Development and use of "stress" animal models of depression to study the mode of action of antidepressant drug treatments

Thesis

DEVELOPMENT AND USE OF "STRESS" ANIMAL MODELS OF DEPRESSION TO STUDY THE MODE OF ACTION OF ANTIDEPRESSANT DRUG TREATMENTS

Volume I of II

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DEDICATION

For my parents, Susan and Tony Bate,

and my long-suffering boyfriend, Daniel Howard,

for their constant and boundless belief in me.

DECLARATION

The work described in this thesis was undertaken in the Department of Applied Science at Nescot and the Pharmacology Department at St. George's Hospital Medical School, with the financial assistance of Nescot.

The work described is original and was carried out by myself except where specific contributions of other persons are acknowledged.

Elizabeth Bate

2003

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ABBREVIATIONS

General:

ACTH	Adrenocorticotrophic hormone; Adrenocorticotrophin
ADX	Adrenalectomy
AF2	Transcriptional activation factor 2
AMV	Avian myeloblastosis virus
Arg	Arginine
AVP	Arginine vasopressin
BDNF	Brain-derived neurotrophic factor
B_{max}	A measure of the receptor concentration in a sample
B_o	Maximum binding
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBT	Cognitive behavioural therapy
CDF	Cholinergic neuronal differentiation factor
CLIP	Corticotrophin-like intermediate lobe peptide
CMS	Chronic mild stress
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CORT	Corticosterone
CRE	cAMP response element
CREB	cAMP response element binding protein
CRH	Corticotrophin-releasing factor; CRF
CSF	Cerebrospinal fluid
CUMS	Chronic unpredictable mild stress

DBD	DNA-binding domain
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DRN	Dorsal raphe nucleus
DSM-IV-R	Diagnostic and Statistical Manual of Mental Disorders
DST	Dexamethasone suppression test
DTT	Dithiothreitol
EAA	Excitatory amino acid
ECS	Electroconvulsive shock
ECT	Electroconvulsive therapy
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EPM	Elevated plus maze
EtOH	Ethanol
FGF	Fibroblast growth factor (a, acidic; b, basic)
FST	Forced swim test
x *g*	Gravitational force
GABA	γ-aminobutyric acid
GAD	Generalised anxiety disorder
GDNF	Glial-derived neurotrophic factor
GH	Growth hormone
GR	Glucocorticoid receptor, type II corticosteroid receptor
GR-i	Glucocorticoid receptor-impaired
HBNF	Heparin-binding neurotrophic factor
HCl	Hydrochloric acid
HD	Head-dipping

HPA	Hypothalamic-Pituitary-Adrenocortical
HRE	Hormone responsive elements
Hsp	Heat shock protein
IC_{50}	Concentration of unlabelled compound that results in 50% displacement of specific binding
IEG	Immediate early gene
IGF	Insulin-like growth factor
IL	Interleukin
IPT	Interpersonal therapy
K^+	Potassium
KCl	Potassium chloride
K_d	Equilibrium dissociation constant, ligand affinity
K_i	Inhibition constant, affinity of the competing compound for the receptor
LBD	Ligand-binding domain
LIF	Leukemia inhibitory factor
Lys	Lysine
MAOI	Monoamine oxidase inhibitor
MAP	Mitogen-activated protein
$MgCL_2$	Magnesium chloride
MMLV	Moloney murine leukemia virus, MuLV
mpPVN	Medial parvicellular part of the PVN
MR	Mineralocorticoid receptor, type I corticosteroid receptor
MRN	Median raphe nucleus
mRNA	Messenger ribonucleic acid
MSH	Melanocyte-stimulating hormone; melanotrophin
NGF	Neurotrophin growth factor

NMDA	N-methyl-D-aspartate
NSB	Non-specific binding
NT	Neurotrophin
OD	Optical density
OT	Oxytocin
PCI	Phenol-chloroform-isoamylalcohol
PCPA	d, *l*-p-chlorophenyl-alanine methyl ester
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PI	Phosphatidylinositol
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
POMC	Pro-opiomelanocortin; pro-ACTH/endorphin
PVN	Paraventricular nucleus
RE	Restriction endonuclease
RIA	Radioimmunoassay
rpm	Revolutions per minute
RT-PCR	Reverse transcription PCR
SAP	Stretch-attend postures
SCN	Suprachiasmatic nucleus
SGHMS	St. George's Hospital Medical School
SHR	Steroid hormone receptors
SSRI	Selective serotonin reuptake inhibitor
TAE	Tris-acetate
TBE	Tris-borate
TC	Total counts

