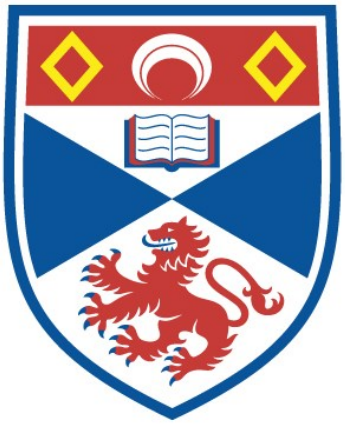
ON THE ACTION OF NORADRENALINE MICROINJECTED INTO THE PARAVENTRICULAR NUCLEUS OF RAT HYPOTHALAMUS

Andrew J. M. Clark

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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A thesis submitted to the University of St. Andrews for the degree of Doctor of Philosophy.

by

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August 1989.

DECLARATION

a) I, Andrew John Matthew Clark, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed Date 9-11-89

b) I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 in October 1985, and as a candidate for the degree of Ph.D. in October 1985.

Signed Date 9-11-89

c) We hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D.

Signatures of Supervisors Date

14.11.89

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Finally my most heartfelt thanks to my family, in particular my mother and father for their continuous and unwavering support, and my wife Judith for everything.

ABSTRACT

The microinjection of noradrenaline (NA) into the hypothalamic paraventricular nucleus (PVN) of the rat results in feeding. This response was shown; contrary to previous reports; to be mediated through both α-1 and α-2 NA receptors. Selective blockade of these two receptor sub-types, in conjunction with re-uptake blockade was used to examine the individual contributions of each receptor type to the whole response. It is suggested that the previously reported α-2 receptor specificity of the response to microinjected NA is a result of the location of these receptors. The post-synaptic α-1 receptor being located close to the pre-synaptic re-uptake mechanism, whilst the post-synaptic α-2 receptor is located outside the synapse and thus away from the re-uptake mechanism. The re-uptake mechanism acts to create a concentration difference of microinjected NA between the two receptor sub-types, resulting in a higher concentration and thus a preferential action at α-2 receptors. The involvement of the paraventricular NA system in stress induced eating was examined using a tail pinch procedure. Microinjection of NA antagonists into PVN prior to the onset of the pinch had no effect on the duration or latency of the eating response, thus there was no evidence for the involvement of this system in tail pinch elicited feeding. Further to the suggestion that the NA α-2 receptor is extra-synaptic whilst α-1 is intra-synaptic, the actions of NA were examined at a second site. NA microinjected into the ventral striatum elicited a vigorous locomotor response, although the origins of this showed a clear priming effect. However, this response was unaffected by prior microinjection of NA α- antagonists, preventing an analysis of receptor involvement comparable with that performed in PVN.

LIST OF ABBREVIATIONS.

ACTH	adrenocorticotropic hormone
ADR	adrenaline
AMB	nucleus ambiguus
ANOVA	analysis of variance
AVP	arginine-vasopressin
CNS	central nervous system
CRF	corticotropin releasing factor
DA	dopamine
DBH	dopamine-B-hydroxylase
DMH	dorsomedial hypothlamus
DMI	desmethyl-imipramine
DMV	dorsal motor nucleus of the vagus
DNAB	dorsal noradrenergic bundle
EAA`s	excitatory amino acids
EDTA	ethylene-diamine-tetra-acetic acid
ESB	electrical stimulation of the brain
HPLC-ED	high performance liquid chromatography with electrochemical detection
IBO	ibotenic acid
IML	intermediolateral cell group
i.p.	intra-peritoneal
LH	lateral hypothalamus
LHA	lateral hypothalamic area
MAOI	monoamine-oxidase inhibitor
MFB	medial forebrain bundle
NA	noradrenaline

NMDA	N-methyl-d-aspartatic acid
NST	nucleus of the solitary tract
6-OHDA	6-hydroxydopamine
OXT	oxytocin
PAG	periaqueductal grey
PHA-L	`phaseolus vulgaris` leuco-agglutin
PMV	premammillary nucleus
PNMT	phenyletheanolamine-N-methyl-transferase
PVN	paraventricular nucleus of the hypothalamus
TH	tyrosine hydroxylase
TP	tail pinch
VMH	ventromedial hypothlamus
VMN	ventromedial nucleus of the hypothalmus
VNAB	ventral noradrenergic bundle
VTA	ventral tegmental area

INTRODUCTION

THE REGULATION OF FOOD INTAKE

The study of the mechanisms and regulation of food intake is one of the most intensively researched aspects of behaviour. Feeding and feeding behaviour are ubiquitous in the animal kingdom and would appear to present a common link between all classes of organisms. What controls feeding behaviour and metabolism? It is clearly more than a peripheral, physiological process and since 1849 when Claude Bernard observed an increase in blood sugar following puncture of the floor of the IV ventricle, systematic research has been undertaken to elucidate the control of feeding and metabolism by the brain.

Much research has indicated the involvement of the hypothalamus in the control of food intake. Hetherington and Ranson (1939, 1942) reported that bilateral electrolytic lesions of the ventromedial hypothalamic area (VMH) resulted in a syndrome of hyperphagia and obesity, and further suggested that lesions of other hypothalamic sites did not result in weight gain. This latter statement was contradicted by the results published by Heinbecker et al. (1944) indicating the involvement of the paraventricular nucleus of

the hypothalamus (PVN), but for nearly 30 years the focus of research was the collection of metabolic and behavioural disturbances accompanying VMH lesion. This research led to the concept of a 'satiety' centre located in the VMH (Stellar 1954). Experiments performed by Anand and Brobeck (1951) further strengthened this concept. They discovered that lesions of the lateral hypothalamic area (LHA) resulted in a syndrome of aphagia, adipsia and dramatic weight loss. Thus it was proposed that food intake and metabolism were regulated by two reciprocally antagonistic centres in the hypothalamus, the VMH as 'satiety' centre and the LH as 'feeding' centre. More support for this theory was derived from the electrical stimulation of these areas. Stimulation of the VMH in hungry rats inhibited food intake (Margules and Olds 1962; Hoebel and Teitelbaum 1962), whilst electrical stimulation of the LH resulted in feeding in satiated animals (Andersson and Wyrwicka 1957).

The appeal of such a clearly defined model of feeding regulation is apparent: it accommodated the great majority of the available data, fitted neatly with the role of the hypothalamus as regulatory centre and could be explained simply, in terms of excitation and inhibition of discrete neuronal populations. Indeed such has been the impact of this model that it still appears in current undergraduate texts in biology, despite many apparent difficulties and

contradictions. Not all the experimental data could be explained and further research has exposed shortcomings in the two centre hypothesis. Richard Gold attempted to define exactly the area which, when lesioned, would give rise to the VMH syndrome. The central component of the VMH is the ventromedial nucleus (VMN) and Gold (1973) demonstrated that radio-frequency lesions of VMN which did not damage tissue outside this nucleus were ineffective in producing the VMH syndrome. Examination of those lesions which did produce the syndrome revealed that "*the effective lesions overflow the bounds of the VMN, the largest lesions typically producing the fattest rats*" (p. 488). Furthermore Gold observed that lesions caudal or lateral to the VMN, parasagittal knife cuts rostrolateral to the VMN and even midbrain lesions could all produce obesity even though the VMN was left intact. Thus it appeared that some component of the VMH other than VMN was the primary contributor to the syndrome. Gold suggested that the syndrome might be due to damage of the ventral noradrenergic bundle (VNAB) and this hypothesis was supported by the lesion studies of Kapatos and Gold (1973), in which knife cuts of VNAB were shown to result in hyperphagia and obesity.

Another interesting observation concerning VMH syndrome animals had been made by Teitelbaum (1955). He observed that the palatability of the diet had a greater impact on VMH animals than controls. He described the VMH animals as

finicky, palatable diet being overconsumed compared to controls, and food adulterated with quinine (bitter) or bulked out with cellulose, underconsumed. Graff and Stellar (1962) suggested that it might be possible to dissociate the finickiness and hyperphagia components of the VMH syndrome with selective lesions. Ahlskog and Hoebel (1973) confirmed this using electrolytic lesions and the catecholamine specific neurotoxin 6-hydroxdopamine (6-OHDA). Lesions of the ventral noradrenergic bundle (VNAB) resulted in a hyperphagia leading to obesity, whether the lesion was electrolytic or made with 6-OHDA. The hyperphagia appeared to result from a depletion of forebrain noradrenaline (NA) due to lesion of the VNAB. However lesions in the medial hypothalamus sufficient to induce the traditional VMH syndrome resulted in no detectable loss of forebrain NA, whereas the NA depleting VNAB lesion resulted in >90% depletion. Furthermore, the two procedures were additive, not substitutive, in their effects on eating (Ahlskog et al. 1975). These results indicated that the 'classical' VMH syndrome was due to a combination of damage in the medial hypothalamus and damage to the VNAB caused by extensive lesions. The finickiness component described by Teitelbaum (1955) was demonstrated not to be a consequence of the obesity these lesions produced. Animals prevented from becoming obese after lesion did not show this syndrome (Franklin and Herberg 1974).

FIGURE 1. Diagram of the noradrenergic projections of the locus coeruleus complex viewed in the saggital plane. Abbreviations: AON, anterior olfactory nucleus; AP-VAB, ansa-peduncularis - ventral amygdaloid bundle systems; BS, brainstem nuclei; CC, corpus callosum; CTX, cerebral neocortex; H, hypothalamus; HF, hippocampal formation; S, septal area; SOD, supra-optic decussation; T, tectum; TH, thalamus. The darkly shaded area is the dorsal noradrenergic bundle. (Redrawn from Moore and Bloom 1979.)

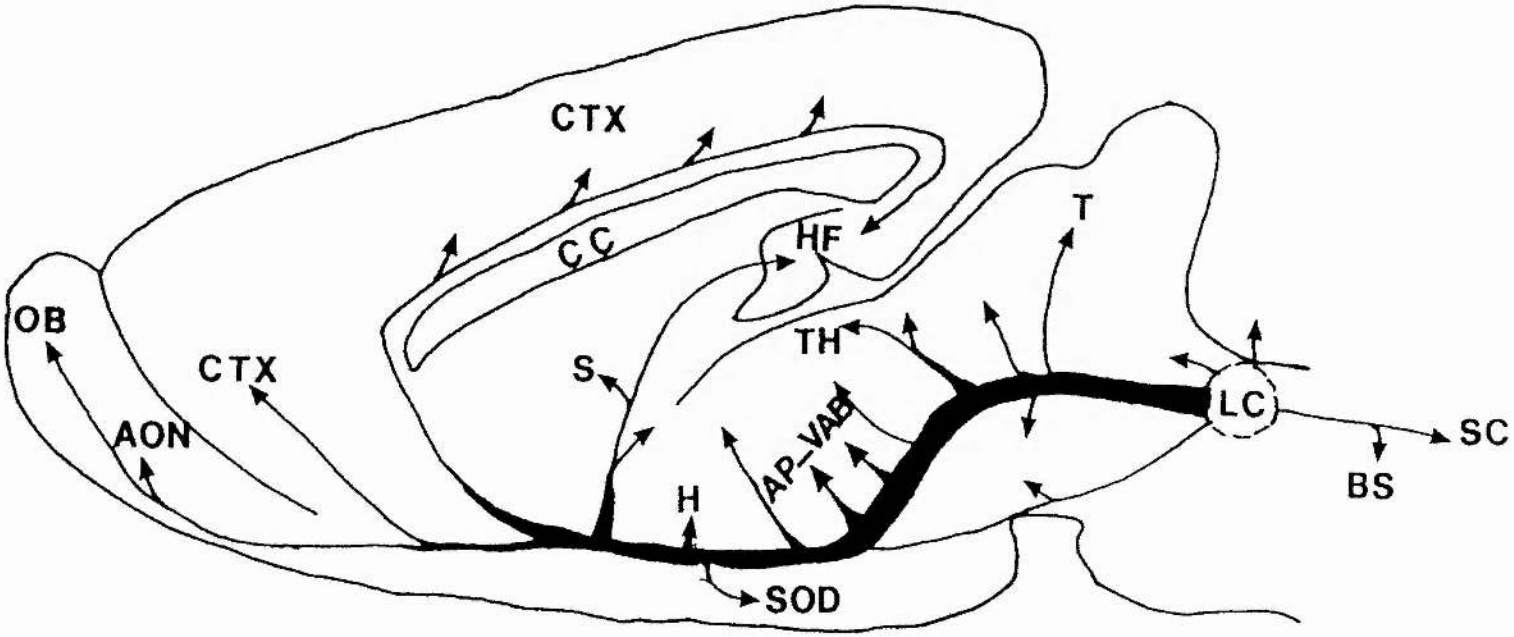
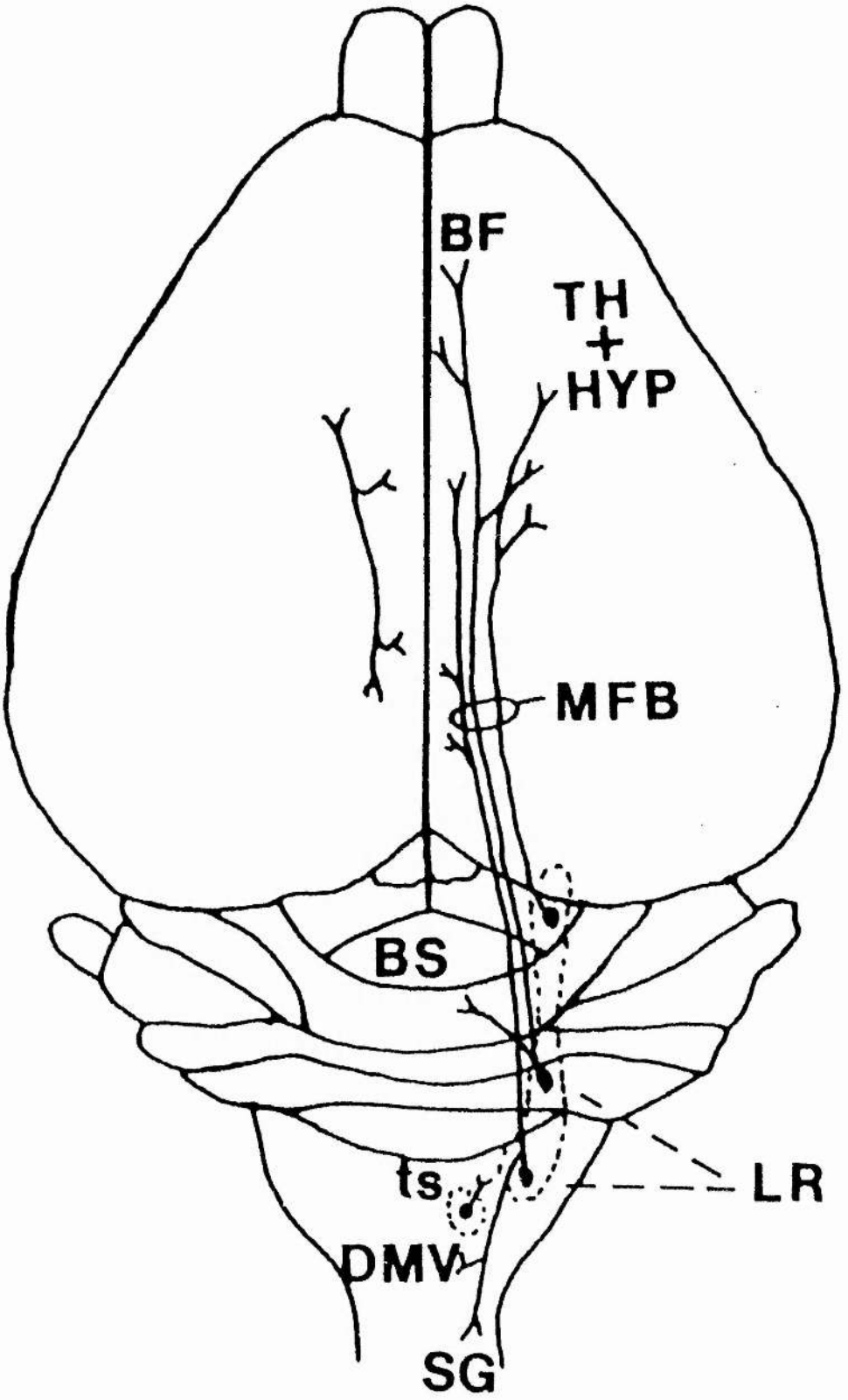

CTX
CC
HF
T
OB
CTX
S
TH
LC
SC
AON
AP_VAB
BS
H
SOD

FIGURE 2. Representation, in horizontal view, of the location and projections of brainstem NA groups excluding the LCC. Abbreviations: BF, basal forebrain; BS, brainstem; DMV, dorsal motor nucleus of the vagus; LR, lateral reticular formation; MFB, medial forebrain bundle, SG, spinal cord grey; TH+HYP, thalamus and hypothalamus; ts, nucleus of the solitary tract. (Adapted from Moore and Bloom 1979.)


BF
TH
+
HYP
MFB
BS
ts
LR
DMV
SG

More recently, evidence has begun to appear suggesting that the lesions made by Ahlskog and Hoebel were not selective for VNAB but in addition damaged the dorsal noradrenergic bundle (DNAB), which originates in and around the locus coeruleus (for details of the anatomy see below p.15 and figures 1 and 2). Experiments by Lorden et al. (1976) and Oltmans et al. (1977) found no significant difference in body weight gain between animals with 6-OHDA lesions of VNAB and controls despite the fact that these lesions damaged not only VNAB but also caused slight damage to DNAB. The deficit produced by lesion of ascending NA fibres was clearly complex in nature, affecting both body weight gain and sensory aspects of the diet. In order to establish which of the ascending NA fibre bundles were responsible for which of the observed deficits Sahakian et al. (1983) made selective lesions of either VNAB or DNAB and contrasted the results. The results of this study serve to indicate the complexity of the interactions between the lesions; DNAB lesions contrast with those of VNAB in that DNAB lesioned animals show no hyperphagia, weight gain or finickiness, all of which were apparent in the VNAB lesioned animals. However the results of the VNAB lesions bear only a superficial similarity to the results of electrolytic VMH lesions in that whilst rats with either lesion show hyperphagia, weight gain and finickiness, the magnitude of some of these effects is far greater in VMH lesioned animals. The finickiness appears to be identical

irrespective of lesion but the hyperphagia and weight gain are reduced in the VNAB lesioned animals when compared to those with a VMH lesion.

The above experiments have all been concerned with the regulation of food intake and metabolism as central processes and have taken little account of metabolic disturbances which may have resulted from the various central manipulations. Analyses of peripheral metabolism in conjunction with central manipulations helped to elucidate the nature of VMH obesity. Animals with VMH lesions become hyperinsulinaemic (Frohman and Bernardis 1968) and as daily administration of insulin in experimental rats results in obesity (MacKay et al. 1940), this is likely to be a component of VMH syndrome obesity. Furthermore Powley and Opsahl (1974) observed that vagotomy abolished the obesity from VMH lesions. However, Vilberg and Beatty (1975) demonstrated VMH obesity in diabetic rats receiving controlled levels of insulin, indicating that hyperinsulinaemia is only a contributory factor in the syndrome. In normal animals the liver and adipose tissue act as parts of an energy store, absorbing nutrients post-ingestionally and then slowly releasing them when food is not available. Friedman and Stricker (1976) suggested that the consequence of a VMH lesion is to affect metabolism in such a way that the animal is fixed in the absorptive phase. Thus instead of performing its normal function, adipose tissue

continuously absorbs nutrients leaving the animal in a state in which it must eat more often to maintain energy balance.

The data presented above demonstrates that far from being a uniform deficit resulting from damage at a specific locus, the VMH syndrome is a collection of deficits due to a variety of damage across several loci. Much of the interst in the VMH syndrome concerned the paradoxical dissociation between 'hunger' and food intake. In an attempt to reconcile all of the VMH effects in one theory Powley (1977) proposed the "Cephalic Phase Hypothesis". This suggested that VMH lesions produced their major effects on feeding behaviour by "*directly heightening the phasic, autonomic and endocrine responses triggered by oropharyngeal contact with food stimuli - the cephalic reflexes of digestion*" (p.89). Further, "*by directly altering the visceral responses in the direction of positive energy balance, the lesion exaggerated cephalic reflexes have the effect of producing anabolic adjustments which in turn produce overeating and weight gain*" (p.90).

As an hypothesis the "Cephalic Phase" was excellent being both parsimonious and testable, however when tested problems were found:

(1). Experiments by Storlien (1985) showed an abolition of cephalic phase reflex in VMH lesioned rats.

(2). Duggan and Booth (1986) claim that the hyperinsulinaemia

(central to the cephalic phase hypothesis) is caused by changes in the rate of gastric emptying. Hyperinsulinaemia is therefore a secondary consequence. Thus Powley's hypothesis which provided a theory of VMH function could not be correct.

The finickiness produced by VMH lesion is also produced by VNAB lesion and may result from a loss of NA input to the amygdala, and the autonomic effects may result from damage to descending fibres from other medial hypothalamic sites.

As a result of the experiments performed to identify the exact locus mediating VMH obesity interest was revived in the paraventricular nucleus of the hypothalamus (PVN). This area had been implicated in hypothalamic obesity by Heinbecker in 1944 but had been largely ignored by researchers until the knife cut studies of Gold and his colleagues (Gold, 1977; and Sclafani, 1971). The PVN is a major target site for the VNAB and also receives a small (<20%) NA input from DNAB. The PVN is also the site most responsive to feeding elicited by the microinjection of exogenous NA (Booth 1968, Leibowitz 1978a). Further, the anatomical connections of the PVN, such as strong reciprocal connections with structures in the brainstem known to be involved in autonomic regulation, indicate a possible role in the control of visceral events. Finally, the demonstration that electrolytic lesions of the PVN produced

increased levels of food intake and excessive weight gain in rats (Leibowitz et al. 1981; Aravich and Sclafani 1983; Sclafani and Aravich 1983) indicated that this site had a major role in the regulation of food intake.

The recognition of the importance of the PVN and the demonstrations of noradrenergic involvement in the functioning of this nucleus represented a significant advance in the understanding of hypothalamic regulation of food intake and metabolism. However, in order to appreciate fully the involvement and interaction of this nucleus with the ascending NA systems and the resulting effects on food intake and metabolism, it is necessary to examine the anatomical interactions and effects of NA manipulation in considerably more detail.

ANATOMY

Behavioural patterns, in particular those concerned with the survival of the organism, appear to be organised by the limbic system. The hypothalamus is a diencephalic component of this system and it is hard to overstate its importance in complex limbic integration. To quote from P.Luiten et al. (1987) "*Speaking in general terms the hypothalamus guards a part of the channels of entry and the majority of the outflow of information to and from the internal environment of the mammalian body. It makes the first overall selection of*

certain inputs and a major selection of output to and from the 'higher' integration centres of the limbic system. As such, the hypothalamus is not only a centre of relay of information, but acts as a centre of integration as well. Focusing such a large number of important functions in a structure of such moderate size leads to two easily discernable conclusions: its anatomy is complex and the effects of even minor damage are dramatic" (pp. 27-28).

PARAVENTRICULAR NUCLEUS

The PVN can be sub-divided into two basic divisions: the magnocellular portion and the parvocellular portion. These are illustrated in figures 3 and 4. A detailed analysis using both connectional and cytoarchitectonic criteria revealed the presence of eight possible sub-divisions within the nucleus (Swanson and Kuypers 1980). The magnocellular portion is composed of three parts, anterior, medial and posterior zones which all project predominantly to the posterior lobe of the pituitary gland. A projection of arginine-vasopressin (AVP) containing fibres arises from this region to the median eminence where terminals contact the hypophyseal portal capillaries (data from the guinea pig) (Sofroniew et al. 1979). The parvocellular portion of PVN can be divided into five areas; anterior, medial, lateral, dorsal and periventricular. The parvocellular part of PVN contains small oxytocin (OXT) and AVP producing neurons and gives rise to a

FIGURE 3. (A) Organisation of ascending, predominantly noradrenergic projections (asterisks) to the PVN. Note that A1 innervates both magnocellular and parvocellular divisions of PVN. (B) Organisation of efferents from PVN involved in central control of autonomic function. Abbreviations: A1, the noradrenergic cell group A1; DVC, dorsal vagal complex; ME, median eminence; IML, intermediolateral cell column of the spinal cord; IX, glossopharyngeal nerve; X, vagus nerve; LC, locus coeruleus; P, posterior pituitary; *, oxytocin and *, vasopressin-containing components of magnocellular PVN. (Adapted from Sawchenko and Swanson 1981.)

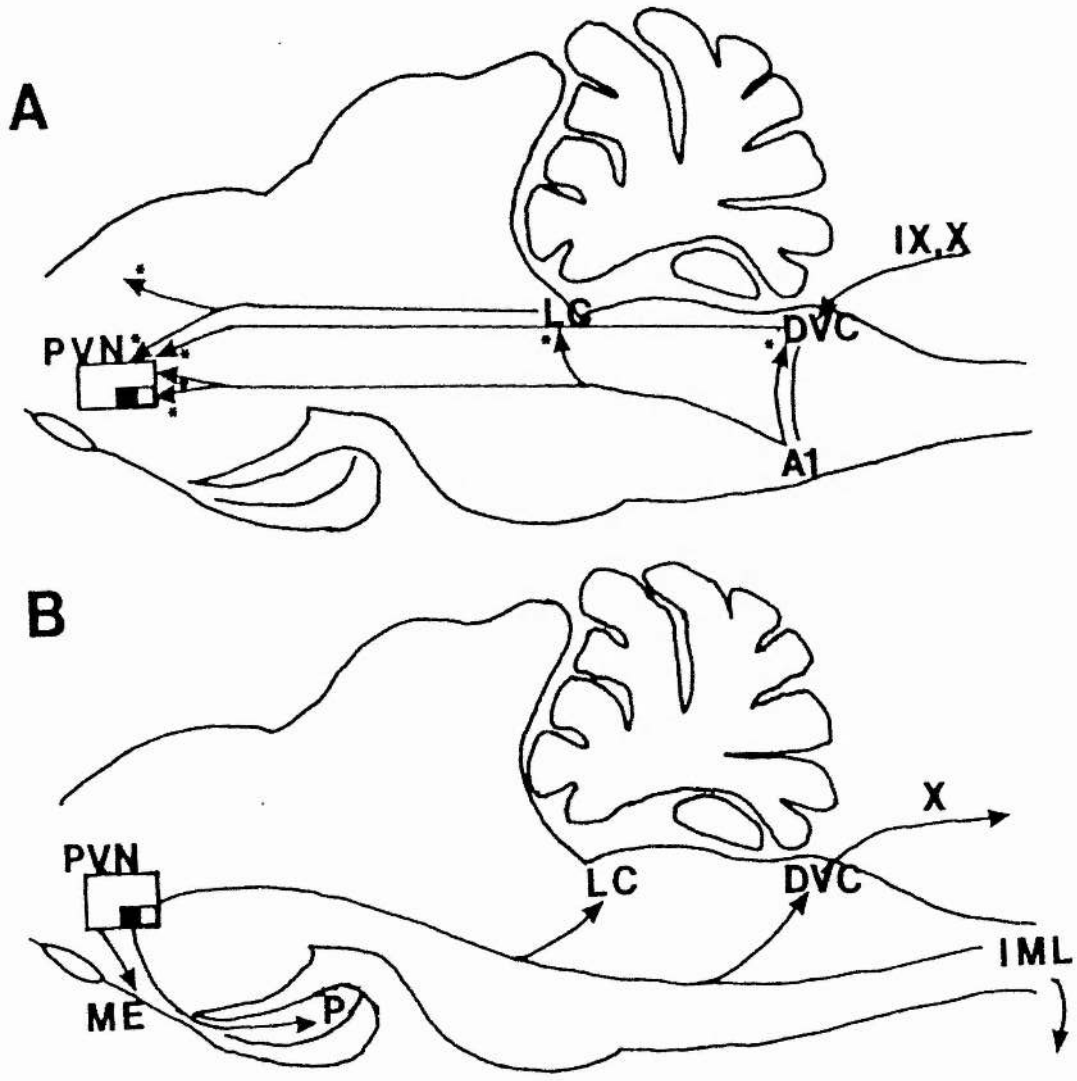
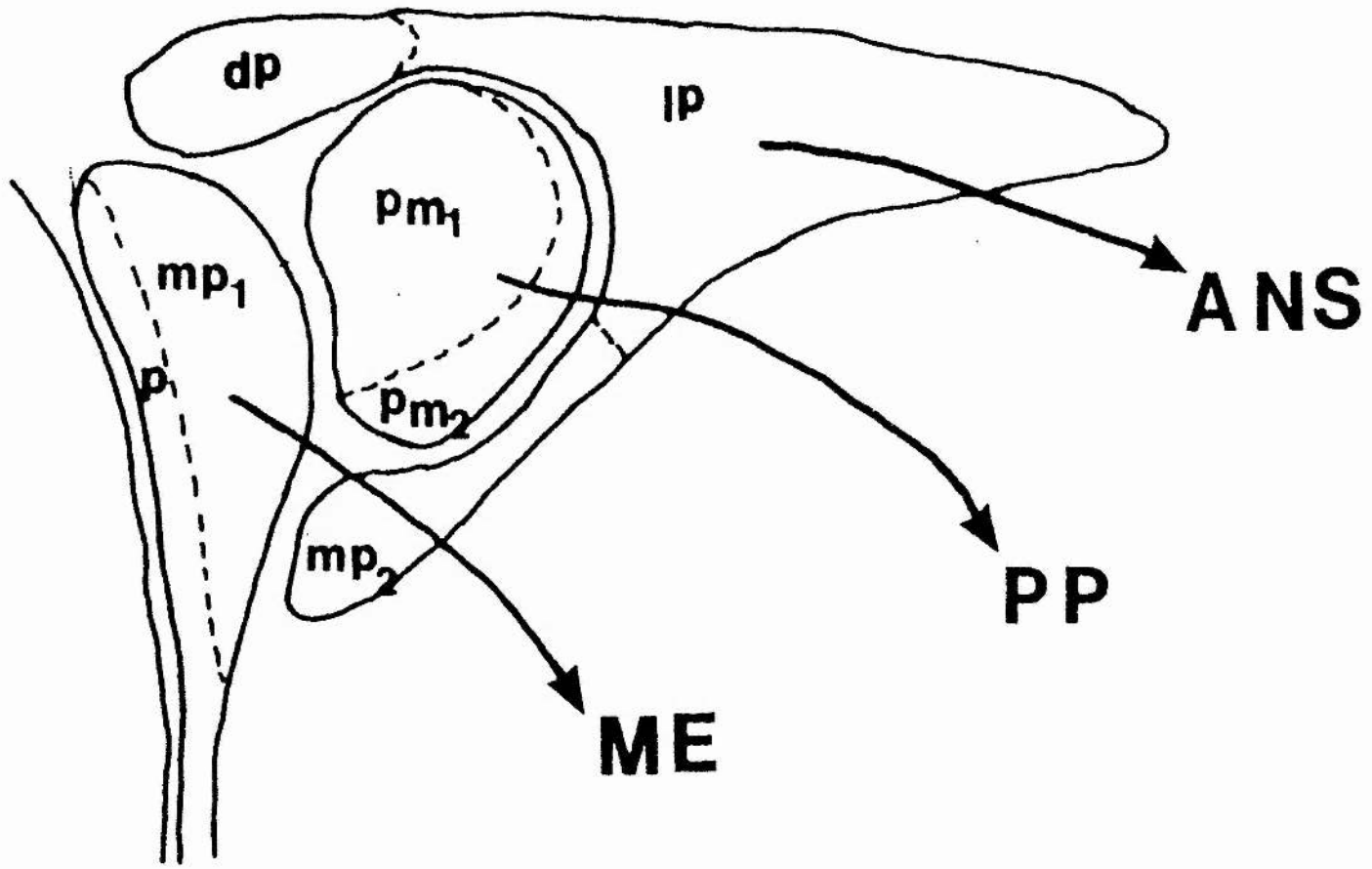

A
IX,X
LC
DVC
PVN
A1
B
X
PVN
LC
DVC
IML
ME
P

FIGURE 4. Representative summary of the major sub-divisions of the PVN. Abbreviations: ANS, autonomic nervous system; ME, median eminence; PP, posterior pituitary; dp, dorsal parvocellular part; lp, lateral parvocellular part; mp1, dorsal and mp2, ventral subdivisions of the medial parvocellular part; pm1, lateral and pm2, medial subdivisions of the posterior magnocellular part; pv, periventricular part (from Swanson and Sawchenko 1983).


dp
lp
pm1
mp1
p
pm2
mp2
ANS
PP
ME

descending projection which distributes fibres to the locus coeruleus, the parabrachial nuclei, the dorsal motor nucleus of the vagus, and the nucleus of the solitary tract (Swanson and Hartman 1980, Sawchenko and Swanson 1982). Some of these fibres enter the dorsal part of the lateral funiculus of the spinal cord. Within the spinal grey matter these fibres terminate in the central grey, the marginal zone of the dorsal horn and the intermediolateral column (Swanson and McKellar 1979).

The function of these AVP and OXT projections from PVN to the autonomic centres in the brain stem and spinal cord may be related to the regulation of the processes with which these peptides are involved in the periphery. A coupling of the central and peripheral actions of hypothalamic peptides has been suggested as a general principle (Swaab 1982).

Localisation studies of cells containing posterior pituitary hormones revealed that AVP stained cells were concentrated in the posterior zone of the magnocellular cell group whilst OXT containing cells were found predominantly in the anterior and medial zones.

Swanson et al. (1981) have interpreted the results of localisation studies of the catecholamine containing neurons in PVN, combined with the anatomy of peptidergic neurons

within this nucleus. They conclude that adrenergic input to PVN may influence the cells projecting to preganglionic autonomic centres, whilst the NA projection influences predominantly AVP cells that project to the posterior lobe of the pituitary, as well as cells in the periventricular region that project to the median eminence. However, in the light of the data presented by Mefford (1987) which suggests that PNMT containing cells are not adrenergic neurons in the accepted sense (see p. 21) Swanson's interpretation must be considered oversimplified.

NORADRENERGIC INNERVATION OF PVN.

The neurons that synthesise NA appear restricted to the pontine and medullary regions. Dahlstrom and Fuxe (1964) described seven NA cell groups in rodents - designated A1-A7 and most of these have been recognised in primates as well (see Nieuwenhuys, 1985 for review). As this thesis is concerned with the PVN only the cell groups and projections relevant to this nucleus will be considered.

The cells of groups A1 and A2 are both situated in the lower portion of the medulla oblongata. The cells of A1 surround the lateral funiculus and extend dorsomedially into the lateral part of the reticular formation, whilst those of A2 lie predominantly in the nucleus of the solitary tract, the dorsal vagal nucleus and the intervening area (Nieuwenhuwys,

1985). A6 NA cells are found within the locus coeruleus, it is a densely packed accumulation of cells, and some evidence suggests that all of the cells situated in the central part of this structure are noradrenergic (Swanson, 1976). The A6 cell group can be described in terms of three extensions, caudolateral, ventral and rostral. It is the rostral projection that is of relevance to the PVN. It consists of cells lying in the caudolateral part of the mesencephalic central grey (A6cg). This A6cg group is usually combined with the A4 group and the ventral extension of A6 (A6sc or subcoeruleul group) and classified as the noradrenergic locus coeruleus complex (LCC).

DORSAL BUNDLE

The LCC is quantitatively the most important site of NA projection, containing about half the total number of NA synthesising neurons in the brain. The major efferent of this system was called the dorsal noradrenergic bundle (DNAB) by Ungerstedt (1971) [see figure 1], but it is a secondary and much smaller efferent that is of interest here. This is the projection that forms the rostral limb of the dorsal periventricular pathway, which ascends to the diencephalon within the ventromedial part of the periaqueductal grey, forming part of the dorsal longitudinal fasiculus complex. Rostrally this pathway continues into the diencephalic periventricular fibre plexus. Lindvall and Bjorklund (1974)

suggested that fibres from this projection innervate the PVN. However subsequent analysis by Sawchenko and Swanson (1981) has shown that it is only the parvocellular part of this nucleus that receives any sizeable input from the LCC.

VENTRAL BUNDLE

The efferents of the dorsal medullary (A2) and lateral tegmental (A1, A5, A7) noradrenergic cell groups were described by Ungerstedt (1971) as giving rise to a long, ascending fibre system called the ventral noradrenergic bundle (VNAB) [see figure 2]. The VNAB ascends through the reticular zone of the brain stem and continues rostrally within the medial forebrain bundle. According to Ungerstedt (1971) the VNAB has widespread terminal areas including the reticular formation, the entire hypothalamus (particularly dorsomedial, periventricular, infundibular, supraoptic and paraventricular nuclei), the preoptic area and bed nucleus of the stria terminalis. However, more recent studies (Lindvall and Bjorklund, 1974, Swanson et al. 1981) have shown that the VNAB and DNAB as described by Ungerstedt are not as clearly distinct from each other as he proposed. They form one complex which has been termed the 'central tegmental tract' in which ascending fibres are mixed with descending ones and axons of the 'lateral tegmental group' feed into it at successive levels through radially coursing transverse fibres. However, the non-coerulean NA projections to the hypothalamic

cell masses originate exclusively from the caudal medullary A1 and A2 cell groups (Ricardo and Koh 1978, Sawchenko and Swanson 1981, 1982a).

The regions innervated by each of the A1, A2 cell groups have been sub-divided as follows:-

A1 innervation.

(i) Bed nucleus of the stria terminalis.

(ii) Medial preoptic area.

(iii) Anterior, lateral and posterior hypothalamic areas.

(iv) The dorsal hypothalamic area, the dorsomedial nucleus and the median eminence.

(v) The magnocellular and parvocellular portions of the PVN. In the magnocellular portion of PVN the projection from A1 terminates predominantly in areas that show high levels of vasopressin-like immunoreactivity.

A2 innervation.

i) Medial preoptic area.

ii) Anterior, lateral and posterior hypothalamic areas and the dorsomedial nucleus.

iii) The parvocellular portion of the PVN.

A large part of the long, ascending projections from A1 and A2 are bilateral, but with an ipsilateral predominance.

ADRENERGIC INNERVATION OF PVN

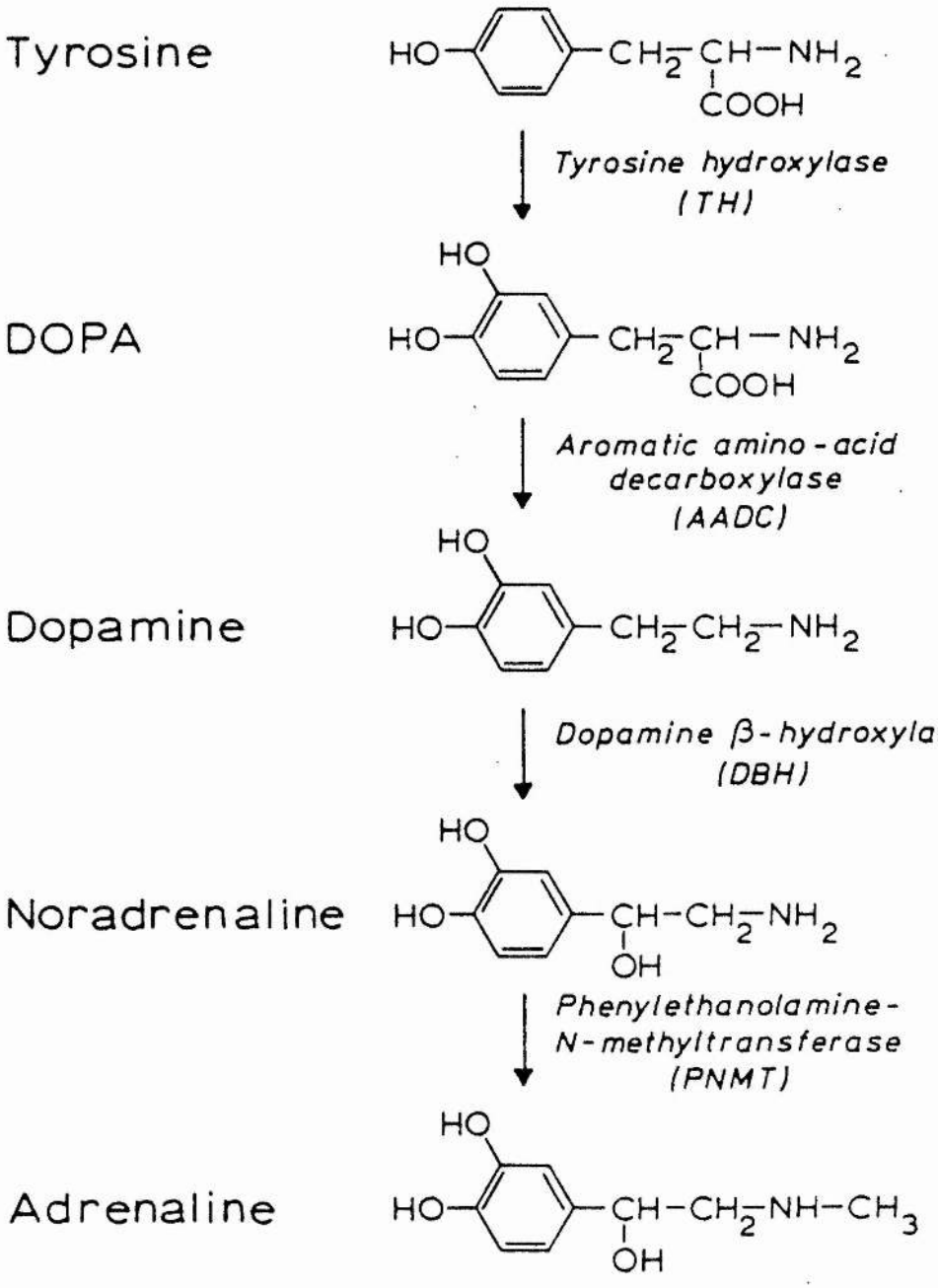
Adrenaline (ADR) represents the final stage in the synthesis chain of the catecholamines, in which noradrenaline is converted by the enzyme phenylethanolamine - N - methyl - transferase (PNMT). [See figure 5]. As it is a catecholamine ADR can be visualised by the fluorescence methods that apply to NA and dopamine (DA) but no distinction can be made between these compounds. However, antibodies against the enzyme PNMT, which is present only in ADR synthesising neurones have enabled selective anatomical tracing of PNMT containing neurones. These studies purport to show the distribution of ADR neurones in the brain.

