the cross inhibitory dynorphins . A significant increase in plasma vasopressin has been reported at the end of pregnancy which may account for the increase in fluid retention observed during the final week of pregnancy (Summy-Long, 1989 ; Landgraf *et al.*,

1991) . Douglas *et al.* (1992 in press) however reported that there was no change, and Schriefer (1991) an increase, in the neural lobe content of dynorphin during pregnancy . This leads to the conclusion that the reduction in kappa-receptor binding in the neural lobe of 16 day pregnant rats is more likely due to desensitisation by co-released opioids from OT neurones themselves and not the result of increased release of dynorphin from neighbouring vasopressin neurones .

The increased endogenous opioid tone on the OT system that develops during pregnancy may restrain its release during the final week and as a result promote sodium retention . This would account for the 33% decrease in sodium excretion reported, 59% of which was found in the products of conception (Churchill *et al.*, 1980) . This sodium retention therefore appears to supply an increased foetal demand . Alternatively the primary reason for restricting OT release at this point could be to facilitate pituitary hormone storage, found towards the end of pregnancy, in preparation for parturition . The changes in fluid balance observed may therefore result from this course of action which restricts OT release which produces a reduction in sodium excretion .

4.4.2 Opiate inhibition of oxytocin secretion in response to i.p. hyperosmotic saline

In our experiments using the mu-receptor agonist morphine we have attempted to investigate whether these changes in endogenous opioid activity produce tolerance in the OT system to exogenous opiates when challenged by a hyperosmotic stimulus . In virgin rats a threshold dose of 1mg/kg (i.v.) morphine proved effective at attenuating the rise in plasma OT concentration obtained after the administration of i.p. 1.5M NaCl (4ml/kg) . In

21 day pregnant rats, which show a decrease in mu-receptor binding in the SON, although we were unable to obtain a significant increase in plasma OT concentration sensitivity to morphine inhibition was apparently similar to that of virgin rats . The OT response to hyperosmotic stimulation in the rat is mediated by a complex of osmoreceptors including the supraoptic neurones themselves (Chapter 3) . Excitatory osmoreceptive input to the SON comes from the AV3V region nucleus and subfornical organ both of which contain opioid receptors (Sharif and Hughes, 1989 ; Fregoneze *et al.*, 1988) . Tolerance to morphine at the level of the SON could therefore reveal morphine sensitivity at one of these input sites .

The non selective opioid antagonist naloxone produced a large increase in plasma OT concentration when administered (i.v.) to hyperosmotically stimulated 21 day pregnant and virgin rats . Naloxone however does not further increase the firing rate of OT neurones, in virgin rats at least (Shibuki *et al.*, 1988), and an action at the neuronal terminals is therefore proposed . This can be explained in view of the fact that the hyperosmotic stimulus used also stimulates the release of vasopressin and presumably the dynorphins with which it is co-localised . Dynorphins are known to inhibit OT secretion from the posterior pituitary as are [met]enkephalins co-localised in the OT neurones themselves (Bicknell and Leng, 1982a and b ; Panula and Lindberg, 1987) . It is therefore possible that hyperosmotic stimulation not only produces cross inhibition of OT secretion from the neighbouring vasopressin terminals but also auto inhibition by co-localised opioids as well . The inhibitory actions of both would be antagonised by naloxone and hence increase the release of OT from the neural lobe .

Despite equivalent increments in plasma sodium concentration in virgin and 21 day pregnant rats plasma OT concentration increased significantly less after naloxone in the 21 day pregnant rat (Fig 4.5) . This leads to the conclusion that the increase in endogenous opioid activity seen during pregnancy is not the reason for the decrease in response to i.p. hyperosmotic stimulation . It could however account for the increased sensitivity of OT neurones to morphine in the 16 day pregnant rat whose apparent threshold for morphine inhibition is reduced to 0.25mg/kg (i.v.) . There is a reduction in the kappa opioid receptor binding in the posterior pituitary at this time . In order to compensate for this reduction it is possible that a compensatory increase in endogenous mu-opioid tone occurs at the level of the cell bodies in the SON or on an excitatory input to them . Addition of an exogenous mu-opiate at this stage in pregnancy would then have a cumulative effect with the endogenous opioids . At this time point their activity may have increased before there is any significant compensatory change in receptor numbers . Addition of an exogenous opiate therefore may act synergistically with the increase in endogenous opioid activity and thereby reduce the threshold for morphine inhibition .

4.4.3 Osmotic regulation of oxytocin secretion in chronic morphine treated rats

Additional evidence that it is not opioid restraint that is attenuating the OT response to hyperosmotic stimulation during pregnancy was obtained in the rat after 5 days of i.c.v. morphine infusion . Chronic morphine infused rats develop a reduction in mu-opioid receptor binding in the SON (Sumner *et al.*, 1990), a similar circumstance as that found in 21 day pregnant rats . Such rats develop tolerance to the exogenously infused morphine revealed

by the resumption of near normal activity (Russell, 1989) . However, we have found that these rats do not develop tolerance to the morphine infusion with respect to their response to hyperosmotic stimulation . This is displayed in their significantly attenuated response to hyperosmotic stimulation, via i.p. 1.5M NaCl, compared to vehicle infused controls (Fig 4.6) .

Urethane has been reported to have a dehydrating effect by elevating plasma osmolality (Hartman *et al.*, 1987). Under basal urethane anaesthetised conditions we have found that chronic morphine infused rats have a significantly lower plasma OT concentration . This may be due to the reduced response of OT neurones in chronic morphine treated rats to the hyperosmotic effects of urethane .

Basal plasma sodium concentration of rats after 5 days of i.c.v. morphine infusion is significantly increased (Fig 4.6B) . It is possible that the reduced responsiveness of the OT system to hypernatraemic stimulation may lead to sodium retention during morphine infusion . Basal plasma osmolality was however similar to that of vehicle infused rats (Fig 4.6C) indicating that the rat is able to compensate for this by altering other haemodynamic parameters . We obtained a similar result in lactating rats which also show a decreased OT response to hyperosmotic stimulation . Basal plasma sodium concentration of lactating rats was significantly greater than virgin rats, in contrast plasma osmolality was found to be significantly less . The increase in plasma sodium concentration may result from the lack of responsiveness of the OT system to fluctuations in plasma osmolality . Again the rat seems to be able to control other haemodynamic parameters to over compensate for this increase in sodium concentration as plasma osmolality in the lactating rat was also significantly less than virgin . However the method used for measuring plasma osmolality in these

experiments has been shown to be unreliable (see page 42, GM4 Blood Sampling) . Differences in plasma osmolality between groups may therefore be the result of experimental error as a result of freezing and thawing samples prior to assay .

In contrast to pregnant rats chronic morphine treated rats display tolerance to i.v. morphine at a cumulative dose of 2.75mg/kg when stimulated by i.p. 1.5M NaCl (Fig 4.6) . It is possible that the small but significant response of the OT system to hyperosmotic stimulation in the morphine treated rat is a result of the intrinsic osmosensitivity of the OT neurones themselves . A fully functional response however requires the excitatory input of other regions . It is possible that tolerance to i.c.v. morphine does not develop at one of these sites, for example the lamina terminalis and will therefore attenuate the activity of the OT neurones in response to hyperosmotic stimulation . At the level of the cell bodies in the SON however tolerance does develop, exhibited by the reduced mu-receptor binding, enabling a reduced OT response to osmotic stimulation compared to that of control rats . Tolerance at the level of the SON would also explain the attenuated inhibitory effect of i.v. morphine in chronic morphine treated rats compared to i.c.v. vehicle infused controls .

Naloxone given to chronic i.c.v. morphine infused rats after hyperosmotic saline stimulation produced a significantly greater increase in plasma OT concentration than in hyperosmotically stimulated i.c.v. vehicle treated controls (Fig 4.6) . This is explained by the finding that naloxone not only has an action at the posterior pituitary in chronic morphine treated rats but also increases the firing rate of OT neurones in the SON (Bicknell *et al.*, 1988a) . Thus naloxone precipitates a withdrawal from the inhibitory effects of chronic morphine treatment revealing the development of dependence

within the OT system to the exogenously applied opiate and this presumably summates with the hyperosmotic stimulation . In contrast naloxone in the hyperosmotically stimulated pregnant rat, although producing a significant increase in plasma OT concentration, does not restore non pregnant responsivity . These results therefore add further evidence to the hypothesis that it is not endogenous opioids that are restraining the hyperosmotic stimulation of OT release at this point in pregnancy .

4.4.4 Do ovarian hormones alter the osmoreception of OT neurones in pregnancy ?

We also investigated the effect of acute withdrawal of the ovarian hormones on the OT response of 21 day pregnant rats to i.p. 1.5M NaCl (Fig 4.7) . Ovariectomy is known to reduce basal plasma vasopressin concentration in the conscious rat from the first day after surgery (Peysner and Forsling, 1990) . A stimulatory action of ovarian steroids upon vasopressin secretion, and presumably a subsequent cross inhibition of OT release by dynorphin, is therefore implicated . Several reports provide evidence of a possible central action of ovarian steroids in modulating neurohypophysial release . A controversial report by Pelletier *et al.* (1988) showed that cells in the SON were labelled with a complementary mRNA probe coding for the oestradiol receptor . Also neurones involved in the regulation of vasopressin secretion have been found to concentrate ovarian steroids (Rhodes *et al.*, 1981) . However neither short-term nor long-term replacement with oestrogen or progesterone restored the plasma concentration of vasopressin in ovariectomised rats (Peysner and Forsling, 1990) . At that time they postulated one explanation for this lack of effect could be the result of an inappropriate steroid replacement protocol . In a

subsequent study Forsling *et al.* (1991) reported that chemical ovariectomy using an analogue of luteinising hormone releasing hormone reduced the OT response of rats to i.p. 1.5M NaCl . They proposed that ovarian steroids may influence osmoreceptor sensitivity involving an inhibitory action of progesterone . Roberts (1971) reported an inhibitory action of progesterone on OT release in response to vaginal distension of the rat . Progesterone is known to increase during the first two weeks of pregnancy before falling off before parturition at which point there is a rise in oestradiol .

We investigated whether acute removal of the ovarian hormones on the morning of the expected day of parturition would influence the response of the OT system to hyperosmotic stimulation . Surgical ovariectomy was performed 4 hours before the administration of i.p. 1.5M NaCl . This period of time was proposed to allow sufficient metabolism of circulating ovarian hormones without compromising the pregnancy of the rat .

Basal plasma OT concentration of ovariectomised rats was found to be significantly increased under urethane anaesthesia compared to sham operated controls . This contrasts with results obtained with vasopressin indicating a possible inhibitory function of ovarian steroids on OT neurone activity . However, after subsequent hyperosmotic stimulation there was no increase in OT secretion compared to sham operated animals . It would therefore appear that acute withdrawal of ovarian hormones has no effect in modifying the attenuated response of the OT system in the 21 day pregnant rat to hyperosmotic stimulation . It is possible that 4 hours after surgery is not long enough to reveal a response of withdrawal and a more prolonged period after ovariectomy may therefore be desirable . Relaxin is a peptide hormone secreted by the corpus luteum of the rat from day 10 of pregnancy . Its role in parturition is documented in a review by Weiss (1984) and

includes inhibition of uterine contraction and softening of the cervix . A naloxone reversible inhibitory action of relaxin on reflex milk ejection in lactating rats has been reported (O'Byrne *et al.*, 1987) . It has therefore been proposed that in pregnancy relaxin could inhibit oxytocin secretion via activation of endogenous opioid tone . A rise in relaxin levels has been reported in pregnancy (Sherwood *et al.*, 1980) . Summerlee (1989) postulated that dependence on this rise in endogenous relaxin could occur during normal rat pregnancy . The fall in plasma relaxin concentration the day before delivery could then trigger a withdrawal response and release OT which might initiate birth . However Musah *et al.* (1989) reported that relaxin caused an acute release of OT . In addition Way and Leng (1991) reported that the inhibition of milk ejection in the lactating rat was probably due to a reduction in the high frequency bursting activity of the OT neurones during reflex milk ejection . Superimposed upon this is an increase in basal firing rate of these neurones which stimulates OT release causing an increase in the plasma concentration of OT in a manner that does not produce milk ejection . Ovariectomy as a result may therefore remove this excitatory effect of relaxin although this is contradicted by the increase in basal OT seen which is not compatible with an excitatory action .

4.4.5 Are angiotensin II responses reduced ?

A blunted pressor response to angiotensin II has been reported during pregnancy in the rat (Nakamura *et al.*, 1988) and systemic administration of angiotensin II is known to stimulate OT release (Lang *et al.*, 1981) . The renin-angiotensin system is known to be activated during pregnancy but prior receptor occupancy by endogenous angiotensin II was found not to be responsible for the attenuated pressor response to

exogenous i.v. administration of the hormone (Conrad *et al.*, 1989) . In addition Naden *et al.* (1985) concluded that the reduced pressor response to infused angiotensin II in pregnancy was not due to an increase in metabolic clearance rate nor to lower plasma angiotensin II concentration . It is therefore possible that stimulation of the renin angiotensin II system during pregnancy produces a down regulation of receptors mediating its responses including the stimulation of OT release . To test this we measured the response of the OT system to an i.v. infusion of angiotensin II in virgin and 21 day pregnant rats . A literature survey revealed that in previous studies angiotensin was infused at varying rates, 0.2-1.0μg/kg/min, and over varying time periods, from 20 to 30 minutes (Ferguson and Kasting, 1988 ; Gutman *et al.*, 1988) . We chose a mid range dose and infused 0.5μg/kg/min for 20 minutes . This produced a significant increase in the plasma OT concentration of pregnant and virgin rats before and after naloxone (Fig 4.9) ; naloxone was given to eliminate complicating actions of endogenous opioids which are known to inhibit angiotensin II stimulation of OT release (Coira and Chlordera, 1991) . There was however no significant difference between pregnant and virgin rats in their responses to angiotensin II . It would appear from this result that a desensitisation to angiotensin II stimulation of OT release does not occur during pregnancy . This therefore would presumably not be the mechanism by which the OT response to hyperosmotic stimulation is blunted . Alternatively we may have chosen an inappropriate infusion protocol and a higher dose or longer infusion period may have revealed an attenuated response .

Central administration of angiotensin II was also not investigated and it may be that this route of administration could reveal a different response . The SFO and OVLT have both become accepted as receptor sites for

angiotensin II in the brain (Phillips *et al.*, 1982) . Intracerebroventricular administration of angiotensin II is found to act synergistically with hyperosmotic saline with respect to increasing the firing rate of neurosecretory cells in the SON and PVN (Akashi *et al.*, 1980) . Hines and Porter (1989) reported that central administration of angiotensin II to pregnant rats produced a significantly augmented pressor response . It is therefore possible that increased central activity of excitatory angiotensin II inputs to the OT neurones during pregnancy results in their down regulation and hence loss of synergistic action in response to hyperosmotic stimulation .

4.5 Results

4.5.1 Effect of hyperosmotic saline administered by i.v. infusion on plasma OT in 21 day pregnant and adult virgin female rats (Fig 4.10) .

The low dose hyperosmotic saline infusion produced a significant rise in the plasma OT concentration of virgin rats ($P < 0.05$ paired t test) from a mean of sample 3+4 at 51.9 ± 16.8 to a maximum of 281.7 ± 70.9 pg/ml . An equivalent hyperosmotic stimulus in pregnant rats also produced a significant rise in plasma OT concentration ($P = 0.02$ paired t test) from a mean of sample 3+4 at 56.7 ± 12.7 to a maximum of 229 ± 38.2 pg/ml .

The high dose hyperosmotic saline infusion of 0.525g/kg produced a larger increase in plasma OT concentration than the low dose infusion and the concentrations reached were significantly different at sample 5 (35min) in virgin rats ($P < 0.03$ two sample t test) . There was a significant rise in the plasma OT concentration compared to the mean of sample 3+4, in virgin rats ($P < 0.02$ paired t test) from 51.7.± 8.8 to 574.8 ± 145.8 pg/ml and in pregnant rats ($P < 0.02$ paired t test) from 39.5 ± 10 to a maximum of 351.2 ± 59.8 pg/ml . There were no significant differences between virgin and pregnant rat responses to the hyperosmotic stimulations used (two sample t test and ANOVA) .

LEGEND : FIGURE 4.10

Plasma OT was measured, by ria, on femoral arterial blood samples from urethane anaesthetised virgin and 21 day pregnant rats that had received an i.v. infusion for 30 minutes of either low or high dose hyperosmotic saline . Low dose hyperosmotic saline infused rats received 262.5mg NaCl/kg body weight (4.4mmol/kg) in a fixed infusion volume of 0.84ml . High dose hyperosmotic saline infused rats received double the salt load of the low dose infused rats at 525mg NaCl/kg body weight (8.8mmol/kg) again in a fixed volume of 0.84ml . All groups received an i.v. injection of isotonic saline vehicle (0.5ml/kg) at 10min .

Values are mean ± s.e.m. : ❍, are low dose hyperosmotic saline infused virgin rats (n = 5) ; ❑, are high dose hyperosmotic saline infused virgin rats (n = 6) ; ●, are low dose hyperosmotic saline infused pregnant rats (n = 5) and ■, are high dose hyperosmotic saline infused pregnant rats (n = 7) .