TCA	Tricyclic antidepressant
TEDGM	Incubation buffer for receptor binding assay
Tfl	*Thermus flavus*
TG	Transgenic
TGF	Transforming growth factor
TPE	Trisphosphate
Tris-HCl	Tris-hydroxymethylaminomethane hydrochloride
TrK	Tyrosine receptor kinase
5-HT	5-hydroxytryptamine, serotonin
5-HT1AA	5-hydroxyindoleacetic acid
5-HTP	5-hydroxytryptophan
5-Me-ODMT	5-methoxy-N, N-dimethyltryptamine
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetralin

Statistical:

Df	Degrees of freedom
F	The statistic calculated by Analysis of Variance, which reveals the significance of the hypothesis that Y depends on X.
n	number
One-way ANOVA	One-way Analysis of Variance
r	Correlation coefficient
SD	Standard deviation
SEM	Standard error of the mean
Two-way ANOVA	Two-way Analysis of Variance
t-test	Student's *t*-test

ABSTRACT

The effect of the exposure of male BALB/c mice to sensory stimuli from male Brown Norway rats was assessed using plasma corticosterone (CORT) concentrations and glucocorticoid receptor (GR) binding in the cortex, and a number of behavioural measures (sucrose preference, food intake and elevated plus-maze tests). Following 8 weeks of exposure, stressed mice showed a 97% increase in plasma CORT concentrations but GR binding, sucrose preference, food intake, and the elevated plus maze (EPM) measurements were unaltered. In conclusion, this study suggests that chronic exposure of mice to sensory stimuli from rats increases stress hormones and may provide a natural predator model of stress.

The effects of acute and chronic restraint stress with the administration of paroxetine (10 mg/kg p.o.), a selective serotonin reuptake inhibitor (SSRI), on the hypothalamic-pituitary-adrenocortical (HPA) axis were studied in adult male Wistar rats. Three weeks of restraint stress induced a 700% increase in plasma CORT and a 100% increase in adrenocorticotrophin (ACTH) concentration, whilst decreasing GR binding in the cortex by 34% and hippocampus by 30%, and a 24% decrease in food intake. Administration of paroxetine induced a 166% increase in plasma CORT concentration, whilst decreasing GR mRNA levels by 45% in the cortex and food intake by 25%. Paroxetine administration decreased the stress-induced plasma CORT by 80% but enhanced ACTH concentration by 78%, partially reversing the stress-induced downregulation of GR in the cortex by 34%, increasing GR mRNA in the cortex by 58%, reducing BDNF mRNA in the cortex by 30%, and reversed the stress-induced decrease in food intake by 22%. Taken together, these results suggest that restraint stress provoked a stress response, which remained elevated after three weeks, and feedback inhibition on the HPA axis following stress was facilitated by treatment with paroxetine.

PUBLICATIONS ARISING FROM THIS AND RELATED WORKS

Presentations at National Conferences

BATE E, THEODOROU AE, OOZEER F, HORTON RW. (2002). Plasma corticosterone and adrenocorticotrophin hormone concentrations following chronic restraint stress, and the subsequent effect of long-term administration of paroxetine. *The 5th International Congress of Neuroendocrinology*. Bristol, UK.

CHAPTER 1. INTRODUCTION

1.1. Depression

1.1.1.Characteristics and origins

The aetiology of depressive illness is complex and variable. The origin of depression may include social, developmental, and genetic factors as well as aberrations in brain biochemistry and possibly in anatomy. A genetic vulnerability has been identified as a predisposing factor in some cases of bipolar depression – with a 43% concordance among monozygotic twins (Wells & Hayes, 1989; Weissman *et al*, 1984). However, it is most likely to be an interaction of social stressors, genetics, and possibly age that causes a depressive episode at a given point.

In the United States, the DSM-1V (1994) represents the most widely used system of psychiatric diagnosis for research purposes. The manual's criteria reflect the view that major depression is a syndromal illness with both psychological and biological components - depressed mood, withdrawal of interest, feelings of worthlessness, changes in patterns of eating and sleeping.

1. Types

Major affective disorders can be subdivided into unipolar and bipolar illness, whereby the bipolar type is manifested by mania or by both mania and depression, and unipolar illnesses are manifested only by depression. An exception to this is melancholic depression, a highly consistent association of symptoms that includes hyposomnia, anorexia, and diurnal variation in mood (DSM-IV, 1994), of which the hallmark is an intensely painful arousal and an obsessional preoccupation with personal inadequacy and the inevitability of loss. This syndrome therefore crosses the boundaries of unipolar and bipolar illness.

