ANATOMICAL AND PHYSIOLOGICAL RELATIONSHIPS BETWEEN CENTRAL SEROTONIN AND VASOPRESSIN

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To my parents who instilled in me the curiosity and desire to understand by exploration, the work ethic to complete the task and who gave me the opportunities and encouragement to make this possible.

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DECLARATION

This research was carried out over a period of three years in the Departments of Medicine and Psychiatry in the University of Newcastle upon Tyne and in the MRC Neurochemical Pathology Unit. I performed all the studies of Sections 3 and 4 on normal volunteers and depressed patients and, in collaboration with DR J Charlton, the studies of Section 5.

The assay of vasopressin was carried out by myself (Section 5), Mrs PA Rooke (Section 3) and Mrs C Holmes (Section 4). The study of serotonin receptors in rat brain was carried out by me. Mr A Leake (MRC Neurochemical Pathology Unit) performed homogenate ligand binding analyses and Mr M Leitch (Department of Psychiatry) carried out the HPLC analyses.

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ABSTRACTS

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ABSTRACT

The role of serotonin (5HT) in the physiological regulation of AVP secretion is controversial. Neuroanatomical studies, largely in rats but also in human brains, have suggested that 5HT may have a direct modulatory effect on magnocellular vasopressin (AVP) secretion. Pharmacological and neurophysiological studies in animals have provided further evidence to support this and suggest that increase in 5HT neurotransmission leads to a rise in plasma AVP and that 5HT may be important in osmoregulated AVP secretion

Studies investigating the importance of 5HT as a modulator of AVP release in humans have not be undertaken. Indirect evidence of a putative role derives from the occurrence of hyponatraemia, and possible inappropriate AVP secretion, associated with the clinical use of drugs, particularly antidepressants, which have effect on 5HT neurotransmission. In addition there has been some suggestion that AVP secretion may be abnormal in depression where there is a putative abnormality of the 5HT nervous system.

This research has approached the study of anatomical and physiological relationships between 5HT and AVP in 3 ways. Firstly through studies in normal man, secondly by studies in depressed patients, as a putative disease model of 5HT neurotransmission, and thirdly to more extensively explore the effect of pharmacological manipulation of 5HT neurotransmission using an animal model of osmoregulation.

Studies in man found no evidence that 7 days treatment with a 5HT reuptake inhibitor (Fluoxetine) had a significant effect on osmoregulated AVP secretion. Studies in elderly depressed patients showed that there was an apparent deficiency of osmoregulated AVP secretion with normal ageing but found no evidence that either moderate depressive illness, or treatment of the depression with Fluoxetine, had significant effect on water balance.

Studies in the rat model of osmoregulation showed that acute 5HT reuptake inhibition stimulated basal AVP secretion and increased the osmotic sensitivity of AVP secretion but had no effect on the osmotic threshold of secretion. Chronic treatment (21 days) with the reuptake inhibitor had no significant effect on basal AVP secretion or on the osmotic threshold but significantly decreased

the osmotic sensitivity of AVP secretion.

Studies with the 5HT2/5HT1c antagonist, Ritanserin, and the 5HT2 agonist, DOI, suggested that this modulatory effect was not mediated through these receptor subtypes. Autoradiographic studies identified a low density of 5HT2 and 5HT1a receptors in the vicinity of the magnocellular neurons of the rat hypothalamus.

The results suggested that 5HT modulates AVP secretion indirectly, possibly by inhibition of inhibitory afferent stimuli. This is of little physiological consequence in the normal rat and probably in healthy man where there is rapid accommodation and autoregulation. In situations where there is a dysfunction of the normal adaptive mechanisms, such as in depression, the role of 5HT may be more important and occasionally may lead to severe hyponatraemia.

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6. The protocol for Study 4.1
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8. Consent forms and letters to consultants and GP's of patients and volunteers in Studies 4.1 and 4.2
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10. Case reports of depression and hyponatraemia
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12. The mathematical Model of Piece-Wise regression analysis

LIST OF ABBREVIATIONS

5,7-DHT	5,7-dihydroxytryptamine
5HIAA	5-Hydroxyindole acetic acid
5HT	5-Hydroxytryptamine (Serotonin)
5HTP	5-Hydroxytrytophan
Ach	Acetylcholine
ACTH	Adrenocorticotrophic hormone
AD	Antidepressant
ANOVA	Analysis of variance
ANP	Atrial natriuretic factor
AVP	Arginine vasopressin
C_{creat}	Creatinine clearance
CDI	Cranial diabetes insipidus
C_{H2O}	Free water clearance
CNS	Central nervous system
CRF	Corticotropin releasing factor
CSF	Cerebro-spinal fluid
D	DOI
DA	Dopamine
DAB	Diaminobenzidine
DOI	(1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane hydrochloride
DPAT	8-Hydroxy-2-(di-n-propylamino) tetralin
DRN	Dorsal raphé nucleus
DSM-IIIR	Diagnostic and statistical manual of mental disorders (3rd Edition revised)
ECF	Extracellular fluid
ECT	Electroconvulsive therapy
F	Fluoxetine
GABA	γ-aminobutyric acid
GH	Growth hormone
GP	General practitioner
H	Hyperosmotic stimulus
HCT	Haematocrit
HNC	Hypothalamic neurohypophysial complex
HNT	Hypothalamo-neurohypophysial tract
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase

HRSD	Hamilton rating scale for depression
ICC	Immunocytochemistry
ICV	Intracerebro-ventricular
IP	Intraperitoneal
IV	Intravenous
L	Hypotonic stimulus
MRN	Median raphé nucleus
N	Normo-osmotic stimulus or vehicle control
NA	Noradrenaline
NTS	Nucleus of the tractus solitarius
OVLT	Organum vasculosum lamina terminalis
OXY	Oxytocin
PBS	Phosphate buffered saline
pCPA	para chlorophenylalanine
pNa	Plasma sodium
pOS	Plasma osmolality
PRL	Prolactin
PVN	paraventricular nucleus
R	Ritanserin
REM	Random eye movement
RIA	Radioimmunoassay
SC	Subcutaneous
SCN	Suprachiasmatic nucleus
SD	Standard deviation
SEM	Standard error of the mean
SFO	Subfornical organ
SIAD	Syndrome of inappropriate antidiuresis
SON	Supraoptic nucleus
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TD	Trytophan decarboxylase
TH	Trytophan hydroxylase
uOS	Urine osmolality

SECTION 1

INTRODUCTION

SUMMARY

Neuroanatomical studies, largely in rats but also in human brains, have suggested that serotonin (5HT) may have a role in regulation of vasopressin (AVP) secretion. Pharmacological and neurophysiological studies in animals have provided further evidence to support this hypothesis suggesting that increase in 5HT neurotransmission leads to a rise in plasma AVP and that 5HT may be important in osmoregulated AVP secretion

Studies investigating the importance of 5HT as a modulator of AVP release in humans have not been undertaken. Indirect evidence of a putative role derives from the occurrence of hyponatraemia, and possible inappropriate AVP secretion, associated with the clinical use of drugs, particularly antidepressants, which have effect on 5HT neurotransmission. In addition there has been some suggestion that AVP secretion may be abnormal in depression where there is a putative abnormality of the 5HT nervous system.

This section discusses the literature pertinent to the regulation of AVP secretion with particular discussion of normal and abnormal osmoregulation and hyponatraemia. The 5HT neurotransmitter system is discussed with the evidence for its modulation of AVP secretion and involvement in the pathophysiology and phenomenology of depression. The evidence for abnormalities of water balance and AVP secretion in mental illness is also discussed.

The hypotheses drawn from the literature review are presented together with the aims of this research.

1.1 VASOPRESSIN AND THE MAINTENANCE OF A NORMAL OSMOTIC ENVIRONMENT

Arginine vasopressin (AVP) is a nine aminoacid peptide hormone, secreted from the posterior pituitary together with its associated carrier protein neurophysin I. It has been found to have important actions in the maintenance of water balance and cardiovascular homeostasis. AVP-secreting neurons have been identified throughout the brain as have AVP receptors (Sofroniew 1985; Lawrence et al 1988; Tribollet et al 1988). This suggests that the peptide has a multitude of functions each of which may be regulated by separate systems and be responsive to independent stimuli (Buijs et al 1983). In the hypothalamus AVP-containing neurons have a variety of functions. Some have a role in the regulation of the pituitary-adrenal axis through synergistic action with corticotropin releasing factor on adrenocorticotrophic hormone (ACTH) release (Zimmerman & Silverman 1983; Schatzberg & Nemeroff 1988). AVP may also have a role in circadian rhythmicity since some neurons in the suprachiasmatic nucleus (SCN) have a high AVP content (Zimmerman 1976). Swanson & Sawchenko (1980) suggested that the AVP neurons in the paraventricular nucleus (PVN) may be involved in the integration of the autonomic nervous system as axons project to the brain stem and spinal cord.

This review will concentrate on AVP-secreting neurons of the hypothalamic neurohypophysial axis, the magnocellular AVP system (Figure 1.1.1).

1.1.1 ANATOMY

The majority of research on the anatomy of the magnocellular AVP system has been carried out in rat and other animal brains. This will be discussed first and the differences in man will then be considered.

Retrograde tracing experiments with dyes or horseradish peroxidase placed in the posterior pituitary have shown that large cell bodied neurons, the magnocellular neurons, in the supraoptic nucleus (SON) and PVN, together with accessory groups of similar neurons, are the source of the majority of nerve fibres in the hypothalamo-neurohypophysial tract (HNT) (Sherlock et al 1975;

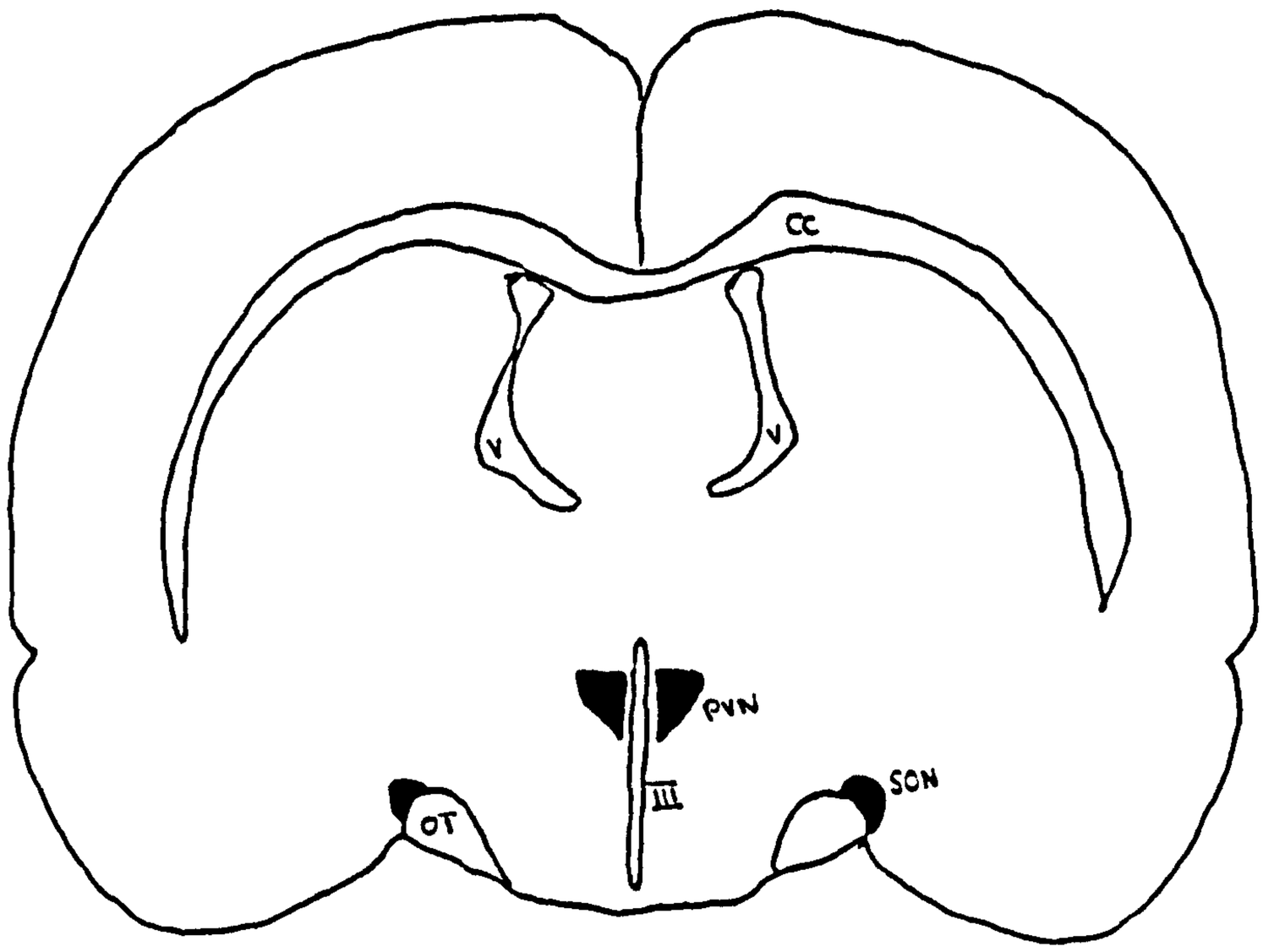


Figure 1.1.1

The location of the magnocellular AVP secreting neurons of the hypothalamo-neurohypophysial axis of the rat.

SON: Supraoptic nucleus; PVN: Paraventricular nucleus;

III: Third ventricle; OT: Optic tract; V: Ventricle; CC: Corpus callosum

Armstrong & Hatton 1980; Sofroniew 1982). The accessory groups contribute about 50% of the AVP fibres to the HNT (Fisher et al 1979; Rhodes et al 1981). Of the rest the majority originate in the SON.

The magnocellular neuronal system comprises both oxytocin (OXY)-and AVP-secreting neurons. Research has shown that only one hormone type is secreted in any one neuron (Aspeslagh et al 1976; van Leeuwen & Swaab 1977; Morris et al 1977; Diericlix et al 1978) and that the neuronal cell bodies for each hormone type are unevenly distributed within the hypothalamic nuclei (Sternberger 1974; Vandesande & Dierickx 1975; Swaab et al 1975a+b). OXY cells are more concentrated in the rostral part of each nucleus. At the mid rostro-caudal level of the SON, AVP cells are generally ventral, whilst OXY cells form a dorsal rim. The magnocellular neurons of the PVN lie at the lateral edge of the nucleus. AVP cell bodies form the central core of this neuronal cluster, rimmed by OXY neurons. The majority of the accessory groups of magnocellular neurons are OXY secreting (Rhodes et al 1981).

Efferent fibres from the PVN, so called Greving tracts, pass laterally around and through the fornix, then move ventrally to join axons from the SON and form a tract converging on the zona interna of the median eminence. The pathway continues down the pituitary stalk to end on the basement membranes of the capillaries in the neurohypophysis (Silverman & Zimmerman 1975; 1983). Neurons from the PVN do not appear to synapse in the SON but some, mostly AVP secreting, terminate in the median eminence (Vandesande et al 1977). It is largely the OXY-containing fibres of the PVN, together with fibres from the SON, which innervate the neurohypophysis.

Within the neurohypophysis there is specific arrangement of neuronal fibres from each nucleus. SON neurons are concentrated in the centre of the gland whilst PVN axons terminate in the periphery. Essentially this means that neurons secreting AVP are in the centre of the gland and those containing OXY are around the periphery (van Leeuwen & Swaab 1977; Van leeuwen et al 1979).

The anatomy of the HNT in man differs in many respects from that of the rat (Defendini & Zimmerman 1978). The SON is divided into anterior and posterior parts by the optic tract and the PVN is a long, thin column of cells

lying in the wall of the third ventricle. Although cell bodies of the SON are large, similar to those in the rat, identification of magnocellular neurons in the PVN is less obvious. Immunocytochemical studies have shown that neurons exhibit specificity of hormone type secreted (Dierickx & Vandesande 1977; Sofroniew et al 1981). In the SON, unlike the rat, the majority of neurons are AVP secreting and are located in the ventral portion of the nucleus. Regional topography of each neuronal type is not defined in the PVN. The efferent fibre tracts of the two nuclei appear to be similar to those described in the rat brain. Thus although study of the anatomy of animal brains may give some insight into possible function of magnocellular AVP secretion some caution must be taken into extrapolation of these findings to man since there are important differences, particularly in the anatomy of the PVN and in the relative loss of OXY neurons.

1.1.2 PHYSIOLOGICAL REGULATION OF AVP RELEASE IN HUMANS

AVP is known to have both antidiuretic and pressor effects and the osmotic environment and circulatory pressure and volume have major roles in regulation of neurohypophysial secretion. In healthy humans the principal regulatory influence is the osmolality of extracellular fluid (Robertson & Athar 1976).

1.1.2.1 Osmoregulation

In 1947 Verney found that injection of hypertonic solutions into the carotid arteries of conscious dogs produced a prompt antidiuresis. This system was sufficiently sensitive to respond to changes in plasma osmolality (pOS) of 2% or less. Verney suggested that intracranial sensors of osmolality changes, osmoreceptors, directly influenced AVP secretion and allowed regulation of water excretion by the kidney to maintain osmotic homeostasis.

Research has since found that osmotically sensitive cells lie within the circumventricular organs of the anterior hypothalamus, either in the organ vasculosum of the lamina terminalis (Thrasher et al 1982) or in the subfornical organ (Iovino & Steardo 1984; Ferguson & Kasting 1986). Changes in the osmotic mileux of these cells modulate firing activity of nerves projecting to the magnocellular neurons of the SON and to some extent to the PVN (Bicknell et al 1984; Mason et al 1988). The magnocellular neurons themselves have also been

shown to be osmotically sensitive but the role of this in osmoregulation is less clear (Mason 1980; Leng et al 1982).

In healthy adults plasma osmolality (pOS) is maintained at 287±2 mOsm/kg (Robertson et al 1973). For any individual the basal pOS is very tightly regulated with daily variation of ±1.8% (Robertson & Athar 1976).

Initial work on the osmoregulation of AVP in humans was carried out subsequent to the development of a sensitive radioimmunoassay for AVP (Robertson et al 1970). Infusion of hypertonic saline leads to a rise in plasma AVP. The rate of rise, and the relationship to pOS was dependent on the rate of infusion of the saline ie. the rate of change of pOS (Robertson et al 1977). Fast infusion rates of infusion with an increase in pOS of >2% per hour caused exaggerated AVP secretion with a curvilinear relationship to pOS (Weitzman & Fisher 1977). A rise in pOS of <2% per hour lead to a linear relationship between pOS and AVP (Robertson et al 1976; Thompson et al 1986).

Linear regression analysis of the change in AVP with change in pOS provides data on the *sensitivity* of AVP secretion to unit change in pOS and allows calculation of the theoretical pOS (x-axis intercept) at which AVP might first be secreted, the *osmotic threshold.* This threshold is theoretical in part because most assays will not detect such low concentrations of AVP, but also because in very sensitive bioassay systems it has been shown that AVP is still detectable below this theoretical threshold (Baylis et al 1986). Nevertheless the osmotic threshold is a useful concept in physiological investigation and assessment of dysfunction (see Section 1.2.2.3).

It has been found that for normal healthy adults the sensitivity of AVP secretion is 0.41 ±0.05 pmol/l AVP per mOsm/kg change in pOS and the theoretical threshold of release is 285±1 mOsm/kg (Thompson et al 1986). Estimates of these values by other groups with different assays are of much the same magnitude (Robertson et al 1973; Robertson & Athar 1976; Baylis & Robertson 1980; Baylis 1983). This means that a rise or fall in pOS of 1% (2.9mOsm/kg) leads to a change in AVP of over 1pmol/l and marked changes in water excretion (see Section 1.1.2.1.1). Maximal antidiuresis occurs at AVP concentration of 4pmol/l (Baylis 1987) with pOS of 295mosm/kg. Maximal diuresis occurs with pOS <285mOsm/kg, the theoretical threshold for AVP release.

Further studies have shown that for each individual the response to a

standardised rate of infusion of hypertonic saline (and rate of change of pOS) is highly reproducible although large differences between individuals are apparent (Robertson & Athar 1976; Thompson et al 1991). It is interesting that Zerbe (1985)b showed that there was concordance in monozygotic but not dizygotic twins. These studies of normal osmoregulation have also shown that the basal pOS of any individual is highly correlated with the theoretical osmotic threshold for AVP release (Robertson 1977). It appears that a sensitivity of AVP release of <0.2 pmol/l per mOsm/kg or >0.7 pmol/l per mOsm /kg is abnormal. The normal osmotic threshold for AVP release lies between 282mOsm/kg and 289 mosm/kg (Figure 1.1.2).

The regulation of AVP by change in pOS is, however, dependent on solute type (Zerbe & Robertson 1983). Hypertonic sodium chloride and mannitol have been shown to be equipotent at stimulating AVP (Athar & Robertson 1974; Robertson et al 1976), even though during mannitol infusion plasma sodium concentration falls. Infusion of hypertonic urea, despite causing a similar rise in pOS, has little or no effect on AVP concentration (Robertson 1977). Infusion of hypertonic glucose in non-insulinopoenic subjects, leads to a rise in pOS, a fall in plasma sodium and a significant fall in AVP (Athar & Robertson 1974). It is suggested that this apparent paradox is explained by the differential ability of solutes to diffuse into cells. Thus the effect of a particular solute on AVP will be a function of its net effect on the osmotic gradient across a selectively permeable cell membrane in the brain (probably the osmoreceptor cell wall). Any solute which penetrates the barrier slowly (sodium and mannitol) will cause a net increase in extracellular osmotic pressure, dehydrating osmosensitive areas and stimulating AVP release. Solutes which diffuse rapidly across cell membranes (eg. urea) have little effect on the osmotic gradient and provide little stimulus to AVP release, providing plasma sodium remains unaltered. Glucose, despite readily diffusing into brain cells in the presence of insulin, causes a drop in plasma sodium because of an osmotic gradient across somatic cell membranes. The net effect on the osmoreceptor is therefore cellular hydration, decreased neuronal firing and inhibition of AVP secretion.

Alcohol has more complex influence on AVP release. Moderate plasma concentrations of ethanol (100mg\dl) appear to blunt the AVP response to osmotic stimulation by an elevation of the osmotic threshold by about

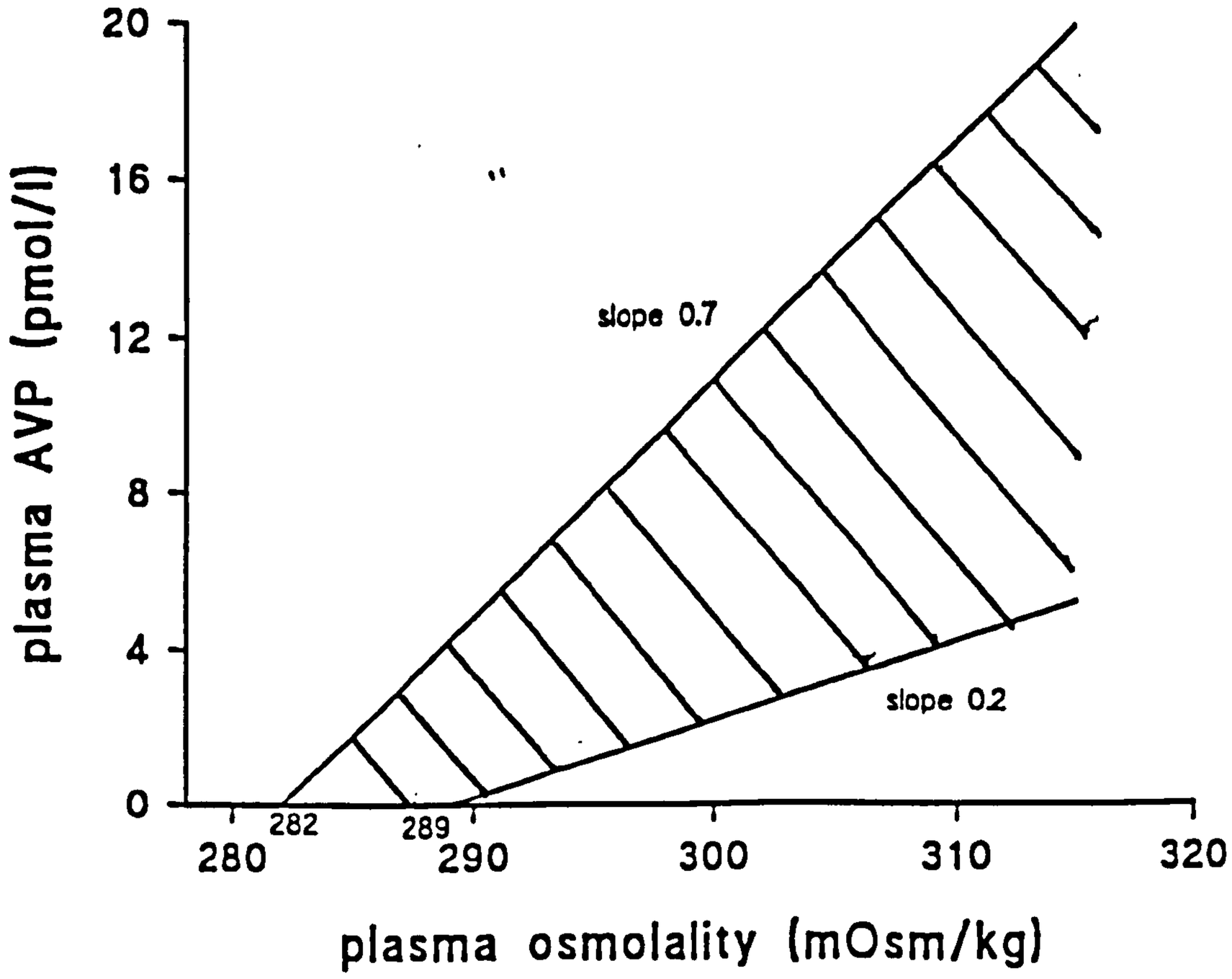


Figure 1.1.2

The 95 percentiles of the relationship between plasma AVP and plasma osmolality during the infusion of hypertonic saline (855mmol/l) in healthy man. The osmotic threshold lies between 282 and 289 mOsm/kg and the sensitivity of AVP secretion lies between 0.2 and 0.7 pmol/l per mOsm/kg.

Adapted from Thompson et al (1986).

6mOsm/kg (Eisenhofer & Johnson 1982). Thus diuresis occurs as AVP concentration falls when pOS is normal. As pOS increases antidiuresis may occur.

Studies of patients with apparent loss of osmoregulatory input to magnocellular neurons have a shown basal secretion of AVP to plasma concentrations of 1-1.5pmol/l (Robertson 1987). This suggests that both excitatory and inhibitory afferent inputs are required for normal osmoregulatory function and maintenance of osmotic homeostasis.

1.1.2.1.1 *Water metabolism*

The maintenance of normal water balance requires an ability to regulate both intake of fluids and excretion of water and solutes. In normal man maintenance of osmotic homeostasis relies on adjusting urinary excretion to daily fluid intake since the latter is regulated more by social factors than physiological requirement.

a) *Control of urine output by AVP*

AVP has a powerful facilitatory effect on the permeability of the distal renal tubule to water, allowing urinary concentration when present. An increase in plasma concentration of 1pmol/l will cause a rise in urine osmolality of 235mOsm/kg (Robertson 1977; Baylis & Robertson 1985). This `gain' in the osmoregulatory system allows pOS to be maintained within very fine limits. Above 4.5pmol/l AVP has no further effect on urinary concentration. Between the pOS of 286mOsm/kg and 296mOsm/kg, with associated AVP concentrations of 0.3-4.5 pmol/l, there is extremely efficient osmoregulation

b) *Thirst and control of fluid intake*

Thirst is a complicated desire in man, modified by voluntary control. The conscious sensation derives from higher cortical centres where stimuli from the oropharynx, upper gastrointestinal tract and afferent inputs from the hypothalamus are integrated. The hypothalamus itself integrates afferent inputs from pressor and volume sensors, the renin-angiotensin system and osmotic stimulation.

The osmotic regulation of thirst is closely associated with the osmoregulation of AVP. Osmotic sensitive cells for thirst reside in the anterior hypothalamus close to the osmoreceptors for AVP regulation. They do not appear to be the

same cells as absence of one function with preservation of the other has been documented (Halter et al 1977; Hammond et al 1986).

The osmotic threshold for severe thirst was noted to be on average 298mOsm/kg (Robertson et al 1976; Baylis & Robertson 1980;

Robertson 1984). This is just above the pOS which stimulates an AVP concentration sufficient to maximally concentrate urine (4.5pmol/l). Thus when no further body water can be preserved by concentration of urine, osmotic homeostasis depends on fluid intake and the desire to drink. The osmotic threshold for initiation of thirst was noted to be on average 293mOsm/kg (Thompson et al 1981) and has a very close relationship to the threshold for AVP secretion in each individual (Thompson et al 1986).

Osmotic stimulus to thirst is, like AVP secretion, solute specific and dependent on the net osmotic gradient across the osmoreceptor cell membranes. (Zerbe & Robertson 1983).

Thus in healthy man the osmotic mileux is maintained by alteration of the plasma AVP concentration and adjustment of free water excretion by the kidney. Thirst stimulation is probably only truly important when pOS has increased outside of the range of normal homeostatic control.

1.1.2.2 Non-osmotic regulation

1.1.2.2.1 *Baroregulation*

The stimulation of high-pressure baroreceptors in the carotid sinus and aortic arch, sensitive to changes in blood pressure, has been shown to reproducibly regulate AVP release (Schrier & Berl 1975; Schrier et al 1977). Change in activity of low-pressure baroreceptors in the left atrium, sensitive to change in blood volume without change in blood pressure, has also been shown to alter AVP release (Share et al 1974). These haemodynamic stimuli are conveyed to the brain in the glossopharyngeal and vagal nerves to the tractus solitarius (NTS) in the brain stem.

It is a matter of some controversy as to whether changes in blood volume or blood pressure are the more sensitive and more important baroregulatory system in man. A fall in mean arterial blood pressure by as little as 5% stimulates

significant AVP release whereas a 10-15% reduction of central blood volume is required for the same effect (Robertson 1977). Blood pressure changes have a curvilinear relationship to AVP release however, since a 15-20% drop is needed to achieve maximal antidiuresis. Changes in blood volume are difficult to assess and leads to further difficulty in conclusion to this debate.

Alteration in these cardiovascular parameters influences the secretory activity of magnocellular neurons through second order afferent neurons from the NTS (see Section 1.1.3.2.)

1.1.2.2.2 *Nausea*

Nausea, produced by a variety of chemical and physical stimuli, is an extremely potent stimulus to AVP release leading to plasma AVP concentrations of up to 500pmol/l. The purpose of, and mechanisms underlying this massive release of AVP are not well understood (Faull & Baylis 1991). AVP concentrations of this magnitude will have pressor effects as well as antidiuretic action. It may also have effect on gluconeogenesis and possibly direct effect on the gut.

Dopamine (DA) may play some role in the neuroendocrine mechanism since apomorphine, a DA2 agonist is a potent stimulus to both nausea and AVP. However the effect on AVP is dependent on the degree of nausea not on the drug dose (Rowe et al 1979).

Ipecachuana, a potent stimulus to nausea, has been shown not to stimulate concomitant AVP release (Nussey et al 1988) accentuating the very limited nature of the understanding of this physiological stimulus to AVP release.

1.1.2.2.3 *Hypoglycaemia*

Hypoglycaemia in man causes a 3-4 fold rise in plasma AVP (to 8pmol/l) 15 minutes after the blood glucose falls to 2.2 mmol/l (Baylis & Robertson 1981). The response is independent of concomitant changes in pOS. There is great variability in individual response and there is also poor intra individual reproducibility of response (Thompson et al 1991).

The mechanism of AVP stimulation is thought to be intra-neuronal glycopaenia since administration of 2-deoxy-D glucose produces a similar AVP response (Thompson et al 1981). The significance of hypoglycaemia-stimulated AVP response is unclear. Although AVP may effect hepatic glycogenolytic activity

(Hems et al 1976), circulatory AVP concentrations of the order of magnitude of 8pmol/l would appear to have no effect on plasma glucose (Spruce et al 1985).

1.1.2.2.4 *Oropharyngeal reflex*

Oral fluid ingestion causes an immediate, and probably total, suppression of AVP release irrespective of pOS (Thrasher et al 1981; Thompson et al 1987). This does not appear to be due to a rise in blood pressure or a volumatic inhibitory stimulus (Thompson et al 1987), nor to gastric filling. It is also independent of changes in portal blood osmolality since it occurs after ingestion of hypertonic fluid and in dogs with gastric fistulae (Thrasher et al 1981). It is explained by an inhibitory stimulus subsequent to the oropharyngeal stimulation of fluid ingestion (Seckl et al 1986; Thrasher et al 1987) and cold rather than warm fluids are more effective (Salata et al 1987).

1.1.2.2.5 *Stress*

The role that stress may have in AVP regulation is subject to much controversy. Rydin & Verney (1937) suggested that non-specific and emotional stresses lead to antidiuresis in dogs. The findings from many other studies have been difficult to interpret due to the confounding variables of pain and alteration in blood pressure (Husain et al 1979). Some authors would suggest that since AVP is involved in the stimulation of ACTH secretion and its release is provoked by the physical stressors of hypoglycaemia, hypoxia and exercise, it does have a role as a hormone of the stress response (Gibbs 1986). A number of studies in animals have suggested that AVP is not a stress hormone in the same way as cortisol and prolactin (Brennan et al 1975; Keil & Severs 1977; Lang et al 1983; Alexander et al 1987; Parrott et al 1988). A recent study has suggested that emotional stress suppresses AVP secretion in rats (Yagi & Onaka 1991). There have been few studies on man to investigate stress as a stimulus to AVP release. Raskind et al (1977) showed that hospitalised patients with anxiety disorders had normal plasma AVP concentrations.

1.1.2.2.6 *Other influences*

Acute hypoxia has been shown to stimulate baroreceptor-mediated AVP release, possibly through a general increase in sympathetic tone (Anderson et al 1978). Pain may also be associated with AVP release (Hayward + Jennings 1973; Yagi & Onaka 1991.

Visceral afferents from the kidney have also been shown to influence AVP secretion,as have afferents from hepatic cells which are apparently osmosensitive and gastric mechanoreceptors.

1.1.2.3 **The interaction of osmotic and non-osmotic regulation**

It seems likely that osmotic and non-osmotic stimuli have afferent input to the same magnocellular AVP secreting neurons since there is sophisticated interaction of stimuli. In addition electrophysiological studies *in vivo* of single neurons have shown response to both types of stimuli (Kannan & Yagi 1978).

a) *Osmotic and barometric stimuli*

Osmotic stimulation has no apparent effect on the sensitivity of baroregulated AVP. Mild hypovolaemia alters the osmoregulation of AVP with a reduction in threshold of 3-4mOsm/kg (Dunn et al 1973; Robertson & Athar 1976; Goldsmith et al 1987). There is no effect, however, on the sensitivity to change in pOS. A 15% drop in blood pressure will similarly effect the osmotic threshold but not the sensitivity (Robertson et al 1977). Hypervolaemia (eg secondary to hyperaldosteronism) may lead to a rise in osmotic threshold of AVP release (Ganguly & Robertson 1980).

These two stimuli to AVP secretion may interact through summation and integration of afferent inputs in the hypothalamus (Sawchenko & Swanson 1983), or possibly through the effects of each system on, and sensitivity to, other hormones such as atrial naturietic peptide (ANP) which may effect AVP release. For example an increase in blood volume causes a rise in ANP which may alter osmoregulated AVP (Williams et al 1988).

b) *Nausea and osmotic stimuli*

The emetic stimulus to AVP release will override both osmotic and baroregulated control of AVP secretion leading to inappropriate antidiuresis and hyponatraemia (Robertson 1977; Rowe et al 1979). There have been no studies to explore further the functional nature of the effect on osmoregulated AVP but

it seems likely that the linear relationship to pOS is lost. Both hypo-osmolality and ethanol appear to attenuate the emetic stimulus to AVP release (Rowe et al 1979).

In the clinical setting it has been shown that loss of one modality of AVP stimulation may occur without apparent effect on the others (Halter et al 1977; Puritz et al 1983; Bannister et al 1984).

1.1.2.4 Species differences in physiological regulation of AVP

It has been found that in humans, monkeys, sheep, dogs and rats pOS, blood volume and blood pressure are major influences on AVP release. The functional properties of each stimulus differ slightly between the species but the pattern of evoked AVP release and interaction of the stimuli appear to be fairly constant between the species although there are some important differences.

a) *Osmoregulation*

Each animal species has a different mean basal pOS but all are subject to control within fine limits. Each species does, however, have a different threshold and osmotic sensitivity of AVP release. In rats there is an increase in osmotic threshold and increase in sensitivity compared to humans (Dunn et al 1973). In rats, and possibly other rodent species, OXY may have a role in maintenance of osmotic homeostasis and is secreted in response to osmotic as well as other stimuli. This is not so in man (Nussey et al 1986). In rats, but not in other animals and humans, glucose, at very high concentration, exerts an osmotic stimulus and hyperglycaemia causes a rise in AVP (Charlton et al 1989; Robertson & Vokes 1991). The osmoreceptor cells in rats may have differences to those of other animal species (see Section 1.1.2.1)

b) *Non-osmotic regulation*

The relative importance of stimuli from intravascular volume receptors and of blood pressure may differ between the species and, similar to osmoregulatory function, the threshold and sensitivity to baroregulatory stimuli also varies between animal species.

In humans, monkeys, dogs and some other animals nausea is a potent stimulus

to AVP release. In rats however, the emetic reflex is incomplete (food avoidance but no vomiting) and OXY and not AVP is released by such stimuli (Verbalis et al 1986b).

In addition to these differences in regulation there are some species differences in the biological action of AVP. There are many similarities in structure between the species in particular rat and human AVP is essentially identical. The potency of antidiuretic effect is also similar between species however, synthetic antagonists to AVP do not block effects with equal potency in different animal species (Allison et al 1988). It is therefore important that care is taken in the use of animal models of AVP regulation, function and pathology to explain physiology and dysfunction in humans.

1.1.3 NEUROENDOCRINE REGULATION OF AVP RELEASE

In comparison to the understanding of physiological stimuli to AVP release, knowledge of the nature of neuroendocrine regulation is sparse (Renaud & Bourque 1991). Neurotransmitters may have direct effect on magnocellular cell bodies or on AVP secreting axons and dendrites in the hypothalamus and neuropituitary. In addition many neurotransmitters may have indirect, multisynaptic influence on AVP release through effects on locally acting pathways.

1.1.3.1 Afferent pathways to AVP magnocellular neurons

There is a large, clearly defined noradrenergic projection arising from A1 neurons in the ventro-lateral medulla (Swanson & Sawchenko 1983; Cunningham & Sawchenko 1988). Both catecholamine (Swanson & Sawchenko 1983) and peptidergic neurons (Sawchenko et al 1990) project from the nucleus tractus solitarius to the SON and PVN. Histaminergic fibres arise from the tuberomamillary nucleus and innervate both SON and PVN (Pannula et al 1984). Cholinergic neurons have been shown to innervate SON and PVN AVP secreting neurons (Armstrong 1985; Hatton & Mason 1985). Little is known of the origin of these cholinergic fibres but it seems that those to the SON may originate immediately dorsal to the nucleus (Hatton & Mason 1985). As

discussed below (Section 1.4.1) serotonin fibres project from raphé nuclei in the brain stem to the SON and PVN (Sawchenko et al 1983).

Both the SFO and OVLT innervate the SON and PVN (Miselis 1981; Swanson & Sawchenko 1983; Tribollet et al 1985). The chemical nature and functional aspects of the OVLT fibre tract are unknown. The SFO projection is possibly of angiotensin II (Jhamandas et al 1989).

Efferent fibres from the median preoptic nucleus in the anterior third ventricle also project to the SON and PVN (Swanson & Sawchenko 1983; Tribollet et al 1985; Weiss & Hatton 1990). These appear to be GABA type neurons (Nissen & Renaud 1989). Other GABA fibres from the nucleus accumbens also project to the SON, and many GABA neurons arise immediately adjacent or intrinsic to the SON (Meyer et al 1980). Both SON and PVN neurons receive a modest DA innervation. These fibres arise from cells in the periventricular zone and in the perinuclear area around the SON and PVN (Buijis et al 1984).

Several other regions of the brain are also known to have direct projection to the magnocellular nuclei, but the chemical nature of these connections is not well established. Of particular relevance to this thesis is the evidence that there is a direct afferent pathway from the limbic system to the PVN (Ferreyra et al 1983; Oldfield et al 1985). Many other direct neuronal inputs have been identified but their source is as yet uncertain (Zaborsky et al 1975; Sladek 1983; Renaud et al 1991[a+b]).

1.1.3.2 Neurotransmitters and secretion of AVP (Table 1.1.1)

The neuroendocrine regulation of AVP secretion is an extremely complex integration of multiple excitatory and inhibitory afferent stimuli which may have effects on the magnocellular neurons in the hypothalamus, on their axons or on neuronal terminals in the neurohypophysis. They may also have indirect effects through pituicytes or by alteration of blood flow. Some neurotransmitters may also act as autoregulators due to co-secretion with AVP.

The role of serotonin in AVP secretion will be discussed in Section 1.4.

1.1.3.2.1. *Noradrenaline* (NA)

Evidence initially pointed to an inhibitory role for NA on AVP release (Sladek 1983) but more recent work indicates a complex but predominantly facilitatory

Table 1.1.1 The effect of neurotransmitters on AVP secretion

Transmitter	Magnocellular neurons	Neurohypophysis	Involvement in osmoregulation	Involvement in baroregulation	Evidence for role in man
NA	↑ α_1 ↓ β	↑ β	Yes (required to be intact)	Yes stimulates release	Shy-Drager Syndrome loss NA: ↓ Baroregulated AVP
Ach	↑ nicotinic (SON) ↑ muscarinic (PVN)	↑ muscarinic	Yes (↑ nicotinic)	Yes (↑ nicotinic)	nicotine infusion ↑
DA	↑ DA_2 ↓ ?	↓ ↑	Yes (required intact)	?	L-Dopa infusion ↓ baroregulated release ? posterior pituitary
Opioids	↑ ↓	↑ ↓	↑ ↓	?	Naloxone infusion ↓ baroregulted release
AII	↑	↑ ↓	↑	?	Stimulates at pharmacological doses
GABA	↓	↓	↓	↓	?

role (Renaud et al 1991). Electrophysiological studies have shown $\alpha 1$ mediated excitatory response and β adrenoreceptor depression of magnocellular AVP neurons *in vivo* (Day et al 1985). *In vitro* (Randle et al 1986) and *in vivo* (Willoughby et al 1987) studies have confirmed these effects of $\alpha 1$ and β agonists applied to the SON on AVP release from the neurohypophysis.

Studies of the neurohypophysis *in vitro* have suggested that norepinephrine stimulates AVP release, possibly through β-adrenoreceptors (Racke et al 1982; Bridges et al 1976).

NA appears to have little effect on osmotically stimulated AVP (Armstrong et al 1982) although destruction of NA neurons by selective toxin reduces the AVP response to osmotic stimulus (Miller et al 1979).

Norepinephrine neurons from the A1 cells in the brainstem are excitatory to AVP release. Selective toxin lesion of these neurons reduces the AVP response to hypovolaemic stimulus (Miller et al 1979) and lesion of the dorsal NA pathway inhibits the AVP rise subsequent to haemorrhage (Lightman et al 1984). Stimuli from baroreceptors have been shown to excite these neurons (McAllen & Blessing 1987; Leng et al 1991).

Studies in man have been limited because of confounding effects of NA on cardiovascular status and the fact that circulating monoamines do not cross the blood brain barrier (Carter & Lightman 1985). Patients with Shy-Drager syndrome, with loss of central ascending catecholamine pathways, have been shown to have normal osmoregulated AVP (Bannister et al 1984) but blunted baroregulatory responses (Puritz et al 1983).

1.1.3.2.2. *Acetylcholine* (Ach)

It is well recognised that Ach stimulates AVP release both in the hypothalmo-neurohypophysial complex (HNC) *in vitro* model (Sladek & Knigge 1977, Gregg 1985; Gregg 1986) and *in vivo* (Iitake et al 1986). Some studies have suggested that nicotinic receptors mediated the stimulus (Sladek & Joynt 1979b) but more recent studies have shown a role for muscarinc receptors (Shoji et al 1989). It is possible that both receptor subtypes are facilitatory to AVP release and that there is a complex interaction of stimuli. It may also be that nicotinic receptors predominate on the SON neurons and muscarinic receptors on magnocellular neurons in the PVN. This latter is supported to some extent by

autoradiographic receptor studies (Michels et al 1986).

Ach has also been demonstrated to stimulate AVP release from the isolated neurohypophysis. The response is mediated through muscarinic cholinergic receptors (Gregg 1985).

The release of osmotically stimulated AVP in the HNC model is blocked by the nicotinic receptor antagonist hexamethonium (Sladek & Joynt 1979a). Atropine, a muscarinic receptor antagonist, does not have this effect. *In vivo* studies in the rat have not confirmed that Ach nicotinic receptors are important in osmo-regulated AVP release (Bisset et al 1988).

Hexamethonium has been shown to inhibit the AVP release in response to hypotension in rats (Bisset & Chowdry 1984).

Studies in man have confirmed that nicotine acutely stimulates AVP release (Burn et al 1945; Lightman et al 1982). However, chronic administration (ie smoking) does not have similar effects (Lightman et al 1982; Rowe et al 1980).

1.1.3.2.3. *Dopamine* (DA)

Electrophysiological studies of the SON have shown that DA modifies firing of magnocellular neurons both *in vitro* (Sakai et al 1974) and vivo. DA2 receptor subtypes appear to mediate this response (Yang et al 1989). *In vitro* HNC models have shown that DA and DA agonists cause dose-related AVP release (Bridges et al 1976). *In vivo* studies have been contradictory with both inhibitory and excitatory effects shown. This may be in part because of confounding effects of DA on other monoamine systems, but species differences and differing effects in the hypothalamus and neurohypophysis may also play a part (Carter & Lightman 1985).

Dopaminergic neurons from the arcuate nucleus of the hypothalamus have been shown to innervate AVP-secreting axons and pituicyte process in the neurohypophysis (Baumgarten et al 1972). Studies of the effect of DA on AVP release from the pituitary have been contradictory but generally support an inhibitory role (Carter & Lightman 1985). It has also been suggested that DA1 receptors function as autoreceptors facilitatory to AVP release and DA2 receptors inhibitory to release (Racke et al 1982).

DA antagonists prevented the AVP response to plasma hyperosmolality in rats suggesting that DA is important in osmotically stimulated AVP release (Moos &

Richard 1982).

Lightman & Forsling (1980b) suggested that L-DOPA suppressed basal AVP release in man and inhibited baroregulated AVP secretion probably by effect on the median eminence or neurohypophysis. Nomura et al (1984) and Norbiato et al (1986) have shown that metaclopramide causes a rise in plasma AVP concentration independent of pOS or blood pressure. However, Coiro et al (1989) have suggested that this is mediated through muscarinic Ach receptors and not by DA antagonist effects.

1.1.3.2.4. *Opioids*

The role that opioid peptides have in AVP release is relatively well explored; the conclusions are, however, contradictory. Initial studies indicated a stimulatory role but more recent work have revealed inhibitory actions (Forsling 1985; Sladek 1983). It is clear that opioids have effects on AVP release from the neurohypophysis (Arnauld et al 1983; Carter & Lightman 1985) and since certain opioids are located within AVP secreting neurons (Watson et al 1982; Whitnall et al 1983), it has been suggested that they may act as autoregulators (Brownstein et al 1980). Supportive evidence for this role is lacking (Carter & Lightman 1985). In the Brattleboro rat, where there is a deficiency of AVP, opioid concentrations in the neuropituitary are lower than in normal rats, suggesting a functional interaction of opioids and AVP (Weber et al 1983).

The evidence that opioids have a functional effect in the hypothalamus has been more difficult to establish (Carter & Lightman 1985) since both inhibition and potentiation of osmotically stimulated AVP release has been found (Forsling 1985).

Observations of the effects of opioids on AVP release in man are no less confusing (Forsling 1985). Naloxone has been shown to inhibit baroregulated AVP (Lightman & Forsling 1980a) but has no significant effect on osmoregulated AVP or basal AVP secretion (Lightman et al 1980). Other studies however, have not confirmed this (Forsling 1985).

1.1.3.2.5. *Angiotensin II* (AII)

AII is a potent stimulus for AVP release (Bonjour & Malin 1970) The major mode of its action appears to be through its effects on the SFO and OVLT

(Simpson et al 1979). Direct application of AII to the SON does provoke AVP release and excites magnocellular neurons (Simmonet et al 1979). Sladek & Joynt (1980) have shown that AII stimulates AVP release from HNC models but a functional direct role of AII in AVP release remains controversial since systemic AII does not cross the blood-brain barrier and no neuronal pathways have been characterised in the hypothalamus (Chowdry & Lightman 1989). It has been argued that AII mediates AVP release by direct effects on receptors on AVP axon terminals in the neurohypophysis (Sladek 1983) but the results are contradictory.

Both infusion of renin and administration of AII have been shown to potentiate osmotically stimulated AVP release (Shimizu et al 1973; Claybaugh 1976; Andersson & Westbyte 1979; Simonnet et al 1979; Akaishi et al 1980). Similar results have been found in *in vitro* models where osmotically stimulated AVP release was inhibited by the AII antagonist saralasin (Sladek & Joynt 1980). It does not, however, inhibit suppression by plasma hypo-osmolality (Henolch et al 1979).

Infusion of AII in dogs caused an increase in AVP despite hypertension (leading to AVP suppression) (Ramsay et al 1978)

AII infusions in man have been shown to stimulate AVP release (Uhlich et al 1975; Padfield & Morton 1977) but only at pharmacological concentrations.

1.1.3.2.6. *Gamma amino butyric acid* (GABA)

It has been show that GABA has inhibitory effects on AVP neuronal firing (Bioulac et al 1978; Sakai et al 1974;). It has inconsistently been shown to decrease AVP release from HNC models (Sladek & Armstrong 1983) but antagonists repeatedly stimulate AVP release in this model (Sladek & Armstrong 1987). GABA also has been shown to have inhibitory effects on AVP release from the neurohypophysis, possibly secondary to effect on pituicytes (Mathison & Dreifuss 1981).

GABA appears to have inhibitory effects on osmoregulated AVP release (Iovino et al 1983; Haywood & Brennan 1985).

GABA may be involved in baroregulation of AVP by inhibitory influences on the A1 cells and other neurons mediating the cardiovascular stimuli since intra-

cerebro-ventricular injection of GABA suppressed hypovolaemic induced AVP secretion *in vivo* (Knepel et al 1980).

1.1.3.2.7. *Other putative regulators*

Histamine and substance P have both been demonstrated to increase AVP release (Sladek 1983). Aspartate and glutamate are also excitatory to AVP secreting neurons (Carter & Lightman 1985).

Sex steroids modulate AVP release but the mechanism is not well understood. Prostaglandins have a modulatory effect on AVP release *in vivo* and *in vitro* (Carter & Lightman 1985). Brain naturietic peptide may have a direct inhibitory effect on magnocellular neurons (Yamamoto et al 1991) and ANP may inhibit AVP secretion, but this is contentious (Baylis & Burrell 1991).

The release of AVP is regulated by the integrated sum of inhibitory and excitatory afferent inputs and autoregulatory processes. Dynorphin or other transmitters, co-secreted with AVP from magnocellular neurons, may mediate local negative feedback and AVP itself may have local inhibitory effects (Abe et al 1983; Carter & Lightman 1985), or inhibitory effects through alteration of CSF concentration (Bhargava et al 1977). AVP has also been shown to have a facilitatory effect on its own release (Ramirez et al 1990). These autoregulatory processes are still relatively unexplored and not well understood (Renaud & Bourque 1991; Renaud et al 1990).

Studies using HNC models have allowed some exploration of the interaction and physiological roles of these neurotransmitters although knowledge of those transmitters that have functional importance is still very much in its infancy. That the integration of the system is so complex ensures that osmotic homeostasis is maintained within very fine limits even if there is an imbalance in known stimulants eg. cigarette smoking, ingestion of neuroactive drugs.

1.2 VASOPRESSIN AND ABNORMAL OSMOREGULATION

Abnormalities of AVP secretion may result in a plasma insufficiency or an "excessive" plasma concentration. The former, diabetes insipidus, results in hypotonic polyuria and osmotic homeostasis relies on ingestion of large quantities of fluid. Excessive or osmotically inappropriate secretion of AVP results in hyponatraemia and is the subject of this section.

1.2.1. HYPONATRAEMIA

Hyponatraemia is a common medical problem and if severe (< 120mmol/l) has a substantial mortality, independent of any underlying disease (Arieff et al 1976). Even if moderate and asymptomatic there is an increased risk of death (Anderson et al 1985). It is plasma hypo-osmolality that is of prime importance since hyponatraemia with normal pOS, which occurs where there is an increase in other osmotically active plasma constituents (eg. glucose), is not clinically as dangerous.

Hyponatraemia with hypo-osmolality reflects an increase in plasma water relative to sodium. It can be due to solute loss or water excess but is usually due to a combination of both.

1.2.1.1. Solute loss

Extracellular fluid (ECF) losses such as diarrhoea, vomiting and inflammatory exudates do not lead to hyponatraemia since there is loss of isotonic solute. Replacement of such losses is most often with hypotonic fluids such as water and this leads to hyponatraemia as fluid, but not solute, is replaced. Such patients are usually hypovolaemic but some may have replaced fluid sufficiently to be normovolaemic. In these patients urinary sodium is conserved and the urine osmolality is low.

Renal sodium loss due to tubular defects, hypocortisolaemia, cerebral salt wasting or most commonly diuretic therapy, leads similarly to hypovolaemic hyponatraemia but with a relatively high urinary osmolality and sodium excretion.

1.2.1.2 Water excess

The kidneys have the capacity to excrete up to 30l of water a day (Robertson & Berl 1986) and under normal circumstances excess fluid intake is seldom a cause of dilutional hyponatraemia (see Section 1.6). When the renal excretory capacity is impaired, however, fluid intake may be in excess of functional body needs and insensible loss and hyponatraemia and hypo-osmolality ensue.

A reduction in the volume of renal tubular fluid due to nephron loss or poor glomerular perfusion, leads to increased reabsorption of both sodium and water in the proximal tubule (enhanced by increased aldosterone concentration in some disease states), and decreased distal nephron load with consequent decreased free water excretion.

Another important mechanism of water retention is the impairment of the distal nephron to dilute urine due to increased AVP concentration. Hypocortisolaemia also inhibits free water excretion in the distal tubule independently of AVP concentration (Linas et al 1980).

In disorders where solute retention in the proximal tubule is the primary abnormality, there is a tendency to hypervolaemia and oedema, whereas patients with distal tubular impairment of free water clearance are usually normovolaemic.

The volume status of patients with hypovolaemia is, inevitably, not asstraight forward as described above since once established the primary dysfunction often leads to a mixture of compensatory mechanisms. A primary solute loss with hypovolaemia leads to poor renal perfusion with decreased free water clearance and exaggeration of the solute depletion hyponatraemia. Excess plasma AVP will expand ECF with consequent renal sodium loss and a further lowering of plasma sodium. Diseases such as cardiac failure, with low effective intravascular volume will have primary solute retention and decreased free water clearance but will also have elevated AVP because of hypovolaemic, non-osmotic stimulation, leading to further inhibition of distal tubular free water excretion (Bichet & Schrier 1985; Bichet 1989).

1.2.2 THE SYNDROME OF INAPPROPRIATE ANTIDIURESIS

Schwartz et al (1957) described the syndrome of inappropriate antidiuresis (SIAD) in patients who despite plasma hypo-osmolality, produced hypertonic urine with inappropriate natriuresis. They suggested that this syndrome resulted from endogenous release of AVP which was inappropriate to the pOS, since it was identical to the features observed in people given neuropituitary extract (Leaf et al 1953). The group further defined the criteria for the syndrome (Bartter & Schwartz 1967).

Decreased plasma osmolality (<275mOsm/kg)

Inappropriate urine osmolality (>100mOsm/kg)

Absence of hypo or hypervolaemia

Inappropriate urine sodium excretion

Absence of other possible causes (eg. hypocortisolaemia, renal failure, hypothyroidism, diuretic therapy).

Measurement of basal AVP is not part of the diagnosis since, as discussed above, AVP may be elevated or inappropriately high in many causes of hyponatraemia (Verbalis 1989). As discussed below the absence of AVP in the plasma does not refute the diagnosis.

1.2.2.1 Aetiology

There are four major groups in the aetiology of SIAD; tumour related, CNS disorders, pulmonary disease related and drug related.

Tumour related SIAD in many cases is due to the production of ectopic AVP or AVP-like peptide by malignant cells. As discussed below it may also arise through other mechanisms.

A great variety of localised and diffuse, apparently unrelated CNS disorders are associated with SIAD. It seems likely the these diseases cause disruption to the inhibitory afferent inputs to magnocellular neurons with consequent dysregulation of AVP secretion (Verbalis 1989).

SIAD associated with pulmonary disease in many cases is probably due to the hypoxia and/or hypercapnia of respiratory failure. However some specific lung

pathologies have be found to secrete AVP-like factors or stimulate AVP release from the neurohypophysis (Zerbe 1985). The occurrence of the syndrome in milder pathologies such as viral and atypical pneumonias is poorly understood.

1.2.2.2 Drug induced SIAD

There are a great many drugs which have been associated with hyponatraemia (Moses 1985). Some have been fully investigated and the pathophysiological mechanisms are understood. Others, such as the tricyclic and other antidepressants have a less clear mode of action (Section 1.6). The data is discussed in detail by Moses (1985).

Some drugs directly stimulate AVP release and have similarities to neurotransmitter systems known to modulate AVP release. Nicotine and opiate drugs would be examples. Some antineoplastic agents (vincristine and cyclophosphamide) have been shown to cause antiduresis due to elevated plasma AVP levels. This may be due to a direct neurotoxic effect on magnocellular neurons (Rufener et al 1972; Moses & Miller 1974). Other drugs have been shown to potentiate the action of AVP on the distal renal tubule (eg. prostaglandin inhibitors), or to have a direct effect on the kidney (eg dDAVP, oxytocin).

Many drugs have a combination of effects. Carbamazepine has been shown to increase AVP in patients with CDI (Kimura et al 1974). The mechanism of this is not known. In normal people carbamazepine appears to cause a reset of the osmostat and to enhance the renal effects of AVP (Stephens et al 1978). Chlorpropramide also causes AVP release and potentiates renal tubular effects (Moses et al 1973b). Tolbutamide, but not other sulfonylureas has a similar but less potent effect. The central stimulatory mechanism of these drugs is not understood but its action on the tubule is clearer (Lozada et al 1972). Clofibrate also stimulates AVP release through an unknown mechanism (Moses et al 1973a).

1.2.2.3 Pathophysiology

Hypertonic saline infusion studies in patients with clinically diagnosed SIAD have shown that there are four patterns of AVP secretion (Figure 1.2.1).

a) *Random hypersecretion* (A)

In this type of SIAD secretion, AVP has no apparent relationship to pOS. Although this is often seen in cases of ectopic AVP production, only about 30% of those patients with this pattern will have malignant disease (Zerbe et al 1980).

b) *Reset osmostat* (B)

In this, the commonest form of the syndrome (Zerbe et al 1980), AVP has a linear relationship to change in pOS, and is secreted with normal sensitivity. The osmotic threshold for initiation of the secretion is, however, lowered. Thus for any given pOS AVP concentration will be higher than normal.

Patients who have hypovolaemic hyponatraemia (by definition not SIAD), may have a reset osmostat due to non-osmotic AVP stimulation (Robertson & Athar 1976; Robertson et al 1982; Bichet & Schrier 1985; Uretsky et al 1985, Bichet 1989). A reset of the osmostat also occurs in nomovolaemic patients. It has been proposed that chronic hypo-osmolality leads to a readjustment of the osmoreceptor cells, although studies in animals have not found this (Verbalis et al 1986a). Resetting may occur due to an interruption of inhibitory pathways to the osmoreceptor cells or magnocellular neurons.

Patients with this form of SIAD differ from the other three groups since they can dilute urine when plasma becomes sufficiently hypo-osmolar (Defronzo et al 1976)

c) *Hypersecretion/normoresponsive (leaky hypophysis)* (C)

In this group of patients AVP is secreted at a high level below the normal osmotic threshold for release, but above this threshold there is a normal response to change in pOS. This could be due to an ectopic source secreting a `background 'concentration, however it is seldom seen in patients with malignant disease. It seems more likely that this pattern of secretion is due to a selective loss of inhibitory neurons.

d) *Undetectable AVP* (D)

Clinically diagnosed SIAD occasionally occurs in the apparent absence of

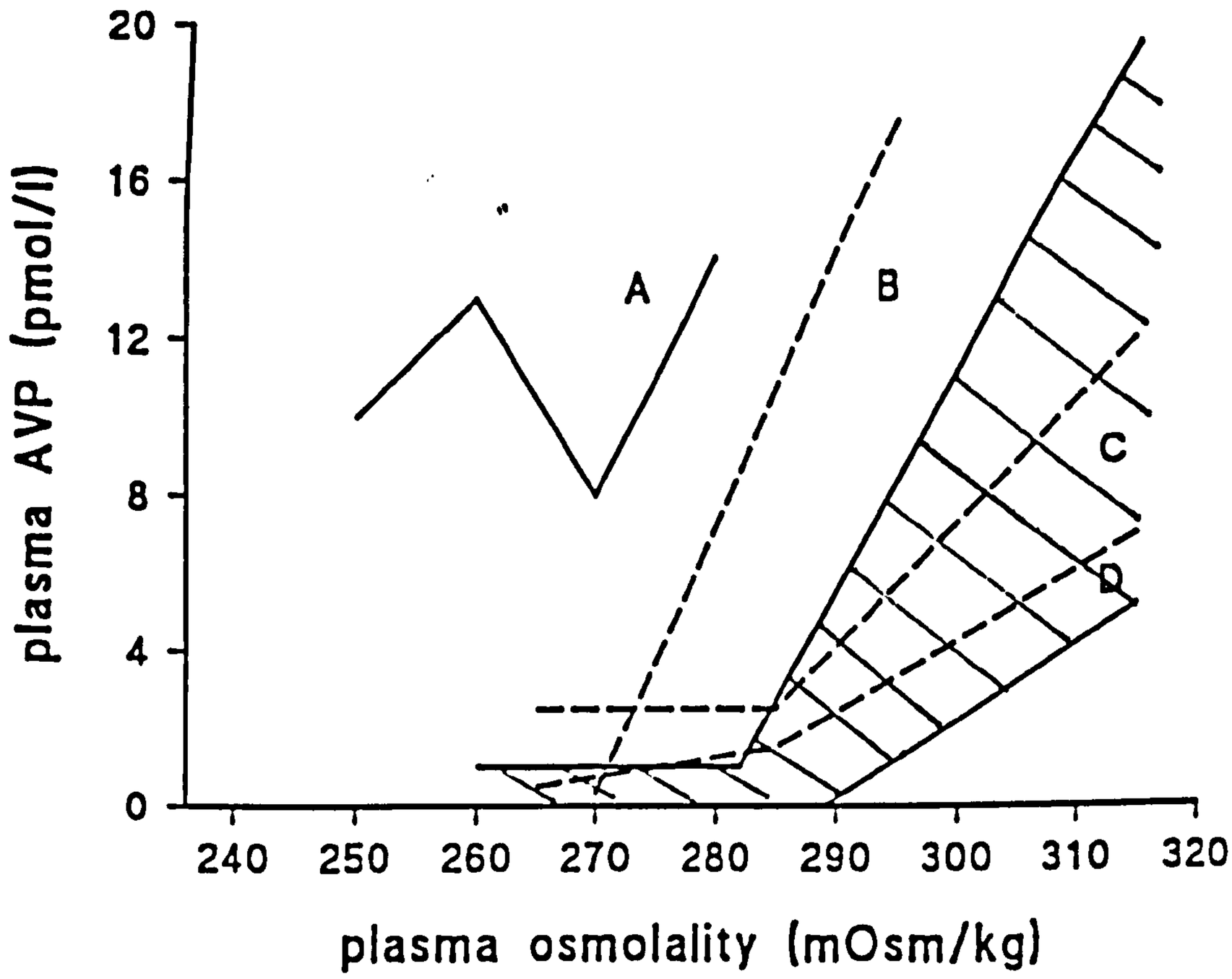


Figure 1.2.1

A schematic representation of the patterns of AVP secretion during infusion of hypertonic saline observed in patients with the syndrome of inappropriate AVP secretion (SIAD). A; Random hyper secretion. B: Reset of the osmostat. C: Leaky hypophysis. D: Undetectable. The shaded area represents the 95 percentiles of the normal response to the infusion (see Figure 1.1.2) Adapted from Robertson et al (1976).

plasma AVP (Zerbe et al 1980). This may be due to altered immunoreactivity of the peptide, the presence of another antidiuretic factor or increased renal tubular sensitivity to AVP. This pattern of the syndrome is poorly understood.

No good correlation has been found between the aetiology of SIAD and the pattern of AVP secretion. Indeed bronchogenic carcinoma has been found to be associated with any of the four types in different patients (Robertson 1978; Zerbe 1985). In addition more than one type of defect may coexist in some patients (Baylis & Robertson 1979; Zerbe 1985).

1.3 THE SEROTONIN NEUROTRANSMITTER SYSTEM

Serotonin is so named because of its vasoconstrictive properties. It was recognised in the late 19th century that there was a factor in serum which had tonic properties and in the 1940's its structure was identified as 5-hydroxytryptamine (5HT) (Rapport 1949). It was found to be chemically identical to `enteramine', an amino acid found in the enterochromaffin cells of intestinal mucosa (Erpsamer & Asero 1952). The synthetic production of 5HT allowed an explosion of research into the physiological function of the compound and localisation studies soon showed the presence of high concentrations in the brain, with regional variations, highly suggestive of a functional role in the central nervous system (Amin et al 1954). This section discusses the 5HT nervous system with particular reference to 5HT receptors and function and regulation of the system.

1.3.1 ANATOMY

The 5HT nervous system is phylogenetically ancient and has shown remarkable stability through evolution. The anatomical detail and classification was first outlined using new histochemical techniques by Dahlstrom & Fuxe (1964; Fuxe 1965). Since then there have been many reviews of the subject in various species of animal, primate and more latterly in man (Bjorklund & Hokfelt 1984; Azmitia & Gannon 1986; Azmitia 1987; Tork 1990). The cell bodies of the system are clustered into nuclei in the brainstem and form a rostral group, with ascending projections, and a caudal group with descending projections. Initially the nuclei were classified B1-B9 (Dahlstrom & Fuxe 1964) but it is more usual now to name them as specific raphé nuclei (Table 1.3.1 Figure 1.3.1). The rostral nuclei comprise 4 groups of serotonergic cells of which the dorsal raphé nucleus (DRN) is the most prominent.

The ascending projections from the rostral nuclei have an extensive and complex pattern of innervation to almost all cerebral areas, although the frontal lobe appears to have a low density of fibres. This comprises the most expansive neuronal network yet described. High fibre density is found in the cerebral cortex, basal ganglia, limbic system structures and diencephalon (Steinbusch

Table 1.3.1 The brainstem nuclei with 5HT-containing cell bodies

Rostral nuclei	**Classification by Dahlstrom & Fuxe (1964)**
Dorsal raphé	(B6&7)
Median raphé	(B5&8)
Caudal linear	(B8)
Supralemniscal region	(B9)
Caudal nuclei	
Raphé obscuris	(B1,2,4)
Raphé magnus	(B3)
Caudal ventrolateral medulla	(B1)
Rostral ventrolaterial medulla	(B3)
Raphé pallidus	(B1)

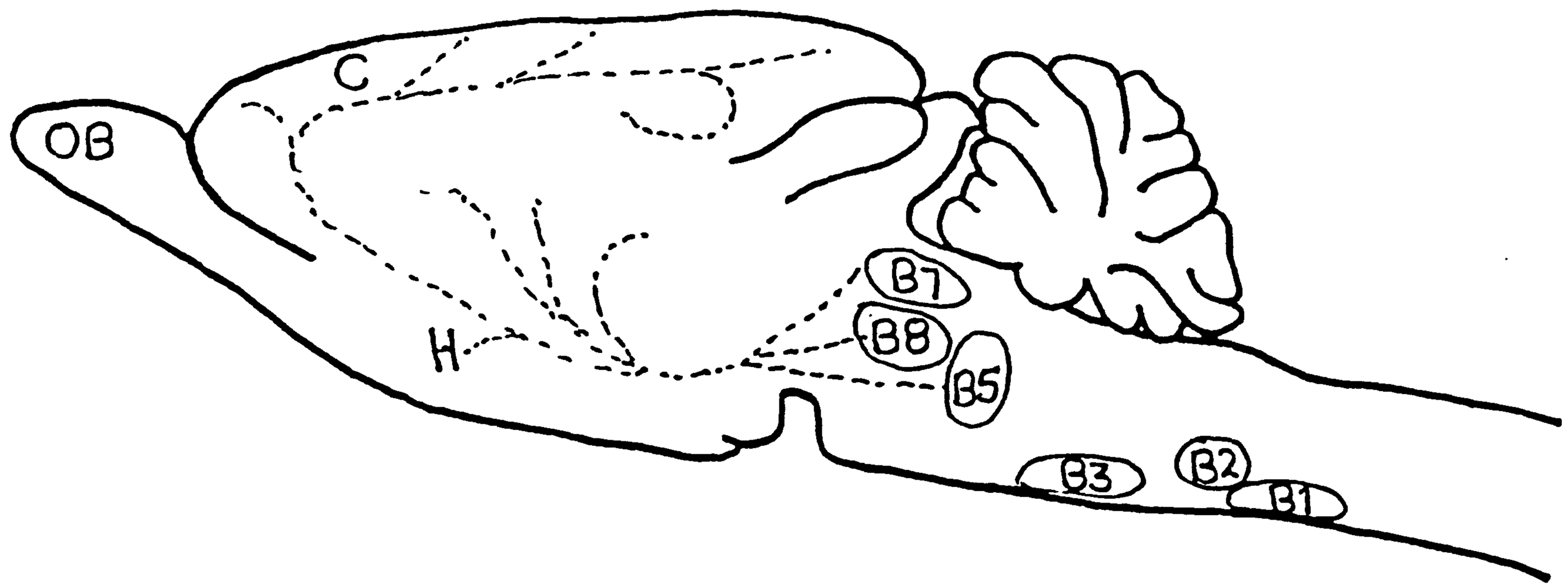


Figure 1.3.1

The raphé nuclei and serotonergic system of the rat brain in saggital section.

B: Raphé nucleus; B7: Dorsal raphé nucleus (DRN); B8: Median raphé nucleus (MRN);

OB: Olfactory bulb; C: Cerebral cortex; H: Hypothalamus;

1981; Azmitia & Gannon 1986). These fibres travel in the median forebrain bundle and in the dorsal raphé cortical tract and ascend through the hypothalamus to the cortex and subcortical areas. In rats most of the nerve fibres are unmyelinated but in higher animals and primates an increasing percentage of fibres are myelinated (Azmitia & Gannon 1983). It has also been found that in some mammals, primates and man there are at least three types of axon present in the forebrain. The most common, thin with fusiform varicosities, extend profusely over large areas of the forebrain and arise from the DRN. The second type of fibre, which appears to arise from the median raphé nuclei (MRN), have very thin axons with weak immunocytochemical staining for 5HT but have large, round or oval varicosities which stain very strongly for 5HT. These beaded axons innervate highly restricted, discrete cortical layers, possibly having selective contact with GABA cells. The third type of fibre, which is relatively thick and non-varicose, is found only in small numbers and usually in or close to the major 5HT tracts (Kosofsky & Molliver 1987). Not only are these fibres dissimilar in anatomical respects but they have differing susceptibility to neurotoxins (Mamounas et al 1991).

Although the 5HT nervous system has been well preserved through the animal species there have been some subtle and potentially important changes. In lower invertebrates there is one neuronal cell body with extensive branching and connections to motor and sensory ganglia. Any stimulus to the cell body will cause widespread ramifications. The system has become more organised through the animal hierarchy and in rats there are tight clusters of many cell bodies (the raphé nuclei) each with interconnecting projections, functioning as one unit but with more subtle interactions and regulatory features. In higher animals, and man, the system has developed further. The cellular organisation is more distinct, the projecting pathways are more varied and some are myelinated, and the innervated tissues have very intricate and subtly varied architecture (Soghomonian et al 1988). It seems that there is cytoarchitectural evidence for at least two 5HT subsystems which may act independently, at different speeds, and each may have highly specific and discrete roles (Tork 1990; Mamounas et al 1991). Although the lower mammals, such as the rat,

may act as a relatively good model for the 5HT system in man, there are important differences in the anatomy which mean that results must be interpreted with caution.

1.3.2 SYNTHESIS AND METABOLISM

5HT is a monoamine formed, intraneuronally, from the neutral amino acid tryptophan, of which the major source is dietary protein. The majority (85%) of plasma tryptophan is bound to albumin and only free tryptophan is available for transport across the blood brain barrier. The uptake system for this transportation is not specific to tryptophan and the ratio to other large, neutral amino acids, and the unbound percentage of tryptophan, determines the concentration of tryptophan entering the brain.

The peripheral availability of tryptophan is controlled in part by metabolism in the liver to kynurenine, and other metabolic products, by the enzyme tryptophan pyrrolase. Most dietary tryptophan enters this pathway and a minority is actually converted to plasma 5HT. Influences on this metabolic pathway (eg corticosteroids) may alter central nervous concentrations of tryptophan, 5HT and 5HIAA by altering the ratio of tryptophan to other neutral amino acids or by altering the protein binding of tryptophan.

Following uptake into the brain tryptophan is converted to 5HT by a two-step process (Figure 1.3.2). Firstly hydroxylation by tryptophan-5-hydroxylase (TH) to 5-hydroxytryptophan (5HTP) and then by decarboxylation to 5HT. High concentrations of TH have been found in the brain stem, hypothalamus, caudate nucleus , amygdala, pineal and pituitary glands. The enzyme is localised at 5HT specific nerve endings, has low activity and is not normally saturated. TH appears to be specific for tryptophan and is the rate-limiting step in its synthesis. 5-hydroxytryptophan-decarboxylase (TD) is also located in nerve terminals. It has 80-100 times higher activity than TH but has been shown to be non-substrate specific as both TD and the decarboxylase enzyme present in dopamine nerve terminals can metabolise both 5-HTP and L-DOPA. Thus infusion of 5-HTP increases 5HT in both 5HT and dopamine neurons, likewise infusion of L-DOPA leads to an increase of dopamine in both neuronal types.

Following release into the synaptic cleft, 5HT, like other monoamines, is

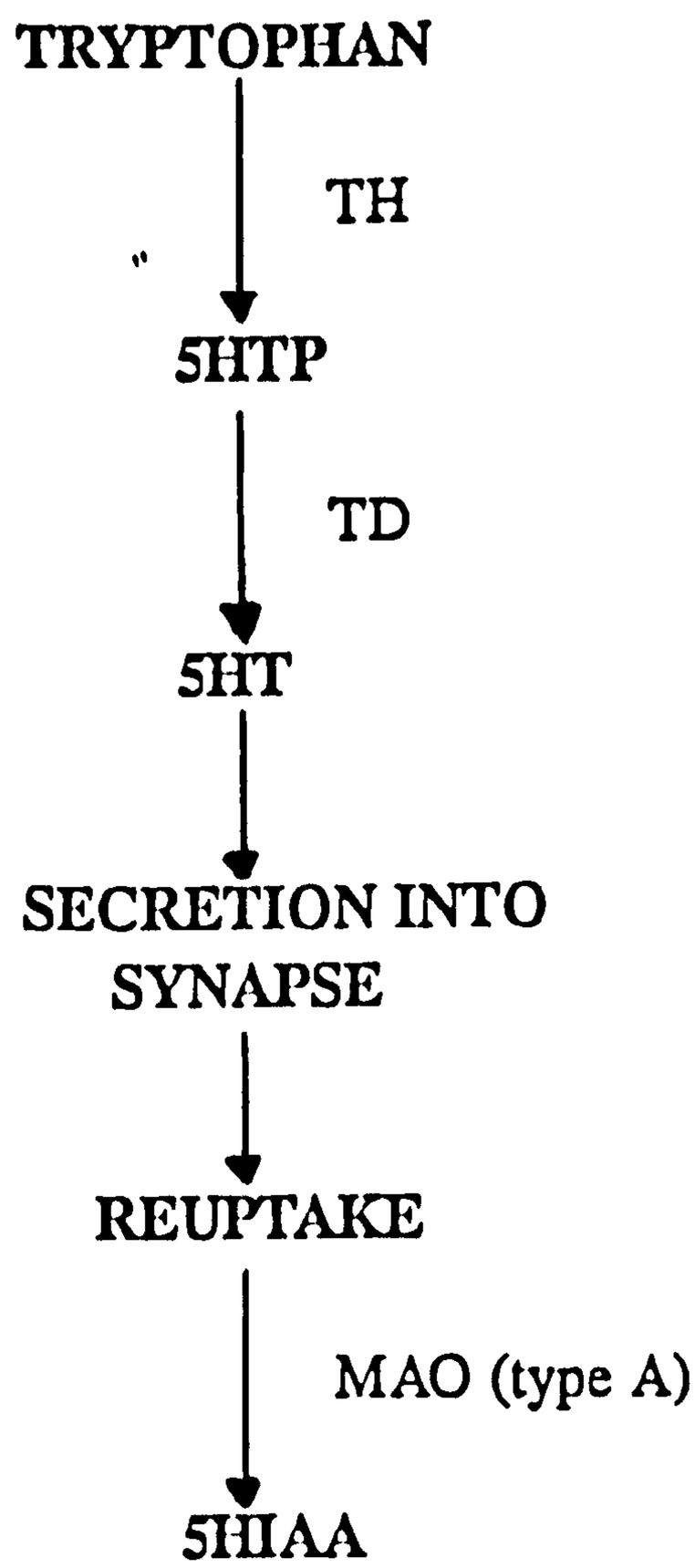


Figure 1.3.2

The synthesis and metabolism of serotonin (5-hydroxytryptamine) (5HT).

TH: Tryptophan-5-hydroxylase; 5HTP: 5-hydroxytryptophan;

TD; 5-hydroxytryptophan decarboxylase; 5HIAA: 5-hydroxyindole acetic acid;

MAO; monoamine oxidase.

inactivated via a high affinity reuptake process into the nerve ending (Iversen 1975). 5HT is then metabolised by a two step-process to its major metabolite 5-hydroxyindole acetic acid (5HIAA), firstly through oxidation by monoamine oxidase (MAO) and secondly rapid dehydrogenation takes place by aldehyde dehydrogenase (Figure 1.3.2). MAO is located on or in close proximity to mitochondria. There is some, but limited substrate specificity. MAO type A is able to deaminate 5HT and noradrenaline whilst MAO type B is more specific for dopamine. 5HT neurons probably contain both enzyme subtypes but under normal circumstances metabolism occurs predominantly by MAO type A. Astro glial cells also deactivate 5HT by active reuptake (Kimelberg 1988) but the importance of this in physiological and pathological function is as yet unclear. Increasing the concentration of tryptophan in the brain by peripheral infusion has been found to increase the rate of 5HT synthesis (Grahame-Smith 1971). It has been proposed however, that 5HT is synthesised in excess of functional needs, and is metabolised intraneuronally without release (Green & Grahame-Smith 1975), since despite marked increases in 5HT synthesis no behavioural changes are seen in rats unless MAO is also inhibited (Marsden et al 1979; Grahame-Smith 1971). Thus studies on changes in synthesis and metabolism do not necessarily reflect or have clinical implication for alteration in function of the 5HT nervous system.

Table 1.3.2 shows the drugs which have been developed on the basis of knowledge of 5HT synthesis and metabolism. Many of these are used as tools in research for further understanding of function and pathology of the 5HT nervous system.

1.3.3 SEROTONIN RECEPTORS

Since the first pharmacological characterisation of 5HT receptors (Gaddum & Picarelli 1957) considerable progress has been made in knowledge of subtypes, physiology and anatomical localisation as well as the beginnings of understanding of regulatory processes and changes in pathological states and therapeutic interventions. Much of this knowledge stems from the development of ligand binding techniques both *in vitro* and increasingly in and ex vivo

Table 1.3.2 Pharmacology of 5HT neurotransmission related to synthesis and degradation processes.

1. Precursors
   - Tryptophan
   - 5-hydroxytryptophan (5HTP)

2. 5HT depleting drugs
   - Parachlorophenylalanine (pCPA)
     - synthesis inhibitor - blocks TH
   - 5,7-Dihydroxytryptamine (5,7 DHT)
     - neurotoxin
   - Fenfluramine
     - releases 5HT with store depletion
     - neurotoxic effect
     - inhibits TH
   - Reserpine
     - releases 5HT with store depletion
   - Tetrabenazine
     - releases 5HT with store depletion
   - p-Chloramphetamine (pCA)
     - inhibits TH
     - releases 5HT with store depletion

3. MAO type A inhibitors
   - Clorgyline
     - irreversible
   - Hormaline
   - FLA336
     - selective to 5HT neurons

4. Uptake inhibitors
   - Selective serotonin reuptake inhibitors (SSRI)
   - Tricyclic antidepressants

(Snyder 1983; Hamon 1984).

The absolute identification of a receptor requires it to have a unique amino acid sequence and cloned sequences must mimic the actions of natural receptors (Peroutka 1988). This is available for some 5HT receptors but many putative ones have not been structurally identified as yet (Hartig 1989; Huang & Julius 1991). A high degree of probability that a receptor is present depends on the identification of functional correlates such as 2nd messenger linkage, delineation of physiologic effects at neuronal membranes and behavioural effects *in vivo*. The suggestion that a receptor exists rests on pharmacological identification of a binding site with high affinity (nM) for a ligand which is saturable, reversible and stereoselective; that there is a specific pharmacological profile of binding to agonist and antagonist drugs; that there is a distinct regional distribution of the site concurrent with innervation and species variations; that there is equipotency of ligand binding affinity and pharmacological and physiological effects.

Such criteria have been used to describe a series of 3, or possibly 4, 5HT receptor families for which the basis of classification depends on pharmacological characteristics (Leysen 1984; Peroutka 1991). Each family also shares similarities in molecular biology and physiological properties. In 1986 a committee presented suggestions of a consensual classification and nomenclature based on pharmacological and functional properties (Bradley et al 1986). The speed of development in the field has meant that this has had to be updated (SEROTONIN CONFERENCE 1991, to be published) particularly because of new developments in molecular biology (Hartig 1989; Peroutka 1991) which have lead to some differences in classification (Hartig 1989). Frazer et al (1990) have proposed a scheme that would classify receptors of all neurotransmitter and neuropeptide systems and would emphasise similarities in neurotransmitter type and second messenger systems . This has not been adopted as yet.

1.3.3.1 Radioligand binding studies (Table 1.3.3)

The first successful ligand binding analysis of 5HT receptors was reported by Bennett & Aghajanian (1974) who showed [^{3}H]LSD binding to be saturable, reversible and stereoselective with a high affinity for the membrane prepared from neuronal material. Brain areas with a high density of 5HT neuron

Table 1.3.3 The potency and selectivity of drugs (and the radioligands commonly used for labelling) 5HT receptor subtypes

DRUG POTENCY	$5HT_{1A}$	$5HT_{1B}$	$5HT_{1D}$	$5HT_{1C}$	$5HT_{2A}$	$5HT_{2B}$	$5HT_{3}$
<10nM	5HT 8-OH DPATΔ 5CT Ipsapirone• Gepirone• Buspirone• dLSD	5HT RU24969 5CT Cyanopindolol (CYP)Δ Methiothepin m-CPP	5HT Sumatriptan•* 5CT Metergoline*	Mesulergine Metegoline Methysergide• m-CPP	5HT DOB DOI DOM Ketanserin Ritanserin•* Spiperone Mianserin• Cinarisen dLSD Mesulergine	dLSD Spiperone Clozapine• Cinarisen Ketanserin* Ritanserin•* Mianserin Mesulergine	ondansetron*Δ• MDL72222Δ* 2-methyl-5HT GR65630Δ QuipazineΔ?
10-1000nM	Methysergide Spiperone Metergoline Mesulergine RU24969	Methysergide dLSD Metergoline	Methysergide Mianserin Methiothepin 8-OHDPAT dLSD RU24969	Ritanserin DOI 5HT dLSD Mianserin RU24969 Ketanserin Mk212		Metergoline Quipazine Mk212 m-CPP	5HT
>1000nM	Mianserin Ketanserin	Mianserin Spiperone Mesulergine Ketanserin	Mesulergine Spiperone Ketanserin	Spiperone 8-OHDPAT Ipsapirone	8-OHDPAT Ipsapirone	RU24969 5HT 8-OHDPAT Ipsapirone	8-OHDPAT 5CT Ipsapirone dLSD Ketanserin Methysergide
Radioligand	[^{3}H]DPAT [^{3}H]Ipsapirone [^{3}H]5HT	[^{125}I]CYP [^{3}H]5HT	[^{3}H]5HT [^{125}I]GTI	[^{3}H]Mesulergine [^{3}H]Ketanserin	[^{3}H]DOB [^{3}H]Ketanserin	[^{3}H]Spiperone [^{125}H]dLSD	[^{3}H]GR65360 [^{3}H]Zacopride

* antagonist, Δ selective, • used clinically

terminals were found to have a high density of ligand binding sites.

The second radioligand employed for receptor studies was [^{3}H]5HT. This too showed binding sites suggestive of 5HT receptor function but discrepancies between the binding of [^{3}H]5HT and [^{3}H]LSD were noted (Bennett & Snyder 1976; Fillion et al 1978). It was proposed that the ligands labelled two different `states' of the same receptor. [^{3}H]spiperone, a dopaminergic ligand, was found to label a population of receptors where it was potently displaced by 5HT antagonist drugs but was only weakly displaced by 5HT and related tryptamines (Leysen et al 1978; Creese & Snyder 1978) suggesting that there was a third `state' or type of receptor.

The ligand binding and displacement characteristics of [^{3}H]5HT, [^{3}H]LSD and [^{3}H]spiperone lead Peroutka and Snyder (1979) to describe 5HT1 and 5HT2 receptor types. The binding sites of [^{3}H]5HT were designated 5HT1 sites and those labelled by [^{3}H]spiperone 5HT2 receptors. [^{3}H]LSD could label both receptor types with equal affinity. Unlabelled dLSD displaced all three ligands with equipotency (10nM). 5HT was 300 times as potent at displacing [^{3}H]5HT from binding sites than displacing [^{3}H]spiperone. Its potency at the [^{3}H]LSD site was intermediate between these, approximately 50 times less potent than [^{3}H]5HT displacement. The converse pattern of displacement potency was true for unlabelled spiperone.

Using similar principles of rank potency of ligand binding and displacement and an increasing variety and specificity of agonist and antagonist ligands, 5HT1 receptors have now been classified into four (possibly 5) subtypes. [^{3}H]5HT binds to all subtypes with nM affinity but is displaced from the different sites by varying concentrations of other ligands (Schmidt & Peroutka 1989) (Table 1.3.3). Although there is still some debate, 5HT2 receptors appear to have two subtypes.

1.3.3.1.1 *The 5HT1 receptor family*

a) 5HT1a

5HT1 binding sites were divided into two subpopulations on the basis of the binding of [^{3}H]spiperone. Those with a high affinity for [^{3}H]spiperone were named 5HT1a and those with a low affinity 5HT1b (Pedigo et al 1981). Highly

specific ligands for 5HT1a binding sites have since been developed (Gozlan et al 1983) (Table 1.3.3).

5HT1a receptors in the rat are of high density in the CA1 and dentate gyrus of the hippocampus, the septum, cerebral cortex and raphé nuclei (Deshmukh et al 1983; Marcinkiewicz et al 1984; Pazos & Palacios 1985). The hypothalamus has been found to contain a moderate density of receptors. In the human high density has been found in the CA1 hippocampus and the raphé nuclei as in the rat. High levels are also in the cerebral cortex but in layers different from the rat. Some nuclei of the thalamus and amydala also have high 5HT1a receptor density. The claustrum and posterior hypothalamus have a moderate density of 5HT1a receptors (Hoyer et al 1986a; Pazos et al 1987a). In the human the density of binding sites does not correlate well with the concentration of endogenous 5HT in the area but there is a good correlation with the density of neuronal terminabidentified by histochemical techniques (Pazos et al 1987a).

Palacios et al (1990) have concluded that the anatomical information would suggest that 5HT1a receptors have functional importance in emotional mechanisms. Localisation on the raphé cell bodies would suggest a role in regulation of 5HT function. Other sites of high density would suggest involvement in hypothalamic function, nocioception and integrative functions of the neocortex.

The effect of lesion of 5HT neurons on 5HT1a binding sites is contradictory. A decrease on the raphé cell bodies occurs (Verge et al 1985; Verge et al 1986). No change in density in the neocortex or hippocampus was shown by one group(Weisman-Nanopoulos et al 1985), but Verge et al (1986) have shown a decrease in 5HT1a receptors in both cortex and hippocampus. This would perhaps suggest that the density of presynaptic 5HT1a receptors was low, or that a postsynaptic upregulation masks the loss of presynaptic sites. Studies with toxins to postsynaptic neurons have shown that there is a marked decrease in density of 5HT1a sites after kainic acid treatment (Forloni et al 1983; Hall et al 1985) which would confirm that presynaptic 5HT1a sites are not numerous.

Recent studies have suggested that there may be an interaction with 5HT3 site since chronic administration of 5HT1a selective agonists leads to a decreases in density of 5HT3 binding sites (Barnes et al 1991).

b) 5HT1b

The 5HT1b site is selectively labelled by [^{125}I]cyanopindolol (Sills et al 1984; Hoyer et al 1985; Pazos et al 1985b). Unlike other 5HT1 sites it has a relatively low affinity for dLSD. Binding sites have been demonstrated in the rat and mouse brain but are not present in the brains of several other animal species or humans (Heuring et al 1986; Hoyer et al 1986a). In the rat brain the 5HT1b sites are thought to be presynaptic and to act as an autoreceptor (Engel et al 1986; Raiteri et al 1986) (See Section 1.3.4.2) However lesion of 5HT neurons does not apparently affect the density of 5HT1b binding sites (Verge et al 1986).

Palacios et al (1990) have suggested that the 5HT1b site in rats is involved in the control of movement since it has a high density in the basal ganglia.

c) 5HT1d

The 5HT1d binding site was identified in bovine brain in 1987 (Heuring & Peroutka 1987). Specific ligands for the 5HT1d receptor subtype are in their infancy. Some studies of ligand binding and molecular biology have been carried out in the human and animal brain (Pazos et al 1987a; Waeber et al 1988a+b; Palacios et al 1990). These have suggested a high density in the basal ganglia implying that, similar to 5HT1b in the rat, the 5HT1d receptor may have an important role in control of movement.

