Modulation of central noradrenergic function by the anti-obesity agent, sibutramine

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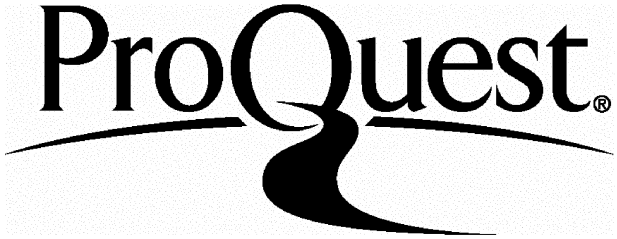

Abstract

The active metabolites of the anti-obesity agent, sibutramine, inhibit the reuptake of noradrenaline *in vitro*. The first aim of this project was to establish whether sibutramine also increases central extracellular noradrenaline concentration *in vivo* and, if so, whether this effect can be distinguished from that of the anti-obesity agent, *d*-amphetamine, which is a noradrenaline-releasing agent. Changes in extracellular noradrenaline concentration were measured using *in vivo* microdialysis in rat frontal cortex or hypothalamus. Since presynaptic α_2-adrenoceptors regulate noradrenergic neuronal activity, the role of these receptors in modulating extracellular noradrenaline concentration following sibutramine or *d*-amphetamine administration was investigated.

The second aim was to investigate the density and function of α_2-adrenoceptors in the lean and obese Zucker rat in order to determine whether an abnormality in central noradrenergic systems existed in the obese state. The density of α_2-adrenoceptors in the cortex and hypothalamus were investigated using radioligand binding studies. The functions of pre- and post-synaptic α_2-adrenoceptors were investigated using clonidine-induced hypoactivity and mydriasis, respectively.

Sibutramine induced a gradual increase in extracellular noradrenaline concentration in the cortex of anaesthetised rats whereas *d*-amphetamine induced a rapid increase. Blockade of α_2-adrenoceptors with the antagonist, RX821002, enhanced the sibutramine-induced increase in extracellular noradrenaline by 5-fold but enhanced the effect of *d*-amphetamine by only 2-fold. Sibutramine also increased extracellular noradrenaline concentration in the cortex and hypothalamus of freely-moving rats although this effect declined in the hypothalamus 40 min post-injection. Reverse dialysis of RX821002 into the cortex or hypothalamus enhanced the sibutramine-induced increase in extracellular noradrenaline in these areas and reversed the decline in extracellular noradrenaline in the hypothalamus 40 min after sibutramine administration. This indicates that central α_2-adrenoceptors restrict the accumulation of extracellular noradrenaline induced by sibutramine, but that this effect is greater in the hypothalamus.

No differences in the density of [^{3}H]RX821002 binding sites or post-synaptic α_2-adrenoceptor function were found between lean and obese Zucker rats. However, the affinity of α_2-adrenoceptors for [^{3}H]RX821002 in the cortex of obese rats was higher compared with lean rats. Obese rats also had a higher hypoactivity score under drug-free conditions than did lean rats. Furthermore, the increase in hypoactivity score induced by clonidine in lean Zucker rats reached a plateau between 0.1-0.3 mg / kg, whereas obese rats showed no sign of reaching a plateau at these doses. All these findings indicate that presynaptic α_2-adrenoceptor function is enhanced in obese Zucker rats.

The findings of this thesis show that the increase in extracellular noradrenaline concentration induced by sibutramine *in vivo* can be differentiated from that of *d*-amphetamine. However, the time-course of sibutramine's effect varies in different brain regions depending on the density and/or function of α_2-adrenoceptors. Finally, the function of presynaptic α_2-adrenoceptors appears to be enhanced in obese Zucker rats, possibly due to an increased affinity of these receptors for α_2-adrenoceptor ligands. This finding suggests that central noradrenergic activity could be impaired in obese Zucker rats.

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Experiments in Chapter 3 involving *d*-amphetamine administration to halothane-anaesthetised rats were carried out by Zoë Hughes. Statistical analysis of data from radioligand binding experiments in Chapter 6 was carried out by Richard Brammer of Knoll Pharmaceuticals Statistics Department. All other work was carried out by the candidate.

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Abbreviations

5-HT	5-hydroxytryptamine, or serotonin
6-OHDA	6-hydroxydopamine
8-OH-DPAT	8-hydroxy-2-(di-*n*-propylamino)tetralin
ATP	adenosine triphosphate
B_{max}	total no. of binding sites
aCSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
AP	anterior-posterior
BAT	brown adipose tissue
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribosenucleic acid
CNS	central nervous system
d.p.m.	disintegrations per minute
DBH	Dopamine-β-hydroxylase
DSP-4	*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine
DV	dorsal-ventral
ECD	electrochemical detection
ED_{50}	dose causing 50% of maximum effect
g	gram
GABA	γ-aminobutyric acid
GDP	guanosine diphosphate
GTP	guanosine triphosphate
h	hour
[^{3}H]	tritium
HPLC	high pressure liquid chromatography
i.c.v.	intra cerebroventricular
i.d.	inner diameter
i.p.	intra peritoneal
kD	kilo Dalton
K_d	dissociation constant
K_i	inhibitor constant

l	litre
m	metre
mol	moles
min	minute
M	molar
ML	medial-lateral
mRNA	messenger ribonucleic acid
n	sample size
NMDA	*N*-methyl-*D*-aspartate
NPY	neuropeptide Y
o.d.	outer diameter
P	probability
p.o.	per os
PVN	paraventricular nucleus
REDOX	oxidation-reduction potential
s.c.	subcutaneous
SEM	standard error of the mean
SSRI	selective serotonin reuptake inhibitor
V	Volt

Prefixes

m	milli	($\times 10^{-3}$)
μ	micro	($\times 10^{-6}$)
n	nano	($\times 10^{-9}$)
f	femto	($\times 10^{-12}$)
p	pico	($\times 10^{-15}$)
k	kilo	($\times 10^{3}$)

Publications arising from this thesis

Wortley, K. E., Hughes, Z. A., Heal, D. J. & Stanford, S. C. (1999). Comparison of the effects of sibutramine with *d*-amphetamine on changes in cortical extracellular noradrenaline concentration *in vivo* and the role of α_2-adrenoceptors. *Br. J. Pharmacol.* **127:** 1860-1866.

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Chapter 1

General Introduction

Obesity has been described as the most important nutritional disorder of the developed world and its prevalence has reached epidemic proportions in Western societies over recent decades. The disorder results from a combination of low energy expenditure and an increased energy intake, often consequences of genetic predispositions and / or environmental factors such as high-calorie diets and sedentary life styles. In some patients, obesity has serious adverse effects on health, promoting a number of co-morbidities including diabetes, hypertension, coronary heart disease and cancer. Initial treatment strategies employ low-calorie diets and increased physical activity programmes in an effort to reduce energy intake and increase energy expenditure. However, in a major proportion of obese patients, this treatment is ineffective and has no satisfactory long-term benefits. For some of these patients, drug therapy provides an additional approach for the long-term management of their body weight.

d-Amphetamine was introduced over 80 years ago as an appetite suppressant to treat obesity. Unfortunately, this drug also had prominent central stimulant and euphoriant properties. In an attempt to overcome the problems associated with psychostimulation, a number of other centrally acting anti-obesity agents were developed for the treatment of obesity. These included agents such as aminorex, phentermine, and *d*-fenfluramine. A popular treatment comprised both phentermine and *d*-fenfluramine, a combination known as 'PhenFen'. These drugs came to be the most commonly used anti-obesity agents in countries such as the United States and Great Britain. However, the use of *d*-fenfluramine

and phentermine became associated with adverse side effects such as primary pulmonary hypertension and valvular heart disease. This eventually led to the removal of these drugs from the market of anti-obesity treatments in September 1997.

Around the same time *d*-fenfluramine and PhenFen were removed from the market of anti-obesity treatments, two other drugs were in the advanced stages of development as potential new anti-obesity agents. These drugs were the centrally acting noradrenaline and 5-HT reuptake inhibitor, sibutramine and the peripherally acting lipase inhibitor, orlistat. This study focuses on the central nervous system pharmacology of sibutramine.

At the start of this PhD study, the pharmacology of sibutramine *in vitro* had been well documented. Pharmacologically, the most interesting findings from these studies were that two metabolites of sibutramine were potent inhibitors of noradrenaline and 5-hydroxytryptamine (5-HT) uptake in both human and rat brain tissue. However, no studies existed on the effects of this drug on central noradrenergic transmission *in vivo*. Such studies are of importance considering *d*-amphetamine is a noradrenaline-, as well as a dopamine- and 5-HT-releasing agent *in vivo*. Some evidence implicates a role for *d*-amphetamine's effects on central noradrenergic systems in its ability to increase behavioural activation. Therefore, it is of interest to establish whether sibutramine enhances central noradrenergic transmission *in vivo* and, if it does, to establish whether this is due to noradrenaline-reuptake inhibiting properties, which are indicated *in vitro*, or whether it is due to a noradrenaline-releasing effect of this drug. To address these issues, the primary aim of this study was to establish the effects of sibutramine on extracellular noradrenaline concentration in the rat brain *in vivo*.

The rest of this General Introduction will describe the background on which the present study has built. The noradrenergic innervation of the rat brain will be described first. Then, as sibutramine and *d*-amphetamine, as well as many other centrally acting anti-obesity agents, increase the extracellular concentration of one or more of the monoamines, the role of noradrenaline and 5-HT in the control of food intake will be discussed. The role of dopamine, as well as other endogenous substances that have been found to modulate food intake will be discussed briefly. Following this, the pharmacology of sibutramine and *d*-amphetamine, as well as *d*-fenfluramine (for the purpose of

comparisons) will be described. Since α_2-adrenoceptors were found to be important for the control of sibutramine's effects on extracellular noradrenaline accumulation, these receptors and their role in regulating central noradrenergic activity will also be discussed. Finally, as the last chapter of this thesis investigated central α_2-adrenoceptor systems in lean and obese Zucker rats, some of the physiological abnormalities of the obese Zucker rat will be highlighted. Particular attention will be given to abnormalities in central noradrenergic function in these rats.

1.1. Noradrenergic innervation of the rat brain

The presence of catecholamine-containing neurones in the rat brain was first demonstrated using a fluorescence histochemical technique by Dahlström and Fuxe in 1964. Regions containing catecholamine cell bodies were divided into twelve groups according to their topographical conditions and morphology: groups A1-A4 located in the medulla oblongata; groups A5-A7 located in the pons; groups A8-A10 located in the mesencephalon; groups A11-A12 located in the diencephalon. Within these brain regions, two subgroups of noradrenergic cell bodies exist, the lateral tegmental system, comprising groups A1-A3, A5 and A7, and the locus coeruleus complex, comprising groups A4 and A6. These two distinct noradrenergic systems are described below.

Following the identification of noradrenaline-containing axons in the brain, pathways of noradrenergic neurones projecting through the central nervous system were soon unravelled (early work reviewed by Moore and Bloom, 1979). A more recent account of the anatomy of noradrenaline-containing neurones in the brain describes four major axon bundles in the brain that consist in part, but not exclusively, of noradrenaline-containing axons: the medial forebrain bundle; the dorsal tegmental bundle; the central tegmental tract; the medullary catecholamine bundle (see Holets, 1990). The dorsal catecholamine bundle carries a major portion of ascending neurones from the locus coeruleus and the lateral tegmental system. This bundle travels as a component of the central tegmental tract and then joins the medial forebrain bundle. The medial forebrain bundle also carries a major portion of ascending noradrenaline-, as well as

dopamine-containing neurones from groups in the pons and mesencephalon. The central tegmental tract primarily carries noradrenaline- and adrenaline-containing axons ascending from groups of cell bodies in the medulla and pons that lie outside of the locus coeruleus. The medullary catecholamine bundle carries descending axons from the A5 and A6 cell groups, as well as ascending axons from noradrenergic and adrenergic neurones from groups of cell bodies in the medulla.

1.1.1. The locus coeruleus

In the rat, the locus coeruleus consists entirely of noradrenergic neurones (Dahlström and Fuxe, 1964). It is the largest nucleus of noradrenergic cell bodies in the mammalian brain and innervates targets throughout the entire central nervous system (see Moore and Bloom, 1979). There are some areas in the brain to which the locus coeruleus system provides the sole noradrenergic innervation. These areas include the cerebellum, the hippocampus and the cerebral cortex. Most other areas in the central nervous system receive a mixed noradrenergic innervation from both the locus coeruleus and lateral tegmental systems. Although it has widespread projections, the size of the locus coeruleus is small, with an estimated 1400-1600 neurones in the rat (Swanson, 1976). These neurones are typically multipolar, with 3-5 dendrites projecting from the soma that branch at least once. The axons show extensive branching with collaterals given off within the nucleus, as well as beyond it (Swanson, 1976).

Noradrenergic innervation of the cortex has been described in both the rat and monkey using a range of techniques including fluoresence histochemistry (Andén *et al.* 1966) and immunohistochemical localisation of dopamine-β-hydroxylase (an enzyme found only in catecholamine-synthesising cells and converts dopamine to noradrenaline) (Morrison *et al.* 1982). Using electron microscopic autoradiography, original studies in the rat frontoparietal cortex indicated that only 5% of noradrenergic terminals in this area formed synapses with postsynaptic elements (Descarries *et al.* 1977). The remaining noradrenergic neuronal terminals did not appear to form identifiable synaptic connections. The authors interpret their findings as evidence that noradrenaline functioned as a neuromodulator, to influence the release of other neurotransmitters. However, later studies provided evidence that contradicted the findings of Descarries *et al.* (1977).

Papadopoulos *et al.* (1987) studied serial ultrathin sections of rat cerebral cortex using antibodies against noradrenaline (these antibodies were actually raised against synthesising enzymes, or against noradrenaline bound to a larger protein, see Papadopoulos and Parnavelas, 1991). Their analyses showed that approximately 90% of labelled noradrenergic variscosities formed synapses with postsynaptic elements. These findings provided some of the first pieces of evidence to show that the noradrenergic system displayed specialised synaptic junctions, similar to other afferent systems in the cortex.

1.1.2. The lateral tegmental noradrenergic system

Like locus coeruleus neurones, lateral tegmental neurones are multipolar, although their variscosities are larger than than those of the locus coeruleus system and their axons are more irregular in size (Moore and Bloom, 1979). Targets for lateral tegmental noradrenergic projections include the basal forebrain, thalamus, hypothalamus, brainstem and spinal cord.

In this thesis, changes in extracellular noradrenaline concentration were monitored in the frontal cortex or in the region of the paraventricular nucleus of the hypothalamus. Evidence from fluorescence histochemical studies suggest that there is no lateral tegmental noradrenergic innervation of the neocortex (Moore and Bloom, 1979), making the frontal cortex a region exclusively innervated by locus coeruleus neurones. In contrast, the hypothalamus is densely innervated by neurones from both the locus coeruleus and the lateral tegmental system, although evidence suggests that the majority of noradrenergic neurones in the hypothalamus derive from the lateral tegmental system. Palkovits *et al.* (1980) measured the concentration of noradrenaline in individual hypothalamic nuclei following surgical transections of the lower brain stem or electrolytic lesions of noradrenaline containing cell groups in the medulla. They found that the largest reduction (45-50%) in hypothalamic noradrenaline content was achieved following unilateral lesioning of the A1 group. Fibres ascending from this group were suggested to cross the midline of the brain as the lesion caused a 52% decrease in noradrenaline content of the contralateral paraventricular nuclei. A relatively smaller noradrenergic innervation of the hypothalamus was indicated by noradrenergic neurones of the A2 group (approximately

20 - 30% decrease in noradrenaline content of the arcuate nucleus, median eminence, paraventricular and periventricular nuclei). Lesioning of the A5 group indicated that this group projected mainly to the ventromedial and arcuate nucleus, with only little or no affect on noradrenaline content of other hypothalamic nuclei. Following lesion of the locus coeruleus, a decrease in noradrenaline content was found in the arcuate, supraoptic, paraventricular and periventricular nuclei only. Although this lesion caused a relatively large depletion of noradrenaline in the arcuate and paraventricular nuclei (52 and 40%, respectively), the authors point out that these estimates could be exaggerated since damage to the locus coeruleus is likely to cause to damage to adjacent noradrenergic pathways projecting to the hypothalamus.

Brain regions innervated by both the locus coeruleus and lateral tegmental systems have a greater tissue concentration of noradrenaline than areas innervated by the locus coeruleus system only. For example, the noradrenaline content of the lateral cortex and hippocampus is estimated to be 2.5 and 4.7 pmol / mg tissue, respectively, whereas that of the hypothalamus is estimated to be 32.4 pmol / mg tissue (Zaczek and Coyle, 1982). Lidbrink and Jonsson (1971) report that the turnover rate of noradrenaline in the hypothalamus is slower than in the cortex. Furthermore synaptosomal studies show that neurones in the hypothalamus have a greater affinity for [^{3}H]noradrenaline uptake compared with neurones in the cortex (40 nM and 100 nM, respectively) (Zaczeck *et al.* 1990). Evidence such as this suggests there could be functional differences between noradrenergic neurones of the locus coeruleus and lateral tegmental systems (see Chapters 4 and 5).

1.2. Endogenous substances affecting food intake

1.2.1. Noradrenaline

In 1960, Grossman demonstrated that administration noradrenaline through cannulae implanted in the perifornical hypothalamus, as well as areas more medial and ventral, induced a marked feeding response in satiated rats. Later experiments carried out

by Booth (1968) showed that the *l*-isomer of noradrenaline was 90% more effective at inducing this feeding response in rats than the *d*-isomer. Further studies using smaller cannulas than those used by Grossman in 1960, suggested that *l*-noradrenaline was more effective at increasing food intake when injected into areas coincident with, or bordering, the paraventricular nucleus of the hypothalamus than when injected into midlateral areas of the hypothalamus (Matthews *et al.* 1978). In a more extensive study, Leibowitz (1978) tested 35 different brain areas of the rat. This study confirmed that local administration of noradrenaline specifically into the paraventricular nucleus is the most effective site for eliciting a feeding and preprandial drinking response in satiated, as well as mildly hungry (24 h with only 80% of normal daily food intake available) rats. In contrast, injection of noradrenaline into lateral perifonical regions induced a reduction in food intake in mildy hungry rats (see below).

In a meal pattern analysis study, injection of noradrenaline into the paraventricular nucleus was found to increase food intake by increasing meal size rather than meal frequency (Shor-Posner *et al.* 1986). The authors suggest that because noradrenaline had no effect on meal frequency, noradrenaline could influence the termination of feeding (*i.e.* satiety) rather than the stimulation of hunger.

Further studies showed that administration of the α_2-adrenoceptor agonist, clonidine, into the paraventricular nucleus also induced a feeding response in satiated rats (Goldman *et al.* 1985). However, Wellman (1992) showed that the effect of local administration of clonidine into the paraventricular nucleus on feeding was biphasic, with low doses facilitating food intake and high doses (20 and 50 nmol) inhibiting food intake. This biphasic effect of clonidine on food-intake is also found following systemic administration of clonidine (Sanger, 1983). However, the decrease in food intake induced by higher doses of clonidine is likely to be due to the sedative effects of this drug (Drew *et al.* 1979). Interestingly, a recent study reports that orally administered clonidine at a dose of 1.5 or 3 μg / kg does not increase food intake in healthy men or women (Crow *et al.* 1998). In fact, food intake was greater in the placebo group compared with clonidine-treated subjects. Although relatively low doses of clonidine were used in this study, the sedation ratings of clonidine-treated subjects were greater than those of the placebo group. Again, this effect of systemically administered clonidine could hinder the

feeding-stimulatory effects that clonidine elicits through pathways in the hypothalamus.

Goldman *et al.* (1985) showed that both noradrenaline- and clonidine-induced feeding responses could be reduced in a dose-dependent manner by local pretreatment with the non-selective α-adrenoceptor antagonist, phentolamine, or the α_2-adrenoceptor antagonists, yohimbine or rauwolscine. These antagonists alone, at doses that inhibited the noradrenaline- and clonidine-induced feeding responses, had no effect on food intake. In contrast, the study shows that the α_1-adrenoceptor antagonists, prazosin or corynanthine, do not affect the noradrenaline- or clonidine-induced feeding response, in fact corynanthine actually enhanced the feeding response elicited by hypothalamic injection of noradrenaline. Earlier studies investigating the feeding response induced by hypothalamic injection of noradrenaline found that β-adrenoceptor antagonists, including *l*-propranolol, did not inhibit this response (Leibowitz, 1975a). Furthermore, the relatively selective β-adrenoceptor agonist, isoproterenol, had no effect on food intake 30 min following its local administration at 10 or 100 nmol. Collectively, these findings suggest that the receptors mediating the feeding response induced by hypothalamic injection of noradrenaline or clonidine include α_2-adrenoceptors.

Goldman *et al.* (1985) also showed that local administration of clonidine into the paraventricular nucleus still elicited a feeding response 2 h after local administration of the catecholamine synthesis inhibitor, α-methyl-para-tyrosine, into satiated rats. α-Methyl-para-tyrosine alone had no effect on food intake. This suggests that the α_2-adrenoceptors mediating the feeding response are located postsynaptically in the region of the paraventricular nucleus. In contrast, activation of α_1-adrenoceptors in the paraventricular nucleus by the local adminstration the α_1-adrenoceptor agonists, cirazoline, methoxamine, phenylpropanolamine or phenylephrine suppressed food intake in freely-feeding rats; an effect that was reversed by local pretreatment with an α_1-adrenoceptor antagonist (Wellman *et al.* 1993).

Noradrenaline (40 nmol) injected into the lateral regions of the hypothalamus produces a reliable, dose-dependent suppression of feeding (Leibowitz, 1978). Local injection of the monoamine-releasing agent, *d*-amphetamine (6 - 400 nmol) into the

lateral hypothalamus also induces a suppression of food intake in food-deprived rats (Leibowitz, 1975b). No effect of *d*-amphetamine on food intake was found following its administration into the medial hypothalamus. The reduction in food intake induced by *d*-amphetamine injected into the lateral hypothalamus was inhibited by pretreatment with systemic administration of the β-adrenoceptor antagonist, *l*-propranolol, or the dopamine receptor antagonists, haloperidol or pimozide, but was not affected by the α-adrenoceptor antagonists, phentolamine or tolazozine. Furthermore, lateral hypothalamic administration of propranolol or haloperidol inhibited the reduction in food intake caused by systemically administered *d*-amphetamine (1 mg / kg) by 45% and 75%, respectively. These findings suggest that the noradrenaline released in the lateral hypothalamus by *d*-amphetamine could act at β-adrenoceptors to mediate part of the reduction in food intake induced by this dose of *d*-amphetamine.

In summary, it appears that activation of both α-adrenoceptor and β-adrenoceptors can modulate feeding behaviour in the rat, with α-adrenoceptor mechanisms appearing to preside in the region of the paraventricular nucleus and β-adrenoceptor mechanisms presiding in the lateral regions of the hypothalamus. Which of these receptor pathways, if any, contribute to the natural, physiological control of food intake in rats is unclear.

1.2.2. 5-Hydroxytryptamine

In 1971, Goldman *et al.* showed that injection of 5-HT into the perifornical region of the hypothalamus induced a suppression of food intake in food-deprived rats. A further study of the suppressive effect of 5-HT on food intake in freely-feeding rats revealed that the injection of 5-HT (5-20 nmol) into the paraventricular nucleus induced a dose-dependent decrease in food intake specifically at the onset of the dark period (Leibowitz *et al.* 1989). No effect of 5-HT on food intake was observed during other stages of the light-dark cycle using this dose of 5-HT. The decrease in feeding behaviour was characterised by a highly selective reduction in carbohydrate intake, although there was a slight enhancement in preference for protein and fat with little overall change in total caloric intake.

This suppressive effect of 5-HT on carbohydrate intake has since been mapped more specifically to the medial hypothalamic nuclei. In a study of 7 different hypothalamic nuclei and 5 extrahypothalamic sights, a 50-70% suppression of carbohydrate intake was observed only when 5-HT (2.5 nmol) was injected into the paraventricular, ventromedial or suprachiasmatic nuclei of freely-feeding rats (Leibowitz *et al.* 1990). Studies investigating the behavioural changes by which 5-HT induces a suppression of feeding behaviour in freely-feeding rats suggest that 5-HT enhances satiety. Shor-Posner *et al.* (1986), injected 5-HT into the paraventricular nucleus of freely-feeding rats and monitored changes in feeding patterns. Injections of 5-HT induced a decrease in meal size, duration and feeding rate, but did not affect the frequency of meals consumed, suggesting that 5-HT influenced the onset of satiety rather than the suppression of hunger.

The hypothesis that central serotonergic transmission mediates a suppressive effect on feeding behaviour is supported by Hutson *et al.* (1986). These authors monitored feeding behaviour in rats following central administration of the 5-HT_{1A} autoreceptor agonist 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT). Activation of these somatodendritic autoreceptors induces a decrease in serotonergic neuronal activity (see Chapter 3, section 3.1.). Infusion of 8-OH-DPAT through cannulas implanted into either the dorsal or median raphé (regions containing the cell bodies of serotonergic neurones) caused an increase in food intake and feeding duration in satiated rats compared with rats infused with saline. Behavioural analysis revealed that infusion of 8-OH-DPAT did not affect behaviours such as locomotion, rearing, forepaw treading or 'wet dog shakes', suggesting that the increase in food intake was not due to an increase in behavioural arousal. The authors suggest that the effect of 8-OH-DPAT is mediated through an action on 5-HT autoreceptors located on serotonergic cell bodies; the ensuing decrease in serotonergic neuronal activity and the increase in food intake is consistent with the hypothesis that that enhanced serotonergic transmission depresses feeding behaviour.

Feeding studies have found that systemic administration of the mixed 5-HT_{1A} and 5-HT_{1B} receptor agonist 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole (RU-24969) reduces food intake in food-deprived rats by decreasing the time spent feeding, without affecting the rate of eating (Simansky and Vaidya, 1990). Further studies

with RU-24969 and the selective 5-HT_{1B} agonist 3-(1,2,5,6-tetrahydropyrid-4-ylpyrolo[3,2,-b]pyrid-5-one (CP 93129) suggest that stimulation of 5-HT_{1B} receptors in the hypothalamus reduces food intake in the rat. When injected directly into the paraventricular nucleus, both these drugs reduced food intake in food-deprived rats and this effect was blocked by propranolol, a β-adrenoceptor antagonist that also blocks 5-HT_{1A} and 5-HT_{1B} receptors at the dose used in this study (20 nmol) (Samanin and Grignaschi, 1996). These findings suggest that activation of hypothalamic 5-HT_{1B} receptors could induce satiety.

Quipazine, is a non-selective 5-HT receptor agonist and its systemic administration induces a dose-dependent reduction in the intake of palatable diet in rats (Hewson *et al.* 1988). This effect is inhibited by systemic administration of the non-selective 5-HT receptor antagonist, methysergide, as well as the non-selective 5-HT_2 receptor antagonist, ritanserin and the selective 5-$HT_{2A/2C}$ receptor antagonist, ketanserin (Hewson *et al.* 1988). However, the 5-HT_3 receptor antagonist, 1,2,3,9-tetrahydro-9-methyl-3[(2-methyl-1H-imidazol-1-yl)methyl]-4H-carbazol-4-one (GR 38032F) had no effect on the suppression of food intake induced by quipazine. The authors conclude that anorectic effects of quipazine are mediated, at least in part, by 5-HT_2 receptors, but also suggest that because none of the 5-HT receptor antagonists alone affected feeding behaviour, 5-HT is not involved in the tonic control of food intake. However, these experiments were performed during the light period and cannot rule out a tonic effect of 5-HT on food intake control during the dark period, when injection of 5-HT into the hypothalamus is reported to be most effective at suppressing food intake (Leibowitz, *et al.*, 1989).

A role for 5-HT_2 receptors in the control of food intake is supported by studies in humans. Systemic administration of a low dose (0.4 mg / kg) of the 5-HT_{2C} receptor agonist, m-chlorophenylpiperazine (m-CPP), to healthy female volunteers reduced food intake by 30% during a test meal (Walsh *et al.*1994). Similarly, chronic administration (14 days) of the selective 5-HT_{2C} receptor antagonist, RS-102221 (a benzenesulphonamide of 8-[5-(5-amino-2,4-dimethoxyphenyl)-5-oxopentyl]-1,3, 8-triazaspiro[4.5]decane-2,4-dione) increased food intake and body weight gain in rats (Bonhaus *et al.* 1997). Furthermore, studies in mutant mice lacking the 5-HT_{2C} receptor

gene showed that body weight and adipose tissue deposition in adult mutant mice were greater than in wild type mice (Heisler *et al.* 1998). Feeding studies suggested that the greater body weight and adipose tissue deposition in mutant mice is due to the increased food intake by these mice.

In contrast to quipazine, the 5-HT receptors mediating the anorectic effects of the 5-HT releasing agent, *d*-fenfluramine, in rats (see section 1.2.3.) are not thought to involve 5-HT_2 receptors. Samanin *et al.* (1989) report that the non-selective 5-HT receptor antagonist, metergoline, dose-dependently inhibited the decrease in food intake following *d*-fenfluramine administration. However, ritanserin, over a similar dose-range to that used by Hewson *et al.* (1988), (0.5-2 mg / kg) only partially prevented the suppression of food intake following *d*-fenfluramine administration. Neill *et al.* (1990), also found the effects of *d*-fenfluramine to be antagonised by metergoline but unaffected by ritanserin. These findings suggested that 5-HT receptors other than the 5-HT_2 receptors are involved in mediating *d*-fenfluramine's effects, possibly 5-HT_{1B} receptors.

The role of 5-HT_3 receptors in food intake control is uncertain. Mazzola-Pomietto *et al.* (1995) found that the 5-HT_3 receptor agonist, m-chlorophenylbiguanide, (m-CPBG), reduced food intake in food-deprived rats only at a relatively high dose of 10 mg / kg. However, the 5-HT_3 receptor antagonist, 3-tropanyl-3,5-dichlorobenzoate (MDL-72222) induced a dose-dependent reduction of food intake in food-deprived rats, whereas the 5-HT_3 receptor antagonist, ondansetron, (0.032 or 0.32 mg / kg) was without effect. In freely-feeding rats, none of these agents affected food intake. The authors concluded that their findings did not support an important role for 5-HT_3 receptors in food intake control. In contrast, Van der Hoek and Cooper (1994) reported that ondansetron reduced the intake of a palatable diet in freely-feeding rats, but not food-deprived rats, at all doses tested over the dose range 3-30 μg / kg. The reduction of food intake in freely-feeding rats was characterised by a reduction in the time spent eating without a change in the frequency of eating, suggesting that ondansetron enhances satiety. Clearly, the role of 5-HT_3 receptors in food intake control requires further investigation.

Other 5-HT receptor subtypes also exist, such as the 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7 receptors, although their role, if any, in food intake control remains to be fully

elucidated.

1.2.3. Other endogenous substances affecting food intake

A variety of other endogenous substances in the brain, as well as the periphery, have been identified as having either a facilitatory or inhibitory effect on food intake. In the brain, these substances include the other monomine, dopamine and the neuropeptides, neuropeptide Y (NPY) and galanin. In the periphery, these substances include the hormones, cholecystokinin (CCK), and leptin.

Studies investigating the receptors through which *d*-amphetamine mediates its effects have indicated that dopaminergic transmission can affect food intake. In 1975, Leibowitz showed that the anorectic effect of peripherally injected *d*-amphetamine was prevented by local injection of the relatively non-selective dopamine receptor antagonist, haloperidol into the lateral hypothalamus (Leibowitz, 1975b). A later study using more selective dopamine antagonists showed that the reduction in food intake induced by a low dose (0.3 mg / kg) *d*-amphetamine in rats was totally reversed by the selective dopamine D_1 receptor antagonist, R-(+)-8-chloro-2, 3, 4, 5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol maleate (SCH 23390), but not affected by the selective dopamine D_2 receptor antagonist, sulpiride (Gilbert and Cooper, 1985). In contrast, the anorectic effect of a higher dose of *d*-amphetamine (1 mg / kg) was not affected by either antagonist. Shukla *et al.* (1990) also found no effect of haloperidol or the dopamine D_2 receptor antagonist, pimozide, on the reduction in food intake induced by a high dose of *d*-amphetamine (3.2. mg / kg). These findings suggest that dopamine D_1 receptors are involved only in mediating the anorectic effect of low doses *d*-amphetamine.

NPY and galanin are synthesised in the hypothalamus and injection of either one into the hypothalamus of satiated rats stimulates feeding behaviour. The effects of these neuropeptides on feeding behaviour can be distinguished by their selectivity to increase the consumption of specific macronutrients: feeding studies show that hypothalamic administration of NPY preferentially increases carbohydrate consumption, whereas galanin preferentially increases the consumption of fat (reviewed by Leibowitz, 1995). Various animal models of obesity, including the obese Zucker rat, exhibit increased

hypothalamic levels of both peptides as well as increased levels of their mRNAs (reviewed by Leibowitz, 1995). Whether these disturbances in hypothalamic peptides are a causal factor in obesity remains to be established.

CCK is found in both the upper gastrointestinal tract and in the brain. Peripheral administration of the peptide reduces food intake in a variety of species including man and feeding studies have characterised this reduction as a decrease in meal size and duration, thus indicating the advancement of satiety (reviewed by Moran, 1996). During feeding, CCK is secreted from the small intestine and, based on findings from studies investigating the effects of peripherally injected CCK, a role for its subsequent influence on satiety has been proposed. However, this idea is challenged by Baldwin *et al.* (1998), who argue that the concentration of CCK released during feeding is probably not sufficient to influence feeding behaviour. These authors also suggest that the decrease in food intake induced by peripheral administration of CCK could result from the induction of aversion and nausea as opposed to satiety. These issues question the physiological relevance of peripherally administered CCK on feeding behaviour.

Although secreted from adipose tissue, leptin is thought to decrease food intake and modulate glucose and fat metabolism *via* leptin receptors in the hypothalamus. Plasma levels of leptin are highly correlated with adipose tissue mass and the concentration of leptin in plasma falls in both humans and mice after weight loss. Conversely, plasma leptin concentrations are increased in obese humans (reviewed by Friedman and Halaas, 1998). In light of the findings correlating leptin and adipose tissue mass, leptin has been suggested to act as a messenger in part of a feedback loop to maintain constant body stores of fat. Consistent with a role for leptin in regulating body fat stores, recessive mutations of both the leptin gene and the leptin receptor gene in mice resemble morbid obesity in humans (reviewed by Friedman and Halaas, 1998).

1.3. Anti-obesity agents

1.3.1. Sibutramine

Sibutramine (BTS 54 524; N-{1-[1-(4-chlorophenyl)cyclobutyl]-methyl butyl}-N,N-dimethylamine HCl monohydrate) was originally developed in a research programme focussed on discovering potential new antidepressant treatments. The rationale for the research programme was based on work by Vetulani and Sulser (1975) which proposed that that β-adrenoceptor down-regulation in the central nervous system was the common therapeutic mechanism for all antidepressant treatments. As preclinical data showed that sibutramine potently down-regulated [^{3}H]dihydroalprenolol binding (a non-selective β-adrenoceptor ligand) in rat cortex after only 3 days of oral administration (Buckett *et al.* 1988) sibutramine was singled out as a potentially powerful antidepressant with a rapid onset of action. However, despite its preclinical profile, sibutramine showed no evidence of antidepressant activity in Phase II clinical trials. Nevertheless, of interest during the trials, it was noted that sibutramine induced weight loss in depressed patients, the magnitude of which paralleled dose. This observation led to the development of this drug as an anti-obesity agent.

Buckett *et al.* (1988) reported that sibutramine preferentially inhibited the uptake of [^{14}C]noradrenaline into rat cortical slices, with only weak effects on [^{14}C]5-HT or [^{14}C]dopamine uptake. In contrast, sibutramine had no effect on monoamine oxidase activity *in vitro*, or *in vivo*. In keeping with its noradrenaline reuptake inhibiting properties, sibutramine dose-dependently inhibited the noradrenaline depleting effects of reserpine, as well as the 5-HT depleting effects of *p*-chloroamphetamine, with 50% prevention of depletion after oral doses of 15.4 or 13.3 mg / kg, respectively. However, of pharmacological importance, sibutramine is rapidly demethylated to the secondary amine, Metabolite 1 (BTS 54 354) and then the primary amine, Metabolite 2 (BTS 54 505) following administration to either animals or humans, (Heal *et al.* 1998a). Later studies showed that these metabolites were at least 20- and 100-fold more potent than the parent compound at inhibiting the uptake of [^{14}C]noradrenaline or [^{14}C]5-HT into rat cortical slices, respectively (Luscombe *et al.* 1989). However, sibutramine and its metabolites had

similar effects on β-adrenoceptor down-regulation. This indicated that the metabolites of sibutramine are involved in mediating the pharmacological effects of sibutramine.

Further studies confirmed that whereas sibutramine has relatively weak activity, Metabolites 1 and 2 are potent inhibitors of [^{3}5-HT] and [^{3}H]noradrenaline uptake in synaptosomal preparations of both rat and human brain tissue (see Table 1.1.). Studies also suggest that Metabolites 1 and 2 are moderate inhibitors of dopamine uptake *in vitro* (Luscombe *et al.*, 1989; Heal and Cheetham, 1997, see Table 1.1.). However, studies investigating sibutramine's profile for dopaminergic function revealed that neither the parent compound nor Metabolites 1 or 2 had any effect on dopaminergic activity *in vitro* or *in vivo* (Heal *et al.* 1992). These studies showed that in contrast to the dopamine reuptake inhibitor and releasing agent, metamphetamine, [^{3}H]dopamine efflux from slices of rat striatum was not potentiated by sibutramine or either of the metabolites. Furthermore, when rats were trained to distinguish between systemically administered *d*-amphetamine or saline in a two-choice lever pressing paradigm, sibutramine generalised to the saline lever. Finally, these studies also investigated circling behaviour in unilateral nigrostriatal lesioned rats. Whereas orally administered metamphetamine induced pronounced circling behaviour in lesioned rats at a dose of 4.2 mg / kg, sibutramine had no effect at a dose of 6 mg / kg. This lack of effect of sibutramine on dopaminergic function led to the description of this drug as a novel 5-HT and noradrenaline reuptake inhibitor.

Neither sibutramine, nor the metabolites stimulate the release of [^{3}H]noradrenaline, [^{3}H]5-HT or [^{3}H]dopamine from rat brain slices *in vitro*, at concentrations up to 10 μM (Heal and Cheetham, 1997). Furthermore, neither sibutramine nor the metabolites have an affinity (K_i) of < 1000 nM for a range of neurotransmitter receptors including adrenergic, serotonergic, muscarinic, histaminergic or glutamate receptors (Heal and Cheetham, 1997). These findings suggest that the pharmacological effects of sibutramine derive solely from reuptake inhibition. Furthermore, considering the roles of 5-HT and noradrenaline in the control of food intake, the potent inhibitory effects of sibutramine's active metabolites on the reuptake of these monoamines suggested that these properties could be responsible for mediating sibutramine's anti-obesity actions.

	NORADRENALINE	5-HT	DOPAMINE
Human brain			
Sibutramine	5451	298	943
Metabolite 1	20	15	49
Metabolite 2	15	20	45
Rat brain			
Sibutramine	283	3131	2309
Metabolite 1	2.7	18	24
Metabolite 2	4.9	26	31
d-Amphetamine	45	1441	132

Table 1.1: *Monoamine reuptake inhibition profiles of sibutramine, Metabolite 1 and Metabolite 2 in rat and human brain in vitro and comparison with d-amphetamine.*

Numbers represent K_i values (inhibition constants) for the inhibition of monoamine reuptake. Data obtained from Heal *et al.* (1998a).

Acute oral administration of sibutramine to rats induced a dose-dependent inhibition of food intake as tested over the dose-range 1-10 mg / kg (Jackson *et al.*, 1997a; 1997b). The ED_{50} (dose causing 50 % inhibition of food intake) for this effect was 2.8 mg / kg (Jackson *et al.* 1997b). Similar effects on food intake were observed after oral treatment with the 5-HT and noradrenaline reuptake inhibitors, venlafaxine or duloxetine, although their effects on food intake were not as potent as sibutramine's (Jackson *et al.* 1997b). A comparable effect on food intake was also observed after combined treatment with the selective noradrenaline reuptake inhibitor, nisoxetine and the selective 5-HT reuptake inhibitor, fluoxetine. Together, these findings strongly supported the hypothesis that the 5-HT and noradrenaline reuptake properties of sibutramine's metabolites mediate this drug's anorectic effect. However, neither fluoxetine nor nisoxetine alone had any effect on food intake over the dose-range 3-30 mg / kg (Jackson *et al.* 1997b). Thus, these findings also highlighted a synergy between 5-HT and noradrenaline in the control of food intake.

Since, over similar dose ranges, sibutramine had profound effects on both food intake as well as noradrenaline and 5-HT reuptake inhibition *in vivo*, it was suggested that the hypophagia was initiated by monoaminergic receptor stimulation as a result of the increased synaptic availability of 5-HT and noradrenaline. Consistent with this idea, the hypophagic effects of sibutramine are inhibited by systemic administration of α_1-adrenoceptor, β_1-adrenoceptor or 5-$HT_{2A/2C}$ receptor antagonists (see Chapter 4, section 4.4.3.). Further evidence suggests that these effects are centrally mediated since intracereboventricular injections of Metabolites 1 and 2 reduce food intake in mice at doses which have no effect on food intake when administered peripherally (Heal and Cheetham, 1997).

In a chronic study of sibutramine treatment in Zucker rats, the initial decrease in food intake seen after acute administration was not apparent in treated animals over a two week period (Connoley *et al.* 1995). Despite this lack of difference in food intake, the body weight of treated rats was less than that of the control group. This suggested that sibutramine could also have thermogenic activity. Subsequent experiments in Wistar rats found that a dose of 10 mg / kg sibutramine induced a 23% increase in oxygen consumption and an increase in colonic temperature of approximately 0.76 °C, which was sustained for at least 2 h (Connoley *et al.* 1995). Experiments with fluoxetine and nisoxetine also showed that when combined, these agents could induce a 25% increase in oxygen consumption, whereas neither compound alone had thermogenic capacity, thus highlighting a synergistic effect between these monoamines in the control of thermogenesis. Furthermore, treatment of rats with the ganglionic blocker, clorisondamine, blocked completely the thermogenic response to sibutramine. This indicated that, like its effects on food intake, sibutramine could induce thermogenesis *via* activation of central pathways (Stock, 1997).