i) Unipolar disorder (*major depression*). A positive diagnosis is made if an individual has five or more of the symptoms of depression during the same two-week period - persistent sad, anxious, or "empty" mood; feelings of hopelessness, pessimism; feelings of guilt, worthlessness, helplessness; loss of interest or pleasure in hobbies and activities that were once enjoyed, including sex; decreased energy, fatigue, being "slowed down"; difficulty concentrating, remembering, making decisions; insomnia, early-morning awakening, or oversleeping; appetite and/or weight loss or overeating and weight gain; thoughts of death or suicide; suicide attempts; restlessness, irritability; persistent physical symptoms that do not respond to treatment, such as headaches, digestive disorders, and chronic pain. Unipolar major depression typically presents in discrete episodes that recur during a person's lifetime.

ii) Bipolar disorder (*manic-depressive illness*) is characterized by cycling mood changes: severe highs (mania) and lows (depression). Sometimes the mood switches are dramatic and rapid, but most often they are gradual. There is abnormally and persistently elevated mood or irritability accompanied by at least three of the following symptoms: overly-inflated self-esteem; decreased need for sleep; increased talkativeness; racing thoughts; distractibility; increased goal-directed activity or physical agitation; and excessive involvement in pleasurable activities with potential for negative consequences.

2. Factors

Evidence from neuroscience, genetics, and clinical investigations demonstrate that depression is a disorder of the brain. Modern brain imaging technologies are revealing that in depression, regulatory neural circuits responsible for mood, thinking, sleep, appetite, and behaviour fail to function properly, and that critical neurotransmitters are out of balance. Genetic research indicates that vulnerability to depression results from the influence of multiple genes acting in combination with environmental factors.

There is much knowledge still to be gained about the aetiology of depression, more specifically, the various pathways to depression. There is evidence that a number of amine transmitter systems are involved in the development of some depressions. Biochemical and neurohormonal data indicate that several neurotransmitter systems may be acting either alone or in combination to produce depressive symptoms.

1.1.2. Treatments

One of the principal treatments of depression is the use of antidepressants. Numerous randomised clinical trials have demonstrated the efficacy of somatic antidepressant therapy for major depressive disorder (Cohn *et al*, 1990; Bech *et al*, 2000).

Research has shown that certain types of psychotherapy, particularly cognitive behavioural therapy (CBT) and interpersonal therapy (IPT), can help relieve depression. CBT helps patients change the negative styles of thinking and behaving often associated with depression. IPT focuses on working through disturbed personal relationships that may contribute to depression.

Research on children and adolescents with depression supports CBT as a useful initial treatment, but antidepressant medication is indicated for those with severe, recurrent, or psychotic symptoms in depression. Studies of adults have shown that while psychotherapy alone is rarely sufficient to treat moderate to severe depression, it may provide additional relief in combination with antidepressant medication (Kornbluh *et al*, 2001; Brown, 2001). For mild depression, however, a recent analysis of multiple studies indicated that combination treatment is not significantly more effective than CBT or IPT alone.

Electroconvulsive therapy (ECT) remains one of the most effective yet most stigmatised treatments for depression. Eighty to ninety percent of people with severe depression improve dramatically with ECT, which involves producing a seizure under general anaesthesia by applying electrical stimulation to the brain through electrodes

placed on the scalp. Repeated treatments are necessary to achieve the most complete antidepressant response. Memory loss and other cognitive problems are common, yet typically short-lived side effects of ECT. Although some people report lasting difficulties, modern advances in ECT technique have greatly reduced the side effects of this treatment compared to earlier decades. Research on ECT has found that the dose of electricity applied and the placement of electrodes (unilateral or bilateral) can influence the degree of clinical improvement and severity of side effects (Flint & Gagnon, 2002; Kornbluh *et al*, 2001).

Recently there has been an enormous growth in public interest in herbal remedies for various medical conditions including depression. For example, St. John's wort (*Hypericum perforatum L.*) is a widely used remedy for the treatment of mild to moderate depressions (for a review see Schultz, 2002). However, the nature of its active principles and the exact mode of antidepressant action are still unknown. It has been suggested repeatedly in preclinical and clinical studies that the content of the acylphloroglucinol hyperforin decisively contributes to the antidepressant efficacy of St. John's wort extracts (Müller *et al*, 2001; Buchholzer *et al*, 2002). Experimental studies *in vivo* also indicate that the naphthodianthrone hypericin may reduce the activity of the HPA axis (Butterweck *et al*, 2002).