5HT1d receptors are thought to act as terminal autoreceptors but, like the 5HT1b sites in rodent brain, lesion of 5HT neurons does not significantly affect the density of binding sites suggesting that the great majority of sites are either post synaptic (Price et al 1991), or have a role in presynaptic regulation of other neurotransmitter systems (Palacios et al 1990).

The 5HT1d receptor is also thought to play a role in the vasoconstriction of cerebral blood vessels in migraine (Doenicke et al 1988).

d) 5HT1c

Pazos et al (1984b) found that [^{3}H]5HT bound with high density to the choroid plexus. The pharmacological characteristics of the site were found to differ markedly in drug rank potency from the 5HT1a and 5HT1b sites. Hoyer et al (1985) and Pazos et al (1984b) characterised the receptor further using a variety

of ligands and displacing agents since there is no site specific ligand. The 5HT1c binding site shares similar characteristics to the 5HT2 receptor with high affinity binding to 5HT antagonists. The 5HT1c site also has physiological and structural similarities to the 5HT2 receptor and is sometime considered as part of that receptor family (Hoyer 1988).

The 5HT1c receptor was one of the first to be structurally characterised and cloned (Hoyer 1988).

In addition to the high receptor density in choroid plexus, the limbic system and regions associated with motor function have a moderately high density in the rat (Pazos & Palacios 1985). In the human there is a high density in the substantia nigra and the globus pallucidus as well as the choroid plexus. The ventromedial hypothalamus also has a high density (Hoyer et al 1986b; Pazos et al 1987a). The location of the receptors would suggest that it has a role in the regulation of motor behaviour.

1.3.3.1.2 *The 5HT2 receptor family*

5HT2 receptor subtypes display a high affinity for serotonergic antagonists (see Table 1.3.3) as well as for spiperone. Recent evidence has suggested that there are two subtypes.

a) 5HT2a

Binding studies using [^{3}H]DOB and related agents have suggested that there are two distinct population of 5HT2 receptors, the 5HT2a site having high affinity for this ligand (Titeler et al 1985; Titeler et al 1987; Peroutka et al 1988; Pierce & Peroutka 1989a). [^{3}H]ketanserin also shows a bimodal binding affinity (Pierce & Peroutka 1989b; McKenna & Peroutka 1989). The 5HT2a site has a much greater affinity for 5HT than the 5HT2b site (Titeler et al 1985; Lyon et al 1987). Although binding studies have not been carried out in the human brain evidence would suggest that the 5HT2 subtypes exist and that the 5HT2a receptor may be involved in the action of hallucinogenic drugs and possibly in psychotic illness (Glennon et al 1984).

b) 5HT2b

5HT2b sites have a similar or slightly lower affinity for [^{3}H]ketanserin than 5HT2a sites (Pierce & Peroutka 1989b) and a very low affinity for DOB and its derivatives. There are no specific ligands for the receptor.

Studies with [^{3}H]ketanserin have shown a high density of 5HT2 (both a and b) receptors in both rat (Pazos et al 1985a) and human cortex in frontal, parietal, temporal, occipital and entorrhinal areas (Schotte et al 1983; Hoyer et al 1986b; Pazos et al 1987b) although there may be some species differences in ligand binding characteristics (Biegon et al 1986; Pazos et al 1984a). Human brains also have a high 5HT2 receptor density in the claustrum and amygdala. There is a moderate density of receptors in the hippocampus and some of the basal ganglia. In the human brain there is a weak correlation of 5HT2 receptor density with 5HT or 5HIAA tissue content. All areas of high receptor density have a high density of 5HT neuronal terminals but not all terminal rich areas have high 5HT2 density (Pazos et al 1987b).

Lesion studies in rats using 5,7-DHT have shown no significant change in post synaptic 5HT2 receptor density in most studies (Blackshear et al 1981; Quick & Azmitia 1983; Seeman et al 1980), although some studies have shown an increase in number. It has been suggested that the receptors are present on intrinsic neurons, possibly of GABA or somatostatin type (Palacios et al 1990).

It is thought that 5HT2 receptors have a role in the limbic system, motor control and visual function.

1.3.3.1.3 *The 5HT3 Receptor Family*

5HT3 receptors were first recognised in the periphery but in 1987 binding sites in brain were identified in the rat (Kilpatrick et al 1987). A variety of specific ligands exist for the 5HT3 receptor (see Table 1.3.3). Regional binding studies suggest a high density in the entorrhinal cortex and lower brain stem in the rat (Kilpatrick et al 1987, 1989).

Lesion of 5HT neurons by 5,7,DHT has shown no subsequent change in hippocampal 5HT3 receptor density, nor after lesion of post synaptic neurons by kainic acid suggesting that 5HT3 receptors, in this area at least, may lie on non-5HT neurons, probably GABA.

There is some evidence that 5HT3 and 5HT1a receptor subtypes may interact since chronic administration of the selective 5HT1a agonist, 8OHDPAT, lead to a decrease in 5HT3 receptors density (Barnes et al 1991).

5HT3 receptors have not been shown in human brain but 5HT3 antagonists have proved to have an important therapeutic role in chemotherapy induced nausea and are presently being discussed as having potential role in a variety of psychiatric and cognitive illnesses (Jones et al 1988).

1.3.3.1.4 *Other receptor subtypes*

In addition to the three major families a variety of other receptors and subtypes have been hypothesised. Some of the data suggests that it may be species differences not receptor class or subtype differences that are being examined (Hoyer & Middlemiss 1989).

5HT4 binding sites were described by Dumuis et al (1988). The pharmacological profile is dissimilar from other families but as yet it is not fully characterised as a receptor (Clarke et al 1989).

It is likely, especially with the application of molecular biology techniques, and with the development of more specific and selective ligands, that many more receptor subtypes and possibly major families will become evident.

1.3.3.2 Physiological correlates of binding sites and assessment of receptor function

1.3.3.2.1. *Second messenger systems*

Biochemical events within the cell are triggered by activation of 5HT receptors. The 5HT1 family, with the exception of the 5HT1c receptor, are coupled to adenylate cyclase with the production of cAMP as an intracellular second messenger. 5HT2 and 5HT1c receptors are coupled to phosphatidylinositol hydrolysis. The 5HT4 receptors may also act in this way. 5HT3 receptors have a much more rapid membrane action as activators of ligand-gated ion channels (Sanders-Bush & Conn 1987; Hamon et al 1988; Sanders-Bush 1988[a+b]; Cornfield & Nelson 1991).

The study of such biochemical events within neurons has lead to increasing

understanding of receptor physiology and pathology a discussion of which is beyond the scope of this review.

1.3.3.2.2 *Monitoring of 5HT release and metabolism*

In vivo monitoring of 5HT and 5HIAA by perfusion collection, voltammetry or CSF sampling allows some assessment of 5HT metabolism and function in a dynamic system. These techniques can be used to study pharmacological drug action and effects of physiological and behavioural stimuli. Microdialysis measurement of 5HT has been shown to closely approximate to firing activity of neurons (Sharp et al 1989). Importantly these techniques can be used to gain insight into dynamic neurotransmitter interactions (Marsden 1985).

1.3.3.2.3 *Electrophysiological studies*

Early studies, using extracellular recordings combined with iontopheresis, examined the effects of 5HT on neuronal firing activity and found predominantly inhibitory responses (Haigler & Aghajanian 1977; Bloom et al 1972). Excitatory responses were observed however, which appeared to act through different receptor populations as they were blocked by 5HT antagonists whilst inhibitory responses were not (Roberts & Straughan 1967). It was proposed that the site which caused neuronal excitation was the 5HT2 site since its affinity for antagonist parallelled ligand binding findings. The inhibitory responses were thought to be due to 5HT1 subtype activation (Peroutka et al 1981). The truth is probably far more complex than this. Aghajanian et al (1987) have put forward an argument that electrophysiological studies have provided evidence of receptor cooperation and modulation such that 5HT2 receptors oppose inhibitory 5HT1 responses but enhance 5HT1 excitatory effects. Based on electrophysiological studies, Aghajanian (1981) has proposed another function of 5HT2 receptors through which 5HT increases the electrical excitability of post synaptic neurons to other neurotransmitter substances, particularly glutamate. This function is apparent in motor neurons and perhaps the reticular formation. The 5HT3 system may be an independent system conducting 5HT transmission with a high velocity (Andrade & Chaput 1991; Aghajanian et al 1990). Further refinement of knowledge of 5HT receptor

function has been hampered by the lack of specific antagonist and agonist compounds.

Electrophysiological studies allow an exposé of intraneuronal events at specific sites (usually examined at raphé and hippocampus) but some studies have assessed a more overall function of 5HT neurotransmission by single unit activity (Jacobs 1987; Fornal & Jacobs 1988; Jacobs et al 1990). These studies have examined the role of 5HT in specific physiological processes and behaviours (see Section 1.5.1). They have also examined the possibility that neuronal firing may not reflect synaptic function when subject to alterations in synthesis and metabolism of 5HT and local influences at nerve terminals. Combining studies of neuronal unit (raphé) activity with microdialysis measurement of changes in ECF 5HT *in vivo*, Jacobs et al (1990) found no evidence that release of 5HT from nerve terminals could be uncoupled form nerve firing activity. In contrast Crespi et al (1990) have shown that stimulation of the neuronal terminal autoreceptor decreased 5HT release *in vivo* without affecting neuronal firing in the DRN.

Neurophysiological studies have provided strong evidence that activation of the 5HT1a receptors on the raphé cell bodies (identified by ligand binding and autoradiographic techniques), completely suppresses activity of DRN neurons (Sprouse & Aghajanian 1987; Dourish et al 1988) and antagonism at this receptor enhances activity. This supports the suggestion that 5HT1a are somatodendritic autoreceptors (Jacobs et al 1990).

Marsden et al (1991) have shown that pharmacological and behavioural modification of 5HT neuronal firing results in both autoreceptor and post synaptic receptor changes. The need for *in vivo* assessment of functional changes in 5HT neurotransmission in experimental procedures is apparent from these elegant studies.

Electrophysiological single cell studies after 5,7,DHT lesion of 5HT neurons have shown a supersensitivity of postsynaptic neurons (Wang et al 1979). The effect of lesioning may be regionally specific however, since no supersensitivity was found in hippocampal cells (de Montigny et al 1980). A component of this supersensitivity may be the added loss of presynaptic reuptake sites in neuronal lesioning (ie loss of degradation regulation) since inhibition of 5HT synthesis

alone does not lead to supersensitivity of post synaptic response (Lorens 1978; Ferron et al 1982).

1.3.3.3 Behavioural studies

Central 5HT stimulation provokes a complex behavioural and motor syndrome in animals (Jacobs 1976; Green 1984) Some aspects of the behaviour are believed to occur because of particular 5HT receptor subtype stimulation. 5HT1a stimulation leads to forepaw treading and flattened body posture (Tricklebank 1985; Green & Heal 1985; Green 1988; Wilkinson & Dourish 1991) whilst 5HT2 receptor stimulation leads to "wet dog shakes" and head twitching (Yap & Taylor 1983). Other behaviour patterns probably arise as a combination of receptor effects. Indeed more than other methods of assessment of 5HT function, behavioural studies identify the complexity of receptor co-operation and modulation. (Green & Heal 1985; Berendsen et al 1988; Backus et al 1990; Wilkinson & Dourish 1991). The effect of pharmacological agents on other neurotransmitter systems will also have a role in the behavioural effects observed.

Behavioural studies are useful pharmacological 'screening' models for the probable effects of drugs Green 1988). As understanding of receptor interaction develops, and drugs of high specificity become available, it may be that behavioural models will become useful in exploring dynamic function of the 5HT neurotransmitter system.

Behavioural studies have also shown that there is functional supersensitivity of 5HT neurotransmission after denervation procedures (Trulson et al 1976).

1.3.4 FUNCTION AND REGULATION OF 5HT NEUROTRANSMISSION

1.3.4.1 What is the function of the 5HT nervous system ?

Voyt (1982) suggested that 5HT plays a role in homeostasis, enabling the organism to modulate excessive stimuli of a wide variety. Several unique anatomical and electrophysiological properties of 5HT neurons support a modulatory role in the CNS. Firstly, the widespread distribution of 5HT terminals in the brain allows influence on nearly every brain function; secondly, the non-synaptic nature of many 5HT terminals suggests that, upon release, 5HT

may potentially interact with several post synaptic neurons (Soghomonian et al 1988; Descarries et al 1990), and thirdly, individual 5HT neurons have been shown to project to more than one target site (van der Kooy & Kuypers 1979). In addition the slow and regular activity of the neurons, together with their slow conduction velocity and long-duration of synaptic action is compatible with a tonic maintenance role rather than rapid and phasic action (Aghajanian et al 1987). 5HT is thought generally to have an inhibitory modulatory effect on behaviours, either directly or via stimulation of GABA secreting or other inhibitory neurons. Some experimental evidence would also suggest that 5HT can modulate the sensitivity of neurons to other afferent inputs.

Numerous specific behaviours appear to be modulated by 5HT (Barchas & Usdin 1973) including mood, appetite, sleep, sexual function, pain, circadian rhythms, body temperature and endocrine functions (see Section 1.5.1). There is evidence which suggests that this modulatory effect on behaviours varies with the general level of arousal (Fornal & Jacobs 1988) which would lead to smooth adjustments of the internal mileux and behaviour patterns to sleep/wake cycles and other influences on the behavioural state.

The 5HT nervous system does not function in isolation, however, and many studies have shown that normally function and responsiveness relies on the integrity of other neurotransmitter systems (Kellar et al 1983; Green & Heal 1985).

1.3.4.2 Autoreceptors

Endocrine and neurotransmitter systems are subject to feedback regulation as a consequence of end organ function or concentration of substance in the immediate environment. This autoregulation may be direct or indirect through inhibitory afferent stimuli.

1.3.4.2.1 *Terminal autoreceptors*

Endogenous 5HT release or exogenously applied 5HT or 5HT-like compounds, leads to inhibition of further stimulated 5HT release in brain slice and synaptosomal preparations, suggesting the presence of an autoreceptor on the terminal nerve process (Gothert & Weinheimer 1979; Cerrito & Raitier 1979). This *in vitro* observation has been confirmed by both neurochemical and

electrophysiological techniques *in vivo* (Moret 1985; Middlemiss 1988). Although the degree of sensitivity of the autoreceptor to synaptic concentration of 5HT is debated (Auerbach & Rutter 1990).

The terminal autoreceptor has been identified in brain preparations from all areas and the receptors appear to have consistent pharmacological characteristics. Studies in the rat have shown that the receptor is probably the 5HT1b subtype since *in vitro* electrically evoked 5HT release is inhibited by drugs with rank potency similar to that of the 5HT1b receptor (Engel et al 1986; Raiteri et al 1986), and 5HT1a agonists do not activate these receptors (Middlemiss & Fozard 1983). In non-rodents and humans the terminal autoreceptor is thought to be of the 5HT1d subtype (Hoyer & Middlemiss 1989). There have been no autoradiographic studies however, that have identified the presence of these receptors subtypes on neuronal terminals (see Section 1.3.3.1.1).

The terminal autoreceptor is thought to exert a tonic inhibitory effect on 5HT release since antagonists to the receptor given alone enhance 5HT release (Gothert 1982) in addition to enhancing stimulated release (Cerrito & Raiteri 1979). Post-synaptic inhibition of firing after 5HT release is also enhanced by 5HT autoreceptor antagonist administration (Chaput et al 1986a).

A close functional link between the terminal autoreceptor and the 5HT reuptake site has been observed since uptake inhibitors act also as terminal autoreceptor antagonists leading to the loss of the inhibition of endogenous 5HT release (Galzin et al 1985: Langer & Moret 1982). However, stimulation of the autoreceptor has no apparent modulatory effect on the reuptake site (Briley & Moret 1983). Other drugs with 5HT activity do not have effects on the terminal autoreceptor (see Section 1.5.3.2). Chronic antagonism of the autoreceptor evidently has no effect on the sensitivity of the receptor (Hagan & Hughes 1983).

1.3.4.2.2 *Somatodendritic autoreceptors*

5HT and LSD were observed to decrease firing activity in the DRN when given systemically or iontophoretically (Wang & Aghajanian 1977; Haigler & Aghajanian 1977). This effect has been found to be mediated through 5HT1a somatodendritic autoreceptors (Verge et al 1985; Sprouse & Aghaganian 1987),

although it has been suggested that the effect may be mediated through 5HT1b receptor subtype in MRN (Pazos & Palacios 1985). Autoradiographic studies have identified a high density of these receptors on raphé cell bodies (Macinkiewicz et al 1984).

Chronic exposure to 5HT1a agonists have produced contrasting results in the changes in somatodendritic autoreceptors. Blier & Montigny (1987) showed that there was a significant decrease in 5HT inhibition of DRN activity. Hamon et al (1990) have suggested that there was no change in biochemical measures of somatodendritic function. The differences in results remain unexplained. No 5HT1a selective antagonist are available at the present time to explore the effects on the somatodendritic receptor.

Administration of drugs which enhance synaptic 5HT have an indirect effect on the somatodendritic receptor. This is thought to be through the action of collateral inhibition from dendrites on DRN cell bodies . Chronic administration of MAOI leads to a decrease in somatodendritic autoreceptor sensitivity as does chronic exposure to uptake inhibitors. Chronic antagonism of the terminal autoreceptor (with increased 5HT release due to loss of inhibition) does not have effect on the somatodendritic autoreceptor (Hagan & Hughes 1983).

The studies of Blier and de Montigny have lead them to believe that the role of the somatodendritic autoreceptor is to control the firing activity of 5HT neurons whereas the role of the terminal autoreceptor is to control the amount of 5HT released by the action potential (de Montigny & Blier 1991).

1.3.4.2.3 *Other autoreceptors*

The presence of $\alpha 2$ adrenoceptor, inhibitory to 5HT release, have been identified on 5HT nerve terminals (Gothert & Huth 1980; Frankhuyzen & Mulder 1980). These receptors are also desensitised after prolonged exposure to 5HT reuptake inhibitors (Blier et al 1990b).

1.3.4.3 **Uptake sites**

After 5HT is released from nerve terminals and has acted at post synaptic receptors it is transported out of the synaptic cleft back into nerve terminals, and into glial cells, by a specific, high affinity membrane uptake carrier. The

uptake site has been found to be ATP dependent and oubain sensitive. Sodium ions, generated by the action of Na^+/K^+ ATPase, may be co-transported with 5HT (Graham & Langer 1988).

Many tricyclic antidepressant drugs non-selectively block the 5HT uptake site. In the past 10 years there has been a great interest in the development of drugs with selectivity for inhibition of the uptake site. This has seen the development of a group of selective serotonin reuptake inhibitors (SSRI) which have proved to have many therapeutic uses and have aided the further understanding of the dynamics of 5HT neurotransmission. Radiolabelled SSRI can also be used in ligand binding and autoradiographic studies of uptake site density and localisation in the brain and other tissues.

Acute inhibition of reuptake has been shown to increase 5HT in the synaptic cleft as measured by histofluorescence (Geyer et al 1978), voltammetry (Marsden et al 1979) and push-pull cannula collection (Guan & McBride 1988) as well as observation of effects on function. Increased synaptic 5HT rapidly leads to autoregulatory changes in the 5HT neuron and within 2 days of treatment there is a marked decrease in firing of DRN (Clemens et al 1977).

Chronic administration of SSRI in animal studies has shown that the uptake site does not apparently undergo upregulation subsequent to inhibition (Graham et al 1980). The firing of DRN returns to normal levels after 14 days of treatment apparently due to two effects of SSRI. Firstly the somatodendritic autoreceptor is desensitised (Chaput et al 1988). This change in sensitivity is parallelled by a decrease in numbers observed in autoradiographic studies (Welner et al 1989). Secondly prolonged exposure to SSRI apparently decreases the ability of the terminal autoreceptor to inhibit 5HT release (Chaput et al 1986b; Blier et al 1988). These studies suggest that the effect of SSRI is an initial increase in synaptic 5HT rapidly followed by a return to normal levels due to inhibition of neuronal firing, followed after longer exposure to SSRI by enhanced 5HT neurotransmission as autoreceptors are inhibited and DRN return to normal firing activity.

There is mixed evidence of the effect of increased 5HT in the synapse on post synaptic function. Chaput et al (1986b) have shown that there was no desensitisation of function, nor have changes in density of 5HT1a or 5HT2

receptors been found in most studies (Walmsley et al 1987). Wong & Bymaster (1981) did find a small decrease in 5HT1 receptors in the frontal cortex.

1.3.4.4. 5HT neurotransmission.

Figure 1.3.3 shows a stylised 5HT neuron with the receptors and regulatory processes involved in neurotransmission.

Increase in concentration of 5HT precursors is known to increase 5HT synthesis, much of which is degraded within the neuron resulting in increased 5HT turnover (increased 5HIAA) but not necessarily an increase in synaptic function (Green & Grahame-Smith 1975).

Release of 5HT into the synapse leads to postsynaptic effects, principally on 5HT1 receptors since these have a much greater affinity for 5HT than 5HT2 receptors, presynaptic effects at the terminal autoreceptor (5HT1b in the rat 5HT1d in human), uptake site and other presynaptic receptors. There is thought to be post-synaptic modulation and co-operation between 5HT1 and 5HT2 receptors.

Administration of receptor subtype selective and non-selective agonists leads to discernible behavioural and physiological changes in animals and humans, whilst administration of 5HT2 antagonists causes little change in behaviour. 5HT2 receptors appear to be unlike other 5HT receptors or receptors of other neurotransmitter systems in that exposure to antagonists causes a decrease in the number of receptors (Leysen et al 1986).

Leysen & Pauwels (1990) have suggested that the 5HT2 receptor has a unique role in neurotransmission. Despite low affinity for 5HT they are extremely sensitive to it and administration of selective agonists causes a rapid and prolonged down regulation (Leysen et al 1989; Buckholtz et al 1988). It may be that under normal circumstances, because of low affinity for 5HT, 5HT2 receptors are not stimulated, thus 5HT2 antagonism causes little discernible change in behaviours. Exposure to 5HT2 agonists cause rapid and marked effects with a compensatory down-regulation.

The regulation of 5HT transmission is through a variety of possible routes:

a) Neuronal cell firing activity. Regulated by somatodendritic 5HT1a autoreceptors. Stimulation of the receptor leads to a decrease in neuronal firing

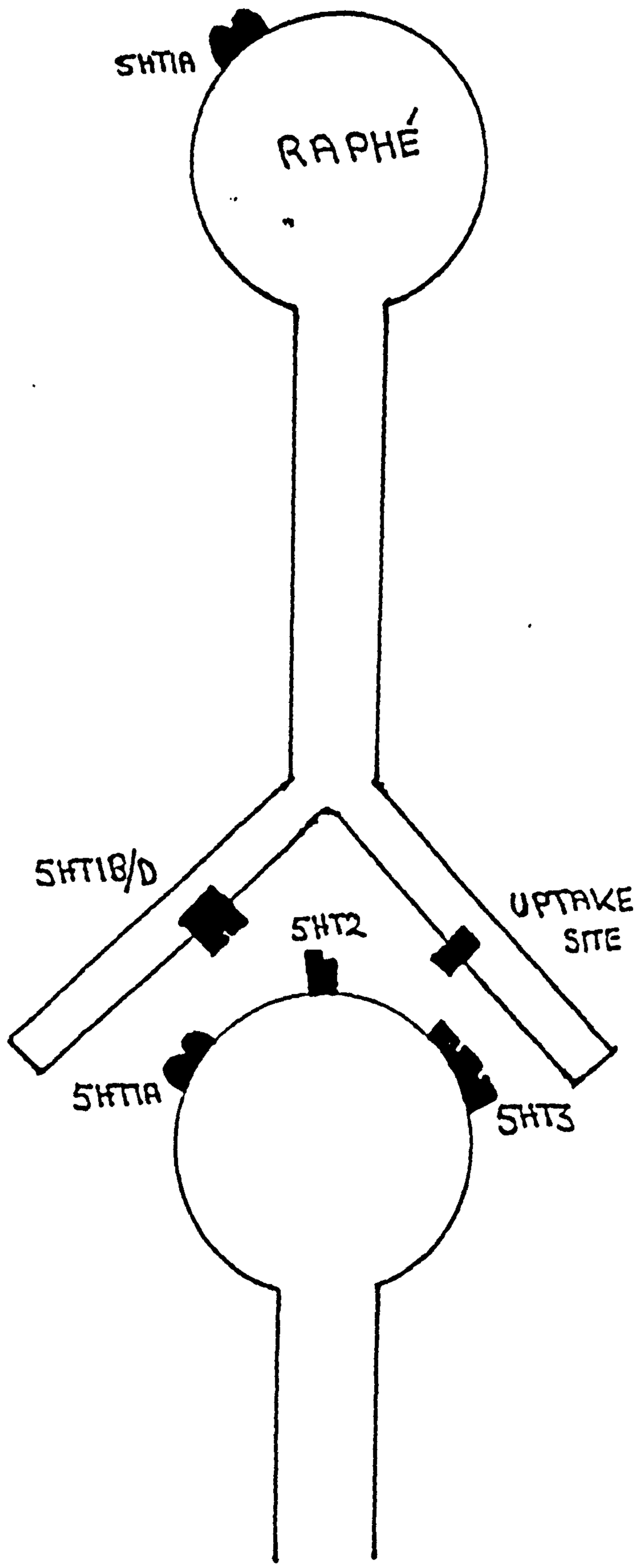


Figure 1.3.3

A stylised serotonin neuron showing the receptors of neurotransmission and regulation.

and a decrease in 5HT release into the synapse.

b) Terminal autoreceptors. Stimulation leads to a decrease in 5HT release from the neuron.

c) Presynaptic uptake sites. The affinity and number of uptake sites does not appear to change in experimental settings. Uptake sites may also influence 5HT release by an effect on the terminal autoreceptor.

d) Post-synaptic receptors. Alteration in stimulation of the post-synaptic receptors leads to a change in receptor number. There is probably a high degree of receptor co-operation and modulation.

e) Other neurotransmitter systems. Alteration in 5HT transmission has been shown to lead to changes in other neurotransmitter systems both indirectly and directly by effect on 5HT receptors present on the neurons of other neurotransmitter systems (Quirion & Richard 1987). In addition to 5HT itself many other neurotransmitters and neuropeptides may influence the release of 5HT through receptors present on 5HT cell bodies and nerve terminals.

It is known that drugs with similar effects on the concentration of synaptic 5HT have differing effects of these regulatory processes (see Section 1.5.3.2). Drugs with apparently the same pharmacological mode of action also have different effects on these processes. It is only by consideration of all of these effects, together with electrophysiological study of the net effect on post-synaptic transmission that we can understand the functional change in 5HT neurotransmission. Examination of only one process may lead to apparently contradictory findings and erroneous conclusions of functional change since it may be that even though drugs may appear to cause different effects in any one part of the system, the net result of these changes allows identical changes in increase or decrease of 5HT function.

Lesion of 5HT neurons appears to cause electrophysiological and behavioural supersensitivity to 5HT, but without apparently major and consistent changes in post synaptic receptor density. The mechanism for this is not fully explained but could be related to changes in receptor affinity.

There is a need in the future study of 5HT function regulation and pathology for the development of a model which takes into account the following:

1. That the anatomy of the 5HT system suggests that there are discrete but interacting subsystems. Each raphé nucleus may serve specific functions and have discrete neuronal type and innervation pattern. It has been suggested that DRN are particularly associated with the 5HT2 receptor (Molliver 1987).

2. That there are a host of receptor subtypes. In the light of point 1 it may be that the subtypes have different function and pattern of response in different anatomical location. Indeed 5HT neurons of the MRN have been reported to be less sensitive to 5HT1a agonists than neurons of the DRN (Blier et al 1990a).

3. That a high receptor density does not necessarily correlate with functional importance of 5HT nor a low density with absence of a functional role for 5HT since receptors may down-regulate with high availability of the monoamine and upregulate where there is a deficiency.

Conclusions about function and pathology must consider all available indicators of synaptic function including measurement of turnover, neuronal and receptor density and physiological and behavioural assessment of receptor processes.

1.4 SEROTONIN AS A MODULATOR OF VASOPRESSIN RELEASE

1.4.1 ANATOMICAL EVIDENCE

5HT nerve fibres innervate almost every area of the brain and pass in two major tracts from the brain stem through the hypothalamus to cortical and subcortical structures (Azmitia & Gannon 1986). There is evidence that 5HT innervates many of the hypothalamic nuclei and specific innervation of the SON and PVN has been shown in the rat, cat and monkey (Conrad et al 1974; Zaborsky et al 1975; Bobillier et al 1976; Azmitia & Segal 1978; Kent & Sladek 1978; Steinbusch 1981). There have been no studies of the human brain which have looked at the 5HT innervation of the SON or PVN specifically.

Van de Kar & Lorens (1979) suggested that the MRN project to the anterior part of the hypothalamus in the rat although others have suggested that the DRN are more important in innervation of the SON (Bobillier et al 1976; Pal kovits et al 1977). Sawchenko et al (1983) found evidence that these hypothalamic nuclei were innervated by both DRN and MRN in the midbrain but not raphé neurons situated more caudally.

Two detailed studies in rats have shown that the 5HT innervation of the PVN is less dense than that of the surrounding cell groups (Steinbusch 1981) and that 5HT terminal are predominantly associated with parvocellular neurons (Sawchenko et al 1983). Although there is a low nerve fibre density in the magnocellular region they have a distinct distribution, which may be preferentially related to the distribution of oxytocin secreting cells (Sawchenko et al 1983) (see Section 1.1.1). Zaborsky et al (1975) estimated that 30% of the afferent fibres to the SON were of noradrenaline or 5HT type. These appear to be associated with oxytocin secreting cells in the anterodorsal part of the nucleus (Sawchenko et al 1983). In the monkey brain 5HT fibres have been found in the lateral PVN and dorsal SON areas (Kawata et al 1984).

In the hypothalamus studies of the 5HT content and the enzymes of synthesis and degradation have shown there to be high concentration in all animal and human brains (Bertler 1961; Azmitia & Gannon 1986). Initial measurement of these substances specifically in the SON proved to be negative (Dahlström & Fuxe 1964) but later studies have identified their presence in both the PVN and

SON (Carlsson et al 1962; Saavedra et al 1974; Brownstein et al 1975; Saavedra 1977). Since surgical isolation of the hypothalamus decreases the concentration of these substances it would seem that 5HT originates from neurons with cell bodies in other areas (Brownstein et al 1976).

5HT nerve fibres have also been found in the neurohypophysis of many animal species (Bjorklund 1968; Steinbusch & Nieuwenhuys 1981; Sano et al 1982; Saland et al 1986). These nerves appear to originate in the DRN (Saland et al 1987). 5HT and the enzymes of synthesis and degradation have also been found in the neurohypophysis in moderate concentrations (Bjorklund et al 1967; Piezzi & Wurtman 1970; Saavedra et al 1975; Payette et al 1985; Koulu et al 1989).

The OVLT and SFO receive a moderately dense 5HT innervation (Bosler 1978; Bouchard & Bosler 1986). Its function is not known but it may be related to the control of LHRH secretion rather than osmoregulation.

Schotte et al (1983) and others (Pazos & Palacios 1985; Pazos et al 1985b; Pazos et al 1987) have found evidence that receptors for 5HT are present in low concentration in the regions of the magnocellular neurons in the hypothalamus of rat and man but in a higher concentration in the neurohypophysis of rats (De Souza 1986).

These anatomical findings suggest that 5HT may have a direct role in the regulation of AVP release. Because of the diffuse nature of 5HT innervation many areas of the brain which have afferent input to the magnocellular neurons may be influenced by 5HT. Thus 5HT could also indirectly modulate AVP release in a variety of ways.

1.4.2 EVIDENCE FOR A PHYSIOLOGICAL ROLE

Early studies to explore the role of 5HT in AVP secretion found that electrical stimulation of the raphé diminished urine output (Sharpless & Rothballer 1961), and lesion of the raphé nuclei increased urine output and water intake (Tangapregassom et al 1974). This appeared to be due to an inability to release AVP since there was an accumulation of neurosecretory material in the magnocellular neurons subsequent to the 5HT neuron loss (Tangapregassom

al 1974). Iovino & Steardo (1985) found that lesion of 5HT neurons with the neurotoxin 5,7-DHT or inhibition of synthesis with pCPA (see Table 1.3.2), prevented the normal rise of AVP in response to water deprivation. This was confirmed in another study using a hypertonic saline stimulus (Brownfield et al 1987). However, depletion of brain 5HT either by neuronal lesion or synthesis inhibition had no effect of basal AVP concentration (Iovino & Steardo 1985; Brownfield et al 1987).

Water deprivation in rats has been found to increase 5HT turnover (5HIAA:5HT ratio) (Handley et al 1991) and cause a depletion of 5HT in the neurohypophysis (Piezzi & Wurtman 1970).

Exogenously applied 5HT has been shown to alter the discharge rate of some magnocellular neurons (Moss et al 1972) although there have been conflicting results as to the excitatory or inhibitory nature of the effect (Barker et al 1971). It has been suggested that only magnocellular neurons secreting oxytocin are stimulated by the 5HT (Barker et al 1971).

It would seem from these studies that 5HT has a role in osmotically stimulated AVP release. 5HT did not appear to be involved in the hypovolaemic stimulation of AVP release in one study in rats since this was preserved after 5HT neuron lesion (Brownfield et al 1987). In another study haemorrhage caused an increase in 5HT concentration in the SON of the rat, suggesting a role in baroregulation responses (Kendrick & Leng 1989).

The work by Tangapregassom . (1974) might suggest that 5HT loss inhibited AVP release but not synthesis. However Carter & Murphy (1989) have shown that 5HT synthesis inhibition blocked the increase in AVP mRNA in the magnocellular neurons which occurs with osmotic stimulation. This would suggest that 5HT was also important in AVP synthesis.

Studies with exogenous 5HT application have provided variable results. Urano & Kobayashi (1982) injected 5HT into the SON and found a biphasic response with an initial brief diuresis followed by a prolonged antidiuresis. Montes & Johns.on (1990) have confirmed this finding and showed that intracerebroventricular (ICV) 5HT stimulated AVP secretion in water-loaded rats. However, Bhargava et al (1972) found that ICV infusion of 5HT had no effect on AVP release in dogs and others have similarly found no effect of urine flow in normal or water-loaded animals (Olsson 1970; Ruoff et al 1974;

Camargo+Saad 1975; Stein et al 1987).

The isolated rat neurohypophysis has been shown to release AVP when stimulated by 5HT (Lemay et al 1979) but incubated hypothalamic fragments did not release AVP with 5HT stimulation (Hillhouse & Milton 1989).

The inconclusive and contradictory nature of these results is compounded by the use of differing animals and techniques of 5HT administration. It is assumed that 5HT given ICV will increase synaptic 5HT which is possibly erroneous. However, even the studies which use locally applied 5HT are not in agreement. Recently it has been suggested that the cardiovascular effects of 5HT (increase in mean arterial pressure, decrease in heart rate) are mediated, at least in part, by AVP since V1 receptor antagonists inhibit the effects (Dedeoglu & Fisher 1991; Pergola & Alper 1991). Plasma AVP was not measured in either of these studies.

1.4.3 PHARMACOLOGICAL STUDIES

The advent of sensitive assays for AVP and selective 5HT agonist and antagonist drugs has allowed further exploration of a possible role for 5HT in the regulation of AVP secretion.

Gibbs & Vale (1983) found a rise in peripheral and portal AVP concentration in anaesthetised rats after acute administration of the SSRI Fluoxetine. In a study using un-anaesthetised rats, Fluoxetine was found to have no effect on urine flow rate (Stein et al 1987). Quipazine and d-fenfluramine, both of which increase 5HT neurotransmission, have been shown to cause a dose-related rise in plasma AVP in conscious rats (Iovino & Steardo 1985). This effect was completely blocked by prior treatment with pCPA. A decrease in urine volume was found after administration of another 5HT stimulator PCA. However, this was <u>not</u> inhibited by prior treatment with pCPA (Stein et al 1987). PCA has also been shown to cause a rise in plasma AVP in rats (Steardo & Iovino 1986; Brownfield et al 1988). This effect was prevented by hypothalamic deafferentation with transection of 5HT and other pathways to the hypothalamus (Brownfield et al 1988). High dose of another agonist (TFMPP) was also been shown to cause a rise in plasma AVP (Hashimoto et al 1982).

Selective 5HT1a and 5HT1b agonists did not cause a rise in plasma AVP but MK212, a mixed 5HT1 and 5HT2 agonist did stimulate the release of AVP in conscious rats (Brownfield et al 1988). Whilst a 5HT2 antagonist had no effect on plasma AVP when given alone, it did block the effect of MK212 on plasma AVP release (Brownfield et al 1988). Bagdy et al (1992) found that ritanserin, a 5HT2 and 5HT1c antagonist, partially inhibited the AVP response to the 5HT agonist mCPP.

It would appear from these studies that an intact 5HT system is necessary for normal osmoregulation of AVP although basal concentration is apparently independent of 5HT neurotransmission. An increase in synaptic 5HT leads to a rise in plasma AVP. This effect is apparently mediated centrally since depletion of neuronal 5HT prevents the agonist stimulated rise. Although many of the drugs used in pharmacological studies do have effect on other neurotransmitter systems the balance of evidence would suggest that 5HT does modulate AVP release. The work by Brownfield et al (1988) has suggested that this is mediated by 5HT2 receptors. Although mCPP, which has 5HT2 agonist properties, did stimulate AVP release in one study (Bagdy et al 1992) it did not stimulate AVP release in another (Saydoff et al 1989). Similarly DOI, a 5HT2a agonist, did not alter plasma AVP concentration (Saydoff et al 1990; Bagdy et al 1992). A recent study has indicated that the 5HT1c receptor subtype may mediate the response (Bagdy et al 1992).

It is by no means conclusive from anatomical and pharmacological studies that 5HT causes a rise in AVP by a direct effect on magnocellular neurons. It does not appear to have an effect which is mediated through alteration of blood pressure (Brownfield et al 1988; Stein et al 1987), nor through the renin-angiotensin system since angiotensin II antagonists did not inhibit the 5HT stimulated rise of plasma AVP (Steardo & Iovino 1986).

1.4.4 STUDIES IN HUMANS

There have been only two studies in humans which have explored the possible role of 5HT in AVP regulation and release. Thompson et al (1992) showed that the 5HT2 and 5HT1c antagonist ICI 169,369, did not alter the osmotically

stimulated AVP response . At high dose however, the antagonist blunted the AVP rise consequent to insulin-induced hypoglycaemia in normal men. The AVP response to hypoglycaemia is not,however, a reproducible phenomena (Thompson et al 1991) and this result must be interpreted with caution.

Coiro et al (1989) have shown that the metaclopramide stimulus to AVP is not mediated through 5HT receptors.

Circumstantial evidence for the possible importance of 5HT in AVP regulation comes from the reports of hyponatraemia associated with the clinical use of some, but not all of the SSRI (Section 1.6.2.1). Hyponatraemia has not been reported with the use of other 5HT active agents such as fenfluramine and 5HTP. It is possible that drugs which are 5HT agonists may non-osmotically stimulate AVP release because of their tendency to cause nausea. However this was not cited as a significant factor in the case reports. Unlike the tricyclic antidepressants the SSRI, and other drugs which increase 5HT neurotransmission, do not cause a drop in blood pressure and would not stimulate AVP through changes in baroregulation.

The role of 5HT in osmotically regulated AVP secretion both in normal man and in states of AVP dysfunction such as cranial diabetes insipidus and SIAD, and in diseases such as depression where there is a putative abnormality of 5HT function, requires further investigation.

1.5 SEROTONIN AND DEPRESSION

It was first proposed that serotonin might have a role in mental illness (Wooley & Shaw 1954; Coppen 1967) after two drugs which had major effects on mood were found to have a profound effect on 5HT neurotransmission. LSD, which caused a variety of disturbances of mental function, was found to have a similar chemical structure to 5HT (Shave 1955), and reserpine, which caused depression in many subjects, was found to deplete brain 5HT (Shave et al 1955; Pletscher et al 1956). Both "deficiency" and "excess" of serotonergic activity have been proposed as predisposing to, or causing depressive illness. With increase in knowledge of the intricacies of 5HT neurotransmission and regulatory processes, particularly receptor physiology and regulation, it has been possible to marry some of the previous, apparently contradictory findings. This section discusses some of the evidence for an abnormality of 5HT neurotransmission in depression.

1.5.1 CHANGES IN BEHAVIOUR AND THE ROLE OF 5HT

Depression is characterised by significant changes in a variety of behaviours. There is evidence to suggest that 5HT may have a role in these changes.

1.5.1.1 Mood

Depressed mood is obviously a major feature of affective illness. There is some evidence that in normal people changes in the availability of plasma tryptophan, by dietary manipulation or l-tryptophan administration, affects mood. Elevation of mood occurs with tryptophan loading (Smith & Prockop 1962; Charney et al 1982) and mild depression may occur with tryptophan deficiency (Young et al 1985). These results have not always been replicated in other studies and, in view of the fact that changes in 5HT synthesis appear to have little effect on function due to a vast excess of synthesis over requirements, (see Section 1.3.2), the results are controversial. 5HTP administration has also been shown to have mood elevating effects in normal subjects (Trimble et al 1975; Purhringe et al 1976).

A recent study in depressed patients showed that the availability of dietary

tryptophan was important in the maintenance of mood improvement during antidepressant treatment (Delgado et al 1990). Inhibition of 5HT synthesis by pCPA was found to block the mood response to antidepressants (Shopsin et al 1976). Thus in depressed patients, and perhaps also in normal people, the synthesis of 5HT has effect on mood with an increase in 5HT leading to mood elevation and a decrease leading to depression.

1.5.1.2 Appetite

Decreased appetite is a frequent symptom of major depression although some patients have increased appetite with a change of desire for specific food types, particularly carbohydrates.

In general increased 5HT activity appears to decrease food intake and decreased activity leads in increased appetite (Blundell 1984). This may suggest that in depression there is an increase in 5HT activity. It is possible that the 'appetite system' is an independent 5HT system and that although other aspects of behaviour in depression suggest a decrease in 5HT activity, this system does have an increase in activity, or it may be that there is much more complex regulation and receptor interaction in the control of appetite. The latter seems more likely in view of recent findings that $5HT_{1a}$ agonists have been shown to increase food intake.

1.5.1.3 Sleep

Disturbed sleep with insomnia and early wakening is a classic feature of major depression, particularly decreased latency of rapid eye movement sleep (REM). Increased 5HT activity promotes total sleep time and decreased 5HT activity produces insomnia (Koella 1988). Many drugs which increase 5HT availability selectively lengthen REM latency.

1.5.1.4 Circadian rhythms

There is much evidence that circadian rhythms are disturbed in affective disorder with abnormalities of sleep cycle, endocrine secretory patterns and temperature rhythms (Healy & Williams 1988; Gold et al 1988). The SCN, thought to be the major co-ordinator of circadian rhythm, is richly innervated by 5HT neurons. Destruction of 5HT pathways in animals is known to ablate

normal light/dark cycles.

5HT may be involved in the abnormalities of circadian cortisol secretion that occur in depression (Meltzer et al 1984; Montange & Calas 1988).

1.5.1.4 Anxiety

Anxiety is a common symptom of depressive disorders. There is evidence from animal studies that "stress" may increase both pre and post synaptic 5HT activity (Kennett et al 1985) . $5HT_{1a}$ agonists, predominantly used as anxiolytic drugs, appear also to have antidepressant action (Kennett et al 1987). Failure of 5HT neurotransmission to respond appropriately to stressors may be a factor in depression

Thus alteration of 5HT activity is consistent with many of the changes in mood, behaviour and somatic function observed in depressed people.

1.5.2 STUDIES OF ABNORMALITIES IN 5HT ACTIVITY IN DEPRESSED PATIENTS

1.5.2.1 5HIAA in the CSF

The concentration of 5HIAA in the CSF has been studied as an indicator of central 5HT activity. Lumbar CSF 5HIAA levels have been found to correlate with brain 5HIAA concentrations in humans, although as much as 50% may originate from the spinal cord. Many other factors such as diet, height, gender, environmental stressors and previous drug treatments may influence lumbar CSF 5HIAA levels. There is thus much disagreement in the data and much controversy with regards to interpretation (Anderson et al 1990). In addition, as discussed above (Section 1.3.2), 5HT metabolism shown by 5HIAA concentration, does not necessarily reflect function at the synapse.

Agren (1980) and Åsberg et al (1984) have found decreased 5HIAA concentrations in large populations of drug free unipolar and bipolar depressed patients compared to normal controls. These findings were not replicated in other studies some of which were examined by Rothpearl et al (1981) who suggested that many studies were not large enough to detect <30% change in 5HIAA concentration, and that there was possibly a subgroup of depressed patients

whose CSF 5HIAA levels correlated with the severity of depression. This had been suggested previously by Asberg et al (1976) and also by Gibbons & Davis (1986).

In follow-up studies of depressed patients Träskman-Bendz et al (1984) found that, whilst CSF 5HIAA levels remained relatively stable in controls and most depressed patients, some of those patients with low 5HIAA levels when ill had a moderate rise on recovery, suggesting that there was a subgroup of patients with an unstable 5HT system. Van Praag & de Haan (1979) showed that those patients who had a persistent low 5HIAA concentration when euthymic, had a higher risk of subsequent relapse than depressed patients with normal CSF 5HIAA.

An inverse relationship between CSF 5HIAA concentration and suicidal behaviour has been found (Asberg et al 1987; Roy et al 1988). Reduced 5HIAA levels have been reported in the CSF of suicide attempters without depression and this may suggest that suicidal behaviour, and not depression per se, correlates with decreased CSF 5HIAA.

Low CSF 5HIAA concentration may predict good response to certain therapies. Van Praag & de Haan (1980) found that treatment with 5HTP, of euthymic patients with persistently low CSF 5HIAA, reduced the risk of subsequent relapse compared to placebo treatment. Maas et al (1982) found that low CSF 5HIAA predicted good clinical response to imipramine but not to amitryptiline.

1.5.2.2 Plasma tryptophan

It has already been discussed that an increase in available plasma tryptophan leads to increased synthesis but not necessarily to increased synaptic function (Section 1.3.2), and that plasma tryptophan concentration may have effect on mood in both normal and depressed people (Section 1.5.1.1).

Moller et al (1983) summarised the 20 or so studies of measurement of plasma tryptophan concentration in depressed patients. About half of these studies showed that there was a decreased concentration of free tryptophan in the plasma of depressed patients, the concentration still, however, fell within the normal range.

Some studies have show a reduced ratio of tryptophan to other neutral amino acids in depression (Joseph et al 1984). De Meyer et al (1981) found an inverse

correlation of this ratio with the severity of depression.

There has been some evidence to suggest that depressed patients with low plasma tryptophan have abnormalities of metabolism after either oral or intravenous administration (Smith & Stromgren 1981; Koyoma & Meltzer 1986). These authors found a larger volume of distribution and longer half life, resulting in a decreased availability in depressed patients. Other studies have failed to confirm this (Hoes et al 1981; Moller et al 1982).

1.5.2.3. Studies in platelets

Blood platelets have many features in common with 5HT neurons and are sometimes used as a model of neuronal function (Sneddon 1973; Stahl 1977). Several studies have reported a reduction in 5HT uptake into platelets from unmedicated depressed patients (Coppen et al 1978; Meltzer et al 1981; Stahl et al 1982). This appears to be due to a decrease in number not a change in affinity of the uptake site. This abnormality appears to remain after recovery and may be a trait marker in some patients (Coppen et al 1978; Scott et al 1979). There is no apparent correlation between uptake site changes and severity of depression nor does it predict response to treatment (Aberg-Wistedt 1982).

If these findings were extrapolated to suggest that uptake sites on central 5HT neurons are decreased in depression this could lead to increased concentration of synaptic 5HT since less was removed by reuptake, or decreased reuptake could lead to diminished presynaptic stores and thus decreased 5HT availability. The decrease in uptake site could, however, be a secondary compensation in response to a primary deficiency in 5HT activity. There is no experimental evidence to support any of these possibilities although a change in uptake site number as a regulatory phenomenon has not been observed in experimental manipulations (Graham & Langer 1988)

1.5.2.4 Neuroendocrine studies

The release of many anterior pituitary hormones is influenced, at least in the experimental situation, by 5HT (Meltzer et al 1982a; Tuomisto & Mannisto 1985; Murphy et al 1986; Van Praag et al 1987; Cowen 1987; Montagne & Calas 1988; Van de Kar 1991). Administration of agents which affect 5HT

neurotransmission, and the measurement of consequent hormone responses has been used to assess three features of psychiatric illness. Firstly abnormalities of neuroendocrine function in mental illness, secondly the function of central 5HT systems in depression or other disorders and thirdly the effects of treatment. Neuroendocrine studies allow a view through the so called "window on the brain" (Checkley 1980; Meltzer et al 1982b). Such studies are made difficult by the absence of 5HT receptor subtype specific agonist and antagonist drugs. Cowen et al (1990) have suggested that 5HT1a and 5HT2/1c receptors facilitate ACTH and PRL release but 5HT1a receptors alone have a more prominent role in GH secretion. These receptor subtypes may have variable pre and post synaptic functions , and opposing influences on hormone release, leading to variability and contradiction in results in different studies using different ligands.

In general the cortisol response to 5HTP has been found to be augmented in unmedicated depressed patients (Meltzer et al 1984) and the response is inversely correlated to CSF 5HIAA concentration (Koyama et al 1987). It has been found to return to normal after antidepressant treatment of the depression (Meltzer et al 1984). A decreased PRL response to serotonergic agents has been found in depression (Henninger et al 1984) and this was normalised by treatment of the depression (Charney et al 1984; Cowen & Anderson 1991; Cowen & Charig 1987). A diminished GH response to both 5HTP and insulin-induced hypoglycaemia has also been found in depression (Koyama & Meltzer 1986).

The involvement of 5HT in the abnormal cortisol regulation and dysfunction of the HPA axis in depression is an important line of research. Brown et al (1986) have suggested that failure of post dexamethasone suppression of cortisol is due to 5HT dysfunction but it is possible that the antithesis is true since cortisol is known to have effect on 5HT receptor subtype sensitivity and 5HT uptake (Biegon 1990).

Meltzer (1990) has suggested that, although there is evidence of dysfunction in the 5HT system, results are difficult to interpret and no clear conclusions can be drawn about the status of pre or post synaptic 5HT mechanisms in depression on the basis of these studies. Further studies are needed to elucidate precise mechanisms and interactions with other neurotransmitter systems.

1.5.2.5 Post mortem neurochemical and receptor studies

The studies discussed so far have given only indirect evidence of abnormalities of 5HT function occurring in depression. Studies of post mortem brain tissue have provided more direct evidence of putative neurochemical disturbances (Mann et al 1989; Mann et al 1986).

Early studies of neurochemical changes in depression relied on brain material from suicide victims. For many reasons study of this material has drawbacks; poor documentation of mental state and previous therapeutic interventions, the possibility of drug overdose at suicide, post mortem delay and the fact that suicide itself, rather than depression, may have distinct associated neurochemical changes (Mann et al 1989) (Section 1.5.2.1). Shaw et al (1967) reported a reduction in 5HT concentration in hind brain of suicide cases who had previously been diagnosed as depressed. Pare et al (1969) replicated this but other studies have failed to do so. The changes in 5HIAA concentration are even more variable (Ferrier et al 1986; Stanley et al 1986).

Studies of receptor changes have perhaps proved more robust. Stanley & Mann (1983) reported an increase in 5HT2 receptors in the frontal cortex of suicide victims and confirmed this in a later study (Mann et al 1986). Cooper et al (1986) found no significant changes in receptor density in frontal cortex or hippocampus of suicide victims and in a further study of depressed patients dying from natural causes (Crow et al 1984). In a study of 16 patients with depression, dying of natural causes but with "active" depression, Ferrier et al (1986) found a non-significant trend of an increase in 5HT2 receptors in the frontal cortex. A more significant increase in 5HT2 receptor concentration was found in a later study of similar patients (Yates et al 1990) and also by another group Arora & Meltzer (1989).

The 5HT1 receptor has not been found to differ in depressed compared to control patient brains (Crow et al 1984; Cooper et al 1986). The 5HT1a subtype has not been found to change in the frontal cortex (Yates & Ferrier 1990) but may be decreased in the hippocampus of depressed patients (Cheetham et al 1991).

5HT1d subtype have been shown to be significantly increased in violent suicide deaths in the globus pallidus in one study (Lowther et al 1991).

Thus post mortem studies would add some weight to the hypothesis that there is a dysfunction of 5HT neurotransmission in depression. An increase in 5HT2 receptors might suggest that there is an upregulation as compensation for diminished synaptic availability of 5HT or the increase could be the primary abnormality, suggesting excessive 5HT neurotransmission occurs in depression. The regulation of 5HT neurotransmission has already been discussed in some detail (Section 1.3.4.) and it is likely that the apparent increase in 5HT2 receptors in depression is a complex interaction of a variety of putative abnormalities of 5HT and dysfunction of other neurotransmitter systems.

1.5.3. STUDIES OF 5HT ACTIVE AND OTHER TREATMENTS IN DEPRESSION

1.5.3.1 5HT precursors

There is little evidence that, given alone, tryptophan has antidepressant effects (Cole et al 1980) but it has been found to facilitate that effect of MAO inhibitors (Glassman & Platman 1969; Baldessarini 1984). It does not, however, potentiate the antidepressant response to tricyclic or other antidepressant treatments. 5HTP does appear to have antidepressant efficacy when given alone or in combination with other 5HT active drugs in major depression (Van Praag 1981;1984; Meltzer & Lowy 1987). It may also be useful prophylactically in a subgroup of depressed patients (Section 1.5.2.1)

1.5.3.2 Studies on the mechanism of action of antidepressant drugs

Many, but not all, effective antidepressant (AD) drugs have acute pharmacological effect on 5HT synaptic concentration and neurotransmission (Ogren & Fuxe 1985). However, it is apparent from clinical studies that therapeutic action takes 14 days or more of continuous treatment. This has been attributed to changes in neurotransmitter systems as a consequence of the acutely enhanced 5HT neurotransmission. Research into the therapeutic mode of action, and the search for understanding of the pathophysiology of depression, has consequently focussed on changes in 5HT neurotransmission after chronic administration of AD therapies.

Studies of changes in monoamine turnover with AD treatment have provided

inconsistent results and no conclusions can be drawn to account for the therapeutic mechanism of action of these drugs (Heninger & Charney 1987). Studies of changes in receptor processes have proved more consistent and the time course of such changes closely mirrors the delay in clinical effects.

1.5.3.2.1 *Receptor binding studies*

Chronic administration of many AD drugs has been found to cause a decrease in β-adrenoceptor binding density in rat cortex and other areas (Charney et al 1981). A decrease in 5HT2 binding sites is also frequently, but not invariably, found (Peroutka & Snyder 1980; Charney et al 1981; Kellar et al 1981;Lloyd et al 1985; McDonald et al 1985;). More variable changes have been observed in the density of ligand binding to other 5HT and neurotransmitter receptor sites (Anderson 1983; Ogren & Fuxe 1985; Heninger & Charney 1987).

Although decreased 5HT2 and β-adrenoceptor binding is a common finding not all AD have the effect and the changes may be regionally specific. In addition ECT has been found to produce a consistent increase in 5HT2 receptor number although it too decreases β-adrenoceptor density (Kellar et al 1981; Green et al 1983; Kellar & Bergstrom 1983). Thus changes in 5HT2 receptor or β-adrenoceptor density can not fully explain the mode of action of AD therapies. It is interesting that 5HT2 antagonists, which paradoxically cause down regulation of 5HT2 receptors, appear to have antidepressant properties (Reyntjens et al 1986).

1.5.3.2.2 *Behavioural studies*

Most AD, except SSRI appear to potentiate α_1-adrenoceptor and postsynaptic DA receptor mediated behaviours, but decrease β-adrenoceptor effects. 5HT1a receptor mediated behaviours are also diminished by chronic exposure to AD (Goodwin et al 1987). The effects of AD on 5HT2-mediated behaviour are less conclusive. Many AD drugs appear to decrease 5HT2-mediated functions but ECT enhances such behaviours (Goodwin et al 1984).

As discussed above (Section 1.3.3.3) behavioural studies are difficult to interpret because of the interaction of multiple receptor, neurotransmitter and physiological effects and it may be for this reason that the functional effects of chronic AD administration are inconclusive.

1.5.3.2.3 *Neurophysiological studies*

Studies of postsynaptic neuronal response have suggested that chronic AD treatment with tricyclic antidepressants (TCA) and ECT leads to potentiation of both inhibitory (De Montigny & Aghajanian 1978; De Montigny 1984) and excitatory responses (Menkes et al 1980). The effect on post synaptic responses is not seen after chronic SSRI or MAOI treatment (Aghjanian 1981; Charney et al 1981; Blier & de Montigny 1985). Chronic treatment with SSRI leads to decreased sensitivity of both the somatodendritic and terminal autoreceptors and treatment with MAOI changes the sensitivity of the somatodendritic autoreceptor alone (see Section 1.3.4.3). Neither of the autoreceptors is apparently affected by chronic administration of other antidepressant drugs or ECT (Blier & de Montigny 1980).

Blier et al (1987) have reviewed electrophysiological evidence that suggests the net effect of any AD treatment is an enhancement of 5HT neurotransmission. TCA, "atypical" AD and ECT sensitise postsynaptic neurons to 5HT but have no effect on the presynaptic neuron, whereas MAOI and SSRI desensitise neuronal presynaptic autoreceptor responses (Table 1.5.1).

In keeping with this hypothesis is the fact that 5HT1a agonists, developed as anxiolytic drugs but which have antidepressant properties, desensitise the somatodendritic autoreceptor after prolonged use and enhance 5HT neurotransmission through an increase in DRN firing activity and direct postsynaptic receptor agonist effects . The drugs have no effect on postsynaptic 5HT1a density or electrophysiological responses to 5HT, although the agonist drugs themselves directly activate postsynaptic 5HT1a receptors (Blier et al 1990a).

Electrophysiological studies have also shown that chronic AD treatment leads to an increased sensitivity of α_1-adrenoceptor responses (Charney et al 1981; Aghajanian 1981) and a decrease in β-adrenoceptor (Aghajanian 1981) and presynaptic DA receptor responses (Willner 1983).

It seems then that in animal models alteration in 5HT neurotransmission is an important mode of action of AD therapies. Neuroendocrine challenge studies in humans have provided some evidence that these conclusions may hold true for

Table 1.5.1 The effect of antidepressant drugs and ECT on aspects of 5HT neurotransmission

Treatment type	Post-synaptic responsiveness	Terminal autoreceptor responsiveness	Somatodendritic autoreceptor responsiveness	Net effect on 5HT neurotransmission
TCA	↑	0	0	↑
ECT	↑	0	0	↑
MAOI	0	0	↓	↑
SSRI	0/↓	↓	↓	↑
$5HT_1a$ agonists	0	0	↓	↑

Adapted from Blier et al (1990)[a]

their action in normal and depressed patients although the results are much more conflicting (Meltzer et al 1982b; Charney et al 1984; Cowen & Andersson 1986). The advent of PET scanning may allow further exploration of their mechanism of action and of the implication that this might have for the pathophysiology and aetiology of depression. The full effect of such treatments undoubtedly is much more complex than an isolated effect on 5HT neurotransmission. Some of the changes in other neurotransmitter systems have been mentioned above. It is apparent that the 5HT neurotransmitter system has intimate links with the noradrenaline neurotransmitter system (Sulser 1987). Changes in β-adrenoceptors with chronic AD treatments requires an intact 5HT system (Nimgaonkar et al 1985; Stockmeier et al 1985; Sulser 1987) and the reduction in 5HT2 receptors with AD is dependent on an intact noradrenaline system (Green & Deakin 1980; Gravel & de Montigny 1987).

Despite enhancement of 5HT neurotransmission being the apparent mechanisms of action of antidepressants it is not possible to predict therapeutic response in depressed patients who appear to have a deficiency of 5HT function (Hasio et al 1987; Joyce & Paykel 1989). There are also other effective antidepressant treatments which do not appear to have a mechanism of action, at least directly, through 5HT transmission. This, together with the fact that selective 5HT reuptake inhibitors have no marked superior clinical effect over less selective drugs, would suggest that the effect of antidepressant drugs is compatible with an important deficiency of 5HT function in depression but it does not mean that it is the only important factor.

1.5.3.3 Lithium and electroconvulsive therapy

Lithium is a useful drug in 50% of patients with major depression who do not respond to single therapy . It appears to have an enhancing effect on post-synaptic 5HT receptors (De Montigny et al 1983; Price et al 1989; Wood & Goodwin 1987). ECT, as discussed above, also has effect on 5HT turnover and receptors although whether it augments or diminishes function is less clear. It also has effect on many other neurotransmitter systems (Grahame- Smith et al 1978; Green & Heal 1985).

1.5.4 CONCLUSIONS

There is substantial evidence that there is a dysfunction of 5HT in depression. It is less clear exactly what this dysfunction is and the mechanism of action of therapeutic interventions. The data would suggest that a deficiency of synaptic 5HT transmission is the most likely pathology and that antidepressant treatments correct this in a variety of different ways. Deakin (1989) has gone further to suggest that there is excessive 5HT2 neurotransmission, probably by increase in receptor numbers in depression, and a deficiency of 5HT1 mediated function although not a decrease in numbers of these receptors. The action of antidepressant therapies is to rectify this imbalance.

5HT may also have an important role in a variety of other psychiatric disorders (Coccoro & Murphy 1990). Thus it is unlikely that dysfunction of 5HT neurotransmission is the full explanation of the pathophysiology or the aetiology of depressive illness. It is clear from anatomical evidence that the 5HT system comprises several subsystems with regional and receptor specificity and independence of function, but with interactions, modulation and co-operation between receptor subtypes and perhaps system subtypes (Aghajanian et al 1987; see Section 1.3.4.4). The 5HT system(s) also interact with other neurotransmitter systems, particularly the catecholamine system (Sulser 1987; Yamamoto et al 1988), and it may be that clues to the further understanding and therapeutic advances may lie in study of these interactions.

It is also clearly important to consider that "depression" may not be a single entity and, although at present defined by phenomenology and symptomatology, it may be that future classification on biochemical or pathological features may show that there are subtypes of the disease and that the syndrome of clinical depression may be the final common pathway of a variety of pathologies.

1.6 MENTAL ILLNESS, WATER BALANCE AND VASOPRESSIN

Abnormalities of fluid intake, and sometimes fluid balance, are a feature of several mental illnesses and have been noted for over 60 years (Sleeper & Jellinek 1936). The importance of the clinical problem is three fold . Firstly that life threatening emergencies may arise with coma, seizures and sometimes death (Raskind 1974); secondly that a significant number of long term complications are associated with polyuria (Blum & Friedland 1983; Illowsky & Kirch 1988); and thirdly that many of the symptoms of milder hyponatraemia may be attributed to the mental illness (eg weakness, lethargy, anorexia, nausea) and consequently both the hyponatraemia and depression or other illness may be inappropriately managed.

Estimates of the prevalence of hyponatraemia associated with mental illness are poor and much of the information and hypotheses of aetiology and pathophysiology rests on case reports. Crammer (1991) noted that 10% of a hospital population of longterm mentally ill (mostly chronic schizophrenia) had polydipsia. Others have shown that polydipsia occurs in 3-18% of chronic psychiatric inpatients and that 25-50% of these patients had episodes of water intoxication (Jose & Perez-Cruet 1979; Hariprasad et al 1980; Jos et al 1986). There is a mortality of about 10% over 2 years in patients with episodic water intoxication. Approximately 20% of deaths in schizophrenic men under 50 years old are attributable to the consequences of abnormal fluid balance (Vieweg et al 1985).

The following disturbances of psychological function have been reported to have associated abnormalities of drinking behaviour and/ or water balance:-

Unipolar depression

Bipolar affective disorder

Schizophrenia

Other psychotic disorders

Anorexia Nervosa

Chronic alcoholism

Organic brain syndromes

In addition abnormal drinking behaviour is seen in the psychogenic polydipsia

syndrome and in some patients with attention seeking behaviours of either neurotic or psychopathic origins (Crammer 1991).

1.6.1 FLUID INTAKE

1.6.1.1 Thirst

Alterations in thirst sensation or perception in association with mental illness have not been researched to any large extent. It is rarely advanced by patients as the reason for drinking, except in patients with lithium toxicity where renal tubular insensitivity to AVP leads to an increase in plasma osmolality and consequent osmotic stimulation of thirst (Singer et al 1972; Gold et al 1983d). Goldman et al (1988) have suggested that in chronic schizophrenic patients with polydipsia and intermittent water intoxication there is evidence of a decreased osmotic threshold for thirst although the 'sensitivity' (Section 1.1.2.1) remains unchanged. This did not explain the degree of fluid ingestion in half of the subjects whose desire for water did not seem to be related to osmotic stimulus.

Many other drugs used in the treatment of mental illnesses (tricyclic antidepressant, phenothiazines) have anticholinergic side effects which cause a dry mouth and often lead to a moderate increase in fluid intake. Interestingly the SSRI are also noted to cause a dry mouth in 11% of patients (placebo 7%) (Cooper 1988) despite the absence of anticholinergic activity in these serotonin selective drugs.

Hypodipsia may be a feature of severe depressive illness and catatonic stupor where fluid intake is much decreased but no documentation has been carried out in these conditions.

1.6.1.2 Non-thirst

It has been documented that excessive fluid intake occasionally occurs in episodes of mania, not apparently explained by increase in physical activity or increase in thirst (Dingman et al 1957; Santy & Schwartz 1983; Zubenko et al 1984; Crammer 1986; Keshavan & Keshavan 1986).

There have been no studies of changes of fluid intake in depression. There are occasional case reports of polydipsia occurring in psychotic depression which resolves with adequate treatment of the affective disorder (Zubenko et al 1984).

Probably about 10% of psychiatric longstay patients exhibit polydipsia. In the search for physiological disturbances in schizophrenia Hoskins & Sleeper (1933) found marked differences in daily urine volumes between schizophrenic patients and normal controls. A greater than normal variation in daily urine output was also found. Other studies have shown that there may be a diurnal pattern with increased fluid ingestion from morning to evening, confirmed by weight increase through the day by as much as 0.5kg per hour, and plasma sodium changes (Delva & Crammer 1988). The reason for this excessive fluid ingestion is not always clear. Patients with persisting delusions may report that they were "told" to or that it is a means of cleansing (Dubovsky et al 1973; Alexander et al 1973; Smith & Clark 1980; Hariprasad et al 1980) . A number of authors have reported that polydipsia increases when the psychosis is worse. This may be as part of the delusional construct, or it has been suggested that fluid ingestion may be anxiolytic (Lee et al 1989). It has also been suggested that polydipsia is a late development in schizophrenic pathology (Vieweg et al 1987). Others have shown that a personal or family history of alcohol abuse is associated with polydipsia in chronic schizophrenia (Ripley et al 1989). These authors suggested that the drinking behaviour may be learned and/or that mild overhydration is pleasurable to these schizophrenic patients. Other have suggested that the drinking behaviour is a facet of institutionalisation and boredom and when distracted the behaviour ceases. Some patients are apparently unaware of their excessive fluid intake (Hariprasad et al 1980). Sleeper & Jellinek (1936) found that schizophrenic patients with polydipsia were more likely than other schizophrenic patients to have a high IQ, fewer negative symptoms and more affective symptoms. These findings were confirmed by Lawson et al (1985) but were not by Kirch et al (1985). This latter group suggested that polydipsic schizophrenics were a subpopulation with more marked neurological disturbances.

Psychogenic polydipsia occurs in a variety of psychological disturbances which are not psychotic or affective disorders. There has been little research done in this very mixed group of patients. It is poorly understood and difficult to manage (Barlow & De Wardener 1959; Crammer 1991).

1.6.2 WATER BALANCE

1.6.2.1. Affective disorder, antidepressants and hyponatraemia.

The psychiatric and medical literature frequently reports the occurrence of hyponatraemia in patients with depression, usually associated with antidepressant or other drug treatments (Sandiffer 1983). The following have been associated with case reports of hyponatraemia and depression.

Fluoxetine	Amitriptyline	Nortriptyline	Trazadone
Fluvoxamine	Phenelzine	Desipramine	
Dothiepin	Lofepramine	Clomipramine	
Imipramine	Amoxapine	Doxepin	

The range of drugs, often with very different putative pharmacological and therapeutic modes of action would perhaps suggest that the pathology arises from the disease rather than the drug.

There are no reports of hyponatraemia occurring with these drugs when used in non-depressive illness (eg. in dysaesthetic pain). This could, however, be a reporting deficit rather than confirmation of disease specificity. Only occasional studies have sought to determine whether drug or illness was responsible for the hyponatraemia. Cohen et al (1990) rechallenged a depressed patient with Fluoxetine after 8 days drug free treatment of hyponatraemia. Serum sodium began to fall after 5 days and returned to normal again 5 days after discontinuation of the Fluoxetine. Staab et al (1990) did not find that hyponatraemia recurred in a patient who was rechallenged with Fluoxetine. It is interesting that in many case reports of hyponatraemia patients are often changed, without adverse effect,to antidepressants which have been previously reported as associated with hyponatraemia (Abbott 1983; Fort et al 1985). This may suggest either an idiosyncratic effect or a disease state-dependent mechanism.

There have been few studies which have considered the possibility that affective illness leads to fluid retention and hyponatraemia. Hyponatraemia has very occasionally been noted to occur in drug free depression, usually when there have been psychotic features (Brown et al 1983; Zubenko et al 1984).

Mania, with psychotic features, has also been reported to be associated with hyponatraemia (Zubenko et al 1984). In both of these disorders the hyponatraemia resolves with appropriate treatment of the disordered mental state, together with fluid restriction.

The mechanism of hyponatraemia associated with affective disorders is not clear although some studies have shown abnormalities in the osmotic regulation of AVP. Gold et al (1983c) looked at the pattern of AVP secretion in response to a hyperosmotic stimulus in drug free patients with different classifications of affective disorder. They found no differences in patients and controls in baseline plasma or urine biochemistry or in plasma AVP concentrations. There was also no difference in 24 hour urine output. Although there was no apparent abnormality in the threshold for, or sensitivity of, AVP release in the depressed group as a whole 50% of those with a bipolar depression showed either a diminished sensitivity or increased threshold of release. The loss of osmotic sensitivity was, however, not clinically important (remained >0.2pmol /ml per mOsm/kg) (Section 1.1.2.1). The subjects with bipolar disorder who were in a manic episode showed no group differences compared to normal controls but 50% of the subjects showed a slight increased sensitivity of AVP release and one subject had an abnormally high threshold. No abnormalities were found in patients who were euthymic or who had unipolar depression. Bipolar patients studied in both affective phases showed significant changes when depressed compared to during hypomania (Gold et al 1984). Laruelle et al (1990) found a decreased concentration of the AVP associated neurophysin in plasma of depressed patients compared to controls and suggested that this gave a more overall assessment of AVP secretion than isolated plasma AVP measurements.

Crammer (1986) and Keshavan & Keshavan (1986) noted marked weight gain, sodium retention and sometimes oedema in some patients with mania. The metabolic abnormality appeared to be reversed in the depressive phase of the bipolar illness with demonstrable sodium excretion. It seemed likely that the metabolic changes were secondary to, and not aetiological in, the mood state since the sodium imbalance was observed shortly after the change in mental state (Crammer 1991). Changes in NA^+/K^+ ATPase activity in both bipolar and unipolar affective disorders have been the subject of much research in the

1970's. The findings from this work are inconclusive but point to a possible abnormality of sodium metabolism in affective disorders in a subgroup of people (El Mallakh 1983; Naylor & Smith 1981).

Thus abnormalities of water balance in affective disorders remain largely unexplained. It is not a common problem and may sometimes be drug associated. It may be that there is a subgroup of people who have abnormalities of either osmotically regulated AVP secretion or sodium metabolism and it is these individuals who are at risk of hyponatraemia during acute illness whether or not they receive antidepressant medication.

1.6.2.2. Psychosis and water intoxication

Water intoxication in psychiatric patients most frequently occurs in association with acute psychotic, non-affective illness (Barahal 1938; Murphy & Zelman 1964; Alexander et al 1973; Dubovsky et al 1973; Raskind et al 1975; Fowler et al 1977; Smith & Clark 1980). Whether these patients have polydipsia at other times and what percentage of those chronic psychiatric patients with polydipsia have intermittent episodes of water retention is not clear from the literature (Crammer 1991). In some of the reported cases AVP is detectable in the plasma (Goldman et al 1988, Delva et al 1990) in others SIAD does not appear to be a feature of the fluid retention (Smith & Clark 1980).

Several drugs have been associated with the occurrence of hyponatraemia in these patients and may cause non-osmotic AVP release (Rao et al 1975; De Rivera 1975; Matuk & Kalyanaraman 1977; Vincent 1978) or possibly other modes of water retention (Deveraux & McCormick 1972; Mendelson & Deza 1976) . Withdrawal and rechallenge studies have often not been able to show alteration in free water clearance after reintroduction of the drug (Miller et al 1973; Fowler et al 1977; Kendler et al 1978; Kosten & Camp 1980). Two studies have however shown impaired free water clearance on rechallenge with thiothixene and haloperidol (Ajlouni et al 1974; Peck & Shenkman 1979). No measurements of AVP or of blood pressure were made in these studies and the results must be interpreted with caution. In contrast it has been noted that the commencement or increase of phenothiazines in acute psychosis has prevented dilutional hyponatraemia occurring in patients with previously documented

episodes (Dubovsky et al 1973; Hariprasad et al 1980).

There have been several studies in normal people to explore further the possibility that these drugs cause AVP release. Dubovsky et al (1973) showed that haloperidol did not increase plasma AVP although plasma PRL did rise. Similarly Rowe et al (1979) showed no effect of fluphenazine on plasma AVP. In both of these studies blood pressure remained constant. Dorsa & Raskind (1985) found that chlorpromazine did not effect plasma AVP unless there was also a hypotensive effect. In a prospective study in five acute psychotic schizophrenic patients Dorsa & Raskind (1985) found that antipsychotic medication did not cause a rise in AVP, and two patients with initially elevated AVP had normal levels 24hr after drug administration. It seems therefore that, in the absence of hypotension, antipsychotic drugs are unlikely to lead to AVP secretion. In an unconfirmed study Kovacs et al (1957) found that chlorpromazine potentiated the peripheral effect of AVP irrespective of its origin. Dyball (1968) found that chlorpromazine inhibited AVP release in animals but other phenothiazines stimulated release. The possibility of drug induced effects on AVP needs still further investigation.

The occurrence of hyponatraemia has been noted in drug free patients (Chinn 1974; Nielsen 1974; Rendell et al 1978). Is it possible then that psychosis itself may cause AVP release? Dorsa & Raskind (1985) have reported that two of five acutely psychotic patients had elevated plasma AVP. Robertson (1979) reported two psychotic patients one with plasma AVP concentrations not related to plasma osmolality and one with a reset osmostat. Several other workers have reported the occurrence of SIAD in drug free psychotic patients (Dubovsky et al 1973; Raskind et al 1975; Hariprasad et al 1980). It could be that the severe psychological distress and anxiety caused by the psychosis and hospitalisation is responsible for the elevated and abnormally regulated AVP (Mandell et al 1964). This seems unlikely as AVP concentrations in hospitalised psychotic patients were found to be much greater than patients who were admitted with anxiety disorders (AVP concentration similar to normal controls) despite similar levels of anxiety shown by rating scales (Raskind et al 1977).

Other authors have suggested that inappropriate release of AVP is a facet of the cerebral pathology of schizophrenia in a subgroup of patients (Vieweg et al 1987). Some case studies have explored more thoroughly the pathology and

shown that an osmostat reset and possibly also non-osmotic reset may occur (Hariprasad et al 1980). Hobson & English (1963) showed not only an abnormal excretion of a water load in a drug free schizophrenic patient but also evidence of acute autonomic dysfunction with increased core temperature and an absence of sweating. This supports the theory of Kirch et al (1985) who suggested that patients with polydipsia and water intoxication are a subgroup of schizophrenics with evidence of neurological damage and dysfunction.

Goldman et al (1988) performed an elegant study of chronic schizophrenic patients who were not acutely psychotic or water intoxicated but had had previous documented episodes of fluid intoxication. The control group were matched schizophrenic patients without fluid balance problems. The authors showed that subjects with polydipsia were unable to excrete a water load normally, despite suppression of AVP. This suggested that there was increased renal tubular sensitivity to AVP in these patients. A 5% saline infusion showed there to be no change in sensitivity of AVP release but a substantial reduction of osmotic threshold in those patients with polydipsia (mean change of 11mOsm/kg).

Thus water intoxication in chronically disturbed psychiatric patients is a fluctuating and unpredictable abnormality although it appears to have some relationship with episodes of acute psychosis. The mechanism of the fluid retention is unclear and may be multifactorial. To overwhelm the excretory ability of the kidney more than 800ml/hr or 20l /day must be ingested. It would seem that excessive drinking is the primary abnormality in these patients but an extra mechanism, possibly abnormal AVP secretion and altered renal tubular sensitivity, leads to water intoxication. The reason for these abnormalities is not clear. Raskind et al (1975) have suggested that psychosis, inappropriate AVP secretion and polydipsia are linked by a common disturbance of CNS function, possibly dopaminergic hyperactivity. Recent reviews of the subject have further expanded the hypothesis (Illowsky & Kirch 1988; Crammer 1991). It is an attractive theory but does not explain all abnormalities, particularly a possible renal tubular hypersensitivity to AVP. Further research in this area is needed since 20% of deaths in young schizophrenics are due to fluid retention and the move towards community care may mean a delay in management.

1.6.2.3 Eating disorders and water balance

Abnormal water balance has been noted in anorexia nervosa. Vigersky et al (1976) have shown that partial CDI occurred in some patients with an inability to concentrate urine adequately and an impaired secretion of AVP. Gold et al (1983b) studied the response of AVP release to 5% saline infusion and showed that some patients with the abnormality had an insensitivity of AVP to osmotic stimulation and others had random fluctuations of AVP release, independent of plasma osmolality. It has been postulated that the abnormal regulation of AVP release may be related to weight loss rather than to the pathology of anorexia nervosa . There have been occasional reports of water intoxication (Silber 1984) which would suggest that there may be more than one pathology of fluid balance in this condition. There have been no studies of osmotic regulation of AVP in bulimia nervosa and no reports of abnormal water balance in these patients.

1.6.3 MENTAL ILLNESS AND AVP SECRETION

In 1978 Gold & Goodwin put forward the hypothesis that AVP was directly involved in affective illness. Their argument was based on the following:-

a) That therapeutic drugs may change AVP activity and sensitivity.

b) That AVP has effects on cognitive function.

c) That central opiate release is increased by AVP, with implications for reward/punishment perception.

d) That AVP has effect on circadian rhythms.

The authors suggested that AVP function may be diminished in depression and augmented in mania. This group went on to try AVP analogs as a treatment for depression. They found subtle, but clinically insignificant improvements in cognitive function. (Gold et al 1979; Weingartner et al 1981). There was improvement in mood in only two of nine patients (Gold et al 1979; 1983c). Despite this apparent absence of therapeutic benefit there is evidence to suggest a role for AVP in the pathophysiology of depression and other mental illnesses.

1.6.3.1 AVP in the CSF of patients with mental illness

AVP is present in the CSF although its origin, regulation and function are poorly understood in the human (Robinson 1983). Studies have suggested that it is probably not derived from plasma since intravenous infusion of large doses does not increase CSF concentration (Luerrson & Robertson 1980; Jenkins et al 1980). CSF concentrations are high in CDI where plasma levels are undetectable (Luerrson & Robertson 1980). This would suggest that AVP in the CSF derives from anatomically distinct population of cells . Immunohistochemical studies have shown that extra-hypothalamic AVP pathways, extending from PVN to 3rd and lateral ventricles, originate in magnocellular neurons different from those which project to neurohypophysis (Sofroniew 1980) and these pathways may be the source of CSF AVP. Animal studies have shown a circadian rhythm of AVP in CSF but not in plasma (Reppert et al 1981; Seckl & Lightman 1987). This is entrained by the light/dark cycle and is reversed in nocturnal animals. It appears that the rhythm may be controlled, and possibly directly secreted into the CSF, by the SCN (Reppert 1985).

CSF AVP, like plasma AVP, increases with haemorrhage and vagal nerve stimulation but does not have a direct relationship with plasma or CSF osmotic change. Inappropriate concentrations of plasma AVP are reflected in elevated CSF levels (Luerrson & Robertson 1980). There are, therefore, some regulatory pathways in common.

The function of CSF AVP is speculatory. It is possible that the CSF acts as a method of conveying peptides to distant areas of the brain (Robinson 1983) and CSF AVP may be involved in memory consolidation or possibly reproductive activity in the female. Because of the circadian rhythm it may be involved in regulation of other behaviours which have a circadian pattern (Reppert 1985). It has also been thought to be involved in regulation of cerebral water permeability (Raichle & Grubb 1978) and in maintenance of intracranial pressure (Noto et al 1978; Sorensen et al 1984).

There have been few studies which have looked at CSF AVP or neurophysin levels in mental illness. All of these have considered data at isolated time points. The data is essentially contradictory and no pattern emerges to suggest

pathophysiological mechanisms.

There is much evidence that in affective disorder there are disturbances of circadian rhythm with abnormalities of sleep cycle, endocrine secretory patterns, mood variations and temperature rhythms (Healy & Williams 1988; Gold et al 1988). It could be postulated, although with no evidence, that CSF AVP maybe related to this circadian dysrhythmia, either directly or indirectly via a common dysfunctional regulator ie the SCN.

It has been noted that in psychotic, bipolar depression, CSF AVP concentrations were higher than in normal controls and in non-psychotic, bipolar, depression the levels were lower than in controls (Gold et al 1983a). The latter group returned to near normal levels on recovery. CSF AVP levels are normal in unipolar depressed patients. Concentrations in mania were not significantly different from controls in this study but were elevated in another (Sorensen et al 1985). These authors found similar levels to controls in depressed and schizophrenic patients (both acute and chronic). Another group have found significantly lower CSF AVP concentrations in both endogenous and non-endogenous depression but no significant changes in manic illness (Gjerris et al 1985). Legros et al (1983) found increased concentration of Neurophysin II (oxytocin-associated) in the CSF of bipolar depressed compared to unipolar depressed patients. This was confirmed in a second study (Linkowski et al 1984) where lower neurophysin I (AVP associated) levels were seen in unipolar depression, and higher levels in bipolar depression, compared to controls.

In schizophrenic patients Neurophysin I levels were found to be decreased but neurophysin II levels increased compared to controls. It is difficult to interpret changes in associated proteins and extrapolate to changes in the peptide since disease states could possibly alter the normal relationships and regulatory mechanisms.

Gold et al (1983c) observed that there was an abnormal plasma:CSF ratio of AVP in anorexia nervosa with much greater levels in the CSF than in plasma. These changes correlated with mild abnormalities in the osmoregulation of plasma AVP. The abnormalities were apparently corrected by weight gain. Abnormalities of CSF AVP in psychosis and anorexia nervosa remain unexplained. Their correction with improvement of the disease state could

suggest aetiological importance but is more likely to suggest secondary changes to underlying pathology.

1.6.3.2 Magnocellular secretion and plasma AVP

AVP is known to be involved in the release of ACTH in conjunction with CRF (Lamberts et al 1984; Antoni 1986). It has been reported that in some depressed patients the cortisol response to AVP is absent (Krahn et al 1985) and it is postulated that the abnormalities of cortisol secretion in depression may be linked to dysfunctional hypothalamic AVP secretion (Schatzberg & Nemeroff 1988; Meller et al 1988; Kathol et al 1989). Laruelle et al (1990) found no evidence of excessive AVP secretion in depressed patients with abnormal dexamethasone suppression of plasma cortisol.

Abnormalities of plasma and osmoregulated AVP secretion in mental illness have been discussed above (Section 1.6.2). The return of plasma AVP concentrations to normal concomitant with improvement of psychosis may suggest a primary role for AVP in psychotic disorders. This is difficult to establish or refute without the availability of AVP antagonists or suppressants.

It would seem than that AVP is important in disturbances of fluid balance, with potentially lethal consequences, in a variety of psychiatric disorders, at least in a subgroup of patients. Whether these marked abnormalities and the minor, less clinically significant changes have any role in the primary pathophysiology of the disturbed mental state is not resolved. It would seem more likely that disturbance of AVP regulation, both in plasma and CSF, is linked to the mental illness by a common abnormality in regulatory pathway. Whether abnormalities in 5HT neurotransmission are the common link is purely speculative since there is no research on this.

1.7 GENERAL HYPOTHESES

1. That 5HT acts as a modulator in the physiological control of AVP secretion in response to changes in plasma osmolality.

2. That manipulation of total brain 5HT content, synaptic availability of 5HT or 5HT receptor sites by pharmacological means may cause abnormalities in the physiological regulation of AVP secretion in response to osmotic stimuli.

3. That this modulatory action is mediated through the 5HT2 or 5HT1c receptors.

4. That there is an abnormality in the 5HT central nervous system of depressed people which, by itself or together with 5HT active agents used as antidepressants, may lead to an abnormality in the modulatory role of 5HT on AVP secretion in response to the osmotic milieu.

The objective of this research was to explore the hypothesis that 5HT is involved in osmoregulated AVP secretion. This was to be approached in three ways. Firstly through studies in normal man, secondly by studies in depressed patients, as a putative disease model of 5HT neurotransmission, and thirdly to more extensively explore the effect of pharmacological manipulation of 5HT neurotransmission using an animal model of osmoregulation .

AIMS

1. To study the effect of pharmacological manipulation of central 5HT neurotransmission on osmoregulated AVP secretion in normal man.

2. To study osmotic homeostasis in depression with reference to the effect of treatment and disease state on water balance, basal osmotic status and osmoregulated AVP secretion.

3. To explore the putative role of 5HT in osmoregulated AVP secretion in an animal model using 5HT active drugs with relevance to antidepressant therapy, with particular reference to the role of the 5HT2 receptor.

4. To explore the possibility that 5HT directly modulates AVP secretion by study of 5HT receptors on the magnocellular neurons in the SON and PVN of the hypothalamus with particular reference to the 5HT2 receptor subtype.

SECTION 2

METHODOLOGY

2.1 THE ASSESSMENT OF OSMOREGULATION AND AVP SECRETION

2.1.1 ASSESSMENT OF OSMOREGULATION IN MAN

The osmoregulation of AVP secretion in man has been discussed in Section 1.1.2.1. Two standard methods of dynamic assessment of osmoregulatory function have been developed for clinical and research purposes.

2.1.1.1 Water load test

The study of osmoregulatory ability after ingestion of a standardised volume of water (20ml/kg) has allowed assessment of disorders of hypersecretion of AVP ie SIAD. The test has proved particularly useful where baseline pOS and pNa are within normal limits (Robertson 1981).

Ingestion of the fluid leads to a fall in pOS of ≥5mOsm/kg within 15-30min (see Figure 2.1). In normal man this inhibits AVP secretion. The time to minimal AVP concentration in plasma will depend on basal concentration since circulating AVP has a half life of 12-20min. Under normal circumstances this is reached about 60min after plasma hypo-osmolality (75-90min after ingestion of fluid load). The suppression of AVP secretion allows diuresis which reaches a peak, with urine osmolality ≤100mOsm/kg, at 60-90min after fluid ingestion. In normal man approximately 90% of the ingested water load is excreted within 4 hours. Failure of either urinary dilution to < 100mOsm/kg or a cumulative urine output of <90% at 4hr is considered to be abnormal.

<u>Free water clearance</u>

Free water is the volume of urine which is excreted over and above that required for the excretion of the solute content of the urine. Solute excretion is controlled by proximal tubular function whilst free water excretion is dependent on the permeability of the distal tubule ie on AVP concentration.

Calculation of free water clearance (C_{H2O}) is from the equation Uvol/time x(1-uOS/pOS).

C_{H2O} is a helpful measure of distal tubular function and as such an indirect measure of AVP function.

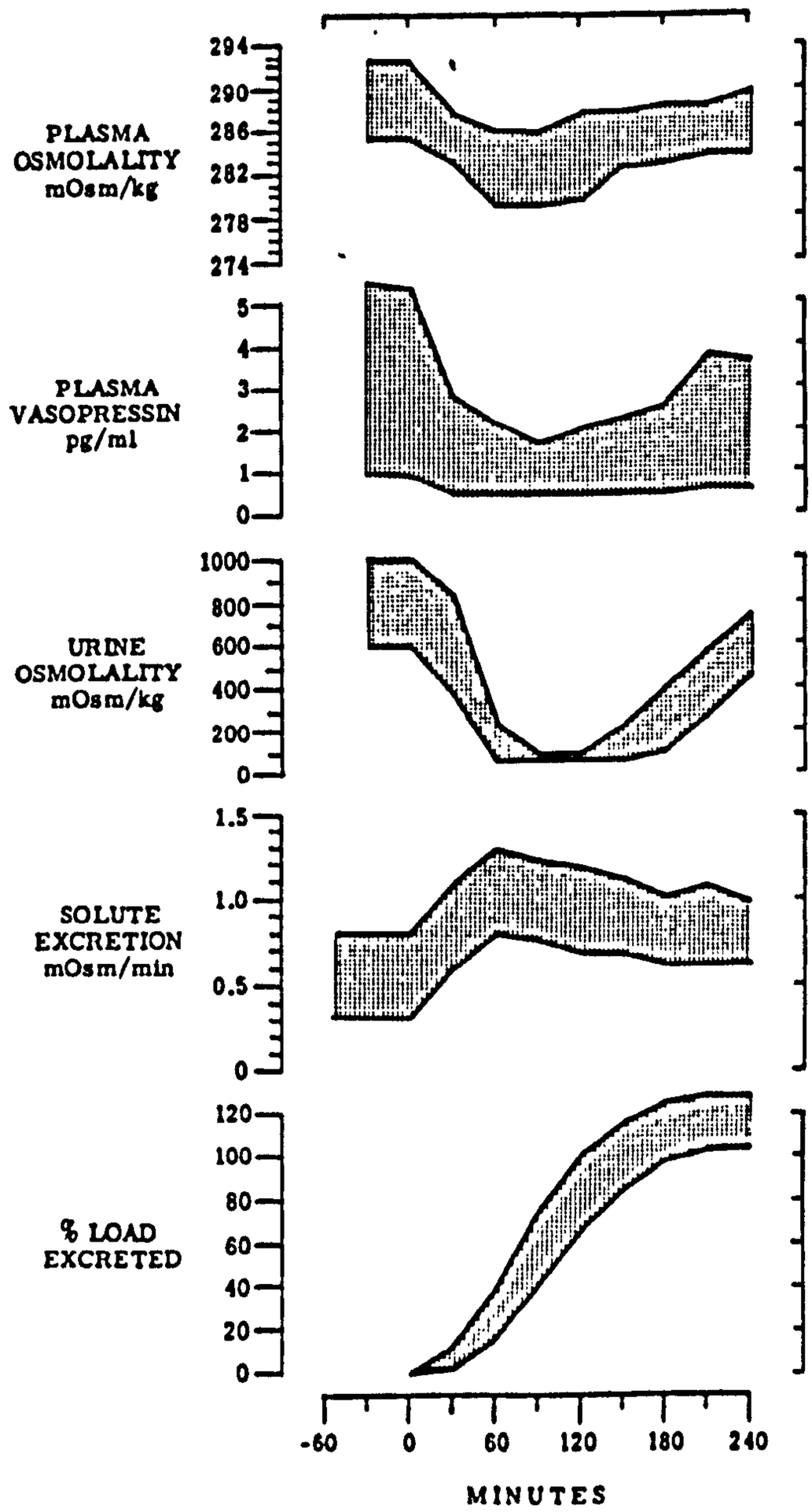


Figure 2.1

The 95 percentiles in normal man of the effect of a standard oral water load (20ml/kg) on plasma osmolality (a), plasma AVP (b), urine osmolality (c) and free water clearance (d). The cumulative excretion expressed as % water load ingested is also shown (e).

