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FACTORS AFFECTING BEHAVIOUR AND CHANGES IN MONOAMINES INDUCED BY NOVEL STIMULI IN RODENTS

by

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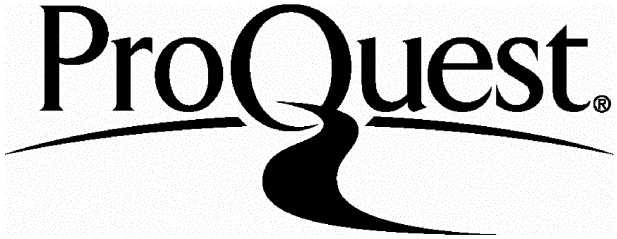

ABSTRACT

Although stress causes neurochemical changes in central noradrenergic and serotonergic neurones, how these changes relate to the response and behavioural adaptation to stress is poorly understood. One prominent theory has suggested that cortical β-adrenoceptor down-regulation underlies resistance to stress. This theory is based on studies of the effects of noxious stimuli such as immobilization. Whether this relationship holds for non-noxious stimuli such as novelty as well, or whether different types of stimuli have different neurochemical effects has been little considered. This project addresses this important question and has compared noxious and non-noxious stress. Both the short- (0-3 h) and long-term (7 days) effects of swim stress and novelty on indices of central noradrenergic and serotonergic function were evaluated, and behaviours induced by these forms of stress measured. Parallels between stress-induced neurochemical and behavioural changes were further evaluated by investigating the effects of treatments known to alter stress-induced behavioural responses. To achieve this, the effects of repeated stress (once-daily saline injection) or administration of a monoamine reuptake inhibitor (sibutramine) on the neurochemical effects of novelty or swim stress were examined.

Neither short-term effects of novelty or swim stress, nor long-term effects of swim stress involved changes in indices of noradrenergic function (e.g. cortical noradrenaline levels, β-adrenoceptor binding), regardless of animals' pretreatment. However, a positive correlation between β-adrenoceptor density and behavioural indices of resistance to novelty was seen, as has been previously described; this was the opposite to the correlation reported in the literature for noxious stressors. The data also suggested differences in estimates of β-adrenoceptor density in mouse cortex when different radioligands ([^{3}H]-CGP 12177, [^{3}H]-dihydroalprenolol) were used. However, an investigation of the displacement of [^{3}H]-dihydroalprenolol by 5-HT receptor ligands indicated that this was not due to binding of this radioligand to 5-HT$_{1B}$ 5-HT$_{1C}$ or 5-HT$_2$ receptors in mouse cortex; binding of [^{3}H]-DHA to 5-HT$_{1A}$ receptors could not be ruled out.

Cortical 5-HT$_2$ receptor binding was unchanged immediately after a 6 min swim. However, long-term effects of the swim included increases in both 5-HT$_2$ receptor density and 5-HT synthesis. These changes, which were attenuated by repeated stress

pretreatment (once-daily saline injection), were still evident in sibutramine-pretreated animals. In contrast to the different effects of these pretreatments on neurochemical effects of the swim, both repeated stress and sibutramine had similar effects on certain behavioural changes induced by swim stress (immobility) and novelty (locomotor activity).

It is concluded that

(i) the nature of the correlation between β-adrenoceptor density and resistance to stress depends on the form of stress studied

(ii) differences between the binding of [^{3}H]-dihydroalprenolol and [^{3}H]-CGP 12177 might be due to binding of the former radioligand to 5-HT1A receptors in mouse cortex

(iii) a single exposure to swim stress has long-lasting effects on central 5-HT2 receptors

(iv) repeated saline injection can alter both neurochemical and behavioural responses to acute stress

These data have evident implications for studies of the long-term effects of stress, for instance post-traumatic stress disorder. Furthermore, although vehicle injection is used as a control for studies of the effects of injected drugs, the marked effects of this procedure suggest that vehicle injection alone is an inadequate control for such studies.

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1.0 INTRODUCTION

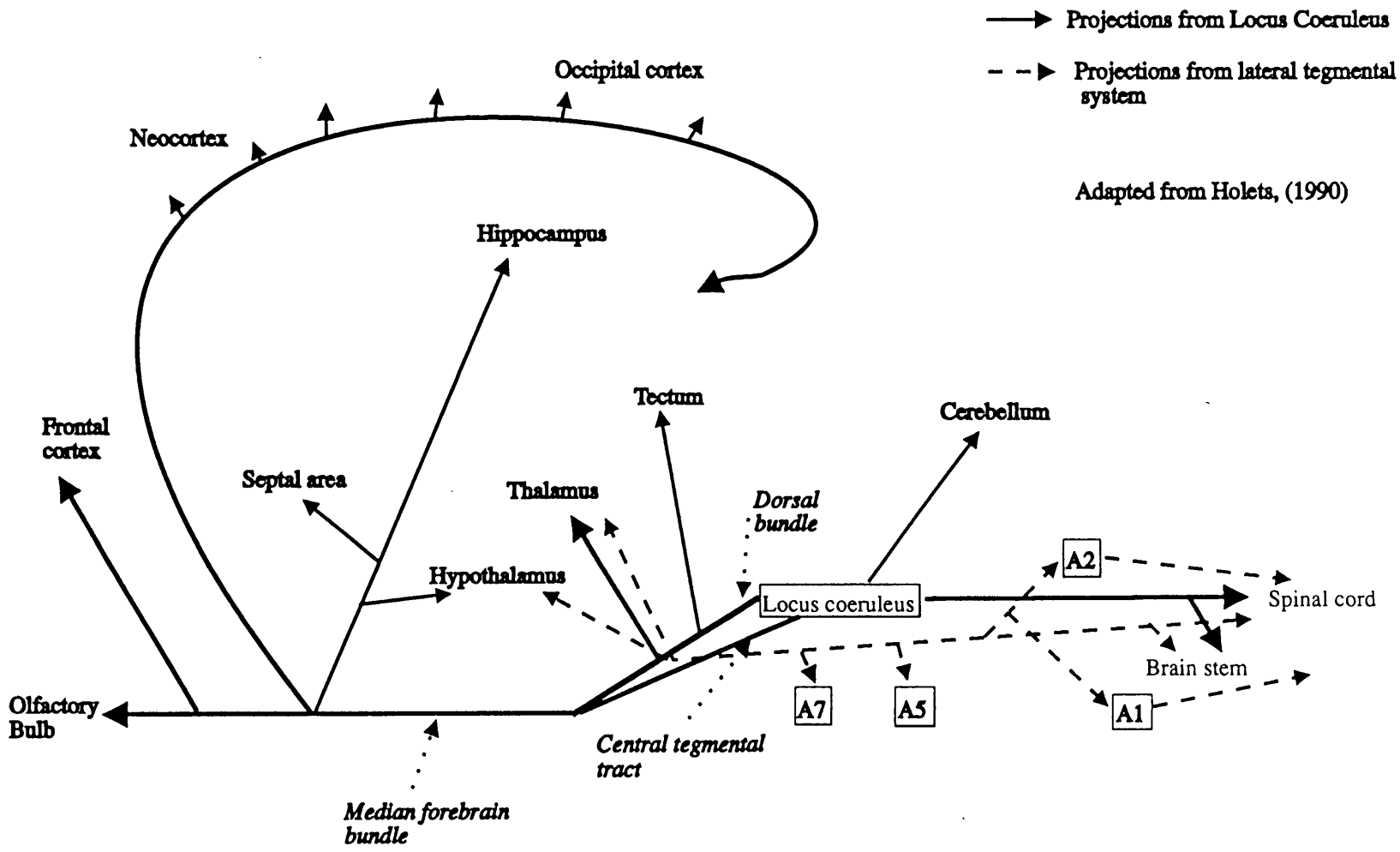
Neurochemical changes in central noradrenergic and serotonergic (5-hydroxytryptamine; 5-HT) neurones parallel behavioural responses to stress, but the exact role of these neurones in response and adaptation to stress is poorly understood. Certain neurochemical effects of stress, for instance increased noradrenaline turnover, have been studied widely: however, other aspects of the neurochemical response to stress have been largely ignored. For instance, that different forms of stress might have different neurochemical effects has rarely been considered. The possibility of long-term changes evoked by exposure to stress has also received virtually no attention, although in man, long-latency effects of stress have been demonstrated e.g. post-traumatic stress disorder. The present study has compared the effects of two different forms of stress, novelty and swim stress, on both behaviour and indices of central noradrenergic and serotonergic function. Both stress and antidepressant administration can have similar effects on the central noradrenergic system; indeed, one theory has linked adaptation to repeated stress with the effects of repeated antidepressant administration, since both treatments down-regulate β-adrenoceptors in this region. This theory has been evaluated in the present study by examining the effects of repeated stress and antidepressant administration, alone and in combination with acute stress exposure.

In order to clarify the role of noradrenaline and 5-HT in response and adaptation to stress, and to investigate whether there is a link between the effects of repeated stress and antidepressant administration, it is important to set in context the anatomy, physiology and neurochemistry of the relevant neurones. Therefore the introduction to this thesis is devoted to reviewing this background literature. The rationale for procedures used to assess central noradrenergic and serotonergic function are also described. Evidence for the involvement of noradrenaline (NA) and 5-HT in stress responses will then be presented. Finally, the influence of repeated exposure to stress or antidepressant administration on behavioural and neurochemical responses to stress will be reviewed.

1.1 Noradrenergic and serotonergic pathways in the brain

In 1964 Dahlstrom & Fuxe described a series of nuclei which contained noradrenergic cell bodies and were located in the brain stem. The noradrenergic innervation of the central nervous system arises from these nuclei which can be divided into two groups. First, there is the locus coeruleus (A6 in the classification of Dahlstrom & Fuxe, 1964) which contains almost 50% of the cell bodies of noradrenergic neurones in the rat brain

Figure 1.1 **NORADRENERGIC PROJECTIONS IN RAT BRAIN**



(Swanson & Hartman, 1975). Fibres from the locus coeruleus form three major ascending pathways (Figure 1.1; reviewed by Moore & Bloom, 1979):

(i) the dorsal noradrenergic bundle

(ii) the periventricular system; this system can be divided into the dorsal and ventral components: fibres ascend in the dorsal longitudinal fasciculus, and in the periventricular region of the hypothalamus, respectively

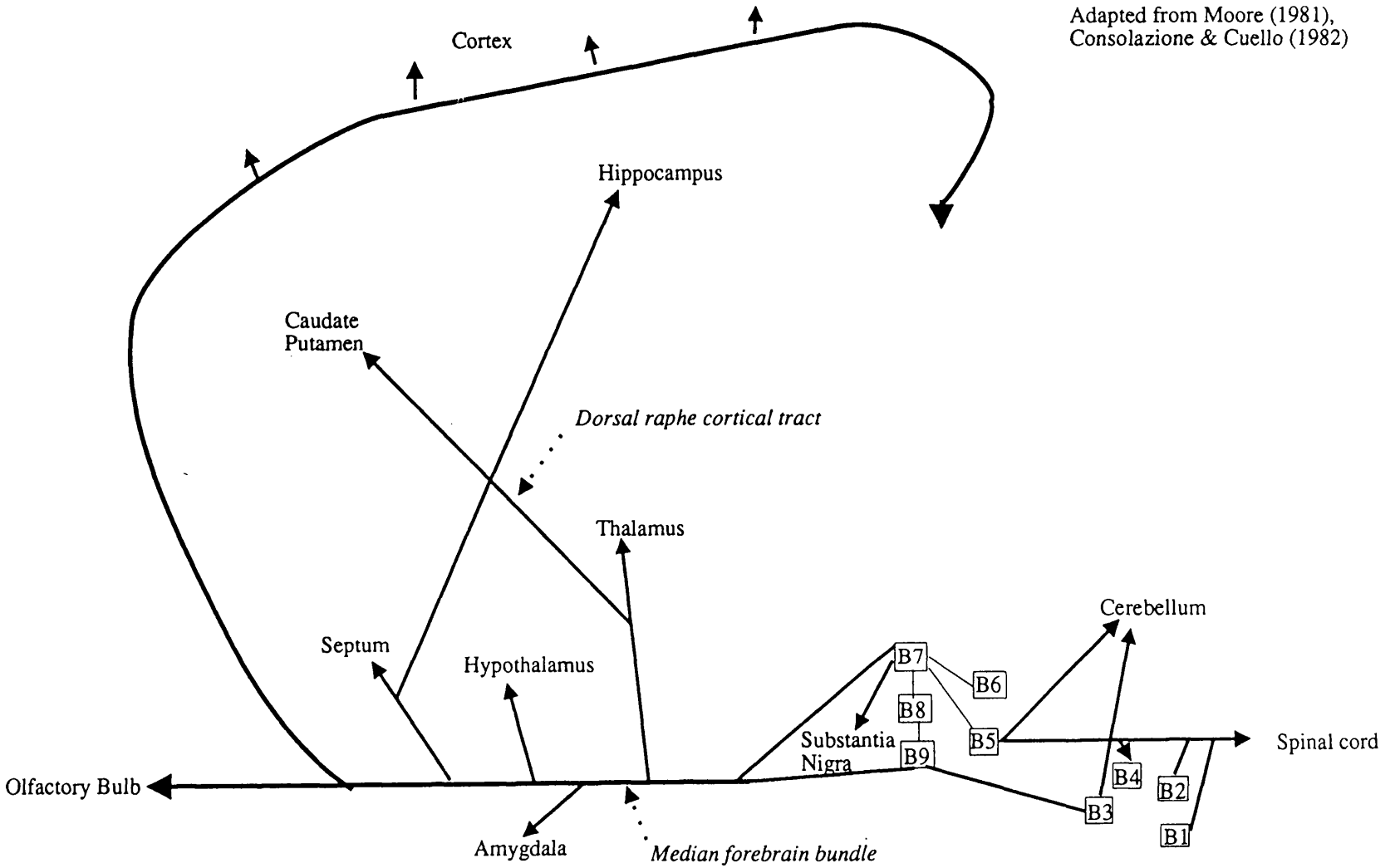
(iii) the central tegmental tract

The second group, comprising nuclei A1-5 and A7, is the lateral tegmental system. Fibres from the lateral tegmental system also ascend in the central tegmental tract. Fluorescence histochemical studies of the terminal fields of noradrenergic neurones reveal that noradrenergic fibres reach almost all areas of the brain. The cerebral cortex and hippocampus are innervated by fibres from the locus coeruleus only. All other areas e.g. basal forebrain, thalamus, cerebellum, brain stem and spinal cord are innervated by neurones from both the locus coeruleus and the lateral tegmental system (Moore & Bloom, 1979). However, the proportion of innervation of a given area is not necessarily the same from both systems; for instance, the hypothalamus is largely innervated by the lateral tegmental system, with only sparse innervation from the locus coeruleus.

There are only a relatively small number of noradrenergic cell bodies in the brain and the terminal field of each axon is diffuse and extensive, with divergent collaterals projecting to different brain regions (reviewed by Holets, 1990). However, there is a crude topographical organization of efferent noradrenergic projections from the locus coeruleus. Cells in different areas or sub-divisions of the locus coeruleus project to defined terminal areas. Furthermore, sub-regions of the locus coeruleus contain cells of different morphological types and these cell types are associated with innervation of particular terminal fields (Loughlin et al., 1986a,b).

Clusters of 5-HT cell bodies in the brain stem were demonstrated in histochemical fluorescence studies almost 30 years ago (Dahlstrom & Fuxe, 1964). More recently, these nuclei, designated B1-9, have been divided into two groups (reviewed by Jacobs & Azmitia, 1992). The superior group contains the median and dorsal raphe nuclei (B6 and B7, B5 and B8, respectively), as well as neurones in cluster B9. The inferior group contains the nucleus raphe magnus (B3), nucleus raphe pallidus (B1 and B4) and nucleus raphe obscurus (B2).

Figure1.2 **SEROTONERGIC PROJECTIONS IN RAT BRAIN**



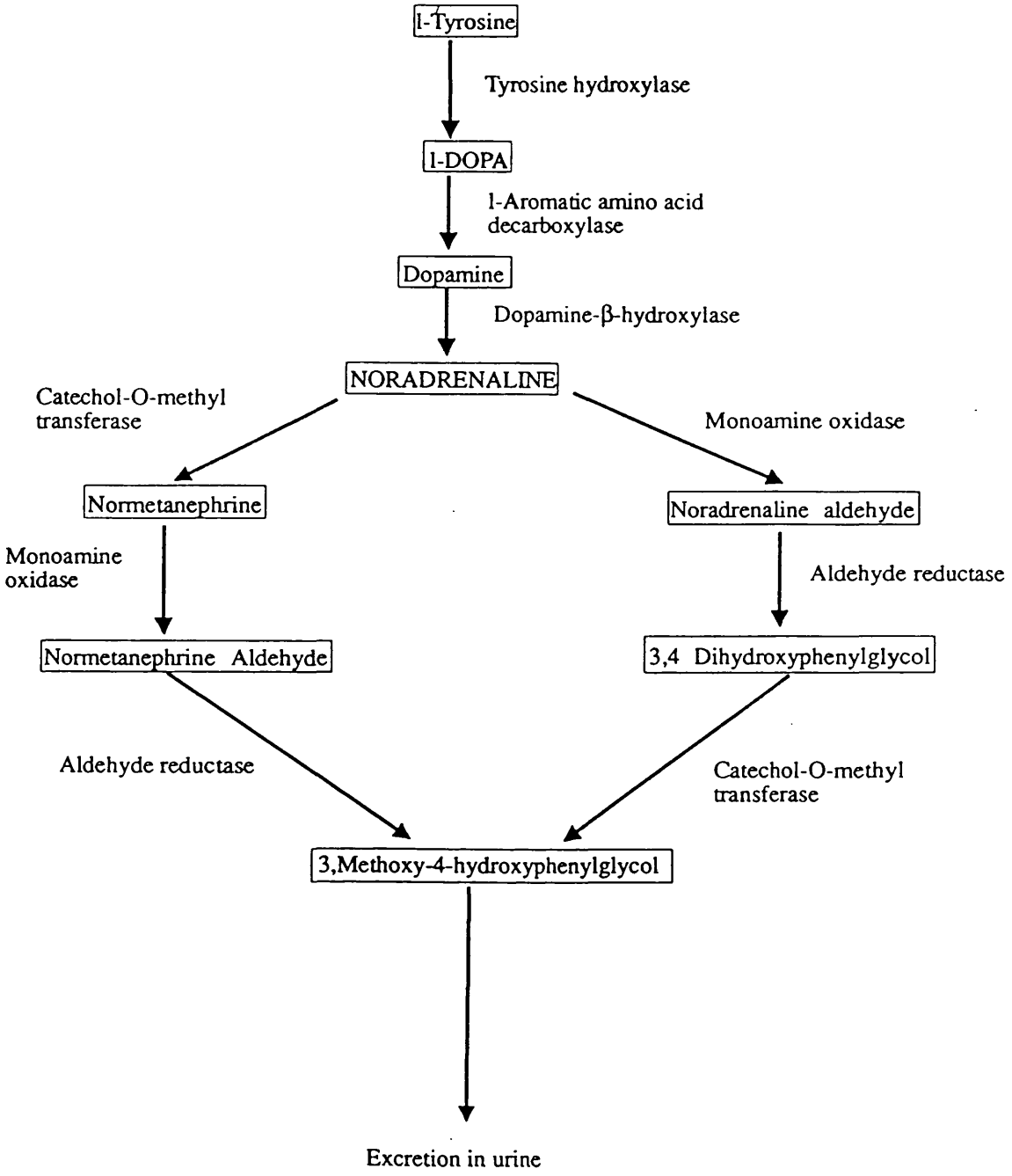
Descending 5-HT neurones arise mainly in the cell bodies of the inferior group of 5-HT nuclei and project to the ventral horn, substantia gelatinosa and other parts of the spinal cord (Figure 1.2). Ascending systems of 5-HT neurones originate mainly in the superior group of 5-HT nuclei. The two main ascending bundles are the median forebrain bundle and the dorsal raphe cortical tract (Figure 1.2). Immunocytochemical methods have shown that terminal projections of these neurones are found in virtually every area of the brain. There is little topographical organization in the innervation of brain regions by neurones from specific brain stem serotonergic nuclei; generally, extensive collateralization of neurones is seen, innervating more than one terminal region (reviewed by Consolazione & Cuello, 1982; Jacobs & Azmitia, 1992).

1.2 Synthesis and metabolism of noradrenaline and 5-HT

1.2.1 Noradrenaline

The formation of noradrenaline from the amino acid tyrosine involves a number of enzymatic conversions (Figure 1.3; reviewed by Fillenz, 1990). Tyrosine is concentrated in the brain from the bloodstream and taken up into neurones by an active transport mechanism. The first step in noradrenaline synthesis is the conversion of l-tyrosine to l-dihydroxyphenylalanine (l-DOPA); this is catalyzed by tyrosine hydroxylase, an enzyme found only in catecholaminergic neurones. This cytoplasmic enzyme is the rate-limiting factor in the synthesis of noradrenaline and shows reasonable specificity for tyrosine. Molecular oxygen and a reduced pteridine co-factor are necessary for the activity of the enzyme, and there are binding sites on the enzyme for both these factors. The activity of tyrosine hydroxylase can be increased by phosphorylation by protein kinase (Morgenroth et al., 1975; reviewed by Fillenz, 1990). Enzyme activity is believed to be altered by conformational changes which affect the kinetic parameters of the enzyme; for instance, increased substrate or cofactor affinity and increased V_{max} of the enzyme. A number of different protein kinases have been shown to increase tyrosine hydroxylase activity; these include both calcium- and cyclic adenosine monophosphate (cAMP)-dependent-protein kinase. These different kinases may have different effects on kinetic parameters of tyrosine hydroxylase (Andrews et al., 1983; Niggli et al., 1984). Tyrosine hydroxylase activity can also be increased by increasing enzyme levels ('induction'). Induction involves synthesis of new enzyme and can be seen after stress, e.g. prolonged cold exposure (reviewed by Fillenz, 1990). Both dopamine and noradrenaline, products of this biosynthetic pathway, can compete for the co-factor binding site to cause 'feedback inhibition' of tyrosine hydroxylase activity.

Figure 1.3 **SYNTHESIS AND METABOLISM OF NORADRENALINE IN THE C.N.S.**



l-DOPA is converted to dopamine by another cytoplasmic enzyme, l-aromatic amino acid decarboxylase, which is relatively non-specific for amino acids. Vitamin B_6 is required as a co-factor for the activity of this enzyme.

Dopamine-β-hydroxylase, the enzyme responsible for the conversion of dopamine to noradrenaline, is found only in noradrenaline storage vesicles (Figure 1.3). This enzyme catalyses the formation of noradrenaline following uptake of dopamine into the vesicles. Noradrenaline is released from nerve terminals along with the vesicle contents, by an exocytotic process which follows an influx of calcium into the terminal (Stjarne, 1989).

The action of noradrenaline released into the synaptic cleft is predominantly terminated by the reuptake of released neurotransmitter into nerve terminals. Following reuptake, noradrenaline may re-enter storage vesicles in the terminal; the majority is deaminated by the enzyme monoamine oxidase (Farah et al., 1977), which is bound to the outer membranes of mitochondria. Monoamine oxidase (MAO) exists in two different forms (A and B) which have specificity for different substrates; MAO_A is predominantly responsible for the metabolism of noradrenaline in the rat brain, though noradrenaline can also be deaminated by MAO_B at sufficiently high transmitter concentrations (Youdim, 1983).

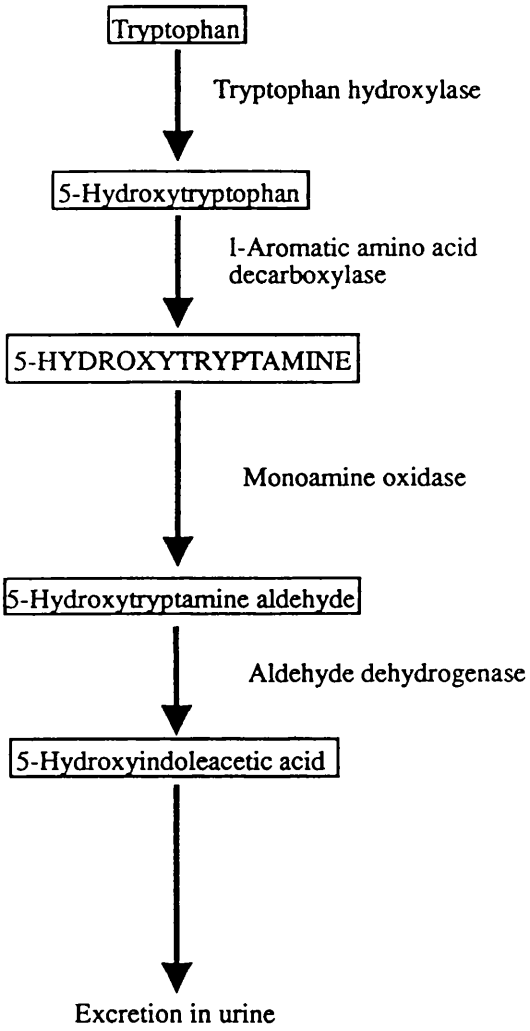
A second enzyme of importance in the metabolism of noradrenaline is catechol-O-methyl transferase (COMT). This enzyme catalyses the transfer of a methyl group to molecules containing the catechol group, and acts on a variety of endogenous substrates including adrenaline and l-DOPA, as well as noradrenaline (Axelrod & Tomchick, 1958). In the brain, COMT is located mainly in the cytoplasm of neurones and glial cells, but it has been suggested that COMT may also be present in neurones in a membrane bound form (Rivett et al., 1983; Kaakkola et al., 1987).

Noradrenaline may be metabolized by either MAO or COMT; the products of these enzymic reactions are substrates for further metabolism by both MAO and COMT. In the brain the final breakdown product of noradrenaline is 3-methoxy,4-hydroxyphenylglycol (MHPG; Figure 1.3), which may be further sulphated in some species (e.g. rat, but not mouse).

1.2.2 5-Hydroxytryptamine

Like noradrenaline, the synthesis of 5-HT involves a series of enzymatic reactions

Figure 1.4 **SYNTHESIS AND METABOLISM OF 5-HYDROXYTRYPTAMINE IN THE C.N.S**



(Figure 1.4; reviewed by Osborne, 1982). The amino acid precursor tryptophan is taken up into the brain via an active uptake process. 5-hydroxytryptophan (5-HTP) is then formed from tryptophan; this step is catalysed by tryptophan hydroxylase. Tryptophan availability can be important in determining the rate of 5-HT synthesis, although tryptophan hydroxylase is generally the rate-limiting step in 5-HT synthesis. Tryptophan hydroxylase is thought to be a cytoplasmic enzyme, but a particulate form of this enzyme associated with 5-HT containing terminals has been isolated. Studies of tryptophan hydroxylase activity *in vitro* using the endogenous co-factor tetrahydrobiopterin suggest that this enzyme is not saturated with substrate in the brain *in vivo*. Changes in substrate or co-factor availability can influence the activity of tryptophan hydroxylase therefore. The activity of this enzyme can also be increased in response to electrical stimulation of the raphe nuclei in a frequency-dependent manner (Boadle-Biber et al., 1986). Since this increase in enzyme activity is reversed *in vitro* by the action of alkaline phosphatase, phosphorylation is presumed to be responsible for the increase in enzyme activity. Under normal physiological conditions, feedback inhibition of tryptophan hydroxylase activity by 5-HT does not occur; however, feedback inhibition may occur following a large increase in cytoplasmic 5-HT levels e.g. after inhibition of monoamine oxidase *in vivo* (reviewed by Boadle-Biber, 1982).

The low levels of 5-HTP present in the brain indicate that the conversion of 5-HTP to 5-HT occurs almost immediately after 5-HTP synthesis. This reaction is catalysed by the enzyme 5-HTP decarboxylase, which is associated with the synaptosomal fraction of brain homogenates. 5-HT is released from vesicles in nerve terminals by an exocytotic process triggered by an influx of calcium into the nerve terminal. The action of released 5-HT is terminated by the reuptake of 5-HT into the nerve terminal. Only one pathway exists for the metabolism of 5-HT: 5-HT is broken down to 5-hydroxy~~tryptamine~~ indoleacetic acid (5-HIAA) by the action of MAO and aldehyde reductase; 5-HIAA is then removed from the brain via an acid transport system.

1.3 **Noradrenergic and serotonergic receptors: sub-types, localization and distribution**

1.3.1 Noradrenergic receptors

Sub-types of noradrenaline receptor were originally described by Ahlquist (1948), who proposed two different receptor subclasses (α- and β-adrenoceptors) on the basis of relative potencies of noradrenaline, adrenaline, isoprenaline and other catechol derivatives for producing any particular physiological response. Further sub-division of α-adrenoceptors arose from the discovery of α-adrenoceptor-mediated inhibition of

noradrenaline release in tissues thought not to contain α-adrenoceptors (reviewed by Langer, 1974). Since α-adrenoceptors were thought to be pre-synaptic, these receptors were sub-divided into α1- (post-synaptic) and α2-adrenoceptors (pre-synaptic). Subsequent evidence has demonstrated that the majority of α2-adrenoceptors in the central nervous system are located post-synaptically (U'Prichard et al., 1979); it has also been shown that ~~and that~~ neither α1- nor α2-adrenoceptors represent homogeneous populations of receptors which can be distinguished pharmacologically (reviewed by Ruffolo et al., 1991). Early studies of the effects of β-adrenoceptor agonists showed different rank orders of potency, depending on the tissue and response examined, leading to the sub-division of β-adrenoceptors into β1- and β2-adrenoceptors (Lands et al., 1967). More recent advances have shown the existence of the β3-adrenoceptor in peripheral tissues (Bond & Clarke, 1988).

α1-Adrenoceptors are located post-synaptically (Janowsky et al., 1984), and are found in particularly high concentrations in the olfactory bulb, thalamus, amygdala and hippocampus (Young & Kuhar, 1979). α1-Adrenoceptor activation is associated with phosphoinositol hydrolysis (Brown et al., 1984; reviewed by McGrath et al., 1989). Studies of the displacement of the α1-adrenergic radioligand [^{3}H]-prazosin by α1-adrenoceptor antagonists have shown the existence of the α1A- and the α1B-adrenoceptor sub-types (reviewed by McGrath et al., 1989; Ruffolo et al., 1991) However, a different classification of α1-adrenoceptor sub-types has been proposed on the basis of functional studies, and there is as yet no consistent system of sub-classification of this receptor (reviewed by Ruffollo et al., 1991).

It was initially thought that α2-adrenoceptors were entirely located pre-synaptically on noradrenergic nerve terminals, and that their stimulation inhibited noradrenaline release (autoreceptors: Starke, 1972; Dubocovitch & Langer, 1974). More recently, pre-synaptic α2-adrenoceptors have been detected on various central non-adrenergic neurones however (e.g. serotonergic neurones: Raiteri et al., 1983); activation of these receptors regulates transmitter release from these neurones (heteroceptors). α2-Adrenoceptors are found in highest concentrations in the locus coeruleus and the limbic system (Young & Kuhar, 1979). Lesioning studies have suggested that presynaptic α2-autoreceptors constitute only a small proportion of central α2-adrenoceptors (U'Prichard et al., 1979; Dooley et al., 1983; Heal et al., 1993). Since application of α2-adrenergic agonists has no effect on cell firing in the central nervous system (e.g. Bradshaw et al., 1984), it is thought that post-synaptic α2-adrenoceptors are heteroceptors on terminals of neurones containing other

neurotransmitters e.g. 5-HT (Maura et al., 1982). It has been shown that α2-adrenoceptor activation inhibits adenylate cyclase activity (reviewed by McGrath et al., 1989). Studies of antagonist affinities for α2-adrenoceptors and molecular biological techniques support the existence of at least 4 different subtypes of α2-adrenoceptor (reviewed by Ruffollo et al., 1991).

β-Adrenoceptors are distributed relatively homogeneously in rat brain, bearing little relationship to the noradrenaline content of particular brain regions (Alexander et al., 1975). It was therefore suggested that some central β-adrenoceptors may be associated with non-neuronal cells e.g. glial cells or blood vessels. Studies of β-adrenoceptor sub-type distribution has shed some light on this anomaly. The amount of β1-adrenoceptors as a proportion of total β-adrenoceptors present has been shown to vary enormously, depending on the brain region examined (highest density in the cerebral cortex and hippocampus); β2-adrenoceptor density varies very little between brain regions (highest density in cerebellum, lowest density in the hippocampus and cortex; Minneman et al., 1979a). This was taken to indicate that β2-adrenoceptors are associated with non-neuronal elements, while β1-adrenoceptors are associated with heterogeneously distributed noradrenergic neurones (Minneman et al., 1979a). Lesion studies have demonstrated that β1-receptors are located post-synaptically to noradrenergic nerve terminals (Minneman et al., 1979b). The existence of pre-synaptic β-adrenoceptors was first suggested by Adler-Graschinsky & Langer (1975) based on studies of peripheral tissue; subsequent studies have shown that the peripheral pre-synaptic β-adrenoceptor has a pharmacological profile consistent with that of the β2-adrenoceptor (reviewed by Misu & Kubo, 1986). Since agonists at β-adrenoceptors facilitate catecholamine release in tissue slices from a variety of brain regions, the existence of pre-synaptic β-adrenoceptors on noradrenergic nerves in the central nervous system has been proposed (Dietl et al., 1981; Ueda et al., 1983, 1985; reviewed by Misu & Kubo, 1986). Both β1- and β2-adrenoceptor stimulation is linked with activation of adenylate cyclase activity (Robison et al., 1965; reviewed by Fillenz, 1990).

Based on a distinctive order of potency for β-adrenergic drugs, the existence of a third sub-type, the β3-adrenoceptor, has been proposed (reviewed by Emorine et al., 1991). The existence of β3-adrenoceptors has been demonstrated in the heart (Kaumann, 1989) and adipocytes (Muzzin et al., 1992), but their existence in the brain has not been demonstrated.

Many radioligands have been used to define α_1-, α_2- and β-adrenoceptor binding (for instance: 2-(2',6'-dimethyloxy)phenoxyethylamino)methylbenzo-1,4-dioxane ([^{3}H]-WB 4101) and [^{3}H]-clonidine for α_1- and α_2-adrenoceptors respectively: U'Prichard et al., 1977; [^{3}H]-dihydroalprenolol for β-adrenoceptors: Bylund & Snyder, 1976). In addition, both β_1- and β_2-adrenoceptor-selective compounds can be used to displace radioligands from their respective β-adrenoceptor sub-type, enabling β-adrenoceptor subtypes to be distinguished (e.g. Minneman et al., 1979a; Dooley et al, 1986). However, some older radioligands commonly used to measure β-adrenoceptor binding are not selective for this receptor, and care must be exercised in the combination of radioligand and displacing ligand chosen for the definition of β-adrenoceptors. In particular, questions have been raised about the the selectivity of [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) which is probably the most widely used β-adrenergic ligand. Despite this, the use of newer, more selective ligands such as [^{3}H]-CGP 12177 (Staehelin et al., 1983) to define β-adrenoceptors is still not widespread.

1.3.2 Serotonergic receptors

The existence of more than one type of 5-HT receptor was first proposed in 1957 by Gaddum and Picarelli, based on studies of the effects of antagonists of the 5-HT-induced contraction of the isolated guinea-pig ileum. Twenty years later, radioligand binding demonstrated the existence of sub-types of 5-HT receptor, which were defined as 5-HT$_1$ receptors, labelled using [^{3}H]-5-HT and 5-HT$_2$ receptors, labelled using [^{3}H]-spiperone (Peroutka & Snyder, 1980a). Since then, the heterogeneity of 5-HT$_1$ receptors, as well the existence of 5-HT$_3$, and most recently 5-HT$_4$ receptors has been described (reviewed by Hoyer & Schoeffter, 1991).

The 5-HT$_{1A}$ receptor, found in high density in the locus coeruleus, hippocampus and cortex as well as the raphe nuclei, is labelled using the highly selective agonist [^{3}H]-8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT; Gozlan et al., 1983). 5-HT$_{1A}$ receptors have been shown both to stimulate and to inhibit adenylate cyclase activity (reviewed by Frazer et al., 1990). Pre-synaptic 5-HT$_{1A}$ receptors are located on serotonergic cell bodies and dendrites (Hall et al., 1985; Verge et al., 1985). Lesioning studies suggest that not all 5-HT$_{1A}$ receptors are pre-synaptic, and that pre- and post-synaptic 5-HT$_{1A}$ receptors have different distributions (Hall et al., 1985). 5-HT$_{1A}$ receptors in the striatum and dorsal raphe nucleus are almost entirely pre-synaptic (Hall et al., 1985; Verge et al., 1985). In contrast, hippocampal 5-HT$_{1A}$ receptors are entirely post-synaptic and those in the cerebral cortex are both pre- and post-synaptically located (Hall et al., 1985).

Receptor binding has demonstrated 5-HT1B receptors in rat and mouse brain, but not in guinea-pig, cow or human brain (reviewed by Peroutka, 1990). These receptors are found in the greatest density in the amygdala, cortex, hypothalamus and substantia nigra in rats (reviewed by Palacios & Dietl, 1988). 5-HT1B receptors are located pre-synaptically and have been identified as the serotonergic pre-synaptic autoreceptor in the rat (Verge et al., 1985; Engel et al., 1986). 5-HT1B receptors are also present as heteroceptors on terminals of cholinergic and dopaminergic neurones in specific brain regions (reviewed by Palacios & Dietl, 1988). 5-HT1B receptors may also be located post-synaptically. Stimulation of 5-HT1B receptors inhibits adenylate cyclase activity in cultured cells (reviewed by Frazer et al., 1990), and in homogenates of rat substantia nigra or cortex membranes (Bouhelal et al., 1988; Engel et al., 1986).

5-HT1C receptors are found in extremely high density in the choroid plexus (Pazos et al., 1985a) but have also been identified in other brain regions such as the cortex (Peroutka, 1986) in a number of species. 5-HT1C receptor activation stimulates phosphoinositide hydrolysis (Conn & Sanders-Bush, 1986). Since 5-HT1C receptor-stimulated phosphoinositide hydrolysis in the choroid plexus is increased after serotonergic denervation, it has been suggested that 5-HT1C receptors are post-synaptic (Conn et al., 1987).

5-HT1D receptors were initially identified as a component of [^{3}H]-5-HT (5-HT1) receptor binding not blocked by ligands with affinity for 5-HT1A and 5-HT1C receptor sub-types in the caudate from bovine brain, which does not contain 5-HT1B receptors (Heuring & Peroutka, 1987). 5-HT1D receptors are found in the highest proportion (of total 5-HT1 receptor sub-types present) in the substantia nigra, globus pallidus and nucleus accumbens in human brain (Waeber et al., 1988a). 5-HT1D receptors have since been described in the brains of a number of other species which do not have 5-HT1B receptors, including humans (Waeber et al., 1988b), but not in rats or mice (Waeber et al., 1989). In the species where 5-HT1D receptors are found, this receptor sub-type is thought to be the 5-HT terminal autoreceptor (reviewed by Hoyer & Middlemiss, 1989). Like 5-HT1B receptors, 5-HT1D receptors are linked to inhibition of adenylate cyclase (Waeber et al., 1988b).

5-HT2 receptors have been described in all mammalian species studied, with an uneven distribution in the brain (reviewed by Peroutka, 1990; Apud, 1991). The highest densities are found in the frontal cortex, amygdala and parts of the olfactory system in rats

(Fischette et al., 1987). The pharmacological profile of this receptor suggests that this receptor is the 5-HT D receptor originally described by Gaddum & Picarelli (1957). Lesioning of serotonergic fibres does not alter 5-HT2 receptor number in rats (Blackshear et al., 1981; Fischette et al., 1987), although in mice, an increase in 5-HT2 receptor binding is seen after denervation (Heal et al., 1985). It has been suggested that 5-HT2 receptors are located post-synaptically to serotonergic neurones, therefore. 5-HT2 receptor activation has been shown to stimulate phosphatidylinositol hydrolysis; a high correlation between affinity of a range of drugs for 5-HT2 receptors and the potency of these drugs to block phosphatidylinositol hydrolysis and ability to block binding of [^{3}H]-ketanserin to 5-HT2 receptors has been shown (Conn & Sanders-Bush, 1984; reviewed by Sanders-Bush, 1988). Sequencing of 5-HT1C and 5-HT2 receptors has revealed that there is 50% sequence homology between the two receptors, with 80% homology seen in the seven transmembrane domains (discussed by Apud, 1991); this suggests that these two receptors are very similar.

The majority of radioligand binding studies have used antagonist radioligands such as [^{3}H]-ketanserin or [^{3}H]-spiperone, to define 5-HT2 receptors (e.g. Buckholtz et al., 1988; Peroutka & Snyder, 1980b). However, 5-HT2 receptor agonists displace such radioligands from two sites (e.g. Battaglia et al., 1984). In addition, 5-HT2 receptor density defined using agonist radioligands is considerably lower than that defined with antagonist radioligands (Peroutka et al., 1988; Lyon et al., 1987). This has been interpreted as evidence of two sub-types of the 5-HT2 receptor; 5-HT2A, and 5-HT2B, with high and low affinity for agonists respectively (Peroutka et al., 1988; Pierce & Peroutka, 1989). Alternatively, the 5-HT2 receptor may exist in two affinity states, with high and low affinity for agonists, but with equal affinity for antagonists (Shannon et al., 1984). This classification problem has as yet not been resolved (Leonhardt & Titeler, 1989; Strange, 1990). Until it is, use of antagonist radioligands such as [^{3}H]-ketanserin is preferable, since they bind to both binding sites/affinity states with equal affinity.

5-HT3 receptors have been shown to represent the 5-HT M receptor described by Gaddum and Picarelli (Richardson et al., 1985), but have only recently been described in the brain (Kilpatrick et al., 1987). This receptor sub-type occurs with the highest density in cortical areas, particularly the entorhinal cortex, in the amygdala and in areas of the lower brain stem (Kilpatrick et al., 1987, 1989). 5-HT3 receptors do not appear to be linked to either adenylate cyclase or phosphoinositide turnover; electrophysiological studies indicate that 5-HT3 receptor stimulation activates fast non-selective monovalent cation

channels (Neijt et al., 1988; Peters & Lambert, 1989). In various brain regions such as the cortex and hypothalamus, presynaptic 5-HT3 receptors modulate the release of 5-HT (Blier & Bouchard, 1992) and other transmitters such as dopamine (Blandina et al., 1988).

5-HT4 receptors were first described in cultured cells from mouse embryo colliculi as a type of 5-HT3 receptor positively coupled to adenylate cyclase (Dumuis et al., 1988a). However, further characterization of this receptor showed that the pharmacological profile of this receptor in both mouse colliculi cells and guinea-pig hippocampal membranes was inconsistent with that of 5-HT3 receptors (Dumuis et al., 1988b). Therefore it was proposed that this receptor was a novel 5-HT receptor sub-type, termed the 5-HT4 receptor. The location and distribution of this receptor sub-type have not been described and the classification of this receptor has not been yet generally agreed (Clarke et al., 1989).

Selective ligands have been described for the 5-HT1A receptor (e.g. 8-OH-DPAT, ipsapirone; Van Wijngaarden et al., 1990) and for 5-HT3 receptors (e.g. ondansetron, zacopride; Van Wijngaarden et al., 1990). However, the majority of ligands for serotonergic receptors have similar affinities for several serotonergic receptor sub-types (Van Wijngaarden et al., 1990; Hoyer & Schoeffter, 1991). Classification of serotonergic receptor sub-types must be deduced from studies of the affinity of a number of different serotonergic ligands. Definition of 5-HT receptor subtypes is therefore not straightforward. For instance, the majority of supposedly selective 5-HT2 receptor ligands such as ritanserin or mesulergine display equal or greater affinity for the 5-HT1C receptor. However, the commonly used radioligand [^{3}H]-ketanserin shows selectivity for 5-HT2 receptors compared with other 5-HT receptors. Spiperone, a compound which has high affinity for 5-HT2 and 5-HT1A receptors, but only weak affinity for 5-HT1C receptors, has been used widely in the differentiation of 5-HT1C and 5-HT2 receptors (Peroutka, 1990).

1.4 Receptor regulation

The receptor-mediated response to a fixed concentration of agonist may not be constant; rather, the response may be either increased or decreased under certain circumstances. It is generally held that prolonged decreases in neurotransmitter concentrations in the cleft, i.e. decreased receptor stimulation, induces an increase in sensitivity of receptors for that transmitter (supersensitivity). In contrast, an increase in transmitter levels leads to decreased responsiveness of the receptors to receptor activation

(desensitization). However, this relationship does not always hold; for instance, 5-HT2 receptors are down-regulated by repeated administration of either 5-HT2 agonists or antagonists (Blackshear & Sanders-Bush, 1982; Leysen et al., 1986; Buckholtz et al., 1988)

Receptor supersensitivity has not been extensively studied since it is not possible to model this phenomenon *in vitro*. In contrast, prolonged (min-h) agonist incubation has been widely used to model desensitization *in vitro*. This phenomenon has been studied using β-adrenoceptors since there are several cell types in which this receptor type can be easily studied. Non-mammalian cells such as frog erythrocytes, which have been shown to contain β-adrenoceptors and functional adenylate cyclase, have been used. For investigations of the role of individual components of the β-adrenoceptor/adenylate cyclase system in desensitization, mammalian cell lines which express β-adrenoceptors, with mutants that are lacking in other components of the system such as the stimulatory G protein or protein kinase A have been used. Most recently, since the cloning of the human β2-adrenoceptor gene, this gene or mutants of this gene have been expressed in mammalian cell lines which do not naturally express β-adrenoceptors, to determine the importance of specific sequences on the β2-adrenoceptor to desensitization.

Agonist-specific or ~~heterologous~~ homologous desensitization of β-adrenoceptors (reviewed by Collins et al., 1990) occurs in response to prolonged β-adrenoceptor stimulation. After as little as a 15 min incubation with isoprenaline, a reduction in β-adrenoceptor-stimulated adenylate cyclase activity is apparent (e.g. Mukherjee & Lefkowitz, 1976; Su et al., 1979; Wagner & Davis, 1979). This has been shown not to be dependent on the presence of the stimulatory G protein or adenylate cyclase (Shear et al., 1976); rather, a structural change in the β-adrenoceptor itself is responsible (Stadel et al., 1982). This change has been shown to be phosphorylation of the receptor, over a time course identical to that of the decline in adenylate cyclase activity (Sibley et al., 1984). At low agonist concentrations (nanomolar isoprenaline), protein kinase A (PKA) is responsible for phosphorylation of β-adrenoceptors; at higher (micromolar) agonist concentrations, both β-adrenoceptor kinase (βARK) and PKA phosphorylate β-adrenoceptors (Lohse et al., 1990). βARK is a cAMP-independent protein kinase which has been shown to specifically phosphorylates the agonist-occupied β-adrenoceptor (Benovic et al., 1986).

Following the agonist-induced reduction in adenylate cyclase activity, β-adrenoceptors are lost from the cell surface; this is seen as a reduction in binding of hydrophilic radioligands such as [^{3}H]-CGP 12177 to whole cells. These receptors have been

sequestered into the cell since, if binding in whole cells is measured using a hydrophobic radioligand such as [^{3}H]-dihydroalprenolol, total receptor number is unchanged (Hertel et al., 1983). The sequestered receptors are associated with a light membrane fraction; sequestration is reversible once the agonist is removed from the incubation medium (Harden et al., 1980). Two lines of evidence suggest that sequestration is not dependent on receptor phosphorylation. If either PKA and/or βARK activity are inhibited or site-directed mutagenesis renders the phosphorylation sites on the β2-adrenoceptor non-functional, receptor sequestration still occurs (Lohse et al., 1990; Hausdorf et al., 1991).

Following agonist incubation of several hours, β-adrenoceptors are down-regulated (Su et al., 1980); this is characterized by a loss of total receptor binding sites from the cell. This process can be stimulated by addition of cyclic AMP analogues and by drugs which indirectly elevate intracellular cyclic AMP concentrations such as forskolin, as well as by agonist incubation, suggesting that adenylate cyclase and the formation of cyclic AMP are required for down-regulation (Collins et al., 1989). Studies of β2-adrenoceptors show that β-adrenoceptor down-regulation is paralleled by a reduction in β2-adrenoceptor mRNA i.e reduced β2-adrenoceptor expression (Collins et al., 1989). Receptor phosphorylation by PKA is also involved in, but is not essential for β-adrenoceptor down-regulation (Bouvier et al., 1989).

Recently, studies of β-adrenoceptor down-regulation have been carried out in a mammalian cell line transfected with the genes for either β1- or β2-adrenoceptors, in order to examine the effects of agonist incubation on the different receptor sub-types (Suzuki et al., 1992). A 15 min incubation with isoprenaline increases the number of sequestered β2-adrenoceptors; this brief treatment has no effect on β1-adrenoceptors. After longer incubations, both receptor subtypes are down-regulated; β2-adrenoceptor down-regulation is more rapid (80% reduction after 24 h incubation) than that of β1-adrenoceptors (Suzuki et al., 1992). These findings suggest that mechanisms regulating these two β-adrenoceptor sub-types are not the same. Differential regulation of β1- and β2-adrenoceptors could play an important role in situations where one or other sub-type is altered e.g. after exposure to acute or repeated stress, or repeated antidepressant administration.

Heterologous desensitization of β-adrenoceptors has also been described; this is defined as a reduction in the response of adenylate cyclase to stimulation of any receptor which is positively coupled to this enzyme. For instance, β-adrenoceptor-stimulated adenylate cyclase activity may be reduced in cells which have been incubated with prostaglandins

(Johnson et al., 1978; Green & Clark, 1981). This process also involves receptor phosphorylation, but is less well characterized than homologous desensitization.

1.5 Neurochemical indices of neurotransmitter function

Studies of central noradrenergic and serotonergic transmission in man examine different aspects of transmission than those which can be studied in animals. Moreover, in animal studies, carefully controlled environmental conditions can be used when the effects of a specific treatment are to be studied.

One of the most useful indices of transmitter function would be to measure transmitter release. Since this cannot be done directly, various methods have been used to estimate this. The first method to be used widely was to measure changes in neurotransmitter and metabolite levels either in whole brain or in selected brain regions (e.g. Rüther et al., 1966; Welch & Welch, 1968). Although simple, measurement of transmitter levels alone is of limited significance. The amount of transmitter present indicates only the balance between release and synthesis; increased levels of a transmitter could reflect decreased release, or increased synthesis of that transmitter. To overcome these problems, measurements of transmitter turnover were developed. Such studies are often used as indirect measurements of release and are generally based on the assumption that synthesis rate balances the rate of release or metabolism, or that if synthesis or metabolism of a transmitter is blocked using an enzyme inhibitor, the rate of depletion or accumulation of transmitter reflects the rate of release.

1.5.1 Noradrenaline

Enzymatic methods to measure synthesis rates normally investigate the rate-limiting step i.e. the conversion of tyrosine to l-dopa by tyrosine hydroxylase (reviewed by Shaman, 1981). Either the rate of decline of noradrenaline is investigated after tyrosine hydroxylase inhibition, or the rate of accumulation of l-dopa after dopa decarboxylase inhibition is studied. Since noradrenaline and dopamine are both products of the same biosynthetic pathway, these methods cannot distinguish between noradrenergic and dopaminergic neurones in the brain, unless regions relatively deficient in one of the two transmitters is studied. This limitation also applies to the study of [^{14}C]-noradrenaline formation from injected [^{14}C]-tyrosine; inaccurate rates of synthesis of noradrenaline may be estimated as the labelled tyrosine will also be taken up by dopaminergic neurones.

Studies of the utilization of [^{3}H]-noradrenaline have also been used to investigate

noradrenaline turnover; a major limitation of this method is that, as noradrenaline cannot cross the blood-brain barrier, it must be administered intracerebroventricularly (i.c.v.). This is a procedure which, in rats, requires anaesthesia, which may interfere with basal levels of noradrenaline turnover.

Studies using the rate of metabolism of noradrenaline as an index of turnover have concentrated on the depletion of glycol metabolites of noradrenaline (DHPG and MHPG) after monoamine oxidase inhibition. However, the estimated turnover rate depends on the monoamine oxidase inhibitor used, so results obtained with this method are variable. (Sharman, 1981)

Recently, Heal and co-workers proposed that the level of 3-methoxy-4-hydroxyphenylglycol (MHPG) in mouse brain could be used as an index of noradrenaline utilization/turnover (Heal et al., 1989a). A number of experiments were carried out to validate this suggestion. Inhibition of tyrosine hydroxylase (i.e. inhibition of noradrenaline synthesis) by intravenous injection of α-methyl-p-tyrosine decreased both noradrenaline and MHPG levels in a time-dependant manner. MHPG levels were also decreased after administration of clonidine, an α2-adrenoceptor agonist, which acts on presynaptic α2-adrenoceptors, decreasing noradrenaline release. Conversely, yohimbine and idazoxan, α2-adrenoceptor antagonists, increased MHPG levels as predicted on the basis of their known effects on α2-adrenoceptors. Further treatments such as monoamine oxidase inhibition, noradrenaline uptake inhibition, and dexamphetamine, which causes noradrenaline release as well as inhibiting noradrenaline uptake, also decreased MHPG levels in mouse brain, findings consistent with the known pharmacological effects of these compounds. All these findings were taken to indicate that MHPG levels provided an reliable index of noradrenaline turnover in mice.

One objective of the present study was to examine changes in noradrenaline turnover in mice after exposure to acute (swim stress) and repeated stress (once-daily intraperitoneal saline injection). Of the above methods for estimating noradrenaline turnover, many were not suitable because they entailed administration of drugs by intraperitoneal injection. As this is the same stress as that under investigation, drug administration by this route is undesirable. However, measurement of MHPG levels involves no drug pretreatment (Heal et al., 1989a), so this method was chosen to study the effects of stress on central noradrenaline turnover.

1.5.2 5-HT

Studies of 5-HT synthesis rate as an index of 5-HT turnover (reviewed by Curzon, 1981) have again concentrated on the rate-limiting step (the conversion of tryptophan to 5-HTP by tryptophan hydroxylase). Inhibition of tryptophan hydroxylase has not been used widely, since inhibitors of this enzyme are slow-acting. Measurement of the rate of accumulation of [^{14}C]-5-HT after an intraventricular injection of [^{14}C]-tryptophan has many limitations. For instance, since the 5-HT precursor 5-HTP is present in negligible amounts in untreated brain, it is thought that 5-HT appears to be formed rapidly from 5-HTP. However, in studies of [^{14}C]-5-HT formation, a significant peak of [^{14}C]-5-HTP is seen, suggesting a delay in the conversion of labelled tryptophan to labelled 5-HT (Lane & Aprison, 1978), and this method is not used widely.

Inhibition of 5-HTP decarboxylase by m-hydroxybenxylhydrazine (NSD 1015) causes an increase in 5-HTP concentration which is linear with respect to time. As the 5-HTP concentration in untreated brain is negligible, estimation of 5-HT turnover can be achieved by measuring 5-HTP levels at a single time-point after decarboxylase inhibition. Although inhibition of 5-HTP decarboxylase should lead to a decrease in 5-HT levels as a result of decreased synthesis, NSD 1015 also inhibits MAO, so 5-HT levels remain virtually constant; this avoids the possibility of changes in feedback control of 5-HT synthesis by altered levels of this transmitter.

Studies of utilization of radiolabelled 5-HT are not used, as when 5-HT is given i.c.v it tends to accumulate locally in the periventricular regions. The rate of accumulation of 5-HT after MAO inhibition has also been used to investigate 5-HT turnover (reviewed by Curzon, 1981). However, the rate of accumulation decreases with time; this is thought to be due to an increase in feedback inhibition of 5-HT synthesis caused by the increasing levels of 5-HT present.

The rate of accumulation of 5-HIAA after inhibition by probenecid of the acid transporter which removes 5-HIAA from the brain has also been examined. The validity of this method depends on several factors, including the completeness of the blockade of 5-HIAA exit from the brain and the inability of extracerebral 5-HIAA to enter the brain.

Since 5-HIAA is the only major metabolite of 5-HT in the brain, levels of 5-HIAA in untreated brain are also commonly used as an index of 5-HT turnover. However, it must

be borne in mind that the use of 5-HIAA levels to index turnover has never been validated, unlike studies of the noradrenaline metabolite, MHPG.

Since in the present study, 5-HT turnover was to be estimated after exposure to repeated stress (once-daily intraperitoneal saline injection), many of the methods discussed above, which also involve intra-peritoneal injection of drugs are not ideal. In the absence of a definitively ideal method, 5-HT turnover was estimated in the current experiments using two techniques: the accumulation of 5-HTP after 5-HTP decarboxylase inhibition (involves intraperitoneal administration of the enzyme inhibitor), and measurement of 5-HIAA levels in untreated brain (not well validated).

1.6 **Other indices of noradrenergic and serotonergic function**

As well as estimates of transmitter release, other indices of central transmitter function are of relevance to the current studies. Measurement of neurotransmitter turnover gives information about the effects of experimental treatments on indices of presynaptic function, but reveals nothing about effects on the post-synaptic cell. One of the most common ways to study post-synaptic neuronal responses is to examine post-synaptic receptors. Radioligand binding *in vitro* is widely used to investigate the effects of experimental treatments on receptor number and affinity. In the present study, saturation binding was used to study the effects of stress and antidepressant treatments on cortical noradrenergic and serotonergic receptors; β-adrenoceptors and 5-HT2 receptors were examined since these receptors are mainly located post-synaptically. β-adrenoceptors were measured using [^{3}H]-dihydroalprenolol, a commonly used β-adrenergic radioligand; however, questions about the selectivity of this ligand have been raised (Stone & U'Prichard, 1981; Riva & Creese, 1989a).

A limitation of receptor binding studies is that no differentiation is made between pre- and post-synaptic receptors; this is especially relevant where receptors of one sub-type exist at both these sites. In addition, they provide no information about the functional state of the receptors (i.e. the physiological response to receptor activation). This is important, since receptor binding cannot predict the response of the receptor to stimulation. For instance, if `spare receptors' are present (i.e. more receptors than are needed to evoke the maximal response to a full agonist), then a large reduction in receptor number could be seen, without any change in receptor function. Alternatively, a change in the coupling of the receptor to the second messenger system could occur; this would be seen as a reduction in receptor function, but with no change in receptor

number. Studies of receptor function are therefore necessary to provide a fuller picture of the effects of experimental treatments on post-synaptic receptors.