Three PNMT containing cell groups have been identified, all situated in the caudal rhombencephalon. In concordance with the nomenclature of Dahlstrom and Fuxe (1964) these groups are referred to as C1, C2 and C3. They are named in order of magnitude, C1, the largest group, contains about 69% of the total number of PNMT immunoreactive cells (Howe et al. 1980). It is located between the lateral funiculus and the inferior olivary complex in the ventrolateral myelencephalon. Group C2, comprising 22% of PNMT reactive cells, is located in intimate association with the NST. Some cells falling within the boundaries of this nucleus, and others directly adjacent

FIGURE 5.Synthesis pathway of the catecholamines.

Figure from Nieuwenhuys (1985).

Catecholamines :



to it. Group C3, which is the remaining 9%, lies adjacent to the dorsal raphe region, its cells interspersed amongst the fibres of the medial longitudinal fasiculus. Groups C1 and C2 are not, as suggested by Hokfelt et al (1974), located within the NA groups A1 and A2 but lie rostral, although adjacent, to these areas. It is interesting to note that, whilst the cells of C1 and C2 are clearly labelled by antibodies to PNMT most of these cells do not show catecholamine histofluorescence (Howe et al 1980).

From the general region of areas C1 - C3 an ascending tract of PNMT-reactive fibres can be traced through the reticular formation, the ventral tegmental area and into the lateral hypothalamus (LH). These fibres follow the VNAB almost exactly. Terminal fields of PNMT containing neurons appear to be distributed quite widely throughout the CNS, innervating in particular; the dorsal motor nucleus of the vagus (DMV), the NST, LC, ventral periaqueductal grey, the dorsomedial nucleus of the hypothalamus, some thalamic midline nuclei and a particularly dense field in the PVN. Exactly which group of cells (C1-C3) projects where is hard to define but Sawchenko and Swanson (1982a) suggest that C1 projects to magno- and parvocellular portions of PVN whilst C2 also projects to the parvocellular portion.

PVN AFFERENTS

There appears to be very little input to the PVN from the telencephalic limbic areas, apart from a few prelimbic cortical afferents (Luiten et al. 1987). In addition to the catecholaminergic afferents already discussed the major afferents appear to be from the dorsolateral septum and bed nucleus of the stria terminalis, as well as from the subfornical organ and the organum vasculosum lamina terminalis (Sawchenko and Swanson 1983). The routes from higher limbic centres such as the hippocampus, amygdala and prefrontal cortex, to the PVN are indirect. These areas undoubtedly influence 'emotional state' and behaviour, involving adrenal hormone release. It is probable that the ventral premammillary nucleus (PMV) acts as a relay station between these structures and the PVN (Luiten et al. 1987).

INTRAHYPOTHALAMIC PROJECTIONS OF PVN.

From the parvocellular area of PVN there are some projections to the medial and ventral peripheral zones of the LHA. Further, some efferents project to the medial aspects of VMH and a well developed output is present to the dorsomedial area (DMH). A projection is present to the perifornical column throughout the hypothalamus from anterior to posterior and there is a dense projection to the periventricular layers of the third ventricle, to the arcuate nucleus and the median eminence (Luiten et al. 1987).

It appears that all hypothalamic nuclei concerned with the control of food intake and metabolism receive input from PVN. Furthermore, the PVN must be considered one of the main hypothalamic outflow stations. Despite the presence of dense projections from other hypothalamic nuclei, particularly DMH, to the PVN, reciprocal pathways are quantitatively limited. This is taken to imply that PVN exerts its wide ranging influence on food intake and autonomic activity not as a result of intrahypothalamic integration and processing but as a result of extensive PVN output. (Gold et al. 1977, Weiss and Leibowitz, 1983).

PVN EFFERENTS

Hypothalamic outflow via the neuroendocrine system is undoubtedly dominated by the PVN projections to adeno- and neuro-hypophesis. Corticotropin releasing factor (CRF) neurons in the parvocellular PVN, via their projections to the median eminence, constitute a major pathway in the expression of motivational drive and emotional state (Bassett 1984). These releasing factors can activate secretion of adrenocorticotropic hormone (ACTH) from the posterior pituitary, which in turn leads to corticosteroid release from the adrenal glands (Merchenthaler, 1984, Merchenthaler et al. 1984). Further, Leibowitz and her colleagues have demonstrated the interaction of steroid hormones and NA within

the PVN (see p. 37 below) and shown that the presence of these hormones is necessary for aspects of PVN function. Thus a feedback loop exists between PVN neurons releasing CRF and levels of circulating steroid hormones.

The outflow pathways from PVN to the autonomic nervous system form two main streams of fibres. One tract maintains a periventricular position whilst the other runs lateral over the substantia nigra and finds a position on the floor of the brainstem. These two pathways provide synaptic endings in the periacqueductal grey (PAG) and Edinger-Westphal nucleus, ventral mesencephalic tegmentum, pedunculo-pontine tegmental nucleus and parabrachial nucleus, locus coeruleus and the raphe nuclei. Continuing to the lower brainstem, PVN projections form highly organised terminations in the nucleus ambiguus (AMB) and DMV, NST, and area postrema. Via these nuclei the PVN has a direct and powerful influence on the parasympathetic cell groups of the autonomic nervous system (Rogers and Hermann 1985, Rogers and Nelson 1984).

Descending through the lower medulla many fibres move laterally in the dorso-lateral funiculus. These PVN efferents can be followed over the entire length of the spinal cord, giving off collaterals that branch extensively in the intermediolateral column of the thoraco-lumbar segments. After entering the intermediolateral cell group (IML) most

fibres continue medially and terminate finally in area 10 of Rexed (the midline extension of IML) which consists of pre-ganglionic sympathetic neurons (Luiten et al. 1984).

Comparing the anatomical data provided by Luiten and his colleagues, generated using the 'phaseolus vulgaris' leuco- agglutinin (PHA-L) tracing methods (Gerfen and Sawchenko 1984) with the immunohistological data from Swanson, remarkable similarity is apparent. The PVN projection to the parasympathetic cell groups in the lower medulla shows high levels of vasopressin like immunoreactivity, whilst in the sympathetic PVN-IML projection the immunoreactivity was exclusively to oxytocin indicating that these two compounds may be parasympathetic and sympathetic transmitters respectively.

ARE PNMT CONTAINING CELLS ADRENALINE NEURONS?

Since the discovery and mapping of PNMT-containing neurones the assumption has been made that these cells are specifically adrenergic. However, a recent review by Ivan Mefford (1987) suggests that this assumption may be erroneous. Several manipulations of ADR and PNMT in brain are difficult to rationalise in terms of classical catecholamine neurons:-

(i) Lesions made with the specific neurotoxin 6-OHDA fail to cause degeneration of PNMT containing cells in the

hypothalamus (Fuller 1982).

(ii) It has not proved possible to demonstrate co-localisation of PNMT with other catecholamine synthesising enzymes (tyrosine hydroxylase [TH] or dopamine-B-hydroxylase [DBH]) within the hypothalamus.

(iii) Transection of fibres from C1, C2 causes a depletion of ADR but no lowering of PNMT activity in hypothalamus (Langer et al. 1980).

(iv) Chronic administration of monoamine oxidase inhibitors (MAOI) causes a disproportionate increase in ADR compared to NA (Mefford 1987).

(v) Acute stress causes a marked depletion of ADR whilst having a considerably smaller effect on NA (Roth et al. 1982).

In order to account for these differences, special properties have been ascribed to ADR neurones. However Mefford proposes an alternative explanation. He suggests first, that the majority of PNMT neurones in the hypothalamus are not capable of specific uptake, storage, or *'de novo'* synthesis and are thus not catecholaminergic neurones; and second, that ADR formed in the hypothalamus by PNMT is a post-synaptic catabolite of NA and diffuses out of the PNMT neurone. It acts either as a local diffuse transmitter or hormone, possibly interacting with glial systems, or it can be taken up into NA neurones and act as a co-transmitter with NA. Finally, in the medulla, PNMT exists with TH and DBH, and ADR

is produced but stored and released as a co-transmitter with NA.

This hypothesis raises many questions about the monoamine involvement in hypothalamic function and serves to indicate the difficulty in separating the NA and ADR actions in the PVN. These questions are further addressed in the discussion section.

NORADRENALINE AND FEEDING

In 1962, Grossman reported that hypothalamic injection of NA could elicit feeding in satiated rats. The effects of injections of NA were examined using operant techniques; rats were trained to press one lever for food reward and another for water reward on a variable interval, 30 second schedule of reinforcement. The injection of NA at perifornical sites in satiated animals significantly increased the rate of lever pressing for food reward, whilst the rate of pressing for water was unaffected. This finding stimulated considerable research into the possibility that central adrenergic systems might mediate natural hunger. A mapping study performed by David Booth (1967) indicated that the most effective sites for eliciting NA induced eating were in the anterior hypothalamic areas. The lateral and caudal hypothalamus were in general ineffective, while injections into certain extra-hypothalamic sites, such as the nucleus accumbens, the subfornical area and the olfactory tubercle were effective in eliciting feeding.

Support for NA involvement in feeding regulation was provided by the results of Slangen and Miller (1969) who demonstrated that eating was stimulated by intrahypothalamic injection of drugs which enhance endogenous NA release and further, that the introduction of NA α-receptor blocking drugs led to a suppression of NA induced feeding. Further evidence for the site specificity of NA involvement was the response to NA injections into the mesencephalic reticular formation which suppressed operant responding for food reward (Grossman, 1968). The response to hypothalamic injections was also found to be dependent upon the internal state of the animal. Coons and Quatermain (1970) confirmed the results reported by Grossman (1962) but found that the facilitatory effect of the NA was prevented by 6 or 24 hour periods of food deprivation prior to testing on both fixed ratio and variable interval schedules. The results suggested that "*the facilitatory action of NA on operant responding for food reward may have been dependent upon a low rate of responding and was reversed by conditions which created a high rate of responding*" (Matthews et al. 1986).

The data presented above served to indicate the involvement of NA in some aspects of feeding behaviour and moreover, indicated that a possible locus for this involvement might be the hypothalamus. In order to clarify further the anatomical basis, pharmacology, and specificity of NA involvement in

feeding or its regulation, detailed studies of all these aspects were required. Over the last fifteen years Sarah Leibowitz, working at the Rockefeller University in New York, has carried out an exhaustive and consistent programme of research aimed at understanding the mechanisms underlying feeding in the rat. The work of Leibowitz and her colleagues is central to this thesis and is thus considered below as a separate chapter.

SARAH F. LEIBOWITZ

The major research effort into the actions of NA microinjected into the PVN has come from Dr. Sarah Leibowitz and her colleagues. Initially, NA was injected at a variety of brain sites and in various doses and the behavioural response observed. Three major effects were noted; i) adrenergic stimulation of the 'perifornical' hypothalamus, at the level of the anterior hypothalamus, had profound effects on ingestive behaviour. Feeding was promoted by stimulation of alpha NA receptors and suppressed by stimulation of beta NA receptors; ii) Injection of NA into other structures, from the pons to the caudate, revealed a consistent pattern with respect to ingestive behaviour. From the rostral midbrain to the preoptic area, the medial portion of the brain appeared sensitive only to alpha stimulation, whilst the lateral areas appeared sensitive only to beta stimulation; iii) Injection of dopaminergic agents at these sites had no effect on ingestive behaviour (Leibowitz, 1973). From these data she hypothesised a reciprocal feeding mechanism: feeding was elicited by the action of NA at medial α-receptors and inhibited by the action of NA at lateral ß-receptors, the effective location of these mechanisms being hypothalamic, this hypothesis was subsequently demonstrated (Leibowitz 1975a).

The pattern of feeding and drinking produced by NA injections

was examined further (Leibowitz 1975b). This examination produced six major components to the response; (i) The feeding and drinking responses to NA injection were dose dependent; (ii) The drinking response (1-4ml) had a latency of around 1.5 min. and lasted for 2-3 min. It was followed closely by eating (2-4gm) that lasted for about 20 min. (Data for 40nm NA in 0.5ul injection). (iii) The drinking response was clearly time related whilst satiation appeared to be important for termination of the feeding response. (iv) These responses were similar in appearance to normal pre-meal drinking and subsequent feeding. (v) The NA elicited responses could not be observed following LH stimulation by NA and only feeding, without the pre-prandial drinking was observed from VMH injection. vi) In contrast to drinking induced by peripheral ß-adrenergic stimulation, that induced by NA was not abolished by nephrectomy. These results together with previous findings (Booth 1968, Slangen and Miller 1969, Leibowitz 1973) convinced Leibowitz that NA played a vital role in the regulation of feeding behaviour and that this effect was primarily mediated via a medial hypothalamic site. Thus, she undertook an extensive mapping study of the hypothalamus to identify the site which produced the greatest feeding response to microinjection of NA (Leibowitz 1978a). This study identified the PVN as the most responsive site to NA injection, in the satiated rat. In mildly hungry rats NA was shown to potentiate ongoing feeding, the most potent site

again being the PVN. Potentiation in these animals was observed along the periventricular hypothalamus adjacent to the third ventricle. Injection of NA into the LH of these hungry animals resulted in a suppression of feeding. This finding, in parallel with those discussed in the earlier part of the introduction, further implicated the PVN in the control of feeding. Further work (Leibowitz, 1978b) demonstrated that the effects of NA injection were consistent despite variation in the time of injection - 4 hrs. into the light cycle, or 4 hrs. into the dark - or the baseline food intake or diet. Moreover, a response could be obtained from very low (4.2ng) doses of NA. Injections of NA or DA into the lateral hypothalamus suppressed feeding, as did injection of amphetamine. (For a review of the mechanisms and sites of actions of anorectic drugs see Leibowitz 1978c). When catecholamine re-uptake blockers were injected the suppressant effect of LH stimulation was reliably strengthened; ß- but not α- blockade inhibited the suppression of feeding. These data provided further support for a lateral, ß-adrenoceptor mediated feeding inhibitory mechanism (Leibowitz and Rossakis 1978).

That NA microinjected into the PVN resulted in feeding behaviour was now an accepted result but it remained to be demonstrated that the endogenous compound acted in the same way at this site. It was necessary to show that the actions

of endogenous NA could result in feeding behaviour. Whilst it was difficult to measure spontaneously released levels of endogenous NA it was possible to use pharmacological manipulations to induce the release of the endogenous transmitter. Many antidepressant compounds were believed to exert their effects through actions on endogenous catecholamine systems. One such drug, tranylcypromine, acts as a monoamine oxidase inhibitor, granule depleting agent, and partial re-uptake inhibitor. Injection of this compound into the PVN, it was hypothesised, would result in the release of endogenous catecholamines from their storage granules and a reduction in the rate at which the catecholamines thus released would be inactivated. This would result in high levels of catecholamine availability at receptor sites. Within the PVN the predominant catecholamine is NA and it was argued that injection of tranylcypromine would mimic the effects of injection of exogenous NA as a result of increased endogenous NA release (Leibowitz et al. 1978a). [Recent work by Irie and Wurtman (1987) examined the release of NA from a slice preparation following electrical stimulation. Both spontaneous and evoked release were greater in a DMI containing than free media. This suggests that without DMI, the NA released from the slices was largely taken up into nerve terminals or glia. Furthermore, high levels of stimulation (1800 pulses in 10s trains, 60mA with 10 sec. intervals) release only 8.79 +/-0.25 % tissue content of NA.

This is not very much, certainly far less than a 40nM injection. Overall these experiments suggest that only about 5% of NA in terminals is available for release. Given these data it is a considerable assumption that pre-synaptic manipulations can be exactly equivalent to pharmacological manipulations.] The results of Leibowitz et al. (1978a) experiment demonstrated that the injection of tranylcypromine into the PVN resulted in feeding behaviour, albeit on a palatable diet, but that this feeding was less potent than that produced by exogenous NA. Two other aspects of these experiments indicated that endogenous NA was responsible for the feeding response; (i) local pre-treatment with NA synthesis inhibitors blocked the response to tranylcypromine induced feeding but not that induced by exogenous NA, and (ii) the feeding to both exogenous NA and tranylcypromine could be selectively blocked by local injection of NA alpha-antagonists. It is of great interest to note that the most potent blockade of the tranylcypromine feeding response was obtained with the α-antagonist phenoxybenzamine, not phentolamine. Phenoxybenzamine had previously been reported as effective in the blockade of feeding induced by exogenous NA microinjection (Leibowitz 1975a). Other monoamine antagonist drugs were not effective in inhibiting this response; these were the ß-blockers propranolol and sotalol, DA blockers haloperidol and fluphenazine, the serotonin blocker cinanserin, and the cholinergic blocker atropine

sulphate. All these compounds were administered in concentrations previously demonstrated to be effective in providing receptor blockade without affecting feeding baseline levels.

Thus NA induced feeding in the medial hypothalamus was elicited most potently from the PVN, could result from exogenous microinjection or drug-induced release of the endogenous transmitter, and was susceptible to blockade by α-adrenergic antagonists but not by other antagonists active at other monoamine receptors. The use of the α-blocking agent phenoxybenzamine in these experiments has caused some difficulty in subsequent analysis of the results. Phenoxybenzamine is an irreversible antagonist selective for the α-1 subtype. Repeated injections of irreversible antagonists, as in Leibowitz et al. (1978a), would lead to an increasingly severe blockade in each trial for each individual dose. The α-1 selectivity of this compound, unlike phentolamine, would lead to a preferential depletion of this receptor subtype.

Further experiments involving antidepressant drugs which block NA re-uptake and perhaps enhance release, demonstrated that they elicited eating when injected into PVN. This eating correlated positively with the eating produced by injection of exogenous NA. Again this feeding could be blocked by

pre-treatment with synthesis inhibitors or α-receptor blockers (Leibowitz 1978b).

The involvement of NA mechanisms within the PVN with the feeding response having been demonstrated, the next area for investigation was the anatomical basis of the endogenous NA involved. Electrolytic lesions in the dorsal and ventral midbrain tegmentum produced a variety of effects relating to feeding and drinking, and also greatly altered the responses to feeding-associated drug manipulations within the hypothalamus (Leibowitz et al 1980). Therefore, Leibowitz and Brown (1980a) applied the techniques of: (i) selective lesions of catecholamine containing neurons with 6-OHDA; (ii) fluorescence histochemistry and (iii) brain cannulation to analyse the NA projections to PVN and their function in stimulating feeding. It must be remembered that much of the anatomical data previously referred to in this thesis was not available at this time. Leibowitz and Brown conclude that "*midbrain lesions are found to have dramatic effects on the responsiveness of the hypothalamic PVN to adrenergic drug manipulations. Associated with these effects are damage to specific ascending catecholamine projections and degeneration of catecholamine varicosities within the PVN. The convergence of histochemical, pharmacological and behavioral evidence supports the hypothesis that brain synthesised catecholamines, specifically in the PVN have a function in stimulating feeding*

behaviour; that the neuroanatomical substrate for this stimulatory function includes noradrenergic or adrenergic fibres of the dorsal CTT which originate from pontine (A5 or A6) or medullary (A1) cell groups, course in the dorsal tegmentum immediately dorsal to the superior cerebral peduncles, and then project ventrorostrally through the medial TR system into the ventral tegmentum; and that these adrenergic projections contribute to the feeding stimulatory effect of antidepressant drugs" [p.308] Leibowitz and Brown demonstrated that damage to either the A1 - A2 system or the A5 - A6 system affected the feeding response to adrenergic manipulations within the PVN, both systems being involved in some part. Further experiments (Leibowitz and Brown 1980b), demonstrated the involvement of the dopaminergic A8 and A9 systems in the feeding inhibition response to certain agents. However, accurate, quantitative data on the amount of NA depletion within the PVN produced by these lesions, were not described.

These results increased the evidence suggesting that the PVN was an area of the hypothalamus of major importance in the regulation of food intake and that this regulation involved NA from the ascending dorsal and ventral bundle systems. Electrolytic lesions of the PVN itself result in overeating and obesity (Leibowitz et al. 1981) as do coronal or parasagittal knife cuts in the vicinity of the PVN (Grossman

1975, Sclafani and Berger 1977). However, injections of the neurotoxin 6-OHDA into the PVN produced little change in feeding behaviour [Fahrbach and Leibowitz unpublished (in Leibowitz et al. 1981)p.1039], although more recently published results show a deficit in ingestion of energy-rich carbohydrate diets in the early dark period, or in response to food deprivation (Shor-Posner et al. 1986). Many other compounds have been demonstrated to affect feeding when administered into the PVN; GABA (Grandison and Guidotti 1977), ß-endorphin (Leibowitz and Hor 1980), enkephalins (McLean and Hoebel 1980), serotonin and cholesystokinin (McCaleb and Myers 1980). The response to manipulations within the PVN was clearly complex, but NA systems continued to provide the best avenues for further exploration.

Peripheral administration of the α-adrenergic agonist clonidine has been demonstrated to induce feeding in a variety of animals, as has intercerebroventricular, anterolateral hypothalamic, or PVN injection (see McCabe et al. 1984). Feeding induced by peripherally administered clonidine can be blocked by peripheral administration of the α-2 selective antagonist yohimbine but not the α-1 selective antagonist prazosin (Schlemmer et al. 1981). This indicated that clonidine exerted its effects through an α-2 receptor mechanism. An examination of the effects of microinjecting selective α-1 and α-2 blockers into the PVN was performed

(Marino et al. 1983). These experiments demonstrated that "*the general α-adrenergic antagonist phentolamine was found to essentially abolish (-82%) the NA eating response. Clonidine induced feeding was also significantly attenuated by phentolamine. Tests with the specific α-1 antagonists, corynanthine and prazosin, failed to reveal any blocker induced attenuation of NA and clonidine feeding.....in contrast the α-2 antagonists yohimbine and rauwolscine significantly attenuated, by 50-75%, both NA and clonidine induced feeding*" [p.467]. A separate experiment in the same report showed that PVN injection of the α-2 antagonist yohimbine significantly suppressed the effects of systemically administered clonidine by 40-80%.

Leibowitz and her colleagues hypothesised that the feeding elicited by PVN microinjection of NA or clonidine was mediated via the α-2 receptor and attempted to define whether or not this receptor was pre- or post-synaptic. High levels of α-receptors had already been demonstrated within the PVN (Leibowitz et al. 1982) but their precise location was not defined. The α-2 receptor had recently been demonstrated to occur post-synaptically as well as pre-synaptically (Paciorek et al. 1984, Unnerstall et al. 1984, Pimoule at al. 1985). It is possible that they may even predominate in the post-synaptic location (Starke 1981a, 1981b, Cerrito and Preziosi 1985, Gothert 1985). Also the actions of clonidine

were believed to be post -synaptic as well as pre-synaptic (Spyraki and Fibiger 1982). Thus as clonidine displays a selectivity for the α-2 receptor Leibowitz and her colleagues suggested that NA injected into the PVN was acting through a post-synaptic α-2 receptor to stimulate feeding behaviour. The following evidence in support of this hypothesis is cited in Leibowitz et al. (1982): (i) eating induced by PVN NA is antagonised by α-2 adrenergic, in contrast to α-1 adrenergic, receptor blockers (Marino et al. 1983). (ii) Eating elicited by peripheral doses of clonidine occurs at doses below 100ug/kg that are believed to act upon α-2 rather than α-1 receptors. (iii) Eating produced by peripheral clonidine can be blocked by PVN injection of the α-2 antagonist yohimbine (Marino et al. 1983) but remains unaffected by PVN injection of the selective catecholamine neurotoxin 6-OHDA (Leibowitz, Shor-Posner and Azar, unpublished, cited in Marino et al. 1983 p.467). Eating induced by ventricular NA injection is essentially abolished by electrolytic PVN lesions (Leibowitz et al. 1983). (iv) Eating induced by PVN injection of NA occurs independently of presynaptic stores of endogenous NA (Leibowitz and Brown 1980a). That is, disruption of NA afferents to PVN or local administration of α-methyl-p-tyrosine significantly enhances NA induced eating (a post-synaptic response) while attenuating the eating response to PVN injection of antidepressant drugs (a pre-synaptic response). (v) In contrast to clonidine's hypothesised

pre-synaptic pattern of action associated with a decrease of NA turnover (Langer 1977), clonidine's effects in the PVN include a reduction of NA levels and an apparently stable NA turnover (Jhanwar-Uniyal et al. 1983).

The hypothesis presented, that NA induced feeding in the PVN was mediated via a post-synaptic α-2 receptor was further tested using a range of α-adrenergic antagonists (Goldman et al. 1985). Again the conclusion was made that the feeding elicited by NA or clonidine injected into PVN was the result of these compounds acting at a post-synaptic α-2 adrenergic receptor. Leibowitz and her colleagues have performed many further experiments to determine the role of the PVN in the regulation of feeding behaviour which will be reviewed briefly below, but it is the conclusion that this feeding results from an action at post-synaptic α-2 receptors that is of major importance to this thesis.

The feeding that can be elicited by microinjection of NA into the PVN can be suppressed by compounds other than α-adrenergic antagonists. The peptidergic transmitter neurotensin has been demonstrated to have a suppressant effect on NA elicited feeding in PVN (Stanley et al 1985a). Similarly, compounds other than NA can elicit feeding when microinjected to this site. The most potent feeding response so far tested is produced by microinjection of neuropeptide Y (Stanley et al.

1985b). Interestingly this peptide has been demonstrated to co-exist with NA in certain ascending fibres (Lundberg et al 1984). The demonstrations that such a variety of compounds can influence feeding when microinjected into PVN serve to demonstrate the complexity of such a fundamental response as food intake.

Leibowitz and her co-workers have recently attempted a more complete analysis of the feeding behaviour seen as a result of NA injected into the PVN and attempted to describe the role that the intact structure plays in normal feeding. After PVN NA injection rats demonstrate a clear preference for high carbohydrate diets, (Leibowitz et al. 1985). This effect is robust and occurs with both chronic and acute NA infusion. Similarly, 6-OHDA infusion into PVN results in an initial increase (24hrs) in food intake, particularly carbohydrate, followed by a long term deficit in carbohydrate intake (-42%). Although these rats maintained a normal diurnal feeding pattern they failed to exhibit the increased ingestion of an energy rich carbohydrate diet which rats normally show during the dark cycle (Shor-Posner et al. 1986). Indeed, rats with 6-OHDA lesions of PVN displayed a syndrome of deficits concerned with the compensatory eating of energy rich foods after periods of deprivation or rapid energy expenditure, suggesting that the PVN α-2 noradrenergic system might be involved in such compensatory mechanisms. The feeding to

microinjected NA was found to be dependent upon the integrity of the adrenal system, and further study demonstrated that it was the presence of corticosterone that was a requirement in producing this effect (Roland et al. 1986). Corticosterone levels have been demonstrated to show a circadian rhythm which closely resembles the periodicity of natural feeding in the rat, being at a peak just after the onset of the dark cycle. Radioligand binding studies by Jhanwar-Uniyal et al. (1986) demonstrated a similar pattern of periodicity in the number of α-2 binding sites in rat PVN. A monophasic peak of PVN NA receptor binding is detected at the onset of the dark cycle, when corticosterone levels are highest and feeding is initiated. Leibowitz suggests that there may be an interaction between circulating corticosterone levels and α-2 noradrenergic receptor binding in the PVN and that this may be of importance in the initiation of natural feeding behaviour.

The work of Leibowitz and her colleagues forms an impressive and powerful argument indicating the involvement of the PVN in the control of feeding in the rat. Pharmacological, biochemical and behavioural data have been combined to present a strong case in support of this theory. It is not my intention to dispute the conclusions drawn by Leibowitz from her programme of experiments, but rather to offer an alternative interpretation of some of this data and, more

importantly, to show how the pharmacological and behavioural manipulations she has used in this paradigm may be applied to provide an explanation of how NA is transmitted within this nucleus and possibly the brain as a whole.

THE PVN AS A FEEDING REGULATORY CENTRE

The evidence that the PVN can influence feeding behaviour is very strong, although it must be remembered that feeding is not a simple reflexive type of behaviour: many physiological measures, such as blood glucose levels, insulin levels, glycogen stores, fat stores and energy requirements must be calculated. However, any purely physiology driven model of feeding is obviously inadequate. A multitude of other variables must be considered: the location and availability of food, palatability, other ongoing activity, and the motor control of feeding related behaviour. Even such factors as habit and 'what the rat next door is doing' may influence feeding in the laboratory rat. Yet it is clear that injection of NA into PVN results in feeding behaviour in a satiated animal. Thus the questions to be addressed regarding the PVN and feeding regulation are threefold: (i) why the PVN, what aspects of PVN anatomy and chemistry make it suitable for such a complex regulatory function ; (ii) how does the PVN exert influence over such physiological mechanisms ; and (iii) do the results of laboratory manipulation of PVN by microinjection and other techniques indicate the role of this

nucleus under normal conditions ?

To answer the first question, it is necessary to reconsider the anatomy of the hypothalamus in general terms. It is also necessary not only to study the mechanisms of visceral or sensorimotor control systems but also their integration and consider what is meant by a regulatory system. Behavioural responses are initiated by both internal and external signals and the problem faced by the scientist was summed up by C.J. Herrick who observed " *The living body...its continual welfare is conditioned upon a nicely balanced adjustment between its own activities and those of surrounding nature, some of which are beneficial and some harmful. The great problem of neurology is the determination of the exact part which the nervous system plays in this adjustment*" (Herrick 1918 p.13). The hypothalamus as a whole has long been regarded as playing a particularly important integrative role in the expression of adaptive behaviour related to homeostasis (Hess 1947; Ranson and Magoun 1939). Homeostasis, the maintenance of a constant internal environment, is of fundamental importance in the survival of an organism. Two points are of particular relevance here; first the hypothalamus is the most rostral part of the CNS in which lesions can abolish the performance of integrated adaptive responses; second, all levels of the CNS contribute to the expression of adaptive behaviour. The goal of the

neuroscientist working in this area is expressed by Swanson as being "*to clarify the precise role of specific parts of the hypothalamus in different types of response, and to determine how these [centres] are related to the rest of the CNS. In essence....to establish the neural basis for Hess's observation that electrical stimulation of the hypothalamus can lead to integrated, goal-oriented behavioural responses*" (Swanson and Mogenson 1981 p.2). Whilst microinjection of NA into the PVN may not be stimulatory in Hess's terms it still results in the generation of goal-oriented behavioural responses, namely feeding and furthermore this occurs in a satiated and thus, prior to injection, 'unmotivated' animal.

It is apparent that any regulatory mechanism must have two features; first it must be supplied with the relevant afferent information concerning the existing state of the organism and second it must have efferent connections such that it can effect changes to this state. The hypothalamus is perfectly equipped for this role. Certain discrete hypothalamic nuclei are completely without a blood-brain barrier giving these areas access to circulating blood glucose, osmolarity, and insulin and other hormones. Such areas include the sub-fornical organ, the median eminence and the nucleus circularis, and each may be involved in a different aspect of physiological homeostasis. Other hypothalamic nuclei are

involved in cyclic regulation, the supra-chiasmatic nucleus in long term cycles and the medial pre-optic area in short term circadian rhythms. The hypothalamus is located above the neurohypophysis to the pituitary and controls the release of many hormones and releasing factors; an important component of hypothalamic efferent activity. The PVN has a central location within the hypothalamus, and the afferent projections to this nucleus suggest that it may receive information as to the state of the periphery from the vagus and NST. Similarly the efferent connections of PVN indicate a possible role in the regulation of autonomic activity and hormonal balance. Whilst it is not possible to state for certain the function of a brain area based on a knowledge of its anatomy and connections, such information is a powerful indicator. Certainly the anatomy of the PVN is consistent with this region having a major role in energy balance and homeostasis. Thus the PVN fulfils the "why this area" question.

The second question about the involvement of the PVN in the control of feeding was, how does it exert its influence? Again the anatomy of this region provides the major clue. The major PVN efferents are; (i) to brainstem nuclei and spinal cord and (ii) to the neurohypophysis and pituitary. It has therefore, two means of influencing the state of the periphery, via direct neuronal influence on the autonomic nervous system and by the manipulation of circulating hormone

levels. The empirically derived information that lesions of the PVN, or microinjection of NA, affect food intake is not sufficient to prove that feeding regulation is the function of PVN under normal conditions, although it may be taken as an indication of such. The anatomy indicates how the PVN might exert control over feeding behaviour by altering the state of physiological components in the periphery but there is a second possible mechanism. The microinjection of NA into the PVN might give rise to the brain state 'hunger' which itself triggers feeding behaviour without there being a physiological requirement. Indeed, the feeding behaviour elicited by NA is prominent in satiated animals which by definition have no physiological need. However, the fact that there is a preference for certain macronutrients, particularly carbohydrate indicates that the animal feels some sort of physiological need. In contrast, the fact that the rat is satiated prior to the test (during which the characteristics of the diet do not change) should by definition exclude the possiblity that feeding is triggered by a 'physiological' deficit in some macronutrient; maybe NA induced feeding is the result of a particular brain state in the animal. There still remain certain physiological requirements however, such as the presence of adrenocorticosteroids for feeding to NA to be expressed. To attempt to explain feeding caused by PVN manipulation in terms of either brain state or physiology contradicts the function of the hypothalamus. The

hypothalamus is a complex integrator receiving and integrating information of both cognitive and physiological import. It is composed of nuclei themselves serving an integratory function. Some of these have predominantly efferent influences whilst others have afferent but all are predominantly concerned with the integration of multiple inputs which bear on the maintenance of homeostasis.

Earlier the concept of why the PVN should affect feeding behaviour was examined with respect to the anatomy of this nucleus. This has suggested why the PVN might be involved in the regulation of food intake as a result of it's anatomical organisation but the experiments of Sarah Leibowitz beg a different answer to the question "why the PVN?". This is "why?" in terms of; what physiological or 'mental' processes does the PVN modulate such that manipulations of this nucleus result in changes in feeding behaviour ?

It is clear that Leibowitz herself conceives the PVN as being an important component in the normal, day-to-day control of food intake in the rat. The integration of high α-2 binding capacity, a peak in the diurnal pattern of corticosterone levels and concurrent NA release is seen as the 'normal' signal that in a rat initiates food intake at the beginning of the dark cycle. Furthermore, as this signal is also

associated with a preference for carbohydrate intake, dietary selection of this macronutrient is also at a peak at this time. As the dark cycle progresses, α-2 binding capacity and levels of corticosterone begin to fall and the rat reduces meal size and preference for carbohydrate. The demonstration by Smyth et al. (1984) of a positive correlation between hypothalamic NA levels and blood glucose levels can be seen as direct support for this argument at the biochemical/ physiological level. If this is indeed the case then the state into which the injection of NA puts the animal must be 'hunger', because such an injection would create a state within the brain indiscriminable from that experienced under 'normal' conditions of hunger. However, this does not appear to be the case as rats after intra-PVN injections of NA display certain differences from normal, food deprived controls. If rats which have been injected with NA into the PVN are prevented from gaining access to food for 1 hour, the subsequent feeding is suppressed compared to animals with immediate access to food following an identical injection (Swiergel and Peters 1987). Similarly, rats are less willing to work for food on an operant schedule following PVN NA than mildly food deprived animals (Matthews et al. 1985) and are more sensitive, or 'finicky', to the palatability of the diet than controls (Sclafani and Toris 1981). Furthermore, Swiergel and Peters (1987) have demonstrated that following PVN NA injections rats will gnaw at wood chips, but only if

these are presented in the animals' normal food hoppers and shaped like food pellets. Food deprived animals did not gnaw these chips, neither did sham injected animals. It seems most probable that the injection of NA into PVN is in some way acting on processes which are indirectly involved in normal regulatory feeding, but it is clearly not the 'whole story' of normal food intake.

If the PVN is a brain area concerned with the regulation of feeding and energy balance it is interesting to ask how and on which processes it exerts it's influence. If it is not as Leibowitz suggests responsible for the brain state 'hunger' then feeding must be brought about by some PVN regulated process, acting on the physiology of the rat, which results in it 'believing' itself to be, however temporarily, in need of food. In these circumstances the term 'hungry' is used to represent a state in which the ingestion of food is currently top in a hierarchy of freely selectable behaviours. There are two possible mechanisms whereby the physiology of the rat might be affected by manipulations of the PVN. First, the PVN is involved in components, particularly endocrine, of the response to stress. It is widely held that stress and feeding are in some way linked (Robbins and Fray 1980) and thus the PVN might exert an influence over food intake through an indirect 'stress induced eating' mechanism. Second, the PVN by its spinal connections is only one or two synapses from the

liver where it may influence the control of glycogen stores and manipulation of these reserves could rapidly alter blood glucose levels and thus indirectly affect food intake. These two hypotheses must be examined in detail in order to assess their impact on the role of the PVN in the regulation of food intake.

PVN CONTROL OF LIVER FUNCTION

As has been suggested previously in this introduction, the hypothalamus is the CNS region most involved in the regulation of the sympathetic and parasympathetic nerves. In addition to the control of endocrine status, the hypothalamus acts via these nerves to control the response to physiological emergency requirements, and to maintain homeostasis. If the hypothalamus is the CNS homeostasis regulator, the liver amy be the peripheral effector mechanism through which it might exert its control. In the maintenance of glucose homeostasis, for example, the liver plays a significant role. It acts as a major temporary store capable of removing large quantities of glucose from the circulation in times of excess and releasing it in times of need. This glucose is stored in the parenchymal hepatocytes in the form of glycogen. The conversion of glucose to glycogen is controlled by the enzyme glycogen synthetase, whilst that of glycogen to glucose is controlled by glycogen phosphorylase. These are not the only two enzymes involved in the conversion but represent the rate limiting steps in the process (Shimazu and Fukuda 1965). Thus whilst the optimal level of blood glucose may be 'determined' or 'defined' by hypothalamic mechanisms, it is the liver which physiologically regulates actual plasma glucose concentration.

The nerves innervating the liver fall into three main categories, sympathetic, parasympathetic and afferent

components (for full review see Sawchenko and Friedman 1979). The sympathetic fibres to the liver are derived from the splanchnic nerves; the pre-ganglionic fibres reaching the celiac ganglia through the greater and lesser splanchnic nerves. The post-ganglionic fibres originate in the celiac ganglia and form the celiac plexus. The pre-ganglionic fibres of the parasympathetic system also reach the celiac plexus, via the anterior and posterior vagus. Extending from the celiac plexus into the hepatic portal region is the hepatic plexus containing post-ganglionic sympathetic and pre-ganglionic parasympathetic fibres. The post-ganglionic parasympathetic fibres arise in the ganglion cells located closer to the liver in the hepatic hillus (Shimazu 1983). There is good evidence that individual hepatic parenchymal cells receive innervation from both sympathetic and parasympathetic branches of the peripheral nervous system, although the density of this innervation is species dependent. In the rat the number of synapse-like contacts is low but there are many gap junctions between neighbouring hepatocytes and synaptic innervation may be widely propagated (Hartmann et al. 1982). Afferent innervation of the liver may be important in terms of its osmoreceptive, chemoreceptive and baroreceptive functions (Sawchenko and Swanson 1979). Whilst the afferent pathways are poorly defined there seems little doubt that the liver is supplied with information from a variety of peripheral receptor mechanisms. This afferent

information courses along fibres forming a proportion of the splanchnic and vagus nerves. Because of the intimate involvement of the liver in the metabolism of ingested nutrients, signals from hepatic chemoreceptors are presumed to inform the CNS about the caloric content of ingested food, probably through afferent fibres of the vagus (Mei 1978). This information is presumed to reach the hypothalamus (Schmitt 1976, Jeanningros and Mei 1980) which is thus constantly informed about glucose status and hepatic carbohydrate metabolism.