Adapted from Robertson (1981).

2.1.1.1.1 *Protocol*

a) Subjects fasted and refrained from drinking from midnight.

b) At 9AM venous cannula was inserted for blood sampling.

c) After an equilibration period, sitting for 30min, a baseline blood sample was taken. The bladder was emptied and urine sample collected.

d) Subjects drank 20ml/kg water over 15-20min.

e) Blood samples were taken at 30, 60, 90, 120, 180 and 240min after the start of fluid ingestion.

f) Urine was collected at 60, 90, 120, 180 and 240min after the start of water load ingestion.

g) Blood pressure was monitored at 10min intervals in the first hour and thereafter at 30min intervals.

h) A subjective assessment of nausea was made at standardised intervals during the test by a visual analogue scale (10cm line) (Appendix 1).

i) Subjects remained seated throughout the study except when voiding urine.

Blood samples

Blood samples were collected as described in Section 2.2.5. Aliquots of plasma were stored at 4^0C for measurement of pOS and pNa in batch. Aliquots of plasma for AVP measurement were stored at -40°C for extraction and assay.

Urine samples

At each time point the volume of urine collected was measured and a cumulative account was made as percentage of oral load ingested .

Aliquots of urine for measurement of osmolality (uOS) and calculation of C_{H2O} were stored at 4°C.

2.1.1.2 Hypertonic saline infusion

Section 1.1.2.1 has discussed in some detail the use of infusion of hypertonic fluids to assess AVP secretion. The rise in AVP concentration is linearly proportional to the change in pOS provided that the rate of change of pOS is <2% per hour (Robertson et al 1976; Thompson et al 1986). The measurement of pAVP and pOS during an infusion of hypertonic fluid allows calculation of the sensitivity of AVP secretion to change in pOS and the theoretical osmotic

threshold of AVP secretion. In normal man the sensitivity of AVP secretion lies between 0.2 and 0.7pmol/l per mOsm/kg and the threshold of release is between 282 and 289mOsm/kg (see Figure 1.1.2).

Thompson et al (1986; 1991) have shown that a reproducible, linear response of AVP to change in pOS ($r=0.96$ $p<0.001$) is achieved during infusion of 5% NaCl (855mmol/l) at 0.06ml/kg/hr. Infusion at this rate increases pOS by 15mOsm/kg over 2hr (approximately 2.5%).

The test can be used to assess both hyper and hyposecretion of AVP (Baylis & Robertson 1980; Baylis 1983).

2.1.1.2.1 *Protocol*

a) Subjects fasted and refrained from drinking from midnight.

b) At 9AM two venous cannulae were inserted, one for collection of blood samples and one for infusion.

c) After 30min recumbent equilibration a baseline blood sample was taken.

d) 5%NaCl (855mmol/l) was infused at 0.06ml.kg/hr for 120min.

e) Blood samples were taken at 30min intervals.

f) Blood pressure was taken at 5min intervals.

g) A subjective assessment of nausea was made at standardised intervals during the test by a visual analogue scale (10cm line) (Appendix 1).

h) Subjects remained recumbent throughout the study.

<u>Blood samples</u>

Blood samples were collected as described in Section 2.2.5. Aliquots of plasma were stored at 4^0C for measurement of pOS and pNa in batch. Aliquots of plasma for AVP measurement were stored at -40°C for extraction and assay.

In all studies of osmoregulation in this research subjects abstained from alcohol for 24hr before assessment. None of the subjects in Section 3 smoked cigarettes. Those in Section 4 who did smoke abstained from cigarettes from midnight prior to the study until after completion of the study. None of the subjects in Section 3 took any medication other than study drugs. Any concomitant drug therapy was noted for subjects in Section 4.

2.1.2 AN ANIMAL MODEL OF OSMOREGULATED VASOPRESSIN

The osmoregulation of AVP in the rat is relatively well understood. As discussed in section 1.1.2.4 the regulation of AVP secretion in the rat has many similarities to man but there are some differences. There is perhaps some difference in the solute specificity of the osmoreceptor cells in the two species. In rats, but not in man, glucose stimulates AVP secretion but this occurs only at very high plasma concentrations (Robertson & Vokes 1991). AVP secretion in both rat and man is sensitive to change in plasma osmolality consequent to alteration of plasma sodium concentration.

Dunn et al (1973) investigated dynamic osmoregulation of AVP secretion in the rat by intraperitoneal (IP) administration of fluid of differing sodium content and showed reproducible changes in pOS and pAVP. Basal pOS in this strain of rats was found to be 293.6±1.4 mOsm/kg and basal pAVP was 2.3±0.9pg/ml.

The estimated osmotic threshold of AVP release in the rat was higher than in man, but remained remarkably constant and reproducible, at least within a particular strain (mean 292±1.3 mOsm/kg in Sprague-Dawley rats). The sensitivity of AVP release was shown to be a more variable feature but it was consistently greater in rat than in man (mean 1.3±0.5 pmol/l per mOsm/kg).

This has become a well accepted model of osmoregulation in the rat (Robertson & Vokes 1991) and has been used repeatedly by my fellow researchers (eg Charlton et al 1989).*

The time course of changes in pOS and pAVP, derived from Dunn et al (1973) is shown in Figure 2.2. A hypertonic stimulus causes a significant rise in pOS by 15min and there is still a marked elevation of pOS at 30min. Plasma AVP is similarly elevated at 15 and 30min but, unlike pOS, has returned to basal concentration by 60min after the IP stimulus.

A hypotonic stimulus does not lead to a significant change in pOS until 30min after IP injection, however pAVP is significantly suppressed at both 15 and 30min.

*The injection of intraperitoneal fluid is obviously not solely an osmotic stimulus but causes pain, abdominal distension and perhaps psychological distress. These stimuli may perhaps have effect on osmoregulated AVP secretion but they have not been found to be a significant factor by these authors.

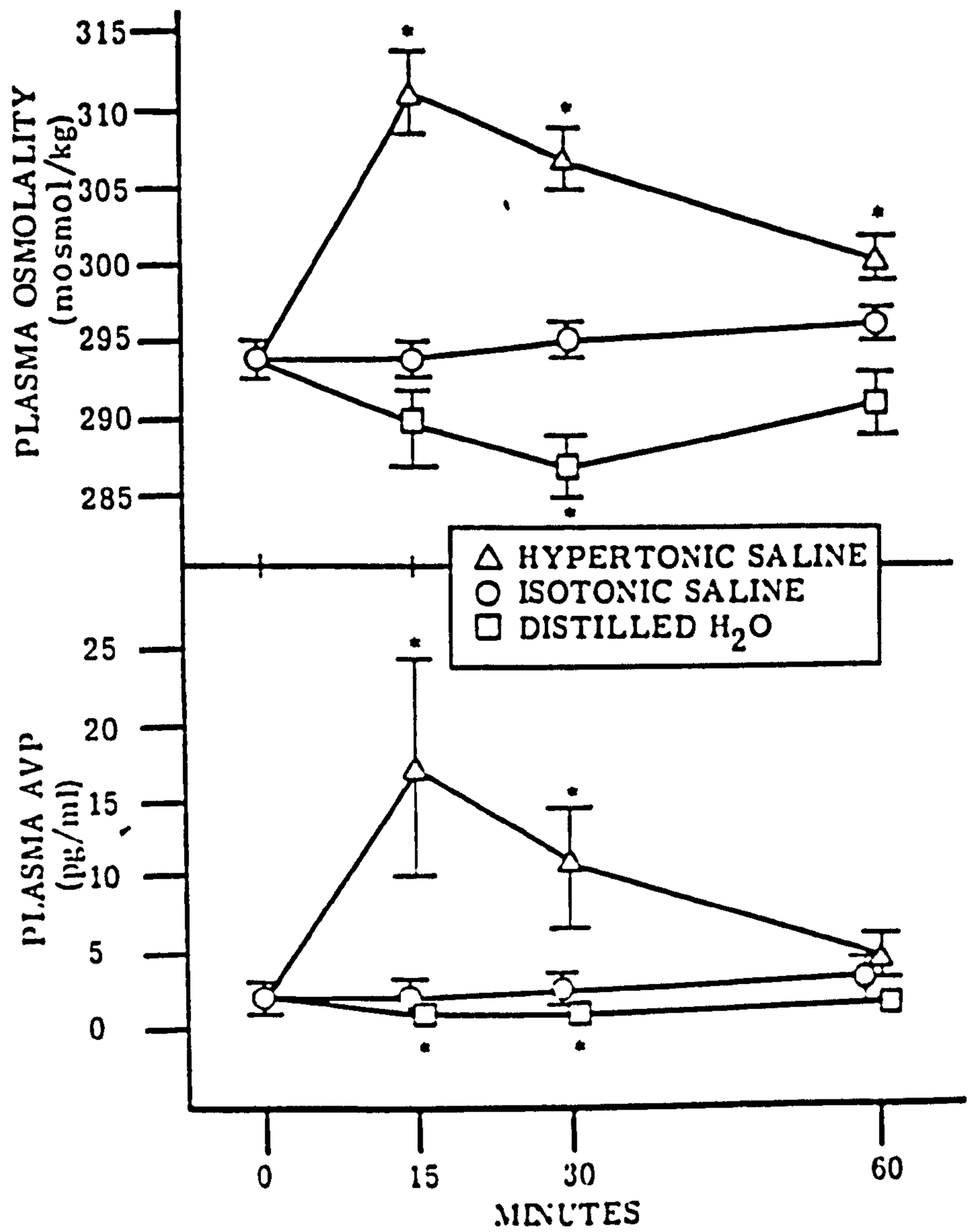


Figure 2.2

The effect of 20ml/kg IP 3 % NaCl (1000 mOsm/kg), 0.9 % NaCl (290 mOsm/kg) or distilled water on plasma AVP and plasma osmolality in rats.
Adapted from Dunn et al (1973).

2.1.2.1 The protocol

Intraperitoneal administration of 20ml/kg fluid using 23G needle into left iliac fossa.

Hypotonic stimulus; distilled water

Hypertonic stimulus; 3% NaCl approximately 1000mOsm/kg

Normal control; 0.9% NaCl approximately 290mOsm/kg

30min after fluid administration rats were killed by guillotine decapitation. No anaesthetic was administered since this may cause elevation of pAVP due to effect of drugs or hypoxia (Toth 1937;Rose et al 1984).

Trunk blood was collected by a heparinised funnel into chilled, heparinised tubes. Duplicate haematocrit samples were taken into heparinised microhaematocrit tubes (Hawksley, Surrey) and the remaining blood centrifuged within 30min at 2000g, 4°C for 15min for separation of plasma and cells. Plasma was aliquoted into 1.5ml eppendorf tubes and stored as appropriate for analysis.

Plasma samples

0.5ml aliquots of plasma were stored at 4°C and pNa and pOS were measured within 24hr in batches.

1.2ml aliquots of plasma were stored at -40°C for pAVP measurement. Samples were extracted and assayed within 3 weeks in batches of 20. (See Section 2.2.6 for assay data).

Collection of brain samples

Guillotined rat heads were immediately dissected in the following manner.

Scalp skin was cut to expose the cranium.

Bone was removed using nail clippers to expose undamaged brain in situ.

The olfactory bulb and optic nerves were severed from the forebrain using a sharpened spatula.

The brain was then gently lifted intact from the skull and placed on a chilled petri dish.

The frontal pole was removed using a scalpel blade. Olfactory cortex was

removed and remaining sample of frontal pole placed in two eppendorf vials, chilled over solid CO_2. Samples were frozen in liquid N_2.
The pituitary remained in the skull and the neurohypophysis was removed using forceps, placed in chilled eppendorf vials and frozen in liquid N_2.
Samples were stored at -80°C.

Unless otherwise stated male, albino Wistar rats were used in this research work. The University Comparative Biology Centre provided some of the rats but some were `imported', supplied by Bantin and Kingman ltd (Aldbrough, Hull). All were housed in the University facilities and kept in sawdust bed cages, in conditions of 12hr light/dark cycle, constant humidity and temperature with ab libitum food and fluid supplied.
The research was carried-out under Home Office regulations according to project licence numbers PPL 50/00237 and PPL 50/0201. All Studies except Study 5.6 were carried out with Dr JA Charlton, appointed as the Project Licence Holder`s deputy.

2.2 THE MEASUREMENT OF PLASMA AVP

The plasma concentration of AVP was measured by means of a radioimmunoassay (RIA) the details of which are described by Rooke & Baylis (1982).

2.2.1 RADIOIMMUNOASSAY

The principle of measurement of a substance by RIA requires competitive binding of a specific antibody to an unknown concentration of antigen in a sample with a known concentration of radiolabelled antigen.
A specific and sensitive antibody is added in excess to the sample containing antigen of unknown concentration. Labelled antigen of known concentration is added and binds in competition to the unlabelled antigen to the antibody sites. A second antibody is added which binds only to the first antibody and allows formation of a precipitate of antigen and antibody complex. After centrifugation the supernatant, with any unbound labelled antigen, is aspirated and the radioactivity of the precipitate is measured. The level of radioactivity, in comparison to that of the total labelled antigen added to the sample, is inversely related to the concentration of unlabelled antigen in the sample. Comparison of the radioactivity of the sample to radioactivity of standard concentrations of antigen allows estimation of the antigen concentration in the sample.

2.2.2 ANTIBODY

The antibody used in the assay of AVP was raised `in house'. Synthetic AVP (Ferring AB, Sweden) was conjugated to bovine thyroglobulin using a carbodiimide reaction and injected into rabbits. Resultant antisera exhibited high cross-reactiviity to synthetic AVP (100%) and very low cross-reactivity to AVP related analogues (LVP, DDAVP,OXY) and had minimal reactivity with other hormones.

2.2.3 EXTRACTION FROM PLASMA

The physiological plasma concentration of AVP is very low (0.3-10pmol/l) and extraction with consequent concentration of AVP is required to measure the concentration in small volumes (1-2ml) of plasma. In this research AVP was extracted by absorption to magnesium trisilicate (Florisil 100-200 mesh: Sigma Chemical CO.) Details of the procedure are in Appendix 2. At normal physiological AVP concentrations there is no significant loss of AVP in the extraction from 2ml of plasma. Recovery progressively declines with increasing plasma concentration (86% @10pmol/l; 72% @ 100pmol/l) and with decreasing volume of plasma sample.

2.2.4 THE ASSAY OF AVP

2.2.4.1 Standard curve

Estimation of unknown concentration in samples requires reference to AVP standards of known concentration. A standard curve is constructed with the following components:

a) Total Count: The total radioactivity of the labelled antigen added to each standard and sample.

b) Non-specific binding (NSB): The binding of the second antibody to labelled antigen and assay tube.

c) Initial binding (B_0): The binding of labelled antigen to the specific antibody when no competing, unlabelled antigen (sample or standard) is present.

d) Standards: Serial dilution of synthetic AVP of known concentration (final concentration 0.03-16 pg/assay tube)

e) Interassay control: Sample of synthetic AVP prepared in batch. Final concentration 2pg/tube

2.2.4.2 Samples

Sample extract resuspended in assay buffer (800μl).

2.2.4.3 Protocol

a) Aliquots (200μl) in triplicate of sample and AVP standards, and equivalent volume of assay buffer in B_0 tubes, incubated with 100μl specific antisera for 24hr, 4°C (final dilution antisera 1:500,000).

b) 100μl radiolabelled AVP incubated with all samples and standards 18hr, 4°C (0.5-1fmol ^{125}IAVP per tube, 2000 cpm).

c) 100μl goat antirabbit IgG incubated with samples and standards for 48-72hr, 4°C (final dilution antisera 1:200).

d) Precipitate separated by centrifugation and supernatant, containing the free fraction, removed by aspiration. Residue ie the bound fraction, is counted on τ counter.

e) Ratio of specifically bound ^{125}IAVP to total ^{125}IAVP activity calculated for all standards and plotted as percentage of B_0 against AVP standard concentration. (Standard Curve)

f) Sample binding calculated and 200μl extract concentration estimated with reference to the standard curve. Actual sample concentration calculated as 4xassay value in the volume of plasma extracted.

Details of the assay protocol and reagents are found in Appendix 2.

2.2.5 STORAGE OF SAMPLES

All blood samples from humans were taken in chilled syringes, transferred to chilled heparinised tubes and centrifuged immediately at 2000RPM, 4°C 15min. Plasma was separated from cells and stored at -40°C. Plasma collected in this way, and extract residues can be stored for at least 3 months with no significant decrease in concentration of immunoreactive AVP.

2.2.6 DETECTION LIMITATIONS AND REPRODUCIBILITY

The sensitivity of the antibody allows detection of as little as 0.1fmol/assay tube. Using 2ml plasma samples this allows a detection limit of 0.2-0.3pmol/l. The interassay control sample allows between assay comparison of standard curves and the use of extraction controls (@5pmol/l) ensures validation of

results. The coefficients of variation (cv) of extracted plasma will vary between assayeurs.

The AVP samples in Section 3 were measured by Mrs P Rooke with an intra-assay cv of 9.7% and interassay cv of 15.3% @5pmol/l. Samples in Section 4 were measured by Mrs C Holmes with intra-assay cv of 10% @2pmol/l and 8.5% @10pmol/l. Inter assay cv was 9.4% @5pmol/l.

The measurement of AVP concentration in rat plasma samples in Section 5 was carried out by me using 1ml plasma aliquots. The extraction recovery was reduced to 85-90% and the limit of detection was raised to 0.6pmol/l. Intra-assay cv was 22% @2pmol/l and 20% @ 14pmol/l. Interassay cv was 17% @5pmol/l.

For the purpose of data analysis all samples with AVP concentration below the limit of assay detection were attributed the value of the detection limit.

2.2.7 DRUG INTERFERENCE WITH MEASUREMENT OF PLASMA AVP

Experiments were carried out with pooled plasma, spiked with synthetic AVP, to study the effect of addition of the drugs to be used in this research on the assay and measurement of AVP concentration.

2.2.7.1 Extraction experiments

2.2.7.1.1 *Fluoxetine*

Plasma concentrations of $\leq$ 1000ng (1μg) /ml of Fluoxetine and Norfluoxetine are found in humans during therapeutic use and do not exceed this concentration even in overdose (see tables 3. and 3.). Addition of 1μg/ml and 10μg/ml of these drugs alone or in combination had no significant effect on the recovery of AVP measured @1pmol/l or @5pmol/l.

Since rats will receive much greater doses of fluoxetine (10mg/kg) higher plasma concentrations would be anticipated. Assuming full absorption and 7% blood volume a plasma concentration of approximately 140μg/ml might be found. However, in a small study, plasma concentration in rats was found to be 0.486μg/ml at 30min after 10mg/kg IP and declined thereafter (Fluoxetine investigators manual 1990). Addition of 140μg/ml of Fluoxetine to AVP spiked

plasma lead to a 32% greater estimation of AVP @1pmol/l ($p<0.001$) and 12% higher estimation @10pmol/l ($p<0.001$).

2.2.7.1.2 *Ritanserin*

The addition of 14μg/ml of Ritanserin to pooled, AVP spiked plasma had no effect on the measurement of low concentrations of AVP but increased the measurement of high concentration (@10pmol/l) by approximately 10% ($p=0.006$).

2.2.7.1.3 *DOI* (1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane hydrochloride)

DOI, 14μg/ml plasma, had no effect on the measurement of AVP by RIA assay at high or low concentration of AVP.

2.2.7.2 **Addition to AVP standard curve**

Fluoxetine (140μg/ml), Ritanserin (14μg/ml), DOI (14μg/ml) or equivalent volume of assay buffer were added to each concentration of AVP in the standard curve and the AVP assay carried out in the usual way.

Figure 2.3 shows the effect of each drug on the binding of labelled AVP. Whilst Ritanserin and DOI appear to have no significant effect on binding, Fluoxetine decreases binding, most noticeably at low concentrations of AVP.

2.2.7.3 **Discussion**

The effect of high concentration of Fluoxetine on the measurement of AVP by RIA is intriguing. It is known that measurement of Fluoxetine in plasma requires careful storage and assay because the compound binds to a great variety of substances including plastic. It is unlikely that Fluoxetine increased the assay measurement of AVP by an increase in extraction recovery. It is more probable from these experiments that at least some Fluoxetine was extracted with AVP by binding to florisil, and then bound to the specific antibody to AVP in the assay and consequently lowered binding to AVP. This interference lead to a falsely high estimation of AVP concentration, particularly at low plasma concentrations of AVP and high concentrations of Fluoxetine.

In the research that follows it is likely that in samples collected from humans

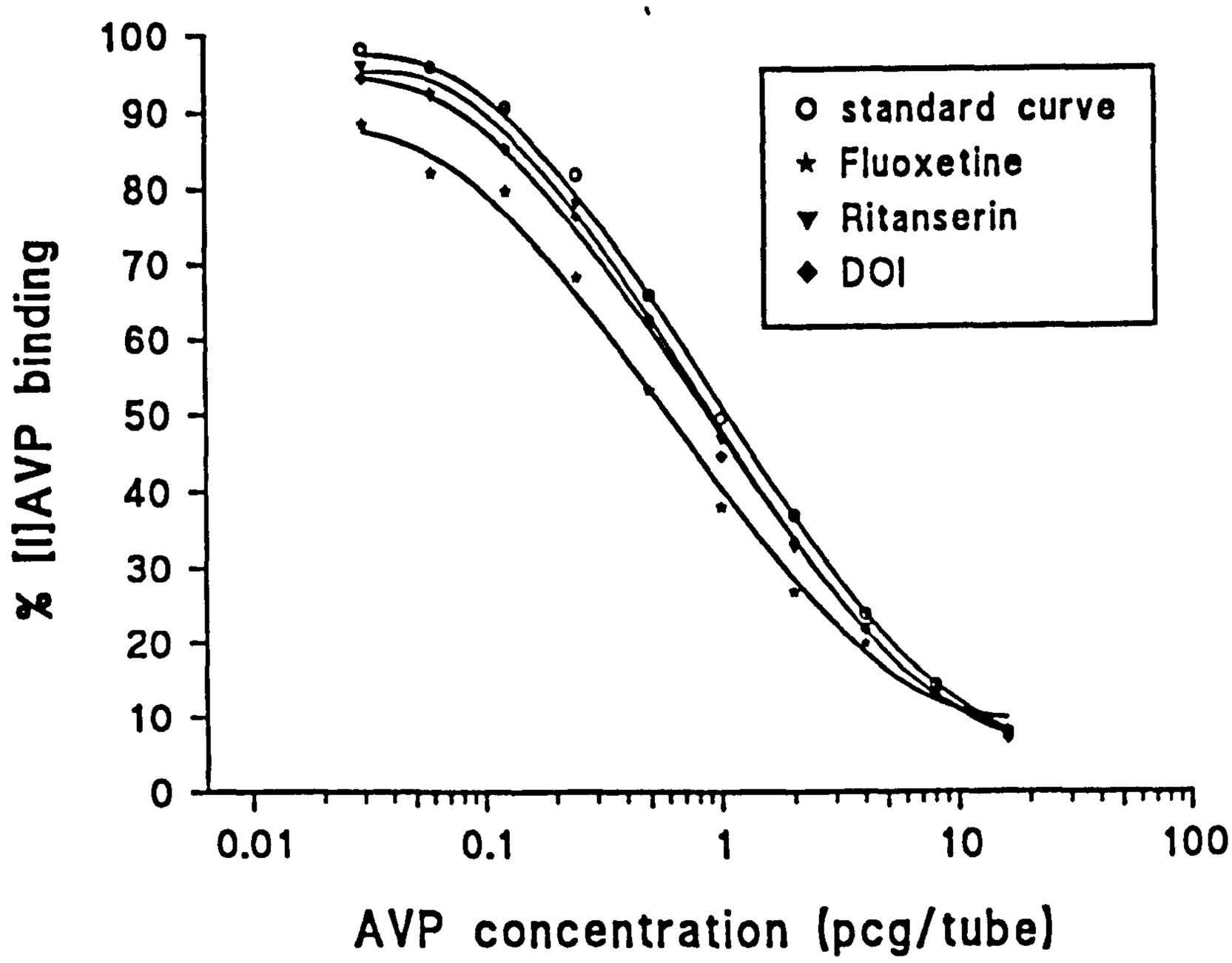


Figure 2.3

The effect of the addition of Fluoxetine (140μg/ml) (*), Ritanserin (14μg/ml) (▾) or DOI (14μg/ml) (♦) on the binding of [^{125}I]AVP in the standard curve for the assay of AVP.

and rats much of the Fluoxetine in plasma has bound to the plastic sample tube and little remained to interfere with the assay of AVP. In addition Fluoxetine appeared to interfere with the assay of AVP only at high concentrations, approximately 700 fold that in human plasma and probably 300 that in rat plasma. The effect of Fluoxetine on the measurement of AVP must, however, be taken into consideration in all experiments in this research. It is possible that Fluoxetine in plasma samples may cause up to a 30% increased estimate of AVP concentration @1pmol/l (ie assay value of 1.3pmol/l when true plasma concentration 1pmol/l), and a 12% higher estimate of plasma concentration @10pmol/l (ie assay value of 11.2pmol/l when true plasma concentration 10pmol/l). The effect is likely, however, to be very much less than this in both human and rat samples.

The effect of Ritanserin on the measurement of plasma AVP has not been explained by interference with the assay of AVP. The effect was numerically very small, although statistically significant, and may well have occurred by chance. In the experiments that follow some consideration must be given to the effect of Ritanserin on the measurement of plasma AVP. It could be anticipated that a maximum error of 10% overestimation of AVP concentration may be made @10pmol/l (ie an assay value of 11pmol/l when true plasma concentration was 10pmol/l).

2.3 SEROTONIN ACTIVE DRUGS

2.3.1 FLUOXETINE

Nomenclature

Fluoxetine hydrochloride

(±)-N-methyl-3-phenyl-3-[(α,α,α-trifluoro-p-tolyl)-oxy] propylamine hydrochloride

$C_{17}H_{18}F_3NO{\cdot}HCL$

LY110140

Fluoxetine is one of a group of chemically unrelated drugs which selectively inhibit the reuptake of 5HT from the synaptic cleft into the neuron, so called selective serotonin reuptake inhibitors (SSRI).

2.3.1.1 **Absorption, metabolism and pharmacokinetics** (Benfield et al 1986)
Studies in man have shown that Fluoxetine is well absorbed with >75% bioavailability after oral administration. Fluoxetine is metabolised in the liver, by N-demethylation, to Norfluoxetine. This metabolite is also a SSRI but has approximately 25% of the potency of its parent compound. Peak plasma concentration of fluoxetine occurs 6-8hr after a single oral dose but peak norfluoxetine concentration occurs after 24-96hr. Steady state plasma concentrations are reached after 2-4 weeks of daily oral dosing.
Administration of a single dose of 10mg/kg IP to rats resulted in highest plasma concentration (0.486μg/ml) at 30min after dosing declining to 0.039μg/ml at 24hr. Norfluoxetine concentration increased slowly to a similar concentration as fluoxetine at 4hr after dosing. Peak tissue concentration of fluoxetine occurred 1hr after IP administration and were 200-1000 times that of plasma.
In man after a 40mg oral dose the $t_{½}$ Fluoxetine is 1-4 days and $t_{½}$ Norfluoxetine 7-9 days. In rats after 10mg/kg IP $t_{½}$ is 26hr for Fluoxetine and 40hr for norfluoxetine.

The drug is excreted mostly in the urine and only 2.5% is excreted as unmetabolised Fluoxetine.

2.3.1.2 Pharmacodynamics

The effect of Fluoxetine on 5HT reuptake has been demonstrated in vitro by the complete inhibition of [^{3}H]5HT uptake into rat brain synaptosomes (Wong et al 1975) and into human brain slices (Harms 1983). Fluoxetine has been shown to have a potency of effect similar to clomipramine and to have little effect on NA and DA uptake processes with 2-300 fold lower potency for these sites that the 5HT uptake site (Wong et al 1974).

In vivo studies in animals have confirmed the effect on 5HT uptake by demonstration of prevention of pCA induced depletion of brain 5HT, an effect which depends on the reuptake site as a means of penetrating into the 5HT neuron (Fuller et al 1975). In vivo studies have also shown the highly specific nature of the effect on 5HT uptake sites compared to NA sites and in comparison to other drugs such as clomipramine (Wong et al 1975; Fuller et al 1975; Bowsher et al 1985). Phase I clinical studies in normal man have shown that Fluoxetine is a specific inhibitor of 5HT uptake in vivo (Lemberger et al 1978; Bowsher et al 1985; Bergstrom et al 1988).

Dose response studies in animals have shown that fluoxetine has significant effect on 5HT uptake into rat blood platelets at 1mg/kg with maximal effect (50% inhibition) at 10mg/kg (Horng & Wong 1976). In mice an ED_{50} of 0.4mg/kg was found to prevent pCA brain 5HT depletion. Complete prevention was achieved with doses of 2mg/kg (Fuller et al 1974a).

Time response studies have shown significant effect of Fluoxetine on 5HT uptake (20% inhibition) within 15min of intraperitoneal (IP) injection with 10mg/kg. Maximal inhibition (57%) occurred at 4hr after drug administration and persisted for a minimum of 24hr after one dose administration (Wong et al 1975).

Wong et al (1975) have shown that Fluoxetine has significant regional variation in effect on 5HT uptake. Synaptosomes prepared in rats pretreated with Fluoxetine showed that cortical and brainstem were most affected with 70% and 50% reduction in 5HT uptake respectively. Uptake inhibition in the diencephalon was 23% whilst in the cerebellum there was no apparent effect on 5HT uptake. The regional concentration of Fluoxetine in brain tissue is not, however, variable (Fluoxetine investigators manual 1990).

2.3.1.2.1 *Receptor affinity*

In vitro studies in rats have shown that Fluoxetine has very weak affinity for adrenergic, histaminergic, opioid, muscarinic, DA, GABA and 5HT receptors (Wong et al 1983).

2.3.1.2.2 *Effect of Fluoxetine on 5HT metabolism*

In vivo studies have shown that synaptic 5HT concentration is increased after Fluoxetine administration (Marsden et al 1979; Guan & McBride 1988). Fluoxetine appears to reduce 5HT turnover (ratio 5HIAA to 5HT) after acute treatment. Fuller et al (1974b) found that brain concentration of 5HIAA was significantly reduced one hour after 10mg/kg IP with maximal reduction at 4hr. The effect on 5HT turnover of this single treatment lasted 24hr. Dose response studies showed that 2.5mg/kg had a significant effect but 10mg/kg produced maximal effect of 5HT turnover. These effects were regionally specific with significant reduction in 5HIAA concentration occurring only in the cerebral hemispheres and midbrain.

Fluoxetine has also been shown to inhibit 5HT synthesis from tryptophan, in part due to non-competitive inhibition of tryptophan uptake (Hwang et al 1980; Bymaster & Wong 1977).

The effects of chronic treatment will be discussed in Section 5.7.

2.3.1.3 **Adverse effects** (Wernicke 1985)

The most frequently occurring adverse effects in man are nausea, nervousness and insomnia. These occur in 15-25% of drug users. Headache is occasionally a moderately severe side effect but occurs infrequently. The occurrence of hyponatraemia will be discussed in Section 3.1. There are no apparent adverse effects in rats of daily doses up to 10mg/kg other than a decrease in daily food intake with reduction in weight gain. This observation has now been exploited clinically with the use of Fluoxetine as an appetite suppressant.

The data sheets for Fluoxetine are presented in Appendix 3.

Fluoxetine and Norfluoxetine were generously provided for the purposes of this research by Lilly Research (Eli Lilly & Co), Windelsham, Surrey.

Solution of fluoxetine used for studies in Section 5 were made from crystalline solid as follows:-

1mg/ml in 0.9%NaCl pH 6.9 (Study 5.2)

10mg/ml in water pH 6.8 (Study 5.2)

10mg/ml in 10% ethanol in 20mMol tartaric acid pH 3.0 (Study 5.4 and 5.7)

Plasma concentration of Fluoxetine and Norfluoxetine were measured in Study 3.1 by reverse phase HPLC with UV detection based on Lilly protocol HPA-O (Orsulak et al 1988), and in Study 3.2 by Gas chromatography with electro capture detection based on Lilly protocol NHC-3 (Nash et al 1982). Measurements were made on 2ml plasma samples stored at -20°C. All samples were analysed by Simbec research Ltd, Glamorgan (courtesy Lilly Research).

2.3.2 RITANSERIN

Nomenclature

Ritanserin

6-[2-[4-[bis(4-fluorophenyl0methylene]-1-piperidinyl]ethyl]-7-methyl-5H-thiazolo[3,2-a]pyrimidin-5-one

R 55667

2.3.2.1 Pharmacodynamics

Ritanserin is a derivative of methylenepiperidine and has been characterised by receptor binding studies as a potent 5HT2 antagonist (Leysen et al 1985). The most sensitive expression of central 5HT2 antagonism in vitro has been found to be reversal of tryptamine-induced lesions. The ED_{50} of Ritanserin for inhibition of clonic seizures of forepaws in rats was 0.037mg/kg and body tremors 0.11mg/kg. Both phenomena were completely inhibited by a mean dose of Ritanserin 3.5 times greater than the ED_{50} (Awouters et al 1988).

Time response studies showed that Ritanserin had significant antagonist effect 30min after subcutaneous injection (SC) but peak responses were achieved 2-4hr after SC injection (Awouters et al 1988).

2.3.2.1.1 *Receptor binding*

Leysen et al (1985) showed that Ritanserin had low affinity in rat frontal cortex tissue for 5HT1a and 5HT1b receptors and very low affinity for other neurotransmitter receptor types. It has been shown to have a relatively high affinity for the rat 5HT1c receptor subtype (Hoyer 1988). Receptor subtype specificity is maintained in rats up to doses of 10mg/kg (Awouters et al 1988) and perhaps central functional 5HT2 receptor specificity up to 160mg/kg in animal studies (Janssen 1985).

Ritanserin was obtained from Cambio, Cambridge. The data sheets are presented in Appendix 3.

Solution of Ritanserin for use in the studies of Section 5 were made from powder in the following manor:-

1mg/ml in 4.3% ethanol 50mMol tartaric acid pH 2.7 (Study 5.3)

1mg/ml in 10% ethanol 20mMol tartaric acid pH 3.0 (Study 5.7)

2.3.3 DOI

Nomenclature

DOI hydrochloride

(±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane hydrochloride

2.3.3.1 **Pharmacodynamics**

DOI is a derivative of the hallucinogen 1-(2,5-dimethoxyphenyl)-2-aminopropane. It provokes reproducible, dose dependent behaviour effects in rats, termed "wet dog shakes". ED_{50} 0.17mg/kg SC with maximal effect of 0.4mg/kg SC (Arnt & Hytel 1989). Acute IV administration in vivo in rats decreased DRN firing with complete inhibition at 0.1mg/kg (Wright et al 1990). IV administration has been found to stimulate ACTH secretion with peak effect at 10min after administration continuing for 60min. Dose response studies showed ED_{50} of 0.2mg/kg iv with maximal effect of 1mg/kg (Bagdy et al 1989).

2.3.3.1.1 Receptor binding

Studies in rats have shown that DOI has a high affinity for binding sites labelled by the 5HT2 antagonist ketanserin and a very low affinity for 5HT1a and 5HT1b sites (Shannon et al 1984; Pierce & Peroutka 1989). These studies suggested that DOI might have higher affinity for a subpopulation of 5HT2 receptors, the 5HT2a site. DOI also has affinity for the 5HT1c receptor (Hoyer & Karpf 1988; Hoyer 1988) although this is probably 40-fold lower than its affinity for the 5HT2 receptor (Titeler et al 1988). It has a very low affinity for receptors of other neurotransmitters (Pierce & Peroutka 1989).

2.3.3.1.2 *Effect of DOI on brain 5HT*

Wright et al (1990) have shown that acute administration of DOI 0.1mg/kg decreased frontal 5HT concentration by 40% and 5HIAA concentration by 32%.

The data sheets for DOI are presented in Appendix 3. DOI was obtained, under Home Office licence, from Semat, St Albans, Herts.

Solution for use in experiments in Section 5 were made from solid as follows:

1mg/ml in water pH 6.3 (Study 5.5)

2.4 OSMOLALITY

The osmolality of plasma and urine samples was measured by depression of freezing point using a Roebling osmometer. The osmometer was standardised to 300mOsm/kg. Intra-assay cv 0.32% @293mOsm/kg.

2.5 PLASMA SODIUM

Plasma sodium concentration was measured by an ion selective electrode (Hitachi 718) by the Department of Clinical Biochemistry, Royal Victoria Infirmary, Newcastle upon Tyne.

2.6 HAEMATOCRIT

Blood haematocrit was measured by drawing duplicate samples of blood into heparinised microhaematocrit tubes (Hawksley, Lancing, Sussex) and centrifugation in Hawksley microhaematocrit centrifuge . Samples were measured on a Hawksley microhaematocrit reader.

2.7 MEASUREMENTS ON RAT NEURAL TISSUE SAMPLES

2.7.1 5HT CONTENT AND TURNOVER

5HT and 5HIAA were measured in homogenates of rat brain samples and neurohypophyses by HPLC with electrochemical detection according to the method of Wagner et al (1982). All assays were carried out by Mr M Leitch, Department of Psychiatry, University of Newcastle upon Tyne. The ratior of 5HIAA;5HT is considered to be a good index of the rate at which 5HT is released into brain synapses and thus an indirect measure of 5HT neurotransmission (Reinhard & Wurtman 1977).

2.7.1.1 Membrane preparation

Tissue samples were homogenised in mobile phase buffer using a ultrasonic cell disrupter.

An aliquot of homogenate was taken for protein estimation. The remaining homogenate was centrifuged @10,000g for 5min and the supernatant collected and stored at -80°C until assay. The pellet was discarded.

2.7.1.2 HPLC conditions

Autosampler: Gilson 231/401

Injection loop: 20μl

Pump: Gilson 302 flow 1.5ml/min

Inline filter: Rheodyne 0.2μm

Column: Spherisorb 5μm ODS2 25cmx4.6mm

Detector: AmoR ECD Potential +0.75V Range 10nA

Standards: 5HT and 5HIAA 10pmol/20μl (Sigma

Detection limit: 0.5pmol/20μl

Mobile Phase : 50mmol NaH_2PO_4

1mmol NaCl

0.3mmol Octane sulphuric acid

14% methanol

pH 3.3 (H_3PO_4)

2.7.2 HOMOGENATE LIGAND BINDING

The binding of radiolabelled ligands to 5HT receptor subtypes in rat brain homogenate preparations was performed by Mr A Leake, MRC Neurochemical Pathology Unit, Newcastle General Hospital.

The assessment of the 5HT1a and 5HT2 receptor site binding was by an adaptation of the method of Leake et al (1991). The variations in buffer constituents, incubation conditions and specific high affinity displacers are outlined below.

2.7.2.1 Membrane preparation

Brain tissue was homogenised in 50mmol Tris 0.25m sucrose pH 7.4 at 4°C using a teflon pestle. The suspension was centrifuged @1000g 10min 4°C and the aspirated supernatant was centrifuged @35,000g 10min 4°C. The supernatant was discarded and the pellet resuspended 1:5 vol/wt buffer using an

ultrasonic cell disrupter.

An aliquot of homogenate was taken for protein estimation and remaining sample stored at -40°C until assay.

2.7.2.2 Receptor binding assay

All incubations were performed in quadruplicate with total assay volume of 400μl.

5HT1a: [^{3}H]8OHDPAT binding (8-hydroxy-2(di-n-propylamino) tetralin) (Amersham International).

Homogenate was diluted to 1:50 vol/wt with buffer A (Appendix 4).

8nmol/l [^{3}H]8OHDPAT (240Ci/mmol) was used to estimate 5HT1a binding. Non-specific binding was measured in the presence of 2μmol/l unlabelled 5HT. Samples were incubated for 30min at 25°C.

5HT2: [^{3}H]ketanserin binding (NEN).

Homogenate was diluted to 1:50 vol/wt with buffer B (Appendix 4).

8nmol/l [^{3}H]ketanserin (60Ci/mmol) was used to estimate 5HT2 binding. Non-specific binding was determined in the presence of 50μmol/l methysergide. Samples were incubated for 15min at 37°C.

All samples were filtered through Whatman GF/B filters with vacuum assistance and washed with 3x5ml of buffer B 4°C. Filters were equilibrated with scintillant for 48hr 20°C prior to counting on β-scintillation counter.

Results were expressed as fmol/mg protein.

2.7.3 PROTEIN ASSAY

The measurement of the protein content of samples for HPLC and ligand binding was carried out using the method of Bradford (1976). This relies on measurement of the change in optical density, proportional to the protein concentration, of a protein sensitive dye (Appendix 5).

SECTION 3

STUDIES OF THE EFFECT OF A 5HT REUPTAKE INHIBITOR ON OSMOREGULATED AVP SECRETION IN NORMAL MAN

INTRODUCTION

The evidence discussed in Section 1.4 has suggested that 5HT may have physiological importance in the maintenance of water balance. As yet there has been little work in humans to explore further the physiological implications either in normal man or in possible disease states. This has, to some extent, been because of the lack of specific and safe serotonergic or 5HT antagonist compounds. The availability of SSRIs (eg. Fluoxetine), used clinically in the management of depression, has allowed further exploration of this area. The recent reports of hyponatraemia and possible SIAD associated with clinical use of Fluoxetine has made research into the role that 5HT may have in physiological AVP release particularly timely .

Hyponatraemia occurring, sometimes with profound severity (<110mmol/l), in association with the treatment of depression by Fluoxetine is a rare occurrence. During intensive drug monitoring in the USA 20 cases were reported to the drug company (Eli Lilly, Indianapolis). The Committee on Safety of Medicines (London) have received fewer than 10 reports. The literature contains six reports of nine patients (Hwang & Magraw 1989; Cohen et al 1990; Gommans & Edwards 1990; Staab et al 1990; Marik et al 1990; Vishwanath et al 1991).

The reports suggest that hyponatraemia occurs early and persists, with presentation of patients from 1-60 days after the commencement of 20-80mg Fluoxetine treatment. Approximately 40% of reported patients were taking concomitant diuretic therapy.

Many of the reports have occurred in patients over 50yr suggesting that age is an additional risk factor. However, since Fluoxetine has few anticholinergic and antihistaminergic side effects it has been particularly recommended for the treatment of depression in the elderly, and consequently there may be over-representation of older people in reports of hyponatraemia. Some cases of younger people with Fluoxetine associated hyponatraemia have been reported.

There has been very little exploration of the possible mechanism(s) for the occurrence of hyponatraemia. Most literature reports are of patients who appeared to be clinically normovolaemic with excretion of urine which was inappropriately concentrated with respect to plasma osmolality, thus suggestive

of SIAD. Patients usually responded to fluid restriction alone although oral sodium was given to some who had a particularly slow rate of rise of plasma osmolality. Some patients responded to fluid restriction with return to normal plasma sodium concentration in 48hr, but most took 5 days to return to normal osmotic status.

The majority of patients were noted to have normal renal, adrenal and thyroid function and none were noted to be polydipsic. In addition nausea was not specifically mentioned as a prominent symptom in any of the case reports.

In one of four patients reported by Cohen et al (1990) rechallenge with Fluoxetine was carried out. Hyponatraemia recurred within 5 days and worsened to 10 days when Fluoxetine was ceased. Staab et al (1990) reported the rechallenge of a patient who had developed pNa 125mmol/l within 20 days of treatment with 80mg Fluoxetine daily. Reintroduction of this dose had no significant effect on pNa up to two months of subsequent follow-up. Vishwanath et al (1991) documented the occurrence of pNa 122mmol/l two month after the start of Fluoxetine treatment at the same dose which had been used in a previous episode of depression with no adverse effect.

None of these studies explored water balance and osmoregulated AVP secretion in these patients either during the episode of hyponatraemia or after apparent recovery. In addition there has been no attempt to identify whether the drug or disease state (depression) alone may be responsible for the occurrence hyponatraemia or whether the two are additive.

The first step in further exploration of this phenomena is to study the effect of Fluoxetine in normal man.

GENERAL HYPOTHESES

1. That Fluoxetine increases central 5HT neurotransmission in normal man.

2. That increase in 5HT activity modulates osmoregulated AVP secretion in normal man.

STUDY 3.1 THE EFFECT OF A 5HT REUPTAKE INHIBITOR, FLUOXETINE, ALONE AND IN COMBINATION WITH A DIURETIC, ON WATER BALANCE IN NORMAL MAN.

SPECIFIC AIMS

1. To investigate the effect of an increase in 5HT neurotransmission, by reuptake inhibition, on the excretion of an oral water load in normal man.

2. To investigate the possible additive effect of a diuretic on 5HT modulated water balance in normal man.

MATERIALS AND METHODS

Subjects

Thirteen healthy male volunteers aged 18 to 40 yrs gave written, informed consent for the study. Exclusions were made on the basis of serious illness (past or present), previous head injury or bacterial meningitis, regular medication or clinical abnormality of serum electrolytes, liver function tests, lipids, blood glucose, full blood count or urinalysis.

Treatments and study design

The treatment order for each volunteer was randomised independently of the researchers. Each subject took a treatment before breakfast, once daily for 7 days. There was a minimum washout period between treatments of 4 weeks. All adverse symptoms were recorded and a direct measure of nausea was made daily by a visual analogue scale (10cm line, Appendix 1), before taking the day's treatment dose.

Identical capsules for each of the 4 treatments were pre-packed (Eli Lilly).

Treatments were:-

Fluoxetine 40mg (F)

Placebo (P)

Hydrochlorothiazide 50mg (D)

Hydrochlorothiazide 50mg and Fluoxetine 40mg (C)

Plasma samples for measurement of drug and metabolite concentration were taken at the start of each drug period and at the start of water load assessment. Samples were analysed as described in Section 2.3.1.

Water load assessment

Twenty four hours after the last of 7 doses of treatment subjects underwent the protocol for water load assessment as described in Section 2.1.1.1.

Ethical approval

Ethical approval was obtained from Newcastle Health Authority Joint Ethics Committee.

Statistical analysis

The results of biochemical analysis were compared by randomised blocks analysis of variance with patients as blocks and factorial treatments (with and without Fluoxetine, with and without diuretic). This has allowed separate estimation of the effect of diuretic, of Fluoxetine and of any interactive effect. Except where stated results are shown as mean with standard error.

RESULTS

Nine subjects completed all 4 treatment studies. One subject completed only three treatment studies, the study omitted was with Fluoxetine alone. The reason for non-completion for this subject was not because of adverse effects. One subject declined to continue the study after two assessment. Two subjects withdrew from the study because of adverse effects. One had marked nausea after the first dose (Fluoxetine and diuretic) which did not abate after 3 daily doses. The other subject developed a severe headache after the first dose of treatment (Fluoxetine). The data for the nine subjects who completed the study and the tenth subject who completed three parts is included in the analysis the follows.

Excretion of water load

Figure 3.1 shows the mean percentage of the water load cleared after 4hr in each treatment group. Subjects excreted 92.8±7.3% of the ingested water load after placebo treatment. After factorial analysis of this data a significant effect of diuretic treatment on excretion of the water load was shown (19±6.9% reduction $p<0.001$). The reduction in load excretion after combination treatment (C) is entirely attributable to the effect of the diuretic. Fluoxetine alone or in combination with a diuretic had no apparent significant effect on the ability to excrete a water load ($p=0.372$ and $p=0.391$ respectively).
Similarly free water clearance was significantly reduced at 90 and 120min in those taking diuretic with or without Fluoxetine (Figure 3.2a).

Plasma sodium and osmolality changes

Basal data (Table 3.1)

There was no significant difference between treatments in pOS at the start of the water load test after 7 days treatment. Although mean pOS after Fluoxetine treatment was lower this just failed to reach significance ($p=0.055$).
There was a statistically significant decrease in pNa of approximately 1 mmol/l attributable to diuretic treatment.
There was no apparent interaction of the effects of Fluoxetine and diuretic treatments (for pNa $p=0.531$, for pOS $p=0.959$).

Dynamic data

During the 4hr assessment pOS changed in a similar pattern after all treatments (Figure 3.2c). The lowest pOS achieved was significantly affected by Fluoxetine treatment (Table 3.2), however the <u>change</u> in pOS during the water load was not significantly affected by Fluoxetine. By the end of the 4hr test period pOS was no longer different between treatments.
The baseline significant difference in pNa after diuretic treatment persisted throughout the water load and there was a slight increase in magnitude (Figure 3.2e: Table 3.2).
In none of the measurement comparisons was any significant interaction apparent between Fluoxetine and diuretic treatment. Although there were trends

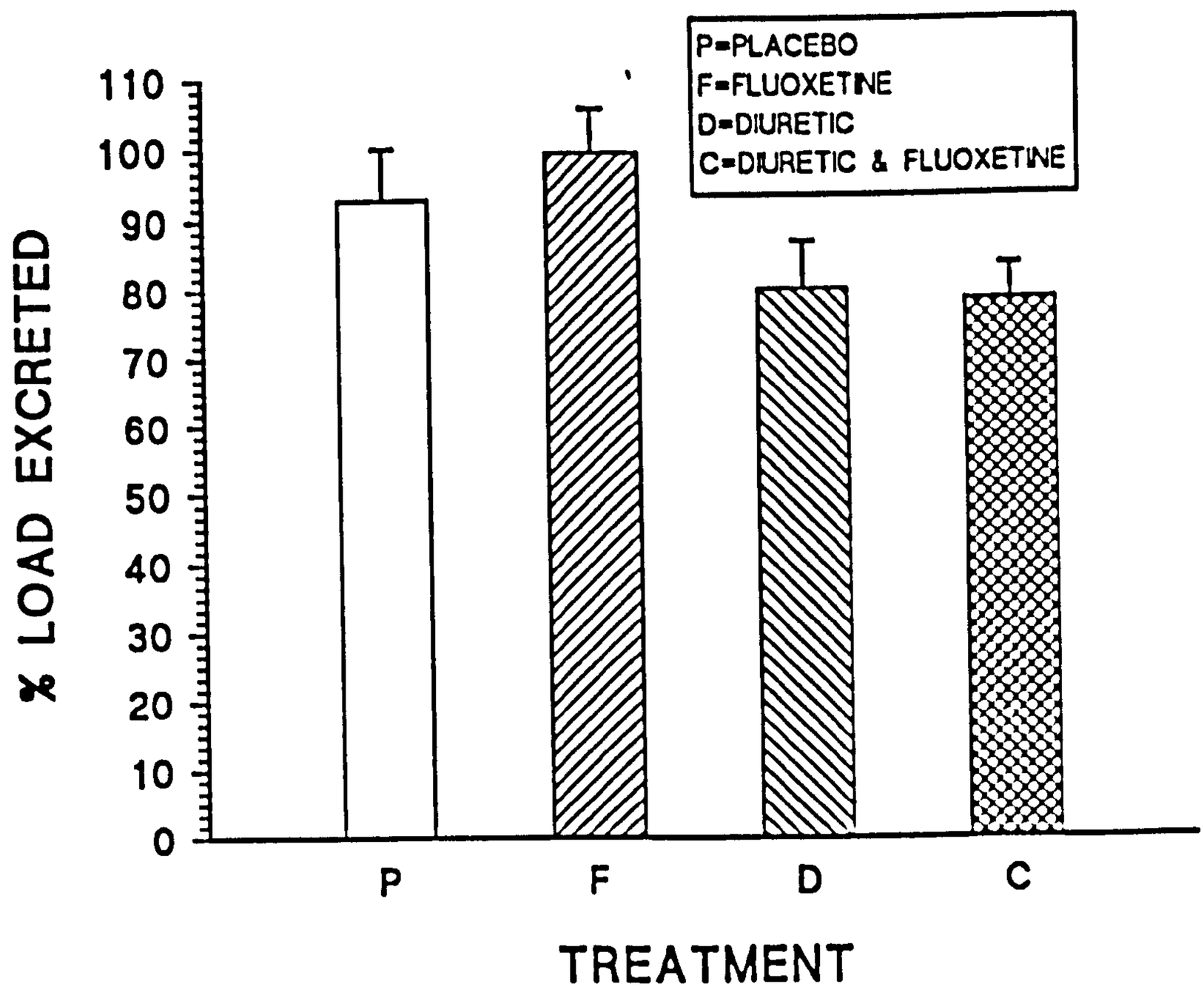


Figure 3.1

The percentage of the oral water load excreted in the 4hr after ingestion after 7 days treatment with each drug (Mean+SEM). Factorial analysis showed a significant reduction in excretion after Diuretic treatment ($p<0.001$). Fluoxetine treatment alone or in combination with the diuretic had no significant effect on the volume excreted.

P: Placebo; F: Fluoxetine; D: Diuretic; C: Fluoxetine and Diuretic.

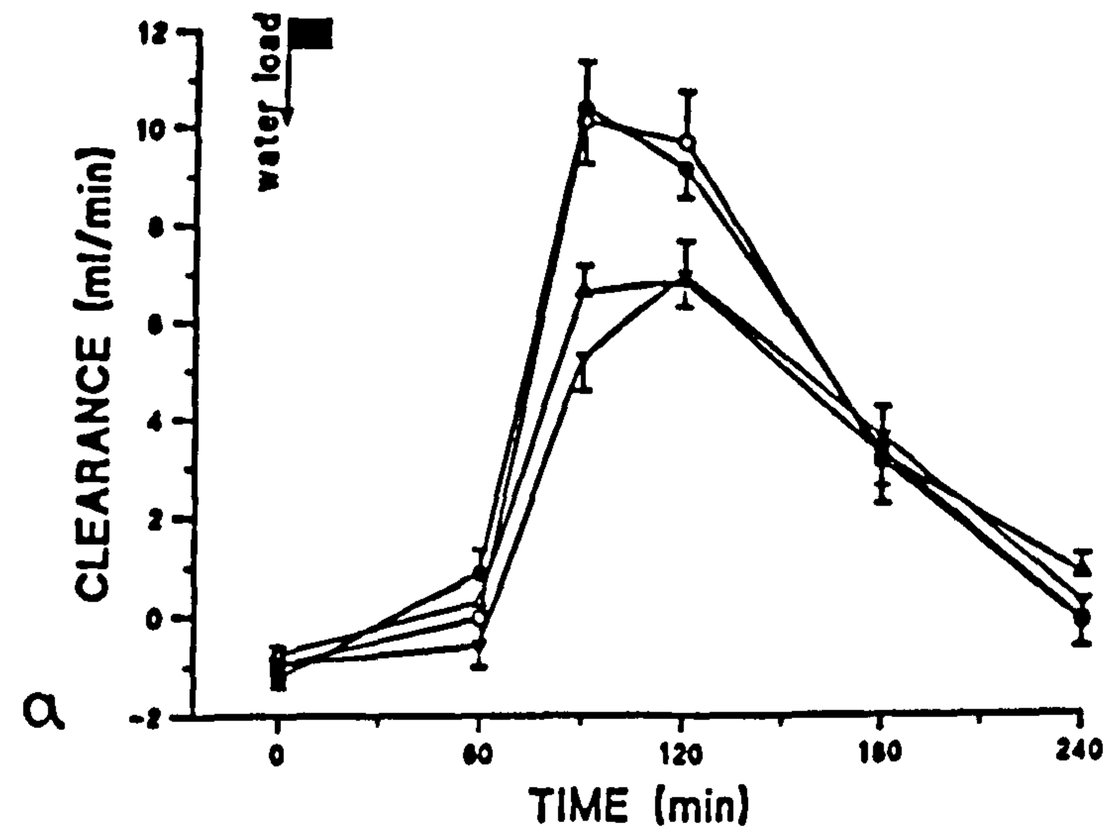
Figure 3.2

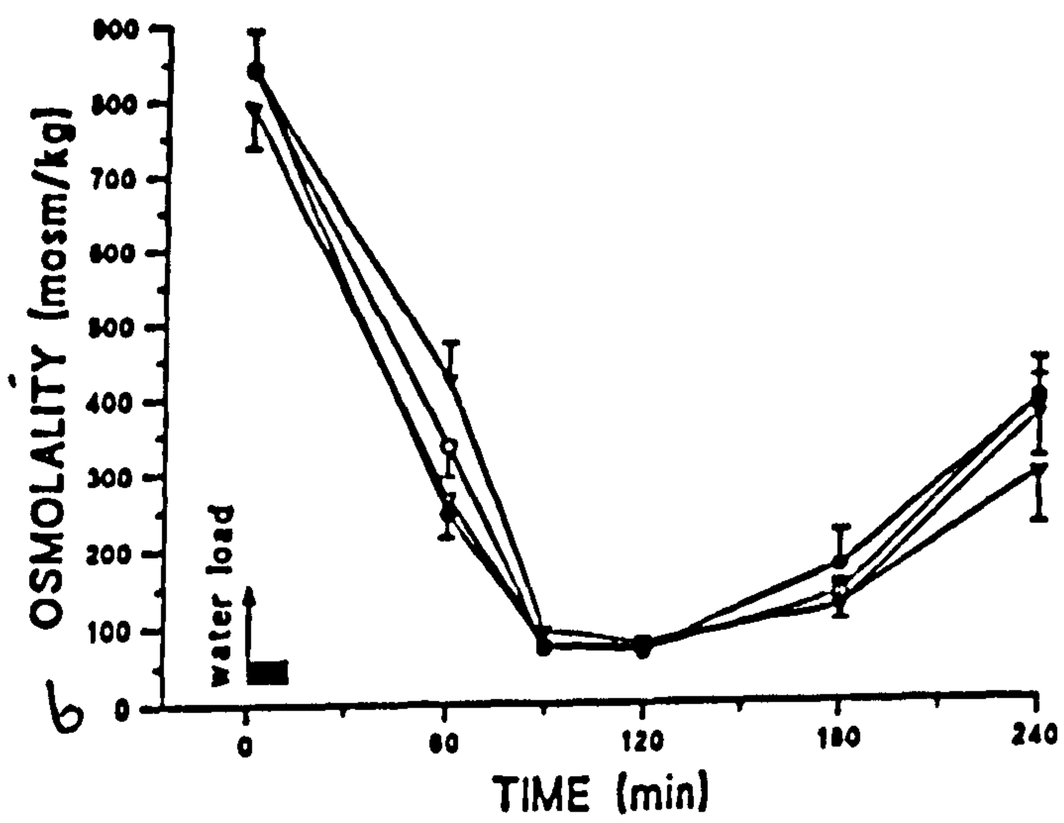
The effect of an oral water load (20ml/kg) on renal function, plasma biochemistry and plasma AVP after 7 days treatment with Placebo (O); Fluoxetine (●); Diuretic (▽); or Fluoxetine and Diuretic (▼). See text for statistical details.

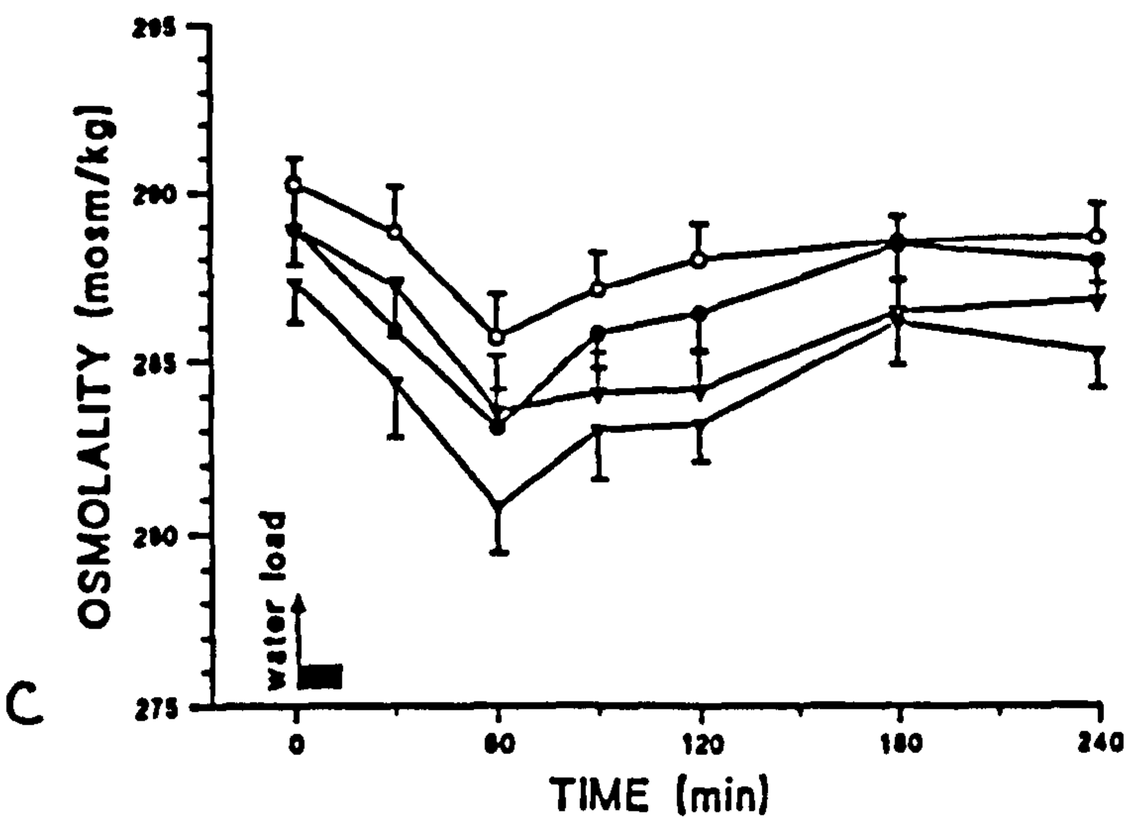
a) Free water clearance; b) Urine osmolality; c) Plasma osmolality; d) Plasma AVP;

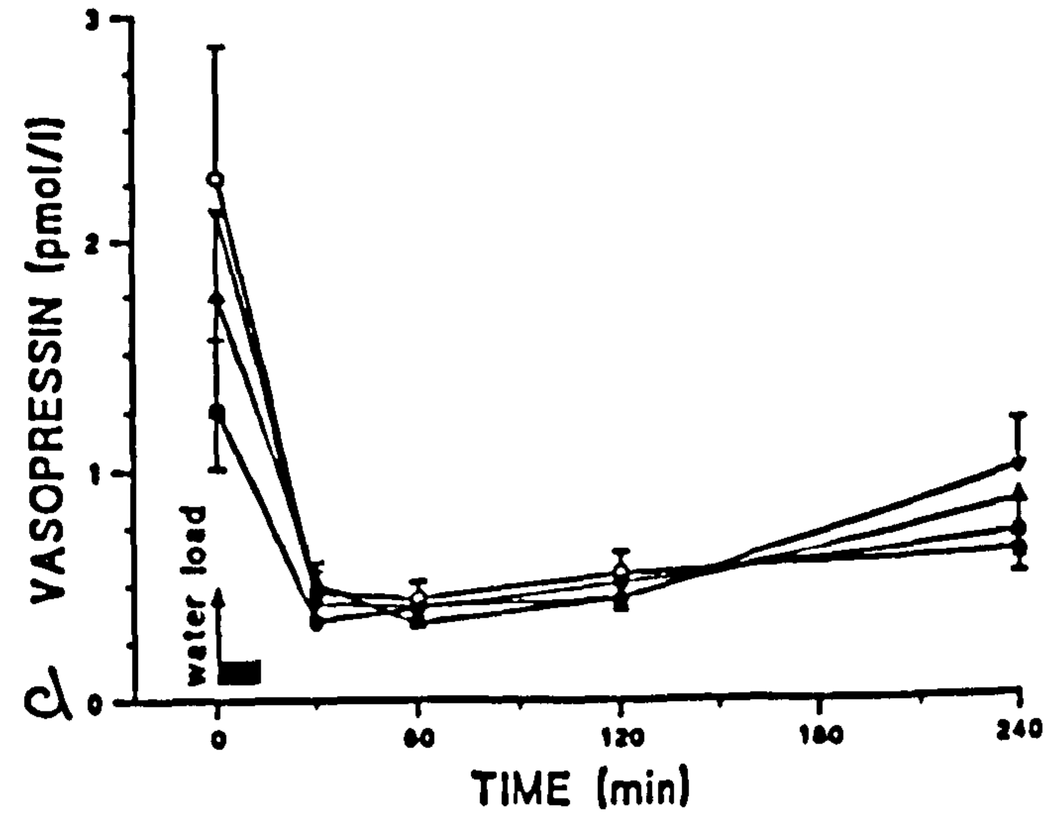
e) Plasma sodium.

Results are presented as mean ± SE.


a
CLEARANCE (ml/min)
water load
TIME (min)


b
OSMOLALITY (mosm/kg)
water load
TIME (min)


c
OSMOLALITY (mosm/kg)
water load
TIME (min)


d
VASOPRESSIN (pmol/l)
water load
TIME (min)

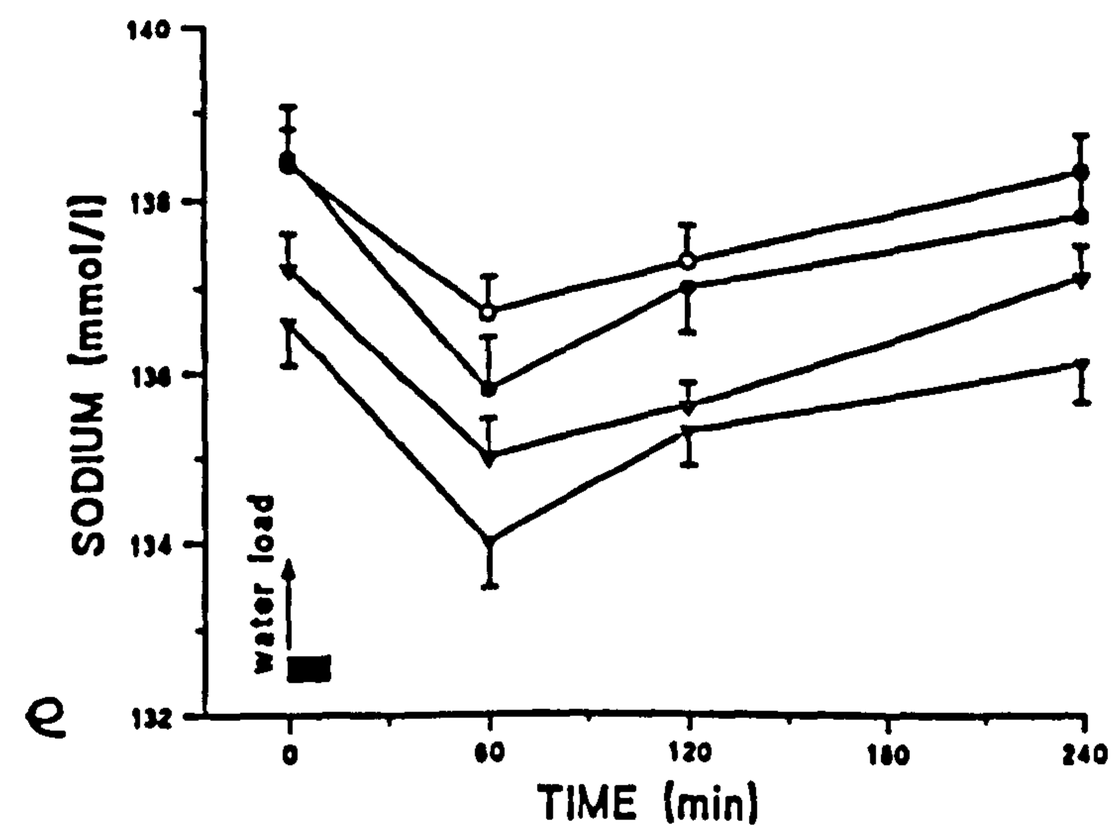

e
SODIUM (mmol/l)
water load
TIME (min)

Table 3.1 **The effect on plasma and urine biochemistry of 7 days treatment with Fluoxetine, diuretic or both treatments combined. Analysis by randomised block analysis of variance with factorial treatments.**

	MEAN				
	with Fluoxetine	without Fluoxetine	difference	95%CI	p value
POs(mosm/kg)	288.0	289.7	1.7	-0.1-3.5	0.055
PNa(mmol/l)	137.6	137.8	0.2	-0.6-1.0	0.642
Pavp(pmol/l)	1.8	2.0	0.2	-0.6-1.0	0.577
UOs(mosm/kg)	862	825	37	-63-137	0.462
	with diuretic	without diuretic			
POs(mosm/kg)	288.4	289.3	0.9	-0.9-2.7	0.278
PNa(mmol/l)	137.0	138.4	1.4	0.6-2.2	0.002
Pavp(pmol/l)	1.9	1.9	0.0	-0.8-0.8	0.845
UOs(mosm/kg)	834	853	19	-81-119	0.697
	mean effect of Fluoxetine alone	mean effect of combination			
POs(mosm/kg)	-1.8	-1.7	0.1	-3.4-3.6	0.959
PNa(mmol/l)	0.1	-0.4	0.5	-1.1-2.1	0.531
Pavp(pmol/l)	-0.9	0.3	1.2	-0.4-2.8	0.147
UOs(mosm/kg)	23.0	52.0	29.0	-171-229	0.765

Table 3.2 **The effect of treatments on nadir plasma and urine biochemical changes showing mean data(±SEM) for each treatment group and by factorial analysis the differences between treatments and the combination treatment.**

	PLACEBO	FLUOXETINE	DIURETIC	COMBINATION
POs(mosm/kg)	286.6±1.3	282.1±1.1	282.5±1.6	280.8±1.4
PNa(mmol/l)	136.6±0.4	135.9±0.6	134.7±0.5	134.0±0.5
Pavp(pmol/l)	0.4±0.1	0.4±0.1	0.3±0.1	0.4±0.1
UOsmosm/kg)	62.4±5.3	64.5±5.6	71.9±3.8	77.1±6.4

	mean effect of Fluoxetine alone	mean effect of combination	difference	95%CL	p value
POs(mosm/kg)	-4.5	-1.7	2.8	-2.8-8.4	0.325
PNa(mmol/l)	-0.7	-0.7	0.0	-1.7-1.7	
Pavp(pmol/l)	0.0	0.1	0.1	-0.1-0.3	0.345
UOs(mosm/kg)	2.1	5.2	3.1	-7.9-14.1	0.579

MEAN

	with Fluoxetine	without Fluoxetine			
POs(mosm/kg)	281.4	284.6	3.2	0.4-6.0	0.036
PNa(mmol/l)	135.0	135.7	0.7	-0.1-1.5	0.104
Pavp(pmol/l)	0.3	0.4	0.1	-0.1-0.3	0.874
UOs(mosm/kg)	70.8	67.1	3.7	-1.9-9.3	0.197

	with diuretic	without diuretic			
POs(mosm/kg)	281.7	284.3	2.6	-0.2-5,4	0.067
PNa(mmol/l)	134.4	136.3	1.9	1.1-2.7	<0.001
Pavp(pmol/l)	0.4	0.4	0.0	-0.2-0.2	0.430
UOs(mosm/kg)	74.5	63.53	11.0	5.4-16.6	<0.001

to lower pOS and pNa after combined treatment than after either treatment alone factorial analysis has shown these effects to be due to the individual treatments (Table 3.2).

Plasma vasopressin

Initial pAVP showed large variation between individuals but there was no significant difference between treatments nor was there an interactive effect of combined diuretic and Fluoxetine treatment ($p=0.147$ Table 3.1). After the water load subjects suppressed AVP in a similar way after all treatments. There was no indication of failure of suppression in response to plasma hypo-osmolality after any of the treatments (Figure 3.2d: Table 3.2).

Urine osmolality

There were no significant differences between treatments in the uOS at the start of the water load (Table 3.1). All treatments allowed minimal dilution of urine (<100mOsm/kg) although slightly less dilute urine was achieved after diuretic treatment (74.5mOsm/kg compared to 63.5mOsm/kg standard error of difference 2.76 $p<0.001$). There was an initial delay in urine dilution after treatment C but the time to maximal dilution was not delayed (Figure 3.2b).

Blood pressure

There was no significant fall in blood pressure during any of the 39 studies. There was no significant difference between the treatment groups in the mean arterial pressure at time zero.

Nausea

One subject included in this data analysis experienced nausea during the first three days of treatment with the combination of diuretic and Fluoxetine but by the time of water load test no nausea had been experienced for 48hr. During the water load some subjects recorded significant nausea on the analogue scale (> 10mm). Excluding their data from the analysis did not alter the results of statistical analysis. Since our findings have shown no evidence of SIAD and significant nausea would, by non-osmotic stimulation of vasopressin, have produced a false positive result, we have included all data in our calculations.

Plasma Drug concentration

Measurement of the plasma drug level at the start of each treatment period and during the water load test showed there to be no carry-over effect between treatment periods.

DISCUSSION

There was no evidence from this study that Fluoxetine modifies osmoregulated AVP secretion. There is no apparent alteration in the ability of normal man to maintain osmotic homeostasis in basal conditions since 7 days of Fluoxetine treatment had no significant effect on baseline pOS or pNa. Fluoxetine alone or in combination with a diuretic had no significant effect on the ability of normal man to excrete a water load or on the suppression of AVP in response to plasma hypo-osmolality achieved by an oral water load.

Statistical analysis by randomised blocks with factorial treatments has allowed evaluation of any interactive effect. As this was shown to be not significant we were able to look at the effects of Fluoxetine and Diuretic by factorial analysis. The small numbers in this study may however be insufficient to allow interactive effects to reach significance.

There is a trend in the data to suggest that Fluoxetine in combination with a diuretic leads to lower pOS and pNa and higher pAVP concentrations relative to pOS after seven days treatment, and possibly increased plasma biochemical changes after a water load, than after either treatment alone. This trend however failed to reach significance and the changes in plasma biochemistry seem to be attributable to diuretic treatment. It is interesting to speculate, however, that there might be an interactive effect. One possible mechanism for this might be that, by the non-osmotic stimulus of hypovolaemia, diuretic treatment might "prime" magnocellular AVP release so that in this situation 5HT may stimulate inappropriate AVP secretion and lead to hyponatraemia.

Plasma osmolality has been found to be non-significantly reduced (from 290mOsm/kg to 288 mOsm/kg) after 7 days treatment with Fluoxetine. It is possible that this small reduction after 7 days may continue with further regular administration and become statistically and clinically significant.

The effects of diuretic treatment on water balance and plasma biochemistry are

intriguing. 24hr after the last of 7 daily doses there was significant (19%) retention of a water load. This was apparently due to mechanisms other than dehydration or inappropriate AVP secretion since there were no differences in initial pOS, uOS or pAVP to suggest fluid depletion and no failure of suppression of AVP in response to the hypo-osmotic stimulus. Presumably the mechanism of fluid retention is due to increased activity of the renin-angiotensin-aldosterone system.

There was a statistically significant reduction in pNa after 7 days treatment with diuretic. This is in keeping with other studies which have associated thiazide diuretics with reduction in pNa concentration (Abramow & Cogan 1984: Friedman et al 1989). In this study the actual change was just over 1 mmol/l and has little biological significance.

There was a suggestion from the data that diuretic treatment prevented maximal urine dilution although mean nadir uOS was still <100mOsm/kg. At such low osmolality there is increased inaccuracy in measurement and this result can be interpreted as no more than an interesting finding.

STUDY 3.2 THE EFFECT OF 5HT REUPTAKE INHIBITION BY FLUOXETINE, ON THE AVP RESPONSE TO HYPEROSMOTIC STIMULUS

SPECIFIC AIMS

1. To study the effect of 7 days treatment with a 5HT reuptake inhibitor, Fluoxetine on basal osmotic status and AVP secretion in normal man.

2. To study the effect of Fluoxetine on the sensitivity of AVP response to increase in plasma osmolality in normal man.

3. To study the effect of Fluoxetine on the theoretical osmotic threshold of AVP release in normal man.

MATERIALS AND METHODS

Subjects

Eleven healthy male volunteers aged 20 to 35yr gave written, informed consent for the study. Exclusions were made on the basis of serious illness, previous head injury or bacterial meningitis, regular medication or clinical abnormality of serum electrolytes, liver function tests, lipids, blood glucose, full blood count or urinalysis.

Treatments and study design

Each subject took, in random order, Fluoxetine 40mg (F) or placebo (P) before breakfast, once daily for 7 days. There was a minimum washout period between treatments of 4 weeks. All adverse symptoms were recorded and a direct measure of nausea was made daily by a visual analogue scale (10cm line, Appendix 1), before taking the day's treatment dose.

Placebo capsules were identical to Fluoxetine (courtesy Eli Lilly). Plasma samples for measurement of drug and metabolite concentration were taken at the start of each drug period and at the start of hyperosmotic stimulus assessment. Samples were analysed as described in Section 2.3.1.

Hyperosmotic stimulus

Subjects were studied 24hr after the last dose of treatment. The protocol is described in Section 2.1.1.2.1.

Ethical approval

Ethical approval was obtained from Newcastle Health Authority Joint Ethics Committee.

Statistical analysis

The relationship between pAVP and pOS for each subject was analysed by linear regression. Results were compared by ANOVA for cross over design studies for investigation of treatment, order and carry-over effects. Unless other wise stated results are shown as mean±SEM.

RESULTS

Ten subjects completed both studies. One subject withdrew from the study after the first dose of treatment (Fluoxetine) due to marked nausea. No other adverse effects were experienced by subjects. All subjects tolerated the hyperosmotic studies well with no significant ill-effects.

Basal data (Table 3.3)

Seven days of treatment with Fluoxetine caused no significant change in baseline pOS, pAVP or pNa. Table 3.3 shows similar data for study 3.1 for comparison of similar groups. (These results are slightly different to those presented in Table 3.1 due to the factorial analysis of the data in study 3.1).

Dynamic data

The infusion of hypertonic saline caused a rise in pOS of 15.8±0.42 mOsm/kg after placebo and 15.9±0.38 mOsm/kg after Fluoxetine treatment (p=0.879). There was no significant difference in the magnitude of osmotic change between the first and second assessment for each subject.

Table 3.3 The effect on plasma and urine biochemistry of 7 days treatment with Fluoxetine or placebo in studies 3.1 and 3.2. Plasma drug concentrations at the start of each osmotic stimulus are also shown (ND=not detected). Results are presented as mean ± SEM.

	STUDY 3.1			STUDY 3.2		
	Placebo	Fluoxetine	p value	Placebo	Fluoxetine	p value
POs(mosm/kg)	290.6±0.9	288.9±1.0	0.282	289.6±0.9	288.7±1.3	0.343
PNa(mmol/l)	138.3±0.4	138.4±0.6	0.865	139.8±0.4	139.4±0.5	0.565
Pavp(pmol/l)	2.0±0.6	1.3±0.2	0.080	0.67±0.13	0.66±0.24	0.974
UOs(mosm/kg)	818± 156	847± 144	0.704			
Fluoxetine (ng/ml)	ND	104.4±11.4		ND	97.4±11.32	
Norfluoxetine (ng/ml)	ND	84.6±8.9		ND	137.6±15.9	

Vasopressin response

The AVP response to change in pOS is shown for each subject after each treatment in Figure 3.3. There was a total increase in pAVP of similar magnitude after both treatments in response to increase in pOS (5.6±1.1pmol/l (P), 6.0±0.9pmol/l (F), p=0.337).

Calculation of the linear regression equation for each test allows comparison of the sensitivity of the AVP response (slope of the regression line) and the theoretical osmotic threshold of AVP release (abscissal intercept) as discussed in Section 1.1.2.1. Tables 3.4 and 3.5 show these parameters for each subject. There was no apparent significant effect of the Fluoxetine on either the sensitivity (0.33±0.06 (P), 0.36±0.06 pmol/l per mOsm/kg (F), p=0.347) or the threshold (287.0±1.2 (P), 286.9±1.1 mOsm.kg (F), p=0.700) of AVP release. Nor was there any evidence of significant order or carry-over effects.

Blood pressure

There was no significant fall in blood pressure during any of the 20 studies. There was no significant difference between the treatment groups in the mean arterial pressure at time zero.

Nausea

None of the subjects experienced nausea during the 7 days of treatment. During the saline infusion significant nausea (> 10mm) was experienced on both occasions to a similar degree by subjects 2, 8 and 9.

Exclusion of their data from the analysis did not alter any of the results as described above.

Haematocrit

Haematocrit values fell slightly in all subjects in both tests (-5.2±0.2% (P), -6.0±0.3% (F)) reflecting the increase in intravascular fluid consequent to hypertonicity. There was no significant effect of Fluoxetine on this change (p=0.053) and in none of the tests was there any suggestion of a fall in blood volume.

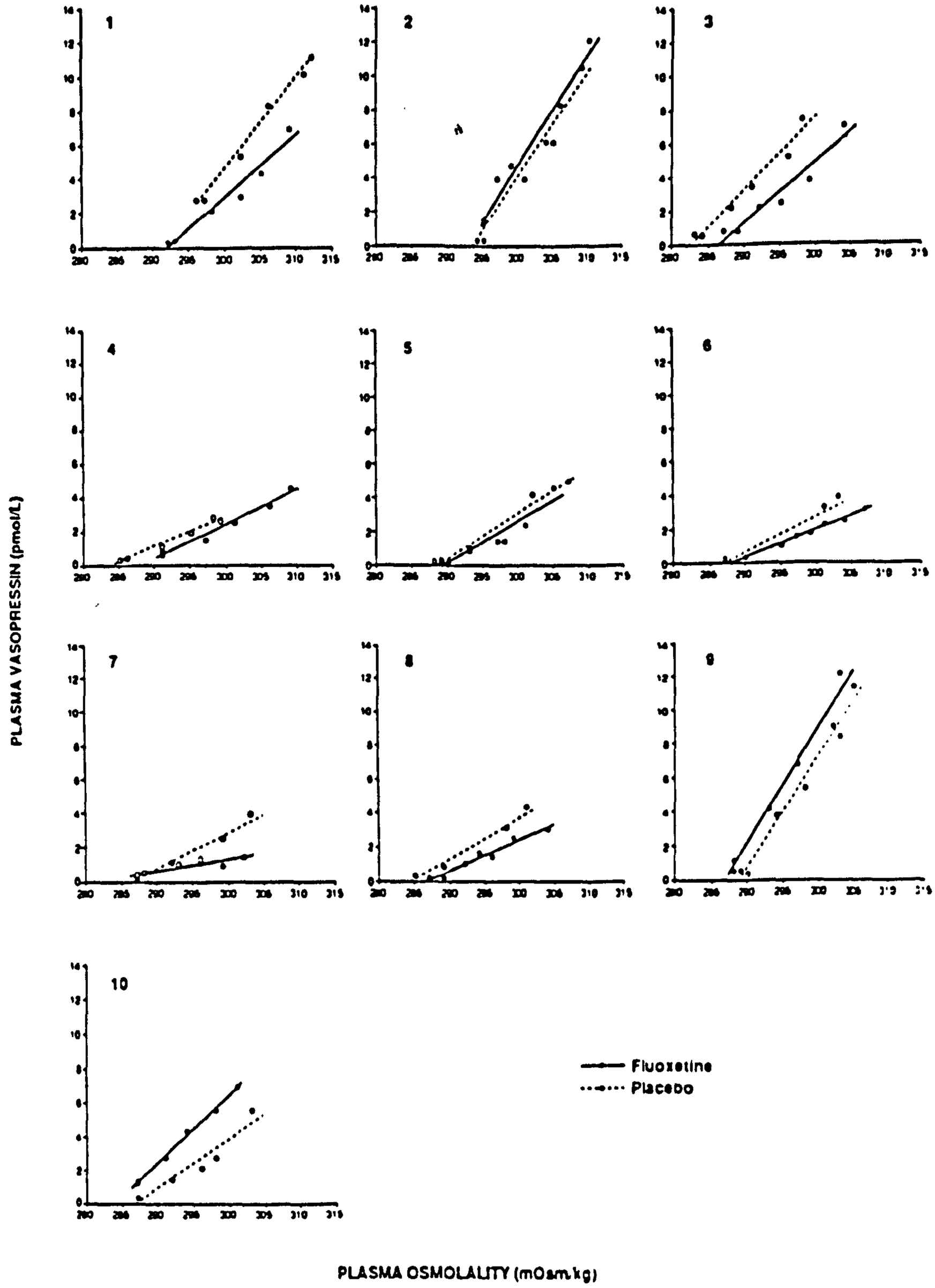


Figure 3.3

The relationship between plasma AVP and plasma osmolality during infusion of hypertonic saline (855mmol/l) for 2hr after 7 days treatment with Placebo O or Fluoxetine ●.

Table 3.4. **The sensitivity of each individual's AVP response per unit change in plasma osmolality during infusion 5%NaCl (855mmol/l) after each 7 day treatment. P=0.347 for treatment effect and p=0.128 for order effect.**

SUBJECT	PLACEBO	FLUOXETINE	DIFFERENCE
01	0.37	0.53	0.16
02	0.67	0.62	-0.05
03	0.35	0.43	0.08
04	0.20	0.18	-0.02
05	0.24	0.26	0.02
06	0.16	0.21	0.05
07	0.18	0.24	0.06
08	0.07	0.21	0.14
09	0.67	0.65	0.02
10	0.40	0.29	-0.11
MEAN	0.33	0.36	0.03

Table 3.5 **The theoretical osmotic threshold of AVP release for each subject after each 7 day treatment.**
Treatment effect p=0.700 and order effect p=0.631

SUBJECT	PLACEBO	FLUOXETINE	DIFFERENCE
01	291.9	291.4	-0.5
02	292.9	293.1	0.2
03	286.1	282.2	-3.9
04	287.5	283.0	-4.5
05	288.6	288.1	-0.5
06	287.0	286.3	-0.7
07	285.9	284.7	-1.2
08	279.4	285.8	6.4
09	286.8	288.6	1.8
10	283.8	286.1	2.3
MEAN	287.0	286.9	-0.1

DISCUSSION

There was no evidence from this study that Fluoxetine had modulatory effect on the release of AVP in response to a rise in pOS. Nor does Fluoxetine have significant effect on basal osmotic status since 7 days treatment had no significant effect on pOS, pNa or pAVP.

The reproducibility of individual response to hypertonic saline stimulation is established (Thompson et al 1991). The magnitude of inter and intra individual variation in our study is remarkably similar to the work of these authors reinforcing the finding that increase in 5HT neurotransmission has no significant effect on osmotically stimulated AVP release. The sample size that we have used in this study with the standard deviation of sensitivity and osmotic threshold of AVP release as shown would have allowed us to discern a difference of sensitivity of AVP response of $\pm$0.07pmol/l per mOsm/kg (20%) and difference in threshold of AVP release of $\pm$2.4 mosm/kg (0.9%).

The AVP response to nausea is rapid and usually to plasma concentrations of >20pmol/l. Nausea of a mild degree may cause a distortion of the physiological AVP responses not only because of high plasma concentrations but also because nausea is a more potent stimulus to AVP release than other stimuli and will take dominance in effect (Rowe et al 1979: Faull & Baylis 1991).

Subjects 2 and 9 have good linear correlation of the AVP response to change in pOS and like other, non-nauseated, subjects the regression lines after Fluoxetine and placebo treatment are very similar. Subject 8 has a less linear pattern of AVP response with coefficients of correlation of 0.95 (placebo) and 0.87 (Fluoxetine). There is a possibility that the AVP response to increase in pOS may be modified by the nausea experienced by this subject. However exclusion of this subject from data analysis does not alter any of the results as described above. The effect of nausea in this studies is therefore likely to be of little significance.

3.3 DISCUSSION

The apparent absence of an effect of 5HT on AVP release is in contrast to *in vitro* and *in vivo* animal studies which suggest that 5HT has a stimulatory effect on AVP release (Section 1.4). These studies have largely been in rats and have perhaps used higher doses of drugs which increased 5HT neurotransmission than we have done in this study in normal men which could explain the difference in findings. Some of the agents administered in the animal studies have been shown to have effects on other neurotransmitter systems in addition to their serotonergic or 5HT antagonist properties. Fluoxetine, however, is a very specific 5HT uptake inhibitor with minimal effects on other neurotransmitter systems at the dose used in these studies. In addition some, but not all, of the animal experiments were performed under anaesthetic which is known to have an effect on the hypothalamo-neurohypophysial-axis in its own right (Toth 1937: Rose et al 1984).

In this study we have given 7 days of treatment with a 5HT reuptake inhibitor. Animal studies have manipulated central 5HT concentrations by administering a single dose of pharmacological agent. It is possible that an initial disequilibrium occurs in the system which is quickly compensated by receptor down regulation or other mechanism similar to the regression of many other adverse drug effects thought to be due to increased synaptic availability of 5HT (eg. nausea, sleep disturbance). The effect of acute and more prolonged SSRI administration on 5HT neurotransmission has been discussed in Sections 1.3.4.3 and 1.5.3.2.3. The literature would suggest that Fluoxetine administration acutely increases 5HT neurotransmission. However, there is rapid adaptation of the system and within 2 days a significant reduction in 5HT neuronal firing with a decrease in 5HT neurotransmission (Clemens et al 1977). An increase in 5HT neurotransmission returns after 10-14 days continued treatment due to specific effects of the drug on autoregulatory processes. These studies, in normal healthy men after 7 days treatment with Fluoxetine, show either that there is no physiologically relevant interaction between 5HT and osmoregulatory AVP release, or that equilibrium has been adequately re-established. However, Fluoxetine associated hyponatraemia has been reported after 5 to 60 days of treatment (over 50% within 10 days) suggesting that, at least in its clinical usage

in depression, the hyponatraemia occurs rapidly and persists.

If the mechanism of this hyponatraemia in depression is failure of normal AVP suppression in response to hypo-osmolality it could be that, in this group of people, equilibrium of 5HT transmission and AVP release cannot be re-established.

There was no evidence from these two studies that Fluoxetine might have non-osmotic stimulatory effects on AVP secretion, although this was not specifically assessed. There were no significant differences in blood pressure during Fluoxetine treatment nor were there changes in blood volume as assessed by haematocrit measurements. The incidence of nausea, about 13%, was perhaps less than might have been anticipated from the literature (Wernicke 1985). It would have been interesting but obviously not possible nor ethical to have studied osmoregulated AVP secretion in the two subjects who withdrew from the study because of nausea. The subject who continued with the study, despite initial nausea, exhibited no significant differences in response to oral water load after that particular treatment (C) than after other treatments, nor were his responses significantly different from other subjects.

SECTION 4

STUDIES OF OSMOREGULATION IN THE ELDERLY, THE ELDERLY DEPRESSED PATIENT AND THE INFLUENCE OF THE SELECTIVE 5HT REUPTAKE INHIBITOR FLUOXETINE

INTRODUCTION

Abnormalities of water balance associated with depression and its treatment by antidepressant medications, have been discussed in Section 1.6.2.1. It is clear that hyponatraemia occurring in depressive illness is poorly understood. Clinically serious hyponatraemia is rare, usually arising in association with drug therapy and may be related to bipolar affective disorder and psychotic depression.