Receptor function may be studied *ex vivo* or *in vivo*, both approaches having advantages and disadvantages. Studies of receptor function *ex vivo*, such as measurement of noradrenaline-stimulated adenylate cyclase activity in cortical slices, provide information about changes in the coupling of receptors to their second messenger system in a defined brain region. However, nothing is revealed about the overall response to receptor activation (e.g. behavioural response), which may be influenced by other neurotransmitter systems. An additional problem of relevance to the current experiments is that assays of receptor function *ex vivo* commonly use fresh tissue, which limits the number of samples which can be handled simultaneously.

An example of the measurement of receptor function *in vivo* is the 5-HT2 receptor-mediated head-twitch. This behaviour can be evoked in mice by administration of non-selective 5-HT or selective 5-HT2 receptor agonists or by 5-HTP loading, and is mediated by central 5-HT2 receptors (reviewed by Heal et al., 1992). Such models are useful in assessing the overall output elicited by central receptor stimulation. Studies of hormonal responses to stimulation by receptor agonists in man, for instance adrenocorticotrophic hormone (ACTH) response to ipsapirone infusion, can also be included in this group. However, studies of receptor function *in vivo* treat the brain as a `black box' and reveal little about regional differences in receptor function, or the modulatory influence of other transmitter systems on the behavioural response evoked by receptor stimulation.

Despite these limitations, in the current studies 5-MeODMT-induced head-twitches were used as an index of central 5-HT2 receptor function *in vivo*, since a reasonably large number of animals can be studied on the same day. Models of β-adrenoceptor function *in vivo* have been proposed (salbutamol-induced sedation: Frances & Simon, 1978; isoprenaline-induced drinking: Lehr et al., 1967). However, these responses have been shown to involve multiple adrenoceptor types (reviewed by Heal, 1990). Since it was not possible to measure noradrenaline-stimulated adenylate cyclase activity (β-adrenoceptor function *in vitro*) in all animals within an experiment simultaneously, measurements of β-adrenoceptor function were considered unfeasible.

1.7 Involvement of noradrenaline and 5-HT in the effects of stress

1.7.1 Historical aspects

Since the early twentieth century, studies of stress have reported an involvement of catecholamines in stress responses. In 1911, Cannon & de la Paz demonstrated activation of the adrenal glands when cats were exposed to barking dogs. Later, Cannon described a series of physiological reactions, e.g. an increase in heart rate, evoked in animals exposed to `emotional' stimuli, such as exposing a cat to an aggressive dog, or to a mouse (Cannon & Britton, 1926). These reactions were modulated by secretion of adrenin (*sic*) from the peripheral sympathoadrenal system; it was suggested that

> "these functions are made to operate in times of stress, when they facilitate and reinforce the labouring muscles.... the sympathico-adrenal (*sic*) mechanism is called into action *only* at times of violent emotion. According to the evidence now in hand, the greater the emergency, as measured by intensity of excitement and struggle, the more is that mechanism utilized." (Cannon & Britton, 1926)

Ten years later, Selye examined changes in adrenal glands and other organs after exposure to various `acute non-specific nocuous (*sic*) agents' such as cold or exercise (Selye, 1935). He described two phases of the stress response,

> "the symptoms of which are independent of the nature of the damaging agent... and represent a response to damage as such." (Selye, 1935)

The first phase, appearing 6-48 h after the stimulus, was termed the general alarm reaction, involving loss of material from the adrenals, formation of acute erosions in the stomach, and loss of fatty tissue. The second phase, appearing 48 h after the stimulus, involves changes of the altered tissues to their normal state. If stimulation is repeated, this second phase is reduced in length, as resistance to the stimulus is developed. The two phases of the response together were termed the `general adaptation syndrome' since

> "the syndrome as a whole seems to represent a generalised effort of the organism to adapt itself to new conditions.....more or less pronounced forms of this reaction represent the usual response of the organism to stimuli such as temperature changes, drugs, muscular exercise, etc., to which habituation or inurement (*sic*) can occur." (Selye, 1935).

1.7.2 Noradrenaline

All the work described above concerned the effects of stress on adrenaline in the periphery, since it predates the discovery of noradrenaline. It was suggested in 1937 that noradrenaline was the neurotransmitter in sympathetic neurones in the periphery (Stehle & Ellsworth, 1937); this was confirmed by subsequent work by von Euler (1946, 1951). The presence of noradrenaline in the brain was also shown by von Euler (1946), but it was almost 20 years later before noradrenergic neurones were described in the central nervous system (Dahlstrom & Fuxe, 1964). Once techniques to examine indices of central neurotransmitter function e.g. transmitter turnover, receptor binding, had been described, studies of the effects of both a single, and repeated exposure to stress on central noradrenergic neurones were possible.

Surprisingly, it was not until the 1980's that electrophysiological studies of the effects of stress on noradrenergic neurones were reported. Neurones in the locus coeruleus of freely moving cats have been shown to be responsive to non-noxious and aversive stimuli such as a flash of light, exercise, threat or tail pinch (Rasmussen et al., 1986). On exposure to such stimulation, an initial increase in firing rate is seen, followed by a period of inhibition, the largest responses being evoked by aversive stimuli such as tail pinch, and by threat, a psychological stress. In contrast, there is no change in the firing rate of neurones when animals are shown inaccessible food or a rat (frustration; another psychological stress: Gray, 1971), although the cats are clearly aroused by the stimuli, since they attempt to reach the rat or food. The firing rate of neurones in the locus coeruleus is also unchanged when animals are involved in `vegetative' activities e.g. grooming, feeding.

Abercrombie and Jacobs (1987a,b) examined plasma noradrenaline levels, reported to be increased during exposure to a variety of stressful stimuli (e.g. Kvetnansky et al., 1977, 1978; Benedict et al., 1979) in parallel with firing rates of locus coeruleus neurones. During a 15 min exposure of cats to either white noise, or restraint, but not inaccessible rats, plasma noradrenaline levels were increased, as was the firing rate of noradrenergic neurones in the locus coeruleus. In contrast, if animals were exposed to 15 min white noise once every hour for 5 h, then by the fifth presentation, no change in either plasma noradrenaline or firing rate was seen.

The abolition of the stress-induced increase in plasma noradrenaline on repeated stimulus presentation may be due to a failure in noradrenaline release, as a result of

exhaustion of transmitter stores (Fillenz et al., 1979). However, since central noradrenergic neurones respond to the first, but not repeated stimulus presentation, these neurones may be involved in adaptation to repeated stress exposure. An alternative interpretation is that on repeated stimulus presentation, central noradrenergic neurones are no longer stimulated to fire, due to the development of a tachyphylaxis in neurones which signal these neurones to fire. This possibility cannot be ruled out.

Evidence that adaptation to stress involves functional changes in central noradrenergic neurones comes from studies of noradrenaline levels and their receptors. A single exposure to stress induces a transient increase of noradrenaline in dialysate from several brain regions as shown by *in vivo* microdialysis studies in rats (Kokaia et al., 1989; Tanaka et al., 1991; Nisenbaum et al., 1991). If a single period of noxious, or prolonged exposure to stress is used, then a transient reduction in tissue levels of noradrenaline can also be seen, depending on the brain region examined and the stress protocol used (Ida et al., 1984; Lehnert et al., 1984; Irwin et al., 1986; Adell et al., 1988). In contrast, repeated exposure to stress does not alter, and may even increase noradrenaline levels in a variety of brain regions in both the rat and the mouse (Weiss et al., 1975; Irwin et al., 1986; Anisman et al., 1987). This suggests that changes in noradrenaline turnover may be involved in responses to an acute stress and adaptation to repeated stress.

The involvement of β-adrenoceptors (post-synaptic noradrenergic receptors) in response and adaptation to stress has also received much attention. In general, β-adrenoceptors are unaltered by a single exposure to stress, although this has not been extensively studied (reviewed by Stanford, 1990). In contrast, after repeated exposure to one of a number of forms of stress, rat cortical β-adrenoceptor down-regulation has been reported. Less consistent effects are found in other brain regions, where the effects of repeated stress on β-adrenoceptor density appear to depend on the stress, protocol and sampling time used (reviewed by Stanford, 1990).

1.7.3 5-HT

5-HT has been implicated in the response to aversive stimuli. For instance, Graeff and co-workers have shown that stimulation of the dorsal midbrain central grey induces behaviour like that seen evoked by aversive stimuli (termed `aversive behaviour') in the shuttle box; injection of 5-HT or 5-HT agonists into the central grey suppresses `aversive behaviour', possibly via 5-HT2 receptor activation (Graeff et al., 1986). Deakin has suggested that the dorsal raphe nucleus 5-HT system, and projections from the median

raphe nucleus to the hippocampus are activated by conditioned aversive stimuli and by repeated exposure to aversive stimuli, respectively (discussed by Deakin & Graeff, 1991).

However, results of electrophysiological studies do not support a role for 5-HT in the response and adaptation to stress/aversive stimuli. Studies of firing rates in serotonergic dorsal raphe neurones in freely moving cats revealed two levels of activity in these neurones in undisturbed animals; the `quiet waking' state, when animals were still, and the `active waking' state, when animals were moving. Exposure of cats to stimuli such as white noise or restraint stress for 15 min induced an increase in heart rate, and behavioural changes indicating aversion; however, firing rate of dorsal raphe serotonergic neurones was not increased above that seen in the `active waking' state (Wilkinson & Jacobs, 1988). Simple sensory stimuli such as clicks, or light flashes experienced by cats in the `quiet waking' state also increased the levels of firing in dorsal raphe neurones to that seen in the `active waking' state. On repeated presentation of the stimulus, there was no habituation of the neuronal response, unlike the phasic changes in firing rate of locus coeruleus neurones, which declined with repeated stimulus presentation (Rasmussen et al., 1986). This suggests that activation of these neurones is associated with arousal generally, with no specific involvement in the response or adaptation to stress.

However, studies of other indices of serotonergic function indicate that 5-HT is involved in stress responses. Although a single stress exposure does not alter 5-HT levels in a variety of brain regions (Morgan et al., 1975; Kennett & Joseph, 1981; Lehenert et al., 1984; Adell et al., 1988), 5-HIAA levels, often used as an indicator of 5-HT turnover, are generally transiently increased after a single exposure to stress (Welch & Welch, 1968; Kennett & Joseph, 1981; Kennett et al., 1986; Adell et al., 1988). Results of studies using other methods to assess 5-HT turnover (such as 5-HT accumulation after monoamine oxidase inhibition) have also shown that a single exposure to various forms of stress increases 5-HT turnover (Mueller et al., 1976; Mitchell & Thomas, 1988).

Repeated exposure to stress generally has no effects on 5-HT levels in rat cortex; in the hypothalamus and hippocampus effects on 5-HT levels depend on factors such as the stress, protocol and sampling time (Roth et al., 1982; Kennett et al., 1986; Adell et al., 1989; Hilakivi et al., 1989). Repeated stress does not generally affect 5-HIAA levels (Roth et al., 1982; Kennett et al., 1986; Adell et al., 1989); other methods of 5-HT turnover measurement, such as 5-HT accumulation after administration of a monoamine oxidase inhibitor, have not been examined after exposure to repeated stress.

Despite this suggestion that both a single and repeated exposure to stress change 5-HT turnover, little is known about the role of 5-HT receptors in the response and adaptation to stress. The large number of 5-HT receptors demonstrated in the central nervous system complicate this field still further. 5-HT2 receptors, which like β-adrenoceptors are post-synaptic, are reported to be increased by a single period of immobilization in a single study in the rat (Torda et al., 1990). Two further reports have shown no change in either 5-HT1A or 5-HT2 receptor binding in rat cortex after repeated exposure to footshock (Kellar & Bergstrom, 1983; Ohi et al., 1989). The current lack of information means that the role of 5-HT receptors in the effects of both acute and repeated exposure to stress requires further investigation.

Despite the evidence linking changes in central noradrenergic and serotonergic neurones with response and adaptation to stress, the role of the changes described above is poorly understood. The changes described are in general induced by exposure to physically noxious forms of stress such as footshock or immobilization in rats. However, it has been held for many years that in man, the effects of physical and psychological stresses were different and efforts have been made to attribute separate peripheral responses to each type of stress (e.g. Mueller et al., 1974; Dimsdale et al., 1980; discussed by Ward et al., 1983). However, the possibility that different forms of stress may induce different responses to stress in the central nervous system has received little attention.

Another aspect of the effects of stress which has so far been neglected are long-latency effects of stress exposure. The initial phase of the general adaptation syndrome described by Selye (1935) was observed hours after exposure to the stimulus, and the late phase 2 days later. However, subsequent studies of the effects of acute stress have examined only transient neurochemical effects measured generally 0-30 min after cessation of stress; that there may be subsequent neurochemical effects of acute stress has rarely been tested. There have been isolated reports of long-latency (days-weeks) effects of stress on behaviour (Antelman et al., 1983; Van Dijken et al., 1990). However, concomitant neurochemical changes, which may underlie these behavioural changes, have not been investigated. More importantly, investigation of long-latency effects of stress in animals may provide some insight into clinical conditions relating to long-latency effects of stress. For instance, post-traumatic stress disorder is a condition which develops months after a single exposure to an atypical stressful stimulus.

1.8 Antidepressant treatments amd central noradrenergic and serotonergic function

As well as the involvement of central noradrenergic and serotonergic neurones in the effects of stress, various theories have implicated these neurones in depressive states. Relative deficiencies of noradrenaline and 5-HT have both been associated with depression (Schildkraut, 1965; Dencker et al., 1966; Shaw et al., 1967); evidence for these theories comes from human studies *in vivo* and *post mortem* (reviewed by Schaffer et al., 1981; Wehr & Goodwin, 1977; van Praag, 1977). Further evidence supporting this link comes from studies of the mechanism of action of antidepressant treatments.

Acute administration of the majority of tricyclic monoamine uptake inhibitors and antidepressant drugs from other generic groups *in vitro* increases noradrenergic and/or serotonergic function. This may be achieved in a variety of ways, e.g. inhibition of noradrenaline and/or 5-HT reuptake or monoamine oxidase inhibition. A comprehensive review of the effects of antidepressant drugs is beyond the scope of this thesis and this subject has been recently reviewed by various authors (McNeal & Cimbolic, 1986; Rudorfer & Potter, 1989). It should be noted that therapeutic effects of antidepressant drugs are seen in man only after 2-3 weeks of treatment; for this reason, it is relevant to examine the effects of long-term antidepressant administration on central noradrenergic and serotonergic neurones.

Development of rat cortical β-adrenoceptor sub-sensitivity is the most consistent effect seen after repeated administration of the majority of antidepressant drugs in rats (e.g. Asakura et al., 1982; Sugrue, 1983; Heal et al., 1989b). It was originally thought that atypical antidepressants such as mianserin, and selective 5-HT uptake inhibitors such as fluoxetine did not alter β-adrenoceptors (Mishra et al., 1980, 1981). However, more recent studies have reported time- or region-specific changes in β-adrenoceptor density after some of these drugs: mianserin reduces β-adrenoceptors at 6, but not 24 h after the final drug injection (Asakura et al., 1987), and autoradiographical studies have shown reductions in β-adrenoceptors in specific layers of the frontal cortex after fluoxetine administration (Byerley et al., 1988). However, even after such detailed investigation, some 5-HT uptake inhibitors e.g. paroxetine (Nelson et al., 1990, 1991) have not been shown to alter β-adrenoceptors.

Although the majority of studies have reported that an antidepressant-induced down-regulation of cortical β-adrenoceptors is seen after repeated administration only (e.g. Sarai et al., 1978; Suzdak & Gianutsos, 1985), these studies have used the non-selective

radioligand [^{3}H]-DHA. One recent study which has used the selective β-adrenergic radioligand, [^{3}H]-CGP 12177, has demonstrated that *a single injection* of the monoamine uptake inhibitor DMI significantly down-regulates rat cortical β-adrenoceptors, 24 h but not 2 h after the injection (Riva & Creese, 1989b). In the same study, significant down-regulation was not seen until after 7 days of DMI treatment when binding was measured using [^{3}H]-DHA. These findings suggest that the binding of [^{3}H]-DHA is not exclusively to β-adrenoceptors and that changes in the non-β-adrenergic component of [^{3}H]-DHA binding may mask the effect of experimental treatments on β-adrenoceptor binding. Additionally, this finding indicates that the time-course of β-adrenoceptor down-regulation is not linked with the development of therapeutic effects of antidepressant drugs in man.

Studies of the effects of repeated antidepressant administration on central 5-HT receptors have mainly been limited to 5-HT2 receptors. Both cortical 5-HT2 receptor density, and central 5-HT2 receptor function *in vivo* (5-MeODMT-induced head-twitches) are decreased after repeated administration of tricyclic monoamine uptake inhibitors and other antidepressant drugs in rats and mice (Peroutka & Snyder, 1980b; Stolz et al., 1983; Goodwin et al., 1984; Eison et al., 1991). As for β-adrenoceptors, some selective 5-HT uptake inhibitors such as citalopram are thought not to alter 5-HT2 receptors (Hytell et al., 1984). However, such suggestions are based on a handful of studies which have not investigated the time course of any effects, or looked at different doses or specific cortical layers/regions. Until such studies are carried out, firm conclusions about the effect of these drugs on cortical 5-HT2 receptors are premature.

Sibutramine hydrochloride (sibutramine) is a noradrenaline plus 5-HT reuptake inhibitor (Buckett et al., 1988). Repeated sibutramine administration down-regulates cortical β-adrenoceptor density and, like other noradrenaline plus 5-HT reuptake inhibitors, decreases the sensitivity of cortical noradrenaline-stimulated adenylate cyclase activity in the rat (Buckett et al., 1988). The effects of repeated sibutramine administration on central 5-HT2 receptors have not been reported.

Although theories of depression implicate a functional deficit of noradrenaline and 5-HT, repeated administration of most antidepressant drugs *decreases* the density of post-synaptic noradrenergic (β) and serotonergic (5-HT2) receptors. The relevance of 5-HT2 receptor and β-adrenoceptor down-regulation in the therapeutic effects of these drugs is therefore unclear.

1.9 Stone's hypothesis

Both repeated exposure to forms of stress such as restraint, footshock or food deprivation, and repeated antidepressant administration over a time course similar to that needed for therapeutic effects in man induces sub-sensitivity of β-adrenoceptors in rat cortex. This sub-sensitivity is seen as a reduction in noradrenaline-stimulated adenylate cyclase activity, and/or a down-regulation of β-adrenoceptor density. Stone (1979b) noted that physiological effects of a single stress exposure e.g. anorexia and weight loss, were reduced when rats were repeatedly exposed to stress. Such changes were interpreted as the development of adaptation to stress; the time-course of these changes was similar to that over which β-adrenoceptor subsensitivity developed. These findings led Stone to propose that β-adrenoceptor sub-sensitivity is the mechanism which underlies stress adaptation (Stone, 1979b). The time-course of adaptation to stress was also similar to that for the reduction in depressive symptoms which is seen in man during antidepressant therapy. This observation, together with the findings that both repeated stress and repeated antidepressant treatment reduce β-adrenoceptor density, led Stone to suggest that repeated antidepressant administration is "a unique form of adaptation to stress" (Stone, 1979b). It is noted that although some physiological effects of stress e.g. anorexia may resemble signs of depression in man, exposure to stress cannot be assumed to mimic the psychological or emotional effects of depression, however.

The hypothesis that β-adrenoceptor down-regulation facilitates stress adaptation was further supported by a subsequent report from the same group (Stone & Platt, 1982). Repeated restraint stress induced a down-regulation of β-adrenoceptors. In addition, the incidence of gastric lesions and the decrease in food intake seen after a single stress exposure were greatly reduced in animals which had previous experience of repeated stress. Together, these findings were interpreted as showing that cortical β-adrenoceptor down-regulation conferred stress resistance. Studies of the group mean scores for cortical β-adrenoceptor binding and adaptation to stress revealed highly significant positive correlations between β-adrenoceptor density and the incidence of gastric lesions. Thus the lowest reduction in food intake, and the lowest incidence of gastric lesions were seen in the groups of animals with the lowest β-adrenoceptor density. Again, this was seen as support for the hypothesis that a reduction in β-adrenoceptor number underlies behavioural resistance to stress.

A major limitation of Stone's hypothesis is that it is based purely on studies of forms of stress such as footshock and restraint. The effects of such stimuli may differ from less

noxious forms of stress such as novelty or loss, which are more relevant to stressors experienced by man (Brown & Harris, 1989). Although it is generally assumed that Stone's hypothesis is relevant to all forms of stress, recent studies of novelty (Salmon & Stanford, 1989) and nonreward (Stanford & Salmon, 1989) found that the relationship between β-adrenoceptor density and behavioural resistance to these forms of stress differed from that predicted from Stone's hypothesis. Although β-adrenoceptor density was correlated with stress resistance, the relationship was opposite to the positive correlation predicted from Stone's hypothesis (discussed in Chapter 3). To date therefore, although several lines of evidence suggest a relationship between sensitivity to stress and cortical β-adrenoceptor density, the exact nature of this relationship is unclear.

1.10 Influence of previous exposure to repeated stress or antidepressant administration on behavioural and neurochemical responses to an acute stress

Repeated exposure to the same stress can modify animals' behavioural responses to exposure to a novel form of stress (e.g. Stone, 1979b; Platt & Stone, 1982; Garcia-Marquez & Armario, 1987). In addition, behavioural responses to stress can be modified by repeated administration of antidepressant drugs. One widely studied behavioural model in which behaviour is altered by stress and antidepressants is the swim test ('Porsolt test'; Porsolt et al., 1977a, b). Briefly, animals are placed in a cylinder of water from which escape is impossible; animals develop a characteristic immobile posture after an initial period of vigorous swimming, and the time spent immobile is measured. Immobility of rodents in the swim test is reduced after sub-chronic and repeated administration of tricyclic monoamine uptake inhibitors and antidepressants from other generic classes (reviewed by Borsini & Meli, 1988); in mice, acute antidepressant administration also reduces immobility (Porsolt et al., 1977b). Immobility is also altered by repeated exposure to stress, although the changes seen are less consistent (Platt & Stone, 1982; Garcia-Marquez & Armario, 1987; Cancela et al., 1991). Both repeated stress and antidepressant administration also down-regulate β-adrenoceptors, but the relationship between immobility and receptor density has been little studied. One study has shown parallel reductions in cortical β-adrenoceptor density and in immobility after repeated DMI administration in separate groups of animals (Kitada et al., 1986). However, studies showing parallel changes in β-adrenoceptors and behaviour *in the same animals* would provide far stronger evidence in support of a relationship between receptors and behavioural resistance to stress. More generally, studies of neurochemistry and behaviour in the same animals could provide indications of neurochemical changes underlying behavioural responses; such studies are very rare, however.

As well as the `Porsolt' test, a whole range of behavioural paradigms used to assess animals' psychological status have been described in which behaviour is modified by stress or antidepressants e.g. learned helplessness (Weiss et al., 1975), the open field (Kulkarni & Dandiya, 1973; Kennett et al., 1985). The arguable question of whether or not such paradigms reflect animals' emotional status is not crucial to the current experiments. However, these behavioural models induce hormonal changes which are used widely as an index of the response to stress e.g. increased plasma noradrenaline or ACTH levels (Hennessy & Levine, 1978; Benedict et al., 1979; de Boer et al., 1990); it is possible that behaviour in such stressful models may reflect resistance to stress. Behaviour in these models has been widely studied and could be used to investigate the relationship between behavioural resistance to stress and cortical β-adrenoceptor density, therefore. Since both repeated stress and antidepressant administration can alter behavioural responses to stress and down-regulate cortical β-adrenoceptors, these treatments could be valuable in assessment of the relationship between behavioural resistance to stress and β-adrenoceptors.

1.11 Objectives of the study

1) To investigate the effects of an acute stress on behaviour and on indices of central noradrenergic and serotonergic function, and the influence of previous exposure to repeated stress or repeated administration of monoamine uptake inhibitors on the behavioural and neurochemical responses to an acute stress.

2) To investigate the relationship between cortical β-adrenoceptor density and behavioural resistance to stress.

3) To investigate the possibility that the non-selectivity of the β-adrenergic radioligand, [^{3}H]-DHA, may influence the interpretation of results of studies examining the effects of stress or antidepressant administration on β-adrenoceptors.

2.0 METHODS

2.1 Animals

Animals used were either adult male Sprague-Dawley rats (Charles River), weighing 250-300 g on arrival in the animal house, or adult male CD1 mice (Charles River), weighing 18-20 g on arrival in the animal house. Animals were housed in groups of not less than 3, under a 24 hour light/dark cycle (light on 08.00 to 20.00 h). Food and water were freely available at all times.

Animals were left undisturbed (apart from routine husbandry) for at least 5 days before experimental procedures were begun. Animals receiving drug/saline treatments were weighed and injected daily (between 09.00 - 10.00 h) for the stated number of days. 'Uninjected' control animals remained unhandled during this time. 24 h after the final injection, animals were killed by cardiothoracic shock and cervical dislocation, or subjected to behavioural testing before killing, between 09.00 to 13.00 h.

2.2 *In vivo* methods

2.2.1 Open Field

Apparatus:

The open field consisted of a circular white perspex floor, 600 mm radius, surrounded by an aluminium wall, 360 mm high. Lines were marked on the floor to give a central circle, 160 mm in radius, divided into quadrants. Beyond this, 16 radii were drawn at equal angles; additional concentric circles were drawn at 275, 390 and 500 mm from the centre. The field was brightly illuminated by 2 x 100 W bulbs mounted 1.3 m above the floor of the field.

Procedure:

Rats were placed individually into the centre of the open field, and removed after 4 min. The floor of the field was cleaned and wiped dry after each animal was removed. A video camera mounted above the field was used to record the activity of the animal.

After testing, individuals' activities were scored for the following behaviours:

(i) Arc movements - the crossing of radially drawn lines

(ii) Radial movements - the crossing of concentric lines

(iii) Latency - the time (in seconds) taken for the rat to cross the outermost circle following placement in the centre of the field.

(iv) Time in the centre - the total time (in seconds) spent by the rat in the inner three circles

(v) Rearing (with both forepaws off the ground)

(vi) Grooming - the number of grooming episodes
- the time (in seconds) spent grooming

(vii) Defecation - the number of boli dropped

The criterion for line crossing was that the rat crossed a line with all 4 paws.

2.2.2 Swim Stress

Apparatus:

2 x 1 l pyrex beakers were used. The beakers were filled with water (temp. 21.5 - 22.5°C) to a depth of 9-10 cm to ensure that mice were prevented from putting their hindpaws on the bottom of the beaker.

Procedure:

Mice were individually placed in the water for 6 min; immobility (defined as the time spent by the animal making only the smallest movements in order to remain afloat) was recorded using a stopwatch during the final 4 min of the test (Porsolt et al., 1977b). On removal from the water, the mice were either killed immediately, or dried (by wrapping with cotton wool to remove excess water, then placing in a cage under a 60 W bulb for warmth until dry). When dry, the mice were returned to the home cage for a predetermined time before killing.

2.2.3 5-HT2 receptor-mediated head-twitches

Procedure:

Mice received an injection of 5-MeODMT (2 mg/kg i.p.) and were placed in a small cage, with a layer of wood chippings on the floor; head-twitches were counted during the 6 min following injection of 5-MeODMT.

Generally, twitches were observed by 2 observers, one of whom was blind to the drug or experimental pretreatment of the animal. Scoring of head-twitches (a rapid rotational movement of the head/ears) was learned from an experienced observer, to standardize scoring criteria.

2.3 *In vitro* methods

2.3.1 Radioligand binding

Tissue collection:

Animals were killed by cardiothoracic shock and cervical dislocation, and brains removed. Brain regions were dissected over ice, and frozen immediately on dry ice, before storing at -20°C.

Membrane preparation:

Membranes were freshly prepared for each assay. Each assay included at least one sample from each experimental group; within any assay, the number of samples from each experimental group was balanced where possible. All samples within an assay contained the same concentration of tissue.

2.3.1.1 β-*adrenoceptor binding measured using [^{3}H]-dihydroalprenolol*

Membrane preparation:

Membranes were prepared according to the method of Stanford *et al* (1984). Tissue was weighed and homogenized (12 strokes of the pestle) in 6 ml Tris-HCl buffer (pH 7.6, 50 mM). Homogenates were spun at 20,000 x g for 20 min at 4°C, using a Beckman L8-70M preparative ultracentrifuge. The resulting pellet was washed twice with 2 ml Tris-HCl, then resuspended by homogenization in 6 ml Tris-HCl. Homogenates were again spun at 20,000 x g for 20 min at 4°C. The final pellet was washed twice with 2 ml Tris-HCl and resuspended by homogenization in a volume of Tris-HCl equivalent to 16-20 mg original wet weight tissue/ml.

Displacement binding:

[^{3}H]-Dihydroalprenolol ([^{3}H]-DHA) binding was displaced by (±)-isoprenaline sulphate, (-)-isoprenaline HCl or (-)-noradrenaline HCl to determine the concentration of these drugs to be used in subsequent saturation or single point binding experiments.

To each of 18 tubes, 50 µl of [^{3}H]-DHA and 50 µl of Tris-HCl or displacing ligand (17 different concentrations in the range 10^{-11}-10^{-2} mol/l) were added. It was aimed to measure displacement binding at a radioligand concentration of ~1 nM, the approximate binding Kd for this ligand at β-adrenoceptors. In practice, the concentration of [^{3}H]-DHA used ranged between 0.8 - 1.5 nM in different assays, although each assay used a fixed radioligand concentration. Assays were started by addition of 150 µl tissue suspension to each tube. Tubes were incubated in duplicate at 26°C for 40 min; incubation time and

temperature were chosen to allow specific binding to reach equilibrium according to previously published findings (Bylund & Snyder, 1976). Incubation was terminated by rapid filtration using a membrane harvester and washing of the filters with 6 ml Tris-HCl. The amount of bound radioligand was measured by liquid scintillation counting; the scintillation fluid used was Ultima Gold (Canberra Packard).

'Total binding' was defined as the amount of radioligand bound in the absence of displacing ligand; the amount bound in the presence of the highest concentration of displacing ligand was defined as 'non-specific binding', since displacement curves indicated that all specific binding of [^{3}H-DHA has been displaced. 'Specific binding' at each displacing ligand concentration was then calculated as the difference between 'total' and 'non-specific' binding. Specific binding curves were analyzed using the computer package 'Graphpad'. The mean Hill coefficient for the displacement of [^{3}H]-DHA by isoprenaline SO4 was significantly less than unity (Table 2.1), suggesting that ~~more than~~ [^{3}H]-DHA is displaced from more than one site. Analysis of each of the 9 curves for the displacement of [^{3}H]-DHA by isoprenaline SO4, each of which was determined in cortices from separate rats, showed that 5 curves were best described by a 1 site model (typical curve shown in Figure 2.1a). However, the remaining 4 curves were best described by a 2 site model, although these curves did not show 2 distinct saturable binding sites (Figure 2.1b). This evidence suggests that isoprenaline may displace [^{3}H]-DHA from two sites in rat cortex. In mouse cortex, the mean Hill coefficients for the displacement of [^{3}H]-DHA by isoprenaline HCl and by noradrenaline HCl were also significantly less than unity (Table 2.1), suggesting that [^{3}H]-DHA is displaced by both these ligands from more than one site. However, all individual displacement curves were best described by a 1 site model (Figures 2.2, 2.3). It is therefore unclear whether or not more than one site is involved in the binding of this radioligand in mice. The concentration of displacing ligand chosen for use in subsequent saturation or single point analyses lies on the plateau reached at high concentrations of displacing ligand. This was to ensure that in these analyses, the amount of displacing ligand will be sufficient to displace all specific binding at the highest radioligand concentrations used. The actual concentrations chosen were 200 µM (±)-isoprenaline SO4, 100 µM (-)-isoprenaline HCl and 30 µM (-)-noradrenaline HCl.

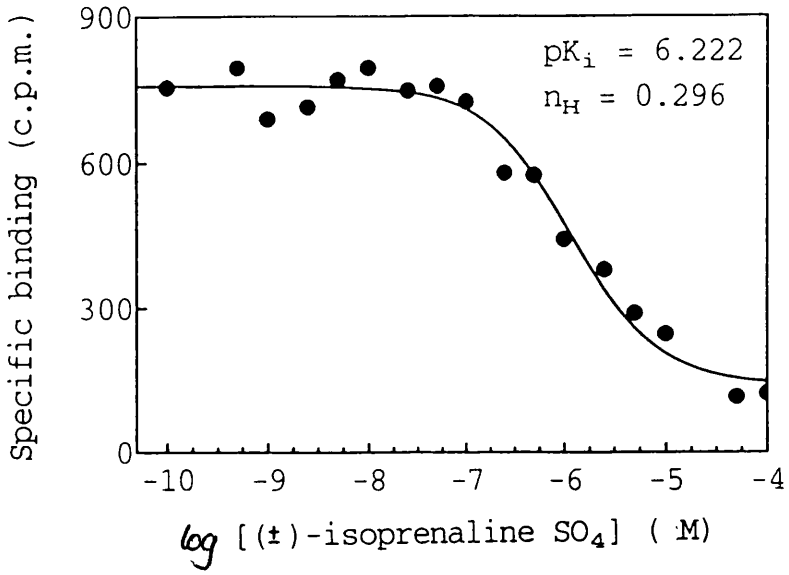
Table 2.1 **MEAN HILL COEFFICIENTS FOR THE DISPLACEMENT OF [^{3}H]-DHA AND OF [^{3}H]-CGP 12177 BY DIFFERENT ADRENERGIC LIGANDS IN CEREBRAL CORTICAL MEMBRANES**

Radioligand	Displacing ligand	Species (n)	Hill coefficient
[^{3}H]-DHA	(±)-isoprenaline SO_4	Rat (9)	0.603 ± 0.074**
	(-)-isoprenaline HCl	Mouse (5)	0.429 ± 0.058*
	(-)-noradrenaline HCl	Mouse (5)	0.626 ± 0.106*
[^{3}H]-CGP 12177	(-)-isoprenaline HCl	Mouse (8)	0.890 ± 0.114

Table 2.1 Data shown are mean ± s.e.m. of 5-9 Hill coefficients for the displacement of [^{3}H]-DHA or [^{3}H]-CGP 12177 by the displacing ligands shown. Each curve was measured in membranes from a separate animal, and the Hill coefficient determined for each individual curve. Hill coefficients compared with unity using the Wilcoxon signed-rank test; $^*p < 0.05$ $^{**}p < 0.01$ *cf* 1.

Figure 2.1 DISPLACEMENT OF [^{3}H]-DHA BINDING BY (±)-ISOPRENALINE SO4 IN RAT CORTEX

a) 1 Site displacement curve



b) 2 Site displacement curve

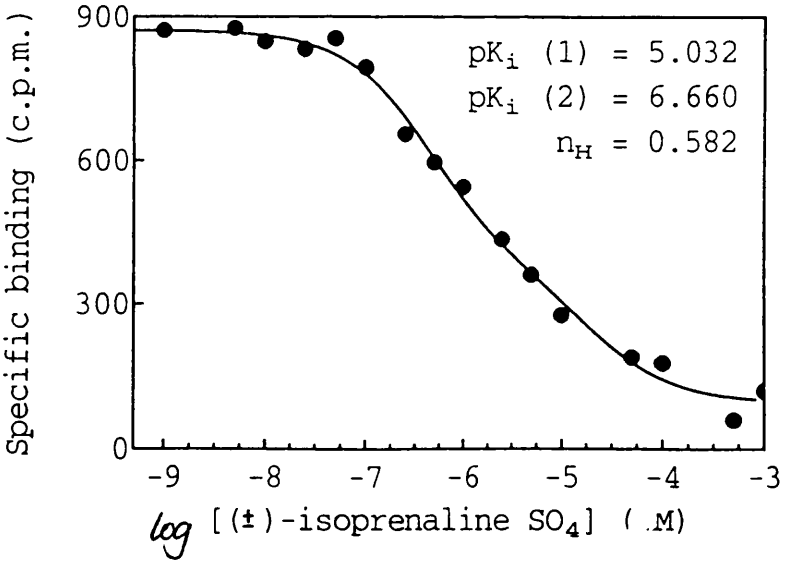


Figure 2.1 Displacement of [^{3}H]-DHA in rat cortex using (±)-isoprenaline SO4. Data expressed as specific binding of [^{3}H]-DHA (the difference between total [^{3}H]-DHA binding and [^{3}H]-DHA binding in the presence of 10^{-2}M (±)-isoprenaline SO4) at each concentration of (±)-isoprenaline SO4. n_H = Hill coefficient

a) Data best described by a 1-site fit ($F = 1.734$; d.f. = 2, 13; $p > 0.05$)

b) Data best described by a 2-site fit ($F = 9.556$; d.f. - 2, 13; $p < 0.01$)

Figure 2.2 **DISPLACEMENT OF [^{3}H]-DHA BINDING BY (-)-ISOPRENALINE HCl IN MOUSE CORTEX**

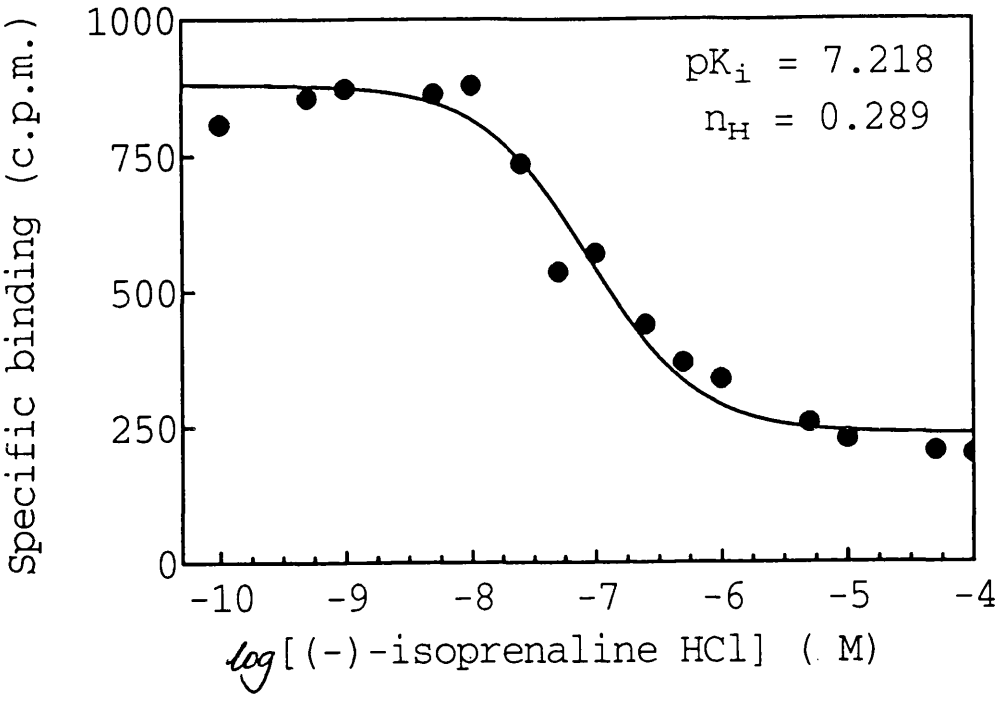


Figure 2.2 Displacement of [^{3}H]-DHA by (-)-isoprenaline HCl measured in mouse cortex. Data expressed as specific binding of [^{3}H]-DHA (the difference between total [^{3}H]-DHA binding and [^{3}H]-DHA binding in the presence of 10^{-2}M (-)-isoprenaline HCl) at each concentration of (-)-isoprenaline HCl. n_H = Hill coefficient. Data best described by a single site model ($F = 0.243$; d.f. = 2, 16; $p > 0.05$).

Figure 2.3 **DISPLACEMENT OF [^{3}H]-DHA BINDING BY (-)-NORADRENALINE HCl IN MOUSE CORTEX**

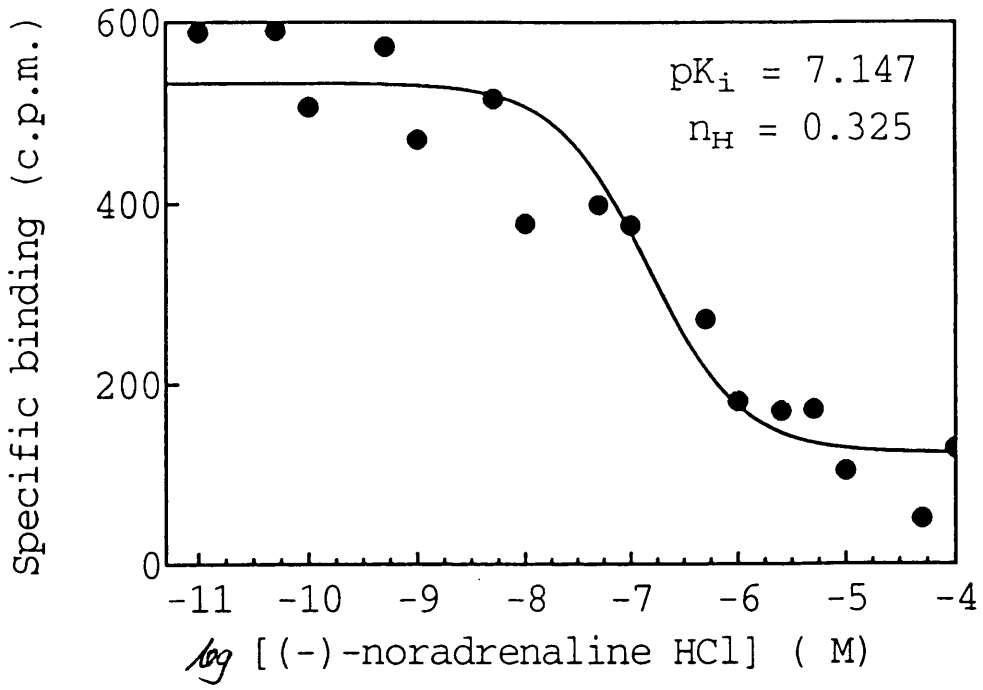


Figure 2.3. Displacement of [^{3}H]-DHA by (-)-noradrenaline HCl measured in mouse cortex. Data expressed as specific binding of [^{3}H]-DHA (the difference between total [^{3}H]-DHA binding and [^{3}H]-DHA binding in the presence of 10^{-3}M (-)-noradrenaline HCl) at each concentration of (-)-noradrenaline HCl. Data best described by a single site model ($F = 0.485$; d.f. = 2. 13; $p > 0.05$).

In investigations of the specific binding of [^{3}H]-DHA, displacement of this radioligand was also investigated in mice using one of a range of serotonergic ligands. The ligands used were: 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT), 1-[3-(trifluoromethyl) phenyl] piperazine (TFMPP), 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)-pyrrol [1,2a]quinoxaline dimaleate salt (CGS 12066 B) and ritanserin. Each ligand was used over the concentration range 10^{-11} - 10^{-3} mol/l. Experiments and analysis were carried out as described above.

Saturation binding:

6 or 8 concentrations of [^{3}H]-DHA, over the range 0.01 - 6.0 nM were used. In rats, cortical β-adrenoceptor binding was measured in an experiment which was a continuation of previous work from this laboratory; for consistency with this work, non-specific binding of [^{3}H]-DHA in rats was assessed by the displacement of total bound radioligand by 200 μM (±)-isoprenaline SO_4. For later studies using mice however, non-specific binding of [^{3}H]-DHA was assessed using 100 μM (-)-isoprenaline HCl, since the displacement of [^{3}H]-DHA by isoprenaline is stereospecific in rat cortex (Bylund & Snyder, 1976).

50 μl [^{3}H]-DHA, and 50 μl displacing drug (non-specific binding) or Tris-HCl (total binding) were added to each tube. Assays were started by the addition of 150 μl tissue to each tube. Tubes were incubated in duplicate at 26°C for 40 min. Incubation was terminated by rapid filtration with a membrane harvester, and rapid washing of the filters with 6 ml Tris-HCl. Bound radioligand was measured by liquid scintillation counting and expressed in terms of protein content, which was measured by Lowry's method.

Specific binding was calculated by subtraction of non-specific binding from total binding at each radioligand concentration (Figure 2.4, 2.5). Analysis of specific binding was carried out using the iterative, weighted least-squares curve-fitting program LIGAND (Munson & Rodbard, 1981) modified for use on an Apple IIe computer (Jackson & Edwards, unpublished). This program uses an exact mathematical model of the ligand binding system to fit data to 1 or more saturable binding sites. Saturation binding curves for [^{3}H]-DHA, defined using (±)-isoprenaline sulphate in rat cortex, or (-)-isoprenaline HCl in mouse cortex were fitted to a 1 site model, since displacement curves for both these combination of ligands are monophasic (see above).

Figure 2.4 SATURATION BINDING OF [^{3}H]-DHA IN RAT CORTEX, DEFINED USING (±)-ISOPRENALINE SO_4

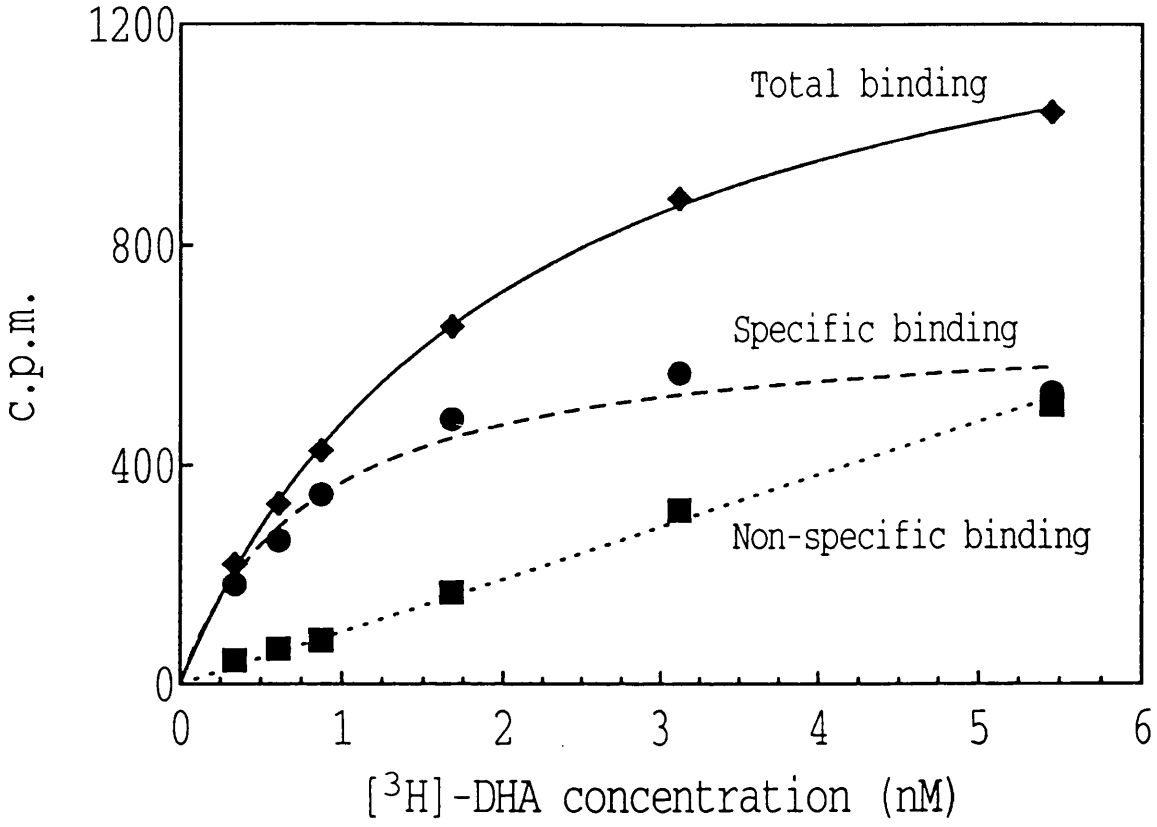


Figure 2.4 Saturation binding of [^{3}H]-DHA in rat cortex. Total binding determined as radioligand binding in the absence of displacing ligand. Non-specific binding of [^{3}H]-DHA measured in the presence of 200 µM (±)-isoprenaline SO_4. Specific binding calculated as the difference between total and non-specific binding at a given radioligand concentration.

Figure 2.5 **SATURATION BINDING OF [^{3}H]-DHA IN MOUSE CORTEX, DEFINED USING (-)-ISOPRENALINE HCl**

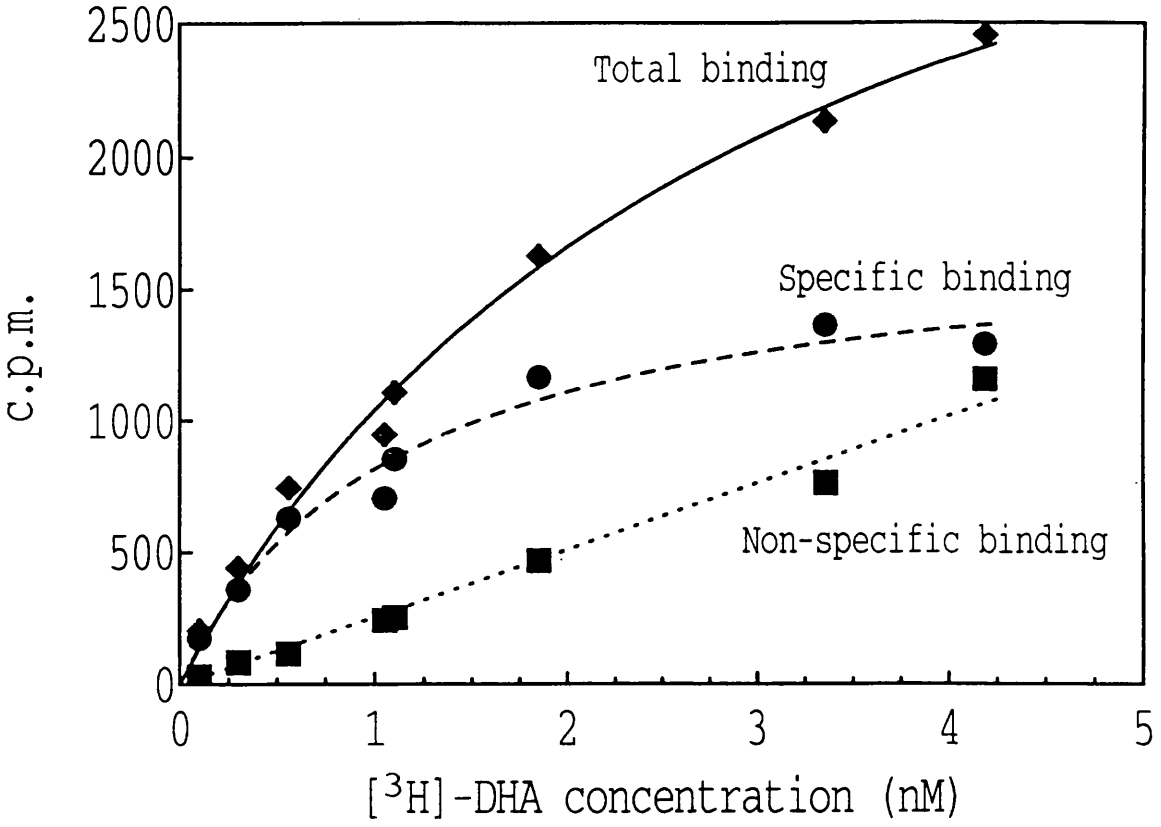


Figure 2.5 Saturation binding of [^{3}H]-DHA in mouse cortex. Total binding determined as radioligand binding in the absence of displacing ligand. Non-specific binding of [^{3}H]-DHA measured in the presence of 200 µM (-)-isoprenaline HCl. Specific binding calculated as the difference between total and non-specific binding at a given radioligand concentration.

Single point binding:

This method was used in investigations of the specific binding of [^{3}H]-DHA in mice, a small proportion of which may represent binding to a 5-HT receptor, in rats at least (Riva & Creese, 1989a; Gillespie et al., 1989; Stockmeier & Kellar, 1989). Although this method is far from ideal, since no estimates of binding parameters can be made, it was used in a situation where measurement of saturation binding proved unsatisfactory, since the site being measured comprised only a small proportion of total [^{3}H]-DHA binding. Single point binding was used as a check to show whether or not binding to the 5-HT site was present, therefore.

To each tube was added 50 µl of [^{3}H]-DHA at a fixed concentration (1 nM), 50 µl of either 50 mM Tris-HCl (pH 7.6) or 30 µM noradrenaline (to block binding to adrenoceptors) and 50 µl of displacing ligand or Tris-HCl. The displacing ligand was one of a range of serotonergic ligands, added in a fixed concentration determined in displacement studies. Assays were started by the addition of 150 µl membrane suspension to each tube; tubes were incubated in octuplicate at 26°C for 40 min. Incubation was terminated by filtration with a membrane harvester and rapid washing of the filters with 6 ml Tris-HCl. The amount of radioligand bound was determined using liquid scintillation counting: the scintillation fluid used was Ultima Gold (Canberra Packard).

[^{3}H]-DHA binding in the absence of any displacing drug was defined as 'total binding'. β-Adrenoceptor binding was defined as the difference between total binding and binding in the presence of noradrenaline. Specific binding (to β-adrenoceptors and to a serotonergic receptor) was defined as the difference between total and non-specific binding ([^{3}H]-DHA binding in the presence of noradrenaline and the serotonergic displacing ligand).

2.3.1.2 *β-adrenoceptor binding measured using (-)-4-(3-t-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]-benzimidazol-2-one ([^{3}H]-CGP 12177)*

Although the current study showed monophasic displacement of [^{3}H]-DHA by (±)-isoprenaline SO_4 (Section 2.3.1.1), other studies have shown that [^{3}H]-DHA may be displaced from 2 binding sites by either propranolol or isoprenaline in rat cortex (Gillespie et al., 1988; Riva & Creese, 1989a). This indicates that [^{3}H]-DHA is not ideal as a radioligand to define β-adrenoceptor binding, since changes in specific binding may reflect changes in the second, as yet undefined binding site, as well as or instead of

changes in β-adrenoceptors. Although biphasic displacement of [^{3}H]-DHA by isoprenaline could not be detected either in rat or mouse cortex in this study, it was decided to compare the binding of this radioligand with that of another β-adrenergic radioligand, [^{3}H]-CGP 12177 in mice. This comparison was made in order to determine if differences in binding estimates defined using the different radioligands which may be seen in tissues from both control and from drug-treated rats (Riva & Creese, 1989a,b) were also apparent in mice. [^{3}H]-CGP 12177 binds with high affinity and low non-specific binding to β-adrenoceptors (Staehelin et al., 1983) and since it is displaced monophasically by (-)-isoprenaline HCl, it is considered to be a more suitable ligand for the definition of β-adrenoceptors. Comparison of [^{3}H]-DHA and [^{3}H]-CGP 12177 binding should indicate therefore whether the specific binding of these radioligands is significantly different in mice, as has been shown in the rat.

Membrane preparation:

Membranes were prepared according to the method of Stanford *et al* (1984). Tissue was weighed and homogenized (12 strokes of the pestle) in 6 ml Tris-HCl buffer (pH 7.6, 50 mM). Homogenates were spun at 20,000 x g for 20 min at 4°C, using a Beckman L8-70M preparative ultracentrifuge. The resulting pellet was washed twice with 2 ml Tris-HCl, then resuspended by homogenization in 6 ml Tris-HCl. Homogenates were again spun at 20,000 x g for 20 min at 4°C on the Beckman centrifuge. The final pellet was washed twice with 2 ml Tris-HCl. The pellet was then resuspended by homogenization in a volume of Tris-HCl equivalent to 16-20 mg original wet weight tissue/ml; all samples within an assay contained the same concentration of tissue.

Displacement binding:

Since saturation binding of this radioligand was to be measured in mice, (-)-isoprenaline HCl was to be used to define non-specific binding, for consistency with studies of [^{3}H]-DHA binding in this species. However, it was first necessary to determine the concentration of (-)-isoprenaline HCl to be used in these studies, by construction of curves for the displacement of [^{3}H]-CGP 12177 by this drug.

To each of 18 tubes was added 50 µl of a fixed concentration of [^{3}H]-CGP 12177 and 50 µl Tris-HCl (pH 7.6, 50 mM) or (-)-isoprenaline HCl (17 different concentrations, to give a final concentration in the range 10^{-10} - $5x10^{-3}$ M). Assays were started by the addition of 150 µl membrane suspension to each tube. The concentration of radioligand was chosen to give a final concentration (0.1 nM) approximating to the published K_d (0.17

nM in rat cerebral cortical membranes: Guyard et al., 1990).

Tubes were incubated in duplicate for 120 min at 26°C; incubation time and temperature were chosen to allow specific binding to reach equilibrium according to previously published data (Wilkinson & Wilkinson, 1985). Incubation was terminated by filtration with a membrane harvester and rapid washing of the membranes with 6 ml Tris-HCl. The amount of bound radioligand was measured by liquid scintillation counting: the scintillation fluid used was Ultima Gold (Canberra Packard).

'Total binding' was defined as the amount of radioligand bound in the absence of isoprenaline; the amount bound in the presence of the highest concentration of isoprenaline was defined as 'non-specific binding'. 'Specific binding' at each isoprenaline concentration was then calculated as the difference between 'total' and 'non-specific' binding. Specific binding curves were analyzed using the computer package 'Graphpad'. Both 1- and 2-site models were fitted to each curve; comparison of the goodness of fit of each model revealed that all individual curves were best described by a 1 site model (Figure 2.6). Moreover, the mean Hill coefficient for the displacement of [^{3}H]-CGP 12177 by isoprenaline HCl was not significantly different from unity (Table 2.1). Clearly this radioligand is displaced from one site only in mouse cortex. This contrasts with the binding of [^{3}H]-DHA in mice where, although curves were best described by 1 site models, the Hill coefficients suggest the presence of more than one site. This suggests that [^{3}H]-CGP 12177 is a more suitable ligand to measure β-adrenoceptor binding in mouse cortex. The concentration of (-)-isoprenaline HCl chosen for use in saturation binding assays was set at 100 μM.