1.1.3. Neurochemistry

Many of the biological theories of depression, have been closely associated with the chemical effects of clinically effective first generation antidepressant drugs; - monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs), discovered in the late 1950s. The biological theories of depression centred largely on the ability of MAOIs and TCAs to alter the release or uptake of monoamines into nerve terminals. The monoamine neurotransmitters include the catecholamines - noradrenaline (Schildkraut, 1965; Bedawy & Evans, 1981) and dopamine (Willner, 1983), and the

indolamine serotonin, or 5-HT (Glassman, 1969; Curzon, 1988). TCAs inhibit the reuptake of amines released from the nerve, and MAOIs act via inhibition of monoamine degradation, and therefore potentiate the effect of these neurotransmitters. The discovery of the antidepressant activity of imipramine and of the MAOI iproniazid (Crane, 1957) intensely stimulated biochemical-pharmacological research on the pathophysiology of depressive illnesses and the mechanism of action of the antidepressants.

Other theories suggest that an interaction of amine systems may cause depression or that amine-specific subtypes of depression may exist. Mendels *et al* (1975) proposed that affective disorders might result from a complex interaction between cholinergic and adrenergic neurotransmitter activity. Depression appears to occur when cholinergic activity is elevated in comparison to adrenergic activity.

Lesioning the serotonergic system with a serotonin neurotoxin results in the failure of TCAs to down-regulate β-adrenergic receptors or to decrease the sensitivity of the β-adrenergic coupled adenylate cyclase (Janowsky *et al*, 1982). Therefore, the discovery of the down-regulation of certain monoamine receptors by antidepressants prompted a different hypothesis, the neurotransmitter receptor hypothesis (Charney *et al*, 1981; Stahl, 1984). It postulated monoaminergic hyperfunction mediates the clinical effects of depression and held that antidepressants work by down-regulating postsynaptic monoamine receptors, especially adrenergic and/or serotonergic receptors. In parallel with the molecular changes in β-adrenergic receptors, following prolonged administration of antidepressant drugs to rats, 5HT-2 receptors are also down-regulated (Peroutka & Snyder, 1980). Evidence from neuroendocrine research suggests that some antidepressants may exert effects on 5-HT pathways through intimate connections with noradrenaline pathways (Charney *et* al, 1984). Others have proposed that acetylcholine pathways may have complex connections with other transmitter systems and the sum of events leads to depression.

According to Stahl and Palazidou (1986) a number of problems exist with these hypotheses, which relate antidepressants or depressive illness to neurotransmitter receptors. Firstly, they are predominantly based on biochemical observations in normal rat brain. Secondly, numerous difficulties arise because there are limitations with the techniques available for assessing central nervous system (CNS) receptors in humans. Thirdly, psychopharmacologists must not assume that "correlations" infer "causation", since receptor changes observed after antidepressant drug treatment could be well-timed epiphenomena rather than the causative molecular mechanism of antidepressant action.

1.1.4. Clinical Studies

Studies of the amine hypotheses have been conducted using a variety of different experimental approaches. For example, (i) Changes in biogenic amine neurotransmitters in post-mortem brains from suicide victims and depressive patients who died from natural causes (Lloyd *et al*, 1974; Coppen & Doogan, 1988); (ii) Changes in cerebrospinal fluid (CSF) and urine concentrations of monoamine metabolites from patients suffering from depression (reviewed by Kapur & Mann, 1992); (iii) Changes in neurotransmitter receptor function and density on platelets and lymphocytes from patients before and following effective treatment (Sneddon, 1973; Paul *et al*, 1982); (iv) The dexamethasone suppression test (DST) is used to determine the sensitivity of the HPA axis to negative feedback. HPA activity is abnormally high in some melancholic patients: cortisol concentrations are elevated in plasma (Carroll, 1978) and CSF (Carroll *et al*, 1976; Traskman *et al*, 1980). The cause of this abnormality is a failure of the brain to inhibit the release of ACTH (Carroll & Mendels, 1976). Dexamethasone is a synthetic corticosteroid, which suppresses ACTH release.

1.1.5. Animal models and Behavioural Tests

A model is defined as any experimental preparation developed for the purpose of studying a condition in the same or different species. Typically, models are animal preparations that attempt to mimic a human condition. In developing and assessing an animal model, it is critical to consider the explicit purpose intended for the model, because the intended purpose determines the criteria that the model must satisfy to establish its validity.

Responsiveness to antidepressant drugs is usually taken to be a basic requirement for an animal model of depression. Yet some depressed patients fail to respond to antidepressants, and in addition to their antidepressant effects, TCAs appear to be highly effective anxiolytics (Deakin & Lader, 1991) and after chronic treatment appear to be effective in at least one animal model of anxiety (Bodnoff *et al*, 1988).

It is widely believed that because the pharmacotherapy of depression requires continuous drug treatment, for a period of weeks, the validity of an animal model is called into question by an acute antidepressant response only. Therefore the real test for a simulation of depression is that tolerance must not develop to the antidepressant response: irrespective of how it responds to acute antidepressant treatment, the model must respond to chronic treatment.