The reports of hyponatraemia associated with the clinical use of Fluoxetine have increased awareness of this clinical problem in the treatment of affective illness, but have done little to increase understanding of its aetiology or pathophysiology. The reports in the literature, to the drug manufacturers and Committee on Safety of Medicines, as discussed in Section 3, are generally of patients over 50yr who were often taking other medications suggesting that many have physical illness in addition to their mental illness. The literature reports have included patients with major depression (unipolar), bipolar affective disorder and depression with associated obsessive-compulsive features. Thus it is far from clear if patients who developed hyponatraemia with Fluoxetine had a particular mental illness, a vulnerability due to other factors, such as age, concurrent physical illness or additive effects of other medications, or if the problem was more idiosyncratic

The putative abnormalities of 5HT neurotransmission that occur in depression have been discussed in Section 1.4. The balance of evidence falls strongly in favour of a dysfunction of the 5HT system, although the manner of the dysfunction is less clear.

The SSRIs are a group of antidepressant medications which have recently been developed and have shown good therapeutic results (Lader 1988). The highly specific mode of action of Fluoxetine (Section 2.3) and its association with hyponatraemia might suggest that alteration of 5HT neurotransmission is the factor that leads to hyponatraemia by direct or indirect stimulation of AVP secretion from magnocellular neurons.

In Section 3 the effect of 7 days treatment with Fluoxetine on osmoregulation was explored in normal man and no significant changes were found. As discussed in Section 3.3 it is possible that the abnormal effects of Fluoxetine on

water balance, and possibly also other antidepressant associated with hyponatraemia in depression, are present only following their administration to depressed people. Neuroendocrine and other abnormalities related to hypothalamic dysfunction are well documented in depression (Brown et al 1988). It has been suggested that AVP, through its synergistic action with CRF on ACTH release, is, at least in part, responsible for the abnormal secretion of cortisol which occurs in depression (Schatzberg & Nemeroff 1988). It would not be surprising therefore if other functions of the hypothalamus, including osmoregulatory function, were disturbed in depression.

The objective of this section of the research was to examine osmoregulation in depression and the effect of treatment on osmoregulated AVP secretion by the SSRI, Fluoxetine, as a putative model of a "disease state" of 5HT neurotransmission.

GENERAL HYPOTHESES

1. That there is an abnormality in 5HT biochemistry in the central nervous system of depressed people.

2. That this abnormality is affected by the SSRI Fluoxetine.

3. That 5HT has a modulatory role in the physiological control of AVP secretion in response to osmotic stimuli.

4. That this modulatory role is affected by Fluoxetine which may be only apparent or more pronounced in depressed people due to their underlying abnormality of central 5HT biochemistry.

5. That the hyponatraemia occasionally observed in depressed people taking Fluoxetine is due to inappropriate secretion of AVP consequent to the influences of depression and Fluoxetine on 5HT balance.

STUDY 4.1 A RANDOMISED SINGLE-BLIND STUDY TO DETERMINE THE EFFECT OF FLUOXETINE, COMPARED TO DOTHIEPIN, ON WATER BALANCE IN ELDERLY, UNIPOLAR DEPRESSED PATIENTS.

SPECIFIC AIMS

Major

1. To study the response of drug free older unipolar depressed patients to plasma hypo-osmolality achieved by water loading.

2. To study the effect of Fluoxetine , in comparison to Dothiepin, an antidepressant with putative low central 5HT activity (Ishikawa et al 1986), on the ability of older, unipolar depressed patients to respond to hypo-osmolality achieved by water loading.

Minor

3. To see if there is any relationship between dexamethasone non-suppression and abnormality in control of water balance in unipolar elderly depressed patients.

Study design

A randomised, single blind study. Psychiatrists and patients were "unblinded" to treatment type but the researcher was "blinded".

This was to be a longitudinal, prospective study of water balance in drug free, unipolar depression and the subsequent effects of treatment with Fluoxetine in comparison with Dothiepin, an equally efficacious antidepressant with low central 5HT activity. The recruitment of patients was from psychiatric in and outpatient departments. The comprehensive study design, presented in Appendix 6, would have provided data to answer questions about the effects alone, and in interaction, of the disease state and antidepressant treatments, on water balance in depression.

Unfortunately no patients were enrolled into the study during the four months of recruitment.

There were a variety of reasons for this:-

a) Most patients with major depression, of the severity required to fulfil the inclusion criteria of this study, were commenced on antidepressant medication by their General Practitioner (GP) before referral to a psychiatrist, thus drug free patients rarely attended psychiatric outpatients.

b) Elderly patients were frequently taking a variety of other medications, particularly diuretics, which excluded their entry into the study.

c) At the time of the study Fluoxetine was a relatively new drug in the UK and psychiatrists were not using it as a first line of treatment.

The study design was modified to adapt to these practical problems. In doing so the aims of the study were simplified.

STUDY 4.2 THE STUDY OF THE RESPONSE TO AN ORAL WATER LOAD OF ELDERLY PATIENTS WITH UNIPOLAR DEPRESSION, TREATED WITH FLUOXETINE OR DOTHIEPIN, COMPARED WITH A CONTROL GROUP OF HEALTHY ELDERLY VOLUNTEERS

SPECIFIC AIMS

To study the effect of Fluoxetine, in comparison with an antidepressant of lower serotonergic activity (Dothiepin), on the ability of elderly depressed patients to respond to hypo-osmolality achieved by water loading.

MATERIALS AND METHODS

Study design

An open, unblinded study of three groups:

1. Depressed patients treated with Fluoxetine (FLUOXETINE).
2. Depressed patients treated with Dothiepin (DOTHIEPIN).
3. Normal, healthy elderly controls (ELDERLY).

Patients

Men or women aged 50 to 75yr, referred to outpatient psychiatry clinic with an episode of major depression defined by DSM-R III criteria (Appendix 7), with no history of mania.

Patients were taking Fluoxetine or Dothiepin at the time of attendance at outpatient clinic or their management required change to either of these medications.

Patients continued taking prescribed medication of either Fluoxetine or Dothiepin and each patient was studied after a minimum of two weeks treatment.

Normal controls

Men or women aged 55 to 75 yr, were recruited from the general public through contact with Age Concern (Mea House, Newcastle and High Street, Gateshead).

Exclusions

Patients and **Controls** were excluded from the study if they were currently taking any of the following medications:-

Diuretics

ACE inhibitors

Anticonvulsants

MAOI

Lithium

In addition **Controls** could not be taking, or have taken in the previous 4 weeks:-

Antidepressants

Neuroleptics

Benzodiazepines except a small dose as an hypnotic

Patients or **Controls** could have no significant other illnesses in particular; ischaemic heart disease, respiratory disease, liver disease or central nervous disorder, nor clinical abnormality of serum electrolytes, liver function tests, blood glucose, full blood count or urinalysis. Other exclusions applied as for Study 4.1 (Appendix 6).

Ethical approval

Ethical approval was obtained from Newcastle Health Authority Joint Ethics Committee.

PROTOCOL

Initial assessment

After written, informed consent had been obtained a physical examination was carried out for all subjects to exclude significant physical illness and a blood sample was be taken for analysis of electrolytes.

The consent forms and information given to each subject are included in Appendix 8.

Water load assessment

The protocol for the water load assessment is described in Section 2.1.1.1.1. In addition urine and plasma was collected for calculation of creatinine clearance (C_{creat}) during the period of maximal diuresis (60-120min after the start of water load ingestion). Creatinine was measured in both urine and plasma by an alkaline picrate spectrofluorometric method, Department of Clinical Biochemistry, Royal Victoria Infirmary.

During the water load test the severity of depression of all patients was assessed by a standard interview rating scale (Hamilton Rating Scale for depression (HRSD) (Hamilton 1960), (Appendix 9). The HRSD is the most widely used scale for patient selection and follow-up in research studies of treatments for depression. In numerous studies the total HRSD score has proved to have a high degree of concurrent validity (Carroll et al 1973). The scale relies on interviewer rating of the intensity and frequency of 17 depressive symptoms yielding a total possible score of 52. For the purposes of this research symptoms pertaining to sexual function were omitted and only 16 items were used with a total score of 50.

The interviewer was trained in the use of the scale by rating videoed subjects and comparing HRSD score with that of a psychiatrist, and by reference to a structured interview guide (Williams 1988). Appropriate revisions to interview technique were made until there was 90% agreement in the scores of the

interviewer and the trainer.

Due to the variability of depressive symptomatology during the course of the illness and its improvement it was decide to elicit from the patients symptoms pertaining to the 2 weeks prior to the water load assessment.

Follow-up

The clinical management of the **Patients** was not altered by this study and patients continued to attend outpatient psychiatry clinics in the usual way. If however the water load test was abnormal advice was given to both the patient and the Consultant psychiatrist about fluid intake and the biochemical status of the patient was monitored by the researcher.

The GP of all **Patients** and **Controls** was informed of their participation in the study. Appendix 8 contains letters relevant to the psychiatrist of **Patients** and the GPs of **Patients** and **Controls.**

After three months of recruitment into this amended study protocol only two patients had been enrolled. At this time the inclusion criteria were broadened to include:-

a) Patients with a current episode of major depression, treated with Fluoxetine or Dothiepin.

b) Patients with a *previous* episode of major depression who were taking Fluoxetine or Dothiepin as a maintenance therapy.

The exclusion criteria were unchanged.

4.2.1. THE NORMAL ELDERLY

The incidence of both hyper and hyponatraemia increases in the elderly population (Leaf 1984) . There are currently two lines of thought emerging from the literature on the changes in homeostatic mechanisms of water balance with increasing age.

It has been repeatedly documented that there is a decrease in the thirst response to osmotic change with age and it is possible that perception of other stimuli to fluid ingestion are also diminished (Phillips et al 1984). There is, however, more controversy regarding changes in osmoregulation of AVP secretion and the ability to conserve and excrete water appropriately. Early studies by Helderman et al (1978) suggested that there was in an increase in sensitivity of AVP secretion in response to a rise in pOS. More recent studies have failed to confirm this, and indeed have suggested perhaps the converse may be true (Mclean et al 1991).

Studies which have explored the ability of elderly people to excrete a water load have indicated that it is impaired and that more time is required for full excretion of the load (Crowe et al 1987).

Since this research has provided important data on the normal elderly (ELDERLY) the results of this group are presented in comparison to the normal young (YOUNG) previously presented in Section 3.1.

Statistical analysis

The results of each group were compared by two tailed t-tests.

Unless otherwise stated results are shown as mean ± SEM.

RESULTS

21 healthy normal elderly completed a water load assessment. All subjects tolerated the test well with no adverse effects. The mean age of the ELDERLY was 68yr (58-74yr).

Basal data

At the start of the water load the **ELDERLY** had a significantly higher pOS and lower uOS than the **YOUNG**. Plasma AVP concentration was significantly lower in the **ELDERLY** than in the **YOUNG** despite the higher pOS (Table 4.1). The relationship between baseline plasma AVP and uOS is shown for the two groups in Figure 4.1.

Excretion of the water load

Figure 4.2 shows the pattern of excretion of the water load as the 95 percentiles of excretion of the water load (mean $\pm$ 2SD) for the two groups. There was close approximation at most time points, but at 1hr, there was a significant difference between the groups at which time the **YOUNG** excreted 12$\pm$3% but the **ELDERLY** 21$\pm$2% ($p=0.031$). By 90 mins there was no longer a significant difference and this persisted throughout the rest of the study. At 4hr both groups had excreted over 90% of the load ingested (**YOUNG** 92.8$\pm$7.6% **ELDERLY** 91.9$\pm$4.6%, $p>0.05$).

The *pattern* of water load excretion showed marked differences between the groups in the changes in C_{H2O} and uOS (see below) as demonstrated in Figure 4.3 (c and d) . The **ELDERLY** had a significantly higher baseline C_{H2O} (**ELDERLY**-0.35$\pm$0.18 ml/min, **YOUNG** -0.95$\pm$0.1 ml/min, $p=0.036$), consistent with lower urinary concentrating ability. The C_{H2O} of the **ELDERLY** is significantly greater at 60min than in the **YOUNG** (**ELDERLY** 1.6$\pm$0.4, **YOUNG** -0.03$\pm$0.4 ml/min, $p=0.028$) consistent with the higher %water load excreted at 60min by the **ELDERLY**. After this time, however, the **YOUNG** achieved a significantly greater C_{H2O}. The increased C_{H2O} persisted for longer in the **ELDERLY** and this group was still excreting free water at 4hr.

The **YOUNG** therefore achieved excretion of the water load by a sharp increase in C_{H2O} but of short duration, the **ELDERLY** achieved the same end result by a lesser but more prolonged increase in C_{H2O}.

Table 4.1. Plasma and urine biochemistry at the start of water load in normal YOUNG and ELDERLY subjects (mean±SEM).

	pOS mosm/kg	pNa mmol/l	uOS mosm/kg	pAVP pmol/l
YOUNG	290.5±0.8	138.4±0.4	842±52	2.3±0.6
ELDERLY	293.5±0.5	139.4±0.3	508±47	0.5±0.1
	p=0.004	p=0.076	p<0.001	p<0.001

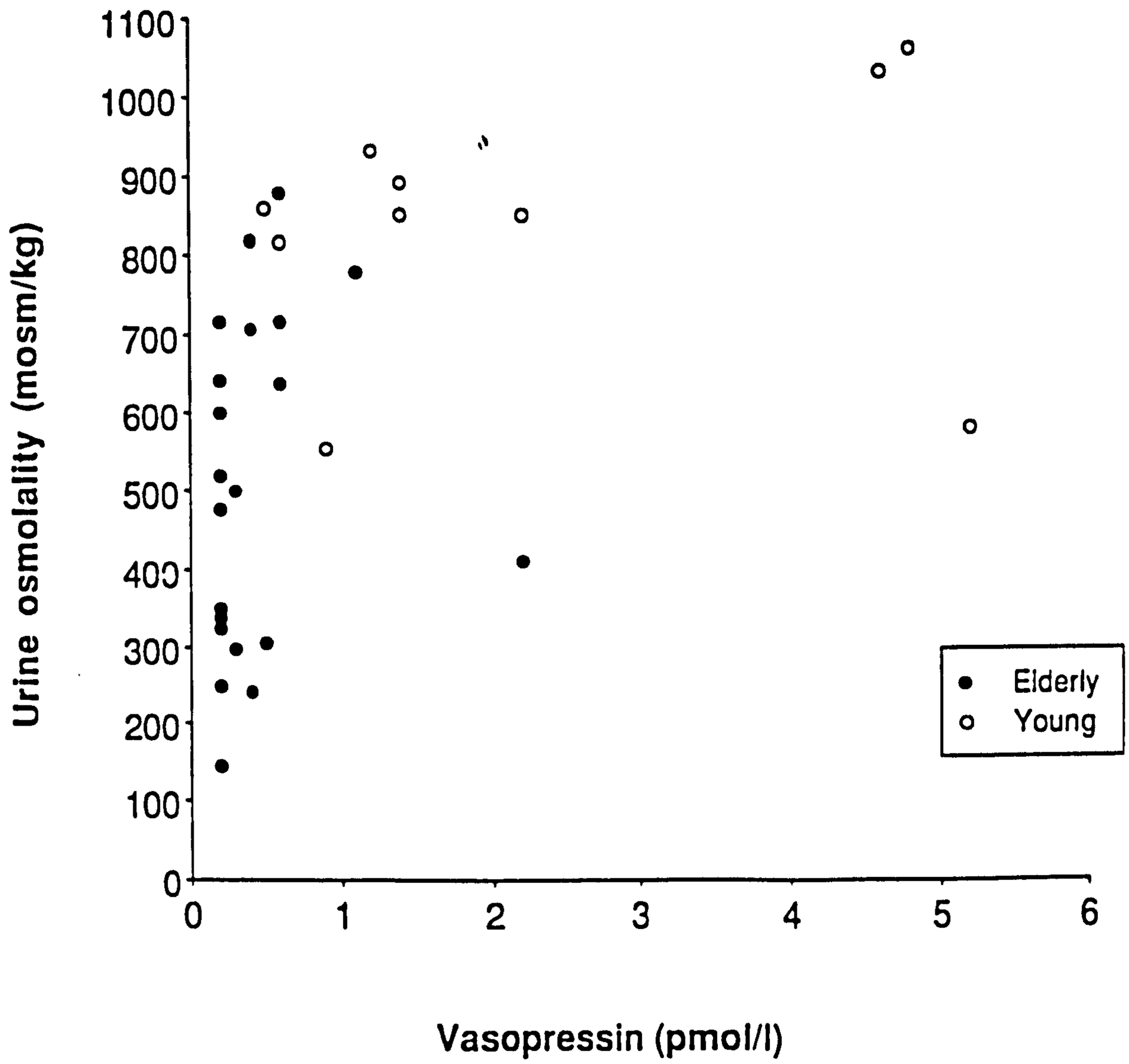


Figure 4.1

The relationship between urine osmolality and plasma AVP after overnight abstinence from drinking in ELDERLY (●) and YOUNG (O).

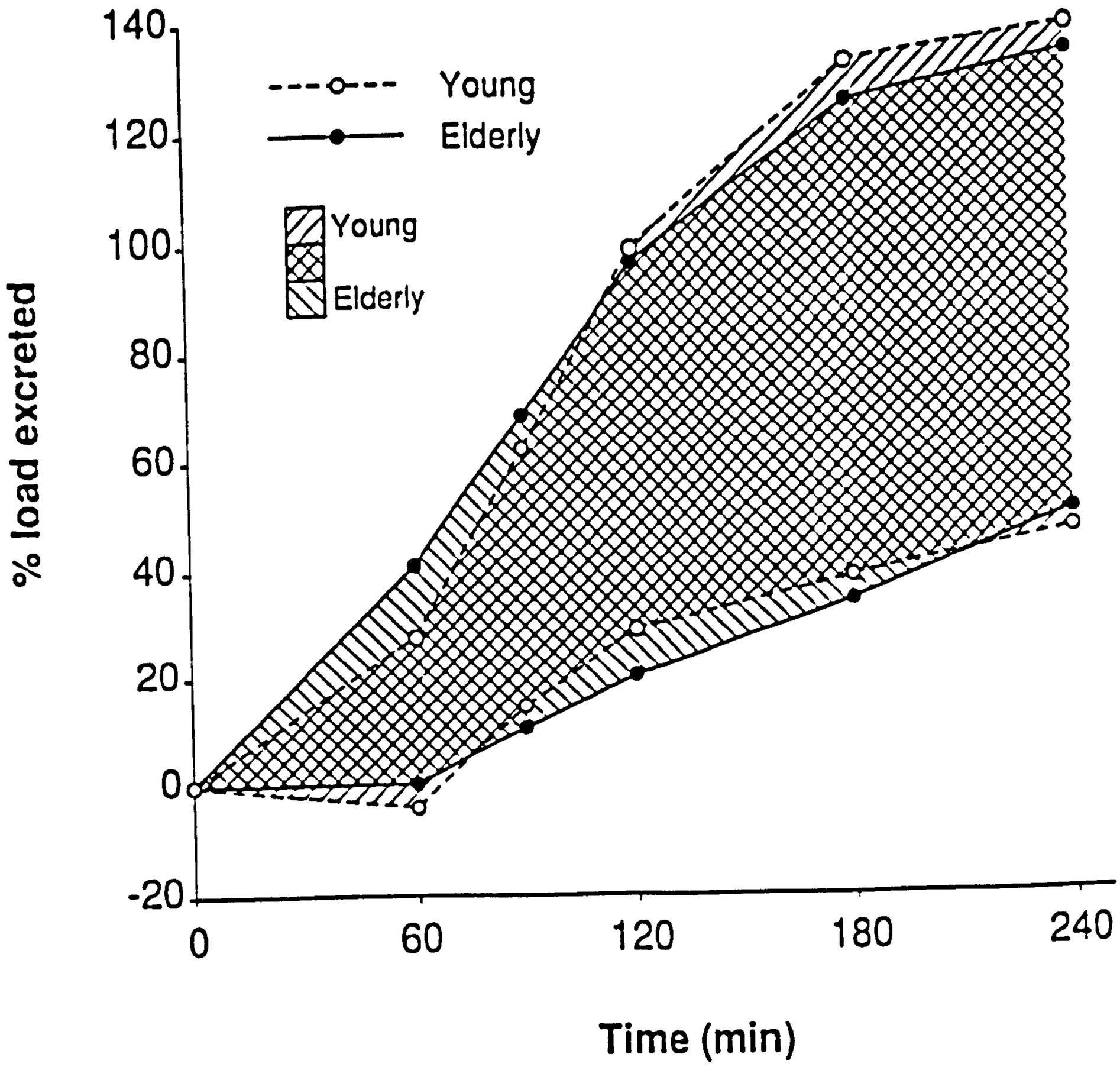


Figure 4.2

The 95 percentiles of cumulative urine excretion of the YOUNG and ELDERLY in the 4hr after water load ingestion (20ml/kg) expressed as a percentage of the oral water load. Only at 60min was there a significant difference between the two groups ($p=0.031$).

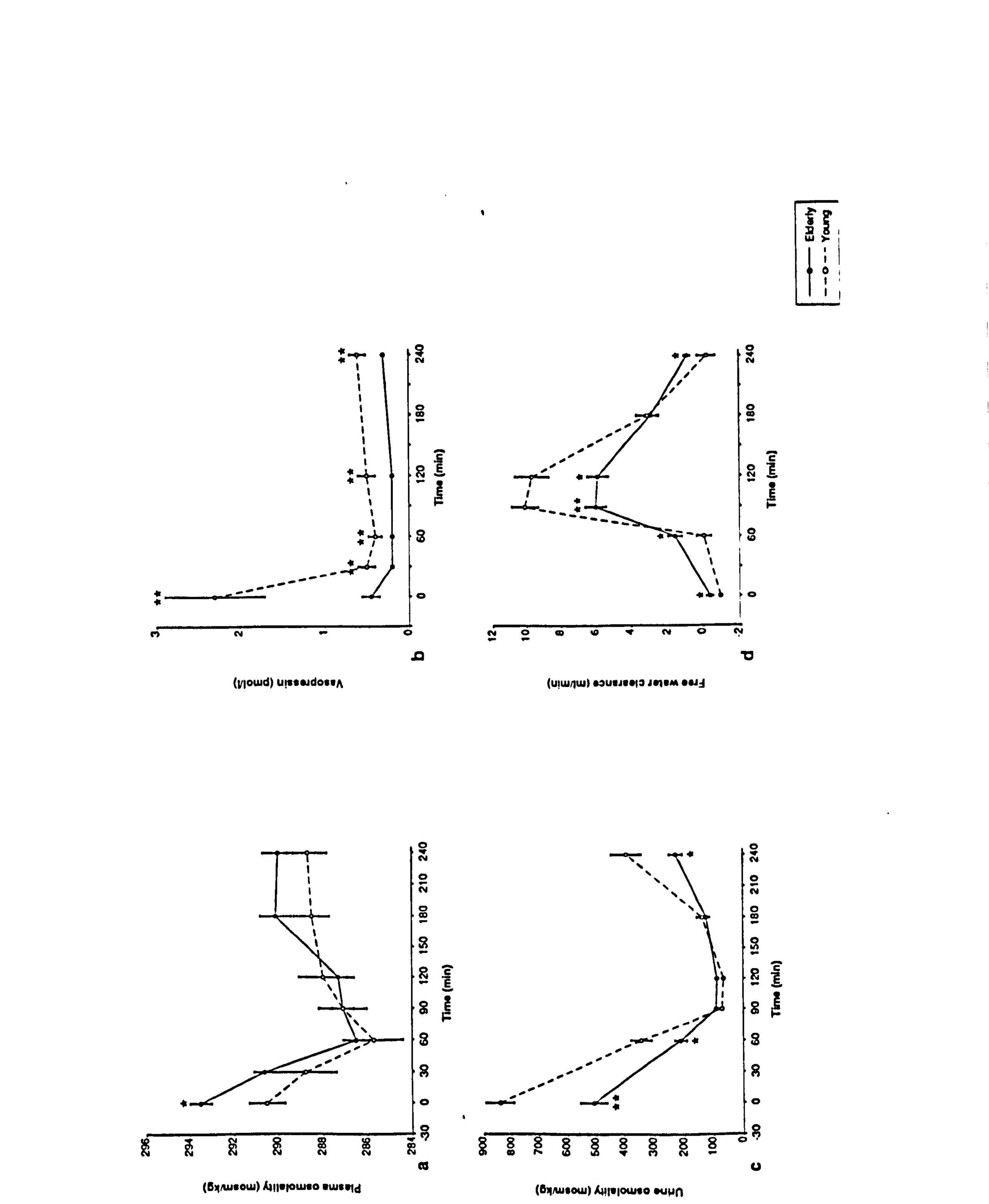
Figure 4.3

The effect of an oral water load (20ml/kg) on renal function, plasma osmolality and plasma AVP in YOUNG (O) and ELDERLY (●) subjects.

a) Plasma osmolality; b) Plasma AVP; c) Urine osmolality; d) Free water clearance.

Results compared by t-test $^{*}p<0.05$ $^{**}p<0.001$.

Results are presented as mean ± SEM.


Elderly
Young
Vasopressin (pmol/l)
Free water clearance (ml/min)
Plasma osmolality (mosm/kg)
Urine osmolality (mosm/kg)
Time (min)
a
b
c
d

Plasma AVP

At all time points AVP in the **ELDERLY** was significantly lower than in the **YOUNG**. There also appeared to be a longer suppression of AVP to minimal levels in the **ELDERLY** than the **YOUNG** (Figure 4.3b)

Plasma and osmolality sodium

Figure 4.3a shows that the initial significant difference in plasma osmolality was not apparent after ingestion of water load and did not recur after the load was excreted. The magnitude of fall in pOS after ingestion of water load was however significantly different in the two groups (**YOUNG** 5.1±0.7mOsm/kg **ELDERLY** 7.7±0.4 mOsm/kg $p=0.003$). The **YOUNG** returned to a mean pOS 1.8±0.7 mosm/kg lower than that at starting ($p=0.035$) but the **ELDERLY** remained at a pOS 3.5 ± 0.5 mosm/kg lower than baseline ($p<0.001$). The difference between the two groups was not, however, significantly different.

Plasma sodium concentrations were not significantly different between the two groups at any point in the study (data not shown).

Urine osmolality

Figure 4.3c shows the change in uOS with respect to time after the water load. The **YOUNG** achieve a greater urine dilution than **ELDERLY** but this failed to reach significance (**YOUNG** 72±6, **ELDERLY** 91±8 mosm/kg, $p>0.05$). The **ELDERLY**, however, maintained a lower urinary osmolality for a longer period than the **YOUNG** and there was again a significant difference in uOS at 4hr (**YOUNG** 392±53, **ELDERLY** 228±23, $p=0.002$).

Blood pressure

There was no significant fall in blood pressure in any of the studies of **ELDERLY** or **YOUNG**.

Nausea

During the water load one **YOUNG** subject experienced a significant degree of nausea (> 10mm on linear scale). Excluding his data from the analysis did not

alter the statistical significance of any of the results. None of the **ELDERLY** experienced more than a very mild degree of nausea.

Sex differences

As expected, since all female subjects were post-menopausal, there were no significant differences in the **ELDERLY** between male and female subject results. Data was thus combined for the statistical analysis.

DISCUSSION

There were three remarkable observations from this study. Firstly, there was no effective difference in the ability of healthy **ELDERLY** or **YOUNG** to excrete an oral water load except in the very early stages when the **ELDERLY** began to excrete water more rapidly than **YOUNG**. Secondly, the osmoregulatory mechanisms of achieving this result were quite different between **YOUNG** and **ELDERLY**, and thirdly, the baseline differences between the two groups were substantial.

After ingestion of the water load the **ELDERLY** achieved a concentration of AVP sufficient to allow urinary dilution more rapidly than the **YOUNG** and consequently diuresis began more rapidly in this group. The pattern of water excretion then changed between the two groups. The **YOUNG** had a short but marked increase in C_{H2O}, the **ELDERLY** a less marked but more prolonged change. The magnitude of maximum C_{H2O} in **YOUNG** and **ELDERLY** is similar to that found in other studies (Lindeman et al 1966; Crowe et al 1987). This might be solely a consequence of a reduction in glomerular filtration rate in the **ELDERLY** or it could be secondary to a reduction in maximal dilutional ability with age . Crowe et al (1987) found that if the reduction with age in C_{creat} was taken into account, no significant differences in C_{H2O} were found between young and elderly. Figure 4.4 shows the C_{H2O}max for each group and C_{H2O} adjusted for C_{creat} in the **ELDERLY**. It can be seen from this that there is no simple relationship in this study between reduction in renal function and reduction in C_{H2O}max since there is still a significant difference between **YOUNG** and **ELDERLY**.

There was no significant difference between the **YOUNG** and **ELDERLY** in

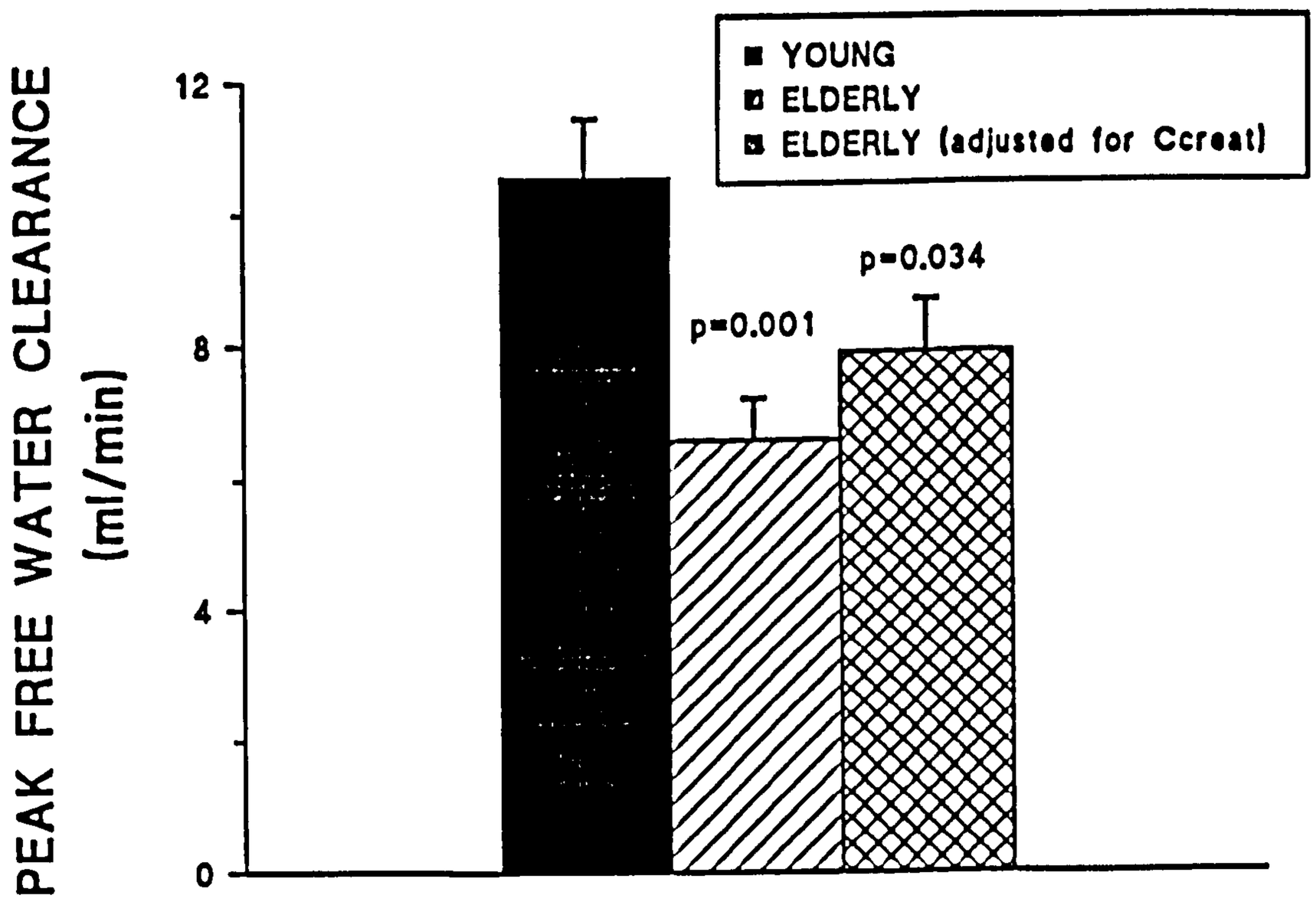


Figure 4.4

The peak free water clearance after the water load of YOUNG (■), ELDERLY (▨) and the mean ELDERLY value adjusted for variation in renal function (C_{creat}) (▩). Comparison by t-tests of the two ELDERLY groups to the YOUNG shows that only some of the significant difference in peak free water clearance is explained by a deterioration of renal function with age.

Results are presented as mean ± SEM.

minimum urine concentration but there was a trend for the **ELDERLY** to have a significantly higher urine concentration during maximal C_{H2O}. Other studies have found similar results after a water load (Lindeman et al 1966) and there is evidence that the aging kidney has a diminished ability to dilute urine due to an impaired sodium conservation mechanism (Macias 1980; Weinstock Brown et al 1985).

It would seem that the potential problem of incomplete bladder emptying was not a source of error in this study since this would lead to an under estimate of water load excretion in the **ELDERLY**. The dilution of urine to <100mOsm/kg would also militate against incomplete urine collection at each time point.

Despite these changes in osmoregulatory mechanisms with age there was no net effect on the ability to excrete the water load. It might be anticipated, however, that a large volume of water or salt deplete fluids (eg. 5% dextrose) would lead to a deficit in excretion by the **ELDERLY** compared to the **YOUNG** due to the diminished maximal C_{H2O}. This may be particularly relevant to the occurrence of hyponatraemia in the hospitalised elderly (Sunderam & Mankikar 1983: Anderson et al 1985).

As expected the **ELDERLY** had a substantially higher baseline pOS than the **YOUNG**. Baseline AVP is, however, significantly *lower* in the **ELDERLY**. This is in contrast to other studies which have shown either no difference in basal AVP concentrations in the elderly (Helderman et al 1978; Rowe et al 1982; Chiodera et al 1991) or higher concentrations (Frolkis et al 1982; Kirkland et al 1984). One study has found a lower concentration of AVP in the elderly than in the young (Li et al 1984). These differences in findings may be explained in part by differences in the age group studied. The AVP concentrations observed in the **ELDERLY** would not be expected to achieve urinary concentration greater than 400mOsm/kg (Thompson 1989). In this study we found a mean urinary concentration of over 500mOsm/kg at baseline in **ELDERLY** which suggests that urine was concentrated by a mechanism independent of AVP, or that renal AVP receptors were upregulated.

Low plasma AVP concentration, high normal pOS and greater than expected uOS would suggest that **ELDERLY** are in a similar biochemical and hormonal state as to that seen in patients with partial cranial diabetes insipidus (CDI)

where despite "inadequate" plasma concentrations of AVP compared to normals urinary concentration can occur to a limited extent (Miller et al 1970; Zerbe & Robertson 1981). This is thought to be due to an increased renal tubular sensitivity to AVP due probably to AVP receptor up-regulation (Block et al 1981). This increased tubular sensitivity to AVP was reported in early studies of water balance in the elderly where infusion of submaximal concentration of vasopressin during sustained maximal diuresis lead to a much greater rise in urine osmolality in the elderly than in a young population (Lindeman et al 1966). Patients with mild partial CDI pass 2.5-4 litres of urine per day and maintain, by adequate fluid intake, a high normal plasma osmolality.

This is the first study to our knowledge that has investigated physiological osmoregulation in the elderly in such a large group of healthy, non-hospitalised subjects. The biochemical and hormonal findings which suggest that partial AVP deficiency develops with age are controversial. Previous research has shown that there maybe an increased AVP response to rise in pOS (Helderman et al 1978; Phillips et al 1984; Bevilacqua et al 1987) and this is the view expressed in many standard texts (Miller 1985). Not all studies have confirmed this however. Li et al (1984) showed that the elderly had a smaller increase in plasma AVP than the young after 14hr water deprivation and these authors suggested that the elderly may be in a state of incomplete CDI. Two recent reports have also indicated that there is perhaps a decreased sensitivity of AVP release to rise in pOS in the elderly (Mclean et al 1991; Duggan et al 1992).

Studies of non-osmotic stimulation of AVP by orthostatic stimulus have shown a failure of AVP secretion with age (Segar & Moore 1968; Rowe et al 1982; Bevilaqua et al 1987). Other studies have shown an age related augmentation in AVP release in response to metaclopramide stimulation (Bevilaqua et al 1987; Chiodera et al 1991) but no age effect on AVP release stimulated by hypoglycaemia (Chiodera et al 1991). Miller (1985) and others (Chiodera et al 1991) have argued that the apparent changes with age of osmotic regulated AVP secretion are related to an imbalance of afferent inputs to AVP secreting neurons in the hypothalamus secondary to reduction of afferent impulses from arterial baroreceptors. This imbalance leads to altered sensitivity to other stimuli to AVP secretion , possibly mediated by increased endogenous opioid activity. Such an hypothesis is not incompatible with the findings of this study

and further work to explore all facets of AVP secretion in individual elderly rather than population studies may elucidate the complex and probably variable interrelations and pathophysiology.

Many elderly people complain of polyuria, especially at night . As well as to urological problems such as prostatism, this has been usually attributed to a decrease in urinary concentrating ability subsequent to a diminished GFR which is well recognised to occur with age (Lindeman et al 1960; Rowe et al 1976; Phillips et al 1984). This study has provided evidence that at least part of the diminished urinary concentrating ability may be due to a deficiency of AVP. Miller & Shock (1953) suggested that renal tubular concentrating ability in the elderly was relatively insensitive to AVP administration. This was not confirmed however, in a later study (Lindeman et al 1966) . This difference in findings remains unexplained. Similar to the argument used to explain the inadequacy of urine concentration after exogenous AVP in both CDI and primary polydipsia (Zerbe & Robertson 1981), failure of exogenous AVP to increase further urine osmolality in the elderly (Monson & Richards 1978) may be explained by a relatively hypo-osmolal renal medullary interstitium (a consequence of both renal aging and polyuria itself, de Wardener & Herxheimer 1957). It does not necessarily confirm either a renal resistance to AVP nor does it necessarily imply that the senescent loss of concentrating ability can not be due to inadequate plasma AVP concentrations.

Together with the well documented decrease in thirst perception with age, and the difficulty many elderly people may have with the practicalities of toiletting the possibility that a deficiency of osmotically stimulated AVP occurs in the elderly has very important implications for the maintenance of fluid balance and further research in the normal elderly is called for.

4.2.2 THE DEPRESSED ELDERLY PATIENTS

Ten patients treated with Dothiepin and six patients treated with Fluoxetine were recruited into the study. Unless otherwise stated results were compared by unpaired t-tests and presented as mean±SEmean.

4.2.2.1 PATIENTS TREATED WITH DOTHIEPIN (DOTHIEPIN)

Four male and six female patients volunteered to take part in the study. Mean age was 65yr (52-77yr). The mean age and age range were not significantly different from the **ELDERLY**.

All patients tolerated the water load without adverse effect. None experienced a significant fall in blood pressure or a significant change in degree of nausea during the water load test.

Basal data

The plasma and urine biochemistry and plasma AVP concentration of the **DOTHIEPIN** and **ELDERLY** groups are shown in Table 4.2. There were no significant differences between the groups in any of these parameters.

Excretion of the water load

Both the **ELDERLY** and **DOTHIEPIN** groups achieved a mean excretion of over 90% of the water load in the 4hr after ingestion (**ELDERLY** 91.9±4.6%, **DOTHIEPIN** 92.0±6.1%, P=0.988). At no time point were there significant differences between the two groups in the percentage of the load excreted. Similarly, at no time point were there significant differences in the mean C_{H2O} of the two groups (Figure 4.5).

Plasma osmolality

Figure 4.6a shows the pattern of change in pOS of the **DOTHIEPIN** group compared to the **ELDERLY**. There were no significant differences between the two groups at any time point.

Table 4.2 The baseline plasma and urine biochemistry and AVP in normal ELDERLY controls and depressed patients treated with DOTHIEPIN (mean±SEM)

	pOS mosm/kg	pNa mmol/l	uOS mosm/kg	pAVP pmol/l
ELDERLY	293.5±0.5	139.4±0.3	508±47	0.5±0.1
DOTHIEPIN	291.9±1.2	138.6±0.6	608.9±60	1.0±0.6
	p=0.238	p=0.258	p=0.202	p=0.401

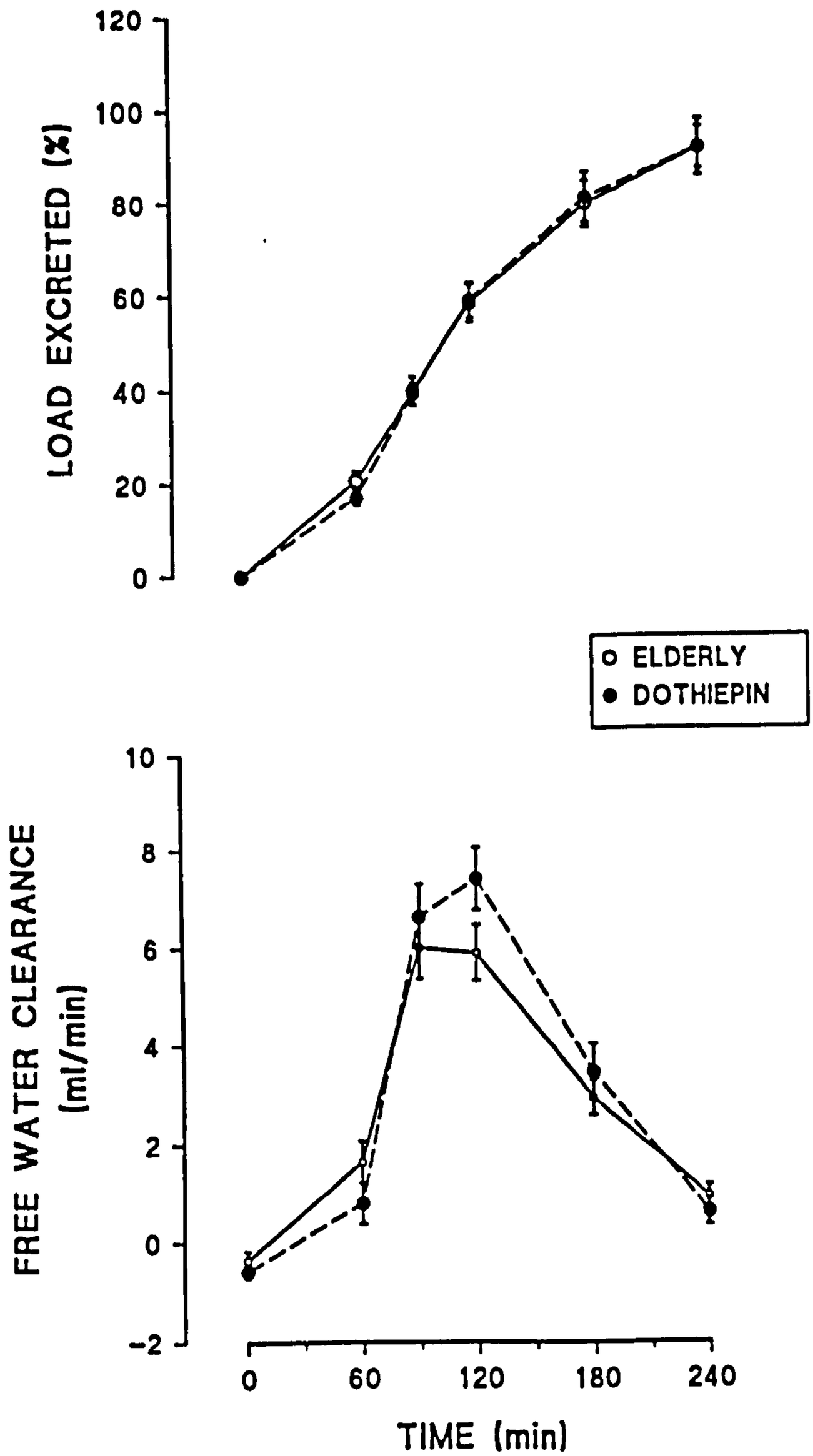


Figure 4.5

The excretion of an oral water load (20ml/kg) by healthy **ELDERLY** subjects and depressed subjects treated with **DOTHIEPIN.**

a) The cumulative urine excretion in the 4hr after the oral water load expressed as a percentage of the water load.

b) The change in free water clearance after the oral water load.

At no time point were there any significant differences between the two groups.

Results are presented as mean ± SEM.

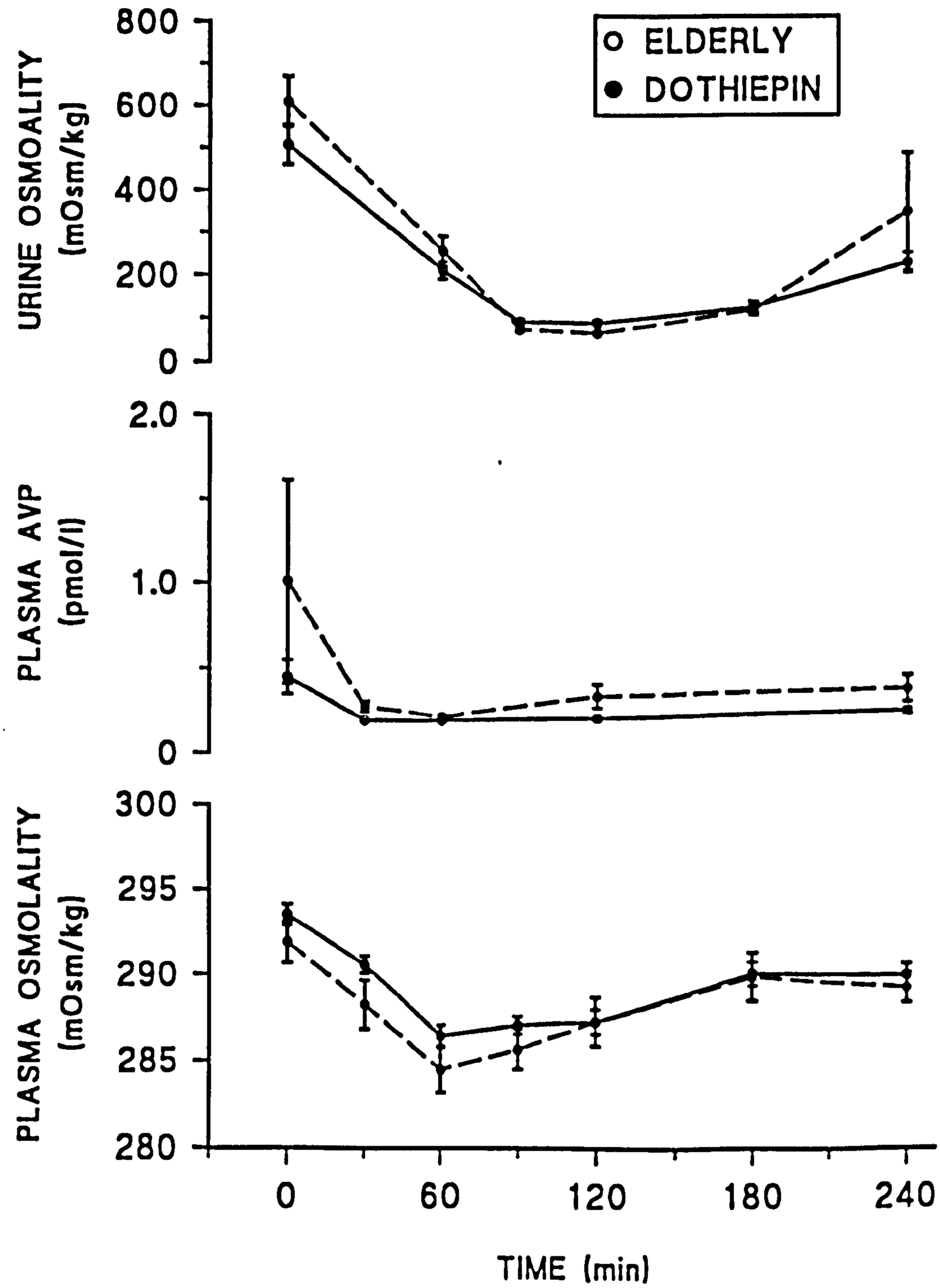


Figure 4.6

The effect of an oral water load (20ml/kg) on plasma and urine osmolality and plasma AVP in the 4hr after fluid ingestion in healthy **ELDERLY** and depressed elderly treated with **DOTHIEPIN.**

Results are presented as mean ± SEM.

Plasma AVP

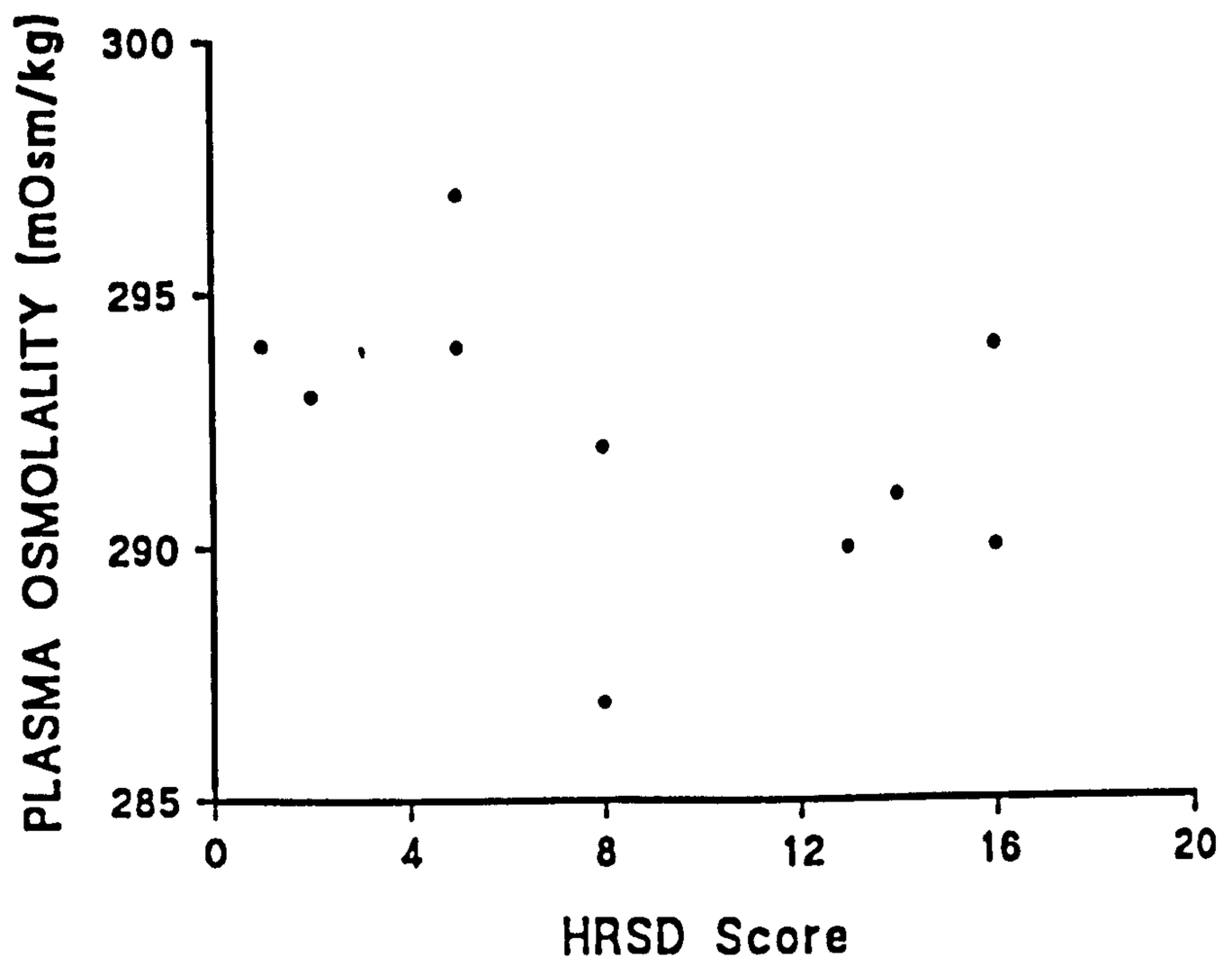
The changes in pAVP are shown for the **DOTHIEPIN** and **ELDERLY** groups in Figure 4.6b. There was a statistically significant difference in pAVP between the two groups at 30min after the start of ingestion of the water load (**ELDERLY** 0.2±0.01pmol/l, **DOTHIEPIN** 0.28±0.09pmol/l, p=0.031). The limit of detection of pAVP by RIA used in this study was 0.2pmol/l (Section 2.2.6). For the purposes of data analysis in this research any individual measurement of pAVP below the limit of detection was attributed the value of the detection limit ie.0.2pmol/l. Thus statistical analysis was perhaps not valid at such low concentrations of AVP, where many individual measurements were below the detection limit of the assay. What is important, however, is that both groups appropriately and adequately suppressed AVP in response to plasma hypo-osmolality.

Urine osmolality

The **DOTHIEPIN** group showed changes in urinary concentration in response to oral water loading which were remarkably similar to those of **ELDERLY** (Figure 4.6c). At 120min after the start of water load ingestion uOS was significantly different between the two groups (**ELDERLY** 87.7±7.7mOsm/kg, **DOTHIEPIN** 65.8±2.7mOsm/kg, p=0.013). At such low osmolalities there is considerable error in measurement and the results must be interpreted with caution. What is important is that both groups diluted urine to <100mOsm/kg (Section 2.1.1.1).

Depression and water balance

The HRSD score was used as an estimate of the severity of depression of each patient. The **DOTHIEPIN** group had HRSD scores ranging from 2 to 16 out of a possible score of 50, suggestive of mild to moderate depression at the time of water load assessment. Figure 4.7 shows that there was no linear relationship between this score and either the basal osmotic status as measured by pOS (r=-0.410, p>0.05), or the percentage of the water load excreted after 4hr (r=-0.284, p>0.05).



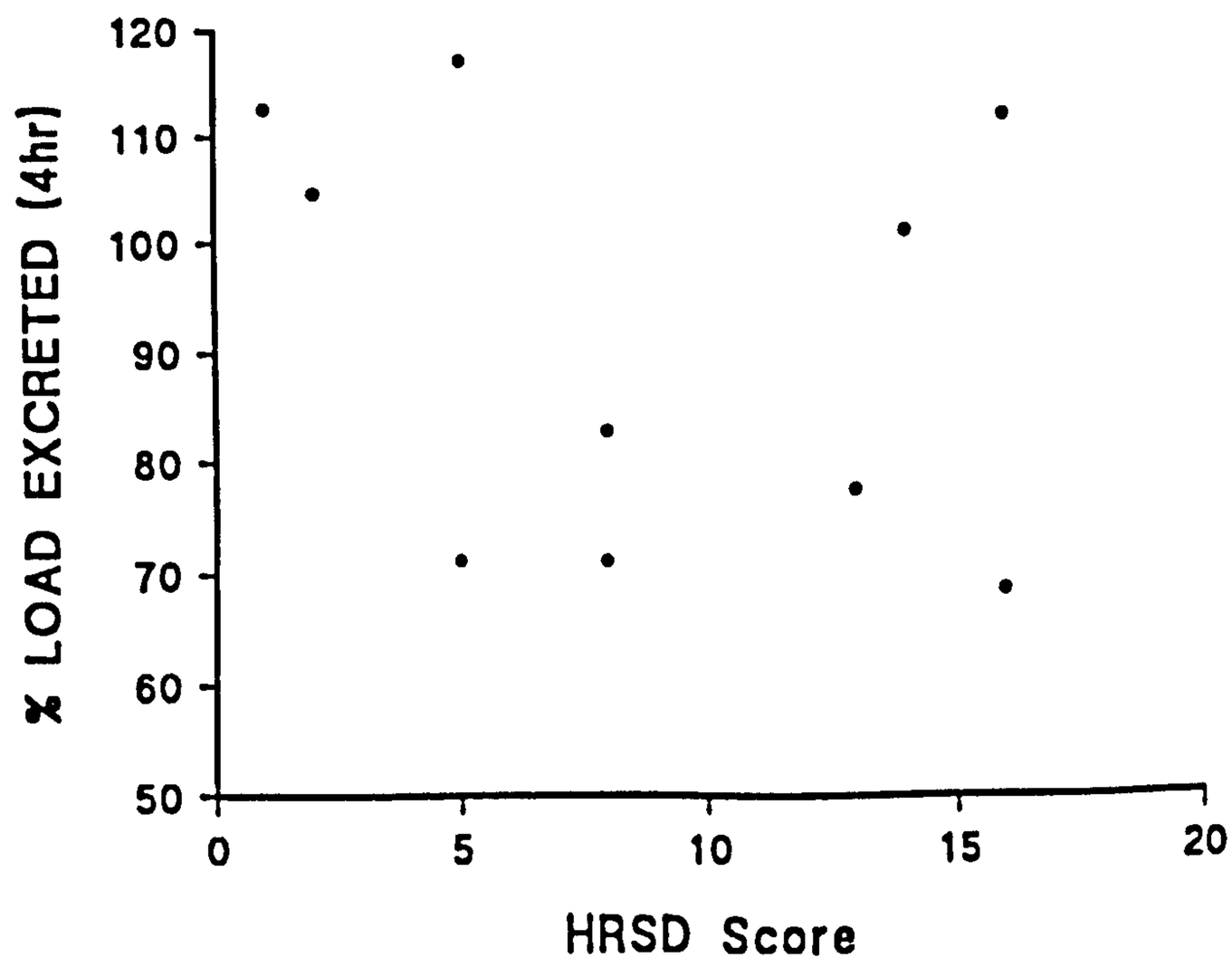


Figure 4.7

a) The relationship between the basal plasma osmolality and the severity of depression (HRSD score) for each patient treated with Dothiepin.

b) The relationship between the percentage of the water load excreted after 4hr and the severity of depression (HRSD score) for each patient treated with Dothiepin.

DISCUSSION

There was no evidence from this study that patients with depression, treated with Dothiepin, had any abnormality in basal osmotic status or in their ability to respond to an oral water load and consequent plasma hypo-osmoality, when compared to a group of healthy people of similar age. Indeed the two groups had remarkable similarities in osmoregulatory function.

There was no evidence that the severity of depression was related to any change in osmoregulation. However, since the number of patients in this study was small and the HRSD scores were over a very small range of mild to moderate depression, it is not possible to conclude that osmoregulatory function is not related to severity of depression.

4.2.2.2 PATIENTS TREATED WITH FLUOXETINE (FLUOXETINE)

Four female and two male patients were recruited to take part in the study. Fluoxetine treatment had usually been commenced after a trial of other medications and therapies. Thus some of the patients had complex and resistant psychiatric illness. Others were treated with Fluoxetine because of physical frailty and the occurrence of adverse effects with other antidepressants. Some patients therefore had a variety of physical illnesses in addition to their depression.

Subjects had received Fluoxetine for 20-300 days.

The results of the individual subjects are presented here as brief case histories since the complex nature of their illness and the small numbers make statistical analysis of the group as a whole inappropriate.

Subjects

<u>F1.</u>

64yr female.

Medical problems: partial gastrectomy for peptic ulcer disease, glaucoma.

Other medications: Thioridazine 25mg QID. Ranitidine 300mg nocte.

Psychiatric problems: Several episodes of unipolar depression previously treated with tricyclic antidepressants. Current episode resistant to tricyclic medications.

HRSD score 17.

Fluoxetine treatment: Dose 20mg BD, duration 20day.

F2.

60yr male.

Medical problems: None.

Other medications: Thioridazine 100mg nocte.

Psychiatric problems: Recurrent unipolar depression over 5yr. Current episode prolonged and resistant to other medical treatments.

HRSD score 12.

Fluoxetine treatment: Dose 20mg BD, duration 300day.

F3.

64yr female.

Medical problems: Hypertension, duodenal ulcer, arthritis.

Other medications: Aetenolol 50mg OD, Distalgesic 2tab QID, Temazepam 10mg nocte.

Psychiatric problems: No previous episode of depression. Equivocal diagnosis of dementia or pseudodementia secondary to depression. CT scan suggested generalised atrophy with some suggestion of multiple small infarcts.

HRSD score: 20.

Fluoxetine treatment: Dose 20mg nocte, duration 157day.

F4.

52yr female.

Medical problems: Arthritis.

Other medications: Naproxen 250mg BD.

Psychiatric problems: No previous episode of depression. Treated with ECT prior to Fluoxetine.

HRSD score: O

Fluoxetine treatment: Commenced as maintenance or prophylactic therapy after ECT. Dose 40mg, duration 140day.

F5.

69yr female.

Medical problems: Arteriopath. Previous bi-femoral bypass graft. Partial gastrectomy for peptic ulcer disease.

Other medications: None.

Psychiatric problems: No previous episode of depression.

HDSR score 13.

Fluoxetine treatment: Dose 20mg OD, duration 112day.

F6.

50yr male.

Medical problems: Peptic ulcer disease.

Other medications: Temazepam 10mg nocte.

Psychiatric problems: Longstanding anxiety. First episode of depression.

HDSR score: 10.

Fluoxetine treatment: 20mg OD, duration 72day.

Basal data

The plasma and urine biochemistry and plasma AVP of the six subjects at the start of the water load are shown in Table 4.3. The data for **ELDERLY** and **DOTHIEPIN** from Table 4.2 are included for ease of comparison. The pOS of subject **F4** was just below the 95 percentile of the **ELDERLY** (288.4-298.4mOsm/kg). This subject was the youngest of the group (52yr) and, since pOS increases with age (Section 4.2.1) this may have accounted for the difference. The pAVP concentration of subject **F5** was greater than the 95 percentile of the **ELDERLY** (0.2-1.4pmol/l). This subject also had a high basal pOS and the pAVP concentration appeared to be appropriate to this.

There was no evidence of true hypo-osmolality or SIAD in the basal measurements of any of the subjects.

Excretion of the water load

The percentage of the oral water load excreted after 4hr is shown for each subject in Figure 4.8. The data for **ELDERLY** and **DOTHIEPIN** groups are also shown. Subject **F5** excreted a significantly lower percentage of the load

Table 4.3 Basal osmotic status of patients treated with FLUOXETINE in comparison to DOTHIEPIN and ELDERLY groups (mean±SEM)

	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	uOS (mosm/kg)	FLUOXETINE (day)
ELDERLY	293.5±0.5	139.4±0.3	0.5±0.1	508±47	-
DOTHIEPIN	291.9±1.2	1386.6±0.6	1.0±0.6	608.9±60	-
Fluoxetine					
1	292	139	0.5	487	20
2	294	139	1.2	739	300
3	293	141	0.6	-	157
4	287	139	0.7	658	140
5	298	139	2.5	544	112
6	293	140	0.4	652	72

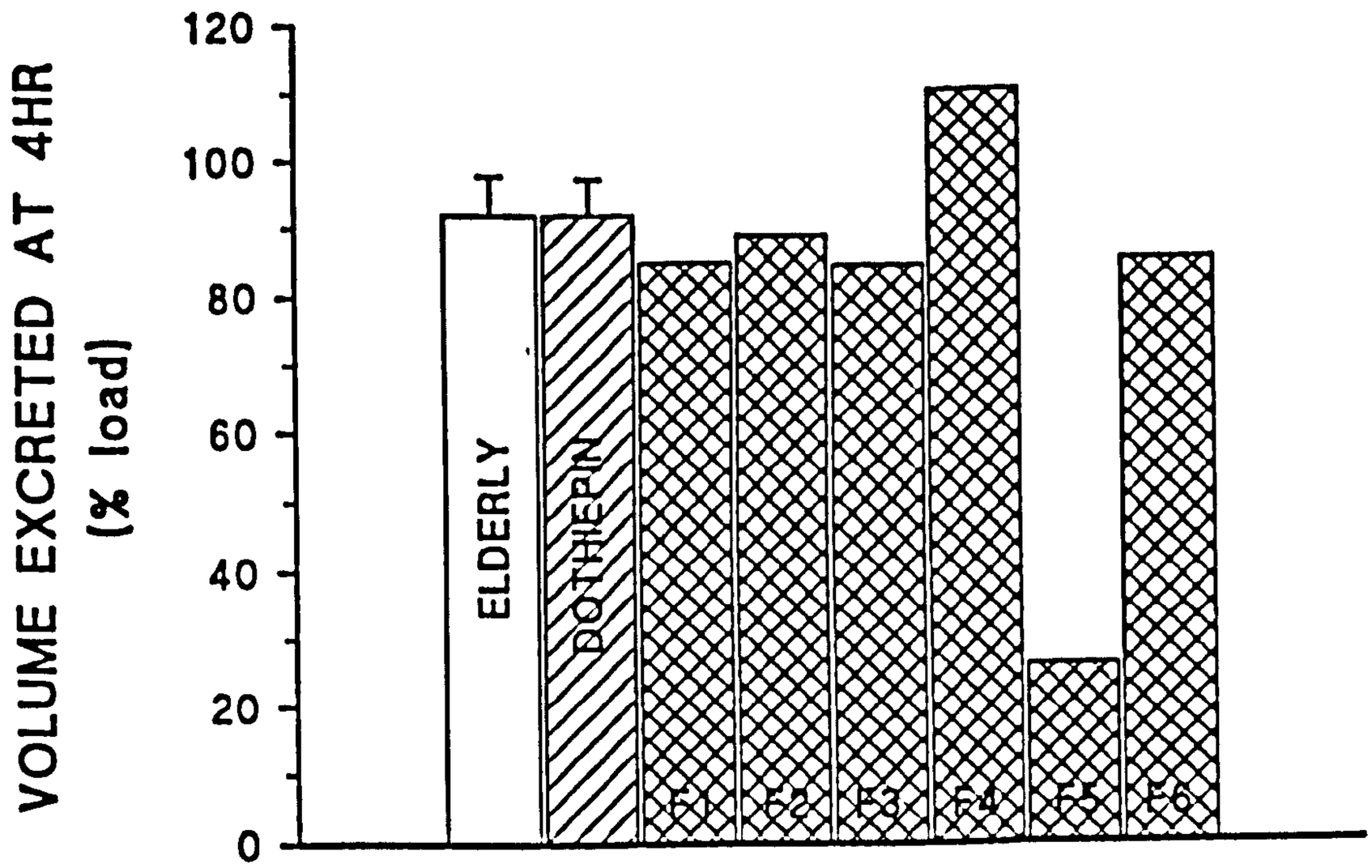


Figure 4.8

The percentage of the oral water load (20ml/kg) excreted in 4hr by each depressed elderly subject treated with Fluoxetine (F1-F6) compared to healthy ELDERLY and depressed patients treated with DOTHIEPIN.

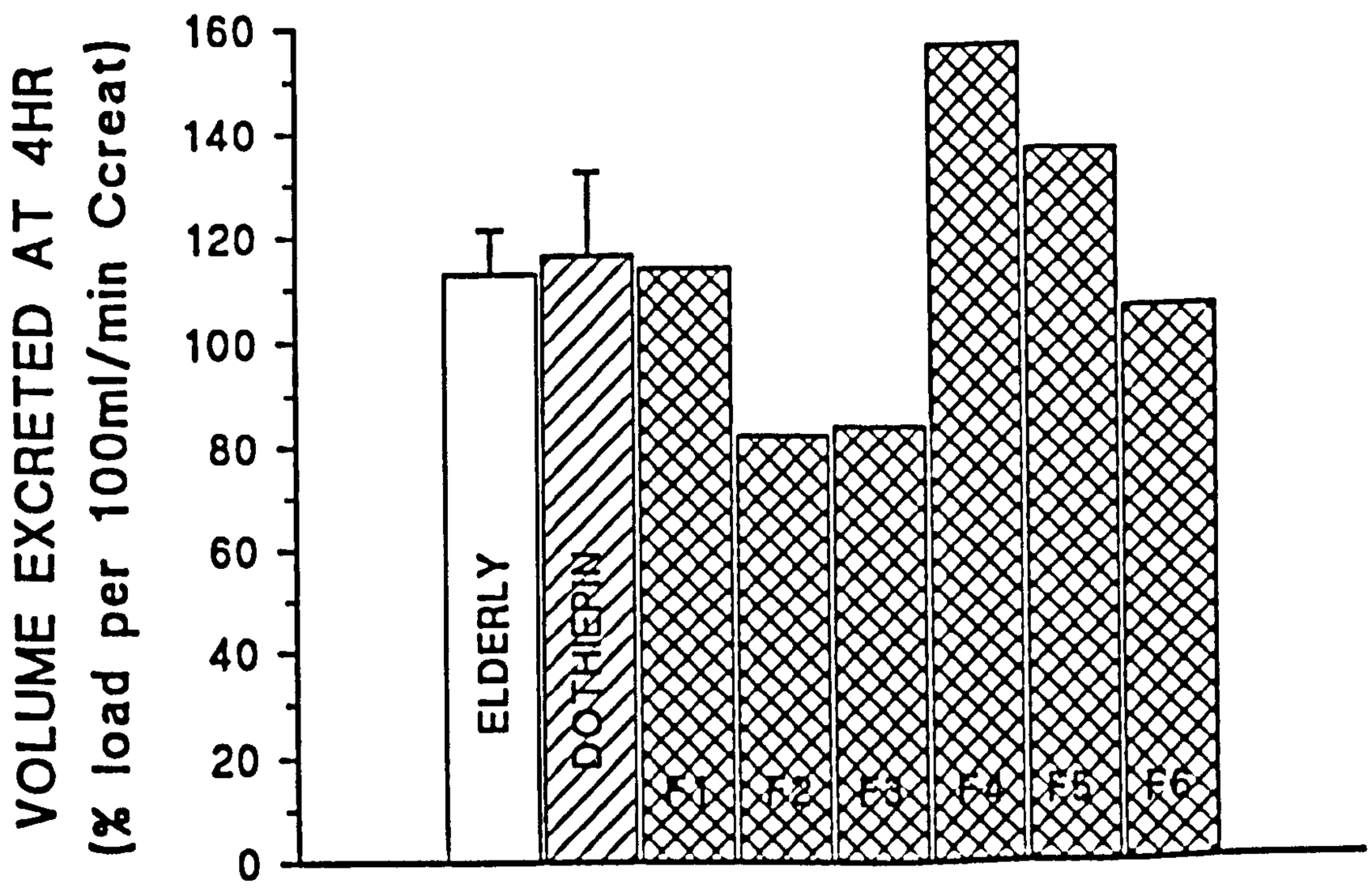


Figure 4.9

The percentage of the oral water load excreted after 4hr by healthy ELDERLY, depressed patients treated with DOTHIEPIN and depressed patients treated with Fluoxetine (F1-F6) adjusted for variation in renal function (C_{creat}).

than that of **ELDERLY** (95 percentiles 50.1-133.7%). All other subjects achieved a total excretion volume within these limits. Figure 4.9 shows the percentage of the water load excreted adjusted for C_{creat} for all **FLUOXETINE** subjects and the **DOTHIEPIN** and **ELDERLY** groups. The abnormality volume of the water load excretion by subject **F5** was largely explained by her very abnormal C_{creat} (19ml/min).

The pattern of water load excretion for each individual in comparison to the 95 percentiles for the **ELDERLY** at each time point is shown in Figure 4.10. There was much inter-individual variation in the pattern of excretion. Subject **F5** showed an abnormal excretion of the water load from 90min after the start of water load ingestion. None of the other subjects showed any apparent delay in excretion of the water load.

Changes in plasma and urine

The changes in response to the water load of pOS and uOS, plasma AVP and C_{H2O} for each individual are shown in figures 4.11-4.16. The 95 percentiles of response of the **ELDERLY** are shown for comparison.

Subject **F1:** (Figure 4.11)

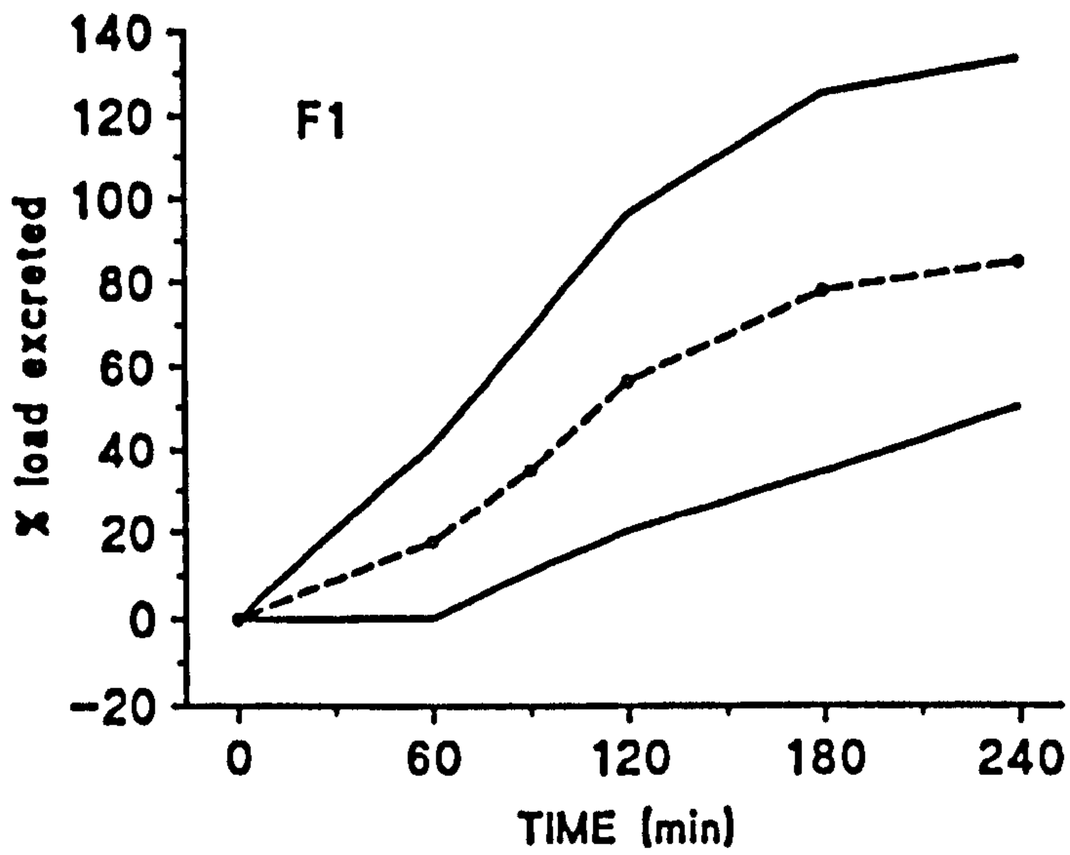
The pattern of change of all parameters was similar to that of the **ELDERLY** at all time points. There was no evidence of any abnormality of response to the oral water load and no evidence of inadequate suppression of AVP in response to plasma hypo-osmolality.

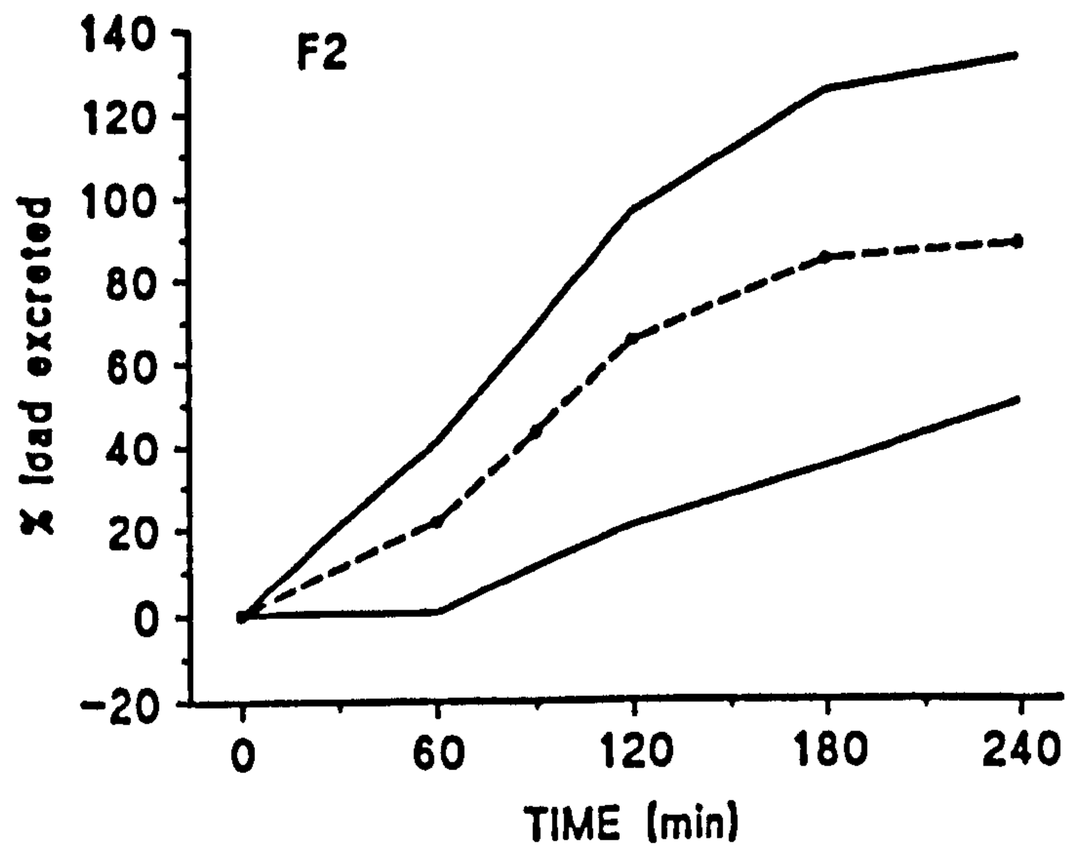
Subject **F2:** (Figure 4.12)

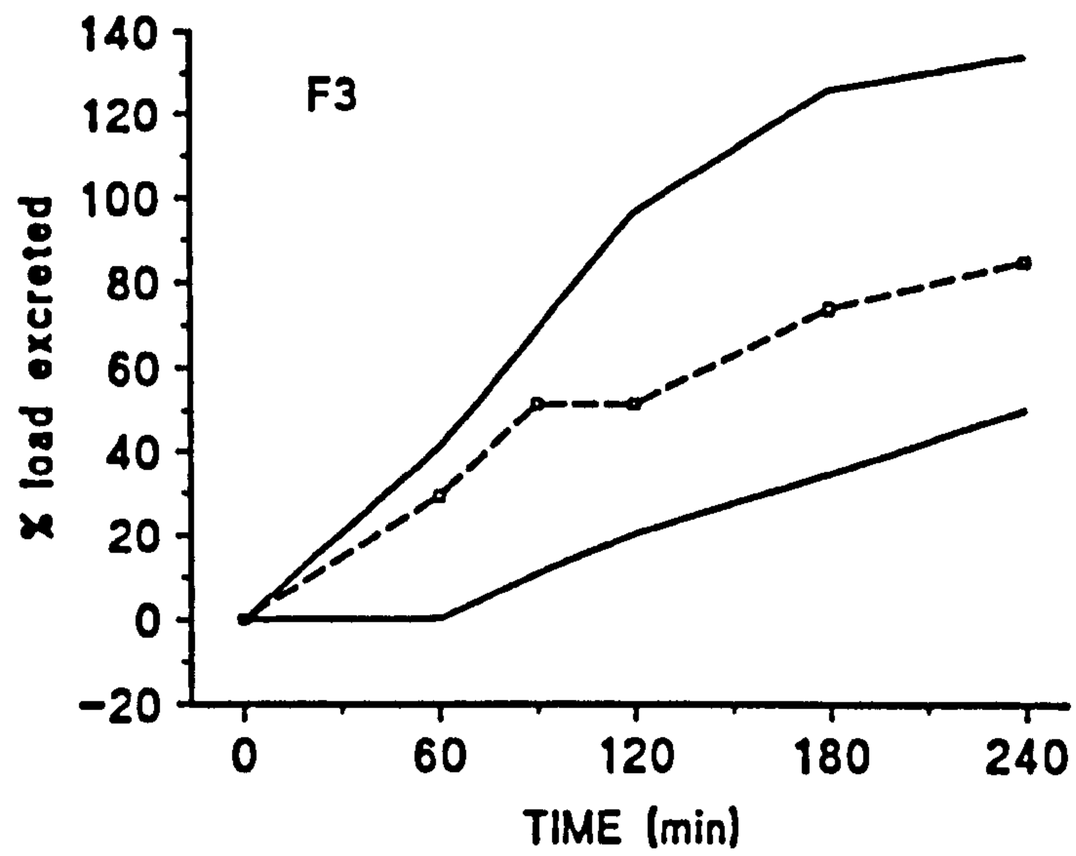
The change in pAVP in response to decrease in pOS was slightly abnormal in this subject. pAVP concentration at 120min was inappropriately elevated at 0.7pmol/l. This subject had not been nauseated nor experienced a fall in blood pressure to account for the rise in pAVP. At 4hr the pAVP had suppressed to below the limit of detection. The subject achieved excretion of 89% of the water load at 4hr and although his pOS remained 5mOsm/kg lower than at the start of the water load test, this fell within the distribution of the **ELDERLY** (pOs 3.5±0.5mOsm/kg less than baseline at 4hr) (see Section 4.2.1). Thus

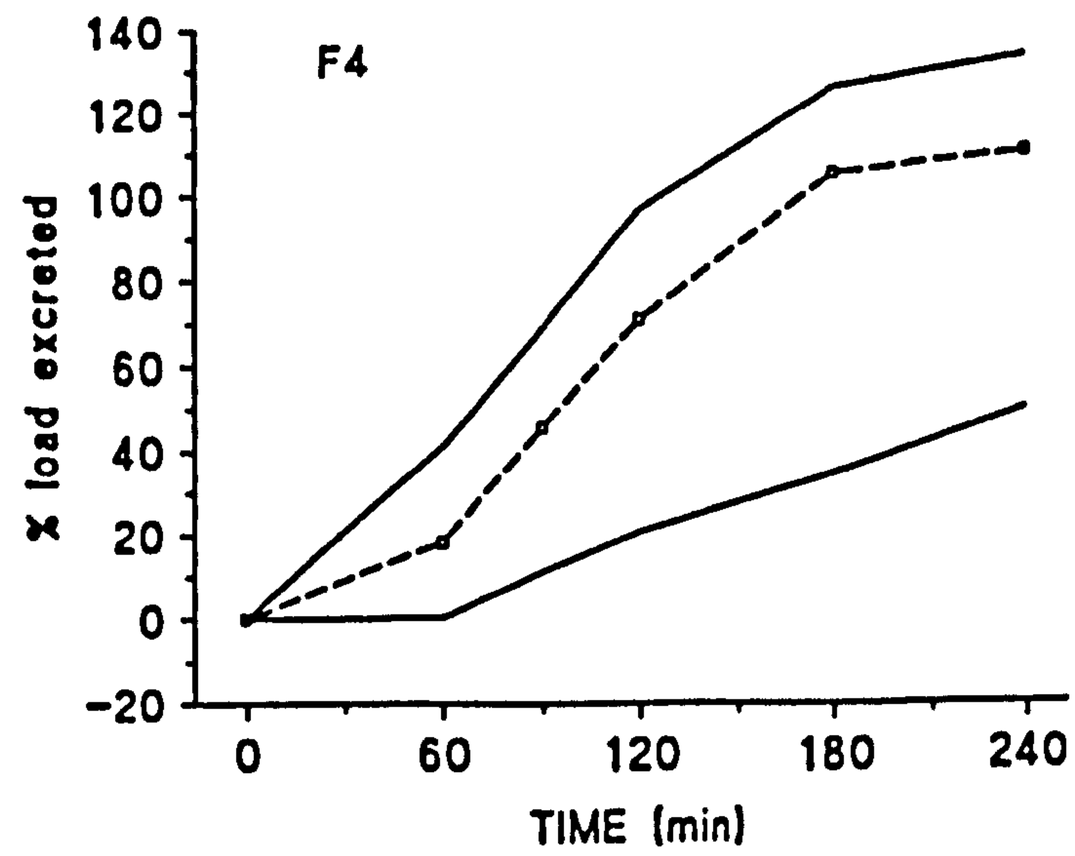
Figure 4.10

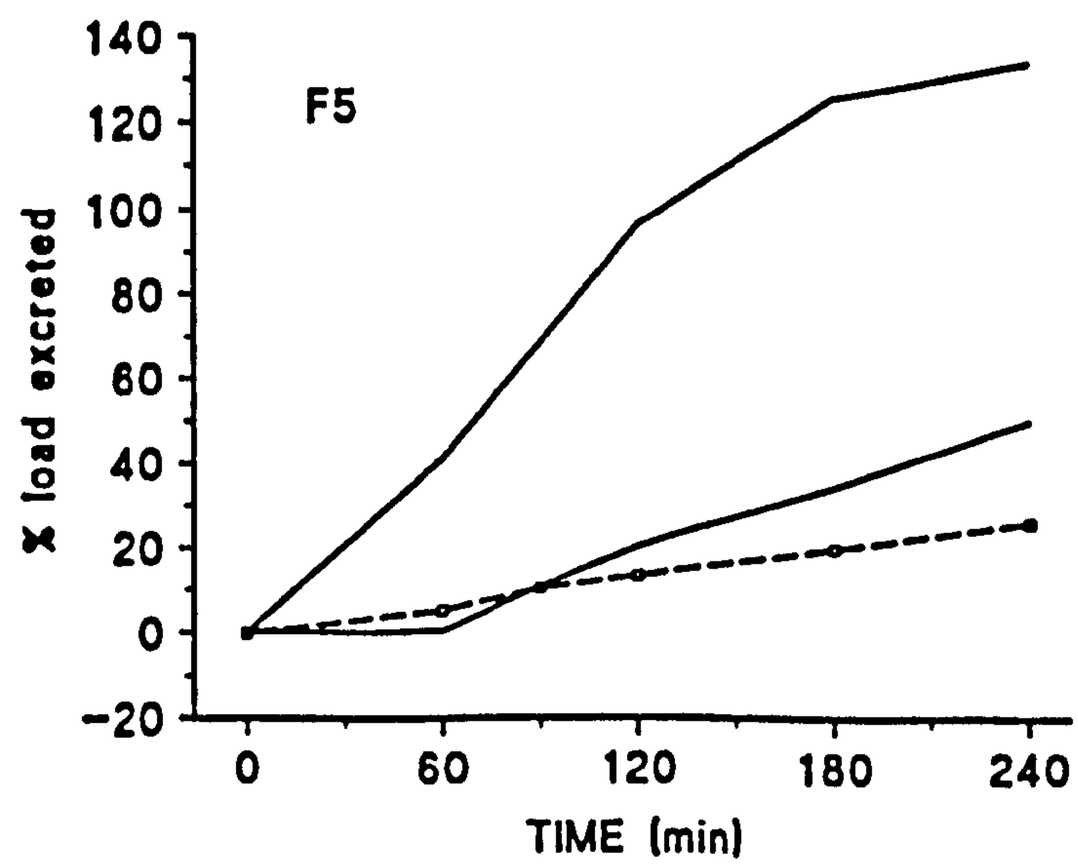
The cumulative excretion of the water load (20ml/kg) in the 4hr after ingestion expressed as a percentage of the water load for each depressed subject treated with Fluoxetine (F1-F6).
The 95 percentiles for the healthy ELDERLY are shown for comparison (--). Subject F5 showed an abnormal pattern of excretion from 90min after fluid ingestion.

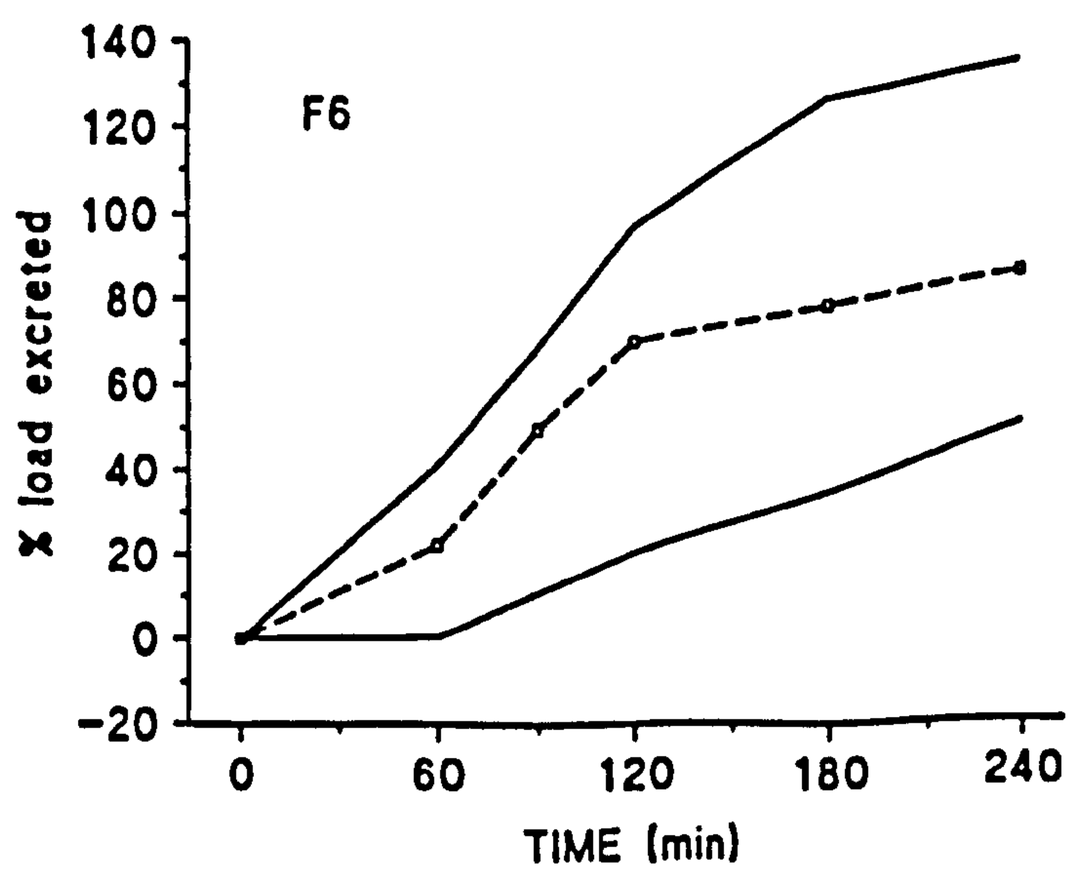

F1
% load excreted
140
120
100
80
60
40
20
0
-20
0
60
120
180
240
TIME (min)


F2
% load excreted
140
120
100
80
60
40
20
0
-20
0
60
120
180
240
TIME (min)


F3
% load excreted
140
120
100
80
60
40
20
0
-20
0
60
120
180
240
TIME (min)


F4
% load excreted
140
120
100
80
60
40
20
0
-20
0
60
120
180
240
TIME (min)


F5
% load excreted
140
120
100
80
60
40
20
0
-20
0
60
120
180
240
TIME (min)


F6
% load excreted
140
120
100
80
60
40
20
0
-20
0
60
120
180
240
TIME (min)

Figures 4.11-4.16

The effect of an oral water load (20ml/kg) on function, plasma osmolality, plasma AVP and the changes in free water clearance and urine osmoality in the 4hr after fluid ingestion for each elderly depressed subject treated with Fluoxetine (F1-F6). The 95 percentiles of the normal, healthy ELDERLY are shown for comparison. The individual responses are discussed in the text.

a) Urine osmolality; b) Plasma AVP; c) Free water clearance; d) Plasma osmolality.