*, # = $P \leq 0.05$; ** = $P \leq 0.005$

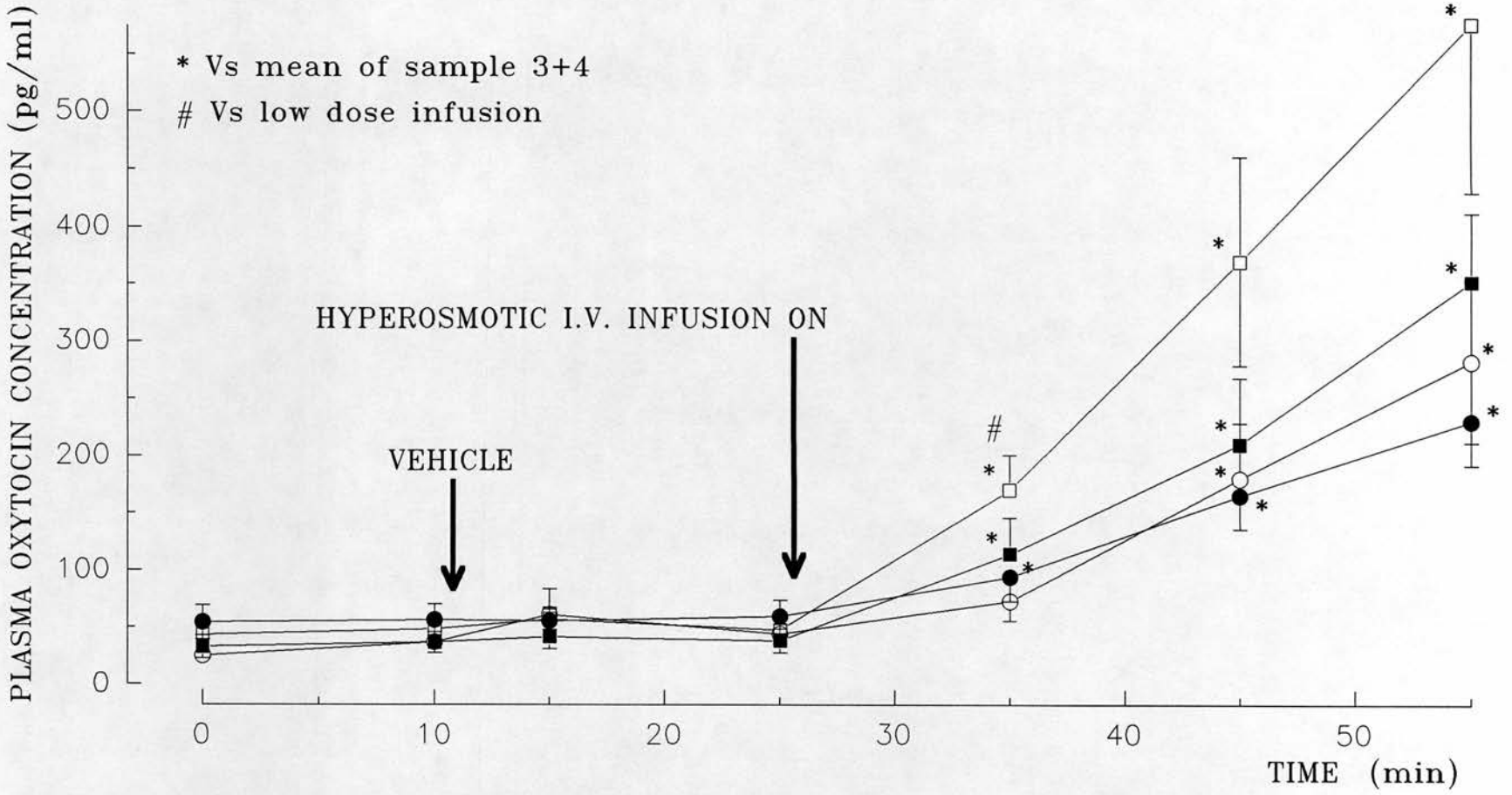


FIGURE 4.10 Effect of hyperosmotic saline administerd by i.v. infusion on plasma OT in 21 day pregnant and adult virgin female rats

4.5.2 Plasma osmolality and sodium concentration in virgin and 21 day pregnant rats during i.v. infusions of hyperosmotic saline (Fig 4.10B) .

The low dose hyperosmotic saline infusion produced a significant increase in the plasma sodium concentration in virgin rats ($P < 0.03$ paired t test) from 136 ± 0.7 mmol/l at sample 4 to a maximum of 146.5 ± 2.9 mmol/l . Pregnant rats responded similarly with a significant increase in plasma sodium concentration ($P < 0.02$ paired t test) from a mean at sample 4 of 131.4 ± 2 mmol/l to a maximum of 143.2 ± 3.7 mmol/l . This infusion also produced a significant increase in the plasma osmolality in virgin rats ($P < 0.05$ paired t test) from 293.3 ± 5.1 to 315.8 ± 8.8 mosmol/kg and in pregnant rats a non significant rise from 290.6 ± 2.8 to 304.4 ± 4.6 mosmol/kg .

The high dose hyperosmotic saline infusion produced greater increases in plasma osmolality and sodium concentration compared to the low dose infusion in pregnant rats . There was an identical trend in virgin rats with a significant difference in plasma sodium concentration by sample 7 ($P = 0.03$ two sample t test) . This infusion produced significant rises in plasma sodium concentration in virgin rats ($P< 0.02$ paired t test) from a mean at sample 4 of 136.5 ± 1.6 to 153.8 ± 3.2 mmol/l and in 21 day pregnant rats ($P < 0.05$ paired t test) from 135.1 ± 1.5 to 153.1 ± 2.1 mmol/l . Plasma osmolalities responded similarly to plasma sodium concentration with a significant increase , compared to sample 4, in virgin rats ($P< 0.05$ paired t test) from 295.5 ± 3.8 to 325.2 ± 7.1 mosmol/kg and in pregnant rats ($P < 0.02$ paired t test) from 277.7 ± 4.5 to 310 ± 5.4 mosmol/kg .

There were three significant differences during the i.v. hyperosmotic saline infusion of plasma osmolality and sodium concentration between virgin and pregnant rats all at sample 6 . Plasma sodium concentration in low

dose hyperosmotic saline infused rats was significantly lower in pregnant rats compared to virgin ($P < 0.05$ two sample t test) . Also plasma osmolality in low and high dose hyperosmotic saline infused pregnant rats were significantly lower compared to similarly infused virgin rats (ANOVA $P < 0.02$ followed by Duncan's range test) .

LEGEND : FIGURE 4.10B

Plasma osmolality and sodium concentration measurements were made on four (samples 4, 5, 6 and 7, Fig 4.10) femoral arterial plasma samples that were stored frozen until assay . Values are mean ± s.e.m. . Graph (i) denotes the plasma sodium concentration and graph (ii) denotes plasma osmolality . ❍, are low dose hyperosmotic saline infused virgin rats (n = 6) ; ❑, are high dose hyperosmotic saline infused virgin rats (n = 6) ; ●, are low dose hyperosmotic saline infused 21 day pregnant rats (n = 5) and ■, are high dose hyperosmotic saline infused pregnant rats (n = 7) .

As in previous experiments (Figs 4.2B and 4.2C) mean basal plasma sodium concentration was lower and plasma osmolality significantly lower in pregnant (▲, n = 12) compared to that in virgin rats (Δ, n = 12), (P = 0.02 two sample t test) .

$*, \#, \dagger = P \leq 0.05$; $** = P \leq 0.005$; $*** = P \leq 0.0005$

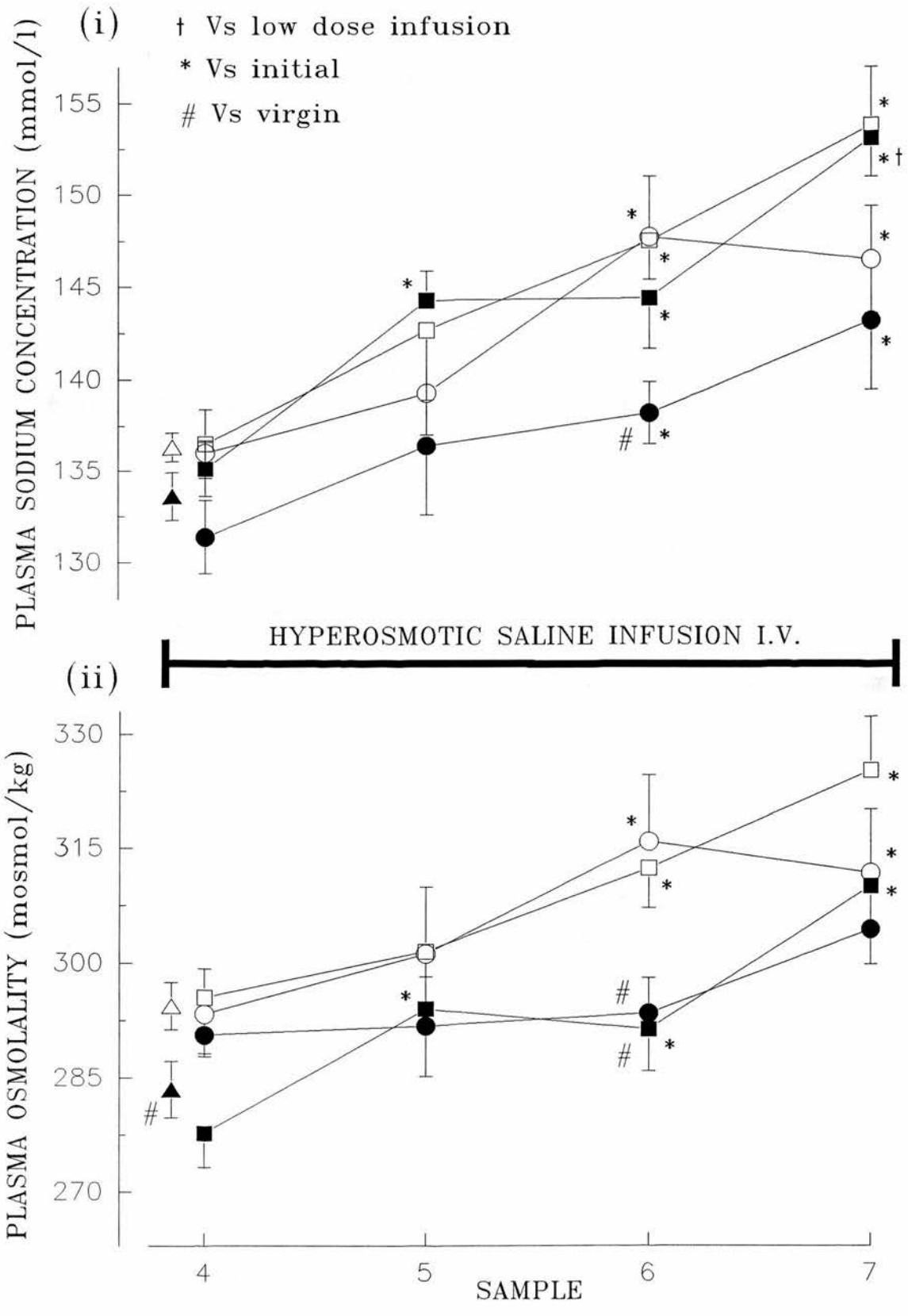


FIGURE 4.10B Plasma osmolality and sodium concentration in virgin and 21 day pregnant rats during i.v. infusions of hyperosmotic saline

4.5.3 Change in plasma sodium and oxytocin concentration with hyperosmotic saline infusions (i.v.) in virgin and 21 day pregnant rats (Fig 4.10C) .

The low dose hyperosmotic saline infusion produced a significant increase in the plasma sodium concentration compared to zero, similar in virgin ($P < 0.0001$ paired t test) and pregnant ($P < 0.02$ paired t test) rats . Plasma OT concentration increased in like manner with a significant change in virgin ($P < 0.03$ paired t test) and pregnant ($P < 0.02$ paired t test) rats .

With the high dose hyperosmotic saline infusion there was also a significant increase in plasma sodium concentration in virgin ($P < 0.0001$ paired t test) and pregnant ($P < 0.0001$ paired t test) rats . This was accompanied by significant increases in plasma OT concentration in virgin ($P < 0.02$ paired t test) and pregnant ($P < 0.02$ paired t test) rats .

The change in plasma sodium and OT concentrations in high dose hyperosmotic saline infused pregnant rats was significantly greater than that in low dose hyperosmotic saline infused pregnant rats ($P < 0.05$ two sample t test) at sample 7 . Also the increase in plasma OT concentration in high dose hyperosmotic saline infused virgin rats was significantly greater than that of low dose hyperosmotic saline infused rats at sample 5 ($P < 0.05$ two sample t test) . However there were no significant differences at any time point in the changes in plasma sodium and OT concentrations between virgin and pregnant rats with either the low or high dose hyperosmotic saline infusions (two sample t test), although OT concentration tended to be greater in the hyperosmotic saline infused virgin rats .

LEGEND : FIGURE 4.10C

This figure compares the change in plasma OT concentration with that of plasma sodium concentration in virgin and 21 day pregnant rats infused with low and high dose hyperosmotic saline (data Fig 4.10, Fig 4.10B) . Values are mean changes from sample 4 ± s.e.m. in the case of plasma sodium concentration and mean changes from an average of sample 3+4 ± s.e.m. for plasma OT concentration .  , are low dose hyperosmotic saline infused virgin rats (n = 5) ;  , are low dose hyperosmotic saline infused 21 day pregnant rats (n = 5) ;  , are high dose hyperosmotic saline infused virgin rats (n = 6) and  , are high dose hyperosmotic saline infused 21 day pregnant rats (n = 7) .

*, # = $P \leq 0.05$; ** = $P \leq 0.005$; *** = $P \leq 0.0005$

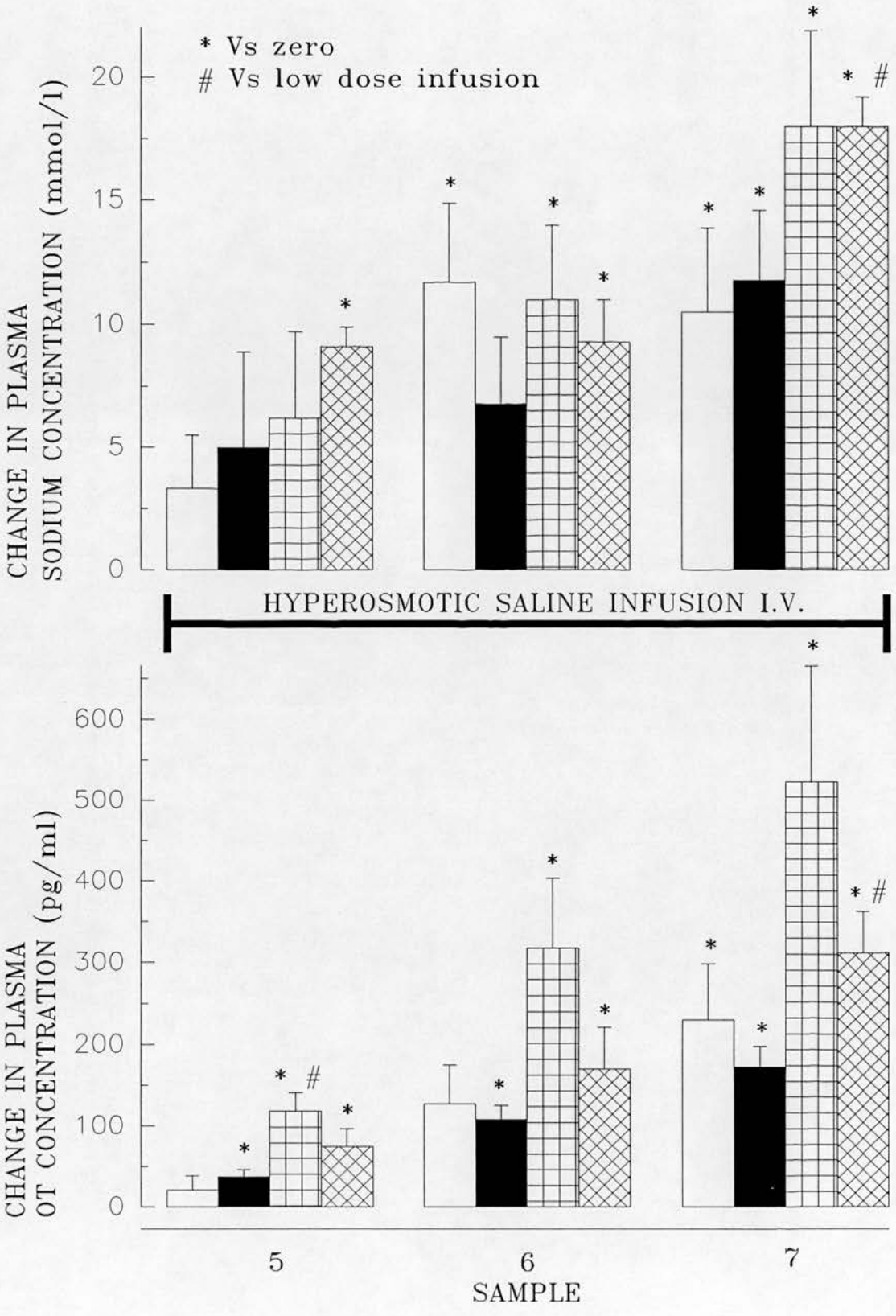


FIGURE 4.10C Change in plasma sodium and oxytocin concentration with hyperosmotic saline infusions (i.v.) in virgin and 21 day pregnant rats

4.5.4 Effect of morphine on hyperosmotic stimulation (i.v.) of OT release in virgin and 21 day pregnant rats (Fig 4.11) .