The major group of models of depression are based on responses to stressors of various kinds. The theoretical rationale underlying these models is usually derived from the well-established finding that the risk of depression is increased substantially by a stressful life event (Paykel *et al*, 1969).

1. Predator Stress

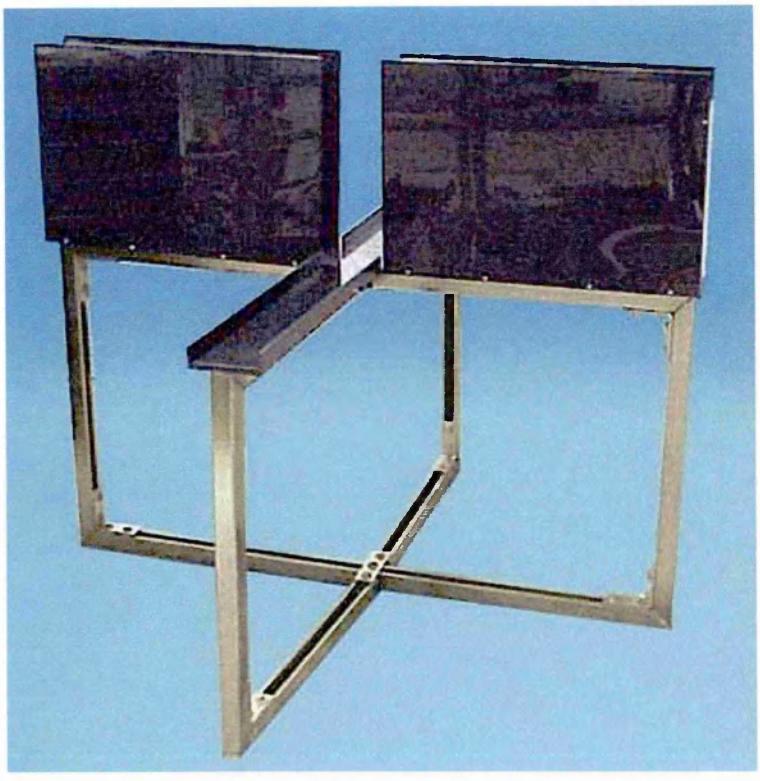
Most studies on stress are based on the use of physical stressors such as electric shocks or restraint. However, animals do no confront these kinds of stimuli in their natural environments. As a consequence, other procedures involving natural contexts such as using predatory stimuli have been developed (Hendrie & Neill, 1991; Blanchard *et* al, 1998). Predatory stimuli are ecologically relevant for an animal's survival and, consequently, induce responses quite similar to those shown in natural contexts. It is claimed that the recognition of such stimuli depends on innate mechanisms (Kavaliers, 1988; Zangrossi & File, 1992b).

While not assessed to a comparable extent, psychogenic stressors, such as predator exposure or cues associated with a predator, lead to behavioural and neuroendocrine variations (Adamec & Shallow, 1993; Blanchard *et al*, 1995a).

2. Elevated Plus-Maze (EPM)

The elevated plus-maze (EPM) test is based on the aversion of rodents for open spaces (Treit *et al*, 1993) and uses a plus-shaped maze elevated above the floor, consisting of two open and two enclosed arms (**Figure.1.1.**). This test of anxiety developed from the work of Montgomery (1955) and of Handley and Mithani (1984), was validated in rats by Pellow *et al* (1985), and in mice by Lister (1987).

Figure.1.1.



A photograph of an elevated plus-maze.

The test involves placing the animal in the centre of the apparatus and allowing it to explore for a short period (usually 5 min). From the beginning, the primary indices of anxiety in the plus-maze have been spatiotemporal in nature (i.e., the proportion of entries that are made on to the open arms of the maze, and the time spent on the open arms of the maze expressed as a percentage of the total time spent on both the open and closed arms) and are highly correlated, while the total number of arm entries has frequently been employed as a measure of general activity. Plus-maze paradigms involve the study of spontaneous exploratory patterns in individual animals exposed to unfamiliar environments, which invariably include areas of relative safety. In this paradigm, the animal is initially placed not in the most aversive area (i.e., the open arm) but at a choice point, the central platform. Although a small proportion of animals may freeze on introduction and an even smaller proportion may display rapid escape, the vast majority

initially engage in high levels of risk assessment from the centre platform towards the arms. Such behaviour dominates the first few minutes of the test, with most time spent on the centre platform and roughly equal time spent exploring the open and closed arms. Thereafter, risk assessment levels decline rapidly, coincident with the emergence of a spatiotemporal preference for the enclosed arms (thigmotactic cues). Therefore, ranges of defensive behaviours are observed (freezing, risk assessment) and these are usually ignored in favour of a relatively simple spatiotemporal measure.