Saturation binding:

Saturation binding of [^{3}H]-CGP 12177 was to be compared with the binding of [^{3}H]-DHA. In order to eliminate the possibility of batch differences in binding to either β-adrenoceptors or any other site to which [^{3}H]-DHA binds, binding of each ligand was measured in simultaneous experiments using duplicate aliquots of membrane preparations derived from the same animals.

8 concentrations of [^{3}H]-CGP 12177 over the range 0.01 - 3.6 nM were used. Non-specific binding was assessed by the displacement of total bound [^{3}H]-CGP 12177 by 100 μM (-)isoprenaline HCl, the unlabelled competing ligand. 50 μl radioligand and 50 μl isoprenaline (non-specific binding) or Tris-HCl (total binding) were added to each tube.

Figure 2.6 **DISPLACEMENT OF [^{3}H]-CGP 12177 BINDING BY (-)-ISOPRENALINE HCl IN MOUSE CORTEX**

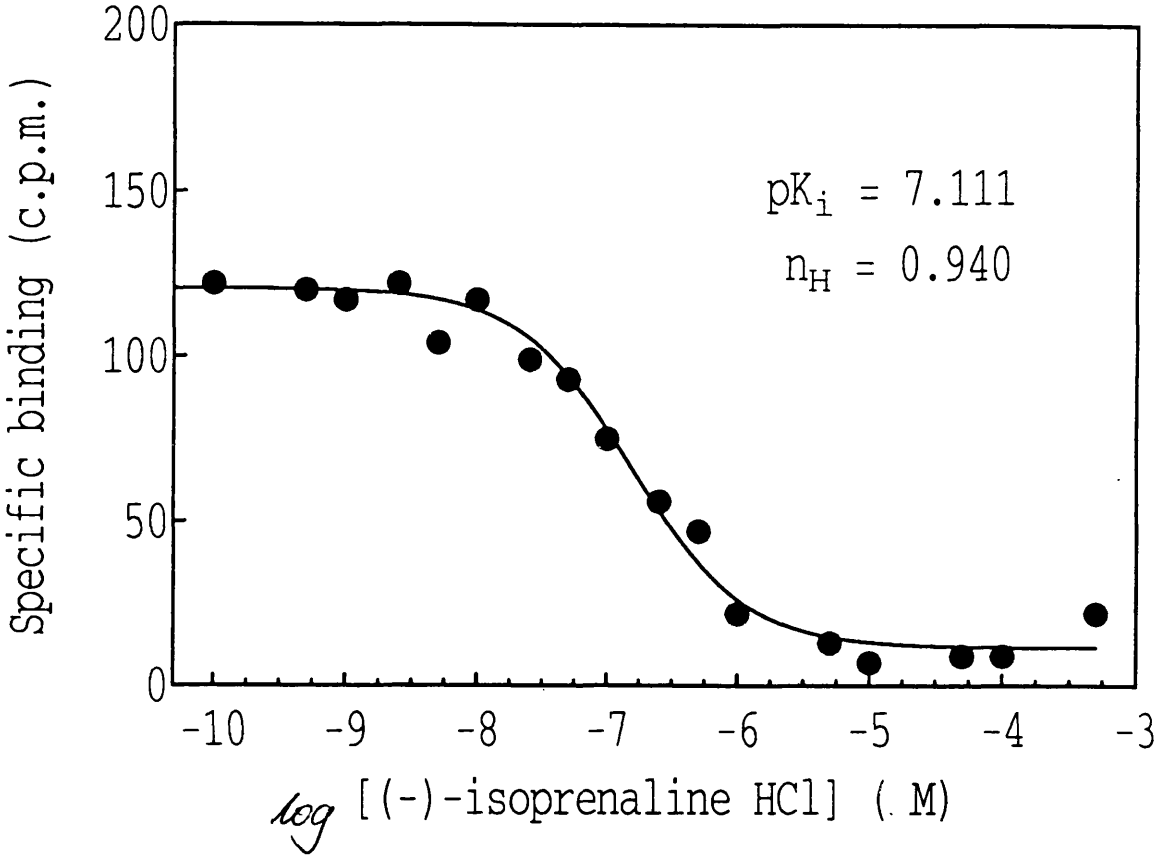


Figure 2.6. Displacement of [^{3}H]-CGP 12177 by (-)-isoprenaline HCl measured in mouse cortex. Data expressed as specific binding of [^{3}H]-CGP 12177 (the difference between total [^{3}H]-CGP 12177 binding and [^{3}H]-CGP 12177 binding in the presence of 10^{-2}M (-)-isoprenaline HCl) at each concentration of (-)-isoprenaline HCl. n_H = Hill coefficient. Data best described by a single site model ($F = 0.659$; d.f. = 2, 13; $p > 0.05$).

Figure 2.7 **SATURATION BINDING OF [^{3}H]-CGP 12177 IN MOUSE CORTEX, DEFINED USING (-)-ISOPRENALINE HCl**

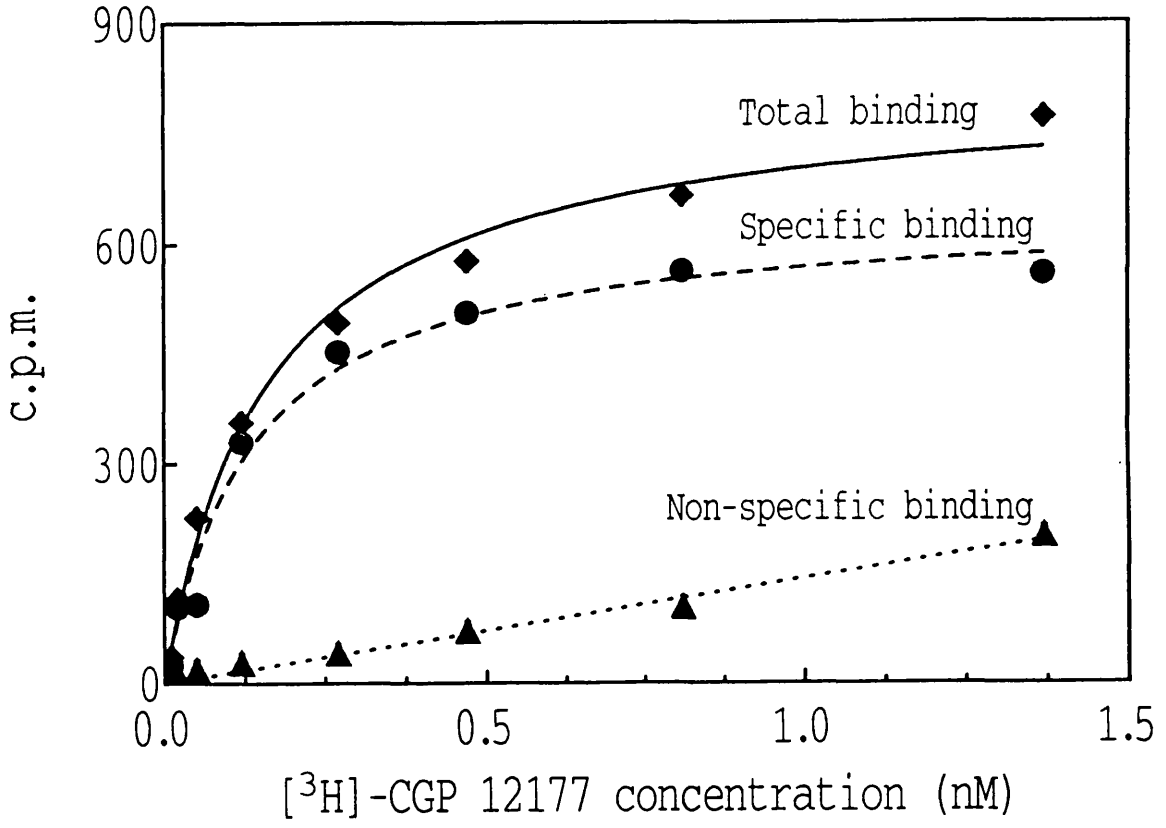


Figure 2.7 Saturation binding of [^{3}H]-CGP 12177 in mouse cortex. Total binding determined as radioligand binding in the absence of displacing ligand. Non-specific binding of [^{3}H]-CGP 12177 measured in the presence of 100 μM (-)-isoprenaline HCl. Specific binding calculated as the difference between total and non-specific binding at a given radioligand concentration.

Assays were started by the addition of 150 µl membrane suspension to each tube; tubes were incubated in duplicate for 120 min at 26°C (Wilkinson & Wilkinson, 1985). Incubation was terminated by rapid filtration with a membrane harvester and rapid washing of the filters with 6 ml Tris-HCl. Bound radioligand was measured by liquid scintillation counting and expressed in terms of protein content.

Specific binding at each [^{3}H]-CGP 12177 concentration was calculated by subtraction of non-specific binding from total binding (Figure 2.7). Analysis of specific binding was carried out using the iterative weighted least-squares curve-fitting program LIGAND (Munson & Rodbard, 1981), modified for use on an Apple IIe computer (Jackson & Edwards, unpublished). This program uses an exact mathematical model to fit data to 1 or more saturable binding sites. Data were fitted to a 1 site model since curves for the displacement of [^{3}H]-CGP 12177 were monophasic, and Kd and Bmax of the receptors estimated.

2.3.1.3 *5-HT2 receptor binding measured using [^{3}H]-ketanserin*

Membrane preparation:

Membranes were prepared essentially according to the method of Leysen *et al* (1982). The tissue was weighed and homogenized (12 strokes of the pestle) in 2 ml ice-cold sucrose (0.25 M). Homogenates were spun at 1,000 x g for 10 min at 4°C, on a Mistral MSE 2L centrifuge. The resulting supernatant was drawn off using a glass pasteur pipette, and stored in a separate tube on ice. The pellet was resuspended by homogenization in 2 ml ice-cold sucrose (0.25 M) and spun at 750 x g for 10 min at 4°C on the Mistral centrifuge. The resulting supernatant and the stored supernatant were then combined, diluted with 2 ml Tris-HCl buffer (pH 7.7, 50mM) and spun at 28,000 x g for 12 min at 4°C on a Beckman L8-70M preparative ultracentrifuge. The pellet was washed twice with 2 ml Tris-HCl, and resuspended by homogenization in 6 ml Tris-HCl. Homogenates were spun at 28,000 x g for 12 min at 4°C on the Beckman centrifuge. The final pellet was washed twice with 2 ml Tris-HCl and resuspended by homogenization in a volume of Tris-HCl equivalent to 12.5 mg original wet weight tissue/ml.

Saturation binding:

Saturation binding to cortical 5-HT2 receptors was defined according to the method of Leysen *et al* (1982). The radioligand used was [^{3}H]-ketanserin at 8 different concentrations over the range 0.05 - 6.0 nM. Non-specific binding was assessed by the displacement of total bound radioligand by a fixed concentration of an unlabelled competing ligand: 5 µM

methysergide (dissolved in 10% ethanol). This concentration of methysergide was chosen on the basis of a well validated method (Leysen et al., 1982; Cheetham et al., 1988).

50 µl [^{3}H]-ketanserin and 50 µl methysergide (non-specific binding) or 10% ethanol (total binding) were added to each tube. Assays were started by the addition of 400 µl membrane suspension to each tube. Tubes were incubated in duplicate at 37°C for 15 min; incubation time and temperature were chosen to allow specific binding to reach equilibrium (Leysen et al., 1982). Incubation was terminated by rapid filtration with a membrane harvester and rapid washing of the filters with 8 ml Tris-HCl (pH 7.7, 50 mM). Bound radioligand was measured by liquid scintillation counting and expressed in terms of the protein content.

Specific binding was calculated at each [^{3}H]-ketanserin concentration by subtracting non-specific binding from total binding (Figure 2.8). Analysis of specific binding was carried out using the iterative weighted least-squares curve-fitting program LIGAND (Munson & Rodbard, 1981), modified for use on an Apple IIe computer (Jackson & Edwards, unpublished). This program uses an exact mathematical model to fit data to 1 or more saturable binding sites. Data were fitted to a 1 site model, and K_d and B_{max} of the receptors calculated.

2.3.1.4 *Protein assay*

The protein content of aliquots of membrane preparations taken during binding assays were determined using Lowry's method (Lowry et al., 1951). Sodium hydroxide (0.5 M) was added to the membrane preparation, to dissolve the protein. Four protein standards containing known concentrations of bovine albumin were used to construct a standard calibration curve, with which the unknown protein samples could be compared.

200 µl sodium hydroxide (0.5 M; blanks) or 200 µl standard/sample in 0.5 M sodium hydroxide was mixed with 4 ml Reagent A (2% sodium carbonate, 0.02% sodium potassium tartrate, 0.01% copper sulphate) and incubated at 37°C for 15 min. 0.6 ml Reagent B (20% Folin & Ciocalteau's phenol reagent) was added to each tube; tubes were left to stand at room temperature for 30 min. The standards were assayed in duplicate, the samples in triplicate; tubes were read on a colorimeter through a 670 mµ Ilford 608 filter.

Figure 2.8 SATURATION BINDING OF [^{3}H]-KETANSERIN IN MOUSE CORTEX, DEFINED USING METHYSERGIDE

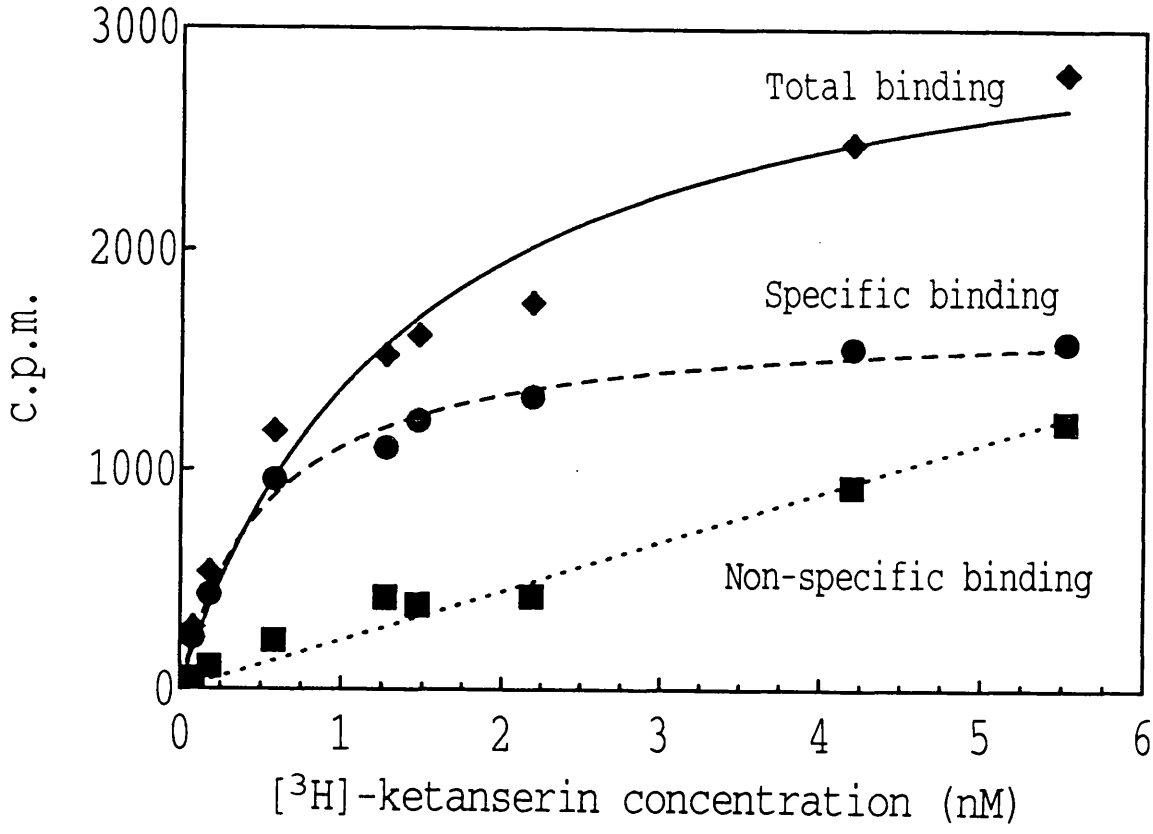


Figure 2.8 Saturation binding of [^{3}H]-ketanserin in mouse cortex. Total binding determined as radioligand binding in the absence of displacing ligand. Non-specific binding of [^{3}H]-ketanserin measured in the presence of 5 μM methysergide. Specific binding calculated as the difference between total and non-specific binding at a given radioligand concentration.

2.3.2 Measurement of monoamines and metabolites

High pressure liquid chromatography with electrochemical detection was used to determine levels of monoamine transmitters and their metabolites in the brain. Different methods of tissue preparation, stationary and mobile phases were used depending on the compounds being measured.

2.3.2.1 *Measurement of noradrenaline, 5-HT and metabolites*

Tissue preparation:

Once cerebral cortices had been dissected, they were frozen in liquid nitrogen and stored on dry ice. Tissue was prepared according to the method of Heal and coworkers (1989a). Cortices were homogenized in 5 volumes perchloric acid (0.1 M), containing 400 µM sodium metabisulphite (antioxidant) and 0.8 µM isoprenaline (internal standard). Homogenates were spun at 1,000 x g for 15 min at 4°C. The resulting supernatants were drawn off using a glass pasteur pipette and spun at 11,600 x g for 5 min at room temperature. 30 µl of the final supernatant was injected onto the column for measurement of noradrenaline, 5-HT and 5-HIAA content.

Stationary phase:

A 5 µ Hypersil ODS 1 reversed phase column, of 25 cm x 4.6 mm internal diameter was used. The main column was protected by a Brownlee Aquapore RP 300 precolumn of 3 cm x 4.6 mm internal diameter. The column was connected to a BAS LC-4A detector, with a glassy carbon electrode. The reference electrode was an Ag/AgCl electrode set at +750 mV.

Mobile phase:

The mobile phase used was a 0.1 M sodium dihydrogen orthophosphate - orthophosphoric acid buffer in 16% methanol, with 2.8 mM octanesulphonic acid and 0.7 mM EDTA added. The pH of the buffer was adjusted to 3.2. The mobile phase was pumped through the system at a flow rate of 0.85 ml/min.

Calculation of monoamine and metabolite content of samples:

The amounts of noradrenaline, 5-HT and 5-HIAA present in the samples were calculated by comparing the heights of the peaks recorded with those obtained from external standards of known concentrations. Recoveries from the column were assessed by comparison of peak heights for the internal standard with the height of the peak obtained from an external standard of the same concentration.

2.3.2.2 *Measurement of 3-methoxy-4-hydroxyphenylglycol (MHPG)*

Tissue preparation:

Once cerebral cortices had been dissected, they were frozen in liquid nitrogen and then stored on dry ice. Tissue was prepared according to the method of Heal and coworkers (1989a). Cortices were homogenized in 5 volumes perchloric acid (0.1 M) containing 400 µM sodium metabisulphite (antioxidant) and 100 µM iso-MHPG (internal standard). Homogenates were spun at 1,000 x g for 15 min at 4°C. The supernatants were drawn off using a glass pasteur pipette and spun at 11,600 x g for 5 min at room temperature. 1 ml of the resulting supernatant was drawn off and added to 5 ml ethyl acetate and vortexed for 1 min, then spun at 1,300 x g for 10 min at room temperature. The organic phase was drawn off using a glass pasteur pipette and mixed with 1 ml potassium bicarbonate (0.08 M). This mixture was vortexed for 1 min then spun at 1,300 x g for 10 min at 4°C. The final organic phase was pipetted off and evaporated to dryness using a Rotavapor. The residues were dissolved in 100 µl mobile phase; 50 µl of this solution was injected onto the column for measurement of MHPG content.

Stationary phase:

A 5 µ Spherisorb ODS 1 reversed phase column, of 25 cm x 4.6 mm internal diameter was used. The main column was protected by a Brownlee Aquapore RP 300 precolumn of 3 cm x 4.6 mm internal diameter. The column was connected to a BAS LC-4B detector, with a glassy carbon electrode. The reference electrode was an Ag/AgCl electrode set at +750 mV.

Mobile phase:

The mobile phase used was a 0.1 M sodium acetate - citric acid buffer in 8% methanol, with 4.6 mM octanesulphonic acid added. The buffer was adjusted to pH 4.4. The mobile phase was pumped through the system at a flow rate of 0.85 ml/min.

Calculation of MHPG content:

The amount of MHPG present in the samples was calculated by comparing the height of the peak recorded with the peak height for the internal standard. Peak height for the internal standard was compared with that for an external standard of MHPG of the same concentration as the internal standard, in order to determine the correction factor to be used if the ratio of peak height for the two compounds was not 1:1.

2.3.2.3 *Measurement of 5-hydroxytryptophan (5-HTP)*

Tissue preparation:

Thirty minutes before killing, animals received injections of 3-hydroxybenzylhydrazine (NSD 1015), a 5-HTP decarboxylase inhibitor (Carlsson 1964). After animals were killed, brains were removed and cortices dissected over ice, before freezing in liquid nitrogen. Cortices were then stored on dry ice until required; they were then homogenized in 5 volumes perchloric acid (0.1 M) containing 0.4 mM sodium metabisulphite (antioxidant). The homogenates were spun at 8,000g for 10 min at 4°C. The resulting supernatant was drawn off using a glass pasteur pipette and spun at 15,000g for 5 min at room temperature. 50 µl of the final supernatant was injected onto the column for determination of 5-HTP content.

Stationary phase:

A 5 µ Spherisorb ODS 1 reversed phase column, of 25 cm x 4.6 mm internal diameter was used. The main column was protected by a Brownlee Aquapore RP 300 precolumn, of 3 cm x 4.6 mm internal diameter. The column was connected to a BAS LC-4B detector, with a glassy carbon electrode. The reference electrode was an Ag/AgCl electrode set at +750 mV.

Mobile phase:

The mobile phase used was a 0.03 M sodium acetate - citric acid buffer in 4% methanol, with 0.01% dibutylamine added. The buffer was adjusted to pH 3.3. The mobile phase was pumped through the system at a flow rate of 0.85 ml/min.

Calculation of 5-HTP content:

The amount of 5-HTP present in the sample was calculated by comparing the height of the peak recorded with those obtained from an external standard of known concentration.

2.4 Materials

The following compounds and reagents were obtained from Sigma, Poole:
Trizma HCl, 5-methoxy-N,N-dimethyltryptamine, (-)-isoprenaline HCl, (-)-noradrenaline HCl, desipramine HCl, bovine albumin, anhydrous sodium carbonate, sodium potassium tartrate tetrahydrate, copper sulphate pentahydrate, l-octane sulphonic acid sodium salt, (-)-arterenol free base, 5-hydroxytryptamine HCl, 5-hydroxy-3-indoleacetic acid dicyclohexylammonium salt and NSD 1015.

The following compounds and reagents were obtained from BDH, Poole: Ethanol (AnalaR grade), sucrose, sodium hydroxide (AnalaR grade), Folin & Ciocalteau's reagent, perchloric acid (Aristar grade), sodium metabisulphite (AnalaR grade), sodium dihydrogen orthophosphate (AnalaR grade), orthophosphoric acid (AnalaR grade), ethylenediaminetetraacetic acid (EDTA: AnalaR grade), dibutylamine (HiPerSol grade), potassium bicarbonate (AnalaR grade), methanol (HiPerSol grade).

Additionally, [^{3}H]-dihydroalprenolol HCl (90-120 Ci/mmol), (-)-[^{3}H]-CGP 12177 (30-60 Ci/mmol) and ethylene-[^{3}H]-ketanserin HCl (60-90 Ci/mmol) were obtained from New England Nuclear, Stevenage. (±)-8-Hydroxy-2-(dipropylamino)tetralin hydrobromide, trifluoromethyl-piperazine HCl and CGS 12066B dimaleate salt were obtained from Research Biochemicals Inc., Natick, MA, U.S.A. Sodium acetate (HPLC grade) and citric acid (A.R. grade) were obtained from Fisons, Loughborough, and ethyl acetate (HPLC grade) from Rathburn Chemicals, Walkerburn. Iso-MHPG was synthesized by Boots Pharmaceuticals Research Department.

The following compounds were generous gifts from the manufacturers: ritanserin (from Janssen Pharmaceutica, Beerse, Belgium), sibutramine HCl (from Boots Pharmaceuticals Research Department, Nottingham), methsergide (Sandoz, Basle, Switzerland).

2.5 Statistics

Throughout this thesis, data are expressed as the mean ± standard error for each experimental group unless otherwise stated. In experiments which contained only two experimental groups, group means were compared using the Mann-Whitney `U' test. Where Hill coefficients were calculated, values for each group were compared with unity using the Wilcoxon signed-rank test. These non-parametric tests avoided making the assumption normally associated with Student's t test; namely, that data are normally distributed. All other experiments used analysis of variance (ANOVA) to compare all experimental groups simultaneously. When ANOVA revealed a significant F ratio, individual groups were compared using Student's unpaired t test. In all tests, $p < 0.05$ was taken as indicating significance.

3.0 BEHAVIOUR IN AND CORTICAL β-ADRENOCEPTORS AFTER EXPOSURE TO THE OPEN FIELD IN RATS; INFLUENCE OF PREVIOUS EXPERIENCE OF REPEATED STRESS (ONCE-DAILY SALINE INJECTION) OR REPEATED SIBUTRAMINE ADMINISTRATION.

3.1. Introduction

Both a single and repeated exposure to stress induces neurochemical changes in central noradrenergic neurones (reviewed by Anisman & Zacharko, 1991). Several theories have been developed to explain how these changes may be involved in behavioural adaptation to stress. One such theory, which focuses on changes in β-adrenoceptors, has suggested that reduced cortical β-adrenoceptor density and/or function (β-adrenoceptor-stimulated adenylate cyclase activity) underlies behavioural adaptation to stress (Stone, 1979b). However, results from this laboratory have given cause to question the general validity of this theory (Salmon & Stanford, 1989; Stanford & Salmon, 1989). The present experiment has investigated this problem further by looking at the effects of treatments aimed at altering either the behavioural response to stress or cortical β-adrenoceptor density on the behavioural response to an acute stress. If cortical β-adrenoceptors and resistance to stress are related in the way suggested by Stone, then increased resistance to stress should be paralleled by a decrease in cortical β-adrenoceptor density. Conversely, treatments which reduce β-adrenoceptor density would be predicted to increase behavioural resistance to stress.

3.1.1 β-adrenoceptors and behavioural resistance to stress

The theory that β-adrenoceptor down-regulation underlies behavioural adaptation to stress (Stone, 1979b) is based on experiments showing parallel changes in receptors and behaviour in groups of animals exposed to repeated stress. Recently it has been argued that, if there is a causal link between changes in β-adrenoceptors and behavioural resistance to stress, then the relationship between β-adrenoceptors and behavioural adaptation should also apply *within* groups of animals (Salmon & Stanford, 1989, 1992: Stanford & Salmon, 1989). That is, within any group of rats, those with the lowest density of β-adrenoceptors should show the greatest behavioural resistance to stress. In addition, since Stone's hypothesis is based on studies of the effects of noxious stress such as immobilization or foot shock, it has been suggested that this theory may not apply to stressful procedures which may be more appropriate models for the forms of stress commonly experienced by humans e.g. 'loss' (Brown & Harris, 1989).

In experiments designed to investigate these points, both β-adrenoceptor density and

behavioural responses to stress were studied in the same animals using the 'frustrative nonreward' paradigm. This mimics a form of stress commonly encountered by humans: namely, 'loss' (Brown & Harris, 1989). In this paradigm, animals are first trained to run down a runway for a food reward ('acquisition'). Once stable running times are achieved, the reward is withdrawn; as a result, animals progressively stop running ('extinction'). Detailed investigations of 'frustrative nonreward' have led to the inference that animals which extinguish slowly (i.e. continue running) are those which are most resistant to the stress of nonreward (Gray, 1971, 1982). It was therefore predicted, on the basis of Stone's hypothesis, that individuals with the lowest density of β-adrenoceptors would be those which showed greatest resistance to nonreward i.e. slow extinction rate. However, when once-daily extinction trials were given, the opposite result was obtained: greatest resistance to stress was linked with *greatest* numbers of β-adrenoceptors (Stanford & Salmon 1989).

There are three possible explanations for the discrepancy between the results predicted from Stone's hypothesis and those obtained using nonreward. First, it is possible that Stone's hypothesis applies only to noxious forms of stress. Secondly, Stone examined the effects of repeated stress, whereas in the non-reward experiments, animals did not have previous experience of stress. It is possible therefore that the neurochemical coding of behavioural resistance to stress may be altered by previous experience of repeated stress. Finally, the relationship between β-adrenoceptors and behavioural resistance to stress seen for group mean differences may not apply when individuals are considered (Salmon & Stanford, 1992).

These possibilities were addressed in further experiments which examined animals' responses to novelty. The aim of this study was to test whether the relationship between β-adrenoceptors and behavioural resistance to stress seen for 'frustrative nonreward' applied generally to other forms of stress. Exposure to a novel environment ('open field') was chosen since this is another non-noxious form of stress during which behavioural responses can be measured, with the advantage that no training is required. In the open field, increased activity in the centre was taken to reflect resistance to stress (Gentsch et al., 1987; see section 3.1.2). However, as for nonreward, there was again a positive correlation between β-adrenoceptor density and behavioural resistance to stress (Salmon & Stanford, 1989).

In the course of this work, a complication became apparent: it appeared that exposure

to the open field itself may have affected β-adrenoceptor density. However, this could not be confirmed as the relevant controls, namely animals which had not been exposed to the open field, had not been included in this experiment. The first objective of the current experiment was to ascertain whether rapid changes in β-adrenoceptors were induced by exposure to the open field, therefore. This was carried out by comparing cortical β-adrenoceptor density in animals exposed to the open field with that in unstressed animals.

A further objective of the current experiment was to examine whether the relationship between β-adrenoceptors and resistance to stress suggested by Stone's hypothesis was affected by treatments designed to increase stress resistance. Previous experience of repeated stress has been shown to alter behavioural responses to a range of different forms of acute stress (e.g. Platt & Stone, 1982; Kennett et al., 1986; Hata et al., 1988). Such studies have commonly examined the effects of repeated exposure to noxious stress such as immobilization. However, even repeated once-daily saline injection has been shown to alter behaviour in the open field (Salmon & Stanford, 1989). The current experiment examined whether repeated injection-induced changes in resistance to stress were paralleled by changes in β-adrenoceptor density, therefore.

Conversely, a further objective of the current experiment was to examine whether changes in β-adrenoceptor density would be paralleled by changes in resistance to stress. Repeated antidepressant administration decreases cortical β-adrenoceptors (e.g. Sellinger-Barnette et al., 1980; Sugrue, 1983; see section 1.8). Here, the relationship between behavioural resistance to stress and β-adrenoceptors was determined in animals after repeated sibutramine hydrochloride (sibutramine) administration. This noradrenaline and 5-HT reuptake inhibitor has antidepressant-like activity in preclinical tests predictive of antidepressant effects in man and causes a rapid and pronounced reduction in cortical β-adrenoceptors in rats (Buckett et al., 1988; Heal et al., 1989b).

3.1.2 The open field

The open field test was first used by Hall almost 60 years ago to examine differences in 'emotionality' (Hall, 1934) between individual rats. In this study, two physiological responses were used as indices of animals' emotional status: defecation and urination. Hall also realised that exposure to the open field was itself stressful, noting "how emotionally upsetting the field situation can be for the rats" (Hall, 1934). In a later study, Hall also measured locomotor activity in this apparatus: he found that animals with the

highest levels of locomotor activity were those which defecated the least. It was inferred that the least emotional rats were those which moved around the most (Hall, 1936).

Behaviours in the open field are still widely interpreted in terms of emotionality (e.g. Delini-Stula et al., 1984; Bruhwyler, 1990). However, exposure to a novel open field is also used as a form of stress (e.g. Gentsch et al., 1987; Gomez et al., 1989) and increases the levels of plasma corticosterone and catecholamines (Hennessy & Levine, 1978; Gentsch et al., 1987; De Boer et al., 1990); these hormonal changes are commonly used as criteria for a stress response. Behaviour displayed in the open field may therefore be interpreted in terms of resistance to stress.

The validity of defecation as an index of an individuals' response to stress in a novel open field rests on three factors. First, for individual animals, the frequency of defecation in the open field is remarkably consistent across repeated trials (Hall, 1934). Secondly, since it is the stress of novelty that supposedly induces defecation, this response should habituate on repeated exposure to the field. In a series of trials carried out over a period of two weeks, Hall (1934) showed that defecation in the open field declined with successive exposures; this has subsequently been confirmed in independent studies (Paré, 1964; Ivinskis, 1970). Finally, Ivinskis (1970) showed that the frequency of defecation could be manipulated by varying the 'stimulus intensity' (i.e. lighting and background noise). Since the number of boli was increased with both noise and light intensity, it was inferred that a greater intensity of stress would elicit a greater emotional response.

In the same way, for locomotion to be validated as an index of emotionality or stress resistance in the open field, it must be shown to be affected by experimental variables aimed at changing stress intensity. Since locomotion correlates negatively with defecation (Hall, 1936; Ader et al., 1967; Satinder, 1968), then locomotion should decrease with increased stress intensity. This prediction has been confirmed by a number of studies: ambulation is generally decreased as light and/or noise intensity are increased (Livesey & Egger, 1970; Valle, 1970), although this is not invariably the case (Broadhurst, 1957). The negative correlation between defecation and locomotion is consistent with evidence from studies on Maudsley Reactive and Nonreactive inbred rat strains: the Maudsley Reactive strain, inbred for high defecation in the open field also shows less locomotor activity in this apparatus than its Maudsley Nonreactive counterpart, which shows low levels of defecation in the open field (e.g. Abel, 1991; reviewed by Broadhurst, 1975).

Although the evidence cited so far is consistent with a link between locomotion and resistance to stress in the open field, it is based purely on studies of experimentally naive animals. The link between increased locomotion and increased stress resistance would be strengthened if validated pharmacologically: drugs which alter the response or resistance to stress should also alter locomotion in the open field. However, the results of studies examining the effects of drugs on locomotion in the open field are inconsistent. For instance, although the anxiolytic benzodiazepine chlordiazepoxide (Crawley, 1981; Pellow et al., 1985) increases locomotion and reduces defecation in the open field (Bruhwyler, 1990; Bruhwyler et al., 1990), non-sedative doses of other anxiolytic benzodiazepines decrease or have no effect on locomotion in this apparatus (Cooper, 1985; Hata et al., 1988). Moreover, the benzodiazepine inverse agonist, N-methyl-β-carboline-3-carboxamide (FG 7142) has anxiogenic effects in man and in animal models of anxiety (Dorow et al., 1983; Pellow & File, 1986) yet increases locomotor activity in the open field (Bruhwyler et al., 1991).

A further complication is that novelty-induced behaviour is determined by the balance between the aversive nature of the novel environment and exploratory urge evoked by novelty (Montgomery, 1955). Findings that locomotion *decreases* over repeated open field trials (Paré, 1964; Denenberg, 1969; Ivinskis, 1970) illustrate the difficulty in distinguishing whether reduced locomotor activity reflects sensitization (or conditioned aversion) to the environment, or reduced exploratory drive resulting from habituation to novelty. Overall, the number of factors influencing locomotor activity in the open field and lack of consistent effect of benzodiazepines make this behaviour an unreliable index of stress resistance.

To overcome this problem, recent work has examined locomotor activity in different zones of the open field arena (e.g. Lee et al., 1986; Gentsch et al., 1987; Gilad & Shiller, 1989). This idea derives from experiments suggesting that the centre of the field is more aversive than the periphery (Ader & Belfer, 1962; Ader & Conklin, 1963; Archer, 1973). Again, there is wide variation in the protocols used in different laboratories. Different parameters measured include: locomotor activity in the central area of the field (Valle, 1970; Lee et al., 1986), the number of entries into the central area (Delini-Stula et al., 1984, Gentsch et al., 1987) and the time spent in the centre of the arena (McClearn & Meredith, 1964; Valle, 1970). Although use of these different parameters creates problems when comparing results from different studies, the most consistent interpretation of published findings is that, during a single trial, increased centre-field activity reflects increased

resistance to stress. Evidence to support this interpretation comes from several lines of investigation. For instance, over a single trial, entries into the centre of the field increase with time, presumably reflecting habituation to the stressful environment (Archer, 1973). Furthermore, a non-sedative dose of the benzodiazepine, chlordiazepoxide, which has anxiolytic effects in several animal models (Crawley, 1981; Pellow et al., 1985), increases centre-field activity (Bruhwyler, 1990; Bruhwyler et al., 1990). In view of these reports, locomotor activity in the centre of the field was used as an index of resistance to stress in the present experiments. The time spent in the centre of the field, as well as the number of crossings of the concentric circles (radial movements) were measured. As a further test, radial movements directed towards the centre of the field ('centre-directed activity') were distinguished from those directed away from the centre.

Although defecation and locomotion are the most widely studied behaviours in the open field, they are not the only behaviours displayed in this apparatus. Other prominent behaviours which can easily be scored are: latency of movement (i.e. the duration of the initial 'freezing' reaction when animals are first placed in the apparatus), rearing and grooming. Like locomotion and defecation, there is evidence that these behaviours could be related to resistance to stress.

Latency has been studied previously but again there is considerable variation in experimental protocol. This behaviour has been variously measured as: the latency to the first sign of movement (Katz & Sibel, 1982), the time to move from the square in which the animal is initially placed (Paré, 1964; Ader, 1965) and the time to enter the field from a start box attached to the side of the field (Gilad & Shiller, 1989). Since the centre of the field is regarded as the most aversive zone (Ader & Belfer, 1962; Ader & Conklin, 1963; Archer, 1973), the time taken for animals to leave the centre or to reach the outer perimeter of the field (Ivinskis, 1968, 1970) may also be considered as an index of the impact of exposure to the novel open field. However, exposure to a novel situation can induce 'freezing' (Bindra & Spinner, 1958; Woods, 1962) or an initial escape response (Livesey & Egger, 1970). Since latency presumably reflects a balance between freezing and escape behaviour, it is not known how it relates to stress resistance in the open field. To investigate this problem further, the latency for animals to reach the outermost zone of the open field, after placement in the centre, was measured in the current experiment.

Rearing is commonly used as an index of exploratory behaviour in the open field (e.g. Ivinskis, 1970; discussed by Archer, 1973; Kennett et al., 1985; Bruhwyler et al., 1991).

However, this interpretation has not been experimentally validated. Previous studies of exploratory behaviour induced by exposure to a novel situation have examined behaviours such as exploration of a novel maze or locomotion in the open field (Montgomery, 1955; Broadhurst, 1957; Lester, 1968). The conclusions of such studies may therefore not be applicable to novelty-induced rearing. However, rearing increases during a brief exposure to the open field (Archer, 1973). This suggests that rearing in the open field could be related to stress resistance. Another line of investigation of links between rearing in the open field and resistance to stress would be to compare open field behaviour in the Maudsley Reactive and Nonreactive rats. Originally derived from a single strain of rats, the Maudsley Reactive and Nonreactive strains were specifically inbred for low and high levels of defecation in the open field (reviewed by Broadhurst, 1975). Yet studies of these strains still only examine locomotion and defecation. Studies of strain differences in rearing have only used completely separate rat strains e.g Brown-Norway compared with Wistar-Kyoto rats (Gilad & Shiller, 1989) which have been adjudged stress-resistant and stress-reactive on the basis of differences in centre-field activity. However, there is evidence to suggest that neither neurochemical nor behavioural changes induced by stress in one rodent strain can be used to predict behaviour in another strain (e.g. Shanks & Anisman, 1988; Shanks et al, 1991). Comparisons of behaviour in two genetically completely separate strains of rat are therefore of limited validity. A further objective of the current experiment was to determine whether rearing was increased in animals which displayed greater behavioural resistance to stress (i.e. increased centre-field activity).

It has long been known that exposure to a novel environment such as the open field induces grooming activity (Hughes, 1968; Satinder, 1968; Jolles et al., 1979). It is generally assumed that in such circumstances grooming reflects animals' emotionality. This is supported by the positive correlation between grooming and defecation (O'Kelley, 1940; Gray et al., 1965), and findings that non-sedative doses of anxiolytic drugs reduce grooming (Hata et al., 1988). However, other evidence conflicts with this interpretation. For instance, grooming increases progressively during a single open field test (Bolles, 1960; Hughes, 1968). This could indicate that grooming parallels habituation and, therefore, resistance to stress.

Another suggested interpretation of stress-induced changes in grooming is that it represents a 'displacement activity'. These are behaviours seen in a variety of species which seem inappropriate or irrelevant to the situation in which the behaviour is evoked

(Kortland, 1940; Tinbergen, 1946). They include feeding during fighting in cockerels (Kortland, 1940) and footshock-induced fighting in pairs of male rats (Conner et al., 1971; Weinberg et al., 1980). Grooming induced by exposure to a variety of mildly stressful situations e.g. nonreward (Kortland, 1940) and handling (including a saline injection: Green et al., 1979), is thought to be one such displacement activity (e.g. Jolles et al., 1979). The role of displacement activities in stress is as yet unclear but it has been speculated that they are an important 'coping' mechanism (Dantzer, 1989). This interpretation is supported by recent evidence that stress-induced corticosterone secretion is reduced in animals which are allowed to display such activities (Dantzer et al., 1988). A final objective of the present experiments was to test whether measures of behavioural resistance to the stress of exposure to the novel open field is related to increased grooming activity.

3.2 Aims

1) To examine the effects of a brief exposure to non-noxious stress (novel environment) on cortical β-adrenoceptors in the rat.
2) To examine the influence of previous experience of repeated stress (once-daily saline injection) on cortical β-adrenoceptors and on behaviour in the open field.
3) To examine the effects of a treatment aimed at reducing cortical β-adrenoceptor density (once-daily administration of sibutramine hydrochloride) on behaviour in the open field.
4) To establish the relationship (statistical correlation) between individuals' behaviour in the open field and the density of cortical β-adrenoceptors.
5) To determine the relationship between centre-directed movements, a measure of behavioural resistance to stress in the open field and other behaviours displayed in this apparatus.

3.3 Methods

3.3.1 Animals and treatments

Male Sprague-Dawley rats (250-300 g on arrival in the animal house) were housed under a 12/12 h light/dark cycle (lights on from 08.00-20.00 h), with free access to water and food. Animals were divided into three groups of 26, destined for either stress (saline injection) or drug (sibutramine) pretreatment, or no pretreatment ('unhandled'); groups were matched for mean weight. The animals were distributed in cages containing 3 or 4 rats; each cage contained animals from one treatment group only. All animals remained unhandled (apart from routine husbandry) for 9 days to allow acclimatization to the new

surroundings. On the tenth day after arrival in the animal house, animals from the stress and drug pretreatment groups were weighed and their tails marked using a waterproof marker. This was to enable each animal to be identified individually. They then received an injection of either 0.9% sterile saline (2 ml/kg i.p.) or sibutramine hydrochloride (3 mg/kg i.p.). This procedure, carried out between 09.30-11.00 h, was repeated once-daily for a total of ten days; tail markings were renewed when they showed signs of fading. The unhandled group remained undisturbed in the home cage throughout this period.

24 h after the final injection, 18 animals from each group were selected at random and placed individually in the open field. The remainder (at least one from each cage) were killed immediately on removal from the home cage. The order of removal of animals from the cage was randomized; that is, the first animal removed from the cage was not always destined for placement in the open field *etc.*

3.3.2 Open field

Rats exposed to the open field were placed individually in the centre of the circular arena (Salmon & Stanford, 1989; see section 2.2.1) immediately after removal from their home cage. Their behaviour was scored (see below) for 4 min, after which the animals were removed and killed immediately. The floor of the field was cleaned and wiped after each individual trial.

3.3.3 Behavioural scoring

Behaviour of rats in the open field was recorded on tape by a video camera mounted directly above the apparatus. After testing, individuals' activities were scored for the following behaviours (see figure 3.1):

(i) Arc movements - the crossing of radially drawn lines

(ii) Radial movements - the crossing of concentric lines. These were further divided into centre-directed movements and movements directed toward the outside of the field

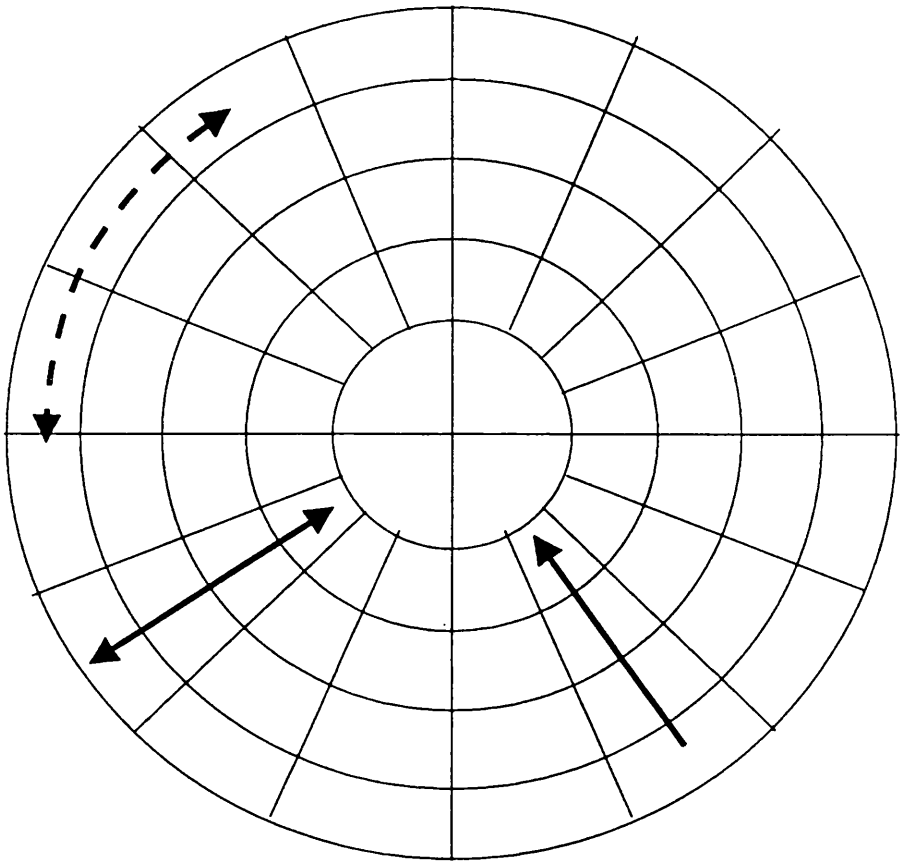
(iii) Latency - the time (in seconds) taken for the rat to cross into the outermost circle following placement in the centre of the field

(iv) Time in the centre of the field - the total time (in seconds) spent by the rat in the inner three circles

(v) Rearing (both forepaws off the ground)

(vi) Grooming - time (in seconds) spent grooming
- number of grooming episodes.

FIGURE 3.1 OPEN FIELD FLOOR PLAN



Radial movements

Centre-directed movements

Arc movements

'Central zone' : area within the inner 4 circles
'Latency' : time to cross out of the central zone after placement in the centre

(vii) Defecation - number of boli dropped

The criterion for line crossing was that the rat crossed the line with all four paws.

There is evidence to suggest that both arc and radial movements in the first minute of the trial may be different from those in subsequent stages of the test (Salmon & Stanford, 1989). For this reason, the following aspects of these movements were considered separately:

1) Movements during the first minute ('initial period') of the test
2) Movements during the remaining 3 min ('final period') of the test
3) Movements during the entire duration of the test ('total')

'Locomotor activity' was calculated as the sum of radial and arc movements. The ratios of radial movements : locomotor activity and centre-directed movements : locomotor activity were also calculated. This was done for both the initial and final periods of the test, as well as for the total test.

3.3.4 Cortical β-adrenoceptor binding

Immediately after removal from the home cage or from the open field, animals were killed in an adjacent room by cardiothoracic shock and cervical dislocation. Brains were removed and cerebral cortices dissected over ice and then frozen on dry ice. These were stored at -20°C.

Washed membranes were prepared freshly for each binding assay, as previously described (see section 2.3.1.1). [^{3}H]-Dihydroalprenolol ([^{3}H]-DHA) was the radioligand used to measure cortical β-adrenoceptor binding; specific binding of the radioligand was defined using 200μM (±)-isoprenaline sulphate (see section 2.3.1.3).

3.3.5 Statistics

F tests were used to assess whether β-adrenoceptor binding data were best fit by a 1- or a 2-site model. Differences in mean behavioural scores and β-adrenoceptor binding of the treatment groups were assessed using analysis of variance (ANOVA). Student's t-tests were carried out where the ANOVA revealed a significant treatment effect.

The statistical correlations between β-adrenoceptor binding dissociation rate constant (K_d) or receptor binding density (B_{MAX}) and individual behavioural measures were evaluated using the MINITAB computer package to calculate the correlation coefficients.

3.4 Results

3.4.1 Behaviour in the open field

3.4.1.1 *Arc movements*

Total arc movements (i.e. all movements involving the crossing of radially drawn lines) carried out by the rats during a 4 min exposure to the open field are shown in Figure 3.2. Since there may be a dissociation between locomotion in the first and the final 3 min of the trial (Salmon & Stanford, 1989), arc movements for these two periods of the test were also recorded separately.

In the saline-pretreated group, total arc movements did not differ from those of the uninjected (control) group ($F = 0.724$; d.f. = 2, 51; $p > 0.05$; Figure 3.2.). When arc movements during the first or the final 3 min of the test were considered independently, there were still no differences between the saline-injected animals and the controls. Arc movements in sibutramine-pretreated animals were also the same as in the control group. This was the case whether total arc movements, or movements carried out in the first or final 3 min of the test were considered.

3.4.1.2 *Radial movements*

Radial movements were defined as crossings of the concentric circles drawn on the floor of the field, i.e. movements to and from the centre of the field. However, one feature of this behaviour is particularly relevant as an index of stress resistance: movements directed towards the centre of the field. Such movements ('centre-directed movements') were therefore counted separately. For all treatment groups, radial movements and centre-directed activity were scored for the initial and final periods of the test, as well as over the total test. The data are shown in Figure 3.3 and 3.4.

Total centre-directed activity in the saline-pretreated group was significantly increased compared with controls (Figure 3.3: $F = 3.471$; d.f. = 2, 51; $p < 0.05$). However, when movements carried out in the first and in the final 3 min of the test were considered separately, it was evident that this increase was not apparent throughout the entire trial. There was a significant stress-induced increase in centre-directed activity over the last 3 min of the trial, ($F = 3.584$; d.f. = 2, 51; $p < 0.05$), but there was no difference in the first minute.

Centre-directed movements in the sibutramine-pretreated group displayed a similar pattern to that seen in the saline group (Figure 3.3). Total centre-directed movements

EFFECT OF SALINE INJECTION OR SIBUTRAMINE PRETREATMENT ON ACTIVITY IN THE OPEN FIELD

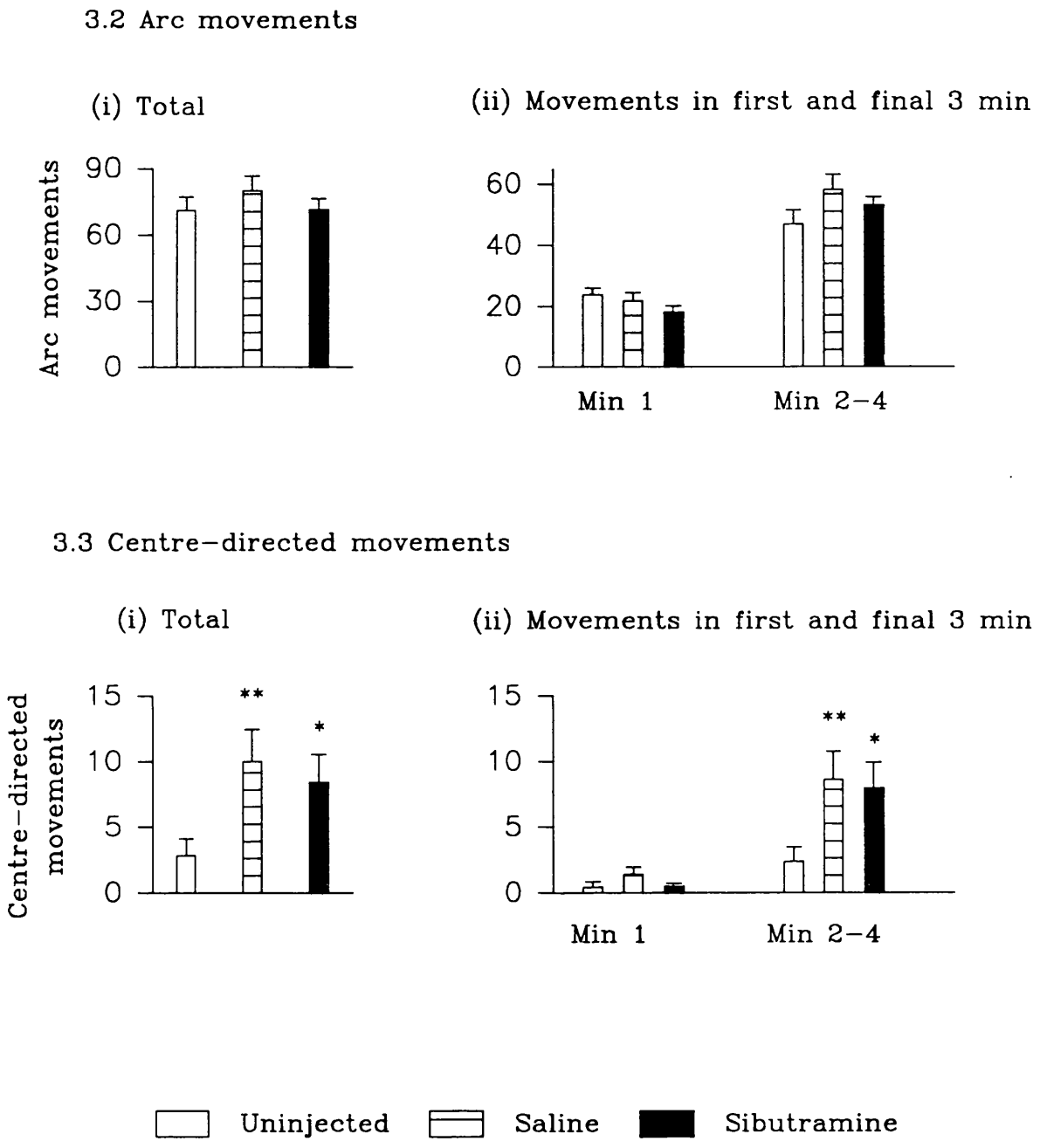


Figure 3.2, 3.3: Arc movements and centre-directed movements during a 4 min open field test and for the first or final 3 min of the test scored in uninjected animals and in repeated saline- and sibutramine-injected animals, 24h after the final injection. n = 18, each group. Data from all treatment groups compared using 1-way ANOVA; following a significant treatment effect, group means were compared using students unpaired t-test: $^{*}p < 0.05$, $^{**}p < 0.01$ *cf* uninjected controls.

EFFECT OF SALINE INJECTION OR SIBUTRAMINE PRETREATMENT ON ACTIVITY IN THE OPEN FIELD

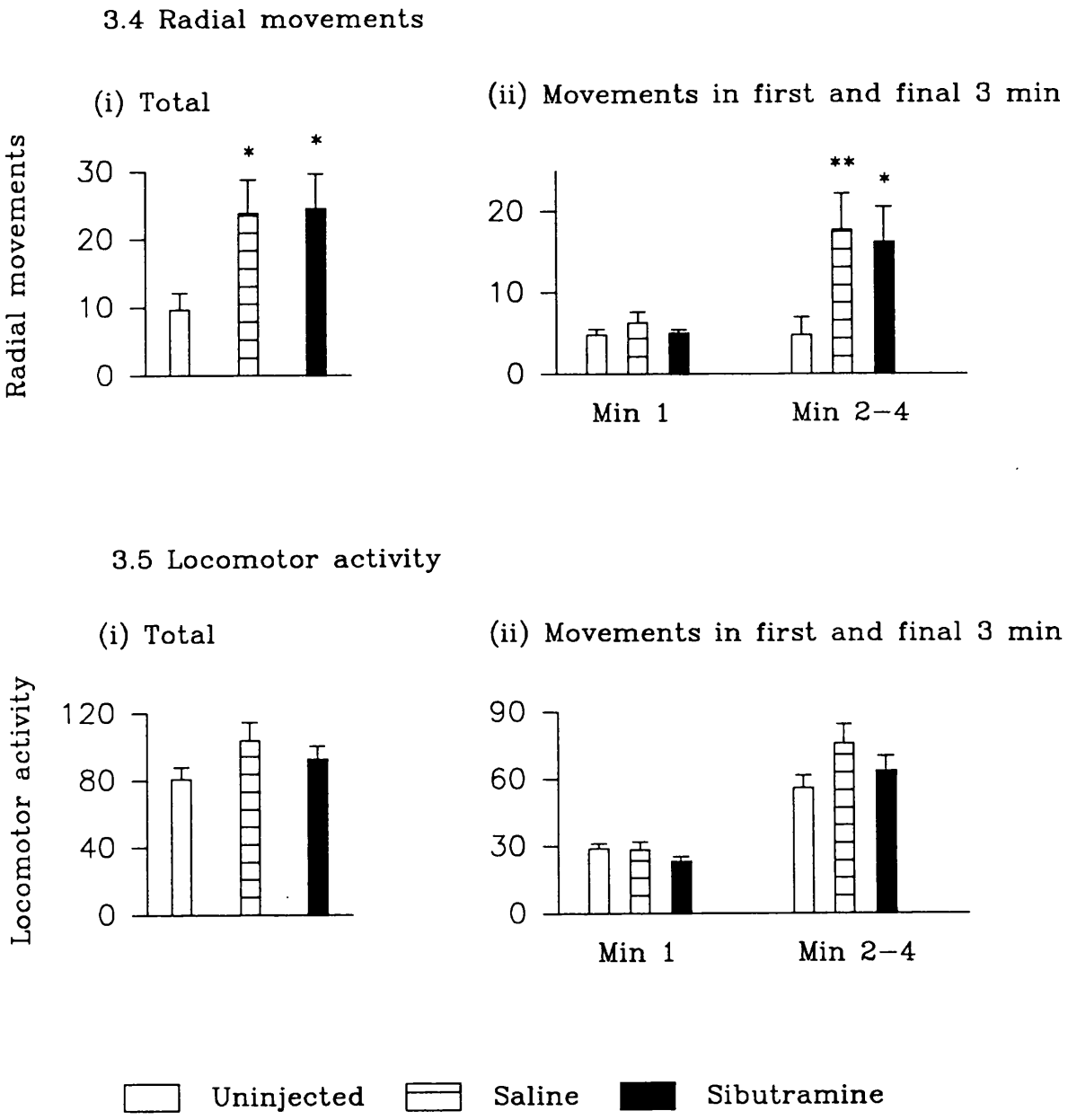


Figure 3.4, 3.5: Radial movements and locomotor activity during a 4 min open field test and for the first or final 3 min of the test scored in uninjected animals and in repeated saline- and sibutramine-injected animals, 24h after the final injection. n = 18, each group. Data from all treatment groups compared using 1-way ANOVA; following a significant treatment effect, group means were compared using students unpaired t-test: $^{*}p < 0.05$, $^{**}p < 0.01$ *cf* uninjected controls.

were increased in drug-pretreated animals. The magnitude of the increase was similar to that seen in saline-pretreated animals ($F = 3.471$; d.f. = 2, 51; $p < 0.05$). Again, movements in the drug-pretreated group were increased in the final 3 min of the test ($F = 3.584$; d.f. = 2, 51; $p < 0.05$), although there was no difference in the first minute.