The thermogenic activity of sibutramine has been suggested to result from sympathetic activation of brown adipose tissue (BAT) mediated *via* the β_3-adrenoceptor. Support for a role of the β_3-adrenoceptor in sibutramine's effects comes from experiments showing that the thermogenic response to sibutramine is blocked by combined treatment with the β_1- and β_2-adrenoceptor antagonists atenolol and erythro-dl-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol (ICI 118551), respectively, at high doses which are

known to also activate β_3-adrenoceptors (Connoley *et al.* 1996). In contrast, low, selective doses of these β_1- and β_2-adrenoceptor antagonists had no effect on the thermogenic action of sibutramine, thus suggesting that β_3-adrenoceptors are involved in mediating this effect. By monitoring glucose utilisation in a range of different tissues, the thermogenic activity of sibutramine has been shown to rely specifically on sympathetic stimulation to BAT. Sibutramine induced a large increase in glucose utilisation specifically in BAT of rats. Smaller increases were seen only in the diaphragm and gastocnemius muscle (Stock, 1997). Since there were no increases in glucose utilisation in other tissues innervated by the sympathetic nervous system that were comparable with the increase in BAT, this suggested that the thermogenic activity of sibutramine did not derive simply from a peripheral action of sibutramine on noradrenaline reuptake.

In summary, sibutramine's anti-obesity actions derive from the enhancement of synaptic availability of 5-HT and noradrenaline in the central nervous system, as a result of reuptake inhibiting properties of its two active metabolites. A synergistic action between the increased serotonergic and noradrenergic function then activates central pathways to reduce food intake and increase resting energy expenditure.

1.3.2. *d*-Amphetamine

Amphetamine was first synthesised in 1887 and has been used clinically as an anorectic agent for many years (reviewed by King and Ellinwood, 1992). However, amphetamine can also cause a number of adverse effects in both the peripheral and central nervous systems. Peripheral effects include an enhancement of systolic and diastolic blood pressure and a slowing of the heart rate. Amphetamine also induces peripheral hyperthermia, whereas centrally it causes hypothermia. There are also many behavioural effects associated with amphetamine administration: low doses enhance locomotor activity while high doses induce stereotypies. Furthermore, amphetamine elicits feelings of euphoria and relief from fatigue, effects that are linked to the abuse liability of this drug. Although a potent anorectic agent, these adverse effects of amphetamine (reviewed by King and Ellinwood, 1992) make this drug an unsuitable candidate for the treatment of obesity.

The effects of amphetamine on food intake have been attributed to its effects on both central dopaminergic and noradrenergic systems. Hollister *et al.* (1975) reported that an intracisternal injection of the catecholamine neurotoxin, 6-hydroxydopamine (6-OHDA) in rats pretreated with desipramine (to protect noradrenergic neurones) reduced the concentration of dopamine in whole brain tissue to approximately 14%. This treatment attenuated the anorectic effect of *d*-amphetamine (0.75-1.5 mg / kg). Although the concentration of noradrenaline in whole brain tissue was reduced in these rats (approximately 20% reduction) a significant correlation was only found between the amount of food consumed and the tissue concentration of dopamine. In contrast, these authors reported that preferential depletion of central noradrenergic neurones (approximately 60% reduction in whole brain tissue) using a low dose of 6-OHDA had no affect on the anorectic action of *d*-amphetamine in rats. These findings suggested that central dopaminergic neurones are involved in the anorectic effect of *d*-amphetamine.

However, Leibowitz (1975b) reported that pretreatment of rats with a β-adrenoceptor antagonist inhibited the anorectic effect of *d*-amphetamine injected into the lateral hypothalamus of rats, suggesting that central noradrenergic pathways could be involved in this drug's effects on food intake. Indeed, a later study showed that electrolytic lesion of the ventral noradrenergic bundle, which decreased the concentration of noradrenaline in the forebrain by approximately 33%, without affecting dopamine or 5-HT, markedly reduced the anorectic effect of *d*-amphetamine (1.25 mg / kg) in rats (Samanin *et al.* 1977).

In 1965, Glowinski and Axelrod showed that *d*-amphetamine induced a 30% release of [^{3}H]noradrenaline from rat brain synaptosomes. By adding, *d*-amphetamine to the synaptosomes before the [^{3}H]noradrenaline, these authors also showed that this drug had a profound inhibitory effect on noradrenaline uptake (70% inhibition). Later studies showed that both stereoisomers of amphetamine could induce a concentration-dependent inhibition of [^{3}H]noradrenaline uptake into rat brain synaptosomes (Thornburg and Moore, 1973). However, Raiteri *et al.* (1975) were unable to show that *d*-amphetamine released [^{3}H]noradrenaline from hypothalamic, cerebellar or pons-medulla synaptosomes and found only a modest release from cortical or striatal synaptosomes. In contrast, *d*-amphetamine induced the release of [^{3}H]dopamine from striatal, cortical or

hypothalamic synaptosomes as well as [^{3}H]5-HT from striatal or cortical synaptosomes. The authors rejected the suggestion that *d*-amphetamine induced noradrenaline release and concluded that the drug increased noradrenaline efflux by inhibiting its reuptake.

In a recent study using COS-7 cells transfected with cDNAs for the human plasmalemmal noradrenaline transporter and/or the vesicular monoamine transporter, Pifl *et al.* (1999) support the suggestion that *d*-amphetamine both releases and inhibits the reuptake of noradrenaline. These authors found that in cells transfected with the plasmalemmal transporter only and preloaded with [^{3}H]noradrenaline, superfusion with *d*-amphetamine stimulated noradrenaline efflux only at concentrations lower than 10 μM; higher concentrations of *d*-amphetamine showed only small, or no effect at all on noradrenaline efflux. However, when the high concentrations of *d*-amphetamine were removed from the perfusion medium, noradrenaline efflux increased. In contrast, in cells transfected with both the plasmalemmal and the vesicular monoamine transporter, superfusion with *d*-amphetamine increased noradrenaline efflux with a maximal effect at 1mM, which was maintained at higher concentrations.

To explain their findings, the authors suggested that *d*-amphetamine interacts with the plasmalemmal transporter in two ways: firstly as a substrate, whereby it is transported into the cell and causes the release of noradrenaline, probably by *d*-amphetamine/noradrenaline exchange on the transporter (see evidence of Rudnick and Wall, 1992, below); secondly, at higher doses, it acts extracellularly to block at low affinity the transportation of substrates in either direction across the plasma membrane. According to this hypothesis, *d*-amphetamine can only reverse the direction of the plasmalemmal transporter from the inside of the cell. This suggestion would account for the reduction of noradrenaline efflux in the presence of high concentrations of *d*-amphetamine in cells transfected with the plasmalemmal transporter only: high doses of *d*-amphetamine bound to a transport-blocking extracellular site and prevented the efflux of noradrenaline. In cells transfected with both the plasmalemmal and the vesicular monoamine transporter, the authors proposed that high doses of *d*-amphetamine trigger release by mobilising an intracellular pool of noradrenaline (to which the vesicular transporter has been directed in the absence of synaptic vesicles). As the mobilised noradrenaline in the cytoplasm creates a steep concentration gradient with respect to the

extracellular medium, the noradrenaline is able to passively diffuse out of the cell. Whether support for this model of *d*-amphetamine's effects on noradrenaline release and reuptake can be gained from studies *in vivo* remains to be elucidated. However, it is of interest that in the study of Raiteri *et al.* (1975), high doses of *d*-amphetamine (10 μM) were used to study its effects on noradrenaline release: according to the hypothesis of Pifl *et al.* (1999) this could account for the lack of *d*-amphetamine's effects on noradrenaline release.

Amphetamine's ability to release not just intracellular stores of noradrenaline, but dopamine and 5-HT as well (Raiteri *et al.* 1975; Zettersrom *et al.* 1983; Heal *et al.* 1998b, see Table 1.2.) has prompted investigations into the exact mechanisms behind this phenomenon. These studies have tended to focus on the ability of *d*-amphetamine and its derivatives to release dopamine and 5-HT. Therefore, suggestions as to the mechanisms by which amphetamine induces noradrenaline release are largely based on evidence arising from such studies.

	NORADRENALINE	**5-HT**	**DOPAMINE**
100 nM *d*-amphetamine	57	-	56
1000 nM *d*-amphetamine	135	-	122
10,000 nM *d*-amphetamine	162	136	138

Table 1.2: Effect of d-amphetamine on [^{3}H]noradrenaline, [^{3}H]5-HT and [^{3}H]dopamine release from rat brain slices in vitro.

Numbers represent percentage release of [^{3}H]monoamine. - = non-significant. Data obtained from Heal *et al.* (1998a).

In their studies using *p*-chloroamphetamine (PCA), Rudnick and Wall (1992) showed that PCA induced 5-HT release from both human platelet plasma membrane and chromaffin granule membrane vesicles. In plasma membrane vesicles, PCA was found to compete with 5-HT as a substrate for translocation across the membrane. The efflux of [^{3}H]5-HT induced by PCA was dependent on the presence of external Na^+ and inhibited

by the 5-HT reuptake inhibitor, imipramine, which binds to the 5-HT transporter and prevents translocation. The authors concluded that as the proposed mechanism for exchange *via* the transporter requires cotransportation of Na^+ with the external substrate, the characateristics of PCA-induced 5-HT efflux could be mediated by 5-HT / PCA exchange.

In contrast, using a pH sensitive fluorescence marker, PCA was found to disrupt the pH gradient across chromaffin granule membranes, which drives 5-HT accumulation within the vesicles. This finding suggested that PCA, as a weak base, caused 5-HT efflux by binding intravesicular protons, thus increasing intracellular pH. If sufficient, the diminished availability of protons could weaken the ability of the proton gradient (generated by an ATPase) to maintain the intracellular accumulation of 5-HT against its concentration gradient: this would lead to the efflux of 5-HT.

Although the findings from studies such as the one of Rudnick and Wall cannot be assumed to generalise to each monoaminergic system, they do provide clues as to potential mechanisms involved in amphetamine's effects on noradrenergic function. In fact, to generalise these mechanisms to other monoaminergic systems may not be unreasonable considering that both plasmalemmal and vesicular transporters appear to be involved in amphetamine's releasing effects on all the monoamines (Rudnick and Wall 1992; Pifl *et al.* 1995; Pifl *et al.* 1999).

*1.3.3. d-F*enfluramine

The introduction of a trifluromethyl group into the phenyl ring of amphetamine (*e.g.* fenfluramine) separates the anorectic from the stimulatory effects of this drug (King and Ellinwood, 1992). As well as these behavioural differences, fenfluramine and amphetamine have markedly different pharmacological activities. Whereas amphetamine mediates its anorectic effects through actions on central dopaminergic and noradrenergic systems, the anorectic effect of fenfluramine is inhibited by 5-HT receptor antagonists. Furthermore, catecholaminergic depleting agents such as 6-hydroxydopamine, which reduce the anorectic effect of amphetamine, do not influence fenfluramine's effects on feeding behaviour (reviewed by Sugrue, 1987).

Feeding studies show that the *d*-isomer of fenfluramine has a greater anorectic effect than the *l*-isomer in both laboratory animals and humans and this effect is blocked by 5-HT receptor antagonists in both animals and humans (reviewed by Silverstone, 1992). Consistent with these findings, *d*-fenfluramine is a potent releaser of [^{3}H]5-HT from rat brain slices, although it has only weak inhibitory effects on [^{3}H]5-HT uptake (Heal *et al.* 1998b). Furthermore, microdialysis studies in rats showed that *d*-fenfluramine releases 5-HT *in vivo*, and that this effect is independent of serotonergic neuronal firing (Gundlah *et al.* 1997; see Chapter 3, section 3.1.).

Feeding studies in rats show that *d*-fenfluramine reduced food intake through the enhancement of satiety (Halford *et al.* 1995; see Chapter 3, section 3.1.). In addition, *d*-fenfluramine enhances diet-induced thermogenesis, although it has no effect on resting metabolic rate (Stallone and Levitsky, 1994). Although it has now been removed from the market as an anti-obesity agents, *d*-fenfluramine remains a useful pharmacological research tool, particularly for the purpose of comparing drugs that act on central serotonergic systems.

1.4. α_2-Adrenoceptors

1.4.1. α_2-Adrenoceptor subtypes

Adrenoceptors are cell membrane receptors belonging to the seven transmembrane spanning G-protein-linked family of receptors. The existence of subtypes for α_2-adrenoceptors was suggested in the mid 1980s by radioligand binding studies comparing the binding characteristics of various α_2-adrenoceptor agonists and antagonists. Experiments showed that prazosin, originally thought to interact with α_1-adrenoceptors only, caused a relatively potent inhibition (K_i <100 nM) of [^{3}H]rauwolscine binding in neonatal rat lung and rat kidney tissue. However, in human platelets, prazosin was relatively insensitive (K_i >1000 nM) at inhibiting [^{3}H]rauwolscine binding (Bylund, 1985; Petrash and Bylund, 1986). These two populations were

designated α_{2A} and α_{2B}, with the human platelet serving as the prototype tissue for α_{2A}-adrenoceptors and the rat neonatal lung serving as the prototype tissue for α_{2B}-adrenoceptors (see Bylund, 1988).

A third α_2-adrenoceptor subtype was identified in a cell line derived from opposum kidney and designated the α_{2C}-adrenoceptor (see Bylund, 1988). The fourth α_2-adrenoceptor subtype was first identified in the bovine pineal gland and rat submaxillary gland by Simonneaux *et al.* (1991). The α_2-adrenoceptors in these tissues had a pharmacological profile distinct from the α_{2A}- α_{2B}- or α_{2C}-adrenoceptor subtypes and was therefore designated the α_{2D}-adrenoceptor. However, later evidence suggested this receptor subtype was a species analogue of the human α_{2A}-adrenoceptor (Harrison *et al.* 1991).

Subtypes of α_2-adrenoceptors have also been cloned and binding studies using recombinant receptors show that they correspond well with the tissue-defined subtypes: the three subtypes cloned from human sources have characteristics of the α_{2A}- α_{2B}- and α_{2C}-adrenoceptor subtypes and those cloned from rat and mouse tissue have characteristics of the α_{2B}- α_{2C}- and α_{2D}-adrenoceptor subtypes (see Hieble and Ruffolo, 1996).

Evidence from functional studies supports the α_2-adrenoceptor subtype classification scheme. Bylund (1988) monitored α_2-adrenoceptor-mediated inhibition of cAMP production (see below) in human adenocarcinoma (HT29 cells) and neuroblastoma glioma hybrid cells (NG-108 cells), which express α_{2A}- and α_{2B}-adrenoceptors, respectively. The study found a high correlation between the ability of various antagonists to inhibit the dose-response curve generated by the α_2-adrenoceptor agonist, 5-bromo-6-(2-imidazoline-2yl)-aminol-quinoxaline (UK-14304) and their K_i values in the same cell line; this correlation did not exist between cell lines. These findings provided evidence to support the functional existence of α_{2A}- and α_{2B}-adrenoceptors.

Trendelenburg *et al.* (1993) suggested that the presynaptic α_2-adrenoceptors in rat cortex are α_{2D}-adrenoceptors, based on the ability of a range of α_2-adrenoceptor ligands

to modulate the release of preloaded [^{3}H]noradrenaline from cortical slices. Other studies employing a range of α_2-adrenoceptor ligands suggest that central postsynaptic α_2-adrenoceptors mediating mydriasis in rats are also α_{2D}-adrenoceptors (Heal *et al.* 1995b).

1.4.2. α_2-Adrenoceptors and second messenger systems

α_2-Adrenoceptors are negatively coupled to the enzyme adenylate cyclase *via* a G_i protein and this is thought to be the major cellular transduction process occurring after α_2-adrenoceptor activation. In 1988, Hertting and Allgaier showed that the inhibitory effects of the α_2-adrenoceptor agonist clonidine on [^{3}H]noradrenaline release from rabbit hippocampal slices is inhibited by treatment with islet-activating protein (IAP) or N-ethylmaleimide (NEM), which inactivate the regulatory G proteins G_i and G_o. Treatment with IAP and NEM alone, increased [^{3}H]noradrenaline release. These findings suggested that α_2-adrenoceptor-mediated signals are transduced across the plasma membrane *via* regulatory G proteins.

In 1991, Osborne showed that clonidine and the α_2-adrenoceptor agonist, UK-14,304 reduced the level of adenylate cyclase activity in rabbit retinal homogenates. In contrast, forskolin, which activates adenylate cyclase, increased the activity of this enzyme in rabbit retinal homogenates. The response to clonidine was blocked by pertussis toxin, which inhibits G_i-proteins exchanging bound GDP for GTP. The decrease in cAMP levels induced by clonidine or UK-14304 was inhibited by the α_2-adrenoceptor antagonists yohimbine or phentolamine but not by the α_1-adrenoceptor antagonist, prazosin. Serotonergic, cholinergic and β-adrenergic receptor antagonists were also without effect. These findings suggested that α_2-adrenoceptors in the rabbit retina exert inhibitory effects on adenylate cyclase activity that are mediated by an inhibitory guanine nucleotide regulating protein. Whether the α_2-adrenoceptor subtypes differ with respect to their interactions with G_i-proteins and the subsequent effects on adenylate cyclase is currently unknown.

1.4.3. α_2-Adrenoceptors and the regulation of noradrenergic neuronal activity

Noradrenergic neuronal activity is subject to autoregulation by α_2-adrenoceptors located on the cell bodies (somatodendritic α_2-adrenoceptors), as well as the axon terminals (terminal α_2-adrenoceptors). Studies demonstrated that clonidine, administered systemically or by direct (microiontophoretic) application, dose-dependently inhibited the spontaneous firing of noradrenergic neurones in the locus coeruleus of anaesthetised rats (Svensson *et al.* 1975; Cedarbaum and Aghajanian, 1976). In contrast, systemic administration of the α-adrenoceptor antagonist, piperoxane, produced a dose-dependent increase in the spontaneous firing rate of locus coeruleus neurones and, furthermore, returned the firing rate of these neurones to pre-injection levels following clonidine administration (Cedarbaum and Aghajanian, 1976). Microiontophoretic administration of noradrenaline or adrenaline to locus coeruleus neurones also reduced the spontaneous firing rate of these cells (Cedarbaum and Aghajanian, 1976; 1977). These findings suggested that locus coeruleus neurones possess α-adrenoceptors on, or in the vicinity, of their cell bodies and that these receptors regulate neuronal firing activity.

Both Svensson and Usdin (1978) and Scuvée-Moreau and Dresse (1979) monitored locus coeruleus neuronal activity in anaesthetised rats and showed that the noradrenaline reuptake inhibitor, desipramine (DMI) reduced the spontaneous firing rate of these neurones. Svensson and Usdin (1978) also showed that this effect was reversed by the α_2-adrenoceptor antagonist, yohimbine, suggesting that the increase in extracellular noradrenaline concentration induced by DMI activated α_2-adrenoceptors and reduced neuronal firing. More recently, Mateo *et al.* (1998) also showed that 1 mg / kg DMI decreased the firing rate of locus coeruleus neurones by approximately 73% in anaesthetised rats. This effect was completely reversed by systemic administration of the highly selective α_2-adrenoceptor antagonist, 2-[2-(2-methoxy-1, 4-benzodioxanyl)] imidazoline hydrochloride (RX821002), confirming that the depression in firing activity caused by DMI in anaesthetised rats is mediated by α_2-adrenoceptors.

α_2-Adrenoceptors have also been implicated in the control of lateral tegmental

noradrenergic neuronal firing. Systemic administration of clonidine has been shown to cause a dose-dependent inhibition of the spontaneous firing rate of cells in the A1, A2 and A5 noradrenergic regions (Andrade and Aghajanian, 1982; Moore and Guyenet, 1983; Kaba *et al.* 1986). Microiontophoretic application of clonidine also inhibited cells in the A5 noradrenergic region, suggesting that α_2-adrenoceptors located in the region of the cell bodies could mediate this inhibitory effect (Andrade and Aghajanian, 1982). In contrast, the α_2-adrenoceptors mediating the inhibitory effects of clonidine on the firing rate of A1 noradrenergic neurones are not thought to be located in the region of the cell bodies (Kaba *et al.* 1986, see Chapter 4, section 4.4.2.).

It is noteworthy that the studies above investigating locus coeruleus and lateral tegmental noradrenergic neuronal firing have all been carried out in anaesthetised rats. An extensive literature search revealed that there have been no studies investigating α_2-adrenoceptor-mediated control of noradrenergic neuronal firing in conscious, freely-moving rats. As anaesthesia can modify noradrenergic neuronal function (see Chapters 3 and 4) it cannot be assumed that the findings of the studies described above also apply to conscious rats.

Also during the 1970s, α_2-adrenoceptors located on the terminals of noradrenergic neurones were proposed to modulate noradrenergic activity. Starke (1972) found that in isolated perfused rabbit heart, preloaded with [^{14}C]noradrenaline, infusion of unlabelled noradrenaline decreased the stimulation-induced overflow of [^{14}C]noradrenaline. This effect was diminished in the presence of the α-adrenoceptor blocking agent, phenoxybenzamine, at doses that do not block noradrenaline reuptake. Vogel *et al.* (1972) measured [^{3}H]noradrenaline release from a culture of rat superior cervical ganglion that had formed axonal sprouts. Electrical- or K^+- induced stimulation of the cultured ganglia caused a Ca^{2+}-dependent increase in [^{3}H]noradrenaline efflux, which was enhanced by phenoxybenzamine. Since only presynaptic sites are present on the axonal sprouts, the site of action for the phenoxybenzamine-induced increase in release must be presynaptic. In isolated rabbit pulmonary arteries, preloaded with [^{3}H]noradrenaline, Starke *et al.* (1974) found that clonidine decreased the efflux of tritium evoked by electrical stimulation, suggesting that the receptors mediating this response were α_2-adrenoceptors. Findings

such as these, and others, led to the proposal that:

> *'presynaptic α-adrenoceptors are involved in the regulation of noradrenaline release through a negative feed-back mechanism mediated by the neurotransmitter itself'* (Langer, 1977).

In 1977, Langer suggested that the mechanism by which α_2-adrenoceptors decrease noradrenaline release involved a reduction in the availability of Ca^{2+} for excitation-secretion coupling. Noradrenaline release induced by electrical stimulation or by K^+ is Ca^{2+}-dependent and noradrenaline released by either of these mechanisms is decreased by stimulation of α_2-adrenoceptors. In contrast, noradrenaline release induced by the noradrenaline-depleting agent, tyramine, is not Ca^{2+}-dependent and presynaptic α_2-adrenoceptors have no influence on the release of noradrenaline induced by this drug (reviewed by Langer, 1977). It is possible that the decrease in cAMP induced by α_2-adrenoceptor activation inhibits the release of Ca^{2+} from intracellular stores or inhibits the influx of Ca^{2+} through Ca^{2+} channels in the membrane. Either of these processes would reduce the amount of intracellular Ca^{2+} available for excitation-secretion coupling. However, electrophysiological studies suggest that α_2-adrenoceptor activation causes a membrane hyperpolarisation *via* an increase in K^+ conductance. North and Surprenant (1985) made intracellular recordings in guinea-pig submucous plexus neurones. Superfusion with noradrenaline or clonidine induced dose-dependent membrane hyperpolarisations, which were inhibited by α_2-adrenoceptor antagonists. The hyperpolarisations induced by noradrenaline or clonidine were associated with an increased membrane conductance, which was abolished by low concentrations of the K^+ channel blockers Ba^{2+} or quinine. Although the α_2-adrenoceptors mediating this effect in guinea-pig submucous plexus neurones are postsynaptic, it is be possible that activation of presynaptic α_2-adrenoceptors also induce a hyperpolarisation though an increase K^+ conductance, which would make the neuronal terminal less excitable.

A number of studies carried out *in vivo* using various α_2-adrenoceptor ligands support Langer's proposal and show that inhibitory presynaptic α_2-adrenoceptors also exist in the central nervous system. Using the technique of microdialysis, local administration of the α_2-adrenoceptor agonists, clonidine or medetomidine at low doses

(0.5 μM) have been found to decrease the concentration of noradrenaline in dialysate samples from rat frontal cortex (Dalley and Stanford, 1995). Similarly, local administration of the α_2-adrenoceptor antagonists, yohimbine, idazoxan, or atipamezole, have been found to increase the concentration of noradrenaline in dialysate samples from rat cortex or hypothalamus (Dennis *et al.* 1987; Itoh *et al.* 1990; Laitinen *et al.* 1995; Dalley and Stanford, 1995; see Chapter 5, section 5.1.).

1.5. The obese Zucker rat

The obese Zucker rat (also called the Zucker-fatty rat) was first described by Zucker and Zucker in 1961. This rat occurred as a spontaneous mutant in a cross between the Merck Stock M and Sherman rats and appeared to be due to a single recessive gene (Zucker and Zucker, 1961). The obese condition of rats that are homozygous for this trait can be detected by body weight and appearance from as early as three weeks of age, whereas the body weight and appearance of heterozygotes and 'normal' homozygotes do not differ (Zucker and Zucker, 1961).

1.5.1. Prominent abnormalities in the obese Zucker rat

Obese Zucker rats have a mutation in the gene encoding the leptin receptor and this results in their insensitivity to this hormone (Takaya *et al.* 1996). Physiological abnormalities in the obese Zucker rats include hyperphagia, hyperlipidemia, and hyperinsulinemia (reviewed by Argilés, 1989). Although hyperphagia is a prominent characteristic of these rats, pair-feeding obese rats with lean rats does not inhibit the development of the obesity, suggesting this is not the only factor contributing to this condition (reviewed by Argilés, 1989). Consistent with this suggestion, brown adipose tissue (BAT) thermogenesis appears to be impaired in obese Zucker rats. Triandafillou and Himms-Hagen, (1983), investigated the activation of BAT thermogenesis by cold-exposure in lean and obese rats by measuring the binding of guanosine diphosphate (GDP) to BAT mitochondria (an index of the thermogenic proton conductance pathway). The authors report that the activation of BAT thermogenesis by cold-exposure (4 °C), as

indicated by an acute increase in GDP binding by BAT mitochondria, occurred normally in obese rats. However, the binding of GDP by mitochondria from obese rats maintained at 28 °C was lower than that of mitochondria from lean rats. The authors suggest that this low thermogenic state of BAT in obese rats maintained at 28 °C reflects a failure of the BAT to respond, not to cold-exposure, but to diet. A second experiment compared GDP binding in mitochondria from lean and obese rats fed a chow or cafeteria diet. The findings of this experiment showed an increase in GDP binding by mitochondria from lean rats on a cafeteria diet relative to lean rats fed chow. In contrast, GDP binding by mitochondria from chow-fed obese rats was not affected by cafeteria feeding. These findings indicate diet-induced thermogenesis is impaired in obese Zucker rats.

Consistent findings were found by York *et al.* (1985). These authors studied tissue noradrenaline turnover rates in both BAT and cardiac tissue of lean and obese rats maintained at 25-27 °C. In these experiments BAT noradrenaline turnover was undetectable in obese rats, whereas a half-life of 4 h was apparent in lean rats. In contrast, cardiac noradrenaline content, half- life and turnover rate were similar between lean and obese rats. These findings suggest mechanisms involved in BAT thermogenesis are impaired in the obese Zucker rat. However, in lean rats maintained at 4 °C, BAT noradrenaline half-life was reduced, whereas the noradrenaline content of the tissue and the turnover rate were increased. Similarly, a rapid turnover of noradrenaline was evident in obese rats maintained at 4 °C. Therefore, it is possible that an abnormality exists specifically in the mechanisms inducing diet-induced thermogenesis in obese rats.

1.5.2. Abnormalities in central noradrenergic function in the obese Zucker rat

Following the implication of noradrenergic transmission in the hypothalamus in the control of food intake, a number of studies focussed on whether changes in this catecholamine could account for the hyperphagia of obese Zucker rats. Cruce *et al.* (1976) measured the concentration of noradrenaline in various regions of hypothalamic tissue from 4-month-old female lean and obese Zucker rats. Noradrenaline concentration was reduced in the paraventricular nucleus of obese rats compared with non-obese, non-littermate controls, although not compared with non-obese, littermate controls. Further studies revealed a reduced noradrenaline concentration specifically in the

paraventricular nucleus of 2-month-old male obese Zucker rats compared with non-obese controls (Cruce *et al.* 1977).

Levin and Sullivan (1979) measured noradrenaline and dopamine concentrations in tissue from various brain regions in 7-month-old male and female Zucker rats using a radioenzymatic assay. These authors reported a reduction in the concentration of noradrenaline (to 51% of lean), as well as adrenaline (to 47% of lean), in the paraventricular nucleus tissue of obese female rats. Furthermore, a reduction in noradrenaline and adrenaline concentrations (to 47% and 42% of lean, respectively), as well as dopamine concentration (to 48% of lean), were found in the dorsomedial hypothalamic nucleus of obese female rats. In contrast, the noradrenaline concentration in the median forebrain bundle was increased in obese female rats to 201% of the female lean controls. The only differences found in obese male rats were a reduction in the dopamine concentration of the dorsomedial hypothalamic nucleus and medulla, to 60% and 43% of lean controls, respectively. However, although not significant, there was a trend for the noradrenaline concentration in the paraventricular nucleus of obese rats to be reduced.

Conflicting evidence is reported by Orosco *et al.* (1980). These authors measured the concentration of noradrenaline in whole hypothalami using a flurometric assay and found the concentration of noradrenaline to be *greater* in obese female Zucker rats compared with lean controls. Kuolu *et al.* (1990) measured central catecholamine concentrations in male Zucker rats aged 12-16 weeks using high-performance-liquid-chromatography. These authors reported no differences in noradrenaline or dopamine concentrations from different hypothalamic nuclei, including the paraventricular and ventromedial nuclei, between lean and obese rats.

The inconsistent findings above make it unclear whether noradrenergic activity in the hypothalamus of Zucker rats is reduced, increased or unchanged in the obese state. Some of the discrepancies could be due to different assay methods used to measure noradrenaline concentrations *in vitro*. However, studies carried out *in vivo* lend support to the hypothesis that noradrenergic activity is impaired in the hypothalamus of obese Zucker rats. Using *in vivo* microdialysis studies, Pacak *et al.* (1995) showed that 12-week-old male lean and obese Zucker rats have similar baseline extracellular noradrenaline

concentrations in the region of the paraventricular nucleus. However, the increase in extracellular noradrenaline concentration induced by immobilisation stress was reduced in obese rats to 60% of the lean response. These findings are in keeping with the reduced concentration of noradrenaline in the paraventricular nucleus of 2-month-old male obese Zucker rats (Cruce *et al.* 1977). However, the possibility that obese rats find immobilisation, induced by taping each limb to a metal frame with adhesive tape, less stressful than their lean counterparts cannot be ruled out.

1.6. Objectives

This study has compared the effects of sibutramine with those of the noradrenaline-releasing agent, *d*-amphetamine, on extracellular noradrenaline concentration in the frontal cortex of anaesthetised rats using *in vivo* microdialysis (Chapter 3). Firstly, a dose-response curve for sibutramine on extracellular noradrenaline concentration was established and compared with the dose-response curve for *d*-amphetamine. Following this experiments investigated whether sibutramine increases extracellular noradrenaline accumulation by inhibiting the reuptake of impulse-mediated noradrenaline release. This involved the blockade of presynaptic α_2-adrenoceptors, which regulate impulse-mediated noradrenaline release, and investigating whether this affected the accumulation of extracellular noradrenaline induced by sibutramine. Again, the effects of sibutramine on extracellular noradrenaline concentration were compared with *d*-amphetamine.

Since anaesthesia has been found to affect central noradrenergic activity, the effects of sibutramine on extracellular noradrenaline accumulation were also investigated, and compared with the effects of *d*-amphetamine, in conscious, freely-moving rats (Chapter 4). Furthermore, considering the hypothalamus has an important role in regulating food intake and thermogenesis, Chapter 4 also investigated the effects of sibutramine and *d*-amphetamine on extracellular noradrenaline concentration in this brain region of conscious, freely-moving rats. Considering the hypothalamus is innervated by noradrenergic neurones from the lateral tegmental system as well as the locus coeruleus system, the effects of sibutramine in the hypothalamus were compared with its effects in

the frontal cortex.

As evidence suggests that there is a greater density of terminal α_2-adrenoceptors in the hypothalamus compared with the cortex, Chapter 5 investigated whether this has functional consequences for the increase in extracellular noradrenaline concentration induced by sibutramine in these two brain areas. The effects of α_2-adrenoceptor blockade on the increase in extracellular noradrenaline concentration induced by sibutramine were investigated in 2 ways. Firstly, by investigating the effects of sibutramine in rats that were pretreated systemically with an α_2-adrenoceptor antagonist. Secondly, to investigate α_2-adrenoceptors specifically in the terminal fields, the effects of sibutramine on extracellular noradrenaline accumulation were monitored during infusion of an α_2-adrenoceptor antagonist into either the frontal cortex or hypothalamus.

The obese Zucker rat is a model of human juvenile-onset obesity and abnormalities in noradrenergic function in the hypothalamus of this rat have been observed. The findings that differences in presynaptic α_2-adrenoceptor function can influence the control of noradrenergic activity led to the comparison of the density and function of α_2-adrenoceptors in lean and obese Zucker rats in Chapter 6. The density of α_2-adrenoceptors was investigated using radioligand binding studies. The function of presynaptic, as well as postsynaptic, α_2-adrenoceptors was investigated using clonidine-induced hypoactivity and mydriasis, respectively.

Further essential background relating to this study is described in the relevant chapters.

Chapter 2

Methods

2.1 Introduction

The growing need to understand neurotransmitter systems in the CNS and their involvement in specific physiological, behavioural and pharmacological events has given rise to a number of techniques that enable measurement of neurotransmitter release *in vivo*. The development of these techniques over the past four decades, along with the concurrent improvement in assay methods, has culminated in two basic approaches for the measurement of neurotransmitter release used in the present day. These approaches are *in vivo* voltammetry and *in vivo* microdialysis and are discussed in relation to the measurement of monoamines in section 2.1.1.

A second approach to understanding neurotransmitter systems is to study the receptors to which the transmitter binds and induces a physiological response. Radioligand binding is a quantitative and qualitative technique developed to identify populations of receptors and their density in a given brain region. This technique exploits the recent development of a range of highly selective ligands for a variety of receptor subtypes. Consequently, radioligand binding has become a powerful tool for studying the pharmacology of subpopulations of receptors in the brain. The technique of radioligand binding as a tool for measuring α_2-adrenoceptors is discussed in section 2.1.2.

Another approach to investigating the pharmacology of α_2-adrenoceptors is to study the receptor function. Behavioural models of α_2-adrenoceptor function have been developed in rodents, making it possible to study the function of these receptors *in vivo*. A

further benefit to the pharmacologist is that these behavioural models allow the exclusive study of either pre- or post-synaptic α_2-adrenoceptor function. These behavioural models of α_2-adrenoceptor function are described in section 2.1.3.

2.1.1. Measurement of monoamine release *in vivo*

One approach to monitoring central monoamine release *in vivo* is to collect samples of cerebrospinal fluid. Substances dissolved in the cerebrospinal fluid can be separated out and quantified using various assay techniques. Monoamines can be separated out using high pressure liquid chromatography (HPLC). Following their separation, monoamines can be oxidised and quantified using electrochemical detection (ECD).

One of the first techniques developed for the collection of cerebrospinal fluid was the cortical cup (MacIntosh and Oborin, 1953). This technique involves removal of part of the skull and the placement of cylinder onto the cortical surface. The cup, formed by the cylinder in tight contact with the cortical surface, is filled with collecting fluid (typically Ringer's solution) into which cerebrospinal fluid from the cortex passes. The fluid in the cup is then collected and analysed for the substance of interest. Although very little tissue damage is encountered with the implantation of the cortical cup, the main limitation of this technique is that sampling is only possible form the surface of the cortex.

Further developments led to the push-pull cannula. This technique, proposed by Gaddum (1961), consisted of two concentric tubes: an inlet to drip perfusion fluid into a chosen brain region and an outlet to withdraw samples from the surrounding extracellular fluid (ECF). The advantage over the cortical cup technique is that sampling is possible from distinct brain regions. Indeed, this technique has been used to measure catecholamines in areas such as the hypothalamus (van der Gugten and Slangen, 1977) and nucleus accumbens (Bartholini, *et al.* 1976) of conscious, freely-moving rats.

One major drawback of the push-pull cannula technique is the extensive tissue damage resulting from the perfusion of relatively large volumes of fluid (typically 150 µl / min) directly into the tissue. More recent developments have led to 2 further

techniques for monitoring monoamine release *in vivo,* which cause less tissue damage than the push-pull cannula method. These are *in vivo* voltammetry and *in vivo* microdialysis.

Monoamines are readily oxidised. The technique of *in vivo* voltammetry harnesses this oxidation reaction by carrying it out at the surface of a carbon working-electrode implanted into a discrete brain region or slice (reviewed by Stamford, 1989). Electroactive species are oxidised at the electrode surface and the ensuing release of electrons is then measured in the form of current. The amount of current is directly proportional to the number of molecules oxidised, thus giving an indirect measure of changes in the concentration of monoamine in the extracellular fluid directly surrounding the electrode surface.

The success of *in vivo* voltammetry is largely due to the small size of the carbon-fibre electrodes (diameter: 8-12 μm), which minimises tissue damage. The degree of resolution between oxidised compounds depends on how the voltage is applied. Slow voltage scans, such as differential pulse voltammetry or linear sweep voltammetry, give high resolution whereas fast cyclic voltammetry or differential pulse amperometry have limited resolution, but can make up to 40 measurements per second. Differential pulse amperometry has been successfully used for the measurement of noradrenaline release in the thalamic nucleus of rats (*e.g.* Brun *et al.* 1993). However, the *in situ* measurement of neurotransmitters, without their prior separation, can create problems with the identification of peaks from compounds that oxidise at similar potentials. In addition, some drugs can oxidise at the same potential as the neurotransmitter of interest and contribute to the signal following injection into the animal, resulting in misleading data.

In vivo microdialysis involves the implantation of a fine dialysis fibre (typically 0.3 - 0.5 mm outer diameter with a molecular weight cut off point of 20 kD) into a selected brain area. Compounds in the extracellular fluid with a lower molecular weight than the cut off weight of the dialysis fibre diffuse down their concentration gradient into the perfusion fluid flowing through the dialysis fibre. The perfusate is collected at regular intervals and analysed for the substance of interest. The predominant improvement of this approach over the push-pull cannula technique is that the dialysis fibre creates a closed

system in which there is no direct contact between the tissue and the perfusion fluid. As well as reducing tissue damage, the dialysis fibre also prevents larger molecules passing into the perfusion fluid leading to cleaner samples for chemical analysis.

One of the earliest accounts of the use of *in vivo* microdialysis to measure neurotransmitter release was in 1966 by Bito *et al.* Small sacks of dialysis membrane, containing a sterile solution of 6% dextran, were chronically implanted into the brains of dogs in order to collect amino acids. The bags were removed 10 weeks later and the perfusate analysed. The technique has since undergone several technical improvements. One particularly beneficial improvement was the development of a system enabling the repeated collection of dialysis samples (Delgado *et al.* 1972). These authors created a dialytrode consisting of a push-pull cannula closed at the tip by a piece of dialysis membrane that could be implanted into any brain area.

Present day microdialysis probes exist in three basic forms. U-shaped probes were developed in the early 1980s and typically had an outer diameter of 600-800 μm. Two cannulae were joined by a piece of dialysis fibre bent into a tight loop (Zetterstom *et al.* 1982). Alternatively, cannulae can be arranged concentrically, with a thin inner cannula carrying fluid to the bottom of the dialysis probe where it leaves and passes back up the probe and is carried out by a lateral tube (outer diameter 520 μm, Tossman and Ungerstedt, 1986). Sandberg *et al.* (1986) created a probe with a side-by-side arrangement of two glass capillaries, serving as the inlet and outlet. The glass capillaries were inserted into a piece of dialysis fibre (outer diameter 300 μm) of which the other end was sealed off with epoxy glue. As well as producing a much narrower probe to the loop design, this arrangement also reduces the dead-space between the area of tissue being dialysed and the perfusion fluid compared with the concentric probes.

For this study, the technique of *in vivo* microdialysis was chosen to monitor extracellular noradrenaline concentration. A particularly useful advantage of this technique over *in vivo* voltammetry is that the microdialysis probe provides a simple way to locally apply drugs to the region of tissue being dialysed. The design of microdialysis probe used throughout the experiments is a modified version of the side-by-side arrangement described by Sandberg *et al.* (1986) (see section 2.2.1.1.).

2.1.1.1. Principle of microdialysis

The technique of microdialysis measures *changes* in the concentration of a given substance in the extracellular fluid directly surrounding the dialysis probe. The size of the dialysis probe does not allow sampling of extracellular fluid directly from a synapse. However, substances released from a neurone into the synapse overflow into the extracellular space and changes in their concentration in the extracellular space are thought to reflect changes in their concentration in the synapse. Support for this hypothesis comes from various experiments involving the manipulation of electrolytes in the perfusion fluid and monitoring subsequent changes in neurotransmitter concentrations (see section 2.2.1.7.).

The dialysis membrane is permeable to water and small solutes and is continually flushed on one side with a perfusion fluid devoid of the substances of interest. The other side of the membrane faces the extracellular space. The constant flow of perfusion fluid through the dialysis fibre maintains the concentration gradient causing the diffusion of substances from the extracellular space into the dialysis probe. The concentration of neurotransmitter in the dialysate reflects the concentration in the extracellular fluid. The constant flow of perfusion fluid through the dialysis fibre prevents the equilibration of substances across the membrane. Consequently, the concentration of substances in the dialysate will be lower than in the extracellular fluid. In an attempt to correct for this, the recovery of microdialysis probes can be assessed *in vitro.* The basic protocol to determine probe recovery is the same for all neurotransmitters. The protocol used to determine the recovery of noradrenaline from the probes used throughout this study was as follows:

1) A microdialysis probe is supported in a vial containing aCSF and is perfused for 30 min with aCSF at the flow rate used in experiments (1 µl / min).

2) The probe is then transferred to vial containing a solution of 5 nM noradrenaline made up in aCSF and is perfused with aCSF for 30 min.

3) A dialysate sample is then collected from the probe over a 20 min period (sampling interval used during experiments).

4) Following collection, the concentration of noradrenaline in the dialysate sample, as well the concentration in the aCSF, is analysed using HPLC-ECD.

5) Probe recovery (%) is calculated as :

$$\frac{\text{Concentration of noradrenaline in the dialysate sample}}{\text{Concentration of noradrenaline in aCSF}} \times 100$$

Using the protocol above, the recoveries of probes used in the experiments performed in this study were calculated to be ~10 %. However, it cannot be assumed that the recovery of a probe *in vitro is* the same as its recovery when implanted into a given brain region. For this reason, the concentrations of noradrenaline in dialysate samples collected during the experiments have not been corrected for probe recovery. In this study, the amount of noradrenaline in dialysate samples is expressed as the concentration of noradrenaline per unit time (*e.g.* fmol / 20 min).