Since the first investigations of Bernard (see above p.1) it has been assumed that it is the sympathetic nervous system that is responsible for initiating hyperglycemia by promoting the mobilisation of liver glycogen, and that this depends almost entirely on neuroendocrine mechanisms, with mediation of adrenaline released from the adrenals and glucagon from the pancreas (Frohman 1971). However, evidence that hepatic glycogenolysis is under direct control of the sympathetic, hepatic innervation was demonstrated by Shimazu and Fukuda (1965). They demonstrated significant increases in the activity of the enzymes responsible for glycogen breakdown (glycogen phosphorylase and glucose-6-phosphatase) with a concomitant decrease in liver glycogen content, following electrical stimulation of the peripheral end of the splanchnic nerve. Since these effects persisted after removal of both

adrenals and pancreas, which also receive sympathetic innervation, they concluded that the release of either adrenaline or glucagon was not necessary for the rapid increase in hepatic glycogenolysis in response to splanchnic nerve stimulation. Thus, these effects were directly dependent upon sympathetic nerve stimulation. Subsequent research has shown that a 300% increase in glycogen phosphorylase activity can occur within 30 seconds of splanchnic nerve stimulation, and that this increase can be counteracted by concurrent vagal stimulation (Shimazu and Amakawa 1968). These data clearly suggest a mechanism by which the CNS might mediate glucose output from the liver via direct neuronal intervention.

In an extensive review of the reciprocal innervation of the liver Shimazu (1983) draws attention to the need for detail in assessing the significance of the intrinsic innervation of this organ. It is therefore unfortunate that in his exploration of the effects of hypothalamic stimulation on liver function his analysis of the hypothalamus is relatively crude. Shimazu divides the hypothalamus into a medial 'satiety' region (VMH) and a lateral 'feeding' region (LH). However, stimulation of the VMH as a whole may involve stimulation of PVN and associated fibre systems as well as VMN, and the results of such stimulation are very similar to the results obtained from splanchnic nerve stimulation.

Electrical stimulation of the VMH causes a rapid rise in circulating glucose and a marked decrease in liver glycogen as a result of increased glycogenolysis; this result has been consistently confirmed (Frohman and Bernardis 1971, Gisel and Innes 1979, Shimazu 1983). In order to identify exactly which hypothalamic site is involved in this sympathetic outflow a more anatomically detailed study is required. It is of interest to note that Weingarten et al. (1985) have conducted a study contrasting the metabolic effects of discrete electrolytic lesions of VMH and PVN. The rats from both lesion groups became obese when fed 'ad lib' but the VMH lesioned animals were hyperinsulinaemic and showed increased levels of gastric acid secretion whereas the PVN lesioned animals did not show any detectable metabolic disturbances. When maintained on a restricted diet, only the VMH lesioned animals became obese, revealing a fundamental difference in aetiology between the two obesity syndromes.

The information presented thus far in this introduction forms the basis of a possible, if speculative, hypothesis concerning the role of the PVN in the regulation of food intake. This hypothesis is as follows: stimulation of the PVN by NA causes depletion of liver glycogen as a result of increased sympathetic outflow. This depletion of its reserves causes the liver to generate a signal indicating rapid metabolic loss, which in turn acts to promote feeding. The assumption

is made that the signal arising from the liver is of sufficient intensity to predominate over other afferent information indicating high levels of plasma glucose (see below). There is an apparent paradox, in that electrical stimulation of the VMH suppresses food intake, whereas NA injection promotes feeding, and both mechanisms apparently involve increasing the rate of glycogen -> glucose conversion in the liver. However electrical stimulation of brain causes complex changes in behaviour in addition to such measurable changes in the physiology of an animal. Eliot Valenstein (1973), using the elicitation of feeding from LH stimulation as his paradigm, pointed out four aspects of electrical stimulation of the hypothalamus which diminish its impact as a measure: (i) In the rat there appeared to be a lack of anatomical specificity; (ii) the responses often lacked specificity at the behavioural level; it was possible to elicit different behaviours from the same stimulus parameters and site, depending upon the presence or absence of goal objects. Such 'mixed' responses are very prevalent following hypothalamic stimulation;

(iii) the responses strengthened with repetition, indicating the possibility of a learning effect; and (iv) the elicited reponses were frequently abnormal in nature. Animals often ignored preferred foods in favour of those that had been presented in previous periods of stimulation.

Robbins (1986) states "*these observations do not encourage the notion of fixed circuitry in the hypothalamus responsible for particular forms of consummatory response which, when activated immediately turn on the appropriate response*" (p.276). The complexities of interpretation of the results of electrical stimulation of the brain are such that the fact that VMH stimulation disrupts feeding behaviour is not a strong argument against the proposed hypothesis.

However, a second paradox also needs explanation: stimulation of the rate of glycogen -> glucose conversion would result in an increase in blood glucose level, normally seen as a satiety signal. It is necessary to examine the possible signals to feed reaching the hypothalamus; (i) the signals produced by food deprivation, might be expected to increase in intensity over a relatively long time course and need to be at a relatively high and sustained level to influence the CNS to initiate feeding behaviour; (ii) the signal produced by sudden depletion of liver glycogen might be both rapid in onset and intense, having a powerful and immediate effect. The signal from the liver may be sufficiently potent to overrule the signal from blood glucose monitors. This explains why preventing a satiated rat from feeding for 6 hours following VMH NA injection suppresses the expected feeding response (Coons and Quatermain 1970). The glucose liberated from the liver would, over this ensuing period, be converted back to

glycogen inhibiting the signal from the liver. Evidence that signals from the liver can be the most potent in the determination of feeding behaviour is provided by the work of Friedman, Stricker and their colleagues. They demonstrated that 'hunger' can be controlled by metabolic products available to liver and not brain. They made rats hypoglycaemic by injection of insulin and then administered injections of several different nutrients. Injections of the sugars glucose, fructose and mannose all inhibited feeding. However, fructose cannot cross the blood-brain barrier and would thus occur in only very low concentrations in the CNS. In contrast, injections of a ketone which can be metabolised in brain but not in the liver failed to inhibit feeding (Friedman et al 1976, Stricker et al 1977).

The hypothesis that the injection of NA into PVN results in feeding because of glycogen -> glucose conversion in the liver has the power to incorporate and explain much of the available data.

(i) The behavioural specificity of the feeding response is explained; glycogen depletion from the liver requires energy intake to make up the deficit.

(ii) Following infusion of NA into the PVN rats preferentially select high carbohydrate diets, which would serve to replace glucose most rapidly.

(iii) Electrolytic lesions of PVN promote obesity in the

absence of hyperinsulinaemia; one consequence of the impairment of control over liver glycogen might be the deposition of excess fat. Also the loss of an effective 'glucose buffering system' might result in more frequent feeding bouts.

(iv) The latency to eat following intra-PVN infusion of NA (2-10 mins.) is consistent with the time necessary to deplete liver glycogen: a 300% increase in glycogen phosphorylase activity can be achieved within 30 seconds following splanchnic nerve stimulation (Shimazu and Amakawa 1975).

(v) The imposition of a delay between infusion of NA and feeding, such as an operant task, reduces the amount of food consumed presumably as a result of re-conversion of glucose to glycogen (Booth et al 1986).

This hypothesis accounts for much of the apparently contradictory data surrounding feeding behaviour and manipulations of the medial hypothalamus, particularly the PVN. However, neither this hypothesis, nor the involvement of the PVN in stress related responses makes clear exactly what role this nucleus plays in the regulation of day to day feeding behaviour. What is apparent is the complexity and variety of signals that can result in feeding behaviour. Far from being the simple, ubiquitous behaviour originally supposed feeding is a complex, integrated behaviour becoming daily more explicable but still not understood.

STRESS INDUCED EATING.

Eating can be influenced by a diversity of stimuli, not all of which are involved in the physiological regulation of energy balance. Certainly the internal signals arising from nutrient or caloric imbalances play a vital role, but even brief introspection reveals the involvement of myriad other factors. The taste, smell or appearance of food all influence ingestive behaviour and it is widely believed that 'stress', often in the form of emotional upset or boredom can also affect food intake. There is a popular concept of stress-induced eating whereby environmental and emotional stimuli, apparently unrelated to food, result in a syndrome of hyperphagia and obesity. Those stimuli that elicit eating and cannot be described as resulting from either food-deprivation induced physiological factors (stomach contraction or low blood glucose) for instance, or food-related stimuli (smell, palatability), have been referred to as 'nonspecific' stimuli by Fass et al. (1981). The factors responsible for 'stress-induced' eating are thus categorised as nonspecific, but some definition of 'stress' is also required. Definition of such a vague concept is hard but Valenstein (1976 p.113) suggests: "*a state when the 'adaptive' mechanisms (of an organism) are taxed beyond their normal range of functioning either because of the intensity or duration of the response required*". There are a great many

nonspecific stimuli reported to result in stress-induced eating. Noises, lights, handling, social influences and isolation are amongst those stimuli reported to result in stress induced eating in rats (for a full review see Robbins and Fray 1980). Other manipulations that are sometimes referred to as producing stress-induced eating, such as electrical stimulation of the brain (ESB), or pharmacological manipulation of transmitter systems can not be clearly categorised as nonspecific as their mode of action might be such as to result in a signal of food deprivation. The use of a term such as stress is in itself confusing, because this term has subtly different implications depending upon the context of its use, (for a discussion of what constitutes stress see Antelman and Chiodo 1983). One of the most intriguing of the nonspecific stimuli to elicit eating in rats is tail pinch (TP). First reported by Antleman and Szechtman in 1975, tail pinch has been widely used as a model nonspecific stimulus. Tail pinch, a sustained, mild pinch to the tail resulted in eating, gnawing and licking in virtually every rat tested (Antelman and Szechtman 1975). The pressure used was such that the animals ate vigorously without vocalising or showing other signs of distress. Initially the rats reared and began sniffing and exploring, orientation to the tail and grooming often occurred. However after several trials the eating became 'stimulus-bound' to the onset and offset of the pinch and the other competing responses were

FIGURE 17.

A. Selective blockade of eating elicited by 40nmoles NA by prazosi and idazoxan. Prazosin (0.2nmoles) did not affect NA elicite eating but 40nmoles idazoxan significantly attenuated eating t NA. When 40nmoles cocaine was added to NA eating was unaffected but 40nmoles idazoxan was completely ineffective in antagonisisn NA/cocaine eating. (:sig. diff. to 40nmoles NA [hatched bar $p<0.05$ at least.)

B. Comparative blockade of eating elicited by 30nmoles NA an 30nmoles NA/ 40nmoles cocaine. Blockade to 30nmoles idazoxan wa attenuated when cocaine was added to NA. Blockade to 30nmole phentolamine was unaffected by the addition of cocaine to NA. :sig. diff. to 30nmoles NA [hatched bar] $p<0.05$ at least.)

disproved:

(i) Eccle's proposal of Dale's principle, in brief that each neuron contains only one transmitter substance*[1]. Co-localisation studies using double labelling with specific antibodies categorically demonstrate the co-existence of different transmitter substances within neurons (Hokfelt et al. 1980).

ii) Eccles principle, that a transmitter substance opens just one type of ionic gate, that being either excitatory or inhibitory, on the post-synaptic membrane. Considerable information is now available to demonstrate that the effects of a neurotransmitter on the post-synaptic cell depend upon the nature of that cell, the type of receptor, the presence of other inputs, and a great many other variables.

However, the assumption that within the CNS neurone-neurone chemical transmission occurs only through discrete synaptic contacts is still widespread. This 'classical' model of transmission has recently been challenged by an alternative hypothesis of 'paracrine' or non-synaptic transmission. This hypothesis suggests that transmitters may be released not into morphologically defined synaptic clefts but into the extra-cellular space. They then diffuse to their site of action, specificity being retained by the presence or absence of appropriate receptors rather than by anatomical constraints.

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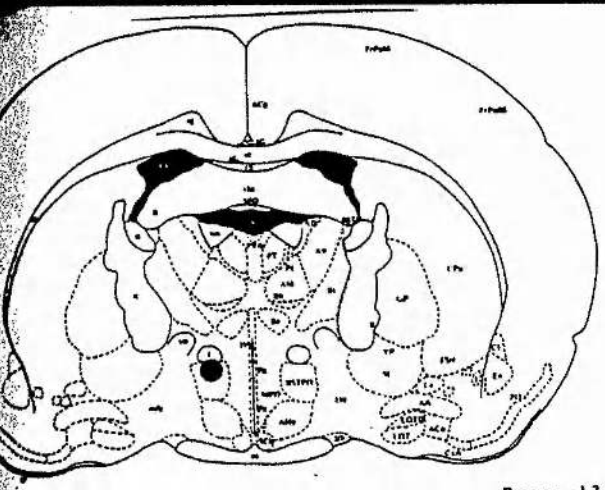
* Unfortunately an inaccurate reproduction of Dale's work - for full discussion see: Multiple Transmitter Status and "Dale's Principle". Potter D. Furshpan E. and Landis S. (1981) Neuroscience commentaries 1, 1: 1-9.



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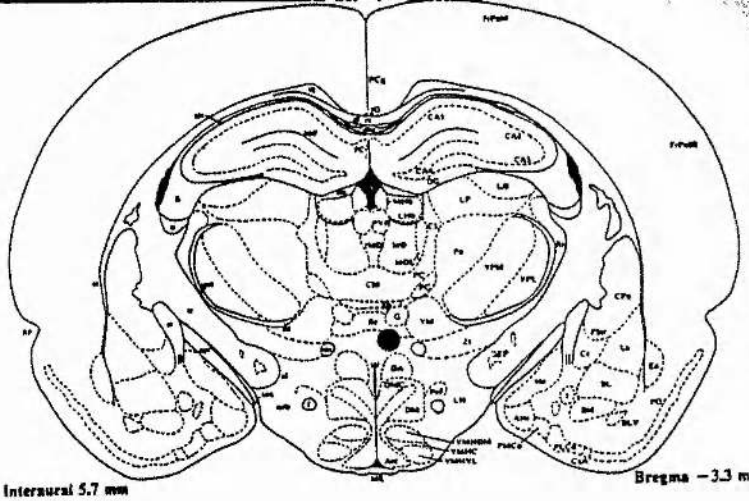

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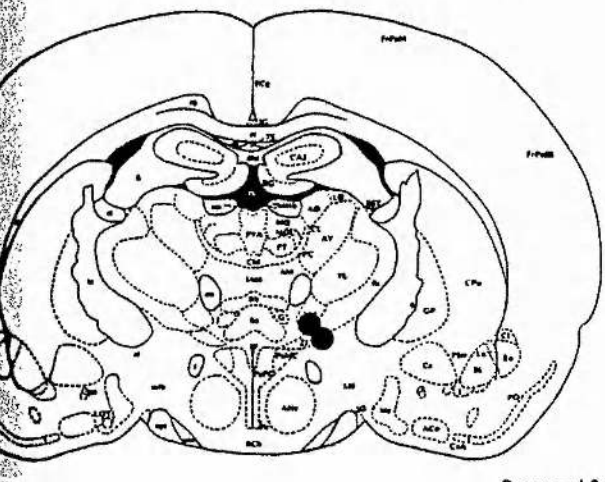

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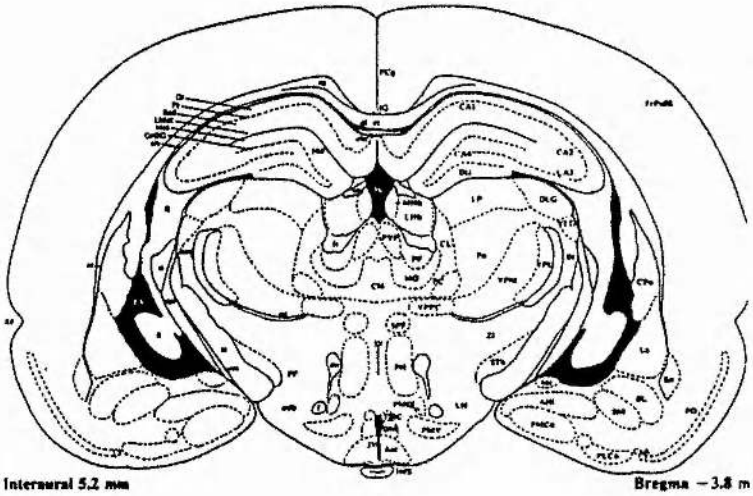

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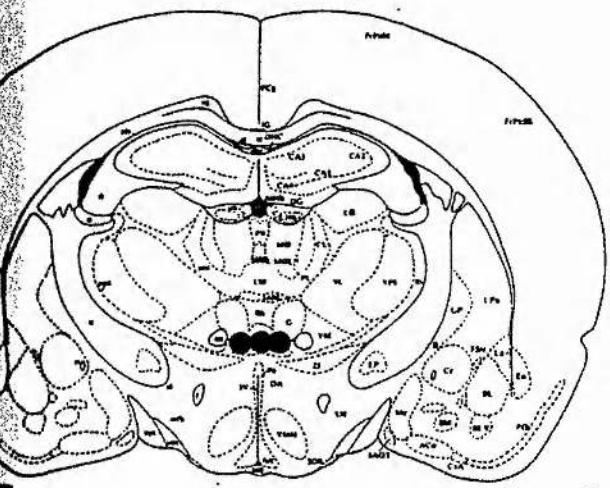

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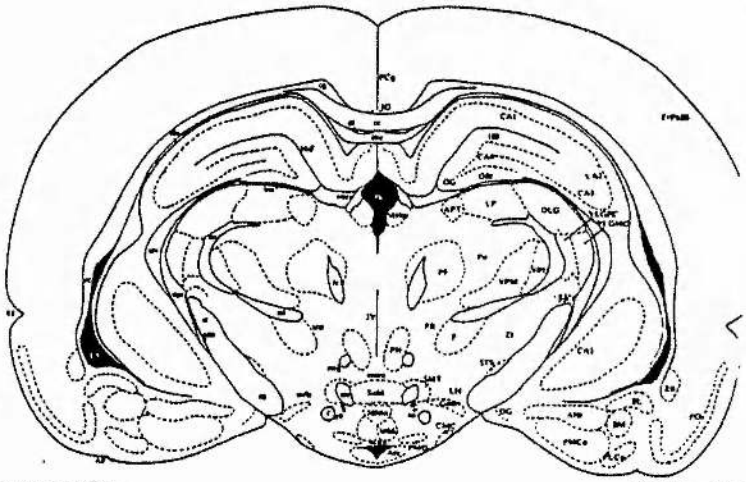

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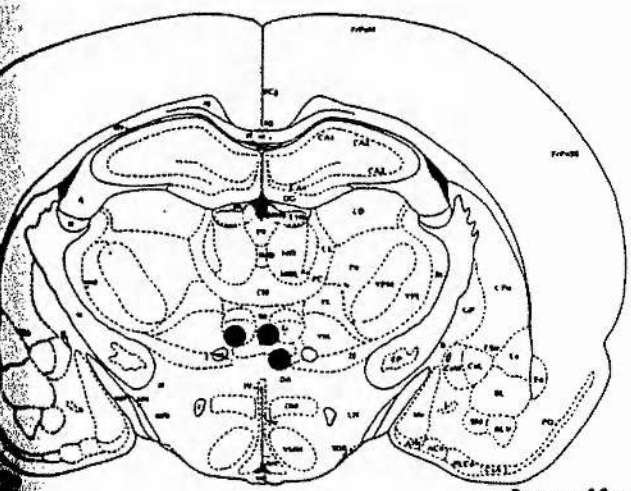


Bregma −2.8 mm

● = the injection sight (n=10)

replaced by eating (Antelman and Szechtman 1975, Antelman et al 1975). Under TP rats demonstrate preferences for certain foods, they will not drink plain water but will consume food pellets, milk and saccharin water. This apparent finickiness was suggested by Antelman et al. (1976 p.746) to result from "*lowered sensory motor thresholds and responsivity to... environment*". In contrast Marques et al.(1979) suggested that the finickiness resulted from a TP induced change in the valence of taste cues but there is little evidence that TP increases the aversive properties of unpalatable foods.

There is clearly an appetitive component to tail pinch as preloading the stomach with milk has been demonstrated to reduce TP induced milk drinking when saline preloading did not (Antelman et al. 1976). However, ingestion under TP was in addition to normal dietary requirements as repeated tail pinch (10 mins. every 4 hours for 5 days) resulted in hyperphagia and obesity (Rowland and Antelman 1976). Eating to TP could be increased by administration of the minor tranquilliser chlordiazepoxide (librium) in doses which had no effect on eating in control trials without TP (Robbins et al. 1977). It has been suggested that TP induced feeding may be a combination of two components, an aversive component and an arousing, appetitive component. The aversive component is masked or reduced by low doses of librium allowing the unmasking of the consummatory component (Robbins and Fray

1980). The consummatory component of TP is dependent upon the salience of external stimuli, thus not only feeding but maternal behaviour, digging, sniffing, licking, and gnawing behaviours have been elicited under TP (Antelman and Caggiula 1977, Szechtman et al. 1977). The consummatory responses performed under TP have been demonstrated to have genuine motivational effect, as rats under TP will learn a T-maze for the opportunity to gnaw on wood or food pellets (Koob et al. 1976, Fray et al. 1978).

The early studies of Antelman and his colleagues on TP suggested that the arousal and motivational component of any TP induced behaviour was dependent upon the ascending DA fibres of the nigro-striatal bundle. Intra-peritoneal (i.p.) injection of the DA receptor blocking agents spiroperidol and pimozide significantly reduced TP-induced behaviour as did 6-OHDA lesion of the nigrostriatal bundle (Antelman and Szechtman 1975, Antelman et al. 1975, 1976). However, these lesions which resulted in a 90% depletion of DA in the caudate blocked TP on only 44% of trials and even if haloperidol was administered post-lesion, TP induced feeding was blocked in only 5 out of 8 animals. Furthermore the lesions were produced by intra-cerebroventricular injection of the toxin, allowing the possibility of widespread depletion not only of DA neurones but also of NA and adrenaline containing neurones. Antelman also reported that even at high doses alpha-

(phentolamine) and beta- (sotalol) adrenergic receptor antagonists failed to antagonise TP elicited eating. However Sahakian et al. (1981) selectively lesioned the DNAB with 6-OHDA, lesions which produced almost total hippocampal and cortical NA depletion, and demonstrated an impairment in response to TP. Eating occurred in only 7 out of 20 animals with DNAB lesions, and the latency of these animals to eat was significantly lengthened. It is possible that some of the effect of TP is mediated through noradrenergic facilitation of the nigrostriatal DA system and NA and DA interact to produce the overall effect. That there is some interaction is demonstrated by the finding that DNAB lesions reduce DA turnover in the striatum (Robbins and Everitt 1982). The dorsal bundle projection provides comparatively little of the NA projection to the hypothalamus (see p.12 above) and thus it is unlikely that TP exerts its effects directly through effects on the hypothalamic NA projection from the DNAB particularly in the absence of TP blockade to i.p. injection of NA antagonists.

The role of the VNAB in TP behaviour was investigated by Sahakian et al. (1983). Lesions of the VNAB enhanced the acquisition of TP induced eating but had no effect on the behaviour once acquired. Food deprivation alters NA turnover in the medial hypothalamus as well as DA activity in the mesolimbic system. The majority of medial hypothalamic NA is

provided by the VNAB. Increase in plasma glucose is positively correlated with NA concentration in the medial hypothalamus (Smythe et al. 1984) from whence Leibowitz (1975,1978a,b) has demonstrated feeding in response to direct microinjection of NA. Leibowitz has also demonstrated the involvement of circulating corticosteroids in the feeding response to NA injection, and that hypophesectomy or adrenalectomy blocks the NA elicited feeding response (Roland et al. 1986). This evidence clearly demonstrates that manipulations of medial hypothalamic NA affect feeding behaviour; TP is known to affect DNAB and VNAB activity, and these pathways provide innervation to the PVN.

Whilst the fact that TP has wide ranging effects on ascending catecholamine transmission might give some clues as to how and where the effects of TP are manifest, other clues might come from analysis of the type of stimulus TP provides. The term 'stress-induced eating' has been mentioned above without explanation of the physiological effects of stress. Some examination of these effects provides further evidence that the medial hypothalamus, particularly the PVN might be involved in TP induced eating.

Physiological response to stress.

Many responses to stress are, in the first instance, endocrine in nature and it is these responses and their consequent

physiological changes that are of interest. Whilst exposure to long term, or very high levels of stress can undoubtedly produce complex behavioural and physiological changes (Antelman and Chiodo 1983) it is the short term endocrine responses that are of interest here. These changes occur along two axes:

i). The sympathetic-adrenal medullary system.

Exposure to stress involves activation of CNS systems. One of the limbic components involved in response to this exposure is the hypothalamus, which generates responses along two neuronal outflows to the periphery. The first of these influences the sympathetic branch of the autonomic nervous system, which innervates the viscera and contains the transmitter NA. The second outflow projects as the splanchnic nerve and innervates the chromaffin cells of the adrenal medulla. When stimulated these cells release mainly adrenaline, but also some NA. This release of catecholamines predominantly affects the peripheral cardiovascular system, preparing the organism for 'fight or flight' (See Fig.6).

ii). The pituitary-adrenocortical system.

'Emotional stress' activates the PVN and supraoptic nucleus of the hypothalamus. This results in the release, from neurosecretory cells in the median eminence, of corticotropin releasing factor (CRF) which travels via the hypothalamic-

FIGURE 6. The pituitary-adrenocortical system. Ach = acetylcholine. 5-HT = 5 hydroxytryptamine. GABA = gamma aminobutyric acid. + = stimulation. - = inhibition.
Figure from Bassett (1984)

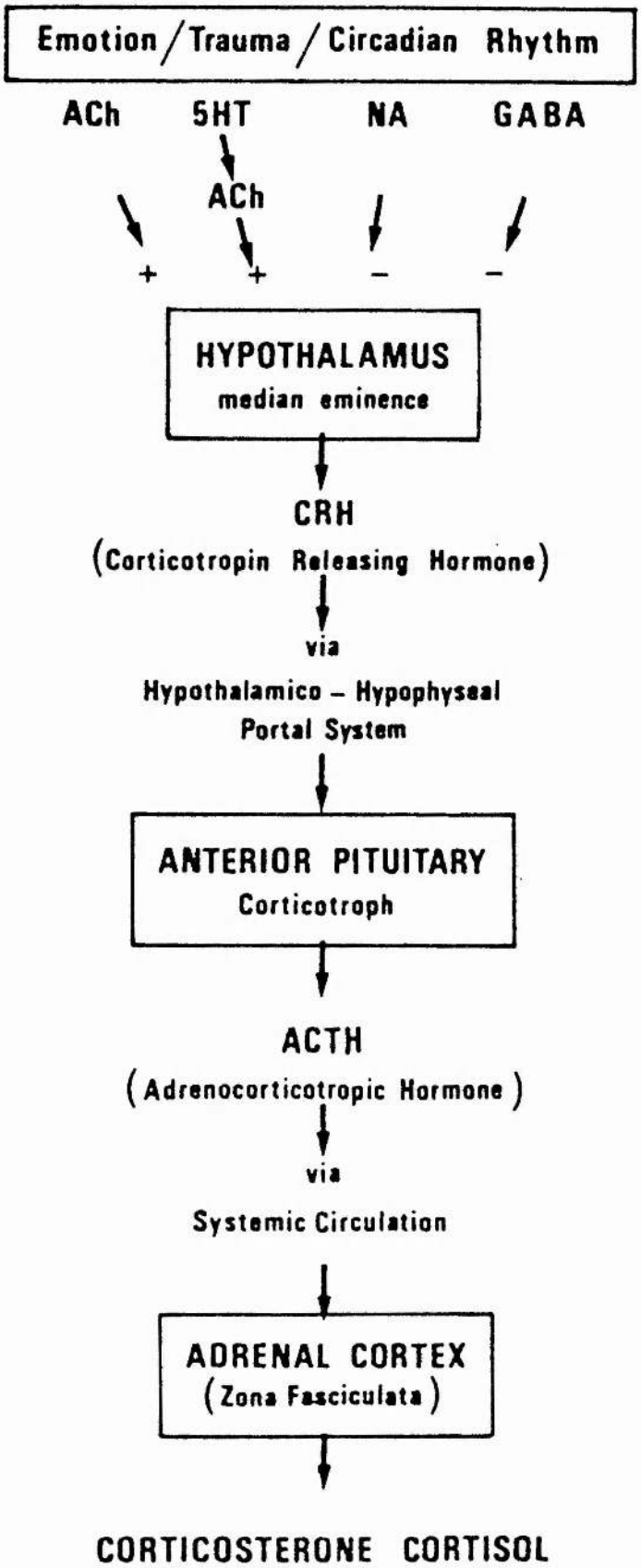
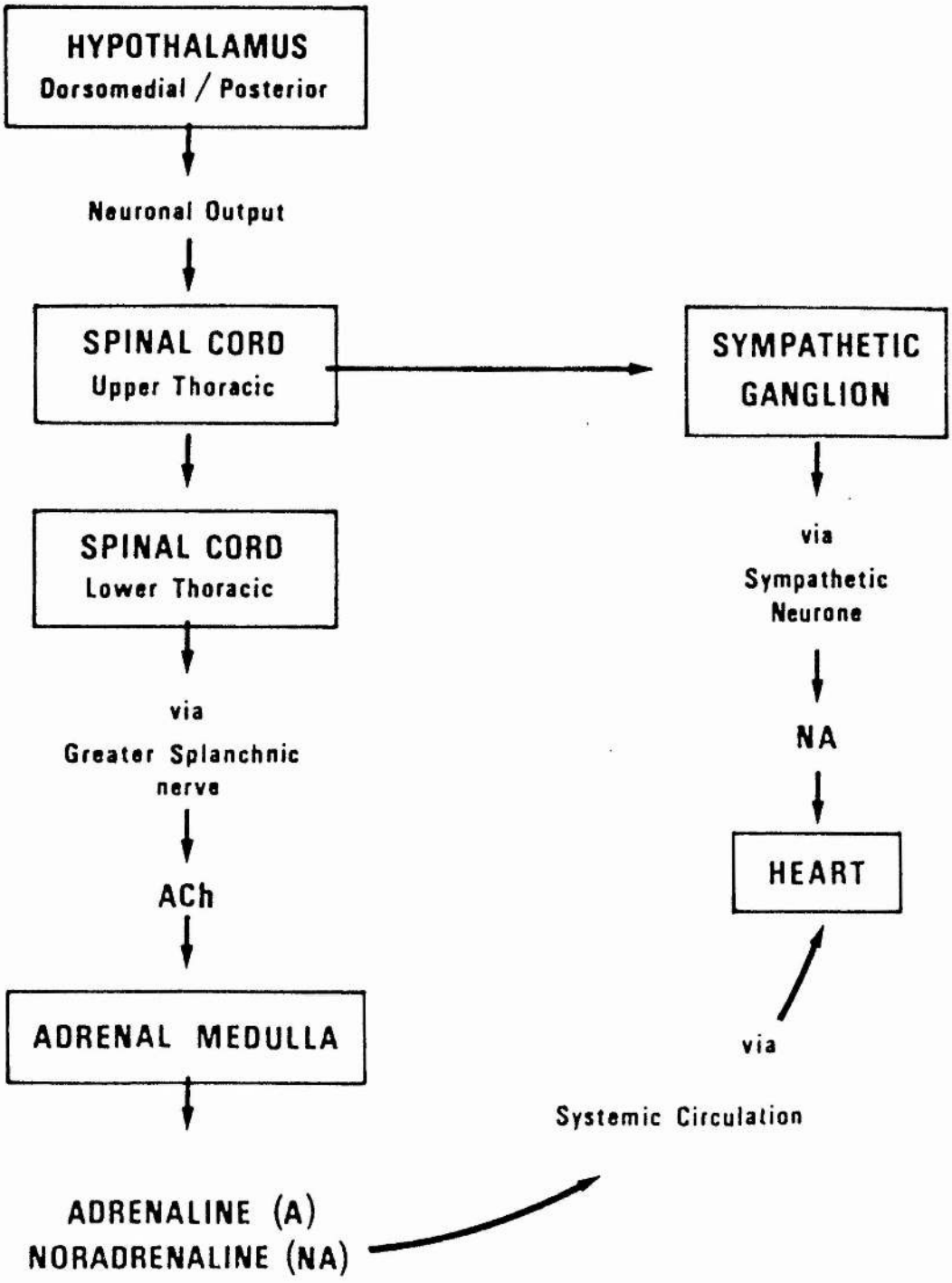

Emotion / Trauma / Circadian Rhythm
ACh
5HT
NA
GABA
ACh
+
+
–
–
HYPOTHALAMUS
median eminence
CRH
(Corticotropin Releasing Hormone)
via
Hypothalamico – Hypophyseal
Portal System
ANTERIOR PITUITARY
Corticotroph
ACTH
(Adrenocorticotropic Hormone)
via
Systemic Circulation
ADRENAL CORTEX
(Zona Fasciculata)
CORTICOSTERONE CORTISOL

FIGURE 7. The sympathetic-adrenal medullary system.

Figure from Bassett (1984)


HYPOTHALAMUS
Dorsomedial / Posterior
Neuronal Output
SPINAL CORD
Upper Thoracic
SYMPATHETIC GANGLION
SPINAL CORD
Lower Thoracic
via
Sympathetic Neurone
NA
via
Greater Splanchnic nerve
ACh
HEART
ADRENAL MEDULLA
via
Systemic Circulation
ADRENALINE (A)
NORADRENALINE (NA)

hypophyseal portal system to the anterior lobe of the pituitary. Here it promotes the release of adrenocorticotropic hormone (ACTH) which is transported in the blood to the zona fasciculata of the adrenal cortex. Here the ACTH stimulates the release of the glucocorticoid hormone corticosterone (Peron 1960) (see Fig.7). Thus situations involving stress are associated with elevation in both catecholamine and glucocorticoid levels, the levels of both rising simultaneously (Mason 1968). Whilst the pituitary-adrenocortical system is activated by a wide range of psychological stressors, "*situations of uncertainty or unpredictability produce a far greater elevation of circulating glucocorticoids*" (Bassett 1984).

In review, the important question is why does the infusion of NA into the PVN elicit feeding in a satiated rat? There are two possibilities: first that NA injected into PVN mimics or affects in some way the natural processes of energy depletion or repletion. The second is that feeding to such injections has nothing to do with 'feeding' in terms of homeostasis or energy balance but is an artefact produced by stimulation of part of the 'stress cascade'. If the first option is examined, the question to be asked is what part of the natural feeding process does the microinjection of NA mimic? The work of Weingarten et al. (1985) indicates that the effects are not mediated through insulin or gastric acid secretion. Further,

there is poor operant responding for food reward following such microinjections. Examination of the anatomy of the PVN in conjuction with the work of Shimazu and colleagues (1965, 1968, 1983) on the regulation of liver function indicates that the PVN may be involved in some aspects of liver function and exert its influence on feeding through this axis. However, this is not incompatible with the second hypothesis, that feeding to PVN microinjection of NA occurs in response to stimulation of part of the 'stress cascade'. The stimulation of medial hypothalamic sites results in both the release of CRF leading to increased ACTH levels and release of glucose from the liver (resulting from glycogen conversion into glucose). This results in an increase in plasma glucose in the absence of intake, presumably to provide energy for 'fight or flight'. However it also results in a very rapid depletion of short term energy reserves which might in turn provide a stimulus to eat. Thus the feeding in response to PVN NA microinjection might be explained in terms of either of the two hypotheses. One series of experiments described in this thesis concerns the microinjection of NA antagonists into the PVN prior to stimulating the rat with TP. These experiments were performed in order to clarify the role of the PVN in stress induced eating.

MECHANISMS OF CHEMICAL INFORMATION TRANSFER

The first part of the introduction to this thesis described some of the progress made in the understanding of feeding behaviour, particularly of the involvement of NA in the PVN. This second section is concerned, not with behaviour but the processes of neurochemical transmission which are believed to underlie the expression of behaviour. Much of the behavioural work described in the first section related to the effects of infusion of neurologically active substances into the CNS of conscious animals. The majority of the work described on feeding and the hypothalamus involved the infusion of NA or adrenergically active ligands into a variety of discrete hypothalamic sites. However, it is not just work on feeding that employs the techniques of microinjection: experiments on drinking, sleep, locomotor activity, sexual behaviour, maternal behaviour, learning and memory, and even visual performance have all employed them (see Myers 1978 for comprehensive but dated review). The infusion of exogenous substances into the CNS, by microinjection, microiontophoresis or pressure ejection are accepted techniques in research aimed at understanding the relationship between neurochemistry, anatomy and behaviour. This being the case it seems particularly pertinant to examine the assumptions that underlie microinjections, for if the theoretical basis of a

technique is unsound, then the results generated by its use must be re-assessed in the light of subsequent advances.

The debate on exactly how nerve cells communicate information has been active for a long time, and indeed the principle components of the argument have themselves changed in the light of continuing advances. Initially the debate was whether or not communication between nerve cells was electrical or chemical in nature. The electrical theory suggested that transmission was brought about by action currents originating from axon terminals and continuing in the post-junctional cell in a way similar to conduction along the axon. The attractiveness of this theory was enhanced by the experiments of Galvani, and later Matteucci, demonstrating that the current from a large mass of muscle may excite a nerve. However, in the physiological state, activation passes not from muscle to nerve but from nerve to muscle and activation of muscle does not occur when an active nerve supply is simply laid upon it. The current from the nerve is insufficient to depolarise the larger mass of muscle tissue. It became clear that the nerve impulse required amplification and that this was somehow provided at the junction between nerve and muscle by means of a chemical process. The theory that nerve cells communicate chemically had been around since the 1900's. Scott stated "*I formed the hypothesis that nerve-cells are true secreting cells and act upon one another*

and upon the cells of other organs by the passage of a chemical substance...This hypothesis has to do with the stimulation of cells, and not with conduction which proceeds in the substance between the neurosomes...When the impulse reaches the nerve ending it causes, I believe, the discharge of some of the neurosomes which are very numerous in that situation." (Scott 1905 pp.521-522). But it was only with the experiments of Loewi (1921) that any direct evidence for the existence of chemical messengers was obtained. There are a few exceptions to the rule of chemical transmission; electrical transmission does occur though it is now referred to as ephatic transmission and the region at which it occurs is called an ephapse. Electrical transmission is not known to occur in the CNS of mammals (Bowman and Rand 1984). This thesis is predominantly concerned with information transfer in the CNS that is chemical in nature. The chemicals involved in information transfer are diverse, and their modes of action complex. Such compounds are referred to below as neurotransmitters; an expanded justification for the use of this term is given in appendix 1.

A recent debate concerns whether or not chemical communication in the CNS is only of a 'classical' synaptic form or whether a non-synaptic or 'paracrine' system of transmission also operates. In recent years many of the 'fundamental principles' of chemical transmission have been challenged and

disproved:

(i) Eccle's proposal of Dale's principle, in brief that each neuron contains only one transmitter substance*[1]. Co-localisation studies using double labelling with specific antibodies categorically demonstrate the co-existence of different transmitter substances within neurons (Hokfelt et al. 1980).

ii) Eccles principle, that a transmitter substance opens just one type of ionic gate, that being either excitatory or inhibitory, on the post-synaptic membrane. Considerable information is now available to demonstrate that the effects of a neurotransmitter on the post-synaptic cell depend upon the nature of that cell, the type of receptor, the presence of other inputs, and a great many other variables.

However, the assumption that within the CNS neurone-neurone chemical transmission occurs only through discrete synaptic contacts is still widespread. This 'classical' model of transmission has recently been challenged by an alternative hypothesis of 'paracrine' or non-synaptic transmission. This hypothesis suggests that transmitters may be released not into morphologically defined synaptic clefts but into the extra-cellular space. They then diffuse to their site of action, specificity being retained by the presence or absence of appropriate receptors rather than by anatomical constraints.