When radial movements were considered (ie crossing of concentric lines both towards and away from the centre of the field) were considered, a similar pattern of findings was seen as for centre-directed movements (Figure 3.4). Total radial movements were increased to a similar extent in both saline- and sibutramine-pretreated groups ($F = 3.706$; d.f = 2,51; $p < 0.05$; ts = 2.298, 2.411, respectively; $p < 0.05$), compared with controls. Both pretreatments increased radial movements in the final 3 min ($F = 3.611$; d.f. = 2, 51; $p < 0.05$; ts = 2.448, 2.163; $p < 0.01$, 0.05 respectively) but not in the first minute of the test.

3.4.1.3 *Locomotor activity*

Locomotor activity (the sum of arc + radial movements) was recorded for the entire test duration, as well as in the first and the final 3 min of the trial; the data are displayed in Figure 3.5.

Locomotor activity over the 4 min test was not altered by either saline or sibutramine pretreatment, compared with uninjected animals. There was also no difference between any of the groups when locomotion in the first and final 3 min of the test were considered separately.

3.4.1.4 *Ratio of centre-directed or radial movements : locomotor activity*

In order to examine whether any changes in radial or centre-directed movements could be explained by changes in locomotor activity, both centre-directed activity and radial movements were expressed as a proportion of total locomotor activity. These ratios were calculated for the entire test, as well as for the first and the final 3 min in the open field. Data are displayed in Figure 3.6 (centre-directed movements : locomotor activity) and Figure 3.7 (radial : locomotor activity).

The proportion of centre-directed movements in the entire 4 min of the test was significantly increased to a similar extent in both the saline- and sibutramine-pretreated groups, compared with uninjected controls ($F = 3.405$; d.f = 2, 51; $p < 0.05$; ts = 2.324, 2.182 respectively; $p < 0.05$). When the first and the final 3 min of the test were examined separately, the effects of stress and sibutramine pretreatment were only apparent in the

EFFECT OF SALINE INJECTION OR SIBUTRAMINE PRETREATMENT ON ACTIVITY IN THE OPEN FIELD

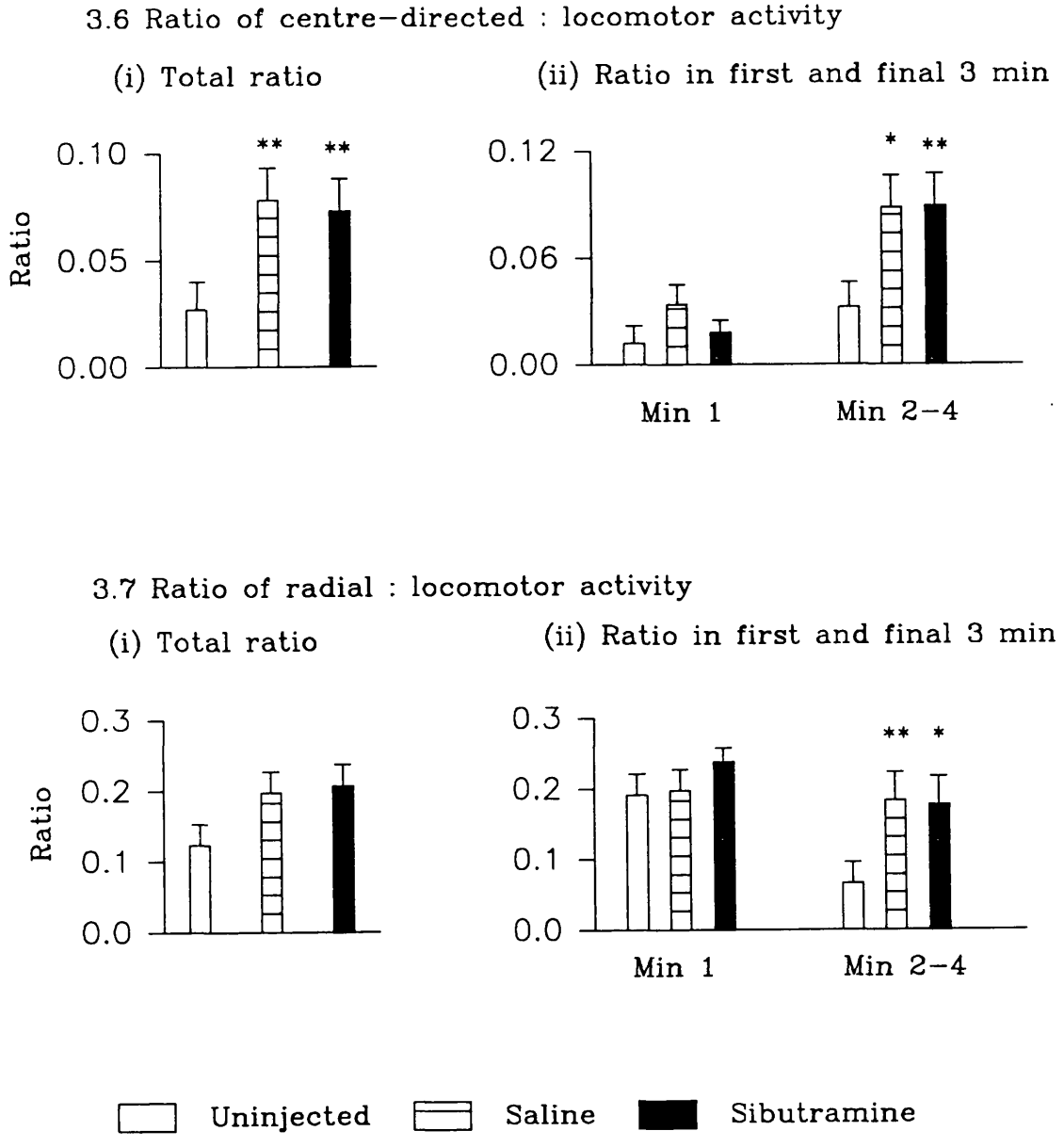


Figure 3.6, 3.7: The ratio of centre-directed movements : locomotor activity and the ratio of radial movements : locomotor activity were calculated for both the entire 4 min and the final 3 min of the open field test. n = 18, each group. Data from all treatment groups compared using 1-way ANOVA; following a significant treatment effect, group means were compared using students unpaired t-test: $^{*}p < 0.05$, $^{**}p < 0.01$ *cf* uninjected controls.

latter period when the proportion of centre-directed movements was increased in both groups ($F = 3.900$; d.f. = 2, 51; $p < 0.05$; ts = 2.376, 2.418; $p < 0.05$, 0.01 respectively). No difference in the proportion of centre-directed : total locomotion was seen in the first minute of the test in either of the groups.

When the ratio of radial movements : total locomotion was analyzed, the ratio for the whole 4 min test was increased by both saline and sibutramine pretreatment, although this effect just failed to reach significance (Figure 3.7: $F = 2.702$; d.f. = 2, 51; $p < 0.1$). Although the ratio of radial : total locomotion in the first minute of the test was the same in all groups, it was significantly increased by both saline and sibutramine pretreatment in the final 3 min ($F = 3.785$; d.f. = 2, 51; $p < 0.05$; ts = 2.443, 2.318; $p < 0.01$, 0.05 respectively). However, this change was due to an apparent reduction in the ratio in the unhandled controls, rather than a pretreatment-induced increase in the proportion of radial movements.

In summary, centre-directed activity is increased by both saline and sibutramine pretreatment. However, neither pretreatment altered total locomotor activity. Both pretreatments specifically increase movement directed towards the centre of the field, rather than inducing a non-selective increase in general levels of activity, therefore. This selective effect of saline injection and sibutramine pretreatment is confirmed by the finding that both pretreatments also increase the proportion of centre-directed movements. However, the effects of these pretreatments are only seen in the final 3 min of the test; no aspect of locomotor activity seen in the first minute of the test was altered in either treatment group.

3.4.1.5 *Latency and time spent in the centre of the field*

The latency (time taken for the animal to enter the outermost circle following placement in the centre of the field) was used as an index of the initial impact of the stress of exposure to the open field. 96% of animals left the centre of the field within the first minute of the test; the mean latency in seconds for each group is shown in Figure 3.8. The latency to cross the outermost circle of the arena was not altered by repeated saline pretreatment, compared with controls. Repeated sibutramine pretreatment also had no effect on this measure.

The time spent in the centre of the field (latency + duration of any additional entries into the centre of the arena) was measured; the mean group scores for this measure are

EFFECT OF REPEATED SALINE INJECTION OR SIBUTRAMINE PRETREATMENT ON OPEN FIELD BEHAVIOUR

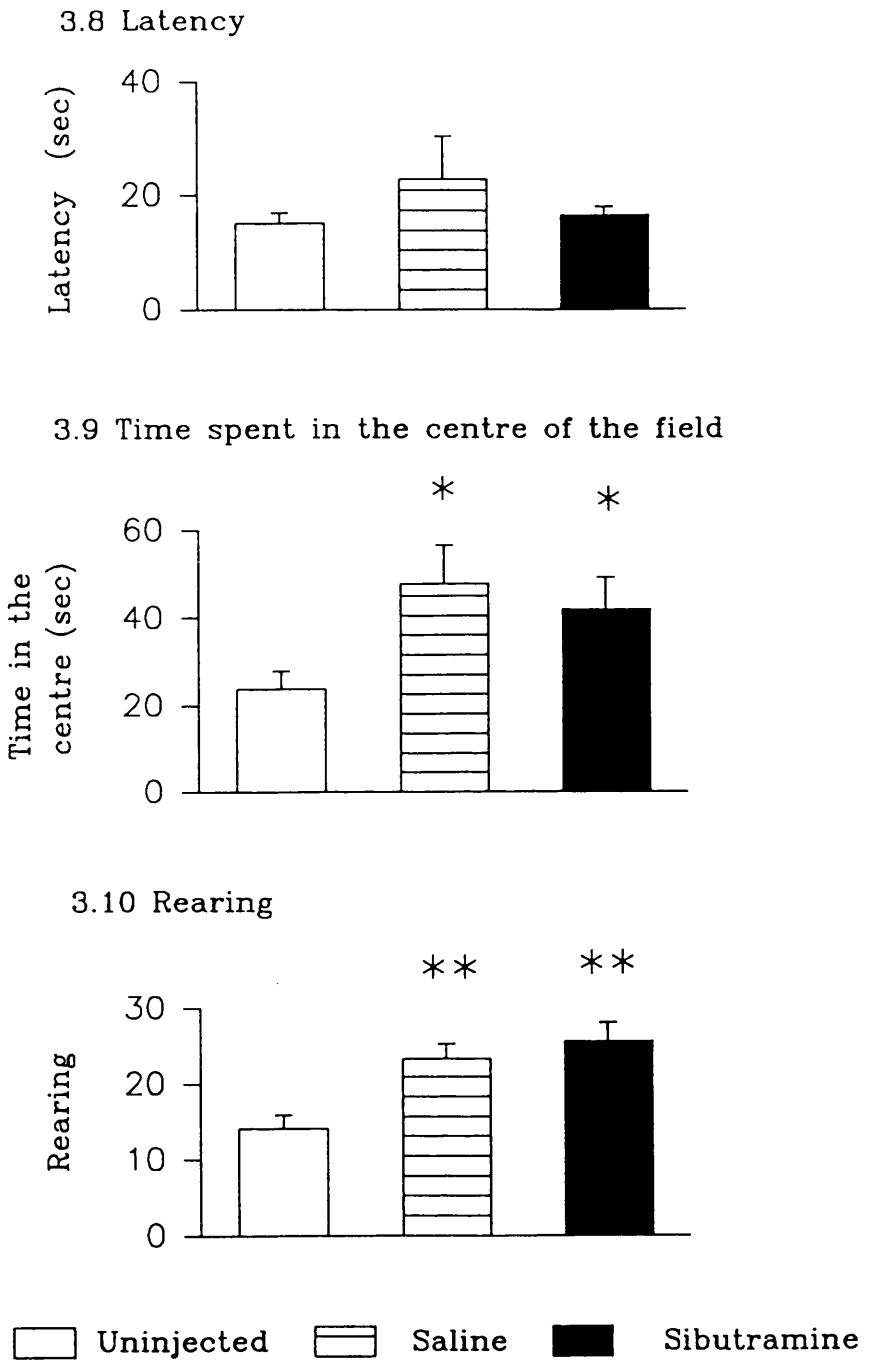
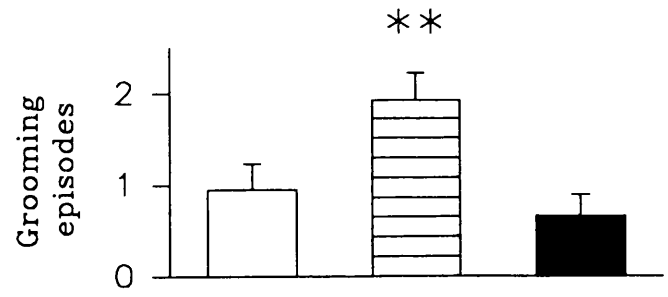


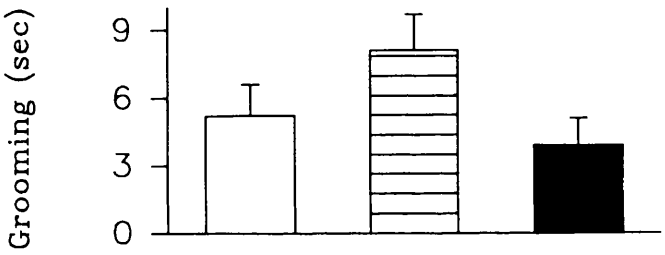
Figure 3.8, 3.9, 3.10: Latency to cross into the outermost circle after placement in the centre of the open field, the overall time spent in the centre of the field, and rearing score during a 4 min exposure to the open field measured in uninjected animals, and in repeated saline- and sibutramine-injected animals, 24h after the final injection. n = 18, each group. Data from all treatment groups compared using 1-way ANOVA; following a significant treatment effect, group means were compared using students unpaired t-test: $^{*}p < 0.05$, $^{**}p < 0.01$ *cf* uninjected controls.

EFFECT OF REPEATED SALINE INJECTION OR SIBUTRAMINE PRETREATMENT ON OPEN FIELD BEHAVIOUR

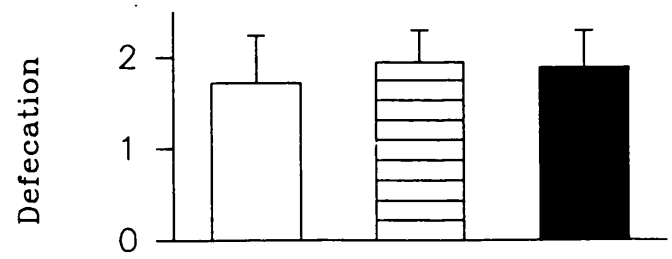
3.11 Episodes of grooming



3.12 Time spent grooming



3.13 Defecation



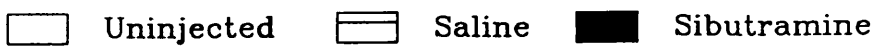


Figure 3.11, 3.12, 3.13: Episodes of grooming, the time spent grooming and defecation (number of boli dropped) during a 4 min open field test scored in uninjected animals and in repeated saline- and sibutramine-injected animals, 24h after the final injection. n = 18, each group. Data from all treatment groups compared using 1-way ANOVA; following a significant treatment effect, group means were compared using students unpaired t-test: $^{**}p < 0.01$ *cf* uninjected controls.

shown in Figure 3.9. The time spent in the centre of the field was significantly increased by repeated once-daily saline injections, compared with controls ($t = 2.417$; d.f. = 51; $p < 0.05$). This measure was increased to a similar extent by sibutramine pretreatment ($t = 1.853$; d.f. = 51; $p < 0.05$)

3.4.1.6 *Rearing, grooming and defecation*

The mean rearing score for each group is shown in Figure 3.10. This behavioural measure was increased to a similar extent by both repeated saline and sibutramine pretreatment, compared with uninjected animals (ts = 3.004, 3.805 respectively; d.f. = 51; p's < 0.01).

Both the total number of grooming episodes and the time (in seconds) were measured; the data are displayed in Figures 3.11 and 3.12. Repeated saline injection significantly increased grooming episodes compared with uninjected animals ($t = 2.565$; d.f. = 51; $p < 0.01$). In contrast, the incidence of grooming episodes in sibutramine-pretreated animals did not differ from uninjected controls. A similar pattern was seen for the time spent grooming, but this did not reach significance ($F = 2.175$; d.f. = 2, 51; $p > 0.05$).

Mean defecation scores for each group are shown in figure 3.13. There was no difference between the number of boli dropped in either treatment group compared with the control group ($F = 0.074$; d.f. = 2, 51; $p > 0.1$).

3.4.2 Cortical β-adrenoceptor binding

When radioligand binding data from each animal were analyzed, several samples were found not to show saturable specific binding; these data were excluded. Analysis of the binding data included only 14 animals from each group exposed to the open field and 7 animals in groups not exposed to the open field, therefore. Comparison of 1- and 2-site models revealed that, in each sample, binding was best described by a single site model.

Exposure to the open field did not alter cortical β-adrenoceptor density in uninjected animals when compared with unhandled animals. In animals which were killed immediately after removal from the home cage, repeated saline injection did not alter cortical β-adrenoceptor binding density compared with uninjected controls (Table 3.1). β-Adrenoceptor density was reduced by 18% after repeated sibutramine administration, but this reduction was not statistically significant. Neither repeated saline nor sibutramine

Table 3.1 **THE EFFECTS OF REPEATED SALINE OR SIBUTRAMINE INJECTION ON CORTICAL β-ADRENOCEPTOR BINDING; INFLUENCE OF EXPOSURE TO THE OPEN FIELD**

a) No exposure to the open field

	Kd (nM)	Bmax (pmol/g prot)
Uninjected	1.65 ± 0.56	90.2 ± 8.8
Saline-injected	1.49 ± 0.49	98.2 ± 12.1
Sibutramine-injected	1.21 ± 0.26	74.1 ± 6.6

b) After exposure to the open field

	Kd (nM)	Bmax (pmol/g prot)
Uninjected	1.24 ± 0.56	86.0 ± 5.7
Saline-injected	1.22 ± 0.18	85.2 ± 5.9
Sibutramine-injected	1.99 ± 0.45	91.8 ± 12.1

n = 7 (not exposed to the open field), 14 (exposed to open field). Cortical [^{3}H]-DHA binding measured 24 h after the final injection, immediately after removal from the home cage or after a 4 min exposure to the open field. Data analyzed using one-way analysis of variance.

administration altered receptor numbers after behavioural testing.

Binding affinity did not differ between any of the groups, regardless of whether or not they had been tested in the open field (Table 3.1).

3.4.3 Correlations between β-adrenoceptor binding and behavioural measures

Although sample sizes were small (n=14 each group), correlations were first calculated for individual treatment groups. In the sibutramine-pretreated animals, there was a statistically significant positive correlation between β-adrenoceptor density and centre-directed activity in the open field ($r = 0.600$; d.f. = 12; $p < 0.05$; Figure 3.14). In this treatment group, β-adrenoceptor density was also significantly positively correlated with the time spent in the centre of the field ($r = 0.507$; d.f. = 12; $p < 0.05$; Figure 3.14). No other behaviours correlated significantly with β-adrenoceptor binding in this group (Table 3.2).

There were no significant correlations between β-adrenoceptor density and behavioural measures in the saline-pretreated or in the uninjected control group. There were also no correlations between β-adrenoceptor K_d and any behavioural measure in any of the treatment groups (Table 3.2).

Since neither saline nor sibutramine pretreatment caused significant changes in β-adrenoceptor binding, correlation coefficients were also calculated for pooled data from all three treatment groups. Again, there was a statistically significant, positive correlation between β-adrenoceptor density and centre-directed movements over the whole 4 min test ($r = 0.267$; d.f. = 40; $p < 0.05$; Figure 3.15). When the two periods of the open field test were analyzed separately, this correlation was significant over the final 3 min ($r = 0.285$; d.f. = 40; $p < 0.05$; Figure 3.15), but not the first minute of the test. No other behavioural measures correlated significantly with β-adrenoceptor binding density or K_d when either the whole test, or the first and the final 3 min were examined.

3.4.4 Correlations between different behavioural measures

Correlations between all measures of behaviour were calculated for data pooled from all treatment groups only; correlation coefficients are shown in Table 3.3. Many behaviours were significantly correlated as would be expected: for instance, total locomotor activity was significantly positively correlated with both locomotor activity in the first and in the final 3 min of the test. However, only correlations of particular

Figure 3.14 **CORRELATIONS BETWEEN CENTRE-DIRECTED MOVEMENTS OR TIME IN THE CENTRE OF THE FIELD AND β-ADRENOCEPTOR DENSITY IN SIBUTRAMINE-TREATED ANIMALS**

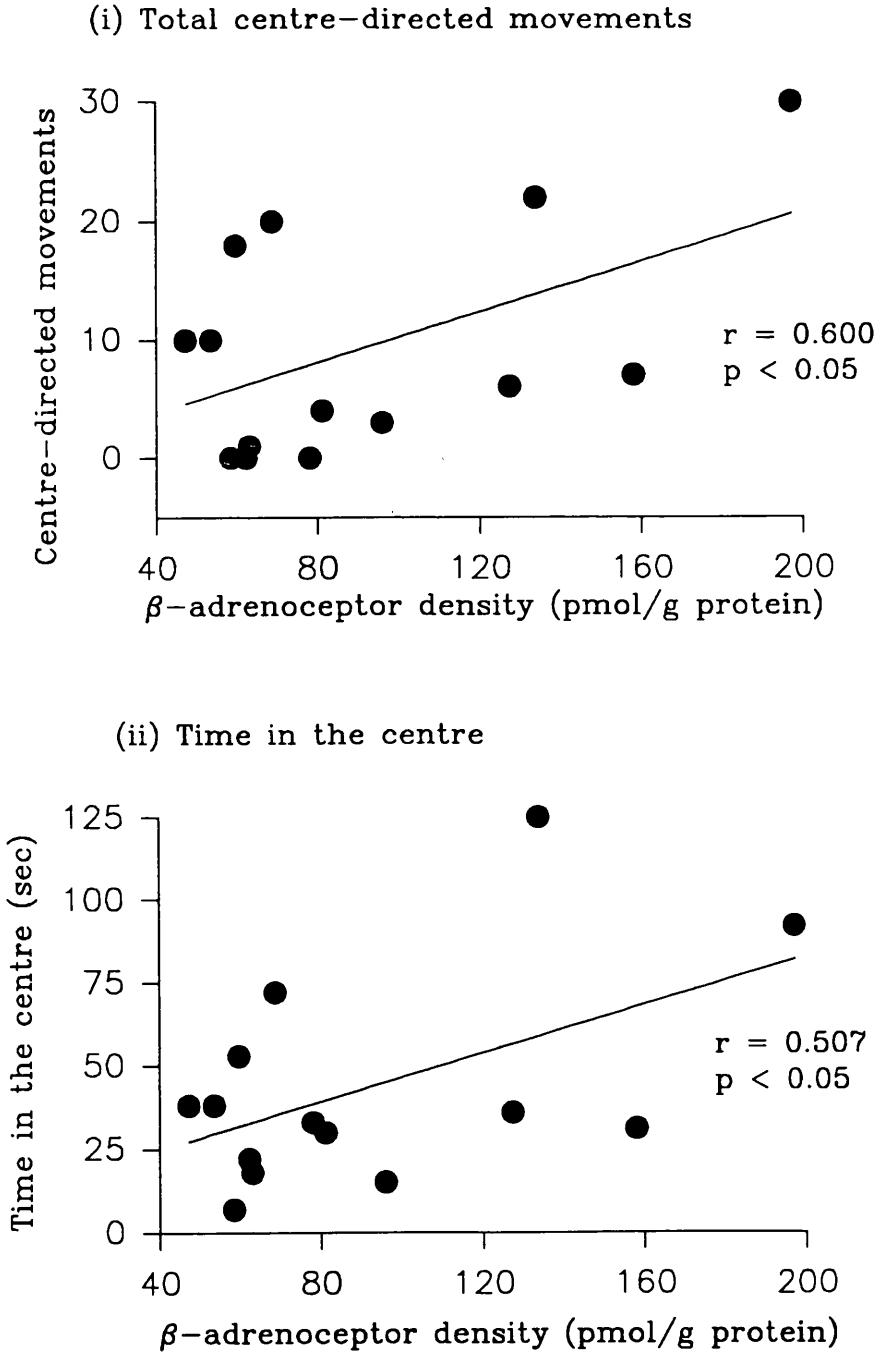


Figure 3.14: Correlations calculated for the sibutramine-pretreated group between cortical β-adrenoceptor B_{max} and

(i) centre-directed movements during a 4 min open field exposure

(ii) time in the centre of the field during open field exposure

n = 14; r > 0.4973, p < 0.05.

Figure 3.15 **CORRELATIONS BETWEEN CENTRE-DIRECTED MOVEMENTS AND β-ADRENOCEPTOR DENSITY FOR ALL TREATMENT GROUPS**

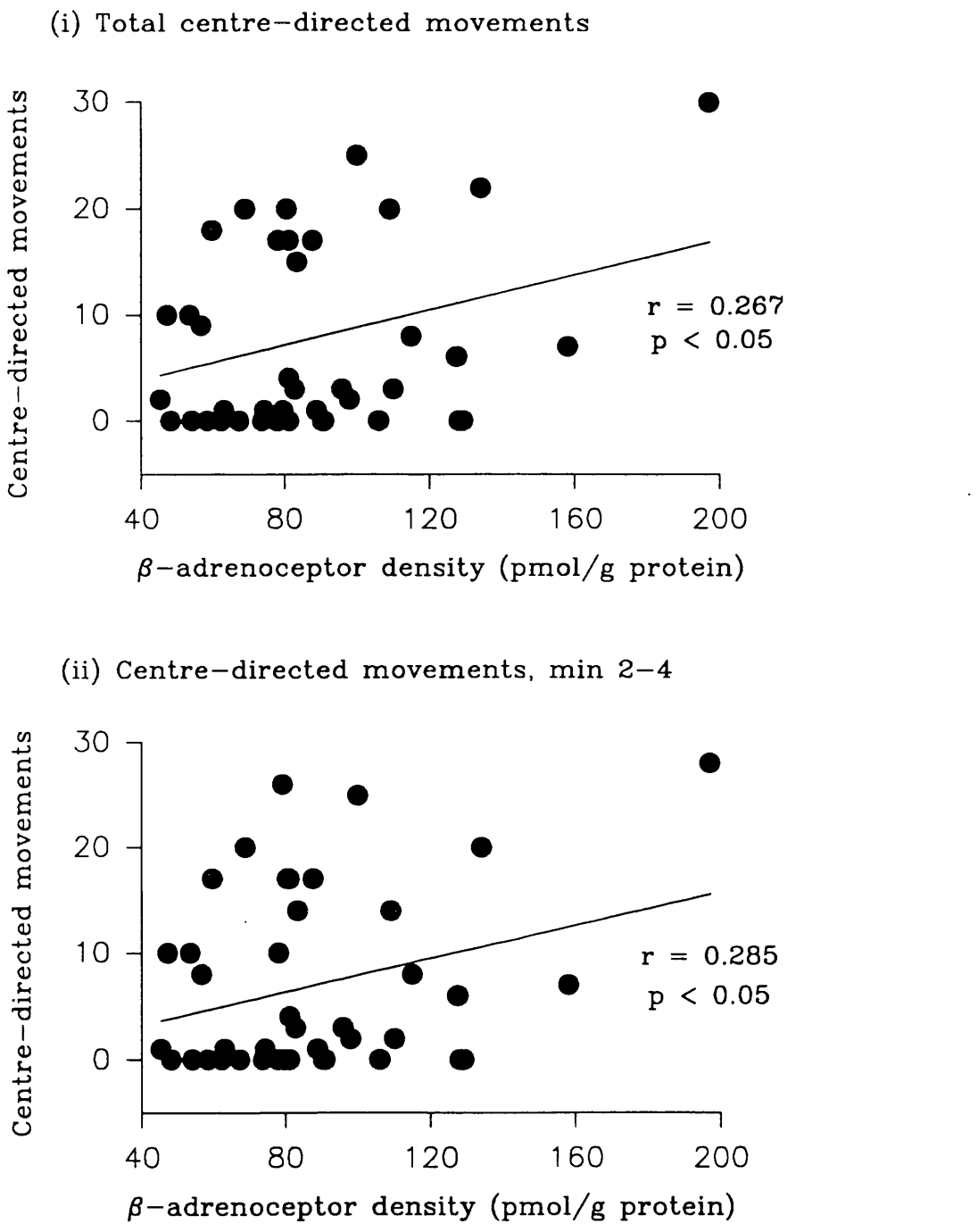


Figure 3.15: Correlations calculated for pooled data from all treatment groups β-adrenoceptor B_{max} and

(i) centre-directed movements during a 4 min open field exposure

(ii) centre-directed movements during the final 3 min of the open field test.

n = 42; r > 0.2638, p < 0.05.

interest to the experiment will be presented. Latency did not correlate with centre-directed activity at any time during the test. In contrast, the time spent in the centre was significantly positively correlated with centre-directed movements at all times. Defecation was not significantly correlated with any behavioural measure. Rearing was positively correlated with both time in the centre and centre-directed movements.

Table 3.2 **CORRELATIONS BETWEEN β-ADRENOCEPTOR BINDING AND BEHAVIOUR IN THE OPEN FIELD.**

		SIBUTRAMINE		SALINE		UNINJECTED		ALL GROUPS	
		Kd	Bmax	Kd	Bmax	Kd	Bmax	Kd	Bmax
Centre-directed movements	Total	0.336	0.600	-0.187	0.090	-0.117	0.013	0.153	0.267
	Min 1	0.222	0.488	-0.281	0.032	-0.282	-0.129	-0.119	0.068
	Min 2-4	0.338	0.504	-0.143	0.097	-0.037	0.062	0.200	0.285
Ratio centre-directed : locomotor activity)	Total	0.325	0.434	-0.215	0.013	-0.214	-0.049	0.167	0.143
	Min 1	0.250	0.461	-0.318	0.114	-0.282	-0.134	-0.064	0.188
	Min 2-4	0.323	0.391	-0.165	-0.039	-0.163	0.004	0.194	0.201
Radial movements	Total	0.412	0.566	-0.187	0.090	-0.187	0.019	0.189	0.315
	Min 1	0.299	0.600	-0.231	0.043	-0.309	-0.131	-0.111	0.075
	Min 2-4	0.415	0.556	-0.150	0.092	-0.072	0.066	0.236	0.334
Ratio (radial: locomotor activity	Total	0.363	0.572	-0.154	0.106	-0.266	-0.021	0.181	0.311
	Min 1	0.574	0.612	-0.027	0.210	-0.228	-0.106	0.226	0.261
	Min 2-4	0.395	0.521	-0.178	0.000	-0.233	0.032	0.207	0.283
Locomotor activity	Total	0.244	0.296	-0.254	-0.029	0.200	0.189	0.066	0.145
	Min 1	-0.325	-0.225	-0.175	-0.123	0.219	0.267	-0.216	-0.085
	Min 2-4	0.355	0.391	-0.244	0.017	0.166	0.178	0.157	0.211
Latency		0.048	0.200	-0.146	0.002	0.115	-0.199	0.001	0.030
Time in centre		0.232	0.507	-0.276	-0.040	-0.166	-0.090	0.096	0.252
Rearing		-0.072	-0.056	-0.248	-0.037	-0.336	-0.023	-0.065	-0.022
Grooming episodes		0.366	0.394	-0.212	0.364	-0.153	0.283	-0.071	0.225
Time grooming		0.262	0.189	0.315	0.572	-0.288	0.239	0.063	0.248
Defecation		-0.070	0.112	-0.319	0.068	0.214	0.063	0.000	0.092
β-Adrenoceptor Kd (nM)			0.900		0.417		0.496		0.761

n = 14, individual treatment groups: $r > 0.4973$, $p < 0.05$; n = 42, for pooled treatment groups: $r > 0.2638$, $p < 0.05$. All significant values underlined

Table 3.3 : Correlations between behavioural measures in the open field were calculated for data pooled from all treatment groups. $n = 42$; $r > 0.2638$, $p < 0.05$. Significant correlations are shown **in bold**.

For clarity in the table, each behavioural measure was assigned a number, as detailed below:

(1) Time in the centre
(2) Latency
(3) Rearing
(4) Grooming episodes
(5) Time spent grooming
(6) Defecation
(7) Arc movements - total
(8) Arc movements - min 1
(9) Arc movements - min 2-4
(10) Radial movements - total
(11) Radial movements - min 1
(12) Radial movements - min 2-4
(13) Centre-directed movements - total
(14) Centre-directed movements - min 1
(15) Centre-directed movements - min 2-4
(16) Locomotor activity - total
(17) Locomotor activity - min 1
(18) Locomotor activity - min 2-4
(19) Centre-directed : locomotor activity - total
(20) Centre-directed : locomotor activity - min 1
(21) Centre-directed : locomotor activity - min 2-4
(22) Radial : locomotor activity - total
(23) Radial : locomotor activity - min 1
(24) Radial : locomotor activity - min 2-4

Table 3.3 CORRELATIONS BETWEEN BEHAVIOURAL MEASURES IN THE OPEN FIELD

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)	(22)	(23)	
(2)	**0.381**																							(2)
(3)	**0.548**	-0.246																						(3)
(4)	0.049	-0.176	-0.076																					(4)
(5)	-0.193	-0.075	-0.225	**0.727**																				(5)
(6)	0.120	-0.112	0.202	-0.180	-0.073																			(6)
(7)	**0.277**	**-0.321**	**0.551**	0.039	-0.070	-0.178																		(7)
(8)	-0.012	**-0.573**	**0.315**	-0.044	-0.067	-0.031	-0.178																	(8)
(9)	**0.361**	-0.134	**0.554**	0.071	-0.122	-0.096	**0.327**	**0.424**																(9)
(10)	**0.802**	-0.106	**0.754**	0.003	-0.092	0.112	**0.541**	**0.322**	**0.538**															(10)
(11)	**0.335**	**-0.318**	**0.488**	0.075	0.082	0.214	-0.001	**0.394**	0.171	**0.583**														(11)
(12)	**0.818**	-0.045	**0.729**	-0.013	-0.122	0.076	0.136	**0.269**	**0.561**	**0.983**	**0.422**													(12)
(13)	**0.805**	-0.110	-0.231	0.013	-0.088	0.111	0.151	**0.338**	**0.553**	**0.995**	**0.600**	**0.974**												(13)
(14)	**0.404**	-0.231	**0.491**	0.152	0.100	0.181	-0.007	**0.349**	0.182	**0.615**	**0.983**	**0.462**	**0.627**											(14)
(15)	**0.815**	-0.075	**0.754**	-0.022	-0.125	0.084	0.171	**0.303**	**0.875**	**0.983**	**0.450**	**0.994**	**0.984**	**0.476**										(15)
(16)	**0.564**	-0.262	**0.722**	0.027	-0.090	-0.004	0.254	**0.349**	**0.875**	**0.833**	**0.462**	**0.823**	**0.842**	**0.472**	**0.840**									(16)
(17)	0.039	-0.165	0.243	-0.141	-0.144	-0.143	**0.963**	**0.312**	**0.278**	0.175	0.176	0.155	0.206	0.156	0.197	**0.303**								(17)
(18)	**0.643**	-0.106	**0.716**	0.037	-0.138	-0.020	**0.272**	**0.400**	**0.905**	**0.836**	**0.322**	**0.860**	**0.841**	**0.350**	**0.866**	**0.933**	0.251							(18)
(19)	**0.845**	-0.025	**0.710**	0.039	-0.070	0.047	0.158	0.185	**0.494**	**0.940**	**0.533**	**0.927**	**0.950**	**0.581**	**0.938**	**0.747**	0.181	**0.781**						(19)
(20)	**0.436**	-0.152	**0.413**	0.115	0.085	0.110	-0.037	0.196	0.158	**0.582**	**0.903**	**0.433**	**0.591**	**0.929**	**0.453**	**0.406**	0.088	**0.325**	**0.614**					(20)
(21)	**0.811**	-0.105	**0.732**	-0.022	-0.091	0.044	0.181	0.250	**0.512**	**0.950**	**0.481**	**0.940**	**0.950**	**0.513**	**0.953**	**0.747**	0.209	**0.799**	**0.986**	**0.536**				(21)
(22)	**0.778**	-0.083	**0.541**	-0.026	-0.053	-0.025	0.090	-0.030	0.097	**0.829**	**0.459**	**0.723**	**0.737**	**0.511**	**0.714**	**0.454**	0.050	**0.431**	**0.754**	**0.533**	**0.745**			(22)
(23)	0.073	-0.056	0.031	-0.086	0.100	0.208	-0.138	**-0.437**	**-0.292**	0.111	**0.433**	0.025	0.107	**0.400**	0.026	-0.207	-0.136	-0.168	0.145	**0.469**	**0.094**	**0.405**		(23)
(24)	**0.832**	-0.058	**0.717**	0.033	-0.086	0.036	0.144	0.222	**0.513**	**0.908**	**0.452**	**0.955**	**0.950**	**0.502**	**0.958**	**0.770**	0.163	**0.807**	**0.895**	**0.514**	**0.996**	**0.754**	0.075	(24)

3.5 Discussion

Although many studies have examined various aspects of behaviour in the open field, the interpretations of behavioural data are often unclear; for instance, locomotor activity has been used both to measure emotionality (e.g. Hall, 1936; Ivinskis, 1970) and as an index of exploration (Lester, 1968). Since this test has also been used as a form of stress, behaviour will also reflect the response to stress (e.g. resistance to stress). However, the relationship between stress resistance and behavioural measures has received little attention. The first part of this experiment aimed to examine the relationship between resistance to stress and various behaviours which can be measured in the open field. Since resistance to stress has been linked with β-adrenoceptor down-regulation, the relationship between behavioural indices of resistance to stress and β-adrenoceptor binding measured *in the same animals* was also examined.

3.5.1 Behaviour in the open field

Exposure to a novel open field is traditionally used as a test for emotionality in individual animals (e.g. Hall, 1934; Ivinskis, 1970; Abel, 1991). More recently, this procedure has been used as a form of stress (e.g. Lee et al., 1986; Gentsch et al., 1987; Gomez et al., 1989). In the present experiment, activity in the centre of the field was used as an index of the individuals' resistance to the stress of the open field (e.g. Gentsch et al., 1987; see Section 3.1.2). This interpretation was based on the idea that the centre of the open field is the most aversive part of the apparatus (Archer, 1973), as indicated by findings that only a small proportion of animals tested enter the centre of the field (Ader & Belfer, 1962; Ader & Conklin, 1963). Further evidence in support of this interpretation is that the increase in centre-field activity induced by benzodiazepines can be attributed to their anxiolytic effects (Hata et al., 1988; Bruhwyler, 1990). Finally, activity in the aversive central zone increases during the course of a single test (Archer, 1973); this change may be interpreted as a sign of habituation to the stress. Collectively, such evidence leads to the inference that activity in the centre of the field reflected animals' resistance to stress.

The main finding of the present experiment was that both previous experience of either repeated stress (saline injection) or pretreatment with the monoamine uptake inhibitor sibutramine increase animals' activity in the centre of the field. This suggests that resistance to stress was increased by these treatments.

Although several studies have investigated the effects of repeated stress pretreatment

on behaviour in the open field, the effects of this treatment on centre field activity have been largely ignored. Hata and co-workers (1988) reported that repeated restraint stress or food deprivation had no effect on centre-field activity in the open field. However, the time of testing in the open field after the final stress exposure differs between Hata's study and the current experiment. The earlier study looked at behaviour immediately after the cessation of stress. In contrast, the present study was concerned with long-lasting changes, and open field activity was measured 24 h after the final stress session, therefore. Another possible explanation for the contrasting findings concerns the form of stress to which animals were repeatedly exposed. Restraint and food deprivation were studied by Hata's group, and it is likely that these have different effects from those of saline injection. It is possible therefore that the effects of repeated stress on behavioural resistance to exposure to the open field depend on the form of stress which animals have previously experienced.

In the current experiment, repeated sibutramine administration had quantitatively the same effect on centre-directed movements as repeated once-daily saline injection. Since sibutramine was administered by repeated injection, it is impossible to distinguish a specific effect of this drug on centre-directed movements, therefore. That is, the increase in activity in the sibutramine-pretreated animals could be due solely to the effect of repeated injection. Alternatively, sibutramine and saline injection may have non-additive effects on centre-directed activity or there may be a ceiling for increases in this behaviour. However, it is clear that both previous experience of repeated saline or sibutramine administration increase centre-directed activity, interpreted as reflecting increased resistance to stress.

Not all published evidence appears to corroborate this interpretation. For instance, the benzodiazepine inverse agonist N-methyl-β-carboline-3-carboxamide (FG 7142), which has *anxiogenic* actions in animal models (File et al., 1985), also increases centre-field activity (Bruhwyler et al., 1991). However, this can be explained by the finding that FG 7142 caused a non-selective increase in locomotor activity in these experiments. This highlights a problem in the interpretation of behavioural changes based on changes in movement in the open field: great care must be taken to distinguish between overall locomotor activity and activity in specific areas of the arena. This also raises the possibility that the changes in centre-field activity induced by stress and antidepressant pretreatment in the current experiment could also merely reflect a non-selective increase in overall activity. To eliminate this, total locomotor activity (i.e. arc + radial movements) was also measured

in the current experiment.

Neither saline nor sibutramine pretreatment significantly altered total locomotion in the open field. Other studies have also found that repeated stress has no effect on locomotion in the open field; these studies have used forms of stress such as restraint and food deprivation (Kennett et al., 1985, 1986; Hata et al., 1988). It is noteworthy that animals were restrained for up to 13 h (Hata et al., 1988), yet this had no more effect on locomotion than did saline injections which took less than 1 min to administer. It is therefore quite unnecessary to use prolonged exposure to forms of stress such as restraint when looking at behaviour in the open field. There are very few studies of the effects of repeated antidepressant administration on locomotion in the open field and the findings of such studies depend on the drug used and the time of open field testing after the final drug administration (e.g. Kulkarni & Dandiya, 1973; Kennett et al., 1987). However, repeated desipramine administration did not alter locomotor activity in the open field when tested 24 h after the final injection (Carli et al., 1989).

It is notable that locomotor activity in the open field has itself been used extensively as an index of emotionality (e.g. Hall, 1936; Ivinskis, 1970; Carli et al., 1989) and even as a measure of exploratory behaviour (Lester, 1968). However, neither the evidence for these interpretations nor evidence that locomotion reflects resistance to stress is consistent. A relationship between locomotion and resistance to stress is supported by the following evidence :

1) Locomotion is decreased when the 'stimulus intensity' is greater during open field testing (e.g. Livesey & Egger, 1970; Valle, 1970)
2) Locomotor activity is increased in stress resistant strains of rat (e.g. Maudsley Non-Reactive) compared with their stress-reactive counterparts (Maudsley-Reactive: Broadhurst, 1975)
3) The anxiolytic benzodiazepine chlordiazepoxide increases locomotion in the open field (Bruhwyler, 1990)

Evidence against a link between locomotion and stress resistance includes the following:

1) Locomotion is *decreased* over repeated open field trials (e.g. Paré, 1964; Ivinskis, 1970)
2) The benzodiazepine inverse agonist FG 7142 increases locomotor activity in the open field (Bruhwyler et al., 1991)
3) Non-sedative doses of the anxiolytic benzodiazepines diazepam and alprazolam have no effect on locomotion in the open field (Hata et al., 1988)

Overall, the evidence for the interpretation of locomotor activity as an index of resistance to stress is not convincing. Indeed, it is not clear what emotion locomotor activity reflects.

Locomotor activity, unaltered in the present experiment, is calculated as the sum of arc movements plus radial movements. Saline and sibutramine pretreatment clearly increased centre-directed and radial movements. This suggests that there should be a reduction of activity around the periphery of the field (arc movements) in saline- and sibutramine-pretreated animals. However, this was not the case. This apparent anomaly is explained by non-significant increases in total locomotion in both saline- and drug-pretreated groups (+28.9%, +15.0% respectively), coupled with the finding that the increases in centre-directed activity by these pretreatments were far larger than the increase in total locomotion (+253%, +198% respectively). The findings of the current experiment, that centre-directed but not overall locomotor activity is increased in both stress- and sibutramine-pretreated animals in the current experiment indicates a selective increase in centre-directed movements in these animals. That is, both previous experience of repeated stress and antidepressant administration apparently increase behavioural resistance to stress without altering general levels of activity.

As a further test of the selective nature of the effects of stress and sibutramine on centre-field activity, centre-directed movements were also expressed as a proportion of total locomotion for each animal. The proportion of centre-directed activity was increased in saline- and sibutramine-pretreated animals. This again supports the conclusion that both pretreatments selectively increase behavioural resistance to the stress of the open field.

When movements during the first and the final 3 min of the test were considered separately, there were no treatment effects on any aspect of locomotor activity during the first minute of the test. During the final 3 min of the test, there were also no pretreatment effects of either locomotor activity or arc movements. During this period, both centre-directed activity and the ratio of centre-directed movements : locomotor activity were significantly increased in both saline- and sibutramine-pretreated animals. This suggests that centre-field activity is increased by stress and sibutramine in the latter portion of the test only. The initial response to placement in the open field is unaffected by saline or sibutramine pretreatment.

When the ratio of radial (movements both into and out of the centre of the field) : locomotor activity was considered, saline and sibutramine pretreatments again increased this ratio only during the final 3 min of the test. However, this appears to be due to a reduction in the ratio in the control group at this time from that in the first minute rather than an increase in the stress- and sibutramine-pretreated groups. This reduction in the control group is easily explained. Animals were initially placed in the centre of the field but, during the first minute of the test, they all crossed into the outermost circle of the field. That is, all animals from all groups crossed concentric lines. In the final 3 min of the test, only a small proportion of uninjected animals re-entered the centre so the ratio in this group was reduced in this period of the test. However, the majority of stress- or sibutramine-pretreated animals re-entered the centre of the field during the final 3 min. The difference in the ratio of radial : locomotor activity in the final 3 min reflects a true increase in this ratio in saline- and sibutramine-pretreated animals, therefore.

Together, these findings suggest the initial impact of exposure to the stress of a novel open field, i.e. behaviour during the first minute, is not altered by previous experience of repeated stress or sibutramine administration. The effect of these treatments, i.e. an increase in centre-directed movements is apparent only in the final 3 min of the test. This suggests that resistance to stress may not be important in determining the initial response to placement in the open field. To further investigate this possibility, latency to cross to the outermost circle of the arena was measured as a specific index of the initial impact of the stress of exposure to the open field.

Latency was unaltered by both stress and sibutramine pretreatment, suggesting that the initial impact of the novel open field is unaltered by these pretreatments. Since stress- and sibutramine-pretreated animals were indistinguishable from controls when behaviour during the first minute of the test was considered, this may not seem unexpected. However, it is not improbable that resistance to stress influences the initial impact of exposure to stress, and both saline and sibutramine pretreatment increased resistance to stress in the open field. However, this increase in centre-field activity was only apparent in the final 3 min of the test. During the first minute of the test, when 96% of animals reached the outermost circle of the arena, there was no difference in any aspect of locomotor activity between any of the groups. Interestingly, there was no correlation between latency and centre-directed activity in individual animals. Together, these findings suggest that the initial impact of open field exposure is unaltered by either previous experience of repeated stress or sibutramine administration. Furthermore, the

initial impact of the stress is influenced by treatments altering resistance to stress.

The time spent in the centre of the field (latency + duration of any further entries into the centre) was measured as a further index of behavioural resistance to stress. Both repeated saline injection and sibutramine administration increased the time spent in the centre of the open field compared with uninjected controls. The time in the centre of the field and centre-directed activity show parallel changes; furthermore, there was a significant positive correlation between centre-directed activity and time spent in the centre of the field in individual animals. It is inferred therefore that the time spent in the centre of the field also reflects resistance to stress in the open field.

In summary, the initial impact of the stress of exposure to the open field appears to be unaltered by either repeated stress or sibutramine pretreatment: the latency to leave the centre of the field, and behavioural resistance to stress in the first minute of the test were unaltered by either pretreatment. Behaviour in the final 3 min of the test appears to differ from that in the first minute: during the final 3 min, behavioural resistance to stress is selectively increased by both pretreatments.

A number of other behaviours have been observed in the open field besides locomotion in specific areas or the whole arena. Rearing, grooming and defecation are among the most commonly studied of these behaviours, but the relationships between these behaviours and stress resistance are not clear.

Defecation was one of the first behaviours to be measured in the open field (Hall 1934), and has been widely used as an index of 'emotionality'. However, in studies where the level of background noise accompanying open field exposure was increased, defecation was increased (Ivinskis, 1970). Consequently, it has been suggested that increasing the aversive nature of the open field increases defecation; it is inferred that defecation in the open field may be inversely related to resistance to stress.

The findings of the current experiment show that defecation was unaltered by saline or sibutramine pretreatment, even though resistance to stress was increased in these treatment groups. In addition, there was no significant correlation between defecation and resistance to stress in individual animals.

Although defecation has been widely studied, the relationship between defecation and

centre-field activity has been examined rarely. Evidence supporting an inverse relationship between defecation and resistance to stress includes the finding that defecation is increased as the aversive nature of the open field is increased (Broadhurst, 1957; Ivinskis, 1970). In addition, the anxiolytic benzodiazepine chlordiazepoxide increases centre-field activity and decreases defecation (Bruhwyler, 1990). However, one study of the effects of repeated stress on open field behaviour does not support this interpretation (Hata et al., 1988). It was shown that repeated restraint or food deprivation, which did not alter centre-field activity, increased defecation in the open field. This study, in combination with the results of the current experiment show that defecation is not related to stress resistance in the open field in any obvious way.

Rearing has been widely used in the open field as an index of exploratory behaviour. However, this interpretation has not been experimentally validated. Indeed, it appears to be based merely on the appearance of this behaviour i.e. standing up on hind legs and looking around. More recent evidence suggests that rearing could be related to resistance to stress, since rearing increases over the course of a single open field test (Blizard, 1971). However, rearing increases when background noise is increased (Ivinskis, 1970), suggesting the opposite: that is, there is an inverse relationship between rearing and stress resistance.

In the present experiment, rearing in the open field was increased by both repeated stress and sibutramine administration. It was not possible to distinguish a specific effect of sibutramine, since the drug was administered by repeated injection. However, the treatment effects on rearing paralleled the effects on centre-directed movements: furthermore, rearing was positively correlated with centre-directed movements in both periods of the test.

A previous study of open field behaviour has reported parallel changes in rearing and centre-field activity in animals with previous experience of repeated stress, although the effect seen depends on the stress used (Hata et al., 1988). There are no studies of the effects of monoamine uptake inhibitors on both rearing and centre-field activity.

It is concluded that rearing reflects resistance to stress in the open field since, in the current experiment, changes in rearing paralleled changes in centre-field activity and rearing was correlated with centre-field activity. However, the lack of studies to which the current findings can be compared expose a general problem in the literature on open

field behaviour. Although early studies investigated a whole range of behaviours in this apparatus (e.g. Ivinskis, 1970; Valle, 1970), more recent studies have examined treatment effects on only one or two behaviours. It is therefore difficult to determine the relationships between different behavioural parameters.

The role of grooming induced by exposure to novelty e.g an open field is unclear. Since grooming increases with time (Bolles, 1960; Woods, 1962; Hughes, 1968) over a single trial, it is possible that grooming could be related to resistance to stress. In the current experiment, grooming was increased by repeated saline pretreatment when compared with uninjected controls. However, levels of grooming in sibutramine-pretreated animals were indistinguishable from the control group. Since sibutramine was administered by repeated injection, and the injection procedure itself increases grooming, sibutramine clearly *decreases* novelty-induced grooming. All the effects of this pretreatment discussed so far were quantitatively the same as repeated vehicle injection, and could be due simply to an effect of repeated injection on open field behaviour. Since grooming is reduced by repeated sibutramine administration, it is clear that this drug can itself have long-term effects on the behavioural response to the stress of exposure to the open field. Both repeated saline and sibutramine pretreatment had similar effects on centre-directed movements, but different effects on grooming, and neither the number of episodes or the time spent grooming correlated with centre-directed movements in individuals. It is therefore concluded that grooming does not reflect behavioural resistance to stress in the open field.

Studies of the effects of acute stress have shown that grooming is induced by a range of different forms of stress e.g. non-reward (Kortland, 1940), novelty (Jolles et al., 1979) and by handling (Green et al., 1979). Since grooming is an apparently inappropriate response to stress, an alternative interpretation of novelty/stress-induced grooming is that it represents a 'displacement activity' (e.g. Jolles et al., 1979). Although the role of these activities in the response to stress is unclear, reduced hormonal and neurochemical effects of stress are found if such behaviours are expressed. For instance, the increase in plasma adrenocorticotrophic hormone (ACTH) and brainstem noradrenaline turnover induced by a single footshock session are attenuated if rats are shocked in pairs and express fighting behaviour on exposure to the shock (Conner et al., 1971; Stolk et al., 1974). Such findings have led Dantzer to propose that 'displacement activities' may represent coping mechanisms, which reduce the impact of stress (Dantzer, 1989). As yet, this interpretation is as yet only speculative, however.

It is not known whether coping mechanisms are increased or decreased with increased stress resistance: indeed the current findings have shown that grooming is not related to centre-directed movements, regardless of whether grooming represents a coping mechanism. If displacement activities do represent coping mechanisms, then sibutramine is reducing coping in the current experiment, since repeated administration of this drug abolishes the increase in grooming induced by repeated saline injection. That a monoamine uptake inhibitor should apparently *decrease* a mechanism for coping with stress is surprising: it is possible that sibutramine is substituting for an intrinsic coping mechanism.

These results highlight the problem discussed earlier: that often only a small number of behaviours are measured in the open field. Whatever the role of grooming in the open field, it is clearly not altered in the same way by stress and sibutramine as are, for instance, centre-directed movements. Such differential effects of experimental pretreatments cannot be detected if only one or two behaviours are measured. A whole range of behaviours must be measured in the open field in order to build up a complete picture of the effects of a given treatment on behavioural responses to the stress.

3.5.2 Cortical β-adrenoceptors

In a previous experiment from this laboratory, exposure to the open field induced rapid changes in cortical β-adrenoceptor binding density in animals which had previous experience of repeated injection; the control group used in this experiment was a previously uninjected group which was exposed to the open field. However, this experiment did not include animals which were not exposed to the open field (injected or uninjected). It was therefore not possible to determine if the increase in binding density was due to an effect of the open field alone, the injection procedure alone or an interaction between the acute stress and repeated injection. The current experiment was designed to examine the effects of the open field on cortical β-adrenoceptor density, and to investigate whether repeated stress or sibutramine pretreatment influence any changes in β-adrenoceptors. Binding was measured in uninjected, saline- and sibutramine-pretreated animals either after open field exposure or immediately on removal from the home cage.

In the current experiment, the density of β-adrenoceptors in the cerebral cortex was unaltered immediately after the open field test: that is, a 4 min exposure to a novel

situation did not induce rapid changes in cortical β-adrenoceptors. In contrast, a previous study from this laboratory reported a rapid up-regulation of β-adrenoceptors in rat cortex following exposure to novelty (see Stanford, 1990). In this case, animals were placed in a novel environment for 40 min, compared with 4 min in the current experiment. It is possible that 4 min novelty stress is simply not long enough to affect the number of cortical β-adrenoceptors in previously unhandled rats therefore. However, the difference in the binding protocol used to define β-adrenoceptors is another difference between the two experiments. Although both studies used [^{3}H]-DHA as the radioligand, specific binding was defined using (±)-propranolol in the earlier report, whereas (±)-isoprenaline was used in the current study. Propranolol displaces [^{3}H]-DHA binding in both rat cortex and brain stem in a biphasic manner (Stone & U'Prichard, 1981; Riva & Creese, 1989a), indicating that these ligands compete for more than one receptor. The second binding site has not yet been identified, but propranolol is now known to bind to both 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors (Middlemiss, 1984; Oksenberg & Peroutka, 1988). Since specific binding of [^{3}H]-DHA defined using propranolol measures binding to more than just β-adrenoceptors, then changes in estimates of binding could reflect changes in β-adrenoceptors, in the second receptor type, or in both. Isoprenaline, used in the current experiment to define [^{3}H]-DHA specific binding, also displaces [^{3}H]-DHA from more than one site in rat cortex (Riva & Creese, 1989a); results of the current study suggest that biphasic displacement of [^{3}H]-DHA by isoprenaline in rat cortex cannot be ruled out (Section 2.3.1.1). The second site has not been characterized, but may be a low-affinity form of the β-adrenoceptor (O'Donnell et al., 1984). Notwithstanding this limitation, this combination of ligands is widely used to define β-adrenoceptors (e.g. Paul et al., 1988; Heal et al., 1989b).