2.1.1.2. Perfusion fluids.

The exact composition of extracellular and cerebrospinal fluid is not known, which makes it impossible to create artificial replicas. There are a variety of perfusion fluids that are used for microdialysis that vary in both electrolyte composition and pH. These range from simple salt solutions containing only sodium, potassium and calcium chlorides (Shimokawa *et al.* 1998; Gundlah *et al.* 1997; Itoh *et al.* 1990), to buffered aCSFs containing magnesium chloride and glucose (Routledge and Marsden, 1987; Done and Sharp, 1994). There is no overriding evidence to suggest that one particular perfusion fluid is preferable to another for the measurement of noradrenaline. In this study, aCSF comprising (mM) NaCl 140, KCl 3, $CaCl_2$ 1.2, $MgCl_2$ 1.0, Na_2HPO_4 1.2, NaH_2PO_4 0.27, glucose 7.2, pH 6.8) was used in all microdialysis experiments except those involving a high K^+ or Ca^{2+}-free perfusion fluid. In these experiments a modified Ringer's solution was used (see section 2.2.1.7. below).

The concentration of Ca^{2+} in the perfusion fluid is particularly important because it can influence basal and drug-evoked changes in extracellular neurotransmitter

concentration. The concentration of Ca^{2+} in the extracellular fluid has been estimated to be 1.2 mM (Benveniste *et al.* 1989; Mogahaddum and Bunney, 1989). Removal of Ca^{2+} from the perfusion fluid results in Ca^{2+} being drained from the extracellular space around the probe (Benveniste *et al.* 1989). This depletion of Ca^{2+} causes dialysate concentrations of various transmitters to decrease (Imperato and Di Chiara, 1984; Westerink and De Vries, 1988; van Veldhuizen *et al.* 1990). Likewise, increasing the Ca^{2+} concentration beyond 1.2 mM in the perfusion fluid causes basal neurotransmitter concentrations to increase (van Veldhuizen *et al.* 1990; Moghaddum and Bunney, 1989). The concentration of Ca^{2+} added to perfusion fluids varies between groups (Shimokawa *et al.* 1998: 2.3 mM; Gundlah *et al.* 1997: 1.8 mM; Done and Sharp, 1994; 1.2 mM). Based on the estimations that the extracellular concentration of free Ca^{2+} is 1.2 mM, this concentration of Ca^{2+} was used in the aCSF throughout this study.

2.1.2 Radioligand binding as a tool for quantifying α_2-adrenoceptor density

The most straightforward approach to identifying receptor sites is to measure the binding of a radioactive neurotransmitter or ligand to a membrane preparation containing the receptor of interest. One of the first accounts of a radioligand binding study was reported by Paton and Rang, who investigated [^{3}H]atropine binding to muscarinic receptors in slices of guinea-pig ileum (Paton and Rang, 1965). Since very small quantities of radiolabelled drugs can be readily and accurately measured, this approach is now widely used for receptor binding studies.

An important consideration in radioligand binding studies is the extent to which the measured binding of a radioligand represents association with the receptor of interest (termed 'specific binding'). Even with radioligands that are highly selective for a particular receptor, there will be some 'non-specific binding'. Non-specific binding could be attributable to the radioligand binding to other receptors, non-receptor tissue components or to non-biological substances used in the experimental procedures.

Non-specific binding has to be accounted for in a radioligand binding experiment in order to derive as an accurate estimate as possible of the specific binding. It is possible

to account for non-specific binding by measuring the amount of binding occurring in the presence an excess concentration of an unlabelled ligand with selectivity for the receptor of interest. The specific binding is then calculated as the difference between the total binding and the binding occurring in the presence of the unlabelled ligand.

As well as being selective, it is also desirable for the radioligand to have a high affinity for the receptor of interest. This allows the binding to be studied at low concentration, which will reduce the non-specific binding. It is also advantageous for the radioligand to have a high specific activity so that very small quantities of bound radioligand can measured accurately. The specific activity of a radioligand is a measure of its radioactivity and is expressed as becquerels or curies per mol of ligand. The more rapidly an isotope decays (*i.e.* the shorter the half-life) the more disintegrations occur per unit time, resulting in a higher specific activity.

The pharmacology and distribution of α_2-adrenoceptors has been extensively studied with a variety of radioloabelled α_2-adrenoceptor agonists and antagonists (*e.g.* [^{3}H]clonidine, Wikberg *et al.* 1987; [^{3}H]*p*-aminoclonidine, Jhanwar-Uniyal *et al.* 1991; [^{125}I]*p*-iodoclonidine, Pesonen *et al.* 1992; [^{3}H]idazoxan Heal *et al.* 1993; [^{3}H]RX821002, Langin *et al.* 1990). An important consideration when choosing a radiolabelled ligand for a binding experiment is whether the ligand is an agonist or antagonist. Agonists and antagonists are thought to bind to different receptor conformational states, therefore giving different measures of the receptor population. In G-protein-linked receptors, such as the α- and β-adrenoceptors, agonists bind to the high affinity conformational state. However, at a high enough concentration, antagonists also bind to the low affinity conformational state of G-protein linked receptors. Therefore, in saturation binding studies, a radiolabelled antagonist gives a better estimate of the total receptor population than a radiolabelled agonist.

Radioligand binding studies were used to compare the total number of α_2-adrenoceptor binding sites in the frontal cortex and hypothalamus of lean and obese Zucker rats. For this reason a tritiated form of the highly selective α_2-adrenoceptor antagonist 2-[2-(2-methoxy-1,4-benzodioxanyl)]imidazoline hydrochloride (RX821002)

(Langin *et al.* 1990) was used. Non-specific binding was determined by adding an excess concentration of the nonselective α-adrenoceptor antagonist, phentolamine. Although phentolamine binds to both α_1- and α_2-adrenoceptors, [^{3}H]RX821002 has a selectivity ratio for α_2-adrenoceptors over α_1-adrenoceptors of 316 (Stillings *et al.* 1985). The vast majority of [^{3}H]RX821002-binding is attributed, therefore, to the binding of α_2-adrenoceptors.

Types of radioligand binding experiments include saturation experiments, inhibition experiments, association kinetic experiments and dissociation kinetic experiments. Each experiment leads to a different type of information. Saturation experiments determine the affinity of a given ligand for a receptor as well as the number of binding sites. Inhibition experiments calculate an inhibition constant for a given inhibitor to displace a radioligand-receptor complex. The inhibition constant is the same as the affinity of the inhibitor for the receptor. Association and dissociation kinetic experiments determine the association and dissociation rate constants for the receptor-ligand complex. In this study, radioligand binding studies were used to estimate and compare the density of RX821002-binding sites (B_{max}) in the frontal cortex and hypothalamus of the Zucker rat brain. For this reason, saturation experiments were performed.

Saturation experiments involve incubating a range of concentrations of the radioligand with a fixed concentration of tissue and measuring the amount of ligand bound. The affinity (usually expressed as the dissociation constant, K_d) and B_{max} are determined by plotting the ratio of bound to free radioligand versus the bound radioligand (Rosenthal plot). The K_d is the negative reciprocal of the slope and the B_{max} is the intercept of the line with the x-axis. In this study, the K_d and B_{max} were determined by non-linear regression analysis fitted to a one-site binding model using the LIGAND computer programm (Munson and Rodbard, 1980)

2.1.3 Behavioural models of monoaminergic receptor function

Behavioural models of receptor function are an important tool for investigating functional changes in receptors following drug treatment as well as providing a means for studying interactions between different neurotransmitter systems *in vivo*. Numerous behavioural models have been developed to study the various receptors of the serotonergic system. For example, activation of 5-HT_{1A} receptors with 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) induces hypothermia and the serotonin behavioural syndrome (including forepaw treading, head-weaving and flat body posture) in rats through an action on presynaptic and postsynaptic 5-HT_{1A} receptors, respectively (Goodwin *et al.* 1987). Activation of 5-HT_{2A} receptors induces head twitches in rats (Schreiber *et al.* 1995) whereas activation of 5-HT_{2C} receptors induces hypophagia (Fone *et al.* 1998)). These approaches have been used to investigate the pharmacology of drugs such as antidepressants (*e.g.* Maj and Moryl, 1992).

With the exception of α_2-adrenoceptors, behavioural models of central α- and β-adrenoceptor functions are not as robust as those 5-HT receptor functions. One particular difficulty with the development of behavioural models for α_1-adrenoceptor function is that the selective agonist, phenylephrine does not cross the blood-brain barrier. A particular advantage of most behavioural studies is that they do not involve any anaesthesia or surgery. This advantage is lost in studies where drugs, such as phenylephrine, have to be injected centrally. The development of behavioural models to investigate the β-adrenoceptor function is limited because few behavioural changes occur following β-adrenoceptor stimulation. Nevertheless, various β-adrenoceptor agonists, such as isoprenaline and salbutamol, have been found to induce a sedative effect and marked drinking response in rodents. However, these β-adrenoceptor agonist-mediated responses are affected by α_2-adrenoceptor agents. For example, the drinking response induced by isoprenaline is decreased by the α_2-adrenoceptor agonist, clonidine, and enhanced by the α_2-adrenoceptor antagonist, yohimbine. Furthermore, yohimbine inhibits the sedative response induced by β-adrenoceptor agonists. This complicates the use of these responses as selective models of β-adrenoceptor function (reviewed by Heal, 1990).

In contrast to α_1-adrenoceptors and β-adrenoceptors, behavioural models of α_2-adrenoceptor functions have been well characterised. Two very prominent effects following administration of the α_2-adrenoceptor agonist, clonidine, are sedation and mydriasis and these responses have formed the basis of two robust models used as functional indices of α_2-adrenoceptors (clonidine-induced hypoactivity and mydriasis, respectively). These two models are described, in turn, below.

2.1.3.1. Clonidine-induced hypoactivity

The sedative effect of clonidine can be assessed in rats either by measuring the inhibition of exploratory behaviour (*e.g.* by measuring the number of lines crossed on the floor of an arena) or by visually assessing the extent of the hypoactivity syndrome. Drew *et al.* described the hypoactivity syndrome in 1979 and devised up to 8 components by which it could be measured. These components were passivity, tactile responsiveness, posture, gait, visual attention, righting reflex, body sag and ptosis. Changes in these components are scored following drug treatment and summed to give a measure of the extent of hypoactivity. A later study suggested that measurement of the hypoactivity syndrome is a more specific measure of α_2-adrenoceptor function than the measurement of exploratory behaviour. By scoring the first five components of the hypoactivity syndrome described above, this study showed that non-noradrenergic agents, such as sodium pentobarbitone and diazepam, which induce sedation, do not induce changes in the hypoactivity syndrome (Heal *et al.* 1981).

Drew *et al.* (1979) reported that both systemically administered and intracerebroventricular injection of clonidine induced hypoactivity in rats in a dose-dependent manner. In contrast, intracerebroventricular injection of the α_1-adrenoceptor agonist, phenylephrine, did not induce hypoactivity. The hypoactivity induced by systemic administration of clonidine was inhibited by intracerebroventricular injection of the α_2-adrenoceptor antagonist, yohimbine, but was not inhibited by the α_1-adrenoceptor antagonist, prazosin. This strongly suggested that the hypoactivity induced by clonidine is mediated by central α_2-adrenoceptors. Nevertheless, the precise location of the receptors within the CNS has never been determined. Tsoucaris-Kupfer and Schmitt (1972) reported that clonidine administered directly into the rostral hypothalamus of rats

induces sedation. However, whether this treatment also induces the hypoactivity syndrome has not been investigated.

Early findings reported that the presynaptic population of α_2-adrenoceptors represented only a small proportion of α_2-adrenoceptors in the brain (U'Prichard *et al.* 1980; Dooley *et al.* 1983) and this promoted the idea that post-synaptic α_2-adrenoceptors mediated clonidine-induced hypoactivity. This theory has since been challenged. Studies have found that the enhancement of noradrenergic activity with amphetamine or desipramine reverses the suppression of clonidine-induced exploratory behaviour (Strömbom and Svensson, 1980). This suggests that clonidine-induced sedation arises from the reduction of noradrenaline release as a result of α_2-adrenoceptor activation. Further support for this theory comes from reports that desipramine injection and intracerebroventricular administration of methamphetamine both reverse clonidine-induced hypoactivity (Heal *et al.* 1983; Heal, 1989b). Although methamphetamine also has some dopaminomimetic actions, this is not thought to be important for the reversal of clonidine-induced hypoactivity because the effect cannot be mimicked by the dopamine receptor agonist, apomorphine (Heal *et al.* 1987).

Although the evidence above supports a strong case for the presynaptic location of α_2-adrenoceptors mediating clonidine-induced hypoactivity, studies in rats involving the lesioning of noradrenergic neurones do not support the theory. The lesioning of noradrenergic neurones with the neurotoxins 6-hydroxydopamine (6-OH-DA) or N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) have no effect on the suppression of locomotor activity induced by clonidine (Spyraki and Fibiger, 1982; Nassif *et al.* 1983). Nevertheless, Nassif *et al* (1983) injected 6-OH-DA into the locus coeruleus only. This produced a 93% and 85% depletion of noradrenaline content in the frontal cortex and hippocampus, respectively. These regions are innervated exclusively by locus coeruleus neurones. However, it is unlikely that the noradrenaline content of regions innervated by noradrenergic neurones of both the locus coeruleus and lateral tegmental system (*e.g.* the hypothalamus) would have been affected to the same extent. Indeed, noradrenaline content in the brainstem was depleted by only 33% following 6-OH-DA lesion. Although Spyraki and Fibiger (1982) used a systemic injection of DSP-4, the

noradrenergic neurones of the lateral tegmental system are more resistant to the toxic effects of DSP-4 than are neurones of the locus coeruleus (Fritschy *et al.* 1990, see Chapter 4, section 4.1). This is reflected in their study, which found that the noradrenaline contents of the cortex and hippocampus were reduced by 70-85% compared with controls, whereas the noradrenaline content of the hypothalamus was reduced by only 26%. Therefore, these studies cannot rule out the possibility that presynaptic α_2-adrenoceptors on noradrenergic neurones of the lateral tegmental system are responsible for clonidine-induced hypoactivity.

However, a later study in mice, using a higher dose of DSP-4 (100 mg / kg) to that used by Spyraki and Fibiger (1982) found that clonidine-induced hypoactivity is markedly attenuated in lesioned mice compared to saline-treated controls (~50% attenuation, Heal *et al.* 1989b). In this study the whole brain noradrenaline content was depleted by 75% in DSP-4 lesioned animals. This discrepancy could be due to the different methods used to rate the sedation induced by clonidine. Spyraki and Fibiger (1982) rated locomotor activity following clonidine adminsitration, whereas Heal *et al.* (1989b) scored the hypoactivity syndrome: the latter approach is a more sensitive measure of α_2-adrenoceptor function.

Considering all the available evidence for the location of α_2-adrenoceptors mediating clonidine-induced hypoactivity (reviewed by Heal, 1990), there is a strong consensus that these receptors are located presynaptically within the CNS.

2.1.3.2 Clonidine-induced mydriasis

Systemic administration of clonidine induces mydriasis in a dose-dependent manner in several species including cats, rats and mice and can be monitored by the simple measurement of pupil diameter (Koss, 1986; Heal *et al.*, 1989a; 1989b; 1995a). This effect of clonidine in cats, rats and mice is inhibited by systemic administration of the α_2-adrenoceptor antagonists, yohimbine, idazoxan or the highly selective antagonist, RX821002, but is not affected by the α_1-adrenoceptor antagonist, prazosin or the β-adrenoceptor antagonist, pindolol (Koss *et al.* 1986; Heal *et al.* 1989a; 1995a; 1995b). Furthermore, clonidine-induced mydriasis in mice is not affected by the systemic

administration of 5-HT or dopamine antagonists (Heal *et al.*1989a). These findings strongly suggest that mydriasis is mediated through α_2-adrenoceptors.

It has been proposed that clonidine-induced mydriasis is mediated through a reduction of parasympathetic tone to the iris (Gherezghiher and Koss, 1979). The iris receives parasympathetic tone from preganglionic cell bodies that are thought to originate from the Edinger-Westphal and anteromedian nuclei of the oculomotor complex (Sillito and Zbrożyna, 1970). Stimulation of the afferent sciatic nerve induces pupil dilation in rats by CNS parasympathoinhibition. Hey *et al.* (1985) showed that pupil dilation evoked by afferent sciatic nerve stimulation in rats is abolished by systemic administration of yohimbine or by treatment with reserpine in combination with α-methyl-para-tyrosine, which reduced the concentration of noradrenaline in the brain by > 95%. These findings suggest that that an ascending noradrenergic pathway could have an inhibitory influence over parasympathetic neurones projecting to the iris. This suggestion is consistent with the proposal that clonidine-induced pupillary dilation is a consequence of a reduction in parasympathetic tone.

Studies involving the removal of CNS parasympathetic tone to the iris, either by using pithed rats or by electrolytic lesions in the CNS efferent parasympathetic pathway, prevent clondine-induced mydriasis (reviewed by Koss, 1986). These findings suggest that clonidine acts centrally to decrease parasympathetic tone to the iris. Further evidence supporting this proposal comes from experiments showing that clonidine administered by intracerebroventricular injection induces a marked mydriasis in mice at a dose that is ineffective when administered peripherally (Heal *et al.* 1989a). The noradrenaline releasing agent, methamphetamine, also induces mydriasis in a dose-dependent manner in rats and mice when administered systemically or by intracerebroventricular injection (Heal *et al.* 1989a; 1989b; 1995a). Both clonidine and methamphetamine-induced mydriasis are inhibited by the intracerebroventricular administration of idazoxan or yohimbine. In contrast, intracerebroventricular administration of the α_1-adrenoceptor antagonist, prazosin or β-adrenoceptor antagonist, pindolol, has no effect on clonidine- or methamphetamine-induced mydriasis (Heal *et al.* 1989a; 1989b).

It is thought that the α_2-adrenoceptors mediating clonidine-induced mydriasis are located on the Edinger-Westphal nucleus since injection of clonidine into this region produces mydriasis in the cat (Sharpe and Pickworth, 1981). Strong evidence suggests that the α_2-adrenoceptors mediating clonidine-induced mydriasis are located postsynaptically. Pretreatment of cats or rats with α-methyl-*p*-tyrosine and reserpine, which results in an almost total depletion of noradrenaline within the brain, has no effect on clonidine-induced mydriasis (reviewed by Koss, 1986). Pretreament of rats or mice with the noradrenergic neurotoxin, DSP-4 also has no effect on clonidine-induced mydriasis, but potently inhibits the mydriasis induced by methamphetamine (72-100% inhibition, Heal *et al.* 1989b; 1995a). This strongly suggests that clonidine acts on postsynpatic α_2-adrenoceptors to induce mydriasis, whereas methamphetamine acts indirectly on these receptors by increasing extracellular noradrenaline concentration.

Furthermore, the extent of methamphetamine-induced mydriasis in mice is additive in combination with clonidine (Heal *et al.* 1989b). Since clonidine decreases extracellular noradrenaline concentration whereas methamphetamine increases it (Glowinski and Axelrod, 1965; Raiteri *et al.* 1975), the additive effect of the two drugs on mydriasis strongly suggest that the α_2-adrenoceptors mediating this response are located postsynaptically.

2.2 Techniques

2.2.1. *In vivo* microdialysis

2.2.1.1. Construction of microdialysis probes

Microdialysis probes were designed as a modification of the single-cannula probe described by Sandberg *et al.* (1986). Figure 2.1. shows a schematic representation of the basic steps involved in constructing the probes. A detailed description of probe construction is described below.

1) Two 3 cm lengths of silica glass tubing (i.d. 75 μm, o.d. 150 μm, Scientific Glass Engineering) were inserted into a 1.5 cm long steel cannula.

2) The coating of the glass was then burnt off to reduce the outer diameter and the two pieces of glass glued together at the base of the cannula with a 4 mm stagger. The shorter piece of glass protruded 2 and 5 mm from the cannula for probes intended for experiments in the frontal cortex and hypothalamus, respectively.

3) A piece of semipermeable membrane (i.d. 240 μm, o.d. 300 μm, molecular cut-off 20 kD, Filtral 12, AN69, Hospal Industrie, France) was then placed over the two pieces of glass protruding from the base of the cannula and secured to the cannula with fast setting epoxy glue (Araldite).

4) The membrane was then cut so that it protruded 2 mm over the end of the longer piece of glass and then the end was sealed with epoxy glue. The resulting dialysis zone of probes intended for the frontal cortex was 5 mm. The upper part of the membrane of probes intended for the hypothalamus was covered with epoxy glue so that the resulting dialysis zone was 1.5 mm.

5) The two lengths of tubing protruding from the upper part of the cannula were inserted individually into two 4 cm lengths of polythene tubing (i.d. 0.28 mm, o.d. 0.61 mm, Portex). The polythene was then secured to the cannula using epoxy glue. The tubing placed over the shorter piece of glass and the longer piece of glass was designated as the inlet and outlet, respectively.

6) Probes were made in batches of 10 and stored in an airtight container.

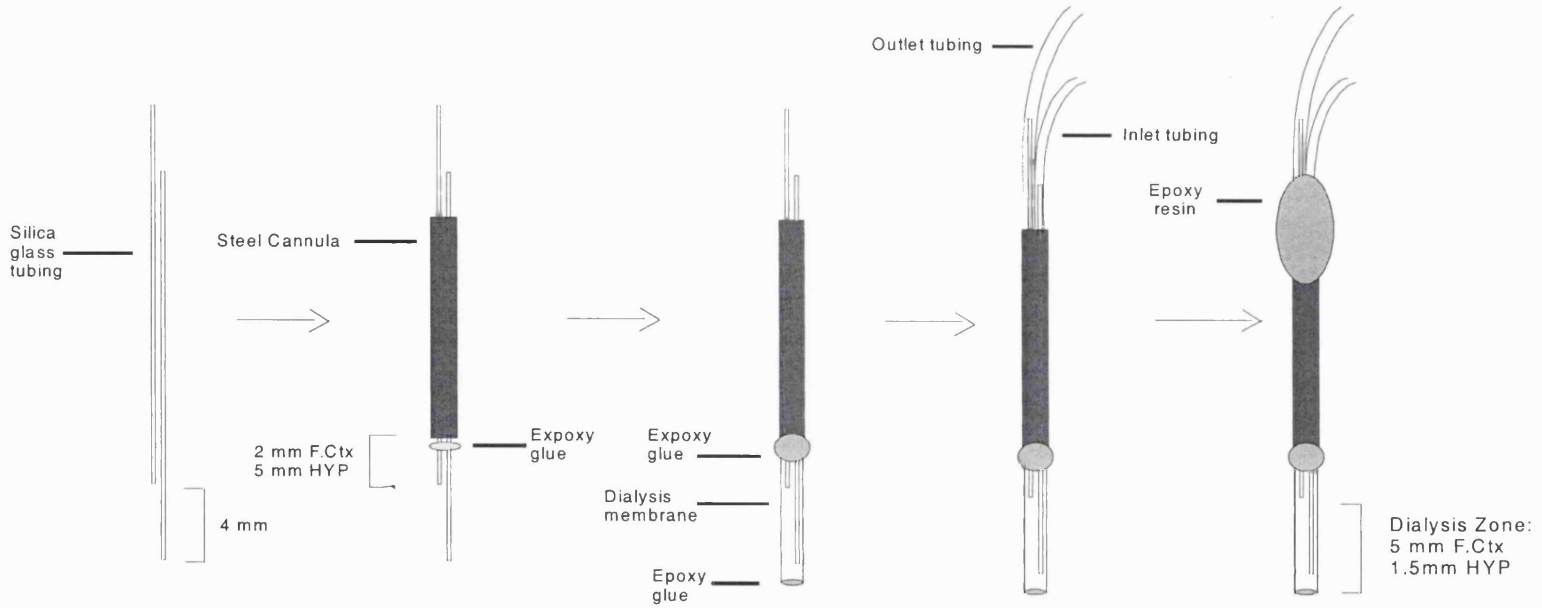


Figure 2.1: *Step-by-step construction of a microdialysis probe for the frontal cortex or hypothalamus*

Figure presents an overview of the basic steps involved in microdialysis probe construction. A detailed description of probe construction is given in the text (section 2.2.1.1.). F.Ctx = Frontal cortex. HYP = Hypothalamus. Drawings are not to scale.

2.2.1.2. Surgical implantation of microdialysis probes

2.2.1.2.1. Animals

Male outbred Sprague-Dawley rats (250-320 g) were obtained from the colony at University College London. They were housed in groups of four at 21°C and 55% humidity with a light-dark cycle of 12 h (lights on at 08.00 h). Animals had free access to food and tap water at all times. Drug-naïve animals were used in each experiment and all procedures complied with the U.K. Scientific Procedures (Animals) Act, 1986.

2.2.1.2.2. Non-recovery rats ('anaesthetised' rats)

Anaesthesia of rats was induced by inhalation of 5% halothane in combination with 95% O_2 / 5% CO_2 delivered through an induction chamber at 2000 ml / min. Following the loss of the righting reflex, the anaesthetic was delivered via a face mask placed over the animal's nose (2.5% halothane in combination with 95% O_2 / 5% CO_2 at 1000 ml / min). Following the loss of the pedal reflex, an incision was made and the trachea exposed. A polythene cannula (2 cm; i.d. 1.67 mm, o.d. 2.42 mm, Portex) was inserted through a small hole made in the trachea and sewn into place with cotton. A Y-piece was then attached to the cannula to enable the direct delivery of the anaesthetic into the trachea (1.5% halothane in 95% O_2 / 5% CO_2 at 500ml / min) and the removal of expired gases.

Following tracheotomy, the rat was placed in a stereotaxic frame in the flat skull position (incisor bar set at 3.3 mm below the interaural line) and a rectal probe inserted to maintain a core body temperature of 37 °C using a homeothermic heating pad. The surface of the skull was exposed to reveal bregma and a small hole (diameter ~2 mm) drilled through the skull (frontal cortex: AP +3.5 mm, ML ±1.5 mm relative to bregma, according to the atlas of Paxinos and Watson, 1986) using a trepanning drill burr. The dura was then broken with a needle and a microdialysis probe, primed with aCSF, was slowly lowered vertically into the brain (frontal cortex: DV -5mm relative to dura, according to the atlas of Paxinos and Watson, 1986). After completion of the implantation, the probe was connected with a piece of portex tubing (i.d. 0.58 mm, o.d. 0.96 mm) to a gas-tight syringe (Hamilton), containing aCSF, fitted to a perfusion pump. The probe was perfused with aCSF at a rate of 1µl / min for the duration of the experiment.

2.2.1.2.3. Recovery rats ('freely-moving'rats)

Anaesthesia of rats was induced by inhalation of 5% halothane in combination with 95% O_2 / 5% CO_2 delivered through an induction chamber at 2000 ml / min. Following the loss of the righting reflex, rats were transferred to a stereotaxic frame and the anaesthetic was delivered via a face mask (1.5-2% halothane in combination with 95% O_2 / 5% CO_2 at 1000 ml / min). The head was held in the flat skull position (incisor bar set at 3.3 mm below the interaural line) using blunted, non-rupture ear bars. Core body temperature was maintained at 37°C using a homeothermic heating pad and rectal probe.

A small incision was made and the surface of the skull was exposed to reveal bregma. A small hole was made through the skull (frontal cortex: AP +3.5 mm, ML ± 1.5 mm; hypothalamus: AP –1.8 mm, ML ± 0.4 mm relative to bregma, according to the atlas of Paxinos and Watson, 1986) using a trepanning drill burr. Two small screws were then screwed into the skull surface on either side of the implantation site. These were used to anchor the probe to the skull with dental cement following implantation. The dura was broken using a needle and a microdialysis probe primed with aCSF slowly lowered vertically into the brain (frontal cortex: DV -5 mm; hypothalamus; DV -9.2 mm relative to dura, according to the atlas of Paxinos and Watson, 1986). A small circle of bone wax was placed around the probe at the surface of the brain to prevent the dental cement entering the brain. The probe was then secured to the skull surface using dental cement. When the cement had dried the inlet and outlet of the probe were sealed with bone wax to prevent blockage prior to the experiment.

When the implantation was complete, rats were transferred to an incubation chamber for recovery from the anaesthesia (~45 min). Rats were then placed individually in plastic cages overnight for the experiment the next day.

2.2.1.3. Verification of probe placement

The position of the microdialysis probe in the brain was verified histologically. At the end of experiments brains were removed and stored in 10% formaldehyde and left for one week. Brains were then sectioned on a freezing microtome and the position of the microdialysis probe in a coronal slice compared to the atlas of Paxinos and Watson 1986).

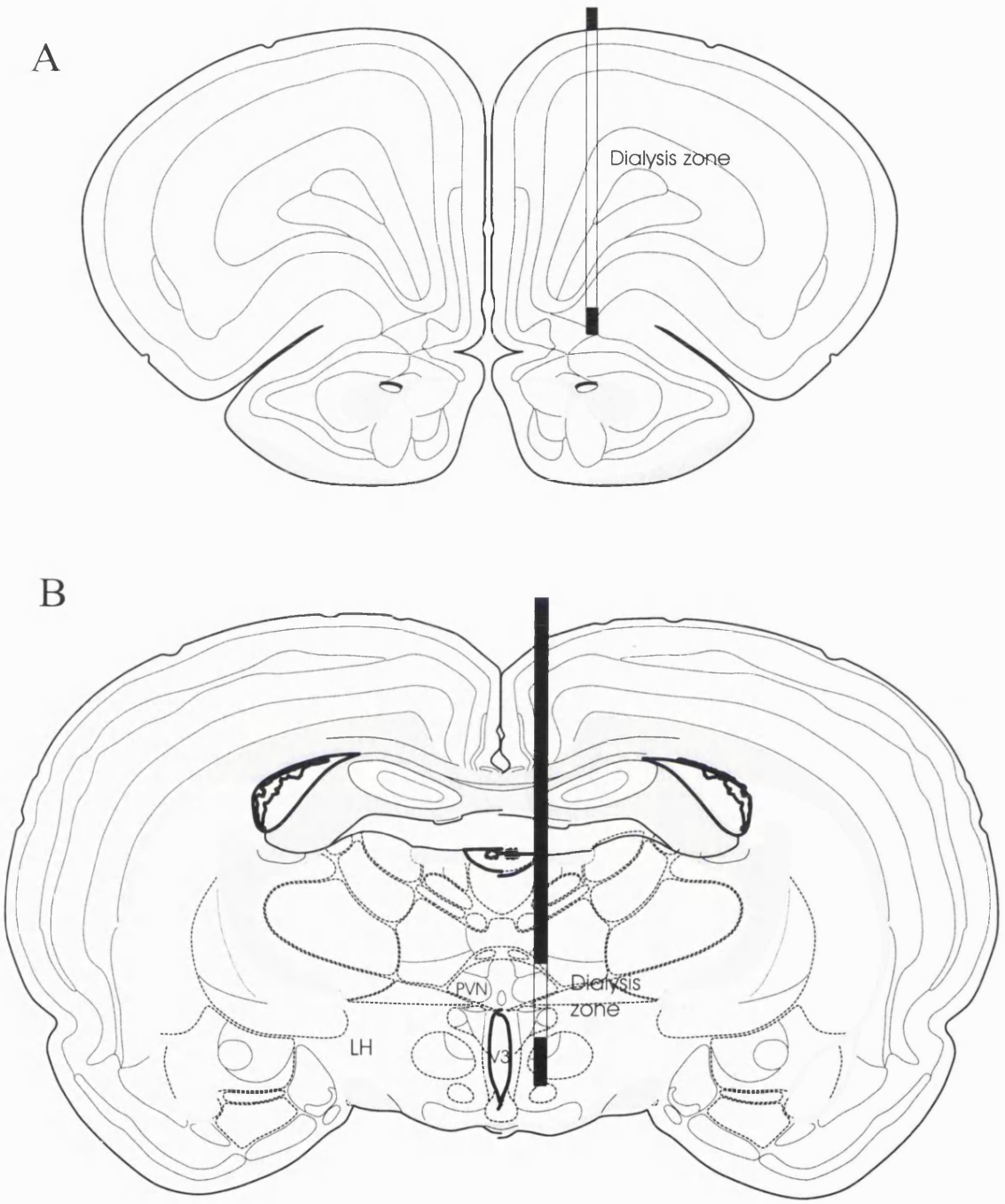


Figure 2.2: *Position of the microdialysis probe in the frontal cortex and hypothalamus.* Diagrammatic representations of coronal sections of rat brain 3.5 mm (A, frontal cortex) and -1.8 mm (B, hypothalamus) from bregma. The position of the microdialysis probe is illustrated. The unfilled portion of the probe represents the dialysing zone. LH, lateral hypothalamus; PVN, paraventricular nucleus; V3 third ventricle. Figures adapted from Swanson (1998/1999).

Figures 2.2a and 2.2b show diagrammatic representations of the position of the microdialysis probe in the frontal cortex and the region of the paraventricular nucleus of the hypothalamus, respectively. Figure 2.3. shows a photograph of a brain slice taken from a halothane-anaesthetised rat with a microdialysis probe implanted into the hypothalamus. The probe was perfused with Evans Blue dye for 160 min to indicate the area of dialysis.

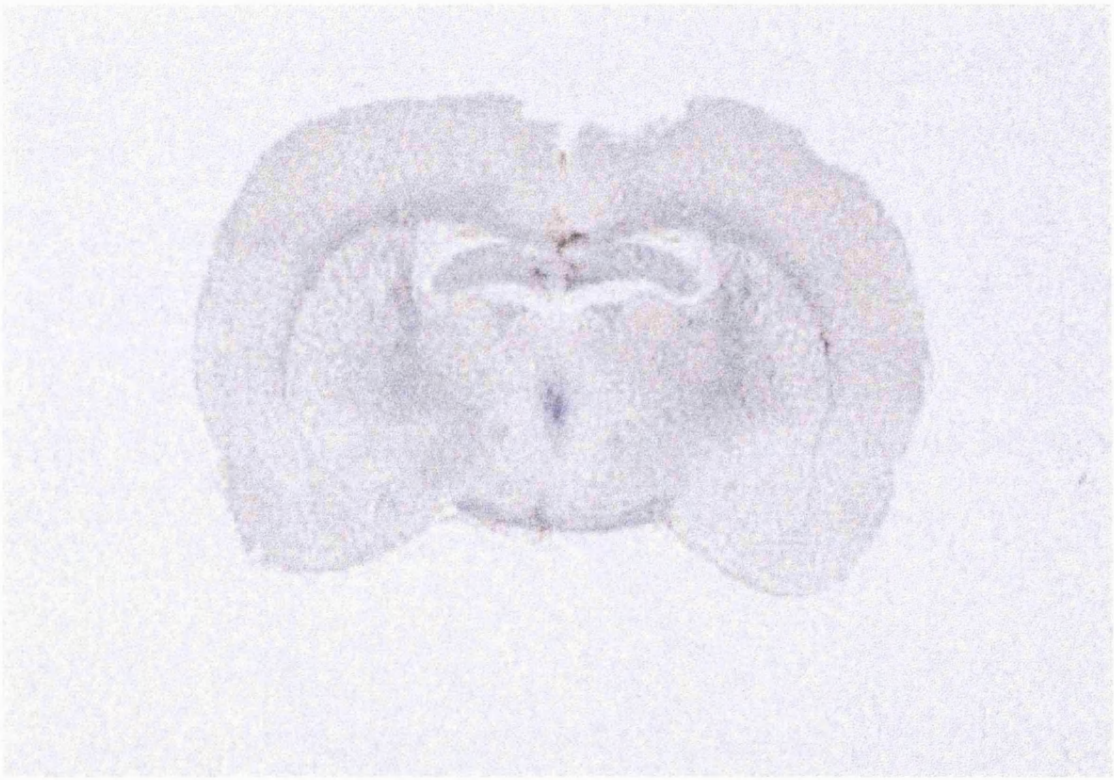


Figure 2.3:*Photograph of a brain slice from a rat with a microdialysis probe implanted into the hypothalamus and perfused with Evans Blue dye.*

Area of blue indicates the dialysing zone of the microdialysis probe.

2.2.1.4. Microdialysis procedure

2.2.1.4.1. Collection of dialysates (anaesthetised rats)

Collection of dialysate started approximately 2 h after probe implantation. Thereafter, samples were collected directly from the probe outlet into polyethyene tubes at 20 min intervals. The polyethyene tubes contained 5 µl perchloric acid (0.01 M) to prevent the spontaneous oxidation of noradrenaline before analysis. Samples taken over the first 2 h were used to estimate resting extracellular noradrenaline concentration. These are referred to as 'basal' samples. When the dialysate noradrenaline concentration in four consecutive samples was stable, a test drug was administered.

Following collection, samples were immediately stored on ice and their noradrenaline content analysed by HPLC-ECD on the same day as collection. On the rare occasion when analysis of samples was not possible on the same day, samples were stored at -70^{o}C until the following day.

2.2.1.4.2. Collection of dialysates (freely-moving rats)

On the day after probe implantation rats were kept in their individual cages and moved into the experimental laboratory. Their probes were connected with a piece of portex tubing (i.d. 0.58 mm, o.d. 0.96 mm) to a gas-tight syringe (Hamilton), containing aCSF, fitted to a perfusion pump. The probe was perfused with aCSF at a rate of 1µl / min for the duration of the experiment. The outlet of the probe was attached to a 20 cm length of fine bore tubing (1.2 µl / 100 mm; Biotech Instruments Ltd) which was extended over the side of the cage to allow collection of dialysates with minimum disturbance to the rat. In experiments involving the administration of *d*-amphetamine, the inlet and outlet tubing was guided through a liquid swivel held in a clamp over the top of the cage. Liquid swivels allow the rat to move freely around their cage without twisting the inlet and outlet tubing. This is obviously desirable with rats experiencing a large dose of *d*-amphetamine. However, as rats often spent long periods of time resting during the experiments, liquid swivels were only used in experiments involving *d*-amphetamine.

Samples were collected at 20 min intervals into polyethyene tubes containing 5 µl perchloric acid (0.01 M) to prevent the spontaneous oxidation of noradrenaline before analysis. Samples from the first 1-2 h were used to estimate resting extracellular

noradrenaline concentration ('basal' samples). When the dialysate noradrenaline concentration in four consecutive samples was stable, a test drug was administered. Samples were stored immediately on ice and their noradrenaline content was analysed using HPLC-ECD on the same day as collection. If samples were unable to be analysed on the same day as collection, they were stored at –70°C.

2.2.1.4.3. Drugs and drug administration procedures

The following drugs were used: atipamezole hydrochloride (Farmos), *d*-amphetamine sulphate (Sigma, UK), sibutramine hydrochloride (Knoll Pharmaceuticals Research, U.K.), 2-[2-(2-methoxy-1, 4-benzodioxanyl)]imidazoline hydrochloride (RX821002; Research Biochemicals Incorporated). Halothane was obtained from Zeneca.

For i.p. administration, drugs were dissolved in 0.9% saline and administered in a volume of 2 ml / kg. For local infusion of drugs *via* the dialysis probe ('reverse dialysis'), drugs were freshly dissolved in aCSF and administered using gas-tight syringes. Inlet tubing was primed with aCSF containing the test drug before its infusion at 1 µl / min. All constituents of the aCSF were AnalaR grade obtained from BDH.

2.2.1.5. Measurement of dialysate noradrenaline concentration

2.2.1.5.1. High performance liquid chromatography coupled to electrochemical detection (HPLC-ECD)

The noradrenaline content of the dialysate samples was analysed using reverse-phase high pressure liquid chromatography coupled to an electrochemical detector. The mobile phase was delivered at constant rate of 1.3 ml / min using a Varian 2010 HPLC pump and passed through a pre-injection guard cell set at +350 mV. This was used to oxidise (or reduce) electroactive substances in the mobile phase. Solutes were separated at room temperature on a Hypersil ODS 5 µm column (250 x 4.6 mm) which was protected by an Aquapore guard column (30mm x 4.6mm; Applied Biosystems). Samples were injected onto the column *via* an injection port (Rheodyne 7125) fitted with a 50 µl stainless steel loop (Anachem). Following separation, noradrenaline was detected using a high performance analytical cell (model 5014A; ESA) controlled by a Coulochem detector (ESA, model 5100A). Potentials were set in REDOX mode (detector 1: -180 mV: detector 2: +180mV). Electrical current was recorded and relayed as a chromatogram

through a Spectra-Physics Chromjet integrator. Dialysate noradrenaline concentration was identified by its retention time and quantified using peak height compared with an external standard.

2.2.1.5.2. The mobile phase

The mobile phase used during HPLC comprised (mM): sodium dihydrogen orthophosphate 83, octane sulphonic acid 2 (Sigma), EDTA 0.85, methanol 12% and was adjusted to pH 4 with orthophosphoric acid. This was filtered and degassed using FTK membrane filters (removal rating 0.1 μm, Pall Proceses Filtration). Mobile phase was recycled and renewed approximately every 2-3 weeks.

2.2.1.5.3. Reagents

Unless stated otherwise, all reagents of the mobile phase were either AnalaR grade obtained from BDH. The noradrenaline used to make external standards was obtained from Sigma and was the bitartrate salt.

2.2.1.6. Statistical analysis

Changes in the noradrenaline content of dialysis samples after drug treatment were tested for statistical significance using the ANOVA repeated measures facility on SPSS PC.$^+$ Analysis was carried out on orthonormalised raw data with 'time' as the 'within-subjects' factor. To compare the effects of different drug treatments, 'drug treatment' was included as the 'between-subjects' factor. The criterion for statistical significance was set at $P < 0.05$. See individual result chapters for further details on statistical analysis used in each study. For characterisation of dialysate noradrenaline content below (section 2.2.1.7.) data were separated into bins of a number of consecutive samples where 'bin 1' comprised basal samples. A split-plot ANOVA was the carried out with 'time' and 'bin' as 'within-subjects' factors.

2.2.1.7. Characterisation of dialysate noradrenaline content

The technique of microdialysis often assumes that, under drug-free conditions, the neurotransmitter content of the dialysate is directly related to neuronal activity. However, when carrying out microdialysis experiments, it is important to validate this assumption. In other words, it is important to establish that the neurotransmitter concentration in the

dialysate decreases when neuronal activity is inhibited. Likewise, it should be established that the neurotransmitter concentration increases if neurones are depolarised. Reverse-microdialysis creates several simple ways of doing this by manipulating the electrolyte make-up of the perfusion fluid. For example, it is possible to depolarise neurones by adding a high concentration of K^+ to the perfusion fluid (Cosford *et al.* 1994; Dalley and Stanford *et al.* 1995) or, similarly, it is possible to decrease exocytotic neurotransmitter release by removing Ca^{2+} (van Veldhuizen *et al.* 1990; Dalley and Stanford, 1995).