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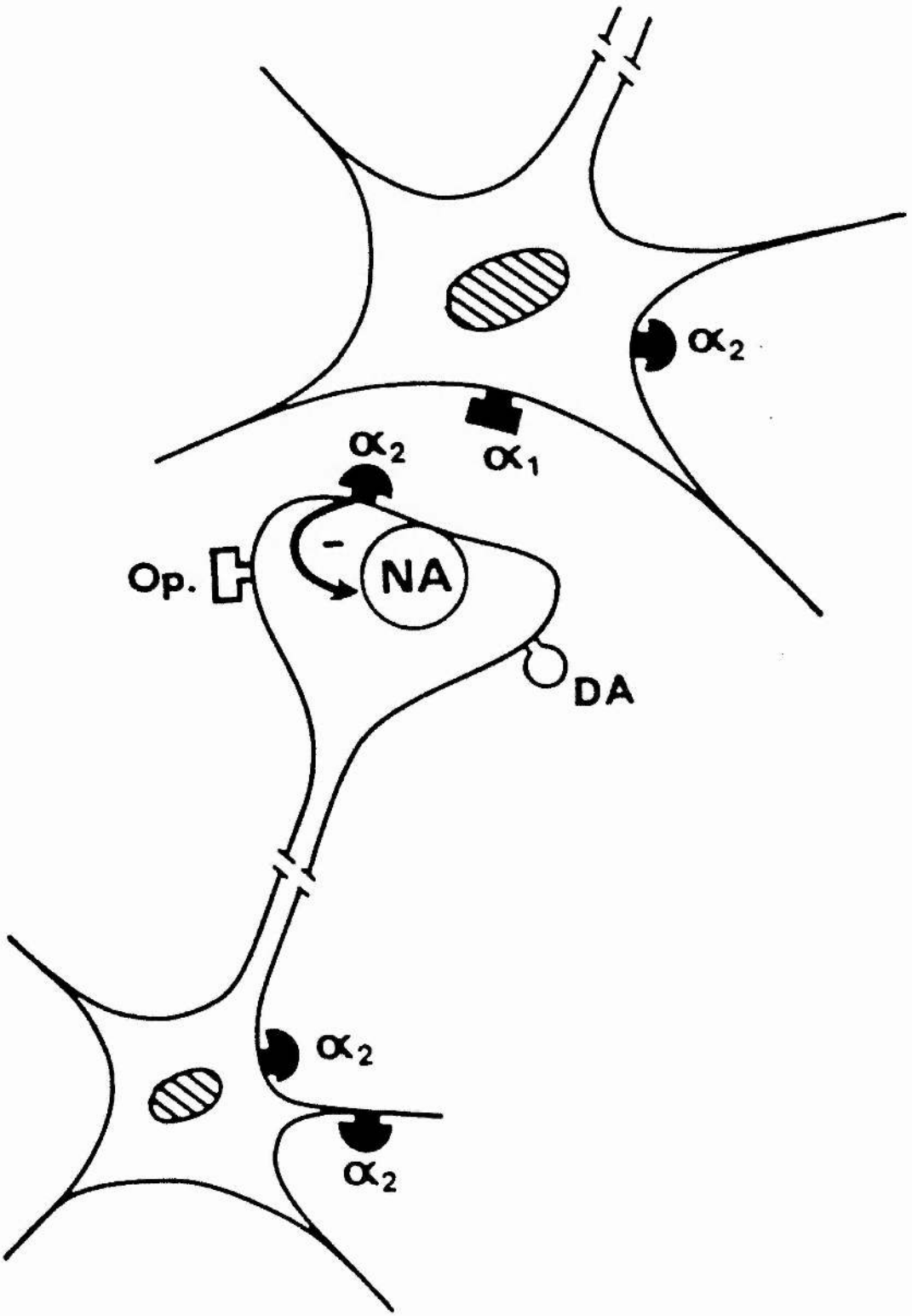
* Unfortunately an inaccurate reproduction of Dale's work - for full discussion see: Multiple Transmitter Status and "Dale's Principle". Potter D. Furshpan E. and Landis S. (1981) Neuroscience commentaries 1, 1: 1-9.

FIGURE 8. The concept of 'chemically addressed' transfer of information between neurones. The cells **A** and **B** release their transmitters **A** and **B** diffusely. These can only have effect at cells equiped with the appropriate receptors (a and b).
Figure from Iversen (1986)

c
d
A
a
d
B
b
c
d
a
b
c

FIGURE 9. α-1 and α-2 adrenoceptors in the central nervous system. Schematic representation of a central noradrenergic neuron. Postsynaptic adrenoceptors are of both α-1 and α-2 sub-types. Figure from Langer et al. (1980b)


α2
α2
α1
Op.
NA
DA
α2
α2

The debate over exactly how chemical transmission occurs within the CNS can be seen to have an important bearing on the theoretical basis of the microinjection technique, for whilst this technique has been used to evaluate the effects of many compounds within the CNS it clearly involves a major implicit assumption. This is that the direct injection of a compound into the CNS results in that compound having equal access to all the sites to which the endogenous ligand might bind.

In isolated tissue preparations, the concentration of a drug in the biophase is taken to be that in the surrounding physiological salt solution (under steady state conditions). However, *'in vivo'* it is known that there are sites of loss, in intimate association with the receptors of certain transmitters, called re-uptake mechanisms. In such cases there may be marked differences in drug concentration between the solution and the biophase unless the site of loss is blocked. The biophase is thus the concentration of the transmitter available to act on a given receptor (Bowman and Rand 1981).

The assumption that direct injection of a compound gives it equal access to all receptors is thus clearly false if some receptors are closer to these sites of loss than others, because the re-uptake mechanism will act to cause a concentration gradient such that exogenous ligand is more available to receptors further from sites of loss. The

structure of the synapse, and the presence of a re-uptake mechanism are the two crucially important components that must be examined to validate the assumptions made in using the microinjection technique.

The structure of the synapse.

A synapse is a morphologically specialised, intimate contact between two neuronal elements. The pre-synaptic component of a synaptic junction is usually formed by the terminal swelling of an axon branch. Often referred to as 'bouton terminaux' these are characterised by the presence of accumulations of small vesicles containing the transmitter substance(s). The pre- and post-synaptic elements are separated by a synaptic cleft, typically about 20nm wide, with both elements showing morphological specialisation. On the pre-synaptic side, electron microscopy has distinguished diffusely outlined dense patches protruding from the membrane into the cleft. These protrusions are arranged into a structure described as the pre-synaptic vesicular grid. The post-synaptic membrane is characterised by local membrane thickening, either continuous or in discrete patches. Freeze-etching studies have revealed the presence of small protrusions from the post-synaptic thickening, which may represent the exposed component of post-synaptic receptor sites (Nieuwenhuys 1985). The sequence of events comprising synaptic transmission is now accepted to be as follows: the arrival of an impulse causes

the synaptic vesicles to move towards the cleft, guided by the vesicular grid. On reaching the pre-synaptic membrane, the membrane of the vesicle fuses with it, releasing it's contents into the cleft. This process is called exocytosis. The transmitter molecules diffuse across the cleft in the extra-cellular fluid and bind to the post-synaptic receptor sites. This receptor - ligand interaction results in a change in the status of the post-synaptic membrane, usually resulting from the opening of particular ionic channels; although this may often be mediated by a 'second messenger' system such as a GTP-binding protein regulating intracellular pathways that result in changes of such specific effectors as ion channels in the membrane, or adenylyl cyclase metabolism (Neer and Clapham 1988). The synaptic cleft contains an extra-cellular matrix of greater density than that surrounding the cell (Nieuwenhuys 1985, Hunter et al. 1989), and this serves to impede the diffusion of transmitter molecules away from the functional zone and into the general extra-cellular space.

Re-uptake of released transmitter.

The best understood re-uptake mechanism is probably that for NA. Study of the peripheral sympathetic nervous system revealed that neurones were capable of taking up both endogenous and exogenous catecholamines by an active transport mechanism. Much of this work was pioneered by Leslie Iversen, and his findings for the rat heart preparation illustrate the

potency of this mechanism well: "*The isolated heart rapidly accumulated NA when perfused with a solution containing a low concentration of [^{3}H]-noradrenaline (10ng/ml). The uptake led to concentrations in the heart about 40 times greater than the perfusion medium, after perfusion for 20 minutes. This means that the NA uptake system is capable of clearing the entire extracellular space of the heart in approximately 10 seconds. If one considers that the uptake sites are restricted to the sympathetic nerve terminals in the heart, which occupy only a minor portion of the tissue volume, this performance is even more remarkable. There is no way of making an accurate estimate of the volume of extracellular space adjacent to the sympathetic terminals, but it seems likely that the uptake system could completely clear NA from this fluid volume in an interval of time measured in milliseconds rather than seconds*" (Iversen 1971 p.573). This re-uptake system, known as uptake-1 is a rapid, high affinity system probably responsible for the majority of NA re-uptake, and is located in the membrane of adrenergic neurones. There is a second system, known as uptake-2 which has a lower affinity for NA than uptake-1 but a higher capacity and is located in post-synaptic cells and glia. Within the CNS these re-uptake processes appear very similar to those of the sympathetic nervous system. The kinetic constants and inhibitor specificities of the central and peripheral processes are very similar (Iversen 1971). Iversen is aware of the possibility that the presence

of a re-uptake mechanism would lower the concentration of compounds, for which it had an affinity, in the biophase: "*under these conditions the concentration of agonist in the extracellular space adjacent to the receptor sites will be reduced to a value lower than that applied to the external medium, because of the continuous removal of the agonist from the biophase by uptake*" (Iversen 1971 p.582.)

If they are to act upon intra-synaptic receptors, it can be seen that exogenously administered compounds have barriers to overcome. First, any compound administered directly into the extra-cellular fluid of the brain has to diffuse from a less dense into a more dense medium in order to penetrate the synapse. Second, if the exogenous compound is susceptible to re-uptake it has to diffuse into the synaptic cleft at a rate sufficient to overcome the rate of loss from re-uptake. It is however clear that exogenous compounds can and do have widespread effects following microinjection into the CNS, even those such as NA which are the endogenous ligand for the re-uptake mechanism. It is possible that these exogenously administered compounds are simply given in concentrations so great as to overcome both the restrictions on diffusion and loss from re-uptake. However there is a second possibility, and that is that some exogenously administered compounds exert their effects at receptors outwith the 'classical' synapse, called extra-synaptic receptors.

Extra -Synaptic receptors.

There is a considerable amount of evidence available that the receptors for certain chemical messengers exist outside any form of synapse. Thus the receptors for hormones in the periphery, for example, receive their ligand from the blood supply. The more difficult question is, do certain synaptically released transmitters also operate in a 'paracrine' fashion within the CNS? Using NA as an example once again, the question becomes; are there NA receptors present both inside and outside the classical synapse? A recent review by Miles Herkenham (1987) compares the data generated by localisation studies of receptors and transmitters in an attempt to illuminate this question.

Receptor mismatch data.

As explained above, the hypothesis most commonly held about chemical information transfer in the CNS is that it takes place through defined synaptic contacts. However Herkenham states "*close transmitter/receptor matches are the exception rather than the rule for most well characterised neuro-chemical systems....mismatches are the general rule for these systems*" (Herkenham 1987 p.2). It is not possible here to review the source evidence used by Herkenham but his conclusion as regards NA is definite: "*Lumping all of the adrenergic receptors together and comparing their distribution*

with the adrenergic innervation of brain leads to the conclusion that the receptors are far more widely distributed than the nerve terminals, and that the patterns of adrenergic receptor distributions do not resemble the patterns of adrenergic fibre distribution" (p.12). There are a variety of explanations for this mismatch data:

(i) Mismatches represent technical failures in visualisation or incomplete knowledge of a family of receptors or transmitters. For example, failure to detect transmitter in an area containing dense receptors may represent a lack of sensitivity for very low transmitter levels. The antibody used may fail to recognise all molecular forms of the transmitters that comprise a family of substances recognised by the receptor. However, the use of more sensitive techniques and antibodies directed against newly discovered transmitters have usually served to compound the mismatch not diminsh it.

(ii) Many transmitters are located by labelling anti-bodies to the synthesising enzyme, but this enzyme is often present throughout the cell, not just in terminals. Furthermore, not all receptors occur in outer cell membranes, receptors may be in axonal transport, or even internal to the cell.

(iii) Biochemical explanations include incomplete labelling because of occupied receptors, non-functional, or "drug binding sites" and unrecognised low-affinity sites. The expression of certain receptors may be genetically linked to

the expression of other receptors even if no ligand for these occurs at that site.

(iv) Many receptors, especially high affinity receptors, may be located not in a synapse, but at a site distant from the source of release.

Herkenham examined the first three explanations (i-iii) in detail but concludes that the extent of mismatch is such that (iv), extra-synaptic receptors, must be the reason for many of the observed discrepancies between transmitter containing neurons and receptor localisation.

Whilst Herkenham might be overstating the case when he implies that all transmitters so far discovered may act in both a classical and paracrine manner it is clear that evidence for non-synaptic transmission of receptors exists, and that in such a system "*connectional specificity is replaced by chemical specificity*" (p.27). Such a system had already been proposed by Iversen (1986; see Fig.8) and independently by Schmitt (1986) who states "*alongside of, and in parallel with, synaptically linked, 'hardwired' neuronal circuitry, that forms the basis for conventional neurophysiology and neuroanatomy, and that operates through conventional synaptic junctions, there is a system that I call 'parasynaptic'. In parasynaptic neuronal systems 'informational substances' may be released at points, frequently relatively remote from target cells, which they reach by diffusion through*

extracellular fluid. Such a system has all the specificity and selectivity characteristic of the conventional synaptic mode; in the parasynaptic case the receptors that provide the specificity and selectivity are on the surface of the cells where they can be contacted by, and bind to, the informational substance ligands diffusing in the extra cellular fluid" (p.240).

Data from the peripheral nervous system.

Much of the early research work into the actions of transmitters such as NA was conducted in the peripheral nervous system. Frequently, it has been determined that peripheral modes of action closely resemble those later discovered within the CNS. Thus the work of Iversen (1971) initially concerned the re-uptake of NA from the rat heart, but has been found to generalise well into the CNS. This is not to say that all features of NA neurones or receptors in the peripheral nervous system have a parallel in the CNS but evidence gained from peripheral research may provide clues to the mechanisms underlying the actions of NA in the CNS.

Certain experiments on vascular smooth muscle, indicated to Salomon Langer and his colleagues that there might be a difference between the effects of endogenously and exogenously administered NA (Langer et al. 1980a,1980b). Using prazosin, a highly selective α-1 antagonist, the effects of α-1 blockade

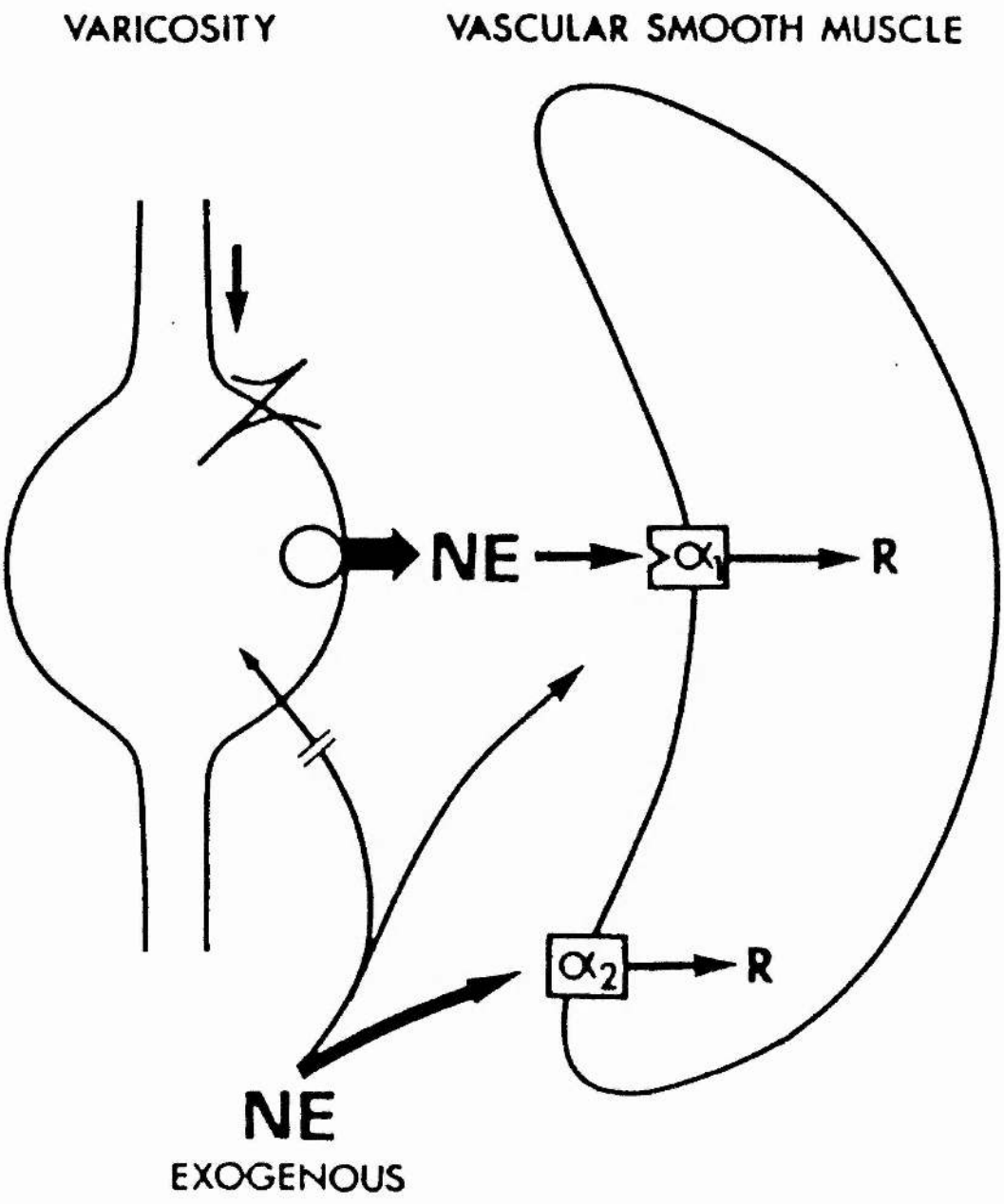
were compared when the tissue was stimulated with NA from an endogenous source (electrical stimulation of the nerve) or from exogenous NA added to the perfusate. They discovered that the blockade of the response achieved by prazosin was far greater to the endogenously released NA than that to NA added to the perfusate. Both α-1 and α-2 sub-types of the NA α-receptor were known to be present post-synaptically in this preparation. Langer and Shepperson (1982) subsequently suggested that this result might be due to the α-1 receptor being synaptic and the α-2 post-synaptic receptor extra-synaptic, both acting to contract the muscle but having different primary sources of their ligand. In order to test this hypothesis they performed a series of blockade experiments using selective α-1 blockade with prazosin, selective α-2 blockade with M-7 and the combined α-1, α-2 blocker phentolamine. In these experiments they once again provided stimulation by both endogenous and exogenous NA, the preparation being was perfused cat spleen. The nerve/muscle junction in this preparation is not a synapse as such but a neuroeffector junction, which shares many of the properties of the synapse. The results of these experiments supported the hypothesis that the α-1 receptor predominated intra-synaptically whilst the α-2 was extra-synaptic. Langer and Shepperson state "*prazosin a selective α-1 adrenoceptor antagonist preferentially antagonised responses to neuronally released NA while it was less effective in blocking responses*

to exogenously administered NA" (p.s10). They further explain that if the NA re-uptake mechanism were to be inhibited with cocaine then the selectivity of these two blockades would be markedly reduced as exogenous NA could now penetrate the neuroeffector junction without being rapidly removed by the re-uptake mechanism and further that the blockade to phentolamine would be unaffected by this re-uptake inhibition. In a review of the role of pre-synaptic receptors in the periphery, Langer (1981) presents a model of a smooth muscle neuroeffector junction and its noradrenergic innervation (see Fig. 9) and further suggests a possible extra-synaptic location for CNS α-2 adrenoceptors (see Fig 10). Referring to the effects of re-uptake inhibition Langer claims that "*when neuronal uptake is inhibited with cocaine, prazosin becomes more effective than in the absence of cocaine in blocking the response to exogenous NA*" (p.343). The findings that the α-1 receptor may predominate intra-synaptically whilst the α-2 is largely extra-synaptic is supported by Yamaguchi and Kopin (1980) and more recently by Lucchelli et al. (1985) who also reported preferential antagonism by prazosin of NA released by nerve stimulation.

However, recent work by Woodman (1987) has indicated that the post-junctional location of α-1 and α-2 receptors may not be as easily separated as Langer suggests. Woodman used tyramine as a noradrenergic releasing agent, this having the advantages

FIGURE 10. Postsynaptic α-1 and α-2 adrenoceptors in vascular smooth muscle. Schematic representation of vascular smooth muscle neuroeffector junction. Endogenous NA acts preferentially at α-1 receptors intra-synaptically, whilst exogenous NA acts at extra-synaptic α-2 receptors.

Figure from Langer and Shepperson (1982)


VARICOSITY
VASCULAR SMOOTH MUSCLE
NE
α_1
R
α_2
R
NE
EXOGENOUS

of not being impulse dependent, thus not involving pre-junctional α-2 receptors, and the release is from all available sites. His experiments on coronary vasoconstrictor responses in the dog indicate the involvement of both α-1 and α-2 post-junctional receptors in the response in this preparation to release from the sympathetic nerves. This he claims, refutes Langer's suggestion that hormonal or circulating NA is responsible for the activation of extra-synaptic α-2 receptors. It is possible that tyramine will release NA from sympathetic nerves both into neuroeffector junctions, and also a limited amount at sites not directly adjacent to a junction. In this case the diffusely released NA would still be free to act at extra-junctional α-2 receptors, indicating when the α-1 receptors are antagonised with prazosin that there is an α-2 component to the response to stimulation of sympathetic nerves with tyramine. The α-2 post-synaptic receptor is available to ligand from any source, diffusely released, hormonal, or synaptically/junctionally released but it is unlikely that synaptically released NA can escape from the re-uptake mechanism associated with the synapse. Thus Woodman's results are not incompatible with Langer's, they differ merely in interpretation. A second and important suggestion made by Woodman is that the relative contributions of α-1 and α-2 receptors to an effect at any site must be determined at that site, attempts should not be made to overgeneralise across

preparations.

Evidence From Ultra-Structural Studies.

The axons of many monoaminergic and peptidergic neurones in the CNS give rise to thin, unmyelinated and profusely branched axons. These axons exhibit varicosities throughout much of their extent, not merely in their terminal region. Ultrastructural studies have determined that these projections do not appear to make synaptic contacts, and yet they contain large numbers of vesicles which resemble those from which transmitter is released at synapses. Such observations led to the speculation that transmitter was released from these varicosities, not into synaptic specialisations but freely into the extra-cellular space (Nieuwenhuys 1985). In a review of the ultrastructural data Beaudet and Descarries (1978) outline the detailed studies which have been undertaken to identify synaptic terminals in monoamine neurones within the CNS. They ".. *examined 1835 noradrenergic terminals in 12 series of 3 thin sections each, selected as representative of all cortical layers. In this extensive sampling, 18% of the varicosities were viewed in more than one thin section, but only 61 (<5%) exhibited the structural features of typical synaptic terminals...The paucity of synaptic junctions formed by noradrenergic...varicosities in the cortex was particularly striking when comparing the labelled terminals with unlabelled nerve endings randomly selected in the surrounding neuropil*

but similarly sampled in serial thin sections: more than 50% of these possessed the morphological characteristics of synaptic terminals" (p.856). Beaudet and Descarries were not the only workers to discover such an apparent shortage of morphologically discrete synaptic specialisations within the CNS. They cite nearly twenty other studies which reach a similar conclusion for NA, 5-HT or DA at different sites, in particular, Swanson et al. (1978) who found only 19% of NA or adrenergic vesicles within the rat PVN formed synaptic contacts with other neurons, whilst a further 5% made contact with blood vessels. Beaudet and Descarries do not, however, imply that all transmission of these amines is non-synaptic, neither could they determine whether the same neurone engaged in both non-synaptic and synaptic release. In addition to all the NA varicosities observed containing vesicles they observed that "*release of the biogenic amines solely by varicosities making synaptic contact could hardly account for the amounts liberated from the superfused cortex during the resting state or upon electrical stimulation*" (p.857). In this they are supported by the study of the changes in endogenous content of NA varicosities after stimulation, as observed by histofluorescent techniques (Arbuthnott et al. 1970). However, the findings of Beaudet and Descarries have been challenged by Molliver et al. (1985) who studied the ultrastructural specialisation of NA and 5-HT varicosities using the technique of immunohistochemical labelling

techniques. They found that about 50% of the positively identified 5-HT and NA terminals displayed synaptic specialisations, and those that did not they argued were merely outside the section plane. Further, in neonatal rats monoaminergic fibres form numerous specialised synapses. The conclusion of this research is that cortical monoaminergic transmission occurs through specialised synapses. The argument that not seeing synaptic specialisations because, if you don't see them they are simply out of the plane of the section, is difficult to refute. Very recent work by Papadopoulos et al. (1989) claims an incidence of 86% of NA vesicles form synaptic contacts within rat cerebral cortex, and suggest that the results reported by Beaudet, Descarries and colleagues resulted from insufficient numbers of serial sections. They report that a given varicosity is present in up to 15 serial sections whilst the post-synaptic specialisation occurs in only two or three. Buma and Roubos (1986) have developed a technique whereby the process of exocytosis can be directly visualised and have presented data which shows non-synaptic release of a substance that is probably NA within the area postrema of rat brain.

Theoretical Considerations

If a theory is a means of explaining empirical data in a way that is parsimonious, complete and testable then the idea that non-synaptic transmission occurs alongside synaptic

transmission within the CNS is an attractive theory, particularly so because it is possible to incorporate all the available empirical evidence regarding synaptic transmission and in addition, explain a considerable number of empirical findings difficult to resolve using only the concept of straight "point-to-point" transmission. These are as follows:

(i) It provides an explanation for the high number of compounds known or suspected of being transmitters within the CNS.

(ii) It suggests an extremely economical use of space within the CNS, by reducing the 'hardware' requirements.

(iii) It allows for specificity to be maintained by the appropriate locating of receptors rather than complex topographical relationships between neurons.

The evidence presented above gives a strong indication that non-synaptic transmission occurs within the CNS of mammals. Unfortunately none of the experiments reported actively tests this hypothesis. It is the purpose of this thesis to demonstrate that it is possible using psychopharmacological methods to test the conclusions presented above using behavioural measures.

HYPOTHESIS

The hypothesis to be tested in this thesis is that non-synaptic transmission occurs within the CNS of rats in

parallel with classical synaptic transmission at least within the PVN. The receptors for the non-synaptically released transmitter are located outwith the conventional synapse; the separation of the two systems being maintained by both the presence of a powerful re-uptake mechanism on the pre-synaptic terminal and the structure of the synapse. The actions of NA at extra-synaptic receptors are of behavioural consequence.

In order to use the available space at the optimum both synaptic and non-synaptic transmission should operate at a given location. However, in order to prevent excessive crosstalk between the two systems they must be kept separate. I have hypothesised that the most efficient way for this to occur is simply for the receptor site for the non-synaptically released ligand to be moved outside the synapse. Overlap is thus prevented, as transmitter released within the synapse fails to diffuse out because of the re-uptake mechanism and the density of the intra-synaptic medium. Similarly non-synaptically released transmitter will not significantly affect receptors within the synapse because these two mechanisms operating in reverse will serve to protect the intra-synaptic receptor population. This arrangement also serves to preserve the 'economy' of the classical synaptic arrangement with regard to the re-use of transmitter and keeps the required concentration of non-synaptic transmitter to a minimum, (see Fig.24). Indeed it is possible to suggest that

the sensitivity of intra- and extra-synaptic receptors might be considerably different.

Experiments were designed to test this hypothesis using as a model system noradrenergic transmission within the PVN. This system was chosen for several reasons:

i) The pharmacology of NA is probably better understood than any other central transmitter system and reliable pharmacological agents are available for manipulation of central NA systems.

ii) The evidence of Langer and others suggested that in the periphery there might be a spatial separation of post-synaptic NA α-1 and α-2 receptors with regard to the synapse.

iii) Iversen has demonstrated the presence of a potent re-uptake mechanism on CNS NA neurones.

iv) Beaudet and Descarries (1978) and Buma and Roubos (1986) have presented evidence in support of non-synaptic release of NA in the CNS of the rat.

v) The work of Leibowitz has demonstrated a reliable behaviour associated with manipulation of post-synaptic α-adrenergic systems within the PVN.

Initially experiments would be performed to replicate the findings of Leibowitz and her colleagues, that the feeding behaviour elicited by microinjection of NA into the PVN results from action at the post-synaptic α-2 receptor. Such replication would serve to validate the methodology being

employed in this laboratory. Subsequent experiments would examine the effects of inhibiting the re-uptake mechanism, either pharmacologically or by physical removal, on the pattern of response to selective pharmacological blockade. The prediction made was that the α-2 selective antagonist idazoxan would markedly reduce the feeding elicited by NA microinjections into PVN, as would the non-selective α-1, α-2 antagonist phentolamine. However, in the absence of a re-uptake mechanism the blockade to the α-2 selective antagonist would be considerably reduced because the intra-synaptic α-1 receptors were made available to the infused NA. In the absence of the re-uptake system the response to phentolamine, the combined α-1, α-2 antagonist would be unaffected.

SCREENING OF PHARMACOLOGICAL AGENTS

EXPERIMENT 1

The purpose of the first experiment was to establish the effects of the pharmacological agents to be used in subsequent studies on baseline food intake and behaviour. If the re-uptake or receptor blocking agents themselves affected food intake, or otherwise affected normal behaviour then their use in a food intake study would be confounded.

METHOD

Animals

A total of 27 male hooded Lister rats, bred 'in house', were used each weighing approximately 320gms at the beginning of the experiment. The rats were individually housed and kept under a 12hr light/dark cycle. The were maintained 'ad lib' on SDS maintenance diet No.1 chow pellets[1] and tap water.

Surgery

1 Cannulae.

Each animal was unilaterally implanted with a permanent, 23gauge stainless steel guide cannula, 11.5mm long, normally

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[1] SDS Maintenance Diet No.1 supplied by Special Diet Services Ltd, 1 Stepfield, Witham, Essex, CM8 3AB. Tel. (0376) 513651

occluded by an indwelling 30gauge wire stylet. The cannulae were manufactured by removing the last 11.5mm of a 23gauge 'monoject' syringe needle[2]. Using syringe needles has the advantage of ensuring a high degree of cleanliness and also complete uniformity of bore and bevelling. The stylets were made from 30gauge surgical steel wire placed inside the cannula, bent over and cut, leaving a 'L' shaped stylet with a projecting arm of about 4mm. Cannulae were implanted using a cannula guide, consisting of about 5cm of straight 23ga tubing with 30ga tubing down the inside[3], projecting exactly 11.5mm. The cannula guide was attached to the side arm of a Kopf stereotaxic frame[4].

2 Implantation.

The animal was removed from its home cage, injected intra-peritoneally with 10ml/Kg Avertin anaesthetic[5] and was then

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[2] Mononject, Sherwood Medical. 0.6x25mm 23x1ga long bevel syringe needles.

[3] Stainless Steel Tubing from; CNW 261/265 Aston Lane, Birmingham, West Midlands. B20 3HS.

[4] David Kopf sterotaxic frame

[5] Avertin anaesthetic - Concentrate solution 100gm of tri-bromo-ethanol crystals + 62ml tertiary amyl-alcohol liquid, dissolved and stored in darkness. Anaesthetic soln. 62.5ml of 0.9% saline + 5.0ml absolute alcohol mixed overnight in a dark bottle

returned to the home cage until reflex testing (tail flick) indicated it to be completely anaesthetised. The animal was then removed and the top of the head shaved with Oster small animal clippers[6]. It was placed in the stereotaxic frame, atraumatic earbars were used, and the skull exposed. Bregma was located and the co-ordinates for entry through the skull recorded. Next, three small stainless steel screws[7] were inserted into the skull spaced at about 5mm around the cannula site, to anchor the implant. A hole was drilled through the skull using a dental drill[8] and dental bit[9]. The dura co-ordinate was established and the cannula lowered into position. Any bleeding was staunched with cotton wool swabs, and the skull surface was wiped down with physiological

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with 1.25ml concentrate. Standard dose 10ml/Kg.

[6] Oster small animal clippers with size 40MN cutting head.

[7] Stainless steel screws 0-80 x 1/16 SS Plastic Products USA.

[8] NSK Volvere 5

[9] Dental bit; Komet, GEBR. BRASSELER GmbH & Co. Size ISO 014, US No. 4.

saline. When the skull was dry, acrylic dental cement[10] was applied to the skull surface and built up as it hardened around the screws until the implanted cannula was firmly held. The cannula guide was then withdrawn and the stylet placed inside the implant. Exposed edges of the wound were dressed with PEP wound dressing powder[11] but not sutured as recovery was found to be optimal if the cut skin grew back around the edge of the cement. Care was taken not to overlap the wound with cement. The animal was then returned to it's home cage and allowed to recover.

The stereotaxic co-ordinates used resulted in the injection cannula terminating in, or directly adjacent to, the magnocellular portion of the PVN. The co-ordinates were according to the atlas of Pellegrino, Pellegrino and Cushman (1979): in the de Groot orientation, nose bar +5mm above the interaural line, 0.6mm posterior to bregma, 0.5mm lateral and 6.0mm below dura. These co-ordinates refer to the placement of the guide cannula, the injection cannula projecting 2mm past the end of the guide to penetrate the PVN. Termination

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[10] Simplex Rapid, Austenal dental Products, The crystal centre, Harrow. HA 1 2HG.

[11] P.E.P. Powder, Intervet Laboratories, Milton Road, Cambridge.

of the guide cannula within the PVN would be too disruptive to the target site.

Test Procedure

Rats were allowed at least 14 days recovery after surgery before testing was started. However, the animals were handled daily and the stylets changed from day 3 after surgery. The animals were tested in wire sided testing cages, 24 x 24 x 39cms. These cages contained lab chow pellets in a food hopper, and water bottles. Animals were acclimatised to the cages with at least three pre-injection trials, in which the animals were placed in the cages for one hour, removed, mock injected and returned to the cages for a further 40 minutes. After acclimatisation the animals were tested following a similar protocol; they were placed in the cages for 1 hour, removed, the stylet extracted and the injection performed. The food and water were weighed and the animal returned to the test cage for a period of 40 minutes. At the end of the test the animal was returned to it's home cage and the food and water re-weighed. The cage floors were metal grids through which spillage could fall onto a paper catch-sheet. All food spillage was added to the unconsumed total.

Injection Protocol

All injections were made through 13.5mm 30ga injection cannulae placed inside the guide cannula. The injection

cannulae were made by attaching a piece of 30ga steel tubing about 2cms long to a 50 cm length of polythene tubing[12], the joint being sealed with 'Araldite' adhesive. The precise length of 13.5mm was ensured by cutting a collar of polythene tubing and placing it on the steel tubing such that exactly 13.5mm projected. This tubing/cannula arrangement was filled with the drug to be injected and attached to a 10ul syringe[13] fitted to a 'Harvard' infusion pump[14]. In attaching the tubing to the syringe a small air bubble, about 1mm long, was allowed to enter the tubing. The position of this bubble was marked before the injection commenced, and the progress of the infusion could be monitored by watching the movement of the bubble along the tubing. This procedure ensured that problems of non-injection due to blocked cannulae could be quickly observed and rectified. The 10ul syringes were always filled with absolute alcohol. All infusions were made over 1 minute, and the volume was always 0.5ul. Constant volume was maintained with all drugs because of the effects on diffusion of adjusting infusion volumes (Myers and Hoch 1978). The

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[12] Polythene tubing, I.D. 0.28mm O.D. 0.61mm (Ref. 800/100/100) from Portex, Hythe, Kent.

[13] S.G.E. 7 Argent place, Ringwood, Australia. Ref. 10-A-RN-GP

[14] Harvard Compact Infusion Pump. Model 975.

animal was removed from the testing cage and held in the left hand, the stylet was removed, and the injection cannula lightly pushed down the guide cannula. When in place the pump and a stopclock were started. After 1 minute the pump was stopped and the cannula left in place for a further 30 seconds before withdrawal.

Drugs

The drugs used in this experiment were as follows:

i) [-]-norepinephrine bitartrate [SIGMA][15].

ii) The a-1 antagonist prazosin hydrochloride [PFIZER][16].

iii) The a-2 antagonist idazoxan [RECKITT & COLEMAN][17].

iv) The mixed α-1/α-2 antagonist phentolamine hydrochloride [CIBA][18].

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[15] Sigma No. A-9512 Lot 65F-0359 Mol. Wt.319.3

[16] Lot No. A.NO.11-8445

[17] Batch No. 800285 Mol.Wt. 240.69

[18] Batch No. 00177 Mol.Wt. 317.8

v) The NA re-uptake inhibitor cocaine hydrochloride [SIGMA][19]. All drug solutions were made up in sterile physiological saline. Two of the drugs posed solubility problems. Phentolamine was only soluble at a maximum of 30nmoles in 0.5ul. Thus in experiments using this compound the dose of NA was reduced to 30nmoles also. Prazosin was used at the maximum solubility obtainable after warming and sonication (approximately 1mg/ml). However, the volume of Prazosin was not increased to allow equimolar injections because of the distortions inherent in increasing microinjection volume (Myers and Hoch 1978).

Histology

In order to confirm the cannula placements, the rats were sacrificed under barbiturate anaesthesia[20] and perfused intra-cardially with 0.9% physiological saline, followed by 10% phosphate buffered formalin solution[21]. The brains were removed and stored individually in jars of the formalin

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[19] Sigma No. C-5776 Lot 35F-0174 Mol.Wt. 339.8

[20] Euthatal May & Baker (200mg/ml pentobarbitone sodium). 1ml/kg.

[21] Phosphate Buffer - pH 7.4 0.1M Sodium Phosphate (dibasic Na2HPO4) plus 0.1M Sodium Phosphate (monobasic NaH2PO4) Approx 4 to 1 dibasic to monobasic. Add Monobasic slowly to dibasic, pH will fall from 9 to 7.4. For 10% buffered formalin add 100ml conc. formalin soln. to 900ml phostphate buffer.

solution for at least 48 hours before cutting. The brains were cut by fixing them to the stage of a bench microtome with Tissue-Tek[22] and freezing with compressed carbon dioxide. The section thickness was 40u and sections were mounted by floating them onto subbed[23] ground glass slides[24]. Sections were stained using a conventional cresyl violet staining procedure[25] and coverslipped using DPX[26] mountant. All rats used in this experiment were found to have injection sites in, or directly adjacent to the PVN. Photographs of representative injection sites are presented in Figure 11.

Statistics

The results of all the experiments were analysed using a repeated measures analysis of variance (ANOVA) computer

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22 Tissue-Tek OCT 4583 embedding compound. Miles scientific.

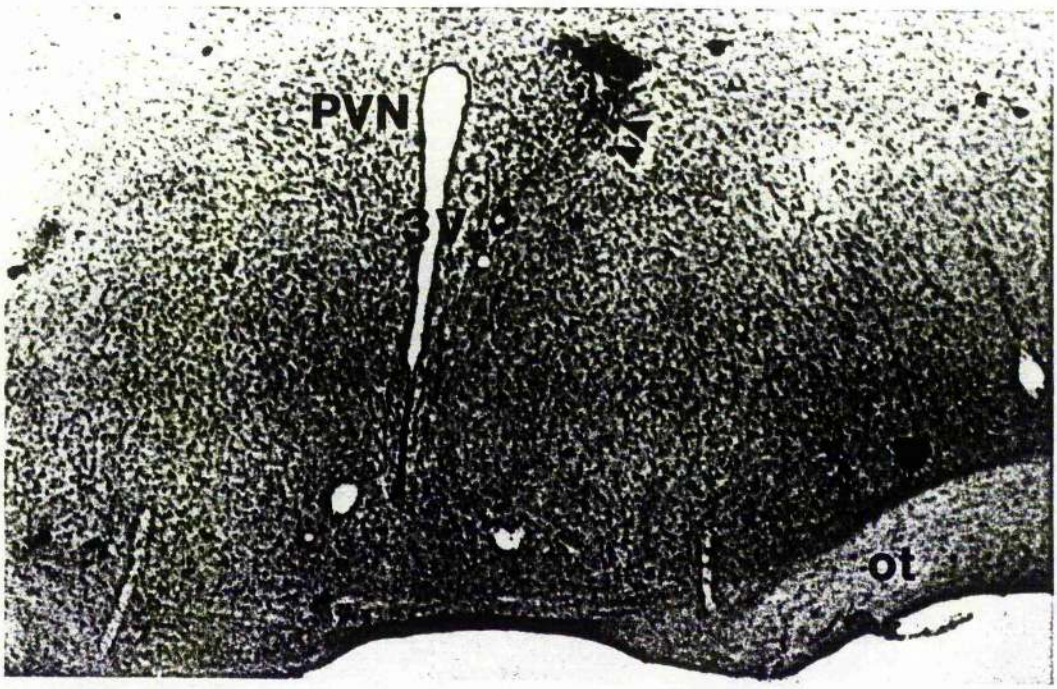
23 23. 4gm Gelatin in 260ml dist. water @ 37'C + 0.4gm Chrome alum in 20ml dist. water. Dissolve both separately, mix and add 120ml 96% alcohol.

24 Chance Propper Ltd. 76-51mm Thickness 1012. Ground edges.

25 Cresyl Fast Violet (aqueous) 0.1gm + 5mls Glacial acetic acid + Distilled water to 100mls. Correct pH to 3.5 using Sodium Acetate

26 D.P.X. Mountant BDH chemicals Product. No.36029

FIGURE 11. Photomicrographs of a representative section showing the PVN and injection site. The top figure is low power (x100), the bottom one is the same section in a higher magnification (x400). Abbreviations: opt, optic tract; 3V, third ventricle; PVN, paraventricular nucleus. The arrow heads indicate the injection site.