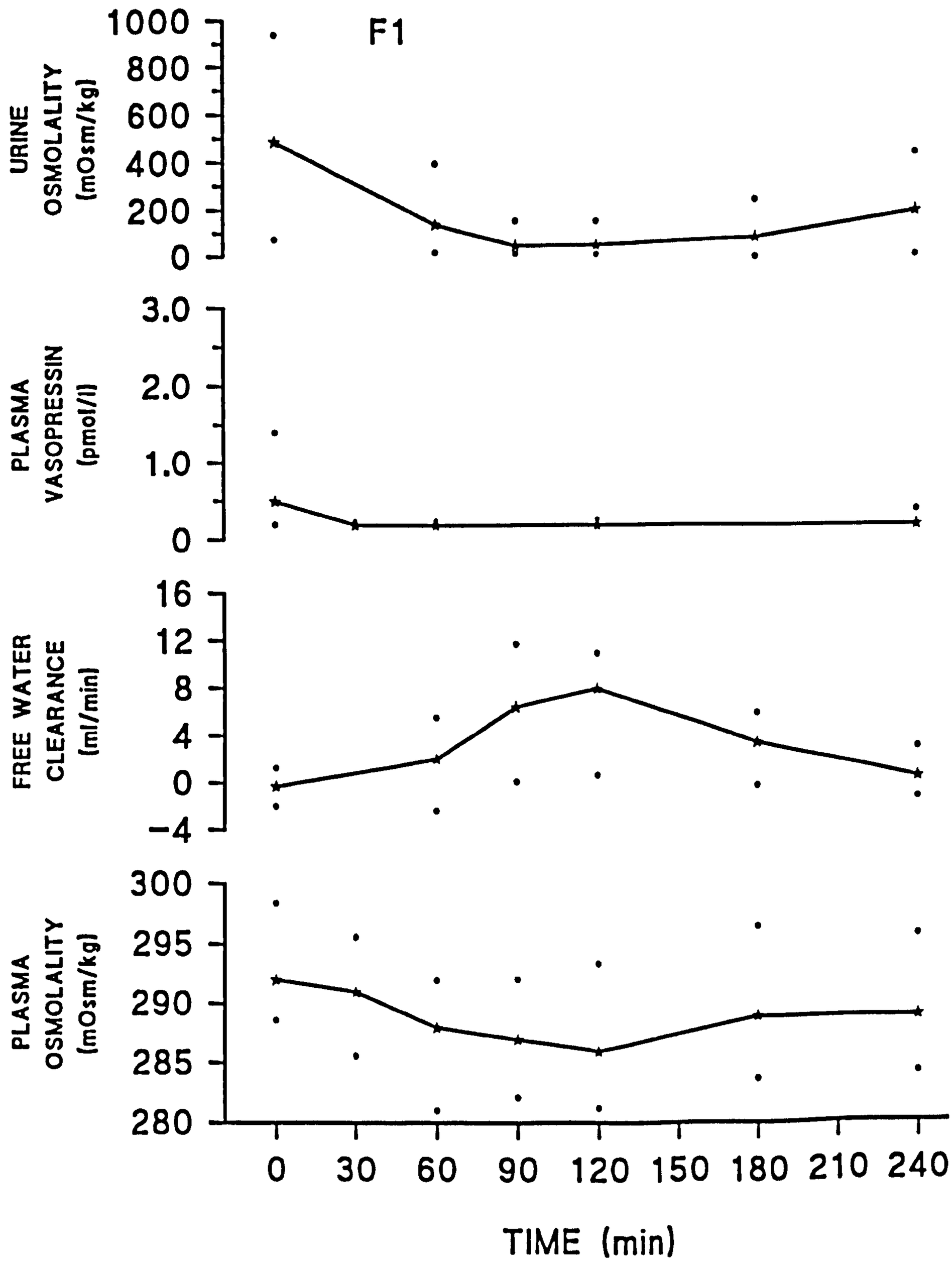

F1
URINE OSMOLALITY (mOsm/kg)
1000
800
600
400
200
0
PLASMA VASOPRESSIN (pmol/l)
3.0
2.0
1.0
0
FREE WATER CLEARANCE (ml/min)
16
12
8
4
0
−4
PLASMA OSMOLALITY (mOsm/kg)
300
295
290
285
280
0 30 60 90 120 150 180 210 240
TIME (min)

Figure 4.11

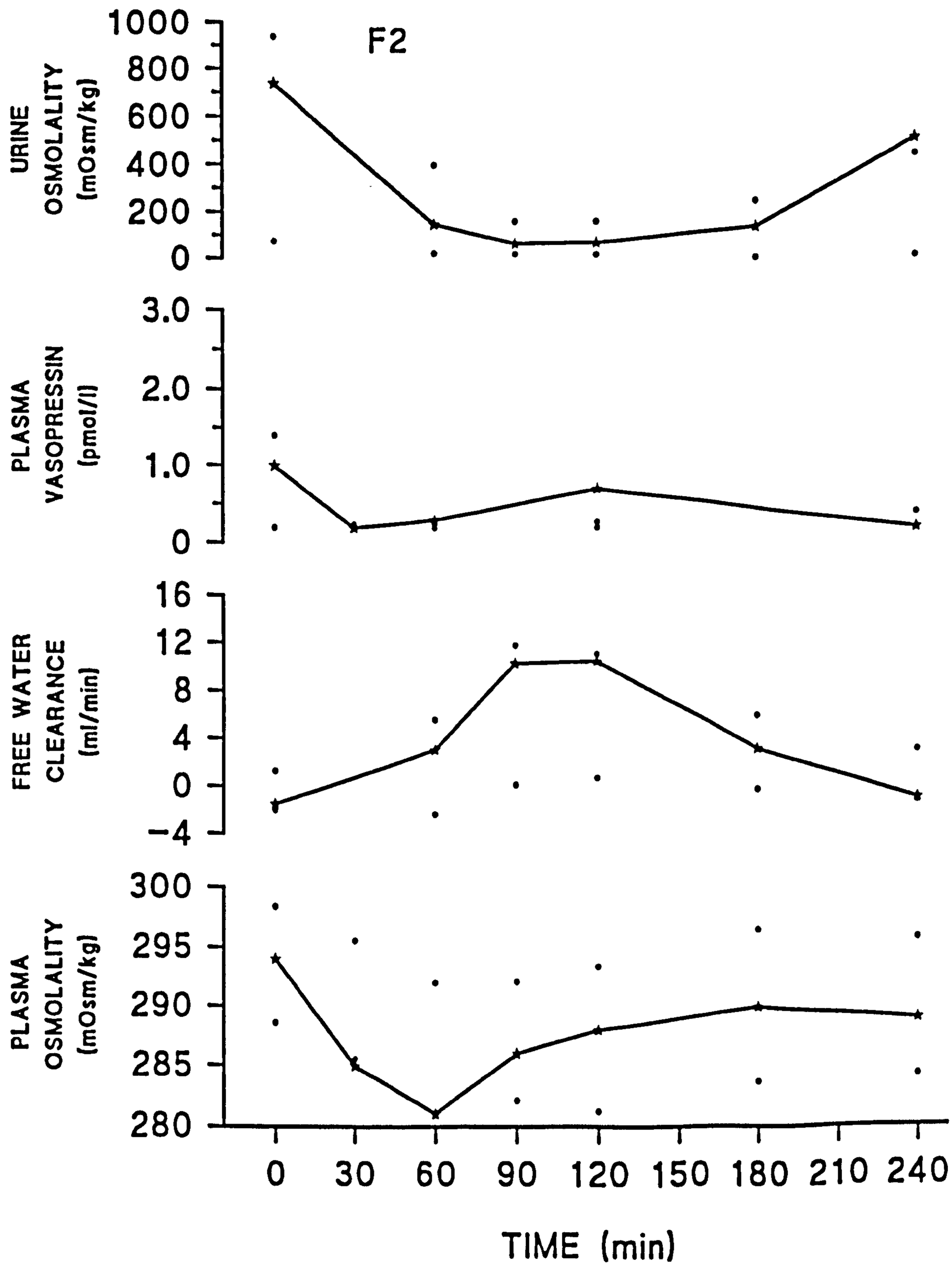

F2
URINE OSMOLALITY (mOsm/kg)
1000
800
600
400
200
0
PLASMA VASOPRESSIN (pmol/l)
3.0
2.0
1.0
0
FREE WATER CLEARANCE (ml/min)
16
12
8
4
0
-4
PLASMA OSMOLALITY (mOsm/kg)
300
295
290
285
280
0 30 60 90 120 150 180 210 240
TIME (min)

Figure 4.12

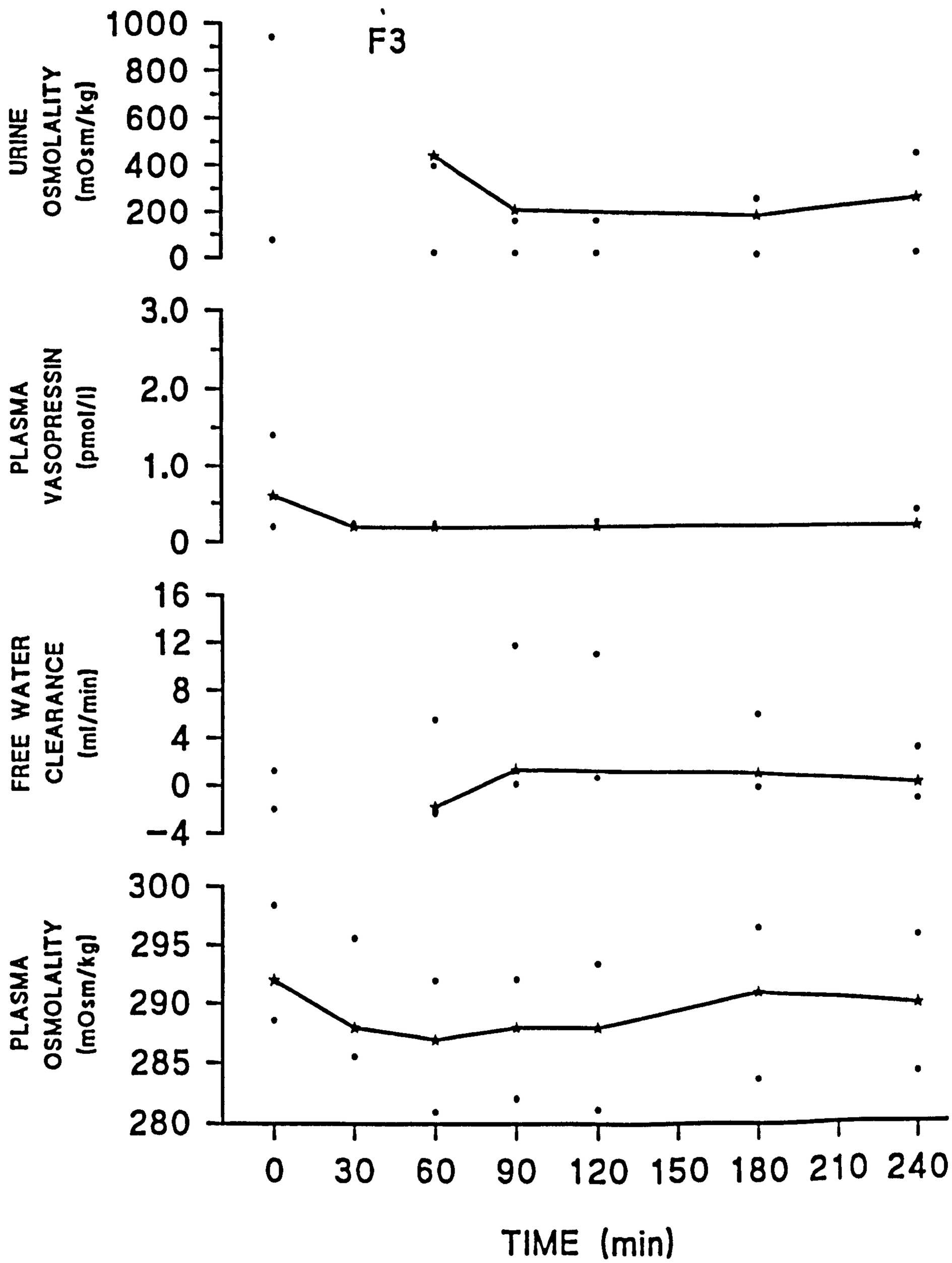

F3
URINE OSMOLALITY (mOsm/kg)
1000
800
600
400
200
0
PLASMA VASOPRESSIN (pmol/l)
3.0
2.0
1.0
0
FREE WATER CLEARANCE (ml/min)
16
12
8
4
0
−4
PLASMA OSMOLALITY (mOsm/kg)
300
295
290
285
280
0 30 60 90 120 150 180 210 240
TIME (min)

Figure 4.13

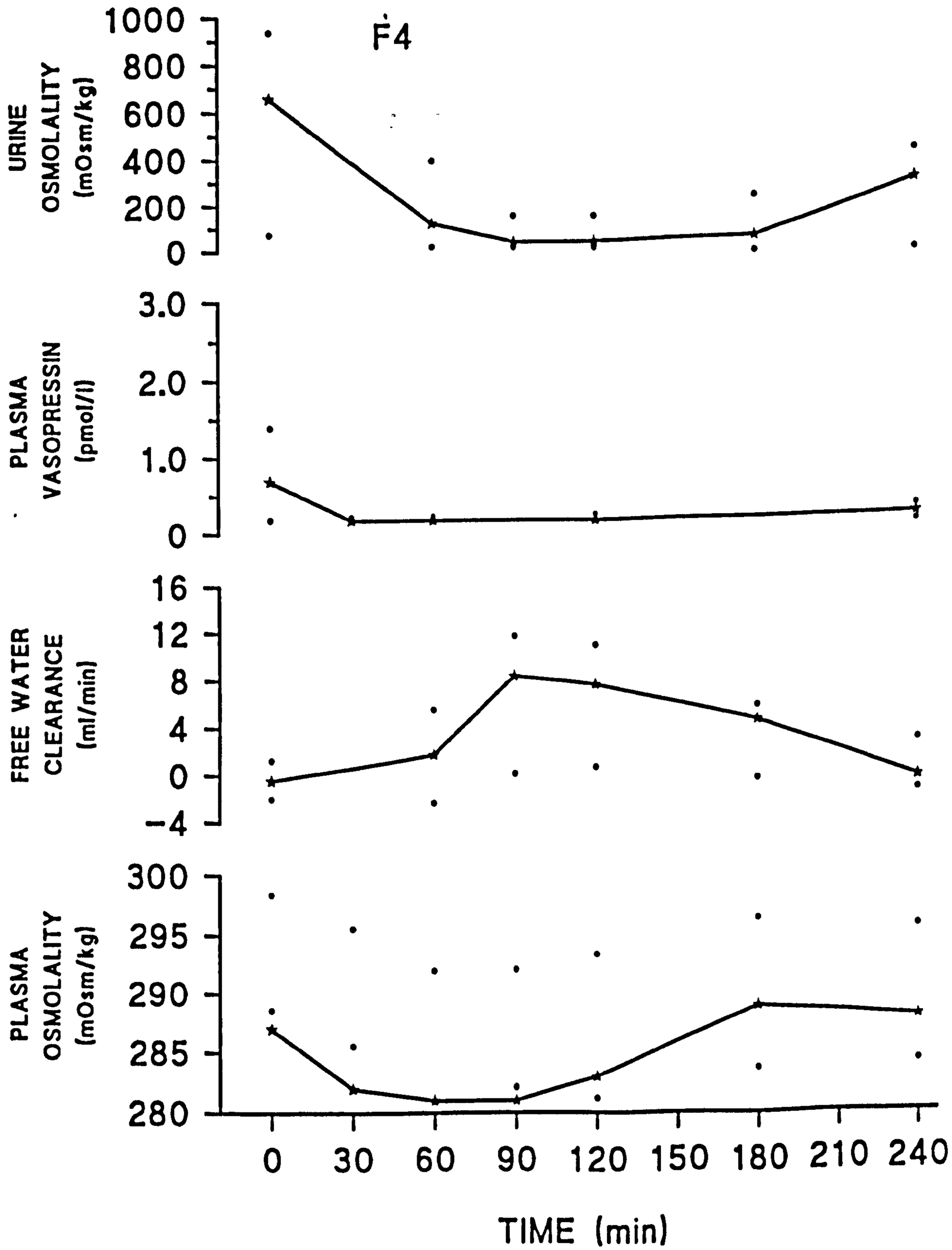

F4
URINE OSMOLALITY (mOsm/kg)
1000
800
600
400
200
0
PLASMA VASOPRESSIN (pmol/l)
3.0
2.0
1.0
0
FREE WATER CLEARANCE (ml/min)
16
12
8
4
0
-4
PLASMA OSMOLALITY (mOsm/kg)
300
295
290
285
280
0 30 60 90 120 150 180 210 240
TIME (min)

Figure 4.14

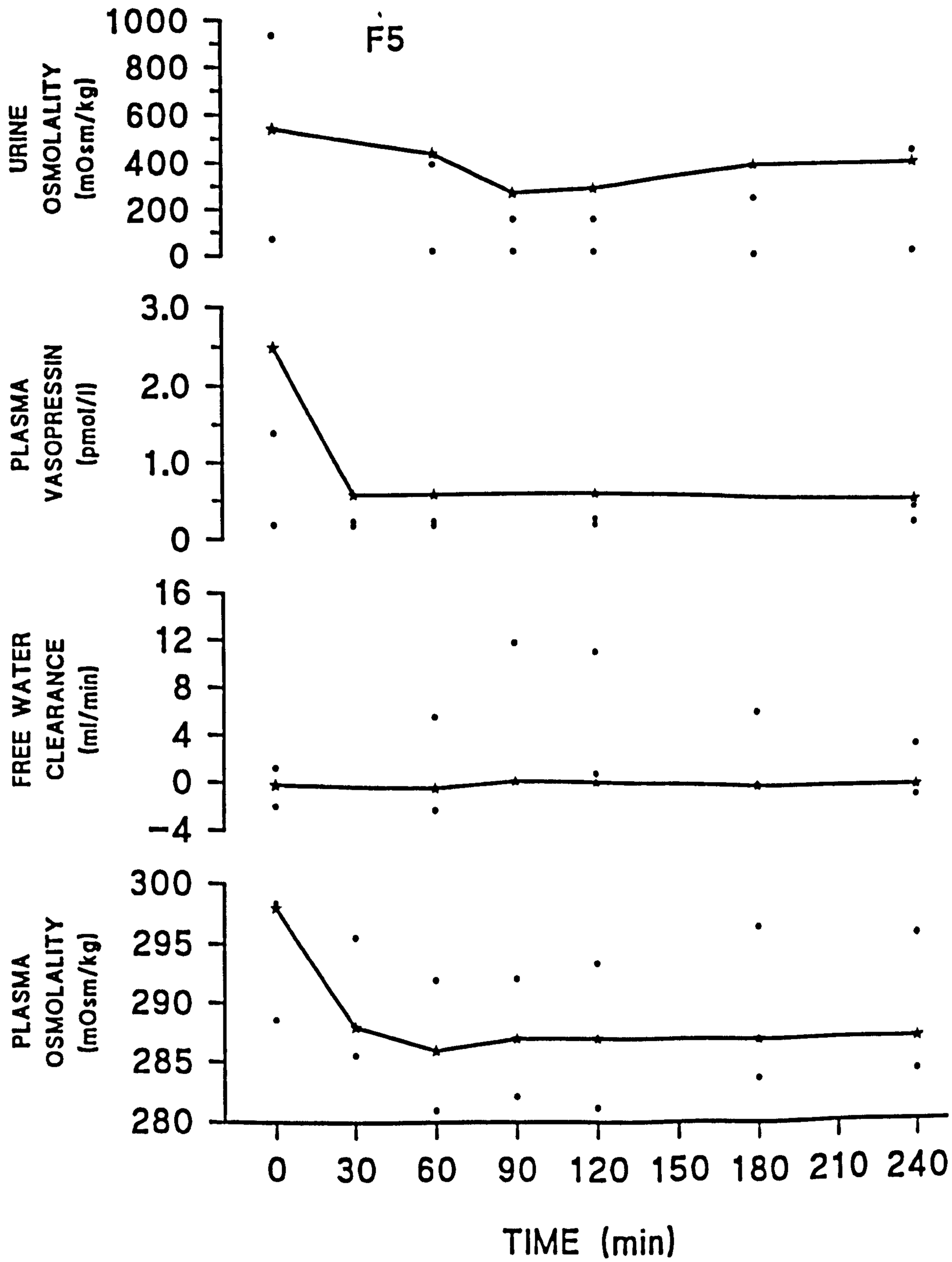

F5
URINE OSMOLALITY (mOsm/kg)
1000
800
600
400
200
0
PLASMA VASOPRESSIN (pmol/l)
3.0
2.0
1.0
0
FREE WATER CLEARANCE (ml/min)
16
12
8
4
0
-4
PLASMA OSMOLALITY (mOsm/kg)
300
295
290
285
280
0 30 60 90 120 150 180 210 240
TIME (min)

Figure 4.15

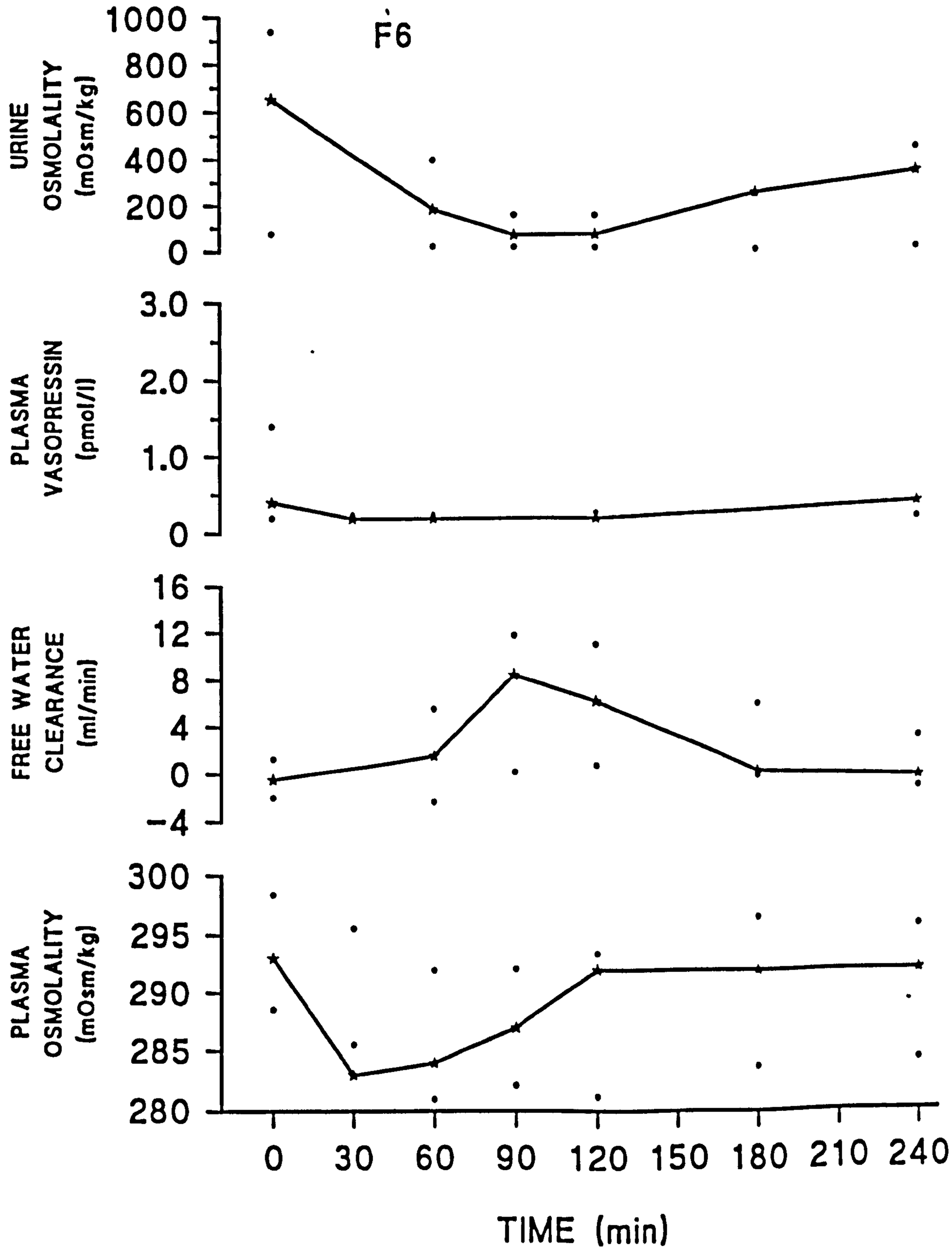

F6
URINE OSMOLALITY (mOsm/kg)
1000
800
600
400
200
0
PLASMA VASOPRESSIN (pmol/l)
3.0
2.0
1.0
0
FREE WATER CLEARANCE (ml/min)
16
12
8
4
0
-4
PLASMA OSMOLALITY (mOsm/kg)
300
295
290
285
280
0 30 60 90 120 150 180 210 240
TIME (min)

Figure 4.16

although the rise in pAVP at 120min remains unexplained it did not appear to have significant effect on osmotic status.

Subject F3: (Figure 4.13)

This subject was unable to pass urine at the start of the water load assessment due to an empty bladder. She also had great difficulty with some aspects of the test, particularly the linear analogue assessment of nausea, due to her depression induced pseudodementia. The dilution of urine, and therefore C_{H2O} were abnormal with uOS_{min} 172mOsm/g and C_{H2O}max 1.39ml/min. These abnormalities could not be attributed to reduced renal function since C_{creat} was 101ml/min. The pAVP response was appropriate to change in pOS and remained below the limit of detection at 4hr. Thus the abnormality of urine dilution remains unexplained but allowed excretion of 85% of the water load. It is possible that the uOS abnormality was related to the hypertension and antihypertensive therapies with salt loss from the proximal renal tubule.

Subject F4 (Figure 4.14)

This subject had a normal excretion of the water load with normal suppression of pAVP and urinary dilution. Basal pOS was lower than predicted from the ELDERLY group but as discussed above this may be attributable to the age of the subject.

Subject F5: (Figure 4.15)

This subject, as discussed above had abnormal renal function with abnormal excretion of the water load. At the time of the water load assessment she appeared clinically hypovolaemic (postural drop in systolic BP >20mmHg) and this was substantiated by the high pOS. The changes in pOS, uOS and pAVP after ingestion of the water load are abnormal. pAVP remained detectable at all time points and C_{H2O} changed only minimally. uOS_{min} was 272mOsm/kg. pOs however, fell to the mid normal range.

Two explanations are possible:

a) That this subject had inappropriate AVP secretion with retention of the fluid due to inability to dilute urine and increase C_{H2O} and that a pOS of 287mOsm/kg was abnormally low for this subject.

b) That the subject was initially hypovolaemic with plasma hyperosmolality and the oral water load merely allowed return to a normal osmotic status. The pAVP concentration of 0.5pmol/l would be appropriate for a pOS of 287mOsm/kg.

Since basal pOS in this subject was 299mOsm/kg with pAVP concentration of 2.5pmol/l it would seem that the second of these two explanations is the more likely.

Subject F6: (Figure 4.16)

This subject had a marked fall in pOS after ingestion of the water load, and consequent appropriate responses in pAVP, uOS and C_{H2O}. He excreted 85% of the water load and pOS returned to basal concentration.

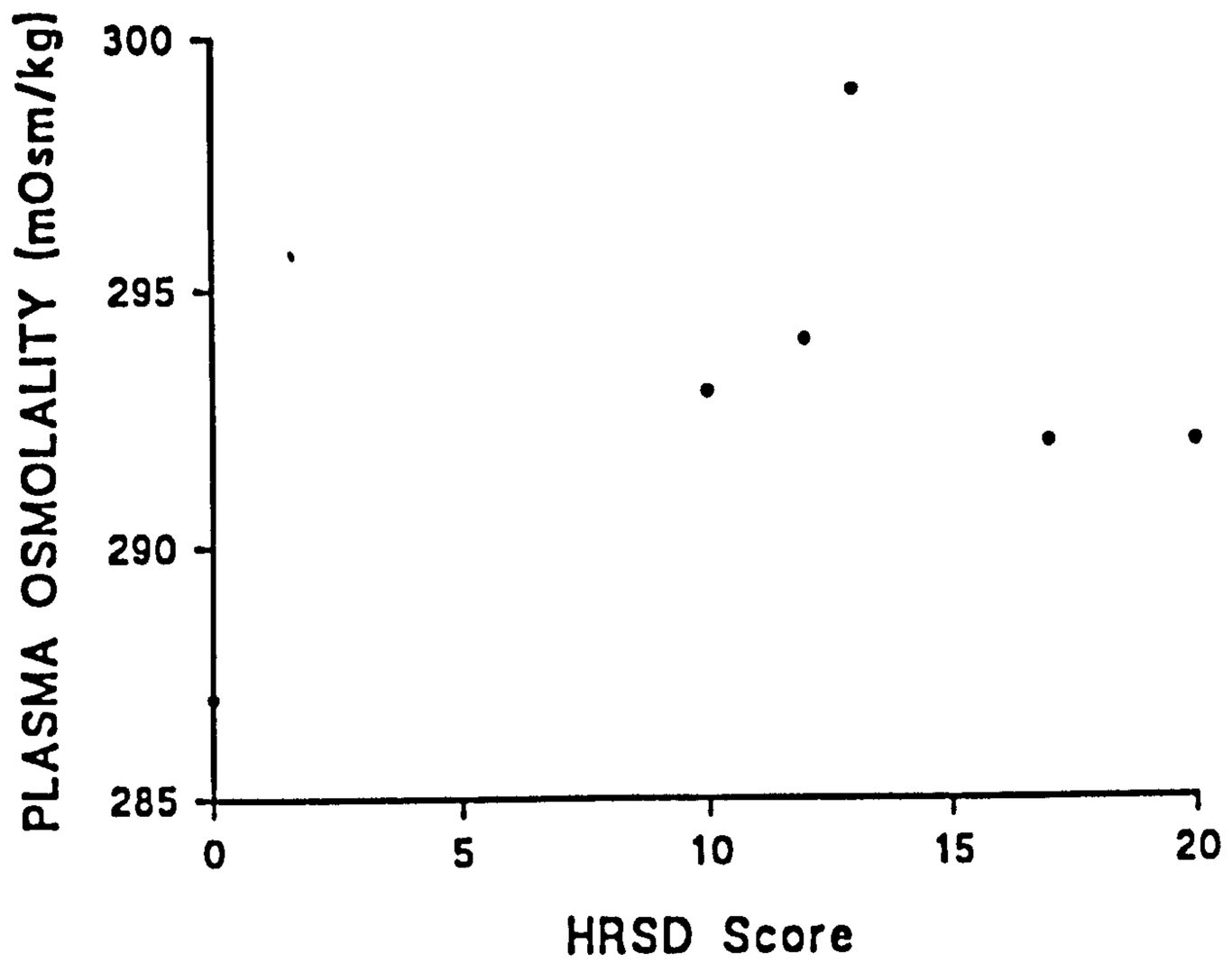
Depression and osmoregulation

Figure 4.17a shows that there was no direct relationship between severity of depression and basal pOS ($r=0.487$, $p>0.05$) and Figure 4.17b shows that the severity of depression was not related to the excretion of the oral water load in these subjects ($r=-0.378$, $p>0.05$).

DISCUSSION

There was no evidence from this study of osmoregulation in six subjects treated with Fluoxetine to suggest an abnormality in basal osmotic status or inappropriate AVP secretion in response to the hypo-osmolality after water loading. Although subject F5 had an abnormal water load assessment it would seem likely that this was not primarily due to abnormal AVP secretion since pOS was elevated at baseline.

There was no evidence from this study that the severity of depression was related to any alteration in osmoregulatory function. The very small number of subjects and the narrow range of severity of depression does not, however,allow any degree of certainty in this conclusion.



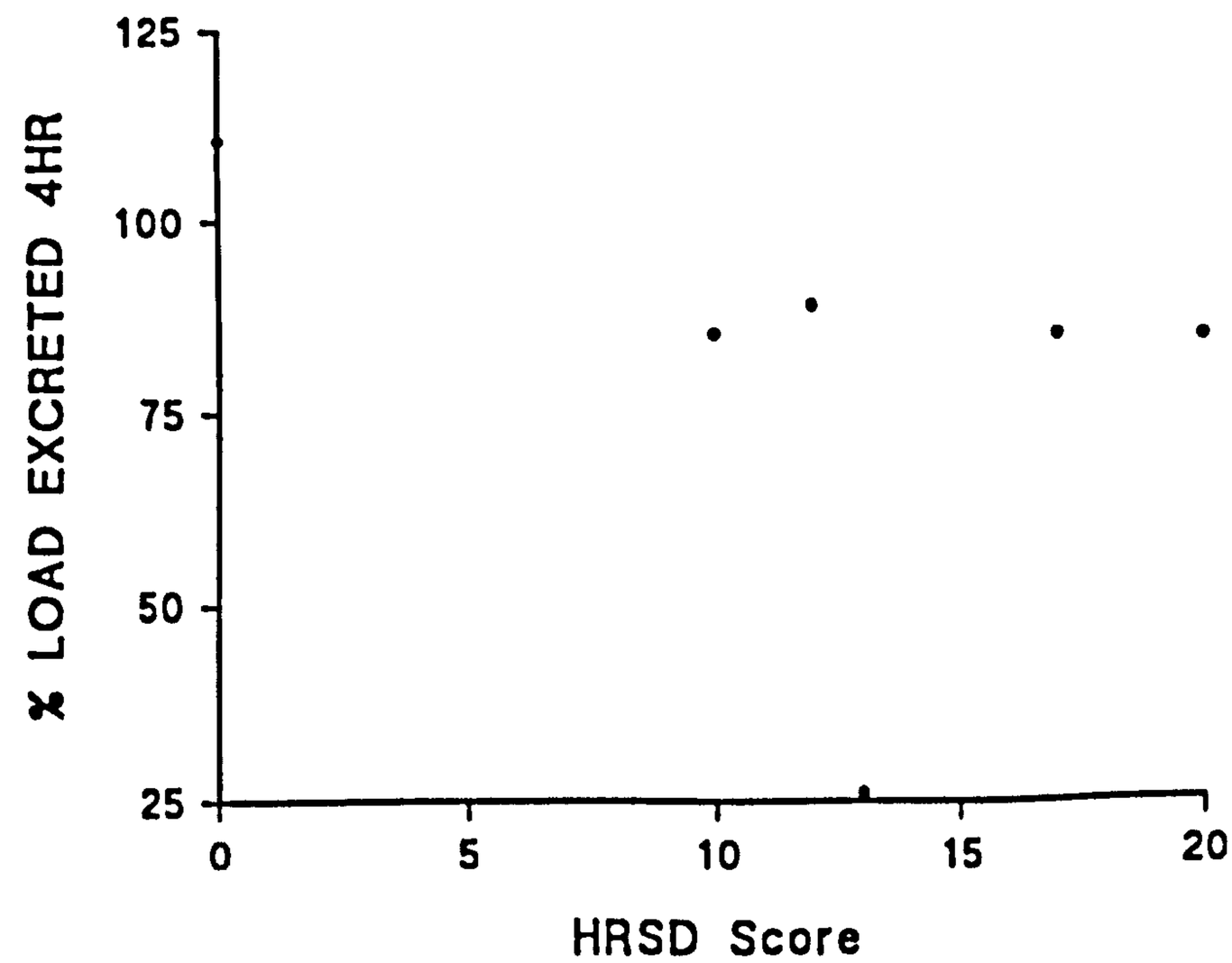


Figure 4.17

a) The relationship between the basal plasma osmolality and the severity of depression (HRSD score) for each depressed patient treated with Fluoxetine ($r=0.487$ $p>0.05$).

b) The relationship between the percentage of the water load excreted after 4hr and the HRSD score for each Fluoxetine treated depressed patient ($r=-0.378$ $p>0.05$).

4.3 DISCUSSION

The methodological difficulties inherent to patient studies, and perhaps particularly to patients with depression, are apparent from this research. Study 4.1 was abandoned because of the problems of patient recruitment discussed above. This study would have answered very important and interesting questions about water balance in depression and the influence of drug treatment. Although it might have been possible to fulfil the aims of the study by approaching patients in the community through general practitioners, it was felt that this would have been impractical for the researcher working alone.

Elderly subjects were selected for this study because of the preponderance of reports of Fluoxetine associated hyponatraemia in patients over 50yr, although the possibility of this being a false over-representation of this age group has already been discussed (Section 3). Study 4.2 has provided new and controversial data on osmoregulation in normal healthy elderly which suggests a deficiency of AVP secretion. It is possible that subjects who volunteered for this study did so because of osmoregulatory dysfunction and polyuria, and a desire to understand and perhaps receive treatment, thus biasing the study population. Even if this is true the presence in the community of such a large group of healthy individuals with dysfunction of AVP secretion requires further study and possibly therapeutic action.

It is possible that this age related osmoregulatory abnormality makes elderly depressed patients particularly vulnerable to the hyponatraemia associated with Fluoxetine and other drugs. As discussed in Section 4.2.1 it is possible that the dysfunction of osmoregulated AVP secretion in the elderly relates to an imbalance of afferent stimuli to the magnocellular AVP secreting neurons. Bevilacqua et al (1987) found that normal elderly had an augmented AVP response to metaclopramide. This was directly related to a diminished AVP response baroregulatory stimuli. This imbalance may be more marked in depression where there is a putative abnormality of 5HT neurotransmission. Fluoxetine, by stimulation of 5HT neurotransmission, may cause further imbalance of these afferent stimuli and thus lead to AVP secretion inappropriate to the osmotic stimulus.

Dothiepin treatment had no significant effect on osmoregulation in the ten

subjects studied. There is one report of SIAD associated with Dothiepin (Fort et al 1985). This patient had liver disease and was taking diuretics in addition to the Dothiepin. Thus it would seem that Dothiepin rarely leads to problems of water balance.

There was no evidence from this study that the treatment of depression with Fluoxetine lead to abnormal osmoregulation in any of the six subjects studied. The small number of subjects, the relatively mild severity of depression and the long duration of treatment with Fluoxetine in most subjects has not allowed full exploration of the hypotheses of the study. In addition the large inter-individual variation in water load assessment enabled only marked abnormalities of osmoregulation to be detected.

There was no evidence from this study that depression per se lead to abnormal osmoregulation. The small number of subjects and the narrow range of HRSD scores does not, however, allow a conclusion about this hypothesis.

The only conclusion that can be made with confidence from this study is that hyponatraemia associated with the Fluoxetine treatment of depression is not a common problem. Appendix 10 presents two brief case reports of patients who were brought to my attention during this research, with hyponatraemia associated with major depression. Both patients developed hyponatraemia during treatment of their depression with a SSRI. One patient had a severe major depressive episode, evidence of physical illness and was taking prednisolone at the time of onset of the hyponatraemia. The other patient had severe psychotic depression and was taking a variety of medications, including diuretics, for her ischaemic heart disease. Both declined further assessment of osmoregulated AVP secretion but fortunately both responded promptly to fluid restriction. It is possible that exclusion of patients with significant physical illness who were taking other medications, and the absence of patients with severe mental illness from this research, has lead to omission of the very patients who are most vulnerable to Fluoxetine associated SIAD. Further work is needed to explore this and to elucidate the markers and predictors of such putative vulnerability.

SECTION 5

STUDIES OF THE EFFECT OF 5HT ON OSMOREGULATED AVP SECRETION IN RATS

INTRODUCTION

This section comprises a series of studies in rats to explore the effect of 5HT on osmoregulated AVP secretion using the model described in Section 2.1.2.1. For clarity the raw data for each Study has been included in Appendix 11. Except where otherwise discussed the results were analysed by un-paired t tests or analysis of variance (ANOVA) as appropriate and have been presented as mean±SD or mean±SE respectively. The standard deviation has been used to indicate the variation in response within the group since this is of some interest in addition to the mean response. Analysis of variance involves multiple significance testing and where this has been used for data analysis a 1% probability level has been accepted as a significant difference between means. For t-test and other analyses a significant difference at a 5% probability level has been accepted.

Where groups of rats have been allotted a two letter label the first letter refers to the treatment type and the second letter refers to the osmotic stimulus type.

5.1 THE EFFECT OF PLASMA HYPEROSMOLALITY ON 5HT NEUROTRANSMISSION IN THE NEUROHYPOPHYSIS

BACKGROUND

Anatomical evidence for the involvement of 5HT in the release of AVP from the neurohypophysis has been discussed in Section 1.4.1. 5HT nerve fibres have been identified in the neurohypophysis and there is a relatively high concentration of 5HT present. 5HT2 receptors have been identified in relatively high density in the neurohypophysis of rats (de Souza 1986) and the isolated rat neurohypophysis has been shown to release AVP when stimulated by exogenous 5HT (Lemay et al 1979). Piezzi & Wurtman (1970) found that the 5HT content of the neurohypophysis was significantly decreased after a period of water deprivation. Handley & McBlane (1991) have also found alteration in 5HT metabolism after water deprivation and suggested that there was an increase in 5HT turnover in the rat whole brain.

These factors would suggest that 5HT may be involved in the release of AVP from magnocellular nerve endings in the neurohypophysis. This study was undertaken to further explore this possibility.

GENERAL HYPOTHESES

1. That 5HT is involved in the release of AVP from the neurohypophysis in response to plasma hyperosmolality.

2. That hyperosmotic stimulation leads to an increase in 5HT turnover with a reduction in 5HT concentration and an increase in the ratio of 5HIAA:5HT.

SPECIFIC AIMS

1. To `pilot' the techniques of the animal model of osmoregulation of AVP described in Section 2.1.2.1.

2. To `pilot' the use of HPLC to measure changes in 5HT and 5HIAA in the neurohypophysis described in Section 2.7.1.

3. To investigate the effect of plasma hyperosmolality on the 5HT content and 5HT turnover in the rat neurohypophysis.

METHODS

Day 1: Female Wistar rats, of two litters were randomly allocated to 2 groups: N(normal controls), H (hypertonic stimulus).
Day 2: Rats were alternately taken from each of the two groups and received 20ml/kg IP 0.9% NaCl(N) or 3% NaCl (H). 30min after the injection rats were killed and trunk blood and neurohypophysis collected as described in Section 2.1.2.1.

RESULTS

The analysis of data has excluded Rat 6, which received hypertonic saline, since it showed a highly atypical AVP response with a rise in AVP to 77.8pmol/l (see discussion).

Plasma measurements

Table 5.1.1 shows the changes in plasma in response to the hypertonic stimulus. The stimulus caused a significant rise in pNa and pAVP but pOS was not significantly different between the two groups. The lack of significant effect of the hypertonic stimulus on pOS was thought to be masked by the large variability within the normal group. Comparison of the pOS results by one tailed t-test (ie consideration only of a significant *increase* in pOS) showed a significant effect of hypertonic saline administration ($p<0.005$).

Neurohypophysial measurements

The Neurohypophysis 5HT content and turnover (ratio 5HIAA:5HT) of the two groups is shown in Figure 5.1. Non-parametric statistical analysis, by Wilcoxon rank sum, found no significant differences between the groups.

DISCUSSION

The first aim of this experiment was as a pilot study of this animal model of osmoregulated AVP. Two problems were apparent. Firstly that one rat had a pAVP response which was very different from the remainder of the group. The magnitude of this response would suggest that the release of AVP was stimulated by other factors, ie. non-osmotic release. This may have been pain, barometric stimulation secondary to blood loss or secondary to stimulation of the gut wall. In order to try to explain this result haematocrit samples would be taken in future studies as an indication of blood volume.

The second problem was the lack of statistically significant effect of hypertonic saline on pOS. This was thought to be due to a greater variability than anticipated. This was attributable to one rat with a very low pOS (282mOsm/kg). If this variability were to persist in other experiments a group

Table 5.1.1 The effect of hypertonic saline on plasma biochemistry and AVP concentration, (mean±SD) comparison by t-test N=normal, H=hypertonic

Group	N	Weight (g)	pNa (mmol/l)	pOS (mOsm/kg)	pAVP (pmol/l)
N	6	200.5±19.4	141.0±1.4	298.5±9.2	2.7±1.6
H	5	199.4±20.7	145.4±0.5	307.6±2.4	12.1±2.7
		P=0.927	p<0.001	p=0.061	p<0.001

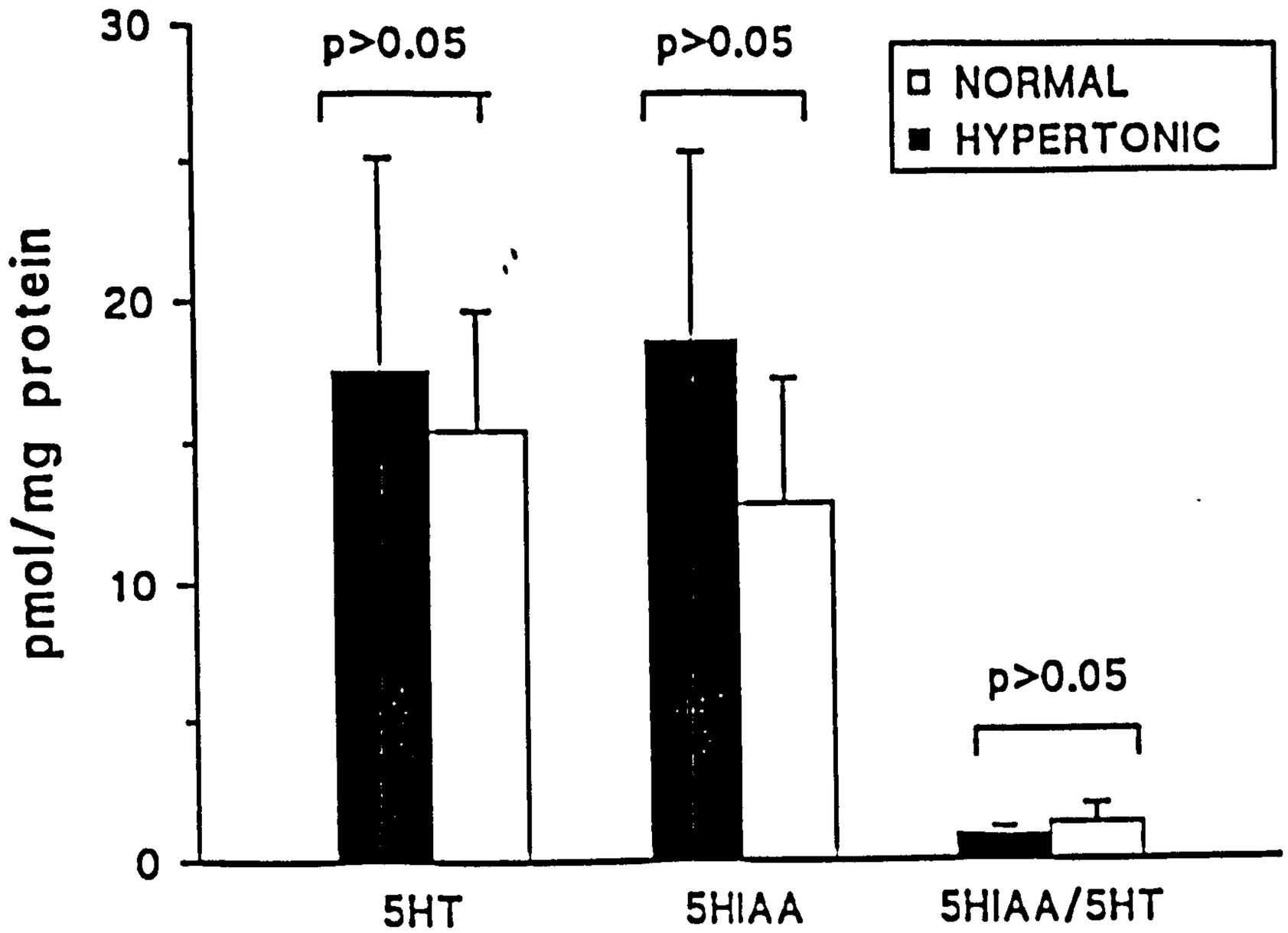


Figure 5.1

The effect of plasma hyperosmolality on 5HT concentration and turnover (5HIAA:5HT) in the neuropituitary (mean ± SEM). There was no significant difference in the pituitary amine content or 5HT turnover in those rats treated with 3% NaCl compared to rats treated with 0.9% NaCl ($p > 0.05$).

size of over 50 (power 90%) would be required to detect a difference in mean pOS of 5mOsm/kg between groups, or a group size of about 15 to detect a difference in mean pOS of 10mOsm/kg.

The second aim of the study was to pilot the HPLC analysis of neuropituitary amine content. The very small amount of tissue present in the neurohypophysis allowed measurement of 5HT and 5HIAA on only one aliquot of homogenate, therefore increasing the possibility of experimental error. In addition there was a large amount of variability observed. It was therefore unlikely that accurate and useful results could be provided by this technique. Three groups have previously measured the 5HT content of the neurohypophysis in rats. Saavedra et al (1977), using an enzymatic-isotopic technique found a concentration of 4.0±1.0ng/mg protein, equivalent to 18.8pmol/mg protein. Koulu et al (1989) found a concentration of approximately 15pmol/mg protein using HPLC, whilst Piezzi & Wurtman (1970) found a concentration in normal rats of 752pmol/mg protein using fluorometric analysis of the homogenate. The reason for this variation in magnitude is likely to be methodological. The mean concentration of 5HT in the neurohypophysis of the normal group of rats in this study (18.5±3.0pmol/mg protein) was very similar to the concentration of the first two studies.

Taking into account these experimental limitations there was no evidence from this study to suggest that 5HT is involved in the release of AVP from the neurohypophysis consequent to plasma hyperosmolality. Piezzi & Wurtman (1970) found a significant decrease in neurohypophysial 5HT content in rats after 5 days of water deprivation. This prolonged period of fluid deprivation would have caused a marked rise of pOS but would also have lead to blood volume depletion. Thus the alteration in 5HT content may have been a consequence of baroregulated AVP secretion. Similarly Handley & McBlane (1991) found that 5HT turnover (in whole brain) was significantly elevated only after water deprivation for 36hr or longer.

5.2 THE EFFECT OF ACUTE FLUOXETINE TREATMENT ON OSMOREGULATED AVP SECRETION.

BACKGROUND

Fluoxetine has been shown to acutely stimulate both pituitary portal and systemic plasma AVP secretion in anaesthetised rats (Gibbs & Vale 1983). However Stein et al (1987), in a study of serotonergic influences on electrolyte and water excretion *in vivo* in rats, found that Fluoxetine had no effect on basal excretion of sodium, potassium or urine. The association of Fluoxetine with hyponatraemia in its clinical use in depression in man has been discussed in Section 3 and Section 4. Since the evidence discussed in Section 1.4 would suggest that 5HT has a role in modulation of AVP secretion in rats and other animals, with 5HT agonism leading to AVP release and antidiuresis, it may be that Fluoxetine stimulates AVP through increase in 5HT neurotransmission. There has been little exploration of the role of 5HT in dynamic, osmoregulated AVP secretion. The purpose of this and following studies was to explore further the influence of 5HT on osmoregulation using Fluoxetine and other 5HT active agents discussed in Section 2.3.

GENERAL HYPOTHESES

1. That increase in synaptic 5HT has a modulatory effect on secretion of AVP in response to experimentally induced change in pOS.

2. That systemic Fluoxetine administration leads to an increase in central synaptic 5HT concentration.

SPECIFIC AIM

To explore the effect of acute treatment with Fluoxetine on osmoregulated AVP secretion.

STUDY 5.2.1

METHODS

Day 1: rats were randomly allocated to 6 groups; NN FN NL FL NH FH. All F groups then received 10mg/kg IP Fluoxetine (1mg/ml in 0.9% NaCl) and all N groups received 10ml/kg IP vehicle (0.9% NaCl).
Day 2: 18hr after the injection rats were given 20ml/kg IP 0.9% NaCl (N), distilled water (L) or 3% NaCl (H) according to their group. 30min after the osmotic stimulus rats were killed and trunk blood collected as described in Section 2.1.2.1.

RESULTS

Plasma biochemistry

The plasma biochemical measurements for each of the 6 groups of rats is shown in Table 5.2.1. Analysis by ANOVA showed that the experimental design provided significant changes in pOS ($p<0.001$). Fluoxetine treatment had no significant effect on the change in pOS ($p=0.441$). Similarly pNa was significantly effected by osmotic stimulus ($p<0.001$) but not by Fluoxetine ($p=0.575$).

Plasma AVP

Figure 5.2.1 shows a scattergram of $\log_{10}$pAVP in each group of rats. This shows that there were many rats, including those in groups NN, NH and NL, which had a pAVP of >30pmol/l, suggestive of a non-osmotic stimulus to AVP secretion. The fact that many rats in the normal control groups had pAVP of high concentration indicates that this should be attributed to an effect of the experimental technique, rather than an effect of Fluoxetine.
Figure 5.2.2 shows the pAVP for each group omitting rats with plasma concentrations >30pmol/l. The relationship between pAVP and pOS has been plotted in Figure 5.2.3 for these rats and the linear regression analysis plotted. If this data can be taken to represent the underlying trend of pAVP response Fluoxetine had no significant effect on basal AVP concentration or on AVP

Table 5.2.1 The effect of acute Fluoxetine (10mg/kg IP) (F) and osmotic stimulus (N, H, L) on plasma biochemistry (mean±SEM). The p value results, from ANOVA, test the hypothesis that osmotic stimulus and Fluoxetine had no effect. The weights of the rats are presented as mean±SD.

Group	N	Weight (g)	pNa (mmol/l)	pOS (mOsm/kg)
NN	10	256.6±8.7	143.5±1.9	298.9±2.4
FN	10	249.1±8.4	142.4±1.9	296.7±2.4
NL	10	251.5±11.8	139.4±1.8	288.8±2.3
FL	10	250.7±13.1	138.2±1.9	288.8±2.3
NH	10	244.7±10.7	147.2±1.9	307.9±2.4
FH	11	250.3±10.1	146.9±1.9	305.5±2.4
EFFECTS				
	Osmotic stimulus		$p<0.001$	$p<0.001$
	Fluoxetine		$p=0.441$	$p=0.575$

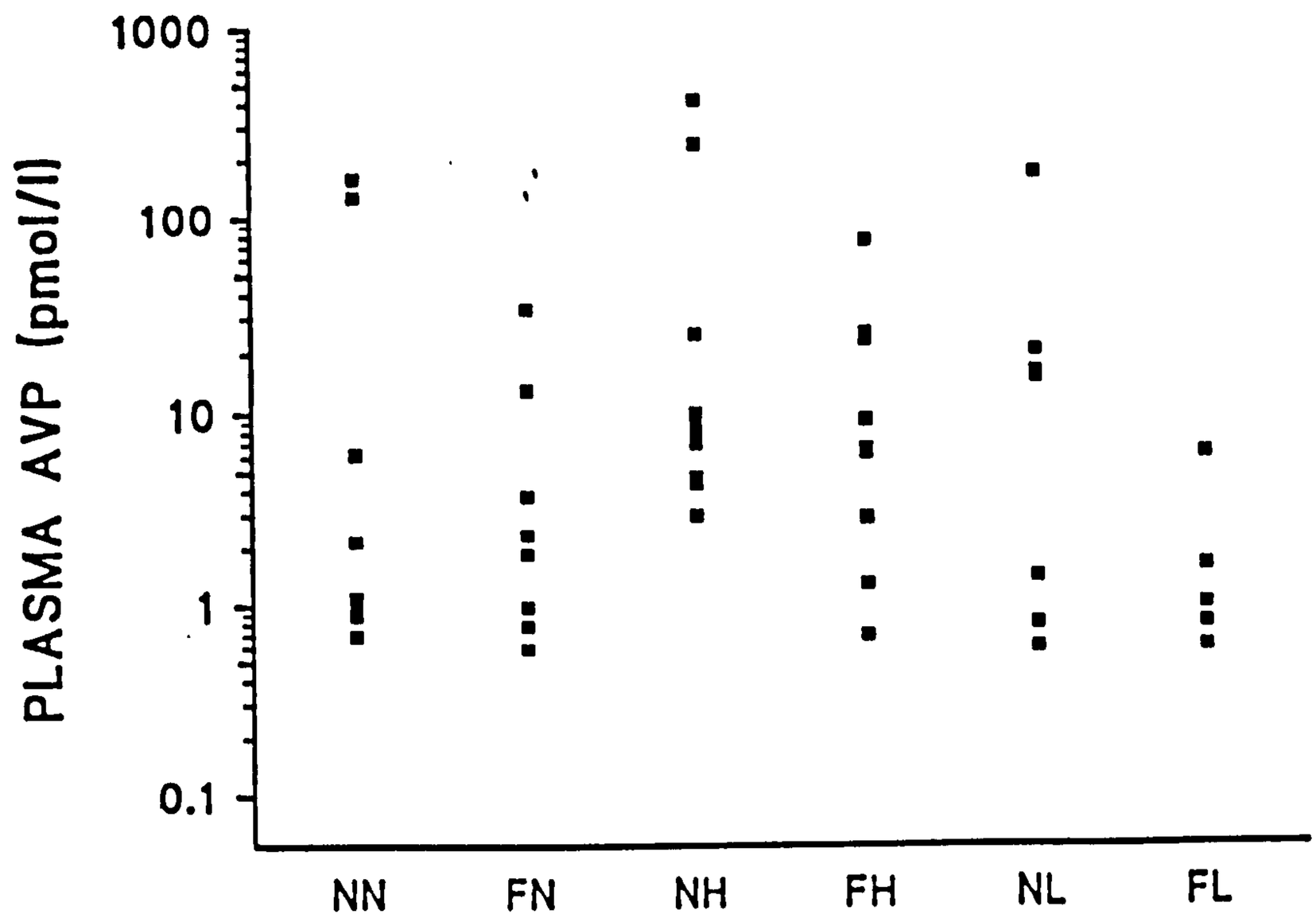


Figure 5.2.1

The effect of Fluoxetine treatment (10mg/kg IP) on plasma AVP 30min after an osmotic stimulus of 20ml/kg IP 0.9% NaCl (N), 3% NaCL (H) or distilled H_2O (L).

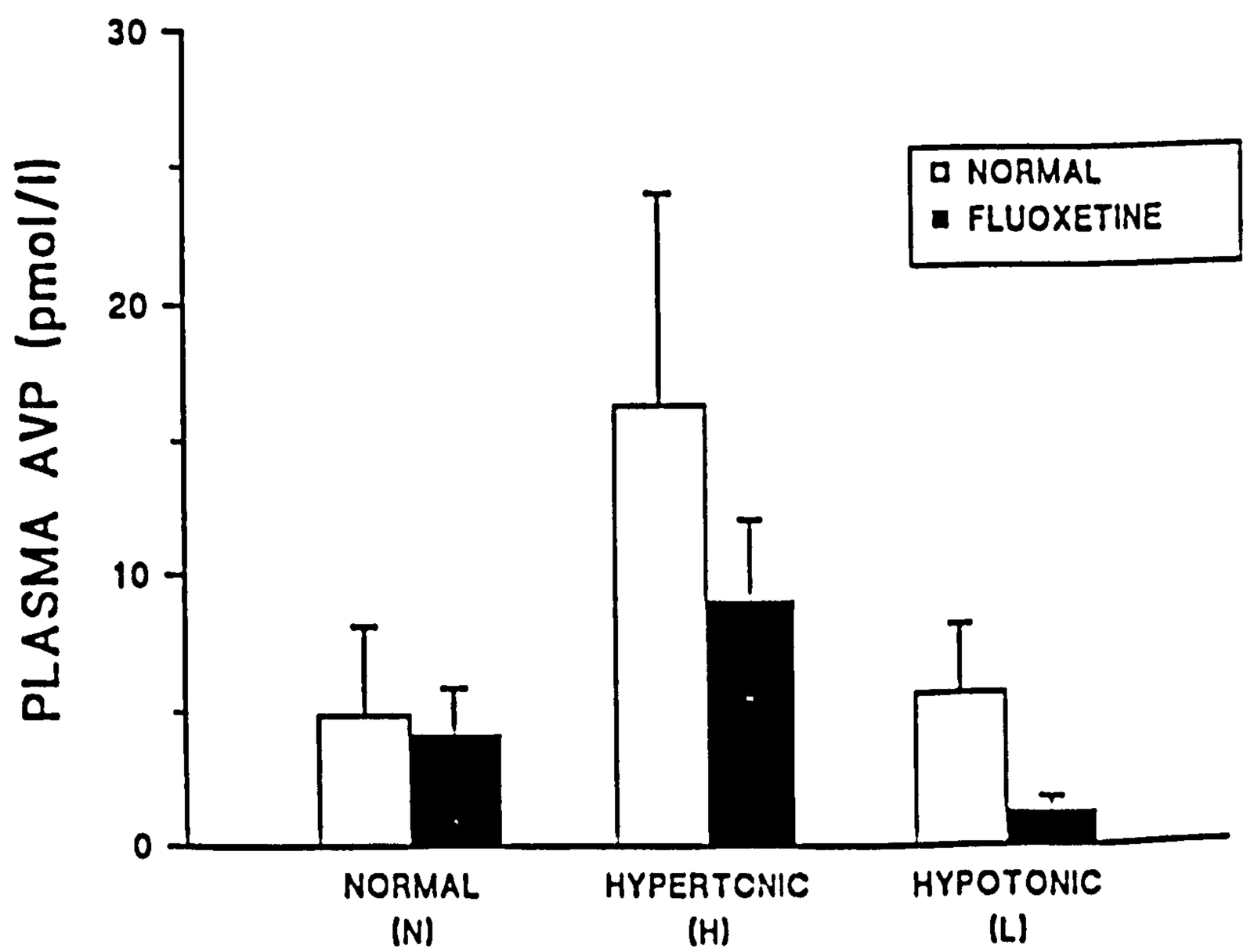


Figure 5.2.2

The effect of Fluoxetine treatment on osmoregulated AVP secretion in each osmotic stimulation group, omitting those rats with a very high AVP concentration (>30pmol/l). Results are presented as mean ± SD.

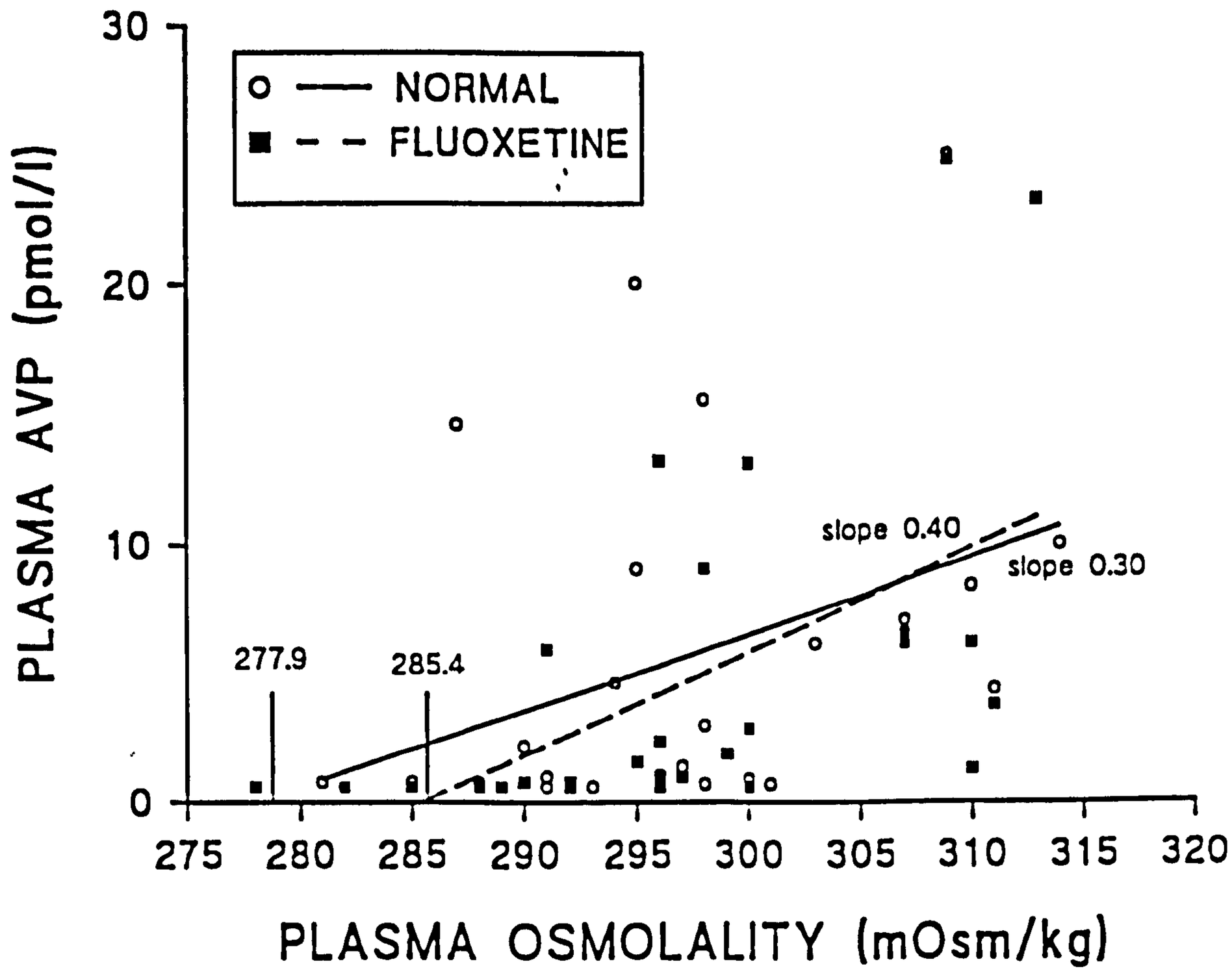


Figure 5.2.3

The effect of Fluoxetine treatment (10mg/kg) on the relationship between AVP and pOS after a normal, hypertonic or hypotonic osmotic stimulus. The analysis by linear regression suggests that there was no significant effect of treatment with Fluoxetine on either the osmotic threshold or the osmotic sensitivity of AVP secretion.

concentration after hypo or hyper-osmotic stimulation. Further more it would seem that Fluoxetine had no significant effect on the sensitivity (slope of regression line) of AVP release in response to change in plasma osmolality (N 0.30, F 0.40 pmol/l per mOsm/kg) or on the osmotic threshold of AVP release (N 277.9, F 285.4 mOsm/kg). Simple linear regression analysis may not, however be the most appropriate method of analysis of the data and is discussed further in Section 5.2.5.

Table 5.2.2 shows the plasma biochemistry of the 6 groups omitting the data for rats with pAVP >30pmol/l.

DISCUSSION

The most striking result of this study was the large, and apparently random, variability in AVP response to osmotic stimulus. The occurrence of this within the normal control groups suggests that this was due to the experimental technique or inherent to the rats. The following were considered as possible explanations:

1. Vehicle and Fluoxetine were administered 18hr prior to the osmotic stimulus. This may have physically damaged rats (eg. by injection into internal organ with subsequent blood loss) and the time interval to collection of blood for analysis had, in comparison to Study 5.1, allowed such disturbance to become apparent in the plasma parameters. However none of the rats appeared unwell at the time of injection of the osmotic stimulus suggesting that severe physical trauma was unlikely.
2. The vehicle and Fluoxetine injection fluids may have been contaminated and rats may have been infected and septicaemic at the time of osmotic stimulation. There was no evidence to support this.
3. The injection of Fluoxetine or vehicle 18hr prior to the osmotic stimulus may have altered osmoregulation of AVP secretion by stimulation of the magnocellular neurons by afferent "stress" impulses. The role of stress in modulation of AVP secretion is controversial (see Section 1.1.2.2.5) but it seemed possible that this may have had a significant role and could explain variability of AVP response, although a recent study would suggest that

Table 5.2.2 The effect of acute Fluoxetine (10mg/kg IP) (F) and osmotic stimulus (N,H,L) on plasma biochemistry omitting rats with high AVP response (mean±SD)

Group	N	pNa (mmol/l)	pOS (mOsm/kg)
NN	8	142.1±7.9	295.9±5.6
FN	9	143.2±4.6	298.3±5.6
NL	10	140.4±5.5	289.7±6.1
FL	10	138.2±4.3	288.8±5.7
NH	8	145.1±4.5	304.8±7.9
FH	9	146.3±5.0	305.1±6.9

emotional stress, at least chronically, inhibits normal AVP responses to dynamic stimuli (Yagi & Onaka 1991).

Exclusion of the extreme AVP responders from the data analysis still, however, showed that the experiment had not provided a good model of osmoregulated AVP secretion since mean pAVP in NL was 5.6pmol/l (Figure 5.2.2). A hypotonic stimulus would be expected to suppress AVP to <1pmol/l. In addition the low correlation coefficient of pAVP to pOS (N $r=0.394$, $p>0.05$) (Figure 5.2.3) suggested that the model was unsuccessful.

It was impossible therefore to draw conclusions from this study about the effect of Fluoxetine on osmoregulated AVP secretion, although Figure 5.2.3 might suggest that the underlying trend was of no significant effect.

STUDY 5.2.2

The hypotheses and aims of this study were identical to Study 5.2. The study design was altered in the light of the discussion above.

METHODS

Day 1: Male Wistar rats randomly allocated to 4 groups; **NH, FH, NL, FL**

Day 2: Rats received in F groups 10mg/kg IP Fluoxetine (1mg/ml in 0.9% NaCl) and in N groups 10ml/kg IP vehicle (0.9% NaCl). This was followed 30min later by osmotic stimulus of 20ml/kg IP 3% Nacl (H) or distilled water (L). Rats were killed 30min subsequent to the osmotic stimulus injection and trunk blood collected.

RESULTS

Plasma biochemistry and haematocrit

The plasma biochemistry and haematocrit (HCT) values for the 4 groups of rats are shown in Table 5.2.3. ANOVA showed that the osmotic stimulus caused significant change in pOS ($p<0.001$) and pNa ($p<0.001$). Fluoxetine had no significant effect on this stimulus ($p=0.240$ for pOS and $p=0.338$ for pNa).

Table 5.2.3 The effect of acute Fluoxetine (10mg/kg IP) (F) and osmotic stimuli (H,L) on plasma biochemistry and haematocrit (mean±SEM). The p value results, from ANOVA, test the hypothesis that osmotic stimulus and Fluoxetine had no effect. The weights of the rats are presented as mean±SD.

Group	Weight (g)	pNa (mmol/l)	pOS (mOsm/kg)	HCT (%)
NH	265.0.0±28.5	155.7±1.2	315.3±2.1	43.1±0.8
FH	270.2±15.9	154.6±1.2	315.8±2.1	44.1±0.9
NL	258.9±24.4	149.2±1.2	298.5±2.1	45.2±0.8
FL	267.9±23.0	149.2±1.4	303.5±2.4	46.8±0.9
EFFECTS				
Osmotic stimulus		$p<0.001$	$p<0.001$	$p=0.006$
Fluoxetine		$p=0.338$	$p=0.240$	$p=0.128$

Haematocrit was significantly affected by the osmotic stimulus (p=0.006) but Fluoxetine had no significant effect (p=0.128).

Plasma AVP

The pAVP concentrations of the 4 groups are shown in Figure 5.2.4. The distribution of pAVP was apparently random and not related to osmotic stimulus or treatment with Fluoxetine.

DISCUSSION

The AVP response to osmotic stimulation was extremely variable in these groups of rats and seemed unrelated to osmotic stimulus or treatment with Fluoxetine. This result was disappointing and perplexing since it implied that the model of osmoregulation of AVP secretion in the rat was incorrect.

Comparison of the pOS changes with those of rats in Study 5.2.1 (Table 5.2.1) showed that the magnitude of change of pOS between groups within each study was similar (5.2.1 NH-NL=19mOsm/kg, 5.2.2 NH-NL=16.8mOsm/kg) but the mean pOS of comparable groups were lower in Study 5.2.1 than Study 5.2.2. This would imply that the basal pOS of rats in Study 5.2.2 was higher than in Study 5.2.1. In retrospect it was unfortunate that a basal group (NN) was not included in the study design. The reason for this basal difference of approximately 10mOsm/kg between the two study groups was not clear. Rats were of the same strain, all imported from the same supplier and housed under similar conditions.

It was possible that the higher basal pOS of rats in this experiment prevented the water load from providing adequate plasma hypo-osmolality to suppress AVP secretion in the NL and FL groups but it would not explain the large variation of AVP concentration in the group. In addition many of the rats had AVP concentrations >30pmol/l, suggestive of a non-osmotic stimulus to secretion.

The total volume of fluid administered to each rat was 30ml/kg. This would present a significant intra-peritoneal volume load and may have lead to non-osmotic AVP secretion by stimulation of pain or nocioceptors in the peritoneal cavity. This hypothesis was explored in Study 5.2.3.

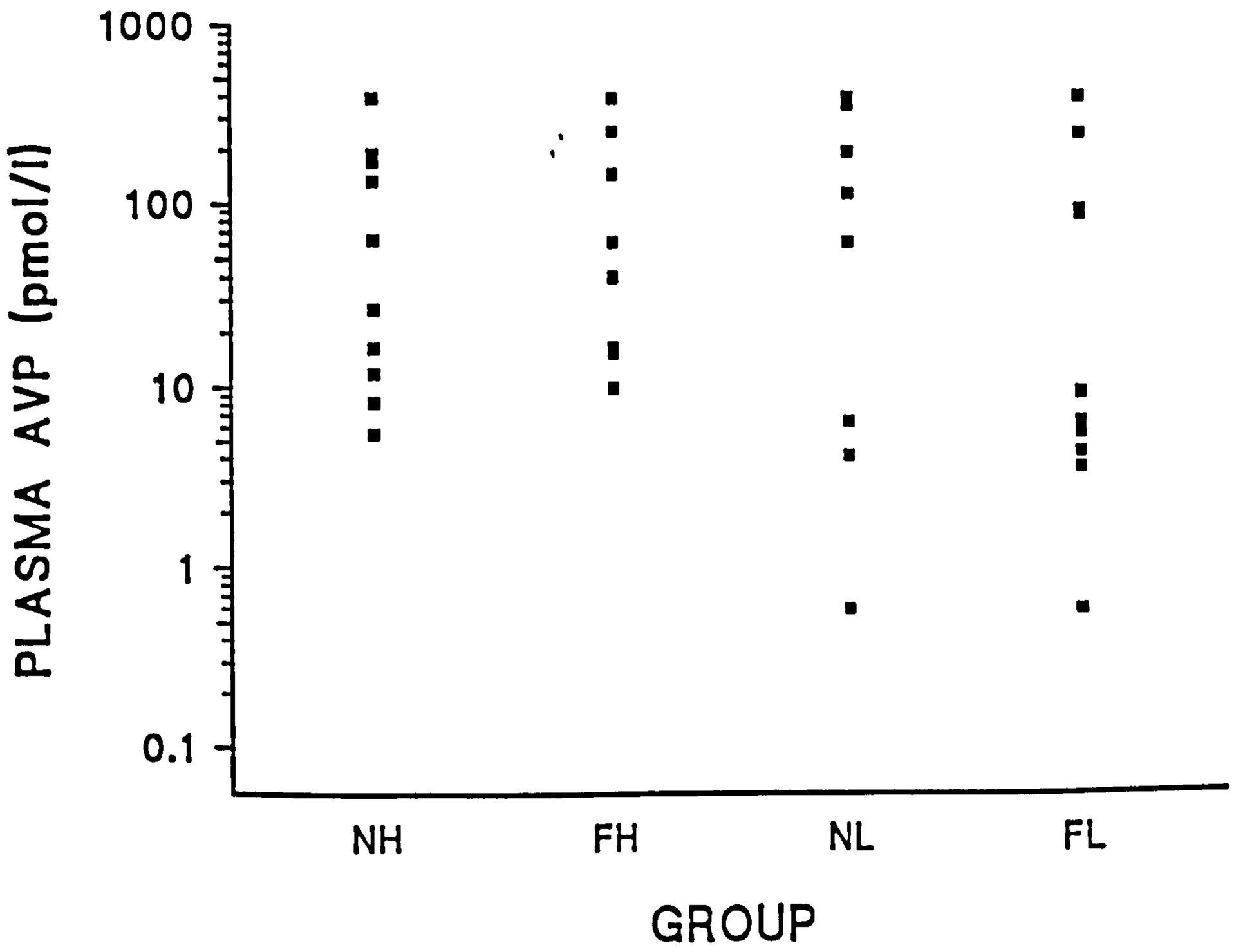


Figure 5.2.4

The effect of Fluoxetine (10mg/kg IP) on the AVP response to an osmotic stimulus of 3% NaCl (H) or distilled H_2O (L).

STUDY 5.2.3 THE EFFECT OF INJECTION VOLUME ON OSMOREGULATED AVP SECRETION

HYPOTHESIS

That the volume of injection of drug or vehicle preceding the osmotic stimulus alters the AVP response to the osmotic stimulus.

AIM

To see if there was a significant effect of volume of Fluoxetine injection on the AVP response to plasma hypo-osmolality.

METHODS

Fluoxetine solutions: 1mg/ml in 0.9%NaCl (L), 10mg/ml in water (H).
Male Wistar rats were randomly allocated to two groups and each were given 10mg/kg of Fluoxetine either as solution L (10ml/kg) or H (1ml/kg). 30min later all rats received a hypo-osmotic stimulus, 20ml/kg IP distilled water, and trunk blood was collected 30min after this injection.

RESULTS

Figure 5.2.5 shows that the volume of Fluoxetine injection had no significant effect on either the degree of plasma hypo-osmolality induced by the water load (L 292.2±3.7, H 292.0±4.5 mOsm/kg, p=0.946), or on the AVP response to this plasma hypo-osmolality (L 0.6±0, H 0.7±0.1 pmol/l, p=0.341). AVP was suppressed to minimal concentration in both groups of rats. This would suggest that Fluoxetine does not prevent the suppression of AVP consequent to plasma hypo-osmolality.

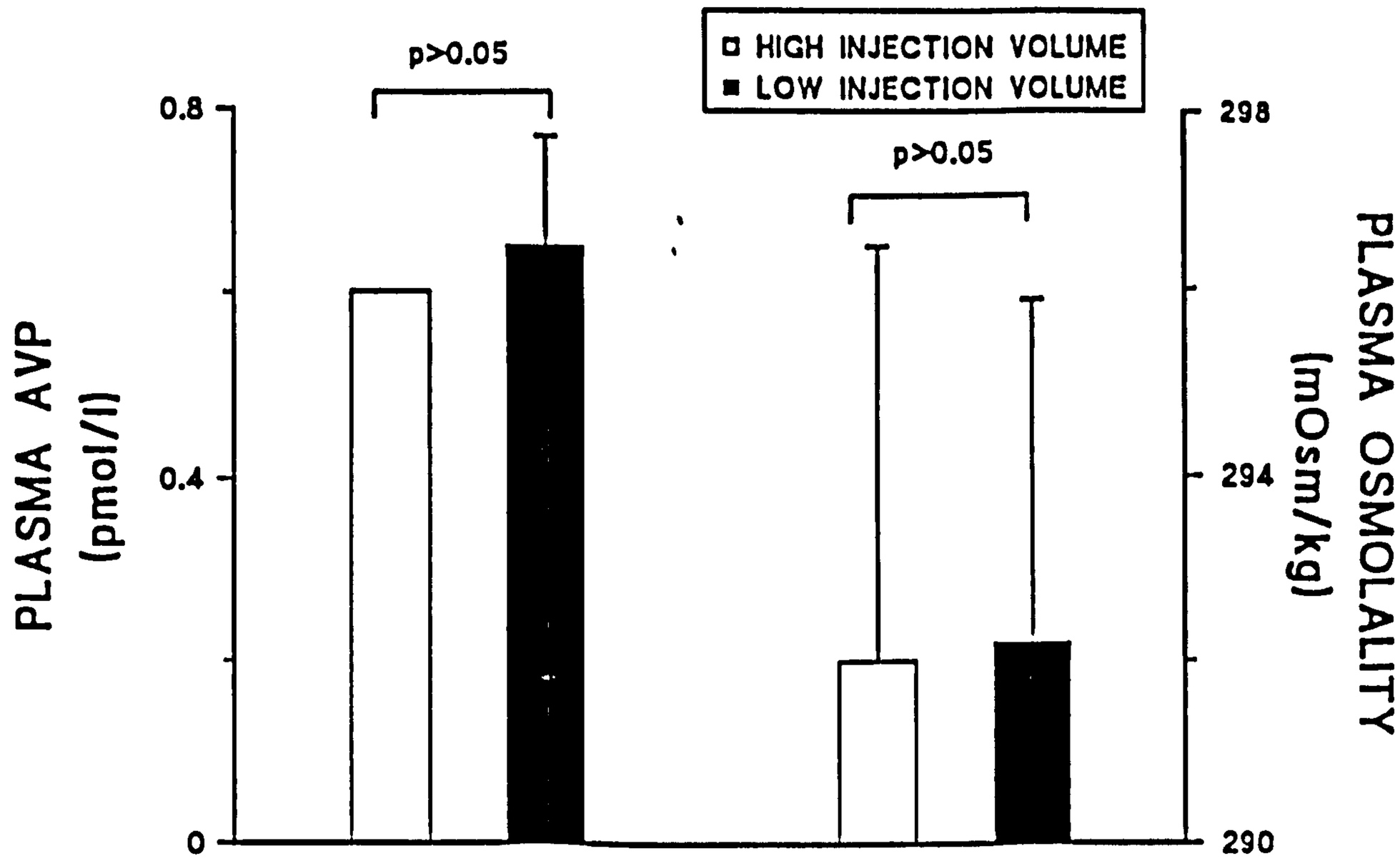


Figure 5.2.5

The effect of volume of Fluoxetine injection (10ml/kg H, 1ml/kg L) on plasma AVP and pOS changes in response to a hypo-osmotic stimulus (20ml/kg IP distilled H_2O). The injection volume had no significant effect on either the AVP response ($p=0.341$) or the change in pOS ($p=0.946$). Results are presented as mean $\pm$ SD.

DISCUSSION

The lack of significant effect of injection volume on the AVP response to plasma hypo-osmoality was unexpected and refutes the hypothesis of this study. Both groups of rats in this study showed very low variability of AVP response. The only apparent reason for this difference to studies 5.2.1 and 5.2.2 was that in this study rats were allocated to groups on the same day as the experiment was carried out. This suggested that randomisation to groups with "alien" rats might have effect on the AVP response to osmotic stimulus, implying that social stress stimulated AVP secretion, both increasing basal release and altering the characteristics of osmoregulated secretion. This was explored further in Study 5.2.4.

It was interesting that the mean pOS of both groups of rats was similar to that of NL in Study 5.2.1 and lower than that of comparable group in Study 5.2.2. The reason for these changes was not clear.

It may be tentatively concluded from this study that Fluoxetine had no significant effect on the AVP response to hypo-osmotic stimulus since it would be anticipated that the normal response of rats would be suppression of AVP to minimal plasma concentration (<0.6pmol/l).

STUDY 5.2.4 THE EFFECT OF HANDLING AND "SOCIAL STRESS" ON OSMOTIC STATUS

HYPOTHESIS

That the handling of rats and random allocation to groups may have effect on basal and osmoregulated AVP secretion.

SPECIFIC AIMS

To see if the basal osmotic status of rats is altered by handling and the "social stress" of contact with alien rats.

METHODS

Day 1: Group (A). Six male Wistar rat were removed from the animal breeding store and randomly allocated to two cages.
Day 3: Group (B). Six male Wistar rats were similarly allocated to 2 cages.
Day 4: Trunk blood was collected from all rats.

RESULTS

The plasma results for the two groups of rats are shown in Table 5.2.4. Although there were no significant differences between the two groups in the mean value of any of the parameters measured, rats in B showed more variability of pAVP concentration. This might suggest that first handling and allocation to a new group on the day preceding the experiment had had effect on basal AVP secretion. Allocation to a group 3 days prior to blood collection apparently allowed return of AVP secretion to normal.

DISCUSSION

The hypothesis that stress modulates AVP secretion is controversial and contradictory (see Section 1.1.2.2.5). Some authors have suggested that AVP is a stress hormone in a similar manor to cortisol and prolactin, although others have not verified this. Such experiments have usually used physical stressors. The role of emotional and social stressors is even less clear and may vary with the animal group. The findings of this study would suggest that social disturbance by allocation to a new group may transiently alter basal AVP secretion. It does not seem that this is mediated through an alteration in drinking behaviour since the pOS of both groups was similar.
Although the evidence for a modulatory effect of stress on osmoregulated AVP secretion was not substantial it seemed that it would be good scientific practice to assume that social disturbance might be a confounding variable in the study of osmoregulated AVP secretion. For this reason all further studies were carried out with randomisation of rats 3 days prior to the experimental procedure.

Table 5.2.4 The effect of handling and "social stress" on basal osmotic status

Results are presented as mean±SD

Group	Weight (g)	pNa (mmol/l)	pOS (mOsm/kg)	pAVP (pmol/l)	HCT (%)
A	266.5±5.6	150.2±4.3	301.1±3.0	1.9±1.7	42.3±2.0
B	275.0±13.3	148.8±5.4	299.2±4.0	3.0±4.5	41.5±1.6
	p=0.179	p=0.647	p=0.373	p=0.587	p=0.444

STUDY 5.2.5

The hypotheses of this study were those of Study 5.2.1.

SPECIFIC AIMS

To study the effect of acute Fluoxetine treatment on basal and osmotically stimulated AVP secretion under "optimal" experimental conditions.

METHODS

Day 1: Male Wistar rats were randomly allocated to 6 groups; NN FN NH FH NL FL.

Day 4: Rats received either 10mg/kg IP Fluoxetine (10mg/ml in water) (F) or 1ml/kg IP vehicle (water) (N). 30min later rats received either no further injection (NN,FN), or 20ml/kg IP 3%NaCl (NH,FH) or water (NL,FL). Trunk blood was collected 30min after the osmotic stimulus. The frontal cortex of groups NN and FN were collected as described in Section 2.1.2.1 for measurement of changes in 5HT related neurochemistry and 5HT receptor density (Section 2.7).

RESULTS

The data analysis was carried out with omission of one rat in group FN. The AVP response of this rat was >40pmol/l and highly atypical of the underlying group mean response. Inclusion of the data from this rat may have lead to a false positive conclusion of the effect of Fluoxetine on osmoregulated AVP secretion.

Plasma biochemistry (Table 5.2.5).

The osmotic stimulus caused a significant change in pOS ($p<0.001$) and pNa ($p<0.001$). Fluoxetine had no significant effect on either the changes in pOS ($p=0.718$) or pNa ($p=0.098$).

Table 5.2.5 The effect of acute Fluoxetine (10mg/kg IP) and osmotic stimuli (N, H, L) on plasma biochemistry and haematocrit (mean±SEM). The p value results, from ANOVA, test the hypothesis that osmotic stimulus and Fluoxetine had no effect. The weights of the rats are presented as mean±SD.

Group	Weight (g)	pNa (mmol/l)	pOS (mOsm/kg)	HCT (%)
NN	268.4±6.8	149.1±1.5	299.0±1.7	40.8±0.9
FN	265.3±7.1	150.1±1.6	300.0±2.1	46.8±0.9
NH	260.8±6.3	154.6±1.5	309.4±1.7	40.4±0.9
FH	259.0±9.2	157.6±1.5	309.5±2.0	44.3±0.9
NL	269.3±7.2	146.4±1.5	293.0±1.8	43.3±0.9
FL	258.4±10.3	149.0±1.5	291.9±2.0	47.0±0.9
EFFECTS				
Osmotic stimulus		$p<0.001$	$p<0.001$	$p=0.007$
Fluoxetine		$p=0.078$	$p=0.721$	$p<0.001$

Haematocrit (Table 5.2.5).

The osmotic stimulus had a significant effect on HCT ($p=0.007$) explained by the increase in HCT with water loading. Fluoxetine significantly increased HCT ($p<0.001$), independent of the osmotic stimulus effect ($p=0.619$).

Plasma AVP

The relationship between pAVP and pOS for each rat is shown in Figure 5.2.6 with simple linear regression analysis. Fluoxetine appeared to significantly increase the sensitivity of AVP secretion per unit change in pOS but had no significant effect on the theoretical threshold of AVP release. The data, however, contains several rats in each treatment group with pAVP$\leq$0.6pmol/l, the detection limit of the assay. All these data points have been attributed the value of the detection limit for the purposes of data analysis (see Section 2.2.6). Thus for any individual rat the attributed AVP concentration will be 0.6pmol/l at any pOS below the osmotic threshold of AVP secretion, whatever the pOS. These data points cause a shift of the x-axis intercept of linear regression and a decrease in gradient of the regression line. Thus there was a potential error in this statistical model for data analysis. Data points with AVP value of 0.6pmol/l would significantly and inappropriately influence the analysis of the relationship of AVP and pOS above the osmotic threshold of AVP secretion. A more accurate analysis of the AVP response to change in pOS above the osmotic threshold was necessary.

This could be perhaps achieved by linear regression analysis, omitting data points with pAVP$\leq$0.6pmol/l. The types of SIAD discussed in Section 1.2.2.3 suggest that Fluoxetine might have any of at least 4 patterns of modulation of osmoregulated AVP secretion either alone or in combination.

a) Effect on basal secretion.

b) Effect on the osmotic threshold of AVP secretion.

c) Effect on the sensitivity of AVP secretion to change in pOS.

d) Loss of linear relationship to plasma osmolality.

The need to consider any of these possible effects of Fluoxetine lead to the analysis of the data by piece-wise regression analysis.