The elevated plus-maze paradigm is currently one of the most widely used animal models in behavioural pharmacology (Handley & McBlane, 1993; Hogg, 1996) and is routinely used for studying the effects of putative anxiolytic drugs, as well as the neurobiological mechanisms of anxiety (Cruz *et al*, 1994; Pellow & File, 1986). It has proven to be bi-directionally sensitive to manipulations of anxiety (Cruz *et al*, 1994; Pellow & File, 1986).

3. Restraint Stress

An animal model of depression based on the biochemical and behavioural effects of a two-hour immobilisation stress in the rat was developed by Curzon (1988). These effects were (a) increased plasma concentrations of corticosterone during the stress, (b) decreased locomotion and increased defecation on placement 24 h later in an open field, and (c) loss of appetite or anorexia. On repeating the stress each day, adaptation occurred (Kennett *et al*, 1985a). Therefore, failure or delay in adaptation was a rational model for depression (Katz *et al*, 1982). Adaptation was associated with increased postsynaptic serotonin function, since some components of the serotonin behavioural syndrome, induced by the serotonin agonist 5-methoxy-N, N-dimethyltryptamine (5-Me-ODMT), were increased when the drug was given 24 h after repeated stress (Kennet *et al*, 1985b), in particular, reciprocal forepaw treading and tremor that are thought to be mediated by postsynaptic 5HT-1A receptors (Trickelbank *et al*, 1985), were increased. These findings

lead to a depression model with high corticoid response to stress and low serotonin functional activity opposing adaptation and therefore predisposing to the illness. This is interesting, since (a) high corticoid production and defective cortisol feedback control are characteristic of depression (Carroll, 1985), (b) there are numerous indications of a serotonin abnormality in depression, and (c) familial studies suggest that defects of both cortisol feedback control (Coryell & Zimmerman, 1987) and of serotonin synthesis (Sedvall *et al*, 1980) may be associated with vulnerability to affective illness.

Behavioural changes following acute uncontrollable stress have been used as animal models of depression (Willner, 1984). Previous results strongly indicate that failure to adapt to repeat restraint may be a valid model of depression since elevated glucocorticoids (Carroll, 1972) and reduced central 5-hydroxyindoleacetic acid (5-H1AA) concentrations (Van Praag, 1982) are recognised features of depression.

4. Sucrose Preference

One of the most significant effects of stress, observed in a number of models, is a reduction in the performance of rewarded behaviour. The hypothesis that depression results from a reduction in the activity of reward systems is central to a number of theories of depression (Ferster, 1973). The influence of stressors upon sucrose or saccharin preference, and self-stimulation phenomena are of particular interest because these processes are believed to reflect changes in motivational and emotional functions of the brain. It is noteworthy that both behaviours are closely interrelated; it was found that rats genetically selected for high self-stimulation rates consumed the most, whilst the genetically low self-stimulators drank the least of a saccharin solution in a two-bottle preference test (Ganchrow *et al*, 1984). This further underlines the similarity of central nervous functions involved in both experimental procedures. The fact that saccharin and sucrose preference is a hedonic-like effect is also demonstrated by its antagonism by small doses, not interfering with motor activity and total fluid consumption, of pimozide (Bailey *et al*, 1986; Towell *et*

al, 1987). This selective D_2 receptor antagonist exerts a potent central influence decreasing the rewarding properties of opiates and psychostimulants (Wise, 1978).

Several animal models of depression, such as the learned helplessness model of Seligman and Maier (1967) and Katz' chronic stress model (1982), attempt to parallel the putative role of stress in producing depressive states. Willner and colleagues (1987) have refined the latter model with the development of the chronic mild unpredictable stress (CMUS) model of depression. Exposure of rats to chronic, unpredictable, and diverse stressors was found to reduce preference for saccharin and sucrose for more than 2 weeks after termination of the stress regime (Katz, 1982; Willner *et al*, 1987). The CUMS model aims to mimic the reduced sensitivity to reward, termed anhedonia, which is a key symptom of many depressive states (Nelson & Charney, 1981), and a core feature of the DSM-IV subtype of melancholia (DSM-IV-R, 1994). The relevance of this model to depression is further supported by the evidence that the decreases in sensitivity to reward are reversed by chronic administration of all the major classes of antidepressant drugs, and by electroconvulsive shock, but not by a variety of non-antidepressant drugs (D'Aquila *et al*, 1994).

1.2. The HPA Axis

The CNS, through its control of secretion of releasing and inhibiting factors from the neuroendocrine hypothalamus into the hypothalamo-hypophysial portal system, regulates hormonal synthesis and secretion from the anterior pituitary, and, via its control of the pituitary hormones, regulates activity of peripheral target endocrine glands (**Figure.1.2.**).