PVN
3V
ot

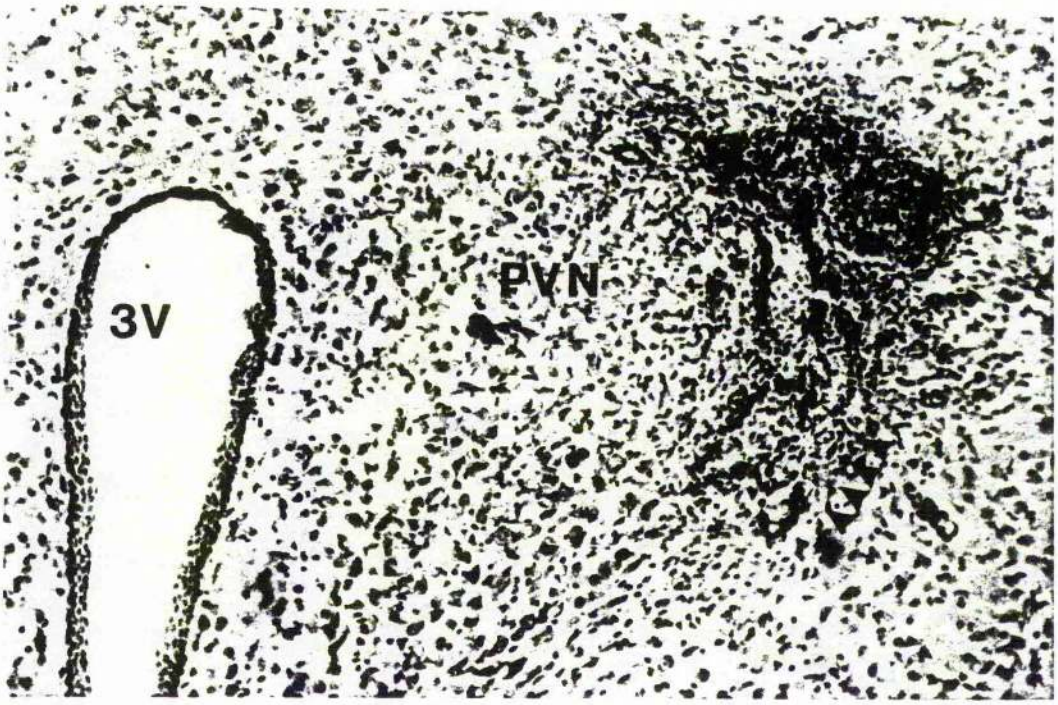
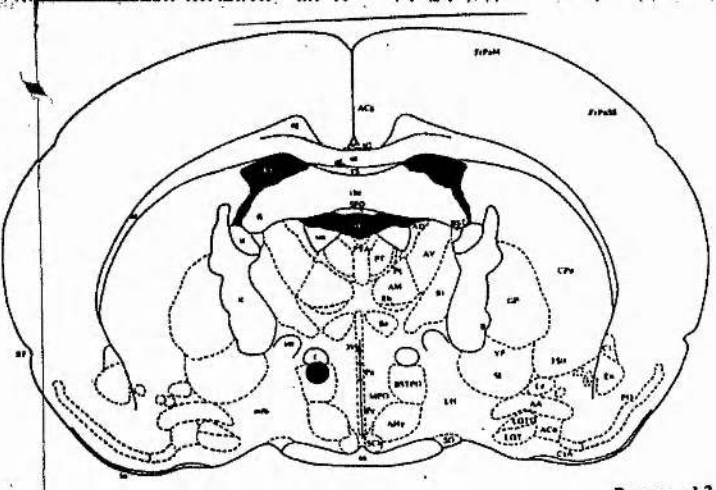
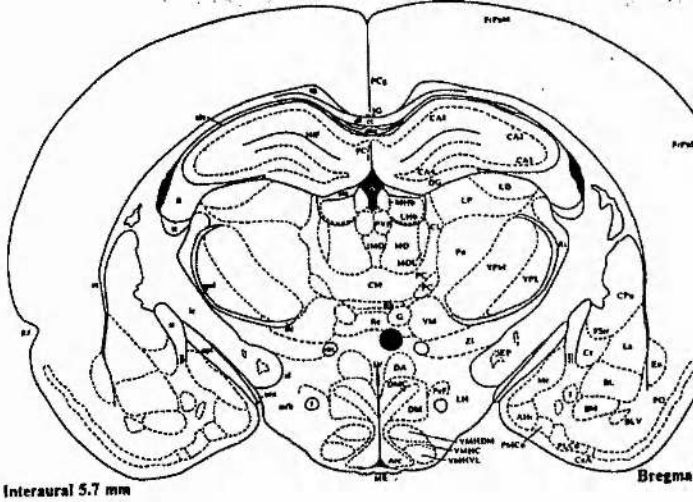

3V
PVN

FIGURE 11a. Representative sections showing typical injection sites which failed to elicit the 1.5gms. feeding response following injection of 40nm NA. All sites are at least 0.75mm from the borders of PVN. Typical "miss" sites were dorsal and caudal to PVN ending in VM thalamic nucleus. These errors probably occured as a result of guide cannula deflection during surgery.

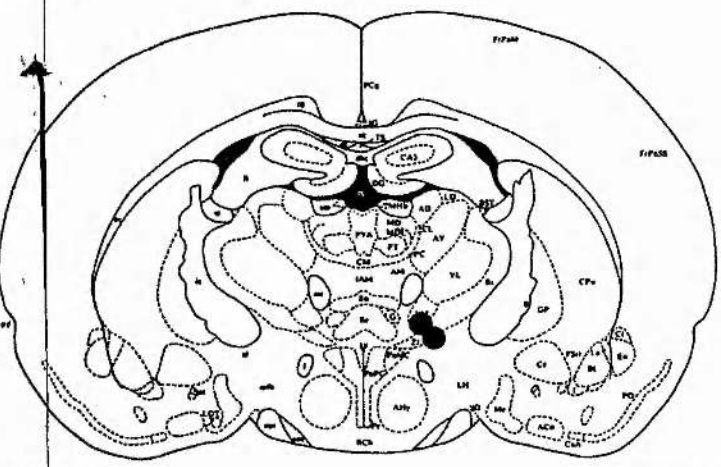


eraural 7.7 mm

Bregma −1.3 mm Interaural 5.7 mm

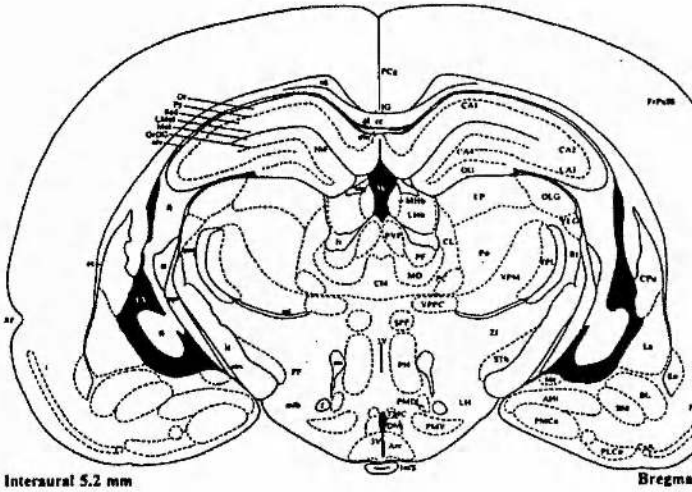


Bregma

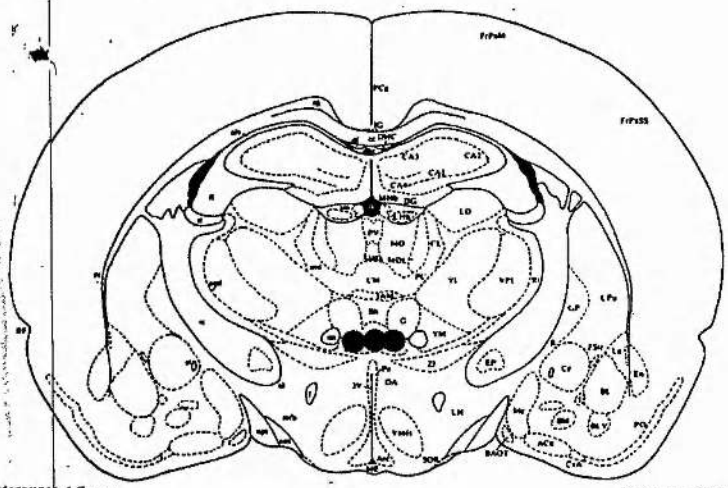


eraural 7.2 mm

Bregma −1.8 mm Interaural 5.2 mm

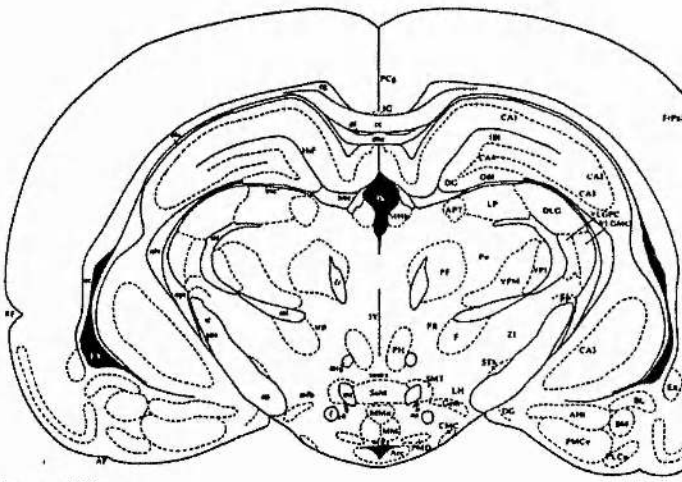


Bregma

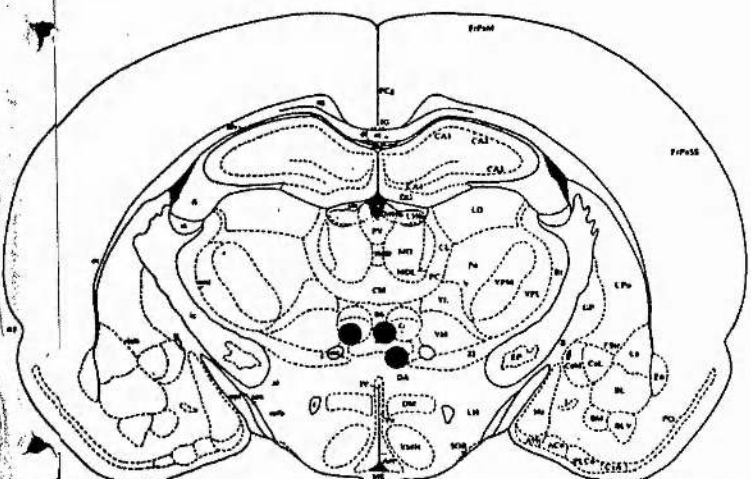


teraural 6.7 mm

Bregma −2.3 mm Interaural 4.7 mm



Bregm



Interaural 6.2 mm

Bregma −2.8 mm

● = the injection sight (n=10)

package ALICE running on the St. Andrews VAX A 11/780 computer. Post-hoc testing between the groups was performed using the Newman-Keuls test (unless otherwise specified). In the cocaine dose response experiment the 200nmoles condition is excluded from the analysis as 7 of the 8 scores in this condition were 0 and would otherwise have confounded the ANOVA. Further explanation of this condition is presented on the appropriate results section.

RESULTS

Experiment 1: Independent effects of Idazoxan, Phentolamine, Cocaine and Prazosin.

This experiment was designed to establish whether or not the drugs to be employed in subsequent blockade experiments have an independent action on food intake when microinjected into the PVN. Each drug was tested on a separate group of rats.

(i) Idazoxan

The effects of a variety of a-2 antagonists in the PVN have been reported (Leibowitz 1978a) but not those of Idazoxan. This compound was chosen as a result of studies demonstrating that it has a greater a-2 specificity than previously used agents such as yohimbine, rauwolscine and corynanthine (Doxey et al. 1984). A second advantage of using this compound is

that it is easily soluble and can be used in high concentrations and low volumes, allowing direct comparison with the doses of NA previously reported. A total of 11 rats with implanted guide cannulae were used in this experiment. The animals were first injected with 40nmoles NA to establish a feeding response. The criterion for acceptance was that the animal ate at least 1.5gms of lab chow in the 40 minute test period following injection. This figure was chosen because it was above the greatest amount eaten by any rat when mock injected. The second injection administered was 0.9% physiological saline, the vehicle for both NA and Idazoxan; third and fourth injections were 80nmoles and 0.1nmoles Idazoxan, and the fifth injection was a further saline injection. All injections were separated by a minimum of 48 hours.

Overall significance was found in these data ($F=5.82$ $df=5,50$ $p<0.001$; see figure 12). Post-hoc tests show that neither 80nmoles nor 0.1 nmoles Idazoxan had an independent effect upon ingestion when injected into the PVN. The only condition which differed significantly from the others was 40nmoles NA which had an effect significantly greater than Idazoxan 80nmoles ($p<0.0015$), Idazoxan 0.1nmoles ($p<0.0005$), first saline ($p<0.001$) and second saline ($p<0.0011$). The effect Idazoxan did not differ significantly from that of either saline injection.

(ii) Phentolamine

Phentolamine is soluble in warmed, sonicated physiological saline up to a maximum of just over 30nmoles in 0.5ul. For this reason the dose of NA used in this experiment was reduced to 30 nmoles in order to equate with the maximum available dose of Phentolamine, which was administered in doses of 1, 10, and 30 nmoles to a group of 8 rats. Overall significance was found in these data ($F=21.13$ $df=6,36$ $p<0.001$ see figure 12). NA was given as both first and last injection and each of these had significantly greater effects ($p<0.001$ in each case) than any of the Phentolamine conditions, none of which differed significantly from saline ($p>0.05$). However it was found that the two NA conditions differed significantly ($p<0.003$) and this is considered further in the discussion.

(iii) Cocaine

Cocaine blocks the re-uptake of released NA into the pre-synaptic nerve terminal and might therefore cause an increase in NA availability at the post-synaptic membrane. In order to establish whether this is sufficient to generate the same feeding response as the injections of exogenous NA a dose response to Cocaine was established in a group of 8 animals using three doses: 10, 100 and 200 nmoles. Overall significance was found in these data ($F=8.24$ $df=4,28$ $p<0.001$ see figure 12). There was no significant difference between

the two NA conditions ($p=0.287$) but both of these were significantly larger than any of the Cocaine or saline conditions ($p<0.005$ in each case). The two lower doses of Cocaine, 10 and 100 nmoles did not differ significantly to saline. The 200 nmoles dose produced a response lower than that of saline or any of the other drug conditions, in fact 7 of the 8 scores in this condition were zero. The inclusion of this number of zero scores in an ANOVA would have confounded the assumptions about the shape of the data employed in this test and thus the results are reported to be significantly lower by inspection only. The inhibition by 200 nmoles Cocaine of even small feeding bouts, commonly found in the saline control group, probably results from the local anaesthetic action of high doses of Cocaine. Five of the rats in this condition appeared incapable of voluntary movement during the test period, whilst the other 3 were severely impaired.

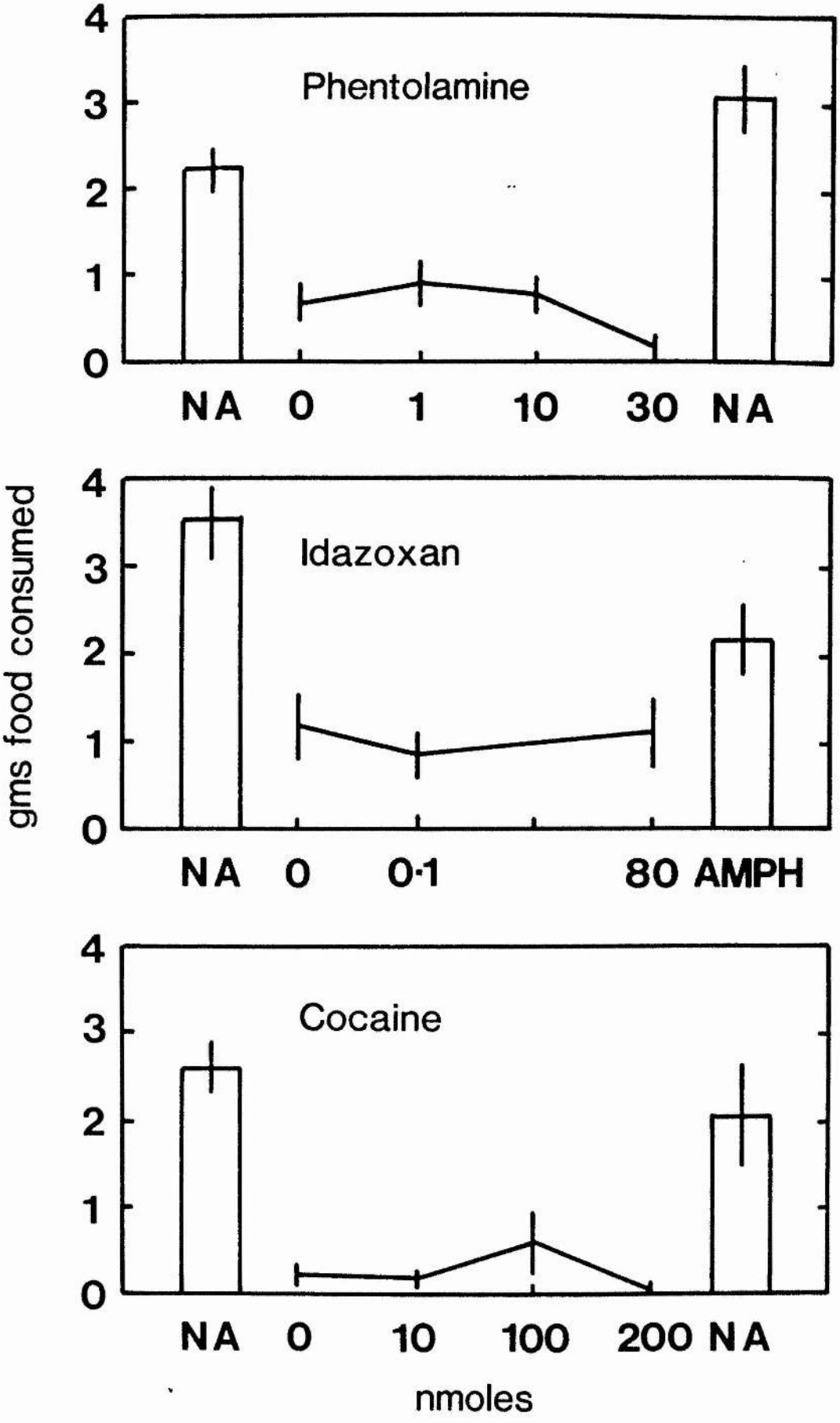
(iv) Prazosin

A dose response to Prazosin was not established due to its low solubility. It was always used at the maximum available concentration after warming and sonication, this dose being independently tested in the animals prior to its use as a blocking agent. Prazosin was found to have no independent effect on food intake, in agreement with the findings of

Leibowitz's group (Leibowitz 1975a, 1978b, Goldman et al. 1985).

Of the four pharmacological agents screened (idazoxan, phentolamine, prazosin and cocaine), only cocaine was found to have an independent effect on food intake. At high doses cocaine suppressed food consumption by disabling the animal. The three antagonist compounds did not cause any observable change in the rats normal behaviour, or cause any change in food consumption as compared to vehicle injection.

FIGURE 12. Mean (+/- SE) gms of food consumed in 40min following microinjection of 40 nmoles NA, and various doses of either phentolamine, idazoxan or cocaine in three separate groups of rats. NA was administered as first and last injection, except in the idazoxan group when 40nmoles d-amphetamine was the last injection. NA or d-amphetamine induced significantly more eating than saline in every condition (at least $p < 0.05$). No dose of phentolamine, idazoxan or cocaine induced significantly more eating than control.


Phentolamine
4
3
2
1
0
NA
0
1
10
30
NA
Idazoxan
4
3
2
1
0
NA
0
0·1
80
AMPH
gms food consumed
Cocaine
4
3
2
1
0
NA
0
10
100
200
NA
nmoles

EXPERIMENT 2

The purpose of this experiment was to establish that a small injection of the catecholamine neurotoxin 6-OHDA made via a cannula implanted into PVN could, at least temporarily, deplete this site of NA. Such a lesion, if effective, could be used to inhibit the re-uptake mechanism on pre-synaptic NA terminals by the physical destruction of such terminals.

METHOD

Animals

A total of 31 male hooded Lister rats were used in this experiment. Of these 6 were unoperated control animals and 23 underwent surgery. These all weighed approximately 300gms at the time of surgery, and were maintained in a the manner described for experiment 1.

Surgery

The co-ordinates for the cannula placement were adjusted in an attempt to avoid a blood vessel located just below dura and frequently ruptured when the guide cannulae were lowered into position. The new co-ordinates were according to the atlas of Paxinos and Watson (1982): flat skull position, nose bar 3.3mm below the interaural line, 1.8mm posterior to bregma, lateral 0.2mm, and 5.5mm below the skull surface. These co-ordinates again resulted in the injection canula terminating in, or directly adjacent to, the magnocellular

portion of PVN. This change of co-ordinates apart, the cannulae were implanted in the same way as for experiment 1.

Test Procedure

The animals were allowed at least 14 days to recover from surgery and then underwent the acclimatisation procedure described previously. Using the injection protocol described for experiment 1 animals were screened with NA and saline injections and only those exceeding the criterion of 1.5gms of lab chow in the 40 minute test after NA infusion were retained. Two animals failed to reach this criterion and were discarded from the experiment. The remaining 21 animals subsequently underwent the 6-OHDA lesioning procedure.

6-OHDA Lesion

The rats were placed in the testing cages and allowed to acclimatise for 30 minutes then removed and injected intra-peritoneally with pargyline [SIGMA][27] at a concentration of 50mg/kg/ml. The rats were then replaced in the testing cages for a further 30 minutes before receiving a microinjection of 6-OHDA. Because 6-OHDA oxidises readily, it was made up freshly for each animal in ice cold 0.02%

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[27] Pargyline [SIGMA] (N-methyl-N-benzyl-2-propynylamine) No. P-8013 Lot. 33F-08051 Mol. Wt. 195.7

ascorbate saline[28]. The 6-OHDA was made up at 4mg/ml of the free base, as the hydrobromide salt [SIGMA][29] was used, a correction factor of 1.48 was applied to the dry compound to account for the hydrobromide. The injected volume was 0.5ul and the injection protocol the same as that described above. The rats were then replaced in the testing cages and food and water weighed 40 minutes later. Finally the animals were returned to their home cages.

Lesion assessment

The effects of the lesion were to be assessed by measuring the amount of NA present in the lesioned PVN at four time points; 24 hours, 48 hours, 72 hours and 8 days post-lesion. The amount of NA present in the tissue of the PVN was assessed by high performance liquid chromatography with electrochemical detection (HPLC-ED) according to the method of Mefford (1981). In order to assess PVN NA levels by HPLC-ED the animal must be sacrificed, the brain quickly removed, micro-dissected on ice, and the tissue samples stored frozen at > -20'C until the assay. This is in order to prevent the breakdown of the

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[28] 0.0002gms per ml ascorbic acid added to ice cold physiological saline.

[29] 6-Hydroxydopamine [SIGMA] (2,4,5-Trihydroxyphenethylamine Hydrobromide) No. H-6507 Lot. 43F-0909 Mol. Wt. 250.1

catecholamines in the tissue by the monamine-oxidases present.

HPLC

Catecholamines were separated and detected by reverse phase HPLC with electrochemical detection. An isocratic 100mM citrate/acetate buffer (pH adjusted to 5.2 using sodium hydroxide) containing 1mM ethylenediaminetetra-acetic acid (EDTA), 100mg/L octanesulphonic acid and 10% methanol, was pumped at 1ml/min using a Gilson 303 HPLC pump. Separation was achieved using a 5um C-18 Ultrasphere column (250 x 4.6mm; Altex). Catecholamines were detected using a Bioanalytic Systems LC4B electrochemical detector connected to a LC-17 thin layer analytical cell. An oxidation current of 0.7 V (with reference to a silver / silver chloride electrode) was used to oxidise the catecholamines. The system was calibrated by injection of 2ng of each relevent catecholamine.

(i) Brain removal and micro-dissection.

The rats to be sacrificed were lightly anaesthetised with halothane[30] and then decapitated with a guillotine. The brain was rapidly removed (<100sec) and placed on an aluminium plate 10cm x 10cm resting in a box of crushed ice. Two cuts were made in the coronal plane with a razor blade, slightly

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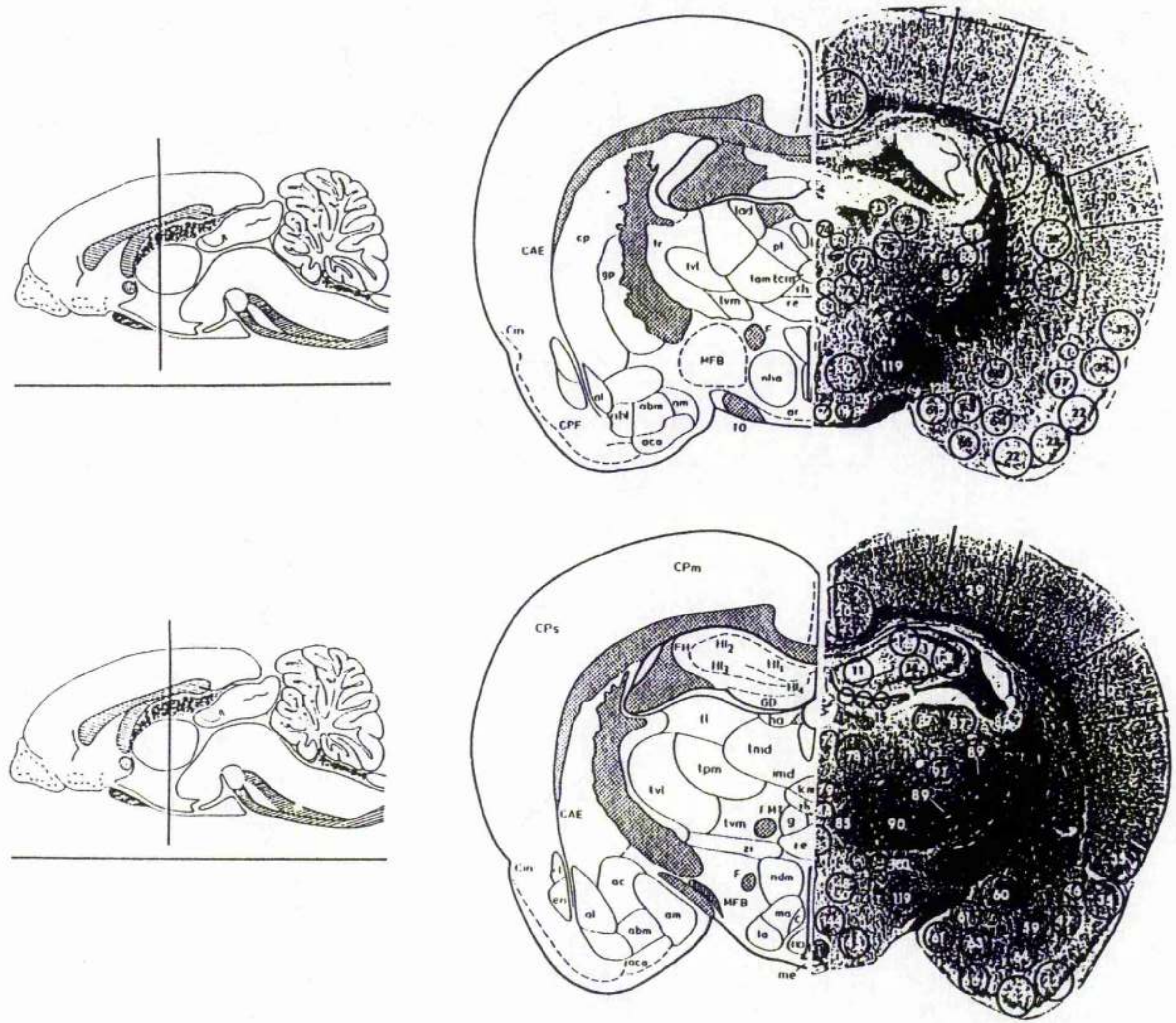
30 Halothane Fluothane inhalation anaesthetic ICI lot UP 686.

anterior and posterior to the PVN, at the levels depicted in plates 24 and 27 of Palkowits and Brownstein (1988). This left a coronal section about 1.5mm thick containing the PVN, which was placed flat on the plate and dissected down the midline. As the 6-OHDA had been injected unilaterally the side contralateral to the injection acted as a matched control to the lesioned side. However, two unoperated control rats were sacrificed at each time to provide absolute control levels of NA. The PVN was removed by means of three further cuts, the first a horizontal cut just dorsal to the top of the third ventricle, the second vertical cut made parallel with the midline at the level of the fornix and the final cut horizontal at the level of the supra-optic nucleus (see figure 13). This left a block of tissue about 2mm x 2mm x 1.5mm containing the majority of the PVN. This block of tissue was wrapped in tin foil and frozen rapidly in a container of dry ice, before being stored in a deep freeze at -25'C until assay. Rats were sacrificed at 24, 48, 72 and 192 hours, 7 animals in the first three groups, 8 in the last.

(ii) Assay with HPLC-ED

The HPLC-ED assay was performed at Edinburgh University, Department of Pharmacology by Dr. S. Butcher. The methodology was as described by Mefford (1981). Unfortunately, when the first series of tests were performed the aluminium

FIGURE 13. Plate from Palkowits and Brownstein showing the lines of cut for dissection of the PVN.



CAE
cp
gp
tr
lad
pl
tvl
tam
tvm
re
MFB
nha
Cin
CPF
abm
aco
10
119
CPm
CPs
GD
ll
tmd
tpm
tvl
imd
tvm
zi
ndm
MFB
ac
al
am
abm
la
me
89
90

silicate, used to bind the catecholamines during the washing stages, had become 'de-activated' and thus retained only very low levels of NA. This resulted in very poor recovery of NA from these samples and consequently the number of effective results was greatly reduced. Subsequent samples were analysed by direct injection of prepared tissue homogenate onto the HPLC column. The methodology was as follows:

Samples were stored at -20'C prior to analysis. They were then weighed before being homogenised in 400ul of 0.6M perchloric acid using a teflon-glass homogeniser. Samples were then centrifuged at 5000g for 2 minutes and 20ul aliquots of the resulting supernatent were injected onto the HPLC column using a Rheodyne 7125 injection valve.

Only the results for the 17 animals that gave acceptable levels of recovery are presented. The criteria for acceptance were: (i) that the results were from samples measured by injection of the homogenate, (ii) that one of the pair of samples is from the results generated using the aluminium silicate process, but resulted from a test in which the internal standard could be determined. No results are presented for pairs of samples tested by the silicate process. All samples were tested at random, and Dr. Butcher was unaware of the experimental procedure applied to each sample. However

it is recommended that the data generated as a result of this experiment be taken as indicative of a trend only.

RESULTS

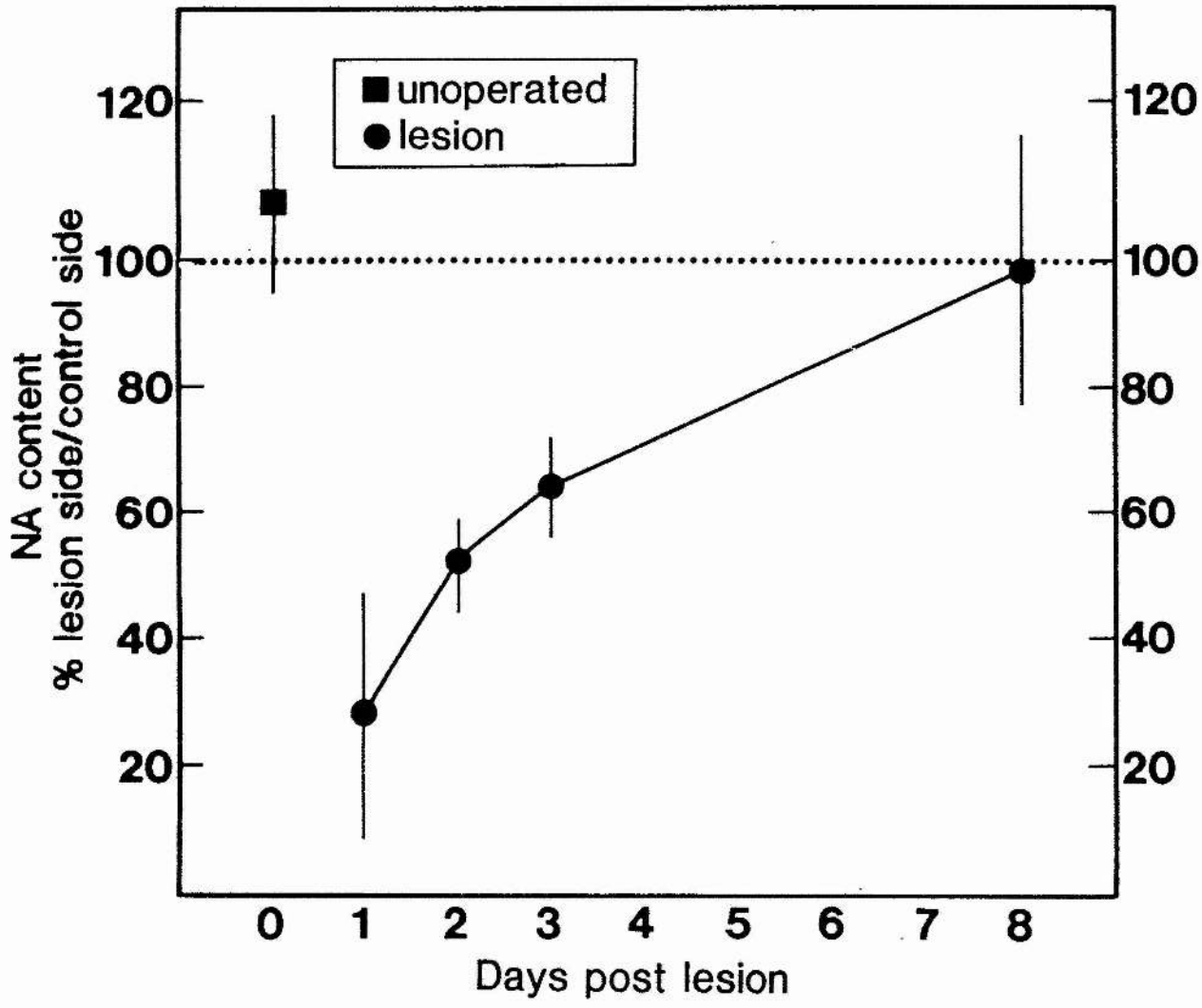
Experiment 2: 6-OHDA lesion control study

This experiment was designed to assess the effects of a small (0.5ul, 4mg/ml) injection of 6-OHDA into the PVN, on the level of NA in this nucleus. If the level were to be significantly reduced then it could be concluded that such an injection physically disrupted the NA projection to the PVN. Thus such a lesion might act as a means of permanently, or temporarily, inhibiting NA re-uptake into pre-synaptic terminals.

As explained in the methods section above this data should not be taken as absolute but indicative of a trend. The data was analysed as 4 lesion groups (24, 48, 72, 192 hours) with 3, 4, 3, 3 subjects in each group respectively. The control animals were taken as a group (N=4) at time zero pre-lesion. The data was entered as a percentage score: NA lesion side / NA control side. The control animals were entered NA,lhs / NA rhs. Analysis of the lesion groups over time reveals overall significance [$F=5.178$ $df=4,12$ $p<0.02$]. Post-hoc testing with the Tukey test reveals that this difference lies between the 24hrs condition and the control animals ($p<0.02$) and 24hrs and 192hrs ($p<0.04$). These data are presented

pictorially in figure 14. The data demonstrate that injection of this dose of 6-OHDA into the PVN produces a temporary depletion of within the PVN which has recovered within about 8 days.

FIGURE 14. Mean (+/- SE) percentage NA loss from PVN following treatment with 0.5ul 6-OHDA (4 mg/ml free base). Values shown are for unoperated rats (percentage loss calculated as L/R x 100) and lesioned rats sacrificed at 24, 48, 72 hours or 8 days post-lesion (percentage loss calculated as lesioned side / non-lesioned side x 100 for each rat). Significant differences between the groups over time were found ($F=5.178$, $df=4,12$, $p<0.02$).


unoperated
lesion
NA content
% lesion side/control side
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
Days post lesion

EXPERIMENT 3

The pharmacological inhibition of the re-uptake mechanism for NA might result in there being more NA available in the synaptic cleft to bind to post-synaptic receptors. In order to examine whether or not this hypothesised increase in NA concentration has an effect on feeding behaviour the following experiment was performed. Two dose response curves were established, one to NA alone and the other to NA in the presence of 40 nmoles cocaine. This experiment would also demonstrate whether inhibition of re-uptake potentiates the effects of exogenous NA by slowing the rate of loss of NA from injection site.

METHOD

Two groups of 8 male hooded Lister rats were used. These weighed approximately 340gms before surgery and were implanted with guide cannulae in the manner described above, using the co-ordinates for experiment 2.

Testing.

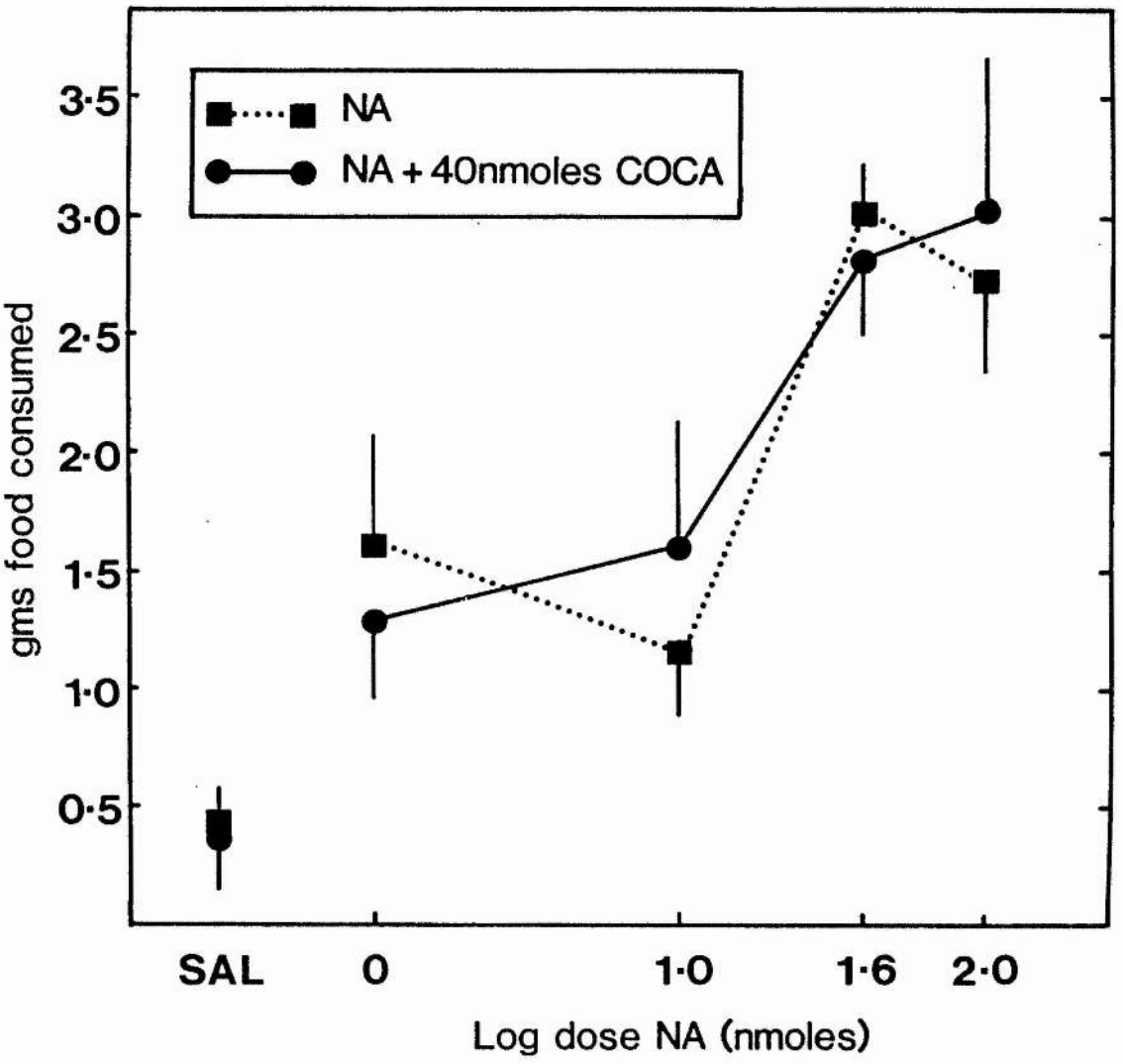
The animals were acclimatised to the test procedure as described previously. One group received 0.5ul injections of NA in the following doses: 1, 10, 40, and 100 nmoles presented in a counterbalanced order, plus a saline control injection.