Collectively, these findings suggest that the increase in the specific binding of [^{3}H]-DHA when defined using propranolol does not reflect a change in β-adrenoceptors. However, the possibility that changes in [^{3}H]-DHA binding are dependent on the duration of exposure to novelty cannot be ruled out.

In animals which were not exposed to the open field, repeated once-daily saline injection did not alter rat cortical β-adrenoceptor density compared with uninjected controls. This procedure is commonly used as a control group in studies of the effects of repeated drug administration, so this may not seem surprising. However, a previous study from this laboratory has reported a reduction in cortical β-adrenoceptors after repeated saline injection (Stanford et al., 1984). Differences in experimental protocol may

account for these contrasting findings. In the current study, animals received once-daily injections for 10 days, compared with 14 days in the earlier study. However, the ligand used to define the specific binding of the radioligand, [^{3}H]-DHA differed between the two experiments. Again, (±)-propranolol was used in the earlier report; as described above, this compound displaces [^{3}H]-DHA from sites which are not β-adrenoceptors (Riva & Creese, 1989a). Since specific binding of [^{3}H]-DHA was reduced by repeated saline injection only when propranolol was used, this could be explained by a down-regulation of the second receptor type defined by this ligand combination. This inference has important implications for studies which have examined β-adrenoceptor binding after exposure to repeated stress, most of which have used [^{3}H]-DHA and propranolol to define binding (e.g. Nomura et al., 1981; Stone, 1981; Cohen et al., 1986). Since repeated saline injection apparently alters only the non-β-adrenergic component of the binding of these ligands, the effects of repeated exposure to other forms of stress on cortical β-adrenoceptors needs to be re-evaluated using a more selective combination of ligands.

After exposure to the open field, cortical β-adrenoceptor density in saline-pretreated animals did not differ from uninjected animals. It has previously been reported that exposure to the open field increases β-adrenoceptor density in injected animals (Salmon & Stanford, 1989). Both experiments used the same pretreatment schedule and open field apparatus and both measured β-adrenoceptors immediately after exposure to the open field. The only difference between the two experiments was the use of propranolol to define specific binding of [^{3}H]-DHA in the earlier experiment. Since specific binding of this radioligand was increased after exposure to the open field in saline-injected animals when using propranolol but not isoprenaline, it is inferred that exposure to the open field has no effect on β-adrenoceptors. Rather, a rapid increase in the second receptor type defined by [^{3}H]-DHA and propranolol is induced in saline-pretreated animals.

In animals which were not exposed to the open field, cortical β-adrenoceptor density was not significantly reduced after repeated sibutramine administration compared with either uninjected or saline-injected animals, despite an 18% reduction in binding B_{max} compared with uninjected controls. Other studies which have shown significantly reduced β-adrenoceptors have used the same rat strain, binding protocol, dose and, in one case, the same dosing schedule as the current experiment (Buckett et al., 1988; Heal et al., 1989b). There is no apparent explanation for the lack of significant effect of sibutramine on β-adrenoceptors in the current experiment; it is probable that large variation in the data is responsible.

After exposure to the open field, there was no difference in β-adrenoceptor binding density in the repeated sibutramine-pretreatment group compared with the uninjected group. The effects of an acute stress on changes in β-adrenoceptor binding induced by chronic antidepressant administration on β-adrenoceptor density after a single stress exposure has been previously been examined in a study of swim stress (5 min) in imipramine-pretreated animals (Duncan et al., 1985). In this study, the reduction in β-adrenoceptor density in imipramine-pretreated animals was not altered by a single swim stress. This could suggest a floor for the reduction in β-adrenoceptors. However, 4 min novelty did not alter β-adrenoceptor density in sibutramine-pretreated animals, a pretreatment which alone did not significantly alter these receptors in the current experiment. Since novelty alone did not alter β-adrenoceptor density, this finding shows that there is no significant interaction between acute stress and the effects of repeated antidepressant administration on cortical β-adrenoceptors.

Although there were no significant effects of saline or sibutramine injection or of open field exposure on β-adrenoceptor binding in rat cortex, there are limitations to the binding data obtained from this experiment. There is a positive correlation between β-adrenoceptor K_d and B_{max} in sibutramine-injected animals, which is also apparent in pooled data from all treatment groups, suggesting that specific binding curves do not saturate. Indeed, closer examination of specific binding curves (typical curve shown in Figure 3.16) shows that this is the case. Inspection of the binding curve for the displacement of [^{3}H]-DHA by (±)-isoprenaline sulphate (Figure 2.1, reproduced with Figure 3.16 for ease of study) suggests a possible explanation for this lack of saturation. Even at the highest isoprenaline concentrations used (1 mM), a clear plateau has not been reached, i.e. not all the specific binding of the radioligand has been displaced. The concentration of (±)-isoprenaline sulphate chosen to define specific binding in subsequent saturation binding studies was however set at 200 μM, a concentration used in published studies (e.g. Begg & Wade, 1983; Heal et al., 1989b; de Paermentier et al., 1989), as it was felt that use of higher concentrations would attract justified criticism. Since even at such a high concentration of (±)-isoprenaline sulphate not all specific binding has been displaced, then a specific binding curve that is not clearly saturated is perhaps not surprising. This could account for the large variability in the binding data.

The implications of the large variability in the current β-adrenoceptor binding data are that the effects of sibutramine on β-adrenoceptors are masked. Significant reductions of less than 20% in β-adrenoceptor density have been previously reported after

Figure 3.16 **SPECIFIC BINDING OF [^{3}H]-DHA DEFINED USING (±)-ISOPRENALINE SO4 IN THE RAT CORTEX (SAMPLE A5)**

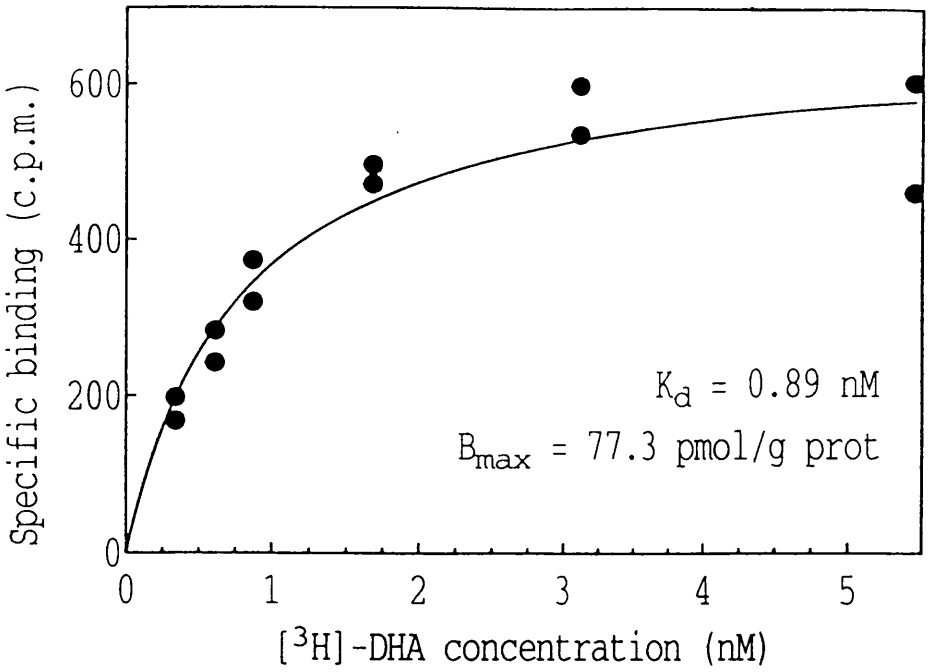


Figure 3.16 Specific binding calculated by subtracting non-specific binding ([^{3}H]-DHA binding in the presence of isoprenaline) from total [^{3}H]-DHA binding at each radioligand concentration. Data analysed using `LIGAND'; Kd and Bmax as indicated.

Figure 2.1a **DISPLACEMENT OF [^{3}H]-DHA BINDIING BY (±)-ISOPRENALINE SO4 IN RAT CORTEX**

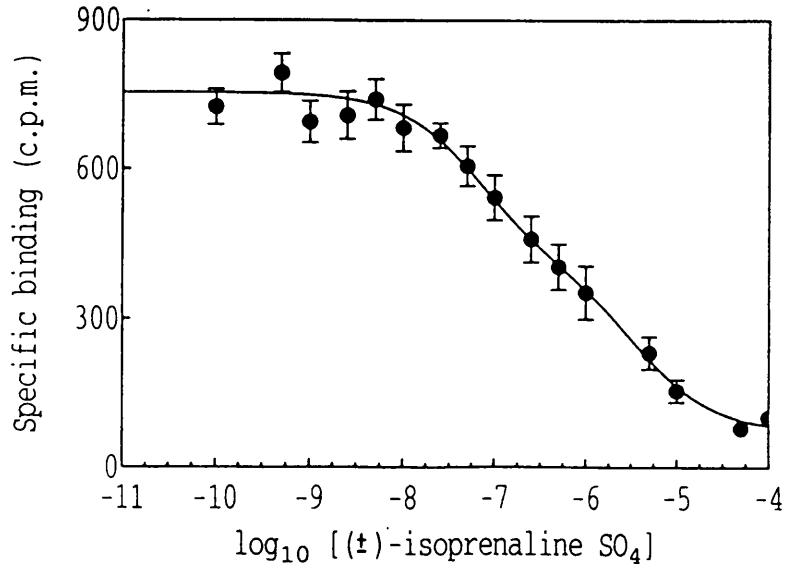


Figure 2.1a Displacement of [^{3}H]-DHA in rat cortex using (±)-isoprenaline SO4. Data shown were best described by a 1-site fit (F = 1.734; d.f. = 2,13; $p > 0.05$)

administration of other tricyclic monoamine uptake inhibitors e.g. amitriptyline, desipramine (Asakura et al., 1982; Sugrue, 1983). Yet in the current experiment, an 18% reduction in density after repeated sibutramine administration failed to reach significance. The 24% increase in β-adrenoceptor density in sibutramine-injected animals after exposure to the open field is also not significant, therefore. This increase suggests an interaction between sibutramine and exposure to novelty on β-adrenoceptors, yet this cannot be proven. The large variability arising from the non-saturability of the isoprenaline-defined specific binding of [^{3}H]-DHA suggests strongly that even isoprenaline and [^{3}H]-DHA is not the ideal ligand combination to define β-adrenoceptor binding.

A number of behaviours reflecting resistance to stress were increased by both saline and sibutramine administration in the open field. Therefore, on the basis of Stone's hypothesis linking β-adrenoceptor down-regulation with increased stress resistance, it would be predicted that both pretreatments would reduce β-adrenoceptor density. However, the above results show that receptor numbers in saline- or sibutramine-pretreated animals did not differ significantly from uninjected animals. The lack of parallelism between group mean differences in β-adrenoceptor density and resistance to stress contrasts with Stone's findings. However, Stone's hypothesis was based on studies of forms of stress such as food deprivation and immobilization. It would seem therefore that Stone's hypothesis does not apply to forms of stress such as novelty at least.

In summary, neither a single exposure to novelty nor repeated saline injection altered cortical β-adrenoceptors in the rat. This contrasts with earlier findings. Although differences in duration of stress exposure may be responsible for these discrepancies, it is also likely that the previously reported changes reflect stress-induced changes in an as yet unidentified receptor (possibly one or more 5-HT$_1$ receptor subtypes) which is a component of the specific binding of [^{3}H]-DHA defined with propranolol. Neither previous experience of repeated stress nor antidepressant administration affected cortical β-adrenoceptors after exposure to the open field. Collectively, these findings do not support Stone's hypothesis.

3.5.3 Cortical β-adrenoceptors and behavioural indices of resistance to stress

In the current experiment, where cortical β-adrenoceptor binding density was defined using isoprenaline to define specific binding of [^{3}H]-DHA, correlation coefficients were calculated to determine if individual differences in β-adrenoceptors were related to stress

resistance.

In a previous report from this laboratory, when propranolol was used to define specific binding of [^{3}H]-DHA (Salmon & Stanford, 1989), a significant positive correlation was found between [^{3}H]-DHA binding and resistance to stress. In the current experiment, when all treatment groups were considered together, a similar correlation between stress resistance and [^{3}H]-DHA binding when specific binding is defined using isoprenaline was found. This indicates that β-adrenoceptors and resistance to stress are indeed related; animals with the highest levels of β-adrenoceptors show greatest levels of stress resistance. This correlation was significant only for the final 3 min of the test. This further confirms the dissociation between behaviour in the first and the final 3 min of the 4 min open field exposure.

The positive correlation between β-adrenoceptor number and behavioural resistance to stress is again the opposite to that predicted on the basis of Stone's hypothesis. Since group mean β-adrenoceptor density did not parallel changes in centre-field activity, this indicates that for the same group of animals, correlations *between* groups and *within* groups are not the same. For this reason, within group correlations cannot be reliably predicted on the basis of comparisons between groups, and *vice versa.*

When individual treatment groups were considered separately, the only significant correlations were found in sibutramine-pretreated animals. In this group, β-adrenoceptor density was positively correlated with both centre-directed activity and the time spent in the centre of the field. The lack of correlation between these parameters in uninjected and saline-pretreated groups could indicate that the link between β-adrenoceptors and stress resistance depends on the animals' previous experience. However, when correlations were calculated for individual treatment groups, there were only 14 animals in each group. It is likely therefore that the lack of correlation between β-adrenoceptors and resistance to stress in uninjected and saline-pretreated animals is simply due to the small sample size.

In the current experiment, both treatment effects on group mean behavioural scores and the `within groups' correlations between β-adrenoceptors and stress resistance are only apparent in the final 3 min of the open field test. This suggests a dissociation between the first and the final 3 min of the test. Such a dissociation confirms previous findings from this laboratory (Salmon & Stanford, 1989), in which the correlation between

receptors and stress resistance was also apparent in the last portion of the test only.

In contrast, this earlier report found treatment effects on group mean behavioral scores, namely an increase in the ratio of radial : total locomotion and a reduction of arc movements in injected animals, in the first minute of the test only. Although there is no obvious explanation for this disparity, both experiments indicate that behaviour in the first minute of the test is clearly different from that in the final 3 min. It is also apparent that the effects of a given treatment on behaviour at different times of the test do not always parallel those over the entire course of the test. This highlights the importance of characterizing both the time course and magnitude of changes in behaviour in any behavioural test.

3.5.4 Conclusions

1) A 4 min exposure to the open field had no effect on cortical β-adrenoceptor binding density
2) The initial behavioural impact of exposure to the open field was unaltered by stress or sibutramine pretreatment.
3) Measures of behavioural resistance to stress during the final 3 min of the test were increased by previous experience of repeated stress and by sibutramine administration.
4) Neither previous experience of repeated stress nor repeated sibutramine administration altered cortical β-adrenoceptors after exposure to the open field.
5) There was no relationship between group mean scores of β-adrenoceptor density and behavioural resistance to stress.
6) When 'within groups' comparisons are made, β-adrenoceptor density is correlated positively with behavioural resistance to stress in the open field.
7) This infers that the neurochemical coding of behavioural resistance to stress in groups of animals are not the same as when individuals are studied.
8) Stone's hypothesis is not supported by studies of either 'between group' or 'within group' behavioural differences in the open field.

4.0 A COMPARISON OF THE EFFECTS OF REPEATED STRESS (ONCE-DAILY SALINE INJECTION) AND REPEATED SIBUTRAMINE ADMINISTRATION ON CENTRAL β-ADRENOCEPTORS AND 5-HT$_2$ RECEPTORS.

4.1. Introduction

In the previous chapter, it was shown that the behavioural response to the acute stress of exposure to a novel open field is modified by previous experience of repeated stress (once-daily saline injection). Repeated administration of the drug, sibutramine, also altered the behavioural response to the open field. Sibutramine is a noradrenaline plus 5-HT reuptake inhibitor which rapidly down-regulates cortical β-adrenoceptors in rats. In preclinical tests predictive of antidepressant activity in man, in the Porsolt test for instance, this drug has effects similar to established antidepressants which also block monoamine reuptake (Buckett et al., 1988).

Parallels between behavioural adaptation to stress and the therapeutic effects of antidepressants have been highlighted by Stone (Stone, 1979b). However, experiments using the open field paradigm give cause to question whether this parallelism applies universally, as is generally presumed. For instance, whereas both sibutramine and repeated stress increased centre-directed movements and rearing, the increase in grooming induced by repeated stress was prevented by sibutramine pretreatment. One obvious explanation for the discrepancy between behaviour in the open field and that predicted on the basis of Stone's hypothesis could be that the effects of stress or drugs on the response to psychological stress (e.g. novelty) do not generalize to the forms of physical stress (immobilization and footshock) used by Stone.

One factor limiting interpretation of results from the open field is the variation in the methods described in published literature. In further appraisal of Stone's theory, it was therefore considered important to compare the effects of stress and sibutramine on behaviour using a paradigm with an accepted standard protocol and for which behavioural changes have been consistently validated. The paradigm chosen was the swim test, for which the behavioural effects of a wide range of psychotropic drugs have been characterized (e.g. Porsolt et al., 1977b; reviewed by Porsolt, 1981). Moreover, this test is used routinely to screen for putative antidepressant drugs including sibutramine (e.g. Buckett & Luscombe, 1985; Buckett et al., 1988). The ultimate objective therefore was to compare the effects of repeated sibutramine administration and repeated saline injection on behaviour in the swim test.

One problem with adopting the swim test is that the standard protocol for rats involves *two* sessions of swimming: a conditioning swim followed by a test swim 24 h later (Porsolt et al., 1977a). However, the aim of the present study was to examine the effects of repeated saline injection or sibutramine administration on behaviour during a *single* stress exposure. In mice though, measurement of the behavioural effects of psychotropic drugs in the swim test involves only a single 6 min swim (Porsolt et al., 1977b). For this reason, it was considered more appropriate to use mice in these experiments.

Although the effects of drugs on behaviour in the swim test have been widely studied, the neurochemical effects of this stressful procedure have largely been ignored. A further objective was to investigate certain neurochemical effects of the swim test, and to examine the influence of repeated saline injection or sibutramine administration on these effects. A further complication is that, unlike rats, effects of repeated stress and antidepressants have been studied very little in other species, and in the few existing reports, results are inconsistent. For instance, repeated administration of the monoamine uptake inhibitor, desipramine (DMI; Horn. 1976; Harms, 1983), does not consistently down-regulate cortical β-adrenoceptors in mice (*cf* Suzdak & Gianutsos, 1986; Stanford et al., 1987), and is reported to have no effect in guinea-pigs (Hu et al., 1980). It is essential therefore first to establish the neurochemical effects of repeated saline injection and sibutramine administration in mice, before going on to investigate the interaction of these treatments with the neurochemical effects of an acute swim.

Although the neurochemical effects of swim stress have been little studied, it is apparent that the response to swim stress involves both noradrenergic and serotonergic neurones. A swim-induced increase both in 5-hydroxyindoleacetic acid (5-HIAA) and in 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in various brain regions has been reported (Miyauchi et al., 1981; Ikeda & Nagatsu, 1985), indicating increased turnover of 5-HT and noradrenaline. However, the effects of swim stress on noradrenergic and serotonergic receptors have not been studied.

Changes in cortical β-adrenoceptors are of particular interest since these receptors are consistently down-regulated by both repeated stress (reviewed by Stanford, 1990) and administration of monoamine reuptake inhibitors (e.g. Asakura et al., 1982; Heal et al., 1989b) in rat cortex; moreover, these receptors are also the focus of Stone's theory (Stone, 1979b). However, one problem highlighted by results from the experiment described in Chapter 3 concerns the combination of ligands commonly used to define β-adrenoceptor

binding. Many studies have reported a reduction in cortical β-adrenoceptor density after repeated exposure to one of a range of different forms of stress (e.g. tailshock: Nomura et al., 1981; food deprivation: Stone & Platt, 1982), including once-daily saline injection (Stanford et al., 1984). The majority of these studies have used [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) as the radioligand, with (±)-propranolol to define specific binding. It has been shown that binding density (B_{max}) when specific binding of [^{3}H]-DHA was defined using propranolol is significantly higher than when isoprenaline is used, in rat cortex at least (Atterwill et al., 1984; Riva & Creese, 1989a). The findings reported in Chapter 3, which used the more selective β-adrenoceptor ligand isoprenaline, suggest that a non-β-adrenoceptor exposed by [^{3}H]-DHA and propranolol could account for the reduction of [^{3}H]-DHA specific binding induced by repeated saline injection. β-adrenoceptors therefore are apparently not altered by repeated saline injection when specific binding of [^{3}H]-DHA is defined using the more selective ligand isoprenaline. To test this further, the effect of stress or monoamine uptake inhibitors on specific binding of [^{3}H]-DHA defined using isoprenaline was compared with binding of (-)-4-(3-t-butylamino-2-hydroxypropoxy)-[5,7,-^{3}H]-benzimidazol-2-one ([^{3}H]-CGP 12177), which has high affinity for β-adrenoceptors and low non-specific binding (de Paermentier et al., 1989). Differences between binding estimates obtained with these two different radioligands have been shown in rats and have been attributed to the binding of [^{3}H]-DHA to a 5-HT receptor (Riva & Creese, 1989a). The current study was carried out in mice, for reasons described above; however, the use of mice also allows us to determine if the binding of [^{3}H]-DHA and [^{3}H]-CGP 12177 are the same in mice, given that it is not clear whether or not [^{3}H]-DHA can be displaced by isoprenaline from more than one binding site in this species (Section 2.3.1.1). Only if quantitatively similar changes in β-adrenoceptor binding density were seen with these two radioligands could it be concluded that stress- or drug-induced changes in the binding of [^{3}H]-DHA were explained by changes in β-adrenoceptors alone.

In comparison to β-adrenoceptors, practically nothing is known about the effects of either acute or repeated exposure to stress on serotonergic receptors. This neglected aspect of stress research is surprising since it has long been claimed that 5-HT transmission is increased by stress. The large number of 5-HT receptor subtypes (reviewed by Hoyer & Schoeffter, 1991) complicates this field, but the role of 5-HT2 receptors is particularly relevant to the present experiments. These receptors, which are predominantly postsynaptic, may be functionally analogous to β-adrenoceptors, which are also postsynaptic. In the current experiments, cortical 5-HT2 receptor binding studies were carried out to determine the effects of repeated saline injection and sibutramine

administration on these receptors.

A common criticism of radioligand binding studies is that they provide no information about the functional state of the receptor. Studies of receptor function either *ex vivo* or *in vivo* are therefore often used to complement information obtained from binding studies. In the current experiments radioligand binding studies of 5-HT2 receptors were paralleled by measurement of central 5-HT2 receptor function *in vivo*, therefore.

Measurement of phosphoinositide (PI) turnover, the second messenger linked to 5-HT2 receptors, is used to measure 5-HT2 receptor function *ex vivo*. However, such measurements of receptor function provide no information about the overall response to receptor activation, which may be influenced by other neurotransmitter systems. In addition, since fresh, not frozen tissue is necessary for this assay, it is only possible to handle a small number of animals simultaneously. This was impractical in the current experiment, since it was necessary to carry out behavioural testing (swim test) and, subsequently, measurement of 5-HT2 receptor function in a large number of animals on the same day. A further problem with some methods for measuring phosphoinositide turnover is that they involve intracerebroventricular (i.c.v.) injection of radiolabelled inositol, 24-48 h before animals are killed (Hide et al., 1989; Whitworth et al., 1990). Such methods were not suitable for use in the current experiments, since it was felt that the i.c.v. injection procedure could interfere with the repeated stress (saline injection) pretreatment which some animals received. However, the 5-MeODMT-induced head-twitch is a simple alternative model of 5-HT2 receptor function *in vivo*, which avoids these problems. The head-twitch response induced in rodents by 5-HT agonists and precursors has been shown to depend on 5-HT2 receptor activation (reviewed by Heal et al., 1992). Consequently, this model is often used as an index of central 5-HT2 receptor function *in vivo*. Unfortunately, available models of β-adrenoceptor function *in vivo* have been shown to involve multiple central adrenoceptors (reviewed by Heal, 1990). It was therefore not possible to carry out an equivalent evaluation of of central β-adrenoceptor function *in vivo*.

4.2 Aims

1) To determine the long-lasting effects (+24 h) of repeated once-daily saline injection on cortical β-adrenoceptor binding in the mouse.
2) To determine the long-lasting effects of repeated sibutramine administration on cortical β-adrenoceptor binding in the mouse, using the established antidepressant

desipramine as an active control.

3) To compare the binding of [^{3}H]-DHA with that of the more selective β-adrenergic radioligand [^{3}H]-CGP 12177, and to establish whether stress- and drug-induced changes in [^{3}H]-DHA binding are explained by changes in β-adrenoceptors alone.
4) To determine long-term effects (+ 24 h) of repeated saline injection and sibutramine administration on cortical 5-HT2 receptors and 5-HT2 receptor-mediated head-twitches in the mouse.

4.3 Methods

4.3.1 Animals and treatments

Male CD1 mice (18-20 g on arrival in the animal house) were housed under a 12/12 h light/dark cycle (lights on from 08.00-20.00 h), with free access to food and water. Animals were assigned to treatment groups, matched for mean weight, destined for stress (saline injection) or drug pretreatment (sibutramine or desipramine), or no pretreatment ('uninjected'). The size of the groups depended on the experiment. Each cage contained not less than 3 mice from one treatment group only. All animals remained unhandled (apart from routine husbandry) for at least 5 days before the start of the experiment to allow acclimatization to the new surroundings. Following this acclimatization period, animals from the stress- and drug-pretreatment groups were weighed. They then received an injection of either 0.9% sterile saline (10 ml/kg i.p.), sibutramine hydrochloride (sibutramine; 3 mg/kg i.p.) or desipramine (DMI; 10 mg/kg i.p.). All injections were given between 09.30-10.00 h. This procedure was repeated once-daily for a total of 10 days. Unhandled groups remained undisturbed in their home cage throughout this period.

The experiments described in this chapter comprise three separate studies. First, the effects of repeated stress and repeated administration of monoamine uptake inhibitors on cortical β-adrenoceptor binding were determined. In this study, the binding of two different radioligands, [^{3}H]-DHA and [^{3}H]-CGP 12177 was compared in uninjected, saline-DMI- and sibutramine-pretreated groups. The second study examined the effects of stress and sibutramine on cortical 5-HT2 receptors. Binding in saline- and sibutramine-injected groups was measured in separate experiments; in each of these experiments, binding in the treated group was compared with that in a parallel uninjected control group. The final study examined the effects of stress and sibutramine administration on central 5-HT2 receptor function *in vivo*. 5-MeODMT-induced head-twitches in saline- and sibutramine-

injected animals were measured in the same experiment, which also included an uninjected group as a control.

4.3.2 Receptor binding

24 h after the final injection all animals, including the uninjected group, were removed in their home cages to an adjacent room, and killed by cardiothoracic shock and cervical dislocation. Brains were removed and cerebral cortices were dissected over ice then frozen on dry ice. These were stored at -20°C.

Washed membranes for β-adrenoceptor binding were prepared freshly for each binding assay as previously described (see section 2.3.1.2). [^{3}H]-DHA or [^{3}H]-CGP 12177 were used as β-adrenergic radioligands. Specific binding of both radioligands was defined using 100 μM (-)-isoprenaline HCl (see section 2.3.1.2). The binding of each of these radioligands was measured simultaneously in duplicate aliquots of membrane preparations. Each binding assay contained at least one sample from each treatment group. Typical binding curves from one sample showing the specific binding of these two β-adrenergic radioligands are shown in Figures 4.1 and 4.2.

Washed membranes for 5-HT2 receptor binding were prepared freshly for each binding assay as previously described (see section 2.3.1.3). [^{3}H]-Ketanserin was used as the radioligand, and specific binding to 5-HT2 receptors was defined using 5 μM methysergide. Each binding assay contained at least one sample from each treatment group.

4.3.3 5-HT2 receptor-mediated head-twitches

24 h after the final injection, animals from all groups, including the 'unhandled' group, were individually removed into an adjacent room. Head-twitches were induced by i.p. injection of 5-methoxy-N,N-dimethyltryptamine (2 mg/kg) and counted for 6 min after the injection, as described previously (see section 2.2.3). Head-twitches were scored independently by two observers, at least one of whom was "blind" to the treatments which the animals had received.

4.3.4 Statistics

β-Adrenoceptor K_d and B_{max} values, and head-twitch data were analyzed using a 1-way ANOVA. Where a significant treatment effect was seen, all group means were subsequently compared using unpaired t-tests.

Figure 4.1 **SPECIFIC [^{3}H]-DHA BINDING DEFINED USING 100 µM (-)-ISOPRENALINE HCl (SAMPLE D1)**

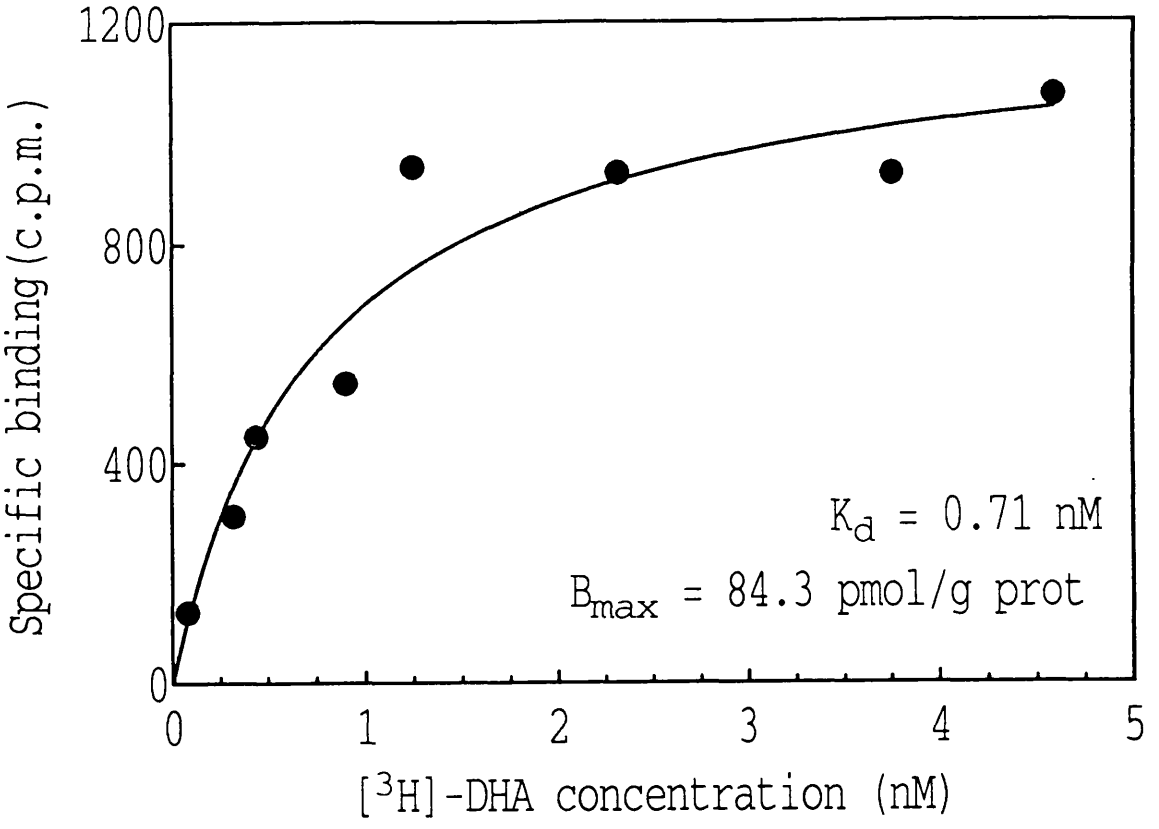


Figure 4.1 : Specific binding calculated by subtracting non-specific binding ([^{3}H]-DHA binding in the presence of isoprenaline) from total [^{3}H]-DHA binding at each radioligand concentration. Data analysed using 'LIGAND'; K_d and B_{max} values as indicated

Figure 4.2 **SPECIFIC [^{3}H]-CGP 12177 BINDING DEFINED USING 100 µM ISOPRENALINE HCl (SAMPLE D1)**

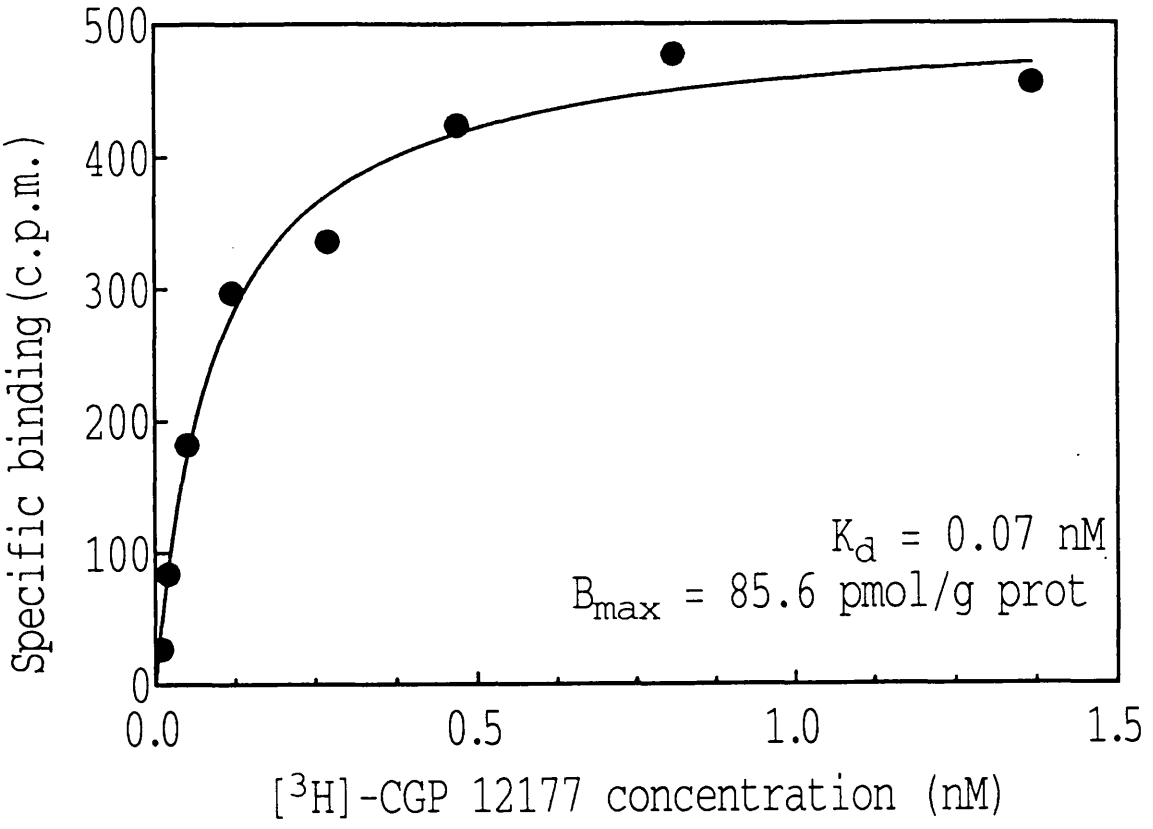


Figure 4.2: Specific binding calculated by subtracting non-specific binding ([^{3}H]-CGP 12177 binding in the presence of isoprenaline) from total [^{3}H]-CGP 12177 binding at each radioligand concentration. Data analysed using `LIGAND'; Kd and Bmax values as indicated.

For a direct comparison of binding defined using [^{3}H]-DHA or [^{3}H]-CGP 12177, a 3-way ANOVA was used. This tested for:

(i) a significant 'treatment' effects

(ii) a significant 'ligand effect'; that is, whether there were differences between binding defined using the two ligands

(iii) any 'treatment x ligand' interaction; that is, whether the size of treatment-induced changes in binding depend on the ligand used to measure binding.

A third factor was included to account for the fact that binding of the two ligands was measured in tissues derived from the same animals. Again, where significant effects were found, group means were subsequently compared using unpaired t-tests.

Since experiments studying 5-HT2 binding contained only two treatment groups (an uninjected group and either a saline- or a sibutramine-pretreated group), 5-HT2 receptor K_d and B_{max} values were analyzed using the Mann-Whitney 'U' test.

4.4 Results

4.4.1 Cortical β-adrenoceptors

4.4.1.1 *Repeated saline injection*

β-Adrenoceptor density measured using [^{3}H]-DHA was not significantly altered by repeated once-daily saline injection ($F = 4.837$; d.f. = 3,36; $p < 0.01$: $t = 1.604$; d.f. = 36; $p > 0.05$). This was despite an 18% reduction in binding B_{max} in this group compared with uninjected animals (Figure 4.3). In contrast, [^{3}H]-CGP 12177 binding density in membranes prepared from the same tissues was unaltered (+1%) in saline-pretreated animals, compared with the uninjected group ($F = 3.439$; d.f. = 3,40; $p < 0.05$: $t = 0.109$; d.f. = 40; $p > 0.05$). The B_{max} value in control tissues was slightly higher when measured using [^{3}H]-DHA compared with that defined using [^{3}H]-CGP 12177. This accounts for the apparent, albeit non-significant, effects of repeated saline injection when [^{3}H]-DHA binding was measured.

β-Adrenoceptor K_d was unaltered in saline-pretreated animals compared with uninjected animals, when measured with either [^{3}H]-DHA ($F = 0.838$; d.f. = 3, 36; $p > 0.05$) or [^{3}H]-CGP 12177 ($F = 0.443$; d.f = 3, 40; $p > 0.05$: Table 4.1).

Figure 4.3 **THE EFFECT OF REPEATED SALINE INJECTION OR ANTIDEPRESSANT ADMINISTRATION ON CORTICAL β-ADRENOCEPTOR BINDING DENSITY**

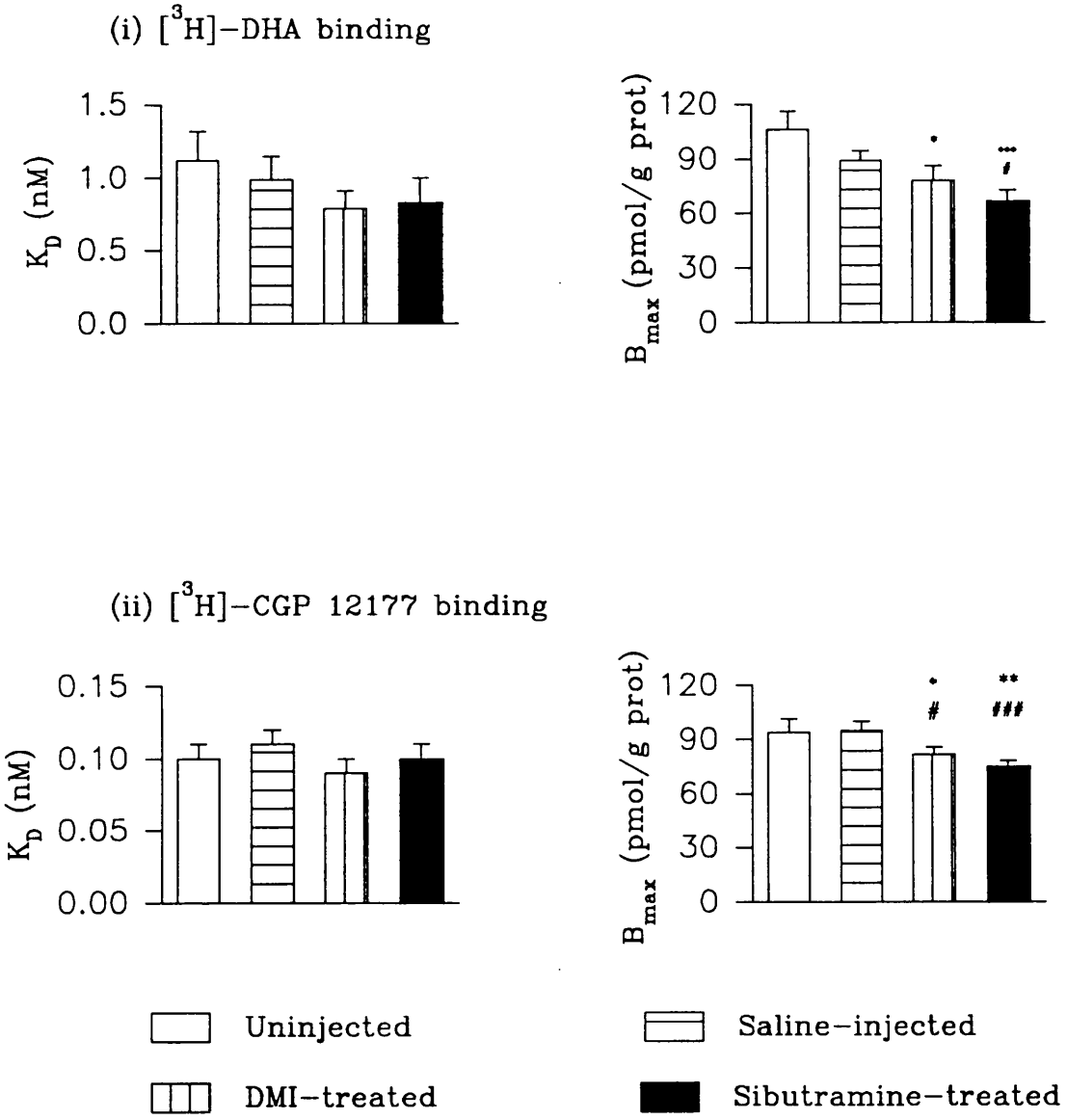


Figure 4.3: n = 10, each group, [^{3}H]-DHA binding; n = 11, each group, [^{3}H]-CGP 12177 binding. β-adrenoceptor binding measured 24 h after the final injection; [^{3}H]-DHA and [^{3}H]-CGP 12177 binding carried out in simultaneous assays using duplicate aliquots of tissue from the same animals. Data for each ligand analysed separately using 1-way ANOVA. Following a significant treatment effect, group means were compared using unpaired t-tests: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *cf* uninjected controls; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *cf* saline-injected group.

Table 4.1 **CORTICAL β-ADRENOCEPTOR Kd: EFFECT OF REPEATED SALINE INJECTION OR ADMINISTRATION OF MONOAMINE UPTAKE INHIBITORS**

	β-adrenoceptor Kd (nM)	
	[^{3}H]-DHA	[^{3}H]-CGP 12177
Uninjected controls	1.12 ± 0.20	0.10 ± 0.01
Saline-injected	0.99 ± 0.16	0.11 ± 0.01
DMI-injected	0.79 ± 0.12	0.09 ± 0.01
Sibutramine-injected	0.83 ± 0.17	0.10 ± 0.01

[^{3}H]-DHA binding : n = 10, each group. [^{3}H]-CGP 12177 binding: n = 11, each group. Radioligand binding was measured 24 h after the final injection; [^{3}H]-DHA and [^{3}H]-CGP 12177 binding were measured simultaneously in duplicate aliquots of membrane preparations from the same animals. Data obtained with each ligand was analyzed separately by 1-way ANOVA.

4.4.1.2 *Repeated administration of sibutramine or DMI*

When compared with uninjected animals, both repeated DMI and sibutramine pretreatment significantly reduced β-adrenoceptor density measured using [^{3}H]-DHA ($F = 4.837$; d.f. = 3, 36; $p < 0.01$: ts = 2.636, 3.696; d.f. = 36; ps < 0.01, < 0.001 respectively: Figure 4.3). Both drugs also significantly reduced binding density measured using [^{3}H]-CGP 12177 ($F = 3.439$; d.f. = 3, 40; $p < 0.05$: ts = 1.713, 2.549; d.f. = 40; ps < 0.05, < 0.001 respectively). Although these data are derived from the same animals, the magnitude of DMI- and sibutramine-induced reductions in β-adrenoceptor B_{max} estimated with [^{3}H]-CGP 12177 (-9%, -16%, respectively) were considerably smaller than those seen using [^{3}H]-DHA (-25%, -34% respectively).

Neither DMI or sibutramine altered binding K_d compared with uninjected animals, whether binding was defined using [^{3}H]-DHA ($F = 0.838$; d.f. = 3, 36; $p > 0.05$) or [^{3}H]-CGP 12177 ($F = 0.443$; d.f. = 3, 40; $p > 0.05$: Table 4.1).

The effects of drug treatment on receptor binding were also compared with the saline-injected group. Repeated sibutramine administration significantly reduced β-adrenoceptor binding density measured with either [^{3}H]-DHA ($F = 4.837$; d.f. = 3, 36; $p < 0.01$: $t = 2.036$; d.f. = 36; $p < 0.05$) or [^{3}H]-CGP 12177 ($F = 3.439$; d.f. = 3, 40; $p < 0.05$: $t = 2.458$; d.f. = 40; $p < 0.001$) when compared with the saline-injected group. However, repeated DMI administration significantly reduced [^{3}H]-CGP 12177 binding only ($F = 3.439$; d.f. = 3, 40; $p < 0.05$: $t = 1.823$; d.f. = 40; $p < 0.05$); [^{3}H]-DHA binding in the same tissues was not significantly reduced compared with the saline-injected group ($F = 4.837$; d.f. = 3, 36; $p < 0.01$: $t = 1.032$; d.f. = 36; $p > 0.05$).

4.4.1.3 *Direct comparison of binding defined using [^{3}H]-DHA and [^{3}H]-CGP 12177*

The above results suggest that binding defined using [^{3}H]-DHA is not identical to that defined with [^{3}H]-CGP 12177. To further investigate this possibility, a 3-way ANOVA was used in which multiple comparisons were made between β-adrenoceptor binding density defined with the two different radioligands.

The 3-way ANOVA confirmed the significant effect of treatment on β-adrenoceptor density ($F = 12.47$, d.f = 3, 48; $p < 0.001$). When differences in group means were subsequently evaluated, it was confirmed that, compared with uninjected controls, repeated saline injection did not alter mouse cortical β-adrenoceptor density, regardless of the ligand used ($t = 0.877$; d.f. = 48; $p > 0.1$). In contrast, both DMI and sibutramine

decreased β-adrenoceptor density (ts = 3.869, 5.331; d.f. = 48; ps < 0.001, < 0.001 respectively), regardless of the ligand used. Unlike the results of the 1-way ANOVA, both drugs reduced β-adrenoceptor density compared with saline-injected animals (ts = 2.990, 4.453; d.f. = 48; ps < 0.001 respectively).

There was no significant difference between β-adrenoceptor density defined with the two different ligands (i.e. no significant 'ligand' effect: F = 0.21; d.f. = 1, 48; p > 0.05). In addition, there was no treatment x ligand interaction i.e. the size of the change in β-adrenoceptor density after saline or drug treatment did not depend on the radioligand (F = 0.245; d.f. = 3, 48; p > 0.05). This is despite the finding that the drug-induced reductions in [^{3}H]-DHA binding were almost twice that found using [^{3}H]-CGP 12177.

4.4.2 Cortical 5-HT2 receptors

Once-daily saline injections did not alter mouse cortical 5-HT2 receptor density or affinity, measured 24 h after the final injection ('U's = 31, 36 respectively; d.f. = 9, 9; p > 0.05: Table 4.2).

Administration of sibutramine once-daily for 10 days did not change cerebral cortical 5-HT2 receptor affinity or density compared with an unhandled control group ('U's = 41, 47; d.f. = 9,9; p > 0.05: Table 4.2).

4.4.3 5-MeODMT-induced head-twitches

Once-daily saline injection for 10 days did not alter the frequency of 5-MeODMT-induced head-twitches when measured 24 h after the final injection (F = 0.134; d.f. = 2, 27; p > 0.05: Table 4.3).

Repeated sibutramine administration (once-daily for 10 days) had no effect on the frequency of head-twitches induced by 5-MeODMT, 24 h after the final sibutramine treatment (F = 0.134; d.f. = 2, 27; p > 0.05: Table 4.3).

Table 4.2 **THE EFFECTS OF REPEATED SALINE INJECTION OR SIBUTRAMINE ADMINISTRATION ON MOUSE CORTICAL 5-HT2 RECEPTORS**

	[^{3}H]-Ketanserin binding	
	Kd (nM)	Bmax (pmol/g prot)
Uninjected control	0.88 ± 0.12	154.3 ± 14.5
Saline-injected	0.90 ± 0.12	180.1 ± 17.3
Uninjected control	0.41 ± 0.07	146.8 ± 13.2
Sibutramine-injected	0.39 ± 0.06	148.5 ± 22.5

n=9, all groups. Separate experiments carried out to measure [^{3}H]-ketanserin binding in saline- and sibutramine-pretreated groups; each experiment included an uninjected control group. Data from treatment groups compared with simultaneous control group using the Mann-Whitney 'U' test.

Table 4.3 **THE EFFECT OF REPEATED SALINE OR SIBUTRAMINE HCl ADMINISTRATION ON 5-MeODMT-INDUCED HEAD-TWITCHES IN THE MOUSE**

	Total head-twitches over 6 min
Uninjected controls	4.1 ± 0.6
Saline-injected	4.0 ± 0.5
Sibutramine-injected	3.7 ± 0.4

n=10 all groups. 5-HT2 receptor-mediated head-twitches measured for 6 min after 5-MeODMT administration (2 mg/kg i.p.), 24 h after the final saline or sibutramine injection. Data analyzed using 1-way ANOVA.

4.5 Discussion

The experiments described here were carried out to determine the effects of repeated saline injection or the administration of monoamine reuptake inhibitors on mouse cortical β-adrenoceptors and 5-HT2 receptors and on central 5-HT2 receptor function *in vivo*. Measurements were made 24 h after the final injection to ensure that prolonged, not immediate effects of these treatments were investigated. This sampling time was also chosen as these experiments were designed to form the basis of subsequent work studying the effects of saline or sibutramine pretreatment on neurochemical effects of an acute swim stress, 24 h after the final injection.

4.5.1 Cortical β-adrenoceptors

4.5.1.2 *Repeated saline injection*

A reduction in cortical β-adrenoceptors in rats has been reported after repeated exposure to any one of a range of different forms of stress (reviewed by Stanford, 1990). Such a reduction has even been reported after once-daily saline injection (Stanford et al., 1984). However, data discussed in Chapter 3 suggested that this effect of repeated saline injection may not involve β-adrenoceptors. Rather, the reduction may be due, at least in part, to a down-regulation of a second, non-β-adrenergic binding site to which [^{3}H]-DHA also binds and which is exposed when propranolol is used to define specific binding. To test whether or not this was the case, the effect of repeated saline injection on cortical β-adrenoceptors, defined using the selective β-adrenergic radioligand [^{3}H]-CGP 12177, was compared with binding measured with [^{3}H]-DHA.

When [^{3}H]-CGP 12177 was used, β-adrenoceptor binding density in mouse cortex was unaltered (+1% compared with uninjected animals) after repeated saline injection. However, there was an 18% reduction, albeit insignificant, when β-adrenoceptor binding density was measured in the same tissue using [^{3}H]-DHA. A difference in binding in the tissues from uninjected controls accounts for this apparent stress-induced reduction in β-adrenoceptor B_{max} when using [^{3}H]-DHA. Since receptor density in the control group was 12% greater, albeit nonsignificantly, when using [^{3}H]-DHA compared with [^{3}H]-CGP 12177, it may suggest that isoprenaline displaces a higher proportion of [^{3}H]-DHA than [^{3}H]-CGP 12177. One possible explanation for this is that [^{3}H]-DHA may be displaced by isoprenaline from a second site to which [^{3}H]-CGP 12177 does not bind.

The results obtained using [^{3}H]-CGP 12177 show clearly that repeated stress (once-daily saline injection) does not alter mouse cortical β-adrenoceptor density. Although this

cannot be assumed to be the case in all species, it is notable that in rats, apparent repeated stress-induced reductions in cortical β-adrenoceptor density have been shown using [^{3}H]-DHA (Nomura et al., 1981; Stone & Platt, 1982; Cohen et al., 1986). If indeed repeated stress does not alter β-adrenoceptor density in this species, then the reduction in [^{3}H]-DHA binding may be due to a reduction in binding to the 5-HT site to which this radioligand binds in rats. It is important therefore to reevaluate the effects of repeated stress in rat cortex using a more β-adrenoceptor selective combination of ligands, to determine whether repeated stress causes a reduction in β-adrenoceptor density in this species. However, repeated exposure to stress does reduce cortical β-adrenoceptor-stimulated adenylate cyclase activity (Stone, 1979a; Stone et al., 1985) suggesting that cortical β-adrenoceptors are involved in the response to repeated stress; this has not been measured after repeated saline injection, however.

4.5.1.2 *Repeated antidepressant administration*

There are numerous reports that monoamine uptake inhibitors and antidepressants from other generic groups down-regulate cortical β-adrenoceptors in the rat after repeated administration. However, this change has not been consistently found in other species (guinea-pig: Hu et al., 1980; CD1 mice: Stanford et al., 1987). In the current experiment, repeated administration of sibutramine, like the established monoamine uptake inhibitor antidepressant, DMI, reduced [^{3}H]-DHA binding in mouse cortex compared with uninjected controls. This was explained by a reduction in the number of binding sites since there was no change in affinity. That this change reflects reduced β-adrenoceptor density is corroborated by the parallel changes in the binding of the selective β-adrenergic radioligand, [^{3}H]-CGP 12177.

Since both drugs were administered by repeated injection, changes in binding were also compared with the saline-injected group. In this case, [^{3}H]-DHA binding was reduced after repeated sibutramine, but not DMI administration. This supports the finding from an earlier study that DMI has no effect on [^{3}H]-DHA binding when compared with that in vehicle-injected mice (Stanford et al., 1987). However, when binding was measured using [^{3}H]-CGP 12177, β-adrenoceptor density was reduced by both sibutramine and DMI compared with the saline-injected group. The [^{3}H]-CGP 12177 binding data suggests that repeated administration of monamine uptake inhibitors reduces cortical β-adrenoceptor density in mice compared with both uninjected and vehicle-injected groups, therefore. This highlights the difference in [^{3}H]-DHA binding in DMI treated animals which was not significantly reduced compared with saline-injected animals. In explaining this

difference, it must be noted that the reduction in binding induced by DMI as a percentage of binding in saline-injected animals is similar whether [^{3}H]-DHA or [^{3}H]-CGP 12177 binding is considered (-12%, -14% respectively). The lack of significance of the DMI-induced reduction in [^{3}H]-DHA binding compared with the saline-injected group probably results from higher variability of the data, whihc may arise from the lipophilicity of this ligand.

Changes induced by sibutramine and DMI in [^{3}H]-DHA binding were almost twice those seen with [^{3}H]-CGP 12177; this finding suggests differences in the binding of these two radioligands. It has not been conclusively shown that [^{3}H]-DHA is displaced by isoprenaline from only one site in mouse cortex (Section 2.3.1.1). If indeed a second site is involved in the binding of this radioligand, then it is possible that a reduction in the density of this second site, in addition to the β-adrenoceptor down-regulation induced by these treatments, could account for the larger changes seen with [^{3}H]-DHA binding. If this is the case, then the effects of antidepressants as well as stress on β-adrenoceptor binding need to be re-evaluated using a more selective combination of ligands, since the binding of [^{3}H]-DHA to the putative second site, which is altered by these treatments, may influence conclusions from studies using this radioligand.

It has been suggested above that the specific binding of [^{3}H]-DHA differs from that of [^{3}H]-CGP 12177 and that this may influence the interpretation of stress- and monoamine uptake inhibitor-induced changes in [^{3}H]-DHA binding. To test this suggestion, the binding of these two radioligands which were assessed in the same tissues was compared directly (see below) using a 3-way ANOVA.

4.5.1.3 *Comparison of the effects of repeated saline injection and antidepressant administration on mouse cortical β-adrenoceptors: binding defined with 2 different radioligands.*

Direct comparison of binding of the adrenergic radioligands [^{3}H]-DHA and [^{3}H]-CGP 12177 in mouse cortex revealed a significant treatment effect on β-adrenoceptor density. Regardless of the ligand used, β-adrenoceptor density was reduced by DMI and sibutramine but not saline compared with uninjected animals; compared with saline-injected animals, receptor density was reduced by both DMI and sibutramine. This pattern of treatment effects is identical to that seen when [^{3}H]-CGP 12177 binding data was analyzed alone; however, when [^{3}H]-DHA binding was considered separately, DMI *did not* reduce binding compared with saline-injected animals. However, the 3-way ANOVA did not show a significant difference in [^{3}H]-DHA and [^{3}H]-CGP 12177 binding

in control tissues, nor was there any significant ligand x treatment interaction. That is, the effects of repeated stress and of repeated antidepressant administration were quantitatively the same, regardless of the ligand used.

The findings of the 3-way ANOVA confirm that cortical β-adrenoceptor density is reduced by repeated DMI or sibutramine administration, but not by repeated saline injection in mice. Moreover, it indicates that there is no significant difference between the binding of [^{3}H]-DHA and [^{3}H]-CGP 12177. It is extremely likely therefore that the lack of significant effects of DMI on [^{3}H]-DHA binding when compared with saline-injected animals is due to the greater variability in binding of this ligand when compared with that of [^{3}H]-CGP 12177. However, it does not explain the differences in size of stress- and drug-induced changes in binding of [^{3}H]-DHA and of [^{3}H]-CGP 12177, which may indicate a difference between the binding of these two β-adrenergic radioligands. This possibility led on to experiments aimed at investigating the identity of any second site to which [^{3}H]-DHA binds in mouse cortex; the effects of both stress and antidepressants on this second binding site were also studied (Chapter 6).

4.5.2 Cortical 5-HT2 receptors

4.5.2.1 *Repeated saline injection*

Several measures of 5-HT turnover such as tryptophan hydroxylase activity or 5-HIAA levels are increased by repeated stress in rat cortex (e.g. Adell et al., 1988, 1989; Boadle-Biber et al., 1989). However, little attention has been given to the effects of repeated stress on 5-HT receptors.