In this study, the response of dialysate noradrenaline concentration to the electrolyte manipulations described above was tested in the frontal cortex of anaesthetised rats using a modified Ringer's solution (mM: NaCl 145, KCl, 4, $CaCl_2$, 1.3, pH 6.6). Firstly, a depolarising pulse of K^+ (80 mM) was added to the Ringer's solution for 40 min (Ringer's solution: (mM) NaCl 71, KCl 80, $CaCl_2$ 1.3, pH 6.6, the concentration of NaCl was reduced to maintain the tonicity). Infusion of 80 mM K^+ caused an ~2.5-fold increase in dialysate noradrenaline concentration (20 min following start of perfusion: $F = 10.89$; d.f. 5, 25; $P < 0.01$) (Figure 2.4). When the perfusion medium was returned to the original Ringer's solution containing 4mM K^+, dialysate noradrenaline concentration returned to baseline concentration (basals vs time points 7, 8 and 9: $F = 0.02$; d.f. 1,8; $P = 0.90$). The removal of Ca^{2+} from the Ringer's solution for 60 min (mM: NaCl 145, KCl 4, pH 6.6) decreased dialysate noradrenaline concentration to approximately 50% of the mean basal concentration (basals vs time points9, 10, 11 and 12: $F = 9.23$; d.f. 1,10; $P = 0.01$) (Figure 2.4). Dialysate noradrenaline concentration returned to baseline concentration when the original Ringer's solution containing 1.3 mM $CaCl_2$ was restored (basals vs time points 14 and 15: $F = 0.454$; d.f. 1,9; $P < 0.52$).

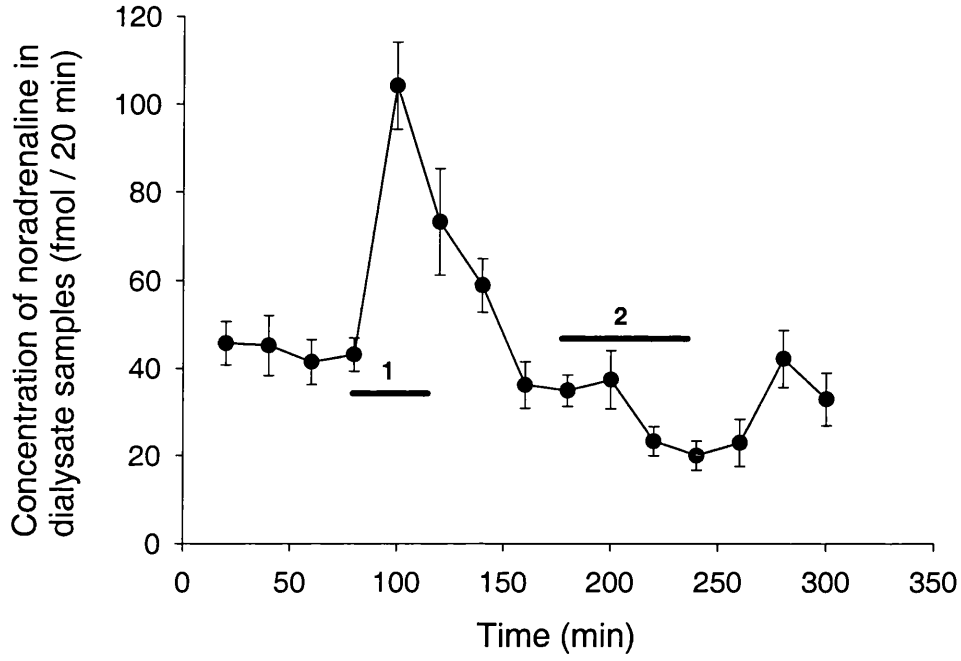


Figure 2.4. Effect of 1) a depolarising pulse of K^+ and 2) Ca^{2+} removal on dialysate noradrenaline concentration.

Figure shows an increase in dialysate noradrenaline concentration in the frontal cortex of anaesthetised rats during a 40 min pulse of 80 mM K^+ (1). Ca^{2+} removal from the perfusion fluid for 60 min caused a decrease in dialysate noradrenaline concentration (2). Points represent mean ± s.e. mean dialysate noradrenaline concentration (n = 6).

2.2.2 Radioligand binding

2.2.2.1. Animals

Male obese Zucker rats (188-231 g, Charles River, France) and their lean male littermates (170-185 g) aged 8 weeks old were used. Subjects were housed two per cage on a 12 h light / dark cycle (lights on 06.00 h) at 21°C and 55 % humidity. The rats were allowed free access to food and tap water at all times. Drug-naïve animals were used in each experiment and all procedures complied with the U.K. Scientific Procedures (Animals) Act, 1986.

2.2.2.2. Sample preparation

Rats were killed between 14.00-16.00 h by stunning and cervical dislocation and the brains rapidly removed. Frontal cortices and hypothalami were dissected and immediately frozen on dry ice. Samples were stored at -20°C until assayed.

2.2.2.3. [^{3}H]RX821002 binding assay

The following method has been adapted from that of Heal *et al.* (1995b).

2.2.2.3.1. Tissue preparation.

Brain tissue was thawed and homogenized in ice-cold 0.25M sucrose (1:30 w / v) and centrifuged at 1000 × g for 12 min. The supernatant was removed and stored on ice and the pellet resuspended in 0.25M sucrose (1:15 w / v) and centrifuged at 850 × g for 12 min. Combined supernatants were made up to 1:80 w / v with 5 mM Tris-HCl (pH 7.5, containing 5 mM EDTA) and centrifuged at 39000 × g for 10 min. The resulting pellet was homogenized in 50 mM Tris-HCl buffer (pH 7.5, containing 0.5 mM EDTA and 0.1% L-ascorbic acid) (1:80 w / v) and centrifuged at 39000 × g for 10 min. The final pellet was homogenized in 50 mM Tris-HCl (pH 7.5 containing 0.5 mM EDTA and 0.1% L-ascorbic acid) (equivalent to 3.125 mg / ml) and used immediately in assays. All centrifugations were carried out between 2-7 °C.

2.2.2.3.2. Binding assay.

Saturation studies were carried out at 8 concentrations of [^{3}H]RX821002 (0.036 - 3.6 nM). One frontal cortex or one hypothalmus was used per saturation experiment. All samples were prepared in duplicate and each sample contained 50 µl [^{3}H]RX821002 and 50 µl of 50 mM Tris-HCl buffer (pH 7.5, containing 0.5 mM EDTA and 0.1% L-ascorbic acid) (total binding) or 5 µl phentolamine (non-specific binding). Binding was initiated by the addition of 400 µl aliquots of freshly prepared membranes, following which samples were incubated for 75 min at 0°C. Remaining membrane preparations from each frontal cortex and hypothalamus was stored at -80 °C for subsequent protein analysis (see section 2.2.2.5.).

Following the incubation period, membrane-bound radioactivity was recovered by rapid filtration through Skatron 11734 filter mats using a Skatron cell harvester (suction

setting 9, 9, 0) with ice-cold 50 mM Tris-HCl buffer, pH 7.5. 1 ml Ultima Gold MV scintillant (Packard) was added to each filter mat and membrane-bound radioactivity was determined by liquid scintillation counting using a Packard emulsifier scintillator 299. Background radioactivity was automatically subtracted and disintegrations per minute (d.p.m.) were calculated from counts per minute (c.p.m) at a counting efficiency of approximately 45%.

2.2.2.4. Drugs and reagents

[^{3}H]RX821002 hydrochloride ([^{3}H]2-[2-(2-methoxy-1,4-benzodioxanyl)] imidazoline hydrochloride) (49 Ci / mmol) was obtained from Amersham International UK. Phentolamine mesylate was obtained from Research Biochemicals. All other reagents were obtained from BDH or Sigma.

2.2.2.5. Protein determination

The protein content of each frontal cortex and hypothalamus used was determined using the method of Lowry *et al.* (1951). A standard curve using bovine serum albumin was generated at 5 unit intervals from 0-50 µg protein / 100 µl. Bovine serum albumin was made up using the incubation buffer from the binding assay. A 100 µl aliqout of each standard (in duplicate) was added into assay tubes. Remaining membrane preparations from each frontal cortex and hypothalamus were thawed and a 100 µl aliquot of each sample (in duplicate) was added into assay tubes. Each tube was diluted with 1 ml 1 M NaOH and left at room temperature for 40 min. Following this, 1 ml of freshly prepared copper solution (1 ml 0.5% copper sulphate and 1% sodium citrate mixed with 50 ml 2% sodium carbonate) was added to each tube and left at room temperature for 15 min. Each tube then received 100 µl Folin-Ciocalteau phenol reagent (diluted 1 : 1 with distilled water). The contents of each individual tube were then well mixed and left at room temperature for 40 min. The ensuing colour density of the tubes was then read by a colorimeter at 750 nm using the standard with 0 µg protein as a reagent blank.

The protein content of each sample was used to convert B_{max} values from pmol / l to fmol / mg protein. This was done by converting the B_{max} from pmol / l to fmol / assay tube and dividing this by the protein content in 0.4 ml of tissue to give fmol / µg protein.

This figure was then multiplied by 1000 to give fmol / mg protein.

2.2.2.6. Statistical analysis

Data were analysed by Richard Brammer (Knoll Pharmaceuticals Statistics Department) using a two-way ANOVA with group (lean or obese) and assay date as factors. The criterion for statistical significance was set at $P < 0.05$.

2.2.3 Hypoactivity and mydriasis measurements

2.2.3.1. Animals

Male obese Zucker rats (148-191 g, Charles River, France) and their lean littermates (135-146 g) aged 7 weeks at the start of the study were used. They were housed in groups of 2 on a 12 h light / dark cycle (commencing 06.00 h) at a temperature of 21°C and 55% humidity. Rats were allowed free access to food and tap water.

2.2.3.2. Experimental design

The effects of a range of doses of clonidine (0.001 - 0.3 mg / kg i.p.) on mydriasis and hypoactivity over a period of 20 min and 1 h, respectively were investigated. These test periods have shown to be appropriate for monitoring mydriasis and hypoactivity in rats (Heal *et al.* 1981; 1995a). Each rat was used to test 3 doses of clonidine, with each test separated by a one-week drug washout period.

Rats were moved to the laboratory in their home cages 30 min before the start of the tests. Eight rats were tested per session and were numbered 1-8 on their tails with a permanent marker pen. Tests began at time 0 with the measurement of pupil diameter in rat number 1, followed by the administration of a randomly assigned dose of clonidine or saline. Rats 2-8 followed the same procedure at 1 min intervals. After treatment, rats were returned to their home cages. At 10 min, pupil diameter measurements were taken in each rat again at 1 min intervals, starting with rat number 1. Immediately after pupil diameter measurement, each rat was placed in an arena (length 1 m; width 0.6 m; height 0.28 m) and scored for hypoactivity. After scoring (see section 2.2.3.4.2. below) rats were again returned to their home cages. The same procedure was carried out at 20 min. Following

this, rats were individually scored for hypoactivity at 1 min intervals at 30, 40, 50 and 60 min post-treatment.

2.2.3.3. Drugs and drug administration procedures

Clonidine HCl was obtained from Sigma. Drugs were dissolved in 0.9% (w/v) sodium chloride solution (saline) and injected intraperitoneally in a volume of 2 ml / kg body weight. Control rats received saline (2 ml / kg body weight).

2.2.3.4. Test procedures

2.2.3.4.1. Pupil diameter measurement

The pupil diameter of the right eye was measured using a Wild M1 binocular microscope containing a graticule scale in one eyepiece and illuminated by a Swift light box (light intensity 2500 lux). The rat was carefully held underneath the microscope and its pupil diameter was read off the graticule scale in eyepiece units. This reading was then converted to millimetres.

2.2.3.4.2. Assessment of clonidine-induced hypoactivity

Clonidine-induced hypoactivity was measured using four behavioural parameters, using the protocol of Heal *et al* (1981). The behavioural parameters scored were as follows:

1) *Passivity*. Rats were picked up in progressively more stressful ways; from vertically by a fold of skin at the nape of the neck through to lifting the rat by a single hindlimb.

2) *Tactile responsiveness*. Rats were tested for their escape reflex. This involved gently squeezing the rat's body between a thumb and forefinger and assessing the speed with which the rat escaped.

3) *Posture*. Clonidine causes a progressive lowering of the tail and abdomen and this was assessed.

4) *Gait.* Rats were rated for their change from normal locomotion to a pronounced rolling gait.

Each behavioural parameter was rated on a scale of 0 – 3 points, where 0 = absent, 1 = slight, 2 = moderate and 3 = severe, using the score chart shown in Figure 2.5. Scoring of tactile responsiveness, posture and gait started 10 min following treatment, whereas scoring of passivity started 20 min following treatment. A passivity score was not taken at 10 min because pilot studies found the response to this test to be marred by defensive behaviour in some rats, as a result of the prior injection. The scoring was carried out by an observer who was unaware of the treatment administered to each rat.

2.2.3.4.3. Statistical analysis

Data were analysed using the ANOVA facility on SPSS PC$^+$. Analysis was carried out using 'dose' as the 'within-subjects' factor. To compare the effects of lean animals to obese animal, 'group' was included as the 'between-subjects' factor. The criterion for statistical significance was set at $P < 0.05$. (See Chapter 6, section 6.2.2. for further details).

TEST	0	1	2	3
PASSIVITY	Rat can be held by fold at nape of neck.	Can be inverted onto back.	Can be held by hind left limb.	Can be held by fore left limb.
TACTILE RESPONSIVENESS	Rat walks away when squeezed by a thumb and finger.	Slowed walk.	No walk.	Rat will not move when tester brushes fur the wrong way.
POSTURE	Rat exhibits normal posture with tail, hindquarters and abdomen raised off bench surface.	Tail touches bench.	Hindquarters and tail touch bench.	Abdomen, hindquarters and tail touch bench.
GAIT	Rat shows fast, agile smooth gait.	The gait slows.	The gait slows and has rolling character.	Rat does not move.

Figure 2.5. *The hypoactivity score sheet used to rate the level of hypoactivity in rats following clonidine administration.*

Chapter 3

Comparison of the effects of sibutramine and *d*-amphetamine on extracellular noradrenaline concentration in the frontal cortex of anaesthetised rats.

3.1 Introduction

In vivo microdialysis experiments in rats show that acute administration of sibutramine produces a moderate, gradual and sustained increase in extracellular 5-HT in the hypothalamus (Gundlah *et al.* 1997, Heal *et al.* 1998b). The accumulation of extracellular 5-HT induced by sibutramine or the selective serotonin reuptake inhibitors (SSRIs), paroxetine or fluoxetine, is decreased by co-administration of the 5-HT_{1A} autoreceptor agonist, 8-hydroxy-2-(di-*n*-propylamino) tetralin, (8-OH-DPAT) (Gundlah *et al.* 1997). 5-HT_{1A} autoreceptors are located on the cell bodies of serotonergic neurones and their activation decreases serotonergic neuronal firing (Hajós *et al.* 1995). These findings suggest that sibutramine, paroxetine and fluoxetine increase the concentration of extracellular 5-HT by inhibiting its reuptake following impulse-mediated release.

The findings above are consistent with those of Gartside *et al.* (1995). Using *in vivo* microdialysis studies, these authors found that systemic administration of a low dose of the SSRI, paroxetine, did not affect extracellular 5-HT concentration in rat frontal cortex but, instead, completely suppressed the firing rate of dorsal raphé neurones. However, in rats pretreated systemically with the 5-HT_{1A} autoreceptor antagonist,

WAY 100635, the same dose of paroxetine induced an increase in extracellular 5-HT concentration in the frontal cortex that was approximately 200% above baseline values. Furthermore, extracellular electrophysiological recordings showed that the inhibition of firing activity in dorsal raphé neurones induced by paroxetine, was reversed by the co-administration of WAY 100635. Similar findings are reported by Romero *et al.* (1996) who report that systemic administration of the selective 5-HT_{1A} antagonist, WAY 100635, augmented the increase in extracellular 5-HT concentration in the frontal cortex of rats pretreated systemically with the SSRIs, citalopram, fluoxetine or fluvoxamine. Together, these findings suggest that, because the increase in extracellular 5-HT induced by 5-HT reuptake inhibitors is derived from impulse-dependent release, the magnitude of effect of these drugs is limited by the subsequent activation of 5-HT_{1A} autoreceptors.

The progressive effect of sibutramine on extracellular 5-HT accumulation in the hypothalamus contrasts with the greater, more rapid and comparatively short-lasting increase in extracellular 5-HT evoked by 5-HT-releasing agents such as fenfluramine, *d*-fenfluramine and *d*-amphetamine (Gundlah *et al.* 1997, Heal *et al.* 1998b). Administration of 8-OH-DPAT in combination with fenfluramine had no effect on the increase in extracellular 5-HT concentration induced by fenfluramine alone (Gundlah *et al.* 1997). Similarly, *d*-amphetamine induces a rapid increase in extracellular noradrenaline concentration in the rat hippocampus (Florin *et al.* 1994). Administration of the α_2-adrenoceptor agonist, clonidine, which decreases locus coeruleus neuronal firing (see Chapter 1, section 1.4.3.) had no effect on the *d*-amphetamine-induced increase in extracellular noradrenaline (Florin *et al.* 1994). These findings lend support to the view that releasing agents induce a rapid increase extracellular transmitter concentration *via* an impulse-independent mechanism.

Previous microdialysis experiments in this laboratory have demonstrated that *d*-amphetamine also induces a rapid increase in extracellular noradrenaline concentration in the frontal cortex of anaesthetised rats (experiments performed by Z.A. Hughes). The magnitude of the increase in extracellular noradrenaline paralleled dose, with a peak effect occurring between 40-60 min post-injection. The rapid increase in extracellular noradrenaline is comparable with the rapid increases in extracellular dopamine and 5-HT

induced by this drug (Zetterström *et al.* 1983; Heal *et al.* 1998b), supporting the view that *d*-amphetamine is a monoamine-releasing agent.

Studies indicate that, in contrast to *d*-fenfluramine and *d*-amphetamine, neither sibutramine nor its active metabolites appear to have noradrenaline or 5-HT releasing properties *in vitro* (Heal *et al.* 1998a, see also Chapter 1, section 1.3.1.). Although sibutramine and its metabolites have been characterised as noradrenaline reuptake inhibitors, there have been no previous studies to investigate sibutramine's effects on central extracellular noradrenaline concentration *in vivo*. Such an investigation is of interest because experiments *in vivo* have indicated that sibutramine exerts its anti-obesity effects through actions on both noradrenergic and serotonergic systems (see Chapter, 1, section 1.3.1.).

Like sibutramine, the anti-obesity effects of *d*-amphetamine have been attributed, in part, to its activity on central noradrenergic neurones (see Chapter 1, section 1.3.2.). However, evidence suggests that central noradrenergic neurones could also be involved in the behavioural activation induced by this drug. Early studies suggested that the locomotor hyperactivity induced by *d*-amphetamine was mediated by the release of dopamine in the nucleus accumbens as injection of the dopamine antagonist, haloperidol, into this area inhibited this effect. Microdialysis studies in the 1980s supported this proposal by showing that the locomotor hyperactivity induced by *d*-amphetamine correlated well with the increase in extracellular dopamine concentration in the nucleus accumbens (Sharp *et al.* 1987).

However, a more recent study suggests that noradrenaline also has a role in the locomotor hyperactivity response induced by *d*-amphetamine (Blanc *et al.* 1994). These authors show that systemic administration of the α_1-adrenoceptor antagonists, prazosin or 2(2,6-dimethoxyphenoxyethyl) amino-methyl-1,4-benzodioxane hydrochloride (WB-4101) inhibited the locomotor hyperactivity induced by systemic administration of *d*-amphetamine. This effect was specific for the increase in locomotor activity induced by *d*-amphetamine as prazosin did not inhibit the locomotor hyperactivity induced by the antimuscarinic agent, scopolamine. α_1-Adrenoceptor antagonists have poor penetration across the blood-brain-barrier. Nevertheless, the study of Blanc *et al.* (1994) showed that

local administration of prazosin (0.16 pmol) into the medial prefrontal cortex completely inhibited the locomotor hyperactivity induced by local administration of *d*-amphetamine into the nucleus accumbens. These findings strongly suggest that α_1-adrenoceptors are involved in the locomotor hyperactivity response to *d*-amphetamine.

Darracq *et al.* (1998) investigated the effect of prazosin further. These authors found that reverse dialysis of *d*-amphetamine (3 μM) bilaterally into the nucleus accumbens did not induce an increase in locomotor activity even though the extracellular dopamine concentration increased by 5-fold. Furthermore, systemic administration of prazosin had no affect on the extracellular dopamine concentration. However, systemic administration of *d*-amphetamine (0.5 mg / kg) injected into rats already perfused with 3 μM *d*-amphetamine in the nucleus accumbens, caused a further 64% increase in dopamine concentration and induced a locomotor hyperactivity. Pretreatment of rats with prazosin, either systemically (0.5 mg / kg) or by bilateral injection into the prefrontal cortex (500 pmol), prevented both the further increase in extracellular dopamine and locomotor hyperactivity induced by systemic administration of *d*-amphetamine. The findings suggest that the increase in dopamine release in the nucleus accumbens associated with locomotor activity is under the control of prefrontocortical α_1-adrenoceptors. The authors suggest that the increase in noradrenergic transmission induced by *d*-amphetamine in the prefrontal cortex could modify the activity of glutaminergic neurones projecting from the cortex to the nucleus accumbens, which in turn increases dopamine release in this area. Alternatively, the increase in noradrenergic transmission could lead to an indirect effect on dopaminergic cell bodies to increase their firing rate.

Evidence suggests that it is the psychostimulant properties of *d*-amphetamine, rather than any direct effect on satiety, that accounts for the reduction in food intake induced by this drug. Antin *et al.* (1975) observed that satiety in the rat is characterised by a specific sequence of behaviours: following the cessation of feeding, a rat engages in grooming and exploration and then rests or sleeps. Although the sequence was fixed, the time spent engaged in non-feeding activities would vary. This sequence of behaviours that occurs following a meal is considered a better reflection of satiety than the cessation of feeding alone. In support of this suggestion, Antin *et al.* (1975) found that rats stopped

eating when they tasted quinine-adulterated food, but failed to exhibit the sequence of behaviours associated with satiety. Using this approach, Halford *et al.* (1995) monitored behavioural activities in rats following systemic administration of the ED_{50} dose of *d*-amphetamine (1.5 mg / kg i.p.) or sibutramine (2 mg / kg p.o.) to reduce food intake. The study found that, compared to the effect of placebo, *d*-amphetamine markedly increased the duration of time spent sniffing and decreased the duration and frequency of time spent resting. These findings suggest that *d*-amphetamine disrupts the satiety sequence and that the increase in behavioural activation accounts for the suppression of food intake caused by this drug. Sibutramine suppressed food intake by reducing the duration and frequency of eating episodes and accelerating the onset and duration of resting. This indicates that, in contrast to *d*-amphetamine, sibutramine decreases food intake by enhancing the natural development of satiety.

Considering the evidence that suggests central noradrenergic systems could have a role in eliciting the increase in locomotor activity induced by *d*-amphetamine, it is of further interest to investigate whether sibutramine increases extracellular noradrenaline concentration *in vivo* and to compare these effects with those of *d*-amphetamine.

Monitoring changes in central extracellular noradrenaline concentration in response to a novel anti-obesity agent is one approach to investigating its effects on central noradrenergic systems. Therefore, the first aim of this investigation was to use the technique of microdialysis to determine whether systemic administration of sibutramine increases the extracellular concentration of noradrenaline in the brain. To address this question, *in vivo* microdialysis was carried out in the frontal cortex of anaesthetised rats. This allowed the direct comparison of the findings of this investigation with those of the previous study on *d*-amphetamine.

One limitation of microdialysis is that changes in the concentration of extracellular transmitter can be attributed to either an increase in noradrenaline release, inhibition of its reuptake, or both. Nevertheless, it was predicted that the increase in extracellular noradrenaline concentration caused by sibutramine, like that of 5-HT, would be progressive and sustained, as a result of the extracellular accumulation following impulse-evoked release and inhibition of reuptake. In contrast, *d*-amphetamine increases monoamine release directly through an impulse-independent mechanism. This involves

effects on both plasmalemmal and vesicular monoamine transporters (see Chapter 1, section 1.3.2.). In view of these different neurochemical actions of sibutramine and *d*-amphetamine, it was predicted that the rate and magnitude of the increase in the concentration of extracellular noradrenaline caused by these two compounds would differ.

Another factor that influences the accumulation of extracellular noradrenaline is activation of somatodendritic and terminal α_2-adrenoceptors on noradrenergic neurones. When activated by extracellular noradrenaline, these receptors depress the firing rate of noradrenergic neurones (Svensson *et al.* 1975) and release of transmitter from their terminals (Langer, 1977), respectively (see Chapter 1, section 1.4.3.). Therefore, accumulation of extracellular noradrenaline, as a result of the inhibition of its reuptake by sibutramine, should activate these receptors and blunt its impulse-evoked release. In contrast, direct, impulse-independent release of noradrenaline by *d*-amphetamine should be less susceptible (if at all) to activation of α_2-adrenoceptors by noradrenaline. Considering this, the second aim of the investigation was to compare the effects of systemic administration of sibutramine or *d*-amphetamine on the amplitude and rate of increase in extracellular noradrenaline in rats pretreated systemically with an α_2-adrenoceptor antagonist.

The α_2-adrenoceptor antagonist chosen for this study was the highly selective α_2-adrenoceptor antagonist, 2-[2-(2-methoxy-1,4-benzodioxanyl)]imidazoline hydrochloride (RX821002) (Langin *et al.* 1990). This compound is a derivative of the previously more commonly used α_2-adrenoceptor antagonist, idazoxan, and has an α_2 / α_1-adrenoceptor binding selectivity ratio of 316 (*c.f.* idazoxan, 151) (Stillings *et al.* 1985). Binding studies show [^{3}H]RX821002 has a high affinity for α_2-adrenoceptors with little selectivity between the α_2-adrenoceptor subtypes (O'Rourke *et al.* 1994). Furthermore, consistent with its greater α_2 / α_1-adrenoceptor selectivity ratio, binding studies in human adipocytes show that [^{3}H]RX821002 does not label nonadrenergic idazoxan binding sites (Langin *et al.* 1990).

3.2 Methods

3.2.1. Experimental design

Experiments were carried out in halothane-anaesthetised rats. For details on procedures, refer to Chapter 2, section 2.2.1. Drugs were administered by the i.p. route.

To establish the dose-response curve for sibutramine, a range of doses of this drug (0.25 – 10 mg / kg) were tested following the collection of four basal samples (at 20 min intervals). Each rat was used to test only one dose of sibutramine and samples were collected for a further 4 h. This was the same procedure employed to establish the dose-response curve for *d*-amphetamine. The doses of sibutramine were chosen from previous studies showing that, over this dose-range, sibutramine has dose-dependent effects on food-intake as well as extracellular 5-HT accumulation (Jackson *et al.*, 1997b; Gundlah *et al.*, 1997).

In experiments involving pretreatment of rats with the selective α_2-adrenoceptor antagonist, RX821002, rats were divided into four groups. The first group of rats was used in a pilot study to find an appropriate dose of RX821002 to use for pretreatment of rats in groups 2-4. Rats in group 1 were administered 1 mg / kg RX821002 and, two hours later, they received a second dose of 3 mg / kg RX821002; samples were collected for a further 2 h. Since neither 1 or 3 mg / kg RX821002 had any effect on dialysate noradrenaline concentration, the second, third and fourth group of rats received a dose of 3 mg / kg RX821002 and, 1 h later, were given a further injection of sibutramine, *d*-amphetamine or vehicle, respectively; samples were then collected for a further 3 h.

3.2.2. Statistical analysis

Changes in the noradrenaline content of dialysis samples after drug injection were tested for statistical significance using analysis of variance (ANOVA) with repeated measures. Analysis was carried out with 'time' as the 'within-subject' factor. To compare the effects of sibutramine and *d*-amphetamine, 'drug treatment' was added as the 'between-subjects' factor. To assess RX821002-induced changes in the latency to reach peak dialysate noradrenaline concentration, the mean latency to reach the peak drug effect

for individual rats was determined and one-way ANOVA used to compare the effects of different treatment groups.

3.3 Results

3.3.1. The effects of sibutramine and *d*-amphetamine on dialysate noradrenaline concentration.

Mean noradrenaline content in basal samples from these experiments was 24.8 ± 0.8 fmol 20 / min (pooled data from all experiments in this section; n = 41).

3.3.1.1. Sibutramine.

There was no change in dialysate noradrenaline concentration in rats injected with saline (Figure 3.1a and b). However, following administration of sibutramine at 0.25, 0.5 or 1 mg / kg (Figure 3.1a) or 1, 3 or 10 mg / kg (Figure 3.1b), there was a gradual and sustained increase in the noradrenaline concentration of dialysis samples.

Sibutramine (0.25, 0.5, or 1 mg / kg) increased dialysate noradrenaline concentration within 40 min post-injection, (0.25 mg / kg: $F = 4.68$; d.f. 5,20; $P < 0.01$; 0.5 mg / kg: $F = 4.13$; d.f. 5,10; $P = 0.03$; 1 mg / kg: $F = 5.79$; d.f 5,10; $P < 0.01$). A dose of 3 mg / kg sibutramine increased dialysate noradrenaline concentration within 80 min post-injection ($F = 5.22$; d.f. 7,28; $P < 0.01$). 10 mg / kg sibutramine increased dialysate noradrenaline concentration only within 180 min post-injection ($F = 2.88$; d.f. 8,24; $P = 0.02$).

The relationship between the magnitude of the increase and dose of sibutramine followed a bell-shaped curve. The largest increase was induced by a dose of 0.5 mg / kg (maximum of 278%, *c.f.* mean basal concentration) and occurred at 240 min post-injection. A plateau, representing an approximately 2.5-fold increase in noradrenaline (*c.f.* mean basal concentration), was attained within 2 h of injection. Slightly smaller increases were found after administration of 1 or 3 mg / kg sibutramine (1 mg / kg: maximum of 245% at 100 min post-injection: 3 mg / kg: maximum of 266% at 200 min post-injection).

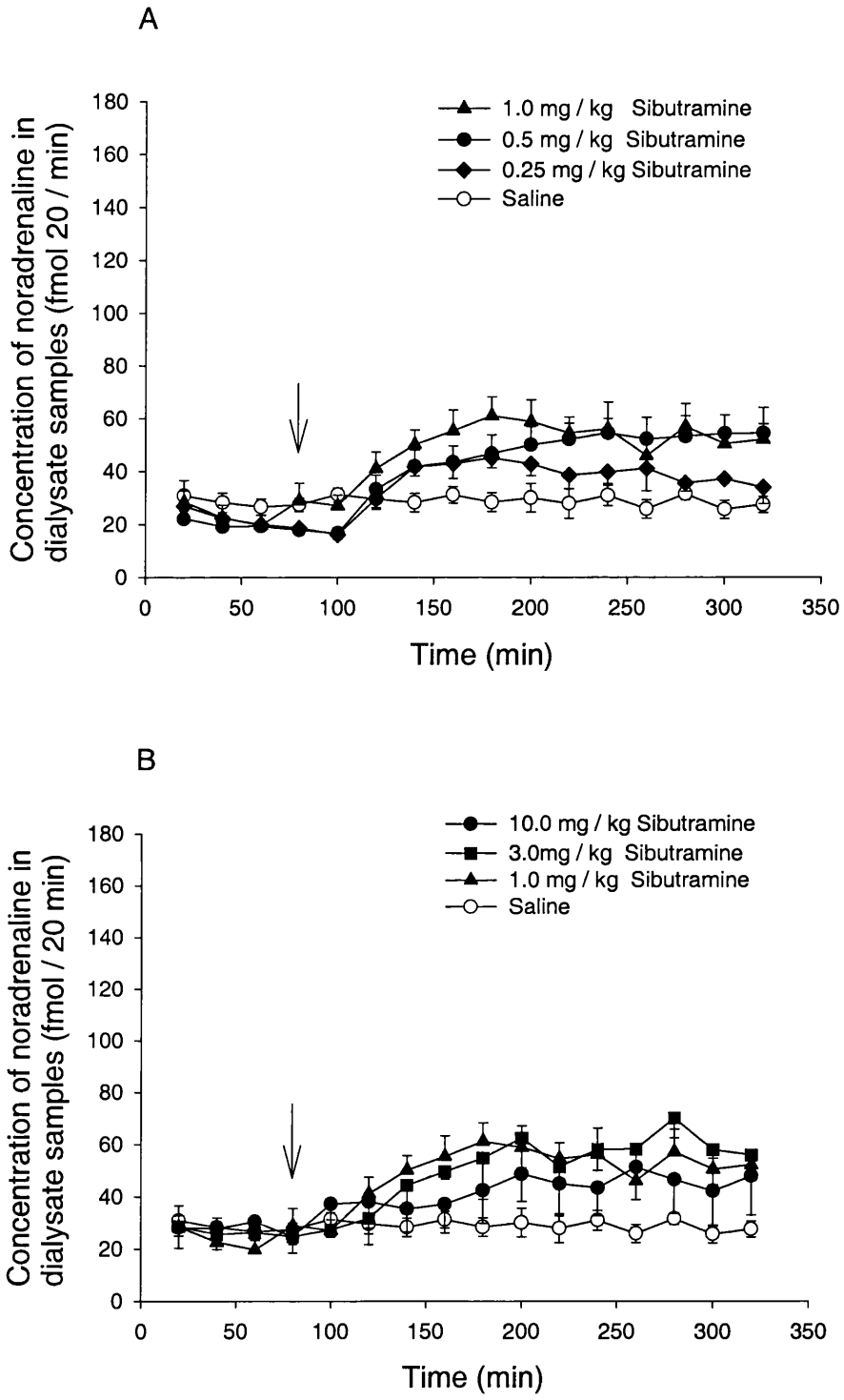


Figure 3.1: *The effect of sibutramine (a: 0.25, 0.5, or 1 mg / kg i.p. b: 1, 3 or 10 mg / kg i.p.) on dialysate noradrenaline concentration in the frontal cortex.*

Arrow represents drug administration. Data points represent mean ± s.e. mean dialysate noradrenaline concentration (fmol / 20 min; n = 4-5).

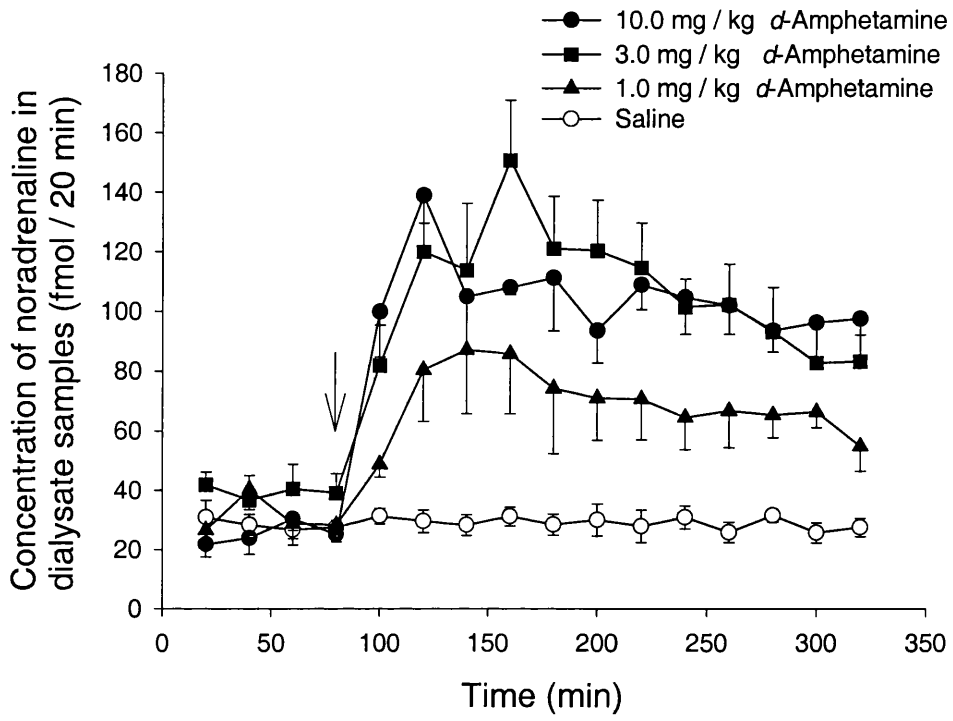


Figure 3.2: The effect of d-amphetamine (1, 3 or 10 mg / kg i.p.) on dialysate noradrenaline concentration from the frontal cortex.

Arrow represents drug administration. Data points represent mean ± s.e. mean dialysate noradrenaline concentration (fmol / 20 min; n = 4-5).

3.3.1.2. d-Amphetamine (experiments performed by Z.A. Hughes).

All three doses of *d*-amphetamine increased the concentration of extracellular noradrenaline within 20 min of injection (1 mg / kg: F = 4.85; d.f. 4,12; P = 0.02; 3 mg / kg: F = 6.14; d.f. 4,16; $P < 0.01$; 10 mg / kg: F = 15.04; d.f. 4,8; P = 0.01) and the magnitude of the increase paralleled drug dose (Figure 3.2). Doses of 1 or 3 mg / kg produced maximum increases of 281% at 60 min post-injection and 381% at 80 min post-injection, respectively. The largest increase was induced by a dose of 10 mg / kg (549% at 40 min post-injection).

3.3.1.3. Comparison of the effects of sibutramine and d-amphetamine.

There was no main effect of drug or drug × time effect when 1 or 3 mg / kg sibutramine were compared with the equivalent dose of *d*-amphetamine over the whole time course of the experiment. However, 10 mg / kg *d*-amphetamine caused a greater increase in dialysate noradrenaline concentration than did the equivalent dose of sibutramine at all times post-injection (1^{st} h: $F = 24.03$; d.f. 1,6; $P < 0.01$, 2^{nd} h: $F = 13.84$; d.f. 1,6; $P = 0.01$, 3^{rd} h: $F = 12.34$; d.f. 1,6; $P = 0.01$, 4^{th} h: $F = 7.02$; d.f. 1,7; $P = 0.03$). There was also a marked difference in the time course of the responses to a 10 mg / kg dose of these drugs (drug × time interaction: $F = 14.59$; d.f. 13,65; $P < 0.01$) reflecting the more rapid increase caused by *d*-amphetamine.

3.3.2. Effects of RX821002-pretreatment on dialysate noradrenaline concentration following sibutramine or *d*-amphetamine administration.

Mean noradrenaline content of basal samples from these experiments was 22.6 ± 1.2 fmol / 20 min (pooled data from all experiments; n = 30).

3.3.2.1. Magnitude of response.

Neither RX821002 alone (1 mg / kg followed by 3 mg / kg) (Figure 3.3), nor when followed by a saline injection 1 h later (Figure 3.4), affected dialysate noradrenaline concentration.

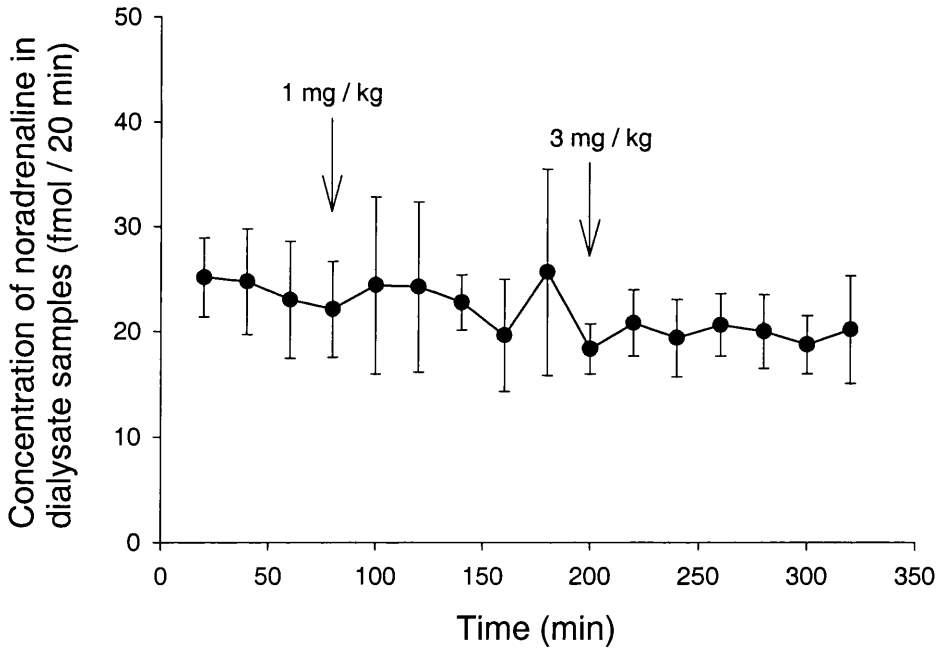


Figure 3.3: *The effect of RX821002 (1 mg / kg followed by 3 mg / kg i.p.) on dialysate noradrenaline concentration from the frontal cortex.*

Arrow represents drug administration. Data points represent mean ± s.e. mean dialysate noradrenaline concentration (fmol / 20 min; n = 5).

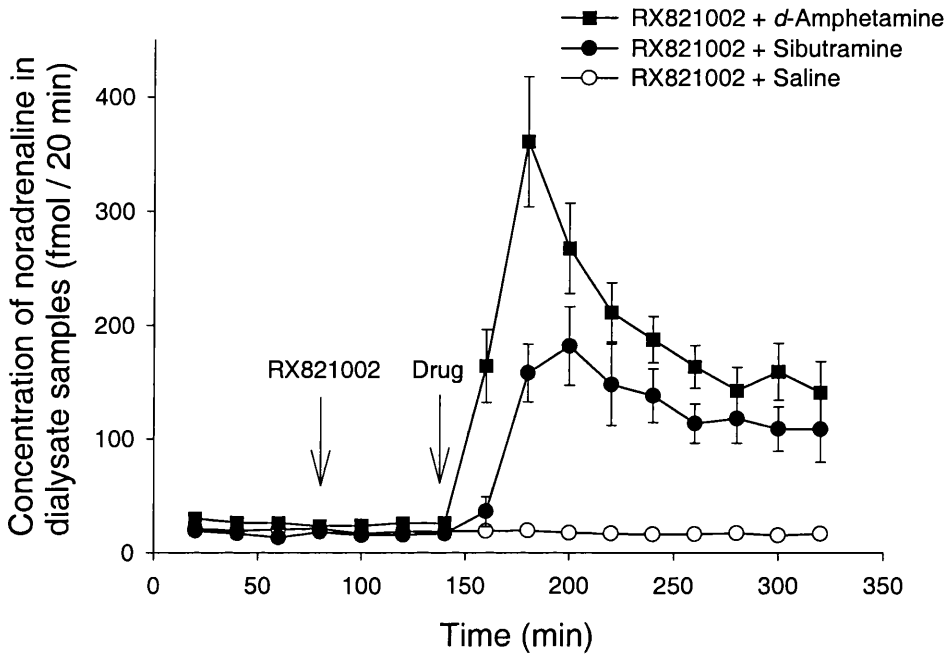


Figure 3.4: *The effect of sibutramine or d-amphetamine (10 mg / kg i.p.) on the concentration of noradrenaline in samples of cortical dialysate from rats treated 1 h previously with RX821002 (3 mg / kg i.p.).*

Arrows represent drug administration. Data points represent mean ± s.e. mean dialysate noradrenaline concentration (fmol / 20 min; n = 5).