The other group received the same doses of NA but prior to each dose they received a pre-injection of 40nmoles cocaine.

RESULTS

The results of this experiment, when combined with those generated from the use of cocaine alone are intended to demonstrate the effects of cocaine manipulations on NA induced feeding within the PVN. The data are presented in figure 15. The two curves do not differ significantly (Groups x Conditions interaction $F=0.41$ $df=4,56$ $p<0.80$). Thus in this preparation pre-treatment with cocaine does not affect the feeding response to exogenous NA. Whilst it is accepted that cocaine is a drug with a wide variety of effects within the CNS these experiments demonstrate that it has neither independent effects on NA elicited feeding within the PVN, nor does it affect the response to exogenous NA.

FIGURE 15. Mean (+/- SE) gms of food consumed in 40min following microinjection of various doses of NA or the same doses of NA directly preceeded by 40nmoles cocaine. All doses of NA or NA/cocaine produce significantly more feeding than saline (at least, $p<0.05$). There is no significant difference between the NA and NA/cocaine groups at any dose.


NA
NA + 40nmoles COCA
3·5
3·0
2·5
2·0
1·5
1·0
0·5
gms food consumed
SAL
0
1·0
1·6
2·0
Log dose NA (nmoles)

EXPERIMENT 4:

Selective blockade of NA elicited eating.

(i): 6-OHDA study.

This is the first part of a three part experiment, with each part performed on a separate group of animals. It was designed to identify the receptor sub-type apparently responsible for mediating the feeding response to infusion of exogenous NA and then to examine the effects of blocking NA re-uptake either by 6-OHDA lesion of pre-synaptic terminals or pharmacologically with cocaine

Method

A total of 8 male hooded lister rats, weighing about 350gms, were implanted with permanent cannulae as described above. These animals were acclimatised to the test cages and given the following series of injections: NA 40nmoles, saline and NA 40nmoles / idazoxan 40nmoles, presented in a counter-balanced order. Following these injections the animals were injected with 0.5ul 6-OHDA at a concentration of 4mg/kg free base (as above p 106). The animals were retested 24 hrs later with either NA 40nmoles or NA 40 / idazoxan 40 and the reciprocal condition applied 48 hours later. Finally these animals received a saline injection.

Twenty four hours after the last injection the animals were sacrificed and the PVN prepared for HPLC-ED assay as described

above. This assay was once again performed by Dr. S. Butcher in Edinburgh. The results of all 3 sections of this experiment are presented together below (p.118).

(ii) Selective blockade cocaine study.

This second part of the experiment was intended to examine the effects of inhibition of the re-uptake mechanism pharmacologically, by means of cocaine. It was hypothesised that this would result in an inhibition of α-2 selective blockade as a result of enabling the exogenous NA to penetrate the synaptic cleft.

Method

A group of 8 male hooded lister rats were implanted with cannulae and acclimatised to the test procedure as in the previous experiments. These rat received the following injections in a counter-balanced order (all drugs 40nmoles except prazosin): NA, NA / cocaine, saline, prazosin / NA, idazoxan / NA, idazoxan / cocaine / NA. These injections were performed at 48 hour intervals and the rats were subsequently perfused intra-cardially with 10% phosphate buffered saline and the brains submitted for histological verification of the cannula position. The results of this experiment are presented below (p.118).

(iii) Simultaneous blockade cocaine study.

The final part of this experiment was designed to determine whether or not simultaneous blockade of α-1 and α-2 receptors with phentolamine; and the subsequent inhibition of the feeding response to exogenous NA; was affected by the presence of cocaine. The hypothesis would suggest that the inhibition of re-uptake would have no effect on the blockade produced by phentolamine as the receptors within the synapse which are made available would also be blocked by this compound.

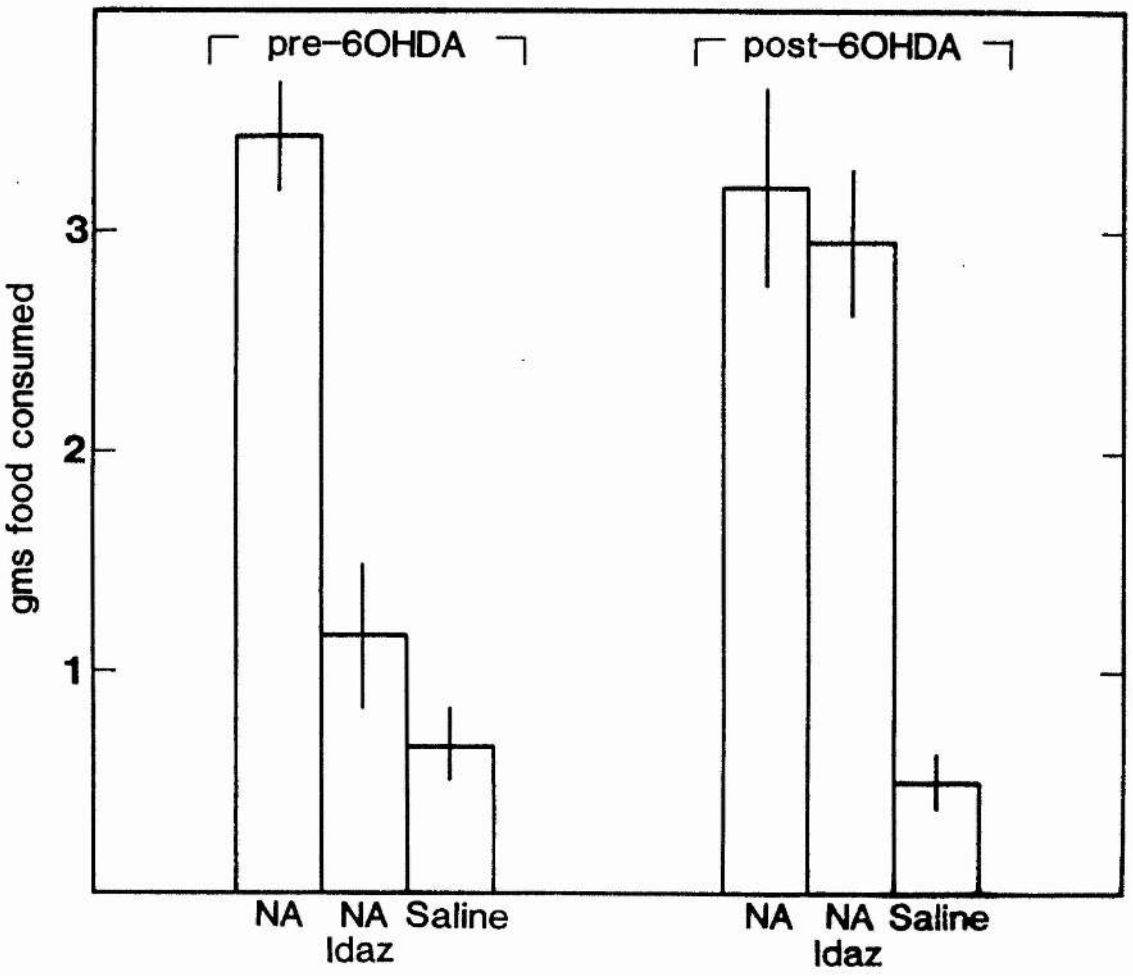
Method

A group of 7 male hooded lister rats (approx. 370gms) were implanted and acclimatised as described above. These rats received the following injections: NA, saline, phentolamine, NA / idazoxan, NA / cocaine / idazoxan, NA / phentolamine, NA / cocaine / phentolamine. All injections were at a dose of 30nmoles in 0.5ul as this corresponds to the maximum solubility of phentolamine. Following the injection series the animals were perfused intra-cardially with 10% phosphate buffered formalin, their brains removed and submitted for histological verification of the cannula position. The results are presented below, see figure 16.

Histology

The brains were cut as described above for experiment 1, and the sections stained with cresyl violet. Sections were

FIGURE 16. Mean (+/- SE) gms food consumed in 40 mins following microinjection of 40nmoles NA, saline or NA preceded by 40nmoles idazoxan before and after administration of 6-OHDA (0.5ul, 4 mg/ml free base). Overall analysis showed significant differences between the conditions ($F=20.484$, $df=6,60$, $p<0.001$). No differences were found between the effects of NA before and after 6-OHDA ($p=0.9977$), but the effects of idazoxan were significantly different after 6-OHDA lesion ($p<0.002$).


pre-6OHDA
post-6OHDA
gms food consumed
3
2
1
NA
NA Idaz
Saline
NA
NA Idaz
Saline

examined using a Leitz Diaplan microscope (eyepieces x10, objectives x1.6, x2.4). All animals reported had injection sites in or directly adjacent to the PVN. Representative sections are presented in figure 17.

It should be noted that the histological verification of injection sites is, in these experiments, a secondary requirement. Each animal receives both control and test injections, the use of a repeated measures design means that if a certain animal performs the behaviour then the manipulations of that behaviour within that animal are internally valid. Thus the screening for effect of NA and the subsequent manipulations are more important than the exact location of the injection. However, the strength of the response to NA was clearly related to the proximity of the injection site to the PVN as previously reported (Leibowitz 1978a), and it was thus considered important to maximise the behavioural effects by injecting as accurately as possible into PVN.

RESULTS

Experiment 4

(i):Selective blockade and re-uptake inhibition by means of a 6-OHDA lesion.

The purpose of this experiment was to examine the effects of re-uptake inhibition on the selective blockade established to

exogenous NA by idazoxan. The results were analysed by ANOVA and post-hoc testing using the Tukey test [$F=20.484$ $df=6,60$ $p<0.001$]. Prior to the 6-OHDA lesion idazoxan significantly reduced the effects of exogenous NA ($p<0.0001$). This replicates the findings of Goldman et al. (1985) that the feeding response is mediated by the α-2 receptor system. NA conditions before and after the 6-OHDA lesion do not differ significantly ($p=0.9977$), neither do the saline conditions pre- and post-lesion ($p=0.9997$). However, the NA / idazoxan conditions before and after the 6-OHDA lesion do differ significantly ($p<0.002$). The NA / idazoxan condition post-lesion does not differ from either NA alone condition (pre- $p=0.9137$, post- $p=0.9972$). Thus the 6-OHDA lesion can be seen to have had a profound effect on the blockade established by idazoxan to the actions of exogenous NA.

Experiment 4

(ii): Selective blockade and re-uptake inhibition by administration of cocaine.

This experiment examined the effect of selective α-1 and α-2 blockade with and without cocaine. Overall significance was found in these data ($F=9.05$ $df=5,35$ $p<0.001$) ANOVA, post-hoc Newman-Keuls. There was no significant difference between NA and NA / cocaine ($p=1.0$; see also expt.1 iii) or between NA and NA / prazosin ($p=1.0$); both of these conditions were significantly different to saline ($p<0.0005$ and $p<0.001$

respectively). However, there was a significant difference between NA and NA / idazoxan ($p<0.02$), the addition of idazoxan having a significant blocking effect on the actions of NA. There is no significant difference between saline and NA / idazoxan ($p=0.21$). When cocaine was added to the NA / idazoxan condition this blockade was abolished. NA / idazoxan / cocaine does not differ significantly from NA alone ($p=1.0$) but is significantly greater than NA / idazoxan ($p<0.005$). This demonstrates that the actions of infused NA appear to act through α-2 receptors when tested by selective blockade (see Goldman et al 1985) but re-uptake inhibition by cocaine attenuates α-2 blockade.

Experiment 4

(iii): Simultaneous blockade and re-uptake inhibition by administration of cocaine.

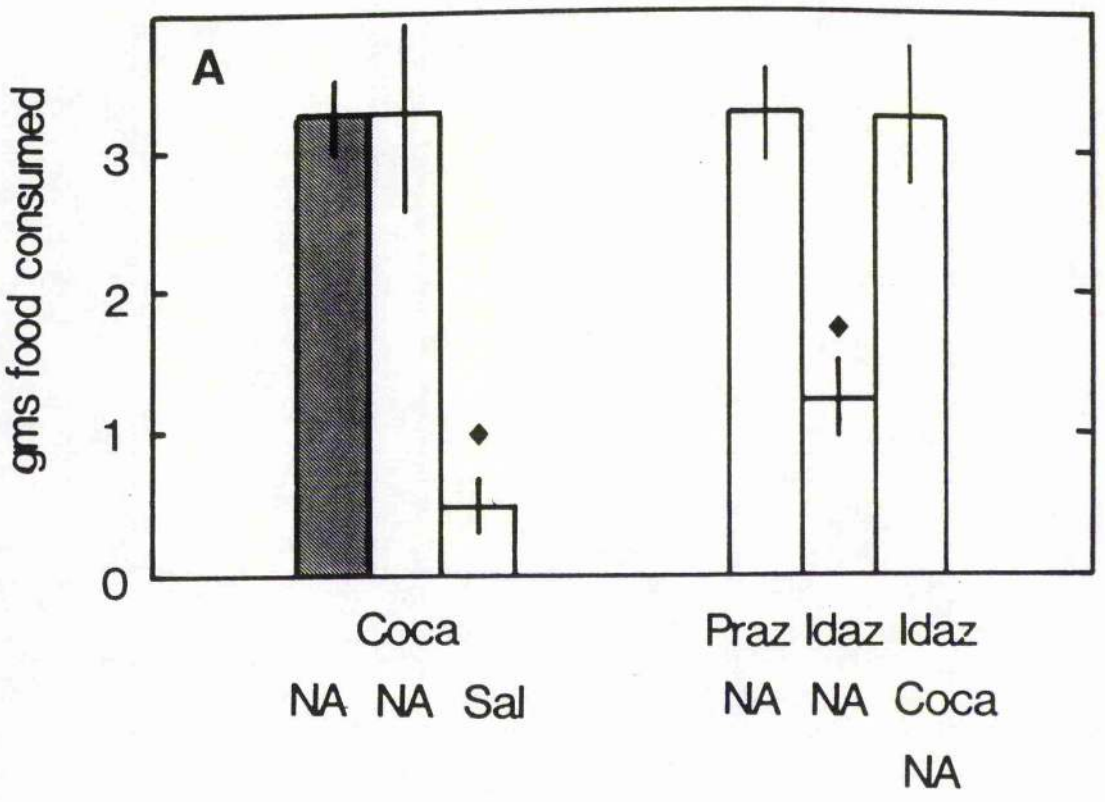
This final part of the experiment was designed to examine whether simultaneous blockade of α-1 and α-2 receptors was affected by re-uptake blockade with cocaine. The data provided significant differences between conditions overall [$F=21.13$ $df=6,36$ $p<0.001$]. The actions of infused NA were significantly inhibited by the actions of phentolamine (NA vs NA / phentolamine $p<0.001$) or by the addition of idazoxan (NA vs NA / idazoxan $p<0.01$). However, the effects of blockade by phentolamine were significantly greater than the effects of blockade by idazoxan (NA / phentolamine vs NA / idazoxan

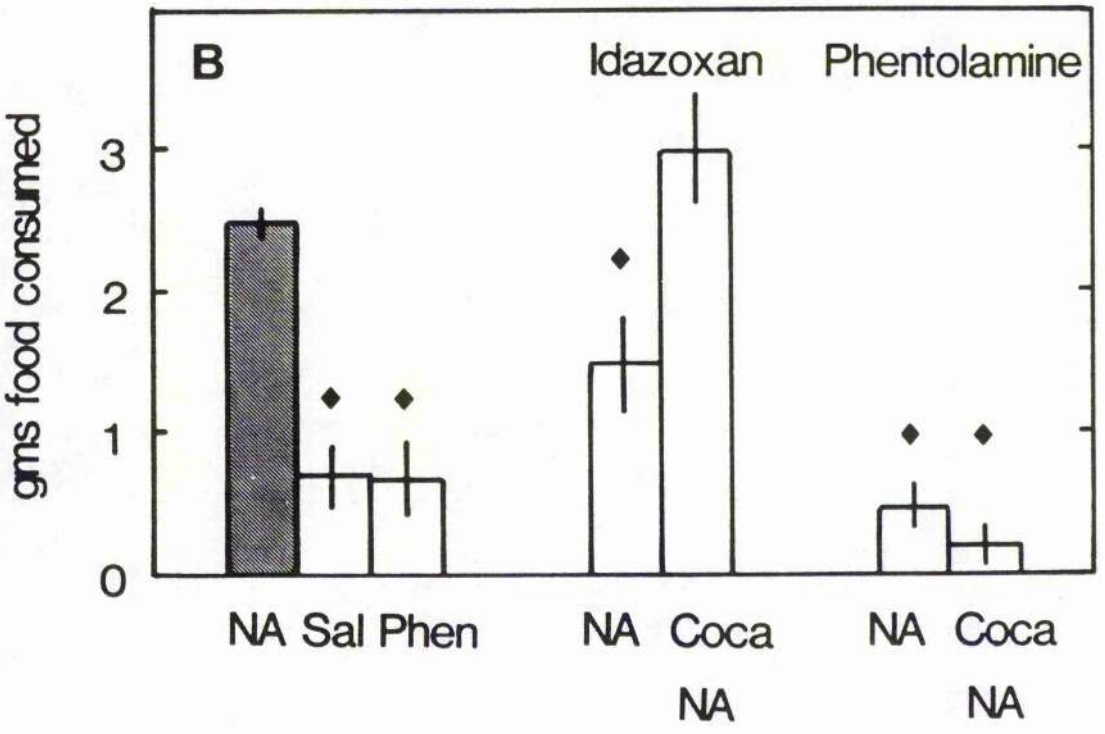
$p<0.05$). The blockade exerted by idazoxan was abolished by the addition of cocaine, the effect of NA / idazoxan / cocaine being significantly greater than NA / idazoxan ($p<0.0002$) while NA / idazoxan / cocaine did not differ from NA alone ($p>0.05$). However, the addition of cocaine to NA / phentolamine had NO significant effect on phentolamine blockade (NA vs NA / phentolamine / cocaine $p=0.33$). These data are presented pictorially in figure 18.

FIGURE 17.

A. Selective blockade of eating elicited by 40nmoles NA by prazosin and idazoxan. Prazosin (0.2nmoles) did not affect NA elicited eating but 40nmoles idazoxan significantly attenuated eating to NA. When 40nmoles cocaine was added to NA eating was unaffected, but 40nmoles idazoxan was completely ineffective in antagonisisng NA/cocaine eating. (:sig. diff. to 40nmoles NA [hatched bar] $p<0.05$ at least.)

B. Comparative blockade of eating elicited by 30nmoles NA and 30nmoles NA/ 40nmoles cocaine. Blockade to 30nmoles idazoxan was attenuated when cocaine was added to NA. Blockade to 30nmoles phentolamine was unaffected by the addition of cocaine to NA. (:sig. diff. to 30nmoles NA [hatched bar] $p<0.05$ at least.)


A
gms food consumed
0
1
2
3
Coca
NA NA Sal
Praz Idaz Idaz
NA NA Coca
NA


B
Idazoxan
Phentolamine
gms food consumed
0
1
2
3
NA Sal Phen
NA Coca
NA
NA Coca
NA

EXPERIMENT 5

In the introduction evidence was presented for the involvement of NA and DA in promoting eating in response to tail pinch. The evidence for the involvement of NA in feeding behaviour was also extensively reviewed, particularly with reference to the actions of NA within the PVN. The PVN was identified, not only as a site clearly involved in the feeding response to NA, but also as a nucleus with considerable influence in the mediation of both neurocrine and endocrine responses to stress. The following series of experiments were performed to examine the hypothesis that the feeding response to tail pinch is in part the result of an increase in endogenous NA release within the PVN. This was examined by means of bi-lateral infusions of NA selective antagonists into the PVN prior to the application of tail pinch, in animals already identified to respond to the pinch.

METHOD

Animals

Two groups of male hooded lister rats were employed in this study. Group 1 consisted of 12 rats each weighing approx. 350gms at the time of surgery. Group 2 consisted of a group of 7 older rats each weighing about 450gms. at the start of the experiment. All rats were single housed and maintained under a 12 hour light/dark cycle with 'ad lib' access to SDS

No.1 chow pellets and tap water. All animals were tested whilst food and water satiated.

Tail-pinch Protocol

The animal to be tested was removed from it's home cage and placed in the testing arena, the floor of which was entirely covered with lab chow pellets. The arena itself was a four sided frame surrounding an area 60cm square and with sides 40cm high. Three of the sides were aluminium, the fourth was clear perspex. The arena was uncovered, see figure 19.

(i) Habituation: Animals were placed in the test arena for an habituation period of 120 seconds during which time they were free to explore. This habituation was performed on alternate days for 5 trials, one per day, at which time the animals were judged to have acclimatised to the handling and experimental conditions.

(ii) Testing: Following the habituation trials animals were placed in the arena as before and allowed 120sec habituation. At the end of this time a pneumatic cuff, made of brass and padded with sticking plaster, was placed around the animal's tail approximately 1 inch (2.5cm) from the tip, see figure 20. Pressure was applied to the tail by means of a cylinder of compressed air forcing the plunger within the cuff against the rat's tail. The pressure of the pinch was regulated by means of a control valve attached to the cylinder. The pressure started at 10 psi and was raised by 5 psi after three

FIGURE 18. Photograph of the Tail Pinch arena. The compressed air cylinder, and controls for the pneumatic cuff are also shown.

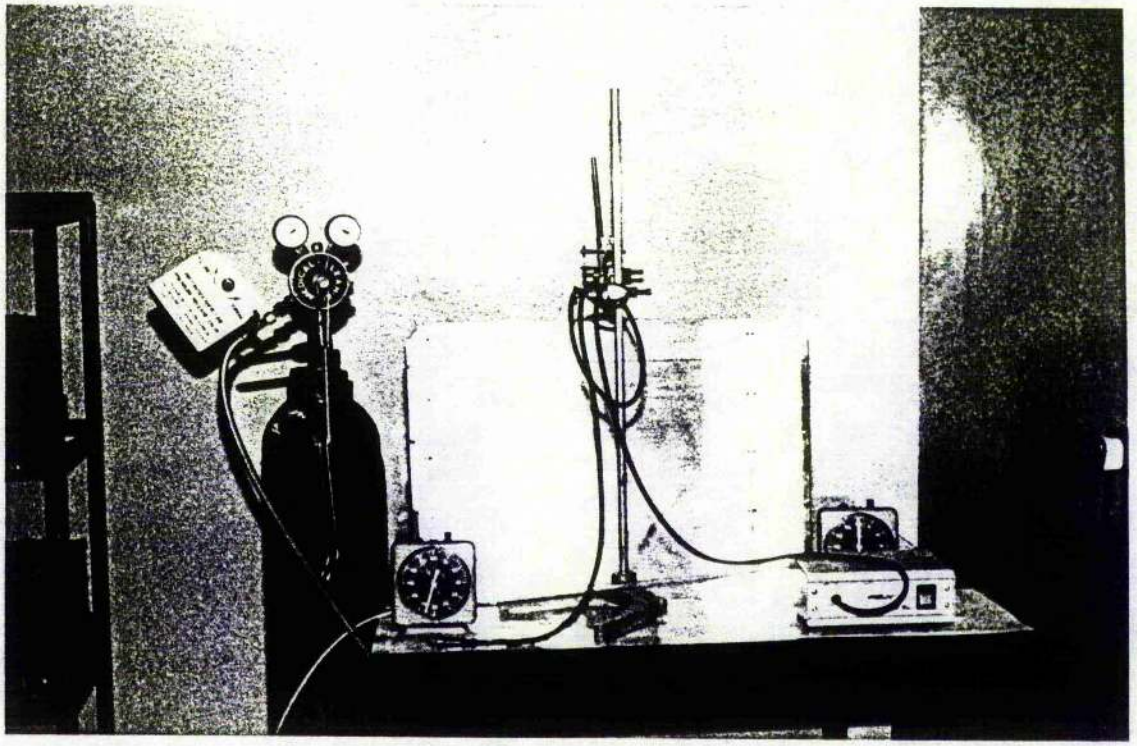
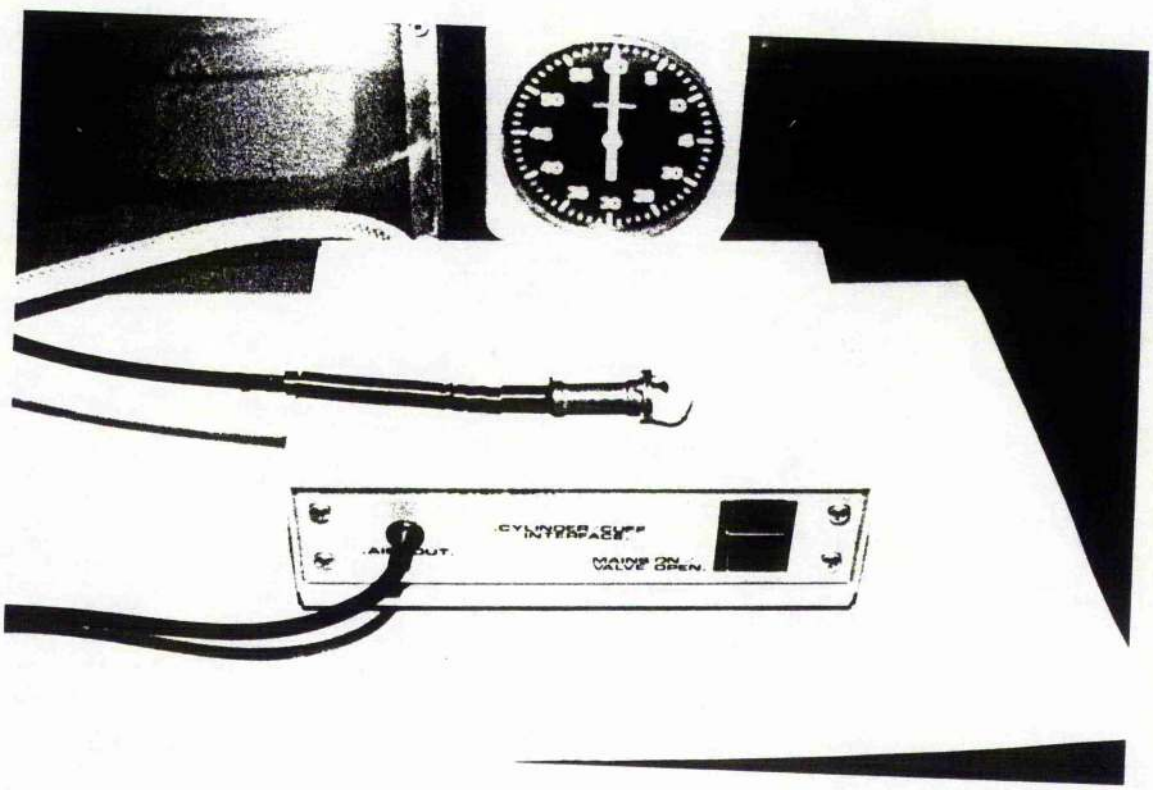


FIGURE 19. Photograph of the pneumatic cuff used for Tail Pinch. The air tube is protected at the cuff-end by a metal spring to prevent it being punctured by a bite.


CYLINDER CUFF
INTERFACE
AIR OUT
MAINS ON
VALVE OPEN

consecutive trials on which the animal did not eat. No pressures below 10 psi were considered as the pinch was dislodged by almost any movement of the animals tail at such low pressures. Pinch pressure was maintained for 120 sec unless the animal started to feed during the pinch, in which case the clock was re-started for 120 sec from the initiation of the feeding bout. Feeding was defined as a period of 3 sec of continuous chewing and swallowing of a pellet. Simply shredding the food pellets was not counted. The time at which feeding was started was recorded and represents the latency. Other salient behaviours were recorded idiosyncratically for each animal. If the pneumatic cuff was dislodged, the clock was stopped and the cuff replaced, in some cases at low pressures it was lightly held in place with sticking plaster if frequently removed.

The objective of the pre-testing of animals with tail pinch was to obtain a population of rats which ate on three consecutive trials. Once an animal reached this criteria TP was suspended until after surgery. The results of the pre-trials are presented below on p.123.

Surgery

The surgical procedure was similar in all respects to that described for experiments 1 - 4 excepting that these animals were implanted with bi-lateral guide cannulae. This was

achieved by affixing two cannula guides to the stereotaxic arm. However, the physical dimensions of these guides required that the lateral co-ordinate be increased to 0.3mm lateral. Injections were thus 0.1mm more lateral than in previous experiments.

Injection Protocol

The injection protocol was similar in most respects to that used in experiments 1 - 4 except that two injection cannulae were used, both injections being performed simultaneously. The Harvard pump employed during these experiments was designed to mount two syringes simultaneously and no significant change in protocol was required.

Drugs.

The drugs used in ths experiment were as follows:

(i) [-]-norepinephrine bitartrate [SIGMA].

(ii) The combined α-antagonist phentolamine hydrochloride [CIBA].

(iii) The DA antagonist haloperidol [SIGMA][31].

(iv) The noradrenergic ß-antagonist propranolol [SIGMA]. All drug solutions were made up in sterile physiological saline, except haloperidol which was made up in 0.02% ascorbate saline.

---

[31] Haloperidol, Sigma No. H-1512 lot 76F-0016 mol wt. 375.9

Cannula placement screening.

In order to determine whether the implanted cannulae were located correctly, the animals were screened by injection of 40nmoles NA unilaterally down each of their cannulae. Thus each animal received two injections separated by 48 hours, one into the left cannula and one into the right. The criteria for acceptance of the cannula placement was that the animal ate 1.5gms or more in the 40 minute test period following the injection. Thus animals could be classified in terms of cannula placement: bi-laterally correct; left correct; right correct; neither correct. No saline injection was administered at this stage in order to minimise the overall number of injections performed. The results of this screening are presented below on p127.

Central drug injections

The purpose of this experiment was to determine whether central injection of adrenergic or dopaminergic antagonists into the PVN could affect the eating response to tail pinch.

The tail-pinch protocol was as described above, except that the animal received a bi-lateral injection (0.5ul) of one of the following drugs into the PVN 30 minutes before tail-pinch: NA (40nm), propranolol (40nm), phentolamine (30nm), or saline.

Latency to eat was recorded and the results are presented below on p128.

Intra-peritoneal drug injections

A group of 8 rats, all of which had been demonstrated to eat to tail-pinch post-surgery, were injected with either 0.02% ascorbate saline (1ml/kg) or haloperidol (4mg/kg/ml) 30 minutes prior to tail-pinch testing. This dose of haloperidol has previously been demonstrated to be non-sedative (Taha and Redgrave 1980). All animals received both injections in a randomised order. The results for this experiment are presented below on p128.

Cold Water Stress

As a final experiment the effect on feeding of a known stressor, cold water immersion, was examined. The 19 rats were randomly assigned to groups; group A (N=10) received an injection of 30nmoles phentolamine into the PVN, whilst group B (N=9) received a saline injection. Following these injection animals were returned to their home cages for 30 minutes before being removed and placed into the Morris water maze (Morris 1978), which was filled with cold water (4'C), for 45 seconds. Food and water in the home cage was weighed at this time. The rat was then removed from the water maze and returned to it's home cage, and a measure was taken of food and water consumption 1 and 3 hours after the exposure

to cold water. The results for this experiment are presented on p.129.

RESULTS

(i) Baseline TP testing

Of the 12 animals tested (group 1) 8 ate on 3 consecutive trials by the eighth trial. Of the animals which ate; 3 ate when a pressure of 10 psi was applied, the remaining 5 rats ate at a pressure of 15psi. ANOVA of log latency scores for each animal was not significant. There was no decrease in latency over the first 3 consecutive trials ($F=1.52$, $df=2,21$ $p>0.25$). Of the rats which did not reach criterion, 1 never ate, 1 ate on three trials and 2 ate on four trials but never on consecutive days.

(ii) Post-operative TP testing

Both groups of rats were tested post operatively (N=19) and the latency for the first three trials was analysed by ANOVA. There was no significant difference between the three trials ($F=1.15$, $df=2,21$ $p>0.25$). Comparison of the pre- and post-operative trials shows no significant differences ($F=1.17$, $df=5,42$ $p>0.25$). It thus appears possible to establish a baseline latency for the onset of TP induced eating. No rat that failed to eat consistently in the pre-operative screening ate consistently post-operatively.

(iii) NA screening procedure

The criteria of consuming 1.5gms of lab chow within 40 minutes was applied as for experiments 1-4. Of the left cannulae 13 from the 19 animals were correct. Of the right cannulae 15 of the 19 were correct. A total of 10 animals were found to have bi-laterally correct cannulae. When the second criterion of eating on three consecutive trials was applied 6 animals fulfilled all the requirements and these 6 animals were subsequently used in the microinjection experiments.

(iv) Microinjection studies

None of the noradrenergic agents phentolamine, propranolol or NA had a significant effect on TP induced eating. ANOVA of the pre-operative, post-operative and drug microinjection trials revealed no significant differences ($F=1.77$, $df=4,25$ $p>0.25$).

Table 1.

Condition	Pre-op.	Post-op.	Phen.	Prop.	NA	Sal
No. rats eating	8/12	8/12	6/6	6/6	6/6	6/6
Latency (secs)	64.2	55.7	44.6	75.8	63.7	88.8
SEM	8.0	7.4	18.5	15.4	15.6	11.4

(v) Intra-peritoneal drug injection study

None of the 8 animals injected i.p. with haloperidol ate under TP. However 6 of these 8 animals ate when injected i.p. with

vehicle (ascorbate saline). Injections were performed in a counterbalanced order.

(vi) Cold water swim

Food consumed 1 and 3 hours after exposure to cold water was calculated. No significant difference was found between the phentolamine microinjected animals and saline injected controls (Student's t test $t=-1.32$, $df=14.6$, $p=0.21$).

EXPERIMENT 6

Experiments 1 - 4 were designed to examine the hypothesis that there is a separation of post-synaptic alpha-receptors within the PVN. The α-2 receptor was hypothesised to be predominantly outside the 'classical' synapse, whilst the α-1 receptor was intra-synaptic. The results of these experiments appeared to justify further examination of this hypothesis. In order to examine whether extra-synaptic α-2 receptors and 'paracrine' release of NA might be a generalisable principle it was necessary to look at the effects of NA at a different site and generating a different behaviour. The site chosen for further investigation was the ventral striatum, and the behaviour to be examined was locomotion. In order to explain the reasoning behind both this choice of site and behaviour, some analysis of the anatomy and function of the ventral striatum is required. This is presented below.

VENTRAL STRIATUM

General Anatomy

The striatum is both a prominent and easily localised component of the basal telencephalic grey matter in all mammals. It is a mosaic of two interlocked but neurochemically distinct compartments known as patches and matrices. The patches demonstrate high opiate receptor binding and enkephalin- and substance-P like immunoreactivity. The

matrices have high acetyl- cholinesterase activity and are dense with fibres displaying somatostatin like immunoreactivity (Gerfen 1984). Graybiel (1983) suggests that this patch and matrix organisation may be a sub-cortical arrangement similar to the layers and columns organisation of the neocortex and be important in the organised channelling of information through this tissue. The lateral ventricle and subcallosal fasiculus clearly define the dorsal extent of the striatum. However, few such landmarks are available to demarcate the ventral extent of this structure. At many points in the basal forebrain it is extremely difficult to distinguish neo-striatal elements from areas such as the nucleus accumbens septi and the olfactory tubercle. Indeed it has been suggested (Heimer and van Hoesen 1979) that it is appropriate to view these latter structures as part of the ventral striatum. Consequently, in this chapter the term 'ventral striatum' includes the nucleus accumbens, the olfactory tubercle and the ventral subcommissural part of the main body of the striatum. These structures are continuous with each other through 'striatal' cell bridges and are characterised by a 'striatal' pattern of connections. However, they are distinguished by certain hodological features which differ from the rest of the striatum. The boundary between the ventral olfactory tubercle and the underlying olfactory cortex seems sharp and clearly defined in both acetylcholinesterase (AChE) and Nissl-stained

preparations in the rat. In contrast, the dorsal boundary between the tubercle and the nucleus accumbens is difficult to define particularly at more rostral levels. Many cell bridges provide a continuity between tubercle and accumbens which reaches the surface between the fibre bundles of the deep olfactory radiation (Heimer and van Hoesen 1979).

Anatomical connections

(i) Afferent connections.

The ventral striatum, as defined above, is largely characterised by the same pattern of connections as the dorsal striatum. That is, the major afferent pathways to the accumbens and tubercle originate in dopaminergic cells in the midbrain, non-specific thalamic nuclei and cerebral cortex. However there are also significant differences between the inputs to dorsal and ventral striatum. In particular, the dorsal striatum appears to receive no input from the amygdala whereas the ventral striatum receives a major amygdaloid projection, originating from the basolateral and basomedial amygdaloid nuclei. Afferents from the piriform cortex terminate in the olfactory tubercle and ventral part of the accumbens and afferents from the hippocampus (originating predominantly from the subiculum) terminate in the medial zone of the nucleus accumbens (Bjorklund and Lindvall 1986). Recently the projection from subiculum to the nucleus

accumbens has been identified as glutaminergic (Totterdell and Smith 1986).

(ii) Efferent connections.

The efferent projections of the ventral striatum again show similarities with those of the dorsal striatum; neither striatal area having reciprocal connections with the cerebral cortex. Fibres from the nucleus accumbens ascend to the anterior septum, hippocampal rudiment, the anterior part of the dorsal striatum and the lateral septal nucleus. Descending fibres run to the globus pallidus and the medial and lateral pre-optic areas. There also appears to be a sparse projection to the PVN. Some labelled nucleus accumbens fibres remain in the MFB before becoming diffusely scattered in the ventral tegmental area, or spreading laterally to the substantia nigra where they distribute medially into the pars compacta and dorsally and medially into the pars reticulata (Conrad and Pfaff 1976).

Organisation of Striatal Circuitry

(i) Dopamine.