In the current study, repeated stress (once-daily saline injection) did not alter 5-HT2 receptor binding in mouse cortex, 24 h after the final injection. The few studies which have looked for stress-induced changes in 5-HT2 receptor binding have used rats, but also found no changes in cortical 5-HT2 receptor density (Kellar & Bergstrom, 1983; Ohi et al., 1989; Cancela et al., 1990). Together, these studies suggest that neither repeated exposure to stress of either short duration (less than 1 min for saline injection) nor longer duration (1 h for immobilization: Ohi et al., 1989; 2 h exposure for restraint: Cancela et al., 1990) affects cortical 5-HT2 receptor density.

This finding is remarkable because the evidence that stress increases 5-HT turnover in the cortex is undisputed. A reduction in 5-HT2 receptors might be predicted as a consequence of this increase. The failure of stress to reduce 5-HT2 receptor density is hard

to explain. Although repeated exposure to stress lasting at least 1 h are found to cause sustained changes in 5-HT turnover (e.g. Adell et al., 1988, 1989), it is possible that repeated saline injection is simply too brief to affect 5-HT2 receptors. Alternatively, any injection-induced change in 5-HT turnover may simply be insufficient to alter 5-HT2 receptors. However, cortical 5-HT2 receptors are atypical in that their density is not obviously related to receptor stimulation or transmitter availability. For instance, they are down-regulated by repeated administration of either 5-HT2 receptor antagonists or agonists (e.g. Leysen et al., 1986; Buckholz et al., 1988; Pranzatelli, 1991). Such findings have even provoked the suggestion that 5-HT is not the endogenous ligand for 5-HT2 receptors (Apud, 1991). The lack of effect of repeated stress on cortical 5-HT2 receptor binding does not rule out a change in 5-HT2 receptor function however. To investigate this further, the effects of repeated saline injection on central 5-HT2 receptor function *in vivo* was investigated in the present study (Section 4.5.3.1).

4.5.2.2 *Repeated sibutramine administration*

In the rat, repeated administration of the majority of, but not all, antidepressant drugs including noradrenaline plus 5-HT reuptake inhibitors such as imipramine or amoxapine, down-regulates cortical 5-HT2 receptors, 24 h after the final injection (e.g. Peroutka & Snyder, 1980b; Kellar & Bergstrom, 1983; Hamon et al., 1987; Nelson et al., 1990). In contrast in the present study, repeated administration of the non-selective monamine uptake inhibitor sibutramine did not alter cortical 5-HT2 receptors in the mouse, 24 h after the final drug injection. Previous studies in mice have reported down-regulation of cortical 5-HT2 receptors after repeated administration of the monoamine uptake inhibitor DMI but this effect was seen up to 90 min after the final drug injection (Goodwin et al., 1984; Metz & Heal, 1986). This effect could be influenced by the presence of the drug, therefore; however, there was a concomittant increase in 5-HT2 receptor Kd in only one of these two studies (Goodwin et al., 1984). The manufacturers estimate that the half-life of the active metabolite of sibutramine is 6-8 h in rats; the half-life in mice is not known but is probably less than that in rats. Since the affinities (Ki) of the active metabolites of sibutramine for 5-HT2 receptors are ≥ 100 μM (Cheetham, unpublished observation), then by 24 h after the final sibutramine injection, it is unlikely that there is sufficient active compound present to affect the parameters for the binding of [^{3}H]-ketanserin to 5-HT2 receptors. Notwithstanding, the important aspect of this data from the point of view of the current and subsequent experiments is that repeated administration of sibutramine did not alter cortical 5-HT2 receptor binding in the mouse, 24 h after the final drug injection.

4.5.3 5-HT2 receptor function *in vivo*

A lack of change in binding does not preclude the possibility of changes in receptor function. For this reason, central 5-HT2 receptor function *in vivo* was assessed in the current experiments.

In rodents, a characteristic head-twitch is induced by the 5-HT precursor, 5-HTP, and by 5-HT agonists (e.g. Corne et al., 1963; reviewed by Heal et al., 1992). Head-twitches are induced by non-selective 5-HT agonists e.g. 5-MeODMT (Friedman et al., 1983), quipazine (Malick et al., 1977) and 5-HT2 agonists e.g. 2-(4-iodo-2,5-dimethoxyphenyl)-1-methylethylamine (DOI; Darmani et al., 1990). In contrast, agonists with selectivity for 5-HT1 receptors, e.g. 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT; Peroutka, 1986) or 5-methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl)-1H-indole succinate (RU 24969), which is relatively selective for both 5-HT1A and 5-HT1B receptors (e.g. Hoyer & Schoeffter, 1991) do not induce head-twitches (Goodwin & Green, 1985). Moreover, the head-twitch response is inhibited by non-selective 5-HT antagonists e.g. methysergide (Malick et al., 1977). 5-HT2 receptor antagonists also attenuate head-twitches; however, several of these e.g. ritanserin (Goodwin & Green, 1985) also have high affinity for 5-HT1C receptors (e.g. Hoyer & Schoeffter, 1991). This suggests that 5-HT1C receptors could be involved in the head-twitch response. However, this is unlikely as head-twitches are attenuated by 5-HT2 receptor antagonists such as ketanserin and spiperone (Darmani et al., 1990), which show little activity at 5-HT1C receptors (Hoyer & Schoeffter, 1991).

Although 5-HT2 receptor activation is generally regarded as being responsible for head-twitches, many other neurotransmitters have a modulatory role. Noradrenaline and GABA have been most widely investigated in this respect (e.g. Handley & Brown, 1982; Handley & Singh, 1986b; Moser & Redfern, 1986; reviewed by Heal et al., 1992). Head-twitches appear to be under tonic control by central α2-adrenoceptors. This is evident from the increase in head-twitches seen after lesioning of noradrenergic neurones (Heal et al., 1986). Moreover, head-twitches are increased by α2-adrenoceptor antagonists and decreased by α2-adrenoceptor agonists (Handley & Brown, 1982; Heal et al., 1986)

Head-twitches are though to be hindbrain/spinally mediated (reviewed by Heal et al., 1992), and therefore cannot be expected to reflect changes in cortical 5-HT2 receptor binding. However, this behaviour is a useful indicator of central 5-HT2 receptor function *in vivo* where it is not feasible to measure phosphoinositide turnover, and so this procedure was used in the current experiments.

4.5.3.1 *Repeated saline injection*

Repeated once-daily saline injection had no effect on 5-MeODMT-induced head-twitches measured 24 h after the final injection. Only one previous study has investigated the effects of repeated stress on 5-HT2 receptor-mediated head-twitches: repeated exposure to 2 h immobilization had no effect 24 h after the final immobilization (Cancela et al., 1990). This suggests that repeated exposure to stress has no effect on 5-HT2 receptor function *in vivo*.

Since head-twitches are generated in the hindbrain and spinal cord, they cannot be expected to faithfully reflect binding in higher brain regions. However, neither cortical 5-HT2 receptor binding nor central 5-HT2 receptor function were altered by repeated saline injection, 24 h after the final injection. Both these findings argue against involvement of central 5-HT2 receptors in the effects of repeated stress at this time.

4.5.3.2 *Repeated sibutramine administration*

In the current experiment, repeated sibutramine administration had no effect on 5-MeODMT-induced head-twitches. The effects of repeated administration of monoamine uptake inhibitors on central 5-HT2 receptor function *in vivo* have been examined in both rats and mice. In rats, such drugs decrease head-twitches 24 h after the final injection (e.g. Lucki & Frazer, 1985; Eison et al., 1991), with a rebound increase at later times (e.g. Mogilnicka & Klimek, 1979). In mice, head-twitches have generally been measured between 0-18 h after the final drug injection, when they are decreased after non-selective uptake inhibitors such as imipramine and amitriptyline (e.g. Friedman et al., 1983; Goodwin et al., 1984; Godfrey et al., 1988). However, 24 h after the final drug administration, a rebound increase in head-twitches is seen after amitriptyline and imipramine (Friedman et al., 1983). The effects of sibutramine, another non-selective monoamine uptake inhibitor, did not correspond to this pattern. There are three possible explanations for this finding. First, the effects of sibutramine on head-twitches may be shorter lasting than those of other non-selective uptake inhibitors. Alternatively, sibutramine simply has no effect on 5-MeODMT-induced head-twitches. A final possibility is that any changes in 5-HT2 receptor binding or function may be offset by changes in the modulation of this behaviour by other neurotransmitters; this is unlikely to involve β-adrenoceptors, however. Head-twitches are potentiated by β-adrenoceptor agonists (Ortmann et al., 1981; Handley & Singh, 1986b), and repeated sibutramine administration down-regulates mouse cortical β-adrenoceptor density (Section 4.5.1.2). However, the modulation of head-twitches by

β-adrenoceptors is not tonic, unlike that by α2-adrenoceptors; a *reduction* in β-adrenoceptor density may not alter the head-twitch response, therefore.

Notwithstanding, since neither central 5-HT2 receptor-mediated head-twitches nor cortical 5-HT2 receptor binding were altered by repeated sibutramine administration 24 h after the final drug injection, there is no evidence to suggest that sibutramine has any effects on central 5-HT2 receptors at this time.

Summary:

Repeated saline injection had no significant effect on the specific binding of either [^{3}H]-CGP 12177 or [^{3}H]-DHA binding defined using (-)-isoprenaline HCl. Repeated administration of the monoamine uptake inhibitors sibutramine or DMI reduced [^{3}H]-CGP 12177 binding compared with saline-injected animals. However, [^{3}H]-DHA binding was reduced by sibutramine but not DMI when compared with the saline-injected group. The magnitude of drug-induced changes in binding also depended on the radioligand used. Cortical [^{3}H]-ketanserin binding and 5-MeODMT-induced head-twitches were not altered by either repeated saline injection or by repeated sibutramine administration, 24 h after the final injection.

4.5.4 Conclusions

1) β-Adrenoceptor binding is not altered by repeated stress (once-daily saline injection) in mouse cortex, 24 h after the final injection.

2) Both DMI and sibutramine reduce β-adrenoceptor density in mouse cerebral cortex.

3) Discrepancies between estimates of binding measured using [^{3}H]-DHA and [^{3}H]-CGP 12177 support the suggestion that [^{3}H]-CGP 12177 is a more suitable ligand to measure β-adrenoceptor binding; however, the possibility that these discrepancies are due to the binding of [^{3}H]-DHA to a second receptor site, which is influenced by saline injection, cannot be discounted.

4) Neither repeated stress nor repeated sibutramine administration alter cortical 5-HT2 receptor binding or central 5-HT2 receptor function *in vivo*, 24 h after the final injection in mice.

5.0 BEHAVIOUR INDUCED BY A BRIEF SWIM, AND EFFECTS ON CENTRAL NORADRENERGIC AND SEROTONERGIC NEURONES: INFLUENCE OF REPEATED SALINE INJECTION OR SIBUTRAMINE ADMINISTRATION

5.1 Introduction

The previous chapter describes the effects of repeated stress (once-daily saline injection) or repeated sibutramine administration on cortical β-adrenoceptor and 5-HT2 receptor binding *ex vivo*, and on central 5-HT2 receptor function *in vivo* in mice. It is now possible to determine how saline or sibutramine pretreatment influences the effects of a single exposure to stress on these receptors, therefore. Since these effects were to be compared with effects on behavioural responses to stress, a well-documented paradigm with a standardized protocol was used in order to facilitate comparison of the current behavioural measurements with the published literature.

The swim test ('Porsolt test') was first described in 1977 (Porsolt et al., 1977a). The first report used rats; subjects were exposed first to a 15 min 'conditioning swim' then 24 h later, behaviour was measured during a 5 min 'test swim' (Porsolt et al., 1977a, 1978). Subsequently a protocol using mice was published; for this species, only a single 6 min swim was required and behaviour was measured during the final 4 min of this period (Porsolt et al., 1977b). The behavioural index measured during the swim test was 'immobility': that is, the time animals were quiescent, making only the smallest movements necessary to stay afloat (Porsolt et al., 1977a, 1978). Although the experimental protocol used in different laboratories is standard, the interpretation of swim-induced immobility is not. Porsolt and co-workers suggested that 'immobility' reflects a despairing or lowered mood, which develops when animals realize that escape from the aversive situation is impossible (Porsolt et al., 1977a, 1978). However in rats at least, immobility during the 'test swim' is not altered when escape is possible during the 'conditioning swim' (O'Neill & Valentino, 1982). Moreover, it has been shown that in rats, development of immobility may depend on previous experience of the environment (Hawkins et al., 1978; Borsini et al., 1986). This led to the inference that 'immobility' may represent an adaptive response to the aversive situation.

The common use of the swim test is to screen antidepressant drugs. Immobility is reduced after acute or subchronic administration (usually 3 injections in rats, 1 injection in mice) of monoamine uptake inhibitors and the majority of antidepressants from other generic groups in both rats and mice (reviewed by Borsini & Meli, 1988). Notable

exceptions include the selective 5-HT uptake inhibitors citalopram (Platznik & Kotowski, 1985) and trazodone (Porsolt, 1981), although other antidepressants of this type do reduce immobility after subchronic administration e.g. sertraline (Cervo et al., 1991), zimelidine (Satoh et al., 1984). However, immobility is also reduced by subchronic administration of one of a number of other types of psychotropic agents such as neuroleptics (Gorka & Janus, 1985; Kawashima et al., 1986), anticholinergics (Browne, 1979; Kitada et al., 1981) and amphetamine (Porsolt et al., 1977a, 1978).

Since, in man, the therapeutic effects of antidepressants are not seen before at least 2 weeks of repeated administration, the effects of repeated drug administration are more relevant to the understanding the basis of therapeutic effects of these drugs. Indeed, the reduction in immobility seen in rats after repeated antidepressant administration is at least the same as, if not larger than, that induced by subchronic administration (Miyauchi et al., 1981; Berettera et al., 1986; reviewed by Borsini & Meli, 1988). Only one study of repeated antidepressant administration has been carried out in mice, where it was reported that repeated administration of the monoamine uptake inhibitor, desipramine (DMI) had a greater anti-immobility effect than a single dose (Poncelet et al., 1986). The effects of repeated administration of other psychotropic drugs on immobility have received little attention. However, the anti-immobility effects of neuroleptic drugs appear to be abolished on repeated administration (Gorka & Janus, 1985; Kawashima et al., 1986). Measuring immobility after repeated drug administration may therefore clarify the effects of psychotropic drugs on immobility, reducing the incidence of 'false positives' seen after subchronic drug administration.

If, as suggested by Stone (1979b), repeated antidepressant administration has neurochemical and behavioural effects which mimic adaptation to stress, then the effects of repeated stress on swim-induced immobility should resemble those of repeated antidepressant administration. Indeed, Stone's group showed that repeated restraint stress reduced immobility to the same extent as repeated DMI administration; a single period of restraint was without effect (Platt & Stone, 1982). However, other groups have reported that swim-induced immobility may be increased by repeated exposure to stress such as restraint (Cancela et al., 1991) or tailshock (Garcia-Marquez & Armario, 1987). These findings suggest that the effects of repeated exposure to noxious stress on immobility are unpredictable. One aim of the current experiments therefore was to determine the effects on immobility of the repeated stress of once-daily saline injection, generally used as a control procedure.

Despite the use of the swim test to predict the psychotropic effects of drugs in man, the neurochemical effects of the test procedure itself have received little attention. A brief swim stress has been shown to increase plasma levels of both noradrenaline (Benedict et al., 1979) and corticosterone (Tuominen & Korpi, 1991), changes which are commonly interpreted as an index of the physiological response to a stressful stimulus. In addition, limited information about the central effects of swim stress suggests that, in rats, both central noradrenaline and 5-HT turnover are increased in a number of brain regions, immediately after a single exposure to swim stress (Miyauchi et al., 1981; Ikeda & Nagatsu, 1985). However, the effects of swim stress on post-synaptic serotonergic and noradrenergic function have not been investigated. A further aim of the current experiments was to investigate effects of the swim stress on cortical β-adrenoceptor and 5-HT2 receptor binding, and on central 5-HT2 receptor function *in vivo*, therefore.

Receptor binding was measured immediately after the swim test; noradrenaline and 5-HT turnover are altered at this time, increased transmitter turnover being seen up to 30 min after an acute stress, but it is not known how these changes affect receptors. In order to examine whether there were any effects of the swim on these parameters beyond transient effects, the effects of the swim stress on 5-HT and noradrenaline turnover were measured 3 h after exposure to the swim stress. Cortical 5-HT2 receptor binding was measured in corresponding groups of animals at this time. 5-MeODMT-induced head-twitches, used to provide information about the functional state of central 5-HT2 receptors, were also measured 3 h after the swim. There was no corresponding measurement of β-adrenoceptor function in these experiments, since existing models are not selective for central β-adrenoceptor function *in vivo* (Heal, 1990).

There are isolated reports of long-latency changes (days-weeks) in behavioural and neurochemical indices seen after a single experimental treatment. For instance, a single brief period of footshock induces long-lasting (~1-3 weeks) reductions in locomotion in the open field (Van Dijken et al., 1990). Two reports of particular interest suggest that both the serotonergic and noradrenergic systems can show long latency changes. First, a long-latency increase in 5-HTP-induced head-twitches in the rat has been reported, 11 days after 2 injections of the noradrenaline and 5-HT reuptake inhibitor, amitriptyline (Antelman et al., 1983). Secondly, immobility during the swim test in mice is increased 7 days after a single injection of the benzodiazepine inverse agonist, (N-methyl-β)-carboline-3-carboxamide (FG 7142: Chopra et al., 1988); mouse cortical β-adrenoceptor density is also increased at this time (Stanford et al., 1987). These findings suggest that

a single exposure to either monoamine uptake inhibitors or to FG 7142, a drug which may mimic the effects of stress (Leidenheimer & Schechter, 1988), has long-latency effects on certain aspects of serotonergic and noradrenergic function. Despite these reports, the delayed effects of stress have rarely been considered. An important aim of the current experiments was therefore to determine whether cortical β-adrenoceptors or central 5-HT2 receptors were involved in any long-latency effects of stress. To this end, receptor binding, and central 5-HT2 receptor function *in vivo* were measured 7 days after swim stress. Neurotransmitter turnover was also measured at this time to determine whether any changes in receptors could be explained by changes in transmitter supply.

5.2 Aims

1) To investigate the effects of a single period of swim stress on neurochemical and behavioural indices of central noradrenergic and serotonergic function in the mouse.

2) To compare the effects of repeated stress (once-daily saline injection) and administration of a monoamine uptake inhibitor (sibutramine) on swim-induced immobility.

3) To examine the influence of previous experience of repeated stress or sibutramine administration on swim-induced neurochemical changes

4) To investigate the possibility that there are long-latency (up to 7 days) effects of a brief swim on neurochemical and behavioural indices of central noradrenergic and serotonergic function.

5.3 Methods

5.3.1 Animals and treatments

Male CD1 mice (18-20 g on arrival in the animal house) were housed under a 12/12 h light/dark cycle (lights on from 08.00-20.00 h), with free access to food and water. Animals were divided into 4 groups, matched for mean weight; the size of the groups varied between experiments, but within any given experiment, all groups were of equal size. Two groups were destined for either repeated stress (saline injection) or drug (sibutramine) pretreatment; the remaining two groups received no pretreatment (uninjected). Each cage, of not less than 4 mice, contained animals from one treatment group only. All animals remained unhandled (apart from routine husbandry) for 5 days

before the start of the experiment to allow acclimatization to the new surroundings. Following this period, animals from the stress and drug pretreatment groups were weighed, then received an injection of either 0.9% sterile saline (10 ml/kg i.p.) or sibutramine (3 mg/kg i.p.). This procedure, carried out between 09.30-10.30 h, was repeated once-daily for a total of 10 days. Uninjected groups remained undisturbed in their home cage throughout this period.

24 h after the final injection, mice from the stress and drug pretreatment groups, and those from an uninjected group ('swim only'), were individually given a 6 min swim. The swim test was carried out as described previously (Section 2.2.2) in a room to which the animals were not accustomed.

Following the swim test, indices of central noradrenergic and serotonergic function were measured. Cortical β-adrenoceptors, cortical 5-HT2 receptors, 5-MeODMT-induced head-twitches, 5-HT turnover (5-HTP levels), noradrenaline turnover (MHPG levels) and cortical monoamine and metabolite (noradrenaline, 5-HT and 5-HIAA) concentrations were all measured in separate experiments. Within each experiment, there were two or three from each treatment group, so that measurements could be made at different times after the swim test (0 h, 3 h, 7 days: Table 5.1).

A separate experiment was carried out to measure the time-course of stress- and sibutramine-induced changes in immobility. This experiment contained animals from only 3 treatment groups (saline- and sibutramine-pretreated, and 'swim only' groups). Animals from each treatment group were exposed to the swim test either 1 or 6 h after the final injection; separate groups were used at each time point so that each animal was exposed to the swim once only. No uninjected controls were included in this experiment since no neurochemical or behavioural indices were to be measured after the swim test.

Table 5.1: Experimental protocol

	+ 0 h	+ 3 h	+ 7 days
Cortical noradrenaline		✓	✓
Cortical MHPG		✓	✓
Cortical β-adrenoceptors	✓		✓
Cortical 5-HT and 5-HIAA		✓	✓
Cortical 5-HTP		✓	✓
Cortical 5-HT2 receptors	✓	✓	✓
Head-twitches		✓	✓

Table 5.1 : Experimental protocol. The swim test was carried out 24 h after the final injection; indices of central noradrenergic and serotonergic function were measured 0 h or 3 h and 7 days after the swim test as indicated (✓).

5.3.2 Immobility

Immobility was defined as the time spent by the animal making only the smallest movements in order to remain afloat. This was recorded during the final 4 min of the swim, using a stop watch.

5.3.3 Neurochemical and behavioural measurements: sampling times

a) 0 h

Immediately after removal from the water, animals were killed and brains removed. Cerebral cortices were dissected over ice, frozen on dry ice and then stored at -20°C for subsequent determination of cortical β-adrenoceptor binding (Section 2.3.1.1) or 5-HT2 receptor binding (Section 2.3.1.3). The remaining uninjected group was also killed at this time ('control'). In one experiment, the binding of the β-adrenergic radioligands [^{3}H]-DHA and [^{3}H]-CGP 12177 was to be compared. To achieve this, binding of each radioligand was compared in separate aliquots of membrane preparations derived from the same animals, with binding assays carried out simultaneously (Section 2.3.1.2).

b) 3 h

On removal from the water, animals were dried and returned to the home cage. 3 h later, animals were removed to an adjacent room in their home cages, together with the uninjected control group. In experiments measuring 5-HT2 receptor binding, noradrenaline or 5-HT turnover (Section 2.3.2.2, 2.3.2.3) or neurotransmitter levels (Section 2.3.2.1), animals were killed and cortices removed for subsequent analysis. For the determination of 5-HT2 receptor-mediated head-twitches, animals received an injection

of 5-methoxy-N,N-dimethyltryptamine (5-MeODMT; 3 mg/kg i.p.) and head-twitches were scored in the 6 min after the injection (Section 2.2.3). It was aimed to measure these parameters 3 h after the swim; in fact the time taken to make certain measurements meant that these parameters were assessed 2-4 h after the swim.

c) 7 days

On removal from the water, animals were dried and returned to the home cage. 7 days later, animals were removed to an adjacent room in their home cages, along with the remaining uninjected group. In experiments to measure cortical β-adrenoceptor or 5-HT2 receptor binding, or neurotransmitter levels or turnover, animals were killed and brains removed. Cortices were dissected over ice and frozen on dry ice, then stored at -20°C for subsequent analysis. In the experiment to measure 5-HT2 receptor-mediated head-twitches, animals received an injection of 5-MeODMT (3 mg/kg i.p.) and head-twitches scored as previously described (Section 2.2.3).

5.3.4 Statistics

1-way ANOVA was used to compare data from all treatment groups at a single time-point, to look for any treatment effects on the parameter measured. This analysis was used for immobility scores, as well as for each neurochemical measurement and head-twitch scores. For the experiment examining the binding of two different β-adrenergic radioligands, 1-way ANOVA was used to look for treatment effects for each radioligand individually. Where a significant treatment effect was found, comparisons were made subsequently between all group means using Student's unpaired t-tests, using the error term from the ANOVA.

Where a given parameter was measured at more than one time point within a single experiment, 2-way ANOVA was used to compare data from all treatment groups at the different times. This analysis looked for

(i) a treatment effect

(ii) a time effect

(iii) any treatment x time interaction.

Where significant effects were found in the ANOVA, comparisons between group means were subsequently made using Student's unpaired t-tests, using the error term from the ANOVA.

Data from the experiment comparing binding obtained with the two β-adrenergic

ligands immediately after the swim test was analyzed using a 3-way ANOVA. This analysis looked for

(i) a treatment effect

(ii) a ligand effect, i.e. any difference between the binding defined with the two ligands

(iii) any treatment x ligand interaction i.e. whether the treatment effect seen depends on the radioligand used to define binding

Since for each sample, binding was measured using both ligands, an additional factor was included to take this into account. Again, significant effects were subsequently examined using Student's unpaired t-tests.

5.4 Results

5.4.1 Immobility during the swim stress

Immobility displayed by the mice during the final 4 min of the swim test is shown in Figure 5.1. In the saline-pretreated group, immobility was significantly reduced 24 h after the final injection, compared with the uninjected ('swim only') group ($F = 3.697$; d.f. = 2, 138; $p < 0.05$: $t = 2.136$; $p < 0.05$). Immobility was also significantly reduced in the sibutramine-pretreated group ($F = 3.697$; d.f. = 2, 138; $p < 0.05$: $t = 2.582$; $p < 0.01$; Figure 5.1). However, immobility did not differ between the stress- and sibutramine-pretreated groups (-8.5%, -10.0% *cf* 'swim only' group, respectively).

The effects of saline and sibutramine pretreatment on immobility at different times after the final injection was measured in a separate experiment; immobility measured in mice 1 and 6 h after the final injection is shown in Table 5.2. When data from each time point was analyzed separately using 1-way ANOVA, it was seen that immobility measured 1 h after the final injection was not significantly altered by either saline or sibutramine pretreatment, despite 12.5% and 9.4% reductions in immobility in these groups respectively ($F = 1.669$; d.f. = 2, 15; $p > 0.05$). 6 h after the final injection, there was again no significant treatment effect on immobility despite a 9.6% reduction in the sibutramine-pretreated (Table 5.2; $F = 2.840$; d.f. = 2, 15; $p = 0.053$). When a 2-way ANOVA was used to compare immobility at both time-points, there was again no treatment effect on immobility ($F = 2.77$; d.f. = 2, 30; $p > 0.05$). Nor was there any significant time effect ($F = 1.22$; d.f. = 1, 30; $p > 0.05$) or any treatment x time interaction ($F = 1.32$; d.f. = 2,30; $p > 0.05$).

Figure 5.1 **IMMOBILITY IN THE SWIM TEST: EFFECT OF REPEATED SALINE INJECTION OR SIBUTRAMINE ADMINISTRATION**

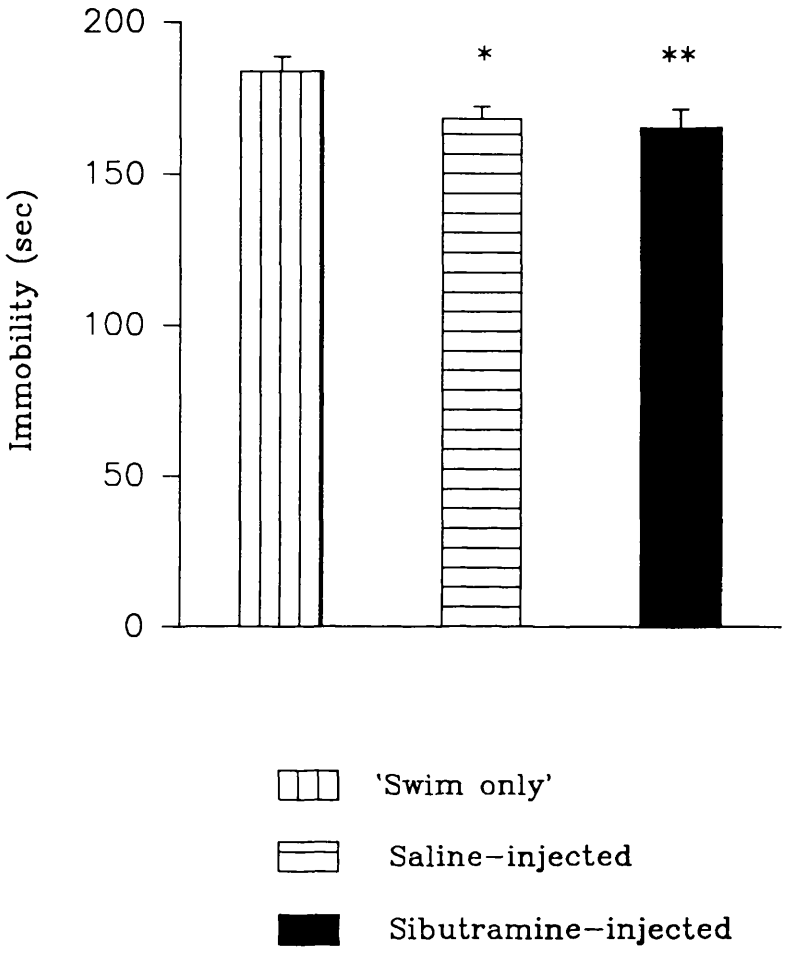


Figure 5.1 : n = 47, each group. Immobility measured 24 h after the final injection. Data from all three treatment groups compared using 1-way ANOVA. Following a significant F value for a treatment effect, group means were compared using unpaired t-tests: $^{*}p < 0.05$, $^{**}p < 0.01$ *cf* 'swim only' group.

Table 5.2 **TIME-COURSE OF CHANGES IN SWIM-INDUCED IMMOBILITY AFTER REPEATED SALINE INJECTION OR SIBUTRAMINE ADMINISTRATION**

	Immobility (secs)	
	+ 1 h	+ 6 h
'Swim only'	203.8 ± 6.4	205.5 ± 7.5
Saline-injected	177.8 ± 5.4	202.3 ± 5.8
Sibutramine-injected	185.6 ± 15.8	183.3 ± 8.1

n = 6, each group. Immobility was measured during the final 4 min of the swim test, 1 or 6 h after the final injection in separate groups of animals. Data from individual time points analyzed separately using 1-way ANOVA. Data from both time-points compared using 2-way ANOVA.

5.4.2 Noradrenergic system: neurochemical measurements.

Cortical noradrenaline levels, and MHPG levels, an index of noradrenaline turnover, 3 h and 7 days after the swim test are shown in Table 5.3. Both 3 h and 7 days after the swim test, neither noradrenaline (Fs = 0.742, 0.794 respectively; d.f. = 3, 36; ps > 0.05) nor MHPG levels (Fs = 0.810, 0.295 respectively; d.f. = 3, 28; ps > 0.05) differed significantly from uninjected controls in any treatment group.

Cortical β-adrenoceptor density measured immediately after the swim test is shown in Figure 5.2. 1-Way ANOVA of [^{3}H]-CGP 12177 binding data revealed a significant treatment effect on binding density (F=4.057; d.f. = 3,36; p< 0.05). Subsequent t-tests showed that a 6 min swim did not alter cortical β-adrenoceptor density in uninjected animals (t = 0.249; d.f. = 32; p > 0.05). However, in saline- or sibutramine-pretreated groups, cortical β-adrenoceptor density was significantly reduced immediately after a 6 min swim compared with uninjected controls (-13%, -20%; ts = 1.935, 2.999; d.f. = 32; ps < 0.05, 0.01 respectively). In contrast, when [^{3}H]-DHA binding was considered, there was no significant treatment effect; that is, none of the groups differed significantly from the control group (F= 1.436, d.f. = 3,36; p > 0.1). This was despite reductions in binding B_{max} in the saline- and sibutramine-pretreated groups of a similar size to those seen for [^{3}H]-CGP 12177 binding (-20%, -14% respectively).

Cortical β-adrenoceptor K_d defined using either [^{3}H]-DHA or [^{3}H]-CGP 12177 did not differ from controls in any of the treatment groups, immediately after the swim test (Fs = 0.310, 0.680 respectively; d.f. = 3, 36; p > 0.05: Table 5.4).

Comparison of the binding of the two β-adrenergic ligands immediately after the swim using a 3-way ANOVA, revealed a significant treatment effect (F= 3.97; d.f. = 3,36; p< 0.05). Subsequent t-tests showed that, regardless of the ligand used to define β-adrenoceptor binding, a 6 min swim alone did not alter cortical β-adrenoceptor density. As for [^{3}H]-CGP 12177 binding alone, there was a significant reduction in cortical β-adrenoceptor density after a 6 min swim in animals which had received repeated saline or sibutramine pretreatment. However, there was no significant difference between β-adrenoceptor densities defined using the two different ligands (F = 2.51; d.f. = 1, 36; p > 0.05). There was also no treatment x ligand interaction: that is, the size of treatment-induced changes in β-adrenoceptor density did not depend on the radioligand used (F = 0.51; d.f = 3, 36; p > 0.05).

Table 5.3 **CORTICAL NORADRENALINE AND MHPG LEVELS AFTER A 6 MIN SWIM TEST: INFLUENCE OF REPEATED SALINE OR SIBUTRAMINE ADMINISTRATION**

a) Cortical noradrenaline levels

	+ 3 h	+ 7 days
Uninjected	286.5 ± 15.2	201.5 ± 10.1
'Swim only'	259.3 ± 14.1	225.2 ± 11.2
Saline-injected	262.8 ± 10.9	208.6 ± 5.2
Sibutramine-injected	275.8 ± 17.0	211.0 ± 15.5

n = 10, each group. Noradrenaline content (nmol/g original wet weight tissue) measured 3 h or 7 days after the swim test in separate groups of animals. Data for each individual time point analyzed using 1-way ANOVA.

b) Cortical MHPG levels

	+ 3 h	+ 7 days
Uninjected	45.8 ± 2.8	37.9 ± 3.7
`Swim only'	41.9 ± 3.1	41.4 ± 3.3
Saline-injected	40.0 ± 1.5	38.1 ± 2.7
Sibutramine-injected	44.5 ± 3.6	41.1 ± 3.9

n = 8, each group. MHPG content (nmol/g original wet weight tissue) measured 3 h or 7 days after the swim test in separate groups of animals. Data for each individual time point analyzed using 1-way ANOVA.

Figure 5.2 **CORTICAL β-ADRENOCEPTOR DENSITY AFTER A 6 MIN SWIM: INFLUENCE OF REPEATED SALINE INJECTION OR SIBUTRAMINE ADMINISTRATION**

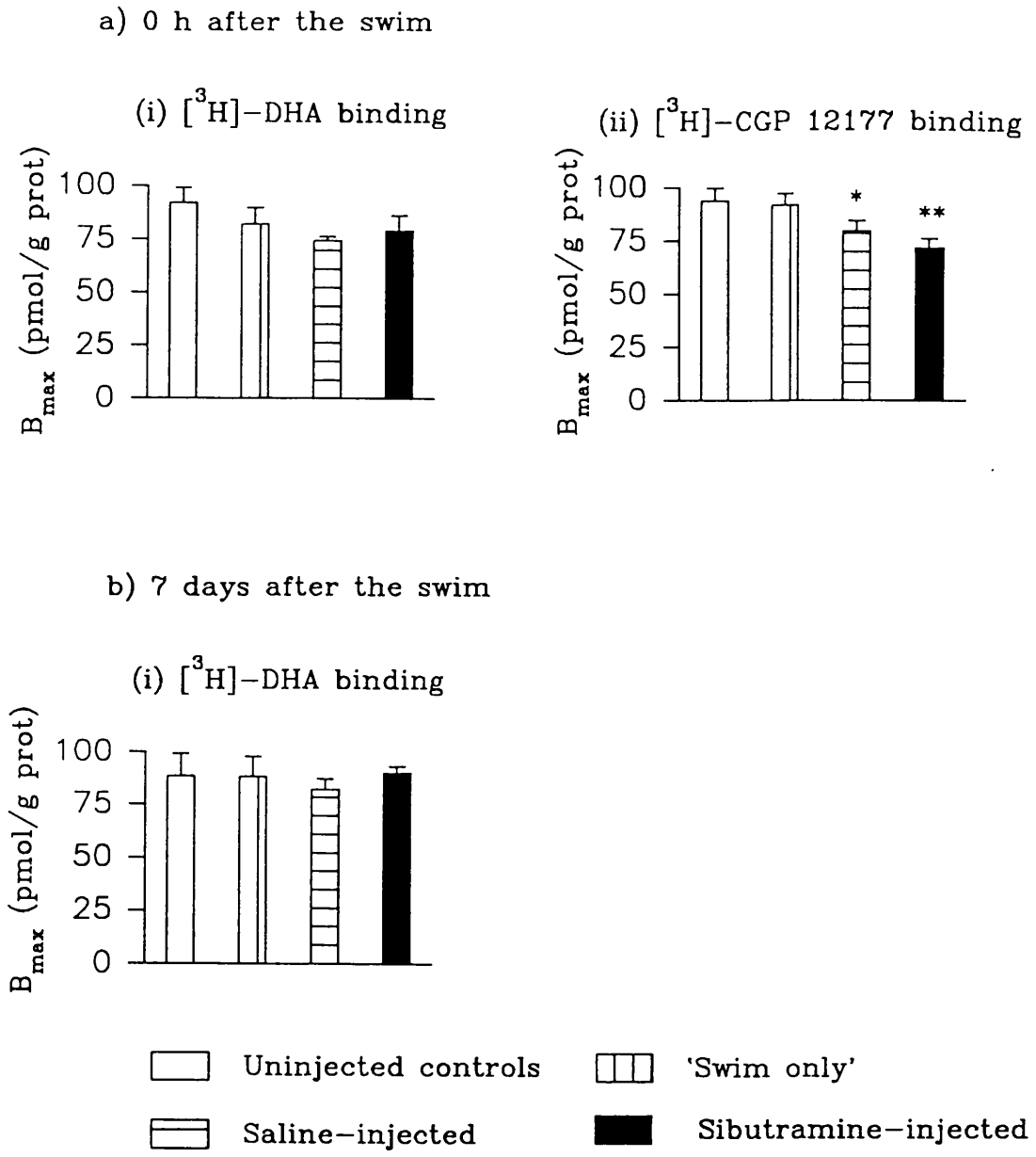


Figure 5.2 : n = 9, each group. [^{3}H]-DHA binding measured immediately and 7 days after the swim in separate groups of animals. [^{3}H]-CGP 12177 binding measured immediately after the swim, in the same animals in which [^{3}H]-DHA binding was measured at this time. Data for each ligand at each time point analyzed separately using 1-way ANOVA. Following a significant F value for a treatment effect, group means were compared using unpaired t-tests: $^*p < 0.05$, $^{**}p < 0.01$ *cf* uninjected control group.

Table 5.4 **CORTICAL β-ADRENOCEPTOR K_d AFTER A 6 MIN SWIM TEST: INFLUENCE OF REPEATED SALINE INJECTION OR SIBUTRAMINE ADMINISTRATION**

	+ 0 h		+ 7 days
	[^{3}H]-DHA	[^{3}H]-CGP 12177	[^{3}H]-DHA
Uninjected	1.03 ± 0.14	0.22 ± 0.07	1.13 ± 0.18
'Swim only'	1.30 ± 0.17	0.29 ± 0.11	1.03 ± 0.07
Saline-injected	1.11 ± 0.19	0.17 ± 0.03	0.94 ± 0.10
Sibutramine-injected	1.20 ± 0.15	0.17 ± 0.03	1.03 ± 0.07

n = 9, all groups. β-Adrenoceptor binding K_d (nM) measured 0 h or 7 days after the swim test in separate groups of animals; immediately after the swim, [^{3}H]-DHA and [^{3}H]-CGP 12177 binding were measured in tissue from the same animals. Data at each individual time point for each ligand analyzed using 1-way ANOVA.

7 days after the swim test, cortical β-adrenoceptor binding was measured using [^{3}H]-DHA. Radioligand binding Kds are shown in Table 5.4; Kd did not differ between any of the groups. B_{max} values are shown in Figure 5.2. 1-Way ANOVA revealed that no treatment group differed significantly from uninjected controls, 7 days after the swim ($F = 0.196$; d.f. = 3, 32; $p > 0.05$).

5.4.3 Serotonergic system: neurochemical measurements.

5.4.3.1 *Cortical 5-HT and 5-HIAA levels*

Cortical 5-HT and 5-HIAA levels are displayed in Table 5.5. There was no change in cortical 5-HT or 5-HIAA levels in any treatment group, compared with controls, either 3 h (Fs = 1.885, 0.194 respectively; d.f. = 3, 36; $p > 0.05$) or 7 days after the swim (Fs = 0.205, 0.124; d.f. = 3, 36; $p > 0.05$: Table 5.5). In addition, the 5-HIAA : 5-HT ratio was calculated for each animal; group mean scores for this ratio are displayed in Table 5.5. 5-HIAA : 5-HT ratios also did not differ between any of the groups, at either time point (Fs = 2.793, 0.413; d.f. = 3, 36; $p > 0.05$).

5.4.3.2 *Cortical 5-HTP levels*

Cortical 5-HTP accumulation after 5-HTP decarboxylase inhibition was used as an index of 5-HT synthesis: data are shown in Figure 5.3. 1-Way ANOVA of data from 3 h after the swim revealed a significant treatment effect ($F = 3.214$; d.f. - 3, 36; $p < 0.05$). Subsequent t-tests showed that 5-HTP accumulation in uninjected animals was unchanged after a 6 min swim ($t = 0.927$; d.f. = 36; $p > 0.05$). However, there was a statistically significant reduction in 5-HTP accumulation in both repeated saline- and sibutramine-pretreated groups compared with uninjected controls (-17.5, -22.0%; t's = 2.235, 2.806; d.f = 36; ps < 0.05, 0.01 respectively).

1-Way ANOVA of data obtained 7 days after the swim showed that there was no significant treatment effect ($F = 2.435$; d.f. = 3, 36; $p > 0.05$). This was despite increases in 5-HTP accumulation of 16% and 25% in the `swim only' and in the sibutramine-pretreated groups respectively at this time (Figure 5.3). Comparison of 5-HTP data obtained 3 h and 7 days after the swim using 2-way ANOVA revealed a significant time effect ($F = 4.08$; d.f. = 1, 71; $p < 0.05$); subsequent t tests revealed that overall, 5-HTP accumulation was greater 7 days after the swim than at 3 h after ($t = 2.012$; d.f. = 72; $p < 0.05$). There was no significant treatment effect ($F = 0.53$; d.f. = 3, 72; $p > 0.05$); the interaction between treatment and time was significant, however ($F = 5.10$; d.f. = 3, 72; $p < 0.01$). Subsequent t tests comparing all treatment

Table 5.5 **CORTICAL 5-HT AND 5-HIAA LEVELS, AND THE 5-HIAA/5-HT RATIO AFTER A 6 MIN SWIM: INFLUENCE OF REPEATED SALINE INJECTION OR SIBUTRAMINE ADMINISTRATION**

a) 3 h after the swim

	5-HT	5-HIAA	5-HIAA/5-HT
Uninjected	607.1 ± 26.8	136.6 ± 15.4	0.224 ± 0.01
'Swim only'	521.7 ± 39.5	138.4 ± 13.2	0.263 ± 0.01
Saline-injected	556.6 ± 13.9	131.6 ± 8.0	0.236 ± 0.01
Sibutramine-injected	548.7 ± 15.2	141.4 ± 6.8	0.256 ± 0.01

b) 7 days after the swim

	5-HT	5-HIAA	5-HIAA/5-HT
Uninjected	363.5 ± 15.4	93.1 ± 5.7	0.257 ± 0.01
'Swim only'	383.2 ± 13.8	94.7 ± 4.3	0.246 ± 0.01
Saline-injected	375.5 ± 18.7	94.9 ± 4.0	0.256 ± 0.01
Sibutramine-injected	378.3 ± 24.8	91.1 ± 5.6	0.243 ± 0.01

n = 10, each group. 5-HT and 5-HIAA levels (nmol/g original wet weight tissue) determined in the same tissues, taken either 3 h or 7 days after the swim test from separate groups of animals. Data from each individual time point analyzed using 1-way ANOVA.

Figure 5.3 **CORTICAL 5-HTP ACCUMULATION AFTER A 6 MIN SWIM: INFLUENCE OF REPEATED SALINE INJECTION OR SIBUTRAMINE ADMINISTRATION**

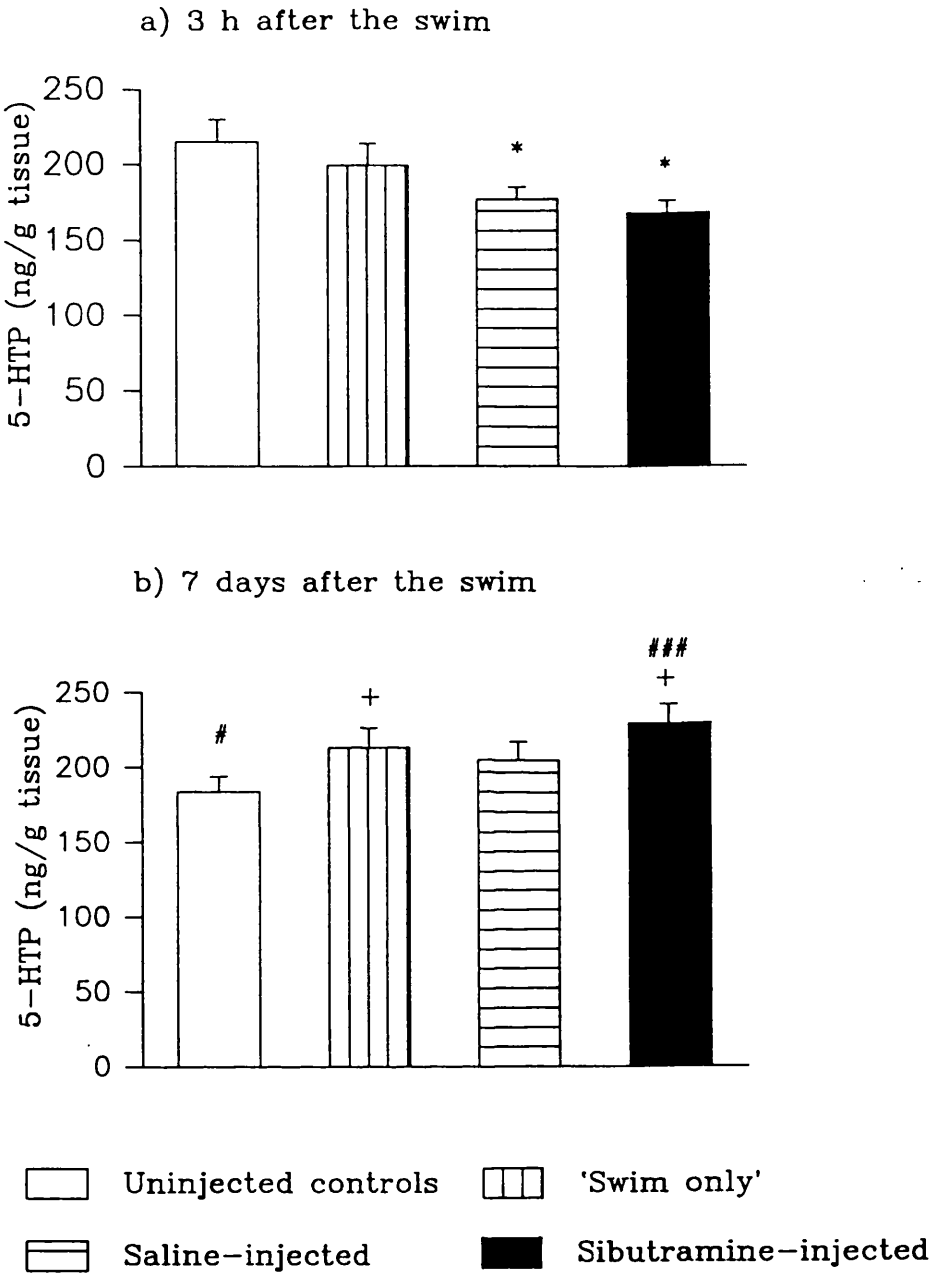


Figure 5.3 : n = 10, each group. 5-HTP accumulation measured 3 h or 7 days after the swim in separate groups of animals. Data at each time point analyzed separately using 1-way ANOVA. Following a significant treatment effect, group means were compared using unpaired t-tests: $^{*}p < 0.05$ *cf* uninjected control group. Data from both time points were compared using 2-way ANOVA. Following a significant treatment x time interaction, group means were compared using unpaired t-tests: $^{+}p < 0.05$, $^{++}p < 0.01$ *cf* simultaneous controls, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ *cf* same treatment group, 3 h after the swim.

group means showed that, 3 h after the swim, 5-HTP accumulation was reduced in saline- and sibutramine-pretreated but not in uninjected animals (ts = 2.199, 2.760, 0.912; d.f. = 72; ps < 0.05, 0.01, > 0.05 respectively), as seen when data at this time were considered separately. In addition, 7 days after the swim, 5-HTP accumulation was increased in uninjected and sibutramine-pretreated animals (ts = 1.713, 2.690; d.f. = 72; ps < 0.05, < 0.01 respectively). This increase was not apparent in saline-pretreated animals (t = 1.228; d.f. = 72; p > 0.05). Each treatment group 7 days after the swim was also compared with its counterpart, 3 h after the swim. 5-HTP accumulation in the control group 3 h after the swim was greater than at 7 days (t = 1.871; d.f. = 72; p < 0.05). There was no difference between either the 'swim only' groups or the saline-injected groups (ts = 0.754, 1.556; d.f. = 72; ps > 0.05) at the two different times. However, 5-HTP accumulation in the sibutramine-pretreated group was greater 7 days after the swim (t = 3.579; d.f. = 72; p < 0.001).

5.4.3.3 *Cortical 5-HT2 receptor binding*

Cortical 5-HT2 receptor B_{max} measured using [^{3}H]-ketanserin, 0 h, 3 h or 7 days after the swim test is displayed in Figure 5.4. 1-Way ANOVA of binding data obtained immediately after the swim did not reveal a significant treatment effect (F = 1,76; d.f. = 3, 40; p > 0.05), despite apparent increases in receptor density in all treated groups compared with the uninjected controls (+ 23%: 'swim only'; +35%: saline injected; +37% : sibutramine-injected). There was also no significant treatment effect on 5-HT2 receptor binding density measured 3 h after the swim test (F = 0.519; d.f. = 3, 40; p > 0.05; Figure 5.4).

7 days after the swim, 1-way ANOVA revealed a significant treatment effect (F = 2.903; d.f = 3, 40; p < 0.05). Subsequent t-tests showed that a 25% increase in B_{max} in the 'swim only' group was significant (t = 2.207; d.f. = 40; p < 0.05). There was also a significant increase in receptor binding density in the sibutramine-pretreated group (t = 2.433; d.f. = 40; p < 0.01; Figure 5.4). However, 5-HT2 receptor density in the saline-pretreated group did not differ from the uninjected controls (t = 0.66; d.f. = 40; p > 0.05).

Cortical 5-HT2 receptor Kds are displayed in Table 5.6. There were no differences in Kd between any of the treatment groups at any time (Fs = 1.76, 0.908, 0.232; d.f. = 3, 40; ps > 0.05)

Figure 5.4 **CORTICAL 5-HT2 RECEPTOR BINDING AFTER A 6 MIN SWIM: INFLUENCE OF REPEATED SALINE INJECTION OR SIBUTRAMINE ADMINISTRATION**

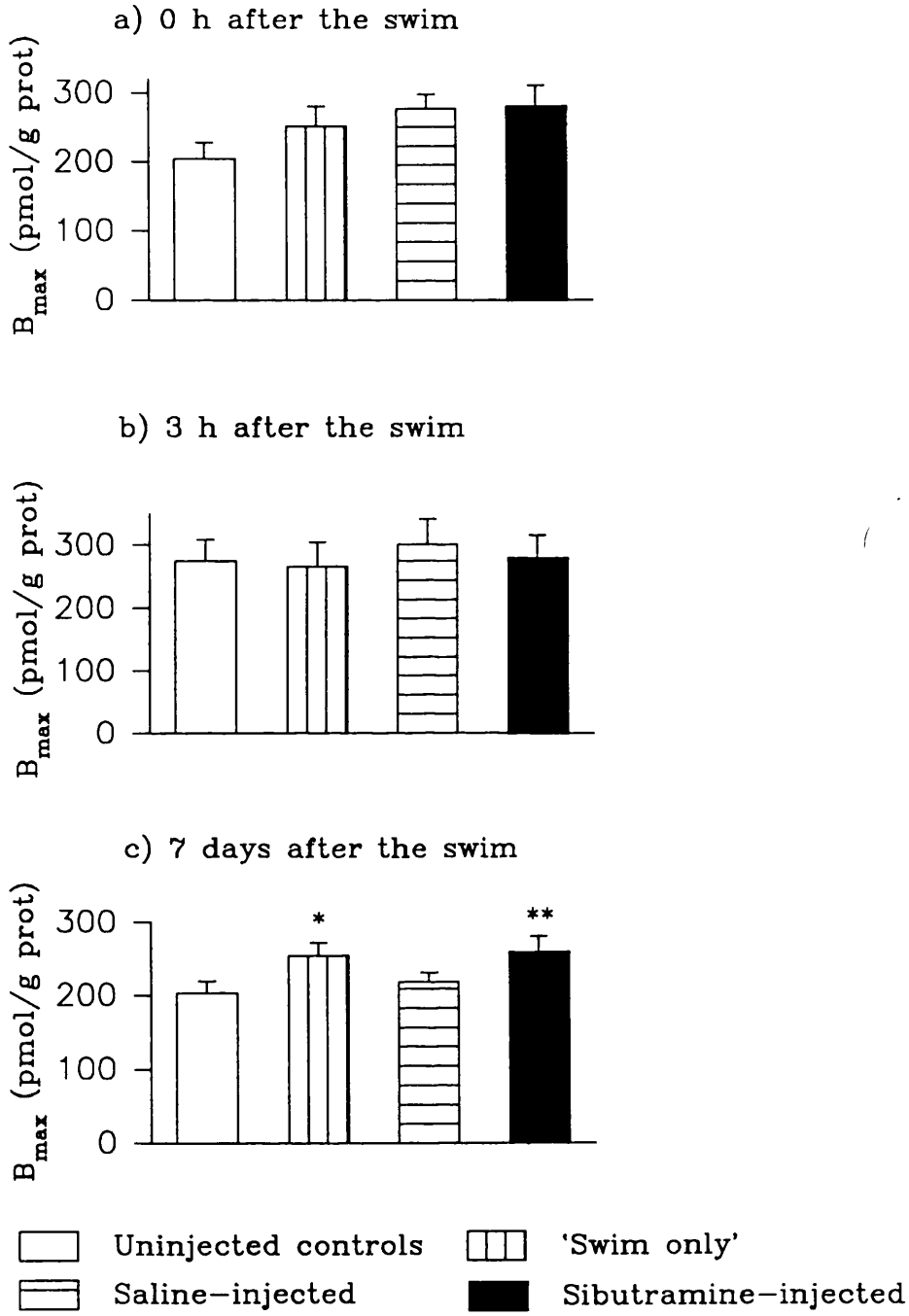


Figure 5.4 : n = 11, each group. [^{3}H]-Ketanserin binding measured 0 h, 3 h or 7 days after the swim, in separate groups of animals. Data from each individual time point analyzed separately using 1-way ANOVA. Following a significant treatment effect, group means were compared using unpaired t-tests: $^*p < 0.05$, $^{**}p < 0.01$ *cf* uninjected controls.

Table 5.6 **CORTICAL 5-HT2 RECEPTOR K_d AFTER A 6 MIN SWIM TEST: INFLUENCE OF REPEATED SALINE INJECTION OR SIBUTRAMINE ADMINISTRATION**

	+ 0 h	+ 3 h	+ 7 days
Uninjected	0.56 ± 0.10	0.50 ± 0.10	0.83 ± 0.32
'Swim only'	0.93 ± 0.21	0.61 ± 0.16	0.65 ± 0.12
Saline-injected	0.96 ± 0.23	0.62 ± 0.13	0.68 ± 0.18
Sibutramine-injected	0.72 ± 0.12	0.56 ± 0.08	0.88 ± 0.23

n = 11, each group. [^{3}H]-Ketanserin binding Kd (nM) measured 0 h, 3 h or 7 days after the swim test in separate groups of animals. Data at each individual time point analyzed using 1-way ANOVA.

5.4.4 Serotonergic system: behavioural measurement

5-HT2 receptor-mediated head-twitches were measured 3 h or 7 days after the swim test; data are displayed in Figure 5.5. 3 h after the swim test, there was a significant treatment effect on head-twitches ($F = 3.667$; d.f. = 3, 36; $p < 0.05$). Subsequent analysis showed that in uninjected animals and in sibutramine-pretreated animals, head-twitches were reduced after a 6 min swim test (ts = 1.704, 2.159; d.f = 36; ps < 0.05). However, head-twitches in saline-pretreated animals did not differ from uninjected controls ($t = 0.682$; d.f. = 36; $p > 0.05$; Figure 5.5).

There was also a significant treatment effect on 5-HT2 receptor-mediated head-twitches measured 7 days after the swim ($F = 4.264$; d.f. = 3,36; $p < 0.05$). Subsequent analysis showed that the large (58%) increase in 5-HT2 receptor-mediated head-twitches seen in the 'swim only' group was significant ($t = 3.091$; d.f. = 36; $p < 0.01$; Figure 5.5). However, this change was not seen in animals which had received either repeated saline or sibutramine pretreatment (ts = 0.818, 0.000; d.f. = 36; ps > 0.05).