According to Checkley (1996), the central drive to the stress response of the HPA axis is organised by the parvocellular component of the paraventricular nucleus (PVN) of the hypothalamus (**Figure.1.2.**). CRH cells project to the external zone of the median eminence and release CRH into the hypophyseal portal system

(Antoni, 1986; Plotsky, 1991), which carries the hormones to the anterior pituitary gland. CRH acts at the pituitary to increase pro-opiomelanocortin (POMC) gene expression and the release of POMC-derived peptides such as ACTH and β-endorphin. The rapid CRH stimulated secretion of ACTH is associated with induction of adenylate cyclase activity and an increase in cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) in corticotropes. ACTH, in turn, induces the synthesis and release of glucocorticoids (principally cortisol in primates and corticosterone in rodents) from the adrenal cortex.

In recent years a large body of evidence has emerged linking stressful life events with an increased vulnerability for affective and anxiety disorders. Stressful events often precede the onset of depression and stress has also been associated with the severity of the illness (Dunner *et al*, 1979; Hammen *et al*, 1992) and depression-like behaviours in animals (Anisman & Zacharko, 1982). This relationship is further supported by the fact that some of the common biological abnormalities observed in depressive illness are related to the HPA axis (Stokes & Sikes, 1988; Lesch & Lerer, 1991), which plays a pivotal role in the response to stress. Exposure to chronic stress is thought to play an important role in the aetiology of depression. In this disorder, a number of studies indicate an abnormal neuroendocrine system and a failure of normal adaptive processes to take place (Gold *et al*, 1988a,b). A reduced negative feedback response to exogenous glucocorticoids, is one of the most consistent findings, and is characterised by the failure in suppression of plasma cortisol levels following administration of the synthetic glucocorticoid, dexamethasone (Carroll *et al*, 1981; Arana *et al*, 1985). A significant number of depressed patients exhibit symptoms indicative of over-stimulation of the HPA axis (Murphy, 1991), with hypersecretion of basal cortisol (Carroll *et al*, 1976) and an increase in adrenal weight (Rubin *et al*, 1995).

An understanding of the normal adaptive changes that occur in brain systems in response to stress forms a necessary foundation for the investigation of the relationship between stress and depressive illness, where such adaptive mechanisms presumably fail. An understanding of neurochemical changes underlying the adaptive response to stress and HPA axis activation is also crucial to the continuing development of effective pharmacological treatments for clinical depression and for the future identification of molecular screens for depressive illness.

The disinhibitory effects of hippocampal lesions on HPA activity implicate the hippocampus in glucocorticoid negative feedback inhibition of ACTH release (Jacobson & Sapolsky, 1991; Sapolsky *et al*, 1986). In support of this hypothesis, hippocampal lesion attenuates dexamethasone inhibition of stress-induced ACTH and CRH secretion in some paradigms (Feldman & Conforti, 1980; Feldman & Weidenfeld, 1993). However, hippocampal damage does not affect the magnitude of HPA responses or negative feedback efficacy following hypoxia, suggesting that feedback effects may be stressor-specific (Bradbury *et al*, 1993). Further, hippocampal damage appears to increase responsiveness to mild but not severe stress (Kant *et al*, 1984). Thus, the influence of the hippocampus on HPA regulation appears to be stressor- and intensity-dependent, inconsistent with an obligatory role in glucocorticoid negative feedback.