The low dose of morphine (graph (i)) completely abolished the increase in plasma OT in pregnant and virgin rats normally associated with the infusion of hypertonic saline and plasma OT concentrations remained non significantly different to basal (paired t test) . There was also no significant difference between the plasma OT concentration of virgin and 21 day pregnant rats at all points (two sample t test) .

With the higher dose of morphine (graph (ii)) there was also a complete inhibition of hyperosmotic saline stimulated OT release . Indeed plasma OT concentration was significantly lower than basal in both virgin ($P < 0.005$ paired t test) and pregnant ($P < 0.05$ paired t test) rats during the i.v. infusion of hyperosmotic saline . Again there was no significant difference in the effect of morphine in virgin and pregnant rats (two sample t test) .

LEGEND : FIGURE 4.11

Plasma OT was measured on femoral arterial blood samples, by ria, from urethane anaesthetised 21 day pregnant and virgin rats that had received an i.v. injection of either 0.25mg/kg or 1mg/kg morphine followed by the low dose hyperosmotic saline infusion, (see 4.5.1) . Morphine was administered 10 minutes before the start of the hyperosmotic saline infusion (i.v.) at a concentration calculated to deliver 0.5ml of solution/kg body weight . Values are mean ± s.e.m. .

Graph (i) denotes the effect of i.v. 0.25mg/kg morphine on hyperosmotic saline (i.v.) stimulated OT release in 21 day pregnant (●, n = 7) and virgin (○, n = 8) rats . Graph (ii) depicts the effect of i.v. 1mg/kg morphine on hyperosmotic saline (i.v.) stimulated OT release in 21 day pregnant (●, n = 7) and virgin (○, n = 8) rats .

$* = P \leq 0.05$; $** = P \leq 0.005$

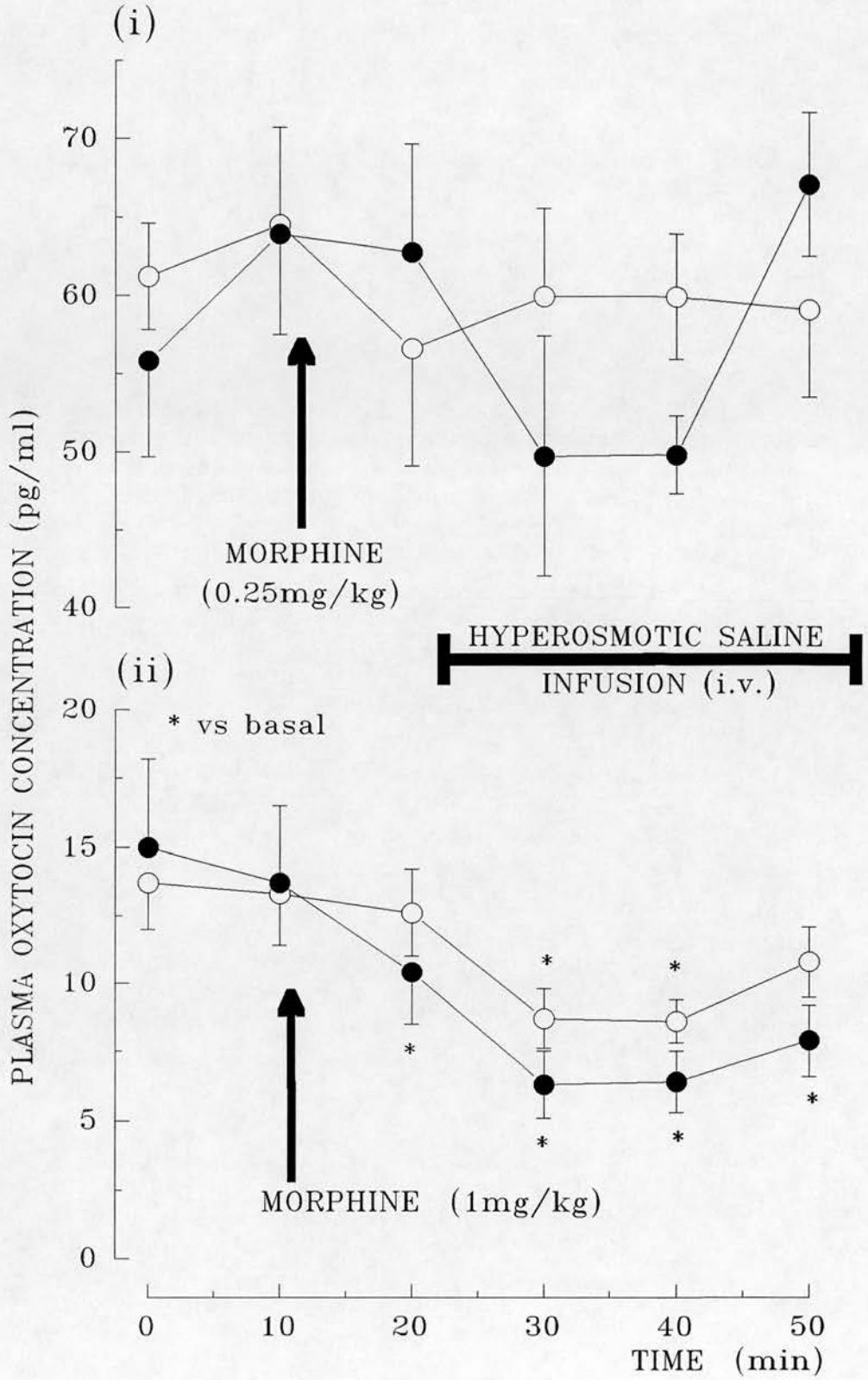


FIGURE 4.11 Effect of morphine on hyperosmotic saline stimulation (i.v.) of OT release in virgin and 21 day pregnant rats

4.5.5 Effect of naloxone and an iso-osmotic saline infusion (i.v.) on plasma OT concentration in virgin and 21 day pregnant rats (Fig 4.12) .

Naloxone produced a significant increase in plasma OT concentration in 21 day pregnant rats ($P < 0.05$ paired t test) from a mean basal of 44.4 ± 8.7 to 281.5 ± 98.3 pg/ml by sample 4 (25min) and a non significant rise in virgin (paired t test) from a mean basal of 35.5 ± 6.4 to 185 ± 74.1 pg/ml at sample 4 (significant with Wilcoxon signed ranks test $P < 0.03$) .

The subsequent i.v. infusion of iso-osmotic saline had no further effect on naloxone stimulated plasma OT concentration in both pregnant and virgin rats when compared to sample 4 (paired t test) .

There were no significant differences between virgin and pregnant rat responses to naloxone and the saline infusion at any point during the experiment .

LEGEND : FIGURE 4.12

Plasma OT concentration was measured by ria on femoral arterial blood samples from urethane anaesthetised 21 day pregnant and virgin rats that received an i.v. injection of naloxone (5mg/kg in 0.5ml/kg) 10 minutes before an iso-osmotic infusion of 0.15M saline at 0.028ml/min for 30 minutes . Values are mean ± s.e.m. : Δ, are virgin rats (n = 6) and ▲, are 21 day pregnant rats (n = 6) .

$* = P \leq 0.05$

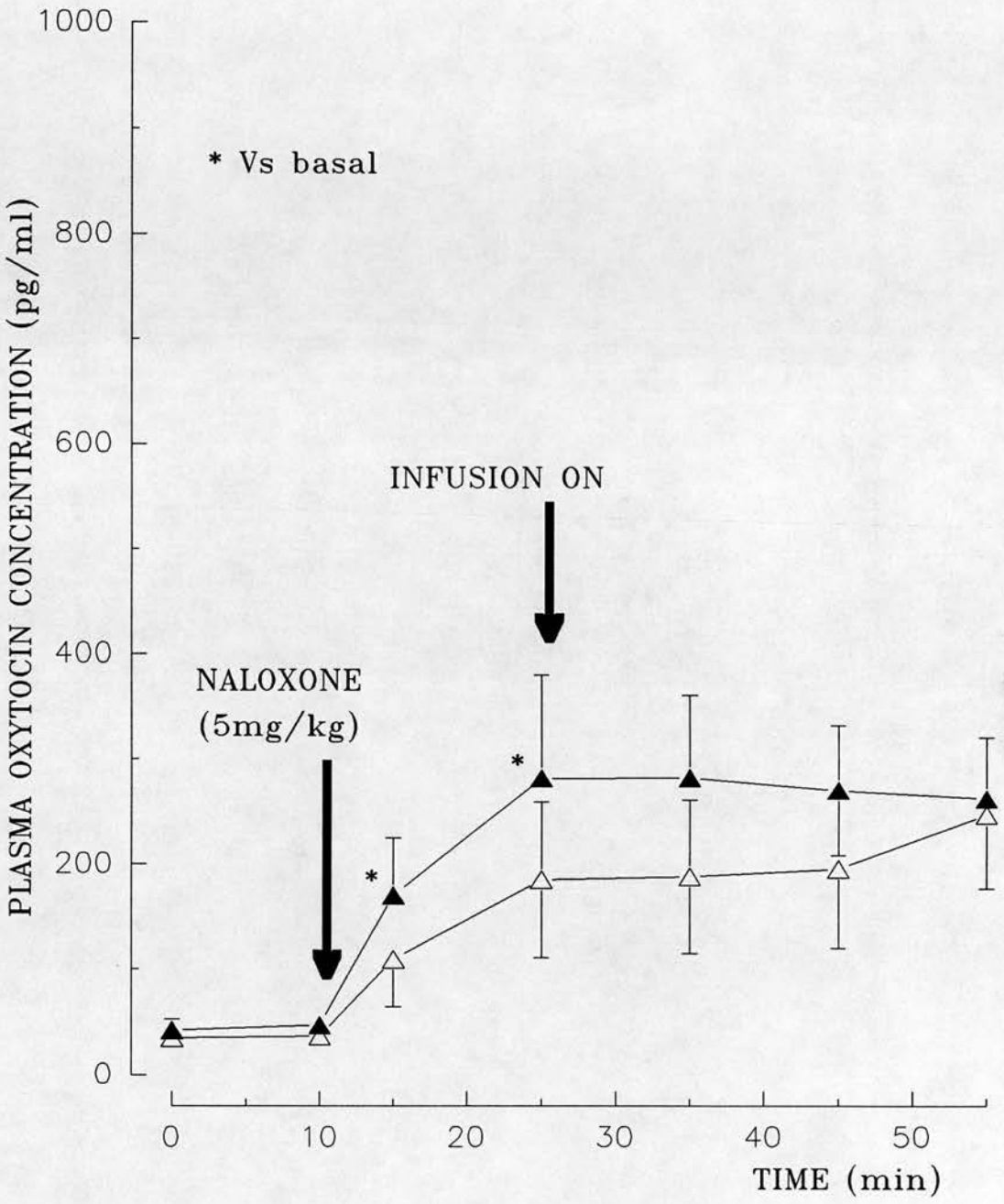


FIGURE 4.12 Effect of naloxone and an iso-osmotic saline infusion (i.v.) on plasma OT concentration in virgin and 21 day pregnant rats

4.5.6 Effect of naloxone on the stimulation of OT release by the low dose hyperosmotic saline infusion (i.v.) in virgin and 21 day pregnant rats (Fig 4.13)

As in Fig 4.12 naloxone produced a significant increase in the plasma OT concentration of pregnant rats ($P < 0.02$ paired t test) from a mean basal of 74.8 ± 7.4 to 204.4 ± 35.1 pg/ml at sample 4 (25min) and in virgin rats ($P < 0.02$ paired t test) from a mean basal of 80.8 ± 5.7 to 172 ± 30 pg/ml at sample 4 .

The ensuing low dose hyperosmotic saline infusion further significantly increased plasma OT concentration compared to sample 4 in virgin rats ($P < 0.03$ paired t test) from 172 ± 30 to 218.8 ± 34.7 pg/ml . There was also a significant increase in plasma OT concentration compared to sample 4 in 21 day pregnant rats ($P < 0.05$ paired t test) from 204.4 ± 35.1 to 363.4 ± 95.5 pg/ml .

Although there were no significant differences between the plasma OT concentrations reached in virgin and 21 day pregnant rats, stimulated plasma OT concentration of pregnant rats was greater at all points compared to virgin . This is a contrast to the previous infusion experiments, without naloxone, in which the hyperosmotic saline stimulated plasma OT concentration of virgin rats was generally greater than pregnant .

LEGEND : FIGURE 4.13

Plasma OT concentration was measured by ria on femoral arterial blood samples from urethane anaesthetised 21 day pregnant and virgin rats that received an i.v. injection of naloxone (5mg/kg in 0.5ml/kg) 10 minutes before the low dose hyperosmotic saline infusion as in Fig 4.10 (262.5mg NaCl/kg body weight) . Values are mean ± s.e.m. : ❍, are virgin rats (n =8) and ●, are 21 day pregnant rats (n = 7) .

*, # = $P \leq 0.05$

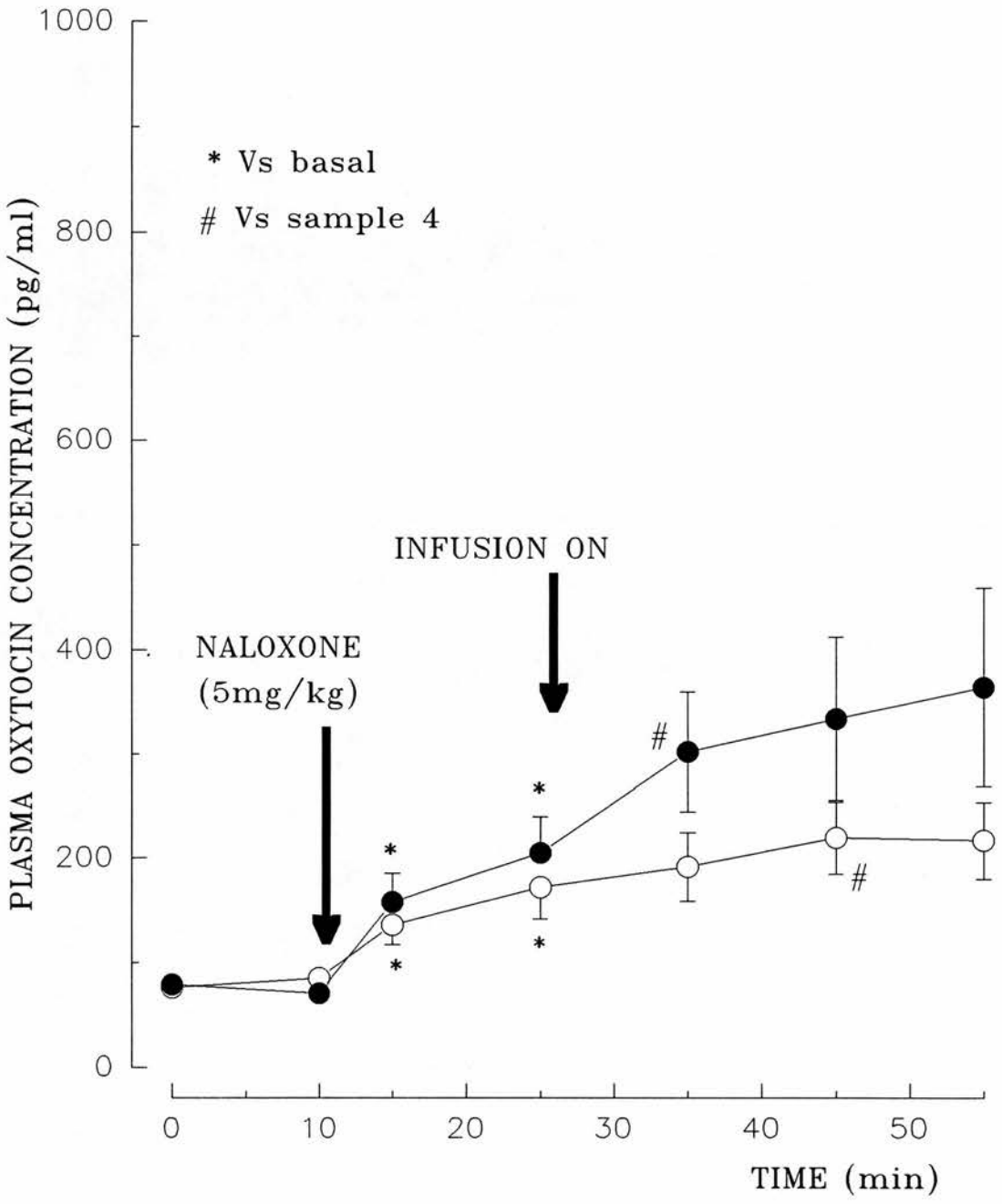


FIGURE 4.13 Effect of naloxone on the stimulation of OT release by the low dose hyperosmtic saline infusion (i.v.) in virgin and 21 day pregnant rats

4.5.7 Effect of naloxone on the stimulation of OT release by the high dose hyperosmotic saline infusion (i.v.) in virgin and 21 day pregnant rats (Fig 4.14).

As in Fig 4.12 and Fig 4.13 naloxone produced a significant increase in the plasma OT concentration of virgin rats ($P < 0.002$ paired t test) from a mean basal of 11.4 ± 3.2 to 139.5 ± 28 pg/ml at sample 4 (25min) . Similarly naloxone produced a significant increase of plasma OT concentration in 21 day pregnant rats ($P < 0.01$ paired t test) from a mean basal 17 ± 3.8 to 127.6 ± 28.4 pg/ml at sample 4 .