The A10 group of the mesencephalic DA cell group, mainly located in the ventral tegmental area (VTA), projects to the entire subcommissural part of the ventral striatum as well as to much of the caudate/putamen (Bjorklund and Lindvall 1986). The substantia nigra in contrast projects DA fibres

FIGURE 20. Comparison of areas within striatal complex receiving afferents from nigral and DA neurons of A10 cell group (left) and from different parts of cortical mantle and amygdala (right). Figure from Bjorklund and Lindvall (1986)

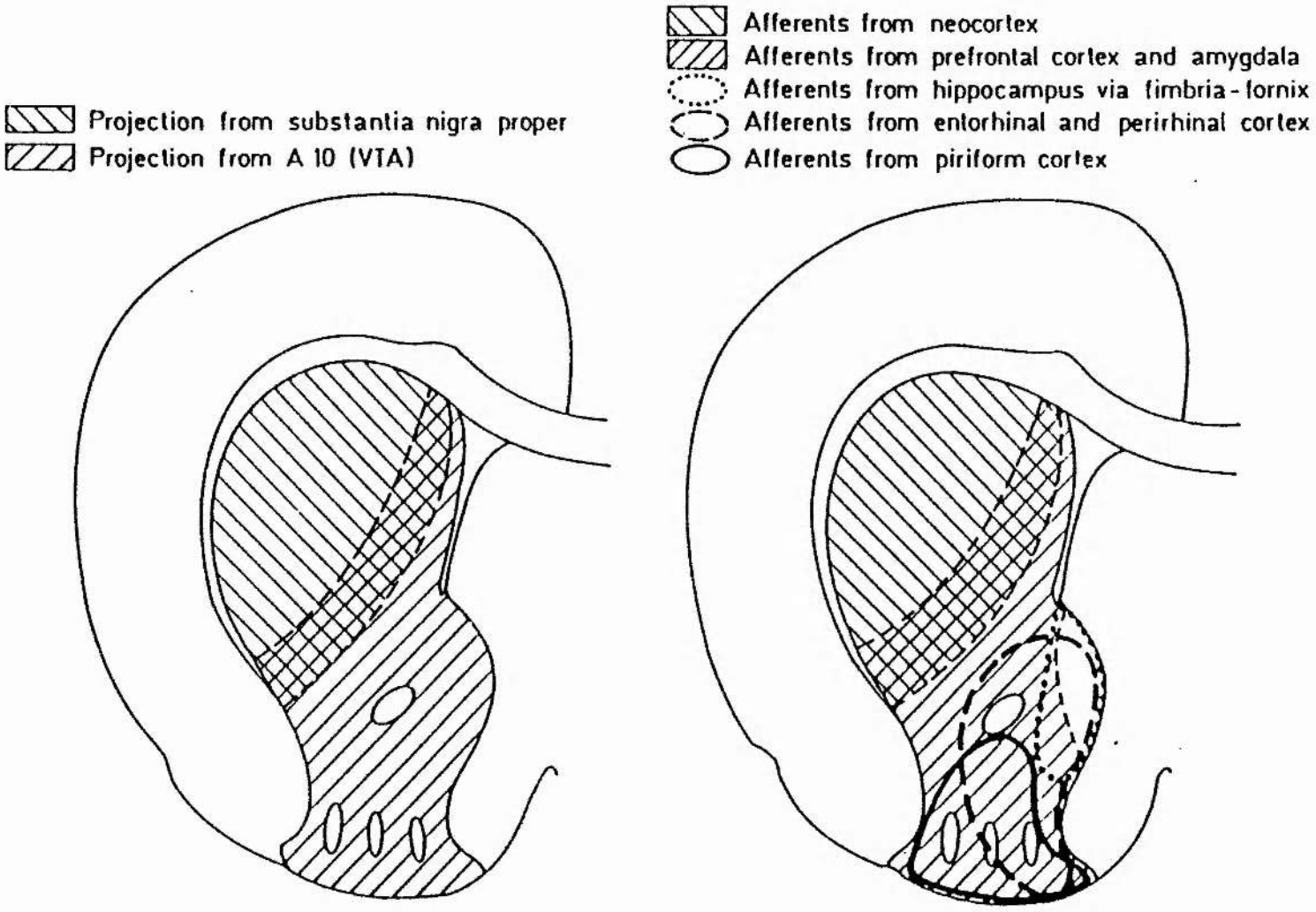
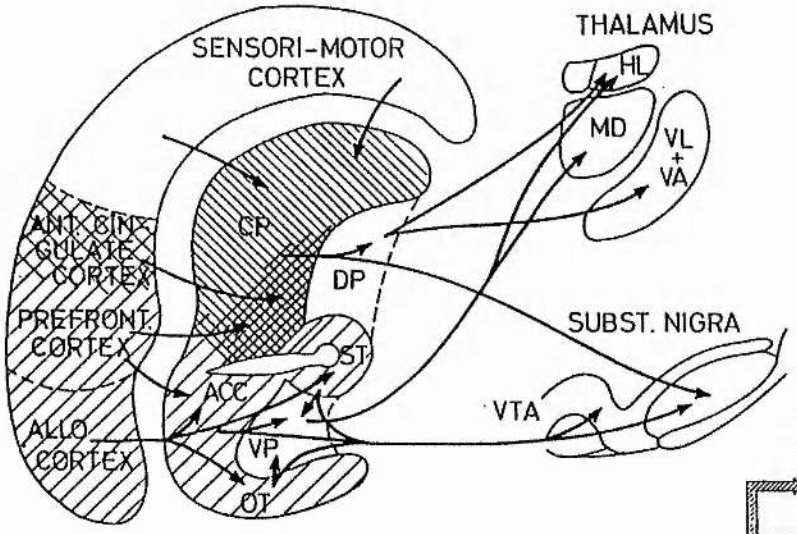
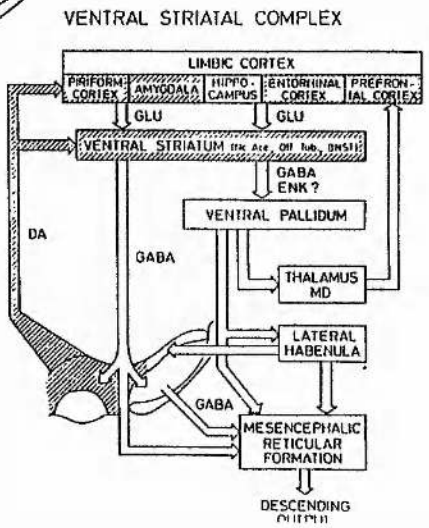

Projection from substantia nigra proper
Projection from A 10 (VTA)
Afferents from neocortex
Afferents from prefrontal cortex and amygdala
Afferents from hippocampus via fimbria - fornix
Afferents from entorhinal and perirhinal cortex
Afferents from piriform cortex

FIGURE 21. A. Anatomical relationship between cerebral cortex, striatal complex, thalamus, and ventral mesencephalon. Left-tilted hatching - areas innervated by substatia nigra; right-tilted hatching - areas innervated by DA neurons of A10 cell group.

B. Some major pathways and transmitter characteristics related to DA projection from VTA and medial substatia nigr to ventral part of striatal complex and to limbic cortex.

Both figures from Bjorklund and Lindvall (1986).


THALAMUS
SENSORI-MOTOR CORTEX
HL
MD
VL + VA
ANT. CINGULATE CORTEX
CP
DP
PREFRONT. CORTEX
SUBST. NIGRA
ST
ACC
VTA
ALLO CORTEX
VP
OT


VENTRAL STRIATAL COMPLEX
LIMBIC CORTEX
PIRIFORM CORTEX
AMYGDALA
HIPPO-CAMPUS
ENTORHINAL CORTEX
PREFRONTAL CORTEX
GLU
GLU
VENTRAL STRIATUM
GABA ENK?
VENTRAL PALLIDUM
DA
GABA
THALAMUS MD
LATERAL HABENULA
GABA
MESENCEPHALIC RETICULAR FORMATION
DESCENDING

predominantly to the caudate/putamen. Reciprocal connections occur with the dorsal striatum projecting to the substantia nigra, and the ventral striatum projecting to the VTA. However, as mentioned above the nucleus accumbens also projects to the substantia nigra which thus seems to represent a convergence point for outflow from both dorsal and ventral striatal complexes. Recent immuno-histochemical studies suggest that a second transmitter substance, cholecystokinin is co-localised in the same neurons as DA in elements of the mesolimbic pathway of the rat (Hokfelt et al. 1980).

(ii) Limbic and non-limbic organisation.

The functional organisation of striatal circuitary is thought to be regionally dissociated into two parts. The ventral or 'limbic' region mediates the involvement of the striatum in affective behaviour. The dorsal 'nonlimbic' region is involved in sensorimotor function. The organisation of striatal input and output systems supports this view. The dorsal striatum receives neocortical inputs from sensorimotor areas and projects to the dorsal pallidum and substantia nigra. The ventral striatum receives 'limbic' inputs from amygdala and allocortex and projects to ventral pallidum and lateral hypothalamic and pre-optic areas. These systems interact by the convergence of output pathways to the thalamus, and midbrain tegmentum as well as the ventral striatal output to substantia nigra which provides the

nigro-striatal 'feedback' loop to the dorsal striatum (Gerfen 1984).

Behavioural Manipulation

Attempts to define the functions of the ventral striatum in behavioural terms have generally taken one of two forms. Either the lesioning of this area or the administration of pharmacological compounds by direct injection. The lesion studies themselves can be sub-divided into two categories: i) non-selective lesions of ventral striatum, ii) 6-OHDA lesions of the monoaminergic innervation of the ventral striatum. Certain studies have included both lesion and pharmacological components. A brief review of some of these studies, predominantly those that are concerned with locomotor behaviour, is presented below.

6-OHDA lesion

Ipsilateral rotation can be generated in rats by unilateral lesion of the ascending nigro-striatal DA pathway, followed by injection with amphetamine and contra-lateral rotation by injection with L-dopa. These drug induced rotational behaviours can be modified by changing activity in the mesolimbic DA system, such as 6-OHDA lesion of the nucleus accumbens. Thus demonstrating the possibility that both mesolimbic and nigro-striatal DA systems are involved in the control of motor behaviour (Kelley and Moore, 1976). More

specifically Fink and Smith (1980a) demonstrated that 6-OHDA lesions of the mesolimbic DA system impair normal exploratory behaviour in rats. This impairment was largely corrected by intra-peritoneal injection of the direct DA agonist apomorphine. The deficits induced by such lesions did not appear to be the result of global motor deficits or changes in emotionality (Fink and Smith, 1979). The restoration of exploratory behaviour was not the result of increased general activity induced by apomorphine and could itself be antagonised by administration of the DA antagonist pimozide (Fink and Smith, 1980b). Furthermore, the increase in locomotion generated by intra-peritoneal administration of 1.5 mg/kg d-amphetamine in the rat, was completely abolished by DA depletion of the ventral striatum. They state "*that we were able to produce an abolition of the amphetamine -induced locomotion by severe denervation that was restricted to the mesolimbicocortical DA system and to adjacent medial and ventral striatal terminal fields is decisive evidence that these terminal fields were necessary for this response*" (Fink and Smith, 1980c p.122). However, it is important to note that denervation of the nucleus accumbens alone is not sufficient for a large decrease in the locomotor response to this dose of d-amphetamine (Fink and Smith 1980c). Furthermore, the lesions made by Fink and Smith which they claim to be selective for the DA innervation of the ventral striatum have been criticised as being too extensive and

causing damage in the caudate/putamen. When selective 6-OHDA lesions of the DA terminal fields in the nucleus accumbens alone were examined exploratory deficits were not observed, and the range and extent of locomotor and drug induced behavioural deficits was greatly reduced (Winn and Robbins 1985).

Neurotoxin lesions

Kainic acid lesions restricted to the dorsal striatum and which left the ventral striatum intact, increased the locomotor response to amphetamine. This indicated that the neuronal elements of the ventral striatum were capable of mediating amphetamine induced locomotion (Mason et al. 1978).

Electrolytic lesions

Small electrolytic lesions confined to the ventral striatum result in animals showing a decrease in d-amphetamine induced locomotion. This is consistent with the suggestion that ventral striatal DA innervation may be more important to amphetamine-induced locomotion than dorsal striatal DA innervation (Neill et al. 1974).

Overall the lesion studies have demonstrated that the ventral striatum may have a role in the control of some aspects of locomotor and exploratory behaviour. However it is most important to note that the majority of 'lesion' studies of the

ventral striatum are NOT lesions of generalised striatal cells or cells + fibres but are in fact lesions of ascending monoaminergic projections. Whilst DA is clearly an important transmitter in this region, the complexity of striatal organisation implies it has a variety of processing tasks to perform and involves a number of different transmitter systems. Thus demonstrations of the effects of manipulation of DA levels show only part of the functions of this region.

Micro-injection studies

The direct injection of pharmacological agents into the CNS is a potent means of establishing behavioural or physiological consequences dependent upon transmitter action in discrete sites. In parallel with the lesion studies, microinjection of compounds into the ventral striatum appears to have been mainly directed at understanding the actions of the monoamine transmitters, particularly DA, at this site. Pijneburg et al.(1973) established that the micro-injection of ergometrine, a potent DA agonist, into the cerebral ventricles induced strong and long-lasting locomotor stimulation. The most potent locus of this behaviour was found to be the nucleus accumbens and these actions could be inhibited by subsequent doses of DA antagonists. Recent studies by Alexander Cools have suggested that whilst locomotor activity is clearly stimulated by injection of DA agonists into the ventral striatum, this activity is from DA actions in the olfactory

tubercle and not the nucleus accumbens (Cools 1985,1986). Interestingly, Pijneburg et al. (1976) suggested that ventral striatal injection of NA also elicited locomotor activity but that this was less pronounced than that elicited by DA. They suggested the possibility that NA elicited locomotion was dependent upon the integrity of the meso-limbic DA system. In animals with DA depleting 6-OHDA lesions of the ventral striatum injection of NA fails to produce an increase in locomotor activity.

As suggested at the beginning of this chapter, in order to confirm the hypothesis about NA action and extra-synaptic α-2 receptors it is necessary to examine more than one site and one behaviour. Pijneburg et al. (1976) state that the injection of NA into the ventral striatum of nialamide pre-treated rats causes an increase in locomotor activity. The ability to demonstrate an effect similar to that found in PVN but at a different site and with a different behaviour would add considerable weight to the suggestion that the effects demonstrated within the PVN were generalisable rather than site specific. Thus the hypothesis for the actions of NA in ventral striatum is; micro-injected NA in ventral striatum produces an increase in locomotor activity. This activity can be blocked by phentolamine and idazoxan but not prazosin. Blockade of NA re-uptake systems does not affect blockade by phentolamine but does affect blockade by idazoxan.

The proposed experiments were as follows:

(i) Micro-inject 40nmoles NA bi-laterally into the ventral striatum of rats and record subsequent locomotor activity.

(ii) If injection of NA at this site resulted in an increase in locomotion, attempt to block this with antagonists to NA α- receptors.

(iii) If such blockade could be established, the receptor apparently mediating this response could be identified. Were this to be of the α-2 sub-type pharmacological manipulations similar to those performed in PVN could be performed.

EXPERIMENT 6

METHOD

Animals

A total of 32 male Hooded Lister rats were used. These animals had been bred 'in house' and weighed approx. 450 gms at the time of surgery. The animals were maintained 'ad lib' on SDS maintenance diet no.1 lab chow and water, and kept under a 12 hour light/dark cycle.

Surgery

All the animals were bilaterally implanted with two permanent, 23 gauge stainless steel guide cannulae which were normally occluded by indwelling stylets (11.5mm in length). The surgical protocol was as previously described in experiment 1, except that the implants were bi-lateral, and the co-

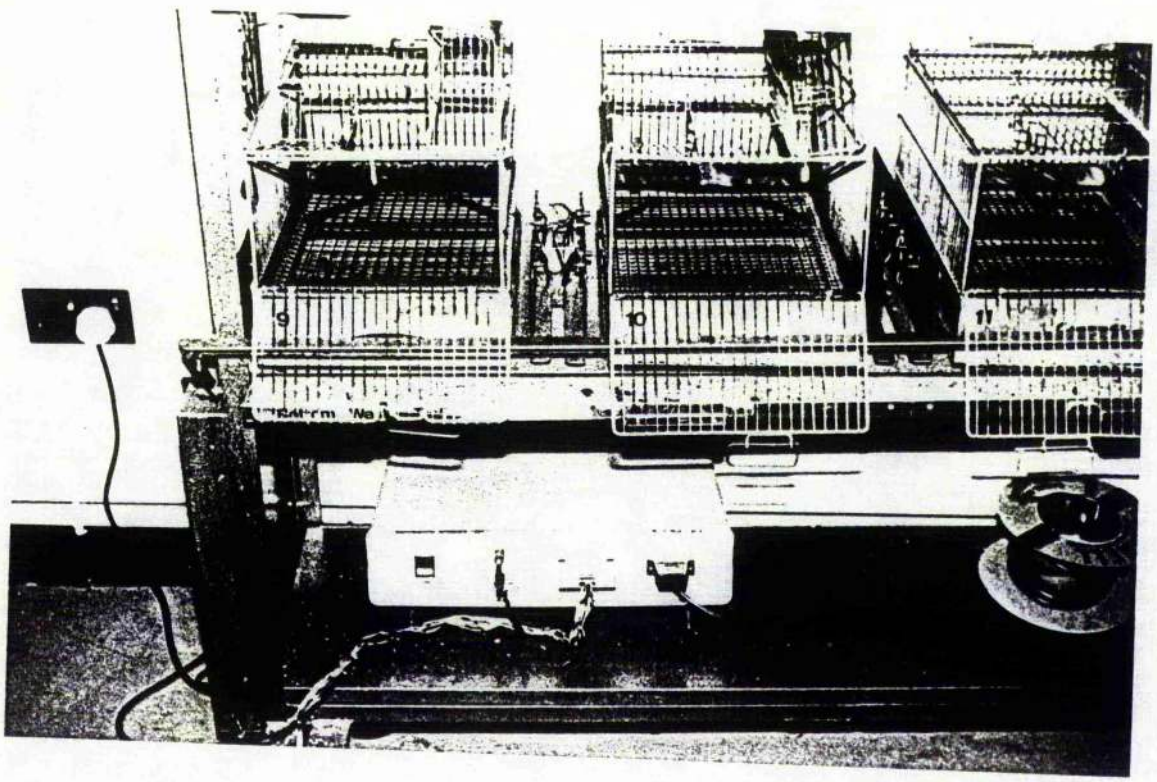
ordinates were changed. The co-ordinates used were according to the atlas of Pelligrino and Cushman; in the de Groot orientation: nose bar +5mm above the interaural line; 3.4 mm anterior to Bregma; 1.5mm lateral to the midline; and 6.0mm below dura. These co-ordinates resulted in injection sites in the ventral striatum.

General Test Procedure

Following at least 3 days recovery from surgery, rats were acclimatised to the test environment. This consisted of an array of 12 wire sided cages each measuring 25cm x 39cm x 18cm, see figure 22. The room containing this array of cages was maintained under red light.Each cage contained four photoelectric cells, arranged in pairs, just above the floor of the cage. These were connected to a BBC microcomputer via a Paul Frey 'Spider' interface, Figure 21. Beam breaks were counted and saved to disc with the computer software filtering out repeated breaks of only one beam. The resulting counts were a reliable measure of locomotion. All animals were acclimatised for at least 7 days prior to drug injection. Stylets were changed each day and the animal handled with the infusion pump running to simulate the injection protocol.

In each test session, the animals to be tested were placed in the test cages for 1 hour prior to injection. They were then returned to their home cages before being microinjected

FIGURE 22. Photograph showing three of the locomotor activity cages and the SPIDER interface.


9
10
11

individually and then placed immediately into the test cage. Each test ran for 100 minutes and all tests were separated by a minimum of 24 hours.

Drug Injections

The drug injection procedure was similar to that previously described for experiment 1. The difference being that the infusions were, in this case, bi-lateral, and of 1ul over 2 minutes. Injection of antagonist was performed 30 minutes prior to NA with the animal being returned to the home cage in between. Injection of cocaine was performed immediately prior to the injection of NA. All the drugs used were administered in a standard dose of 40nM\ul, and all were made up in physiological saline, (for details see expt. 1).

Histology

In order to confirm the cannula placements the animals were sacrificed under barbiturate anaesthesia and perfused intra-cardially with 0.9% physiological saline and 10% phosphate buffered formalin. Sections were cut and stained as described for experiment 1. All animals presented in the analysis had bi-lateral injection sites within the ventral striatum. Table 3a. shows the bi-lateral positioning for each animal.

Pilot Study

This study was intended to determine whether injection of 40nM of NA into the ventral striatum would induce an increase in locomotion. The literature is unclear on the effects of such an infusion with Pijnenburg et al. (1976) finding no effect but Cools (1986) claiming a significant increase in locomotion. This experiment used 8 animals and doses of 20, 40, and 80nM/ul. Rats were split into 2 groups and injections were counterbalanced as shown below:

	DAY1	DAY3	DAY5	DAY7
Group (A)	80nM	Saline	40nM	20nM
Group (b)	40nM	80nM	Saline	20nM

RESULTS

The results of this experiment are presented in figure 23. There is clearly an increase in locomotion as a result of injection of NA. However this increase is not easily linked to the dose of NA administered. It appears that an important factor in the size of the response to NA injection is whether or not there had been a previous infusion of NA. Second or more subsequent injections of NA produce a greater response than that produced to the same dose as a first injection. Histological examination of the injection sites did not reveal a clear answer. There is some indication that the deeper the

injection the greater effect of NA, locations of the sites are presented in Table 3b.

EXPERIMENTAL PROCEDURE

The main experiment involved the use of 24 rats which were split into two groups, A and B in order to examine a wide range of drug manipulations without increasing the number of injections per animal. The injections given to each animal are presented in table 4. Procedures are summarised as follows.

(i) All rats from both groups were given bi-lateral NA (40nm in 1ul).

(ii) All rats from both groups were given bi-lateral injections of both 40nm NA or vehicle (saline) separated by 48 hours. The order of injection was counterbalanced both between and within groups.

(iii) Group B rats only (N=10) received injections of phentolamine/NA and phentolamine/saline. Injections were separated by 48 hours and presented in a counterbalanced order.

(iv) Group A rats only (N=8) received injections of cocaine/NA and cocaine/saline. Injections were presented in a counterbalanced order and separated by 48 hours.

(v) Group B rats received injections of idazoxan/NA or idazoxan/saline. Again injections were separated by 48 hours and counterbalanced.

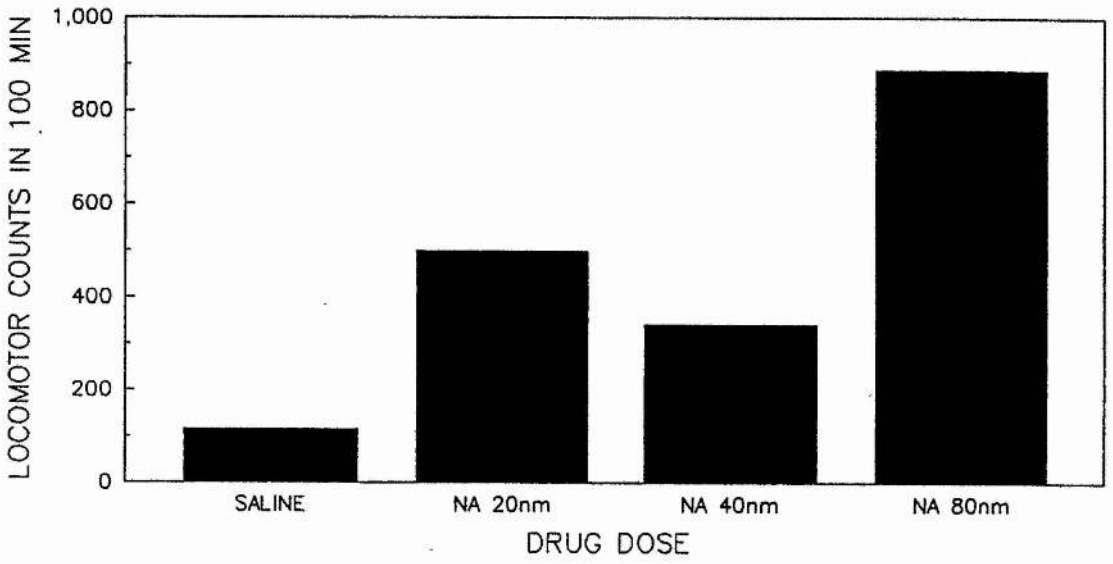
(vi) All rats (N=18) received microinjections of idazoxan/ cocaine/NA.

RESULTS

The pilot study had demonstrated that microinjection of NA, at a dose of 40nm in 1ul, into the ventral striatum produced an increase in locomotor activity. There was also the suggestion that a 'priming' effect; that is subsequent injection of an equal dose of NA being significantly more potent at eliciting this behaviour than the initial dose; occurred following injection of NA into the ventral striatum. The data for this study is presented in figure 24 a. showing the dose response curve and the modified curve if an allowance is made for the priming dose.

The suggestion that there was a priming effect was further examined in the main experiment. The injection of 40nm NA into the ventral striatum elicited an increase in locomotion when compared with saline ($F=5.07$, $df=17$, $p<0.05$). The effect of a second dose of 40nm NA administered 48 hours after the initial or priming dose was significantly greater than saline ($F=14.94$, $df=17$, $p<0.002$) and also significantly greater than the effects of the first dose of NA ($F=9.74$, $df=17$, $p<0.05$). However this priming effect is clearly complex, in both groups A and B whilst the majority of animals showed an increase following the second injection, one or two animals showed a

NA DOSE RESPONSE
PILOT STUDY



NA DOSE RESPONSE
PILOT STUDY
'PRIMED RESPONSES ONLY'

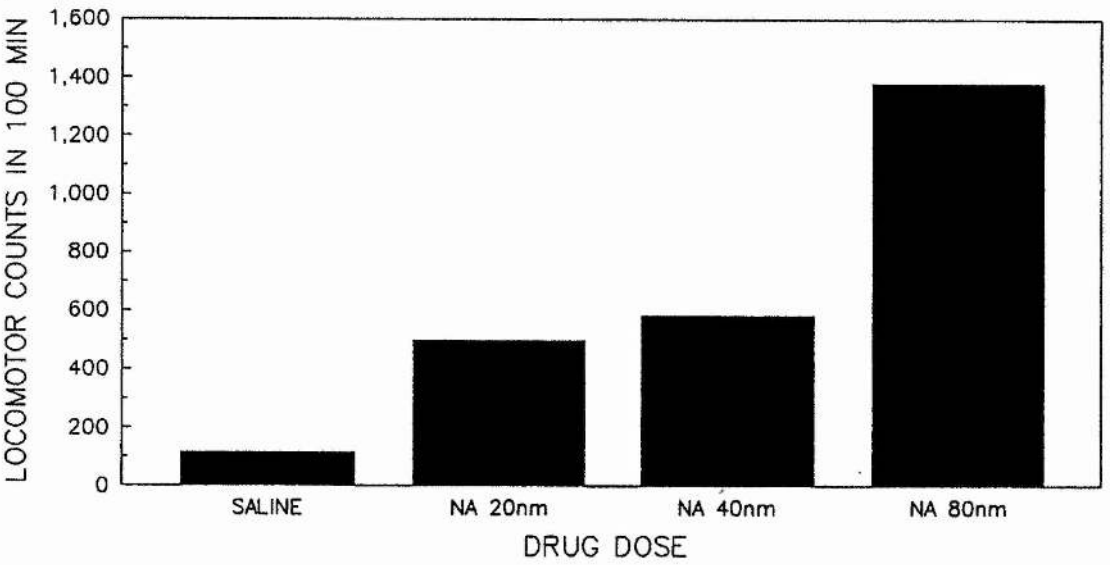
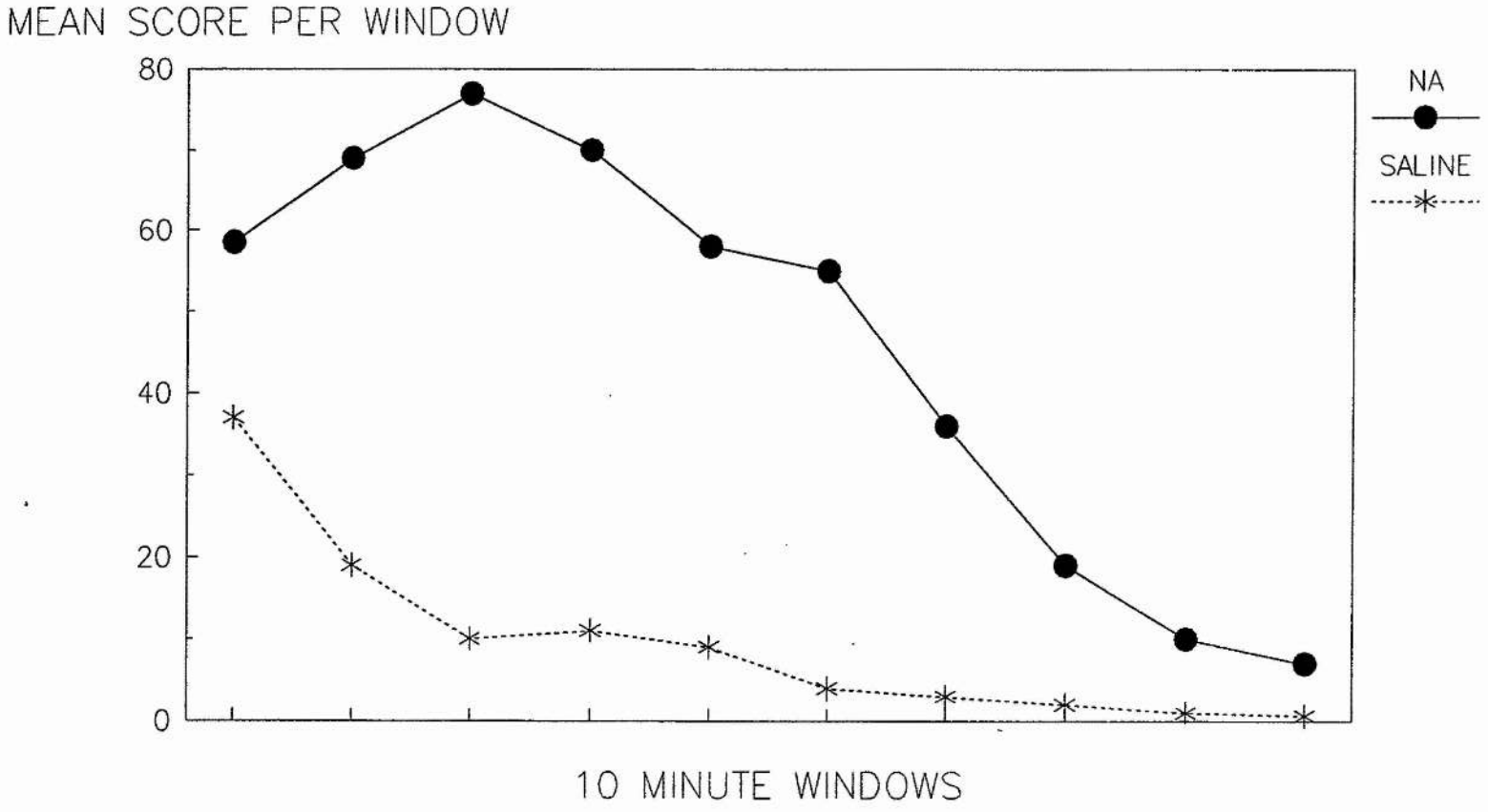


FIGURE 24. Graph showing enhanced locomotion over 100 mins. as a result of NA microinjection into ventral striatum. Data shown represents the second dose of 40nm NA for group B. The increase in activity was significant ($p<0.002$, $F=14.9$)

VENTRAL STRIATAL MICROINJECTION OF NA

NA PRIMED VS SALINE

GROUP B



markedly reversed response. For this reason the complete data is presented in table 5. Figure 24 b. illustrates the 'primed' NA response compared with saline and the initial NA response combined over both groups.

Phentolamine blockade.

The combined α-1, α-2 antagonist phentolamine did not reduce the locomotor stimulant effect of NA. Phentolamine and NA is not significantly different to NA alone ($F=0.413$, $df=9$, $p>0.05$). Phentolamine and saline does not differ significantly from saline alone ($F=0.68$, $df=9$, $p>0.05$). Inspection of the data indicates that, as with the NA priming effects, the majority of the animals show an effect in one direction whilst one or two rats respond strongly the other way. The reasons for this are unclear.

Re-uptake inhibition with cocaine.

The combination of cocaine and NA did not differ significantly from NA alone ($F=1.76$, $df=7$, $p>0.05$). However cocaine alone did differ significantly from saline ($F=6.7$, $df=7$, $p<0.05$). This may have been the result of cocaine actions at NA or other monoamine sites in ventral striatum and will be considered further in the discussion.

Idazoxan blockade.

FIGURE 25. Graph showing the priming effect of a second dose of 40nm NA for all rats. The significance levels for these data are reported in the text.

VENTRAL STRIATAL MICROINJECTION OF NA

PRIMING AND SECOND NA AND SALINE

GROUPS A AND B COMBINED

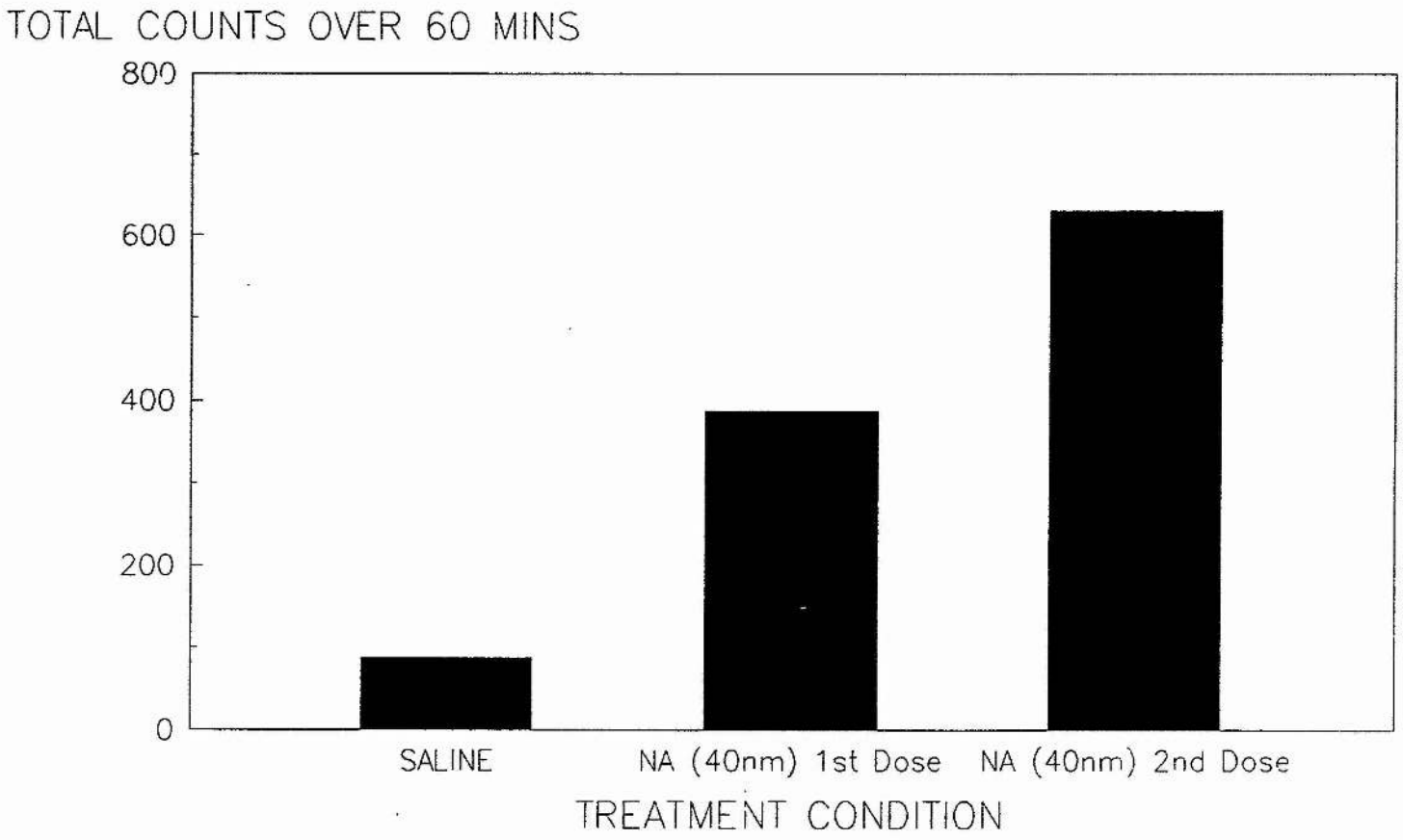
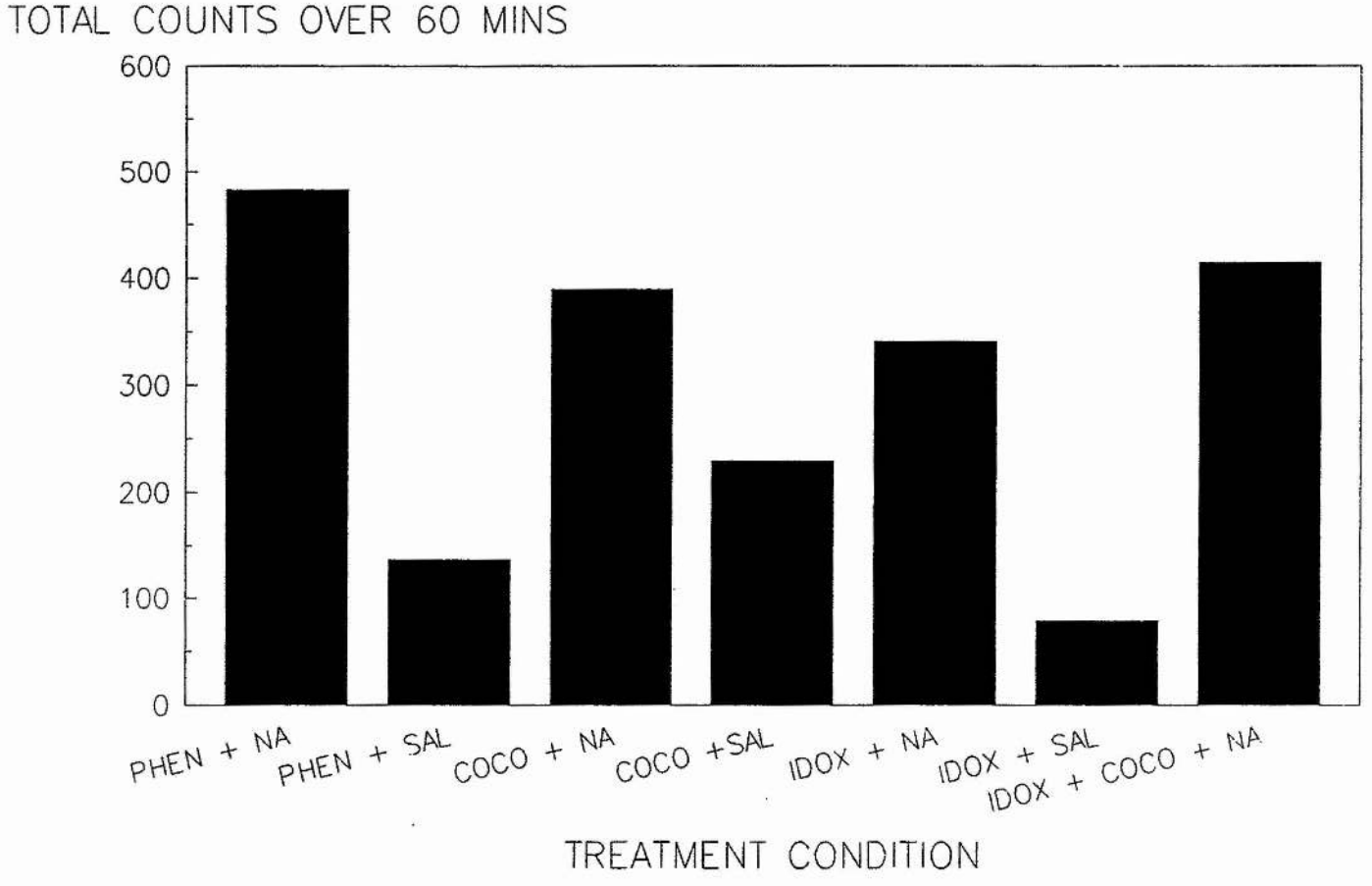


FIGURE 26. Graph showing the effects of NA α- antagonists, and cocaine on the locomotor response to microinjection of 40nm NA into ventral striatum. The significance levels for these data are reported in the text.

VENTRAL STRIATAL MICROINJECTION OF NA

BLOCKADE EXPERIMENT

GROUPS A AND B COMBINED



In the light of the fact that phentolamine produced no significant blockade of NA induced locomotion, it was not expected that the α-2 selective antagonist idazoxan would produce blockade. No significant blockade was established ($F=0.728$, $df=9$, $p>0.05$). Once again two rats showed an opposite trend to the other eight, and interestingly it was the same two animals as with phentolamine. One of these rats had cannulae ending in the pallidum, obviously incorrectly placed. However, the other had bi-lateral cannulae sited in ventral striatum. The results are not simply the result of incorrect cannula placing.

Combined cocaine, idazoxan and NA injection.

As the idazoxan experiment produced no significant blockade the microinjection of cocaine in addition to idazoxan could not show attenuation of blockade. This combined injection did not differ significantly from NA alone ($F=3.77$, $df=7$, $p>0.05$).

The results of these experiments are discussed further below (p159) however it is worth noting that whilst the mechanism by which it acts is unclear, microinjection of NA into the ventral striatum produces a significant increase in locomotion.

TABLE 3a

Table for pilot study animals showing number of counts in locomotor cages over 60 min. following injection of NA. Site refers to the areas shown histologically to be affected by the injections.

Rat No.	NA 20ul	NA 40ul	NA 80ul	Site
1	313	708	---	NAS + OT
2	102	151	259	NAS
4	177	219	111	NAS
5	1019	1121	335	NAS
7	263	70	1377	NAS + OT
8	636	288	938	NAS + OT
9	740	78	1334	NAS
11	743	105	1871	NAS + OT

TABLE 3b

Table showing locations of injection site damage in animals used in main experimental groups.

Rat No.	LHS.	RHS.	
41	NAS	NAS	
43	NAS + OT	NAS + OT	
44	NAS + OT	NAS + OT	
45	NAS + OT	NAS + OT	
46	NAS	NAS	
47	NAS	NAS + OT	
48	NAS	NAS	
49	NAS + OT	NAS + OT	
50	NAS + OT	NAS + OT	
51	PALLIDUM	PALLIDUM	DISCARDED
52	NAS + OT	NAS + OT	
53	OT	NAS + OT	
57	NAS	NAS + OT	
58	NAS + OT	NAS	
59	NAS + OT	NAS + OT	
60	NAS + OT	NAS + OT	

TABLE 4

Data as total counts in 100 min. trial from main experimental groups A and B.

Group A.

Rat No.	Cond. 1	2	3	4	5	6	7	8	9	10
38/3	88	211	155			91	71			73
39/3	141	598	37			266	34			286
41/3	197	571	68			463	310			498
43/3	162	83	62			190	546			317
44/3	2584	2361	176			482	39			846
45/3	232	420	29			744	341			312
46/3	690	1879	89			214	138			475
47/3	88	751	158			666	356			510

Group B.

48/3	136	480	60	326	36			288	88
49/3	120	176	18	13	20			262	33
50/3	265	633	43	383	51			406	65
51/3	461	244	232	1091	895			534	51
52/3	56	184	62	60	32			59	114
53/3	266	443	92	1523	7			718	33
57/3	184	492	73	277	175			228	78
58/3	800	1160	50	477	42			110	98
59/3	100	98	22	158	25			74	71
60/3	400	552	148	526	85			732	159

Conditions: 1= first dose of NA, 2= second dose of NA, 3= saline, 4= phentolamine + NA, 5= phentolamine + saline, 6= cocaine + NA, 7= cocaine + saline, 8= idazoxan + NA, 9= idazoxan + saline, 10= idazoxan + cocaine + NA.