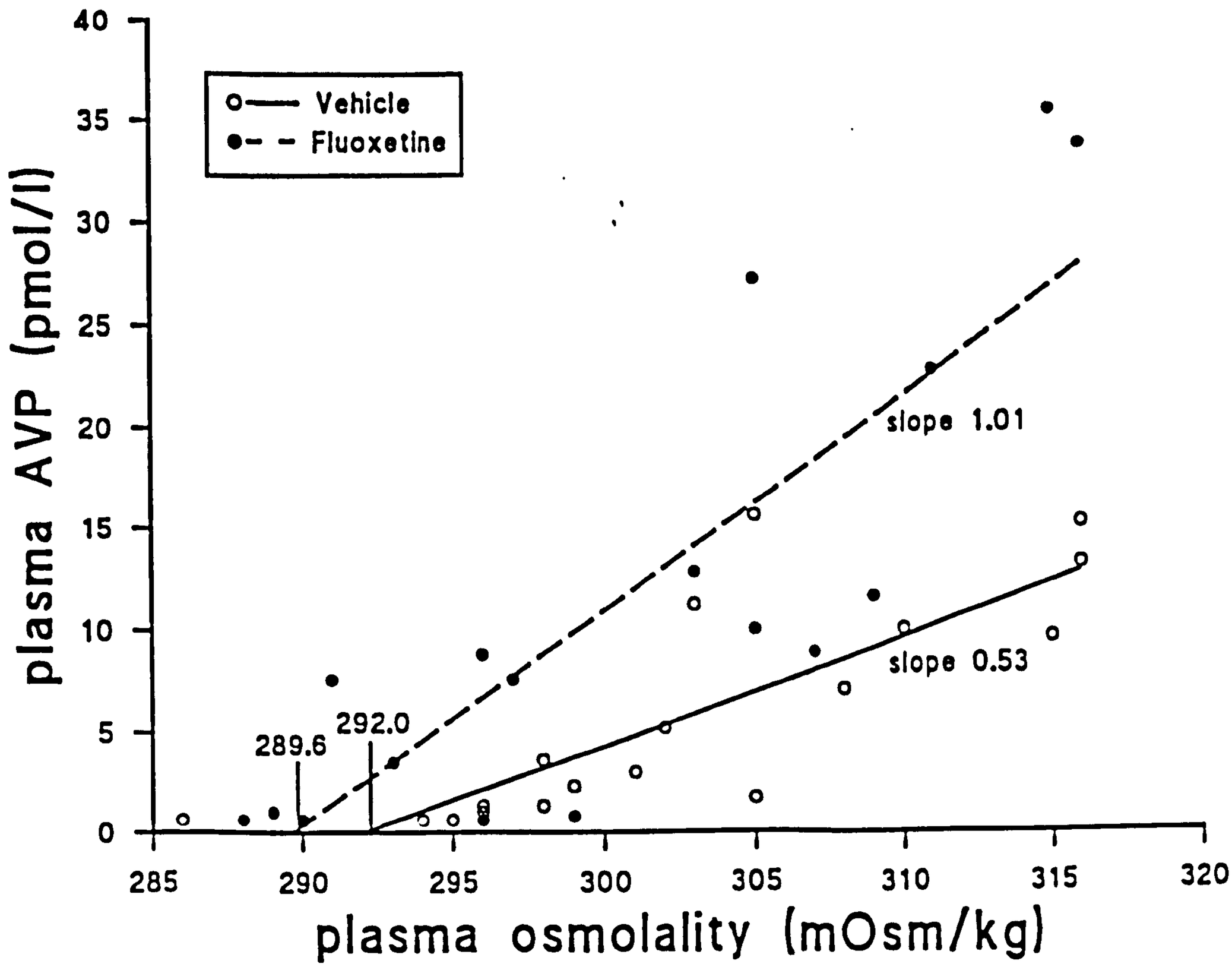


Figure 5.2.6

The effect of acute Fluoxetine treatment (10mg/g IP) on osmoregulated AVP secretion. Linear regression analysis is shown with the estimated osmotic thresholds and osmotic sensitivity of AVP secretion for each group.

Piece-wise regression analysis

Piece-wise regression analysis allows consideration of a linear relationship of two variables which may alter in characteristics consequent to change in one of the variables. Piece-wise regression analysis with one change point fits a regression line to the data with two segments, allowing the characteristics of the relationship to vary either side of this change point. For analysis of the data in the two treatment groups of this study the change point was the theoretical osmotic threshold of AVP secretion. The first line segment represented mean basal secretion and was assumed to have a slope of zero. The second line segment, the sensitivity of AVP secretion per unit change in pOS above the osmotic threshold, was continuous with the first segment at the change point.

The statistical analysis allowed both the basal AVP concentration and the sensitivity of AVP response to be variable for each treatment group. Separate regression lines were estimated for each group in two models.

The analysis was done twice, firstly estimating a common threshold for both the Fluoxetine group and the vehicle group and secondly by allowing different thresholds to be estimated for each group. The optimum threshold values for each model were estimated by repeatedly entering different thresholds in the regression analysis and finding the values which minimised the residual variation (see Figure 5.2.7). The residual variations for each model were then compared by a likelihood ratio test, allowing a statistical test for the effect of Fluoxetine on the threshold.

After the optimum model of common or separate change points for each treatment group had been established, differences in basal secretion and the sensitivity of AVP response were than compared by standard analysis of co-variance based upon that model.

Analysis was performed by Mr T Butler, Department of Medical Statistics, University of Newcastle upon Tyne, using the `GLIM' statistical computing package (Royal statistical society, 1986) with custom written macros to carry out the repeated regression. The mathematical model is shown in Appendix 12. Parameters are expressed as estimate$\pm$SE.

Analysis of the effect of Fluoxetine on osmoregulated AVP secretion by piece-wise regression analysis is shown in Figure 5.2.8.

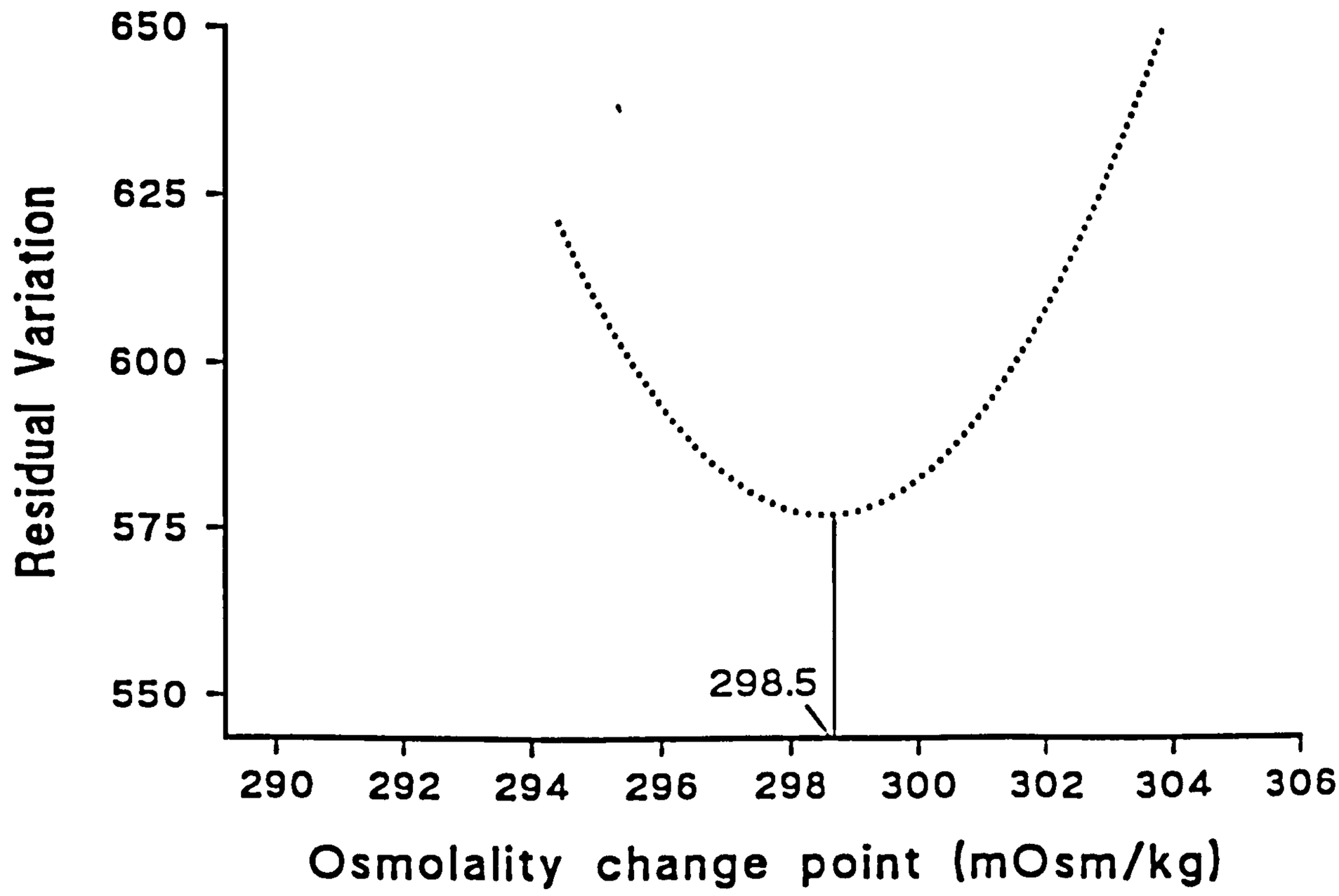
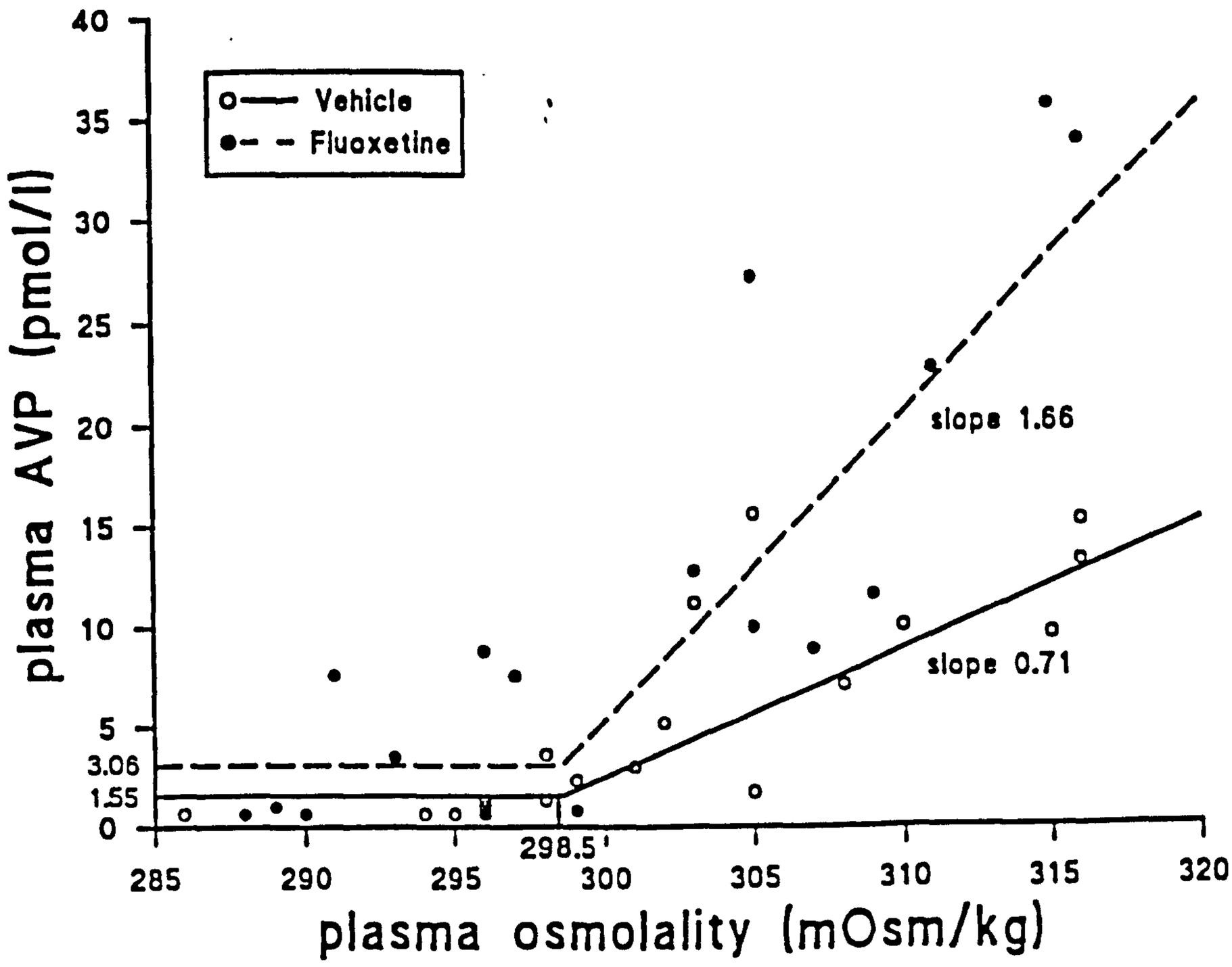


Figure 5.2.7

An example of residual variation of piece-wise regression analysis for estimated osmotic threshold change point assuming a common change point for Fluoxetine and vehicle treated rat groups. The optimum threshold (minimal residual variation) is 298.5 mOsm/kg.



Piece-wise regression analysis

Parameter ± SE

	DRUG	VEHICLE	COMMON	p value
Change Point (mOsm/kg)	299.0	294.6	298.5	0.158
Basal AVP (pmol/l)	3.06 ± 1.25	1.55 ± 1.03		<0.001
Sensitivity AVP (pmol/l per mOsm/kg)	1.66 ± 0.17	0.71 ± 0.14		<0.001

Figure 5.2.8

The effect of Fluoxetine (10mg/kg IP) on osmoregulated AVP secretion analysed by piece-wise regression. Since there was no significant effect of Fluoxetine on the osmotic threshold ($p = 0.160$) a common threshold model has been used. Fluoxetine had a significant effect on basal AVP secretion ($p < 0.001$) and significantly increased the osmotic sensitivity of AVP secretion above the threshold ($p < 0.001$).

Fluoxetine had no significant effect on the osmotic threshold of AVP secretion ($p=0.160$) which was estimated as 298.5mOsm/kg. It had a significant effect on basal secretion of AVP (N 1.55 ± 1.03pmol/l, F 3.06 ± 1.25pmol/l , $p<0.001$) and significantly increased the AVP response to change in pOS (N 0.71 ± 0.41, F 1.66 ± 0.17 pmol/l per mOsm/kg, $p<0.001$).

Neurochemistry

The effect of Fluoxetine on brain neurochemistry was assessed by measurement of changes in 5HT turnover and receptor binding in the frontal cortex of the NN and FN groups of rats (Table 5.2.6 and Figure 5.2.9). There was a non-significant trend in reduction of 5HT turnover in the forebrain samples. Fluoxetine treatment had no significant effect on 5HT1a receptors ([^{3}H]DPAT binding) but significantly increased 5HT2 binding sites ([^{3}H]ketanserin binding) in the forebrain homogenate preparation.

DISCUSSION

Acute Fluoxetine administration to rats significantly increased basal AVP secretion and increased the sensitivity of AVP response to change in pOS within 60min of drug administration. In Section 2.2.7 it was noted that at high concentration Fluoxetine may significantly increase the assay estimate of AVP concentration. This could not explain the approximately 2 fold increase in basal secretion and increase in AVP response to hyperosmolality even assuming the worst scenario of 30% over-estimate of pAVP.

Fluoxetine may stimulate AVP secretion in a variety of ways. Through alteration of barometric stimuli to magnocellular neurons; through stimulation of release of AVP from the pituitary, or by direct or indirect stimulation of AVP secreting magnocellular neurons. In man 5HT agonism may lead to AVP secretion secondary to an emetic effect. In rats, however the emetic reflex is incomplete and stimulates OXY not AVP secretion (see Section 1.1.2.4).

Although blood pressure (BP) was not recorded in the rats in this experiment it is well documented that 5HT agonism either has no effect or a hypertensive effect on BP (McCall et al 1987), and Fluoxetine has been observed to have no effect on BP in normal rats (Fuller, personal communication). It is intriguing

Table 5.2.6 The effect of acute Fluoxetine (10mg/kg IP) on brain neurochemistry in rat groups receiving no osmotic stimulus (Mean±SD)

Group	No	[3H]Ketanserin fmol/mg	[3H] DPAT fmol/mg	5HIAA:5HT
NN	8	300.6±170.6	98.0±33.5	1.32±0.39
FN	8	687.3±285.1	125.5±130.2	1.08±0.28
		p=0.005	p=0.572	p=0.180

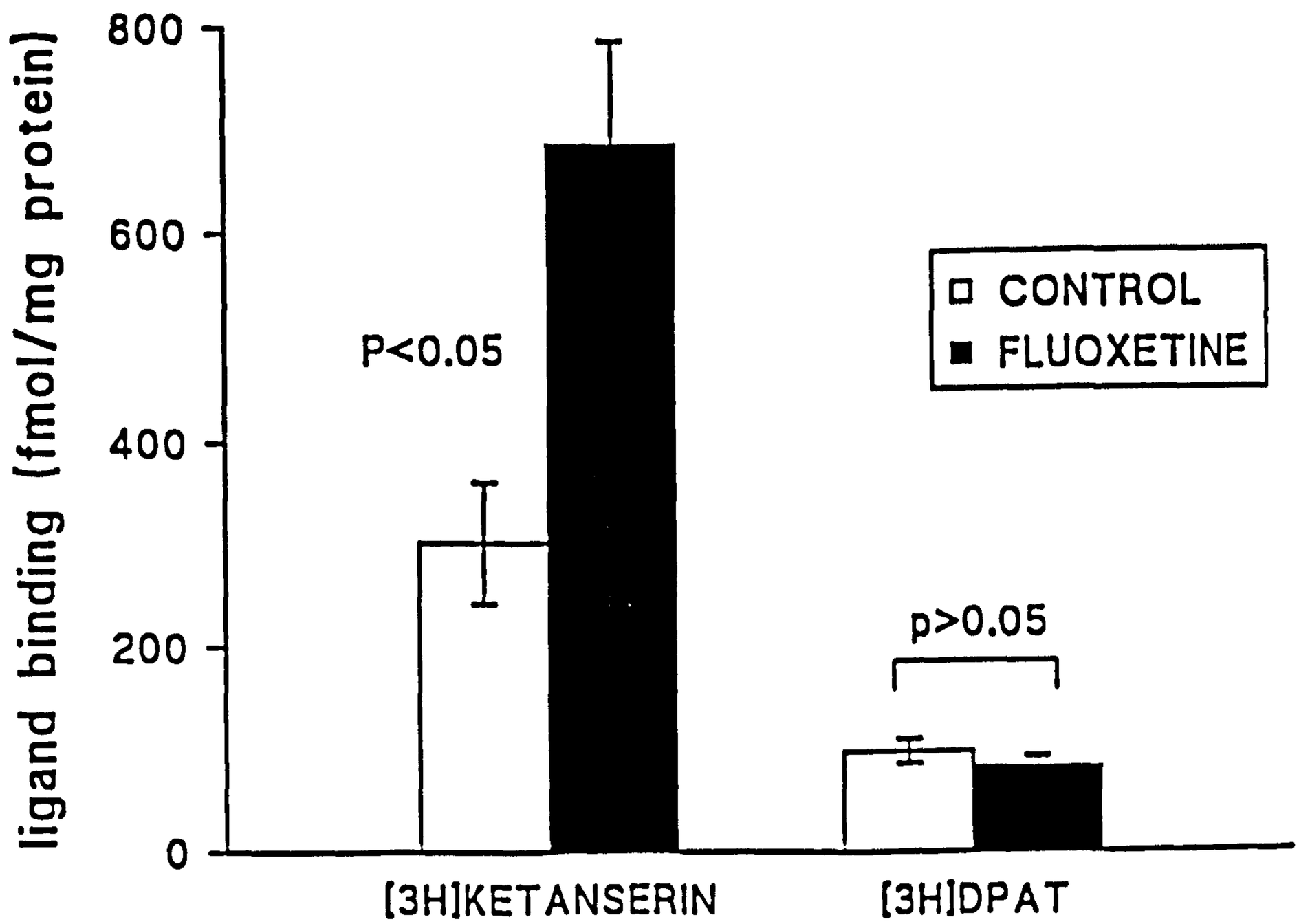


Figure 5.2.9

The effect of acute Fluoxetine treatment (10mg/kg IP) on forebrain ligand binding. [3H]ketanserin with and without the presence of methysergide estimated 5HT2 binding sites and [3H]DPAT with and without the presence of 5HT estimated the density of 5HT1a sites in membrane homogenates. Fluoxetine significantly increased the binding of [3H]ketanserin to 5HT2 sites ($p<0.05$) but had no significant effect on the [3H]DPAT binding to 5HT1a sites. Results are presented as mean $\pm$ SEM

that some authors have postulated that the hypertensive effect of 5HT agonists is mediated through AVP since AVP antagonists block the effect (Pergola & Alper 19 91; Dedeoglû & Fisher 1991).

The effect of Fluoxetine on haematocrit measurements in this experiment would suggest that there was a significant effect either on circulating red cell mass, or an effect on plasma volume. Haematocrit estimation is a very crude estimation of plasma volume. However the effect of Fluoxetine is present in all three treated groups and would suggest that this was a robust result. It was possible than that Fluoxetine decreased plasma volume and caused stimulation of AVP release through firing of atrial stretch receptors. The mechanism of this reduction in plasma volume is not apparent. There was no peritoneal pooling of fluid in dissected rat carcasses and Fluoxetine is not known to have effect on capillary permeability.

Fluoxetine may have direct effect on the neurohypophysis, not requiring penetration of Fluoxetine into the CNS. It would seem unlikely that this could be a 5HT mediated effect since SIAD has not been routinely associated with conditions of high blood 5HT content ie the carcinoid syndrome. There may, however, be an indirect effect of Fluoxetine on the release of AVP from neurons in the neurohypophysis. Similarly Fluoxetine may stimulate magnocellular cell bodies directly or indirectly by influencing the numerous putative afferent pathways (see Section 1.1.3).

The effect of Fluoxetine on AVP secretion, analysed by piece-wise regression, has shown a pattern similar to type C SIAD (Section 1.2.2.3), the "leaky hypophysis", together with a change in the sensitivity of AVP response to rise in pOS above the osmotic threshold. It has been suggested that this pattern of AVP secretion is due to a loss of inhibitory neurons. If it is assumed that Fluoxetine modulates AVP secretion by effect on the magnocellular neurons it is possible that this is through loss of inhibitory tone, or increase in excitatory tone since either effect would permit increase in basal AVP secretion and sensitise neurons to afferent osmotic stimuli.

These hypotheses of the mechanism of the effect of Fluoxetine on osmoregulated AVP secretion require further exploration not only to determine the site and physiology of the effect but also to establish whether the effect is unique to Fluoxetine or a more general effect of 5HT and, if the latter, which 5HT

receptor subtypes are involved. Some of these questions have been explored in the studies that follow.

Section 2.3.1 discussed that pharmacodynamics and neurochemical effects of IP Fluoxetine. This study found no significant effect on 5HT turnover. This is in disagreement with previous studies (Section 2.3.1.2.2) but may be explained by the known regional differences in effect of Fluoxetine. There was, however, evidence that Fluoxetine had penetrated into the brain since there was a significant increase in 5HT1C/5HT2 binding sites in the forebrain homogenate. This was an unexpected finding. It would suggest either an increase in number and/or affinity of 5HT1C/5HT2 receptors. The 5HT2 receptor is a unique receptor with characteristics that differ from receptors of other neurotransmitters (Section 1.3.4). It has been observed that rapid down regulation of 5HT2 receptors occurs with administration of 5HT2a agonists (Buckholtz et al 1988). Change in 5HT2 receptors after acute Fluoxetine administration has not previously been explored and the reason for this finding is unclear. It is possible that the effect was an autoregulatory response to acute increase in 5HT neurotransmission. Further study of this change would be interesting and perhaps increase understanding of the regulation of 5HT neurotransmission.

5.3 THE EFFECT OF ACUTE ANTAGONISM OF 5HT2/5HT1C RECEPTORS BY RITANSERIN ON BASAL AND OSMOREGULATED AVP SECRETION

BACKGROUND

The 5HT2 antagonist, LY5387, has been shown to block the MK212 (5HT1 and 5HT2 agonist) stimulated rise in plasma AVP in rats (Brownfield et al 1988). Ritanserin was found only partially to antagonise the AVP rise in rats induced by the 5HT1c and 5HT2 agonist mCPP (Bagdy et al 1992). As discussed in Section 2.3.2 Ritanserin is a 5HT antagonist with high affinity for 5HT2 receptors but does have some affinity for the 5HT1c receptor subtype. As discussed in Section 1.4.3 it has been postulated that 5HT stimulates AVP secretion through the 5HT2 or perhaps the 5HT1c receptor subtype. This was the first of three studies to explore this hypothesis further.

GENERAL HYPOTHESES

1. That 5HT has a role in the physiological AVP response to change in pOS.

2. That the 5HT2 or 5HT1c receptor mediates this effect of 5HT in osmoregulated AVP secretion.

3. That Ritanserin may cause an abnormality of osmotically stimulated AVP secretion due to antagonism of 5HT at 5HT2 and/or 5HT1c receptors.

SPECIFIC AIMS

To study the effect of acute 5HT2/5HT1c antagonism by Ritanserin on basal AVP secretion and on the AVP response to change in pOS.

METHODS

Day 1: Male Wistar rats were randomly allocated to 6 groups NN RN NH RH NL RL

Day 4: All R groups received 1ml/kg IP Ritanserin (1mg/ml)l and all N groups received 1ml/kg IP vehicle (4% ethanol 0.05M tartaric acid). 30min later rats received 20ml/kg IP 3%NaCl (H), water (L) or no further injection (N). 30min after the osmotic stimulus trunk blood and forebrain samples were collected as described in Section 2.1.2.1.

RESULTS

Seven rats were excluded from data analysis because of an AVP response >30pmol/l. The rats were fairly evenly distributed through the groups (2NN, 1RN, 2NH, 1RH, 1RL). None of these rats appeared unwell at the time of sample collection although one had been demonstrating aggressive behaviour.

Plasma biochemistry

The effect of the osmotic stimuli and Ritanserin treatment on plasma biochemistry is shown in Table 5.3.1. The experimental model caused a significant change in pOS ($p<0.001$) and pNa (<0.001). Ritanserin had no significant effect on the changes in pOS ($p=0.289$). Ritanserin lowered pNa but the effect was significant only at a 5% probability level ($p=0.048$). In addition there was a suggestion of an interaction between osmotic stimulus and the effect of Ritanserin on pNa, but this was not significant ($p=0.044$).

Haematocrit

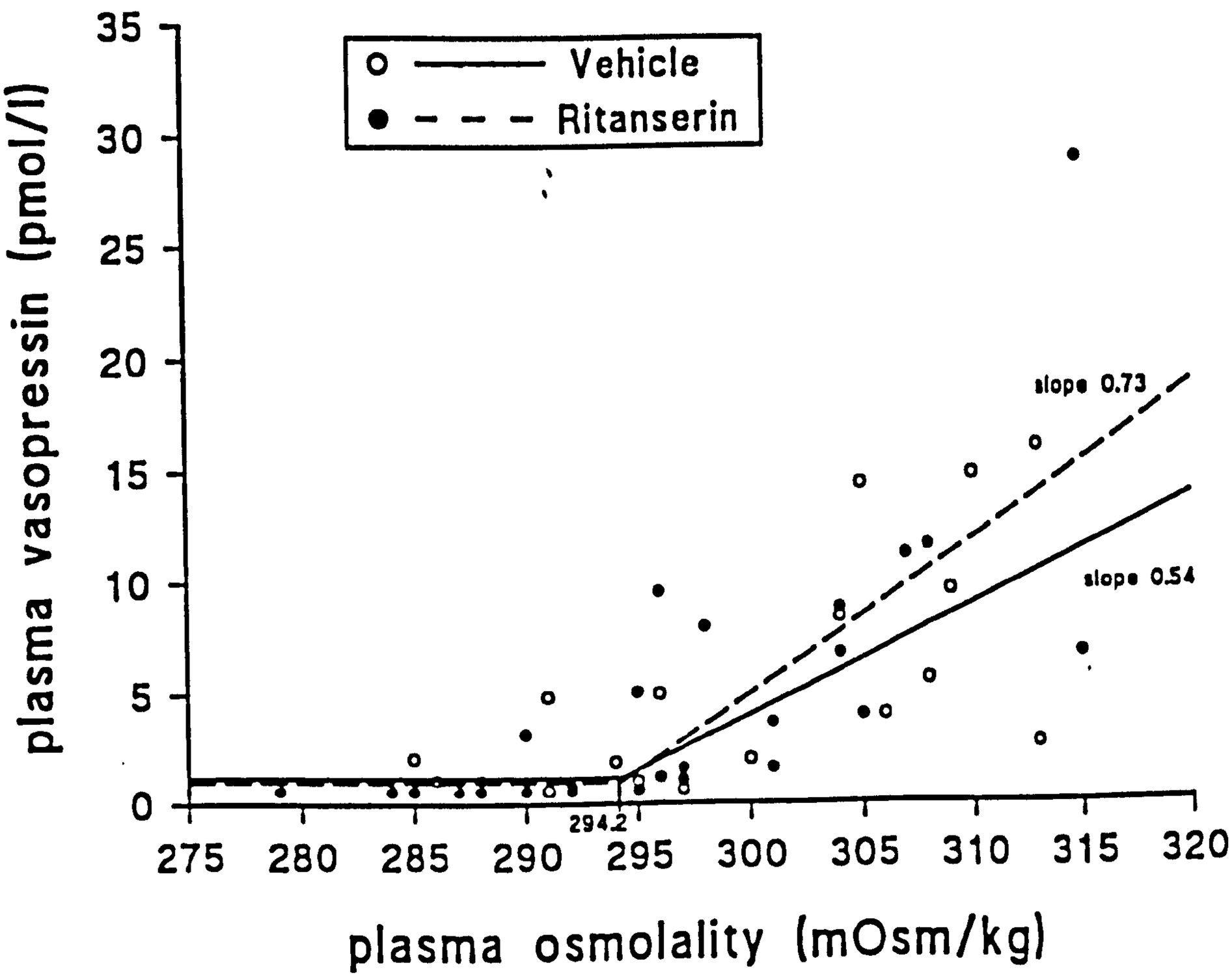
Blood haematocrit was not significantly affected by the osmotic stimulus ($p=0.051$) or Ritanserin treatment ($p=0.470$).

Plasma AVP

Figure 5.3.1 shows the relationship of pAVP to pOS of each rat in the two treatment groups with analysis by piece-wise regression. Ritanserin had no effect on the osmotic threshold of AVP secretion (294.2mOsm/kg, $p=0.570$), on

Table 5.3.1 The effect of acute Ritanserin (1mg/kg IP) and osmotic stimuli (N, H, L) on plasma biochemistry and haematocrit (mean±SEM). The p value results, from ANOVA, test the hypothesis that osmotic stimulus and Ritanserin had no effect. The weights of the rats are presented as mean±SD.

No	N	Wt (g)	pOS (mosm/kg)	pNa (mmol/l)	HCT (%)
NN	8	282.4±9.1	295.6±1.3	140.4±1.3	40.5±0.9
RN	10	281.5±12.5	295.2±1.2	141.0±1.3	41.9±0.9
NH	8	283.1±12.1	308.5±1.3	150.5±1.3	40.5±0.9
RH	9	280.0±8.4	305.3±1.3	146.9±1.3	41.6±0.9
NL	10	280.1±9.1	287.6±3.0	137.6±2.8	43.4±0.8
RL	9	289.9±11.8	286.8±4.7	134.3±5.2	42.7±3.1
EFFECT					
Osmotic stimulus			$p<0.001$	$p<0.001$	$p=0.051$
Ritanserin			$p=0.289$	$p=0.048$	$p=0.470$
Interaction			$p=0.367$	$p=0.044$	$p=0.085$



Piece-wise regression analysis

Parameter ± SE

	DRUG	VEHICLE	COMMON	p value
Change Point (mOsm/kg)	299.9	294.7	294.2	0.568
Basal AVP (pmol/l)	0.95±0.85	1.16±0.88	1.07±0.61	0.238
Sensitivity AVP (pmol/l per mOsm/kg)	0.73±0.11	0.54±0.11	0.63±0.18	0.229

Figure 5.3.1

The effect of Ritanserin treatment (1mg/kg IP) on osmoregulated AVP secretion. Analysis by piece-wise regression showed that Ritanserin had no significant effect on the osmotic threshold of AVP secretion ($p=0.570$) or on the basal concentration ($p=0.240$) or osmotic sensitivity of AVP secretion ($p=0.230$).

the basal concentration of AVP secretion (N 1.16±0.88, R 0.95±0.85 pmol/l, p=0.240) or on the sensitivity of AVP secretion above the osmotic threshold (N 0.54±0.11, R 0.73±0.11 pmol/l per mOsm/kg, p=0.230).

Neurochemistry

Ritanserin treatment significantly increased 5HT turnover in the forebrain (5HIAA:5HT NN 1.44±0.29, RN 2.07±0.46, p=0.001).

DISCUSSION

The results of this study suggest that Ritanserin had no significant effect on either basal or osmotically stimulated AVP secretion. Since Ritanserin is a 5HT2 and 5HT1c antagonist it may be concluded that either 5HT is not involved in basal and osmoregulated AVP secretion, or that these 5HT receptor subtypes do not mediate the effect of 5HT in basal and osmoregulated AVP secretion.

The results of HPLC measurement of 5HT and 5HIAA in the forebrain samples showed that Ritanserin penetrated into the CNS and in this region at least, altered 5HT metabolism. The increase in 5HT turnover might be interpreted as an autoregulatory attempt to overcome the post-synaptic antagonism by Ritanserin. Ritanserin has been shown to have significant effect on central 5HT2 functions within 30min of subcutaneous (SC) administration, with peak effect after 2-4hr (Awouters et al 1988). The absorption from IP administration would probably be faster than after SC administration thus significant effect on central 5HT metabolism would be anticipated within the time period of this study although peak effect on 5HT2 function may not have been achieved.

The effect of Ritanserin on pNa and its interactive effect with the osmotic stimulus is worthy of note although it did not reach the level of significance accepted in this study. The absence of a significant effect on pOS would suggest that the difference in group means was due to experimental variability in the measurement of pNa.

Ritanserin has previously been shown to blunt the pharmacologically stimulated rise in AVP attributed to 5HT agonism in a study which used similar concentration of drug and experimental time scale as this work (Bagdy et al 1992). This would suggest that in rats 5HT may modulate AVP secretion

pharmacologically, and that the 5HT2 and/or the 5HT1c receptor is important in this. In physiological osmoregulated AVP secretion, however, either 5HT does not have a role or it does not have effect through the 5HT2 and/or the 5HT1c receptors.

Nelson et al (1987) found that infusion of Ritanserin into sheep caused a 50% decrease in water intake at 24hr but no significant effect on urine output in this period. Plasma sodium concentration was significantly elevated at 24hr but AVP was not significantly different to pre-infusion plasma concentration. This would suggest that Ritanserin significantly reduced both thirst and AVP secretion and that the normal rise in AVP secretion with increase in pOS was prevented. There was apparent "fatigue" of the effect however since all parameters had returned to normal by 48hr of continued infusion of Ritanserin. It is possible then that the role of 5HT in osmoregulated AVP secretion varies in different animal species.

5.4. DOES THE 5HT2/5HT1C ANTAGONIST RITANSERIN INHIBIT THE FLUOXETINE AUGMENTED AVP RESPONSE TO PLASMA HYPEROSMOLALITY?

BACKGROUND

As discussed in Section 1.4.3 the 5HT2 and/or 5HT1c receptors have been implicated in the stimulation of AVP secretion by 5HT. It was shown in Study 5.2.5 that Fluoxetine stimulated basal AVP secretion and augmented the AVP response to plasma hyperosmolality. If this effect was secondary to increase in 5HT neurotransmission it was possible that Ritanserin might inhibit the augmented stimulation of AVP secretion.

GENERAL HYPOTHESES

1. That Fluoxetine modulate the osmoregulated secretion of AVP by enhancing 5HT neurotransmission.

2. That this effect is through the 5HT2 or 5HT1c receptor.

3. That Ritanserin would inhibit the Fluoxetine augmented AVP response to plasma hypo-osmolality.

SPECIFIC AIM

To explore the effect of Ritanserin on the Fluoxetine augmented AVP response to hyperosmotic stimulus.

METHODS

Day 1: Male Wistar rats were randomly allocated to 4 groups NN RN NF RF
Day 4: At time zero rats in groups RN and RF received 1ml/kg IP Ritanserin (1mg/ml in vehicle) and rats in groups NN and NF received 1ml/kg IP vehicle (10% ethanol 0.02M tartaric acid). This was followed 60min later by 1ml/kg IP

Fluoxetine (NF RF) (10mg/ml in water) or water (NN RN). 30min subsequent to this injection all rats received 20ml/kg IP 3%NaCl and trunk blood was collected after a further 30min (120min after initial injection).

RESULTS

Two rats were excluded from the data analysis because of an AVP response >30pmol/l. Both were in the NF group and inclusion of their results may have lead to a false positive conclusion of the effect of Ritanserin on the Fluoxetine augmented AVP response to rise in pOS.

Plasma biochemistry (Table 5.4.1)

Neither Fluoxetine ($p=0.658$) or Ritanserin ($p=0.152$) or the combination ($p=0.918$) had significant effect on the pOS after the hyperosmotic stimulus. Similar results were found for the effect on pNa.

Haematocrit (Table 5.4.1)

Fluoxetine treatment significantly increased the haematocrit ($p<0.001$). Ritanserin had no effect alone ($p=0.429$ or in combination with Fluoxetine ($p=0.091$) on the haematocrit.

Plasma AVP

The AVP response in each group is shown in Figure 5.4.1 and Table 5.4.1. Fluoxetine significantly augmented the AVP response to plasma hyperosmolality ($p<0.001$). Ritanserin had no significant effect on the AVP response to plasma hyperosmolality ($p=0.060$) and did not significantly alter the Fluoxetine augmented AVP response ($p=0.468$).

DISCUSSION

The effect of Fluoxetine of the AVP response to hyperosmolality was very similar to that found in Study 5.2.5. In addition the effect of Fluoxetine on blood haematocrit was reproduced in this study. Ritanserin had no significant effect on either of these changes attributable to Fluoxetine treatment.

Table 5.4.1 The effect of Ritanserin (1mg/kg IP) on the Fluoxetine (10mg/kg IP) augmented AVP response to hypertonic saline (mean±SEM). The p value results, from ANOVA, test the hypothesis that Ritanserin and Fluoxetine, alone and in combination, had no effect.

Group	N	pAVP (pmol/l)	pOS (mOsm/kg)	pNa (mmol/l)	HCT (%)
NN	8	8.2±2.3	310.3±1.7	151.8±1.1	38.6±1.1
RN	8	13.3±2.3	312.8±1.7	152.7±1.1	38.4±21.1
NF	6	18.5±2.6	311.3±1.9	151.0±1.2	45.3±1.3
RF	9	20.3±2.1	313.3±1.6	151.0±1.0	42.6±1.1
EFFECTS					
Ritanserin		p=0.060	p=0.152	p=0.760	p=0.429
Fluoxetine		p<0.001	p=0.658	p=0.268	p<0.001
Interaction		p=0.468	p=0.918	p=0.690	p=0.091

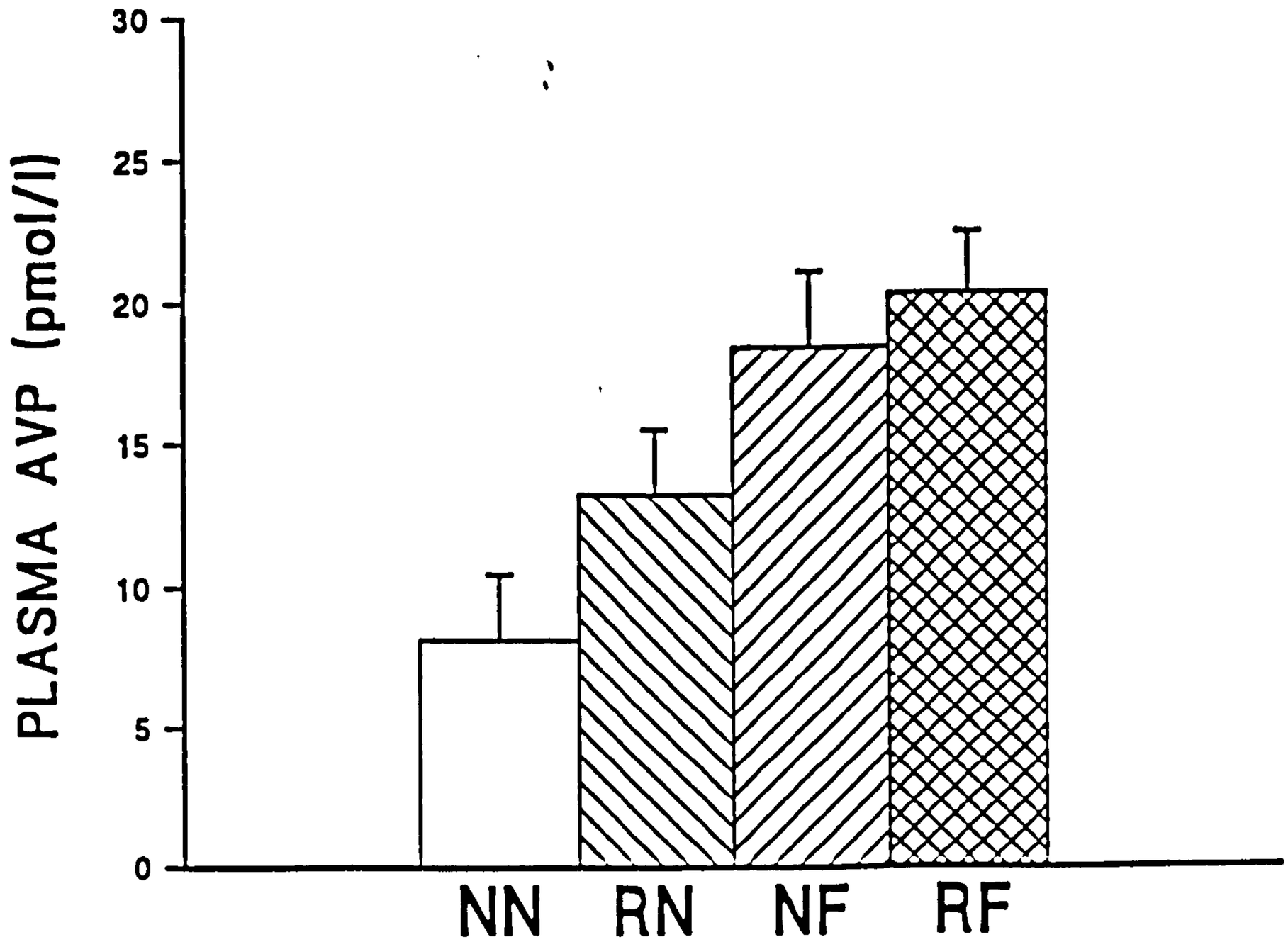


Figure 5.4.1

The effect of Ritanserin (R) (1mg/kg IP) on Fluoxetine (F) (10mg/kg IP) augmented AVP secretion. Fluoxetine significantly increased the AVP response to hyperosmolality ($P<0.001$) but Ritanserin had no significant effect on the response either when given alone ($p=0.060$) or when given with Fluoxetine ($p=0.468$). Results are shown as mean ± SEM with analysis by ANOVA.

These findings would suggest either that the Fluoxetine-augmented osmotically stimulated AVP secretion was not mediated by an effect on 5HT neurotransmission or that the effect was mediated through receptors other than the 5HT2 and 5HT1c subtypes. In addition, as had been found previously in Study 5.3, the normal AVP response to plasma hyperosmolality was not apparently mediated through the 5HT2 and/or 5HT1c receptors since antagonism of these receptors had no significant effect on the response.

Exclusion f m the data analysis of 2 rats in group NF allowed analysis of the underlying mean trend. The AVP response in these rats, >30pmol/l, may have been a facet of the experimental model or may have been a true effect of augmentation of AVP secretion by Fluoxetine treatment.

5.5 THE EFFECT OF THE 5HT2A AGONIST DOI ON OSMOREGULATED AVP SECRETION

BACKGROUND

The hypothesis that an increase in 5HT neurotransmission stimulates AVP secretion and its postulated mechanism through the 5HT2 and/or 5HT1c receptors, has already been discussed in some detail . Bagdy et al (1992), however, found that the 5HT2 agonist DOI did not stimulate secretion of AVP. There has been no previous exploration of the effect of 5HT2 or 5HT1c agonists on osmoregulated AVP secretion.

GENERAL HYPOTHESES

1. That the 5HT2 and/or the 5HT1c receptors mediates the stimulatory effect of 5HT on AVP secretion.

2. That stimulation of the 5HT2 and 5HT1c receptors by the agonist DOI may alter basal and osmotically stimulated AVP secretion.

SPECIFIC AIM

To study the effect of DOI on basal and osmotically stimulated AVP secretion.

METHODS

Day 1: Male Wistar rats were randomly allocated to 6 groups NN DN NL DL NH DH

Day 4: All D groups received 1ml/kg IP DOI (1mg/ml)l and all N groups received 1ml/kg IP vehicle (water). 30min later rats received 20ml/kg IP 3%NaCl (H), water (L) or no further injection (N). 30min after the osmotic stimulus trunk blood and forebrain samples were collected as described in Section 2.1.2.1.

RESULTS

All rats that received DOI and none that received vehicle exhibited "wet-dog shakes" (see Section 2.3.3). This occurred from 15min after the injection of drug.

Two rats in group DN were extremely disturbed at the end of the experiment and the AVP responses were highly atypical of the mean group response (>30pmol/l). One rat in group NH had an absence of AVP response to plasma hyperosmolality. These rats were excluded from the data analysis.

Plasma biochemistry (Table 5.5.1)

The experimental model provided significant changes in pOS ($p<0.001$). DOI treatment had no significant effect on the change in pOS ($p=0.304$) or pNa ($p=0.438$).

Haematocrit

The osmotic stimulus significantly altered the haematocrit ($p=0.007$) but treatment with DOI had no significant effect on ($p=0.394$).

Neurochemistry

Table 5.5.2 shows that treatment with DOI had no significant effect on the forebrain neurochemistry of rats.

Plasma AVP

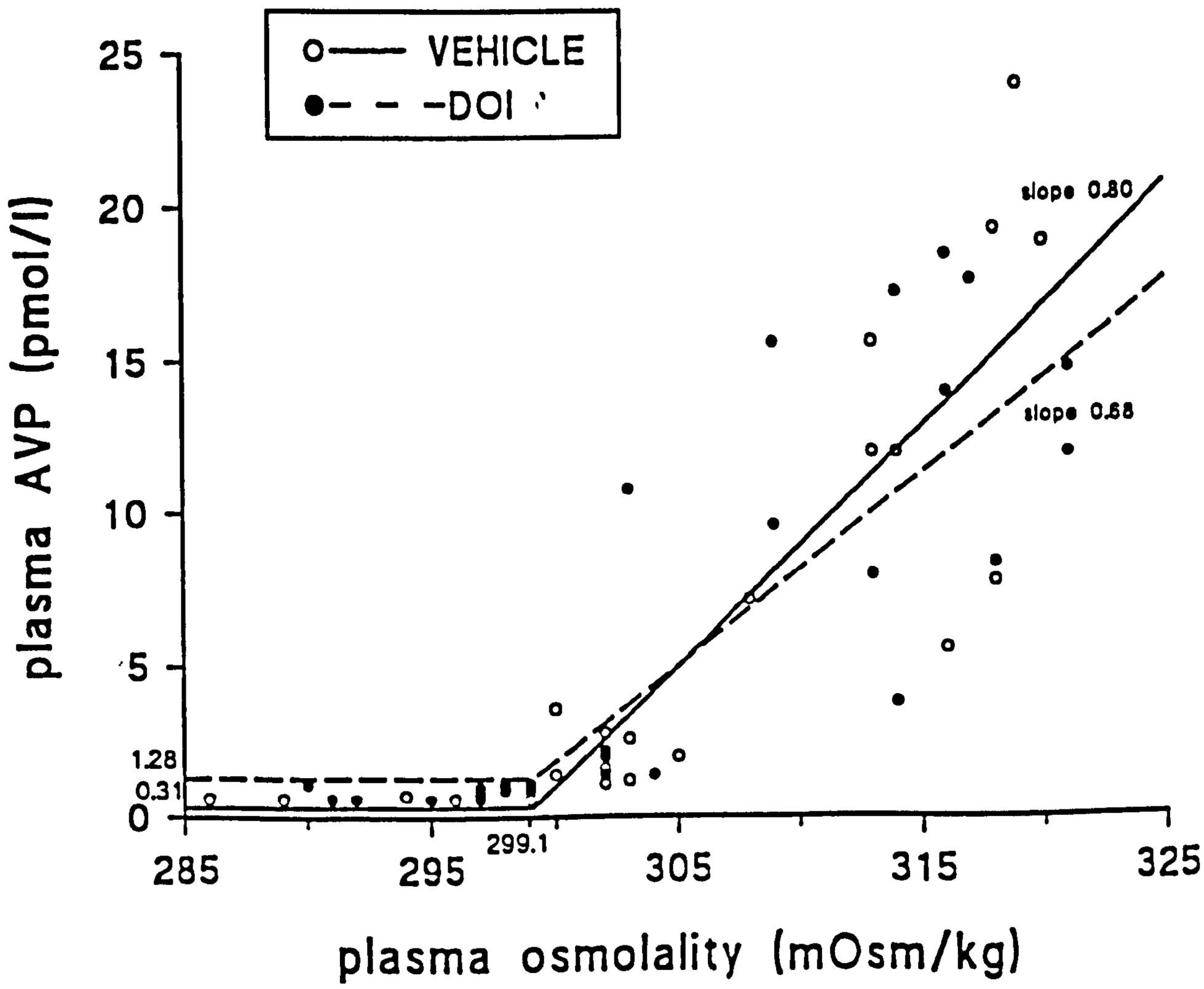
Figure 5.5.1 considers the relationship of pAVP and pOS for the two treatment groups and the analysis of the data by piece-wise regression according to the statistical model explained in Section 5.2.5. DOI had no significant effect on the estimated threshold of AVP secretion which was 299.1mOsm/kg ($p=0.18$). Basal secretion of AVP below pOS 299.1mOsm/kg was not significantly affected by DOI treatment (N 0.31 ± 0.79, D 1.28 ± 0.86 pmol/l, $p=0.879$), nor was the osmotic sensitivity of AVP secretion above the estimated threshold (N 0.8 ± 0.08, D 0.68 ± 0.08 pmol/l per mOsm/kg, $p=0.276$).

Table 5.5.1 The effect of acute DOI (1mg/kg) and osmotic stimuli (N, H, L) on plasma biochemistry and haematocrit (mean±SEM). The p value results, from ANOVA, test the hypothesis that osmotic stimulus and DOI had no effect.

Group	N	pOS (mOsm/kg)	pNa (mmol/l)	HCT (%)
NN	10	301.5±1.2	152.6±1.0	41.2±0.8
DN	8	303.8±1.3	154.2±1.1	42.9±0.9
NL	10	293.8±1.2	149.2±1.0	42.2±0.9
DL	10	294.8±1.2	150.4±1.0	42.4±0.9
NH	10	315.5±1.3	161.9±1.1	40.5±0.9
DH	10	315.4±1.2	160.9±1.0	39.6±0.8
EFFECTS				
Osmotic stimulus		p<0.001	p<0.001	p=0.007
DOI		p=0.304	p=0.438	p=0.394

Table 5.5.2 The effect of DOI (1mg/kg IP) on forebrain neurochemistry in rats receiving no osmotic stimulus (Mean±SD)

Group	No	5HT pmol/mg	5HIAA pmol/mg	5H1AA:5HT
NN	10	15.3±5.1	19.4±5.7	1.38±0.68
DN	10	12.1±3.1	19.5±4.0	1.74±0.48
		p=0.110	p=0.979	p=0.178



Piece-wise regression analysis

Parameter ± SE

	DRUG	VEHICLE	COMMON	p value
Change Point (mOsm/kg)	297.2	302.6	299.1	0.176
Basal AVP (pmol/l)	1.28 ± .86	0.31 ± 0.79	0.76 ± 0.58	0.879
Sensitivity AVP (pmol/l per mOsm/kg)	0.68 ± 0.08	0.80 ± 0.08	0.74 ± 0.06	0.276

Figure 5.5.1

The effect of DOI (1mg/kg IP) on osmoregulated AVP secretion. Analysis by piece-wise regression showed that DOI had no significant effect on the osmotic threshold of AVP secretion (P=0.180) or on the basal concentration (p=0.879) or osmotic sensitivity of AVP secretion (p=0.276).

DISCUSSION

The results of this study would suggest that DOI had no significant effect on basal or osmoregulated AVP secretion.

It was apparent from behaviour observations that DOI had penetrated into the CNS causing "wet-dog shakes" due to 5HT agonism. This was not reflected in biochemical measurements of change in 5HT function. Previous studies have shown that acute administration decreases frontal 5HT and 5HIAA concentrations (Wright et al 1990). The reason for the absence of neurochemical changes in this study is unclear.

The results of this study concur with those of Bagdy et al (1992) and Saydoff et al (1990) who found that DOI had no effect on basal AVP secretion. This would indicate that either 5HT is not involved in basal or osmoregulated AVP secretion, or its effect is not mediated through the 5HT2a receptor subtype. DOI has a moderately high affinity for the 5HT1c receptor subtype and, although this is 40 fold lower than its very high affinity for the 5HT2a receptor, it is likely that administration of 1mg/kg would have had some effect on this receptor. It is therefore unlikely that 5HT stimulates AVP secretion through the 5HT1c receptor subtype. It is possible that the effect of 5HT on AVP secretion is mediated through the 5HT2b receptor subtype although the lack of significant antagonism, by Ritanserin, of Fluoxetine augmented AVP secretion in Study 5.4 would militate against this.

Dedeoglu & Fisher (1991) showed that DOI administered either intra-venously (IV) or intra-cerebro-ventricularly (ICV) significantly increased mean arterial blood pressure. The pressor response of IV administration appeared to be peripherally mediated whilst that after ICV administration was centrally mediated and partially blocked by AVP antagonists. On this evidence the authors suggested that DOI stimulated AVP secretion, possibly indirectly though an increase in sympathetic activity. Plasma AVP concentrations were not measured however, and the inferred difference of these findings and the results of this and other studies discussed above remain unresolved.

5.6. THE EFFECT OF LESION OF 5HT NEURONS OF THE DORSAL AND MEDIAN RAPHÉ NUCLEI ON BASAL OSMOREGULATION

BACKGROUND

The 5HT neurons of the brain may be lesioned in a variety of ways (see Table 1.3.1). The synthesis inhibitor pCPA is 5HT specific but depletes 5HT in all neurons. The neurotoxin 5,7-DHT is selectively transported into 5HT neurons by the 5HT reuptake site. Some non-specific effects in noradrenergic neurons may occur but this is prevented by prior inhibition of noradrenergic reuptake sites by the inhibitor desipramine (Bjorklund et al 1975). Infusion of 5,7-DHT into the raphé nuclei of rats leads to a 75-90% depletion of telencephalic 5HT with little effect on other monoamines (Schallert & Wilcox 1985). One major advantage of this neurotoxin is its use as a site specific neurotoxin when injected into anatomically selected neuron populations.

Tangapregassom et al (1974) found that electrolytic lesion of the DRN increased daily urine output and Iovino & Steardo (1985) found that although generalised lesion of 5HT neurons had no effect on basal plasma AVP concentration it prevented the increase in AVP in response to 24hr water deprivation. Brownfield et al (1987) found that generalised lesion of 5HT neurons by ICV administration of 5,7-DHT significantly inhibited the AVP response to hypertonic stimulus but had no effect on basal AVP concentration or on the AVP response to barometric stimuli (plasma volume depletion).

Section 1.4.1 discussed the controversy of the origin of 5HT neuronal projections to the region of the SON and PVN in the hypothalamus and it is not clear if the DRN and/or the MRN send afferent fibres to these magnocellular nuclei. This study explored the effect of lesion of both raphé nuclei on basal osmoregulation in rats.

GENERAL HYPOTHESES

1. That 5HT has a role in osmoregulated AVP secretion.

2. That lesion of 5HT neurons decreases AVP secretion.

3. That lesion of 5HT neurons of the DRN and MRN may disturb normal osmoregulation subsequent to the decrease in AVP secretion.

SPECIFIC AIM

To study the effect of lesion of the DRN and MRN by 5,7-DHT, on basal osmotic status.

METHODS

Male hooded Wistar rats were randomly allocated to 2 groups (SHAM, LESION). Rats received 25mg/kg IP desipramine and were anaesthetised with 45mg/kg IP pentobarbitol. 25nmol 5,7-DHT in 0.2% ascorbic acid 0.9%NaCl or vehicle was infused into the DRN and MRN 30min after the desipramine injection. The DRN and MRN were identified by streotaxic co-ordinates according to Paxinos & Watson (1982). (7.3mm posterior to the bregma, 6.2mm and 8.4 mm below the dura respectively).
Post-operatively rats were housed with ab libitum food and water for 21 days. Trunk blood was then collected and brain dissected. Forebrain samples were taken for homogenate ligand binding and neurochemistry, and hypothalamic blocks were collected for use in the Study 6.2.
The operative procedures and post operative care were carried out by Mr S Keith, MRC Neurochemical Pathology Unit, Newcastle upon Tyne.
Results were compared by Mann Whitney U tests.

RESULTS

Neurochemistry
The 5HT content of 2 LESION rat brain samples were below the limit of detection by HPLC analysis (0.5pmol/20μl sample). Two other LESION rat brains showed >80% depletion of 5HT compared to SHAM rats but 2 LESION rats had no significant decrease in 5HT concentration, suggesting that the lesion was inadequate in these rats. These latter 2 rats were excluded from further data analysis.

Homogenate ligand binding

The effect of lesion on 5HT1a and 5HT2 ligand binding sites is shown in Figure 5.6.1. There was no significant effect of the lesion on ligand binding to either 5HT receptor.

Plasma biochemistry and AVP

Table 5.6.1 shows the effect of lesion on basal osmotic status. There were no significant differences between the basal pOS of SHAM and LESION nor in the plasma AVP concentration of the two groups.

DISCUSSION

There was no evidence from this small study that normal osmoregulation required the presence of intact 5HT neurons of the DRN and/or MRN. The similar basal pOS and pAVP of the sham and lesioned rats suggested that neither thirst nor osmoregulated AVP secretion were significantly altered by loss of 5HT neurons, at least after a period of 21 days. It would be interesting to pursue this model to see if, as suggested by others, 5,7-DHT lesion of 5HT neurons has effect on dynamically stimulated osmoregulated AVP secretion.

The exclusion of 2 rats from the data analysis resulted in a very small group of lesioned rats. The mean and individual pOS and pAVP of the two groups were so similar that it was unlikely that a significant effect has been missed because of paucity of numbers.

The findings of this study are therefore in agreement with some of the previous work discussed above which involved study of rats with less selective lesioning of 5HT neurons. The increase in urine output observed by Tangapregassom et al (1974) , suggestive of loss of AVP secretion, remains unexplained. There are three possible explanations for the findings of this study; (i) that 5HT is not involved in basal homeostatic osmoregulation; (ii) that 5HT is involved in osmoregulation but adequate compensation for the loss of 5HT neurons had occurred after 21 days; (iii) that 5HT neurons other than those of the MRN and/or DRN are important for the osmoregulatory role of 5HT.

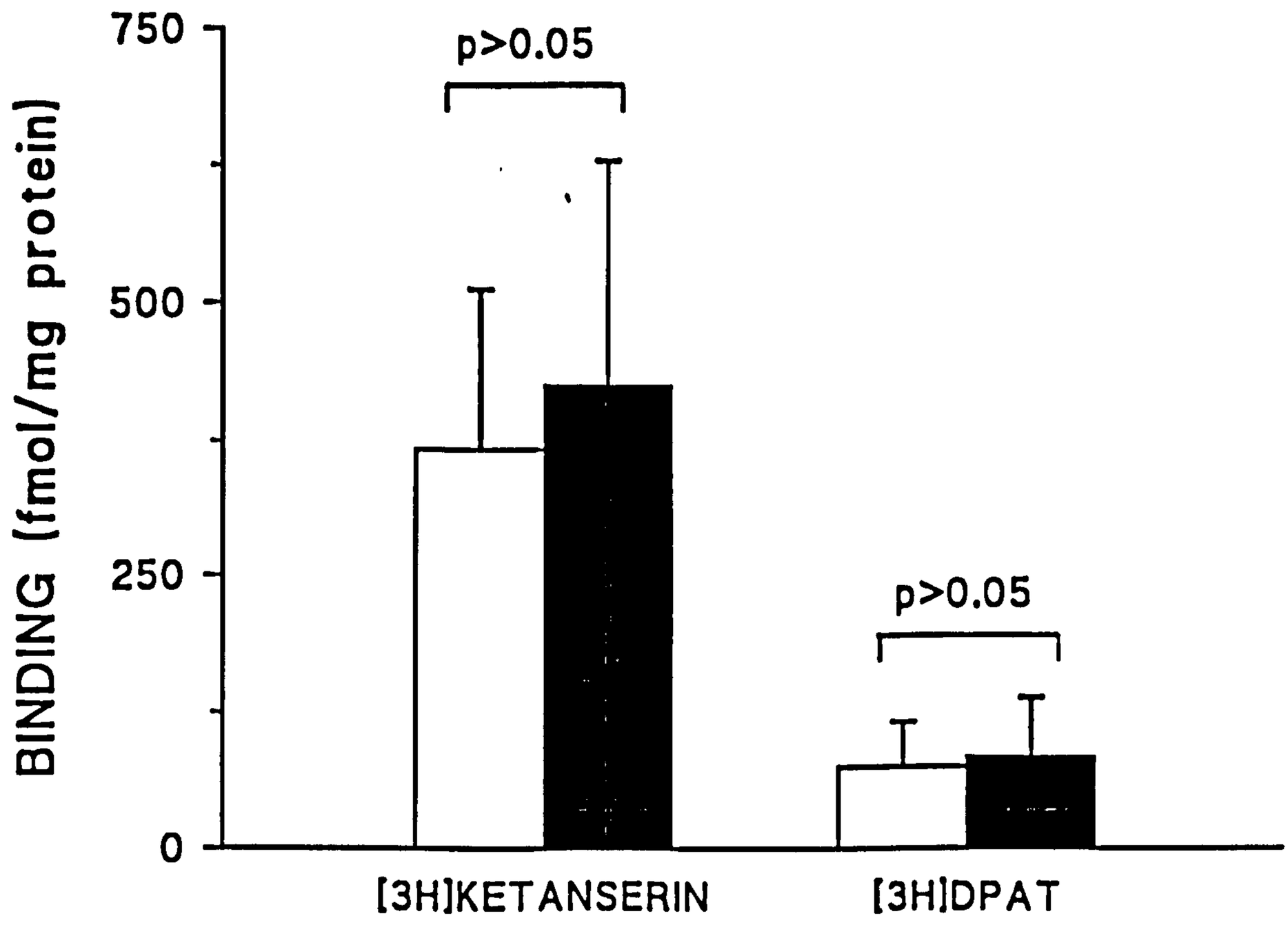


Figure 5.6.1

The effect of lesion of 5HT neurons of the DRN and MRN by 5,7-DHT on forebrain ligand binding. Neither [3H]ketanserin binding to 5HT2 sites of [3H]DPAT binding to 5HT1a sites was significantly altered by the lesion ($p > 0.05$).

Results are presented as mean ± SD.

Table 5.6.1 The effect of lesion of 5HT neurons of the DRN and MRN by 5,7-DHT on basal osmotic status (mean±SD)

Group	No	pOS (mOsm/kg)	pAVP (pmol/l)	HCT (%)
SHAM	6	303.0±4.2	1.4±0.5	36±1
LESION	4	303.3±1.3	2.2±0.8	38±1
		p>0.05	p>0.05	p>0.05

5.7 THE EFFECT OF CHRONIC TREATMENT WITH FLUOXETINE OR RITANSERIN ON OSMOREGULATED AVP SECRETION

BACKGROUND

The 5HT nervous system is capable of many autoregulatory processes consequent to alteration in 5HT neurotransmission and synaptic 5HT concentration (see Section 1.3.4). As discussed in Section 1.5.3.2. recent psychopharmacological research in depression has focussed on the changes in the 5HT system subsequent to chronic administration of antidepressant drugs, given for time periods which mimic therapeutic action (usually 14-21day).

Section 2.3.1.2. discussed the acute effect of Fluoxetine on 5HT neurotransmission. Within 2 days autoregulatory adaptation occurs and a marked decrease in neuronal firing has been found with continued Fluoxetine treatment (Clemens et al 1977). However, after 14 days of continued administration the neuronal firing rate returns to normal and Fluoxetine appears to again augment 5HT neurotransmission. The putative mechanisms for this have been discussed in see Section 1.3.4.3.

The reports of hyponatraemia associated with the clinical use of Fluoxetine in depression have been discussed in Sections 3 and 4 with the evidence that hyponatraemia occurs early in drug administration and persists. In Section 3.3 it was suggested that the mechanism of this hyponatraemia may be due to an inability of some depressed patients to re-establish osmoregulatory homeostasis consequent to the acute stimulation of AVP secretion by Fluoxetine treatment, due to dysfunction of 5HT neurotransmission in depression. In Study 5.2.5 Fluoxetine was found acutely to stimulate AVP secretion. There was a suggestion in the results of Study 5.2.1 that after 18hr, Fluoxetine no longer caused abnormal osmoregulated AVP secretion. This study was to explore the effect of more prolonged treatment with Fluoxetine on osmoregulated AVP secretion using a duration of administration of the drug which mimics its therapeutic effect (21day).

The effect of chronic administration of Ritanserin was also studied. Ritanserin has been identified as having both anxiolytic and antidepressant properties with therapeutic effect in depression after 14-21 days although its anxiolytic effects

may occur more rapidly. Chronic administration of Ritanserin in rats has been shown to lead to 5HT2 receptor down-regulation (Leysen et al 1986). Since it is postulated that 5HT modulates osmoregulated AVP secretion through the 5HT2 receptor it could be that down regulation of these receptors might have an effect on osmoregulated AVP secretion.

GENERAL HYPOTHESES

1. That 5HT modulates osmoregulated AVP secretion.

2. That this is mediated by the 5HT2 receptor.

3. That pharmacological manipulation of brain 5HT receptor numbers and 5HT function by chronic administration of antidepressant drugs may cause observable abnormalities in osmoregulated AVP secretion.

SPECIFIC AIMS

To explore the effect of Fluoxetine and Ritanserin, administered for a time period to mimic their therapeutic effect in depressed patients, on brain neurochemistry, receptor density and osmoregulated AVP secretion.

METHODS

Male Wistar rats were randomly allocated to 9 groups NN,NH,NL, FN,FH,FL, RN,RH,RL.

Each rat received 21 daily SC injections of 1ml/kg vehicle (N), Ritanserin (R) or Fluoxetine (F) according to their treatment group. Drugs were made up in 10% ethanol 0.02M tartaric acid.

Rats were weighed every other day to assess well being and injection volume.

Day 22: The protocol for assessment of osmoregulated AVP secretion was carried out as described previously and in Section 2.1.2.1 according to the group allocation of each rat.

RESULTS

All rats tolerated the daily injections well. There was some scab formation at injection sites in the second week of treatment but this resolved without evidence of infection or distress to the animals.

Rats treated with Ritanserin were noted to be more irritable than either vehicle or Fluoxetine treated rats in which groups no abnormal behavioural effects were noted.

Three rats in group RN and one in group RH were excluded form data analysis due to an atypical AVP response to osmotic stimulus of >30pmol/l.

Weight gain

Rats treated with Fluoxetine gained 13% less weight than rats treated with vehicle (N 123.6±24g, F 108.5±19g, $p=0.031$). There was no significant effect of Ritanserin on weight gain.

Plasma biochemistry (Table 5.7.1)

The osmotic stimulus caused a significant change in pOS ($p<0.001$) and pNa ($p<0.001$). Neither Fluoxetine ($p=0.634$) or Ritanserin ($p=0.399$) had significant effect on the change in pOS. A similar absence of effect on change in pNA was found for each drug.

Haematocrit

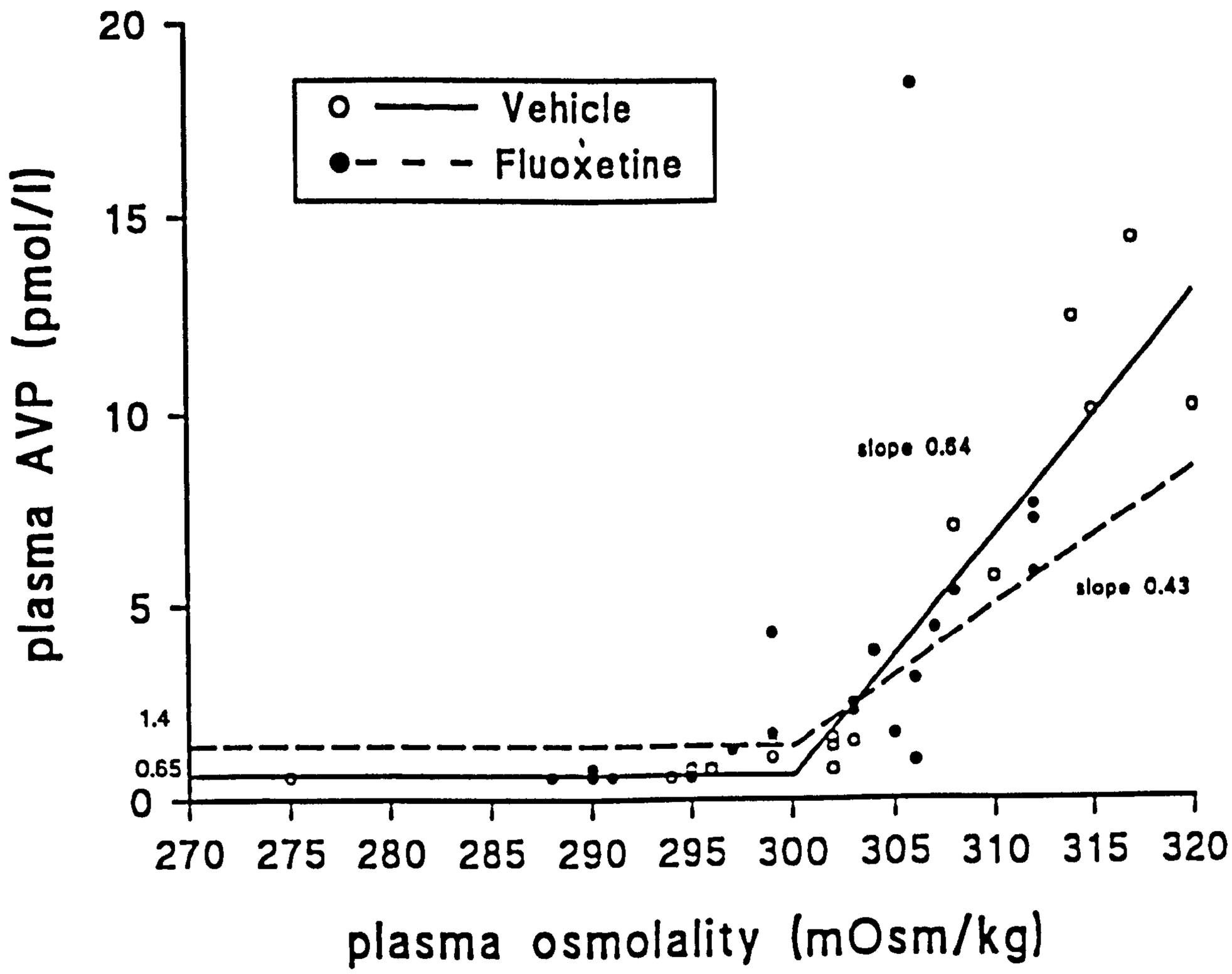
The osmotic stimulus did not significantly affect the HCT ($p=0.02$) but both Fluoxetine treatment ($p=0.004$) and Ritanserin treatment ($p=0.006$) significantly decreased HCT.

Plasma AVP

Figure 5.7.1 shows the piece-wise regression analysis of the relationship between pAVP and pOS for rats treated with Fluoxetine compared to control, rats. Fluoxetine had no significant effect on the osmotic threshold of AVP secretion which was estimated as 300.1mOsm/kg ($p=0.170$). Basal AVP secretion was not significantly effected by Fluoxetine treatment (N 0.65±0.66, F 1.4±0.57pmol/l, $p=0.580$). Fluoxetine treatment did, however, significantly

Table 5.7.1 The effect of chronic treatment (21 day) with Ritanserin (1mg/kg SC) (R) or Fluoxetine (10mg/kg SC) (F) on plasma biochemical and haematocrit changes in response to osmotic stimuli (N, H, L) (mean±SEM). The p value results, from ANOVA, test the hypothesis that osmotic stimulus and Fluoxetine or Ritanserin treatment had no effect.

Group	N	Wt gain (g)	pOS (mosm/kg)	pNa (mmol/l)	HCT (%)
NN	6	108.5±7.2	301.2±2.2	142.2±1.5	43.7±0.9
FN	10	104.4±6.2	303.1±1.9	146.9±1.3	41.9±0.9
RN	10	120.4±6.2	300.6±1.6	145.3±0.9	42.0±1.0
NL	6	117.7±7.2	292.5±2.2	140.3±1.5	45.0±0.9
FL	11	117.7±5.8	292.6±1.8	142.6±1.2	44.0±0.7
RL	10	124.0±6.2	293.9±1.6	141.3±0.9	42.9±1.0
NH	6	134.5±7.1	314.0±2.2	151.7±1.5	44.0±0.9
FH	10	102.2±6.2	313.4±1.9	152.4±1.3	40.9±0.7
RH	10	127.6±6.2	308.0±1.6	149.1±0.9	40.3±1.0
EFFECTS					
Osmotic stimulus			p<0.001	p<0.001	p=0.020
Fluoxetine			p=0.634	p=0.030	p=0.004
Ritanserin			p=0.399	p=0.548	p=0.006



Piece-wise regression analysis

Parameter ± SE

	DRUG	VEHICLE	COMMON	p value
Change Point (mOsm/kg)	295.4	300.9	300.1	0.168
Basal AVP (pmol/l)	1.40±0.57	0.65±0.66	1.1±0.43	0.578
Sensitivity AVP (pmol/l per mOsm/kg)	0.43±0.06	0.64±0.08	0.45±0.06	0.028

Figure 5.7.1

The effect of treatment with Fluoxetine (10mg/kg) for 21day on osmoregulated AVP secretion analysed by piece-wise regression. Fluoxetine had no significant effect on the osmotic threshold of AVP secretion (p=0.170) or on the basal concentration of AVP secretion (p=0.580) but significantly decreased the osmotic sensitivity of AVP secretion above the osmotic threshold (p=0.030).

decrease the osmotic sensitivity of AVP secretion (N 0.64±0.08, F 0.43±0.06 pmol/l per mOsm/kg, p=0.030).

Figure 5.7.2 shows the effect of Ritanserin treatment of the relationship of pAVP and pOS. Ritanserin had no significant effect on osmotic threshold (estimated as 300.5 mOsm/kg, p=0.470), basal AVP secretion (N 0.73±0.4, R 0.75±0.07 pmol/l, p=0.240) or the osmotic sensitivity of AVP secretion (N 0.66±0.04, R 0.52±0.07 pmol/l per mOsm/kg, p=0.120).

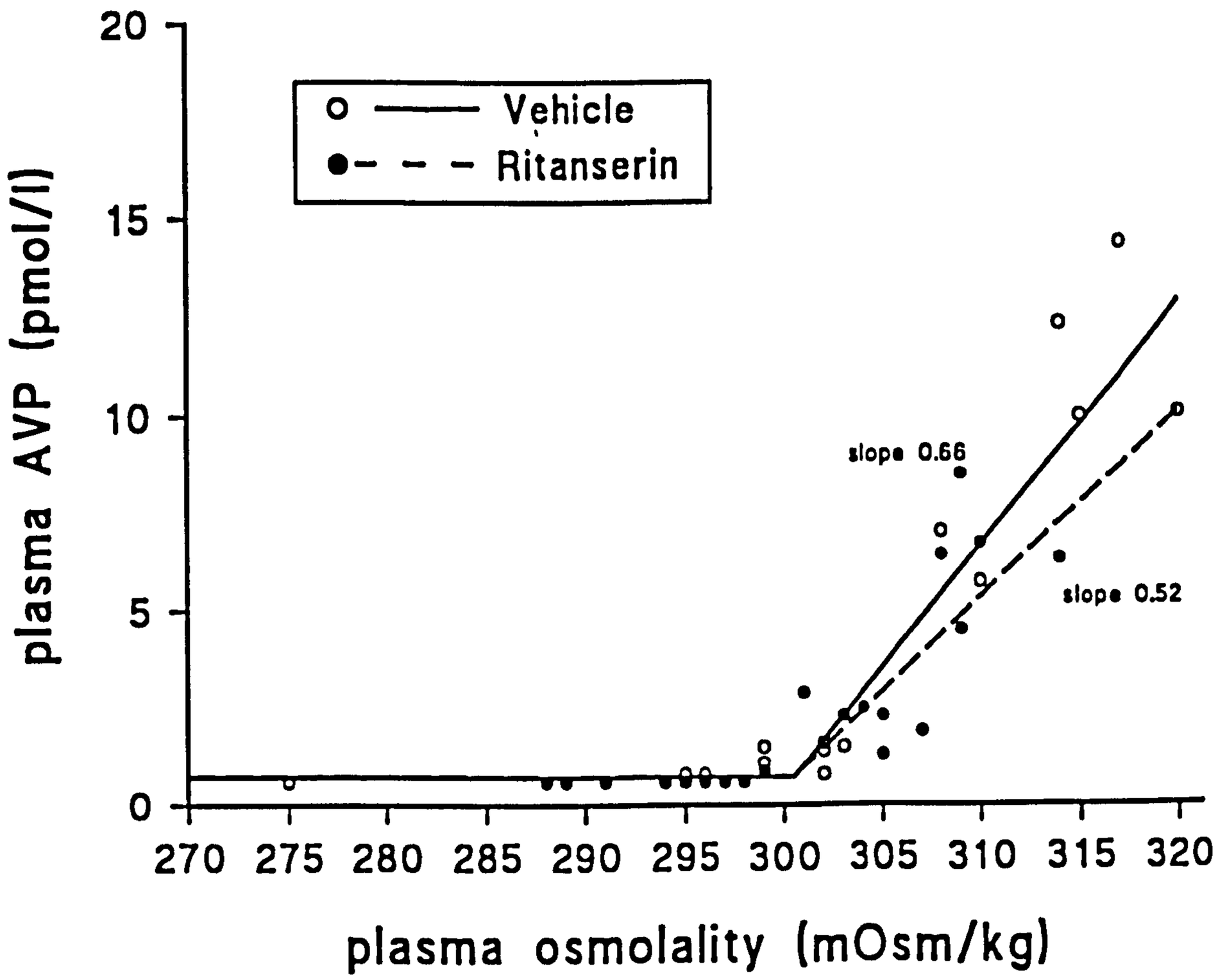
Changes in forebrain samples

Table 5.7.2 shows the effect of treatment with each drug on ligand binding to 5HT1a and 5HT2 receptors and 5HT turnover. Results,compared by Wilcoxon rank sum analysis, showed that neither drug treatment had significant effect on ligand binding density to either receptor type, or on the turnover of 5HT in the forebrain as quantified by the ratio of 5HIAA to 5HT concentration.

DISCUSSION

The results of this study indicate that chronic treatment with Ritanserin had no effect on osmoregulated AVP secretion. Chronic treatment with Fluoxetine caused a significant decrease in the AVP response to change in pOS but had no effect on basal AVP secretion or the osmotic threshold of secretion. The decrease in AVP responsiveness to change in pOS would mean that for an increase in pOS of 10mOsm/kg rats treated with Fluoxetine would have a rise in AVP of 2.1pmol/l less that normal control rats. This would certainly be biologically significant and it is possible that, due to the interference of Fluoxetine with the RIA of AVP (Section 2.2.7), this was an underestimate of the effect. The lack of significant effect of Fluoxetine on pOS, however,would suggest, that either an increase in fluid ingestion or perhaps an increase in renal sensitivity to plasma AVP had adequately compensated for the relative deficiency of AVP in these rats.

In Study 5.2.5 Fluoxetine was found to acutely stimulate AVP secretion and increase the osmotic sensitivity of AVP secretion. This effect has been lost after 21 daily treatments. Consideration of the findings of Study 5.2.1 ,might suggest that the effect was lost 18hr after one dose of Fluoxetine, although it is known



Piece-wise regression analysis

Parameter ± SE

	DRUG	VEHICLE	COMMON	p value
Change Point (mOsm/kg)	299.3	300.9	300.5	0.467
Basal AVP (pmol/l)	0.75 ± 0.40	0.73 ± 0.40	0.64 ± 0.28	0.238
Sensitivity AVP (pmol/l per mOsm/kg)	0.52 ± 0.07	0.66 ± 0.04	0.62 ± 0.04	0.120

Figure 5.7.2

The effect of treatment with Ritanserin (1mg/kg) for 21day on osmoregulated AVP secretion analysed by piece-wise regression. Ritanserin had no significant effect on the osmotic threshold of AVP secretion ($p=0.470$) or on the basal concentration ($p=0.240$) or the osmotic sensitivity of AVP secretion above the osmotic threshold ($p=0.120$).

Table 5.7.2 The effect of chronic treatment (21day) with Fluoxetine (10mg/kg SC) (F) or Ritanserin (1mg/kg SC) (R) compared to vehicle treated (N) rats on forebrain neurochemistry and ligand binding (mean±SD).

Group	No	[3H]Ketanserin fmol/mg	[3H] DPAT fmol/mg	5H1AA:5HT
N	5	360±109	91±46	2.18±0.6
F	8	434±201	108±40	2.29±0.5
R	8	305±176	113±52	2.89±0.6
		p>0.05	p>0.05	p>0.05

that the drug still has significant effect on 5HT reuptake at this time (Wong et al 1975. See Section 2.3.1.2). Thus osmotic homeostasis is apparently acutely disturbed by Fluoxetine treatment but equilibrium is re-established, possibly as rapidly as within 18hr. It could be postulated that the decrease in osmotic sensitivity after chronic Fluoxetine treatment was an overcompensation for the increase in osmotic sensitivity after acute treatment.

Assuming that Ritanserin had significant penetration into the CNS in this study the lack of significant effect of chronic treatment on osmoregulated AVP secretion would suggest that either the 5HT2 receptor was not important in 5HT modulated osmoregulated AVP secretion, or that adequate compensation for any disturbance has occurred. It is of course also possible that 5HT does not modulate osmoregulated AVP secretion and that the effect of Fluoxetine was not mediated through its inhibition of 5HT reuptake and the consequent effects on 5HT neurotransmission.

Neither drug has lead to significant neurochemical change or change in density of 5HT receptors.

The literature contains contradictory results of the effect of chronic Fluoxetine treatment on 5HT receptor density. Some authors have shown no effect on 5HT1 receptors (Peroutka & Snyder 1980b, Maggi et al 1980, Stoltz et al 1983) whilst others have suggested 5HT1 receptors are significantly decreased (Walmsley et al 1987, Wong & By master 1981, Wong et al 1985, Dumbrille-Ross & Tang 1983). Only one group has studied the 5HT1a receptor subtype but this was specifically in the brainstem and hippocampus where there was a differential effect (Welner et al 1989). The effect of chronic Fluoxetine treatment on 5HT2 receptor is no more clear. Peroutka & Snyder (1980)b suggested no significant effect, whilst Dumbrille-Ross & Tang (1983) found a 25% increase in [^{3}H]spiperone binding in frontal cortex and Stoltz et al (1983) found a 27% decrease in the same region with the same tritiated ligand.. Walmsley et al (1987), in an autoradiographic study, found a decrease in 5HT2 receptors in all layers of the frontal cortex but an increase in other brain areas. Some of these differences are undoubtedly due to experimental techniques but some might be explained by differences in the duration of treatment with Fluoxetine since, as discussed above, Fluoxetine decreases 5HT neurotransmission between 2 and 14 days and increases it both acutely and after

14 days continuous treatment.

In addition to the absence of significant change in 5HT1a or 5HT2 receptors the study has shown no neurochemical changes after chronic Fluoxetine treatment. These findings are unexplained since the significant lack of weight gain in Fluoxetine-treated rats, thought to be mediated by a central effect of appetite, would suggest that Fluoxetine had penetrated into the CNS and had discernible effect on 5HT function. It could be that the lack of significant neurochemical and receptor changes are due to a summation of regional differences of effect.

Leysen et al (1986) and Twist et al (1990) found that chronic treatment with Ritanserin caused a 30-50% decrease in [^{3}H]ketanserin binding in frontal cortex. The findings of this study have not shown any change in ligand binding to either of the 5HT receptor types considered, or in 5HT turnover. This is unexplained. The behavioural observations would suggest that Ritanserin had significant central effect but, since the study was not designed specifically to note changes in behaviour, this can be no more than subjective evidence of significant effect of Ritanserin at 5HT2 receptors.

The changes in haematocrit consequent to chronic treatment with Fluoxetine or Ritanserin are different to the effect of acute treatment (Sections 5.2.5, 5.3 and 5.4). The decrease in haematocrit after chronic treatment with Fluoxetine, suggestive of an increase in plasma volume, may have lead to a decrease in barometric stimulation of AVP secretion, with consequent decrease in sensitivity of osmoregulated AVP secretion (see Section 1.1.2.3). However Ritanserin had a similar effect to Fluoxetine on haematocrit after chronic treatment with no significant effect on AVP secretion. It is more likely that the apparent, small differences are largely due to experimental error since the technique is a very rough guide to plasma volume and probably only useful in detecting changes of at least 10%.

5.8 CONCLUSIONS and DISCUSSION

The major conclusions of this series of studies are:

1. Acute Fluoxetine stimulates basal AVP secretion and increases the sensitivity of osmoregulated AVP secretion.

2. This acute effect on AVP secretion is no longer apparent after 21 daily treatments of Fluoxetine and is possibly
lost as early as 18hr after one dose of treatment.

3. Chronic treatment with Fluoxetine allows return of basal AVP secretion to normal concentration and leads to a decrease in the osmotic sensitivity of AVP secretion, perhaps as an overcompensation to the initial increased sensitivity of osmoregulated secretion.

4. The effect of Fluoxetine on osmoregulated AVP secretion is not blocked by antagonism of 5HT2/5HT1c receptors with Ritanserin nor reproduced by the selective 5HT2a agonist DOI which also has some 5HT1c agonist effects.

5. Osmoregulated AVP secretion is not altered by antagonism of 5HT2and/or 5HT1c receptors either acutely or after chronic antagonist administration.

6. Lesion of 5HT neurons of the DRN and MRN does not alter osmotic homeostasis at least at basal pOS.

Each of these conclusions has already been discussed in some detail in the relevant sections.
The work has explored the hypothesis that 5HT has effect on AVP secretion through the 5HT2/5HT1c receptor. Since neither of these receptors appears to influence Fluoxetine-stimulated AVP secretion it could be concluded that the 5HT1a or 5HT3 receptor or a receptor type or subtype as yet uncharacterised is important in this effect. Brownfield et al (1988) found that a putative selective 5HT1a agonist had no effect on AVP secretion and Bagdy et al (1992) found a similar

lack of significant effect of a different, but highly selective 5HT1a agonist. 5HT3 agonists are not available and 5HT3 antagonists have not been used in the study of the neuroendocrinology of AVP secretion, although it has been shown that 5HT3 antagonists block the increase in plasma AVP concentration associated with nausea (Edwards et al 1989). This effect, however, is probably secondary to the anti-emetic effect of such drugs and not a direct effect of the drug on AVP secretion. The clarification of receptor subtypes involved in 5HT modulated AVP secretion awaits further development of pharmacological agents and knowledge of receptor subtypes.

It could be concluded from these studies that Fluoxetine stimulated basal and osmoregulated AVP secretion through a non-5HT mediated mechanism. It is unlikely that this was through barometric or emetic stimulus as discussed in Section 5.2.5 although the effect of Fluoxetine on haematocrit perhaps requires further investigation of changes in plasma volume using a more sensitive technique. Although the pharmacological effects of Fluoxetine are thought to be highly specific to 5HT neurotransmission effects on other neurotransmitter systems have also been shown (Fuller & Wong 1990). Chronic administration to rats has been shown to cause down regulation of β-adrenoreceptors (Walmsley et al 1987), and to decrease noradrenergic mediated growth hormone release in depressed patients (O`Flyn et al 1991). Both these findings suggest that Fluoxetine, at least in chronic administration, may have significant effect on noradrenergic function. It is possible that it stimulates AVP secretion in this way.

SECTION 6

SEROTONIN RECEPTORS IN THE SON AND PVN OF THE RAT HYPOTHALAMUS

6.1 INTRODUCTION

The anatomical evidence suggesting that 5HT might have a direct influence on AVP secretion from magnocellular neurons has been discussed in Section 1.4.1. There is evidence of a sparse but specific 5HT innervation of these neurons. Autoradiographic ligand-binding studies in human brain have shown the presence of a low density of 5HT1a receptors in the vicinity of the SON and PVN but a slightly higher density of 5HT1c receptors in the PVN than the SON (Pazos et al 1987a) . Studies in rat brain have not specifically examined these hypothalamic nuclei but Pazos & Palacios (1985) and Pazos et al (1985a) have suggested that both 5HT1 and 5HT2 receptors are of low density in these areas of the hypothalamus.

This Section of the research has explored the possibility that 5HT may have a direct influence of secretion of AVP from magnocellular neurons by examination of the 5HT receptor density localised to the SON and PVN.

GENERAL HYPOTHESES

1. That 5HT has a direct mode of influence on AVP secretion from magnocellular neurons of the PVN and/or SON through stimulation of 5HT receptors.

2. That the 5HT2 receptor subtype is of particular importance in this mode of action.

3. That 5HT receptors may be identified anatomically and quantified by autoradiographic receptor ligand binding.

4. That the density of receptors identified by autoradiography is directly related to the functional importance of 5HT as a neurotransmitter.

SPECIFIC AIMS

1. To anatomically localise magnocellular AVP secreting neurons in the SON and PVN.

2. To quantify 5HT receptor density in the SON and PVN by autoradiography with particular reference to the 5HT2 receptor.

6.2.1 METHODOLOGY

Tissue preparation.

Rat brains were collected in the manor described in Section 2.1.2.1. The brains were placed intact in a brain mould and a blocks containing the hypothalamus were dissected by cutting anterior to the optic chiasm and posterior to the median eminence. The blocks were immediately frozen in the vapour of liquid N_2. Blocks were stored, air sealed at -80°C

20μm coronal sections were cut by cryostat microtome (Bright instrument company, Cambs) at -15°C using an orientating object holder to an achieve the anatomical plain according to the atlas of Paxinos & Watson (1982). Sections were thaw mounted on to microscope slides, air dried for 1-3hr and stored, air-tight, at -80°C.

Slide preparation.

Washed microscope slides (1.2mm BDH) were coated according to the experimental use.

a) Histology: No coating.

b) Immunocytochemistry: 2% Gelatin coating.

c) Autoradiography: 0.1% Poly-l-lysine coating.

6.3 THE ANATOMICAL LOCALISATION OF THE SON AND PVN IN THE RAT HYPOTHALAMUS

In order to quantify 5HT receptors in the SON and PVN it was important to be able to identify these nuclei with accuracy. There is evidence that AVP and

OXY secreting neurons are differentially distributed in the two nuclei (Sternberger 1974; Vandesande & Dierickx 1975; Swaab et al 1975) (Section 1.1.1). In rats AVP-secreting cells may be more concentrated in the mid and dorsal parts of each nucleus and form a ventral cluster in the mid portion of the SON. In the PVN AVP cells form a cluster in the centre of the outer wing of magnocellular neurons. This difference in anatomical localisation of neurons of the two hormone types could perhaps be important in the study of 5HT receptor localisation since there is some evidence to suggest that the direct 5HT innervation of the magnocellular neurons is preferentially to OXY type neurons (Sawchenko et al 1983).