The subsequent high dose hyperosmotic saline infusion significantly further increased the plasma OT concentration compared to sample 4 of virgin rats ($P < 0.01$ paired t test) from 139.5 ± 28 to 577.1 ± 105.2 pg/ml . Also in 21 day pregnant rats there was a significant increase in the plasma OT concentration of pregnant rats compared to sample 4 ($P < 0.05$ paired t test) from 127.6 ± 28.8 to 983.7 ± 224.4 pg/ml .

Naloxone stimulated plasma OT concentration was greater in pregnant rats compared to virgin and was significantly greater after hyperosmotic saline stimulation at sample 6 (45min), ($P < 0.02$ two sample t test) .

LEGEND : FIGURE 4.14

Plasma OT concentration, measured by ria, of femoral arterial blood samples from urethane anaesthetised virgin and 21 day pregnant rats that received an i.v. injection of naloxone (5mg/kg in 0.5ml/kg) 10 minutes before the high dose hyperosmotic saline infusion as in Fig 4.10 (525mg NaCl/kg body weight) . Values are mean ± s.e.m. : ❑, are virgin rats (n = 6) and ■, are 21 day pregnant rats (n = 6) .

*, #, † = $P \leq 0.05$; **, ## = $P \leq 0.005$

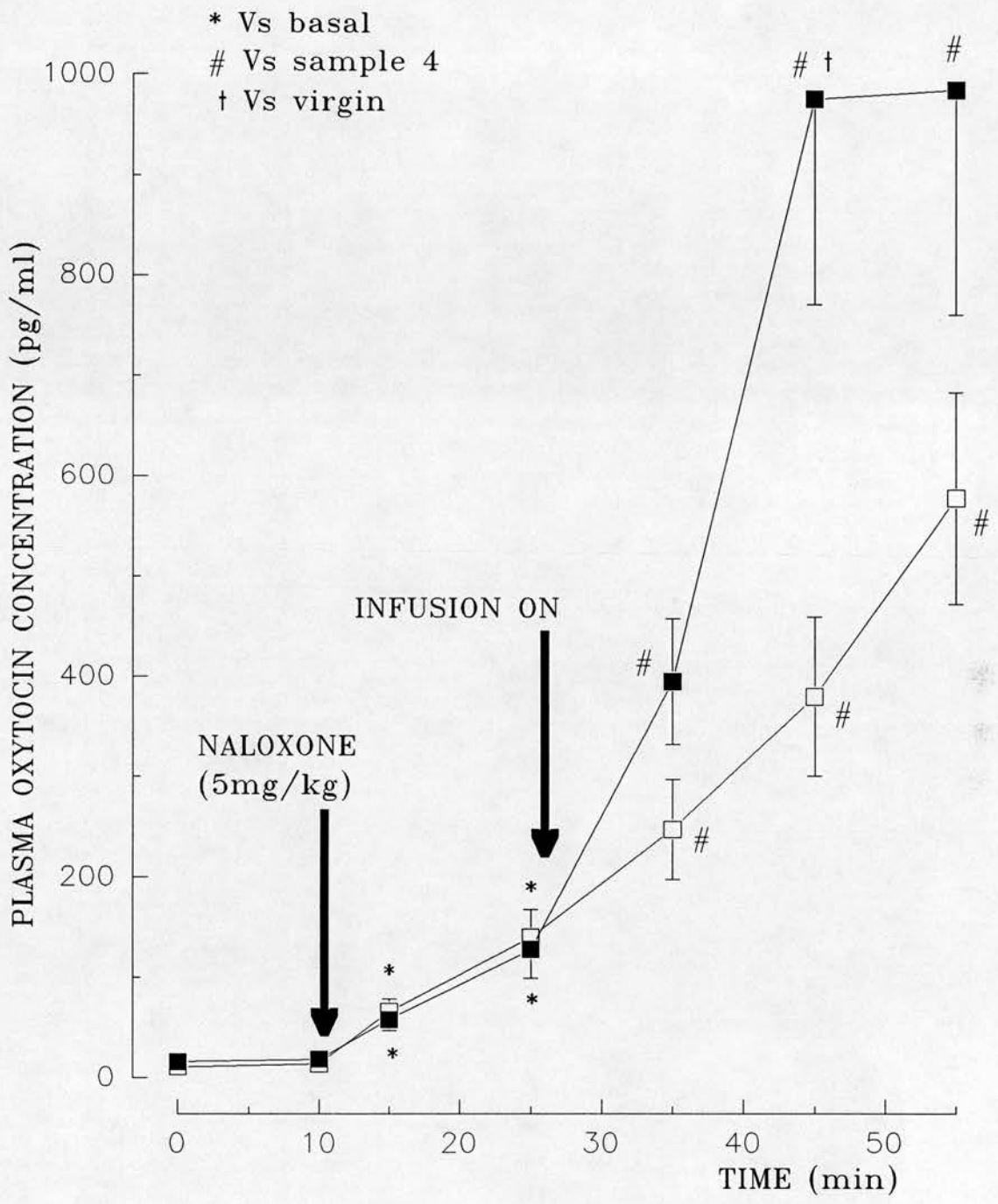


FIGURE 4.14 Effect of naloxone on the stimulation of OT by the high dose hyperosmotic saline infusion (i.v.) in virgin and 21 day pregnant rats

4.5.8 Plasma sodium concentration in virgin and 21 day pregnant rats that received either an iso-osmotic, low or high dose hyperosmotic saline infusion i.v. (Fig 4.15) .

The iso-osmotic infusion had no effect on plasma sodium concentration of virgin and pregnant rats as values remained non significantly different to basal (paired t test) . However the plasma sodium concentration in the pregnant rats of this experiment were significantly less than virgin rats ($P < 0.03$ two sample t test) .

The low dose hyperosmotic saline infusion produced a significant increase in plasma sodium concentration compared to basal in virgin rats ($P < 0.02$ paired t test) from a mean basal of 135.8 ± 0.8 to 146.8 ± 1.5 mmol/l . There was a similar significant increase in pregnant rats ($P = 0.001$ paired t test) from a mean basal of 133.9 ± 1.2 to 144 ± 0.8 mmol/l . Stimulated plasma sodium concentrations of virgin and pregnant rats were not significantly different from each other (two sample t test) .

The high dose hyperosmotic saline infusion produced a significant increase in plasma sodium concentration compared to basal in virgin rats ($P < 0.0001$ paired t test) from a mean basal of 138.4 ± 1.6 to 154.6 ± 1.9 mmol/l . This increase was significantly greater than that produced by the low dose infusion ($P < 0.03$ two sample t test) . This infusion in 21 day pregnant rats also produced a significant increase compared to basal in plasma sodium concentration ($P = 0.01$ paired t test) from a mean basal of 138.3 ± 2.2 to 153.2 ± 1.4 mmol/l . As in virgin rats this increase in pregnant rats was significantly greater than that produced by the low dose infusion ($P < 0.02$ two sample t test) .

LEGEND : FIGURE 4.15

Plasma sodium concentrations were measured at four sample points (25, 35, 45 and 55min) in experiments Fig 4.12 (iso-osmotic saline infusion), 4.13 (low dose hyperosmotic saline infusion) and 4.14 (high dose hyperosmotic saline infusion) . Values are mean ± s.e.m. : Δ, are virgin rats that received an i.v. iso-osmotic saline infusion (n = 6) ; ▲, are 21 day pregnant rats that received an i.v. iso-osmotic infusion (n = 6) ; ❍, are virgin rats that received an i.v. low dose hyperosmotic saline infusion (n = 8) ; ●, are 21 day pregnant rats that received an i.v. low dose hyperosmotic saline infusion (n = 7) ; ❑, are virgin rats that received an i.v. high dose hyperosmotic saline infusion (n = 6) and ■, are 21 day pregnant rats that received an i.v. high dose hyperosmotic saline infusion (n = 6) .

*, #, † = $P \leq 0.05$

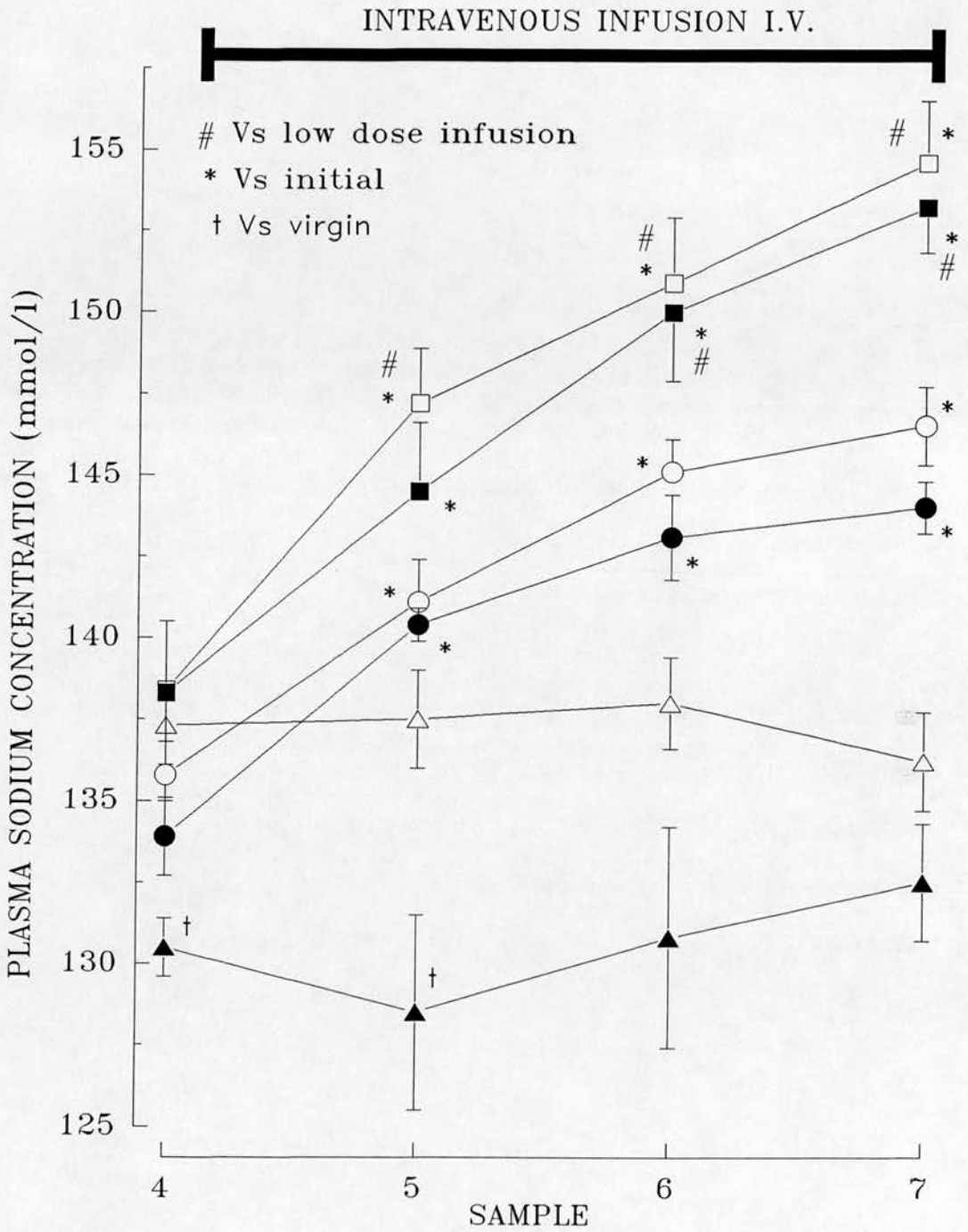


FIGURE 4.15 Plasma sodium concentration in virgin and 21 day pregnant rats that received either an iso-osmotic, low or high dose hyperosmotic saline infusion i.v.

4.5.9 Plasma osmolality in virgin and 21 day pregnant rats that received either an iso-osmotic, low or high dose hyperosmotic saline infusion i.v. (Fig 4.15B) .

The iso-osmotic saline infusion had no effect on plasma osmolality in either virgin or 21 day pregnant rats as values remained non significantly different from basal (paired t test) . The plasma osmolality of pregnant rats however was significantly less than virgin rats at all points in this experiment ($P < 0.05$ two sample t test)

The low dose hyperosmotic saline infusion produced a significant increase in plasma osmolality compared to basal in virgin rats ($P = 0.0003$ paired t test) from a mean basal of 294.4 ± 4 to 313 ± 2.8 mosmol/kg . There was a similar significant increase in pregnant rats ($P < 0.02$ paired t test) from a mean basal of 276 ± 4 to 291.7 ± 2 mosmol/kg . As with the iso-osmotic infusion plasma osmolality of low dose hyperosmotic saline infused pregnant rats remained significantly lower than virgin rats at all points ($P < 0.05$ two sample t test) .

The high dose hyperosmotic saline infusion produced a significant increase of plasma osmolality compared to basal in virgin rats ($P = 0.0007$ paired t test) from a mean basal of 294.1 ± 3.2 to 330.3 ± 4.8 mosmol/kg . This increase was significantly greater than that produced by the low dose infusion ($P < 0.03$ two sample t test) . This infusion in 21 day pregnant rats also produced a significant increase compared to basal in plasma osmolality ($P < 0.02$ paired t test) from a mean basal of 281 ± 4.2 to 310 ± 2.6 mosmol/kg . As in virgin rats this increase in pregnant rats was significantly greater than that produced by the low dose infusion ($P < 0.03$ two sample t test) . As with the previous two infusion protocols plasma osmolality in

pregnant rats of this experiment remained significantly lower than similarly high dose hyperosmotic saline infused virgin rats ($P < 0.05$ two sample t test) .

LEGEND : FIGURE 4.15B

Plasma osmolalities were measured at four sample points (25, 35, 45 and 55min) in experiments shown in Fig 4.12 (iso-osmotic saline infusion), 4.13 (low dose hyperosmotic saline infusion) and Fig 4.14 (high dose hyperosmotic saline infusion) . Samples were stored frozen until assay . Values are mean ± s.e.m. : ▲, are 21 day pregnant rats that received an i.v. iso-osmotic saline infusion (n = 6) ; Δ, are virgin rats that received an i.v. infusion of iso-osmotic saline (n = 6) ; ●, are 21 day pregnant rats that received an i.v. low dose hyperosmotic saline infusion (n = 7); ❍, are virgin rats that received an i.v. infusion of low dose hyperosmotic saline (n = 8) ; ■, are 21 day pregnant rats that received an i.v. high dose hyperosmotic saline infusion (n = 6) and ❑, are virgin rats that received an i.v. infusion of high dose hyperosmotic saline (n = 6) .

$*, \#, \dagger = P \leq 0.05$

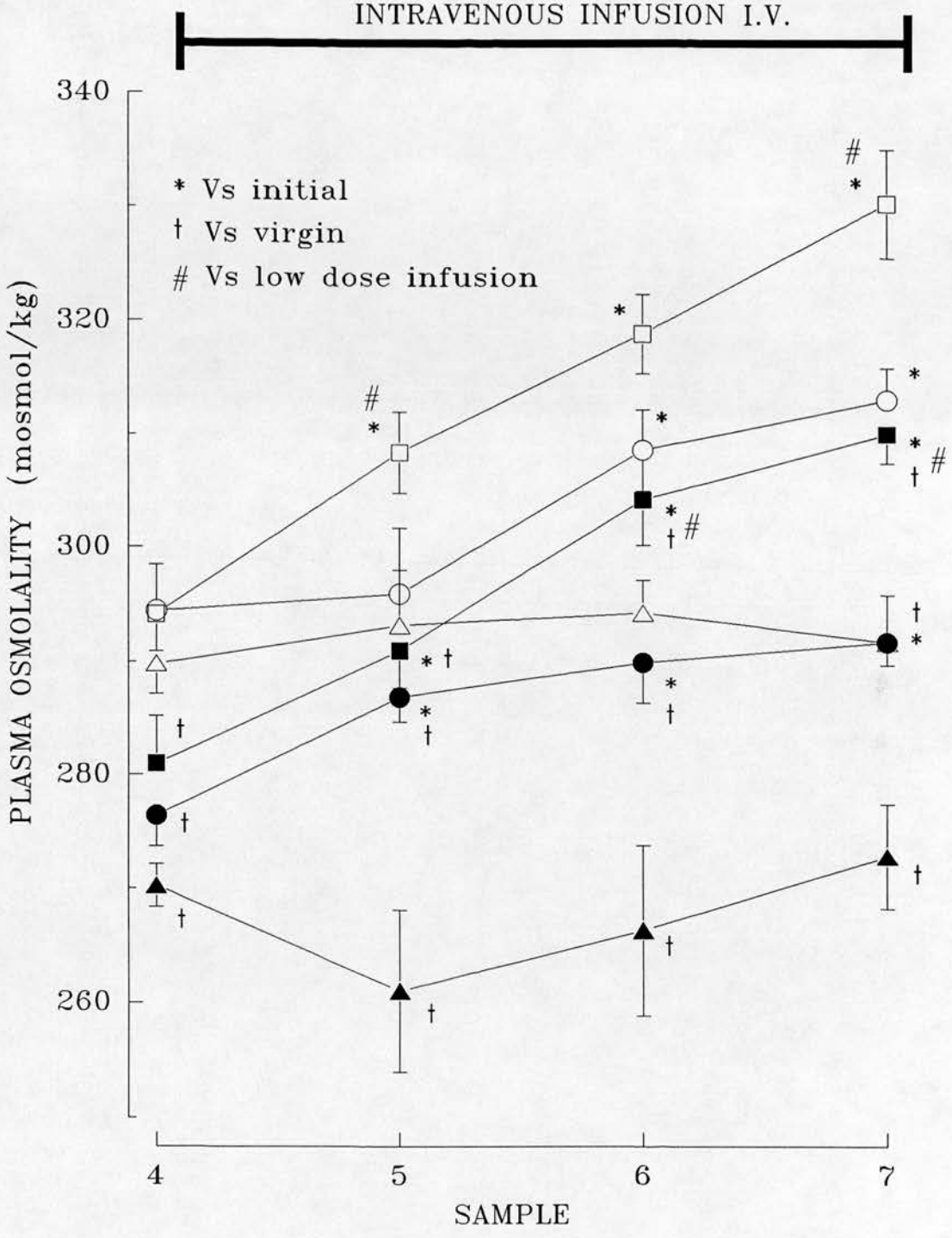


FIGURE 4.15B Plasma osmolality in virgin and 21 day pregnant rats that received either an iso-osmotic, low or high dose hyperosmotic saline infusion i.v.