EXPERIMENT 7

This experiment was designed to examine the possibility of making a selective cell specific neurotoxic lesion of the parvocellular portion of the PVN. A variety of supposedly 'fibre sparing' neurotoxic agents are available and are becoming widely used tools in neuroscience. These agents, in particular ibotenic acid (IBO) and n-methyl-d-aspartic acid (NMDA) are reported to cause death of cell bodies without damage to fibres 'en passant'. It is known that these toxins have their effect through receptors for excitatory amino acids (EAA's) although the exact classification of which toxin acts through which receptor is as yet unclear (Winn et al. in preparation). One frequently noted observation made regarding the use of certain excitotoxins is that magnocellular neurons are reliably and frequently spared in areas of otherwise complete cell death. One reason suggested for this is that magnocellular neurons may lack receptors for EAA's and thus remain unaffected by these toxins.

In the light of this observation it was considered a possibility that a neurotoxic lesion of the PVN might result in destruction of the parvocellular portion without causing significant damage to the magnocellular component. This would permit an examination of the contribution of the separate components of the PVN to the feeding behaviour elicited by microinjection of NA. Such a parvocellular depleting lesion

has been obtained in the Syrian hamster (Hastings; pers. comm.) although the shape of the PVN differs slightly in this animal.

METHOD

Animals

A total of 10 male hooded Lister rats, bred 'in house', were used each weighing approximately 370gms at the beginning of the experiment. The rats were individually housed and kept under a 12hr light/dark cycle. The were maintained 'ad lib' on SDS maintenance diet No.1 chow pellets and tap water.

Surgery.

The rats were anaesthetised with avertin and mounted in a Kopf stereotaxic frame. The surface of the skull was exposed and the location of bregma recorded. The co-ordinates were according to the atlas of Paxinos and Watson (1982): flat skull position, nose bar 3.3mm below the interaural line, 1.8mm posterior to bregma, lateral 0.2mm, and 8.5mm below the skull surface. A hole was drilled in the skull such that the 30gauge injection cannulae could be lowered bi-laterally.

Injection protocol.

The injection was in principle very similar to that described for microinjections in previous experiments. Two 15mm long, 30gauge injection cannulae were mounted on the stereotaxic

frame. These were assembled and connected to the infusion pump in the manner previously described. Once the surface of the brain was exposed these cannulae were stereotaxically lowered into place and infusion of the neurotoxin commenced.

Results

Several concentrations of both ibotenic acid and NMDA were used: Ibotenic 0.12M, 0.06M, and 0.03M and NMDA 0.12M, 0.06M and 0.03M (all injections in 0.5ul). Within approximately 30 seconds of the commencement of infusions of either neurotoxin at either 0.06 or 0.12M, the rat began to hyperventilate and died in less than 1 minute even if the infusion was immediately stopped. At the concentration of 0.03M both toxins still resulted in hyperventilation but only ibotenic acid caused death. Unfortunately, whilst this dose of NMDA did not cause death, no lesion was found when the brains were examined histologically 14 days post-operatively. This experiment was not pursued further following this discovery.blockade (NA vs NA / phentolamine / cocaine p=0.33).

DISCUSSION

Tail Pinch.

This experiment was concerned with the possibility that some component of tail pinch induced eating is mediated via a noradrenergic mechanism within the PVN. This hypothesis was based upon the evidence that the PVN has a major influence over the endocrine responses to stress (Bassett 1984) and that the microinjection of NA into PVN elicits feeding in the satiated rat (Leibowitz 1973,1975a,b,1978a). Furthermore Robbins et al. (1981) selectively lesioned DNAB with 6-OHDA and demonstrated an impaired response to TP.

In this laboratory, applying a pinch to rats' tails reliably elicited eating in satiated animals. Once such eating was established the latency of onset remained stable. This appears to be in contradiction with Antelman et al. (1975) who reported a steady decrease in latency with repeated trials, a feature which led both they and Koob et al. (1976) to postulate that TP is a learned response. However, Judith Clark (Pers. Comm. unpublished) has reported that at some pinch pressures rats with lesions of the lateral hypothalamus responded to TP on the first trial. The fact that a first trial response was possible, albeit in animals with CNS manipulations, suggested that direct physiological or psychological factors might induce this behaviour in circumstances where learning had not yet occured.

The level of pinch pressure was found to be important. No responses were generated by pinch pressures of less than 10 psi. and (unquantified) behavioural observation suggested a pressure of 5 psi. to have no effect different from the habituation conditions of having the cuff in place but no pressure applied. However, in contrast to the reports of Antelman's group (Antelman and Rowland 1976, Antelman et al. 1975) the animals in this study were clearly aware of the cuff even if no pressure was applied. Frequent movements of the tail and cuff directed orienting and biting were observed even in the habituation stage. The effective pinch pressures for eliciting eating were found to be 10 or 15 psi. and the typical latency was around 60 sec. In agreement with other studies (Antelman et al. 1975, Koob et al. 1976) the eating bout finished exactly at the time pinch pressure was released. Animals were observed to allow food to fall from their mouths as the pinch ceased. The four animals which failed to eat at 15 psi. still did not eat when the pressure was increased to 20 psi. but they exhibited marked signs of distress; vocalisation, defecation and pinch directed biting; at these pressures.

As a result of their being two sets of independent criteria for these experiments the number of animals used in the central drug studies was reduced to six. Animals were

required to have bi-lateral cannulae either of which independently supported feeding to the microinjection of NA and also to eat to TP on three successive trials. The microinjection of NA antagonists into the PVN prior to TP revealed the eating elicited by TP to be maintained in the absence of NA activation of PVN sites. Noradrenergic antagonists had previously been administered systemically by Antelman et al. (1975) without producing a blockade to TP induced eating. However, central injections often differ in effect from peripheral injections and in the light of the evidence linking stress and feeding to the PVN such an examination was considered worthwhile. Phentolamine the combined alpha-antagonist, administered at a dose previously shown to abolish feeding to PVN NA injection did not affect TP induced eating. Neither did the beta-antagonist propranolol. That the injection of these antagonists did not affect even the latency of TP induced eating can be taken as evidence that TP is not mediated via the PVN.

In order to compare the action of phentolamine in the PVN on TP and another form of stress induced eating, a second stressor known to elicit eating, was examined. Microinjection of phentolamine into the PVN failed to antagonise eating resulting from the stress of a cold water swim.

Intra-peritoneal injection of the DA antagonist haloperidol, in a dose previously demonstrated not to inhibit normal feeding significantly attenuated the response to TP. Not one of the 10 animals injected with haloperidol ate to TP. This result replicates Antelman's (1974) finding.

In conclusion: TP induced eating is not attenuated by PVN microinjections of the NA antagonists phentolamine or propranolol, nor is the response altered by microinjection of NA. Thus despite the involvement of the PVN and NA both feeding and stress this system is not involved in TP induced eating.

<u>Noradrenergic transmission within the PVN.</u>

Injection of NA into the PVN of rats induced eating even though the animals were satiated. This feeding response was blocked by the administration of the alpha blocking agent phentolamine and by the selective α-2 antagonist idazoxan, although this blockade is not as great as that established by phentolamine. These effects replicate the work previously published by Leibowitz and her colleagues (Leibowitz 1973,1975a,b 1978b, Goldman 1985). In the doses used here, neither of these agents had independent actions on food consumption. The blockade established to idazoxan was greatly attenuated by pre-injection of the re-uptake blocking agent cocaine, whereas that established to phentolamine was

unaffected by this treatment. Injection of cocaine had no independent effect on food intake, nor did it affect the response elicited by NA. Similarly, lesion of NA terminals in the PVN by 6-OHDA had no effect on the eating elicited by microinjection of NA but significantly attenuated the ability of idazoxan to antagonise this effect of NA.

These data can be accommodated in an hypothesis which proposes that the α-1 and α-2 receptors differ in terms of their location on neuronal membranes. As discussed in the introduction the idea that post-synaptic receptor sub-types differ in location with regard to the synaptic specialisation is not new. It has previously been suggested in the periphery by Langer and his colleagues (Langer and Shepperson 1982, Langer et al. 1980a,b)and in the CNS by others (Lucchelli et al. 1985, Nieuwenhuys 1985). The suggestion here is that the α-2 receptor is predominantly extra-synaptic and more accessible because, at this site, it is the receptor for non-synaptically released NA and that α-1 and α-2 receptors may differ in location because the source and means of delivery of their ligand is different. This arrangement is illustrated in figure 27. According to this model the post-synaptic α-1 receptor lies proximal to pre-synaptic elements and is therefore surrounded by a relatively dense medium (Nieuwenhuys 1985) and associated with the re-uptake mechanism of the pre-synaptic element. The post-synaptic α-2 receptor may be

located at a distance from any synaptic bouton and be unprotected by medium or the re-uptake mechanism of a pre-synaptic element. Johnson et al. (1986) have presented electrophysiological evidence in support of a similar system involving DA receptors in the caudate. They suggest that D1 DA receptors are extra-synaptic and the D2 DA receptors are intra-synaptic. The implications of this suggestion will be discussed further below.

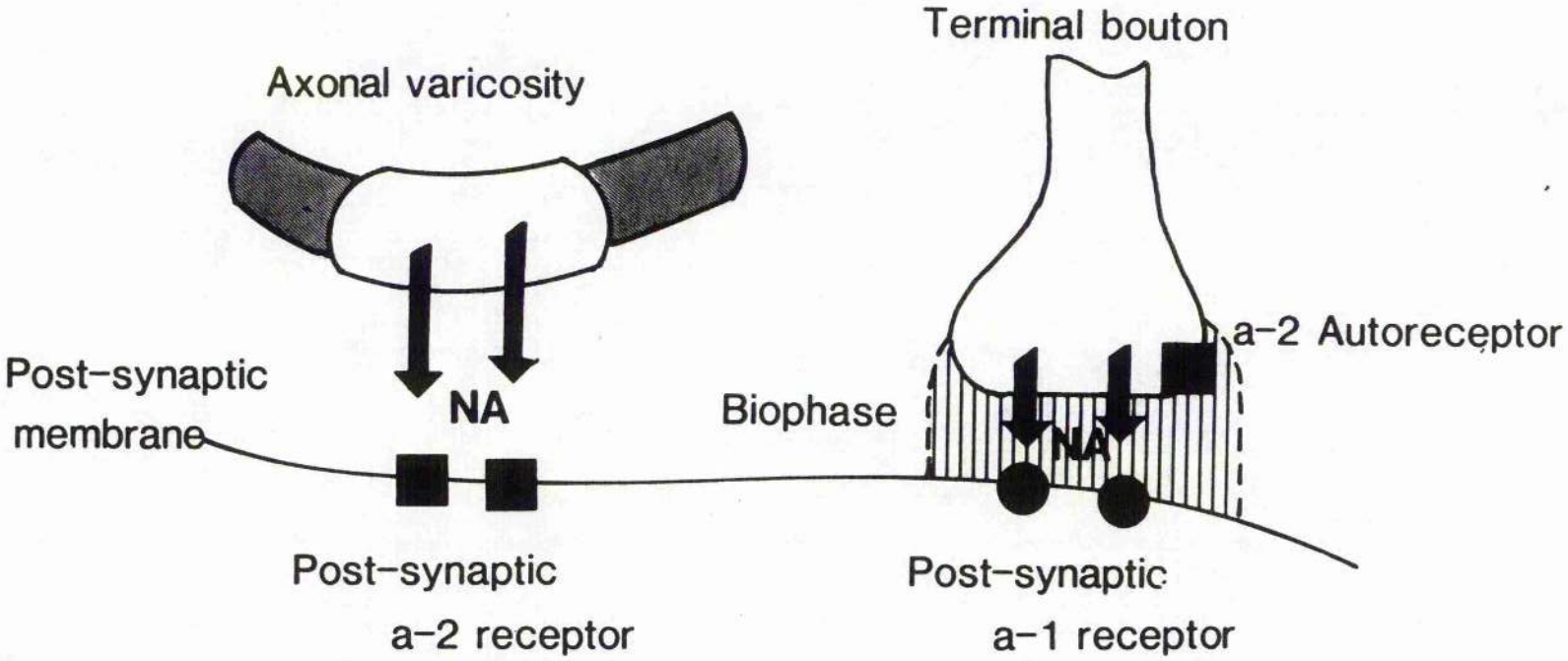
The present data might be incorporated in the hypothesis as follows:

(i) Microinjected NA is largely denied access to the α-1 receptor by the powerful local re-uptake mechanism and therefore acts preferentially on the α-2 receptor.

(ii) Idazoxan is a very effective blocker of the eating induced by NA (though not as effective as phentolamine) because, with the re-uptake mechanism intact, exogenous NA has little opportunity to act through the α-1 receptor. Prazosin is ineffective because it blocks a receptor to which the microinjected NA does not have sufficient access.

(iii) When the re-uptake mechanism is blocked by cocaine the α-1 receptor is 'exposed' and exogenous NA can now act at both α-1 and α-2 making idazoxan much less effective. Phentolamine, which blocks both α-1 and α-2 receptors, continues to exert an almost total blockade of the effects of exogenous NA.

FIGURE 27. Diagramatic representation of the difference in location of post-synaptic α-1 and α-2 receptors. The biophase for the α-1 receptor differs from that for the α-2 because of the presence of the pre-synaptic terminal bouton withits associated re-uptake mechanism. Thus, NA released from axonal varicosities or exogenous NA microinjected in this general region will find the α-2 receptor more readily accessible. Inhibition of re-uptake or removal of pre-synaptic terminal boutons renders both α-1 and α-2 receptors equally accessible.


Terminal bouton
Axonal varicosity
a-2 Autoreceptor
Post-synaptic
membrane
NA
Biophase
NA
Post-synaptic
a-2 receptor
Post-synaptic
a-1 receptor

(iv) Removal of the pre-synaptic terminal with 6-OHDA does not affect the eating response to NA (Goldman et al. 1985, Marino et al. 1983). However, 6-OHDA lesion does significantly attenuate the ability of idazoxan to block responding to exogenous NA, just as re-uptake inhibition by cocaine did. Thus terminal removal and re-uptake inhibition have a functional equivalence in terms of selective blockade.

In order to explain the present data it is necessary to accept that both α-1 and α-2 have an equivalent or compatible function at the post-synaptic site. Stimulation of either type of receptor should generate the same response in terms of excitation or inhibition of the post-synaptic cell, even if the mechanism by which this is achieved differs. However, in contrast to the principle suggested by Eccles (1964) that a neurotransmitter released from a neurone has only one and the same effect on all the 'follower' cells, the co-existance of both excitatory and inhibitory receptors has been described (for review see Szabadi 1978). With regard to NA, it is known that the application of NA to central neurones by microelectrophoresis can evoke both excitatory and depressant responses (Krnjevic 1974). Beven et al. (1977) suggested that NA could stimulate both α- and ß- adrenoceptors on cortical neurones, and that the α- receptors mediate excitatory, and the ß- receptors mediate depressant responses. A similar organisation of receptors is an established property of smooth muscle cells where functionally antagonistic α- and ß-

receptors have been understood to exist for many years (for review see Furchgott 1972). The presence of antagonistic receptors on the same neuronal membrane has been suggested (Szabadi 1978) to create a degree of plasticity for synaptic transmission. However, Szabadi (1978 p.1895) further states that both receptors should occur within the same synapse, a suggestion that is hard to reconcile with present understanding. This point will be discussed further below (p.161).

Receptors may have a variety of descriptions depending on how they are classified: pharmacological (e.g. receptor binding studies), physiological (e.g. growth hormone responses to α-2 agonist infusion), or anatomical (e.g. pre-synaptic, post-synaptic).

Two groups of workers in the early seventies independently arrived at the conclusion that there were two sub-types of NA α-receptor. Starke et al. (1975) discovered that NA overflow (presumably a pre-synaptic response) and the contraction of smooth muscle (presumably post-synaptic) showed different rank orders of potency for the agonists phenylephrine, oxymetazoline and naphazoline. Similarly Langer (1974) demonstrated that the α-selective antagonist phenoxybenzamine was some 30-fold less potent at inhibiting smooth muscle contraction than it was in promoting NA overflow. Langer

categorised these two sub-types as α-1 and α-2 receptors. Initially these were considered anatomically discrete with the α-1 receptor occurring post-synaptically and the α-2 acting as a pre-synaptic autoreceptor. However, this description is simplistic and demonstrably not the case (see below).

One of the major techniques for the description of receptors in terms of : number, location, differentiation and characterisation is direct labelling of the receptors with a radioactively labelled ligand. This technique, known as direct radioligand labelling, involves the binding of a compound with high specific radioactivity (usually [3H]) to the receptor recognition site (and often other associated proteins). This technique has been widely used in the description of NA α-receptors. Unfortunately, whilst simple in theory such a technique has many pitfalls and has prompted Perry and U'Prichard (1984) to write the following 'caveat'. "*Because of the simplicity of the methodology, it has been widely used by investigators from all disciplines. Unfortunately, while subtle complexities of radioligand interactions with the α-receptor complex have been demonstrated, a lag in communication across disciplines has led, in some instances, to the less than optimal use and/or interpretation of radioligand binding assays. In many studies non-optimal preparation of tissue, improper selection of radioligand, blank to define specific binding, assay buffer,*

and/or other assay constituents (e.g. metal ions and nucleotides), as well as incomplete experimental design, have made published results from adrenergic radioligand binding studies difficult to interpret."

Receptor binding studies, such as those of Perry and U'Prichard (1984), have described a 'kinetic' model of α-1 and α-2 receptors. It is proposed that both α-1 and α-2 receptors exist in two (or possibly more) states; a high affinity state and a low affinity state and that these states are in equilibrium. In general it appears that α-agonists are selective for the high affinity state of the receptor whilst α-antagonists are more selective for the low affinity state. However, this is in many respects an oversimplification and each compound used should be verified in this regard.

Far from being relatively stable entities receptors are capable of significant changes in number and sensitivity. Changes have long been shown to occur after prolonged overstimulation, resulting in receptor loss (Cohen et al. 1982) and by prolonged alterations in receptor occupancy, such as occurs in dopamine receptor blockade. This results in a state equivalent to denervation supersensitivity (Clow et al. 1979). Such changes have most commonly been measured using receptor binding techniques and are referred to as 'up or down regulation'. However, studies performed on tissue cultures

demonstrate that rapid changes in responses to agonists and antagonists (minutes) can occur in receptors; these changes being referred to as 'desensitisation'. To complicate the issue still further, in some tissues adrenergic receptor number shows a circadian rhythm, high at night and lower during daylight; for ß-receptors in the pineal (Campbell et al. 1985) and α-2 receptors in PVN (Jhanwar-Uniyal et al. 1986). High during the day and low at night for α-2 adrenoceptors in the supraoptic nucleus (Jhanwar-Uniyal 1986). It is at present unclear whether or not these processes are related. For a complete review of circadian rhythms in mammalian neurotransmitter receptors see Wirz-Justice (1986). Desensitisation may occur by two processes. First, the receptor may migrate out of the membrane fraction into a 'light density fraction' this process being rapid and apparently reversible (Chuang and Costa (1979) , Harden (1983)). Second, the receptor may become uncoupled from its effector system, for example α- or ß-receptors may become uncoupled from their respective N proteins. This would manifest itself in binding studies as a change from high to low affinity state (Campbell et al. 1987). This leads to the problem for a behavioural scientist of whether or not receptors that are labelled in binding studies can be construed as being part of the functionally relevant receptor population. For example in the Jahnwar-Uniyal paper described above, total binding to [^{3}H]p-aminoclonidine ([^{3}H]PAC) was

measured in a variety of areas at set times during the light and dark cycle. However, as discussed above, this α-2 receptor agonist is probably selective for the high affinity state of the receptor complex, also it is not possible to determine the proportion of receptors bound which are of functional consequence, or even how many are pre- and how many post-synaptic. Thus in itself this data says little about the function of α-2 adrenoceptors at these sites.

Receptor/recognition sites that are labelled by radioligands represent only a single link in the transmitter - receptor - effector - cellular response chain. In some systems changes in total α-receptor binding have been observed, with no corresponding change in the proximal biochemical effect (inhibition of adenylate cyclase) (U'Prichard et al. 1982). In other systems, changes in the number of receptor binding sites and effector functioning are temporally dissociated (Su et al.1980). Thus to quote Perry and U'Prichard (1984) "*Functional changes in operationally defined, physiological α-receptors cannot always be inferred from the demonstration of changes in radioligand binding properties.*" Indeed certain studies now report that whilst finding significant levels of binding of antagonists to certain receptors this is not of functional significance. McKernan and Campbell (1986) state "*although phenoxybenzamine has partial inhibitory effects on α-2 adrenoceptors 'in vivo' it does not appear to alter those*

that are functionally active."(p.50). They explain that this lack of effect may be the result of irreversible blockade leading to the unmasking of 'spare' receptors already preformed in the membrane. Alternatively, receptors present inside the cell may be inserted into the membrane.

As stated above, in order to explain the current data it is necessary to assume that stimulation of both α-1 and α-2 post-synaptic receptors results in the postsynaptic cell responding in the same way, although the mechanisms involved may differ. There is considerable evidence in support of this assumption. The data cited by Szabadi (1978) indicates that there are receptors which produce opposite effects to stimulation by the same neurotransmitter.

Further to the classifications of receptors described above a number of receptor proteins have been isolated and characterised. These reports indicate that the structure and mechanism of receptors are interdependent (for review see Strange 1988). Receptors may be distinguished according to the speed of their response. Class 1 are fast responding whilst class 2 are much slower and longer acting. Examples of class 1 receptors are GABA, glutamate, glycine, and cholinergic nicotinic receptors. These receptors mediate very rapid (millisecond) alterations in the ionic distribution across the membrane. Class 1 receptors are linked directly

to an ion channel and there is no evidence for the involvement of G proteins or 2nd. messengers (Strange 1987). Class 2 receptors show slower responses (seconds) and have actions analogous to hormones at surface receptors. The monoamine transmitters are typical of this class. The receptor is coupled to a guanine nucleotide regulatory protein (G protein) acting as a transducer. The whole apparatus is associated with the membrane (Bourne 1986). The two best characterised effector systems are adenylate cyclase, and phosphatidylinositol biphosphate specific phospholipase C (Strange 1987). There are a variety of effector systems called into play in excitable cells many of which may be present in neurons, see figure 28. "*Rigid use of the terms neurotransmitter and modulator will hinder a sound insight into the continuity between transmitters and hormones*" (Fujita et al 1988 p.84).

A frequently cited objection to post-synaptic α-2 receptors having the same function as the α-1 is the evidence that post-synaptic α-2 are inhibitory whilst post-synaptic α-1 are excitatory (Aghajanian and Rogawski 1983). However this paper states that "*to date there has only been one single-cell electrophysiological study [Guyenet and Cabot (1981)] demonstrating the existance of functional post-synaptic a2-adrenoceptors in the CNS.*" Sympathetic pre-ganglionic neurons are inhibited by catecholamines acting via an α-

FIGURE 28

Messenger substances released from neurons may induce a variety of actions.

a. Changes in ion fluxes generate slow depolarisation in some cases. A slow depolarisation may sometimes develop into an action potential, which is propagated to the secretory portion and opens the voltage dependent Ca^{2+} channel.

b. Activation of the inisitol lipid cycle may induce various cellular responses, including the release of Ca^{2+} from the intracellular Ca^{2+} store sites and cell growth.

c. Potentiation of ion pumps requires ATP supply and is responsible for electrogenesis.

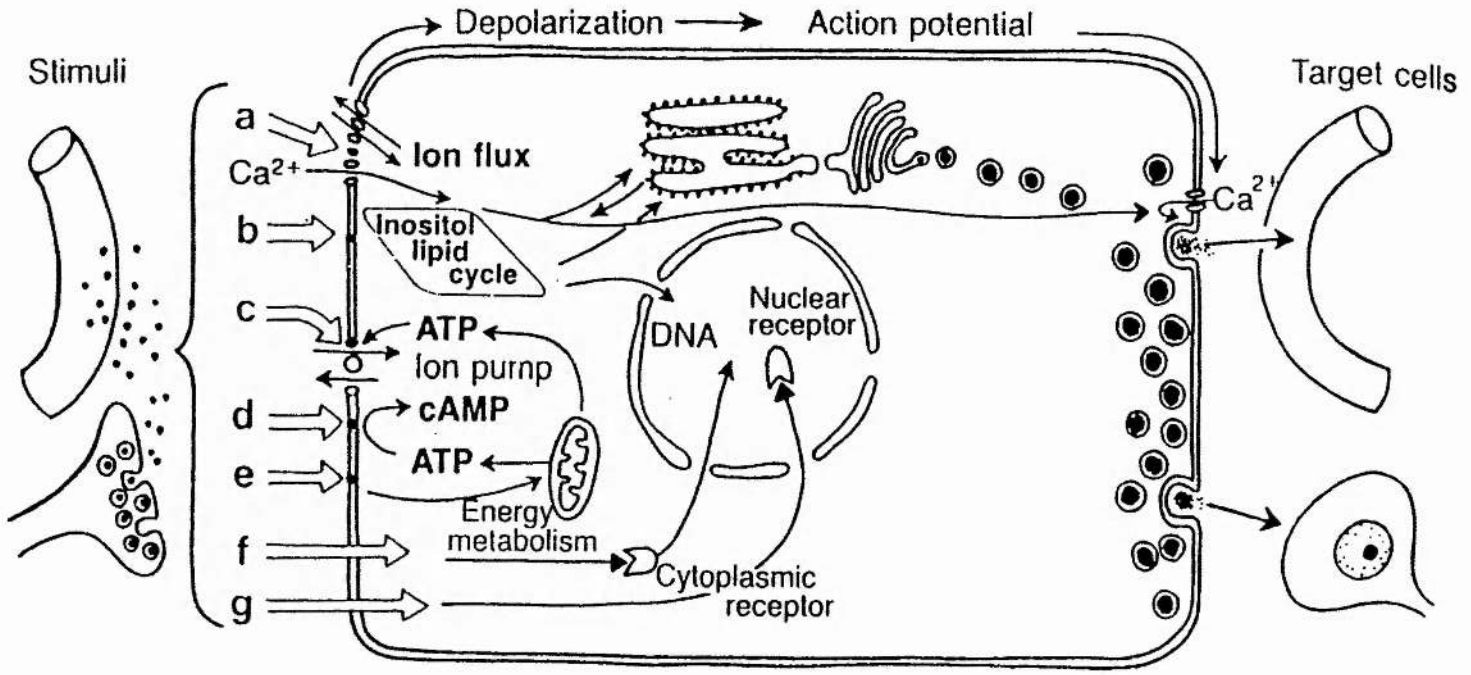
d. Activation of cyclic AMP formation triggers various cellular responses in concert with Ca^{2+} -dependent processes and turnover of the inisitol lipid cycle.

e. Activation of oxidative phosphorylation in mitochondria and the glycolysis system supplies ATP for various cellular responses.

f. Activation of cytoplasmic receptors alters the transcription of mRNA.

g. Action directly on the nucleus increasing the transcription of mRNA.

Figure from Fujita et al. (1988)


Depolarization
Action potential
Stimuli
Target cells
a
Ca2+
Ion flux
b
Inositol lipid cycle
c
ATP
Ion purnp
DNA
Nuclear receptor
d
cAMP
e
ATP
Energy metabolism
f
Cytoplasmic receptor
g
Ca2+

adrenoceptor which shows the same rank order of potency as do pre-synaptic α-receptors. From this single item of data regarding α-2 receptors the following conclusion was levelled "*α-1 adrenoceptors are located postsynaptically and mediate facilitation of excitation, α-2 adrenoceptors mediate inhibition both pre- and post-synaptically and ß-adrenoceptors mediate inhibition post-synaptically.*"

For NA it seems that it is the α- and ß- classes of receptor that have opposing effects, the present data is concerned with α-1 and α-2 receptors. The second messenger systems associated with adrenergic receptor sub-types have recently been presented by Strange (1988) and are reproduced below.

RECEPTOR	SUBTYPE	EFFECTOR
Adrenergic	α-1	PIP_2-PLC
	α-2	AC
	ß-1	AC
	ß-2	AC

Effectors: AC, adenylate cyclase; PIP_2-PLC, phosphatidylinositol biphosphate-specific phospholipase C.

Stimulation of ß-receptors serves to increase adenylate cyclase activity, whereas stimulation of α-2 receptors decreases adenylate cyclase activity and stimulation of α-1 increases PIP_2-PLC activity. Recent work by Gandhi and Ross (1987) has examined the function of α-adrenergic receptors on synaptic plasma membranes from rat brain cortex. They have indeed found that, with regard to the regulation of Ca^{2+}/Mg^{2+} -

ATPase in these membranes, that both α-1 and α-2 receptors have similar function.

In the light of Strange's (1988) data showing that ß-receptors increase adenylate cyclase activity whilst α-2 receptors decrease it, it is hard to see how both of these result in inhibition. It is possible to understand how the same transmitter substance released from two separate neurons with different origins might convey opposite information when released onto different receptor sub-types on the same post-synaptic cell. It is not easy to understand why the same transmitter released from a single pre-synaptic location should have simultaneously opposing effects within the same synapse.

	SYNAPTIC	NON-SYNAPTIC
Communication	One to one	One to many
Delay	Short (100-200 ms)	Long (>1 sec)
Action	Short and phasic	Long and tonic
Discrimination	Topographical organisation	Depends on presence of receptors
Chemical agent has to:	Cross the gap (5 - 20nm)	Diffuse: 20nm to several micrometers
Morphological characteristics	Axo-axonic synapse Post-junctional specialisation	Free nerve endings Axonal varicosities No post-junctional specialisation

Table adapted from Vizi (1984 p10).

The existance of α-2 receptors in both pre- and post-synaptic locations (the pre-synaptic receptor being an auto-receptor), does not affect this explanation of the data. The α-2 auto-receptor may be positioned within the synapse to monitor released NA, and as such should be protected from exogenous NA as the α-1 receptor is. It is unlikely to be involved in accounting for the present data, particularly as it is already known that the α-2 receptors which are involved in the feeding resulting from NA stimulation of the PVN are post-synaptic. The location of an auto-receptor within the synaptic cleft might be part of the explanation why cocaine does not itself elicit feeding. As re-uptake blockade causes the concentration of NA within the cleft to rise, the stimulation of pre-synaptic auto-receptors inhibits further release. Recent work in this laboratory has been unable to replicate the findings of Leibowitz's group that another NA re-uptake blocker, DMI elicits feeding. Despite initially promising pilot data, no significant increase in feeding has been elicited by microinjection of DMI (Winn; pers. comm.). One of the major drawbacks with the testing thus far performed on the proposed model was the failure to examine the effects of α-1 stimulation or blockade directly. The selective α-1 antagonists considered were prazosin and phenoxybenzamine. However prazosin was insufficiently soluble to allow equivalence of concentration and volume with doses of NA found to be effective, and phenoxybenzamine is irreversible and has

some action at α-2 receptors (McKernan and Campbell 1986). Furthermore it is hard to see any merit in the use of an α-1 antagonist in this preparation as the actions of NA at α-1 receptors are only expressed detectably in the presence of α-2 blockade and combined blockade has been achieved using phentolamine. Interestingly, in a preparation where NA release may be predominantly stimulated from pre-synaptic NA terminals; the elicitation of feeding by microinjection of the anti-depressant Tranylcypramine; Leibowitz describes the most potent blockade of this effect as due to phenoxybenzamine (Leibowitz et al. 1978b). Recent work by Winn and Batchelor has, however, concentrated upon the possibility of direct stimulation of α-1 receptors with selective agonists. Such work is necessarily difficult to interpret in view of the proposed model. If the α-1 agonist is a molecule similar in shape to NA itself then it should also be rapidly removed from the biophase by the re-uptake mechanism, and would thus be unable to reach the α-1 receptor. By using two very different α-1 agonists Winn and Batchelor hope to have circumvented this problem. Whilst the α-1 agonist phenylephrine has no apparent effect on food intake, a second α-1 agonist methoxamine has an effect some fourfold that of an equivalent dose of NA. They suggest that this effect is possible because the shape of the methoxamine molecule renders it an unsuitable substrate fo the NA re-uptake mechanism (Winn and Batchelor; pers. comm.). A further experiment currently in progress will

examine the effects of re-uptake inhibition on the ability of phenylephrine to stimulate feeding, the hypothesis predicts that in the absence of re-uptake phenylephrine will stimulate feeding.

Ventral striatum and NA.

The purpose of experiment 6 was to examine the hypothesis; that α-2 receptors are predominantly extra-synaptic whilst α-1 are predominantly intra-synaptic; at a different site and using a different behaviour. The same techniques of selective blockade were attempted as for the manipulations in PVN. In this regard the experiment was manifestly unsuccessful. The noradrenergic antagonists used had no significant inhibitory effects on the increase in locomotor activity stimulated by NA. However, this experiment has generated two interesting findings. First, that NA microinjection into the ventral striatum results in increased locomotor activity and second that this response shows a priming effect whereby a second injection, 48 hours after the first, results in an enhancement of the response. Whilst it is well known that dopamine microinjected into ventral striatum increases locomotor activity (Fink and Smith 1980a, 1980b, 1980c) there has long been debate as to whether or not NA injected into the same sites will produce locomotion (Pijnenburg et al. 1973, Pijnenburg et al. 1976, Cools 1986). The data from experiment 6 clearly indicate that NA alone can increase locomotion but

do not shed any light on the mechanisms underlying this increase. More experiments are clearly required to examine further the 'priming' effect and whether or not the locomotion in response to NA requires an intact dopamine system in ventral striatum.

Excitotoxic lesion of PVN

Successful lesion of the parvocellular portion of PVN would have made possible studies to determine the contribution of the different cytological components of the PVN to the feeding response to microinjected NA. Unfortunately the mechanism of action of the excitotoxins used rendered such a lesion impossible. The excitotoxins are believed to act by stimulating cells to such an extent that they die. The parvocellular cells in the PVN are known to play an important role in the regulation of cardiovascular function and attempted lesion of such cells resulted in the death of the animal. This was probably due to cardiac arrest brought on by overstimulation of the heart resulting from the hyper-excitation of cells in the PVN. Doses of toxin low enough for the animal to survive the infusion were insufficient to generate a lesion. Thus it would appear that at present the development of a selective, neurotoxic lesion of the PVN in the rat is not possible.

THEORETICAL IMPLICATIONS

The data presented in this thesis support the hypothesis that communication between neurons within the CNS occurs both at specialised synapses and via simple diffusion through the extra-cellular fluid. Whether, as suggested by Herkenham (1987), all transmitters use both systems, or as Strange suggests only the slower, class II receptor systems employ diffuse release (Strange 1988) the implications for the study and manipulation of CNS systems are considerable. The use of centrally acting drugs is widespread in the treatment of a variety of CNS disorders. If, as this thesis suggests, merely admitting certain substances to the CNS is not sufficient for them to reach all the receptors at which they might bind then the way in which many drugs are believed to act will have to be re-thought. The majority of anti-depressants do not act directly at receptors but through a combination of re-uptake inhibition and enhanced release of the endogenous transmitter. The behavioural effects of such compounds may become more explicable if an understanding is developed of how such actions might affect the balance between synaptic and non-synaptic communication. A model similar to that suggested here for NA has been suggested for dopamine by Johnson et al. (1986) based upon electrophysiological evidence. Furthermore, Walters et al. (1985) suggest that stimulation of dopamine D1 and D2 receptors is synergistic and that a minimum level of stimulation of either receptor is required for the expression

of the other sub-type. If dopamine receptors are located intra-and extra-synaptically in populations of predominantly D1 or D2 sub-types as would be suggested by the Johnson model then understanding how the ratio of stimulation of the two populations affects the post-synaptic cell might lead to great advances in both Parkinson's and schizophrenia research.

CONCLUSION

Chemical transmission of information within the CNS is achieved through both release at specialised synapses and non-specific diffusion. This has been demonstrated behaviourally for NA within the PVN. The involvement of PVN NA in feeding resulting from tail pinch was not demonstrated. Direct injection of NA at another site can elicit a clear behavioural response, injection into the ventral striatum results in increased locomotor activity but the mechanisms underlying this response remain unclear.

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APPENDIX I

CHEMICAL INFORMATION TRANSFER

There is currently much research into the nature of the chemicals that nerve cells use to communicate with, and influence each other by. This research covers a variety of techniques and disciplines, and has spawned a range of descriptive terms for the chemical substances under examination. Thus in addition to "neurotransmitter", the following terms have been proposed: Neurohumor, (non)synaptic neurotransmitter, neurohormone, classical/ genuine neurotransmitter, possible/putative/suspected neurotransmitter, neuromediator, neuromodulator, neuroregulator, neuroactive principle, neurochemical, chemical messenger, neuroactive substance, and informational substance. This variety has come about as more and more information is generated about the range of chemical substances which can influence neuronal processes and the ways in which an individual chemical can have different effects depending upon a wide variety of release and receptor parameters. There is clearly an enormous overlap in the specificity of many of these terms; some have been coined to cover the actions of a class of chemicals in many situations while others refer to the specific actions of a limited group of similar compounds under particular conditions. From the above list it is possible to describe NA with all but one of the listed terms (one would classify it as a genuine rather

than putative neurotransmitter). It is clear that a definition of the terms used in this thesis, and explanation for their choice, is necessary.

There are conflicting requirements in the choice of term for identifing a compound with actions that affect the state of neuronal tissue. On the one hand, for clear, accurately defined, provable or disprovable statements to be made about the nature and actions of a compound the definition must be tight and constrained. In contrast, it is clear that little is known of the properties, actions and interactions of many of these compounds and for any definition to be valid it must be flexible. Thus it may be better, as Burnstock (1976) suggested, to apply the term transmitter to "*any substance produced and physiologically released from nerve terminals to evoke post-synaptic response via surface receptors.*" However two allowances must be made, first that a change in membrane state is a response, and second that the action may occur pre- or post-synaptically. Similarly Dismukes (1979) states that "*Given the incomplete state of knowledge, there is little advantage...in attempting to distinguish modulators from transmitters.*" For the purposes of the present thesis definition of the following terms is considered important: neurohumor, neurotransmitter, nonsynaptic neurotransmitter, neurohormone. No discussion of the concepts and definition of neuromediation or neuromodulation will be attempted here,

for review of this topic see Dismukes (1979) and commentaries, Iversen (1979), Van Dongen (1981) and Nieuwenhuys (1985).

DEFINITION OF TERMS.

The following definitions are modifications of those proposed by Van Dongen (1981):

NEUROHUMOR

A compound [X] is a neurohumor of a neurone or group of neurones when the following criteria are confirmed:

1. X is present in the neurone.
2. X is synthesised in the neurone.
3. Stimulation of the neurone causes the release of X.
4. X interacts with specific receptors.
5. A system exists which terminates the action(s) of X at its target site (there may be more than one such system).

Van Dongen includes a further requirement, that *'direct application of X mimics the effect of increasing its endogenous concentration: this effect is identical in all respects, including pharmacological.'* However, this appears both unnecessessary and self- defeating and it is in many respects the purpose of this thesis to demonstrate that the application of exogenous ligands does **NOT** mimic the effects of increasing endogenous concentrations. It is also

difficult to determine whether the effects of application of X is 'identical in all respects' to increasing the endogenous levels, when as explained above, the exact effects of many compounds are not clearly understood.

NEUROTRANSMITTER

Compound X is a neurotransmitter of a neurone when:

1. X is a neurohumor.
2. X is released proximal to its site of action and is not transported to this site by CSF or blood.

This class of compounds may be sub-divided into two:

A. Synaptic Neurotransmitter

Compound X is a synaptic neurotransmitter when:

1. X is a neurotransmitter.
2. X is present in the pre-synaptic component of a morphologically distinct synapse; X acts transsynaptically at receptor sites located in the post-synaptic membrane.

B. Nonsynaptic neurotransmitter

Compound X is a nonsynaptic neurotransmitter when:

1. X is a neurotransmitter.
2. X is present in and may be released from terminals without synaptic specialisations.

The categories synaptic and nonsynaptic neurotransmitter are in no way mutually exclusive and the same compound may be said to fit either of these definitions dependent upon the sites of release and action.

NEUROHORMONE

Compound X is a neurohormone when:

1. X is a neurohumor.
2. X is released from a site remote from its site of action; to which it is transported by the action of CSF or blood.

However even when the appropriate definition is applied it is not possible to be absolute as to the function of certain transmitters. As Fujita et al. state "*neuronal secretions, including typical neurotransmitters, are largely common to paraneuronal secretions including local hormones (parahormones) and typical hormones or telehormones. It is impossible to distinguish neurotransmitters, parahormones, or telehormones, either by their chemical nature or by the mechanism of their secretion*" (Fujita et al. 1988, p. 77).

The compound of primary importance in this thesis is NA which, as defined above, is a neurotransmitter in CNS. The terms synaptic and nonsynaptic will be applied to NA as appropriate, but no attempt will be made to describe NA as a mediator or modulator of neuronal function. Such terms are often idiosyncratic and would serve merely to distract attention from the aspects of NA transmission under examination.