However, both sibutramine (10 mg / kg) and *d*-amphetamine (10 mg / kg) increased dialysate noradrenaline concentration when administered to RX821002-pretreated rats (Figure 3.4). Moreover, the increases caused by both these compounds were greater than with either drug alone (sibutramine: ~5 fold increase, $F = 12.85$; d.f. 1,30; $P < 0.01$; Figure 3.5a; *d*-amphetamine: ~2.5 fold increase, $F = 15.85$; d.f. 1,24; $P < 0.01$; Figure 3.5b). However, the increase induced by RX821002 in combination with *d*-amphetamine was still greater than that induced by RX821002 in combination with sibutramine (main effect of drug combination over 1 h following sibutramine or *d*-amphetamine administration: $F = 6.9$; d.f. 1,7; $P = 0.03$).

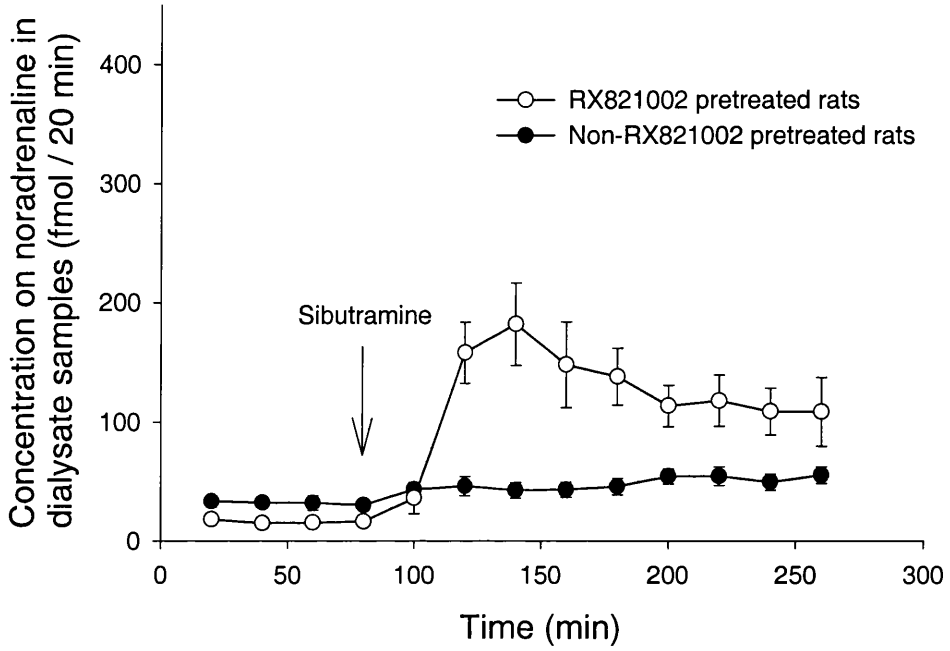


Figure 3.5a: *The effect of sibutramine (10 mg / kg i.p.) on the concentration of noradrenaline in samples of cortical dialysate from non-pretreated rats and rats pretreated 1 h earlier with RX821002 (3 mg / kg i.p.).*

Arrow represents sibutramine administration. Data points represent mean ± s.e. mean dialysate noradrenaline concentration (fmol / 20 min; n = 5).

3.3.2.2. Latency to peak effect.

RX821002 reduced the latency of sibutramine (10 mg / kg) to reach its maximum effect from 144 ± 14 min to only 56 ± 4 min ($F = 33.80$; d.f. 1,8; $P < 0.01$). In contrast, RX821002 had no significant effect on latency to reach mean maximum noradrenaline concentration after treatment with *d*-amphetamine (RX821002 pretreated: 50 ± 17 min, non-pretreated: 40 ± 0 min). Finally, a drug × time interaction ($F = 4.84$; d.f. 12,84; $P < 0.01$) again suggests that, even in the presence of RX821002, the increase in extracellular noradrenaline concentration induced by *d*-amphetamine was more rapid than that caused by sibutramine.

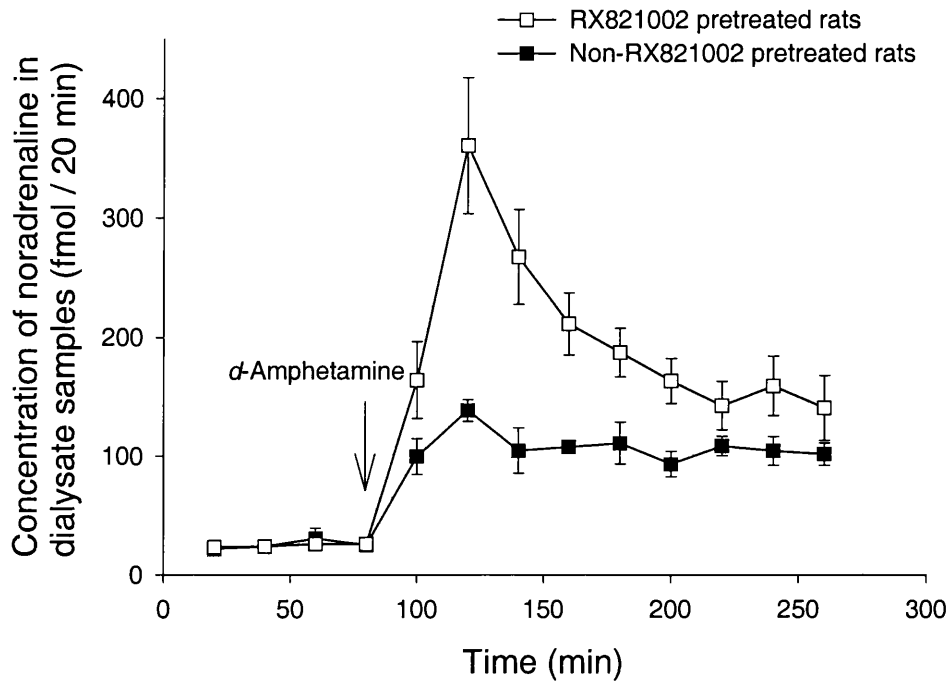


Figure 3.5b: *The effect of d-amphetamine (10 mg / kg i.p.) on the concentration of noradrenaline in samples of cortical dialysate from non-pretreated rats and rats pretreated 1 h earlier with RX821002 (3 mg / kg i.p.).*

Arrow represents *d*-amphetamine administration. Data points represent mean ± s.e. mean dialysate noradrenaline concentration (fmol / 20 min; n = 5).

3.4. Discussion

Sibutramine's active metabolites are potent inhibitors of noradrenaline reuptake *in vitro*, (Cheetham *et al.* 1996) but both the parent compound and its metabolites are devoid of noradrenaline-releasing activity (Heal and Cheetham, 1997). However, nothing is known about the effects of sibutramine on central extracellular noradrenaline concentration *in vivo*. Questions that arise are whether its reuptake blocking activity increases extracellular noradrenaline concentration and whether this action can be distinguished from the effect of *d*-amphetamine?

3.4.1. The effects of sibutramine or *d*-amphetamine on cortical extracellular noradrenaline concentration.

The present results show that sibutramine caused a gradual and sustained increase in the concentration of extracellular noradrenaline in rat frontal cortex. *d*-Amphetamine also increased extracellular noradrenaline concentration. However, at the highest dose tested (10 mg / kg), this increase was both more rapid and of greater magnitude than that caused by the same dose of sibutramine, or by the dose inducing sibutramine's maximal effects (0.5 mg / kg). The magnitude and time-course of effects of these two drugs on extracellular noradrenaline concentration resemble their effects on extracellular 5-HT in the hypothalamus (Gundlah *et al.* 1997). In the study of Gundlah *et al.* (1997) the profile of the increase in extracellular 5-HT concentration evoked by 5-HT-reuptake inhibitors differed markedly from that induced by 5-HT-releasing agents. Based on these findings, Gundlah *et al.* define specific criteria that distinguish between the effects of 5-HT-reuptake inhibitors and 5-HT-releasing agents when using microdialysis. According to these criteria:

1) *Systemic administration of a releasing agent induced a greater increase in extracellular 5-HT concentration than that induced by reuptake inhibitors.* The authors suggest that the modest increase in extracellular 5-HT induced by reuptake inhibitors reflects a balance between reuptake inhibition and the partial decrease in release that is a consequence of autoreceptor activation. In

contrast, the greater increase in extracellular 5-HT induced by high doses of 5-HT releasing agents probably reflects impulse-independent 5-HT release.

2) *Attenuation of the firing of 5-HT neurones, through activation of presynaptic autoreceptors, diminishes the effects of reuptake inhibitors on extracellular 5-HT concentration but does not affect the actions of releasing agents because these depend on impulse-independent transmitter release.* The authors demonstrated that the increase in 5-HT induced by 5-HT-reuptake inhibitors (including sibutramine) is reversed by the subsequent administration of the somatodendritic 5-HT_{1A} autoreceptor agonist, 8-OH-DPAT. This strongly supports the hypothesis that the extracellular 5-HT accumulating following the administration of a reuptake inhibitor is derived from impulse-dependent release. However, 8-OH-DPAT did not reduce the large increase in extracellular 5-HT following fenfluramine administration, supporting the notion that the extracellular 5-HT accumulation following administration of this drug is derived from an impulse-independent process.

Generalising these criteria to the findings of the present study of noradrenaline would suggest that *d*-amphetamine produced a greater and more rapid increase in extracellular noradrenaline concentration, than does sibutramine, because the former drug is a releasing agent whereas the latter is a reuptake inhibitor, only. Consistent with studies showing that sibutramine's metabolites inhibit the uptake of noradrenaline *in vitro,* the gradual increase in extracellular noradrenaline caused by sibutramine reflects the progressive accumulation of noradrenaline after its spontaneous, impulse-evoked release. The rapid increase in extracellular noradrenaline induced by *d*-amphetamine is in keeping with evidence that *d*-amphetamine causes the release of noradrenaline *in vitro* (Glowinski and Axelrod, 1965; Pifl *et al.* 1999; see Chapter 1, section 1.3.2.).

The finding that *d*-amphetamine induced a rapid increase in extracellular noradrenaline concentration in the frontal cortex is consistent with the findings of Kuczenski and Segal (1992). These authors found that *d*-amphetamine induced a rapid increase in extracellular noradrenaline concentration in the prefrontal cortex of freely-moving rats. They also reported that the time-course of the increase in noradrenaline paralleled the time-course of the increase in locomotor activity induced by

this drug. In the present study, the findings that sibutramine induced a smaller and more gradual increase in extracellular noradrenaline than did *d*-amphetamine, suggests that the former drug may not induce the behavioural activation associated with the latter drug. This suggestion is strongly supported by recent studies showing that in contrast to *d*-amphetamine, systemically administered sibutramine does not increase locomotor activity in rats (Rowley *et al.* 2000). Indeed, by monitoring the satiety sequence Halford *et al.* (1995) show that sibutramine actually increases the duration of time spent resting in rats (see section 3.1.).

Several reports suggest that *d*-amphetamine decreases the firing rate of noradrenergic neurones in the locus coeruleus (Graham and Aghajanian, 1971; Ryan *et al.* 1985; Holdefer and Jensen, 1987). This effect of *d*-amphetamine is thought to mediated by noradrenergic activation of α_2-adrenoceptors located on the cell bodies of noradrenergic neurones (Engberg and Svensson, 1979; see Chapter 1, section 1.4.3). Nevertheless, the findings of this chapter so far suggest that *d*-amphetamine induces impulse-independent release of noradrenaline whereas sibutramine inhibited the reuptake of spontaneous, impulse-evoked noradrenaline release. Therefore, it may be predicted that activation of presynaptic α_2-adrenoceptors would blunt the increase in extracellular noradrenaline concentration induced by sibutramine whereas the effects of *d*-amphetamine would be less susceptible, if at all.

3.4.2. The effect of RX821002 alone on cortical extracellular noradrenaline concentration.

When given alone, the α_2-adrenoceptor antagonist, RX821002, did not modify basal dialysate noradrenaline concentration. This suggests that there is little tonic activation of these receptors. This finding is at variance with reports that systemic administration of RX821002 does increase the concentration of extracellular noradrenaline in rat brain (Javier-Meana *et al.* 1997; Nutt *et al.* 1997). However, although Javier-Meana *et al.* (1997) report an increase in extracellular noradrenaline in the cingulate cortex of rats after treatment with RX821002, the results of this study can not be compared with the present study because the noradrenaline reuptake inhibitor,

desipramine, was included in the aCSF. This, in itself, would indirectly increase the tonic activation of α_2-adrenoceptors.

Nutt *et al.* (1997) reported a 2-fold increase in extracellular noradrenaline concentration in the frontal cortex of chloral hydrate anaesthetised rats following RX821002 administration. An explanation for the apparently anomalous finding is not immediately clear. However, the findings indicate that there must be more activation of central α_2-adrenoceptors in the rats used by Nutt *et al.* (1997) compared with those used in the present study. This could be a result of the different anaesthetics used in these experiments. Extracellular noradrenaline concentration is affected in different ways by different anaesthetic agents (*e.g.* chloralose and pentobarbital sodium decrease, whereas urethane increases the concentration of extracellular noradrenaline in the hypothalamus; Shimokawa *et al.* 1998). It is unclear how these anaesthetic agents could modify central noradrenergic activity or why they would have opposite effects on the same system. However, there is evidence to suggest that some anaesthetic agents modify neuronal firing rate in the brain. Studies on halothane and chloral hydrate show that these anaesthetic agents decrease the firing rate of striatal and dorsal raphé neurones in the rat and cat, respectively (Warenycia and McKenzie, 1984; Heym *et al.* 1984). Other studies show halothane to decrease the firing rate of both mesencephalic reticular neurones in the cat (Shimoji *et al.* 1977) and locus coeruleus neurones in the rat (Saunier *et al.* 1993). A reduction in locus coeruleus firing by halothane-anaesthesia would suggest that noradrenaline release in the cortex could have been reduced during experiments performed in this study. This could have caused a reduction in tonic activity at the terminal α_2-adrenoceptors, accounting for the lack of effect of RX821002.

Alternatively, anaesthetic agents could modify neuronal activity by modulating α_2-adrenoceptor function. Evidence suggests that halothane-anaesthesia interacts with α_2-adrenoceptors. Bloor and Flacke (1982) found that the α_2-adrenoceptor agonist, clonidine, decreased the minimal anaesthetic concentration (MAC) of halothane required to anaesthetise dogs by approximately 45%. The effect of clonidine on MAC was rapidly and completely reversed by the non-selective α-adrenoceptor antagonist, tolazoline. These findings suggested that clonidine is able to influence the potency of halothane through an action at α_2-adrenoceptors. In addition, Saunier *et al.* (1993) showed that the

dose of clonidine required to inhibit locus coeruleus neuronal firing by 50% (ED_{50}), is decreased under halothane-anaesthesia compared with 30 min following halothane withdrawal (ED_{50}: 25 mg / kg vs 5 mg / kg under halothane). The findings of Saunier *et al.* (1993) suggest that α_2-adrenoceptors are more sensitive under halothane-anaesthesia. Although this suggests that halothane influences α_2-adrenoceptor function, it does not explain why RX821002 had no effect on basal extracellular noradrenaline concentration in the present study.

Considering evidence that anaesthetic agents can modify central noradrenergic activity in rats, the dose-response curve for sibutramine's effects on extracellular noradrenaline concentration was investigated in conscious, freely-moving rats (see Chapter 4). Either of the proposals described above would predict that the basal concentration of extracellular noradrenaline would be higher in freely-moving rats compared with halothane-anaesthetised rats. These proposals were not fully supported by studies in conscious, freely-moving rats, which found that the basal dialysate noradrenaline concentration in the frontal cortex to be no greater than in anaesthetised rats (see Chapter 4). Nevertheless, this comparison is not ideal because the time interval between the end of surgery and the collection of samples is much greater in freely-moving rats (18-22 h) compared with anaesthetised rats (2 h). However, the effects of RX821002 on extracellular noradrenaline concentration in the frontal cortex and hypothalamus were investigated in conscious, freely-moving rats in Chapter 5. This study found that systemic administration of RX821002 (3 mg / kg) did increase the concentration of noradrenaline in dialysate samples from both these areas. This finding is not consistent with the study of Saunier *et al* (1993) which suggests that α_2-adrenoceptor function is more sensitive under halothane-anaesthesia, but it is consistent with the proposal that locus coeruleus firing is reduced by halothane-anaesthesia. It is possible that the basal concentration of noradrenaline in dialysate samples from anaesthetised and freely-moving rats would differ if compared at the same time following surgery.

In light of the evidence described above, it is not unreasonable to propose that the choice of anaesthesia affects noradrenergic neuronal firing and/or α_2-adrenoceptor function and that this could explain the different of effects of RX821002 in the present study compared with the study of Nutt *et al.* (1997).

3.4.3. The effect of RX821002 in combination with sibutramine or *d*-amphetamine.

Despite its lack of intrinsic activity, RX821002 augmented the increase in extracellular noradrenaline concentration caused by sibutramine. This finding suggests that activation of somatodendritic and / or terminal α_2-adrenoceptors by extracellular noradrenaline, which accumulates after administration of sibutramine, attenuated impulse-derived transmitter release. In keeping with these findings, the increase in cortical extracellular noradrenaline concentration caused by the established noradrenaline reuptake inhibitor, desipramine, is similarly enhanced by idazoxan (Dennis *et al.* 1987). The effect of sibutramine in combination with RX821002 is similar to the effect of an SSRI in combination with a 5-HT_{1A} receptor antagonist on extracellular 5-HT accumulation (Gartside *et al.* 1995; Romero *et al.* 1996). Together, these findings highlight a similarity between the functioning of noradrenergic and serotonergic neurones in the brain.

RX821002 also augmented the effect of *d*-amphetamine (10 mg / kg) on extracellular noradrenaline. This suggests that the subsequent activation of α_2-adrenoceptors by noradrenaline does restrict the magnitude of this drug's effect on extracellular noradrenaline accumulation to some extent. This inference conflicts with evidence that the α_2-adrenoceptor agonist, clonidine, has little effect on the increase in noradrenaline induced by *d*-amphetamine in the hippocampus (Florin *et al.* 1994). This could be because activation of α_2-adrenoceptors after treatment with *d*-amphetamine is already maximal and that co-administration of an α_2-adrenoceptor agonist has no further impact on release rate. However, the comparison of the study of Florin *et al.* (1994) with the present study is complicated by the fact that the former study was carried out in conscious, freely-moving rats. Nevertheless, the present finding suggests that, if noradrenergic neuronal activity is not completely depressed by *d*-amphetamine administration, then impulse-dependent noradrenaline release can contribute to the increase in extracellular noradrenaline accumulation induced by this drug.

The rapid increase in extracellular noradrenaline concentration induced by *d*-amphetamine alone suggests that this drug induces impulse-independent noradrenaline release. However, the finding that RX821002 potentiated the effect of *d*-amphetamine on

extracellular noradrenaline accumulation by approximately 2.5-fold shows that *d*-amphetamine also has characteristics of a noradrenaline reuptake inhibitor. The K_i for the inhibition of noradrenaline uptake into rat brain by *d*-amphetamine is 45 nM (Heal *et al.* 1998a). This value is relatively high compared with the K_i values for this drug's effects on dopamine and 5-HT uptake in rat brain (132 and 1441 nM, respectively, Heal *et al.* 1998a). The 5-HT releasing agent, *d*-fenfluramine, also has K_i values for monoamine uptake into rat brain of greater than 100 nM (260, 279 and 6227 nM for noradrenaline, 5-HT and dopamine, respectively, Heal *et al.* 1998a). The high affinity of *d*-amphetamine for the noradrenaline transporter suggests that the dissociation of this drug from the transporter could be relatively slow. At high concentrations of *d*-amphetamine, which would saturate the noradrenaline transporter, this slow dissociation could lead to some reuptake inhibitor-like properties by preventing noradrenaline in the synapse binding to the transporter. Consistent with this proposal, Pifl *et al* (1999) show that, at low doses, *d*-amphetamine acts as a noradrenaline releasing agent, releasing noradrenaline from intracellular vesicular stores *in vitro*. However, at high doses, *d*-amphetamine prevents the reuptake of released noradrenaline (see Chapter 1, section 1.3.2.).

The present finding that RX821002 enhanced the increase in extracellular noradrenaline concentration induced by 10 mg / kg *d*-amphetamine is consistent with the proposal that high doses of *d*-amphetamine inhibit the reuptake of noradrenaline. Based on this proposal, it may be predicted that RX821002 would have less of an effect, if any, on low doses of *d*-amphetamine that cause noradrenaline release only. The effect of RX821002 in combination with 1 or 3 mg / kg *d*-amphetamine was not investigated in the present study. Investigating the effects of a range of doses of *d*-amphetamine in combination with RX821002 would be able to test the proposal. Ideally, these experiments would be done in conscious, freely-moving rats, so that any interference of the effects by anaesthesia could be ruled out.

Pretreatment of rats with RX821002 not only augmented the amplitude of the increase in extracellular noradrenaline caused by sibutramine, but also reduced the latency to reach its maximum. This is consistent with disinhibition of impulse-derived noradrenaline release by this α_2-adrencoceptor antagonist. There was no apparent

reduction in the latency to reach maximum after treatment with *d*-amphetamine from the 40 min seen in the RX821002-free state. However, it is unlikely that any such reduction would have been detected because the present protocol for microdialysis dictated sampling at 20 min intervals.

3.4.4. Conclusion

The findings of this study support the view that sibutramine is a noradrenaline reuptake inhibitor *in vivo* and that the rate and magnitude of this drug's effects on extracellular noradrenaline are strongly attenuated by indirect activation of α_2-adrenoceptors. In contrast, a high dose of *d*-amphetamine caused a much more rapid increase in extracellular noradrenaline, the magnitude of which is affected to a lesser extent by α_2-adrenoceptor activation.

Chapter 4

Comparison of the effects of sibutramine and *d*-amphetamine on extracellular noradrenaline concentration in the frontal cortex and hypothalamus of conscious, freely-moving rats.

4.1 Introduction

The previous chapter showed that sibutramine or *d*-amphetamine increased the concentration of extracellular noradrenaline in the frontal cortex of anaesthetised rats. Although this supports the view that these two anti-obesity agents enhance central noradrenergic function, noradrenergic transmission in the hypothalamus, in particular the paraventricular nucleus, has been shown to have the most marked effects on food intake (see Chapter 1, section 1.2.1.).

As well as a role in food intake, evidence also suggests the hypothalamus has a role in the regulation of thermogenesis. Perkins *et al.* (1981), showed that unilateral electrical stimulation of the ventromedial hypothalamus produced a biphasic response in the temperature of interscapular brown adipose tissue (BAT). At 1 min following stimulation there was a small decrease in BAT temperature from 36.2 °C to 36.0 °C. This was followed by an increase in BAT temperature that reached a maximum of 37 °C approximately 8 min post-stimulation and returned to baseline levels by approximately 20

min post-stimulation. The effect was abolished by systemic administration of the non-selective β-adrenoceptor antagonist, propranolol. Further investigation revealed that blockade of the sympathetic nerve supply to BAT with local anaesthetic abolished the increase in temperature induced by hypothalamic stimulation. However, under the same conditions, systemic administration of noradrenaline induced changes in BAT temperature of the same duration and magnitude as seen following stimulation of the hypothalamus. Based on their findings, the authors suggest that central stimulation of BAT thermogenesis is mediated by the sympathetic nerve supply and involves β-adrenoceptors. More recent studies using agonists and antagonists selective for β-adrenoceptor subtypes suggest that the β-adrenoceptor that mediates BAT thermogenesis is the β_3-subtype (Manara *et al.* 1996).

Support for a role of hypothalamic noradrenaline in the control of thermogenesis comes from findings of Sahakian *et al.* (1983). These authors depleted hypothalamic noradrenaline by approximately 71% in rats by injecting the neurotoxin 6-hydroxydopamine into the rostral medulla in order to lesion the ventral noradrenergic bundle carrying neurones to the hypothalamus. BAT activity was monitored by measuring the binding of guanosine diphosphate (GDP) to BAT mitochondria (an index of the thermogenic proton conductance pathway). The study found that lesion of the ventral noradrenergic bundle induced a weight gain of 10% above that of sham-controlled rats when fed an identical palatable diet. Furthermore, binding of GDP in BAT mitochondria was reduced in lesioned rats compared with sham-lesioned controls. These results indicate that BAT thermogenic activity is impaired in lesioned rats and suggest that this could contribute to the elevated weight gain in these animals.

Although the findings of Sahakian *et al.* (1983) show that a depletion of hypothalamic noradrenaline content leads to weight gain, these findings are at variance with studies that show acute injection of noradrenaline into the region of paraventricular nucleus of the hypothalamus *increases* food intake in satiated and hungry rats (Leibowitz, 1978). These studies raise the question as to which of noradrenaline's actions on thermogenesis and food intake in the hypothalamus predominate under normal physiological conditions *in vivo*?

The paraventricular nucleus of the hypothalamus, which receives a dense noradrenergic innervation, has been implicated in the control of BAT thermogenesis. De Luca *et al.* (1989) show that rats with an electrolytic lesion of the paraventricular nucleus and fed identical diets have a lower rate of oxygen consumption and decreased core and BAT temperatures compared with sham-lesioned controls. Although both lesioned and sham-lesioned rats showed an increase in energy intake when put on a cafeteria diet compared with a chow diet, the lesioned rats gained more weight over an 18 day period. The authors suggest that the increase in energy intake in lesioned rats on a cafeteria diet may not induce the usual compensatory increase in thermogenesis, which results in a greater increase in body weight in these rats. Further experiments showed that the increase in oxygen consumption, as well as core and BAT temperatures, stimulated by systemic administration of noradrenaline, was also impaired in lesioned rats compared with sham-lesioned controls. This suggests that the decrease in BAT activity in lesioned rats could be due to a reduced function of the sympathetic nervous system as a result of an impaired facilitatory influence from the paraventricular nucleus.

The effects of sibutramine on extracellular noradrenaline concentration in the hypothalamus cannot be assumed to be the same as in the cortex. The reason for this is primarily that these two brain areas differ in respect of the source of their noradrenergic innervation: the cortex receives noradrenergic neurones exclusively from the locus coeruleus, while the hypothalamus is primarily innervated by noradrenergic neurones with cell bodies in the lateral tegmental nulcei (see: Holets 1990 and Chapter 1, section 1.1.). The tissue noradrenaline content, as well as the appearance, of neurones originating in the lateral tegmental system are different from those originating in the locus coeruleus (see Chapter 1, section 1.1.). In addition, evidence suggests that there could be functional differences between these two groups of neurones; Fritschy *et al.* (1990) reported that noradrenergic neurones of the lateral tegmental system are more resistant to the selective noradrenergic neurotoxin (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine), DSP-4, than are locus coeruleus neurones. Thus, in studies visualising noradrenergic neurones stained with antibodies to noradrenaline (antibodies raised against noradrenaline conjugated to a protein carrier), a marked decrease in staining intensity in areas such as the cerebral cortex and hippocampus was found in rats injected with DSP-4 (*c.f.* untreated controls) within 6 h post-injection. This depletion in staining intensity persisted for the two week period of

the investigation. However, DSP-4 treatment caused virtually no reduction in staining intensity in the hypothalamus at any time point in the investigation. In the same study, noradrenergic neurones were also visualised using antibodies to the membrane-bound marker protein of noradrenergic axons, dopamine-β-hypdroxylse (DBH). A decrease in DBH staining occurred four days after DSP-4 treatment (*c.f.* untreated controls) in areas which had experienced a loss of noradrenaline content only.

Heal *et al.* (1993) also reported a differential effect of DSP-4 on neurones innervating the hypothalamus and cortex. This study used twice the dose of DSP-4 (100 mg / kg) to that used by Fritschy *et al.* (1990) and found marked reductions in the tissue content of noradrenaline in both the cortex and hypothalamus 3 days after treatment (8 and 17% of saline treated controls, respectively). However, whereas the depletion in noradrenaline content persisted in the cortex at 15 days post-injection, a partial reversal of the effects of DSP-4 in the hypothalamus occurred at 15 days post-injection, with noradrenaline content being reduced to only 45% of saline controls. The authors suggest their findings indicate that neurones innervating the hypothalamus are only temporarily inactivated by DSP-4 and are able to functionally resist the toxic effects of DSP-4.

The aim of this investigation was to establish whether sibutramine affects the concentration of extracellular noradrenaline in the hypothalamus using *in vivo* microdialysis. Drug-induced changes in extracellular noradrenaline concentration were monitored in the region of the paraventricular nucleus (PVN) of the hypothalamus, based on evidence that changes in noradrenergic activity in this specific area of the hypothalamus show the greatest influence on food intake compared with other brain regions (Leibowitz, 1978). This region of the hypothalamus is densely innervated by lateral tegmental noradrenergic neurones but also receives some locus coeruleus innervation (see Chapter 1, section 1.1.2.). Since evidence suggests that there could be functional differences between noradrenergic neurones projecting to the hypothalamus and cortex, the effects of sibutramine on extracellular noradrenaline accumulation in the hypothalamus were compared with its effects in the frontal cortex. In view of evidence that anaesthesia could influence the activity of noradrenergic neurones (Chapter 3), these studies used conscious, freely-moving rats so that any interference of drug-induced changes in extracellular noradrenaline concentration by anaesthesia could be ruled out.

4.2 Methods

4.2.1. Experimental design

Experiments were carried out in conscious, freely-moving rats. For details on procedures, refer to Chapter 2, section 2.2.1. Drugs were administered by the i.p. route.

To establish the dose-response curve for sibutramine, a range of doses of this drug (0.25 – 10 mg / kg) were tested following the collection of four basal samples. This dose-range of sibutramine was used in the previous chapter and showed that, over this dose-range, sibutramine has dose-dependent effects on extracellular noradrenaline accumulation. *d*-Amphetamine was tested at 10 mg / kg because findings from the previous chapter show that this dose caused a greater and more rapid increase in dialysate noradrenaline concentration than the equivalent dose of sibutramine in the frontal cortex of anaesthetised rats. Each rat was tested with only one dose of drug and samples were collected for a further 4 h.

4.2.2. Statistical analysis

Changes in the noradrenaline content of dialysis samples after drug injection were tested for statistical significance using ANOVA with repeated measures. Analysis was carried out with 'time' as the 'within-subject' factor. To compare the effects of sibutramine and *d*-amphetamine, 'drug treatment' was added as the 'between-subjects' factor.

4.3 Results

4.3.1. Effect of sibutramine or *d*-amphetamine on dialysate noradrenaline concentration from the frontal cortex.

The mean noradrenaline content of the basal samples was 23.1 ± 0.9 fmol / 20 min (pooled data from all experiments carried out in the frontal cortex; n = 26). There was no change in dialysate noradrenaline concentration in rats injected with saline vehicle (Figure 4.1).

4.3.1.1. Sibutramine.

There was a gradual and sustained increase in dialysate noradrenaline concentration following administration of sibutramine at 0.25, 1 or 10 mg / kg (Figure 4.1).

The magnitude of the increase in noradrenaline induced by sibutramine was dose-dependent. A dose of 0.25 mg / kg sibutramine failed to increase dialysate noradrenaline concentrations significantly. However, sibutramine (1 or 10 mg / kg) induced a significant increase in noradrenaline concentration within 40 min post-injection (1 mg / kg: $F = 4.83$; d.f. 5,20; $P < 0.01$; 10 mg / kg: $F = 9.01$; d.f. 5,20; $P < 0.01$). The largest increase was induced by the largest dose (10 mg / kg: maximum of 462%, *c.f* mean basal concentration) and occurred at 140 min post-injection. The maximum increase after 1 mg / kg sibutramine was 289% and occurred at 80 min post-injection. Both 1 and 10 mg / kg sibutramine induced a sustained increase in dialysate noradrenaline concentration, which was still maintained 4 h post-injection.

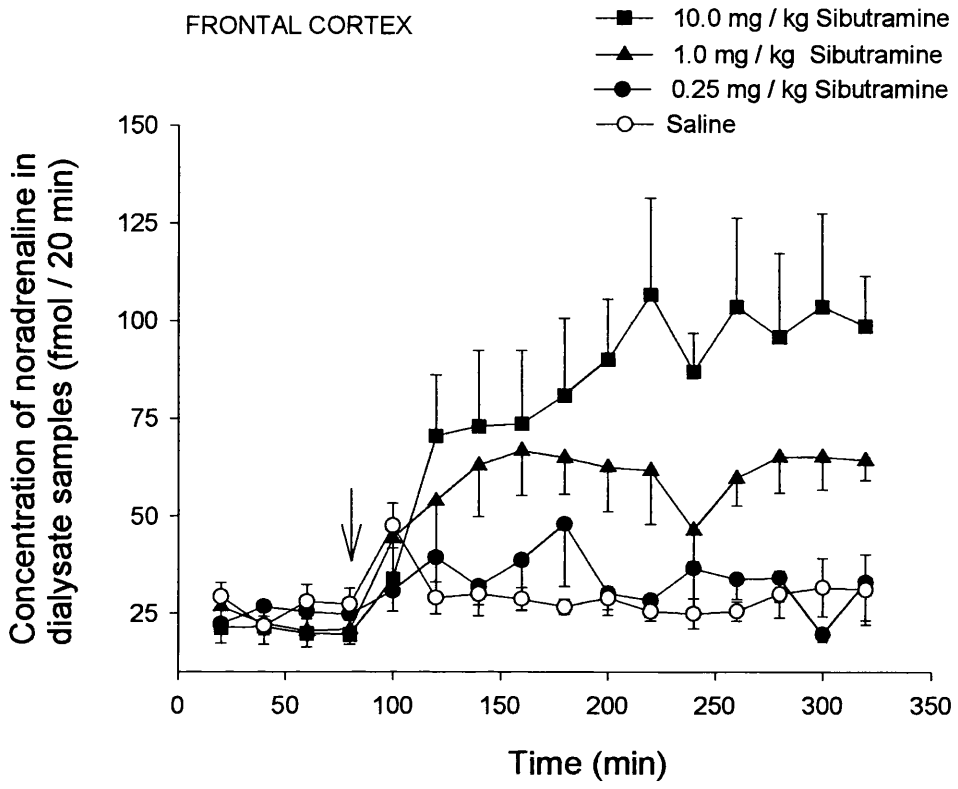


Figure 4.1: *The effect of sibutramine (0.25, 1 or 10 mg / kg i.p.) on dialysate noradrenaline concentration in the frontal cortex.*

Arrow represents drug administration. Data points expressed as mean ± s.e. mean dialysate noradrenaline concentration (fmol / 20 min; n = 4-6).

4.3.1.2. d-Amphetamine.

d-Amphetamine (10 mg / kg) induced a rapid increase in dialysate noradrenaline concentration which was significant within 20 min post-injection ($F = 19.33$; d.f. 4,16; $P < 0.01$) (Figure 4.2). A maximum increase of 1035% was found at 40 min post-injection. Following this, dialysate noradrenaline concentration declined towards basal concentration. The increase in dialysate noradrenaline concentration induced by *d*-amphetamine was greater and more rapid than that induced by the same dose of sibutramine (main effect of drug: $F = 8.93$; d.f. 1,6; $P = 0.02$. Drug × time interaction; $F = 13.76$; d.f. 15,90; $P < 0.01$).

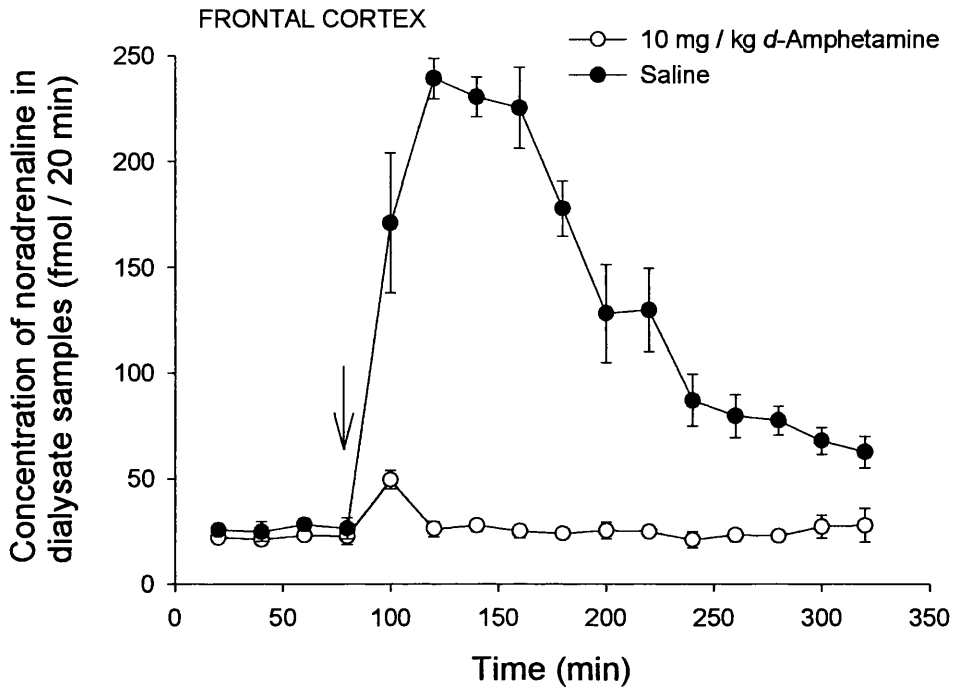


Figure 4.2: *The effect of d-amphetamine (10 mg / kg i.p.) on dialysate noradrenaline concentration in the frontal cortex.*

Arrow represents drug administration. Data points represent mean ± s.e. mean dialysate noradrenaline concentration (fmol / 20 min; n = 5).

4.3.2. Effects of sibutramine or *d*-amphetamine on dialysate noradrenaline concentration from the hypothalamus.

The mean noradrenaline content of the basal samples was 15.4 ± 0.7 fmol / 20 min (pooled data from all experiments carried out in the hypothalamus; n = 29). There was no change in dialysate noradrenaline concentration in rats injected with saline (Figure 4.3).

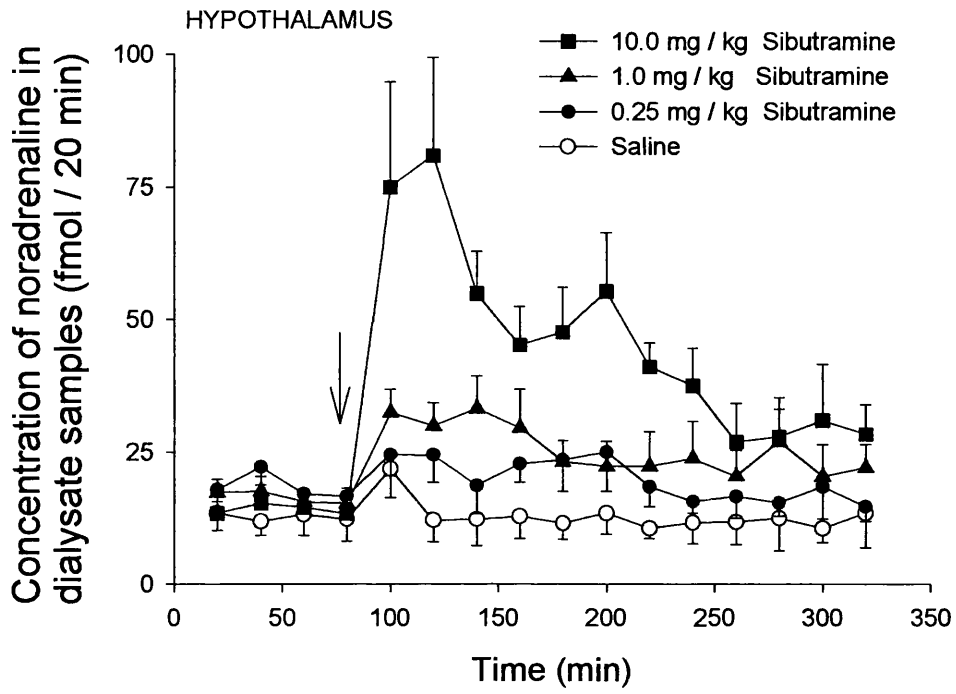


Figure 4.3: *The effect of sibutramine (0.25, 1 or 10 mg / kg i.p.) on dialysate noradrenaline concentration in the hypothalamus.*

Arrow represents drug administration. Data points represent mean ± s.e. mean dialysate noradrenaline concentration (fmol / 20 min; n = 4-8).

4.3.2.1. Sibutramine.

Dialysate noradrenaline concentration increased following administration of sibutramine at 0.25, 1 or 10 mg / kg (Figure 4.3).

The magnitude of the increase in noradrenaline concentration induced by sibutramine was dose-dependent. A dose of 0.25 mg / kg failed to significantly increase noradrenaline concentration. Sibutramine (1 and 10 mg / kg) increased dialysate noradrenaline within 20 min post-injection (1 mg / kg: $F = 15.76$; d.f. 4,24; $P < 0.01$; 10 mg / kg: $F = 11.62$; d.f. 4,28; $P < 0.01$). The largest increase was induced by the largest dose (10 mg / kg: maximum of 525%, *c.f.* mean basal concentration) and occurred 40 min post-injection. The maximum increase after 1 mg / kg sibutramine was 216% and occurred at 60 min post-injection.