4.5.10 Change after naloxone in plasma sodium and oxytocin concentration with hyperosmotic saline infusion in virgin and 21 day pregnant rats (Fig 4.16)

The low dose hyperosmotic saline infusion produced a significant increase in the plasma sodium concentration compared to zero, similar in pregnant ($P < 0.02$ paired t test) and virgin ($P < 0.01$ paired t test) rats . Plasma OT concentration increased correspondingly with a significant change in virgin ($P < 0.03$ paired t test) and pregnant ($P < 0.05$ paired t test) rats .

With the high dose hyperosmotic saline infusion there was also a significant increase in plasma sodium concentration in virgin ($P < 0.0001$ paired t test) and pregnant ($P < 0.01$ paired t test) rats . This was accompanied by a significant increase in plasma OT concentration in virgin ($P < 0.01$ paired t test) and pregnant ($P < 0.01$ paired t test) rats .

The changes in plasma sodium concentration in the high dose hyperosmotic saline infused rats were significantly greater than in the respective low dose hyperosmotic saline infused virgin ($P < 0.05$ two sample t test) and pregnant rats ($P < 0.05$ two sample t test) . This coincided with significantly greater increases in plasma OT concentration in the high dose hyperosmotic saline infused virgin ($P < 0.03$ two sample t test) and pregnant ($P = 0.02$ two sample t test) rats compared to the respective low dose hyperosmotic saline infused groups . The increase in plasma OT concentration in high dose hyperosmotic saline infused pregnant rats was significantly greater at all points than in similarly infused virgin rats ($P < 0.05$ two sample t test) .

LEGEND : FIGURE 4.16

This figure compares the change in plasma OT concentration (graph (i)) with that of plasma sodium concentration (graph (ii)) in virgin and 21 day pregnant rats infused after naloxone with low and high dose hyperosmotic saline (data Fig 4.13 and Fig 4.14) . Values are mean changes from sample 4 ± s.e.m. for plasma sodium concentration and mean changes from an average of sample 3+4 ± s.e.m. in the case of plasma oxytocin concentration .  , are low dose hyperosmotic saline infused virgin rats (n = 8) ;  , are low dose hyperosmotic saline infused 21 day pregnant rats (n = 7) ;  , are high dose hyperosmotic saline infused virgin rats (n = 6) and  , are high dose hyperosmotic saline infused 21 day pregnant rats (n = 5) .

$*, \#, \dagger = P \leq 0.05$

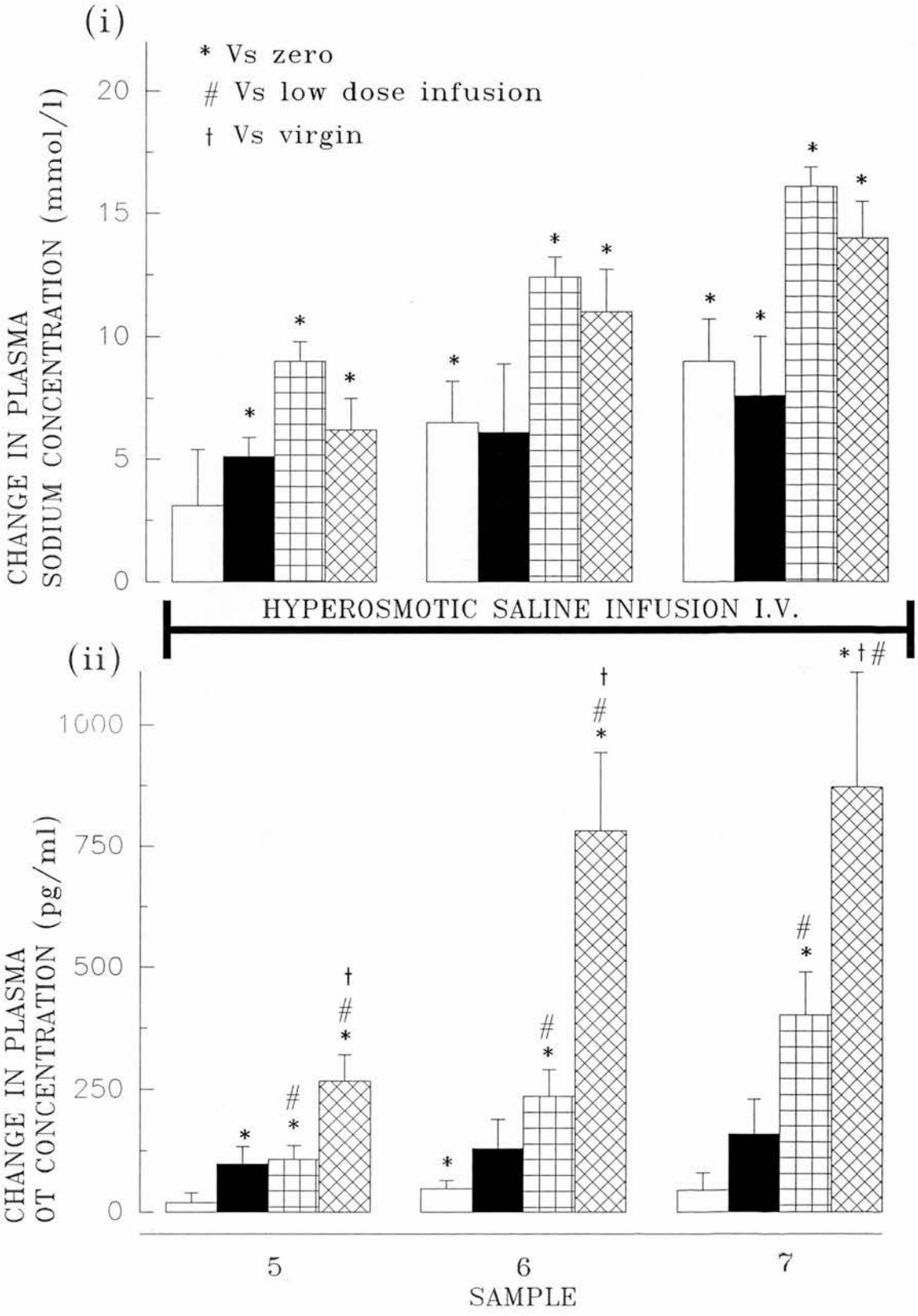


FIGURE 4.16 Change after naloxone in plasma sodium and oxytocin concentration with hyperosmotic saline infusion in virgin and 21 day pregnant rats

4.6 DISCUSSION

Hyperosmotic saline was also administered by intravenous infusion in virgin and 21 day pregnant rats (Fig 4.10), at 4.4 and 8.8mmol NaCl/kg body weight in a fixed volume of 0.84ml over 30 minutes . The lower dose infusion significantly increased the plasma OT concentration of virgin and 21 day pregnant rats, in contrast with the lack of response in pregnant rats to i.p. injection of hypertonic saline . The trends of the responses to i.p. injection were maintained however in that for equivalent hyperosmotic saline infusions virgin rats responded with greater increases in plasma OT concentration compared to pregnant rats . Both infusion protocols produced significant increases in the plasma osmolality and sodium concentration of virgin and pregnant rats (Fig 4.10B) . The changes in plasma sodium concentration were not significantly different between virgin and pregnant rats when the responses to equivalent hyperosmotic saline infusion protocols were compared (Fig 4.10C) . By the end of the infusion the higher dose of 8.8mmol NaCl/kg produced a significantly greater increase in the plasma sodium and OT concentration in the pregnant rats compared to the lower dose infusion . The same trend was obtained in the virgin rats except that the OT and sodium concentration values following low and high dose NaCl infusions were not significantly different from each other (Fig 4.10C) .

4.6.1 Effect of opioids

The stimulation of OT secretion by i.v. hyperosmotic saline was completely abolished by i.v. morphine at a dose of 0.25mg/kg in both virgin and pregnant rats (Fig 4.11) . As with ip administration of hyperosmotic

saline the sensitivity to morphine in pregnant rats was apparently similar to that of virgin .

We also investigated the effect of naloxone on the response to i.v. infusion of saline . Naloxone produced the expected increase in plasma OT concentration in urethane anaesthetised rats . The subsequent infusion of isotonic saline did not produce any further increases in plasma OT concentration (Fig 4.12) . Plasma osmolality (Fig 4.15B) and sodium concentration (Fig 4.15) also remained unchanged in this control study suggesting that the experimental protocol had no further effect after naloxone on any of these parameters . Infusion of the low dose hyperosmotic saline after naloxone had a more pronounced effect on OT secretion in the pregnant rats than in the virgin (Fig 4.13) . This trend was more prominent with the high dose hyperosmotic saline infusion (Fig 4.14) when the difference between the responses in pregnant and virgin rats became significant . These results contrast dramatically with those obtained with identical infusion protocols without naloxone and with i.p. administration of hyperosmotic saline, even after naloxone .

4.6.2 Does the route of administration produce a difference in the stimulus ?

The administration of hyperosmotic saline via i.p. injection increased the plasma sodium concentration by approximately 7.5mmol/l in pregnant and virgin rats within 30 minutes . This was similar in both time course and effect on plasma sodium concentration as the intravenous infusion of the low dose hyperosmotic saline . In the virgin rats plasma OT concentration was significantly increased with both methods of hyperosmotic saline administration . This agrees with the findings of Forsling *et al.* (1988a) who

reported that i.p. and i.v. administration of hypertonic saline to rats produced a similar increase in vasopressin secretion with a similar osmotic threshold for the two routes . Despite similar changes in plasma sodium concentration with the i.p. and low dose i.v. infusion protocols the pregnant rats did not respond to the i.p. stimulus but in contrast had a significant response to the i.v. infusion protocol . It appears therefore that i.v. infusion of hypertonic saline may stimulate OT release by other mechanisms in addition to raising plasma osmolality and sodium concentration .

At the end of the high dose NaCl i.v. infusion there was a significant decrease in haematocrit in both virgin and pregnant rats with a mean of 16.2 ± 2 % ($P < 0.0001$ paired t test versus zero) suggestive of an increase in plasma volume . Alternatively this fall in plasma haematocrit may be the result of blood cell shrinkage in response to the increased plasma osmolality . However other workers have shown that i.p. administration of hyperosmotic saline at doses both similar to ours up to doses that produce increases in plasma osmolality similar to that following our high dose infusion yield very little or no change in haematocrit (Dunn *et al.*, 1973 ; Brimble and Dyball, 1977 ; Durr *et al.*, 1981 ; Hartman *et al.,* 1986) . Considering these data with the OT data in the present experiments it would appear that during pregnancy sensitivity of the OT system to changes in plasma osmolality and sodium concentration do not adjust in accordance with the decreased plasma parameters . It may be that the OT system becomes dissociated from osmoreceptor inputs and responds only to changes in plasma volume . Alternatively the osmotic threshold for OT secretion may not be reset during pregnancy . Administration of hyperosmotic saline via i.v. infusion produces an increase in plasma volume and osmolality which may act synergistically to stimulate OT secretion . With the higher dose of i.v hyperosmotic saline

used in these experiments the osmotic threshold for OT secretion may be reached . Summation between the increased responsiveness to changes in plasma volume in addition to osmolality once the threshold is reached may result in the increased response of the late pregnant rats after naloxone . Further work is therefore required in order to clarify which aspects of the hyperosmotic infusion the OT system of pregnant rats are responding to .

The natriuresis which follows i.v. infusion of hyperosmotic saline depends on OT secretion (Balment *et al.*, 1980) . The decrease in the OT response to fluctuations in plasma sodium concentration may therefore lead to the increase in sodium retention observed during the last week of pregnancy as discussed previously .

The results with naloxone would suggest that the increase in OT activity in response to hypervolaemia is under endogenous opioid restraint . This could be a demonstration of a function for the increase in opioid tone on OT secretion reported to develop during rat pregnancy (Hartman *et al.*, 1986 ; Leng *et al.*, 1988) . Plasma volume is known to increase during pregnancy . One function of this increase in opioid tone could therefore be to restrain OT release in response to the volume increases of pregnancy, and therefore maintain the rat in a state of hypervolaemia . The increase in opioid tone occurs during the second week of pregnancy at the same time as we found a reduced responsiveness of the OT system to hypertonicity using i.p. hyperosmotic stimulation . It would therefore appear that this selective increase in OT sensitivity to the plasma parameters discussed occurs during the second week of pregnancy .

A functional consequence of this could involve the rapid normalisation of body fluids immediately after parturition . OT has both natriuretic and diuretic activities (Fraser, 1937 and 1942 ; Sawyer, 1952 ; Brunner *et al.*,

1956 ; Jacobson and Kellogg, 1956 : Balment *et al.*, 1980 ; Forsling *et al.*, 1982 ; Conrad *et al.*, 1986 ; Verbalis *et al.*, 1991) which would be useful in reducing plasma volume and electrolytes . The stimulatory action of naloxone on OT secretion is known to diminish shortly after parturition (Leng *et al.*, 1988), indicating a reduction in the inhibitory opioid tone at this time . This would then expose the unrestrained hypersensitised OT neurones to the expanded plasma volume that the post parturient rat no longer requires . This would facilitate OT release, and the renal actions of OT would then allow the rat to dump this excess fluid load .

4.6.3 Do ovarian hormones modulate the reduced osmosensitivity in pregnancy ?

Differential effects of ovarian steroids on the vasopressin secretory response of rats to hypertonicity and hypovolaemia have been reported by Forsling *et al.* (1991) . They found that in surgically ovariectomised rats the vasopressin secretory response to hypovolaemia but not hypertonicity was blunted . They proposed that oestrogens potentiate vasopressin release in response to decreases in plasma volume . Lindheimer *et al.* (1985) reported that the vasopressin secretory response to hypovolaemia was blunted during pregnancy in the rat . However, our results suggest that there is a potentiated OT response to hypervolaemia during pregnancy in the rat at a time when there is an increase in circulating plasma oestrogens (Warnock and Csapo, 1975 ; Fuchs, 1978) .

Ovarian steroids have also been shown to modulate the oxytocin receptor density of the uterus in sheep (Zhang *et al.*, 1992 ; Lau *et al.*, 1992), cow (Soloff and Fields, 1989 ; Jenner *et al.*, 1991) and rabbit (Maggi

et al., 1991) . Progesterone caused a down regulation of uterine receptors whilst oestrogen had the opposite effect of up regulating receptor density .

Oestrogen and progesterone also facilitate mating behaviour in the rat by inducing changes in OT receptor density in the ventromedial hypothalamus . Oestradiol benzoate injections have been found to produce a four fold increase in OT receptor binding in this region (Schumacher *et al.*, 1989) over the course of two days (Schumacher *et al.*, 1990) initially in the ventromedial nucleus but also spreading to more lateral areas (Corini *et al.*, 1991) . Kow *et al.* (1991) reported electrophysiological findings which were consistent with the hypothesis that oestrogen potentiates OT action by increasing functional OT receptors preferentially on neurones involved in female reproductive behaviour . Warembourg and Poulain (1991) have also reported the distribution of oestrogen and progesterone receptor immunoreactive cells in the supraoptic and paraventricular nuclei of the guinea-pig . They found that only oestrogen receptors were expressed in the SON and the majority of receptors in the PVN were also of this type . Interestingly all the OT immunoreactive cells revealed oestrogen receptor immunoreactivity . They therefore thought it likely that oestradiol controls the hypothalamo-neurohypophysial OT system by a direct action on the magnocellular neurones . In addition oestradiol and progesterone have also been shown to increase hypothalamic beta-endorphin concentration in the human (Laatikainen, 1991) .

It would therefore appear that the ovarian steroids could modulate OT responses by a number of mechanisms . Both central and peripheral OT receptor density has been demonstrated to fluctuate depending on the ovarian hormone present . We have found cells in the SON to be the most osmoreceptive site (Chapter 3) . Therefore the reduced osmosensitivity of

the system may reflect an action of oestradiol either directly or on inputs to the neurones in the SON . Alternatively modulation may be the result of an interaction of the ovarian hormones with an increased central inhibitory tone of beta-endorphin .