Figure 5.5 **5-MeODMT-INDUCED HEAD-TWITCHES AFTER A 6 MIN SWIM: INFLUENCE OF REPEATED SALINE INJECTION OR SIBUTRAMINE ADMINISTRATION**

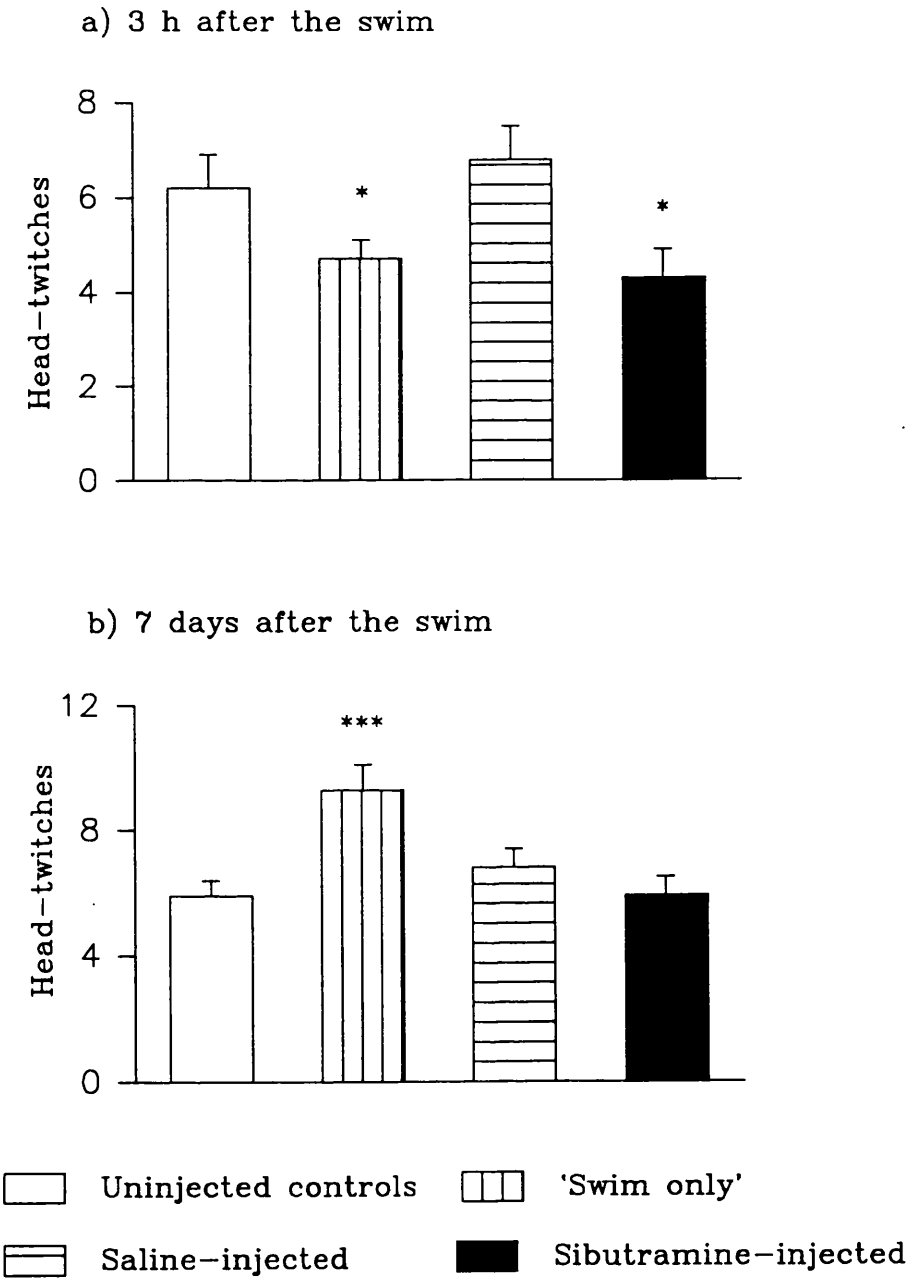


Figure 5.5 : n = 10, each group 3 h after the swim; n = 15, each group 7 days after the swim. Head-twitches measured in separate groups of animals at the two time points. Data from individual time points analyzed separately using 1-way ANOVA. Following a significant treatment effect, group means were compared using unpaired t-tests: $^{*}p < 0.05$, $^{***}p < 0.001$ *cf* uninjected controls.

5.5 Discussion

The experiments described here studied behaviour during a brief swim stress, and the effects of swim stress on indices of central noradrenergic and serotonergic function. The influence of previous experience of repeated stress (once-daily saline injection) or repeated sibutramine administration on behaviour during the swim and subsequent neurochemical changes was also determined. Both short- (+0-3 h) and long-latency (+ 7 days) neurochemical effects were tested for in an attempt to evaluate the duration of effects of a single exposure to stress.

5.5.1 Behavioural response to swim stress.

When animals are placed in water from which escape is impossible, a characteristic hunched, immobile posture is seen after initial vigorous swimming. Immobility does not simply reflect general locomotor activity: a study in naive rats showed that immobility in the swim test does not correlate with open field motor activity (Alonso et al., 1991). Moreover, many antidepressants reduce immobility (i.e. increase swimming) after dosing schedules which reduce or have no effect on locomotor activity in the open field (Porsolt et al., 1979; Borsini et al., 1985). It has been suggested that 'immobility' reflects a despairing or lowered mood which develops when animals realize that escape from the aversive situation is impossible (Porsolt et al., 1977a, 1978). However in rats at least, the inescapable nature of the situation appears not to be important. O'Neill & Valentino (1982) showed that although immobility during the test swim in rats was increased by pre-exposure to a conditioning swim, it was increased to a similar extent whether the pretest swim was inescapable or escapable. Another plausible interpretation of swim-induced immobility suggested is that it develops as an adaptive response to the aversive situation (Hawkins et al., 1978; de Pablo et al., 1989). Familiarity with the aversive environment may play a role in the development of this adaptive response; Borsini's group have shown that previous experience of the cylinders in which swimming is carried out increases immobility in a subsequent test swim even when the cylinders contain no water during the pre-test (Borsini et al., 1986). Since monoamine uptake inhibitors and other antidepressants reduce immobility, this would suggest that such drugs reduce adaptation to an aversive environment. That is, they may substitute for adaptive behaviour, as was suggested earlier for the effects of sibutramine on grooming in the open field (Chapter 3). Whether immobility reflects an adaptive response in mice is not clear; a conditioning swim is not required to induce immobility in this species (Porsolt et al., 1977b), and factors affecting immobility have not been investigated in mice. However, in this experiment, the interpretation of immobility is not a relevant factor since

the swim test is not being used to model a human emotional state. Rather it is being used to determine and compare the effects of repeated saline injection and sibutramine administration on a behavioural response to stress.

In the current experiment, the time spent immobile was significantly reduced by previous experience of repeated stress (saline injection), when this was measured 24 h after the final injection. A previous study has found that immobility in rats is unchanged after repeated saline injection, 1 h after the final injection (Platt & Stone, 1982). An obvious explanation for this discrepancy are the different times at which immobility is measured and could suggest long-latency (+ 24 h) effects of saline injection on immobility. However, when measured 24 h after the final stress exposure, immobility is increased after repeated restraint (Cancela et al., 1991) and tailshock (Garcia-Marquez & Armario, 1987). These changes contrast with the effects of repeated saline injection at this time. One explanation for this discrepancy is that the effects of repeated stress on immobility depend on the duration of stress exposure; saline injections took less than 1 min to administer, whereas animals were restrained for 2 h. Alternatively, saline injection and more severe stress such as restraint may simply have different effects on swim-induced immobility. This suggestion is supported by findings that repeated restraint decreased immobility whereas repeated saline injection had no effect, albeit 1 h after the final stress exposure (Platt & Stone, 1982).

In the current experiment, immobility was also significantly reduced by repeated sibutramine administration, compared with the uninjected controls. A number of other studies have reported a reduction in immobility after repeated administration of monoamine uptake inhibitors (e.g. desipramine: Kitada et al., 1981; Cervo et al., 1990; imipramine, amitriptyline: Zebrowska-Lupina, 1980; Kawashima et al., 1986); however, these drug-induced changes are seen when compared with vehicle-injected groups. In the current study immobility in the vehicle- and drug-injected groups was the same. One interpretation of this finding is that sibutramine itself had no effect on immobility; this seems unlikely, however. Acute sibutramine administration has been shown to reduce immobility (Buckett et al., 1988), and other monoamine uptake inhibitors which reduce immobility in the swim test when given acutely or subchronically have the same effect after repeated administration (e.g. Kitada et al., 1981; Miyauchi et al., 1981; Poncelet et al., 1986;). A more likely explanation for the different effects of monoamine uptake inhibitors relative to vehicle-injected animals in previous and the current experiments is the time at which immobility is measured. Compared with vehicle-injected animals,

monoamine uptake inhibitor-induced reductions in immobility are apparent between 1 and 20 h after the final injection (e.g. Kitada et al., 1981; Miyauchi et al., 1981; Kawashima et al., 1986). However, one study has shown that the reduction in immobility seen 20 h after the final injection of the noradrenaline plus 5-HT reuptake inhibitors amitriptyline or imipramine is 'no longer apparent', compared with saline-injected animals, 24 h after the final injection (Zebrowska-Lupina, 1980). This suggests that the anti-immobility effects of repeated administration of sibutramine, another noradrenaline plus 5-HT reuptake inhibitor, are not long-lasting; that is, they are no longer apparent, 24 h after the final injection.

It has been suggested above that both the effects of repeated saline injection, and the effects of sibutramine relative to the saline-injected group in the swim test, depend on the time at which immobility is measured. This was investigated further by measuring immobility 1 and 6 h after the final injection, to determine the time course of stress- and sibutramine-induced changes in immobility. In this experiment, immobility was unaltered both 1 and 6 h after the final saline or sibutramine injection, however. The lack of significant effects were almost certainly due to the small sample size ($n = 6$ in each group). However, it is noteworthy that, 6 h after the final injection, the treatment effect on immobility only just failed to reach significance ($p = 0.053$, *versus* the chosen criterion for significance of $p < 0.05$). At this time there is a reduction in immobility in sibutramine-pretreated animals, compared with either uninjected or saline-injected animals. Although not conclusive, these observations suggest that the saline injection-induced reduction in immobility develops between 6 and 24 h after the final injection. In addition, sibutramine may reduce immobility, but this effect is no longer apparent 24 h after the final injection; at this time, the apparent anti-immobility effect of sibutramine is due to an effect of repeated injection only.

Summary:

Both repeated saline injection and repeated sibutramine administration reduced immobility during a brief swim, given 24 h after the final injection, to the same extent in mice. While a reduction in immobility after repeated sibutramine is consistent with a drug-induced antidepressant effect, this change may be due entirely to an anti-immobility effect of repeated injection.

5.5.2 Noradrenergic system: neurochemical measurements.

5.5.2.1 *Cortical noradrenaline levels*

A single stress exposure has been shown to decrease cortical noradrenaline levels in the rat; however, a relatively long stress exposure (i.e hours not minutes) is required to effect this reduction (Nakagawa et al., 1981; Tanaka et al., 1988; Adell et al., 1990), which is measurable only immediately after the stress (Tanaka et al., 1988; Ida et al., 1984). However, the possibility that stress may have effects on noradrenergic neurones beyond this initial transient effect has received little attention. The current experiment measured cortical NA levels 3 h after the swim stress, therefore.

Exposure to a 6 min swim did not alter cortical noradrenaline levels in uninjected animals, 3 h after the swim stress. This suggests that any depletion of noradrenaline levels induced by a 6 min swim are transient, as for many other forms of stress.

Cortical noradrenaline levels were also unaltered in animals with previous experience of repeated stress. It has previously been shown that immediately after a single immobilization, cortical noradrenaline levels are increased in rats with previous experience of repeated restraint stress, an effect not seen in previously unhandled rats (Adell et al., 1988). In the current experiment there was no effect on cortical noradrenaline levels in saline-pretreated animals 3 h after the swim; it would appear that previous experience of repeated stress does not prolong the time course of changes in noradrenaline levels induced by an acute swim, therefore.

Repeated administration of the monoamine uptake inhibitor, sibutramine, also did not influence cortical noradrenaline levels, 3 h after a 6 min swim; possible interactions of this type have not previously been studied. It would seem that like repeated stress, repeated sibutramine pretreatment does not alter the duration of changes in noradrenaline induced by an acute stress.

Of the handful of studies examining long-latency neurochemical changes, the majority are generally concerned with receptors not neurotransmitter levels. For instance, rat cortical β-adrenoceptors but not noradrenaline levels were increased 9 days after a single injection of FG 7142 (Stanford et al., 1989). However, it is important to measure transmitter levels, turnover and receptors concurrently, in order to establish whether any long-latency changes in receptors are secondary to any concurrent change in neurotransmitter supply. In the current study, there was no difference between

noradrenaline levels in the unhandled control group and any of the experimental groups, regardless of pretreatment.

5.5.2.2 *Cortical MHPG levels*

Measurement of neurotransmitter levels cannot be used as a measure of turnover since it cannot be determined whether changes in transmitter stores reflect changes in synthesis or utilization of the transmitter. For this reason, levels of cortical MHPG, the final metabolite of noradrenaline, were measured; in the mouse, MHPG levels provide a simple but reliable index of noradrenaline turnover (Heal et al., 1989a).

A single exposure to stress increases cortical noradrenaline turnover in rats, but these changes are rapidly reversed; when measured more than 30 min after exposure to stress (using protocols known to increase noradrenaline turnover, immediately after the stress), noradrenaline turnover is no longer increased above control levels (Ida et al., 1984; Lehnert et al., 1984).

A 6 min swim stress had no effect on cortical noradrenaline turnover, as indexed by MHPG levels in the mouse, 3 h after the swim. This is in agreement with published findings, that stress-induced increases in noradrenaline turnover are rapidly reversed. It suggests also that a 6 min swim does not alter noradrenaline turnover beyond the initial immediate transient increase. Cortical noradrenaline turnover in mice which had received repeated saline or sibutramine pretreatment was also unchanged 3 h after the swim. This suggests that neither pretreatment prolongs stress-induced changes in noradrenaline turnover.

Because these experiments also investigated the possibility of long-latency changes in receptors, it was important to discover whether any such changes might be secondary to changes in transmitter utilization. Cortical noradrenaline turnover was also measured 7 days after the swim stress therefore. At this time noradrenaline turnover was the same in both control animals and those which had received a 6 min swim, regardless of pretreatment. That is, there are no long-lasting effects of the swim stress on noradrenaline turnover.

5.5.2.3 *Cortical β-adrenoceptors*

The effects of a single stress exposure on cortical β-adrenoceptors have received little attention and findings are inconsistent. For instance, novelty (Stanford, 1990), but not

footshock (Cohen et al., 1986), tailshock (Nomura et al., 1981) or immobilization (Stone & Platt, 1982) increase cortical β-adrenoceptors, immediately after a single stress exposure. An important point is that studies of acute stress have generally defined binding using [^{3}H]-DHA with either propranolol or isoprenaline as the displacing ligand (Nomura et al., 1981; Stone & Platt, 1982; Cohen et al., 1986). However, [^{3}H]-DHA binding density (B_{max}) varies with the displacing ligand used (Atterwill et al., 1984; Riva & Creese, 1989a). Moreover, binding of [^{3}H]-DHA defined using either propranolol or isoprenaline may not be only to β-adrenoceptors; both these displacing agents show biphasic displacement of [^{3}H]-DHA in rat cortex (Gillespie et al., 1988; Stockmeier & Kellar, 1988; Riva & Creese, 1989a). Furthermore, results presented in Chapter 4 show that experimentally-induced changes in β-adrenoceptor binding defined using the more selective ligand [^{3}H]-CGP 12177 are not always apparent when [^{3}H]-DHA is used. However, β-adrenoceptor binding density measured with [^{3}H]-CGP 12177 was unchanged, immediately after a 6 min swim stress in the current experiment. [^{3}H]-DHA-defined receptor density was also unchanged. These findings in the mouse are in agreement with studies in rats, which have reported that cortical β-adrenoceptor binding density is unchanged after a single stress exposure (e.g. Nomura et al., 1981; Stone & Platt, 1982; reviewed by Stanford, 1990).

β-Adrenoceptor density measured using [^{3}H]-CGP 12177 was reduced in saline-pretreated animals, immediately after the swim test. Since saline injection alone does not alter [^{3}H]-CGP 12177 binding at this time (24 h after the final injection: Section 4.4.1.2), there is an apparent interaction between acute swim stress and repeated stress pretreatment on cortical β-adrenoceptor binding density. If β-adrenoceptor down-regulation underlies stress adaptation, as suggested by Stone (Stone, 1979b), this interaction may be interpreted as indicating that previous experience of repeated stress facilitated neurochemical adaptation to a subsequent acute stress exposure.

[^{3}H]-CGP 12177 binding in sibutramine-pretreated mice was reduced immediately after the swim test. At this time, repeated sibutramine administration alone down-regulates β-adrenoceptors compared with both uninjected and vehicle-injected mice (Chapter 4). It would appear that the acute stress does not alter cortical β-adrenoceptor density in sibutramine-pretreated animals, therefore. This suggests that no interaction between acute swim stress and repeated sibutramine administration is necessary since β-adrenoceptor down-regulation has already occurred. Alternatively, a further swim-induced down-regulation in sibutramine-pretreated animals is not possible due to a floor for the

reduction of β-adrenoceptor density.

The reduction in [^{3}H]-CGP 12177 binding in both saline- and sibutramine-pretreated animals was of a similar size. Exposure to the swim stress therefore apparently reversed the sibutramine-induced reduction in β-adrenoceptor binding seen when compared with saline-injected animals, by reducing binding in saline-injected animals. The reduction of β-adrenoceptor binding in the saline- and sibutramine-injected groups is only apparent by comparison with the 'swim only' group. This highlights a general problem: namely, the choice of control groups in studies of the effects of repeated drug administration. Commonly, repeated vehicle injection is used as a control in such studies, but this procedure clearly can affect both neurochemical and behavioural parameters, effects which are only apparent by comparison with uninjected controls. A vehicle-injected control group alone may be inadequate for determining effects of repeated drug administration under certain circumstances, and uninjected control groups should be used *in addition* to vehicle-injected animals, therefore.

Although reductions of a similar size in both [^{3}H]-CGP 12177 and [^{3}H]-DHA binding were seen immediately after the swim in saline- and sibutramine-pretreated animals, the reductions in [^{3}H]-DHA binding were not statistically significant. This appears to be due to higher variability in cortical β-adrenoceptor density within each group (larger S.E.M. *cf* [^{3}H]-CGP 12177 binding data). Since binding to each radioligand was determined in the same tissues, it is possible that the higher proportion of non-specific binding of [^{3}H]-DHA relative to [^{3}H]-CGP 12177 (Staehelin et al., 1983; De Paermentier et al., 1989), which may be a result of the lipophilicity of the former radioligand, may contribute to this variability. When binding of each ligand was compared simultaneously (3-way ANOVA), there was no difference between the binding of the two radioligands. This was supported by the lack of significant treatment x ligand interaction. It cannot be concluded, on the basis of these findings, that a second binding site defined by [^{3}H]-DHA and isoprenaline contributes to the increased variability of the binding of this ligand combination. The treatment effect revealed by the 3-way ANOVA showed that, regardless of the radioligand used, cortical β-adrenoceptor binding density was reduced after a single swim stress in both saline- and sibutramine-pretreated animals. These findings parallel those seen when [^{3}H]-CGP 12177 binding was analyzed alone. Although differences in the binding of the two radioligands could not be distinguished statistically, [^{3}H]-CGP 12177 appears to be the more suitable ligand for defining β-adrenoceptor binding; lower variability in the binding of this ligand means that the significance of experimentally-

induced changes is clearer. Although the current findings, which do not support the existence of a second binding site in the binding of [^{3}H]-DHA, are in agreement with the displacement studies of [^{3}H]-DHA in mouse cortex, which showed only displacement by isoprenaline from only 1 site (Section 2.3.1.1). However, Hill coefficients for [^{3}H]-DHA displacement were less than one, whereas those for [^{3}H]-CGP 12177 were not significantly different from unity. The possibility of differences between the binding of the two radioligands still cannot be discounted, therefore.

Seven days after the swim stress, β-adrenoceptor binding density measured using [^{3}H]-DHA was unaltered in either uninjected, saline- or sibutramine-pretreated mice. This indicates that cortical β-adrenoceptors are not involved in any long-latency effects of a brief swim stress.

5.5.2.4 *Cortical β-adrenoceptors and immobility.*

Several reports have shown that immobility in previously unhandled rats is unaltered by pretreatment with either β-adrenoceptor agonists or antagonists (Porsolt et al., 1979; Borsini et al., 1984; Kitada et al., 1983, 1986). These findings indicate that β-adrenoceptors are not involved in the generation of immobility *per se*. However, β-adrenoceptors may play a role in the anti-immobility effects of antidepressants. The reduction in immobility induced by subchronic administration of one of a range of antidepressants (reviewed by Borsini & Meli, 1988) such as desipramine or amitriptyline is potentiated by administration of β-adrenoceptor antagonists (Kitada et al., 1983; Miyauchi et al., 1984). Conversely, co-administration of β-adrenoceptor agonists attenuates the anti-immobility effect of desipramine (Kitada et al., 1983; Miyauchi et al., 1984). Furthermore, the majority of antidepressants tested, including the noradrenaline plus 5-HT reuptake inhibitors imipramine and amitriptyline, both reduce immobility in the swim test (Cervo &Samanin, 1988; reviewed by Borsini & Meli, 1988) and down-regulate rat cortical β-adrenoceptors (Asakura et al., 1982; Heal et al., 1989b). However, repeated administration of the selective 5-HT uptake inhibitor citalopram, which has not been shown to down-regulate cortical β-adrenoceptors (Hytell et al., 1984; Garcha et al., 1985) also has no effect on immobility in the swim test (Platznik & Kotowski, 1985). The effects of other antidepressants which do not alter cortical β-adrenoceptor density (e.g. paroxetine: Nelson et al., 1990, 1991; nisoxetine: Maggi et al, 1980; Asakura et al., 1982) on immobility after repeated administration have not been tested. Together, such findings indicate that cortical β-adrenoceptors may be involved in the anti-immobility effects of antidepressants.

The role of β-adrenoceptors in the effects of repeated exposure to stress on swim-induced immobility has received little attention. Repeated restraint, which alone down-regulates cortical β-adrenoceptors (Stone & Platt, 1982), and repeated saline injection, which down-regulated cortical β-adrenoceptors immediately after swim stress (Section 5.5.2.3), both reduce immobility in the swim test (Platt & Stone 1982; Section 5.5.1). However, repeated restraint has also been shown to increase immobility (Cancela et al., 1991), as does repeated tail-shock (Garcia-Marquez & Armario, 1987), again a procedure which down-regulates cortical β-adrenoceptors (Nomura et al., 1981). These findings do not support a role for β-adrenoceptors in the effects of repeated stress on swim-induced immobility.

Summary:

Effects of a 6 min swim on cortical noradrenaline levels and turnover are transient, with no effects being seen 3 h after the swim. Acute swim stress, in mice, does not affect β-adrenoceptors. However, previous experience of repeated stress appears to facilitate cortical β-adrenoceptor down-regulation in response to an acute stress. Repeated administration of the putative antidepressant sibutramine appears to substitute for this effect of repeated stress. There were no long-latency effects of the swim on noradrenergic neurones.

5.5.3 Neurochemical and behavioural measurements of central serotonergic function.

5.5.3.1 *Cortical 5-HT and 5-HIAA levels*

A single stress exposure increases cortical 5-HT turnover as indexed by 5-HIAA levels or by the 5-HIAA/5-HT ratio, with 5-HT levels being unaltered (Dunn, 1988; Adell et al., 1990). However, the increase in turnover is transient (Lehnert et al., 1984; Adell et al., 1988) and depends on the duration of stress exposure (Kennett et al., 1986; Adell et al., 1988). The effects of acute stress on 5-HT levels and turnover beyond these short-term effects have not been examined.

3 h after a 6 min swim stress, cortical 5-HT levels were unchanged in any treatment group, compared with uninjected controls. 5-HT turnover, as assessed by cortical 5-HIAA levels, or the 5-HIAA/5-HT ratio, was also unaltered in any treatment group. This suggests that beyond any transient elevation of 5-HT turnover after an acute swim stress, there are no short-term effects on 5-HT levels and turnover. Furthermore, any transient effects of acute stress on 5-HT levels and turnover were not prolonged either by previous experience of repeated stress or repeated sibutramine administration.

7 days after the swim, neither cortical 5-HT nor 5-HIAA levels differed from uninjected controls in any treatment group; the 5-HIAA/5-HT ratio was also unaltered in any treatment group. This suggests that there are no long-latency (+7 days) effects of a single brief swim stress on cortical 5-HT turnover at this time. Any changes in cortical 5-HT$_2$ receptors at this time are not secondary to concurrent changes in 5-HT supply, therefore.

5.5.3.2 *Cortical 5-HTP accumulation*

In the current study, information from studies of 5-HIAA levels, used to evaluate 5-HT turnover, was supplemented by measurement of 5-HTP accumulation after 5-HTP decarboxylase inhibition, another index of 5-HT turnover. The two methods were used since neither is ideal. Changes in 5-HIAA levels are not always a reliable index of 5-HT turnover since 5-HIAA may be formed from 5-HT without release occurring: changes in 5-HIAA may not always reflect 5-HT release (Knott, 1988). In contrast, 5-HTP accumulation is a well-validated index of 5-HT turnover (Carlsson et al., 1972; Curzon, 1981). However, it is not ideal for use in the current experiment as the 5-HTP decarboxylase inhibitor, NSD 1015, is administered by i.p. injection. Since i.p. injection is used in the current experiments as a form of stress, any further injections may interact with previous treatment to complicate the results. In the absence of more suitable methods for the assessment of 5-HT turnover, these two methods were compared in the current experiments.

3 h after a 6 min swim, 5-HTP accumulation was unaltered in uninjected animals in the current experiment. This finding is supported by the lack of effect of the swim on cortical 5-HIAA levels, and suggests that 5-HT turnover may be elevated only transiently by an acute swim stress.

5-HTP accumulation was decreased, 3 h after the swim, in saline-pretreated animals. Decreased 5-HTP accumulation reflects decreased activity of tryptophan hydroxylase. However, a previous study has shown that both acute and repeated exposure to sound stress *increase* tryptophan hydroxylase activity in rat cortex, when enzyme activity is measured *ex vivo*. Moreover, the repeated stress-induced increase in activity, present at least 24 h after the final stress exposure, is potentiated if a further stress exposure is given immediately before the animals were killed (Boadle-Biber et al., 1989). However, this potentiation of tryptophan hydroxylase activity *ex vivo* was seen after re-exposure to the same form of stress, whereas in the current experiment animals were exposed to a novel stress (6 min swim, after repeated saline injection) before 5-HT synthesis was measured.

When exposure to a novel acute stress is considered, it has again been shown that the increase in 5-HT turnover is potentiated by previous experience of repeated stress, measured immediately after the acute stress exposure (Adell et al., 1988). It is unclear as to why 5-HT turnover 3 h after acute swim stress should be decreased in animals with previous experience of repeated stress (saline injection).

There are two possible explanations for a reduction in tryptophan hydroxylase activity. First, since tryptophan hydroxylase is not saturated with tryptophan, a reduction in substrate availability may lead to a reduction in enzyme activity. Tryptophan supply may not be compromised when previously unhandled animals are exposed to a single swim stress: that is, the increase in 5-HT release which is compensated for by an increase in 5-HT synthesis (Boadle-Biber et al., 1989) does not deplete tryptophan levels sufficiently to reduce 5-HT synthesis. However, when animals with previous experience of stress are exposure to an acute novel stress, the acute stress-induced increase in 5-HT release is potentiated. The increase in 5-HT synthesis needed to compensate for the reduction in 5-HT levels may ultimately compromise tryptophan levels and therefore reduce tryptophan hydroxylase activity. This explanation is not supported by findings that tryptophan levels in rat cortex are unaltered by either a single stress exposure, or exposure to a novel acute stress in animals with previous experience of repeated stress (Adell et al., 1988, 1990).

The second, more likely explanation concerns end-product inhibition of tryptophan hydroxylase. Inhibition of tryptophan hydroxylase by 5-HT is not important under normal physiological conditions (reviewed by Boadle-Biber, 1982). However, in animals with previous experience of stress, exposure to a novel acute stress induces a large increase in 5-HT release (Adell et al., 1988). The released 5-HT, if taken back up by the neurone, may lead to a large increase in cytoplasmic 5-HT concentrations. This increase may be sufficiently large to cause inhibition of tryptophan hydroxylase activity, in a manner similar to that seen after inhibition of monoamine oxidase, which also increases cytoplasmic 5-HT levels (see Boadle-Biber, 1982). In theory, this explanation could account not only for the reduction in 5-HT synthesis, but for the lack of change in cortical 5-HT levels seen in saline- and drug-pretreated animals after the swim stress.

In the current experiment, 5-HTP accumulation was also reduced 3 h after the swim in cortices from sibutramine-pretreated mice. Since the reductions in the sibutramine- and saline-pretreated groups were similar, the effect of sibutramine may be due merely to an

effect of repeated injection on 5-HT turnover after the swim stress. Alternatively, repeated sibutramine administration and repeated saline injection have similar but non-additive effects on 5-HT turnover after a 6 min swim. Finally, although the effect of repeated sibutramine administration on cortical 5-HT turnover has not been reported, repeated administration of other non-selective monoamine uptake inhibitors e.g. chlorimipramine, decreases 5-HT turnover in rat brain (Van Wijk et al., 1977). The current findings could be explained therefore by a reduction in 5-HT turnover, 24 h after the final sibutramine injection, which is not altered by the swim stress.

The results obtained using 5-HTP accumulation as an index of 5-HT turnover differ from those seen using 5-HIAA levels (Section 5.5.3.1). Such worrying discrepancies between these two methods for estimating 5-HT turnover have been seen previously (Mitchell & Thomas, 1988). The current results obtained using 5-HTP accumulation are probably more reliable since this is a well-validated method for estimating 5-HT turnover. Although this method involves an i.p. injection, this procedure alone would be expected to *increase* 5-HT turnover. The reduction in 5-HTP accumulation seen after the swim in saline- and sibutramine-pretreated animals are highly relevant, therefore.

When 5-HTP accumulation 7 days after the swim stress was analyzed using 1-way ANOVA, cortical 5-HT turnover was unaltered in uninjected animals as well as in saline- and sibutramine-pretreated groups. This is in agreement with the studies of 5-HIAA levels, suggesting that a 6 min swim stress has no long-latency effects on 5-HT turnover, regardless of repeated stress or sibutramine pretreatment. However, although no significant effects were seen, there were 16% and 25% increases in 5-HTP accumulation in the 'swim only' and sibutramine-pretreated groups respectively, 7 days after the swim.

When data from both 3 h and 7 days after the swim test were compared simultaneously (2-way ANOVA), a significant difference between the control groups were found. Although all mice were from the same batch, the difference between controls may result from carrying out the two assays at different times. This should not affect the conclusions since data at each time was compared with simultaneous controls. However, 5-HTP accumulation in sibutramine-pretreated animals was also different at the two time points. If this were merely due to the difference between the two assays, then 5-HTP accumulation would be expected to be reduced in sibutramine-pretreated animals 7 days after the swim compared with 3 h after. However, in the drug-treated group, accumulation was significantly *increased* 7 days after the swim, compared with at 3 h,

suggesting a relevant increase in 5-HT synthesis in this group 7 days after the swim, compared with at 3 h.

3 h after the swim test, treatment effects on 5-HTP accumulation revealed by the 2-way ANOVA were the same as those revealed by the 1-way ANOVA on data from this time point. However, 7 days after the swim, the 16% increase in 5-HT turnover in uninjected animals was now significant. This indicates that any changes in 5-HT2 receptors, 7 days after a 6 min swim stress may be secondary to an increase in cortical 5-HT turnover.

The long-latency swim-induced increase in cortical 5-HT turnover was abolished by previous experience of repeated stress. However, this effect was itself prevented by repeated sibutramine administration. Since 5-HTP accumulation 3 h after the swim was reduced in sibutramine-pretreated animals, the increase seen at 7 days in this group may represent a rebound increase in 5-HT turnover following withdrawal of the drug. This is supported by the significant increase in 5-HTP accumulation in the sibutramine-treated group 7 days after the swim, compared with the same treatment group, 3 h after the swim revealed by the 2-way ANOVA.

Overall, these findings indicate that the long-latency effects of the swim stress on 5-HT turnover are influenced differently by previous experience of repeated stress and sibutramine administration, however.

5.5.3.3 *Cortical 5-HT2 receptor binding*

5-HT2 receptors may be functionally analogous to β-adrenoceptors since they are both thought to be located post-synaptically. However, unlike β-adrenoceptors, little is known about the effects of stress on 5-HT2 receptors.

Immediately after the swim stress, cortical 5-HT2 receptor binding density was unaltered in uninjected animals. It has been shown that a single 2 h immobilization stress increases cortical 5-HT2 receptor density, immediately after cessation of the stress (Torda et al., 1990). However, the current findings are supported by the report that cortical 5-HT2 receptor density is unaltered immediately after a 15 min swim stress in rats (Kawanami et al., 1992). It would appear therefore that cortical 5-HT2 receptors are not down-regulated by a brief (minutes) exposure to stress. Cortical 5-HT2 receptors were unaltered by both repeated saline injection and sibutramine administration, immediately after the swim. Alone, neither saline nor sibutramine alters cortical 5-HT2 receptor density at this

time (24 h after the final injection: Chapter 4). There is no interaction between repeated saline injection or sibutramine administration and an acute swim stress on cortical 5-HT2 receptors measured immediately after the swim stress, therefore.

3 h after the swim stress, cortical 5-HT2 receptor binding density was again unaltered in uninjected animals, suggesting that a single swim stress has no short-latency effects on cortical 5-HT2 receptors. At this time, cortical 5-HT2 receptor density was also unchanged in both pretreated groups, although at this time, cortical 5-HT turnover is reduced in saline- and sibutramine-pretreated animals. If there is an inverse relationship between 5-HT supply and 5-HT2 receptor density, an upregulation of cortical 5-HT2 receptors in these groups would be predicted at this time. However, it seems that 5-HT2 receptors are not regulated in the normal way (reviewed by Apud, 1991). Whether or not this is the case here, any change in 5-HT2 receptors induced by the reduction in 5-HT turnover 3 h after the swim in saline- or sibutramine-pretreated animals has not yet developed at this time.

7 days after the swim stress, cortical 5-HT2 receptor density was increased in uninjected mice. This increase was prevented by previous experience of repeated stress (saline injection). Despite this effect of repeated stress, an increase in 5-HT2 receptor density was apparent in sibutramine-pretreated animals; this suggests that the effects of repeated stress are prevented by repeated sibutramine administration. Since the effects of repeated stress and sibutramine administration were not the same, long-latency effects of an acute stress are apparently influenced differently by previous experience of repeated stress and sibutramine administration. This parallels the difference in effects of repeated saline injection and sibutramine administration on grooming in the open field. It is apparent therefore that previous experience of repeated stress and administration of monoamine uptake inhibitors can have different effects on both behavioural and neurochemical responses to stress.

5.5.3.4 *5-HT2 receptor function in vivo*

As with 5-HT receptor binding, little attention has been paid to the effects of stress on central 5-HT receptor function. In the current experiment, 5-MeODMT-induced head-twitches were used as an index of central 5-HT2 receptor function *in vivo.*

3 h after the swim stress, 5-HT2 receptor-mediated head-twitches were reduced in uninjected animals although cortical 5-HT2 receptor binding density was unaltered at this

time (Section 5.5.3.3). However, head-twitches are hindbrain/spinally mediated and this behaviour cannot be assumed to mirror binding in the cortex. It is possible that swim stress induces region-specific changes in 5-HT2 receptor density, therefore. Alternatively, changes in the coupling of the receptor to its second messenger system (phosphoinositide turnover) could account for different effects on receptor density and receptor function *in vivo*. In fact, it has recently been shown that, in the cortex at least, 5-HT2 receptor-stimulated phosphoinositide turnover but not 5-HT2 receptor binding density was reduced immediately after a 15 min swim stress (Kawanami et al., 1992). Changes in other neurotransmitter systems which modulate head-twitches could also account for differences between cortical 5-HT2 binding density and function *in vivo*.

Although the anatomical location of the receptors involved has not been determined, β-adrenoceptor agonists increase 5-HT2 receptor-mediated head-twitches (Ortmann et al., 1981; Handley & Singh, 1986a, b). However, the involvement of β-adrenoceptors in the swim-induced reduction in head-twitches would seem unlikely since β-adrenoceptors were unaltered after the swim stress in uninjected animals, in the cortex at least (Section 5.5.2.3). Head-twitches are also under tonic control by α2-adrenoceptors, since α2 adrenoceptor agonists attenuate, and antagonists potentiate the head-twitch response (Handley & Brown, 1982; Heal et al., 1986). The anatomical site of these α2-adrenoceptors is not known, but they are thought to be postsynaptic since the effect of α2-adrenoceptor antagonists on head-twitches is not abolished by noradrenergic denervation (Heal et al., 1986; Tadano et al., 1987). However, the effects of acute stress on α2-adrenoceptor binding, which measures predominantly post-synaptic α2-adrenoceptors, are not consistent (reviewed by Stanford, 1990) and the effects of swim stress on these receptors has not been reported. It is not known therefore whether α2-adrenoceptors are involved in the decrease in head-twitches seen 3 h after a 6 min swim. On the basis of the current results, the reason for the difference in effects on receptor binding and 5-HT2 receptor-mediated head-twitches is not clear. Notwithstanding, central 5-HT2 receptor function *in vivo* is clearly reduced 3 h after a 6 min swim stress.

In contrast, 3 h after the swim, the swim-induced decrease in head-twitches was not apparent in saline-pretreated animals. This reflects an interaction between acute and repeated stress since repeated saline injection does not alter head-twitches, measured 24 h after the final injection (Chapter 4). Head-twitches are potentiated by β-adrenoceptor activation, but cortical β-adrenoceptors are down-regulated by swim stress in saline-injected animals (Section 5.5.2.3). It is unlikely that the interaction between the swim

stress and repeated saline injection on central 5-HT2 receptors involves β-adrenoceptors, therefore.

3 h after the swim stress, head-twitches were decreased in sibutramine-pretreated animals. This suggests that the interaction between repeated stress and acute swim stress on head-twitches is prevented by sibutramine. This effect represents a complex interaction since, like saline injection, sibutramine administration alone has no effect on head-twitches measured 24 h after the final injection (Chapter 4). This interaction may involve β-adrenoceptors, since cortical β-adrenoceptors were down-regulated in sibutramine-pretreated animals, immediately after the swim; head-twitches are increased by administration of β-adrenoceptor agonists, so a reduction in β-adrenoceptors may reduce this behaviour. An effect on α2-adrenoceptors is less likely since repeated sibutramine administration has been shown to decrease post-synaptic α2-adrenoceptor function (Heal et al., 1991), which would be predicted to *increase* head-twitches. It is clear however that the short-term effects of acute swim stress on 5-HT2 receptor-mediated head-twitches is influenced differently by previous experience of repeated stress and sibutramine administration.

7 days after a single swim stress, central 5-HT2 receptor function *in vivo* was increased in uninjected animals. Although twitches cannot be expected to reflect cortical 5-HT2 receptor binding, this long-term swim-induced increase in 5-HT2 receptor-mediated head-twitches mirrors the increase in cortical 5-HT2 receptor binding density at this time. These long-term effects are concurrent with an increase in 5-HT turnover (Section 5.5.3.2). These findings again suggest that 5-HT2 receptors are not modulated in the traditional manner.

The swim-induced long-latency increase in 5-HT2 receptor-mediated head-twitches was blocked both by previous experience of repeated stress and by repeated sibutramine administration. This contrasts with previous findings that sibutramine prevented the effects of repeated stress on both the short-term swim-induced decrease in head-twitches and the long-term swim-induced increase in 5-HT2 receptor binding density. Repeated sibutramine administration may have no effect on the swim-induced increase in head-twitches, 7 days after the swim. If this is so, then the similar effects of both saline and sibutramine pretreatment on head-twitches at this time are due entirely to an effect of repeated injection. Alternatively, it is possible that repeated saline injection and repeated sibutramine administration have similar non-additive effects on head-twitches at this time.

The long-latency swim-induced increase in head-twitches may be prevented by sibutramine or saline pretreatment by a change in 5-HT2 receptor binding in the hindbrain/spinal cord, or in the coupling of 5-HT2 receptors to their second messenger system. A change in the modulation of head-twitches is also possible; however, any such change is unlikely to involve noradrenergic neurones since the long-term effects of swim do not involve this transmitter system.

Changes in cortical 5-HT synthesis parallelled the swim-induced changes in twitches in the sibutramine-pretreated group 3 h and 7 days after the swim, and those in the uninjected group 7 days after the swim. That is, both twitches *and* synthesis were decreased by the swim in sibutramine-pretreated animals in the short-term, and both increased in uninjected and sibutramine-pretreated groups, 7 days later. It is generally held that a reduction in transmitter supply induces an increase in post-synaptic receptors, and vice versa, but it is clear that this does not hold for cortical 5-HT2 receptors (see above). This anomaly in 5-HT2 receptor regulation could explain the parallel changes in both 5-HT turnover and 5-HT2 receptor regulation. However, 5-HT synthesis was measured in the cortex, and head-twitches are hindbrain/spinally mediated; the parallel changes in these two parameters could merely reflect region-specific changes.

Summary;

Both the short- and long-term effects of a brief swim involve changes in central serotonergic function. In the short-term (3 h after the swim), no effects were seen on cortical 5-HT2 receptor binding or on 5-HT turnover but central 5-HT2 receptor function *in vivo* was reduced after the swim. Repeated previous experience of stress (once-daily saline injection), but not sibutramine administration abolished the short-term impact of a 6 min swim on 5-HT2 receptor function.

A brief swim induced long-latency increases in cortical 5-HT turnover, 5-HT2 receptor binding density *in vitro*, and central 5-HT2 receptor function *in vivo*. None of these changes were present in mice which had received repeated saline pretreatment. Repeated sibutramine pretreatment abolished the effect of repeated injection on cortical 5-HT turnover and 5-HT2 receptor density but the effect of repeated saline injection on 5-HT2 receptor-mediated head-twitches was either not altered, or was mimicked, by repeated sibutramine pretreatment.

5.5.4 Conclusions

1. The short-term effects of a brief swim stress involve changes in ~~cortical~~ central 5-HT2A receptor function, but not cortical β-adrenoceptors.

2. Previous experience of repeated stress facilitates cortical β-adrenoceptor down-regulation on exposure to an acute swim stress

3. Swim stress induces long-latency changes in cortical 5-HT synthesis and 5-HT2 receptors; noradrenergic neurones are not involved in the long-latency effects of the swim.

4. Both the short- and long-latency effects of the swim on central 5-HT2 receptors and 5-HT synthesis are influenced by previous experience of stress

5. The effects of repeated stress on short- and long-latency effects of the swim on central 5-HT2 receptors and 5-HT synthesis is prevented by or mimicked by repeated sibutramine administration, depending on the parameter under consideration.

6. Apparent reductions in cortical β-adrenoceptors in tissue derived from the same stress- or sibutramine-pretreated animals were significant only when binding was defined using [^{3}H]-CGP 12177, but not [^{3}H]-DHA. This supports the suggestion that [^{3}H]-CGP 12177 is a more suitable ligand than [^{3}H]-DHA to define β-adrenoceptor binding.

6.0 AN INVESTIGATION OF THE BINDING PROFILE OF [^{3}H]-DIHYDROALPRENOLOL IN MOUSE CORTEX: EFFECTS OF REPEATED SALINE INJECTION OR ADMINISTRATION OF MONOAMINE UPTAKE INHIBITORS

6.1 Introduction

In the preceding chapters, various anomalies in the binding of the β-adrenergic radioligand, [^{3}H]-dihydroalprenolol ([^{3}H]-DHA), have been noted. However, not all these anomalies can be accounted for by the greater variation in binding estimates seen with this lipophilic radioligand when compared with that seen when binding is measured using [^{3}H]-CGP 12177, a lipophobic β-adrenergic radioligand. First, there is a possible species difference in the binding of [^{3}H]-DHA. Published studies have shown that [^{3}H]-DHA is displaced by isoprenaline from two binding sites in rat cerebral cortex (Manier et al., 1987a,b) and the results of the current study are not inconsistent with this (Section 2.3.1.1). However, in mouse cortex, [^{3}H]-DHA was displaced from only one binding site by isoprenaline (Section 2.3.1.1). This suggests that this radioligand may be adequate for measuring β-adrenoceptor binding in mice, but not rats, when specific binding is defined using isoprenaline. However, this is not supported by evidence obtained from further analysis of the displacement curves. This evidence reveals an anomaly in the binding of [^{3}H]-DHA in mice: although curves for the displacement of [^{3}H]-DHA by isoprenaline in mice were best described by a 1 site model, the mean Hill coefficient was significantly less than unity, suggesting that [^{3}H]-DHA may be displaced from more than one site (Section 2.3.1.1). On the basis of this conflicting preliminary evidence, the possibility that [^{3}H]-DHA binds to a second binding site in mouse cortex cannot be conclusively discounted.

A further discrepancy was noted when the binding of [^{3}H]-DHA was compared with [^{3}H]-CGP 12177 in cortices from saline- and drug-treated mice. Although the binding of the two radioligands was defined in duplicate aliquots from the same tissues, saline-, DMI- and sibutramine-induced changes in [^{3}H]-DHA binding were almost twice those for [^{3}H]-CGP 12177 binding. If indeed [^{3}H]-DHA is displaced from more than one binding site in mouse cortex, then changes in this second site could account for differences in stress- and drug-induced changes in the binding of these two radioligands.

These findings support the view that [^{3}H]-CGP 12177 is a more suitable ligand to measure β-adrenoceptor binding, as suggested in Chapter 5. However, questions still to be answered include whether [^{3}H]-DHA binds to more than one site in mouse cortex and,

if so, what is the identity of this second site. These issues are of relevance since studies of the effects of monoamine uptake inhibitors on β-adrenoceptor binding, although not often carried out in mice, commonly use [^{3}H]-DHA to measure binding (Suzdak & Gianutsos, 1985; Stanford et al., 1987). If this radioligand is displaced from a non-β-adrenergic site which is altered by antidepressant pretreatment then any effects of the treatment on β-adrenoceptors may be exaggerated, or even masked, by changes in the second site. The current studies aimed to examine the binding of [^{3}H]-DHA in mouse cortex in more detail, therefore.

The binding of [^{3}H]-DHA has rarely been investigated in mice; for this reason, published studies of the binding of this radioligand in rats were used to provide clues as to the possible identity of any second binding site from which [^{3}H]-DHA might be displaced in mice. In rats, the second site from which isoprenaline displaces [^{3}H]-DHA has low affinity for isoprenaline, but is unlikely to be an agonist low-affinity form of the β-adrenoceptor, since changes in the density of this site can be abolished if 5-HT is added to the binding assay (Gillespie et al., 1988). This finding indicates that isoprenaline displaces [^{3}H]-DHA from a 5-HT receptor.

Subsequent studies have used ligands with relative selectivity for the various 5-HT receptor sub-types in an attempt to determine to which receptor type [^{3}H]-DHA binds in rats. In a detailed study by Riva & Creese (1989a), the displacement of [^{3}H]-DHA by a variety of serotonergic ligands in rat cerebral cortex was investigated. The affinities of these ligands (including the relatively selective 5-HT$_{1B}$ ligand, CGS 12066B; Neale et al., 1987) led to the suggestion that [^{3}H]-DHA is displaced from 5-HT$_{1B}$ receptors in control tissues. A similar suggestion was made by another group, who investigated the effect of the addition of one of a range of serotonergic ligands on the 5,7-dihydroxytryptamine lesion-induced increase in the specific binding of [^{3}H]-DHA defined using propranolol in rat cortex (Stockmeier & Kellar, 1989). The lesion-induced increase in binding was abolished by the addition to the binding assay of ligands with high affinity for 5-HT$_{1A}$ or 5-HT$_{1B}$ receptors e.g. RU 24969, although [^{3}H]-DHA binding was not altered in the presence of any serotonergic ligand in control tissues. This study again suggests that [^{3}H]-DHA can bind to a 5-HT$_1$ receptor, although this receptor is not present in a significant proportion in naive animals (Stockmeier & Kellar, 1989). Moreover, these results suggest that changes in the 5-HT$_1$ receptor to which [^{3}H]-DHA binds could underlie experimentally-induced changes in [^{3}H]-DHA binding which have previously been interpreted as changes in β-adrenoceptors. However, these findings are not conclusive:

in the same study, binding of the selective radioligands [^{3}H]-8-OH-DPAT and ^{125}I-CYP, to 5-HT1A and 5-HT1B receptors respectively, showed that these receptors were not increased in rat cortex after 5,7-dihydroxytryptamine lesioning (Stockmeier & Kellar, 1989).

The current experiments concentrated on the displacement of [^{3}H]-DHA in mouse cortex by each of four serotonergic ligands, chosen for their differing selectivities for different serotonergic receptors. This was to determine whether or not [^{3}H]-DHA binds to 5-HT1A, 5-HT1B, 5-HT1C or 5-HT2 receptors in mouse cortex, using a similar approach to that adopted by a previous study in rats (Riva & Creese, 1989a). The ligands chosen were:

(i) 8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT), a 5-HT1A selective ligand (Gozlan et al., 1983; Hoyer, 1988)

(ii) 7-trifluoromethyl-4(4-methyl-1-piperazinyl)-pyrrolo[1,2-a]quinoxaline 1:2 maleate salt (CGS 12066B), a ligand which has high affinity for 5-HT1A and 5-HT1B receptors (Neale et al., 1987)

(iii) Trimethylfluoropiperazine (TFMPP), a ligand with similar affinity for 5-HT1A, 5-HT1B and 5-HT1C receptors (Peroutka, 1986; Hoyer, 1988)

(iv) Ritanserin, a ligand with high affinity for both 5-HT1C and 5-HT2 receptors (Hoyer, 1988; Sanders-Bush & Breeding, 1988).

A major difference between this and the previous displacement study of the binding profile of [^{3}H]-DHA (Riva & Creese, 1989a) was that the binding of this radioligand was examined not only in cortices from uninjected mice, but also in mice which had previous experience of repeated stress (once-daily saline-injection) or repeated administration of a monoamine uptake inhibitor (DMI or sibutramine). The proportion of [^{3}H]-DHA displaced from the putative second site may be relatively small, and it was not expected that the current displacement studies would be able to show stress- or drug-induced changes in the binding of [^{3}H]-DHA from this site. However in rats, there is conflicting evidence as to whether [^{3}H]-DHA is displaced from a 5-HT receptor in untreated animals (*cf* Riva & Creese, 1989a and Stockmeier & Kellar, 1989). Moreover, these studies have inferred that binding to this second site is increased by experimental treatments such as lesioning of 5-HT neurones or DMI treatment (Stockmeier & Kellar, 1989; Riva & Creese, 1989b). The possibility that in mice, [^{3}H]-DHA may be displaced from a 5-HT receptor which is induced by stress or antidepressant treatment, but is not detectable in cortices from uninjected mice was examined therefore.

Following displacement studies aimed at identifying the 5-HT receptor to which [^{3}H]-DHA binds, saturation binding was used with the aim of quantifying stress- and drug-induced changes in this receptor. In these studies, the binding of [^{3}H]-DHA to cortical β-adrenoceptors was masked by the addition of 30 μM (-)-noradrenaline HCl. Noradrenaline was used in these studies since it has been suggested that it does not displace [^{3}H]-DHA from the serotonergic site to which this radioligand binds in rats (Stockmeier & Kellar, 1989). Since it has been shown that isoprenaline may displace [^{3}H]-DHA from a 5-HT receptor in rats (Gillespie et al., 1988), this drug was considered inappropriate to mask binding to β-adrenoceptors in the current experiments.

6.2 Aims

1) To determine whether any part of the specific binding of [^{3}H]-DHA is to one or more serotonergic receptor types in cortices from uninjected mice

2) To determine whether repeated saline, DMI or sibutramine injection influences any component of [^{3}H]-DHA binding which is to a 5-HT receptor in mice

6.3 Methods

6.3.1 Animals and treatments

Male CD1 mice (18-20 g on arrival in the animal house) were housed under a 12/12 h light/dark cycle (lights on from 08.00-20.00 h), with free access to water and food. Animals were divided into 4 groups destined for either stress (saline injection) or drug (desipramine or sibutramine) pretreatment, or no pretreatment ('uninjected'); groups were matched for mean weight. The animals were distributed in cages of not less than 4; each cage contained animals from one treatment group only. All animals remained unhandled (apart from routine husbandry) for 5 days to allow acclimatization to the new surroundings. Following this period, animals from the stress and drug pretreatment groups were weighed and then received an injection of either 0.9% sterile saline (10 ml/kg i.p.), desipramine (DMI: 10 mg/kg i.p.) or sibutramine hydrochloride (3 mg/kg i.p.). This procedure, carried out between 09.30-10.30 h, was repeated once-daily for a total of ten days. The uninjected group remained undisturbed in the home cage throughout this period.

24 h after the final injection, animals from all treatment groups including the uninjected group were removed in the home cage to an adjacent room. Animals were killed by cardiothoracic shock and cervical dislocation; brains were removed and cortices dissected

over ice. Tissue was frozen on dry ice and subsequently stored at -20°C.

6.3.2 Radioligand binding

Separate experiments were carried out to determine the displacement of [^{3}H]-DHA by each of the four serotonergic ligands, and to determine saturation and single-point specific binding of [^{3}H]-DHA to non-β-receptors. Each experiment examined displacement by one ligand only, measuring binding in cortices from individual subjects from each of the 4 groups (uninjected, stress and DMI- and sibutramine-treated). Within a given experiment, all treatment groups contained the same number of animals.

Membranes were prepared freshly for each assay (Section 2.3.1.1); all assays contained at least one sample from each group, and the number of samples from each treatment group was the same within an assay.

[^{3}H]-DHA displacement was studied as previously described (Section 2.3.1.1) using each of the following serotonergic ligands:

(i) 8-OH-DPAT
(ii) CGS 12066B
(iii) TFMPP
(iv) ritanserin

Stock solutions of each displacing ligand were prepared as below:

(i) 8-OH-DPAT was dissolved in 1 ml 100% ethanol, then made up to volume with 10% ethanol to give a final concentration of 5 x 10-3 M
(ii) CGS 12066B was dissolved in 10% ethanol to give a concentration of 5 x 10-3 M
(iii) TFMPP was dissolved in Tris-HCl, to give a concentration of 5 x 10^{-3} M
(iv) Ritanserin was dissolved in a few drops of glacial acetic acid; 1 ml of distilled water was added dropwise to this solution whilst vortexing, then distilled water was used to make the solution up to volume. The final concentration of the stock solution was 5 x 10^{-3} M.

Each ligand was used at 17 different concentrations over the range 10^{-11}-10^{-3} M prepared from the stock solution; a further tube contained no displacing ligand. As data accumulated, the actual concentrations used were adjusted between assays, to enable better definition of inflexions of the displacement curves.

Displacement curves were analyzed using GRAPHPAD; curves were fitted to the data using both 1- and 2-site models. The analysis provided estimates of the Ki

(equilibrium dissociation constant) for the displacement of [^{3}H]-DHA by each displacing ligand. Data were expressed as pKi values (-log Ki); this transformation was made since, in general, published data is expressed as -log Ki. However, for some ligands, in particular CGS 12066B, only IC_{50} values (concentration of cold ligand producing 50% displacement of the radioligand used) or -log K_d (pK_d) values have been published. Although not ideal, it was sometimes necessary to compare the pKi values obtained in the current experiments with published IC_{50} or pK_d values, therefore.

The remaining portion of the study concentrated on 5-HT$_{1A}$ receptors, since displacement of [^{3}H]-DHA from 5-HT$_{1B}$, 5-HT$_{1C}$, or 5-HT$_2$ receptors had been ruled out in the displacement studies described above. Saturation binding of [^{3}H]-DHA to 5-HT$_{1A}$ receptors was carried out (Section 2.3.1.1), but this approach proved unfeasible. Single-point binding studies were therefore used to determine whether binding of [^{3}H]-DHA to 5-HT$_{1A}$ receptors could be distinguished. Studies were carried out as previously described (Section 2.3.1.1). Total [^{3}H]-DHA binding was defined as [^{3}H]-DHA binding in the absence of any displacing ligand. [^{3}H]-DHA binding was also measured in the presence of 30 µM (-)-noradrenaline HCl ('non-β-adrenoceptor binding'), and in the presence of both 30 µM (-)-noradrenaline HCl and 10 µM 8-OH-DPAT ('non-specific binding'). This enabled the two components of specific [^{3}H]-DHA binding to be distinguished as defined below:

(i) β-adrenoceptor binding - the difference between total and 'non-β-adrenoceptor binding'

(ii) 'specific binding' - the difference between total and 'non-specific binding' (i.e. β-adrenoceptor binding and 5-HT$_{1A}$ binding)

(iii) 5-HT$_{1A}$ binding - any difference between 'specific binding' and β-adrenoceptor binding

6.3.3 Statistics

F-tests were carried out to determine whether the data were better fitted by a 1- or 2-site model for displacement curves. 1-Way ANOVA was used to evaluate treatment effects on pKi values for [^{3}H]-DHA displacement for each ligand. Where significant differences were found, differences between group means were evaluated using Student's unpaired t-test.

Significant differences between mean Hill coefficients for each treatment group and unity were tested for using the non-parametric Wilcoxon signed-rank test.

For single-point binding, 1-way ANOVA was used to simultaneously compare total binding, β-adrenoceptor binding and 'specific binding' (to both the β- and non-β-receptor components of [^{3}H]-DHA binding). A significant difference between 'specific binding' and β-adrenoceptor binding was taken to indicate the presence of 5-HT$_{1A}$ receptors.