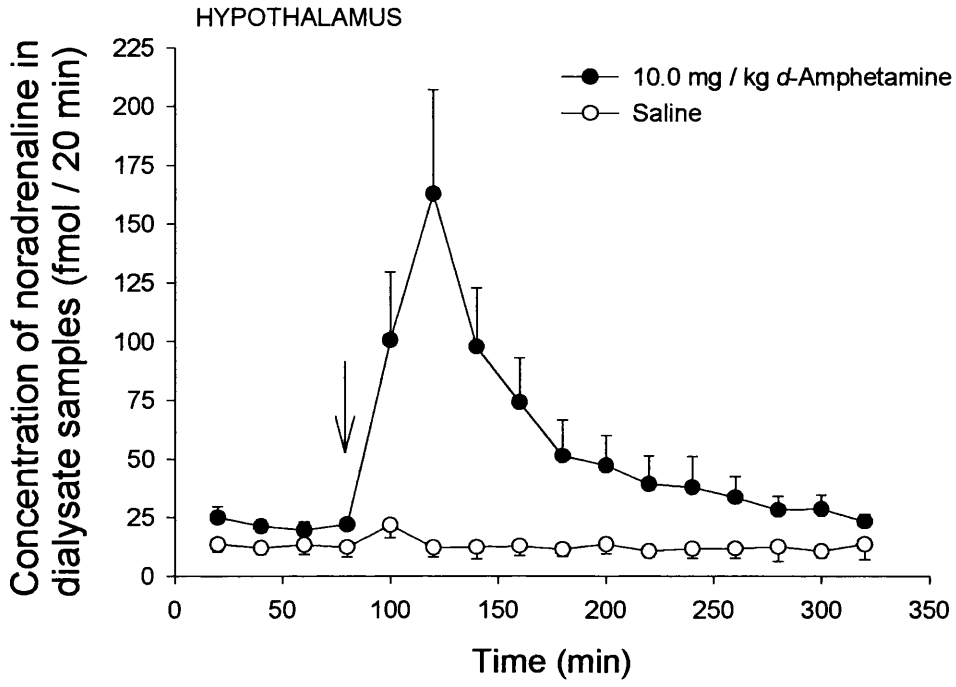


Figure 4.4: *The effect of d-amphetamine (10 mg / kg i.p.) on dialysate noradrenaline concentration in the hypothalamus.*

Arrow represents drug administration. Data points represent mean ± s.e. mean dialysate noradrenaline concentration (fmol / 20 min; n = 5).

4.3.2.2. *d-Amphetamine.*

d-Amphetamine (10 mg / kg) induced a rapid increase in dialysate noradrenaline concentration which was significant within 20 min post-injection ($F = 9.12$; d.f. 4,16; $P < 0.01$) (Figure 4.4). A maximum increase in noradrenaline concentration of 1058% occurred at 40 min post-injection. The increase was greater than that induced by the same dose of sibutramine (main effect of drug: $F = 5.59$; d.f. 1,9; $P = 0.04$).

4.4 Discussion

Since noradrenergic transmission in the hypothalamus, and in particular the paraventricular nucleus, has been implicated in the control of feeding behaviour (Leibowitz, 1978; Wellman, 1992), this study investigated the actions of sibutramine in the hypothalamus. Furthermore, because evidence suggests functional differences between the noradrenergic neurones innervating the hypothalamus and the cortex (Fritschy *et al.* 1990), the effects of sibutramine on extracellular noradrenaline accumulation in the hypothalamus were compared with its effects in the frontal cortex.

4.4.1. The effects of sibutramine or *d*-amphetamine on extracellular noradrenaline concentration in the frontal cortex.

Consistent with the previous chapter, the present results show that sibutramine increased extracellular noradrenaline in the frontal cortex, with maximum concentrations being reached after at least 1 h post-injection and persisted for the duration of the experiment. There were no differences in the basal concentration of noradrenaline between freely-moving and halothane-anaesthetised rats. However, in the previous chapter using anaesthetised rats, the relationship between dose of sibutramine and magnitude of response was described by a bell-shaped curve, with a maximum increase in noradrenaline of 278% at 0.5 mg / kg. In the present investigation, the magnitude of effect of sibutramine paralleled the dose, with a maximum increase of 462% at 10 mg / kg. Thus, halothane-anaesthesia appears to shift the dose-response curve for sibutramine to the left and decreases the maximum accumulation of extracellular noradrenaline attainable following doses of sibutramine greater than 0.5 mg / kg.

The reason for the halothane-induced shift in sibutramine's dose-response curve is unclear. At 0.5 mg / kg, sibutramine induced a similar maximal increase in extracellular noradrenaline concentration in halothane-anaesthetised (278%) to a dose of 1 mg / kg sibutramine in freely-moving rats (289%). This finding suggests that halothane-anaesthetised rats could have a higher release rate of noradrenaline in the frontal cortex compared with freely-moving rats, although this suggestion is not

supported by the finding that basal noradrenaline concentration did not differ between anaesthetised and conscious, freely-moving rats in this study. Nevertheless, as described in Chapter 3, a direct comparison of basal noradrenaline concentration between anaesthetised and freely-moving rats is not entirely satisfactory considering sample collection took place at different times following surgery.

Evidence suggests locus coeruleus neuronal firing is decreased under halothane-anaesthesia. Saunier *et al.* (1993) recorded the activity of locus coeruleus neurones under 1% halothane-anaesthesia and 30 min following halothane withdrawal. They found that under halothane-anaesthesia locus coeruleus neurones exhibited a slow, regular and spontaneous discharge that increased by 87% after the halothane was discontinued. On re-administration of halothane, the firing activity decreased again. The decrease in locus coeruleus neuronal firing rate by halothane could explain the decrease in the magnitude of sibutramine's effects on extracellular noradrenaline concentration at high doses: a reduction in noradrenergic neuronal firing could lead to a reduction in noradrenaline release, which would make less noradrenaline available for accumulation in the extracellular fluid by sibutramine. A comparison of basal dialysate noradrenaline concentration at the same time following surgery in anaesthetised and freely-moving rats would test this proposal. If the reduction in locus coeruleus firing induced by halothane was found to decrease basal dialysate noradrenaline concentration, this would raise the question of how halothane-anaesthesia decreases noradrenergic neuronal firing?

As described in Chapter 3, evidence suggests that halothane interacts with α_2-adrenoceptors, possibly causing an increase in their sensitivity to α_2-adrenoceptor agonists (Saunier *et al.* 1993) report that the dose of clonidine required to inhibit locus coeruleus neuronal firing by 50% is decreased under halothane-anaesthesia, see section 3.4.2). It is possible that in both halothane-anaesthetised and freely-moving rats, sibutramine induces an increase in extracellular noradrenaline concentration in the region of the cell bodies, as well as in the terminal fields, the magnitude of which parallels dose. However, like the effects of clonidine in the study of Saunier *et al.* (1993) the increase in extracellular noradrenaline concentration in the region of the cell bodies could inhibit locus coeruleus firing, through activation of α_2-adrenoceptors, to a greater extent in

halothane-anaesthetised rats than in freely-moving rats. In halothane-anaesthetised rats, the greater accumulation of extracellular noradrenaline concentration in the region of the cell bodies by doses of sibutramine greater than 0.5 mg / kg, could be sufficient to prevent the release of noradrenaline in the terminal fields. This would explain the reduced effects of high doses of sibutramine (> 0.5 mg / kg) on extracellular noradrenaline accumulation in the frontal cortex of halothane-anaesthetised rats.

According to the proposal above, the dose of sibutramine needed to induce an extracellular noradrenaline concentration sufficient to 'turn-off' locus coeruleus neuronal firing rate, through activation of α_2-adrenoceptors, was greater in the present study using conscious, freely-moving rats, than in the previous study using halothane-anaesthetised rats. This proposal would predict that extracellular noradrenaline accumulation would begin to decrease in freely-moving rats at a particular dose of sibutramine greater than 10 mg / kg. However, Gundlah *et al.* (1997) found that the magnitude of sibutramine's effects on 5-HT paralleled dose up to a dose as high as 30 mg / kg in conscious, freely-moving rats. Further experiments investigating the effects of higher doses of sibutramine on extracellular noradrenaline accumulation in the frontal cortex of freely-moving rats would establish whether halothane shifts the dose-response curve to the left. Whatever the effect of halothane on normal central noradrenergic function, it is clear from this study that experiments conducted in halothane-anaesthetised rats can provide misleading information concerning the effects of drugs acting on noradrenergic neurones.

The sustained increase in extracellular noradrenaline concentration induced by sibutramine in the frontal cortex of both anaesthetised and freely-moving rats is consistent with reports showing that systemic administration of the noradrenaline reuptake inhibitor, desipramine (DMI), caused an increase in extracellular noradrenaline in the cerebral cortex of freely-moving rats (Dennis *et al.* 1987). However, the increase in extracellular noradrenaline concentration induced by DMI reached a maximum (3-fold increase in basal noradrenaline concentration) 40 min after its administration. Following this, a stable, 2- to 2.5-fold increase in extracellular noradrenaline concentration was maintained for at least 5 h. The comparison of the findings of Dennis *et al.* (1987) with

the findings of this study suggest that the gradual increase in extracellular noradrenaline induced by sibutramine, which reaches a maximum concentration 80-140 min post-injection, may not be explained by an action of sibutramine on noradrenergic neurones, only.

The simultaneous rise in extracellular 5-HT concentration induced by sibutramine could affect the concurrent increase in extracellular noradrenaline concentration. Evidence suggests that locus coeruleus neurones are tonically inhibited by serotonergic neurones. Thus, M^{c}Rae-Degueurce *et al.* (1985) reported that lesions of raphé nuclei (containing serotonergic cell bodies) or serotonergic terminals increased the activity of tyrosine hydroxylase (the rate-limiting enzyme involved in catecholamine synthesis) in the locus coeruleus. Furthermore, electrophysiological studies indicate that the firing rate of locus coeruleus neurones is enhanced in rats treated with the 5-HT synthesis inhibitor, p-chorophenylalanine (Reader *et al.* 1986). It is possible that the increase in extracellular 5-HT induced by sibutramine increases the inhibitory serotonergic input onto locus coeruleus neurones. This effect is thought to be mediated through 5-HT$_2$ receptors, since systemic injection of the 5-HT$_2$ receptor antagonists, ritanserin or 2-(2-dimethylamino-2-methylpropylthio)-3-phenylquinolinehydrochloride (ICI 170,809) increases extracellular accumulation of noradrenaline in hippocampus of conscious, freely-moving rats (Done and Sharp, 1994). However, this suggestion is complicated by more recent findings that activation of postsynaptic 5-HT$_{1A}$ receptors with 5-HT$_{1A}$ agonists *increases* extracellular noradrenaline concentration in the hippocampus, as well as the hypothalamus (Suzuki *et al.* 1995; Hajós-Korcsok and Sharp, 1996). Although many studies suggest that the activity of central noradrenergic neurones are modulated by 5-HT, further studies are needed to establish whether the increase in extracellular 5-HT induced by a 5-HT and noradrenaline reuptake inhibitor, such as sibutramine, affects the rate and/or magnitude of the simultaneous increase in extracellular noradrenaline.

Also consistent with previous findings, *d*-amphetamine (10 mg / kg) increased extracellular noradrenaline concentration in the frontal cortex. This increase was greater and more rapid than that induced by the equivalent dose of sibutramine, supporting the hypothesis that *d*-amphetamine and sibutramine have noradrenaline-releasing and

noradrenaline-reuptake inhibiting properties, respectively. Although the latency to peak noradrenaline concentration following *d*-amphetamine was the same in both anaesthetised and conscious, freely-moving rats (40 min), the maximum noradrenaline concentration reached was greater in the latter (912% *c.f.* 549% above basal concentration). This finding tentatively supports the idea that impulse-dependent noradrenaline release contributes to the increase in extracellular noradrenaline induced by *d*-amphetamine (see Chapter 3, section 3.4). If central α_2-adrenoceptors are more sensitive to increases in extracellular noradrenaline concentration in the presence of halothane-anaesthesia, noradrenaline released through impulse-independent mechanisms could decrease the impulse-dependent component of *d*-amphetamine's effects to a greater extent than in freely-moving rats. Although studies show that locus coeruleus firing is almost completely inhibited in the presence of *d*-amphetamine at doses of 1-10 mg / kg. (Graham and Aghajanian, 1971; Ryan *et al.* 1985; Holdefer & Jensen 1987, see Chapter 3, section 3.4.) all these electrophysiological studies were carried out in anaesthetised rats. Thus, it cannot be ruled out that *d*-amphetamine's effects on locus coeruleus neuronal firing in anaesthetised rats are different to its effects in conscious, freely-moving rats.

4.4.2. The effects of sibutramine and *d*-amphetamine on extracellular noradrenaline concentration in the hypothalamus.

In the hypothalamus of freely-moving rats, sibutramine increased extracellular noradrenaline in the hypothalamus at 1 and 10 mg / kg, the magnitude of which paralleled dose. However, unlike its action in the cortex, sibutramine induced a more rapid increase in extracellular noradrenaline in the hypothalamus, with a maximum effect occurring 40 min post-injection after a dose of 10 mg / kg. Following this, dialysate noradrenaline concentration gradually declined.

Functional differences between noradrenergic neurones terminating in these two brain areas have been reported before (Fritschy *et al.* 1990; Heal *et al.* 1993: see section 4.1). One possible explanation for the more rapid accumulation of extracellular noradrenaline in the hypothalamus, compared with in the cortex, could be a difference in

the pharmacological properties of the noradrenaline transporter site in these two brain areas. Previous studies show that the selective noradrenergic neurotoxin, DSP-4, causes a substantial loss of noradrenergic neurones in the cortex, but leaves hypothalamic noradrenergic neurones largely intact (Fritschy *et al.* 1990; Zaczek *et al.* 1990). The neurotoxic effects of DSP-4 are thought to depend on the uptake of the drug by the noradrenaline reuptake transporter complex on noradrenergic terminals, as the effects of DSP-4 are completely counteracted by co-administration of the noradrenaline reuptake inhibitor, desipramine (Jonsson *et al.* 1981). Studies have found that DSP-4 has a higher affinity for the noradrenaline transporter site in the cortex compared with the hypothalamus (Zaczek *et al.* 1990). This could explain why neurones in the cortex are more susceptible to the neurotoxic effects of DSP-4 than are neurones in the hypothalamus. Furthermore, Zaczek *et al.* (1990) found that noradrenaline itself, as well as the highly selective noradrenaline reuptake inhibitor, nisoxetine, displayed higher affinities for the noradrenaline transporter site in the cortex than in the hypothalamus.

Alternatively, the differential effects of DSP-4 could be due to fewer noradrenaline transporter sites per noradrenergic neurone in the hypothalamus compared with cortical regions. Limited transportation of DSP-4 into hypothalamic neurones could lead to insufficient DSP-4 accumulation within the neurone to cause long-term damage. Differences in the pharmacology and/or the number of noradrenaline transporter sites in these two brain regions could account for the differences in the rate of accumulation of extracellular noradrenaline in the cortex and hypothalamus following sibutramine administration.

The suggestions above provide possible explanations as to why the increase in extracellular noradrenaline concentration appears to be more rapid in the hypothalamus compared with the frontal cortex. However, these suggestions do not account for the decline in extracellular noradrenaline concentration in the hypothalamus 40 min post-injection of sibutramine. An explanation for this could lie in a difference in the function of terminals α_2-adrenoceptors in the cortex and hypothalamus and/or a difference in the function of somatodendritic α_2-adrenoceptors located on the cell bodies of lateral tegmental noradrenergic and locus coeruleus noradrenergic neurones.

The hypothalamus has a greater proportion of terminal α_2-adrenoceptors than the cortex (Heal *et al.* 1993; see Chapter 5, section 5.4.4.); this could imply that noradrenergic neurones in the hypothalamus are subject to greater feedback-inhibition of release by these α_2-adrenoceptors, than are neurones in the cortex. Nevertheless, a greater level of control over noradrenaline release in the hypothalamus would be expected to restrict the accumulation of extracellular noradrenaline to a particular magnitude: it is difficult to visualise how this would explain the *decline* in extracellular noradrenaline concentration 40 min following sibutramine administration.

Electrophysiological studies in anaesthetised rats show that the firing rate of locus coeruleus, as well as lateral tegmental neurones, is inhibited by activation of α_2-adrenoceptors (see Chapter 1, section 1.4.3.). However, whereas the α_2-adrenoceptors mediating these effects on locus coeruleus appear to be located on the cell bodies (Svensson *et al.* 1975), evidence suggests that the α_2-adrenoceptors mediating these effects on A1 noradrenergic neurones are not. Kaba *et al.* (1986) showed that systemic administration of clonidine dose-dependently inhibited the firing rate of cells in the A1 noradrenergic region that were antidromically stimulated from the medial forebrain bundle. However, whereas microiontophoretic application of noradrenaline inhibited the firing of neurones in the A1 noradrenergic region, microiontophoretic application of clonidine did not. This finding contrasted with similar experiments carried out in the locus coeruleus (Svensson *et al.* 1975) and suggested that systemic administration of clonidine did not act directly on these neurones to inhibit their firing. The lack of effect of microiontophorectically applied clonidine on A1 noradrenergic neuronal firing may be attributed to an effect of the anaesthesia. However, the finding of Kaba *et al.* (1986) are consistent with studies showing that the density of α_2-adrenoceptors labelled with the α_2-adrenoceptor agonist, [^{3}H]*p*-amino-clonidine, was greater in the region of the locus coeruleus compared with the region of A1 noradrenergic neurones (Young and Kuhar, 1980).

Support for the suggestion above came from further experiments in the study of Kaba *et al.* (1986) showing that microiontophoretic application of the α_2-adrenoceptor

antagonists, phentolamine and piperoxane did not block the inhibitory effect of noradrenaline. Instead, the inhibitory effect of noradrenaline on A1 noradrenergic neuronal firing was inhibited by microiontophoretic application of the β-adrenoceptor antagonist, timolol. This suggested that the firing rate of these neurones could be regulated by β-adrenoceptors in the region of their cell bodies, as well as α_2-adrenoceptors in a location outside of this region.

This proposal could go some way to explaining the decline in extracellular noradrenaline accumulation in the hypothalamus 40 min following sibutramine administration. It is possible that the sibutramine-induced increase in extracellular noradrenaline in the region of noradrenergic neuronal cell bodies affects the firing rate of A1 neurones to a greater extent than neurones of the locus coeruleus. The initial increase in extracellular noradrenaline concentration induced by sibutramine could activate β-adrenoceptors in the region of the cell bodies of A1 noradrenergic neurones and directly inhibit their firing to a certain extent. Then, as the concentration of extracellular noradrenaline increases, α_2-adrenoceptors outside of the region of the cell bodies are activated and inhibit (indirectly) the firing activity of A1 noradrenergic neurones further. This greater inhibition of neuronal firing of A1 noradrenergic neurones would withdraw at least a part of the noradrenergic input into the paraventricular nucleus, as well as the surrounding areas: this could account for the decline in extracellular noradrenaline accumulation in this region 40 min following sibutramine administration.

Nevertheless, a proposal such as the one described above is still not satisfactory for explaining the *persistence* of the decline in extracellular noradrenaline accumulation in the hypothalamus 40 min following sibutramine administration. If the firing rate of noradrenergic neurones innervating the hypothalamus is inhibited by the increase in extracellular noradrenaline induced by sibutramine, why do they not recover when the concentration of extracellular noradrenaline is reduced (*e.g.* 60 min post-injection of sibutramine, see Figure 4.3.) and subsequently allow further release of noradrenaline? (*i.e.* why is there no oscillation of extracellular noradrenaline concentration, which reflects the inhibition and recovery of the noradrenergic neurones innervating the hypothalamus?). The possibility that the reuptake inhibiting properties of sibutramine's metabolites have worn off is highly unlikely since studies in the frontal cortex show that

sibutramine promotes extracellular noradrenaline accumulation in this area for up to 4 h. However, it is possible that noradrenaline release is oscillating in the hypothalamus but that this effect is not detectable using *in vivo* microdiaysis. A major limitation of this technique is the low time resolution of events. This is due to the collection of samples at 20 min intervals in order to obtain a sufficient concentration of noradrenaline for detection by HPLC-ECD.

Perhaps noradrenergic neurones innervating the hypothalamus take a long time to recover following an increase in extracellular noradrenaline concentration and the subsequent inhibition of firing. Electrophysiological studies investigating the effects of sibutramine and its metabolites on noradrenergic neuronal firing of lateral tegmental as well as locus coeruleus neurones would shed light on this matter. In addition, electrophysiological studies comparing the effects of sibutramine's metabolites with a selective noradrenaline reuptake inhibitor, such as nisoxetine, on the firing activity of lateral tegmental and locus coeruleus neurones would be useful. Such studies would indicate if / how the simultaneous rise in extracellular 5-HT induced by sibutramine effects the magnitude and /or time-courses of the increase in extracellular noradrenaline concentration in the cortex and hypothalamus. Ideally, these experiments would be carried out in conscious, freely-moving rats.

The time-courses for the increase in extracellular noradrenaline induced by 1 and 10 mg / kg sibutramine are comparable with ED_{50} values for the inhibition of food-intake following a single dose of this drug (ED_{50} values: 0.6, 2.0 & 3.7 mg / kg i.p. at 1, 2 and 4 h post-injection, respectively, 'in house' data, Knoll Pharmaceuticals). These findings are both in agreement and at variance with existing literature. Firstly, they are consistent with *in vitro* evidence that the active metabolites of this drug are potent inhibitors of noradrenaline uptake (Cheetham *et al.* 1996). Nevertheless, the finding that sibutramine increases noradrenaline in the region of the paraventricular nucleus is at variance with a long-standing proposal that enhancing noradrenergic activity in this hypothalamic region increases feeding behaviour (Matthews *et al.* 1978; Leibowitz, 1978). The increase in feeding behaviour induced by injection of noradrenaline into the paraventricular nucleus is mediated by noradrenergic activation of postsynaptic α_2-adrenoceptors (Goldman *et al.*

1985). This raises the question of why does exogenous noradrenaline injected into the paraventricular nucleus mediate an increase in feeding behaviour whilst sibutramine induced an increase in extracellular noradrenaline in this region but causes a decrease in food intake? Other studies show that injection of α_1-adrenoceptor agonists into the PVN reliably and dose-dependently suppresses feeding behaviour in rats (Wellman, 1992). Therefore, it is possible that the anti-obesity effects induced by sibutramine involve noradrenergic activation of α_1-adrenoceptors in the PVN. Indeed, Jackson *et al.* (1997a) found the hypophagic response to systemic sibutramine in rats is fully reversed by coadministration of the α_1-adrenoceptor antagonist, prazosin, but is unaltered by coadministration of the α_2-adrenoceptor antagonist, RX821002.

One limitation of experiments involving the local administration of exogenous noradrenaline or clonidine is that the injected drug is likely to reach receptor sites in addition to those at noradrenergic synapses. These extrasynaptic sites could contain α_2-heteroreceptors on the membranes of other neurones and their activation by noradrenaline or clonidine could modulate their activity. For example, local administration of noradrenaline or clonidine could activate α_2-heteroreceptors on the terminals of serotonergic neurones and decrease 5-HT release (Saito *et al.* 1996). Given the inhibitory role of 5-HT on food intake, it is possible that such an inhibition of 5-HT release could account for the increase in food intake following local administration of noradrenaline or clonidine into the paraventricular nucleus.

The purpose of experiments involving the injection of substances into discrete areas of the brain is to investigate the function of a specific neurotransmitter or it's various receptors in that area. A further limitation of experiments such as these is that the findings cannot be extrapolated to the whole animal unless similar findings are found following systemic administration of the substance. Systemic administration of low doses of clonidine does increase food intake in rats (Sanger, 1983) which supports the findings of studies involving the injection of clonidine into the paraventricular nucleus. Furthermore, systemic administration of the α_1-adrenoceptor agonist, cirazoline, in rats dose-dependently suppresses food intake (Davies and Wellman, 1992) which supports the findings of studies involving the injection of α_1-adrenoceptor agonists into the

paraventricular nucleus. Nevertheless, the selective noradrenaline reuptake inhibitor, nisoxetine, which presumably increases the concentration of noradrenaline in noradrenergic synapses of the paraventricular nucleus, does not affect food intake in rats (Jackson *et al.* 1997b). Perhaps the simultaneous activation of both α_1- and α_2-adrenoceptors by the increase in synaptic noradrenaline concentration cancels out any effect on food intake.

Of further note is that the increase in food intake induced by intrahypothalamic injection of clonidine in satiated rats is only small compared with the increase in food intake induced by other substances that have more recently been found to increase food intake, such as neuropeptide Y (NPY). For example, satiated rats injected with clonidine (20 nmol) into the paraventricular nucleus eat approximately 6 g of food over the following 2 h, whereas satiated control rats eat approximately 0.5 g of food (Goldman *et al.* 1985). Although this is a marked increase in food intake, it is relatively small compared to the 12.5 g of food eaten by satiated rats only 1 h following injection with NPY (78 pmol) into the perifornical hypothalamus at the level of the caudal paraventricular nucleus (Stanley *et al.* 1993). Furthermore, unlike α_2-adrenoceptor antagonists, recent studies showed that i.c.v. administration of the NYP receptor antagonist, 1229U91 (a nonapeptide dimer) alone, inhibited spontaneous food intake in rats (Ishihara *et al.* 1998). However, the subtype of NPY receptor involved in the effects of 1229U91 on food intake in this study was not clear. The less potent action of clonidine on food intake compared with NPY supports the findings of the present study, which suggest that postsynaptic α_2-adrenoceptors in the hypothalamus are unlikely to be involved in the physiological control of food intake. Furthermore, the decrease in food intake induced by sibutramine is not affected by RX821002, suggesting that these receptors are not involved in the this drug's anti-obesity actions (see section 4.4.3. below).

d-Amphetamine (10 mg / kg) increased extracellular noradrenaline concentration in the hypothalamus and, as with sibutramine, this finding is inconsistent with reports that increasing noradrenergic activity in this region increases feeding behaviour. *d*-Amphetamine also increased the extracellular concentration of dopamine in the

striatum and nucleus accumbens and this has been correlated with an increase in stereotypical behaviour and locomotor activity in rats (Sharp *et al.* 1987). Studies show that it is probably these stimulant effects of *d*-amphetamine that cause its anti-obesity effects (Halford *et al.* 1995: see Chapter 3, section 3.1.). Although central noradrenergic activity has shown to be important for *d*-amphetamine's anti-obesity effects (Samanin *et al.* 1977: see Chapter 1, section 1.3.2.) it is possible that the increased noradrenergic activity is involved in the behavioural arousal, which leads to the decrease in food-intake, rather than being directly involved in this effect (see Chapter 3, section 3.1.).

4.4.3. The role of noradrenaline in mediating sibutramine's anti-obesity effects.

The time-courses for the increases in extracellular noradrenaline and 5-HT induced by sibutramine in the hypothalamus are consistent with findings from feeding studies investigating the involvement of noradrenergic and serotonergic receptors in mediating this drug's effects. Jackson *et al.* (1997a) found that the hypophagic effect of sibutramine in rats was completely antagonised by systemic administration of the α_1-adrenoceptor agonist, prazosin, at 2, 4, and 8 h post-sibutramine administration. Blockade of β_1-adrenoceptors with metoprolol induced a partial reversal at 2, 4 and 8 h post-sibutramine administration. Blockade of 5-$HT_{2A/2C}$ or 5-$HT_{2B/2C}$ receptors with ritanserin or N-(1[2,3-dihydro-(7-methyl-1H-inden-4-yl)oxy]-3[(1-methylethyl) amino]-2-butanol hydrochloride (SB200646) respectively, or with the non-selective 5-HT receptor antagonist, metergoline, also partially reversed the hypophagic effects of the sibutramine. However, this partial reversal was not observed until 8 h post-sibutramine administration. Grignaschi *et al.* (1999) also found no effect of systemic administration of metergoline, or ritanserin on the hypophagic effects of sibutramine in rats in the 2 h period following sibutramine administration. The 5-$HT_{1B/1D}$ receptor antagonist, N-[4-methoxy-3-(4-methyl-1-piperazinyl]phenyl]-2′methyl-4′(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]4-carboxamide hydrochloride (GR127935) was also without effect. However these authors did report a small reduction in sibutamine's effects when combined with the 5-$HT_{2B/2C}$ receptor antagonist, 5-methyl-1-(3-pyridylcarbamoyl)-

1,2,3,5-tetrahydopyrrolo [2,3-f]indole) (SB206553).

The findings of both Jackson *et al.* (1997a) and Grignashi *et al.* (1999) led the authors to suggest that noradrenaline plays a prominent role in sibutramine-induced hypophagia in the first few hours following drug administration. Again, this questions the physiological role of hypothalamic α_2-adrenoceptors in the control of food intake. Jackson *et al.* (1997a) suggested that, considering the hypophagic effects of sibutramine are completely reversed by prazosin, the α_1-adrenoceptors involved in this effect are located down-stream of the 5-HT and β_1-adrenoceptors which appear to only partially mediate sibutramine's hypophagic effects. Thus, the increase in extracellular noradrenaline could activate β-adrenoceptors in the lateral hypothalamus to inhibit food intake (see Chapter 1, section 1.2.1.) whilst the increase in extracellular 5-HT activates 5-HT receptors in the medial hypothalamus to decrease food intake (see Chapter 1, section 1.2.2.). It is possible that both these systems send signals to a common downstream pathway to inhibit food intake and that the receptor in this pathway that receives the signals from the β-adrenergic and serotonergic systems is the α_1-adrenoceptor. Whether this suggestion can be supported with further evidence remains to be elucidated.

Although the increase in extracellular noradrenaline induced by sibutramine and *d*-amphetamine in the region of the paraventricular nucleus is difficult to reconcile with evidence that injection of noradrenaline into this area stimulates food intake, this effect is consistent with a role for hypothalamic noradrenaline in the regulation of BAT thermogenesis. Sahakian *et al.* (1983) showed that depletion of hypothalamic noradrenaline induced a reduction in brown adipose tissue activity in rats (see section 4.1.). Thus, it could be possible that the sibutramine- or *d*-amphetamine-induced increase in noradrenergic transmission in the hypothalamus enhances BAT thermogenesis. This suggestion is consistent with the increase in BAT thermogenesis induced by sibutramine and amphetamine (Stock, 1997; Wellman, 1983, respectively).

4.4.4. Conclusion.

Sibutramine and *d*-amphetamine increased the concentration of extracellular noradrenaline in the frontal cortex of conscious, freely-moving rats. The time-courses of the increases in extracellular noradrenaline concentration are consistent with the noradrenaline-reuptake inhibiting and noradrenaline-releasing properties of sibutramine and *d*-amphetamine, respectively. However, this study revealed both quantitative and qualitative differences in the effects of sibutramine on cortical extracellular noradrenaline accumulation in freely-moving rats compared with halothane-anaesthetised rats. This highlights how the effects of anaesthesia should be borne in mind when investigating the pharmacology of centrally acting agents. Sibutramine and *d*-amphetamine also increased the concentration of extracellular noradrenaline in the hypothalamus of conscious, freely-moving rats. However, the increase in extracellular noradrenaline induced by sibutramine in the hypothalamus is more rapid and shorter-lived than in the cortex. The reason why the increase in extracellular noradrenaline concentration in the hypothalamus declines 40 min following sibutramine administration is unclear. However, the difference responses to sibutramine in the cortex and hypothalamus could be due to functional differences between the noradrenergic neurones innervating these brain regions.

Chapter 5

Effect of α_2-adrenoceptor blockade on sibutramine-induced increases in extracellular noradrenaline concentration in the frontal cortex and hypothalamus of conscious, freely-moving rats.

5.1 Introduction

Chapter 3 described how blockade of α_2-adrenoceptors enhanced the sibutramine-induced increase in extracellular noradrenaline concentration in the frontal cortex of anaesthetised rats. This strongly suggests that activation of somatodendritic and / or terminal α_2-adrenoceptors, by the increased extracellular noradrenaline concentration, decreases locus coeruleus neuronal firing and / or exocytotic noradrenaline release, respectively (see Chapter 1, section 1.4.3.). Sibutramine also increased the extracellular concentration of noradrenaline in the hypothalamus (Chapter 4). A question arising is whether noradrenergic neurones innervating the hypothalamus are subject to the same α_2-adrenoceptor-mediated inhibition of neuronal activity, after the administration of sibutramine, as are noradrenergic neurones innervating the cortex.

There have been fewer studies of the effects of drugs on extracellular noradrenaline concentration in the hypothalamus *in vivo* compared with areas innervated

exclusively by locus coeruleus neurones. However, there is evidence to suggest that extracellular noradrenaline concentration in the hypothalamus is affected by α_2-adrenoceptor activation or blockade. *In vivo* microdialysis studies showed that extracellular noradrenaline concentration in the medial hypothalamus of anaesthetised rats is decreased to approximately 20% of basal values by systemic clonidine administration (Itoh *et al.* 1990). In contrast, systemic administration of the α_2-adrenoceptor antagonists, yohimbine or atipamezole, increaseed extracellular noradrenaline accumulation by 64% and 125% above basal values, respectively, in the medial hypothalamus of anaesthetised rats (Itoh *et al.* 1990; Laitinen *et al.* 1995). Similarly, systemic administration of the α_2-adrenoceptor antagonist, idazoxan, increases extracellular noradrenaline accumulation by 229% above basal values in the posterior hypothalamus of anaesthetised rats (Routledge and Marsden, 1987). These findings suggest that somatodendritic and / or terminal α_2-adrenoceptors are capable of modulating neuronal firing and exocytotic noradrenaline release in the hypothalamus of anaesthetised rats.

Few studies have investigated the effects of local administration of the α_2-adrenoceptor antagonists on extracellular noradrenaline concentration in the hypothalamus. One study showed a trend for extracellular noradrenaline concentration to increase during local perfusion of the selective α_2-adrenoceptor antagonist, atipamezole, through a microdialysis probe implanted in the medial hypothalamus (Laitinen *et al.* 1995). However, the authors reported that, in chloral hydrate anaesthetised rats, the increase in extracellular noradrenaline induced by atipamezole was not significant compared with basal values, because of considerable individual differences in the rats' response.

All the studies described above have been carried out in anaesthetised rats. Therefore, it cannot be assumed that these findings generalise to conscious, freely-moving rats. Furthermore, although α_2-adrenoceptors have been found to regulate extracellular noradrenaline concentration in the cortex of conscious, freely-moving rats, it cannot be assumed that these findings generalise to the hypothalamus since, unlike the cortex, this area is innervated by both noradrenergic systems.

Since there have been no previous microdialysis studies comparing the function of α_2-adrenoceptors in the frontal cortex and hypothalamus, the principle aim of this study was to investigate the effect of α_2-adrenoceptor blockade on noradrenergic neuronal activity in these two brain areas. This involved comparing the effects of α_2-adrenoceptor blockade on basal, as well as sibutramine-induced increases in extracellular noradrenaline concentration in the frontal cortex and hypothalamus. Two approaches were taken in this investigation. Firstly, the effects of systemic administration of an α_2-adrenoceptor antagonist on basal and sibutramine-induced increases in extracellular noradrenaline concentration were compared in rat frontal cortex and hypothalamus. Secondly, to investigate the function of terminal α_2-adrenoceptors alone, the effects of local administration of an α_2-adrenoceptor antagonist into the frontal cortex or hypothalamus on basal and sibutramine-induced increases in extracellular noradrenaline concentration were compared. The study was carried out in conscious, freely-moving rats so that any effect of anaesthesia on central noradrenergic activity could be ruled out.

The α_2-adrenoceptor antagonists chosen for this study were the highly selective antagonists, RX821002 (see Chapter 3, section 3.1.) and atipamezole. Atipamezole is an imidazole derivative and is reported to have an α_2 / α_1-adrenoceptor selectivity ratio of 8526 in rat brain membranes (Virtanen *et al.* 1989). A further reason for the choice of atipamezole is that this α_2-adrenoceptor antagonist binds to all α_2-adrenoceptor subtypes, even at low doses (Haapalinna *et al.* 1997).

5.2 Methods

5.2.1. Experimental design

Experiments were carried out in conscious, freely-moving rats. For details on procedures, refer to Chapter 2, section 2.2.1. Pretreatment of rats with an α_2-adrenoceptor antagonist or saline was carried out using i.p. administration. Local administration of RX821002 into the cortex or hypothalamus was carried out using reverse dialysis. In both experiments sibutramine was administered using the i.p. route.

In the first series of experiments, rats were divided into 3 groups. After the collection of 4 basal samples, one group of rats received 3 mg / kg of the selective α_2-adrenoceptor antagonist, RX821002, and 2 h later received sibutramine (10 mg / kg) or saline. RX821002 was administered at 3 mg / kg based on findings from Chapter 1 showing that this drug effectively blocks presynaptic α_2-adrenoceptors at this dose. A second group of rats received 1 mg / kg of the selective α_2-antagonist, atipamezole, following the collection of 4 samples and 2 h later received sibutramine (10 mg / kg). Atipamezole was administered at 1 mg / kg because this induced an increase in basal dialysate noradrenaline concentration that was comparable with that caused by 3 mg / kg RX821002. A third group of rats received saline following collection of 4 basal samples and 2 h later received sibutramine (10 mg / kg). In all groups of rats, samples were collected for 3 h following the second injection. A time period of 2 h was left between each injection to allow a clear discrimination of the increase in extracellular noradrenaline concentration induced by each drug administration.

A second series of experiments examined the effects of local administration of RX821002 into the frontal cortex or hypothalamus on the increases in extracellular noradrenaline concentration induced by 10 mg / kg sibutramine. Following the collection of 4 basal samples, rats were injected with either sibutramine (10 mg / kg) or saline and, 80 min later, RX821002 was administered *via* the dialysis probe at a concentration of 1 μM. A time period of 80 min was left between the injection of sibutramine and the administration of RX821002 to allow a clear discrimination of the initial increase in

extracellular noradrenaline induced by sibutramine and the enhanced increase induced by α_2-adrenoceptor blockade.

5.2.2. Statistical analysis

Changes in the noradrenaline content of dialysis samples after drug injection were tested for statistical significance using ANOVA with repeated measures. Analysis was carried out with 'time' as the within-subject factor. To compare the effects of α_2-adrenoceptor antagonist-pretreatment with saline-pretreatment or the effects of local administration of RX821002 with drug-free aCSF, 'drug treatment' was added as the 'between-subjects' factor. To assess RX821002-induced changes in the latency to reach peak dialysate noradrenaline concentration, the mean latency to reach the peak drug effect for individual rats was determined and one-way ANOVA used to compare the effects of different treatment groups.

5.3. Results

5.3.1. Effects of systemic administration of RX821002 or atipamezole on sibutramine-induced increases in dialysate noradrenaline concentration.

5.3.1.1. Frontal cortex.

The mean noradrenaline content of the basal samples from the frontal cortex was 30.1 ± 8.55 fmol / 20 min (pooled data from all experiments in this section; n = 19). RX812002 (3 mg / kg) induced an approximately 1.7-fold increase in dialysate noradrenaline concentration (Figure 5.1). The increase in noradrenaline concentration was greater than that induced by saline injection (effect of treatment: $F = 25.3$; d.f. 1,8; $P < 0.01$).

At 120 min following the injection of RX821002, when dialysate noradrenaline concentration had become stable again, an i.p. injection of saline had no further effect on dialysate noradrenaline concentration (Figure 5.1.). However, an i.p. injection of sibutramine at this point (10 mg / kg), caused a rapid increase in dialysate noradrenaline concentration. The increase was statistically significant at 20 min ($F = 9.78$; d.f. 3,15; $P = 0.01$) and reached a maximum of 1014% (*c.f.* mean basal concentration) within 40 min. Following this, dialysate noradrenaline concentration decreased to a plateau representing an approximately 4.5-fold increase in noradrenaline (*c.f.* mean basal concentration). In contrast, when sibutramine (10 mg / kg) was administered 120 min after an i.p. injection of saline, there was a gradual increase in dialysate noradrenaline concentration and the maximum (320%) was not reached until 120 min post-injection (Figure 5.1.). At all times following the injection of sibutramine, dialysate noradrenaline concentrations in RX821002-pretreated rats were significantly greater than in saline-pretreated rats (last hour: $F = 7.95$; d.f. 1,7; $P = 0.03$). Furthermore, the latency to reach maximum noradrenaline concentration was less in rats pretreated with RX821002 than with saline ($F = 10.67$; d.f. 1, 6; $P = 0.02$).

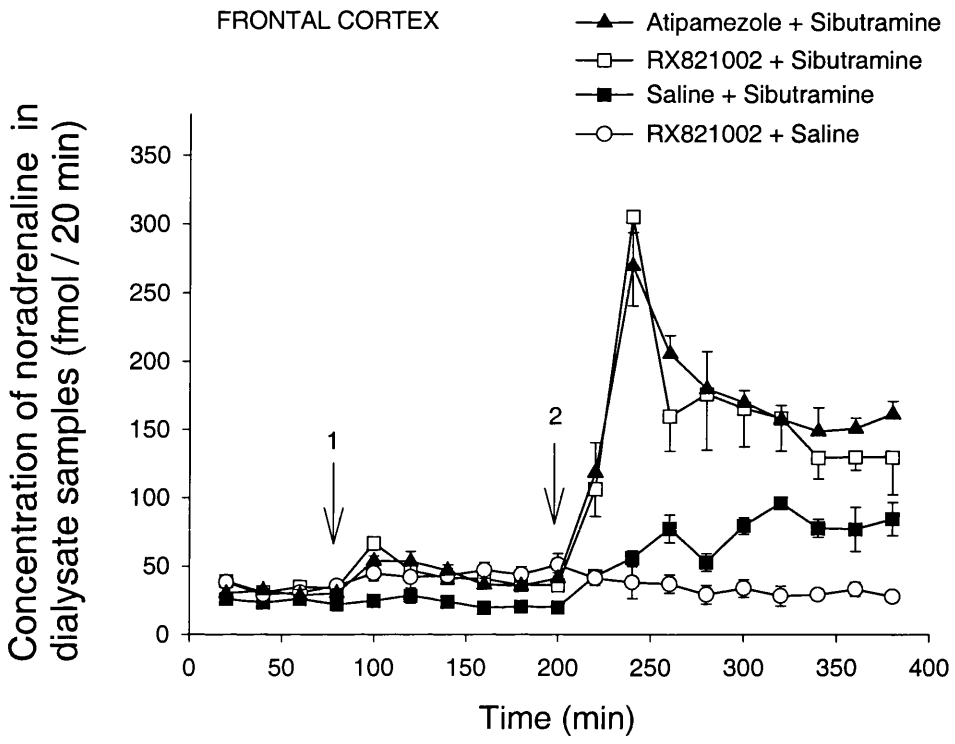


Figure 5.1: *The effect of sibutramine (10 mg / kg i.p.) on the concentration of noradrenaline in dialysate samples from the cortex of rats pretreated with either RX821002 (3 mg / kg i.p.) or atipamezole (1 mg / kg i.p.).*

Arrow 1 represents systemic administration of RX821002, atipamezole or saline. Arrow 2 represents systemic administration of sibutramine or saline. Data points represent mean ± s.e. mean dialysate noradrenaline concentration (fmol / 20 min; n = 4-6).