A spatial segregation of osmoreceptor and baroreceptor inputs to the hypothalamo-neurohypophysial system has also been suggested (Cross and Wakerley, 1977) . Magnocellular vasopressin neurones receive inputs from two brain stem areas ; the A1 noradrenergic cell group in the caudal ventrolateral medulla, and the A2 noradrenergic cell group in the caudal nucleus of the solitary tract (NTS) (Cunningham and Sawchenko, 1988) . A dense pathway extending from the caudal NTS and the adjacent reticular formation has also been described and verified as ending preferentially on OT neurones (Sawchenko *et al.*, 1988) . Neurones that convey information on blood pressure and volume are known to terminate in portions of the caudal NTS (Norgen, 1984) . It has therefore been proposed that the NTS could integrate information on blood pressure and volume and elicit the release of vasopressin and OT in a physiologically appropriate manner (Cunningham and Sawchenko, 1991) . Oestrogen receptors have been found in medullary centres involved in the cardiovascular control of vasopressin release (Sar and Stumpf, 1981) . It would therefore appear quite plausible for differential modification of the brain stem baroreceptor and central osmoreceptor inputs to occur due to the influence of increased ovarian steroid release .

4.6.4 Other possible factors that may modulate oxytocin release

Other factors that may modulate this selective increase in the sensitivity of the OT system to volume changes could involve the structural

changes known to occur in the organisation of these neurones during pregnancy (Montagnese *et al.*, 1988) . This may provide the changes in synaptic input required for the up and down regulation of responses to plasma volume and osmolality respectively .

Forsling *et al.* (1988a) have also reported that beta-adrenergic receptor antagonists potentiate the osmotically stimulated release of vasopressin suggesting that noradrenergic pathways have an inhibitory effect on the osmotic response . Guzek (1987) also reported that alpha-adrenergic mechanisms were implicated in the osmoreceptive response of OT neurones . Song *et al.* (1988) also reported that beta-adrenergic receptors inhibit OT secretion whereas activation of central alpha adrenergic mechanisms are necessary for suckling-induced OT release . Mason *et al.* (1988) reported that cultured neonatal supraoptic neurones under voltage clamp demonstrated a modulatory effect of alpha-adrenergic agonists on potassium and calcium ion channels . Of interest here is the report of Day and Sibbald (1988) that stimulation of the medial nucleus tractus solitarius, an area associated with blood pressure regulation, preferentially excites SON neurones involving an adrenergic input . An increased responsiveness of the OT neurones to this input would produce the potentiation of OT release in response to hypervolaemia reported here . Further evidence of the effects of beta-adrenergic involvement in fluid balance was reported by Janus and Guzek (1990) . Using dehydrated rats after 4 days of water deprivation they reported that the beta-adrenergic agonist isoprenaline intensified OT and reduced vasopressin depletion in the neurohypophysis . Beta-adrenoceptor agonists have also been reported to increase the plasma progesterone concentration of cattle (Kotwica *et al.*, 1991) and to be partly involved in the inhibitory actions of opioids (Zhao *et al.*, 1988c) . Alterations

in the noradrenergic innervation to the OT system during pregnancy could therefore produce the increased responsiveness reported here both directly on the neurones and via indirect interactions with ovarian hormones and opioids .

A decrease in the response of the OT system to osmotic stimulation in pregnancy has also been reported by other workers (Koehler *et al.*, 1991) . In more recent work by this group they have reported an increase in the OT response to hypovolaemia (Koehler *et al.* in press) . They have proposed that exaggerated release of OT in response to hypovolaemia during parturition promotes uterine contractions during expulsion of the placenta, which is associated with a certain amount of haemorrhage, and therefore OT helps to reduce blood loss . They also found that this augmented response of OT had returned to normal by day 6 of lactation . These results are possibly contradictory to ours in that an increased OT response to hypovalaemia was reported whereas we have found an increase in response to hypervolaemia . The results of Koehler *et al.* do however demonstrate the same selective increase in sensitivity of the OT system to volume disturbances and a decreased response to osmotic fluctuations during pregnancy in the rat .

It is possible that the renal actions of OT released in response to increases and decreases in blood volume at this stage of pregnancy may be modified by the levels of circulating vasopressin . Haemorrhage also stimulates vasopressin release and a significant increase in plasma vasopressin concentration has been reported in the pregnant rat in response to 0-5% decreases in blood volume (Barron *et al.*, 1984) . Oxytocin has also been found to significantly potentiate the antidiuretic effects of vasopressin (Balment *et al.*, 1986) whilst vasopressin may potentiate the natriuretic

effects of OT and inhibit its diuretic effects (Balment *et al.*, 1982) . In regulating the ratio of these hormones to each other in the plasma the rat could therefore either conserve or lose fluid and electrolytes from the plasma .

4.7 Conclusions

Hyperosmotic stimulation of OT secretion is less effective during pregnancy in the rat . This contrasts with the activity of the vasopressin system during pregnancy which responds in a similar manner to that of virgin animals when challanged by equivalent hyperosmotic stimuli . This reduced osmosensitivity of the OT system does not appear to involve either a reduced sensitiviy to peripheral angiotensin II or acute inhibitory effects of ovarian hormones, and is not the result of increased endogenous opioid activity .

At the same time as OT secretion in response to hypernatraemia is reduced our results suggest that there is a potentiated response to either hypervolaemia alone or a combination of hypervolaemia with hypernatraemia . This may involve changes in one or a combination of known modulators of OT neurone activity . The increased sensitivity of the OT system is apparently under endogenous opioid restraint . Further work is required before any conclusions can be made about which aspects of the hyperosmotic infusion the OT system is responding to .

Sensitivity to mu-opioids is increased at day 16 of pregnancy but is similar to that of virgin rats at day 21 despite evidence of increased endogenous opioid tone . This contrasts to the tolerance to the inhibitory effects of morphine that develops during chronic exogenous mu-opioid exposure .

CHAPTER 5

GENERAL DISCUSSION

Opiate peptides inhibit OT secretion by attenuating electrical activity via an action on the OT neurone cell bodies, and reduce release from the neural lobe . In addition, inhibitory influences of opiates have been reported on areas that project to the magnocellular OT neurones such as the SFO and AV3V region .

Endogenous opioid innervation on the cell bodies of OT neurones has been reported to arise in the arcuate nucleus and dorsal medulla . However conditions have not yet been reported where antagonism of endogenous opioid actions has produced an increase in the firing rate of OT neurones . For example, naloxone does not further excite the firing rate of OT neurones following hyperosmotic stimulation and the i.v. administration of CCK8 . The facilitatory effects of naloxone on OT secretion have therefore been put down to an action on the neurone terminals . Opioid inhibition at the level of the neural lobe has been attributed to the co-release of opioid peptides from OT and vasopressin neurones, resulting in two independent sources of opioid inhibition of OT secretion in the neural lobe . These involve a cross inhibitory effect of dynorphin opioids released from vasopressin neurones and an auto-inhibitory effect of extended [met]-enkephalins and possibly dynorphins from the OT neurones themselves . We attempted to provide further evidence for inhibition of OT secretion due to co-released opioids by silencing vasopressin neurone activity, with an AV3V lesion, and selectively stimulating OT neurone activity, with iv CCK8, before and after naloxone . As reported in Chapter 1 our results provide evidence for an auto-inhibitory effect of co-localised opioids . However, we were unable to demonstrate that the vasopressin system had been effectively silenced . More experiments are therefore required, possibly involving a refined protocol before further conclusions can be drawn from this experiment .

The density of opioid receptors by which opioids mediate their effects on the neurohypophysial system has been shown to respond to the stimulatory state of the animal . For example, the ingestion of 2% saline in the rat increases the release of vasopressin and OT from the neural lobe and simultaneously depletes the levels of dynorphin . 5 days of such dehydration has been reported to produce a significant reduction in posterior pituitary kappa-opioid receptor binding . We have used a similar experimental protocol incorporating kappa-selective binding conditions to investigate the time course of this receptor down regulation . Due to restrictions of time only a preliminary investigation could be accomplished involving 2 and 5 day saline drinking rats . After 2 days of 2% saline ingestion we found a non-significant 15% reduction in ^{3}H-bremazocine binding . After 5 days however there was a highly significant 76% reduction in kappa-receptor binding in the neural lobe . Down regulation of opioid inhibitory effects during chronic hyperosmotic stimulation may facilitate OT secretion . This would be advantageous to the rat in restoring sodium balance as OT has been demonstrated to act synergistically with vasopressin in promoting natriuresis .

The quantity of receptor down-regulation after 3 and 4 days of saline loading therefore requires further investigation . In addition opioid-receptor down-regulation in the supraoptic and paraventricular nuclei during chronic hyperosmotic stimulation has yet to be investigated .

A decrease in supraoptic nuclei mu-opioid receptor binding has been reported during pregnancy in the rat at a time when endogenous opioid activity and OT secretion is increased . Similarly reduced mu-receptor binding has been measured in the supraoptic nuclei of rats after 5 days of icv morphine infusion . Chronic morphine treated rats develop tolerance to

the effects of morphine explained in part by the down regulation of receptors . We therefore investigated whether down regulation of mu-receptors during pregnancy produces tolerance to the effects of morphine on OT secretion . OT secretion was stimulated in two ways, via electrical stimulation of the AV3V region and by the administration of hyperosmotic saline .

Electrical stimulation of the AV3V region influences the activity of most supraoptic neurones in a complex manner resulting in an increase of plasma OT concentration in the rat . Neurotransmitters thought to be involved in the pathways mediating this response include GABA, acetyl choline, angiotensin II and excitatory amino acids partly acting at NMDA receptors (see Chapter 2) . The results presented in Chapter 2 reveal that morphine inhibition of OT secretion in response to electrical AV3V stimulation was frequency dependent . Nevertheless, both morphine and U50,488 were equally effective in virgin and pregnant rats, suggesting there is no change in central mu- and kappa-opioid mechanisms regulating OT neurones during pregnancy .

In contrast, high frequency AV3V stimulation was more effective after naloxone at stimulating the release of OT in virgin compared with pregnant rats . However, electrically stimulated isolated neural lobes from pregnant rats *in vitro* release more OT than equivalently stimulated pituitaries from virgin rats . This would indicate that during pregnancy in the rat the AV3V region input is less effectively coupled to OT neurones . This may be important in tuning the OT system during pregnancy to the selective stimulation by inputs involved in parturition .

Following hyperosmotic stimulation of OT secretion during pregnancy once again no evidence could be found of tolerance to the mu-agonist morphine . In comparison, the response of the OT system to hypernatraemia

decreases . OT secretion in response to hyperosmotic stimulation has been proposed to be influenced by an osmoreceptor complex of structures . The results of experiments in Chapter 3 would indicate that these osmoreceptors are partly in the lamina terminalis but mainly involve the OT neurones themselves . In addition, reports by other workers indicate that the integrity of both the SFO and AV3V region are vital for normal osmotic release of OT . A reduced excitatory influence of osmoreceptors in the AV3V region, as a result of the uncoupled nature of this input during pregnancy, may be involved in the attenuated release of OT to hypernatraemia . At the same time OT released in response to changes in plasma volume increases, although this is apparently under endogenous opioid restraint . An increased response of OT to changes in plasma volume may be important in controlling haemorrhage during parturition and the subsequent normalisation of plasma osmolality and volume during lactation .

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Oxytocin secretory responses to hypernatraemia and inhibition by morphine in pregnant urethane-anaesthetized rats

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We have examined whether increased endogenous opioid inhibition of oxytocin neurones in pregnancy (Bicknell *et al.* 1988) alters sensitivity to the potent central inhibitory actions of μ-agonists on oxytocin secretion. Urethane-anaesthetized (1.25 g kg^{-1}) Sprague–Dawley rats were given increasing I.V. doses of morphine after oxytocin secretion was stimulated by I.P. injection of 1.5 M NaCl (4 ml kg^{-1}). In femoral arterial blood samples, withdrawn at 10 min intervals and replaced by resuspended blood cells, plasma oxytocin was measured by radioimmunoassay, and $[Na^+]$ by flame photometry. Initial plasma $[Na^+]$ was greater in virgin ($n = 17$), than in pregnant rats on day 21 ($n = 18$), 138.8 ± 0.9 (mean ± S.E.M.) and 135.3 ± 0.8 mM l^{-1} (mean ± S.E.M., $P < 0.05$, t test), but not on day 16 ($n = 14$). The increments (all $P < 0.005$, paired t test) in plasma $[Na^+]$ 65 min after I.P. 1.5 M NaCl were similar in the virgin, 16- and 21-day pregnant rats (8.0 ± 0.7, 6.3 ± 0.6, and 7.6 ± 1.0 mM l^{-1}). Initial plasma oxytocin concentration was 34 % lower in the 21-day pregnant rats *vs.* virgins ($P < 0.05$), opposite to findings in conscious rats (Russell *et al.* 1991). In the virgin and 16-day pregnant rats, but not in the 21-day pregnant rats, 50 min after I.P. 1.5 M NaCl, plasma oxytocin was significantly increased ($P < 0.05$, Wilcoxon) by 114.9 ± 32.3 and 62.9 ± 11.9 pg ml^{-1} (virgin > 16- and 20-day pregnant, Duncan's $P < 0.05$). Naloxone (5 mg kg^{-1}) did not restore normal responses to hypernatraemia in pregnant rats. In the virgin rats, morphine at doses of 1, 2.5 and 5 mg kg^{-1}, but not at 0.25 mg kg^{-1} inhibited the oxytocin response to 1.5 M NaCl I.P., and in the 21-day pregnant rats it reduced plasma oxytocin at the same doses. Morphine was effective at 0.25 mg kg^{-1} in the 16-day pregnant rats. Thus pregnant rats show a reduced oxytocin secretory response to hypernatraemia, not mediated via endogenous opioids. Considering the reduced plasma $[Na^+]$ in pregnancy, and the reduced stimulatory (hyperosmotic) effect of urethane, we conclude that the threshold for hypernatraemic stimulation of oxytocin secretion is not reduced in pregnancy. Morphine inhibited stimulated oxytocin secretion, with increased sensitivity in 16-day pregnant rats, coinciding with the start of inhibitory endogenous opioid tone on oxytocin secretion (Bicknell *et al.* 1988).

Supported by the AFRC. P.M.B. is an AFRC Research Student.

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ogress in Brain Research, Vol. 91

CHAPTER 7

Pregnancy and opioid interactions with the anterior peri-third ventricular input to magnocellular oxytocin neurones

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Rostral circumventricular organs and magnocellular oxytocin neurones

Structures in the lamina terminalis are major sources of input to the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei, and both magnocellular oxytocin and vasopressin neurones receive inputs from these sources (Sawchenko and Swanson, 1983; Blackburn et al., 1987; Ferguson and Kasting, 1987; Russell et al., 1988; Wilkin et al., 1989). Electrophysiological, lesioning and retrograde and anterograde transport studies have shown that the sub-fornical organ (SFO) projects both directly and indirectly, via the nucleus medianus (median preoptic nucleus), to the PVN and SON (Carithers et al., 1981; Miselis, 1981; Renaud et al., 1983; Sgro et al., 1984; Tanaka et al., 1987). Similarly, there are both direct and indirect projections from the organum vasculosum of the lamina terminalis (OVLT) (Saper and Levisohn, 1983; Wilkin et al., 1989). Acute lesion of the region anterior and ventral to the third ventricle (AV3V region), involving the nucleus medianus, silences supraoptic neurones, indicating that this region provides a tonic drive producing the continuous pattern of firing activity of oxytocin neurones (as well as driving the phasic activity of vasopressin neurones) (Leng et al., 1989a). In the rat, magnocellular oxytocin neurones are osmoresponsive (Brimble et al., 1978), and the oxytocin secreted as a result probably has a natriuretic role, acting in concert with vasopressin (Balment et al., 1986). AV3V lesions remove the ability of SON neurones to fire action potentials in response to a hyperosmotic stimulus and consequently oxytocin secretion is not increased (Leng et al., 1989a); an acute lesion of the SFO has a similar effect (Leng et al., 1989a). The lamina terminalis input thus seems to provide activity essential for normal osmoresponsiveness of oxytocin neurones. Supraoptic neurones themselves are directly osmosensitive (Leng et al., 1982), as demonstrated by their depolarization in vitro with increased osmolality (Mason, 1980; Bourque, 1989), and by their increase in firing-rate in vivo after an AV3V lesion in response to a hyperosmotic stimulus if a steady excitation with glutamate is provided (Leng et al., 1989a). It is uncertain whether elements of the lamina terminalis input to oxytocin neurones are themselves osmosensitive, as distinct from showing responses to osmotic changes which may, surprisingly, be at least partly the result of direct or indirect input from SON neurones (Chaudhry et al., 1989; Dyball and Leng, 1989). Cells in the SFO and OVLT region that project directly to the SON are not apparently osmoresponsive (Chaudhry et al., 1989; Dyball and Leng, 1989), although other cells in the OVLT are excited by increased osmolality, especially cells receiving input from the SON (Honda et al.,

1987; Chaudhry et al., 1989). However, nucleus medianus neurones, receiving input from the OVLT and SFO, and projecting to the SON are osmoresponsive (Honda et al., 1989), and some SFO neurones in vitro respond to changes in osmolality by changing their firing-rate (Sibbald et al., 1988). Some SFO neurones project to the nucleus medianus, and may influence magnocellular SON and PVN neurones by this route (Miselis, 1981; Saper and Levisohn, 1983; Tanaka et al., 1987). A further sign of osmoresponsiveness of SFO neurones is that some SFO cells, and some nucleus medianus neurones, show rapid increase in *c-fos* transcription factor gene expression, indicated by an in situ hybridization study of *c-fos* mRNA content (Hamamura et al., 1991). The osmoresponsiveness of oxytocin (and vasopressin) neurones is thus a product of their direct osmosensitivity interacting with excitatory (and inhibitory) input from the lamina terminalis, and with neurones in the SON perinuclear zone (Leng, 1980, 1982). Indirect input from the SFO and OVLT relayed via the nucleus medianus is likely to be from osmosensitive neurones in these circumventricular organs. Reciprocal actions of SON neurones on AV3V neurones lead to the idea of a neural network acting as an osmoreceptor complex to regulate oxytocin and vasopressin secretion (Leng et al., 1988a; Dyball and Leng, 1989).