The immunocytochemistry (ICC) of AVP secreting neurons has previously been performed on brain sections of animals which have been perfused with fixative or immersed as a block in fixative. For the purposes of this research unfixed, thaw mounted cryostat sections would be used since sections from the same tissue block would be used for autoradiography. It was not known if the antigenicity of AVP would be preserved under these conditions.

6.3.1 HISTOLOGY OF MAGNOCELLULAR NEURONS IN THE HYPOTHALAMUS OF THE RAT

A Nissl stain, cresyl violet was used for histological staining of tissue sections. This stain identified the perikarya and proximal dendrites of neurons. A "fast cresyl" preparation was used (0.1% cresyl violet in 1% acetic acid). (Drury & Wallington 1967).

Methods

20μm frozen sections were warmed to room temperature and incubated for at least 12hr in 10% formalin fixative, buffered with marble chips. Sections were washed in tap water, incubated with cresyl fast violet dye 10-20min and then washed in tap water.

Sections were differentiated in 70% methanol and progressively dehydrated by increasing concentration of alcohol before immersion in xylene and coverslip application with DBX xylene based mountant (BDH).

Figure 6.1 show typical sections of the hypothalamus at various points through the SON and PVN.

6.3.2 THE IMMUNOCYTOCHEMICAL LOCALISATION OF AVP SECRETING NEURONS

ICC utilises antibodies to localise specific antigens in tissue sections. In essence a tissue section is incubated with a labelled antibody, the section is then washed and the sites of the antibody-antigen interaction identified by a visual label. In this research an indirect, immunoperoxidase technique was used where the visual label was formed by the oxidation and precipitation of the chromagen diaminobenzidine (DAB) subsequent to the reaction of the enzyme horse radish peroxidase (HRP) with the substrate H_2O_2. The specific antibodies to AVP used in the experiments were polyclonal, derived from rabbit sera during development of the specific anti-AVP antibody for RIA (Rooke & Baylis 1982) (Section 2.2).

6.3.2.1 Basic protocol for ICC by an indirect immunoperoxidase method:

Buffer: 0.01 Phosphate buffered saline (PBS) pH 7.2 (Appendix 4).

a) Pre-incubation of tissues sections: 2x5min, 20°C.

b) Block non-specific antibody binding sites: 5% dried milk powder in PBS.

c) Incubation with 1st antibody: variable

d) Wash: 2x5min PBS, 20°C.

e) Incubation with 2nd antibody: Biotinylated donkey anti-rabbit IgG, 1:100 dilution, 30min, 20°C.

f) Wash: 2x5min PBS, 20°C.

g) Incubation with enzyme-linked avidin: Strep-avidin HRP, 1:200, 30min, 20°C.

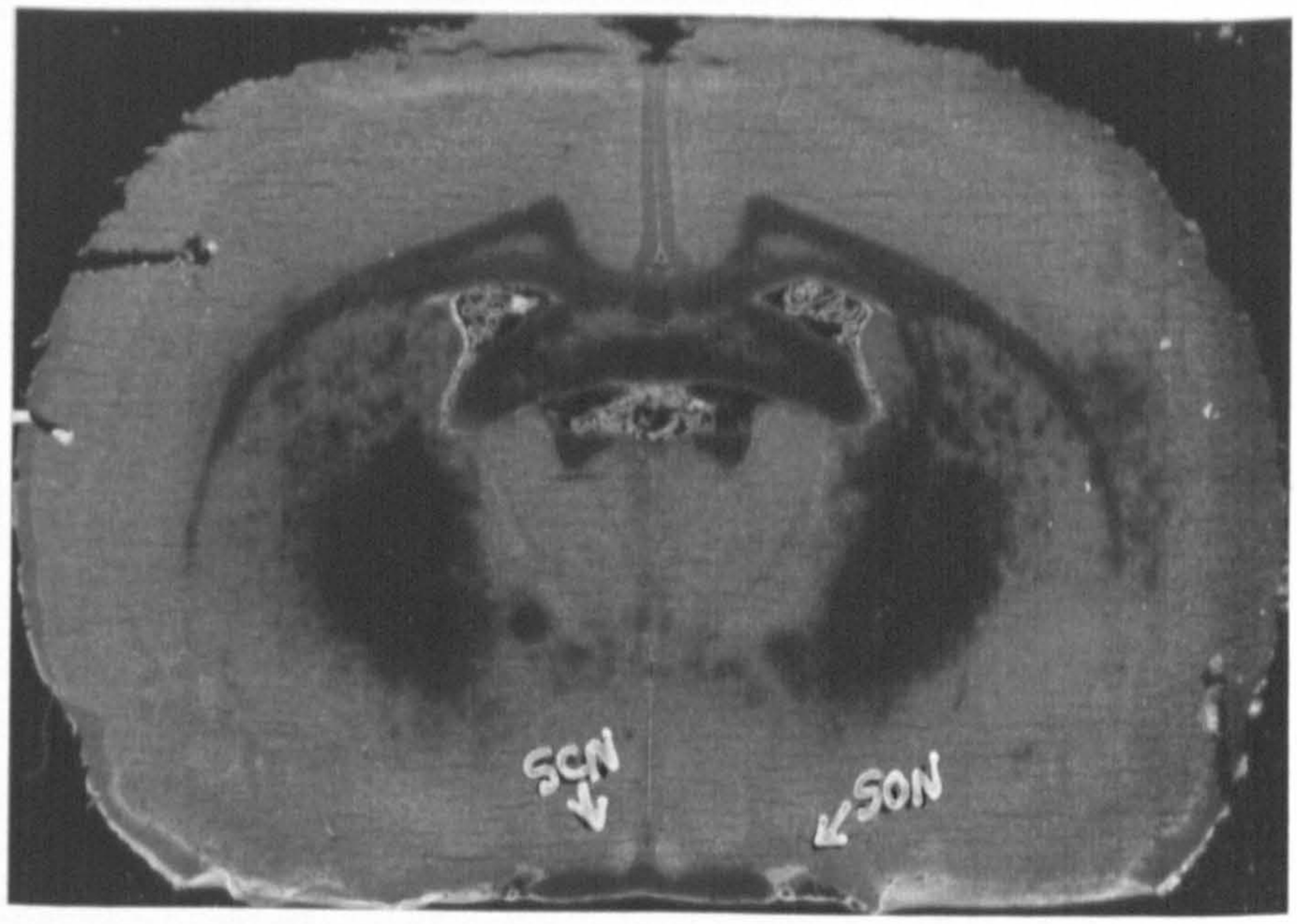
h) Wash 2x5min PBS, 20°C.

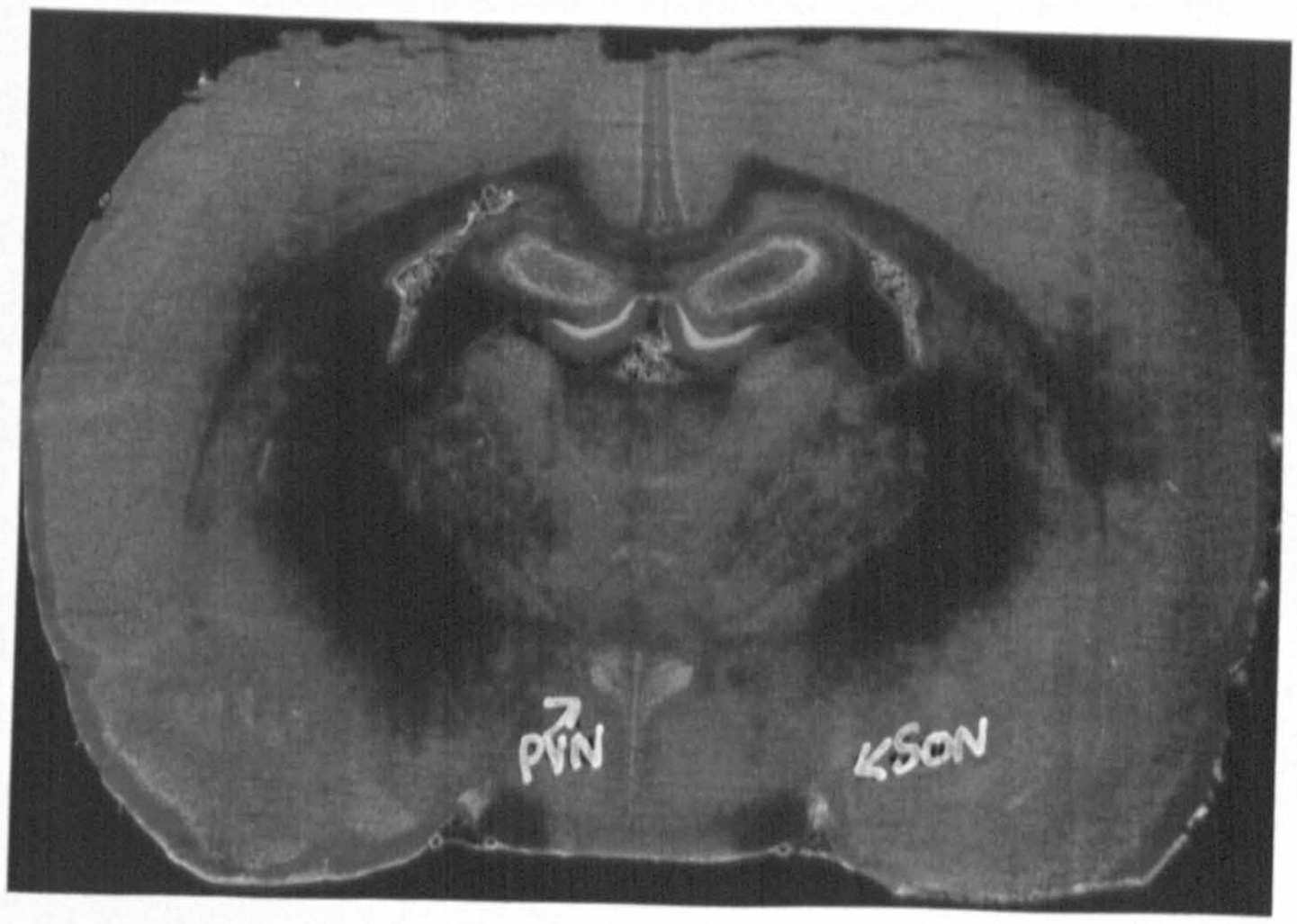
i) Substrate addition: DAB 0.25mg/ml with 2% H_2O_2, 10min 20°C.

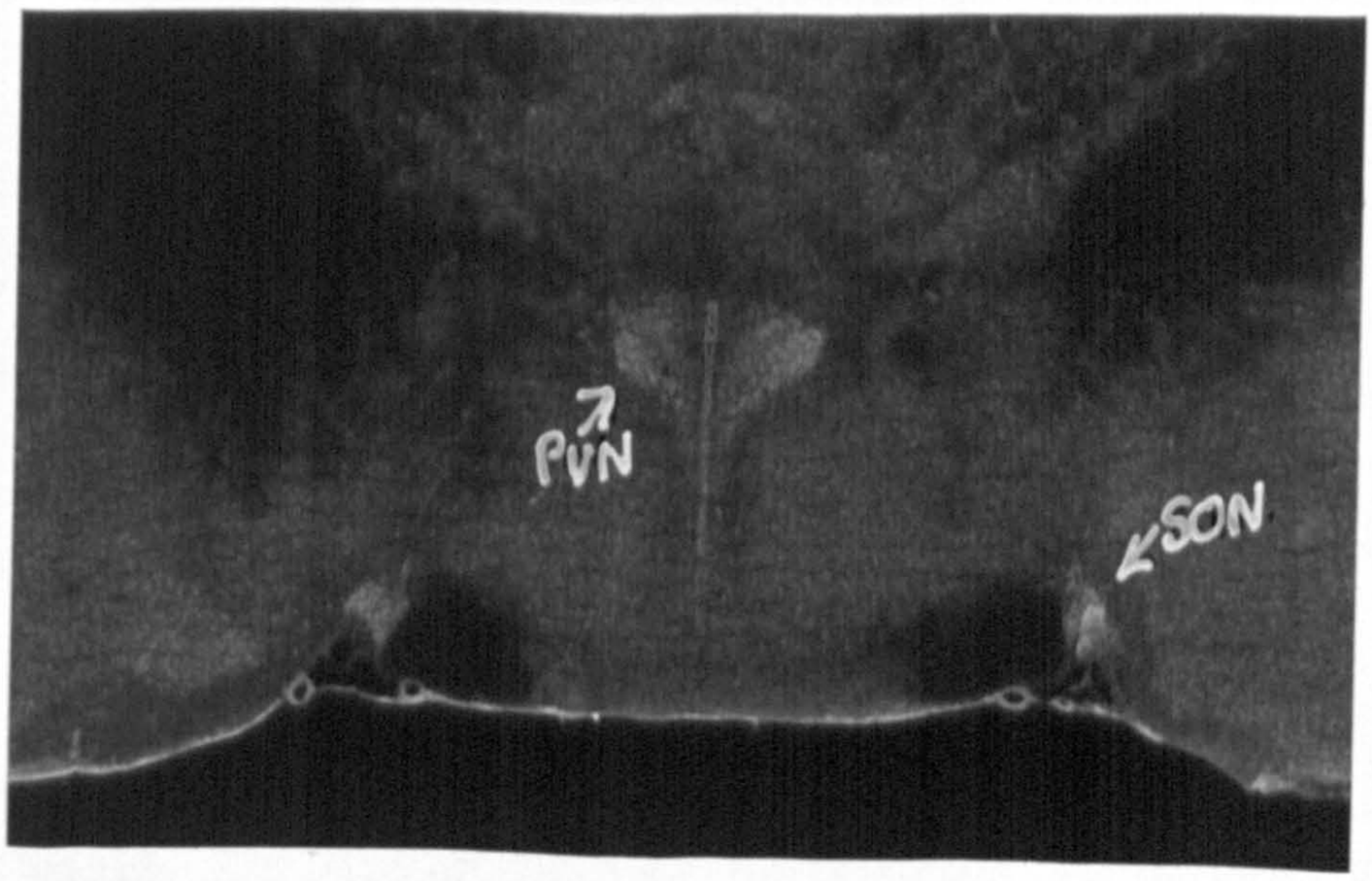
j) Wash: 10min, tap water.

Figure 6.1

Coronal sections of the rat brain to show the anatomical location of the SON and PVN. Frozen sections were thaw mounted and post-fixed in 10% formalin. Nissl substance was stained with cresyl fast violet. The stained section was used as the "negative" for the photographs.


SCN
SON


PVN
SON


PVN
SON

6.3.2.1 The experiments:

The following experiments were carried out to identify AVP-secreting magnocellular neurons the hypothalamus. Frozen tissue sections from human neurohypophysis were used as positive controls and step (c) in the protocol was omitted to generate "negative", non-specific binding, controls.

a) Comparison of ICC in tissue sections from perfusion-fixed hypothalamic blocks (1% gluteraldehyde, 1% formaldehyde, 0.8M PBS) and unfixed, frozen tissue sections.

Results

It was possible to identify AVP secreting neurons in the hypothalamus in frozen sections but the staining pattern was less intense than in perfusion fixed tissue. There was poor preservation of the quality of the unfixed frozen tissue section.

b) Comparison of post-fixation techniques on ICC in frozen tissue sections.

Methods

Absolute alcohol, absolute acetone, 2.5% gluteraldehyde in 0.2M PBS, 0.4% periodate-lysine paraformaldehyde (PLP).

Results

Acetone fixation caused complete loss of AVP antigenicity. This was perhaps not surprising since acetone is used in the extraction of AVP from plasma samples (Section 2.2). 2.5% gluteraldehyde provided best results.

c) Comparison of the effect of different antibodies and antibody dilutions on ICC in frozen tissue sections post-fixed by 2.5% gluteraldehyde.

Methods

4 different antibodies used at dilutions of 1:100, 1:500, 1:1000, 1:5000 in PBS. Incubated with tissue sections 4°C, 18hr.

Results

One antibody (A) appeared to have particular superiority of specificity density and of staining. This was optimal at a dilution of 1:1000.

d) Comparison of 1st antibody (A, 1:1000) incubation times and temperature conditions on ICC in frozen tissue sections post-fixed by 2.5% gluteraldehyde.

Methods

60min, 37°C and 18hr 4°C.

Results

Incubation for 18hr at 4°C gave slightly better staining characteristics. In a further experiment it was found that using antibody dilution of 1:200 similar results could be obtained with shorter incubation at a higher temperature.

Figure 6.2 shows a tissue section stained by ICC and counterstained with cresyl fast violet prepared by this, optimal, methodology.

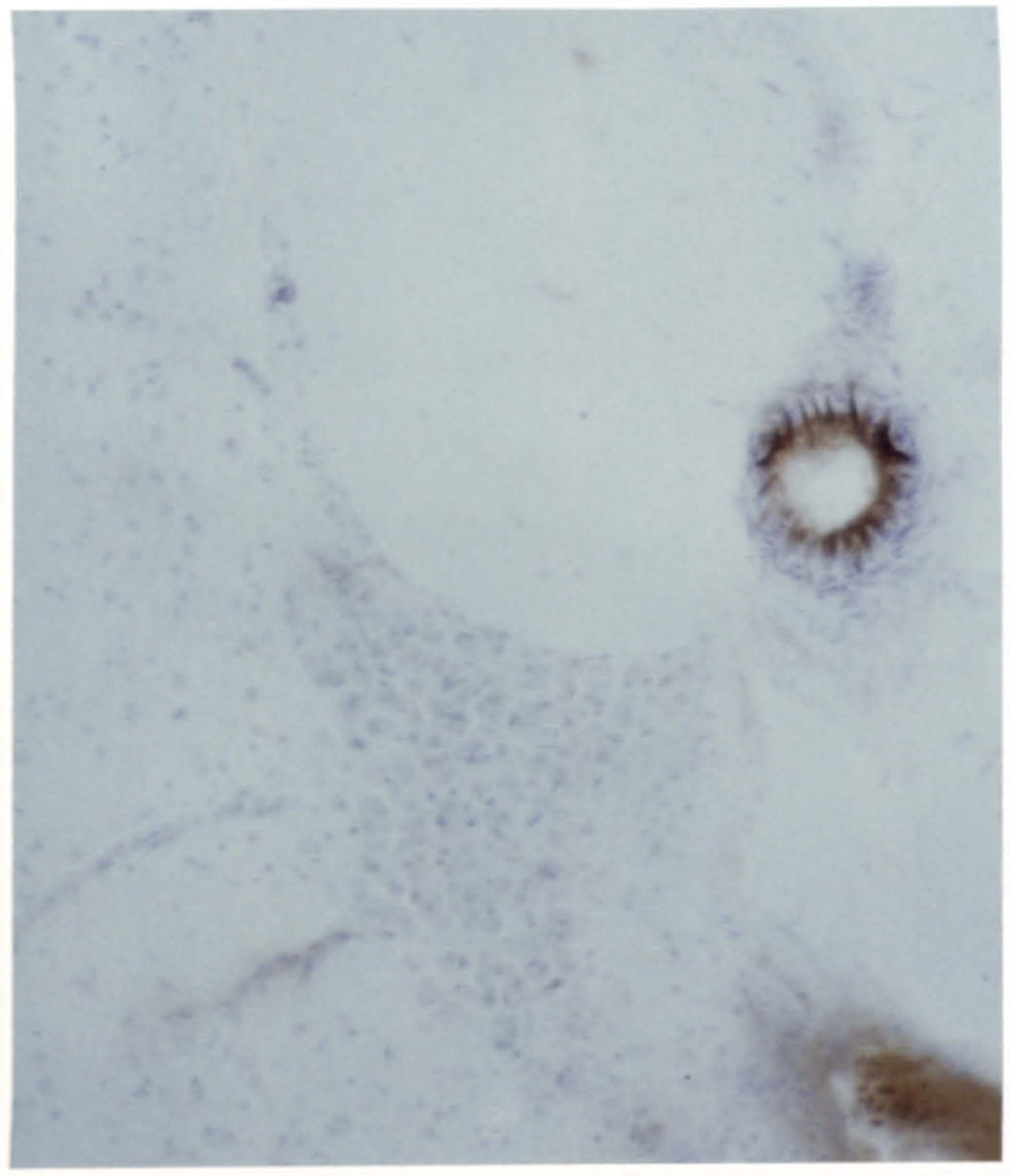
6.3.2.3 Discussion and conclusions

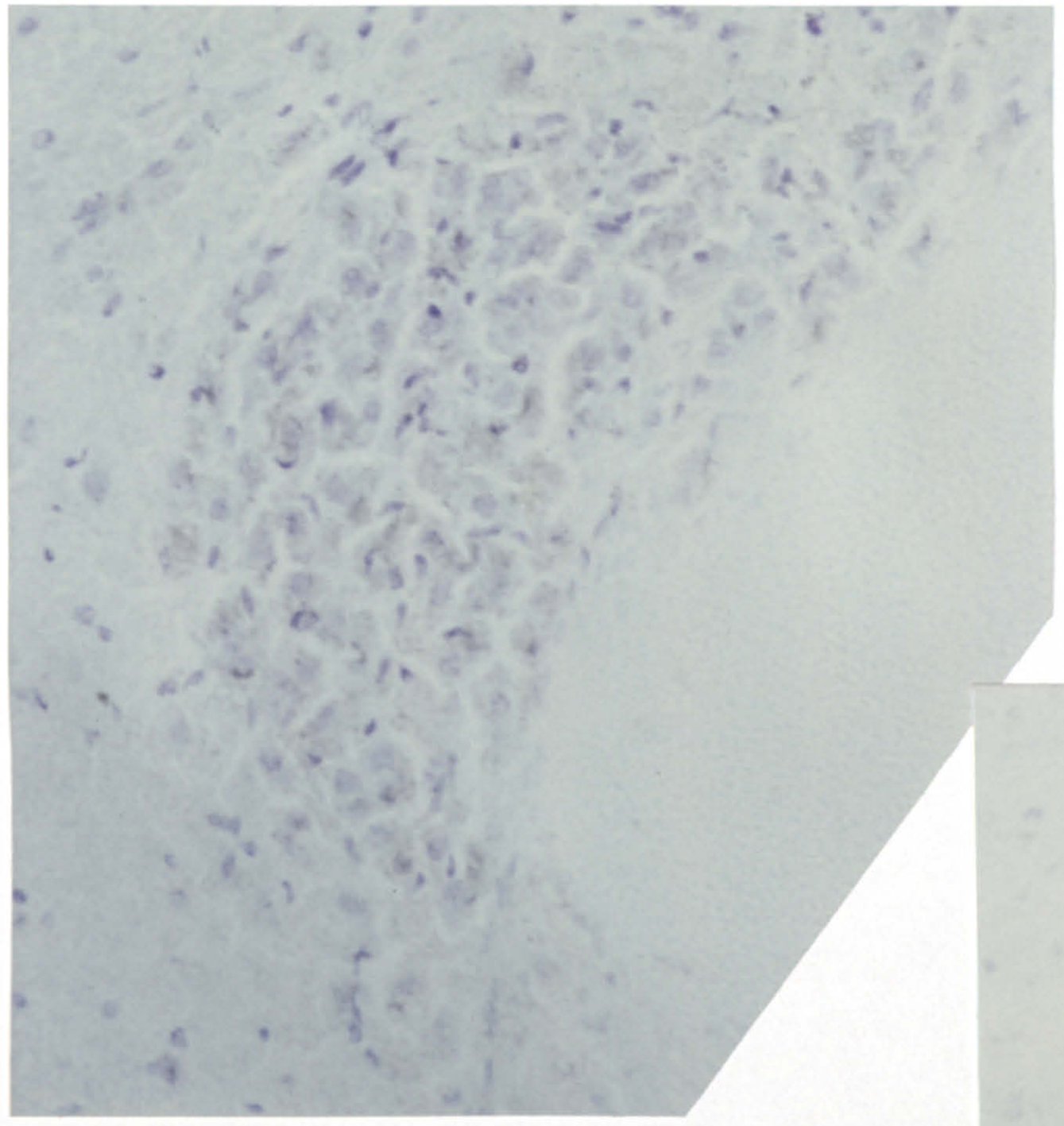
This series of experiments has shown that ICC of AVP may be performed on frozen tissue sections with post-fixation. There was, however, loss of intensity of staining pattern compared to perfusion fixation of tissue. It has been assumed that the antibodies used were specific for AVP. No purification or cross reactivity studies were undertaken and it is possible that at least some of neurons stained may be OXY-secreting. Light microscopic study of the sections suggested that there was more intense staining in the neurons and dendrites in the ventral portion of the mid SON but there was also staining of cells in the most rostral portion of the SON where, it is proposed, OXY neurons predominate.

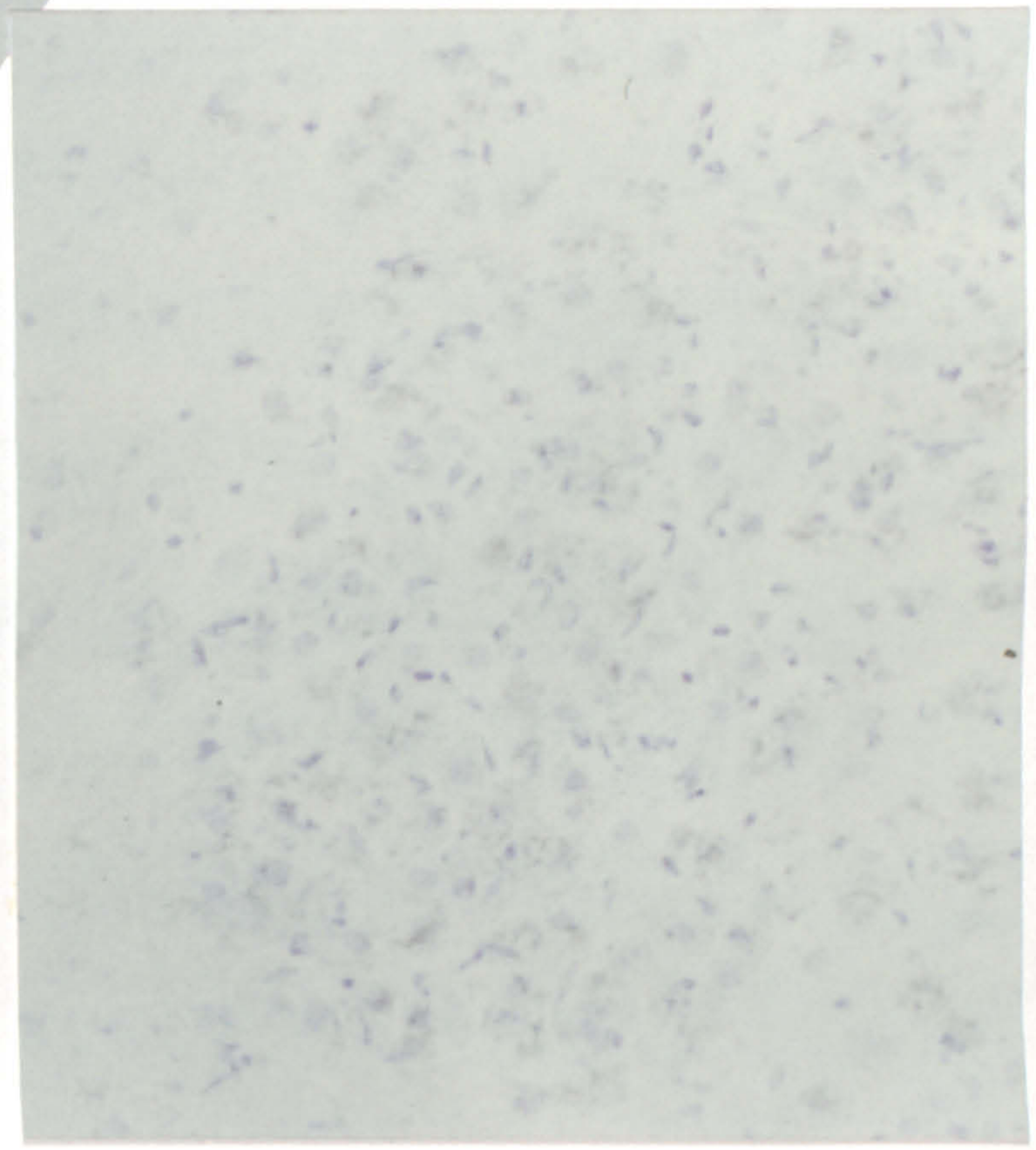
Comparison of staining of magnocellular neurons of the SON and PVN by ICC and cresyl violet suggested that ICC had no advantage over Nissl staining in anatomical identification of these nuclei since they have very distinct anatomical appearance and landmarks. ICC did perhaps yield more information about the hormone type of the individual cell but this was inconclusive without further work on antibody specificity. Since the autoradiographic image would not provide cellular resolution it was decided that Nissl staining of histology sections would be adequate anatomically to localise the nuclei for image analysis.

Figure 6.2

Coronal sections of the rat brain with immunocytochemical staining for AVP in the magnocellular neurons of (a) and (b) the SON and (c) the PVN. Thaw mounted frozen section were post fixed in 2.5% Gluteraldehyde before ICC. Sections were lightly counterstained with cresyl fast violet.







6.4 THE DENSITY OF 5HT RECEPTORS OF THE MAGNOCELLULAR NEURONS OF THE SON AND PVN

6.4.1 LIGAND BINDING

Ligand binding experiments involve reversible and saturable interaction of a specific ligand (L) with a binding site (putative receptor). If the ligand is specific for one binding site (R) then, by the law of mass action,

$$R+L \underset{K-1}{\overset{K1}{==}} RL \text{ and at equilibrium } [R][L]/[RL] = k\text{-}1/k1 = K_D$$

where [R] is the concentration of the binding site.

[L] is the concentration of the ligand.

[RL] is the concentration of the binding site-ligand complex.

K_D is the dissociation constant of the equilibrium.

In any preparation the total number of binding sites $B_{max} = [R] + [RL]$.
Therefore $[RL] = B_{max}/1+(K_D/[L])$.
Since [RL] is the amount of ligand bound (B), therefore

$$B = B_{max}/1+(K_D/[L]) \text{ or } B = B_{max}\,[L]/\,(K_D+[L])\,.$$

When an increasing concentration of a ligand is added to a preparation of binding sites an increasing number of sites are occupied. A graphic representation of the percentage of ligand bound with respect to the concentration of ligand added yields a hyperbolic curve. When $[L] = K_D$ than $B = 50\%\ B_{max}$ ie. 50% of the total binding sites are occupied.
The hyperbolic curve may be transformed to a linearity by plotting B/[L] with respect to B this derives from the Scatchard equation
$B/[L] = B_{max}/K_D - B/K_D$ (Scatchard 1949).
Representation of the data in this way allows linear regression analysis and B_{max} to be estimated from the x-axis intercept of the line and the K_D calculated from the slope of linear regression analysis. This linear relationship only holds true when there is a single population of binding sites with one affinity state. More complex transformations of ligand binding data allows identification of multiple

binding sites and affinity states and allosteric interaction of ligand binding (Cross & Owen 1986).

The introduction of a competitive ligand, at varying concentrations, displaces the specific ligand from the binding site and generates a series of parallel displacement curves which allows specific definition and description of the binding site characteristics and estimation of the equilibrium dissociation constant of the inhibitor K_i.

$$K_i = IC_{50} / (1 + [L]/K_D)$$

Where IC_{50} is the concentration of the inhibitor required to produce 50% inhibition of ligand binding.

The use of multiple competitive ligands with the same specific ligand establishes rank potency and defines the binding site pharmacologically (see Section 1.3.3).

6.4.2 AUTORADIOGRAPHY

Autoradiography is the localisation of a radiolabel within a solid specimen, by placement of the specimen against a detector material. The principle of the autoradiographic process is that the radiolabel within the specimen undergoes decay with emission of particles of radiation which, after a suitable exposure time, cause a change in the detector. The image produced relates to both the distribution and quantity of radiolabel. This has advantages over homogenate radiolabel binding since anatomical resolution is possible. Autoradiography is in addition 10^3-10^4 quantitatively more sensitive than homogenate ligand binding (Kuhar 1988).

The development of *in vitro* autoradiographic techniques has allowed application of the principles of homogenate ligand binding to specific 5HT receptor subtypes in tissue sections (Palacios & Dietl 1988; Pazos et al 1988). The radioligands commonly used for identification of 5HT receptors and receptors subtypes have been outlined in Table 1.2.3.

Initial studies dipped radiolabelled tissue sections in detector emulsion but found that the temperature and moisture of this process allowed dissociation of the ligand (Kuhar 1985). The more recent development of dry detector emulsion on sheet film has lead to more satisfactory results with the added benefit of allowing computer assisted image analysis to quantify the resulting

autoradiographic image (Palacios et al 1981).

The experimental conditions required for *in vitro* autoradiographic receptor labelling are established after biochemical studies of the characteristics of the binding of ligands to mounted tissue sections. Initially the conditions are adapted from those of membrane homogenate binding experiments and tailored to provide the best ratio of specific to non-specific binding and selectivity of binding-site labelled.

6.4.2.1 Choice of radioligand

In order to obtain a good radiographic image the ligand of choice must have a high affinity for the binding site of interest ($K_D < 10nM$). It should ideally also have pharmacological selectivity for the site of interest eg. be a 5HT1a selective ligand with low affinity for other 5HT1 receptor subtypes. These two features will ensure a high ratio of specific to non-specific binding in the resulting autoradiograph. In addition the ligand must be metabolically stable, chemically pure and have a high specific activity to detect a low density of binding sites in the tissue section.

The most commonly used radio isotopes for binding studies are ^{3}H and ^{125}I. The advantages of a tritium-labelled ligand are a very long $t_{1/2}$ (12.5yr) and the lack of disturbance of biological activity of the ligand (^{125}I-labelled ligands may significantly alter the pharmacological properties of ligands due to its much larger structure). Tritium-labelled ligands also produce good autoradiographic image resolution since they emit low energy β-particle radiation with a short track.

In general the use of a ligand at a concentration similar to its K_D for the binding site yields an autoradiograph with optimal ratio of specific to non-specific binding. From the discussion above this will achieve 50% total possible binding-site occupancy by the ligand. If labelling of the total binding site population is required (B_{max}) a concentration of ligand of approximately $10xK_D$ is required to ensure saturation. The disadvantage of this is the increase in non-specific binding.

6.4.2.2 Choice of inhibitor

The choice of competing inhibitor and the concentration at which it is used is an important consideration of autoradiography since specific binding is defined as the difference in amount of radioligand bound in the absence and presence of an excess of competing ligand. Thus best results will be obtained from an inhibitor with high affinity and selectivity for the binding site of interest. If the radioligand has high affinity for two binding sites it is useful to employ an inhibitor with high affinity for only one of these sites. The inhibitor concentration should be high enough to occupy all specific binding sites without displacement of ligand from non-specific binding sites. In practice this requires a concentration of 100-1000xIC_{50}. If the radioligand is used at the K_D concentration then the inhibitor concentration is 200-2000xK_i (see above).

6.4.2.3 Assay conditions

The conditions of autoradiography are optimised to obtain the best ratio of specific to non-specific binding. For quantitative analysis the experiment must be carried out under conditions which ensure equilibrium of binding site-ligand interactions.

a) *Tissue preincubation:*

Initial preincubation of tissue sections in ligand-free buffer is used to dissociate endogenous ligand from the binding site and to stabilise binding sites in a high affinity state. The introduction of ions to the buffer may significantly increase specific binding (Leslie et al 1988).

b) *Incubation buffer:*

Radioligand incubation with binding sites should ideally be carried out in physiological conditions. However, the presence of physiologic ions (eg Na^+) in buffers of agonist radioligands may reduce specific binding due to conformational change of the binding site and therefore a reduction in affinity. Radioligand binding assays are usually carried out at lower than physiological temperatures in order to preserve tissue quality, binding site numbers and metabolic integrity of the ligand. The rate of ligand-binding site interaction reduces with temperature such that longer incubation times are required to reach a state of equilibrium.

The volume of incubation buffer is important since equilibrium assumes that the

concentration of bound ligand is very small compared to the concentration of free ligand. This is particularly important in experiments where ligand is placed over the tissue section rather than the tissue section dipped into a trough of ligand. In such "drop assay" experiments there may be significant ligand depletion if the volume "dropped" is insufficient. This can be estimated and checked by measurement of ligand concentration at the start and end of the incubation time.

c) *Incubation time:*

Initial kinetic experiments should be undertaken to determine the time course of binding site-ligand interaction and the establishment of equilibrium. While specific binding achieves a plateau at equilibrium, non-specific binding continues to increase with time. The optimal incubation period is defined as the minimum time required to reach steady-state binding conditions. While shorter incubation periods may increase the ratio of specific to non-specific binding the quantitative analysis of the resultant autoradiographs is not valid.

d) *Washout conditions:*

Following incubation tissue sections are rinsed to dissociate loosely bound radioligand and to optimise specific to non-specific binding ratios. Since radioligand dissociation rates are inversely related to binding site affinity, non-specific, low-affinity binding dissociates more rapidly than high-affinity, specific binding. The rate of dissociation of specifically bound ligand is diminished by temperature.

6.4.2.4. Standards and controls

The main difficulty in quantification of autoradiographs is the non-linear relationship between autoradiographic grain density in the emulsion and the tissue content of radioactivity ie. binding site density. Radioactive standards are exposed to the detector emulsion together with the radiolabelled tissue sections in order to derive a mathematical relationship between grain density and radioactivity. The exposure time of radiolabelled tissue sections must be such that the change in grain density produced by the tissue section image lie within the linear range derived from the radioactive standards. This can be predicted to some extent from the specific activity of the ligand and approximate density of

binding sites, but test autoradiographs are employed to ensure correct exposure duration.

Inter and intra-assay tissue section controls, for both total and non-specific binding, may be used as a measure of experimental variability.

For the purpose of this research 5HT1a and 5HT2 receptors were localised and quantified by adaptation of the method of Pazos et al (1985a; 1988) and Pazos & Palacios (1985). Some initial experiments were performed with variation in wash characteristics and competitive inhibitor type and concentration to optimise the ratio of specific:non-specific binding by comparison of density of ligand binding in cortex, caudate and hypothalamus.

6.4.2.5 The protocols of optimal autoradiography conditions

	5HT1a	5HT2
Ligand	[^{3}H]DPAT	[^{3}H]ketanserin
Buffer	A	B
Inhibitor	1μM 5HT	1μM methysergide
Preincubation	30min 20°C	15min 20°C
Incubation	60min 20°C	120min 20°C
Wash	1x15min 4°C	2x15min 4°C
		tap water dip 4°C
Dry	Fan assisted 1hr 20°C	

Ligands

5HT1a: The potent 5HT agonist 8-hydroxy-n-n-dipropyl-2-aminotetralin (8OHDPAT) (Arvidsson et al 1981) has been shown to be a selective ligand with high affinity for the 5HT1a receptor subtype (K_D=0.4-4nM depending on assay conditions) (Middlemiss & Fozard 1983; Gozlan et al 1983). The tritiated ligand [^{3}H]DPAT is well established as a useful ligand for 5HT1a autoradiography (Marcinkiewicz et al 1984; Nelson 1988). [^{3}H]DPAT was obtained from Amersham. Specific activity was 240ci/mmol.

5HT2: The 5HT antagonist ketanserin (Leysen et al 1981) has a high affinity for 5HT2 receptor sites (K_D=0.4-3nM in rat frontal cortex). It also has affinity for the 5HT1c site although this is lower than that for the 5HT2 site (Hoyer

1988). The tritiated ligand [^{3}H]ketanserin is well established for use in the autoradiography of 5HT2 receptors although there is high non-specific binding (20%) and approximately 10% binding to α1-adrenergic receptors (Leysen et al 1982). [^{3}H]ketanserin was obtained from NEN. Specific activity was 60ci/mmol.

Inhibitors

5HT1a: 5HT has a high affinity for the 5HT1a receptor and has been shown to reduce [^{3}H]DPAT specific binding by 80-85% at a 1μM concentration, yielding a specific:non-specific binding ratio of at least 8:1 (Marcinkiewicz et al 1984). 5HT was obtained from Research Biochem. Inc.

5HT2: Methysergide has a high affinity for the 5HT2 receptor but, unlike ketanserin, lacks affinity for the α1-adrenergic receptor (Leysen et al 1981). It has a high affinity for the 5HT1c and other 5HT1 receptor subtypes (see Table 1.3.4) (Leysen et al 1982).. Specific binding of [^{3}H]ketanserin is reduced by 80% at a 1μM concentration (Leysen et al 1982). Methysergide was obtained from Sandoz.

Buffers

5HT1a: Buffer A (Appendix 4). 0.17M Tris, pH7.6 with 4mM $CaCl_2$, 0.01% ascorbic acid, 10μM pargyline. $CaCl_2$ enhances specific binding whilst ascorbic acid prevents degradation of 5HT. Pargyline, a SSRI, prevents uptake of the competitive inhibitor, 5HT, into the tissue neurons.

5HT2: Buffer B (Appendix 4). 0.05M Tris, pH7.7. There are no specific requirements of the ligand or competitive inhibitor for this receptor type.

6.4.3 THE DENSITY OF 5HT RECEPTORS IN THE SON AND PVN OF THE NORMAL RAT

Methods

Serial 20μm sections were taken through the SON and PVN of six normal rat brain hypothalamic blocks. Adjacent sections in the mid rostro-caudal portion of each nucleus taken in triplicate and thaw mounted alternately for specific and non-specific binding. Histology sections were also collected from each nucleus.

20μm tissue standards for inter and intra-assay comparisons were cut from a block of normal rat frontal cortex.

Sections and tissue standards were processed as described above. Incubation with ligand was carried out using the "drop-assay" technique with 1ml buffer volume per slide. Tissue sections and radioactive standards ([^{3}H]microstandards, Amersham) were exposed to tritium-sensitive film (Hyperfilm, Amersham) for 26day, 4°C.

The latent image on the detector film was developed by the reduction agent D19 (Kodak) and subsequent fixation (Unifix, Kodak) and hardening (Kodak) of the gelatin film. The autoradiographs were analysed using a Magiscan 2A image analysis system (Joyce Loebl) according to the method of Bell et al (1987). A "best-fit" curve for changes in grain density was calculated from the image of the radioactive standards by polynomial regression analysis according to the equation $y=(a+cx)/(1+bx+dx^n2)$. Results are expressed as fmol/mg tissue.

Average background grain density change was subtracted from each image. The SON and PVN were identified by comparison of image landmarks with the light microscopic view of stained histological sections. Triplicate readings of grain density change in each nucleus were taken in the total and non-specific binding tissue sections and mean values subtracted to calculate average specific binding.

Results

Figures 6.3 and 6.4 show the specific binding of [^{3}H]ketanserin and[^{3}H]DPAT in the SON of five and the PVN of the six rat brains. The mean values are summarised in Table 6.1. There was a low density of 5HT2 receptors in both the SON and PVN. The 5HT1a receptors appear to be even less numerous with a slight preponderance in the SON. Example autoradiographs are shown in Figure 6.5.

6.4.4 THE DENSITY OF 5HT RECEPTORS IN THE SON AND PVN OF RATS WITH SHAM OR 5,7-DHT LESION OF THE DORSAL AND MEDIAN RAPHÉ NUCLEI

Lesion of 5HT neurons by the selective neurotoxin 5,7-DHT has been reported to have variable effects on 5HT receptor subtypes. Some of these studies have

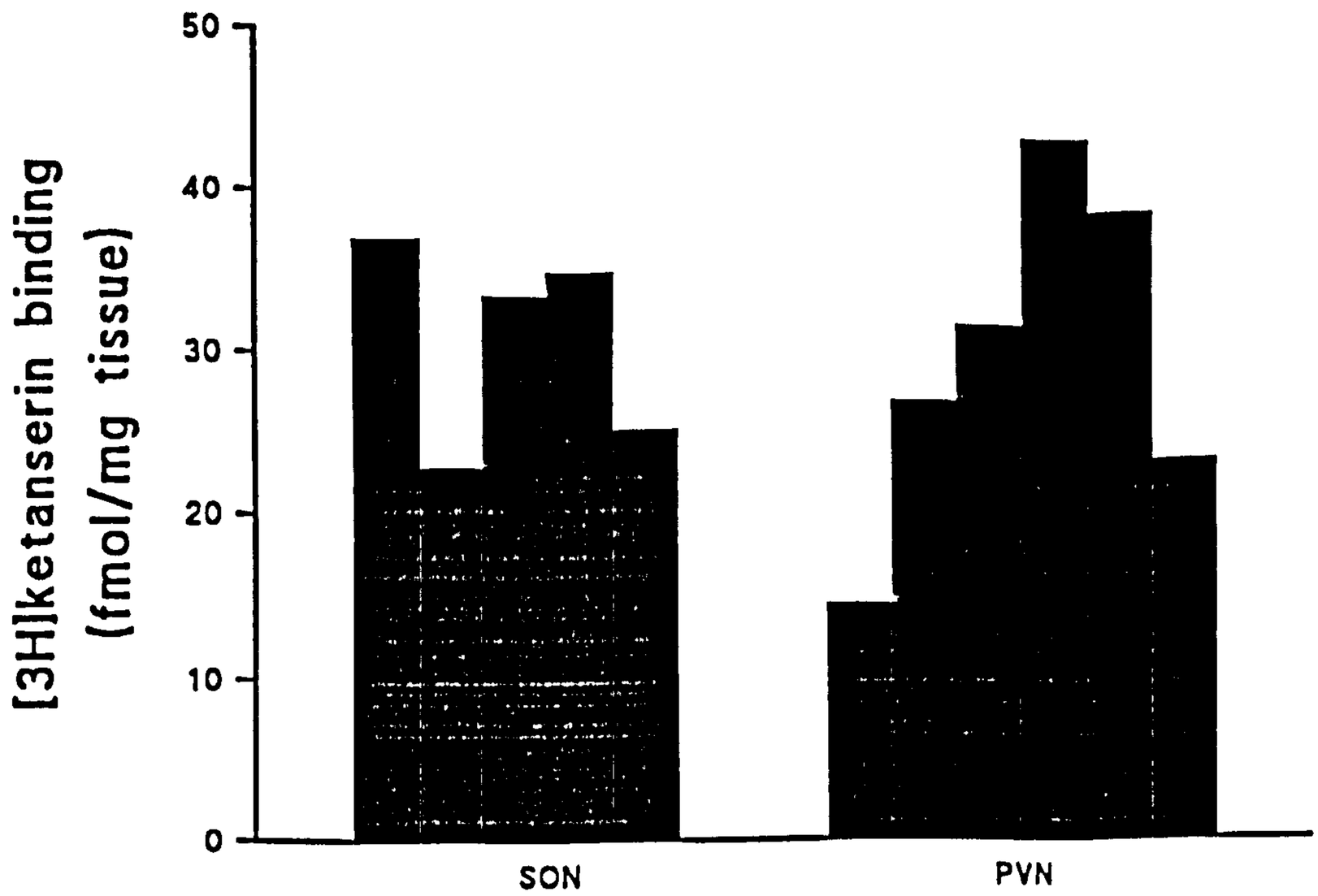


Figure 6.3

The specific binding of [^{3}H]ketanserin to 5HT2 receptors in the SON of five and the PVN of six normal rat brains.

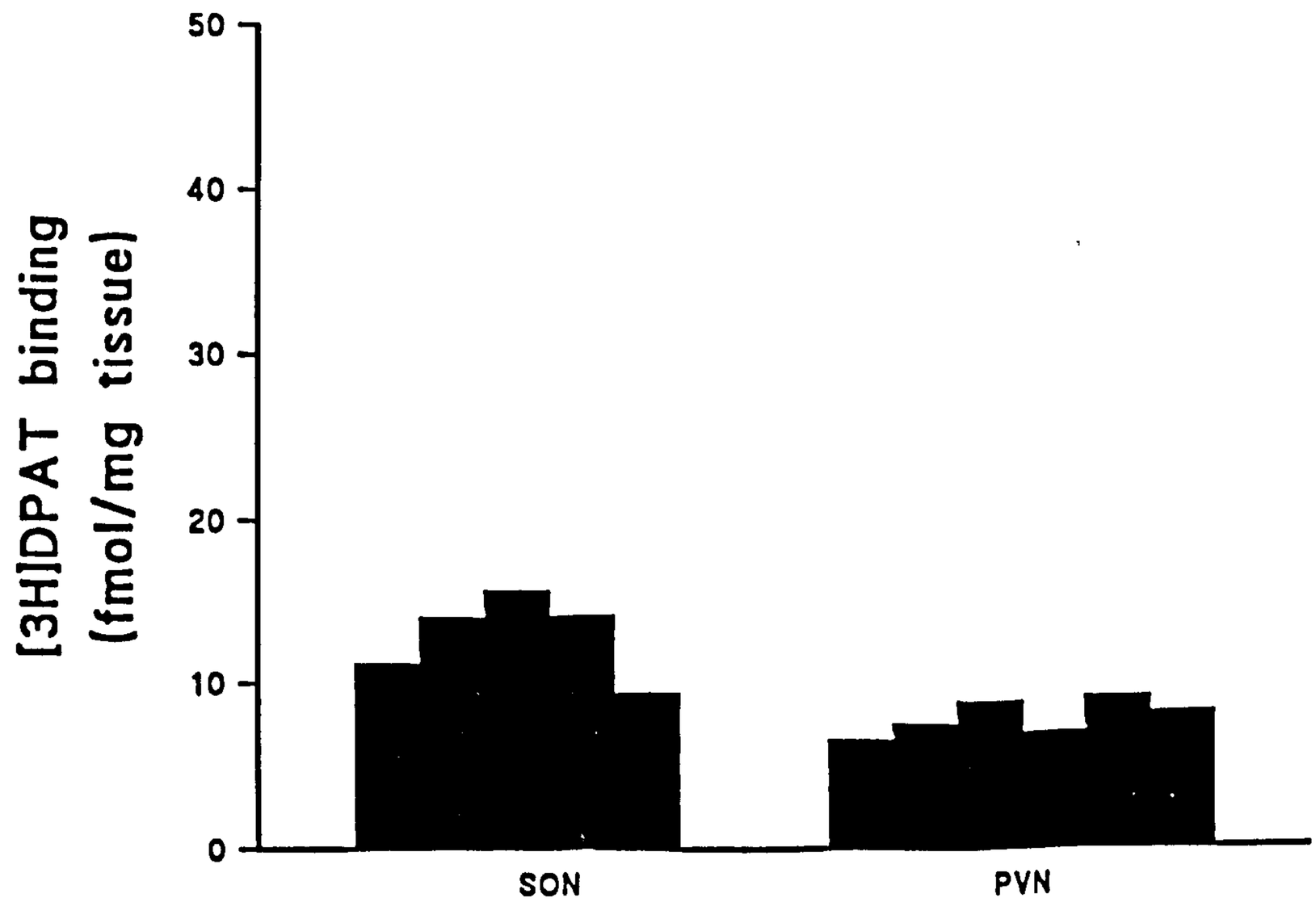


Figure 6.4

The specific binding of [^{3}H]DPAT to 5HT1a receptors in the SON of five and the PVN of six normal rat brains.

Table 6.1 The density of [3H] Ketanserin and [3H] DPAT binding in the SON and PVN of normal rats (mean±SEM)

	[3H]Ketanserin (fmol/mg) tissue	[3H] DPAT (fmol/mg) tissue
SON	30.6±2.8	12.9±1.1
PVN	29.5±4.2	7.9±0.4
Frontal section (control)		
High density area	210.0±12.0	30.2±2.9
Low density area	43.6±4.3	11.2±1.9

Figure 6.5

Examples of autoradiographic images of ligand binding to coronal sections of normal Wistar rat brains. The autoradiograph was used as the "negative" for the photograph.

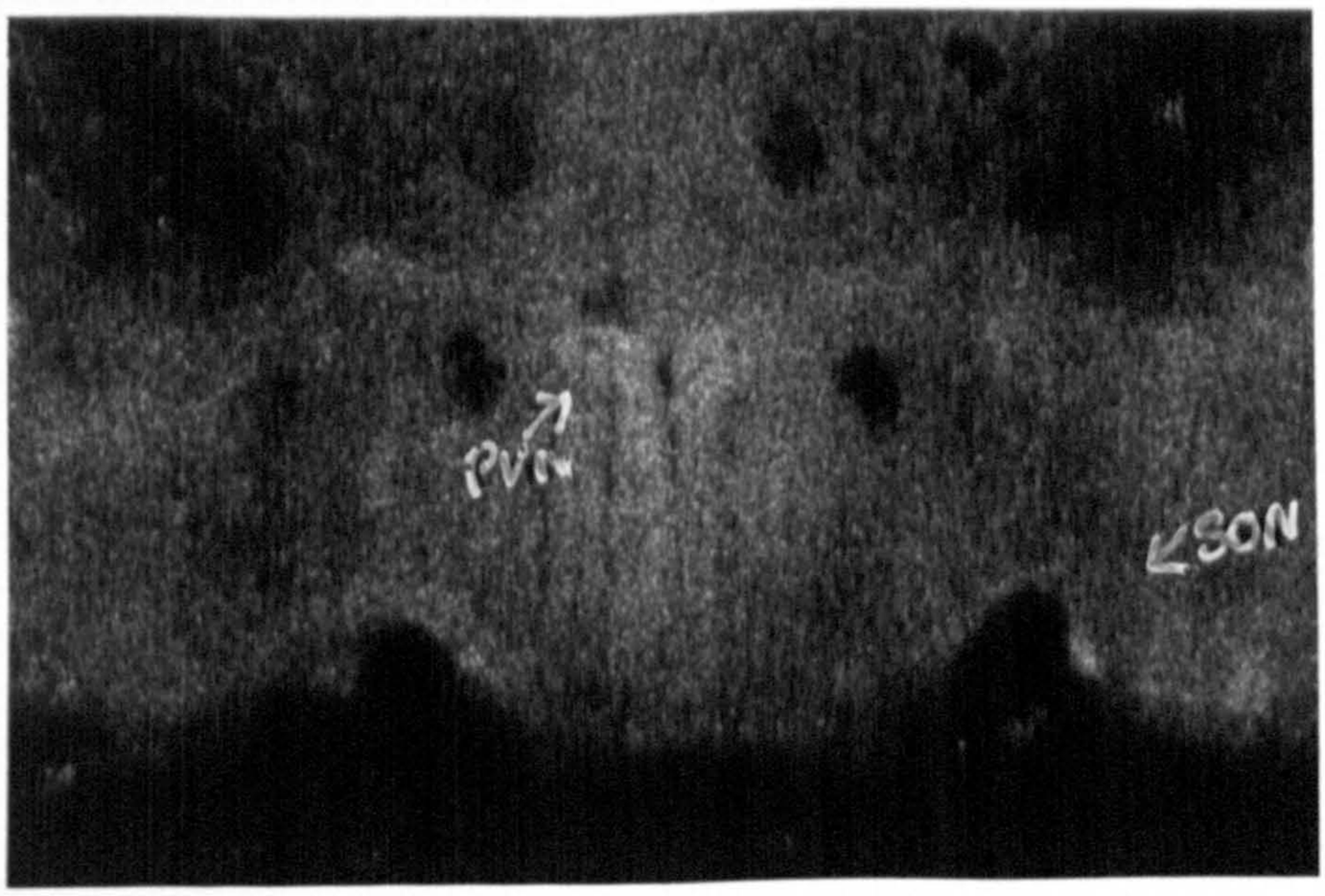
(a) Total [^{3}H]ketanserin binding to a tissue section containing the SON and PVN.

(b) Enlarged image of the hypothalamus of the same section showing the SON and PVN.

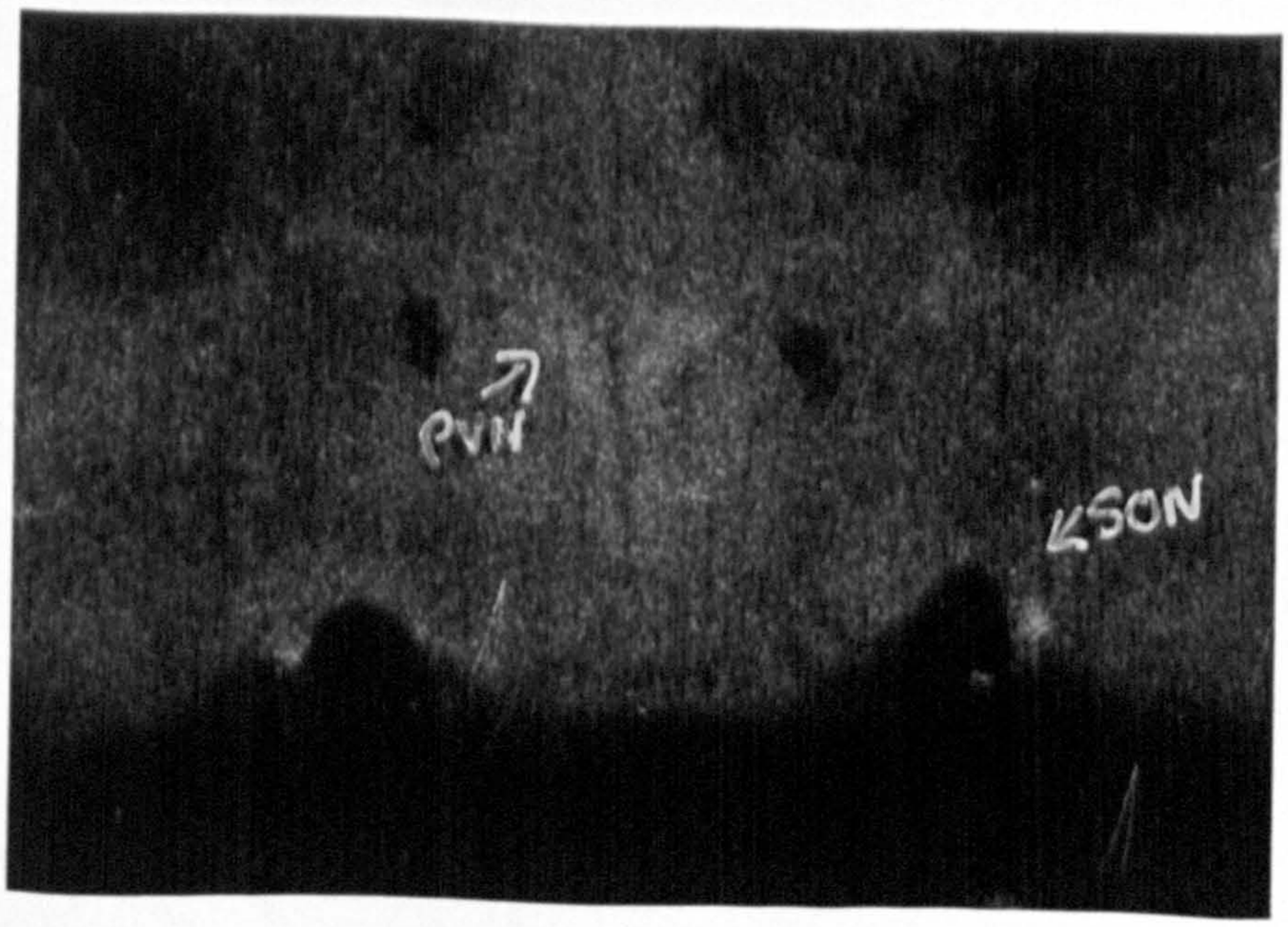
(c) The non-specific binding of [^{3}H]ketanserin in the presence of methysergide to an adjacent tissue section.

(d) The total and (e) non-specific binding of [^{3}H]DPAT in sections of the hypothalamus adjacent to (a).

(a)

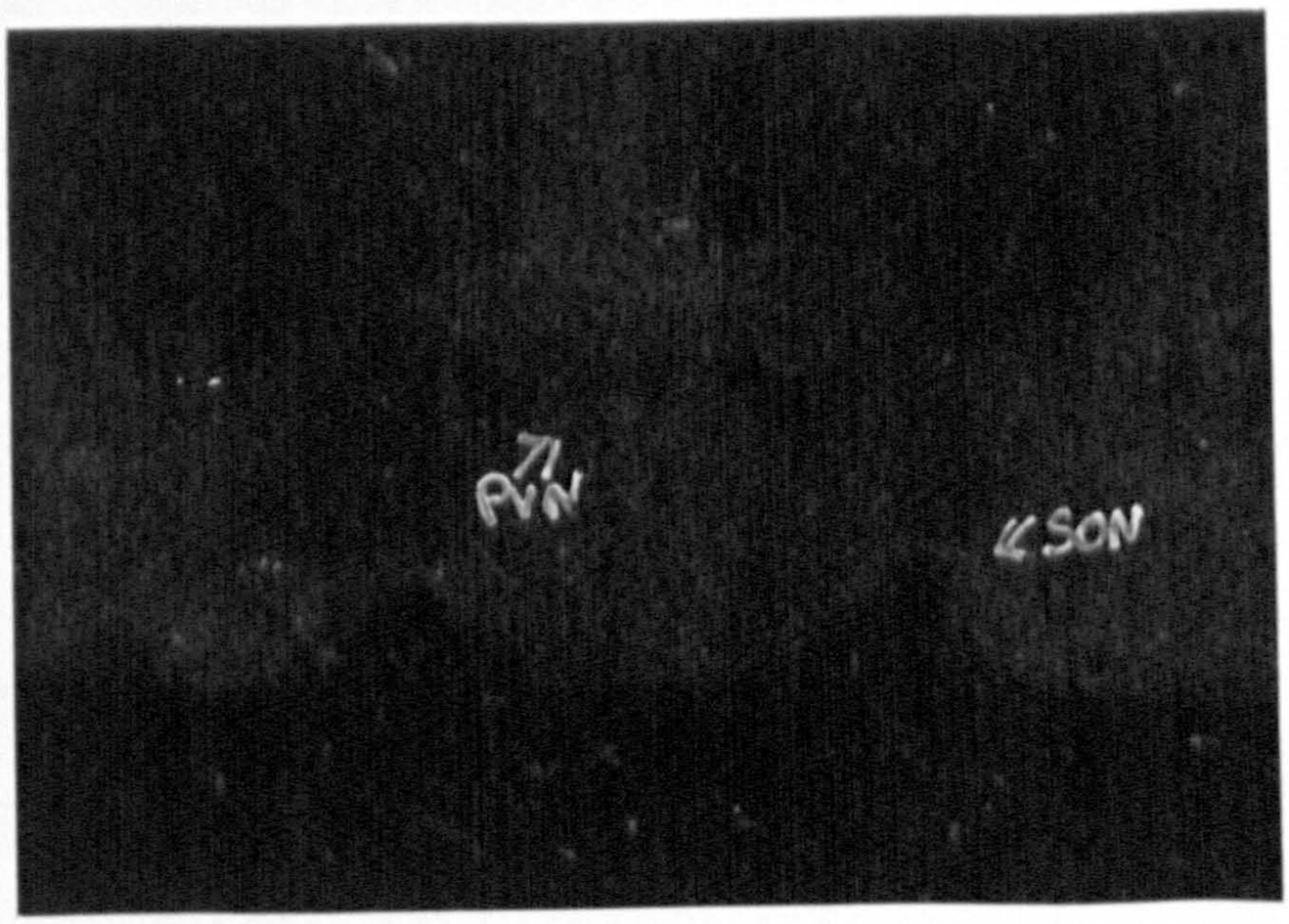


(b)



(c)

(d)



(e)

been discussed in Section 1.3.3.1.

The differences in observation are perhaps dependent on the receptor subtype and brain region examined and the duration of post-lesion receptor modification time.

The purpose of this study was to examine the effect of loss of presynaptic 5HT neurons from the raphé nuclei, on the 5HT receptor density in the SON and PVN.

GENERAL HYPOTHESES

1. That receptors for 5HT are present on magnocellular neurons in the SON and PVN.

2. That these receptors may be increased in density due to up-regulation after lesion of serotonergic neuronal cell bodies in the raphé nuclei.

SPECIFIC AIMS

To study the change in 5HT1a and 5HT2 receptor density in the SON and PVN after bilateral ablation of serotonergic neurons from the dorsal and medial raphé nuclei.

Methods

Hypothalamic blocks and tissue sections were prepared from rats with specific and sham lesion of the dorsal and median raphé nuclei previously described in Section 5.6. The density of 5HT1a and 5HT2 receptors was determined autoradiographically as described in Study 6.4.3.

Results

Figure 6.6 shows the density of [^{3}H]ketanserin binding in the nuclei of rats with lesion of the 5HT neurons of the raphé nuclei together with sham lesioned rats and the "normal" rats of Study 6.4.3. Figure 6.7 shows the mean density of [^{3}H]DPAT binding for the same rat groups.

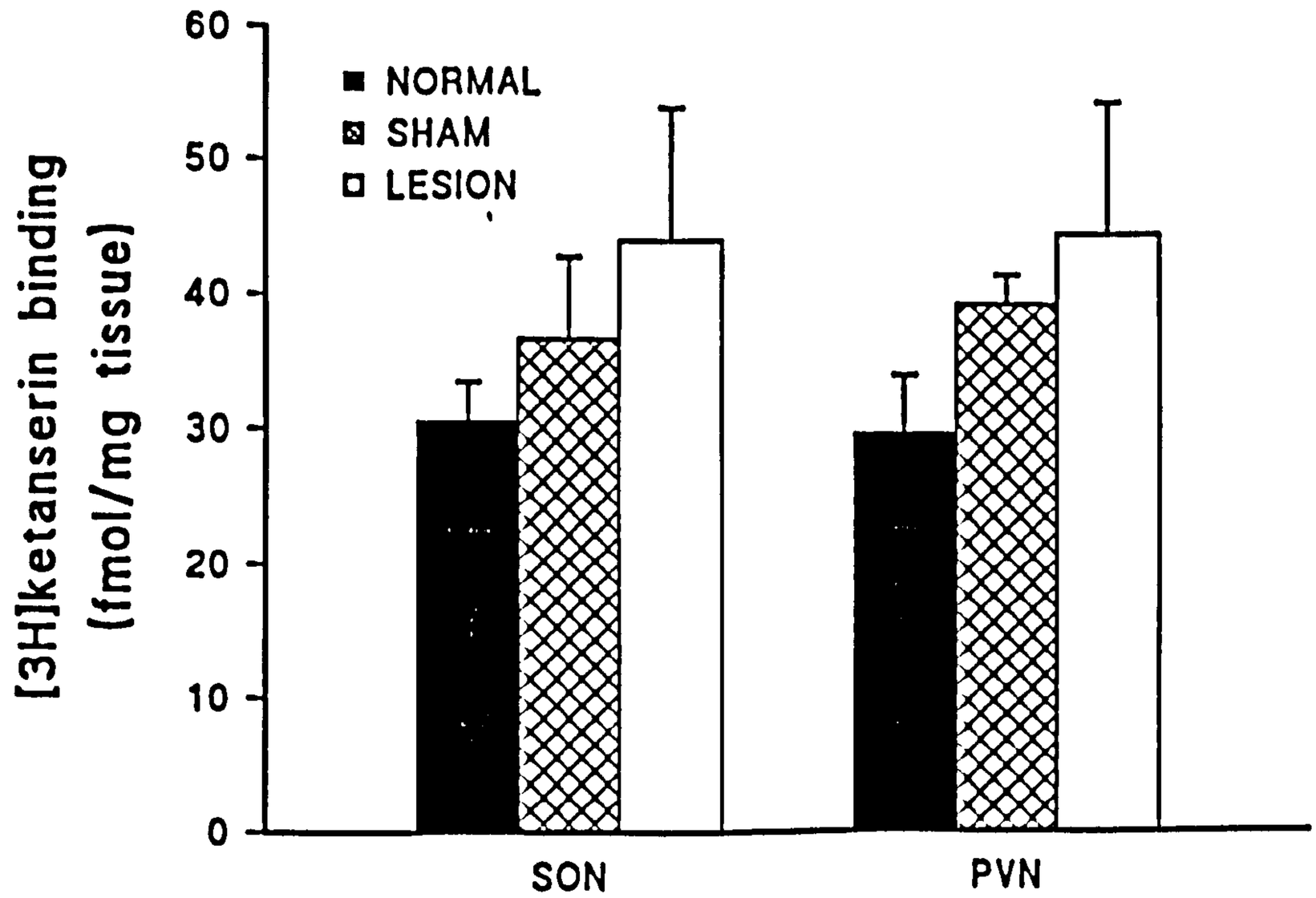


Figure 6.6

The specific binding of [^{3}H]ketanserin to 5HT2 receptors in the SON the PVN of normal Wistar (■) and SHAM (▨) and 5,7-DHT LESION (□) rat brains.
There was no significant differences between the SHAM and LESION groups when compared by the Mann Whitney U Test ($P > 0.05$).

Results are presented as mean ± SEM.

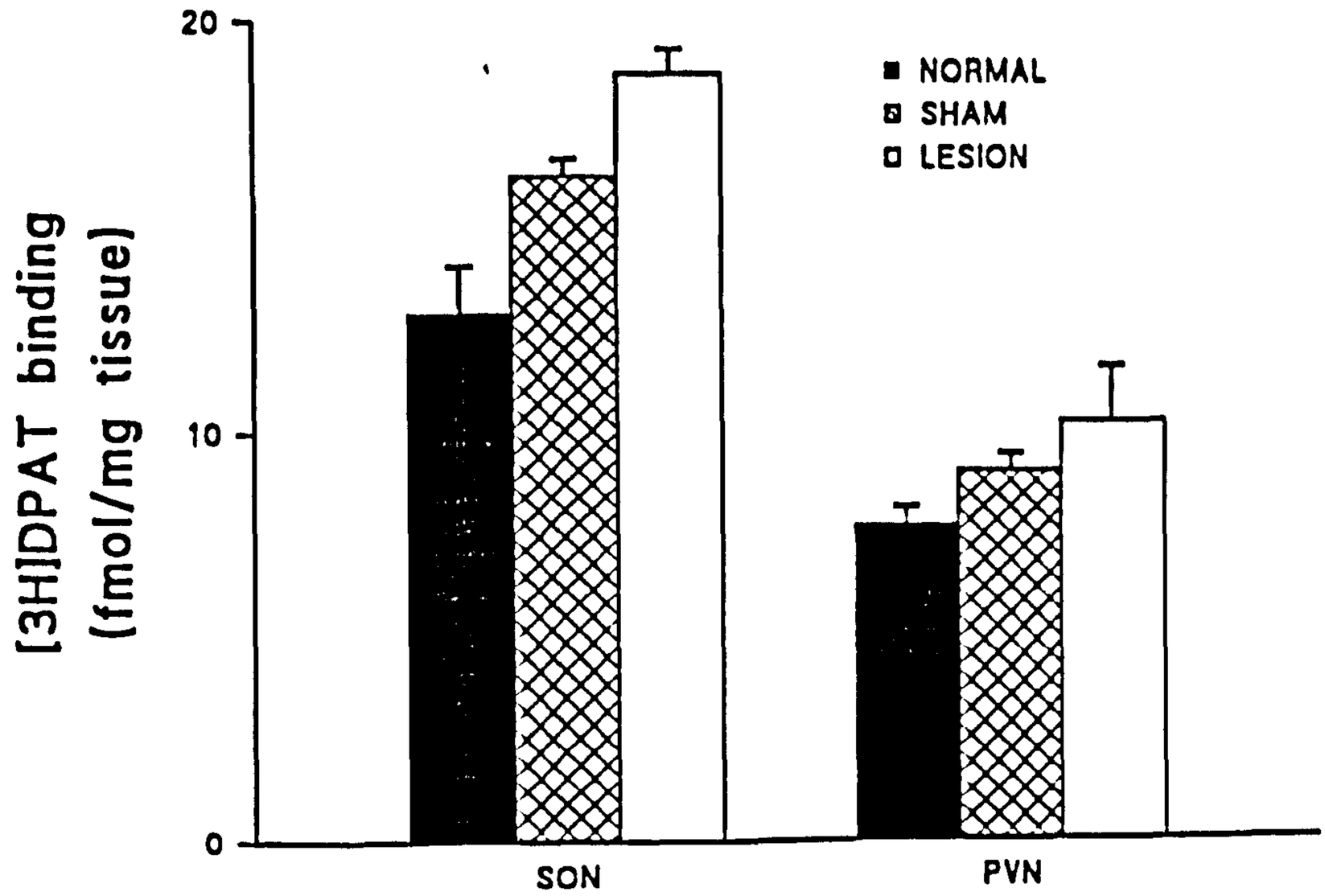


Figure 6.7

The specific binding of [^{3}H]DPAT to 5HT1a receptors in the SON the PVN of normal Wistar (■) and SHAM (▨) and 5,7-DHT LESION (▢) rat brains.
There was no significant differences in binding between the SHAM and LESION groups in either the SON or PVN when results were compared by the Mann Whitney U Test ($P > 0.05$).

Results were compared by the Mann Witney U Test and showed no significant differences between 5HT lesioned and sham lesioned rats and sham lesioned (hooded Wistar) and "normal" rats (albino Wistar).

6.4.5 DISCUSSION

The receptor binding studies suggest that the 5HT2 receptor was present, in low density, in both the SON and PVN and that the density was similar in both nuclei. 5HT1a receptors were also present but at a very low density. This receptor subtype was more numerous in the SON than the PVN. There was no evidence to suggest that either receptor subtype increased (up-regulated) in density consequent to loss of 5HT pre-synaptic neurons.

The localisation and quantification of receptors by autoradiography is fraught with potential for experimental error, of which some have been discussed above. The autoradiography of 5HT receptors in the SON and PVN has, in addition, particular problems. The location of the SON, adjacent to the optic chiasm at the base of the brain, makes it particularly vulnerable to damage during tissue sectioning and autoradiographic processing. This was noted to lead to both decreased and increased ligand binding dependent on the nature of the tissue damage. The anatomical localisation of the SON and PVN on the autoradiographic image was difficult. Images were magnified (16x) for analysis. Further magnification was limited by the resolution of the hyperfilm (approximately 150μm) and the Magiscan system. It is possible to increase anatomic detail by using a high resolution autoradiographic technique, such as detector dipped cover-slip application to the ligand labelled tissue section. Using this method it would perhaps be possible to discretely identify the 5HT receptors for the OXY and AVP secreting neurons of the SON and PVN. The use of other autoradiographic techniques such as ^{125}I labelled ligand or *ex vivo* autoradiography might also aid anatomical resolution and improve the accuracy of quantification of such low receptor density.

Incorporation of tissue standards allowed an estimate of intra-assay variability. There was no inter-assay variation since ligand binding was carried out on tissue from all rat groups in one experiment. For [^{3}H]ketanserin binding @200fmol/mg tissue cv14%, @40fmol/mg tissue cv24% and for[^{3}H]DPAT

binding @30fmol/mg tissue cv23%, @10fmol/mg tissue cv42%. There was therefore a high degree of intra-assay variation at the low density of receptors present in the SON and PVN. It was unlikely that receptor density was overestimated since the binding density was similar to the general "low density binding" of Pazos & Palacios (1985) and Pazos et al (1985a), and measurement of ligand binding in cortical areas of was similar to those found by others. It is possible that the receptor density is lower than that suggested. This is particularly so for 5HT1a binding since there is a high percentage of error at such low binding densities.

If 5HT receptors are present in low density and do not up-regulate consequent to the loss of pre-synaptic 5HT neurons this could mean that 5HT is not important, at least directly, in the regulation of AVP secretion from the magnocellular neurons. However research has shown that occasionally there is a "mis-match" of receptor density and functional importance (Kuhar 1985b) and that lesion of 5HT neurons has been found to lead to functional receptor supersensitivity without change in receptor density (Section 1.3.4). This could be explained by differences in affinity state of receptor subtype but there is, as yet, little understanding of these paradoxes.

The data presented in Section 5.6 has suggested that osmotic homeostasis continues despite lesion of 5HT neurons of the raphé nuclei. The lack of significant changes in 5HT receptor density in the SON and PVN would be compatible with this finding.

These studies have essentially provided very preliminary evidence that 5HT is not important as a direct modulator of magnocellular AVP secreting neurons. Different techniques of receptor identification with greater anatomical resolution are required fully to disprove this hypothesis.

SECTION 7

GENERAL DISCUSSION

AND

CONCLUSIONS

The results of this research have already been discussed in considerable detail in each Section together with some criticism of experimental techniques and methodological sources of error. This discussion brings together the major conclusions in the light of the hypotheses and aims of the research set out in Section 1.7 and puts forward proposals for future work in the area. The major objective of the research was to explore the hypothesis that 5HT modulates osmoregulated AVP secretion.

The first aim of this work was to study the effect of pharmacological manipulation of central 5HT neurotransmission on osmoregulated AVP secretion in normal man. The two studies in Section 3 found no evidence that 7 days treatment with the SSRI, Fluoxetine, significantly altered either basal or dynamically assessed osmoregulated AVP secretion. There are three possible interpretations for this negative finding. Firstly that 5HT does not have a role in osmoregulated AVP secretion. Secondly that Fluoxetine did not increase synaptic 5HT in these normal subjects. Thirdly that 5HT does modulate osmoregulated AVP secretion but this was not apparent after 7 days treatment with Fluoxetine. These hypotheses have been discussed in Section 3.3 and it has been postulated that 5HT may acutely increase AVP secretion but that rapid adaptation occurs to re-establish osmotic homeostasis.

To explore further the putative role of 5HT in osmoregulated AVP secretion in man it would be important to study the effect of very acute increase in 5HT neurotransmission. This would require intravenous administration of a 5HT active agent. There are two such drugs presently available, L-tryptophan and clomipramine, a tricyclic antidepressant with inhibitory activity at the 5HT reuptake site. Unfortunately L-tryptophan has recently been associated with the eosinophilic myalgic syndrome and, although this was probably attributable to a constituent of the oral medication, it would be unethical to use this for research purposes at present. A major problem of using clomipramine, and many other agents which increase 5HT neurotransmission, is the marked nausea inducing effect. This would undoubtedly lead to abnormal osmoregulated AVP secretion (Section 1.1.2.3). It is difficult therefore, to see how progress can be made in the further study of 5HT and osmoregulated AVP secretion in man. It is

possible that agonist and antagonist drugs of the various 5HT receptor subtypes may provide some answers. Investigation of the effect of more prolonged administration of antidepressant or other 5HT active drugs may also prove interesting since, as discussed in Section 1.5.3.2, such drugs initially increase 5HT neurotransmission with a subsequent period of autoregulatory compensation followed by a recurrence of a net increase in 5HT neurotransmission after about 14 days of continued administration.

The modulatory effect of 5HT on osmoregulated AVP secretion was more extensively explored in rats by pharmacological manipulation of 5HT neurotransmission. The studies in Section 5 have shown that Fluoxetine, when administered acutely, significantly altered osmoregulated AVP secretion. The analysis of the data by piece-wise regression has suggested that there was an increase in basal secretion and an increase in the osmotic sensitivity of AVP secretion above the osmotic threshold. The osmotic threshold itself was not apparently altered by acute Fluoxetine treatment. This pattern of alteration of AVP secretion was similar to that described in type C SIAD, thought to be due to loss of inhibitory stimuli to AVP secreting magnocellular neurons (Section 1.2.2.3). Chronic administration of Fluoxetine for 21 days allowed basal secretion to return to normal but significantly decreased the sensitivity of AVP secretion to change in plasma osmolality. The evidence presented in Study 5.2.1 might suggest that this effect was lost as early as 18hr after one dose. The possible reasons for this have been discussed in Sections 5.2.5 and 5.8. If it might be assumed that the effect was mediated through alteration of 5HT function the chronological change in effect is in keeping with the ideas put forward in Section 3.3, that 5HT is involved in osmoregulated AVP secretion but that the two systems can adjust very rapidly to stimulation of 5HT neurotransmission achieving a new equilibrium and re-establishing osmotic homeostasis. Nelson et al (1987) found similar "fatigue" of the inhibition of AVP secretion by the 5HT antagonist, Ritanserin (Section 5.3).

The Studies in rats were unable to identify the 5HT receptor subtypes involved in Fluoxetine-modulated osmoregulated AVP secretion. The results of Sections 5.3, 5.4 and 5.5 would suggest that the 5HT2 and 5HT1c receptors were not important in the effect since Ritanserin did not inhibit the Fluoxetine-augmented

AVP response to plasma hyperosmolality and DOI had no significant effect on osmoregulated AVP secretion. This might indicate that the mechanism of Fluoxetine stimulated AVP secretion was non-serotonergic. It has previously been shown that the stimulatory effect of two 5HT agonists on AVP secretion was inhibited by prior treatment of the animals with the 5HT synthesis inhibitor pCPA (Iovino & Steardo 1985). It would be interesting to see the effect of depletion of central 5HT or lesion of 5HT neurons on Fluoxetine-stimulated AVP secretion.

Since it is possible that the 5HT3, or an as yet uncharacterised 5HT receptor subtype is important in the effect, the identification of the receptor subtypes involved in Fluoxetine-stimulated osmoregulated AVP secretion could be further explored using 5HT agonist and antagonist drugs to other receptor subtypes. The hypothalamic explant system (Section 1.1.3.2.2) would be a useful system to explore this before further *in vivo* studies are performed.

The anatomical studies in rats (Section 6) have suggested that 5HT2, 5HT1c and 5HT1a receptors are present only in low density in the region of the SON and PVN. Although there is some evidence for a direct influence of 5HT on AVP secretion it would seem most likely that the effect is mediated by a complex interaction of several neurotransmitter systems and afferent inputs to the magnocellular neurons. The 5HT neurotransmitter system is extensive and innervates most regions of the brain (Section 1.3.1). It may influence any of the neurotransmitter systems involved in osmoregulated AVP secretion (Section 1.1.3). In addition the SFO and other circumventricular organs have relatively dense 5HT innervation (Bouchaud & Bosler 1986) and 5HT could have affect on afferent stimuli from these osmosensitive areas to the magnocellular neurons in the hypothalamus. 5HT is generally an inhibitory neurotransmitter and it could be that stimulation of 5HT neurotransmission inhibits afferent stimuli to the magnocellular neurons which exert inhibitory control on AVP secretion eg. DA, opioids. This would be a difficult hypothesis to explore but could be studied using neurobiochemical and electrophysiological *in vivo* systems (Section 1.3.3.2).

The third objective of this research was to explore water balance and the effect of 5HT active antidepressant drugs in depression, a putative "disease state" of

5HT neurotransmission. The studies of water balance in elderly depressed patients (Section 4) found all but one of 16 subjects to have remarkably normal osmoregulation in response to water loading. This would suggest that problems of water balance in depression are infrequent. The subjects in this study, however, had only mild to moderate depression. In addition the water load test could only discern large individual differences from the population norm (approximately 40%). A more detailed longitudinal study of osmoregulation in depression, including patients with more acute and severe stages of illness, would be extremely valuable to assess the influence of disease state and its progressive improvement, together with the putative changes in 5HT function. In such a study repeated water load tests in the same individual could probably detect a ≥20% significant change in water load excretion (derived from Section 3.1).

Similar studies have been undertaken on the abnormalities of function and regulation of the HPA axis (Schatzberg & Nemeroff 1988) and useful information has been obtained about a subgroup of patients with implications for prognosis. In addition such research leads to further understanding of the puzzle of the pathophysiology of depression (see Section 1.5.2.4). Abnormalities of AVP secretion associated with depressive illness, particularly psychotic depression have been discussed in Section 1.6. Further information about pathophysiology might be gained from studies of water balance and osmoregulatory function in these patients although the practicalities of even simple assessment such as fluid balance are often insurmountable in such disturbed patients.

Five of the six patients with depression treated by Fluoxetine had no apparent abnormality of water balance compared to depressed patients treated with Dothiepin or normal elderly subjects. The abnormal result of one patient was probably attributable to her impaired renal function. This was a small study and it may be that a larger study would have detected a significant effect of Fluoxetine on water balance. It is possible, however, that by excluding patients taking certain medications such as diuretics and patients with physical illness, the very population that was most vulnerable to the effects of Fluoxetine on water balance had been excluded from study. The reports of Fluoxetine-associated hyponatraemia and the cases presented in Appendix 10 might suggest

that it is elderly patients with severe mental illness, and concomitant physical illness, who are particularly vulnerable to 5HT associated SIAD. This group of patients would be extremely difficult to study either by water load test or infusion of 5% saline. This putative vulnerability has implications for clinical management and it would perhaps be prudent to monitor the plasma sodium of such patients by weekly blood samples during the first 4-6 weeks of treatment with Fluoxetine and perhaps other SSRI.

Other SSRI have not been reported in the literature as associated with SIAD or hyponatraemia although there is one report of fluvoxamine to the Committee on Safety of Medicines and Appendix 10 presents a case of paroxitene-associated hyponatraemia. Although this might suggest that the mechanism of Fluoxetine-associated hyponatraemia is not through its inhibition of 5HT reuptake, other SSRIs have perhaps not as yet been as widely used in the treatment of severe depressive illness. It may be, therefore, that these drugs have not yet been prescribed to patients who are particularly vulnerable to disturbance of osmoregulation by increase in 5HT neurotransmission. It is also known that at least some of the SSRIs have regional variation in effect on 5HT reuptake in the CNS (Wong et al 1975; Section 2.3.1.2). This might also explain the apparent differences of drug effect.

Sections 1.1.1, 1.1.2.4 and 1.3.1 discussed some of the species differences in AVP secretion, osmoregulation and the central 5HT nervous system. If the results of the studies in rats in Section 5 may be extrapolated to man, the following conclusions and hypotheses may perhaps be drawn from this research. Acute increase in 5HT neurotransmission leads to a rise in basal AVP secretion. This modulatory effect of 5HT on osmoregulated AVP secretion is probably mediated indirectly, possibly by inhibition of inhibitory afferent stimuli.

Both the AVP-secreting neurons and the 5HT system rapidly accommodate, by autoregulatory processes, to the increase in 5HT neurotransmission and AVP secretion. The 5HT autoregulatory process have been discussed in Section (1.3.4). The magnocellular autoregulatory processes are less well understood (Section 1.1.3) but the studies of Section 6 would suggest that changes in 5HT receptor density do not play an important role. With continued drug administration the 5HT autoregulatory processes are overcome and there is

again a net increase in 5HT neurotransmission. This does not lead to increased AVP secretion in normal man and the normal rat since the autoregulatory processes of the magnocellular neurons are still apparent, indeed the studies in rats may suggest that some overcompensation occurs (Section 5.7). In the "disease state" of 5HT neurotransmission present in depression, where there is also some evidence of AVP dysregulation (Section 1.6), there may be dysfunction of these regulatory processes and ensuing abnormality of osmoregulation.

The neuroendocrine regulation of AVP is thought to have such adaptive and homeostatic ability and some parallels may perhaps be drawn to the effect of nicotine on AVP secretion. Nicotine has been shown to alter AVP secretion *in vitro* and *in vivo* and is important in osmoregulated AVP secretion (Section 1.1.3.2.2) . Acute administration of nicotine in man leads to secretion of AVP but it has been suggested that chronic administration, by cigarette smoking, leads to blunting of the response (Burn et al 1945; Rowe et al 1980; Lightman et al 1982).

The commencement of antidepressant therapy with 5HT active drugs leads to several clinical adverse effects (eg. nausea, disturbed sleep) most of which habituate within 3-4 days presumably due to autoregulatory features of 5HT neurotransmission (see Section 1.3.4). Continued administration of antidepressant drugs leads to a net increase in 5HT neurotransmission after 14-21 days and the concomitant onset of therapeutic action (Section 1.5.3.2). The adverse effects of treatment do not, however, recur with the onset of therapeutic action. The paradox of how these adjustments to adverse effects occur but therapeutic effect is still evident is probably explained by the putative abnormality of 5HT in depression and a dysfunction of normal regulatory processes. Failure of these compensatory mechanisms might explain why Fluoxetine-associated hyponatraemia occurs early in depression and persists, but does not appear to occur in normal man. The apparent abnormality of osmoregulated AVP secretion that occurs with normal ageing (Section 4.2.1) may play an additional role in the vulnerability of elderly depressed patients to 5HT-associated SIAD. It may be postulated that the more severe the dysfunction of 5HT neurotransmission, ie. the more marked mental disturbance, the greater the vulnerability to 5HT SIAD. As discussed above it would seem to

be important that when Fluoxetine or other potent 5HT active agent is used in the treatment of elderly depressed patients with severe mental illness, perhaps particularly those with psychotic depression, their osmotic status is monitored.

Based on the results of these studies it is my view that 5HT modulation of AVP secretion is of little physiological consequence in normal man and the normal rat. In situations where there is a dysfunction of normal adaptive mechanisms, such as in depression, the role of 5HT is more important and occasionally may lead to severe hyponatraemia and death.

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APPENDIX 1

The nausea scales used in Studies 3.1, 3.2, 4.1 and 4.2

a) Daily nausea scale during 7 days drug treatment

b) Nausea scale used during water load and hypertonic saline infusion assessments.

DATE	Not Nauseated	NAUSEA	Very Nauseated	Not Thirsty	THIRST	Very Thirsty
1						
2						
3						
4						
5						
6						
7						

FLUOXETINE: ORAL WATER LOAD IN DEPRESSED PATIENTS

B1Y-EW-E087	8 0 1	8 0	
Study Code	Investigator No.	Patient No.	Visit No.

PLEASE COMPLETE LEGIBLY USING A BLACK BALL-POINT PEN

Protocol Time (mins)
(Circle one)

Pre

30

60

120

180

240

Actual Time (24 hour clock) H H : M M

LINEAR ANALOG SCALE ASSESSMENT

Please mark each scale (use a small vertical line) to indicate how nauseous and thirsty you feel at the present time.

1. NAUSEA

Not Nauseated ——————————————— Very Nauseated

2. THIRST

Not Thirsty ——————————————— Very Thirsty

OFFICE USE	NAUSEA (mm)	THIRST (mm)

NOTE: Always measure from left end of scale

APPENDIX 2

The extraction and radioimmunoassay of vasopressin

IODINATION OF AVP

Preparation of the reaction vessels:-

Dissolve 1mg of IODOGEN (Pierce Warriner, cat.no.28600) in 1ml of dichloromethane. Take 100ul of this solution and make upto 1ml with dichloromethane (100ug/ml). Aliquot 100ul of this second solution (10ug) into plastic eppendorf tubes (12x75mm) and dry down under nitrogen at room temperature. These vessels can be stored at 4°C for 3months.

Iodination method:-

16ug of AVP in 200ul of 0.25M phosphate buffer (pH 7.5) is added to a reaction vessel. 0.5mCi of $Na^{125}I$ is added to the vessel and the reaction allowed to proceed for 10-15min at room temperature whilst the mixture is gently shaken. The sample is removed from the vessel to stop the reaction procedure and the volume made upto 1ml with column eluant and then applied to the G25 column.

COLUMN ELUANT

1.25ml of conc. acetic acid added to 400ml water . 0.1g sodium azide and 0.625g bovine serum albumin (by sprinkling on top) added. BSA left to dissolve and pH to 2.5 with HCl. Solution made upto 500ml and stored at 4°C.