Atipamezole (1 mg / kg) induced an increase in dialysate noradrenaline concentration that was similar in magnitude and time-course to that seen with RX821002 (3 mg / kg) (Figure 5.1.). An injection of sibutramine (10 mg / kg), 2 h after atipamezole, also induced a increase in dialysate noradrenaline concentration similar to that seen in rats pretreated with 3 mg / kg RX821002 (effect of pretreatment: $F = 0.27$; d.f. 1,4; $P = 0.63$) (Figure 5.1.).

5.3.1.2. Hypothalamus

The mean noradrenaline content of the basal samples from the hypothalamus was 18.9 ± 0.9 fmol / 20 min (pooled data from all experiments in this section; n = 21). RX821002 (3 mg / kg) induced an approximately 2-fold increase in dialysate noradrenaline concentration (Figure 5.2.). This increase in noradrenaline was greater than the increase induced by saline injection ($F = 25.45$; d.f. 1,8; $P < 0.01$).

At 120 min following the injection of RX821002, when dialysate noradrenaline concentration had become stable again, an i.p. injection of saline did not significantly affect dialysate noradrenaline concentration (Figure 5.2.). In contrast, an i.p. injection of sibutramine at this point caused a rapid increase in dialysate noradrenaline concentration, which reached a maximum of 995% (*c.f.* mean basal concentration) at 20 min ($F = 44.67$; d.f. 3,6; $P < 0.01$). Following this, dialysate noradrenaline concentration declined steadily to an approximately 3-fold increase in noradrenaline (*c.f.* mean basal concentration), 80 min later. Sibutramine (10 mg / kg) administered 120 min after an i.p. injection of saline, induced a maximum increase in noradrenaline concentration of approximately 300% at 40 min post-injection ($F = 11.79$; d.f. 4,20; $P < 0.01$). Although this was a smaller initial increase in noradrenaline compared with that induced by sibutramine in RX821002-pretreated rats ($F = 114.10$; d.f. 1,7; $P < 0.01$), there was no difference in noradrenaline concentration between the two treatment groups 2 h after sibutramine injection. There was also no difference in the latency to reach maximum noradrenaline concentration in rats pretreated with RX821002 or saline.

Atipamezole (1 mg / kg) induced an approximately 3-fold increase in dialysate noradrenaline concentration (Figure 5.2.). An injection of sibutramine 2 h after atipamezole induced an increase in dialysate noradrenaline similar to that observed in rats pretreated with 3 mg / kg RX821002 ($F = 0.34$; d.f. 1,5; $P = 0.59$) (Figure 5.2.).

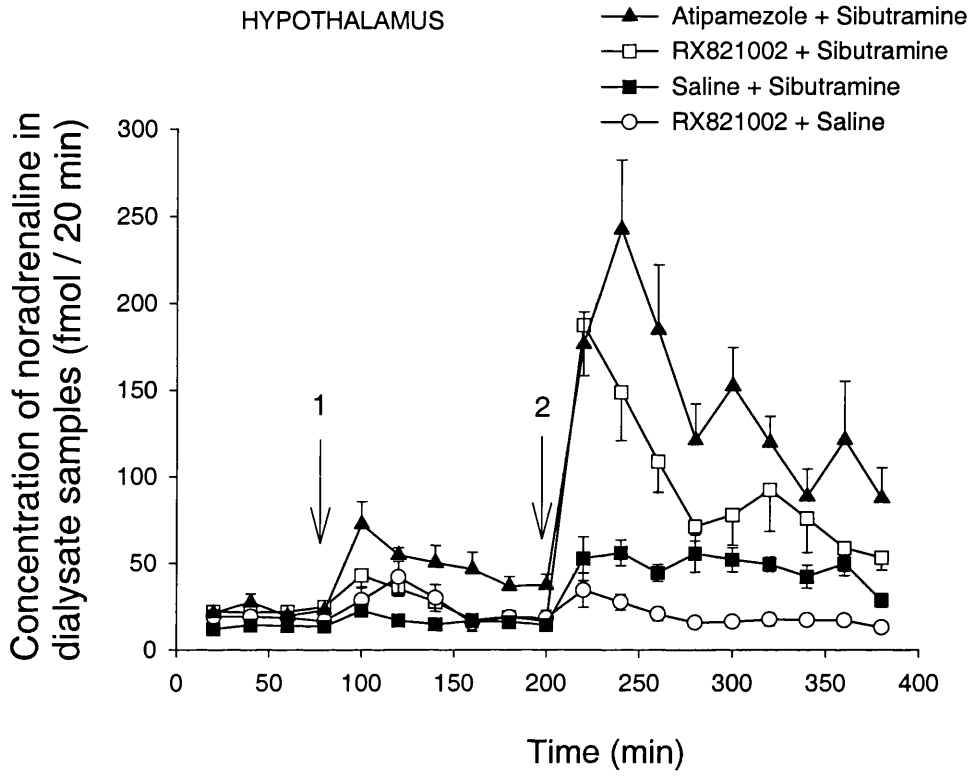


Figure 5.2: *The effect of sibutramine (10 mg / kg i.p.) on the concentration of noradrenaline in dialysate samples from the hypothalamus of rats pretreated with either RX821002 (3 mg / kg i.p.) or atipamezole (1 mg / kg i.p.).*

Arrow 1 represents systemic administration of RX821002, atipamezole or saline. Arrow 2 represents systemic administration of sibutramine or saline. Data points represent mean ± s.e. mean dialysate noradrenaline concentration (fmol / 20 min; n = 5-6).

5.3.2. Effects of local administration of RX821002 on sibutramine-induced increases in dialysate noradrenaline concentration.

5.3.2.1. Frontal cortex.

The mean noradrenaline content of the basal samples from the frontal cortex was 22.7 ± 0.8 fmol / 20 min (pooled data from all experiments in this section: n = 22).

Administration of RX821002 (1 μM) *via* the probe, 80 min after saline injection (2 ml/ kg), increased basal noradrenaline concentration by approximately 2-fold compared with that of rats infused with drug-free aCSF ($F = 22.23$; d.f. 1,9; $P < 0.01$) (Figure 5.3.). RX821002 administered 80 min after sibutramine (10 mg / kg), also increased dialysate noradrenaline concentration compared with rats infused with drug-free aCSF ($F = 6.87$; d.f. 1,7; $P = 0.03$) (Figure 5.3.). Within 80 min of the start of RX821002 infusion, the increase in noradrenaline concentration caused by sibutramine was approximately 1.5-fold greater than the dialysate noradrenaline concentration induced by sibutramine alone.

5.3.2.2. Hypothalamus.

The mean noradrenaline content of the basal samples from the hypothalamus was 12.3 ± 0.6 fmol / 20 min (pooled data from all experiments in this section: n = 23).

Administration of RX821002 (1 μM) *via* the probe 80 min after saline (2 ml / kg), increased basal noradrenaline concentration by approximately 2-fold compared with samples from rats infused with drug-free aCSF ($F = 10.11$; d.f.1,5; $P = 0.03$) (Figure 5.4.). RX821002 administered *via* the probe 80 min after sibutramine (10 mg / kg), also increased dialysate noradrenaline concentration compared with rats infused with drug-free aCSF ($F = 6.90$; d.f 1,6; $P = 0.04$) (Figure 5.4.). Within 80 min of the start of the RX821002 infusion, and for the rest of the experiment, the increase in noradrenaline concentration caused by sibutramine was approximately 3-fold greater than the increase induced by sibutramine alone.

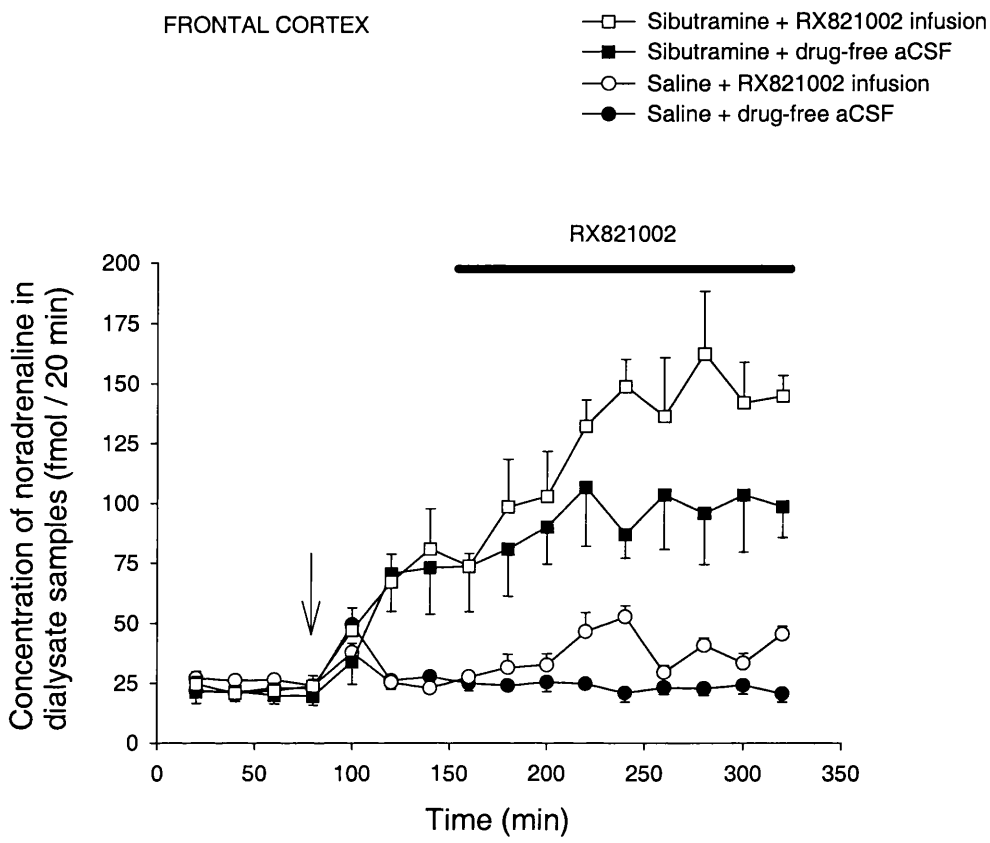


Figure 5.3: *The effect of local administration of RX821002 (1 μM) on basal and sibutramine-induced (10 mg / kg i.p.) increases in dialysate noradrenaline concentration from the frontal cortex.*

Arrow represents systemic administration of sibutramine or saline. Bar indicates the period of RX821002 perfusion *via* the dialysis probe. Data points represent mean ± s.e. mean dialysate noradrenaline concentration (fmol / 20 min; n = 5-6).

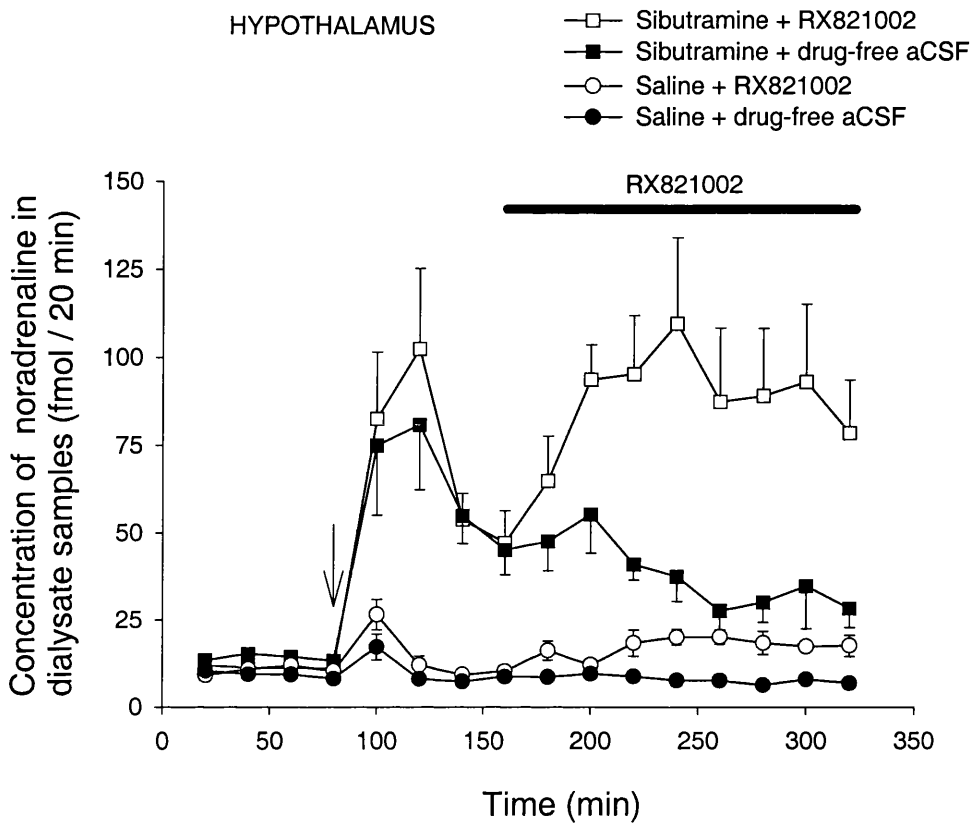


Figure 5.4: *The effect of local administration of RX821002 (1 μM) on basal and sibutramine-induced (10 mg / kg i.p.) increases in dialysate noradrenaline concentration from the hypothalamus.*

Arrow represents systemic administration of sibutramine or saline. Bar indicates the period of RX821002 perfusion *via* the dialysis probe. Data points represent mean ± s.e. mean dialysate noradrenaline concentration (fmol / 20 min; n = 5-6).

5.4. Discussion

In order to investigate the contribution of α_2-adrenoceptors to the control of noradrenaline release in the hypothalamus, this study investigated the effects of α_2-adrenoceptor blockade on noradrenergic function in this brain area. Since studies indicate that cortical regions and hypothalamus differ with respect to their terminal α_2-adrenoceptor density (Heal *et al.* 1993), this study also compared the effects of α_2-adrenoceptor blockade on noradrenergic function in the hypothalamus with the frontal cortex.

5.4.1. Effect of systemic or local perfusion of RX821002 on basal extracellular noradrenaline concentration.

Systemic administration of RX821002 increased resting concentrations of extracellular noradrenaline in the cortex by approximately 2-fold. Similarly, local administration of RX821002 into the cortex approximately doubled basal noradrenaline concentrations. These findings support the suggestion put forward in Chapter 3 (section 3.4.) that halothane-anaesthesia affects central α_2-adrenoceptor function and accounts for the lack of effect of systemic RX821002 administration on basal extracellular noradrenaline in rats under this anaesthetic.

The results of the present study in the frontal cortex of freely-moving rats are consistent with Gobert *et al.* (1997). These authors report an increase in extracellular noradrenaline concentration of 180% above basal values in the frontal cortex of freely-moving rats following systemic administration of atipamezole (0.16 mg / kg s.c.). Dennis *et al.* (1987) report a 200% increase in extracellular noradrenaline concentration in the cerebral cortex following systemic administration of the less selective α_2-adrenoceptor antagonist, idazoxan into freely-moving rats. Mateo *et al.* (1998) infused 1 μM RX821002 into the locus coeruleus of freely-moving rats using reverse dialysis and found a 30% increase in extracellular noradrenaline concentration in the cingulate cortex. This finding supports electrophysiological evidence that activation of α_2-adrenoceptors on the cell bodies of locus coeruleus neurones regulates the neuronal firing rate (see

Chapter 1, section 1.4.3.) and suggests that this contributes to the control of noradrenaline release in the terminal fields.

Similarly, Dennis *et al.* (1987) reported an approximately 2-fold increase in extracellular noradrenaline concentration in the cerebral cortex of freely-moving rats following infusion of a relatively high concentration of idazoxan (10^{-4} M). Dalley and Stanford (1995) infused 0.5 μM atipamezole into the frontal cortex of anaesthetised rats using reverse dialysis and found an increase in extracellular noradrenaline concentration in the frontal cortex of approximately 100% above basal concentration. The findings of the present study, using a highly selective α_2-adrenoceptor antagonist in conscious, freely-moving rats, support the proposal that terminal α_2-adrenoceptors also contribute to the regulation of extracellular noradrenaline release under resting conditions.

As in the frontal cortex, systemic administration of RX821002 (3 mg / kg) increased resting concentrations of extracellular noradrenaline in the hypothalamus approximately 2-fold. This finding strongly suggests that α_2-adrenoceptors also regulate basal extracellular noradrenaline concentration in the hypothalamus of conscious, freely-moving rats. Electrophysiological evidence from anaesthetised rats shows that activation of α_2-adrenoceptors inhibits the firing rate of both locus coeruleus and lateral tegmental (see Chapter 1, section 1.4.3.). Considering the effect of anaesthesia on noradrenergic neuronal function, it cannot be assumed that the findings of these electrophysiological studies also apply to conscious, freely-moving rats. Nevertheless, it is possible that the increase in extracellular noradrenaline concentration in the hypothalamus induced by RX821002 results from changes in noradrenergic neuronal firing.

Local administration of 1 μM RX821002 into the hypothalamus doubled basal noradrenaline concentration. However, Laitinen *et al.* (1995) found that even though there was a trend for extracellular noradrenaline concentration to increase, a dose of atipamezole as high as 50 μM did not significantly increase the concentration of extracellular noradrenaline in the hypothalamus of 7 rats tested under anaesthesia. It is likely that the lack of effect of atipamezole is due an effect of the chloral-hydrate anaesthesia on noradrenergic neuronal activity.

Together, the present findings suggest that α_2-adrenoceptors regulating the release of noradrenaline in the frontal cortex and hypothalamus are activated under resting conditions and restrict basal noradrenaline release to the same extent in both areas. Furthermore, the findings support extensive evidence suggesting that there are α_2-adrenoceptors in the terminal fields of both locus coeruleus and lateral tegmental noradrenergic projections and that some of these are presynaptic.

5.4.2. Effect of systemic RX821002 on sibutramine-induced increases in extracellular noradrenaline concentration.

Systemic pretreatment of rats with RX821002 augmented the increase in extracellular noradrenaline concentration induced by sibutramine in both the cortex and hypothalamus. However, as in Chapter 4, there were differences between the responses of the two brain areas. In the cortex, RX821002 reduced the latency for sibutramine to reach its peak effect. This is consistent with the disinhibition of noradrenaline release by this α_2-adrenoceptor antagonist (see Chapter 3, section 3.4). However, in the present study there was no apparent reduction in the latency for sibutramine to reach its peak effect in the hypothalamus. Nevertheless, it should be borne in mind that it is unlikely a reduction would have been detected, due to the collection of dialysate samples at 20 min intervals.

RX821002 also enhanced the maximum increase in extracellular noradrenaline concentration induced by sibutramine in both brain areas. Gobert *et al.* (1997) also found systemic administration of atipamezole to potentiate the effects of another noradrenaline and 5-HT reuptake inhibitor, duloxetine, on extracellular noradrenaline concentration in the frontal cortex of freely-moving rats. The findings of this study suggest that α_2-adrenoceptors limit the increase in extracellular noradrenaline induced by noradrenaline reuptake inhibitors in areas innervated by lateral tegmental neurones as well as locus coeruleus neurones.

Although RX821002 increased the maximum noradrenaline concentration induced by sibutramine in both brain areas, this effect of RX821002 was shorter-lived in the hypothalamus than in the frontal cortex. Dialysate noradrenaline concentration from the frontal cortex was still significantly greater in RX821002-pretreated rats compared to

saline-treated rats 3 h post-sibutramine administration. In contrast, dialysate noradrenaline concentration from the hypothalamus in RX821002-pretreated rats was no longer greater than in saline-pretreated rats 2 h post-sibutramine administration. It is unlikely that the relatively short-lived effect of sibutramine in combination with RX821002 in the hypothalamus is due to the decreasing activity of RX821002 over the duration of the experiment as experiments in the frontal cortex show RX821002 is still active at this time point. A possible explanation could be that there are more α_2-adrenoceptors regulating the activity of noradrenergic neurones projecting to the hypothalamus than the cortex and that the dose of RX821002 used did not block all of these receptors (see section 5.4.4. below).

5.4.3. Effect of systemic atipamezole on sibutramine-induced increases in extracellular noradrenaline concentration.

Previous reports suggest that RX821002, like the α_2-adrenoceptor antagonists yohimbine and rauwolscine, has moderate affinity for 5-HT_{1A} receptors in rat brain. Javier-Meana *et al.* (1996) show that when rat hippocampal membranes are incubated with 10 nM [^{3}H]RX821002, the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT, displaces [^{3}H]RX821002. To establish that the findings of the present study were a result of an effect of RX821002 at α_2-adrenoceptors, experiments involving the systemic administration of RX821002 were repeated with another highly selective α_2-adrenoceptor antagonist, atipamezole. This drug is reported to have negligible affinity for 5-HT_{1A} receptors since it displaced [^{3}H]8-OH-DPAT binding to rat hippocampal membranes with a K_i of only 13 μM; this compares with K_i values of 52 nM and 74 nM for rauwolscine and yohimbine, respectively (Winter and Rabin, 1992).

Atipamezole produced almost identical effects on extracellular noradrenaline to RX821002 in both brain areas when combined with sibutramine. This strongly suggested that the observed changes in extracellular noradrenaline induced by RX821002 were a result of α_2-adrenoceptor blockade. Subsequently, local perfusion experiments were only carried out with RX821002.

5.4.4. Effect of local infusion of RX821002 into the frontal cortex or hypothalamus on sibutramine-induced increases in extracellular noradrenaline concentration.

Local infusion of RX821002 into the terminal fields augmented the increase in extracellular noradrenaline induced by sibutramine, in both the cortex and hypothalamus. These findings are consistent with a previous report that reverse dialysis of the α_2-adrenoceptor antagonist, idazoxan, into the cerebral cortex increases the accumulation of extracellular noradrenaline induced by desipramine in this brain area (Dennis *et al.* 1987). However, in this study, infusion of RX821002 into the hypothalamus caused a 3-fold increase above the noradrenaline concentration induced by 10 mg / kg sibutramine alone. This compared with a 1.5-fold increase in extracellular noradrenaline concentration above that induced by sibutramine alone, when RX821002 was infused into the cortex. Furthermore, infusion of RX821002 into the hypothalamus prevented the *decline* in extracellular noradrenaline accumulation that occurs in this brain area 40 min post-sibutramine injection. These findings suggest that the increase in extracellular noradrenaline induced by sibutramine in the hypothalamus activates α_2-adrenoceptors and decreases noradrenergic neuronal activity to a greater extent than in the cortex.

One interpretation of the reversal of sibutramine's effects by local administration of RX821002 into the hypothalamus is that α_2-adrenoceptors on the terminals alone are responsible for the decline in extracellular noradrenaline seen 40 min post-sibutramine injection. However, it is puzzling that this effect was not seen when sibutramine was combined with the systemic administration of RX821002. A large terminal α_2-adrenoceptor reserve on the terminals of noradrenergic neurones in the hypothalamus could explain this discrepancy. A dose of 3 mg / kg RX821002 may bind only to a certain proportion of terminal α_2-adrenoceptors in the hypothalamus after its administration. If this is the case, the increase in extracellular noradrenaline concentration induced by sibutramine could still activate the remaining proportion of terminal α_2-adrenoceptors to decrease exocytotic noradrenaline release. This could explain why, when sibutramine is administered to RX821002-pretreated rats, the dialysate noradrenaline concentration falls back to basal values more rapidly than in the cortex. However, continuous local infusion of 1 μM RX821002 into the hypothalamus could block all terminal α_2-adrenoceptors in this region and allow exocytotic noradrenaline release in the presence of a high

extracellular noradrenaline concentration. This would account for the sustained increase in extracellular noradrenaline concentration following sibutramine administration.

The proposal that the hypothalamus could have a terminal α_2-adrenoceptor reserve would be consistent with evidence that there is a greater density of terminal α_2-adrenoceptors in the hypothalamus than in the cortex. Heal *et al.* (1993) measured α_2-adrenoceptor ligand binding in different brain regions of rats three days following treatment with saline, or DSP-4. Measuring α_2-adrenoceptor binding with the α_2-adrenoceptor antagonist, [^{3}H]idazoxan, the authors report that the maximal specific binding (B_{max}) of [^{3}H]idazoxan was approximately 340 and 725 fmol / mg tissue in the cerebral cortex and hypothalamus, respectively, in saline-treated rats. In DSP-4 treated rats, [^{3}H]idazoxan binding was reduced by 20% in cerebral cortex tissue and by 40% in hypothalamic tissue. Since noradrenergic neurones in the frontal cortex are more vulnerable to the toxic effects of DSP-4 than noradrenergic neurones in the hypothalamus (see Chapter 4, section 4.4.2.) this finding cannot be attributed to more neurones surviving in the cortex following DSP-4 treatment. Therefore, the findings of Heal *et al.* (1993) suggest that there is a greater density of α_2-adrenoceptors in the hypothalamus than in the cortex and, furthermore, that a greater proportion of the α_2-adrenoceptor population in the hypothalamus is located on noradrenergic terminals compared with cortical regions. However, although consistent with the present findings, one limitation of radioligand binding studies is that they do not indicate the number of α_2-adrenoceptors per noradrenergic neurone, a potentially critical factor for the proposal of a terminal α_2-adrenoceptor reserve in the hypothalamus.

The proposal of a terminal α_2-adrenoceptor reserve suggests that the decline in extracellular noradrenaline in the hypothalamus 40 min following sibutramine administration is not due to a decrease in the firing of lateral tegmental neurones, as suggested in Chapter 4. Nevertheless, this possibility cannot be ruled out. Kaba *et al.* (1986) reported that systemic administration of the α_2-adrenoceptor agonist, clonidine, inhibited the firing rate of neurones in the A1 noradrenergic region but that the α_2-adrenoceptors, which presumably mediate this effect, are not located in the region of the noradrenergic neuronal cell bodies (see Chapter 4, section 4.4.2.). Although this study was performed in anaesthetised rats, the findings that activation of α_2-adrenoceptors on

locus coeruleus and other lateral tegmental neurones in anaesthetised rats does decrease neuronal firing suggests that there could be a functional difference in A1 noradrenergic neurones. It is possible that these α_2-adrenoceptors are located on the cell bodies of other neurones in the hypothalamus, which project to noradrenergic cell body regions and regulate neuronal activity. For example, using immunohistochemical methods, Swanson *et al.* (1981) showed that the paraventricular nucleus contains dopaminergic neurones, some of which project to the medulla. Perhaps activation of α_2-adrenoceptors on neurones such as these could form part of a negative feedback loop to regulate the firing of noradrenergic cell bodies in the medulla.

In the proposed situation above, the increase in extracellular noradrenaline in the hypothalamus induced by sibutramine would activate α_2-adrenoceptors on the cell bodies of other, non-noradrenergic neurones when the concentration of noradrenaline in the noradrenergic synapses was sufficient to diffuse into adjacent areas. Under resting conditions, these non-noradrenergic neurones could provide an excitatory input to noradrenergic cell bodies. Activation of α_2-adrenoceptors on the cell bodies of the non-noradrenergic neurones could reduce their firing activity and decrease the excitatory input to the noradrenergic neurones. At this point, noradrenergic neuronal firing would decrease and impulse-mediated noradrenaline release in the hypothalamus would be reduced (see Figure 5.4.1.).

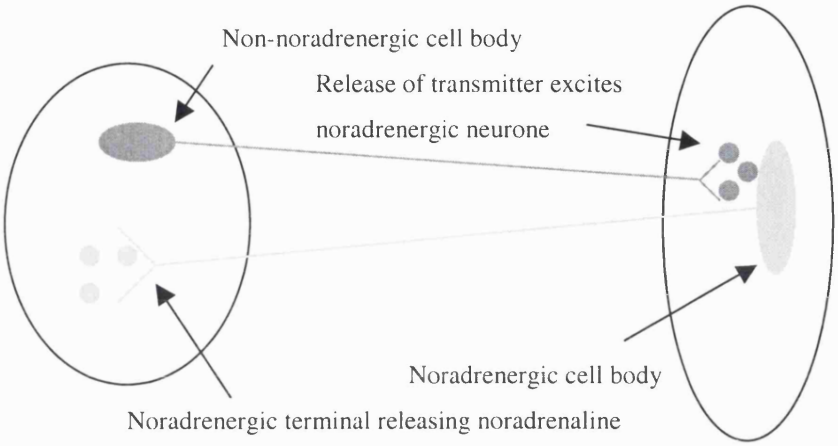
Local infusion of RX821002 into the hypothalamus would block terminal and postsynaptic α_2-adrenoceptors, as well as any α_2-adrenoceptors located outside of noradrenergic synapses such as those described above: blockade of terminal and/or the latter receptors could account for the sustained increase in extracellular noradrenaline induced by sibutramine in the presence of RX821002.

5.4.5. Conclusion

The increases in extracellular noradrenaline concentration induced by sibutramine in both the frontal cortex and hypothalamus are restricted by local populations of α_2-adrenoceptors. Although both populations of α_2-adrenoceptors appear equally effective at restricting basal noradrenaline release, α_2-adrenoceptors in the hypothalamus

cause a greater restriction to sibutramine's effects than do α_2-adrenoceptors in the cortex. This could account for the different time-courses of extracellular noradrenaline accumulation in these two brain areas, following sibutramine administration.

1) RESTING CONDITIONS



2) IN THE PRESENCE OF A NORADRENALINE REUPTAKE INHIBITOR

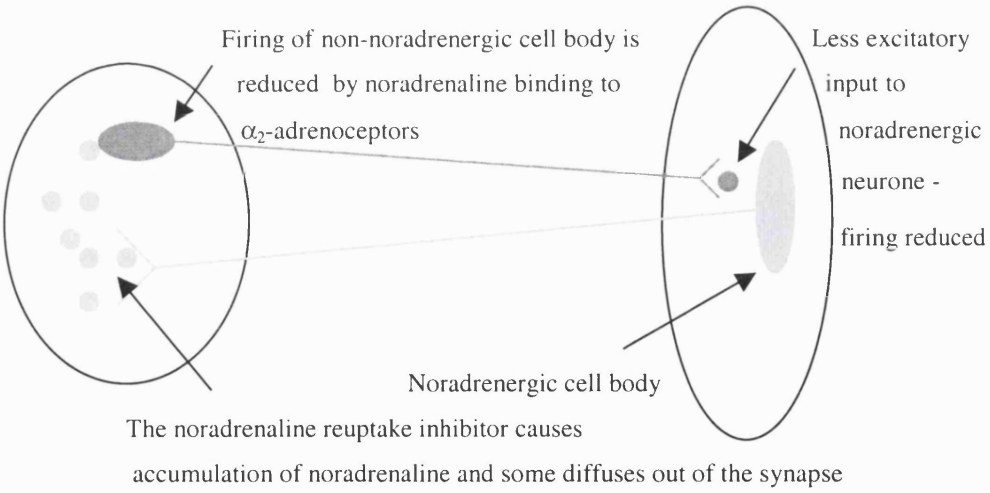


Figure 5.5: *Schematic diagram showing a possible negative feedback loop regulating the firing rate of noradrenergic neurones in the medulla by non-noradrenergic neurones in the hypothalamus.*

Chapter 6

Comparison of the density and function of central α_2-adrenoceptors between the lean and obese Zucker rat.

6.1 Introduction

Obesity in Zucker rats is inherited as a recessive gene (Zucker and Zucker, 1961). Recent studies have shown that these obese rats have a mutation in the gene encoding the leptin receptor (Takaya *et al.* 1996) which results in their insensitivity to this hormone. The obesity of these rats is consistent with a role for leptin in the regulation of body fat in both animals and humans (See Chapter 1, section 1.2.3.). Obese Zucker rats are hyperphagic and have a decreased metabolic rate compared with their lean counterparts (see Chapter 1, section 1.5). The resulting increase in body weight is apparent from as early as 3 weeks of age (Zucker and Zucker, 1961), making the genetically obese Zucker rat a potentially useful model of human, juvenile-onset obesity (Argilés, 1989).

Early studies in the 1970s suggested that noradrenergic activity in the hypothalamus of obese Zucker rats, in particular the paraventricular nucleus, could be reduced compared with their lean counterparts. For example, Cruce *et al.* (1976; 1977) and Levin and Sullivan (1979) found that the tissue concentration of noradrenaline in the hypothalamus of male and female obese Zucker rats was reduced compared with their

lean counterparts (see Chapter 1, section 1.5.2.). It is difficult to deduce the functional significance of tissue content measurements *ex vivo*; although these findings were taken to indicate that noradrenergic activity in the hypothalamus is decreased, a decrease in tissue noradrenaline concentration could mean that noradrenaline release is increased.

Nevertheless, activation of brown adipose tissue thermogenesis in response to a meal is impaired in obese Zucker rats (Triandafillou and Himms-Hagen, 1983; see Chapter 1, section 1.5.1.). Considering the role of hypothalamic noradrenaline in the control of brown adispose tissue thermogenesis (Sahakian *et al.* 1983, see Chapter 4, section 4.1.) the proposal that noradrenergic activity is decreased in the hypothalamus of obese Zucker rats is consistent with these finding. Furthermore, *in vivo* microdialysis experiments show that the increase in extracellular noradrenaline concentration induced by immobilisation stress is reduced in obese Zucker rats compared with their lean counterparts (Pacak *et al.* 1995). This lends further support to the proposal that noradrenergic activity is impaired in the obese Zucker rat.

However, later findings reported in the 1990s were not consistent with the proposal above. Koulu *et al.* (1990) found no differences in the concentration of noradrenaline in the hypothalamus of lean and obese male Zucker rats (see Chapter 1, section 1.5.2.) but found the feeding response induced by a non-sedative dose of clonidine was greater in satiated obese rats compared with lean controls. This suggests that the feeding-stimulatory influence of postsynaptic α_2-adrenoceptors in the paraventricular nucleus of obese Zucker rats is enhanced. This suggestion was supported by Jhanwar-Uniyal *et al.* (1991) who measured α_1- and α_2-adrenoceptor binding in membrane preparations from seven distinct hypothalamic nuclei of female lean and obese Zucker rats. The density of α_1- and α_2-adrenoceptors was measured with the α_1-adrenoceptor antagonist, [^{3}H]prazosin, and the α_2-adrenoceptor agonist, [^{3}H] *p*-amino-clonidine ([^{3}H]PAC), respectively, in freely-feeding rats. In obese rats, [^{3}H]PAC binding was increased to 176% of the lean control value, specifically in the paraventricular nucleus. Also in the paraventricular nucleus, the [^{3}H]prazosin binding was increased to 189% of the lean control value. No other differences were found in receptor density in any of the other regions examined.

Jhanwar-Uniyal *et al.* (1991) proposed that the greater density of α_2-adrenoceptors in the paraventricular nucleus of obese Zucker rats could account for their greater feeding response, compared with lean Zucker rats, following systemic injection of clonidine, as reported by Koulu *et al.* (1990). Furthermore, the authors suggested that this disturbance in the density of α_2-adrenoceptors in obese Zucker rats could contribute to the development or maintenance of obesity in these animals. However, this proposal is based on two assumptions. Firstly, that the release rate of noradrenaline is the same in lean and obese rats although the greater density of α_2-adrenoceptors could reflect their up-regulation as a result of decreased noradrenaline release. The finding that α_1-adrenoceptor density was greater in obese rats lends support to this idea. Secondly, the proposal assumes that the greater density of α_2-adrenoceptors reflects an increase in the density of postsynaptic α_2-adrenoceptors. It is possible that the increase in the density of these receptors reflects an increase in their presynaptic population, which are not involved in the noradrenaline-induced feeding response (Goldman *et al.* 1985). If the density of presynaptic α_2-adrenoceptors is increased in obese rats, this could have implications for the control of noradrenergic firing and / or noradrenaline release: a greater density of presynaptic α_2-adrenoceptors could mean a tighter control over noradrenaline release. This could account for the diminished increase in extracellular noradrenaline concentration in the hypothalamus of obese rats following immobilisation stress (Pacak *et al.* 1995).

Other studies have also investigated the density of α_2-adrenoceptors in the hypothalamus of lean and obese Zucker rats, although the findings are not consistent with those of Jhanwar-Uniyal *et al* (1991). Pesonen *et al.* (1992) measured the density of α_2-adrenoceptors in five forebrain areas of lean and obese male Zucker rats (approximately 21-weeks-old) using autoradiography with the α_2-adrenoceptor agonist, *p*-[^{125}I]iodoclonidine ([^{125}I]PIC). No differences were found in the density of α_2-adrenoceptors between lean and obese Zucker rats in any of the regions studied: the paraventricular nucleus; anterior hypothalamic nucleus; amygdala; cerebral cortex; hippocampus. Miralles *et al.* (1993) also reported no differences in the binding of the α_2-adrenoceptor agonist, [^{3}H]clonidine, to hypothalamic membranes from 10-week-old lean and obese male Zucker rats. The findings of these studies could differ from those of

Jhanwar-Uniyal *et al.* (1991) for a number of reasons, including differences in age and sex of Zucker rat studied, as well as differences in the technique used to determine α_2-adrenoceptor density. Nevertheless, all these studies have one aspect in common; each study used a derivative of clonidine to determine the density of α_2-adrenoceptors. Clonidine, *p*-aminoclonidine and *p*-iodoclonidine are all α_2-adrenoceptor agonists. Therefore, these agents will only bind to α_2-adrenoceptors that are in the high affinity conformational state (see Chapter 2, section 2.1.2.) and, consequently, may not reflect the total density of α_2-adrenoceptors. Furthermore, these ligands have all been shown to have high affinity for imidazoline1 receptors, as well as α_2-adrenoceptors, in rat brain (Kamasaki *et al.* 1990; Heemskerk *et al.* 1998). Thus, the binding data obtained from these studies could also reflect imidazoline1 receptor binding .

The first aim of this study was to re-establish the density of α_2-adrenoceptors in the hypothalamus of lean and obese Zucker rats using [^{3}H]RX821002. Since the early 1990s when the studies above were reported, the highly selective α_2-adrenoceptor antagonist, [^{3}H]RX821002, has become a more preferable radioligand for the study of α_2-adrenoceptor densities. This is because [^{3}H]RX821002 is one of few α_2-adrenoceptor antagonists to show negligible affinity or activity at imidazoline binding sites (Langin *et al.* 1990; Javier-Meana *et al.* 1997), whilst retaining high α_2-adrenoceptor selectivity (see Chapter 3, section 3.1). Furthermore, since this compound is an α_2-adrenoceptor *antagonist*, it will bind to α_2-adrenoceptors in both high- and low-affinity conformational states. Noradrenergic neurones in the hypothalamus are derived primarily from the lateral tegmental area system, although some nuclei, such as the paraventricular nucleus, receive noradrenergic innervation from the locus coeruleus as well (see Chapter 1, section 1.3.). Considering this, it was of further interest to investigate the density of α_2-adrenoceptors in an area exclusively innervated by neurones from the locus coeruleus. Therefore, [^{3}H]RX821002 binding was also investigated in the frontal cortex of lean and obese Zucker rats.

As outlined above, the study by Koulu *et al.* (1990) suggested that postsynaptic α_2-adrenoceptors in the paraventricular nucleus of obese Zucker rats have a greater stimulatory influence on feeding than do α_2-adrenoceptors in the lean Zucker rat. In order

to investigate whether postsynaptic α_2-adrenoceptor function is also enhanced in other brain areas, the second aim of this investigation was to compare postsynaptic α_2-adrenoceptor function in lean and obese Zucker rats using clonidine-induced mydriasis. This is an established and selective model of postsynaptic α_2-adrenoceptor function (see Chapter 2, section 2.1.3.2.) and the α_2-adrenoceptors that mediate this response are thought to be in the Edinger-Westphal nucleus of the midbrain.

The third aim of the study was to investigate the sensitivity of presynaptic α_2-adrenoceptors in lean and obese Zucker rats. The α_2-adrenoceptor binding studies in Zucker rats described above do not indicate the ratio of pre- and postsynaptic α_2-adrenoceptors in the hypothalamus: perhaps the greater density of α_2-adrenoceptors in the paraventricular nucleus of obese Zucker rats, reported by Jhanwar-Uniyal *et al.* (1991) reflects a greater number of presynaptic, as well as, or instead of, postsynaptic α_2-adrenoceptors. In Chapter 5, noradrenergic neurones with different densities of preynaptic α_2-adrenoceptors were shown to respond differently to increases in extracellular noradrenaline concentration induced by sibutramine. Differences in the function of presynaptic α_2-adrenoceptors in the hypothalamus of lean and obese Zucker rats could well contribute to the abnormality in noradrenergic activity in this area. The responses of presynaptic α_2-adrenoceptors in lean and obese Zucker rats were investigated by scoring clonidine-induced hypoactivity. The mechanism through which clonidine induces hypoactivity has been established as presynaptic α_2-adrenoceptor activation (see Chapter 2, section 2.1.3.2.).

6.2. Methods

6.2.1. Experimental design.

6.2.1.1. Experiments involving [^{3}H]RX821002 binding.

Seven male obese Zucker rats (188-231 g) and seven male aged-matched lean controls (170-185 g) were used. Rats were killed between 1400 and 1700 h and the frontal cortex and hypothalamus were dissected from each rat. For details on the [^{3}H]RX821002 binding assay and protein determination, refer to Chapter 2, section 2.2.2.

6.2.1.2. *Experiments involving clonidine-induced hypoactivity and mydriasis.*

Twenty male obese Zucker rats (weighing 148-191 g at the start of the study) and twenty male aged-matched lean controls (weighing 135-146 g at the start of the study) were used. Each rat was used to test both clonidine-induced mydriasis and hypoactivity responses simultaneously. For details on the procedures used and scoring of hypoactitivity, refer to Chapter 2, section 2.2.3.

6.2.2. Statistical analysis.

Data from [^{3}H]RX821002 binding experiments were analysed by Richard Brammer (Knoll Pharmaceuticals Statistics Department). K_d data were log transformed. K_d and B_{max} data were then analysed using a two-way ANOVA with group (lean or obese) and assay dates as factors.

For analysis of mydriasis experiments, the mean score for each dose was calculated from scores at 10 and 20 min to derive a mean value for the extent of the mydriasis over the 20 min sampling period. The dose-response relationship for lean and obese rats were compared using the ANOVA repeated measures facility on SPSS$^+$ PC. Analysis was carried out on orthonormalised data with 'dose' as 'within' and 'group' (lean or obese) as 'between' subject factor.