An acute AV3V lesion renders oxytocin neurones incompetent to respond to an acute hyperosmotic stimulus, and an infusion of the angiotensin II antagonist saralasin into a lateral cerebral ventricle (i.c.v.) achieves the same result, as well as silencing their basal activity (Blackburn et al., 1987; Leng et al., 1989a; Russell et al., 1990). This is consistent with the suggested role of angiotensin II as a neurotransmitter in the pathway from the SFO via the nucleus medianus to the magnocellular nuclei (Jhamandas et al., 1989), although neither the firing-rate nor secretory response of oxytocin neurones to electrical AV3V or SFO stimulation, respectively, is significantly affected by i.c.v. saralasin infusion (Leng et al., 1989a; Russell et al., 1990). This suggests that i.c.v. saralasin acts on the SFO itself. Alternatively, electrical stimulation of the SFO or AV3V region may be expected to activat inputs to the magnocellular nuclei that mediat other signals from the inner chemical world that th SFO detects in the circulation; this includes ci culating angiotensin II (Tanaka et al., 198 Ferguson and Kasting, 1988) and the ovarian ho mone relaxin in pregnancy (Summerlee et al., 1987 the chemical coding for these in the pathways fro the SFO to the magnocellular nuclei is not known

Electrical stimulation of the AV3V region pro duces complex patterns of firing-rate changes i SON oxytocin neurones (Leng et al., 1989a), in dicating activation of synapses using short- an long-lasting excitatory transmitters perhaps in cluding excitatory amino acids, angiotensin II an maybe acetylcholine (Akaishi and Negoro, 1983 Gribkoff and Dudek, 1988; Gribkoff et al., 1988 Jhamandas et al., 1989). Inhibitory synapses ar also activated (Leng et al., 1989a), though opioid are evidently not involved since naloxone does no affect hyperosmotic excitation of SON oxytoci neurones (Shibuki et al., 1988), or responses t AV3V stimulation (unpublished data); GABA ma be involved in the pathway (Randle and Renaud 1987).

Although necessary for normal responsiveness o oxytocin neurones to hyperosmotic stimulation, ar intact AV3V region is not required for norma pulsatile secretion of oxytocin during suckling to ef fect the milk-ejection reflex (Russell et al., 1988) and the process of parturition and oxytocin secre tion intra-partum are undisturbed by an acut AV3V lesion (Russell et al., 1989a). The suckling stimulus reaches oxytocin neurones in the magnocellular nuclei by ascending neural pathways not directly involving lamina terminalis structures (Wakerley et al., 1988), and similar pathways may be used from sensory receptors in the birth canal to reflexly stimulate oxytocin secretion, although little is known of this route. Further evidence of selective function of the lamina terminalis input is that the stimulation of oxytocin secretion by intravenou (i.v.) cholecystokinin-8-S (CCK8S), which does no affect vasopressin secretion, is not affected by a acute AV3V lesion (Blackburn and Leng, 1990); in

mulus acts via gastric vagal afferents and possibly the area postrema (Carter and Lightman, 1987; Renaud et al., 1987).

Opioids and oxytocin neurones

Opioids have powerful inhibitory actions on oxytocin neurones. Given during suckling they block the milk-ejection reflex (Clarke and Wright, 1984), and during parturition the administration of opiates inhibits oxytocin secretion and the delivery of pups is interrupted (Russell et al., 1989b, 1991b; Douglas et al., 1990a). Opioids act at multiple sites in relation to oxytocin neurones, ranging from sensory inputs in the spinal cord (Wright, 1985) to the terminals of oxytocin neurones in the posterior pituitary (Bicknell and Leng, 1982; Zhao et al., 1988). Given systemically both morphine and U50,488, selective μ- and κ-opioid receptor agonists, respectively, inhibit the firing of SON oxytocin neurones (Leng et al., 1990; Pumford et al., 1991), although at the lowest doses of opioid excitatory effects are seen, possibly reflecting inhibition of inhibitory input (Leng et al., 1990). There are both μ- and κ-opioid receptors in the SON, with a predominance of κ-receptors (Mansour et al., 1988; Sumner et al., 1990), and in vitro electrophysiological studies on hypothalamic slices clearly show inhibitory actions of both μ- and κ-opioids (Wakerley et al., 1983; Russell et al., 1989c; Inenaga et al., 1990). Recording in conditions with synaptic transmission blocked shows that both types of opioid can act directly on putative oxytocin neurones, although κ-agonists also act pre-synaptically (Russell et al., 1989c; Inenaga et al., 1990). Despite the clear evidence from these studies that the cell bodies of oxytocin neurones are sensitive to opioids, circumstances have not yet been found in which endogenous opioids restrain the discharge activity of oxytocin neurones. For example, after hyperosmotic stimulation, i.v. CCK8S or i.v. relaxin, the excitatory effects of these stimuli on the firing-rate of oxytocin neurones is not further increased by naloxone (Shibuki et al., 1988; Dyball et al., 1991; unpublished data). Nor is it clear which endogenous opioids might affect the cell bodies of oxytocin neurones: the projection from the arcuate nucleus, a source of β-endorphin neurones, to SON oxytocin neurones does not seem to be opioid-mediated (Sawchenko et al., 1982; Leng et al., 1988c), but there could be local effects from dynorphins or enkephalins released from adjacent dendrites of vasopressin or oxytocin neurones, respectively (Watson et al., 1982; Adachi et al., 1985; Pow and Morris, 1989).

In the posterior pituitary, κ-receptors are the predominant, or only, opioid receptor sub-type (Herkenham et al., 1986; Sumner et al., 1990), and only κ-agonists have actions on oxytocin secretion at this level (Coombes and Russell, 1988; Zhao et al., 1988). Endogenous opioid action at this site is demonstrated by the facilitating action of naloxone on stimulated oxytocin release in vitro from isolated posterior pituitaries or in vivo in circumstances when effects on the firing-rate of oxytocin neurones can be excluded (Bicknell and Leng, 1982; Bicknell et al., 1988). A possible source of endogenous opioid acting on oxytocin terminals are the adjacent vasopressin terminals which contain and release dynorphins which are κ-selective opioids (Corbett et al., 1982; Whitnall et al., 1983; Lorenz et al., 1985); thus activation of vasopressin neurones is proposed to cross-inhibit, by pre-terminal action, the secretion of oxytocin (Summy-Long, 1989). This may be significant in the fine balancing of the water-retaining actions of vasopressin and the natriuretic actions of oxytocin (Leng et al., 1988a, 1989a).

With respect to the input from the lamina terminalis, opiates inhibit the firing of oxytocin neurones excited by electrical stimulation of the AV3V region (Leng and Russell, 1989). In addition, there are opioid receptors in the nucleus medianus and both μ- and κ-opioid receptors in the SFO (Sharif and Hughes, 1989; Sumner et al., 1990), and the discharge activity of some SFO neurones is inhibited by morphine (Buranarugsa and Hubbard, 1979). It is thus possible that in vivo inhibitory opioid action on the SFO or nucleus medianus would lead to inhibition of oxytocin neurones by silencing the input that lesioning and other studies (see above) have shown to be essential for both the basal, continuous activity and the osmorespon-

siveness of oxytocin neurones. Since the milk-ejection reflex does not involve inputs from the lamina terminalis this could partly explain why suckling-induced bursting activity of oxytocin neurones is less easily inhibited by morphine than is their continuous pattern of activity (Clarke et al., 1979; Pumford et al., 1991).

Chronic morphine

During i.c.v. infusion of morphine for 5 days the magnocellular oxytocin neurone system develops both tolerance and dependence (Bicknell et al., 1988; Rayner et al., 1988; Russell, 1989). Tolerance is shown by the resumption of near-normal oxytocin neurone activity and oxytocin secretion (Russell, 1989; Pumford et al., 1991), while morphine withdrawal, provoked by naloxone, produces a large increase in oxytocin secretion (ca. 24-fold), some 8 times greater than the increase seen in control rats in similar conditions, under urethane anaesthesia (Bicknell et al., 1988). The explanation of the different effects of naloxone is that in the control rats naloxone acts only on the posterior pituitary and does not increase the firing-rate of oxytocin neurones but antagonizes endogenous opioid in the posterior pituitary, probably dynorphin released by the terminals of vasopressin neurones excited by the hyperosmotic effects of urethane anaesthesia (Hartman et al., 1987); whereas in morphine-infused rats naloxone increases the firing-rate as well as acting in the posterior pituitary (Bicknell et al., 1988; Leng et al., 1989b). In morphine-dependent rats the withdrawal excitation is probably at least partly in the SON since it is not prevented by acute lesion of the AV3V region (Bicknell et al., 1987), by i.c.v. saralasin infusion (Russell, 1989), nor by cholinergic antagonists (Bicknell et al., 1988).

However, naloxone methyl-bromide, a quaternary form of naloxone that does not cross the blood-brain barrier, excites oxytocin neurones in morphine-dependent rats when given i.v., although less potently than naloxone, and this may reflect morphine withdrawal at a site outside the blood-brain barrier projecting to the magnocellular oxytocin neurones (Leng et al., 1989b). This could be in the lamina terminalis; although an acute AV3V lesion does not prevent withdrawal excitation of oxytocin secretion, the excitation is attenuated in terms of arithmetic increment in oxytocin secretion, but not with respect to the proportional increase from a basal level reduced as a result of the lesion (Fig. 1; Russell et al., 1992). Thus it is possible that the excitation of oxytocin neurones during morphine withdrawal involves a contribution from excited morphine-dependent neurones in the lamina

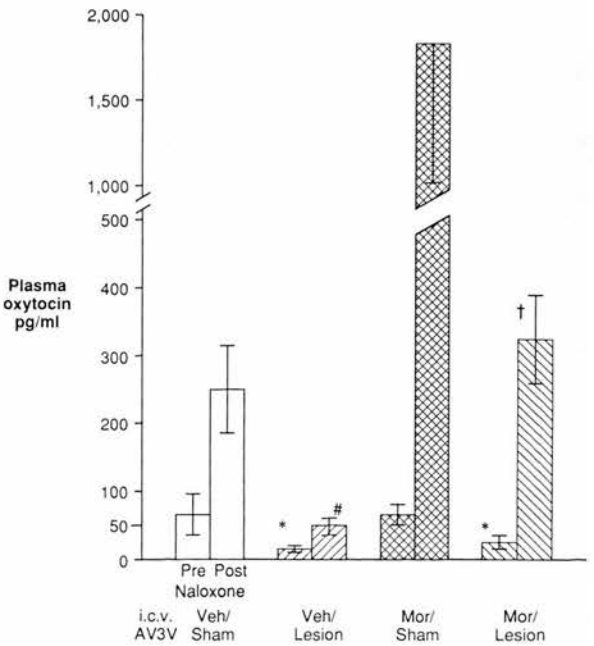

Fig. 1. AV3V lesion and naloxone-induced withdrawal excitation of oxytocin secretion in morphine-dependent rats. After intracerebroventricular (i.c.v.) morphine (Mor) or vehicle (Veh) infusion for 5 days, femoral arterial blood plasma samples were taken under urethane anaesthesia for oxytocin radioimmunoassay. The AV3V region was lesioned electrolytically (Lesion) or a sham procedure was carried out (Sham; see Russell et al., 1992); after a blood sample 60 min later (Pre) naloxone (5 mg/kg i.v.) was injected, 6 min before the second sample (Post). The values are mean ± S.E., $n = 6$ rats per group. Plasma oxytocin decreased after the AV3V lesion ($^{*}P < 0.05$, U-test) and increased after naloxone in all groups ($P < 0.05$, paired t-tests), but rose more in morphine-infused rats ($P < 0.025$); in lesioned rats naloxone increased plasma oxytocin less (arithmetically, but not proportionately) than in the respective sham group ($^{\#}P < 0.005$, $^{\dagger}P < 0.05$).

terminalis, perhaps leading to the type of reciprocal excitation postulated for the normal behaviour of the osmoregulatory complex.

Morphine tolerance in the mechanisms regulating oxytocin neurones involves reduced density of opioid receptors in the nucleus medianus, and specifically of μ-receptors in the SON (Sumner et al., 1990). This may not fully explain the reduced morphine sensitivity of oxytocin neurones since inactivation of an inhibitory G-protein, G_i or G_o, by i.c.v. injection of pertussis toxin 72 h previously also decreases morphine sensitivity of oxytocin neurones (Russell et al., 1991a), suggesting that changes in coupling of receptor to $G_{i/o}$ protein, or in G-protein function could be involved in morphine tolerance. Interestingly, morphine tolerance does not involve changes in κ-receptor density in either the posterior pituitary or the SON (Sumner et al., 1990), nor is the sensitivity to κ-agonist at either site reduced (Leng et al., 1990; Coombes and Russell, 1991). Similarly i.c.v. pertussis toxin pre-treatment does not alter the sensitivity of oxytocin neurone firing-rate to κ-agonist (Russell et al., 1991a).

The changes in opioid sensitivity of oxytocin neurones in the chronic morphine and pertussis toxin models suggest that similar changes might occur in physiological circumstances and could play a role in adaptations of the oxytocin neurone system to functional demands. We have been investigating whether such changes can be seen in oxytocin neurones during pregnancy, in preparation for parturition.

Pregnancy, opioids and oxytocin neurones

In conscious virgin rats or rats in early pregnancy, naloxone has no effect on oxytocin secretion, but after 2 weeks of pregnancy naloxone has an increasing stimulatory action on oxytocin secretion, maximal during parturition and disappearing immediately afterwards (Hartman et al., 1986; Leng et al., 1988b). This effect of naloxone indicates at least an inhibitory action of endogenous opioids on oxytocin secretion during pregnancy, and possibly dependence by analogy with changes in morphine-treated rats. Since either μ- or κ-agonists can interrupt parturition and reduce oxytocin secretion (Russell et al., 1989b, 1991b; Douglas et al., 1990a), it is clear that mechanisms regulating oxytocin neurones retain at least some opioid sensitivity at the end of pregnancy. Opioid tolerance is a quantitative phenomenon (Pumford et al., 1991), so quantitative comparisons of opiate sensitivity between pregnant and non-pregnant rats are needed; since there is no obvious equivalent stimulus in virgin rats to the stimuli driving oxytocin secretion during parturition we have used other stimuli to test opioid sensitivity.

Osmoresponsiveness in pregnancy

Plasma osmolality and $[Na^+]$ are decreased at the end of pregnancy, but in experiments using intraperitoneal (i.p.) hypertonic saline as a stimulus, the osmotic threshold for stimulation of AVP secretion is reduced pari passu, with no change in sensitivity relative to virgin rats (Durr et al., 1981; Atherton et al., 1982); consequently, at the end of pregnancy AVP levels are similar (or even greater) than in virgin rats despite the lower plasma osmolality (Durr et al., 1981; Hartman et al., 1986). Osmoregulation of oxytocin secretion in pregnancy does not appear to have been studied previously; we have found that in urethane-anaesthetized rats i.p. hypertonic saline (1.5 M) produces a non-significant increase at the end of pregnancy, contrasting with a large increase in virgin controls (Fig. 2). This difference in response is not due to endogenous opioid action in pregnancy since naloxone given after the hyperosmotic stimulus increased the plasma oxytocin level more in virgins than in pregnant rats, despite similar increases in plasma $[Na^+]$ (Fig. 2). In lactating rats, although plasma osmolality is not decreased compared with virgins (Russell, 1980), there is also a greatly reduced response to i.p. hypertonic saline, and this has been attributed to depletion of oxytocin from the posterior pituitary in lactation (Hartman et al., 1987; Higuchi et al., 1991). This is not the explanation in late pregnant rats since stronger hyperosmotic stimulation by i.v. infusion