CHROMATOGRAPHY COLUMN

Sephadex G25F and the above column eluant. Collect 2ml fractions. AVP will elute after the iodine peak so it is better to take the fractions from the descending side of the peak, about half way down.

EXTRACTION OF AVP FROM PLASMA

Plasma defrosted, centrifuged 15min, 1500 RPM at 4°C. 2ml sample aliquots placed in small glass test tubes (round bottomed, 5ml capacity, silicon- coated). Approximately 20mg of activated Florisil added to each tube. Tubes mixed on a cell mixer for 20min at room temperature. Florisil manoeverd to the bottom of the tube and plasma aspirated. 2ml distilled water added to each tube and briefly shaken. Water aspirated. 2ml 0.2M HCl added and briefly shaken. Acid aspirated leaving a little remaining in the tube. 500ul of 90% acetone added to each tube, and mixed on cell mixer for 10min. Florisil brought to the bottom of the tube and acetone aspirated with a glass pasteur pipette and transfered to a glass siliconised centrifuge tube. Acetone procedure repeated. 1ml of diethyl ether (Analar) added to acetone in to each centrfuge tube and mixed vigorously. Solution left for 5min until two layers are apparent. The top layer is removed with a fresh glass pasteur pipette for each sample and the bottom layer, containg the extracted, concentrated AVP is dried a gentle stream of air at 37°C. The dried extracts are stored at -40°C until assayed.

ACTIVATION OF FLORISIL

Florisil (100-200 mesh) Sigma F7752 (250g)

Using a 500ml glass beaker, put Florisil in till it reaches the 50ml mark approximately. Add approx. 300ml of dist. water and stir vigorously with a magnetic stirrer for a few seconds. Allow the Florisil to settle and aspirate off the water. Repeat this procedure till the water becomes clear, about 10 washes. Then repeat washing procedure with 1MHCl, 3 water washes, 1M NaOH, 10 water washes until the water is clear. Aspirate off all of the water and cover the beaker with aluminium foil. Place in an oven at 120°C for 18h. When cool, loosen the Florisil and dispense into several small containers.

THE RADIOIMMUNOASSAY OF AVP

STOCK STANDARD SYNTHETIC AVP DILUTIONS

Ferring AVP, 2mg aliquots (Ferring Pharmaceuticals, Malmö, Sweden).

2mg is dissolved in 2ml of distilled water containing 0.25% acetic acid.
Buffer A:- 0.25% acetic acid with 0.02% sodium azide.
Buffer B:- 50mM TRIS + 1mg/ml BSA + 0.02% sodium azide.
Buffer C:- Assay Buffer.

1) 400ul of standard diluted with B 1:100 (10ug/ml). Stored in 1ml aliquots.
2) 1ml of solution 1 diluted with solution B 1:100 (100ng/ml). Stored in 1ml aliquots.
3) 1ml of solution 2 diluted with solution C 1:20 (5ng/ml). Stored in 1ml aliquots.
4) 1ml of solution 3 diluted with solution C 1:100 (50pg/ml). Stored in 1ml aliquots. This is INTRA-ASSAY CONTROL.
5) 1ml of solution 2 added to 77.125ml of solution C (1280pg/ml). Stored in 0.5ml aliquots. This is ASSAY STANDARD.

ASSAY PROCEDURE FOR ARGININE VASOPRESSIN (AVP)

Day 1:

Preparation of standards

Stock standard = 1280pg/ml stored at -20°C in 0.5ml aliquots. Defrost standard and make upto 2ml with assay buffer (1:4 dilution) now 320 pg/ml. Prepare and label standard tubes; 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03. Add 800ul assay buffer to all tubes. To the "16" tube add a further 400ul of assay buffer and 400ul of standard (320pg/ml), total volume now 1600ul, mix. Transfer 800ul of standard from the "16" tube to the "8" tube and mix. Repeat serial dilution to all tubes so that each tube contains 800ul except for the last standard tube (0.03) which contains 1600ul. Add 200ul of each standard in triplicate to each standard tube set up for the standard curve.

Samples

Add 800ul of assay buffer to the dried extracts in the centrifuge tubes and mix vigorously. Centrifuge at 1500 RPM for 15min at 4°C. Take out 200ul in triplicate, from each sample tube and add it to each triplicate sample tube in

assay. Do not disturb the sediment in the bottom of the centrifuge tube when dispensing out the 200ul aliquots, leave the remaining 200ul in the bottom of the centrifuge tube.If you suspect that the sample that you are about to measure will be very high i.e. too high to read accurately from the standard curve then the sample could be resuspended in 1000ul of assay buffer instead of 800ul. Alternatively, the sample could be resuspended in 800ul as usual but 200ul taken in duplicate only, then 100ul is taken and made upto 1000ul with assay buffer, mixed and 3 X 200ul assayed i.e. a 1:10 dilution has been made of the sample so that it can be read accurately from the standard curve.

Intra-assay control (IAC)

Dilute stock intra-assay control (stored at -20°C) from 50pg/ml to 10pg/ml. Take 200ul of 50pg/ml and add to 800ul of assay buffer mix, this is now 10pg/ml. Add 200ul of 10pg/ml, in triplicate, to IAC tubes in assay, this should read 2pg/tube from the standard curve.

Dilution of first antibody(AVP-Ab)

Stock first antibody is stored at -20°C at 1:10000 dilution in 1ml aliquots. Take 1ml of 1:10000 and add to 11.5mls of assay buffer to give an antibody dilution of 1:125000. 100ul of this is added to zero binding tube, all standard tubes, all sample tubes, extraction control and intra-assay control tubes.

Buffer addition

200ul of assay buffer is added to zero binding tubes (Bo). 300ul of assay buffer is added to non-specific binding tubes (NSB).

Incubation

Mix all the tubes from the assay and cover well with plastic. Leave at 4°C for 24h.

<u>Day 2</u>

Dilute stock ^{125}I-AVP with buffer to a count of 2000 in a minute in 100ul. Add 100ul of ^{125}I-AVP to all tubes. Add 100ul to 3 tubes containing nothing else and put a stopper on these, these are the total count tubes and must have nothing else added to them (TC tubes). Mix all tubes and recover. Store at 4°C for 18h.

<u>Day 3</u>

Dilute second antibody (goat anti-rabbit IGG) 1:40 with separation buffer, 0.5ml of second antibody with 19.5ml of separation buffer and mix. Add 100ul of diluted second antibody to all tubes except total count tubes. Mix, recover and store at 4°C for 48-72h.

Day 5-6

Centrifuge all tubes except total count tubes at 4°C for 30min at 2500RPM. Aspirate off all of the supernatant to leave the pellet containing bound fraction at the bottom of the tube. Count every tube including total count tubes, for 10min on a gamma-counter.

Total counts, non-specific binding and zero binding tubes repeated at the end of each assay and counted at the end of the assay to check for assay drift. When the calculations are performed the tubes from the beginning and the end of the assay must be used.

CALCULATIONS

Total counts = TC - Background (bg)

$$\text{Non-specific binding} = \frac{\text{NSB-bg} \times 100\%}{\text{TC-bg}}$$

$$\text{Zero binding} = \frac{\text{Bo-NSB} \times 100\%}{\text{TC-bg}}$$

$$\text{Standard or sample binding (B/Bo)} = \frac{\text{sample or standard -NSB} \times 100\%}{\text{Bo-NSB}}$$

Graph:- Plot on log linear graph paper, x axis (log) AVP concentration pg/tube, y axis (linear) is B/Bo % for standards.

Read B/Bo for samples off standard curve, this is pg/200ul extract (800ul, total volume). If extract is of 2ml of plasma, then AVP (pg/ml)=reading from graph x 4/2. For extract of 1ml volume assay buffer from 2ml of plasma, then AVP(pg/ml)= reading x 5/2. For extract of 800ul from 1ml plasma, then AVP (pg/ml)= reading x 4/1.

ASSAY BUFFER FOR AVP

All chemicals must be ANALAR grade

Dissolve 6.057g TRIS [Tris(hydroxymethyl)methylamine] and 0.2g sodium azide in 800-900ml of distilled water. Sprinkle on the top, 3.5g of bovine serum albumin fraction V (Sigma A 9647) and leave to dissolve. Bring to pH 7.4 by adding 5M HCl. make volume to 1 litre. Store at 4°C.

SECOND ANTIBODY SEPARATION BUFFER

9.306g EDTA (Disodium salt) added to 200ml of assay buffer. Dissolved by stirring aided by 8-10 pellets of NaOH. Leave for 10 min. pH to 7.4 with 2M NaOH. Add 0.5ml of normal rabbit serum (Wellcome Labs. Beckenham, Kent) and make upto 250ml with assay buffer. Store at 4°C.

SILICON COATING OF GLASSWARE

Both the extraction tubes and the conical centrfuge tubes need to be silicon coated every 6 weeks to prevent AVP sticking to the glass. Use "Repelcote" from Hopkin & Wiliams cat. no. 63216 (dimethyldichlorosilane). Add 1-2ml of Repelcote to each tube, rotate and pour back into the bottle. Leave the tubes to dry at room temperature. All new glassware must be acid washed then Repelcoted prior to use for the first time.

APPENDIX 3

The data sheets for Fluoxetine, Ritanserin and DOI

'PROZAC' ▼

DATA SHEET

Name of Product
'PROZAC', fluoxetine hydrochloride.

Presentation
Capsules (green and off-white, coded 3105) each containing 20mg fluoxetine hydrochloride.

Uses
Depression: Prozac is indicated for the treatment of the symptoms of depressive illness, especially where sedation is not required.

Bulimia nervosa: Prozac is indicated for the reduction of binge-eating and purging activity.

Dosage and Administration
For oral administration to adults only.

Depression – adults and the elderly: A dose of 20mg/day is recommended.

Bulimia nervosa – adults and the elderly: A dose of 60mg/day is recommended.

Fluoxetine has a half-life of 2 to 3 days, and its major active metabolite has a half-life of 7 to 9 days. Steady state plasma concentrations are only achieved after continuous dosing for weeks. When dosing is stopped, active drug substance will persist in the body for weeks. This should be borne in mind when starting or stopping treatment.

Children: The use of Prozac in children is not recommended, as safety and efficacy have not been established.

Patients with renal and/or hepatic dysfunction: See 'Contra-indications' and 'Precautions' sections.

Contra-indications, Warnings, etc.

Contra-indications
Hypersensitivity to fluoxetine.
Fluoxetine should not be administered to patients with severe renal failure (GFR <10ml/min) because accumulation may occur in these patients during chronic treatment.

Usage in nursing mothers: Prozac should not be prescribed to nursing mothers. In one breast milk sample the concentration of fluoxetine, plus norfluoxetine, was 70.4ng/ml, compared to 295.0ng/ml in the mother's plasma. No adverse effects on the infant were noted.

Monoamine oxidase inhibitors: At least 14 days should elapse between discontinuation of an MAOI and initiation of treatment with Prozac. At least five weeks should elapse between discontinuation of Prozac and initiation of therapy with an MAOI.

Serious, sometimes fatal reactions (including hyperthermia, rigidity, myoclonus, autonomic instability with possible rapid fluctuations of vital signs, and mental status changes that include extreme agitation progressing to delirium and coma) have been reported with concomitant use or when fluoxetine had been recently discontinued and an MAOI started. Cyproheptadine or dantrolene may benefit patients experiencing such reactions.

Warnings
Rash and allergic reactions: Upon the appearance of rash or of other allergic phenomena for which an alternative aetiology cannot be identified, Prozac should be discontinued.

Pregnancy: The safety of fluoxetine in human pregnancy has not been established; accordingly, the drug should be avoided in pregnancy unless there is no safer alternative. There was no evidence of teratogenicity from animal studies but full testing was limited by maternal toxicity.

Lactation: See 'Contra-indications'.

Precautions
Prozac should be discontinued in any patient who develops seizures. Prozac should be avoided in patients with unstable epilepsy; patients with controlled epilepsy should be carefully monitored. There have been rare reports of prolonged seizures in patients on fluoxetine receiving ECT treatment.

Fluoxetine is extensively metabolised by the liver and excreted by the kidneys. A lower dose, e.g. alternate day dosing, is recommended in patients with significant hepatic dysfunction or mild to moderate renal failure (GFR 10-50ml/min).

Clinical experience in acute cardiac disease is limited, therefore caution is advisable. However, the ECG of 312 patients who received fluoxetine in double-blind trials were retrospectively evaluated; no conduction abnormalities that resulted in heart block were observed.

Prozac may cause weight loss which may be undesirable in underweight depressed patients. Only rarely have depressed or bulimic patients been discontinued for weight loss when treated with fluoxetine.

In patients with diabetes, fluoxetine may alter glycaemic control. Hypoglycaemia has occurred during therapy with fluoxetine and hyperglycaemia has developed following discontinuation. Insulin and/or oral hypoglycaemic dosage may need to be adjusted.

There have been reports of abnormal bleeding in several patients, but causal relationship to fluoxetine and clinical importance are unclear.

Although fluoxetine has been shown not to affect psychomotor performance in healthy volunteers, any psychoactive drug may impair judgement or skills. Therefore patients should be cautioned that their ability to perform potentially hazardous tasks (e.g. driving, operating machinery) may be impaired.

As improvement may not occur during the first two or more weeks of treatment, patients should be closely monitored during this period. The possibility of a suicide attempt is inherent in depression and may persist until significant remission occurs.

Drug Interactions
Monoamine oxidase inhibitors: (see 'Contra-indications').

Caution is advised if the concomitant administration of Prozac and CNS active drugs, including lithium, is required. There have been reports of both increased and decreased lithium levels when used concomitantly with fluoxetine. Cases of lithium toxicity have been reported. Lithium levels should be monitored.

Greater than 2-fold increases of previously stable plasma levels of cyclic antidepressants have been observed when Prozac has been administered in combination.

Agitation, restlessness and gastro-intestinal symptoms have been reported in a small number of patients receiving fluoxetine in combination with tryptophan.

The long elimination half-lives should be borne in mind (see 'Dosage and Administration') when considering pharmacodynamic or pharmacokinetic drug interactions.

In formal testing, no drug interaction of clinical significance has been observed between fluoxetine and chlorothiazide, ethanol, secobarbital, tolbutamide and warfarin

Fluoxetine is bound to plasma protein and concurrent administration may alter plasma concentrations of other plasma protein bound drugs, or conversely fluoxetine

Fluoxetine does not appear to potentiate the effects of alcohol.

Adverse Effects
Body as a whole: asthenia, fever.

Digestive system: nausea, diarrhoea, dry mouth, appetite loss, dyspepsia, vomiting

Nervous system: headache, nervousness, insomnia, drowsiness, anxiety, tremor, dizziness, fatigue, decreased libido, seizures (see 'Precautions'). Hypomania or mania occurred in approximately one per cent of fluoxetine treated trial patients.

Respiratory system: pharyngitis, dyspnoea. Pulmonary events (including inflammatory processes of varying histopathology and/or fibrosis) have been reported rarely. Dyspnoea may be the only preceding symptom.

Skin and appendages: a small percentage of patients developed rash and/or urticaria (see 'Warnings'). Serious systemic reactions, possibly related to vasculitis, have developed in patients with rash, and rarely death has been reported. Excessive sweating, serum sickness and anaphylactoid reactions have also been reported.

Urinogenital system: sexual dysfunction (delayed or inhibited orgasm).

Hyponatraemia (including serum sodium below 110mmol/l) has been rarely reported and appeared to be reversible when Prozac was discontinued. Some cases were possibly due to the syndrome of inappropriate antidiuretic hormone secretion. The majority of reports were associated with older patients, and patients taking diuretics or otherwise volume depleted.

The following have been reported in association with fluoxetine but no causal relationship has been established: cerebral vascular accident, confusion, dyskinesia, ecchymoses, gastro-intestinal haemorrhage, hyperprolactinaemia, pancreatitis, suicidal ideation, thrombocytopenia, thrombocytopenic purpura, vaginal bleeding after drug withdrawal and violent behaviour.

Overdose

On the evidence available, fluoxetine has a wide margin of safety in overdose. Since introduction, a single death, attributed to a large overdose of fluoxetine alone, has been reported.

One patient who reportedly took 3000mg of fluoxetine experienced 2 grand mal seizures that remitted spontaneously. Nausea and vomiting were prominent in overdoses involving higher fluoxetine doses. Agitation restlessness, hypomania and other signs of CNS excitation were also observed.

Management: No specific antidote is known.

An airway should be established. Cardiac and vital signs monitoring is recommended, along with general symptomatic and supportive measures.

Based on experience with animals, fluoxetine-induced seizures which fail to remit spontaneously may respond to diazepam. Due to the large volume of distribution of fluoxetine, forced diuresis, dialysis, haemoperfusion and exchange transfusion are unlikely to be of benefit. Activated charcoal, which may be used with sorbitol, may be as or more effective than emesis or lavage.

Pharmaceutical Precautions
Store at room temperature (15°-25°C).

Legal Category POM

Package Quantities
Calendar packs of 28 capsules: 2 strips of 14 capsules

Further Information
Fluoxetine is chemically unrelated to tricyclic and tetracyclic antidepressant agents. It is a specific serotonin (5-hydroxytryptamine, 5-HT) reuptake inhibitor, whose specificity is unaltered by its major metabolite.

Product Licence Number, Name and Address
0006/0195

Dista Products Limited
Kingsclere Road
Basingstoke, Hampshire RG21 2XA
Telephone: Basingstoke (0256) 52011

Date of Preparation or Last Review April 1991

'PROZAC' is a Dista trade mark

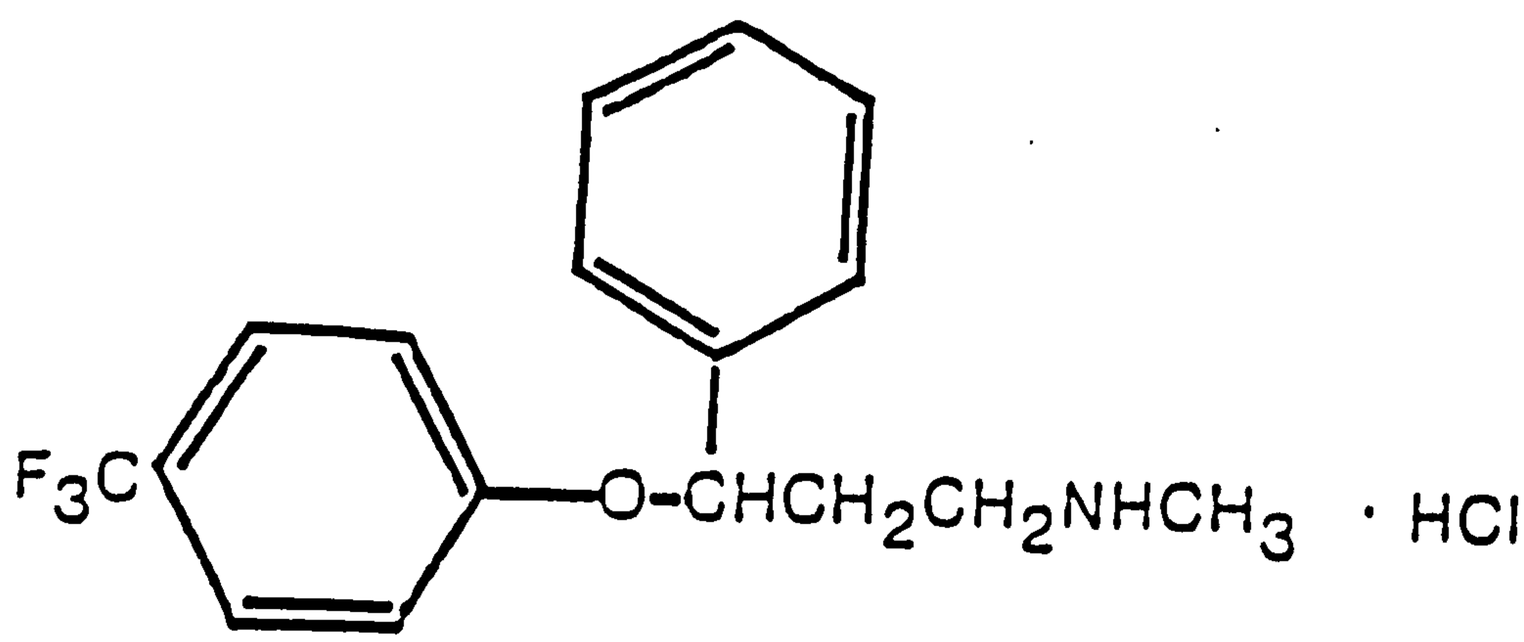
FLUOXETINE HYDROCHLORIDE (LY110140)

CHEMISTRY

Fluoxetine hydrochloride, (±)-N-methyl-3-phenyl-3-[(α,α,α-trifluro-p-tolyl)-oxy] propylamine hydrochloride, has an empirical formula of $C_{17}H_{18}F_3NO \cdot HCl$ and a molecular weight of 345.79. Its formula is shown below.

Fluoxetine is a white to off-white crystalline solid which is soluble at a concentration of 14 mg/ml in water, 250 mg/ml in methanol, and 125 mg/ml in chloroform. Less than 1 percent is soluble in benzene, ethyl acetate, and hexane.

Stability assays have shown fluoxetine to be stable for periods of 24 months when exposed to temperatures of 25°, 40°, or 50°C.



BEST COPY

AVAILABLE

Some text bound close to the spine.

LILLY RESEARCH LABORATORIES
EXPERIMENTAL COMPOUND
SAFETY DATA SHEET

ELI LILLY AND COMPANY • LILLY CORPORATE CENTER • INDIANAPOLIS, INDIANA 46285

ompound: LY110140 - Fluoxetine Hydrochloride

ssue Date: July, 1987 (will be updated July, 1988)

his document should not be used for registration purposes. These data, and the resulting aution statement, are provided so that proper protective measures can be taken by persons ho may be exposed to the compound during initial research and development activities.

aution Statement: DANGER, fluoxetine is a severe eye irritant and toxic by inhalation. gestion and dermal contact should be avoided. Based on human clinical data, fluoxetine hould be handled as a potential sensitizer.

'RELIMINARY TOXICOLOGY DATA:

cute Exposure: Severe and irreversible damage occurred when fluoxetine was placed in the yes of rabbits whether a posttreatment rinse was employed or not. The application of 500 g of fluoxetine per kg of body weight to the backs of rabbits caused a transient loss of ody weight. The median lethal dose (LD_{50}) of fluoxetine, when administered by oral gavage o rats, was approximately 400 mg/kg of body weight. Ante mortem signs of toxicity were remors, convulsions, and myoclonus. Other signs were hyperirritability, limb weakness, nd salivation. The median lethal concentration (LC_{50}) of fluoxetine was 0.8 to 1.0 mg/L f air in rats exposed for one hour to a solid particulate aerosol. Surviving animals xhibited difficult breathing, hypoactivity, nasal mucosal irritation and weight loss.

eproduction: Reproduction studies in rats (2, 5, 12.5 mg/kg) and rabbits (2.5, 7.5, 15 g/kg) indicate that fluoxetine is not teratogenic. In a rat fertility study at doses of , 5, and 12.5 mg/kg/day, there was no impairment of reproductive performance or evidence f teratogenicity. There was a slight decrease in survival and weight gain of offspring at he highest dose level. In a two-generation fertility study in rats fed diets containing .002, 0.005, and 0.0125% fluoxetine, there was no impairment of reproductive performance r evidence of teratogenicity. Only minor effects, which included depressed food onsumption and weight gain of F_0 animals during the premating treatment period and a light decrease in survival and weight gain of the F_1 neonatal progeny, were observed when $_0$ rats were given diets at the highest dose level, ca 7.4 mg/kg/day. The no-effect level n this study was 0.005%, equivalent to ca 3.1 mg/kg/day during the premating and gestation eriod.

ubchronic Exposure: Studies of three months duration have been completed in rats (ca 10, 5, 75 mg/kg) and dogs (5, 10, 20 mg/kg) to evaluate the effects of daily oral dministration of fluoxetine. Compound effects in rats were deaths at the high dose, epressed weight gain and dose-related incidence and severity of pulmonary histiocytosis phospholipidosis). Treatment related changes in dogs included decreased weight gain, transiently increased platelet count, anorexia, transient mydriasis, occasional fine tremors and vomiting. In rats and dogs, 10 mg/kg fluoxetine was tolerated with minimal

vert effects. In a 90-day dietary study in mice at doses nominally equivalent to 1.6, 9, or 31 mg/kg/day, the significant toxicological effects were essentially limited to the igh dose and included hyperactivity; decreased body weight gain; minimal and reversible egenerative hepatic changes and pulmonary phospholipidosis; and focal testicular ypospermatogenesis. This latter effect appeared to be species specific and was ssentially restricted to the high dietary level of 0.02% (ca 31 mg/kg/day), which is pproximately 30 times the clinical dose.

hronic Exposure: In a one year dietary study with a two-month recovery phase in rats at oses nominally equivalent to 0.5, 2.5, or 10 mg/kg/day, significant toxicological effects ncluded phospholipidosis, anorexia, slight hepatic fat deposition, and minimal eticuloendothelial cell hyperplasia; these effects were essentially reversible during the ecovery phase. There were no significant toxicological effects at the lowest dose. In a ne year study with a two month recovery phase in dogs at doses of 1.0, 4.5, or 20 g/kg/day (decreased to 10 mg/kg/day), the most significant effects were associated with he high dose group. These effects consisted of deaths at 20 mg/kg and moderate radycardia, physical signs of toxicity including anorexia and aggressive behavior, and hospholipidosis at 10 (20) mg/kg/day. The effects were reversible in all surviving nimals. At 4.5 and 1.0 mg/kg/day, the effects were minimal and reversible. In a two year hronic toxicity and carcinogenicity study in rats fed diets containing 0.001, 0.0045, and .02% fluoxetine, there was no evidence of carcinogenicity and no adverse effects on urvival. In addition to decreased body weight gain, significant accumulation of luoxetine and norfluoxetine occurred in the lung and was associated with pulmonary hospholipidosis. In the liver, moderate lipid deposition and phospholipid accumulation ere demonstrated at various times during the first 17 months of treatment, but were not pparent after two years of fluoxetine administration. These changes observed at 0.02% in he lung and the liver were without apparent long-term detrimental effects. The effects bserved at the low and middle dose after two years of treatment were limited primarily to he liver changes and were considered to be of minimal toxicologic significance. In a wo-year chronic toxicity and carcinogenicity study, mice received average daily dietary oses of 1.14, 4.46, or 11.87 mg of fluoxetine/kg body weight for males and 1.23, 4.64, or 1.86 mg/kg for females. There was no evidence of carcinogenicity, and no overall effect n survival. Treatment-related effects of chronic fluoxetine administration included yperactivity with associated mortality in high-dose females, minimal to moderate fatty hange in the liver and hepatocellular cytomegaly at the mid- and high-dose, and ose-related handling-induced clonic seizures at all doses in females.

utagenicity Data: Fluoxetine was not mutagenic in the modified Ames bacterial mutagen ssay, the DNA repair assay in primary rat hepatocytes, the sister chromatid exchange assay n Chinese hamster bone marrow cells, and the mouse lymphoma assay.

onclusion: The toxicity seen with chronic fluoxetine administration was consistent with hat expected based on its pharmacology, the toxicity evident in preclinical studies of ther drugs in its therapeutic class, and the effects seen with other hospholipidosis-inducing drugs. Moreover, no mutagenic, carcinogenic, teratogenic, or dverse reproductive effects have been associated with fluoxetine administration to aboratory animals. In human clinical trials, a small percentage of patients developed ashes suggestive of sensitization. Fluoxetine should be handled as a potential ensitizer.

FIRST AID AND HANDLING

luoxetine is an experimental material and no specific antidote is known. If systemic ffects are suspected, consult a physician for supportive treatment as indicated.
ye: Flush immediately with large amounts of water and contact a physician if irritation evelops.
kin: Wash all areas with soap and water. Preferably use a tub or shower. Wash all ontaminated clothing thoroughly before reuse.
nhalation: Remove individual to fresh air. If breathing difficulty occurs, get medical ttention. If not breathing, give artificial resuscitation, preferably mouth-to-mouth.

JANSSEN

: RESEARCH PRODUCTS

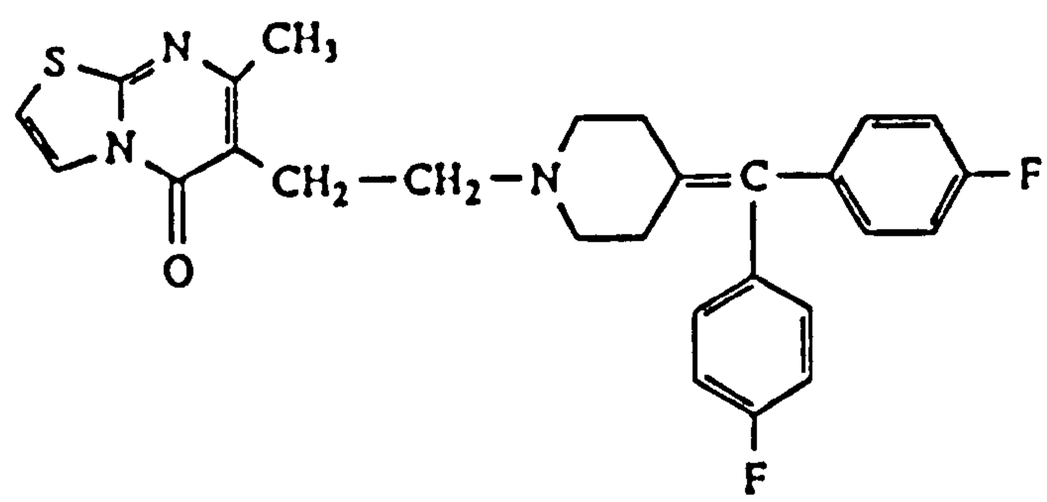
A division of Janssen Biotech N.V.

CHEMICAL REPORT

COMPOUND INFORMATION

R 55667 : Authentic Substance

Graphic formula :



Chemical name : 6-[2-[4-[bis(4-fluorophenyl)methylene]-1-piperidinyl] ethyl]-7-methyl-5H-thiazolo[3,2-a]pyrimidin-5-one.

International non-proprietary name : ritanserin

Molecular formula : $C_{27}H_{25}F_2N_3OS$

Molecular weight : 477.57

Sample Data.

Sample No. : V 880-275

QUALITY DATA

A.1. Appearance : light-yellow powder.

A.2. Melting temperature : 144.1-145.1°C

A.3. Spectroscopic techniques :

A.3.a. UV:

Maxima		Minima	
λ nm	ε	λ nm	ε
228 323	24700 13300	219 286	22400 3570

A.3.b. IR : confirms the structure; polymorph I.

A.3.c. NMR : confirms the structure.

A.3.d. MS : confirms the structure.

B.1. Liquid chromatography : one peak corresponding to R 55667 i.e. 100 % R 55667.

B.2. Colour and clarity.

The solution is clear.

It has a transmittance of 80 % at 450 nm.

C.1. Basic equivalent : molecular weight : 477.39 (100.0 %) one equivalent per mole.

D. Elemental analysis.

Element	Calculated (%)	Found (%)	Difference
Carbon	67.90	67.99	+ 0.09
Hydrogen	5.28	5.21	- 0.07
Nitrogen	8.80	8.79	- 0.01
Fluorine	7.96	7.70	- 0.26
Sulfur	6.71	6.66	- 0.05

APPROVED BY

A. VAN DIJCK

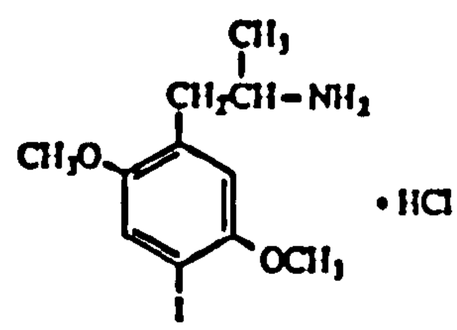
RBI Research Biochemicals Incorporated
One Strathmore Road, Natick, MA 01760 USA
508-651-8151 • 800-736-3690 • Fax 508-655-1359

SEMAT
SOLE UK DISTRIBUTOR

Cat. No. D-101

(±)-DOI HYDROCHLORIDE

Potent and selective 5-HT_2/5-HT_{1C} agonist that crosses the blood brain barrier.



Mol. Formula: $C_{11}H_{16}INO_2 \cdot HCl$

Mol. Wt.: 357.6 (anhyd.)

m.p.: 198-200°C

CAS Registry No.: 82830-44-2

Chemical Name: (±)-1-(4-Iodo-2,5-dimethoxyphenyl)-2-aminopropane hydrochloride

Physical Properties: White solid.

Caution: The pharmacology of this compound is incompletely characterized and due care should be exercised in its use. Avoid skin contact, ingestion or inhalation.

Storage: Store tightly sealed at room temperature.

Solubility: Soluble in water; moderately soluble in ethanol.

Disposal: Dissolve or mix the compound with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber.

References:

1. Sargent, T., 3d, Budinger, T.F., Braun, G., Shulgin, A.T., Braun, U. "An iodinated catecholamine congener for brain imaging and metabolic studies." *J. Nucl. Med.* 19(1), 71 (1978).
2. Glennon, R.A., Young, R., Benington, F., Morin, R.D. "Behavioral and serotonin receptor properties of 4-substituted derivatives of the hallucinogen 1-(2,5-dimethoxyphenyl)-2-aminopropane." *J. Med. Chem.* 25, 1163 (1982).
3. Shannon, M., Battaglia, G., Glennon, R.A., Titeler, M. "5-HT_1 and 5-HT_2 Binding properties of derivatives of the hallucinogen 1-(2,5-dimethoxy-phenyl)-2-aminopropane (2,5-DMA)." *Eur. J. Pharmacol.* 102, 23 (1984).
4. Glennon, R.A., McKenney, J.D., Lyon, R.A., Titeler, M. "5-HT_1 and 5-HT_2 binding characteristics of 1-(2,5-dimethoxy-4-bromophenyl)-2-aminopropane analogs." *J. Med. Chem.* 29, 194 (1986).
5. Glennon, R.A., Seggel, M.R., Soine, W.H., Herrick-Davis, K., Lyon, R.A., Titeler, M. "[^{125}I]-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane: An iodinated radioligand that specifically labels the agonist high affinity state of 5-HT_2 serotonin receptors." *J. Med. Chem.* 31, 5 (1988).

(OVER)

FOR RESEARCH PURPOSES ONLY - NOT FOR HUMAN USE OR CONSUMPTION

Cat. No. D-101 (cont'd)

6. Dabire, H., Chaouche-Teyara, K., Cherqui, C., Fournier, B., Schmitt, H. "DOI is a mixed agonist-antagonist at postjunctional 5-HT_2 receptors in the pithed rat." *Eur. J. Pharmacol.* 170, 109 (1989).
7. Appel, N.M., Mitchell, W.M., Garlick, R.K., Glennon, R.A., Teitler, M. "Autoradiographic characterization of (±)-1-(2,5-dimethoxy-4-[^{125}I]-iodophenyl)-2-aminopropane ([^{125}I]DOI) binding to 5-HT_2 and 5-HT_{1C} receptors in rat brain." *J. Pharmacol Exp. Ther.* 255(2), 843 (1990).

TELEPHONE ORDERS ARE NOT ACCEPTED FOR THIS PRODUCT.

APPENDIX 4

The buffers for immunocytochemistry and radioligand binding

a) Phosphate buffered saline (PBS)

b) Buffer A: [^{3}H]DPAT binding to 5HT1a receptors

c) Buffer B: [^{3}H]ketanserin binding to 5Ht2 receptors

0.01M Phosphate Buffered Saline

0.002M NaH_2PO_4; 0.08M Na_2HPO_4; 0.145M NaCl

Buffer A

0.17M Tris pH7.6

4mM $CaCl_2$

0.01% ascorbic acid

10μM pargyline

Buffer B

0.05M Tris pH7.7

PRODUCT INFORMATION

1. Solubility

a. Solubility in different solvents.

Solvent	Solubility in g/100 ml solution
water (pH = 7.1)	<0.001
methanol	3.5
ethanol	2.3
2-propanol	1.1
dichloromethane	>50
2-propanone	7.7
ethyl acetate	10
diethyl ether	1.7
hexane	0.042
toluene	18
N,N-dimethylformamide	21
tetrahydrofuran	44
4-methyl-2-pentanone	6.2
propylene glycol	0.82
polyethylene glycol 400	5.3
dimethylsulfoxide	6.4
2-butanone	12
acetonitrile	1.7
tetrachloroethene	6.1

b. Solubility in aqueous medium as a function of pH.

Solvent		pH of solution	Solubility in g/100 ml solution
HCl 0.1 N		1.2	0.048
HCl 0.01 N		3.2	0.39
citrate-HCl	buffer pH 2.0	2.4	0.062
citrate-HCl	buffer pH 4.0	4.1	0.007
citrate-NaOH	buffer pH 6.0	6.0	0.003
borate-HCl	buffer pH 8.0	7.9	<0.001
borate-KCl-NaOH	buffer pH 10.0	10.0	<0.001
NaOH 0.1 N		12.5	<0.001

APPENDIX 5

The measurement of tissue protein (Bradford`s Method)

PROTEIN ASSAY

From Bradford, 1976. Anal Biochem 248-254

INTRODUCTION

Protein concentration measured is proportional to blue colour of dye in protein environment range 5-50ug. *

METHOD

1)Set up standard curve in the range 5-50ug of protein. This is done by adding 10,20,50,100ul of 0.05%BSA to clean test tubes which gives 5,10,25,50ug of protein per tube respectively.A reagent blank is also set up by adding 10ul of a buffer (usually the same buffer which is present in the samples to be measured). BSA 0.05% = (0.5 mg ml⁻¹).

2)Add 10ul of sample to a clean test tube.

3)Add 3.3ml of Bradford Reagent [1] and mix.

4)Incubate at room temp for 5-30 mins.

5)Transfer contents to a cuvette and read absorbance at 595nM

6)Draw a graph of OD against PROTEIN for the standard curve and read the amount of protein present for the given OD.

[1] COOMASSIE BRILLIANT BLUE SOLUTION
A)Dissolve 100mg of coomassie brilliant blue-G (Sigma B-1131) in 50ml of ethanol.
B)Transfer to 1L flask on ice.
C)Add 100ml of 85% AnalaR phosphoric acid (85% w/v)
D)Mix until all blue colour disappears leaving a reddish brown solution.
E)Add 870ml of water.
F)Filter through 2 sheets of Whatman No.1 paper. [illegible]
*N.B. In the presence of 1M NaCl the sensitivity seems to be increased 10 fold, (See MELS LAB BOOK 1 P.57. DATE-13,06,86)

REFERENCE

BRADFORD M.M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding.
Analytical Biochemistry 72, 248-254.

APPENDIX 6

The protocol for Study 4.1

Study 4.1. A randomised single-blind study to determine the effect of Fluoxetine, compared with Dothiepin, on water balance in elderly, unipolar depressed patients.

EXPERIMENTAL PLAN

Patients

Forty male or female out-patients. This number is based on the ability to detect a statistically significant difference in water excretion (> 10%) between treatments, as determined from study 3.1.

Inclusions

Patients between the ages of 55 and 75 with major depressive illness defined by DSM IIIR criteria and Hamilton rating equal to or greater than 17 on the 17 point scale.

Biochemical normality (total protein, albumin, calcium, urea, creatinine, fasting blood glucose, total bilirubin, alkaline phosphatase, SGOT, gamma-GT, plasma Na, K, HCO_3, urinalysis, FBC), medical history and physical examination.

Exclusions

Patients with significant hepatic, renal, respiratory, cardiac, haematological or gastrointestinal diseases or requiring concomitant therapy.

Patients with any central nervous disorder other than depressive illness,.

Patients with any history of mania.

Patients considered a high suicide risk.

Patients with narrow angle glaucoma.

Patients with prostatism.

Patients who had taken any of the following medication in the previous 4 weeks.

antidepressants

neuroleptics

diuretics

drugs affecting cardiovascular system except calcium

channel blockers

anticonvulsants

Patients should not have taken monoamine oxidase inhibitors (MAOI's) or lithium within 2 weeks prior to commencing the study.

Patients taking benzodiazepines except in low doses as a hypnotic.

STUDY PROCEDURES

Fluoxetine: Initial dosing will be 20mg daily for first 3 weeks, optionally increasing at the discretion of the psychiatrist to 40 mg daily.

Dothiepin: 25 mg day 1-3

50 mg day 4-6

75 mg day 7-21

Allocation of treatment and randomisation

Randomisation was made by Lilly Research Centre. Allocation was made by the staff pharmacist at the Royal Victoria Infirmary once the patient had entered the trial.

Study period 1

Psychiatric rating (Hamilton Rating Scale) will be conducted at initial consultation.

The patients will undergo a pre-treatment standard oral water load test according to protocol 2.1.1.1.1. This will be followed by the Dexamethasone Suppression Test (see protocol below).

Fluoxetine or dothiepin will be given for one week, after which the patient will be visited or will attend the hospital, and a blood sample taken for urea/electrolytes. Linear analogue scales for nausea and thirst from the previous week will be collected. Any adverse events will be recorded.

After 3 weeks of treatment the patient should be assessed by Global Clinical Impression and a decision taken either to continue or change the treatment. Should a change in treatment (drug therapy) be indicated the patient should be withdrawn from the study and the investigator informed.

Study period 2

After 6 weeks of treatment the water load and Dexamethasone Suppression Test will be repeated. Formal psychiatric re-evaluation and rating will be conducted.

Dexamethasone Suppression Test

On the night of the water load test each patient will take 1 mg of dexamethasone orally on retiring to bed.

At 4pm the next day, a 5 ml blood sample for plasma cortisol will be taken.

APPENDIX 7

The DSM-IIIR criteria for major depression

Diagnostic criteria for Major Depressive Episode continued

B. (1) It cannot be established that an organic factor initiated and maintained the disturbance
(2) The disturbance is not a normal reaction to the death of a loved one (Uncomplicated Bereavement)

Note: Morbid preoccupation with worthlessness, suicidal ideation, marked functional impairment or psychomotor retardation, or prolonged duration suggest bereavement complicated by Major Depression.

C. At no time during the disturbance have there been delusions or hallucinations for as long as two weeks in the absence of prominent mood symptoms (i.e., before the mood symptoms developed or after they have remitted).

D. Not superimposed on Schizophrenia, Schizophreniform Disorder, Delusional Disorder, or Psychotic Disorder NOS.

Major Depressive Episode codes: fifth-digit code numbers and criteria for severity of current state of Bipolar Disorder, Depressed, or Major Depression:

1-Mild: Few, if any, symptoms in excess of those required to make the diagnosis, and symptoms result in only minor impairment in occupational functioning or in usual social activities or relationships with others.

2-Moderate: Symptoms or functional impairment between "mild" and "severe."

3-Severe, without Psychotic Features: Several symptoms in excess of those required to make the diagnosis, and symptoms markedly interfere with occupational functioning or with usual social activities or relationships with others.

4-With Psychotic Features: Delusions or hallucinations. If possible, specify whether the psychotic features are *mood-congruent* or *mood-incongruent*.

Mood-congruent psychotic features: Delusions or hallucinations whose content is entirely consistent with the typical depressive themes of personal inadequacy, guilt, disease, death, nihilism, or deserved punishment.

Mood-incongruent psychotic features: Delusions or hallucinations whose content does *not* involve typical depressive themes of personal inadequacy, guilt, disease, death, nihilism, or deserved punishment. Included here are such symptoms as persecutory delusions (not directly related to depressive themes), thought insertion, thought broadcasting, and delusions of control.

5-In Partial Remission: Intermediate between "In Full Remission" and "Mild," and no previous Dysthymia. (If Major Depressive Episode was superimposed on Dysthymia, the diagnosis of Dysthymia alone is given once the full criteria for a Major Depressive Episode are no longer met.)

6-In Full Remission: During the past six months no significant signs or symptoms of the disturbance.

0 Unspecified

Diagnostic criteria for Major Depressive Episode

Note: A "Major Depressive Syndrome" is defined as criterion A below.

A. At least five of the following symptoms have been present during the same two-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood, or (2) loss of interest or pleasure. (Do not include symptoms that are clearly due to a physical condition, mood-incongruent delusions or hallucinations, incoherence, or marked loosening of associations.)

(1) depressed mood (or can be irritable mood in children and adolescents) most of the day, nearly every day, as indicated either by subjective account or observation by others
(2) markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated either by subjective account or observation by others of apathy most of the time)
(3) significant weight loss or weight gain when not dieting (e.g., more than 5% of body weight in a month), or decrease or increase in appetite nearly every day (in children, consider failure to make expected weight gains)
(4) insomnia or hypersomnia nearly every day
(5) psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down)
(6) fatigue or loss of energy nearly every day
(7) feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick)
(8) diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others)
(9) recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide

APPENDIX 8

Consent forms and letters to consultants and GP`s of patients and volunteers in Studies 4.1 and 4.2

STUDY 4.1 INSTRUCTIONS TO PSYCHIATRISTS.

INITIAL VISIT

Age 55-75
Hamilton rating > 17 on 17 point scale.
Check for exclusions.

If patient seems to be eligible discuss the study briefly and contact Dr Faull bleep 2229

She will:
1. Explain the study in depth.
2. Take a medical and drug history.
3. Perform a brief physical examination.
4. Consent the patient.
5. Organize waterload and Dexamethasone tests.
6. Give the patient the antidepressant medication.
7. Send you a letter about the medication to be filed in the patients notes.
8. Send you a prescription form to complete at the next visit.

Arrange to see the patient in 3 weeks.
If you need to see the patient sooner, or wish that the patient does not have 3 weeks supply of antidepressant please inform Dr. Faull.

3 WEEK REVIEW.

Assess the patient by global clinical impression analogue scale: improved, ISQ, Deteriorated.
Decide whether to continue in the study.
If the patient is to be withdrawn, contact Dr. Faull.

She will
1. Take blood tests.
2. Arrange to do a waterload test if agreed by the patient.
3. Take data about the reason for withdrawal.

If the patient continues in the study:

Decide whether to increase the dose of antidepressant.

Complete the prescription form for your hospital pharmacy for further antidepressant for 4 weeks treatment and record the dose clearly on the patient information study sheet.

Arrange to see the patient in a further 3 weeks.

6 WEEK REVIEW>

Complete the global impression analogue scale.
Find out the time of the second waterload test (this will have been arranged with the patient by Dr. Faull.).
Plan further clinical management for after completion of the study, ie. after the waterload.

Department of Medicine,
Floor 4- Clinical Block,
Medical School,
Framlington Place,
Newcastle. NE2 4HH.

Dear Dr.

As you know your patient..... has been recruited into the study of the effect of Fluoxetine on water balance in depression and underwent the first waterload test today. The dexamethasone suppression test will be completed tomorrow and antidepressant treatment will start that night.

The medication randomly selected for your patient is.....and a four week supply has been given. A further supply for this patient is in your hospital pharmacy. A prescription form for access to this supply is attached. When you review the patient, please complete it for the same dose or increased dose of antidepressant for a further 3-4 weeks*.

As you are aware the protocol involves review by me at 1 and 6 weeks. If however if any of the following occur I should be most grateful if you could contact me on bleep 2229.

a) Patient wishes to withdraw from the study.
b) You decide to withdraw your patient from the study.
c) Medication, other than antidepressant, is altered*.
d) The patient is admitted to hospital.
e) Any other problems occur.

If the patient withdraws from the study prematurely I would like to perform a waterload test at that point, or at least take a blood sample for electrolytes and osmolality before the medication is altered in any way.

*Please note that this is a single blind trial. If you wish to increase the dose of antidepressant please try to do this at three weeks after starting treatment. You do not need to inform me at this point but record the change in dose on the study information sheet in the patients notes. Your pharmacy has tablets available for your patient if they need to be increased.

Please place this information sheet with the consent form in your patients notes for future reference. Thank you for your co-operation.

Yours sincerely,

Christina Faull.
Research Registrar.

APPENDIX C

PATIENT/SUBJECT INFORMATION AND CONSENT FORM

The Effect of Fluoxetine or Dothiepin on Water Balance in Depression Compared to Water Balance in Healthy Subjects

[Protocol No. B1Y-EW-E087]

Purpose of Study

It is thought that depression is in part due to an imbalance of biochemistry in some areas of the brain. Many of the drugs used to treat depression do so by correcting these imbalances. Sometimes these drugs also affect other areas of the brain which are not involved in depression. We are particularly interested in the way the antidepressant drug fluoxetine compared with another called dothiepin, may affect water balance in people with depression. There is some suggestion that very occasionally fluoxetine may disturb water balance, as do many other drugs used in the treatment of depression and other illnesses.

We are seeking 40 male or female patients aged 55 to 75 years for this research study comparing the effects of fluoxetine and dothiepin in water balance. Twenty volunteer subjects within the same age group will also be studied to act as a control panel. This may help us to understand why some drugs upset water balance and therefore prevent it from happening.

Study Description

Your suitability for the study will be assessed by your psychiatrist or the medical researcher. If you consent to participate in the study your involvement will be as follows:-

1. You will have a blood test to measure some biochemistry as a 'baseline'.

2. You will then have a test of water balance. This means coming to the Royal Victoria Infirmary with no breakfast or drink and taking no alcohol for 24 hours beforehand. A needle will be put in your arm through which to sample blood. Then you will drink about 3 pints of water. You will then have blood taken about every half hour and be asked to collect urine during the morning. You will also be asked how thirsty or nauseated you feel every hour. The test will last about 4½ hours. You will not be able to eat until afterwards and will be asked not to smoke before or during the test. You will also be interviewed that morning about your symptoms if you are taking fluoxetine or dothiepin.

3. You should inform the researcher of any medicines taken within 4 weeks before or during the study period.

You should be aware that fluoxetine or dothiepin in common with other drugs used in psychiatry, may impair your driving skills or mental performance.

You should avoid taking other medicines during the study. You should inform the investigator of the names and doses of any medication that is taken.

The study has been approved by the Ethical Committee of the Health Authority and you will be informed of significant new findings during the study which may relate to your willingness to continue participation in the study.

Insurance exists to ensure that compensation can be paid in the rare event of anyone suffering harm or injury. Please ask if you require further information.

Your participation is completely voluntary and you may withdraw your consent at any time during the study. The investigator reserves the right to withdraw you from the study at any time.

Your General Practitioner will be informed about your participation in this study, and if you are currently having treatment with either Fluoxetine or dothiepin the Psychiatrist will also know that you are having this test, which will not affect the treatment you have been prescribed.

If you have any questions concerning the study please do not hesitate to ask. The Consultant in charge of the study is Dr. P. Baylis, and Dr. Christina Faull will be organising the study. Please sign the consent form if you then decide to take part in the study.

CONSENT FORM

The Effect of Fluoxetine Compared with Dothiepin on Water Balance in Depression

[Protocol No. B1Y-EW-E087]

I have received a copy of "Patient/Subject Information' and the purpose, procedures and effects of this study have been explained to me by Dr.

I understand the requirements and agree to cooperate with the study procedures and disclose all the necessary information to the Doctor's involved. I understand that the results of the water load research may not be directly of benefit to me.

I understand that I am under no obligation to take part and may withdraw from the study at any time. Withdrawal from the study will not affect any treatment I may be receiving.

I consent to all information obtained being put at the disposal of medical authorities, should this be required and that this information will remain completely confidential.

I have read and understand this information and consent form. I voluntarily consent to participate in the research outlined.

Full Name: .....

Date of Birth: ..... Telephone No: .....

Present Address: .....

.....

.....

.....

Patient's Signature: ..... Date: .....

Witness' Signature ..... Date: .....

The hormonal and biochemical responses of healthy elderly people to oral water load

Purpose of study

It is thought that as we grow older our bodies are less able to respond to certain situations, in particular there may be some change in the way we excrete water. Our group of researchers has a particular interest in problems of water balance and we have already done a lot of research in people who have known problems. In order to fully understand the problems we need a very clear understanding of the normal changes with age.

This study is to establish the hormonal and biochemical responses of a group of healthy elderly people to an oral water load. We are seeking people in the age group of 55 to 75 years who are physically well.

Study Description

Your suitability for the study will be assessed by the medical researcher. If you consent to participate in the study your involvement will be as follows:-

1. You will have a physical examination and a blood test to exclude any other problems with your health and to make a 'baseline' assessment.
2. You will then have a test of water balance. This means coming to the Royal Victoria Infirmary with no breakfast or drink and taking no alcohol for 24hrs beforehand. A needle will be put in your arm through which to sample blood. Then you will drink about 3 pints of water (20ml/kg body weight). You will then have blood taken about every half hour and be asked to collect urine during the morning. You will also be asked about how thirsty or nauseated you feel. The test will last about 4½hrs. You will not be able to eat until afterwards and will be asked not to smoke before or during the test.

Your family Doctor will know about your participation in the test . If any of your results are not normal you will be told about them by the researchers and you will be given any advice necessary.

Insurance exists to ensure that compensation can be paid in the rare event of anyone suffering harm or injury. Please ask if you require further information. Your participation in the study is completely voluntary and you can withdraw from the study at any time. If you have any question about the study at any point please do not hesitate to ask.

Please sign the consent form if you decide to take part in the study.

The hormonal and biochemical responses
of healthy elderly people to oral water load

I have received a copy of the information about the study and the purpose, procedures and effects have been explained to me.

I understand the requirements and agree to cooperate and disclose all the necessary information. I realise that the results of the water load research may not be directly of benefit to me.

I consent to all information obtained being put at the disposal of medical authorities, should this be required and that this information will remain completely confidential.

I have read and understood this information and consent from. I voluntarily consent to participate in the research outlined.

Full name.....
Date of Birth.....Tel. No.....

Present address: .....
.....
.....
.....

Signature..... Date.....
Witness's signature..... Date.....

Department of Medicine,
Floor 4- Clinical Block,
Medical School,
Framlington Place,
Newcastle. NE2 4HH.

Dear Dr.

Your patient.....has volunteered to take part in a study we are performing, at the Royal Victoria Infirmary, to explore the effect of age on waterbalance. I enclose a copy of the information that has been given to your patient for your interest.

If you have any questions about this work or are in any way concerned about your patient in connection with this study, please do not hesitate to contact me. I can be reached through the Royal Victoria Infirmary switchboard (232-5131) on bleep 2229.

Yours sincerely

Dr.Christina Faull.
Research Registrar.

Department of Medicine,
Floor 4- Clinical Block,
Medical School,
Framlington Place,
Newcastle. NE2 4HH.

Dear Dr.

Your patient.....who was referred to Dr. ..... for treatment of depression has consented to take part in a study we are performing, at the Royal Victoria Infirmary, to explore the interaction of serotonin and waterbalance. I enclose a copy of the information that has been given to your patient for your interest.

If you have any questions about this work or are in any way concerned about your patient in connection with this study, please do not hesitate to contact me. I can be reached through the Royal Victoria Infirmary switchboard (232-5131) on bleep 2229.

Yours sincerely

Dr.Christina Faull.

Research Registrar.

Department of Medicine,
Floor 4- Clinical Block,
Medical School,
Framlington Place,
Newcastle. NE2 4HH.

Dear Dr.

As you know your patient..... has been recruited into the study of the effect of Fluoxetine compared with Dothiepin on water balance in depression and underwent the waterload test today. The results of this test appear to be abnormal. I have discussed this with the patient and will be continuing to follow the course of his/her serum biochemistry on a weekly basis. If the abnormality becomes clinically important I shall obviously be discussing this with you in more detail. If you have any queries about this please do not hesitate to contact me on RVI bleep 2229.

Thank you for your help in this research.

Yours sincerely,

Christina Faull
Research Registrar

Department of Medicine,
Floor 4- Clinical Block,
Medical School,
Framlington Place,
Newcastle. NE2 4HH.

Dear Dr.

As you know your patient..... has been recruited into the study of the effect of Fluoxetine compared with Dothiepin on water balance in depression and underwent the waterload test today. The results of this test appear to be normal and I do not need to follow the patient further. I have discussed this with the patient. I should be very interested to hear if problems with hyponatraemia do arise at any point in this patient's illness.

Thank you for your help with this project.

Yours sincerely,

Christina Faull
Research Registrar

APPENDIX 9

The Hamilton rating scale for depression (HRSD)

FLUOXETINE DEPRESSION -

Study Code | Investigator No. | Patient No. | Visit No. 0 0

PLEASE COMPLETE LEGIBLY USING A BLACK BALL-POINT PEN

HAMILTON RATING SCALE FOR DEPRESSION - (HAMD)

Circle the number of the ONE "CUE" which best characterizes the patient during the past therapy period

1 DEPRESSED MOOD (Sadness, hopeless, helpless, worthless)	0	Absent
	1	These feeling states indicated only on questioning
	2	These feeling states spontaneously reported verbally
	3	Communicates feeling states non-verbally — i.e., through facial expression posture, voice, and tendency to weep
	4	Patient reports VIRTUALLY ONLY these feeling states in his spontaneous verbal and non-verbal communication
2 FEELINGS OF GUILT	0	Absent
	1	Self-reproach feels he has let people down
	2	Ideas of guilt or rumination over past errors or sinful deeds
	3	Present illness is a punishment Delusions of guilt
	4	Hears accusatory or denunciatory voices and/or experiences threatening visual hallucinations
3 SUICIDE	0	Absent
	1	Feels life is not worth living
	2	Wishes he were dead or any thoughts of possible death to self
	3	Suicide ideas or gesture
	4	Attempts at suicide (any serious attempt rates 4)
4 INSOMNIA EARLY	0	No difficulty falling asleep
	1	Complains of occasional difficulty falling asleep — i.e., more than ½ hour
	2	Complains of nightly difficulty falling asleep
5 INSOMNIA MIDDLE	0	No difficulty
	1	Patient complains of being restless and disturbed during the night
	2	Waking during the night — any getting out of bed rates 2 (except for purposes of voiding)
6 INSOMNIA LATE	0	No difficulty
	1	Waking in early hours of the morning but goes back to sleep
	2	Unable to fall asleep again if gets out of bed
7 WORK AND ACTIVITIES	0	No difficulty
	1	Thoughts and feelings of incapacity, fatigue or weakness related to activities, work or hobbies
	2	Loss of interest in activity, hobbies or work — either directly reported by patient, or indirect in listlessness, indecision and vacillation (feels he has to push self to work or join activities)
	3	Decrease in actual time spent in activities or decrease in productivity In hospital, rate 3 if patient does not spend at least three hours a day in activities (hospital job or hobbies) exclusive of ward chores
	4	Stopped working because of present illness In hospital, rate 4 if patient engages in no activities except ward chores, or if patient fails to perform ward chores unassisted
8 RETARDATION (Slowness of thought and speech, impaired ability to concentrate, decreased motor activity)	0	Normal speech and thought
	1	Slight retardation at interview
	2	Obvious retardation at interview
	3	Interview difficult
	4	Complete stupor
9 AGITATION	0	None
	1	Fidgetiness
	2	"Playing with" hands, hair, etc
	3	Moving about, can't sit still
	4	Hand-wringing, nail-biting, hair-pulling, biting of lips
10 ANXIETY PSYCHIC	0	No difficulty
	1	Subjective tension and irritability
	2	Worrying about minor matters
	3	Apprehensive attitude apparent in face or speech
	4	Fears expressed without questioning

FLUOXETINE DEPRESSION -

Study Code | Investigator No. | Patient No. | Visit No. 0 0

PLEASE COMPLETE LEGIBLY USING A BLACK BALL-POINT PEN

HAMILTON RATING SCALE FOR DEPRESSION - (HAMD) (Cont'd)

11 ANXIETY SOMATIC	0 Absent 1 Mild 2 Moderate 3 Severe 4 Incapacitating	Physiological concomitants of anxiety, such as: Gastro-intestinal—dry mouth wind, indigestion diarrhea, cramps, belching Cardio-vascular—palpitations, headaches Respiratory—hyperventilation, sighing Urinary frequency Sweating
12 SOMATIC SYMPTOMS GASTRO-INTESTINAL	0 None 1 Loss of appetite but eating without staff encouragement Heavy feelings in abdomen 2 Difficulty eating without staff urging Requests or requires laxatives or medication for bowels or medication for G I symptoms	
13 SOMATIC SYMPTOMS GENERAL	0 None 1 Heaviness in limbs, back or head Backaches, headache, muscle aches Loss of energy and fatigability 2 Any clear-cut symptom rates 2 Debilitating.	
14 GENITAL SYMPTOMS	0 Absent 1 Mild 2 Severe	Symptoms such as: Loss of libido Menstrual disturbances
15 HYPOCHON-DRIASIS	0 Not present 1 Self-absorption (bodily) 2 Preoccupation with health 3 Frequent complaints, requests for help, etc 4 Hypochondriacal Delusions	
16 LOSS OF WEIGHT	ACTUAL WEIGHT CHANGE (since last visit) 0 No weight loss or weight loss NOT caused by present illness 1 Weight loss probably caused by present illness 2 Definite weight loss caused by present illness	
17 INSIGHT	0 Acknowledges being depressed and ill 1 Acknowledges illness but attributes cause to bad food, climate, overwork virus, need for rest, etc 2 Denies being ill at all	

TOTAL 17 POINT SCORE

APPENDIX 10

Case reports of depression and hyponatraemia

This appendix presents two patients with depression who developed hyponatraemia during treatment with a SSRI. Both patients declined further investigation. These case reports help to illustrate the clinical relevance of some of the work of this thesis and the paucity of understanding of hyponatraemia in mental illness.

1. CH, a 43yr male was diagnosed as having systemic lupus erythematosus (SLE) in 1981. He had received a variety of treatments with little response. There was initially only cutaneous involvement but the disease had gradually progressed to be more systemic with occasional episodes of dysphasia and gate disturbance but with no residual symptomatic deficit. He had been admitted to a dermatology ward with an acute episode of SLE in April 1991 which was complicated by short episode hyponatraemia (lowest pNa 124mmol/l). Because of his physical illness he had not been working for 6 months before he presented to a psychiatric unit in August 1991 with attempted suicide by overdose. This was not physically serious but as he had had marked depressive symptoms for the previous 18 months and these were gradually worsening he was admitted for observation and treatment.

Other significant features of his history and examination were :-

He appeared very agitated.

He had increased his alcohol intake to 10units/day over 6months.

He had no previous history of mental illness despite severe physical illness for many years.

There was no family history of psychiatric illness.

Several neurological features were noted on examination: Mild disorientation in time. Mild impairment of short term memory with confabulation.

Mild hypertension was also noted.

Medications on admission:-

Prednisolone 25mg daily.

Intermittent amitriptyline and temazepam as hypnotic.

Differential diagnosis:-

Major depression.

Adjustment disorder

Organic mood disorder secondary to SLE or steroids.

Alcohol related mood disorder and possible neurological damage.

Investigations:-

Neurological investigations:

CT scan showed multiple small infarcts, mostly old.

LP showed normal protein and cell content of CSF suggestive of quiescent SLE.

Electrolytes:

pNa 136mmol/l. Creatinine 74mmol/l urea 1.7 mmol/l.

Management:-

He was commenced on regular amitriptyline, thiordiazine and lorazepam and his oral steroids gradually reduced.

Progress at 4 weeks:-

There was no improvement in his affect and some worsening of agitation. Some worsening of skin lesions was also noted. He developed severe back pain consequent to a vertebral crush fracture (secondary to steroid induced osteoporosis).

The amitriptyline was stopped and the SSRI paroxitene commenced.

Progress at 6 weeks:-

He had a grand mal fit with prolonged post ictal drowsiness and confusion.

Investigations:-

pNa 115mmol/l pOS 247mOsm/kg urea 1.4 mmol/l Creatinine 79mmol/l.

Volume status: Thought to be normovolaemic.

CT scan showed a small, new frontal infarct.

Diagnosis:-

SIAD probably secondary to worsening of cerebral SLE, although possibly associated with the paroxitene treatment.

Management:-

500ml 1.8%NaCl (50ml/hr).

Fluid restriction: 500ml/24hr

Commenced on phenytoin.

Paroxitene stopped.

Progress:-

Conscious level and pNa returned to normal over 7 days.

He has remained an inpatient because of continued, resistant depression and worsened cutaneous SLE. His pNa remained within the normal range for 3 months until late January 1992 when it was noted to be falling (122mmol/l). He was taking no psychoactive medications at that time. Fluid restriction was reinstituted and the pNa returned to normal in 14 days. Due to persistent depression he was commenced on trazadone on 31/3/92 (metabolite mCPP) with no adverse effect on pNa after 14 days.

2. MD, 60yr female had had multiple episodes of unipolar major depression with psychotic features over 10 years. She also had the physical illnesses of ischaemic heart disease and hypothyroidism, for both of which she was receiving medications. Her electrolytes had been measured in November 1991 and were normal at that time.

Regular medications:-

Frusemide 40mg, Amiloride 5mg, Thyroxine 125mcg, Isosorbide dinitrate 20mg TDS, Aetenolol 75mg, Depixol 60mg 2 weekly, procyclidine 5mg BD.

She was commenced on Fluoxetine 20mg daily in late January 1992 for recurrence of depression. The dose was increased to 40mg daily after 2 weeks due to lack of mood improvement. She was admitted to hospital in mid February due to worsening psychotic symptoms and her pNa was found to be 129mmol/l. She was also noted to have a urine infection and mild urinary retention (possibly

behavioural, secondary to mental illness).

Five days later her pNa was 124mmol/l. Her volume status was not determined.

Management:-

Fluoxetine and were diuretics stopped.

Fluid restriction: 500ml/24hr.

Depression was treated by ECT and chlorpromazine 50mg TDS with good effect.

Progress:-

Her pNa returned to normal over 10 days with no apparent adverse effects.

DISCUSSION

These brief case histories have shown that it is often difficult to discern aetiology of the hyponatraemia which occurs in association with depression. Both subjects had physical illness and were taking multiple drug therapies. SSRIs were associated with the occurrence of hyponatraemia in both cases. This may only reflect the increase in clinical use of theses drugs, particularly in resistant mental illness, or it could indicate that hyponatraemia in depression treated with drugs that increase 5HT neurotransmission is indeed related to dysfunction of central 5HT neurotransmission. Subject one had had a previous episode of hyponatraemia and the hyponatraemia recurred when drug free, suggesting that it was related to his physical and/or mental illness and not to drug therapy. Subject two had a very severe mental illness but had had previous similar episodes without hyponatraemia developing. These had not been treated with Fluoxetine. This fact and the timing of onset of hyponatraemia in relation to the commencement of antidepressant therapy would suggest that there was an association with Fluoxetine treatment in this woman.

APPENDIX 11

The data of the rat studies in Section 5

Study 5.1 **The effect of plasma hyperosmolality on 5HT neurotransmission in the neurohypophysis: N=normal, H=hypotonic**

No	Type	Wt (g)	Injection vol (ml)	pNa (mmol/l)	pOs (mosm/kg)	pAVP (pmol/l)	5HT (pmol/mg)	5HIAA (pmol/mg)	5HIAA:5HT
1	N1	214	4.3	140	300	4.2	18.5	16.0	0.9
3	N2	186	3.7	141	282	3.1	8.7	11.9	1.4
5	N3	185	3.7	142	308	4.7	8.9	23.2	2.6
7	N4	187	3.7	143	306	1.2	9.5	13.7	1.4
9	N5	198	4.0	141	297	0.8	15.1	17.1	1.2
11	N6	233	4.7	139	299	2.0	15.8	15.6	1.0
2	H1	203	4.1	146	309	11.4	15.1	17.7	1.2
4	H2	183	3.7	145	305	12.0	15.4	14.1	0.9
6	H3	186	3.7	151	316	77.8	8.7	8.3	1.0
8	H4	188	3.8	146	311	8.0	28.4	25.3	0.9
10	H5	189	3.8	145	306	13.8	22.5	23.9	1.1
12	H6	234	4.7	145	307	15.1	8.7	11.9	1.4

Study 5.2.1 The effect of acute Fluoxetine on osmoregulated AVP secretion

Group: Normal saline pretreatment : normal saline stimulus (NN)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)
6	250	288	125	0.7
18	242	290	136	2.2
22	260	303	150	6.2
28	263	315	151	130
30	254	291	144	1.0
38	245	296	145	1.1
43	270	301	144	0.7
48	260	298	146	0.7
55	263	307	147	160
60	259	300	147	0.9

Group: Fluoxetine pretreatment : normal saline stimulus (FN)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)
1	245	296	139	2.4
10	239	290	136	0.8
13	240	282	132	34.0
17	244	296	140	13.3
24	244	311	151	3.8
34	250	299	145	1.9
36	250	297	146	1.0
44	260	296	143	0.6
50	255	300	146	13.2
56	264	300	146	0.5

Study 5.2.1 (Cont/d) The effect of acute Fluoxetine on osmoregulated AVP secretion

Group: Normal saline pretreatment : hypotonic stimulus (NL)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)
3	246	280	129	170
9	237	287	134	14.7
12	249	282	133	<0.6
20	240	281	135	0.8
26	255	288	139	0.8
27	250	298	144	15.6
39	249	285	142	0.8
41	243	297	142	1.4
46	266	291	139	<0.6
57	254	293	146	<0.6
61	278	295	150	20.0

Group: Fluoxetine pretreatment : hypotonic stimulus (FL)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)
4	224	282	131	<0.6
7	238	296	133	1.0
16	250	278	133	0.6
23	260	289	139	0.6
31	247	285	140	0.6
35	250	295	143	1.6
47	257	288	139	<0.6
57	271	292	141	0.6
53	261	292	140	0.8
59	249	291	143	6.0

Study 5.2.1 (Cont/d) The effect of acute Fluoxetine on osmoregulated AVP secretion

Group: Normal saline pretreatment : hypertonic stimulus (NH)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)
2	220	309	142	25.0
8	244	295	142	9.1
14	236	294	139	4.7
15	255	298	142	3.0
21	242	311	146	4.4
323	256	314	150	10.0
33	245	310	149	8.4
49	253	328	159	250
52	250	313	152	430
54	246	307	151	7.1

Group: Fluoxetine pretreatment : hypertonic stimulus (FH)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)
5	238	300	141	2.9
11	251	298	141	9.1
19	249	292	140	0.7
25	259	310	144	6.2
29	244	313	151	23.2
37	236	309	152	76
40	250	307	153	6.6
42	251	307	147	6.2
[illegible]	45[illegible]	271[illegible]	310[illegible]	149	1.3
58	254	309	151	24.8

Study 5.2.2 The effect of acute Fluoxetine on osmoregulated AVP

Group: Normal saline : hypertonic stimulus (NH)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
4	304	314	155	5.6	41
5	294	320	157	173	47
12	297	309	154	120	42
18	265	326	-	64	43
20	280	308	157	27.2	41
25	225	304	157	8.4	46
30	244	313	153	192	39
34	268	317	155	16.8	41
35	237	324	158	136	45
39	236	318	155	>400	46

Group: Fluoxetine : hypertonic stimulus (FH)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
1	245	310	154	16.8	40
6	279	321	153	400	43
8	268	314	155	44	33
13	250	322	161	256	45
16	252	312	155	148	42
23	283	312	153	15.6	43
24	279	325	154	256	48
29	275	314	-	40	44
33	292	316	152	10	44
40	276	310	154	16.8	48

Study 5.2.2 (Cont/d) The effect of acute Fluoxetine on osmoregulated AVP

Group: Normal saline : hypotonic stimulus (NL)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
3	288	291	147	6.5	46
7	277	291	-	360	48
11	274	293	145	92	46
15	290	295	141	<0.6	45
17	276	311	155	112	50
22	249	313	150	192	44
26	228	295	149	>400	40
27	238	298	155	400	44
32	236	304	153	4.2	46
37	233	294	148	<0.6	43

Group: Fluoxetine : hypotonic stimulus (FL)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
2	233	299	151	6.5	45
9	250	-	-	84	46
10	241	-	-	240	46
14	262	305	-	3.6	45
19	260	307	152	>400	50
21	293	307	154	9.2	46
28	305	291	144	<0.6	46
31	283	305	149	4.4	46
36	280	309	145	88	49
38	272	305	141	5.6	47

Study 5.2.3 The effect of volume of Fluoxetine injection on AVP response to hypotonic stimulus

Group: High volume fluoxetine : hypo-osmotic stimulus (H)

No	Wt (g)	pOs (mosm/kg)	pAVP (pmol/l)
1	255	288	<0.6
4	242	288	<0.6
5	234	292	<0.6
7	257	297	<0.6
11	225	289	<0.6
12	242	298	<0.6

Group: Low volume fluoxetine : hypo-osmotic stimulus (L)

No	Wt (g)	pOs (mosm/kg)	pAVP (pmol/l)
2	250	287	<0.6
3	262	296	<0.6
6	259	294	<0.6
8	241	288	<0.6
9	266	294	0.9
10	282	294	0.6

Study 5.2.4 The effect of handling and social stress on osmoregulation

Group: Early randomisation (A)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
2	260	154	-	1.4	42
3	270	148	298	1.4	41
6	275	156	306	1.0	39
8	268	144	300	5.2	41
10	264	150	302	<0.6	46
11	262	149	300	1.7	43

Group: Late randomisation (B)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
1	285	155	298	1.4	44
4	269	150	298	2.5	41
5	263	155	307	12.0	40
7	258	145	299	0.7	43
9	288	142	297	0.8	40
12	287	146	296	<0.6	41

Study 5.2.5. The effect of acute Fluoxetine on basal and osmotically stimulated AVP secretion

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)	[^{3}H]DPAT (fmol/mg)	[^{3}H]ketanserin (fmol/mg)	5HIAA:5HT
Group: Vehicle pretreatment : no osmotic stimulus (NN)								
1	263	160	-	<0.6	39	61	802	1.80
5	263	151	298	3.6	41	108	113	1.94
11	267	148	298	1.3	40	140	143	1.48
18	274	148	305	1.7	42	75	419	0.96
29	278	145	299	2.3	43	57	392	1.18
31	270	144	296	1.0	42	96	289	1.16
36	274	146	296	1.3	39	147	534	1.22
43	258	151	301	3.0	40	100	435	0.83
Group: Fluoxetine pretreatment : no osmotic stimulus (FN)								
4	272	153	305	10.0	40	74	901	1.49
9	267	151	-	8.0	48	64	827	1.21
14	262	150	-	2.3	46	443	366	1.44
20	266	145	296	8.8	48	86	1081	0.88
28	250	147	302	40	51	36	381	1.13
32	271	153	309	11.6	49	104	392	0.84
41	264	150	297	7.6	47	101	618	0.83
46	270	149	291	7.6	45	95	932	0.83

Study 5.2.5 (Cont/d) The effect of acute Fluoxetine on basal and osmotically stimulated AVP secretion

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
Group: Vehicle pretreatment: Hyperosmotic stimulus (NH)					
8	264	154	303	11.2	43
12	267	156	316	15.2	41
16	265	151	310	10.0	42
23	268	163	316	13.2	43
26	250	155	315	9.6	41
35	259	150	305	15.6	34
39	256	152	302	5.2	39
45	257	156	308	7.1	43
Group: Fluorexetine pretreatment: Hyperosmotic stimulus (FH)					
3	265	164	-	15.6	44
7	257	156	303	12.8	44
15	270	163	-	33.8	45
21	242	154	316	8.9	44
22	250	154	307	27.2	47
33	266	154	315	12.8	41
37	259	159	311	35.6	46
40	263	159	305	22.7	43

Study 5.2.5 (Cont/d) The effect of acute Fluoxetine on basal and osmotically stimulated AVP secretion

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
Group: Vehicle pretreatment : hypoosmotic stimulus (NL)					
6	267	152	295	<0.6	43
10	262	142	286	<0.6	42
17	261	145	294	<0.6	45
19	269	145	196	<0.6	47
25	272	147	290	<0.6	43
30	267	141	294	<0.6	41
38	272	146	295	<0.6	42
48	284	153	294	<0.6	43
Group: Fluoxetine pretreatment : hypo-osmotic stimulus (FL)					
2	260	159	-	<0.6	51
13	269	149	296	<0.6	43
24	259	150	290	<0.6	48
27	251	146	299	0.8	49
34	256	145	288	<0.6	44
42	239	149	288	<0.6	45
44	272	148	289	1.0	48
47	261	146	293	3.5	48

Study 5.3 To explore the effect of Ritanserin, a $5HT_2/5HT_{1C}$ antagonist, on osmotically stimulated AVP release

Group: Vehicle pretreatment : no osmotic stimulus (NN)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)	5HT:5HIAA
2	290	297	143	1.0	42	1.54
8	292	297	131	<0.6	41	1.44
16	303	293	141	>40	45	1.36
22	276	295	142	1.0	38	1.46
30	270	296	141	1.2	46	1.80
33	285	298	142	>40	44	1.60
40	272	290	138	<0.6	39	1.10
45	281	296	141	5.0	38	1.80
51	285	294	140	1.9	40	1.38
57	293	300	147	2.0	40	0.88

Group: Ritanserin pretreatment : no osmotic stimulus (RN)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)	5HT:5HIAA
5	266	297	142	1.6	41	1.47
7	294	301	140	1.6	42	1.69
13	290	292	139	<0.6	42	2.06
20	270	292	140	0.8	41	1.71
25	288	295	143	<0.6	41	2.32
31	267	295	143	5.1	45	3.14
35	267	297	142	1.1	45	1.84
42	270	293	142	>40	44	2.14
48	197	290	139	3.2	41	1.95
54	285	296	146	1.2	39	1.94
59	291	297	138	1.0	41	2.49

Study 5.3 (Cont/d) To explore the effect of Ritanserin, a $5HT_2/5HT_{1C}$ antagonist, on osmotically stimulated AVP release

Group: Vehicle pretreatment : hypertonic stimulus (NH)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
4	278	308	148	5.6	45
9	276	309	148	9.6	40
15	279	310	150	14.8	34
21	279	307	150	>40	40
26	292	313	152	2.7	40
36	288	306	148	4.0	41
39	277	312	149	>40	42
46	280	313	154	16.0	42
53	264	304	152	8.4	39
58	283	305	152	14.4	43

Group: Ritanserin pretreatment : hypertonic stimulus (RH)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
3	288	304	146	6.8	46
14	277	308	148	11.6	38
18	286	301	146	3.7	38
23	299	311	150	>40	-
28	278	315	150	28.8	45
32	296	305	141	4.0	42
41	281	298	142	8.0	44
43	273	307	149	11.2	41
49	268	304	149	8.8	43
60	276	315	154	6.8	41

Study 5.3 (Cont/d) To explore the effect of Ritanserin, a 5HT$_2$/5HT$_{1C}$ antagonist, on osmotically stimulated AVP release

Group: Vehicle pretreatment : hypotonic stimulus (NL)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
6	284	285	134	<0.6	43
10	287	291	138	<0.6	42
17	299	285	136	2.1	44
27	272	291	139	4.9	48
29	279	290	136	<0.6	43
34	282	284	136	<0.6	41
47	270	290	140	<0.6	42
50	274	286	137	1.1	43
52	284	284	136	<0.6	43
56	270	290	144	<0.6	40

Group: Ritanserin pretreatment : hypotonic stimulus (RL)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
1	293	284	136	<0.6	44
11	294	288	137	<0.6	41
12	276	285	135	<0.6	47
19	278	291	139	>40	42
24	280	296	142	9.6	44
37	297	287	123	<0.6	40
38	287	284	134	<0.6	46
44	295	279	134	<0.6	43
55	275	290	131	<0.6	37
61	312	288	137	1.1	42

Study 5.4 **Does Ritanserin inhibit the fluoxetine augmented AVP response to plasma hyperosmolality?**

Group: vehicle and vehicle (NN)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
4	292	299	147	5.2	37
7	297	309	152	4.8	37
11	280	313	155	12.8	40
15	291	311	154	9.6	41
19	308	314	149	6.8	39
22	291	306	151	6.0	34
27	295	316	153	17.6	41
30	301	314	153	2.6	40

Group: Vehicle and fluoxetine (NF)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
3	285	309	152	27.3	47
5	287	315	156	>32	40
10	284	312	153	27.1	44
13	289	310	151	17.7	48
17	278	306	149	21.6	46
21	295	313	148	6.0	40
25	289	313	154	>32	48
31	293	317	153	11.4	47

Study 5.4 (Cont/d) Does Ritanserin inhibit the fluoxetine augmented AVP response to plasma hyperosmolality?

Group: Ritanserin and vehicle (RN)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
1	283	308	148	8.0	42
6	294	320	158	11.6	38
8	302	316	153	26.1	40
12	286	312	151	13.2	40
20	296	309	154	15.6	34
23	286	310	152	9.2	36
28	289	312	152	8.4	37
29	294	315	153	14.0	40

Group: Ritanserin and fluoxetine (RF)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
2	301	320	153	21.6	37
9	287	309	152	23.1	42
14	295	319	158	20.0	48
16	296	313	148	27.8	41
18	283	314	151	15.2	39
24	298	308	152	12.0	40
26	291	317	144	11.0	48
32	300	306	149	23.6	48
33	303	314	152	28.0	40

***Study 5.5. The effect of the $5HT_2$ agonist DOI on osmoregulated AVP.**

Group: Vechide pretreatment : no osmotic stimulus (NN)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)	5HT:5HIAA
10	283	303	154	2.6	38	0.90
11	297	300	152	3.6	41	0.94
16	296	300	151	1.4	40	0.94
23	305	305	150	2.0	44	1.94
27	308	300	150	1.4	43	1.70
38	288	302	153	1.1	40	1.03
45	288	303	155	1.2	43	1.42
49	290	302	154	1.6	40	0.84
53	293	302	153	2.8	42	2.98
59	296	298	154	1.0	41	1.06

Group DOI: pretreatment: no osmotic stimulus (DN)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)	5HT:5HIAA
6	305	303	153	10.8	44	1.58
13	287	314	153	3.8	43	1.26
20	293	302	156	1.4	43	1.89
25	266	302	156	2.2	40	1.93
29	283	299	154	1.1	44	2.58
36	266	335	168	>50	48	1.37
43	294	304	156	1.4	40	1.78
47	270	302	153	2.0	45	1.18
54	293	298	150	1.1	44	1.41
57	262	210	157	37.3	46	2.45

Study 5.5 (Cont/d) The effect of the $5HT_2$ agonist DOI on osmoregulated AVP.

Group: Vehicle pretreatment: hypertonic stimuls (NH)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
4	299	316	159	5.6	44
5	278	316	163	0.6	44
14	280	320	161	18.8	41
18	280	319	166	24.0	39
30	290	318	166	7.8	44
34	304	308	157	7.2	42
41	304	318	163	19.2	39
42	284	313	163	12.0	38
50	280	314	160	12.0	36
55	280	313	161	15.6	38

Group: DOI pretreatment: hypertonic stimulus (DH)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
2	288	316	161	18.4	39
7	290	318	162	8.4	39
17	288	309	158	9.6	40
21	299	321	164	12.0	40
26	303	316	167	14.0	31
33	280	314	158	17.2	43
37	278	321	162	14.8	40
44	274	313	161	8.0	42
52	293	317	163	17.6	40
58	299	309	153	15.6	42

Study 5.5 (Cont/d) The effect of the $5HT_2$ agonist DOI on osmoregulated AVP.

Group: Vehicle pretreatment : hypotonic stimulus (NL)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
3	274	296	151	<0.6	46
8	310	299	149	<0.6	41
12	290	291	145	<0.6	44
19	288	294	150	0.7	41
24	289	299	153	<0.6	41
32	291	299	154	<0.6	40
40	290	291	149	<0.6	42
48	293	286	145	<0.6	42
51	296	294	149	0.7	41
60	292	289	147	<0.6	44

Group: DOI pretreatment : hypotonic stimulus (DL)

No	Wt (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
1	276	297	154	<0.6	41
9	277	297	151	1.0	39
15	285	297	153	0.8	44
22	260	290	153	1.1	43
28	276	299	151	0.9	45
31	291	298	154	0.9	46
35	295	291	147	0.6	-
39	286	292	151	0.6	41
46	295	295	139	<0.6	37
56	278	292	151	-	46

Study 5.6 **The effect of lesion of 5HT neurons on basal osmotic status and neurochemistry. (ND = not detectable)**

Group: Sham lesion (SHAM)

No	pOs (mosm/kg)	pAVP (pmol/l)	HCT (%)	[^{3}H]DPAT (fmol/mg)	[^{3}H]ketanserin (fmol/mg)	5HT (nmol/mg)	5HIAA (nmol/mg)	5HIAA:5HT (nmol/mg)
1	307	1.8	38	17	455	700.00	784.6	1.121
2	295	1.8	36	31	596	20.8	32.5	1.559
3	305	1.0	35	105	204	51.2	13.9	1.033
4	304	0.7	-	113	344	28.9	45.5	1.572
5	302	1.7	36	80	236	21.4	41.9	1.958
6	305	1.3	-	104	366	35.7	77.1	2.160
Group: 5,7, DHT lesion (LESION)								
7	305	2.3	39	128	143	ND	ND	ND
8	303	3.2	38	11	489	ND	ND	ND
9	306	3.0	37	116	276	31.1	40.5	1.304
10	302	1.8	38	105	450	2.2	3.6	1.633
11	303	3.2	32	82	322	38.6	50.8	1.315
12	330	1.3	36	100	621	ND	3.8	ND

Study 5.7 The effect of chronic administration of Fluoxetine, Ritanserin or vehicle on osmoregulated AVP

No	Wt(1-21days) (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)	[^{3}H]DPAT (fmol/mg)	[^{3}H]ketanserin (fmol/mg)	5HIAA:5HT
Group: Vehicle: normal saline stimulus (NN)								
9	195-315	299	141	1.5	43	106	268	2.77
18	186-283	302	143	1.6	42	125	473	2.39
25	196-313	302	142	0.8	46	43	483	1.19
36	188-265	302	142	1.4	47	139	272	2.00
42	196-328	303	145	1.5	42	42	302	2.54
17	189-297	299	140	1.1	42	-	-	-
Group: Fluoxetine: normal saline stimulus (FN)								
6	193-329	297	140	1.3	39	147	694	2.79
12	183-269	304	146	3.8	44	98	325	2.02
28	185-279	306	144	1.0	42	100	296	1.32
33	186-270	303	145	2.3	46	31	117	2.17
38	190-292	305	145	1.7	39	-	-	2.84
55	188-331	308	146	5.3	42	141	473	2.22
61	194-299	303	154	2.5	42	136	506	2.74
66	179-204	299	155	1.7	41	102	628	2.30

Study 5.7 (Cont/d) The effect of chronic administration of Fluoxetine, Ritanserin or vehicle on osmoregulated AVP

No	Wt(1-21days) (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)	[^{3}H]DPAT (fmol/mg)	[^{3}H]ketanserin (fmol/mg)	5HIAA:5HT
Group: Ritanserin: normal saline stimulus (NN)								
1	183-301	300	144	25.6	44	137	141	6.73
14	188-308	295	143	20	35	101	310	2.54
21	177-265	301	144	25.6	48	152	242	2.16
32	185-311	304	145	2.5	44	77	281	3.60
34	196-358	305	146	1.3	39	111	155	2.07
40	189-301	303	144	2.3	45	77	295	3.00
46	186-314	299	145	0.9	42	42	308	3.02
57	180-289	298	141	0.6	39	208	708	2.75
Group: Vehicle: hypotonic stimulus (NL)								
3	182-306	295	136	0.8	40			
16	187-293	275	136	<0.6	46			
29	185-296	296	141	0.8	45			
45	183-308	295	142	<0.6	46			
52	195-319	294	141	<0.6	44			
65	176-302	294	146	<0.6	49			

Study 5.7 (Cont/d) The effect of chronic administration of Fluoxetine, Ritanserin or vehicle on osmoregulated AVP

No	Wt(1-21days) (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
Group: Fluoxetine: hypotonic stimulus (FL)					
4	186-304	290	137	0.8	46
19	185-318	290	138	<0.6	44
26	184-295	290	143	<0.6	47
31	280-178	288	140	<0.6	42
37	183-284	299	143	4.3	44
49	193-299	295	143	<0.6	44
56	185-315	291	147	0.6	41
64	192-314	299	148	1.7	43
67	188-324	291	144	<0.6	45
Group: Ritanserin: hypotonic stimulus (RL)					
11	195-306	291	138	0.6	40
13	186-330	301	142	2.9	39
22	188-316	289	141	<0.6	46
27	191-300	294	140	<0.6	43
39	196-345	295	140	<0.6	42
47	190-314	297	141	0.6	48
54	199-290	288	143	<0.6	43
62	187-323	296	145	<0.6	42

Study 5.7 (Cont/d) The effect of chronic administration of Fluoxetine, Ritanserin or vehicle on osmoregulated AVP

No	Wt(1-21days) (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
Group: Vehicle: hyperosmotic stimulus (NH)					
8	190-303	315	151	10.0	42
15	190-361	314	151	12.3	44
43	200-365	320	153	10.1	46
50	193-338	310	153	5.7	45
51	191-325	308	148	7.0	42
60	187-311	317	154	14.3	45
Group: Fluoxetine: hypertonic stimulus (FH)					
2	190-317	316	151	18.4	41
10	181-293	312	153	7.1	39
20	190-275	306	147	3.1	40
24	185-271	330	155	8.7	43
41	186-278	312	150	7.7	40
48	182-310	312	150	5.8	41
58	187-277	312	158	7.6	41
63	180-278	317	155	4.4	42

Study 5.7 (Cont/d) The effect of chronic administration of Fluoxetine, Ritanserin or vehicle on osmoregulated AVP

No	Wt(1-21days) (g)	pOs (mosm/kg)	pNa (mmol/l)	pAVP (pmol/l)	HCT (%)
Group: Ritanserin: hypertonic stimulus (RH)					
5	182-294	308	148	6.4	41
7	189-316	307	148	1.9	38
23	191-305	307	145	30.0	41
30	190-331	310	153	6.7	39
35	192-344	314	151	6.3	41
44	189-314	309	149	8.5	40
53	188-323	305	147	2.3	41
59	194-309	309	152	4.5	41

APPENDIX 12

The mathematical Model of Piece-Wise regression analysis

Analysis was performed in the 'GLIM' statistical computing package (© The Royal Statistical Society 1986) with custom written macros to carry out the repeated regressions.

The model

Let y_{ij} be the vasopressin response of the ith rat in the jth group for $i = 1,\ldots\ldots,n_j$ and $j = 1$ or 2 depending on the group.

Similarly let x_{ij} be the measured osmolality of the ith rat in the jth group.

Then the equation of the lines can be given by

$$\hat{y}_{ij} = \alpha_j + \beta_j(x_{ij} - t_j)\mathbb{I}_{(x_{ij} > t_j)}$$

where $\mathbb{I}_{(x_{ij} > t_j)}$ is an indicator function such that $\mathbb{I}$ is zero when $x_{ij} \leq t_j$ and $\mathbb{I}$ is one when $x_{ij} > t_j$.

In the case when a common change point is used $t_1 = t_2$.

Thus the residual variation of a model can be considered in terms of a residual sums of squares given by

$$\sum_{i,j} (y_{ij} - \hat{y}_{ij})^2 \qquad (*)$$

where $\hat{y}_{ij}$ is the fitted value of the $y_{ij}{}^{th}$ observation. Obviously the $\hat{y}_{ij}$'s are functions of the change point and minimising the above equation (*) with respect to the change point gives the required change point. In the case of two separate change points, the two groups are independent and thus the procedure is identical, only separate for each group.