For analysis of the hypoactivity experiments, the dose-response relationships for lean and obese rats were compared using the ANOVA repeated measures facility on SPSS$^+$ PC. Analysis was carried out on orthonomalised data with 'dose' as 'within' and 'group' (lean or obese) as the 'between' subjects factor. Because a significant effect of group was found, each dose was compared separately between the groups using a one-way ANOVA (SPSS$^+$ PC) to test over which dose range the dose-response relationship differed. Since lean and obese rats had different hypoactivity scores following saline administration (baseline hypoactivity score), one-way ANOVAs were also carried out on 'net' hypoactivity scores. These were calculated by subtracting the mean baseline hypoactivity score from each individual data point.

6.3. Results

6.3.1. [^{3}H]RX821002 binding in frontal cortex and hypothalamus.

Saturation binding studies with [^{3}H]RX821002, in the presence of 5 μM phentolamine, did not reveal any differences between lean and obese Zucker rats with respect to the density of binding sites (B_{max}) (Figure 6.1.). However, the affinity ($1/K_d$) of [^{3}H]RX821002 for α_2-adrenoceptors in the frontal cortex of obese rats was higher than that of their lean counterparts (F = 17.3; d.f. 1,9; $P < 0.01$) (Table 6.1.). No differences were found between the K_d values of [^{3}H]RX821002 for α_2-adrenoceptors in the hypothalamus of lean and obese rats.

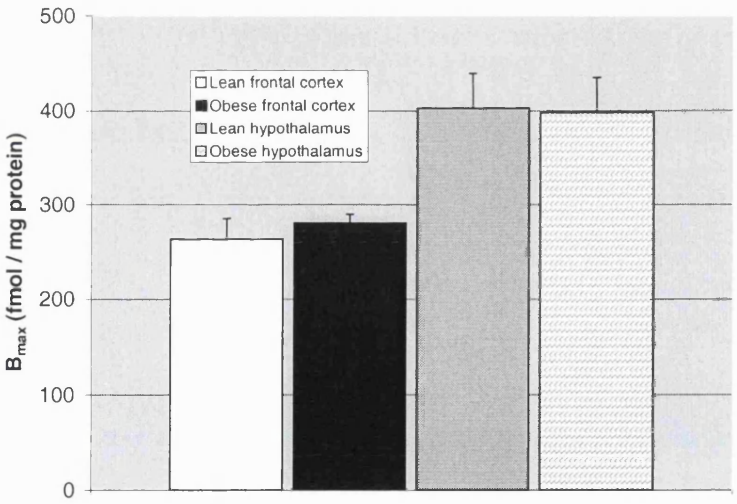


Figure 6.1: The density of [^{3}H]RX821002 binding sites in the frontal cortex and hypothalamus of lean and obese Zucker rats.

Data expressed as mean ± s.e. mean B_{max} (fmol / mg protein, n = 6-7).

BRAIN REGION	LEAN	OBESE
Frontal Cortex	0.28 ± 0.06 (n = 7)	0.22 ± 0.04 (n = 7)**
Hypothalamus	0.29 ± 0.04 (n = 7)	0.24 ± 0.04 (n = 6)

Table 6.1:K_d values (nM) for the binding of [^{3}H]RX821002 to α_2-adrenoceptors in the frontal cortex and hypothalamus of lean and obese Zucker rats.

** $P < 0.01$, compared with lean K_d value.

6.3.2. Clondine-induced mydriasis.

Baseline pupil diameter was 1.33 ± 0.03mm in lean rats and 1.39 ± 0.02 mm in obese rats (mean scores calculated from the baseline diameter from each rat at the start of each experiment: n = 60 for each group). A one-way ANOVA revealed there was no difference in baseline pupil diameter between lean and obese groups ($F = 2.96$; d.f. 1,118; $P = 0.09$). Clonidine induced an increase in pupil diameter in both lean and obese Zucker rats. The increase in pupil diameter reached a plateau of approximately 0.55 mm in both rat types at 0.1 mg / kg clonidine (Figure 6.2.). A repeated measures ANOVA did not reveal a difference in the dose-response relationship between lean and obese rats.

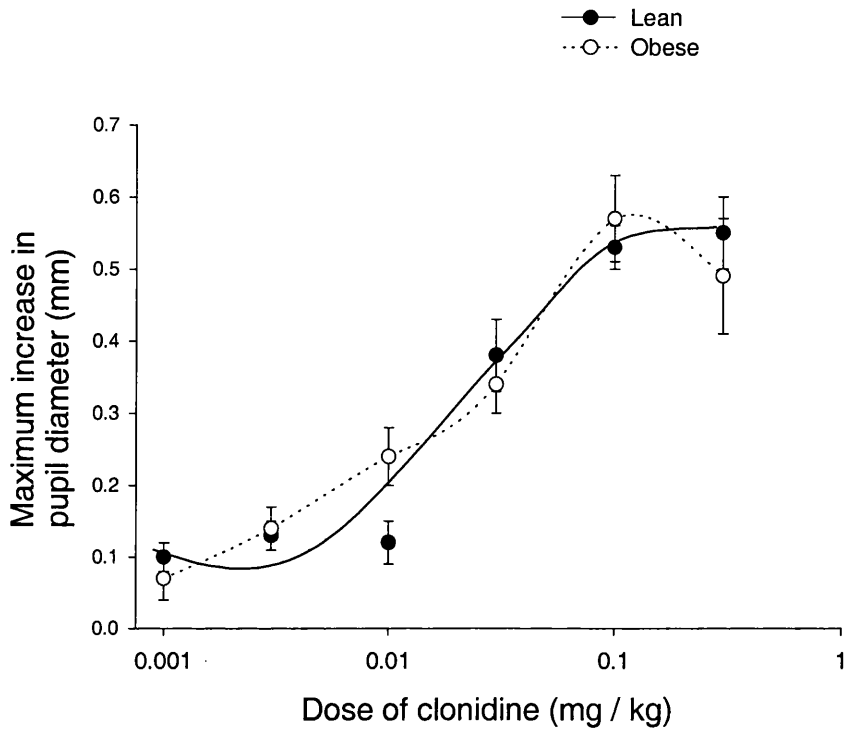


Figure 6.2: *Dose-response relationships for clonidine-induced mydriasis in lean and obese Zucker rats.*

Each point represents mean ± s.e. mean increase in pupil diameter for each dose of clonidine (n = 7-8).

6.3.3. Clonidine-induced hypoactivity.

Obese rats had a higher hypoactivity score following saline administration than did lean rats (lean rats: 4.5 ± 2.3 points *c.f.* obese rats: 8.2 ± 4.8 points, $F = 5.41$; d.f. 1,20; $P = 0.03$) indicating that obese rats are more hypoactive in drug-free conditions than their lean counterparts.

Clonidine induced a hypoactivity response in both lean and obese Zucker rats. The magnitude of the response paralleled dose, with the greatest dose inducing a hypoactivity score of approximately 26 points in both rat types (Figure 6.3a). Although both lean and obese rats reached a similar maximum level of hypoactivity following the highest dose of clonidine (0.3 mg / kg) a repeated measures ANOVA revealed an effect of group (lean or obese) as well as a dose × group interaction, between the dose-response relationships (effect of group: $F = 6.75$; d.f. 1,13; $P = 0.02$, dose × group interaction: $F = 2.87$; d.f. 5,65; $P = 0.02$).

One-way ANOVAs revealed that clonidine induced a greater hypoactivity response in obese rats at the lower doses of 0.003 and 0.01 mg / kg (0.03 mg / kg: $F = 17.26$; d.f. 1,14; $P < 0.01$, 0.01 mg / kg: $F = 6.4$; d.f. 1,14; $P = 0.02$) but not at any other dose. However, considering baseline hypoactivity scores were different in lean and obese rats, one-way ANOVAs were also carried out on net increases in hypoactivity scores to determine the magnitude of the increase in hypoactivity following clonidine administration (Figure 6.3b). There were no differences in net hypoactivity scores between lean and obese Zucker rats over the dose range 0.001-0.03 mg / kg clonidine. However, one-way ANOVAs revealed that clonidine induced greater hypoactivity scores in lean rats compared with obese rats at the higher doses of 0.1 and 0.3 mg / kg (0.1 mg / kg: $F = 8.17$; d.f. 1,13; $P = 0.01$, 0.3 mg / kg: $F = 10.88$; d.f. 1,14; $P < 0.01$).

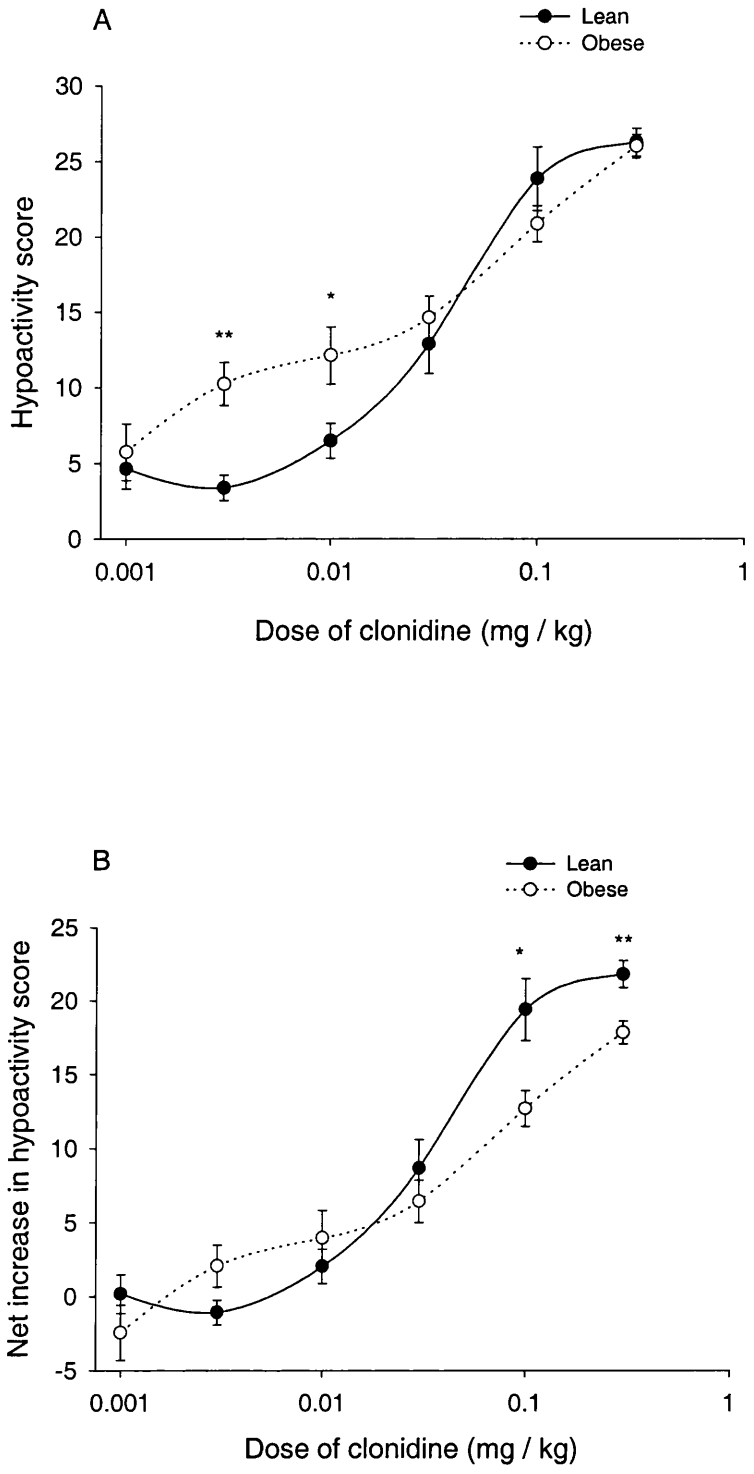


Figure 6.3: Dose-response relationships for clonidine-induced hypoactivity in lean and obese Zucker rats.

Data show a) absolute increases in hypoactivity following clonidine administration and b) net increases in hypoactivity following clonidine administration. Each point represents mean ± s.e. mean hypoactivity score for each dose of clonidine (n = 7-8). * $P < 0.05$, ** $P<0.01$.

6.4. Discussion

Several studies have compared the density of α_2-adrenoceptors in the hypothalamus of lean and obese Zucker rats to investigate whether an increased population of these receptors can account for the hyperphagia of the obese rat. However, these studies have revealed no consistent findings, possibly because of the affinity of the test radioligands for imidazoline receptors (see section 6.1.). This study has used the highly selective α_2-adrenoceptor radioligand, [^{3}H]RX821002, to compare the total density of cortical and hypothalamic α_2-adrenoceptors in lean and obese Zucker rats. In addition, clonidine-induced hypoactivity and mydriasis were used to compare the functions of pre- and postsynaptic α_2-adrenoceptors in these rats, respectively.

6.4.1. [^{3}H]RX821002 binding in the frontal cortex and hypothalamus.

[^{3}H]RX821002 binding studies revealed that α_2-adrenoceptors in frontal cortex tissue from obese rats had a higher affinity for RX821002 than did frontal cortex tissue from lean rats. Although the affinity of RX821002 for α_2-adrenoceptors in the hypothalamus was also higher than in lean rats, this difference did not reach statistical significance. Similarly, Miralles *et al.* (1993) reported that the affinity of RX821002 for α_2-adrenoceptors in the cerebral cortex of lean and obese Zucker rats was higher in obese rats compared with lean rats (0.53 *c.f.* 0.49 nM) although this difference was not significant. These findings could mean α_2-adrenoceptors in obese rats also have a higher affinity for other selective α_2-adrenoceptor ligands or noradrenaline itself. Further experiments investigating the binding characteristics of noradrenaline and other α_2-adrenoceptor ligands in lean and obese rats are needed to establish this. If α_2-adrenoceptors in obese rats do have a higher affinity for noradrenaline, this could mean that their function is also increased.

[^{3}H]RX821002 binding revealed no differences between lean and obese Zucker rats in the density of α_2-adrenoceptors in either the frontal cortex or hypothalamus. These findings are consistent with those of Miralles *et al.* (1993) who investigated the density of α_2-adrenoceptors in cerebral cortical membranes using [^{3}H]RX821002. In agreement with

the present study in the frontal cortex, no differences in the density of α_2-adrenoceptors in the cerebral cortex of lean and obese rats were found.

Jhanwar-Uniyal *et al.* (1991) reported a greater density of [^{3}H]PAC binding sites specifically in the paraventricular nucleus of obese Zucker rats. This report is consistent with the present finding that there is a trend for the affinity of α_2-adrenoceptors for RX821002 to be higher in obese rats compared with lean rats. [^{3}H]PAC is an α_2-adrenoceptor agonist and will therefore bind only to α_2-adrenoceptors in the high affinity state: if there are more α_2-adrenoceptors in the high affinity state in obese rats, this will result in more [^{3}H]PAC binding.

However, Pesonen *et al.* (1992) did not find a difference in α_2-adrenoceptor density in discrete nuclei of the hypothalamus, including the paraventricular nucleus, with the agonist [^{125}I]PIC. It is possible that the different findings could be linked to the time of day animals were killed. Studies show that the density of α_2-adrenoceptors in the paraventricular nucleus of Sprague-Dawley rats follows a diurnal rhythm, with a marked increase (approximately 2.5-fold) in the receptor density just prior to and during the dark phase compared with the light phase (Jhanwar-Uniyal *et al.* 1986). This diurnal rhythm of α_2-adrenoceptor binding was also observed in the perfornical lateral hypothalamus, although to a lesser extent, but was not seen in other brains areas such as the frontal cortex or hippocampus (Jhanwar-Uniyal *et al.* 1986). It is possible that there is a disturbance in this diurnal rhythm in obese Zucker rats and that this is only detectable at particular time points. In the study of Jhanwar-Uniyal *et al.* (1991), rats were killed between 0900 h and 1100 h (3-5 h after the start of the light phase). However, in the study of Pesonen *et al.* (1992) rats were sacrificed from 1600 h onwards ($\leq$ 2 h before the start of the dark phase). If, for example, the diurnal rhythm of α_2-adrenoceptor density is lost in obese rats and the density remains high at all times, it is possible that Jhanwar-Uniyal *et al.* (1991) compared lean and obese rats when the α_2-adrenoceptor density in lean rats was at its lowest (*i.e.* a difference between the lean and obese rats would be evident). In contrast, Pesonen *et al.*(1992) compared lean and obese rats just prior to the dark phase, when α_2-adrenoceptor density is thought to increase; it is possible that at this time point

α_2-adrenoceptor density in lean rats rises to a level comparable with obese rats (*i.e.* no difference between lean and obese rats would be evident).

In this study, rats were killed between 1400 and 1700 h with the dark phase due to start at 1800 h. In retrospect, this may not have been an ideal time to obtain brain tissue if the density of α_2-adrenoceptors increases in lean rats just prior to the onset of the dark phase. If the density of α_2-adrenoceptors increased in the lean rats between 1400 and 1700 h, this will have affected the mean value of their α_2-adrenoceptor density. Considering this, a more thorough investigation of α_2-adrenoceptor densities in lean and obese Zucker rats would involve a comparison of the receptor densities at different time-points over a 24 h period.

6.4.2. Clonidine-induced mydriasis and hypoactivity.

A comparison of the dose-response curves for clonidine-induced mydriasis did not reveal any differences between lean and obese Zucker rats in the function of the α_2-adrenoceptors mediating this response. The α_2-adrenoceptors mediating mydriasis are located postsynaptically (see Chapter 2, section 2.1.3.2.). Therefore, these findings present evidence that there are no differences in postsynaptic α_2-adrenoceptor function between lean and obese Zucker rats. This evidence is at variance with the finding that postsynaptic α_2-adrenoceptors in the paraventricular nucleus of obese Zucker rats are more sensitive to the feeding-stimulatory effects of clonidine than are those in lean rats (Koulu *et al.* 1990). However, the location of the postsynaptic α_2-adrenoceptors mediating mydriasis are thought to be in the Edinger-Westphal nucleus in the midbrain. Therefore, the function of postsynaptic α_2-adrenoceptors mediating mydriasis cannot be assumed to generalise to *all* central postsynaptic α_2-adrenoceptors. Thus, the present findings do not rule out the possibility that a disturbance in postsynaptic α_2-adrenoceptor function exists specifically within the paraventricular nucleus of obese Zucker rats. Indeed, the trend for the affinity of α_2-adrenoceptors for RX821002 in hypothalamus to be higher in obese rats than in lean rats could account for the greater feeding response in obese rats following intrahypothalamic injection of clonidine (Koulu *et al.* 1990).

Hypoactivity experiments revealed that obese rats have a greater baseline level of hypoactivity than their lean counterparts. This finding indicates that, under drug-free conditions, obese Zucker rats are more hypoactive than their lean counterparts, suggesting that the function of the presynaptic α_2-adrenoceptors mediating this effect could be enhanced. The greater hypoactivity of obese rats is unlikely to be due to these rats being more lethargic than their lean counterparts since in the study of Koulu *et al.* (1990) no differences in spontaneous locomotor activity were found between lean and obese Zucker rats. The finding that there is a trend for the affinity of α_2-adrenoceptors for RX821002 in the cortex and hypothalamus of obese rats to be higher than in lean rats provides a possible explanation as to why presynaptic α_2-adrenoceptor function could be enhanced in obese rats. Although the precise location of the α_2-adrenoceptors mediating hypoactivity in the brain is not known, it is possible that their affinity for α_2-adrenoceptors ligands is also higher in obese rats compared with lean rats. If these receptors have a higher affinity for noradrenaline in obese rats, this could mean that more released noradrenaline may bind to these receptors, leading to greater presynaptic α_2-adrenoceptor activation, which would result in greater baseline hypoactivity.

Analysis of hypoactivity responses following clonidine administration revealed that, at doses of 0.003 and 0.01 mg / kg, clonidine induces a greater increase in hypoactivity in obese rats compared with their lean counterparts. Analysis of net increases in hypoactivity scores following clonidine administration showed that although baseline hypoactivity was greater in obese rats, the magnitude of the increase in hypoactivity induced by clonidine was the same in both groups over the dose-range 0.001-0.03 mg / kg. However, at the higher doses of 0.1 and 0.3 mg / kg clonidine, the net increases in hypoactivity in obese rats is less compared with their lean counterparts. It is possible that there is a 'ceiling level' for clonidine's effect on hypoactivity and that this is reached with smaller increases in hypoactivity in obese rats because their baseline hypoactivity level is higher. However, Figures 6.3a and b indicate that whereas the dose-response curve for lean rats begins to plateau over the dose range of 0.1-0.3 mg / kg, the dose-response curve for obese rats does not. This suggests that the dose-response curve for obese rats reaches a plateau at doses of clonidine greater than 0.3 mg / kg. There are two possible explanations for this. Firstly, there could be a greater density of

α_2-adrenoceptors mediating hypoactivity in obese rats and a higher dose of clonidine is required to saturate these receptors, thus causing a plateau in the dose-response curve. Although this study found no differences in the density of α_2-adrenoceptors in the frontal cortex or hypothalamus between lean and obese rats, it is possible that the presynaptic α_2-adrenoceptors mediating clonidine-induced hypoactivity are located in a different brain region. Alternatively, the ratio of pre- and postsynaptic α_2-adrenoceptors could be higher in lean and obese rats (*i.e.* there could be a greater density of presynaptic α_2-adrenoceptors in obese rats but this may not have been detected in the binding studies if the density of postsynaptic α_2-adrenoceptors was lower).

A second explanation is that the function of these receptors could be enhanced so that doses of clonidine greater than 0.3 mg / kg can still induce further increases in hypoactivity in a dose-dependent manner in obese rats. Again, the latter suggestion is supported by the present findings that the affinity of α_2-adrenoceptors for RX821002 in the frontal cortex is higher in obese rats than lean rats. If the α_2-adrenoceptors mediating clonidine-induced hypoactivity have a higher affinity for clonidine in obese Zucker rats, it is possible that this could result in a greater level of activation of these receptors when clonidine binds.

Since the precise location of the presynaptic α_2-adrenoceptors mediating clonidine-induced hypoactivity is not known, the present finding that their function could be enhanced in obese rats cannot be generalised to all central presynaptic α_2-adrenoceptors in the obese rat. Therefore, it is difficult to foresee how, or if, the enhancement in the function of these particular presynaptic α_2-adrenoceptors would affect the activity of noradrenergic neuronal systems in these animals. Nevertheless, it is tempting to speculate on the possible changes in noradrenergic neuronal activity that could result as a consequence of enhanced presynaptic α_2-adrenoceptor function.

In Chapter 4, noradrenaline reuptake inhibition was observed to cause a prolonged increase in extracellular noradrenaline concentration in the frontal cortex of Sprague-Dawley rats. However, in the hypothalamus the increase in extracellular noradrenaline concentration was much shorter-lived following noradrenaline reuptake

inhibition. In Chapter 5, it was suggested that different densities of α_2-adrenoceptors in these brain regions are responsible for the different time-courses of extracellular noradrenaline accumulation following noradrenaline reuptake inhibition. It is possible that an increase in the function of presynaptic α_2-adrenoceptors in a given brain region of obese Zucker rats could also lead to differences in the regulation of extracellular noradrenaline concentration compared with lean rats. For example, during an increase in noradrenaline release, presynaptic α_2-adrenoceptors in obese rats could turn off neuronal firing and / or noradrenaline release to a greater extent than in lean rats. This could account for the reduced accumulation of extracellular noradrenaline in the paraventricular nucleus of obese rats during immobilisation stress (Pacak *et al.* 1995; see Chapter 1, section 1.5.2.).

Cruce *et al.* (1976; 1977) and Levin and Sullivan (1979) found that the concentration of noradrenaline in hypothalamic tissue was less than that of their lean counterparts and proposed that noradrenergic activity in the hypothalamus of obese rats is reduced (see Chapter 1, section 1.5.1.). Although it is difficult to deduce the functional significance of tissue content measurements *in vitro*, the finding in the present study that presynaptic α_2-adrenoceptor function is enhanced in obese rats is consistent with the suggestion above: it is possible that the enhanced function of presynaptic α_2-adrenoceptors, which decrease noradrenergic activity, leads to a decrease in noradrenaline synthesis. Collectively, these findings do not support the idea that enhanced noradrenergic activation of postsynaptic α_2-adrenoceptors in the paraventricular nucleus contributes to the hyperphagia of obese Zucker rats (Koulu *et al.* 1990; Jhanwar-Uniyal *et al.* 1991). Nevertheless, the proposal above that central noradrenergic activity in the obese Zucker rat could be limited to a greater extent by presynaptic α_2-adrenoceptors could contribute to the impaired thermogenesis in these animals. Triandafillou and Himms-Hagen (1983) reported that the binding of GDP by brown adipose tissue mitochondria from obese Zucker rats maintained at 28°C was lower than in lean rats. Furthermore, whereas lean Zucker rats on a cafeteria diet showed an increase in GDP-binding by mitochondria compared with lean rats fed standard laboratory chow, GDP-binding in obese Zucker rats was not affected by a cafeteria diet. These findings indicate that brown adipose tissue thermogenesis is disrupted in obese Zucker rats. A

6-hydroxydopamine-induced reduction of noradrenaline content in the hypothalamus of lean Sprague-Dawley rats impaired brown adipose tissue thermogenic activity (Sahakian *et al.* 1983, see Chapter 4, section 4.1.). In light of all these findings, it is not unreasonable to suggest that the enhancement of presynaptic α_2-adrenoceptor function in the obese rat could limit the concentration of extracellular noradrenaline in the hypothalamus to the extent that this pathway does not activate brown adipose tissue thermogenesis. However, the function of presynaptic α_2-adrenoceptors specifically within the hypothalamus would need to be investigated in order to gain support for this idea.

6.4.3. Conclusion

The present findings suggest that presynaptic α_2-adrenoceptor function is enhanced in the obese Zucker rat. The implications of this finding for the control of noradrenergic activity in the hypothalamus and the subsequent control of food-intake and thermogenesis in these rats remain to be established.

Chapter 7

General Discussion

This thesis has investigated changes in the function of central noradrenergic neurones in rats induced by the anti-obesity agents, sibutramine and *d*-amphetamine. Experiments also included an investigation into the density and function of α_2-adrenoceptors in the genetically lean and obese Zucker rats in order to determine whether an abnormality in these receptors exist in the obese state.

The finding that sibutramine increased extracellular noradrenaline concentration in the rat frontal cortex and hypothalamus supported the proposal that the active metabolites of this drug are potent inhibitors of noradrenaline reuptake. By comparing the effects of sibutramine on extracellular noradrenaline accumulation with those of the noradrenaline releasing agent, *d*-amphetamine, *in vivo* microdialysis experiments were able to provide support for this suggestion. The increase in extracellular noradrenaline concentration induced by sibutramine in the frontal cortex was gradual and sustained, suggesting that this drug could be inhibiting the reuptake of noradrenaline following its spontaneous, impulse-evoked release. *d*-Amphetamine induced a more rapid increase in extracellular noradrenaline concentration in the frontal cortex, suggesting that this drug induced impulse-independent noradrenaline release.

In support of this proposal, blockade of presynaptic α_2-adrenoceptors with RX821002 enhanced the increase in extracellular noradrenaline induced by sibutramine. This indicates that the accumulation of noradrenaline in the extracellular fluid induced by

sibutramine activates presynaptic α_2-adrenoceptors, which decreases noradrenergic neuronal firing and exocytotic noradrenaline release. This negative feedback loop accounts for the gradual and sustained increase in extracellular noradrenaline induced by this drug in the frontal cortex.

Despite being classified as a noradrenaline-releasing agent, blockade of presynaptic α_2-adrenoceptors enhanced the increase in extracellular noradrenaline induced by 10 mg / kg *d*-amphetamine. This finding suggests that α_2-adrenoceptor-mediated feedback inhibition *does* control the accumulation of extracellular noradrenaline induced by *d*-amphetamine to some extent. This suggestion is not consistent with other studies reporting that clonidine has no effect on the increase in extracellular noradrenaline concentration induced by 5 mg / kg *d*-amphetamine (Florin *et al.* 1994). As suggested in Chapter 3, a possible explanation for this discrepant finding could be that α_2-adrenoceptors mediating the negative feedback response are already maximally activated by noradrenaline after *d*-amphetamine administration. Thus, in the study of Florin *et al* (1994) the administration of clonidine cannot influence the accumulation of extracellular noradrenaline further. However, by adding an antagonist, such as RX821002, the contribution of α_2-adrenoceptor activation to the extent of extracellular noradrenaline accumulation induced by *d*-amphetamine is revealed. Consistent with the present finding, a recent study *in vitro* showed that, at low doses, *d*-amphetamine caused the release of noradrenaline, whereas at high doses it acted as a noradrenaline reuptake inhibitor (Pifl *et al.* 1999). The present finding provides evidence that, at a high dose, *d*-amphetamine also has noradrenaline reuptake inhibiting properties *in vivo*. Nevertheless, as a substrate for the noradrenaline transporter, *d*-amphetamine cannot, by definition, be an inhibitor of the transporter as well. However, the relatively high affinity of *d*-amphetamine for the noradrenaline transporter (K_i = 45 nM) could result in its dissociation being relatively slow. It is possible that this phenomenon accounts for *d*-amphetamine's noradrenaline reuptake inhibitor-like properties.

Recent studies have implicated a central noradrenaline-dopamine coupling effect in the enhancement of locomotor activity induced by *d*-amphetamine (Darracq *et al.* 1998, see Chapter 3, section 3.1.). The evidence suggests that the *d*-amphetamine-induced

increase in noradrenergic transmission in the prefrontal cortex activates α_1-adrenoceptors, which facilitates the release of dopamine in the nucleus accumbens, although the pathway through which this effect is mediated is not clear. The anti-obesity agent, phentermine, also evokes noradrenaline and dopamine release from rat cortical slices preloaded with [^{3}H]noradrenaline or [^{3}H]dopamine (Viggers *et al.* 1999; Lancashire *et al.* 1998) and increases locomotor activity in rats (Rowley *et al.* 2000). Considering this, it is probable that sibutramine's lack of effect on dopaminergic function along with its only modest increase in extracellular noradrenaline concentration compared with *d*-amphetamine, explains its lack of ability to increase locomotor activity in rats (Rowley *et al.* 2000).

In halothane-anaesthesised rats the magnitude of response for sibutramine's effects on extracellular noradrenaline accumulation in the frontal cortex is described by a bell-shaped curve, whereas in freely-moving rats the magnitude paralleled dose. This suggests that halothane-anaesthesia changes central noradrenergic function, although the mechanism by which this happens is unclear. However, this finding highlights why conscious, freely-moving rats are preferable for investigating the pharmacology of CNS agents. One question that arises is what happens to the dose-response curve for sibutramine at doses of sibutramine greater than 10 mg / kg. Does halothane-anaesthesia simply shift the dose-response curve for sibutramine to the left, or, like its effects on extracellular 5-HT concentration (Gundlah *et al.* 1997), does the maximum response induced by sibutramine parallel dose in freely-moving rats?

Sibutramine also increased the concentration of extracellular noradrenaline in the region of the paraventricular nucleus. This is at variance with reports that exogenous noradrenaline injected into this region *increases* feeding behaviour in rats through an action on postsynaptic α_2-adrenoceptors. This suggests that the feeding response induced by injection of noradrenaline into the paraventricular nucleus may not be reflecting a system that is involved in the natural, physiological control of food intake. Oral administration of the noradrenaline reuptake inhibitor, nisoxetine, which presumably increases the extracellular concentration of noradrenaline in the hypothalamus, does not affect food intake in rats (Jackson *et al.* 1997b). Furthermore, systemic administration of RX821002 has no effect on the inhibition of food intake induced by sibutramine (Jackson *et al.* 1997a). The finding that the α_1-adrenoceptor, prazosin, completely inhibited the

reduction of food intake induced by sibutramine (Jackson *et al.* 1997a), illustrates the importance of this receptor, over the postsynaptic α_2-adrenoceptor, in the overall control of feeding behaviour. This suggestion is supported by the fact that the feeding studies carried out by Jackson *et al.* (1997a) were performed during the dark phase; a period when the density of α_1-adrenoceptor binding decreases and α_2-adrenoceptor binding increases in the paraventricular nucleus (Jhanwar-Uniyal *et al.* 1986; Morien *et al.* 1999).

Sibutramine's effect on extracellular noradrenaline accumulation in the hypothalamus was shorter-lived than its effect in the frontal cortex. Evidence suggested that α_2-adrenoceptors in the hypothalamus have a greater control over noradrenaline release in this brain area compared with the frontal cortex and that this could account for the more transient effects of sibutramine in the hypothalamus. This is consistent with studies showing that there is a greater density of terminal as well as postynaptic α_2-adrenoceptors in the hypothalamus compared with cortical areas. However, a greater density of terminal α_2-adrenoceptors would be expected to restrict the concentration of extracellular noradrenaline to a particular level following sibutramine administration, but not to cause its decline 40 min post-injection. Questions arising from these findings are how, and why, would α_2-adrenoceptors control noradrenaline release differently in these two brain areas? It is possible that neurones exist in the hypothalamus that project to regions containing noradrenergic cell bodies. These neurones could form a feedback loop to regulate the firing of the noradrenergic neurones. The activation of α_2-heteroreceptors on their cell bodies in the hypothalamus could influence the level of this regulation. There is no evidence that directly supports this proposal. However, Kaba *et al.* (1986) report that systemic administration of clonidine inhibits the firing rate of A1 noradrenergic neurones but that this effect was not mediated through α_2-adrenoceptors in the region of the noradrenergic cell bodies. A proposal such as the one described above could account for how clonidine reduces the firing rate of A1 noradrenergic neurones. Nevertheless, this proposal does not provide an explanation as to *why* the brain would regulate noradrenaline release differently in the frontal cortex and hypothalamus.

Evidence suggests that noradrenergic activity is reduced in obese Zucker rats compared to their lean counterparts (reviewed in Chapter 1, section 1.5.). In light of the

evidence suggesting that differences in α_2-adrenoceptor density can affect noradrenaline release, this study investigated the density of α_2-adrenoceptors in lean and obese Zucker rats using radioligand binding studies. The functions of pre- and postsynaptic α_2-adrenoceptors in lean and obese Zucker rats were also investigated using clonidine-induced hypoactivity and mydriasis, respectively.

No differences in the density of [^{3}H]RX821002 binding sites were found in either the frontal cortex or hypothalamus of lean and obese rats. However, [^{3}H]RX821002 binding studies did reveal that α_2-adrenoceptors in the frontal cortex of obese rats had a higher affinity for RX82100 than lean rats. α_2-Adrenoceptors in the hypothalamus also had a higher affinity for RX821002 than did lean rats, although this difference was not statistically significant.

Clonidine-induced mydriasis did not reveal any differences in the function of postsynaptic α_2-adrenoceptors in lean and obese Zucker rats. The findings of Koulu *et al.* (1990) suggest that postsynaptic α_2-adrenoceptor function in the paraventricular nucleus is enhanced in obese Zucker rats. It is possible that this abnormal functioning of postsynaptic α_2-adrenoceptors in obese Zucker rats exists only in the paraventricular nucleus. The enhanced function of postsynaptic α_2-adrenoceptors in the region of the paraventricular nucleus reported by Koulu *et al.* (1990) could reflect their up-regulation as a result of decreased noradrenergic transmission is this area. Consistent with this proposal, Pacak *et al.* (1995) report that the stress-induced increase in extracellular noradrenaline concentration in the region of the paraventricular nucleus of obese Zucker rats is reduced compared with their lean counterparts. The authors point out that their findings are consistent with reports that the noradrenaline content of the paraventricular nucleus of obese Zucker rats is reduced (reviewed in Chapter 1, section 1.5), thus there is less noradrenaline available to be released on demand. However, their experiments do not rule out the possibility that a difference in the control of noradrenaline release in the hypothalamus of obese rats accounts for the differential effects of stress on extracellular noradrenaline accumulation in lean and obese rats: it is possible that *presynaptic* α_2-adrenoceptor function is enhanced in obese Zucker rats, which restricts the

accumulation of extracellular noradrenaline during stress. Clonidine-induced hypoactivity was used to investigate this possibility.

The baseline hypoactivity score of obese Zucker rats was higher than their lean counterparts. This suggests that the function of the presynaptic α_2-adrenoceptors mediating hypoactivity in obese rats is enhanced under resting conditions compared with lean rats. Although there were no differences between lean and obese rats in the net increase in hypoactivity following clonidine over the dose range 0.001-0.03 mg / kg, lean rats were more hypoactive than obese rats following a dose of 0.1 and 0.3 mg / kg clonidine. It is possible that the ceiling effect for this response is reached at lower doses of clonidine in obese rats because their baseline response is already elevated compared with lean rats. However, unlike the dose-response curve for clonidine-induced hypoactivity in lean rats, the dose-response curve for obese rats did not appear to reach a plateau at the two highest doses, suggesting that the maximum response had *not* been reached in these rats. These findings suggest that there could be functional differences between presynaptic α_2-adrenoceptors mediating hypoactivity in lean and obese Zucker rats. The finding that there was a trend for the affinity of α_2-adrenoceptors for RX821002 to be higher in obese rats compared with lean rats adds some support to this suggestion. The exact location of the α_2-adrenoceptors in the central nervous system mediating clonidine-induced hypoactivity is not known. It is possible that these receptors could also have higher affinities for α_2-adrenoceptor ligands such as RX821002, clonidine or noradrenaline. Perhaps a difference in presynaptic α_2-adrenoceptor affinity could account for the differences in the hypoactivity responses between lean and obese Zucker rats.

The implications of these findings for the control of noradrenaline release in the CNS of obese Zucker rats are not clear. The possibility that the function of presynaptic α_2-adrenoceptors mediating hypoactivity is enhanced in obese Zucker rats cannot be generalised to all presynaptic α_2-adrenoceptors in the brain of obese Zucker rats. At the same time, the possibility that the function of other presynaptic α_2-adrenoceptors in the obese Zucker rat is enhanced cannot be ruled out and this could have consequences for the control of noradrenaline release. Whether such an abnormality would contribute to the obesity of this animal remains unknown.

Future work related to this thesis

The principle findings of this thesis raise several questions that include:

1) Does the increase in hypothalamic extracellular noradrenaline concentration induced by sibutramine contribute to its anti-obesity effects?

Monitoring food intake and / or thermogenesis during local infusion of sibutramine's metabolites into the hypothalamus could provide clues as to whether the increase in extracellular noradrenaline accumulation in this brain area contributes to this drug's anti-obesity effects. The effects, if any, on food intake and thermogenesis could be compared with the effects of local infusion of 1) a selective noradrenaline reuptake inhibitor, 2) a selective 5-HT reuptake inhibitor and 3) a combination of a selective noradrenaline and 5-HT reuptake inhibitor. Local infusion of the drugs could be carried out using reverse dialysis in the hypothalamus either unilaterally or bilaterally. Unilateral infusion would cause less damage to the hypothalamus. However, bilateral infusion may be needed to see an effect of the drugs on food intake.

Firstly, these experiments would reveal whether the hypothalamus is involved in mediating sibutramine's anti-obesity effects. If the hypothalamus does appear to be involved in these effects, the experiments would also reveal whether the reuptake inhibition of noradrenaline, 5-HT or both monoamines in this region have a role in mediating the anti-obesity effects. If the reuptake inhibition of 5-HT and noradrenaline together in the hypothalamus suppresses food intake, this could go some way to explaining why sibutramine induces a decrease in food intake, whilst local injection of noradrenaline or clonidine into the region of the paraventricular nuclues induces an increase in food intake: the simultaneous increase in extracellular 5-HT concentration in the hypothalamus induced by sibutramine could mask the feeding-stimulatory effects of postsynaptic α_2-adrenoceptor activation.

2) Why do α_2-adrenoceptors in the hypothalamus restrict the effect of sibutramine on extracellular noradrenaline accumulation to a greater extent than do α_2-adrenoceptors in the frontal cortex?

Electrophysiological studies could be used to investigate whether activation of α_2-adrenoceptors on the cell bodies of neurones in the hypothalamus affect the firing rate of lateral tegmental or locus coeruleus noradrenergic neurones in the pons or medulla. An α_2-adrenoceptor agonist could be microiontophoretically applied to the hypothalamus and the firing rate of noradrenergic neurones recorded. A decrease in the firing rate of noradrenergic neurones would indicate whether neurones projecting from the hypothalamus to noradrenergic cell bodies are capable of regulating noradrenergic neuronal activity. This could shed light on the reason why extracellular noradrenaline accumulation declines in the hypothalamus 40 min following sibutramine administration.

3) Are α_1- or α_2-adrenoceptors involved in the natural, physiological control of food intake?

Gene knockouts of α_1- or α_2-adrenoceptor subtypes would be one approach to address the question of whether these receptors are involved in the natural, physiological control of food intake. They could also be used to investigate whether these receptors are involved in the central control of thermogenesis. Mutant mice lacking the 5-HT_{2C} gene have revealed the role of this receptor in the control of food intake (Heisler *et al.* 1998). However, the survival of mutant animals lacking α_1- and α_2-adrenoceptor subtypes could be impaired due to their roles in the control of cardiovascular function. Nevertheless, studies report the development of α_{2C}-adrenoceptor knockout mice (Link *et al.* 1995). These mice appear to develop normally, suggesting that the α_{2C}-adrenoceptor is not required for normal development in mice or for its survival in a laboratory environment. Studies involving the control of food intake in these animals do not yet appear to have been carried out.

4) Does an abnormality exist in terminal α_2-adrenoceptor function specifically in the paraventricular nucleus of the obese Zucker rat and does this contribute to the obesity of this animal?

Experiments involving *in vivo* microdialysis could investigate whether any difference in terminal α_2-adrenoceptor (or α_2-heteroceptor) function in the paraventricular

nucleus of obese rats could affect noradrenergic transmission in this area. These experiments would compare basal extracellular noradrenaline concentration in lean and obese Zucker rats before and after local infusion of an α_2-adrenoceptor antagonist. This would reveal whether α_2-adrenoceptor-mediated negative feedback contributes to the regulation of basal extracellular noradrenaline concentration in this region to the same extent. If noradrenaline release is restricted to a greater extent by α_2-adrenoceptors in obese rats, it may be predicted that infusion of an α_2-adrenoceptor antagonist would cause a greater increase in extracellular noradrenaline concentration in these rats compared to their lean counterparts.

It has been 40 years since noradrenaline was first suggested to have a role in the control of food intake. Despite this length of time, the findings of this study and others highlight that there is still a lot to be learned about the role of noradrenaline in the natural, physiological control of food intake. Future work aimed at unravelling the receptor pathways by which noradrenaline influences feeding behaviour will increase the understanding of how anti-obesity drugs such as sibutramine work. Furthermore, a greater understanding of the physiological basis of appetite control could reveal more targets for the anti-obesity agents of the future.

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