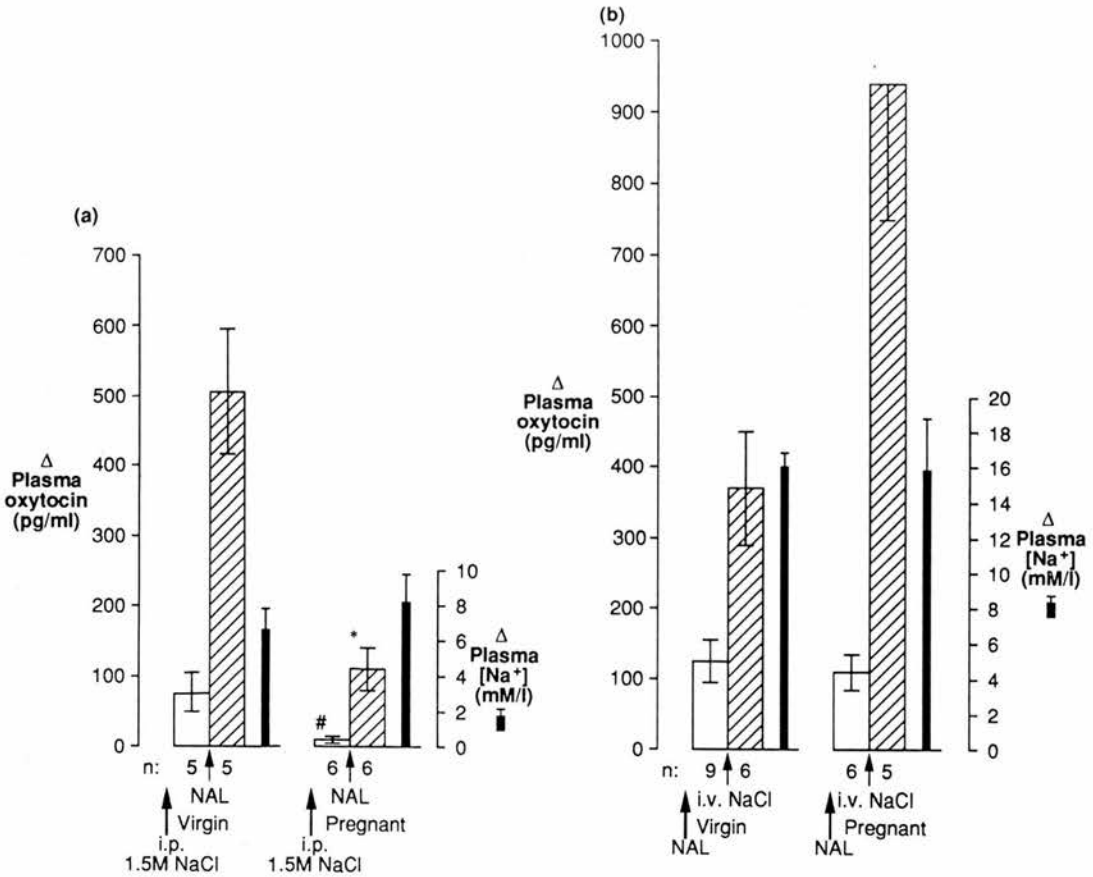

Fig. 2. Hyperosmotic stimulation of oxytocin secretion in pregnancy. Virgin or pregnant (day 21) rats anaesthetized with urethane were blood sampled for oxytocin radioimmunoassay and measurement of $[Na^+]$ by flame photometry. Values are mean ± S.E. differences from basal concentrations; narrow solid bars are differences in $[Na^+]$ between the basal and the last plasma sample; n = number of rats. *a.* An intraperitoneal (i.p.) injection of 1.5 M NaCl (4 ml/kg body weight) was given 50 min before the first blood sample; this increased oxytocin only in the virgins ($P < 0.05$, paired Wilcoxon), $^{\#}P < 0.01$ vs. virgins. Intravenous (i.v.) naloxone (5 mg/kg) was given immediately after the first sample, 15 min before the second sample. Naloxone increased plasma oxytocin in both groups ($P < 0.05$), but by much less in the pregnant rats ($^{*}P < 0.01$ vs. virgins). Plasma $[Na^+]$ increased similarly in the two groups. *b.* Naloxone (5 mg/kg i.v.) was given 15 min before the first sample, and immediately after this sample an i.v. infusion of hypertonic NaCl (0.3 mM NaCl/kg in 28 μl 0.9% NaCl/min) was started, 20 min before the second sample. After i.v. hypertonic NaCl and naloxone, plasma oxytocin increased more in the pregnant rats ($^{*}P < 0.02$ vs. virgins).

of hypertonic saline after naloxone (to remove any opioid inhibition) produced a larger increase in oxytocin secretion compared with virgin rats, despite similar increases in plasma $[Na^+]$ (Fig. 2). These data indicate that in late pregnancy the osmotic threshold for stimulation of oxytocin secretion is not lowered (in contrast with that for vasopressin), but the gain is increased once the threshold is reached (unlike vasopressin). This implies that in late pregnancy basal oxytocin secretion is not stimulated by plasma $[Na^+]$ or osmolality, but by another factor, which may then interact with osmolality above the osmotic threshold. In late pregnant conscious rats, basal oxytocin concentration in blood plasma is greater than in virgin controls (Russell et al., 1991b) indicating that oxytocin secretion is being

riven more strongly in late pregnancy despite the educed stimulation related to plasma osmolality or Na^+].

The reduced plasma osmolality and [Na^+] at the nd of pregnancy will have two consequences with espect to oxytocin neurones: first, oxytocin secretion will be independent of plasma osmolality and an be stimulated selectively by inputs related to parturition, and secondly, reduced osmotic stimulation of oxytocin secretion may, because of its natriuretic action, favour Na^+ retention and sustain the hyperolaemia of pregnancy. How osmoregulation of asopressin and oxytocin neurones are changed in different ways in pregnancy is not clear.

Oxytocin secretion stimulated by i.p. hypertonic aline can be powerfully inhibited by morphine in both virgin and pregnant rats with no evidence of educed morphine sensitivity in pregnant rats (Fig. 3). However, as discussed above, following systemic administration, morphine may act at several sites in

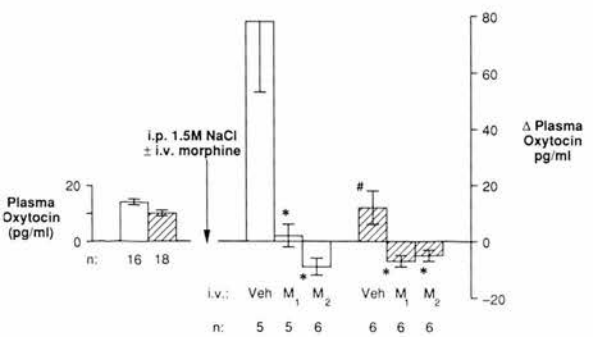

Fig. 3. Morphine and hyperosmotic stimulation of oxytocin secretion in pregnancy. Virgin (open columns) or 21-day pregnant (hatched columns) rats anaesthetized with urethane were blood sampled for oxytocin radioimmunoassay. Values are mean ± S.E.; n = number or rats. A hyperosmotic stimulus (1.5 M NaCl, 4 ml/kg i.p.) was given 50 min before the second blood-sample; at the same time i.v. vehicle or morphine (M_1, 0.25 mg/kg; M_2, 1 mg/kg) were given, then larger doses of morphine were given 20 min before the second sample (M_1, 2.5 mg/kg; M_2, 5 mg/kg). Initially plasma oxytocin was greater in the virgins ($P < 0.025$, U-test); for the second sample differences from respective values in the first sample are shown. Hyperosmotic stimulation did not increase plasma oxytocin in pregnant rats (paired Wilcoxon; $\#P < 0.01$ vs. virgin/vehicle group, U-test; see Fig. 2). Both morphine doses prevented hyperosmotic stimulation in the virgins ($*P < 0.005$, Kruskal-Wallis) and reduced plasma oxytocin in the pregnant rats ($*P < 0.05$).

the osmoreceptor complex and in general the effective doses will be those acting at the most sensitive site. Measurements of opioid receptor density, using in situ ^{3}H-ligand binding in vitro in conditions selective for receptor sub-types followed by quantitative autoradiography, showed reduced density of μ-receptors, but not of κ-receptors, in the SON at the end of pregnancy, with no changes in opioid receptor density in the nucleus medianus (Russell and Sumner, 1990; unpublished data). To test whether opioid actions in the SON are different in pregnancy, opiates were tested against oxytocin secretion evoked by electrical stimulation of the AV3V region (see Blackburn et al., 1987; Fig. 4). With the parameters used morphine (up to 10 mg/kg) was not effective in depressing stimulated oxytocin secretion either in controls or pregnant rats, a result probably of the hyperpolarizing postsynaptic action of morphine being overcome by the evoked excitatory synaptic activity (Leng and Russell, 1989). The results confirm our previous finding that morphine does not inhibit oxytocin secretion by an action at the posterior pituitary (Coombes and Russell, 1988), but leaves the question of whether there is a change in the sensitivity of SON oxytocin neurones to μ-opioids in pregnancy unresolved. In contrast, the selective κ-agonist U50,488 is highly effective at inhibiting oxytocin secretion evoked by electrical AV3V stimulation, with no evident difference in potency between late pregnant and virgin rats (Fig. 4*a*). Together with results from in vitro studies on the posterior pituitary (see below), this indicates no change in the κ-opioid sensitivity in the SON, consistent with the lack of change in SON κ-receptor density in pregnancy.

Without opiate administration the increment in plasma oxytocin concentration following AV3V stimulation was similar in pregnant and non-pregnant rats, but the facilitating effect of naloxone was strikingly less in pregnant rats (Fig. 4*b*). From the results of the experiments with hyperosmotic stimulation, it is clear that the secretory capacity of the oxytocin system is not decreased in pregnancy, so the reduced response to AV3V stimulation after naloxone appears to suggest that, in pregnant rats, the

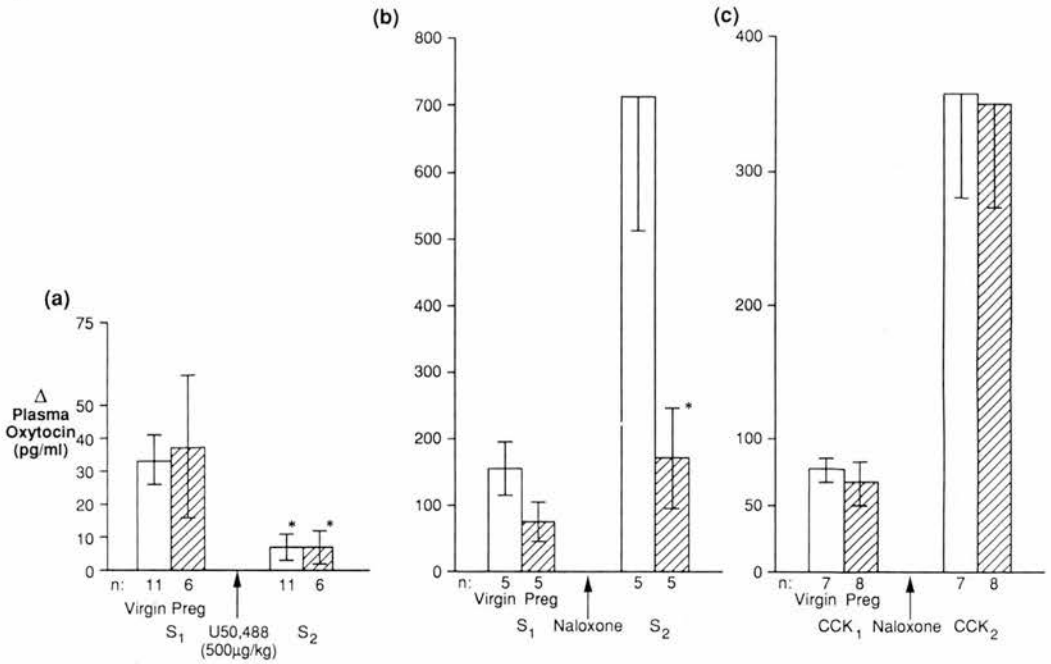

Fig. 4. Opioid actions on oxytocin secretion evoked by electrical AV3V stimulation or i.v. CCK8S in pregnancy. *a.* Urethane anaesthetized virgin or 21-day pregnant rats with a bipolar stimulating electrode stereotaxically located in the AV3V region (0.3 mm posterior to bregma, 8.3 mm ventral to skull surface, mid-line); femoral arterial blood samples were collected before and immediately after two periods (S_1, S_2) of AV3V stimulation 20 min apart (25 Hz, 0.5 mA, 1 msec pulses, 10 sec on 10 sec off for 2 min). The values are mean ± S.E. increments in plasma oxytocin concentration between the pre-/post-stimulation samples; n = number of rats. The κ-opioid agonist U50,488 (500 μg/kg i.v.) injected 15 min before S_2 inhibited the response in both groups similarly (*$P < 0.05$ vs respective S_1 value, paired Wilcoxon). *b.* AV3V stimulation as in *a*. Naloxone (5 mg/kg i.v.) injected 15 min before S_2 increased the response to AV3V stimulation in the virgins ($P < 0.05$, paired Wilcoxon), but not in the pregnant rats (paired Wilcoxon; *$P < 0.0$ vs. virgin S_2, *U*-test). *c.* Conscious virgin or 21-day pregnant rats blood sampled via a jugular venous cannula inserted under ether anaesthesia. Injections of CCK8S (20 μg/kg i.v.) were given 80 min apart (CCK_1, CCK_2), and naloxone (5 mg/kg i.v.) was given 10 min before CCK_2. The values are increments in plasma oxytocin concentration 5 min after CCK8S relative to immediately before CCK8S. CCK8S had similar effects in the two groups, and naloxone had a similar facilitatory action ($P < 0.02$, paired Wilcoxon).

coupling of the AV3V input to oxytocin neurones is less effective. Thus the initial similar response to AV3V stimulation in pregnant and virgin rats seems to be a product of reduced AV3V coupling to oxytocin neurones and decreased inhibitory opioid action at the level of the posterior pituitary (as discussed below). The reduced effectiveness of AV3V stimulation after naloxone in pregnant rats may relate to the reduced plasma osmolality and [Na^+] by diminishing the component from direct osmosensitivity of oxytocin neurones which interacts with excitatory input to determine the osmoresponsiveness of oxytocin neurones (Russell et al., 1988; Leng et al., 1989a). In contrast, in conscious rats the stimulatory effect of i.v. CCK8S on oxytocin secretion is unchanged in late pregnant rats (Fig. 4*c*). Thus, not only are oxytocin neurones normally responsive to this stimulus, but the input path acted on by CCK8S via the vagus and then a central noradrenergic projection appears to be normally coupled to oxytocin neurones in late pregnancy (Kendrick et al., 1991).

Posterior pituitary

We have investigated changes in opioid mechanisms on oxytocin secretion at the level of the posterior pituitary by using an in vitro protocol involving measurement of oxytocin secretion evoked during two periods of electrical stimulation, with opiat

agonist or antagonist added prior to the second stimulation (Bicknell and Leng, 1982). Electrical stimulation releases not only oxytocin (and vasopressin) but also other transmitters in nerve terminals in the posterior pituitary, including opioids whose effects are antagonized by naloxone. Electrical stimulation of posterior pituitaries from late pregnant rats releases more oxytocin compared with posterior pituitaries from virgin females, but naloxone has a smaller facilitating effect on oxytocin release by posterior pituitaries from the pregnant rats (Douglas et al., 1990b). These results indicate that endogenous opioid action on oxytocin terminals is reduced toward the end of pregnancy. Reduced opioid sensitivity of oxytocin terminals is involved since addition of the κ-opioid agonist U50,488 before the second stimulation was found to be less effective at inhibiting oxytocin release by posterior pituitaries from late pregnant rats (Douglas et al., 1990b). This reduced sensitivity to κ-agonists may be a result of decreased κ-receptor number on oxytocin terminals since the density of ^{3}H-bremazocine binding, in κ-selective conditions, is significantly reduced on day 16 of pregnancy, when changes in naloxone and U50,488 actions are evident (unpublished data). The cause of this loss of κ-opioid receptors and of the reduced sensitivity of oxytocin terminals to κ-opioid, is not yet clear. Circumstantial evidence that these changes result from increased endogenous opioid action is that at the end of pregnancy, the content of [met^5]enkephalin in the posterior pituitary is decreased by 33% (unpublished data) with no change in dynorphin content. [Met^5]enkephalin is co-produced in oxytocin neurones (Adachi et al., 1985) and the changes in posterior pituitary content could reflect chronic stimulation of oxytocin neurones and depletion of their opioids. Although [met^5]enkephalin is a δ-opioid agonist, the hepta- and octa-peptide met-enkephalin products of pro-enkephalin A have κ-agonist activity, and these are also present in the posterior pituitary (Castanas et al., 1985; Panula and Lindberg, 1987). It is possible that continuous release of these endogenous opioids toward the end of pregnancy desensitizes the oxytocin terminals to κ-agonists.

Significance of opioid and osmoreceptor changes in pregnancy

Under many conditions when the secretion of both oxytocin and vasopressin is increased, naloxone selectively increases oxytocin release further, without exciting the firing-rate of oxytocin neurones (Bicknell et al., 1988; Shibuki et al., 1988; Summy-Long, 1989). Consequently it has been proposed that through co-released dynorphin$_{1-8}$ vasopressin neurones will attenuate oxytocin release; our results indicate that this cross-inhibition is less effective at the end of pregnancy and so the stimuli that drive oxytocin neurones at parturition will be more effective. Additionally, any auto-inhibitory actions of enkephalins from oxytocin terminals will be reduced. Reduced effectiveness of terminal opioid action will thus further enhance the frequency facilitation of oxytocin release with the bursting pattern of firing of oxytocin neurones during parturition (Summerlee, 1981; Bicknell, 1988).

The degrading of the opioid barrier at the level of the posterior pituitary is not associated with an increase in oxytocin secretion driven by the osmoreceptor complex, since plasma osmolality and [Na^+] are reduced, and this could be considered to be a compensatory adjustment for the opioid changes in the posterior pituitary. The in vitro posterior pituitary studies do not, however, explain why naloxone increases oxytocin secretion in vivo at the end of pregnancy. This may be accounted for by a shift in the primary site of endogenous opioid action to the cell bodies of oxytocin neurones, as indicated by the μ-opioid binding changes in the SON at the end of pregnancy. Such a compensatory central opioid tone may act selectively on some inputs: our studies indicate that the osmoreceptor complex input is not influenced by opioids in pregnancy, but the possibility of endogenous opioid inhibition on other inputs, such as that activated by i.v. CCK8S, requires further investigation with electrophysiological recording.

Acknowledgements

A.J.D. is supported by an AFRC Linked Research

Group Project Grant, which supports collaboration between J.A.R., R.J.B. and G.L.; K.M.P. was supported by an MRC Project Grant; P.B. is supported by an AFRC Postgraduate Research Studentship.

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