6.4 Results

6.4.1 Displacement binding

6.4.1.1 *Displacement of [^{3}H]-DHA by 8-OH-DPAT*

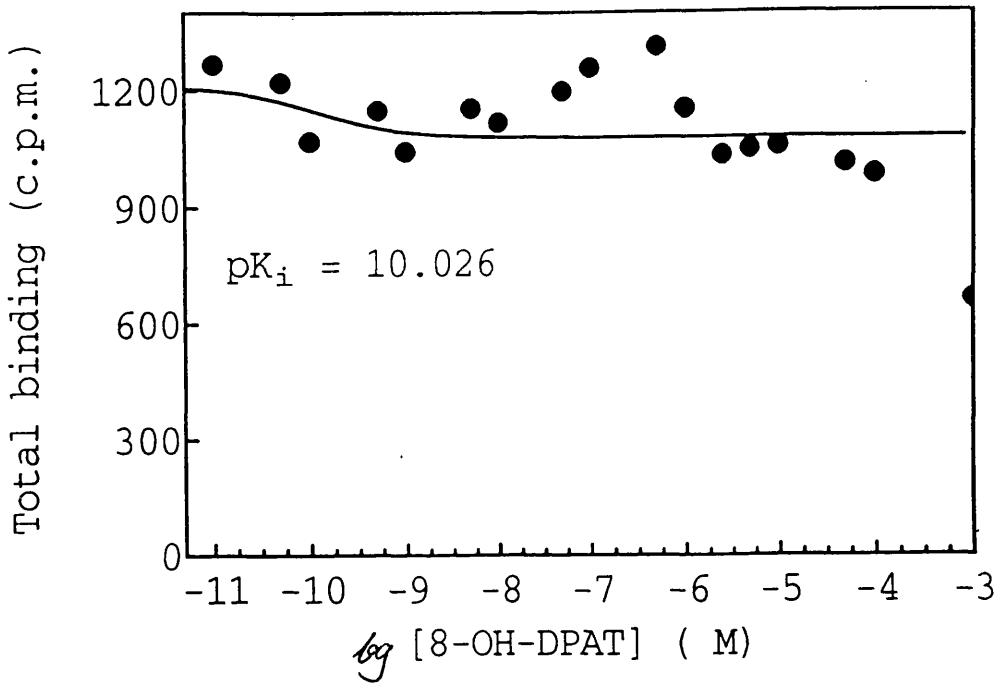
A typical curve for the displacement of [^{3}H]-DHA by 8-OH-DPAT in control tissues is shown in Figure 6.1a. Although displacement curves show considerable scatter, curve-fitting analysis indicated that all curves (each of which was determined in a different subject), were best fit by a 1-site model; the average pKi value (from 8 curves) was 10.1 ± 1.06 (Table 6.1). Displacement curves from saline- and DMI-pretreated animals, although again scattered in appearance, were also best fit by a 1-site model; pKi values in these treatment groups were similar to the control group and are shown in Table 6.1. Displacement curves in cortices from sibutramine-pretreated mice were also best described by a 1-site model (Figure 6.1b). However, 1-way ANOVA comparing pKi values from all treatment groups revealed a significant treatment effect ($F = 9.568$; d.f. = 3, 27; $p < 0.001$). Comparison of group means showed that the average pKi value in the sibutramine-treated group was significantly lower than in all other groups (ts = 4.430, 4.740, 3.570; d.f. = 27; ps < 0.001 *cf* uninjected, saline-injected and DMI-injected groups respectively; Table 6.1). Although Hill coefficients were calculated for each displacement curve, values obtained ranged from less than 0.01 up to greater than 15; in some cases, no Hill coefficient could be obtained from the data. These problems presumably arose from the scatter in the binding curves, and the data are therefore not shown.

6.4.1.2 *Displacement of [^{3}H]-DHA by CGS 12066B*

A typical curve for the displacement of [^{3}H]-DHA binding by CGS 12066B in control tissue is shown in Figure 6.2. Curves were best fit by a 1-site fit; the mean pKi value for CGS 12066B displacement was 5.74 ± 0.10 (Table 6.1). Curves for the displacement of [^{3}H]-DHA by CGS 12066B in tissues from saline-, DMI- and sibutramine-treated animals were also best fit by a 1-site model. The pKi values in these groups were similar to those in the control group (Table 6.1); 1-way ANOVA comparing all 4 groups did not reveal any significant treatment effect on pKi values ($F = 0.550$; d.f = 3, 24; $p > 0.05$). Hill coefficients for the displacement of [^{3}H]-DHA by CGS 12066B for each of the treatment groups did not significantly differ from unity (Table 6.2).

Figure 6.1 **DISPLACEMENT OF [^{3}H]-DHA BY 8-OH-DPAT IN MOUSE CORTEX**

a) Displacement in cortices from an uninjected mouse



b) Displacement in cortices from a sibutramine-treated mouse

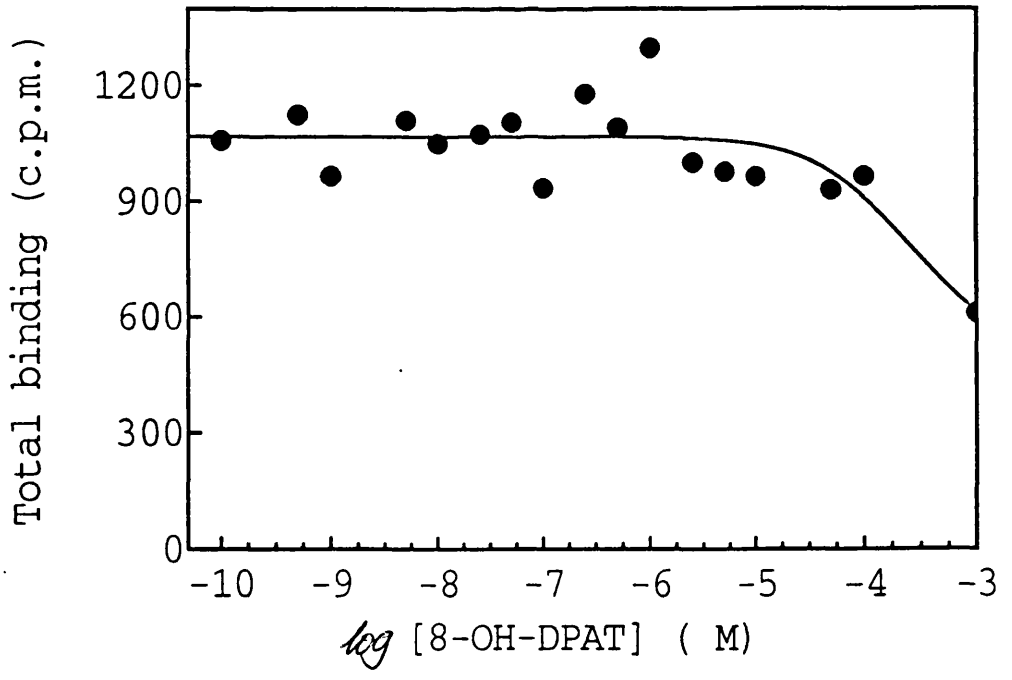


Figure 6.1 Displacement of 1 nM [^{3}H]-DHA by 8-OH-DPAT in cortices from a) an uninjected and b) a sibutramine-treated mouse. Data, analyzed using GRAPHPAD, were best fit by a single site model (Fs = 0.022, 1.050; d.f. = 2, 14; ps > 0.05).

Table 6.1 **MEAN pKi VALUES FOR THE DISPLACEMENT OF [^{3}H]-DHA IN MOUSE CORTEX BY VARIOUS SEROTONERGIC LIGANDS**

	8-OH DPAT pKi	CGS 12066B pKi	TFMPP pKi	Ritanserin pKi
Uninjected	10.1 ± 1.06	5.74 ± 0.10	6.07 ± 0.16	5.17 ± 0.39
Saline	10.6 ± 0.45	5.76 ± 0.07	5.83 ± 0.09	5.13 ± 0.26
DMI	9.4 ± 0.67	5.79 ± 0.09	6.01 ± 0.14	4.87 ± 0.14
Sibutramine	5.7 ± 0.40***	5.90 ± 0.13	5.76 ± 0.12	5.68 ± 0.90

pKi values (-log Ki) shown are the mean ± S.E.M. of values from 7 or 8 curves, which were analyzed separately using GRAPHPAD. Each displacement curve was measured in cortices taken from individual mice killed 24 h after the final saline or drug injection, and from uninjected mice killed simultaneously. Following a significant treatment effect from the 1-way ANOVA, group means were compared using Student's t-tests; ***$p < 0.001$ *cf* uninjected controls.

Table 6.2 **MEAN HILL COEFFICIENTS FOR THE DISPLACEMENT OF [^{3}H]-DHA BY CGS 12066B, TFMPP AND RITANSERIN IN MOUSE CORTEX**

	Displacing ligand		
Treatment group	CGS 12066B (n=7)	TFMPP (n=7)	Ritanserin (n=7)
Uninjected	0.871 ± 0.097	0.917 ± 0.135	2.210 ± 0.722
Saline	0.929 ± 0.081	1.191 ± 0.282	1.094 ± 0.220
DMI	0.889 ± 0.079	0.979 ± 0.245	2.007 ± 0.321
Sibutramine	0.651 ± 0.248	0.742 ± 0.152	1.494 ± 0.319

Data shown are mean ± s.e.m. of 7 Hill coefficients for each treatment group; each curve for the displacement of [^{3}H]-DHA measured in tissues from individual mice. Mean Hill coefficient compared with unity using Wilcoxon signed-rank test.

Figure 6.2 **DISPLACEMENT OF [^{3}H]-DHA BY CGS 12066B IN MOUSE CORTEX**

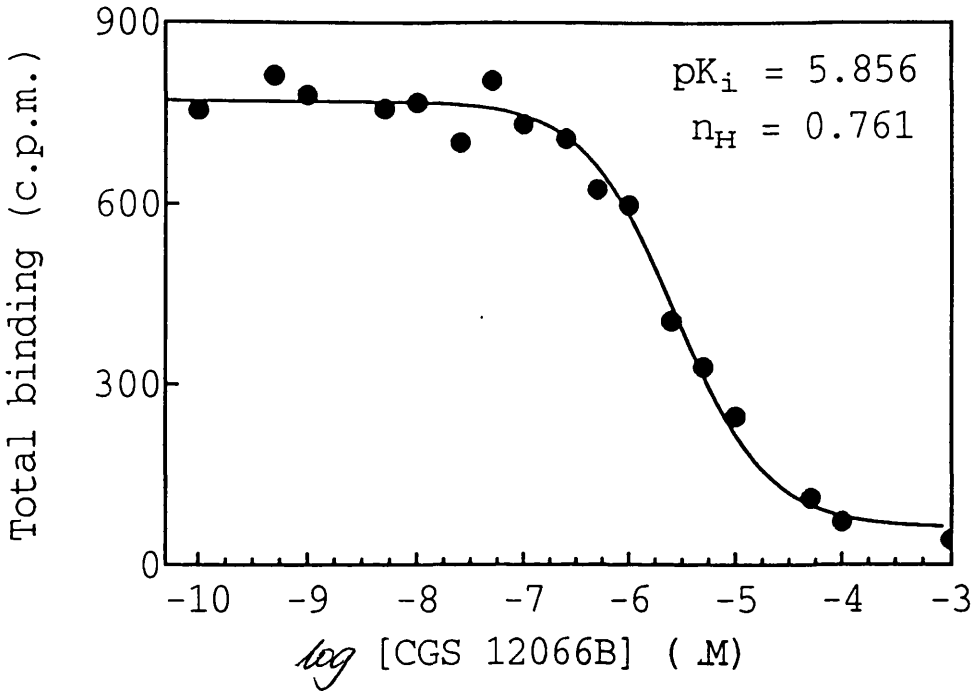


Figure 6.2 Displacement of 1 nM [^{3}H]-DHA by CGS 12066B in cortices from an uninjected mouse. Hill coefficient denoted as nH. Data, analyzed using GRAPHPAD, were best fit by a single-site model ($F = 0.128$; d.f. = 2, 14; $p > 0.05$).

6.4.1.3 *Displacement of [^{3}H]-DHA by TFMPP*

A typical curve for the displacement of [^{3}H]-DHA by TFMPP in cortices from uninjected mice is shown in Figure 6.3. Again, this was best fit by a single site model; the mean pKi value in control tissues was 6.07 (Table 6.1). Similar curves and pKi values were obtained when [^{3}H]-DHA was displaced using TFMPP in cortices from saline-, DMI- and sibutramine-treated mice (Table 6.1). Comparison of all treatment groups using 1-way ANOVA did not reveal any significant treatment effect on pKi values ($F = 1.204$; d.f. = 3, 24; $p > 0.05$). Hill coefficients for the displacement of [^{3}H]-DHA by TFMPP for each of the treatment groups did not significantly differ from unity (Table 6.2).

6.4.1.4 *Displacement of [^{3}H]-DHA by ritanserin*

Figure 6.4 shows a typical curve for the displacement of [^{3}H]-DHA by ritanserin in cortices from uninjected mice. Curves in control tissues and in tissues from saline-, DMI- and sibutramine-treated mice were best fit by a 1-site model. pKi values, which were similar in all groups, are shown in Table 6.1. The 1-way ANOVA to compare all treatment groups simultaneously did not reveal a significant treatment effect on pKi values ($F = 1.382$; d.f. = 3, 24; $p > 0.05$). Although Hill coefficients for the displacement of [^{3}H]-DHA by ritanserin in the uninjected and DMI-treated groups were greater than two, the mean Hill coefficient did not significantly differ from unity in any treatment group (Table 6.2).

6.4.2 Single-point binding

Single-point binding was measured using 1 nM [^{3}H]-DHA, the concentration at which the presence of the non-β receptor component of [^{3}H]-DHA binding was detected in mouse cortex (Section 6.4.1). In these experiments, it was also necessary to determine the amount of [^{3}H]-DHA binding which was to β-adrenoceptors. This was taken as the difference between total [^{3}H]-DHA binding and [^{3}H]-DHA binding displaced by (-)-noradrenaline HCl. To determine the concentration of (-)-noradrenaline HCl to be used, the displacement of [^{3}H]-DHA by (-)-noradrenaline HCl was investigated (Figure 6.5). Although individual curves for the displacement of [^{3}H]-DHA by noradrenaline were best fit by a single site model, the mean Hill coefficient was significantly lower than unity (Section 2.3.1.1). However, in rats, noradrenaline does not displace [^{3}H]-DHA from the 5-HT receptor to which this radioligand binds (Gillespie et al., 1988). Noradrenaline was therefore used to block binding of [^{3}H]-DHA to β-adrenoceptors. The percentage of total [^{3}H]-DHA displaced by (-)-noradrenaline HCl was 77.2 ± 4.3%; the mean pKi value for the displacement [^{3}H]-DHA binding was 6.3 ± 0.22. On the basis of the displacement

Figure 6.3 DISPLACEMENT OF [^{3}H]-DHA BY TFMPP IN MOUSE CORTEX

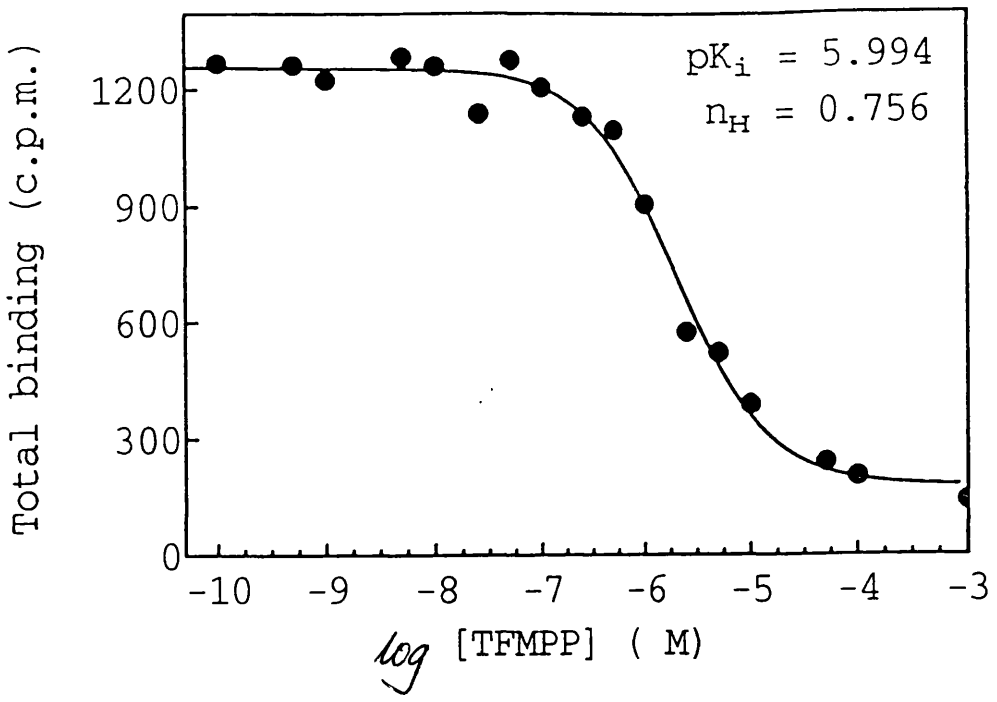


Figure 6.3 Displacement of 1 nM [^{3}H]-DHA by TFMPP in cortices from an uninjected mouse. Hill coefficient denoted as nH. Data, analyzed using GRAPHPAD, were best fit by a single-site model ($F = 0.331$; d.f. = 2, 14; $p > 0.05$).

Figure 6.4 DISPLACEMENT OF [^{3}H]-DHA BY RITANSERIN IN MOUSE CORTEX

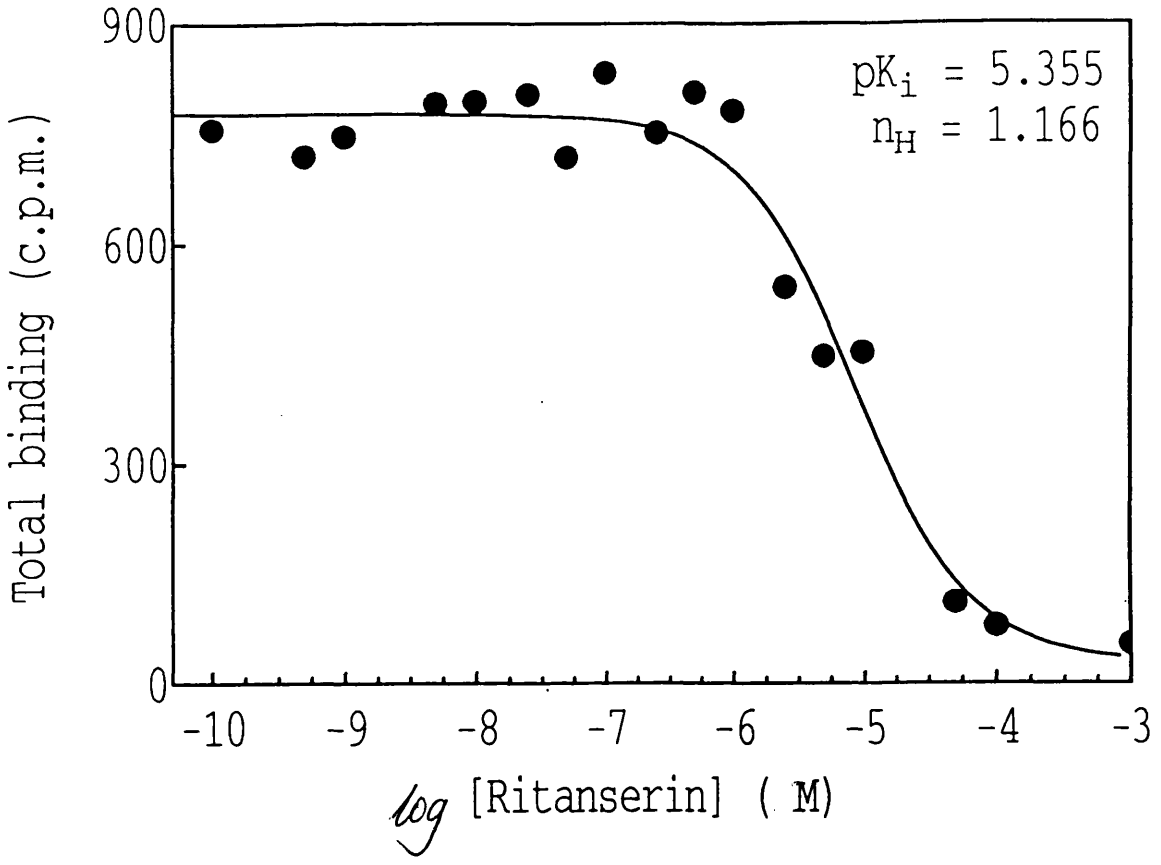


Figure 6.4 Displacement of 1 nM [^{3}H]-DHA by ritanserin in cortices from an uninjected mouse. Hill coefficient denoted as nH. Data, analyzed using GRAPHPAD, were best fit by a single-site model ($F = 1.094$; d.f. = 2, 14; $p > 0.05$).

Figure 6.5 **DISPLACEMENT OF [^{3}H]-DHA BY (-)-NORADRENALINE HCl IN MOUSE CORTEX**

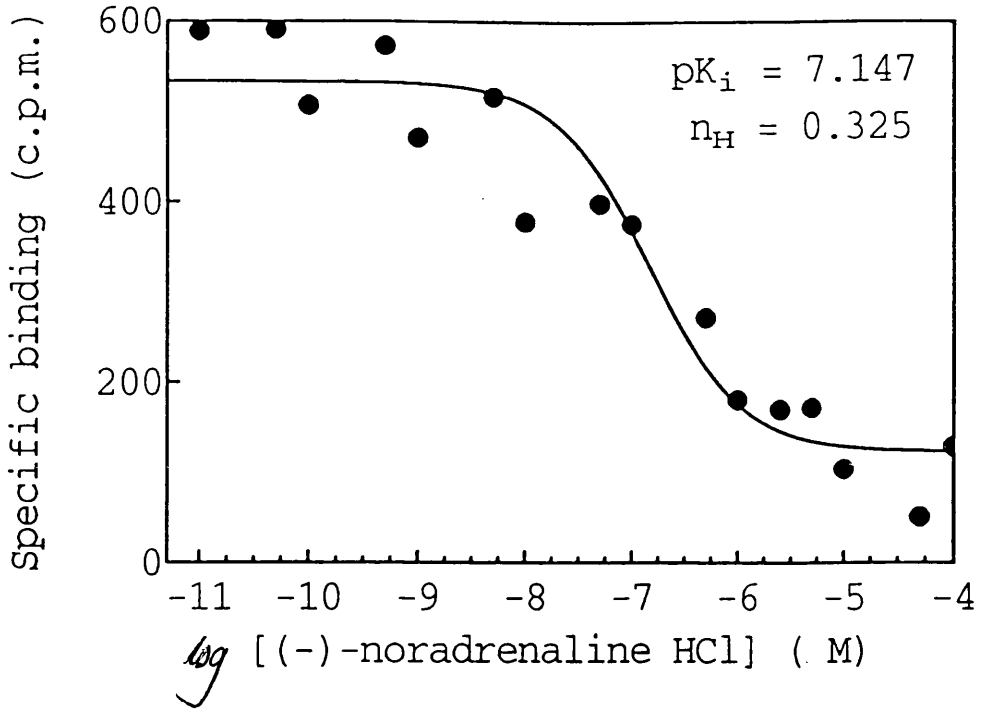


Figure 6.5 Displacement of 1 nM [^{3}H]-DHA by (-)-noradrenaline HCl in cortices from an uninjected mouse. Hill coefficient denoted as nH. The curve, analyzed using GRAPHPAD, was best fit by a single-site model ($F = 0.485$; d.f. = 2, 13; $p > 0.05$). 66% total [^{3}H]-DHA binding was displaced by (-)-noradrenaline HCl.

curves, a concentration of 30 μM (-)-noradrenaline HCl was chosen to define specific binding to β-adrenoceptors in subsequent assays.

Binding to 5-HT1A receptors by [^{3}H]-DHA was calculated as the difference between 'specific binding' and β-adrenoceptor binding. Binding data from a typical control sample are shown in Figure 6.6; the data are shown as 8 separate determinations of each component of binding in aliquots from the same membrane preparation. When single point binding of [^{3}H]-DHA to 5-HT1A receptors was investigated in cortices from each of a number of individual subjects, 1-way ANOVA revealed a significant effect on binding in each sample from uninjected mice. β-adrenoceptor binding and 'specific binding' (the difference between total binding and binding in the presence of both noradrenaline and 8-OH-DPAT) were significantly reduced compared with total binding. However, there was no difference between β-adrenoceptor binding and 'specific binding' in any sample. This pattern of binding was also seen in each individual sample from saline-, DMI- and sibutramine-treated mice.

β-adrenoceptor binding and 'specific binding' (to β-adrenoceptors and 5-HT1A receptors) were expressed also as a percentage of total [^{3}H]-DHA binding (Table 6.3). When 1-way ANOVAs were used to examine the percentage of either β-adrenoceptor or 'specific binding', no significant treatment effect was seen for either parameter.

Figure 6.6 SINGLE POINT BINDING OF [^{3}H]-DHA TO β-ADRENOCEPTORS AND TO (β-ADRENOCEPTORS + 5-HT1A RECEPTORS) IN MOUSE CORTEX

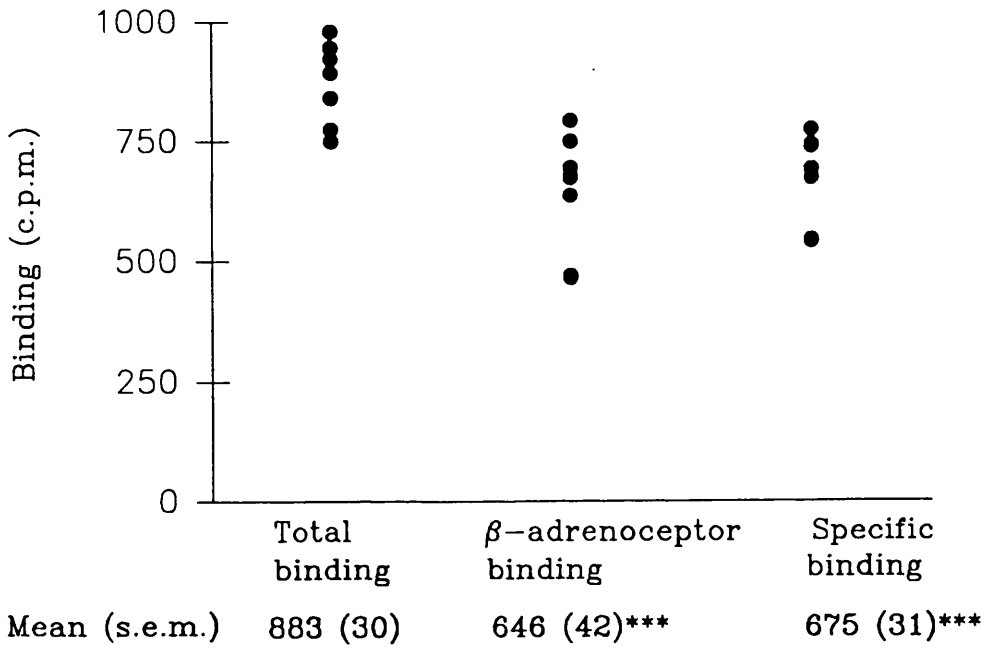


Figure 6.6 Single-point binding determined at 1 nM [^{3}H]-DHA. Each data point represents one of 8 separate determinations of each component of binding in identical membrane aliquots **from a single sample.** Radioligand binding in the absence of any displacing ligand termed total binding. Binding in the presence of 30 μM noradrenaline HCl termed non-β-adrenoceptor binding: total minus non-β-adrenoceptor binding termed β-adrenoceptor binding. Non-specific binding defined using 30 μM noradrenaline HCl *and* 10 μM 8-OH-DPAT: specific binding defined as the difference betweeen total and non-specific binding. Following a significant main effect in the 1-way ANOVA ($F = 13.679$; d.f. = 2, 21; $p < 0.001$), mean values for each component of binding in the individual sample were compared using students t test: ***$p < 0.001$. $t = 4.796$, d.f. = 21, $p < 0.001$ (β-adrenoceptor binding *cf* total binding. $t = 4.209$, d.f. = 21, $p < 0.001$ (specific binding *cf* total binding). $t = 0.587$, d.f. = 21, $p > 0.05$ (β-adrenoceptor binding *cf* specific binding).

Table 6.3 **β-ADRENOCEPTOR BINDING AND SPECIFIC BINDING AS A PERCENTAGE OF TOTAL [^{3}H]-DHA BINDING**

	β-adrenoceptor binding	Specific binding
Uninjected	67.5 ± 2.9%	69.2 ± 3.0%
Saline	65.2 ± 3.0%	69.1 ± 6.1%
DMI	66.7 ± 6.1%	65.6 ± 0.6%
Sibutramine	71.5 ± 1.2%	71.4 ± 1.2%

Single-point binding determined in cortices from mice killed 24 h after the final injection. Data shown as mean ± s.e.m. of 4 separate experiments. In each experiment, measurement of total, β-adrenoceptor binding and specific binding (to β-adrenoceptors and 5-HT1A receptors) were measured in octuplicate in identical membrane aliquots. $^{**}p < 0.01$ *cf* uninjected controls.

6.5 Discussion

Growing evidence over the past decade has suggested that the radioligand [^{3}H]-DHA is not selective for β-adrenoceptors and, even when specific binding is defined using isoprenaline, it may be displaced from a second, possibly serotonergic, binding site in rat cortex. In mice also, the possibility that [^{3}H]-DHA is displaced from a second binding site cannot be discounted. Such findings could potentially explain the anomalous results of β-adrenoceptor binding studies presented in earlier chapters of this thesis. In an effort to identify this second binding site, the displacement of [^{3}H]-DHA by each of a range of serotonergic ligands has been studied. Table 6.4 summarizes the documented relative selectivity of the ligands used. Subsequently it was attempted to quantify any changes in the non-β-adrenoceptor component of [^{3}H]-DHA induced by repeated saline injection or administration of monoamine uptake inhibitors.

6.5.1 Displacement binding

6.5.1.1 *Displacement by 8-OH-DPAT*

8-OH-DPAT displays a high degree of selectivity for 5-HT$_{1A}$ receptors (Hoyer, 1988). In the current experiment, [^{3}H]-DHA was displaced by 8-OH-DPAT in control tissues with a pKi value similar to that for this ligand at 5-HT$_{1A}$ receptors in rat cortex (Table 6.5); pKi values for 8-OH-DPAT at 5-HT$_{1A}$ receptors in mouse cortex are not known. However, the affinity of this ligand for other 5-HT receptor types in rat cortex is at least 1,000 fold lower than that for 5-HT$_{1A}$ receptors (Table 6.6), and it has only micromolar affinity for receptors for other neurotransmitters (Van Wijngaarden et al., 1990). It therefore appears that [^{3}H]-DHA can be displaced from 5-HT$_{1A}$ receptors in cortices from uninjected mice. In saline-injected and DMI-injected animals, the affinity of 8-OH DPAT for the site from which it displaces [^{3}H]-DHA was similar to that in control tissues, suggesting that [^{3}H]-DHA binds to 5-HT$_{1A}$ receptors in these groups also.

Although 8-OH-DPAT displaced [^{3}H]-DHA in cortices from sibutramine-treated mice, the affinity of 8-OH-DPAT for the site from which [^{3}H]-DHA was displaced was in the micromolar range. This suggests that 8-OH-DPAT displaces [^{3}H]-DHA from a site other than the 5-HT$_{1A}$ receptor in sibutramine-treated mice, therefore. It may be inferred from this data that repeated sibutramine administration abolished the binding of [^{3}H]-DHA to cortical 5-HT$_{1A}$ receptors in the mouse. The pKi value for 8-OH-DPAT displacement of [^{3}H]-DHA in sibutramine-treated mice is similar to published pKi values for 8-OH-DPAT at a range of different receptors including β-adrenoceptors (Table 6.5; Van Wijngaarden et al., 1990); it is not possible to identify the site from which [^{3}H]-DHA is displaced in

Table 6.4 **RANGE OF SELECTIVITY OF SEROTONERGIC LIGANDS USED TO DISPLACE [^{3}H]-DHA IN THE CURRENT EXPERIMENT**

	5-HT$_{1A}$	5-HT$_{1B}$	5-HT$_{1C}$	5-HT$_2$
8-OH DPAT	✓			
CGS 12066B	✓	✓		
TFMPP	✓	✓	✓	
Ritanserin			✓	✓

'Selectivity' (✓) defined as 10-100-fold greater affinity of a serotonergic ligand for particular receptor type over other receptor types

✓ indicates similar affinity for several receptor types

Based on published pKi and pKd values in Van Wijngaarden et al., 1990, Schoeffter & Hoyer, 1989, Hoyer, 1988.

Table 6.5 **PUBLISHED AFFINITIES OF 8-OH-DPAT, CGS 12066B, TFMPP AND RITANSERIN AT SEROTONERGIC RECEPTORS AND AT β-ADRENOCEPTORS**

		8-OH DPAT	CGS 12066B	TFMPP	Ritanserin
5-HT1A receptors	pKi	8.61		6.71	6.08
	pKd	8.7	7.16	6.3 6.54	5.2
	IC50	12.7 nM	876 nM	32.5 nM	
5-HT1B receptors	pKi	5.75		7.31	5.76
	pKd	4.2	7.56	6.4 6.88	< 4.0
	IC50	3.0 μM	51 nM	9.0 nM	
5-HT1C receptors	pKi	5.11		7.92	9.26
	pKd	5.2	4.89	7.2 7.30	8.9
	IC50				
5-HT2 receptors	pKi	≤ 5.0		6.11	8.51
	pKd	5.0		6.6	8.8
	IC50	5.2 μM	6.5 μM	153 nM	
β-adreno-ceptors	pKi	≤ 5.0		6.02	≤ 5.0
	pKd				
	IC50	> 10 μM	> 1 μM	2.4 μM	
References		[1] [2] [4]	[3] [4] [5]	[1] [2] [4]	[1] [2]

[1] Hoyer & Schoeffter, 1991: pKd = -log Kd [2] Van Wijngaarden et al., 1990: pKi = -log Ki [3] Koe et al., 1992 [4] Neale et al., 1987 [5] Schoeffter & Hoyer, 1989: pKd = -log Kd

this treatment group on the basis of this data alone, therefore.

The above findings indicate that [^{3}H]-DHA is displaced from 5-HT1A receptors in uninjected mice and in saline- and DMI-injected, but not in sibutramine-injected mice. However, individual 8-OH-DPAT displacement curves showed considerable scatter (compared with scatter on individual curves for the displacement of [^{3}H]-DHA by the other ligands investigated). The large scatter was seen for all 8-OH-DPAT curves even though they were obtained in eight separate binding assays (each of which contained cortices from a single subject from each treatment group). The scatter seen could reflect poor solubility of the displacing ligand; indeed, problems were encountered when attempting to dissolve 8-OH-DPAT. It should have been possible to make up the stock solution in water or buffer, since the quantity of compound to make a solution of 5×10^{-3} M was less than the 5 mg/ml which the manufacturers claim can be dissolved in water. However, when the stock solution was made up in water, the compound did not appear to be completely dissolved. To overcome this difficulty, the stock solution was made by dissolving 8-OH-DPAT in 1 ml 100% ethanol, then further diluted using 10% ethanol. 8-OH-DPAT should be soluble in ethanol up to 10 mg/ml according to the manufacturers; when the stock solution was prepared in this way, the drug appeared to be completely dissolved. Although not evident when solutions were checked visually, it may be possible that some drug came out of solution when the solution of 8-OH-DPAT in 100% ethanol was diluted using 10% ethanol, although this was not seen when solutions were checked visually. Whether or not poor solubility of 8-OH-DPAT is responsible, the scatter in the displacement curves make it unsafe to conclude that 8-OH-DPAT displaces [^{3}H]-DHA from 5-HT1A receptors; studies of the displacement of [^{3}H]-DHA by other, more soluble 5-HT1A-selective compounds are needed to investigate this problem further.

6.5.1.2 *Displacement of [^{3}H]-DHA by CGS 12066B, TFMPP or ritanserin*

CGS 12066B has high affinity for 5-HT1B receptors (Neale et al., 1987). Although it has relatively high affinity for 5-HT1A receptors also (Neale et al., 1987; Schoeffter & Hoyer, 1989), it displays at least 10-fold selectivity for 5-HT1A *versus* 5-HT1B receptors (Neale et al., 1987) and its affinity at other 5-HT receptors is at least 100-fold lower (Neale et al., 1987; Schoeffter & Hoyer, 1989).

Binding studies indicated that in control tissues, [^{3}H]-DHA was displaced by CGS 12066B with micromolar affinity, which was similar to published data for its affinity at both 5-HT2 receptors and β-adrenoceptors (Table 6.2). There is no published evidence to

indicate that [^{3}H]-DHA binds to 5-HT2 receptors; it is suggested therefore that CGS 12066B is displacing [^{3}H]-DHA from β-adrenoceptors in mouse cortex. In saline-, DMI- and sibutramine-injected animals, the pKi values for the displacement of [^{3}H]-DHA by CGS 12066B did not differ significantly from those in uninjected controls, suggesting that CGS 12066B displaces [^{3}H]-DHA from β-adrenoceptors in these samples, as in the uninjected group.

The study of the displacement of [^{3}H]-DHA by CGS 12066B does not implicate 5-HT1B receptors in the specific binding of [^{3}H]-DHA in either uninjected or stress- and drug-treated animals. This is in direct contrast to the findings of Riva & Creese (1989a), who reported that [^{3}H]-DHA was displaced from 5-HT1B receptors in cortices from control rats. The most obvious explanation for the discrepancy between the two studies is the species difference; this may be a species difference in either the specific binding of [^{3}H]-DHA or in the pharmacology of 5-HT1B receptors. The current data has been interpreted on the basis of the affinity of ligands at 5-HT receptors in rat brain; however, there is very little published data on the affinity of ligands at 5-HT receptors in mice. If the pharmacology of 5-HT1B receptors differed between rats and mice, then the interpretation of results in mice may be misleading. Although 5-HT1B receptors are generally accepted to be present in both rat and mouse brain, to my knowledge only one relevant study of 5-HT1B receptors in mice has been published (Ciaranello et al., 1990). This study includes curves for the displacement of binding to 5-HT1B receptors by a range of serotonergic ligands, but is of limited value since actual Ki or Kd values are not quoted. Approximate IC50 values estimated from the published graphs suggest that the affinities of a range of drugs (including TFMPP and 8-OH-DPAT) for displacement of [^{125}I]-ICYP from 5-HT1B receptors in mouse brain are similar to those in rat cortex (Ciaranello et al., 1990). More detailed studies of the pharmacology of 5-HT1B receptors in mice are needed before a species difference in pharmacology or density of 5-HT1B receptors can be ruled out as an explanation for the involvement of this receptor type in the specific binding of [^{3}H]-DHA in the cortex of rats but not of mice.

TFMPP has relatively high affinity for 5-HT1A, 5-HT1B and 5-HT1C receptors; its affinity for 5-HT2 receptors and β-adrenoceptors is between 10-100 fold lower (Table 6.4; Hoyer, 1988). In cortices from uninjected mice, TFMPP displaced [^{3}H]-DHA with a mean pKi value at this site of 6.07. The affinity of TFMPP was comparable to that of this ligand at both β-adrenoceptors and at 5-HT2 receptors. Since it has not been shown that [^{3}H]-DHA binds to 5-HT2 receptors, it appears that [^{3}H]-DHA is displaced from β-adrenoceptors by

TFMPP in mouse cortex. As in control tissues, the pKi value of TFMPP for the displacement of [^{3}H]-DHA binding suggested that this is also the case in cortices from saline-, DMI- and sibutramine-treated groups.

Displacement studies with either CGS 12066B or TFMPP do not implicate 5-HT1A receptors in the binding of [^{3}H]-DHA, even though both these compounds bind to this receptor subtype (Table 6.5). This further suggests that the apparent displacement of [^{3}H]-DHA by 8-OH-DPAT is questionable; it is possible that the displacement may even be an artefact of the curve-fitting program.

Ritanserin has high affinity for both 5-HT1C receptors and 5-HT2 receptors in rat cortex, with at least 100-fold selectivity for these receptor types over 5-HT1A, 5-HT1B receptors and β-adrenoceptors (Table 6.2; Van Wijngaarden et al., 1991). Ritanserin displaced [^{3}H]-DHA binding with an affinity most like that for this ligand at β-adrenoceptors (in the micromolar range). In tissues from saline-, DMI- and sibutramine-treated groups, ritanserin displacement of [^{3}H]-DHA was of a similar affinity to that in control tissues. This suggests that ritanserin displaces [^{3}H]-DHA from β-adrenoceptors in cortices from saline- and drug-treated mice.

Displacement studies with ritanserin have indicated that 5-HT2 receptors are not involved in the binding of [^{3}H]-DHA; this further suggests that both CGS 12066B and TFMPP displace [^{3}H]-DHA from β-adrenoceptors not from 5-HT2 receptors.

6.5.2 Single point binding

The findings discussed above indicate that neither 5-HT1B, 5-HT1C nor 5-HT2 receptors are involved in the binding of [^{3}H]-DHA in mouse cortex. Although this radioligand is apparently displaced from 5-HT1A receptors by 8-OH-DPAT, the findings of displacement studies using CGS 12066B and TFMPP raise questions abount the validity of this suggestion. A different approach was used in order to investigate whether or not [^{3}H]-DHA binds to 5-HT1A receptors, therefore; it was attempted to measure saturation binding of [^{3}H]-DHA to 5-HT1A receptors. However, this approach proved impractical due to the low proportion of [^{3}H]-DHA binding to this site. Similar problems were also encountered by Riva & Creese (1989a) when attempting to measure saturation binding of [^{3}H]-DHA to the non-β component of [^{3}H]-DHA binding (thought to be 5-HT1B receptors) in rat cortex. These authors concluded that "the binding signal of [^{3}H]-DHA at this site is very poor, mitigating against its accurate characterization" (Riva & Creese,

1989a). Since saturation binding could not be used, single point binding of [^{3}H]-DHA to β-adrenoceptors and to 5-HT$_{1A}$ receptors was measured. This approach was adopted in order to maximize the chance of detecting any changes in binding, by making multiple estimates of each component of binding (total, non-β-adrenoceptor and non-specific binding) in order to reduce the variance of the data. In this study, noradrenaline was used to define binding to β-adrenoceptors. Noradrenaline has been little used in radioligand binding studies but early work indicates that it displaces [^{3}H]-DHA from only one binding site in rats (Bylund & Snyder, 1976; Dolphin et al., 1978). In the current study also, displacement of [^{3}H]-DHA by noradrenaline was monophasic, although the mean Hill coefficient was significantly less than unity, suggesting the involvement of more than one site (Section 2.3.1.1). However, evidence that noradrenaline does not bind to any serotonergic component of [^{3}H]-DHA binding comes from the work of Stockmeier & Kellar (1989). This group showed that the 5,7-dihydroxytryptamine lesion-induced increase in [^{3}H]-DHA binding, which was abolished when 5-HT was present in the assay, was not abolished in the presence of noradrenaline. In the current experiments, noradrenaline was therefore considered more suitable to define specific binding of [^{3}H]-DHA to β-adrenoceptors than either isoprenaline or propranolol.

When single point binding was measured, there was no significant difference between β-adrenoceptor binding and 'specific binding' (binding to β-adrenoceptors + 5-HT$_{1A}$ receptors) in any treatment group. Furthermore, when binding was expressed as a percentage of total [^{3}H]-DHA binding (Table 6.4), there was again no difference between β-adrenoceptor binding and 'specific binding'. It is possibile that the ligand used to define β-adrenoceptor binding, (-)-noradrenaline HCl, may also be binding to 5-HT$_{1A}$ receptors, such that in the presence of 8-OH-DPAT no further [^{3}H]-DHA is displaced. This is not supported by evidence that, in rat cortex, the affinity of noradrenaline at 5-HT$_{1A}$ receptors is in the millimolar range (pIC$_{50}$ = 3.77 in rat hippocampus; Gozlan et al., 1983). The current experiments used 30 μM (-)-noradrenaline HCl; this concentration should not displace [^{3}H]-DHA from 5-HT$_{1A}$ receptors. Moreover, displacement of [^{3}H]-DHA by (-)-noradrenaline HCl was monophasic (pK$_i$ = 6.3 ± 0.22) and similar to that in rat cortex (K$_d$ = 1 μM; Bylund & Snyder, 1974: 3.9 μM; Dolphin et al., 1978). Finally, in rat cortex, the non-β-adrenoceptor component of [^{3}H]-DHA binding is not abolished when noradrenaline is present in the assay (Stockmeier & Kellar, 1989). All these findings argue against displacement of the serotonergic component of [^{3}H]-DHA binding by noradrenaline. It is perhaps more likely that binding of [^{3}H]-DHA to 5-HT$_{1A}$ receptors may not have been displaced due to poor solubility of 8-OH-DPAT. Although single-

point binding could not detect binding of [^{3}H]-DHA to 5-HT1A receptors in mouse cortex, the possibility that [^{3}H]-DHA can be displaced fram 5-HT1A receptors in this species cannot be conclusively ruled out until studies using other 5-HT1A receptor-selective compounds have been carried out, therefore.

6.5.3 Summary and conclusions

Published studies indicate that a component of [^{3}H]-DHA binding is to a 5-HT receptor in rat cortex (Riva & Creese, 1989a; Stockmeier & Kellar, 1989). These studies are not consistent with each other in some respects; for instance Riva & Creese demonstrated binding of [^{3}H]-DHA to a 5-HT1 receptor in cortices from untreated rats, whereas Stockmeier & Kellar found that, although [^{3}H]-DHA bound to a 5-HT1 receptor in cortices taken from rats after 5,7-dihydroxytryptamine lesioning, the 5-HT receptor component of binding could not be detected in untreated animals. However, both studies suggest that [^{3}H]-DHA can be displaced from a 5-HT1 receptor in rat cortex. The current findings did not implicate 5-HT1B or 5-HT1C receptors in the binding of [^{3}H]-DHA in mouse cortex; it is also unlikely that 5-HT1A receptors are involved in the binding of [^{3}H]-DHA in mice, although this possibility cannot be conclusively excluded on the basis of the current results. Further investigation of the binding of [^{3}H]-DHA using other, perhaps more readily soluble 5-HT1A receptor-selective compounds is required to determine whether this radioligand is displaced from 5-HT1A receptors. The possibility that displacement of [^{3}H]-DHA from 5-HT1A receptors may account for differences between binding measured using [^{3}H]-DHA and [^{3}H]-CGP 12177, when non-specific binding is defined using isoprenaline in mouse cortex, can then be addressed in more detail.

7.0 GENERAL DISCUSSION

The experiments presented in this thesis were designed to investigate several different aspects of responses to stress, looking at behavioural responses as well as effects on indices of central noradrenergic and serotonergic function. These experiments led on to an investigation of the binding profile of [^{3}H]-DHA, a β-adrenergic radioligand used in this study. The most important inferences of this study are discussed in a broader setting here, and problems raised during the course of this study are highlighted.

An important aim of the first series of experiments was to further investigate the relationship between behavioural resistance to stress and β-adrenoceptors. A reduction in cortical β-adrenoceptor density is the only neurochemical change for which there are claims of a causal link with behavioural adaptation to stress (Stone, 1979b), but this relationship has been questioned in previous reports from this laboratory (Salmon & Stanford, 1989; Stanford & Salmon, 1989). One factor which could account for the differences between findings of this laboratory and of Stone's group was that differences in previous experience of repeated stress might alter the relationship between behavioural resistance to stress and cortical β-adrenoceptor density.

In order to examine this possibility, the approach used previously in this laboratory, namely that of measuring both behaviour (in a novel open field) and β-adrenoceptor binding in the same animals, was adopted in this study. This enabled both 'group mean' and 'within group' comparisons of β-adrenoceptor density and behavioural resistance to stress to be made. That is, the relationship between receptor density and resistance to stress could be examined for group means, and a statistical correlation between the two parameters calculated using information from each animal tested. The possibility that the link between β-adrenoceptors and behavioural resistance to stress differs for 'group mean' comparisons and for 'within group' correlations could be examined, therefore.

The current study replicated the positive 'within groups' correlation between measures of behavioural resistance to novelty and *increased* β-adrenoceptor density reported previously by this group (Salmon & Stanford, 1989). However, there was no relationship between group mean β-adrenoceptor density and behavioural resistance to novelty. This contrasts with the findings of Stone (1979b; Stone & Platt, 1982) who studied forms of stress such as foot shock and food deprivation. It was concluded that the relationship between behavioural resistance to stress and β-adrenoceptor density is dependent on the

form of stress used.

This finding has particular importance with respect to studies of stress in man which, in general, examine the effects of forms of stress which are psychological or involve exercise (e.g. Dimsdale & Moss, 1980; Ward et al., 1983). Novelty was chosen for use in the current experiments since it is a form of stress with a predominant psychological component, and may be relevant to forms of stress experienced by humans. In contrast, the forms of stress studied both by Stone and by the majority of other groups (e.g. Nomura et al., 1981; Cohen et al., 1986) are predominantly physical; where exercise has been used, animals may be forced to exercise almost continuously over long periods of time. However, physical and psychological forms of stress cannot be expected to have the same physiological effects (e.g. Iimori et al., 1982). The continued use of such physical forms of stress cannot, in general, be justified. However, such forms of stress are still used, even though stressors as mild as handling have been shown to have neurochemical effects similar to those seen on exposure to physical stressors (Grahame-Jones et al., 1983; Stanford et al., 1984; *cf* Rossetti et al., 1990 and Kokaia et al., 1989).

A further interesting point raised by this experiment concerns the effects of the monoamine uptake inhibitor, sibutramine. Grooming in the open field was increased by previous experience of repeated stress (once-daily saline injection), but sibutramine blocked or reversed this effect of repeated stress. Novelty-induced grooming, which is presumed to be a displacement activity, may represent a coping mechanism; it is possible therefore that sibutramine is substituting for coping. This speculative interpretation could have important clinical implications. Monoamine uptake inhibitors, a drug class to which sibutramine belongs, are used clinically in the treatment of depression and anxiety, conditions which are known to be triggered by stress in some cases. If treatment with a monoamine uptake inhibitor blocks or substitutes for coping on exposure to a further acute stress, then cessation of drug treatment may be problematic! In fact, current advice to clinicians on cessation of antidepressant treatment is that "reduction in dosage should preferably be carried out gradually over a period of about 4 weeks" (British National Formulary, 1993). Clearly the effects of antidepressants on behaviours representing coping mechanisms deserve further attention.

The current study of behaviour in a novel open field exposed two further limitations of previous studies. First, this study measured a number of different aspects of novelty-induced behaviour although, in general, studies of open field behaviour examine only 2-3

behavioural measures such as locomotor activity and defecation. Yet the findings presented in this thesis indicate that behaviours evoked by a given situation are not all altered similarly by a given treatment and that they cannot all be interpreted in the same way. Differences in stress- and sibutramine-induced changes in behaviours reflecting resistance to stress and a possible coping mechanism illustrate the need to examine more than just one or two parameters. Such an approach is necessary to obtain a clear picture of the effects of a given treatment on different aspects of behavioural responses to stress.

A more general point concerns the use of 'within group' correlations. For a relationship between two parameters to be determined on the basis of individual, not group mean observations, both parameters must be measured in the same animals. This method allows clear determination of whether the relationship between two parameters differs in different treatment groups. The current study shows that relationships determined on the basis of differences between group means are not always the same as those obtained from 'within group' correlations. However, the evidence for a relationship between two parameters is greatly strengthened if it can be shown for groups as well as for individual animals (i.e. using a 'within groups' correlation). This approach should be more widely adopted in the study of neurochemical changes underlying changes in behaviour, therefore.

The second series of experiments presented in this thesis examined behavioural and neurochemical effects of a brief swim stress. The major finding emerging from this study concerned the duration of the effects of stress on serotonergic neurones. The possibility of long-latency neurochemical changes induced by exposure to stress has rarely been considered, although such effects have potential clinical importance. The current study implicated both serotonergic and noradrenergic neurones in the immediate effects of acute stress, as has previously been reported by many groups. Moreover, published findings indicate that a single stress exposure has no effect on indices of noradrenergic or serotonergic function, 24 h later. However, when measured *7 days* after a single 6 min swim stress, cortical 5-HT turnover, 5-HT2 receptor binding and central 5-HT2 receptor function *in vivo* were all increased, although indices of noradrenergic function were unchanged at this time. It appears therefore that serotonergic neurones are involved both in the immediate and the long-lasting effects of a single brief exposure to stress. In contrast, noradrenergic neurones are only involved in the short-term response, since 3 h after the swim stress, there was no longer any change in cortical noradrenaline levels or turnover.

Much wider investigation of the role of 5-HT and other transmitters in long-term effects of a single stress exposure is obviously required to determine possible mechanisms underlying clinical disorders which are known to be triggered by stress. One such condition is post-traumatic stress disorder (PTSD). It is possible that research into causes of this particular disorder may be the only situation where the use of severe stressors may be appropriate; PTSD is triggered by an experience which is outside the range of normal experience - recent examples include the King's Cross fire, and the Herald of Free Enterprise ferry disaster. The need for studies of mechanisms underlying this disorder is not a cue to start using forms of stress such as almost continuous forced exercise or prolonged electroshock which, in man, would be considered to be brutal. However, the use of stressors with a physical as well as a psychological component such as a brief swim stress may possibly be an appropriate approach to tackling this, as yet, unexplained disorder.

This study also highlights a point which is to be generally inferred from the literature, although it is little discussed. It is generally held that an increase in transmitter supply leads to a reduction in post-synaptic receptor density, as seen for β-adrenoceptors. However, in the current study both 3 h and 7 days after the swim, parallel changes in both cortical 5-HT turnover and 5-HT2 receptors were seen. This and previously published evidence (e.g. Blackshear et al., 1986; Leysen et al., 1986; Yocca et al., 1991) indicates that 5-HT2 receptor density is not necessarily inversely related to transmitter supply. The regulation of this receptor type clearly requires further investigation, therefore.

Together, the two studies of acute stress (novelty, swim) raise further important points about the design of such experiments. Firstly, behavioural tests such as those used here are commonly used to assess animals' emotional status; however, it is clear that such tests can themselves alter animals' neurochemical status. If the effects of a given treatment (e.g. previous experience of stress) on any neurochemical parameter are to be measured after behavioural testing, it is inadequate therefore to use an untreated group (e.g. no previous experience of stress) tested in the behavioural paradigm as a control group. A naive control group, which is *not* exposed to the paradigm, must be used in order to determine effects of both the paradigm, the treatment and any interactions between the two. Secondly, it is also clear that repeated saline injection, so often used as a control for studies of repeated drug administration, can have effects on both behavioural and neurochemical responses to stress. In fact, many effects seen in the sibutramine-treated

group in the current experiments were apparently due to the injection and not a drug effect, since there was no difference between saline- and sibutramine-treated groups. However, when a clear drug effect was seen, these effects were only fully appreciated by comparison with both saline-injected and uninjected groups. For instance in the open field, grooming was reduced by sibutramine compared with saline-injected animals. However, it is only by further comparison with an uninjected group that it is clear that sibutramine merely reduces grooming to the basal levels seen in uninjected animals; that is, it prevents the saline injection-induced increase in grooming.

In the course of the open field study (Chapter 3) discussed above, it became clear that results of β-adrenoceptor binding studies in which specific binding of [^{3}H]-DHA was defined using (±)-isoprenaline SO_4 differed from published findings in which specific binding of [^{3}H]-DHA was defined using propranolol. Subsequent studies (Chapters 4 & 5) further highlighted the problems of using [^{3}H]-DHA to measure β-adrenoceptor binding; stress- and drug-induced changes in [^{3}H]-DHA binding were not consistent with the changes obtained when β-adrenoceptor binding was measured using the more selective β-adrenergic radioligand, [^{3}H]-CGP 12177. These inconsistencies could possibly be explained by the binding of [^{3}H]-DHA to a non-β-adrenergic receptor; indeed it has recently been shown that [^{3}H]-DHA can be displaced from an, as yet unidentified, 5-HT receptor in rat cortex. However, preliminary experiments examining the displacement of [^{3}H]-DHA by isoprenaline showed that this radioligand was only displaced from one site in mice, although Hill coefficients for the displacement curves suggested that more than one site may be present. This apparent discrepancy between the binding of [^{3}H]-DHA in rats and in mice was examined in a series of experiments carried out to determine whether [^{3}H]-DHA could be displaced from a 5-HT receptor in mouse cortex.

The results of these experiments indicated that [^{3}H]-DHA could not be displaced from 5-HT$_{1B}$, 5-HT$_{1C}$ or 5-HT$_2$ receptors in mouse cortex. However, displacement of [^{3}H]-DHA from 5-HT$_{1A}$ receptors could not be ruled out. In mice, differences between estimates of β-adrenoceptor binding measured using [^{3}H]-DHA and [^{3}H]-CGP 12177 may possibly be attributed to the binding of the former radioligand to 5-HT$_{1A}$ receptors; further, more detailed investigation is required to confirm this possibility, however. It is clear however, that, as in rats, [^{3}H]-CGP 12177 is a more suitable ligand to use when β-adrenoceptor binding is measured in mice.

In summary, the studies described in this thesis have raised a number of points.

Firstly, various problems in experimental design, which make the majority of published studies hard to interpret, have been highlighted. Perhaps of most importance is the need for careful choice of the control group(s), in order to detect interactions between the different treatments. However, the importance of measuring both neurochemistry and behaviour in the same animals, to provide more concrete evidence to support links between changes in a given neurochemical parameter and in behaviour must also be stressed. The choice of radioligand to measure β-adrenoceptor binding has also been discussed. [^{3}H]-DHA is still widely used, and a number of studies indicate that this is not suitable to measure β-adrenoceptor binding in rats. The current findings show that this is the case in mice also; in cortex, [^{3}H]-DHA may bind to a second site in addition to β-adrenoceptors, whereas [^{3}H]-CGP 12177 is selective for β-adrenoceptors in this species. Finally, this thesis has suggested a substrate for long-latency effects of acute stress, namely serotonergic neurones. More detailed studies of the long-term effects of stress on indices of serotonergic function are required to investigate the importance of this transmitter in clinical disorders which may develop as a long-latency consequence of stress exposure.

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