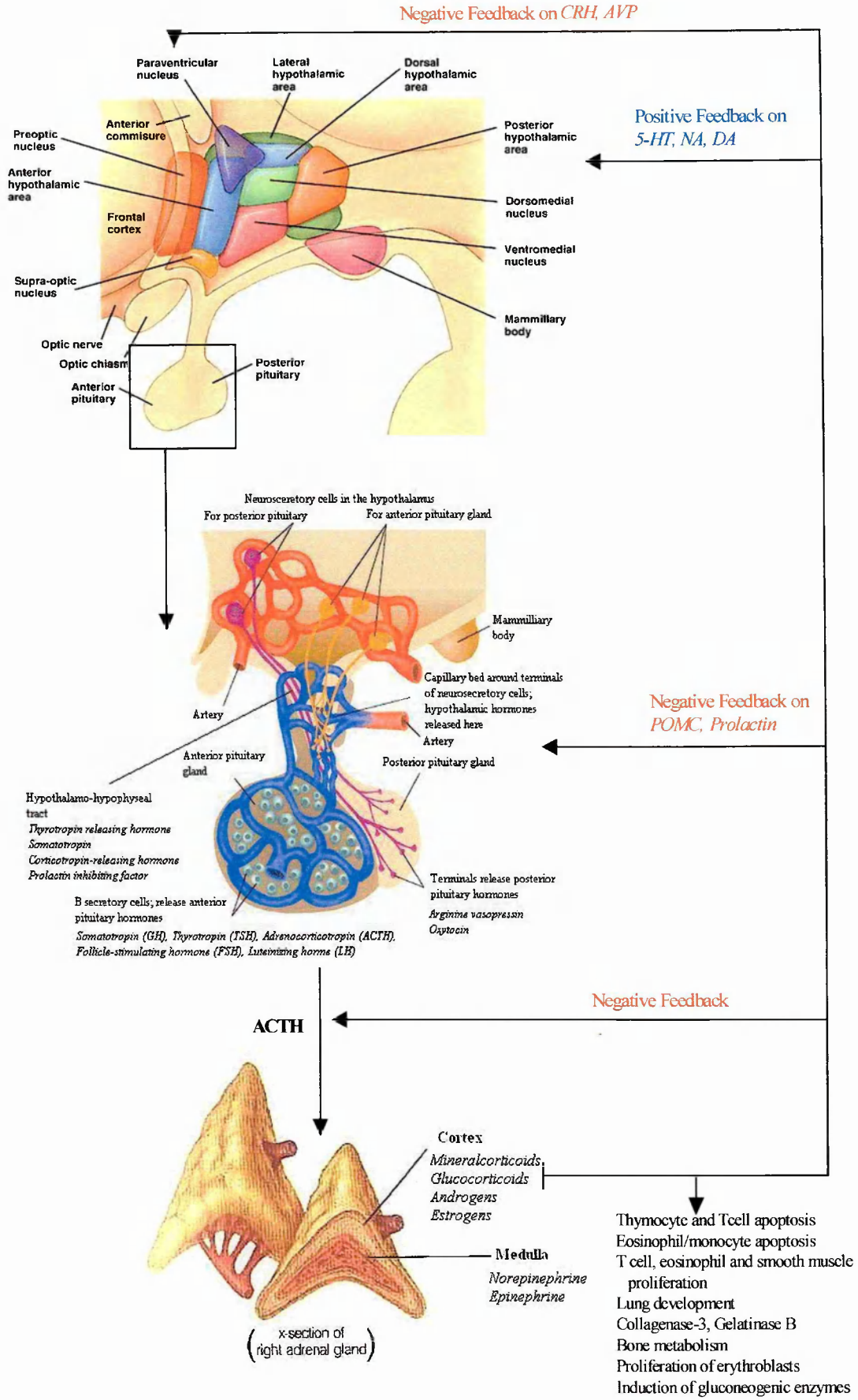


Figure.1.2: Schematic diagram of the HPA axis showing the sites of synthesis and action of the main HPA hormones and the targets of glucocorticoid action (Adapted from Koelling, 1985; http://www.driesen.com/).

1.2.1. Corticotropin-Releasing Hormone (CRH)

CRH, is a 41 amino-acid peptide that has been associated with several different endocrine, physiological, neurochemical and behavioural responses (for a review see Owens & Nemeroff, 1991). It is therefore not surprising to find a wide distribution of this neuropeptide throughout the central nervous system; cell bodies and fibres that stain positive for CRH are heterogeneously but selectively distributed within several distinct structures including the isocortex; central nucleus of the amygdala; bed nucleus of the stria terminalis; arcuate, periventricular, supraoptic, suprachiasmatic, preoptic and paraventricular nucleus of the hypothalamus; and brainstem nuclei (i.e., locus coeruleus, dorsal vagal complex, and parabrachial nucleus; Swanson *et al*, 1983; Cassell & Gray, 1989). A schematic of the distribution of CRH-containing cell bodies and fibers in rat brain is shown in **Figure.1.3.**

The primary neuroendocrine purpose of CRH is to increase the synthesis and release of ACTH from the anterior pituitary (Vale *et al*, 1981; Spiess *et al*, 1981). In addition, it increases pituitary corticotroph density and may directly enhance the action of ACTH upon the adrenal gland. These actions are synergistic with weaker ACTH secretagogues including arginine vasopressin (AVP), oxytocin (OT), angiotensin-II and interleukin 1 (IL-1; Owens & Nemeroff, 1991).

Corticosteroids act at several loci to exert negative feedback inhibition on ACTH secretion (Keller-Wood & Dallman, 1984). Negative feedback effects have been demonstrated not only at the pituitary level, but also at hypothalamic and extrahypothalamic sites in the brain (Sayers & Portanova, 1974; Keller-Wood & Dallman, 1984). Consequently, corticosterone may inhibit ACTH secretion by reducing CRH release from the hypothalamus as well as by direct actions on the corticotroph.

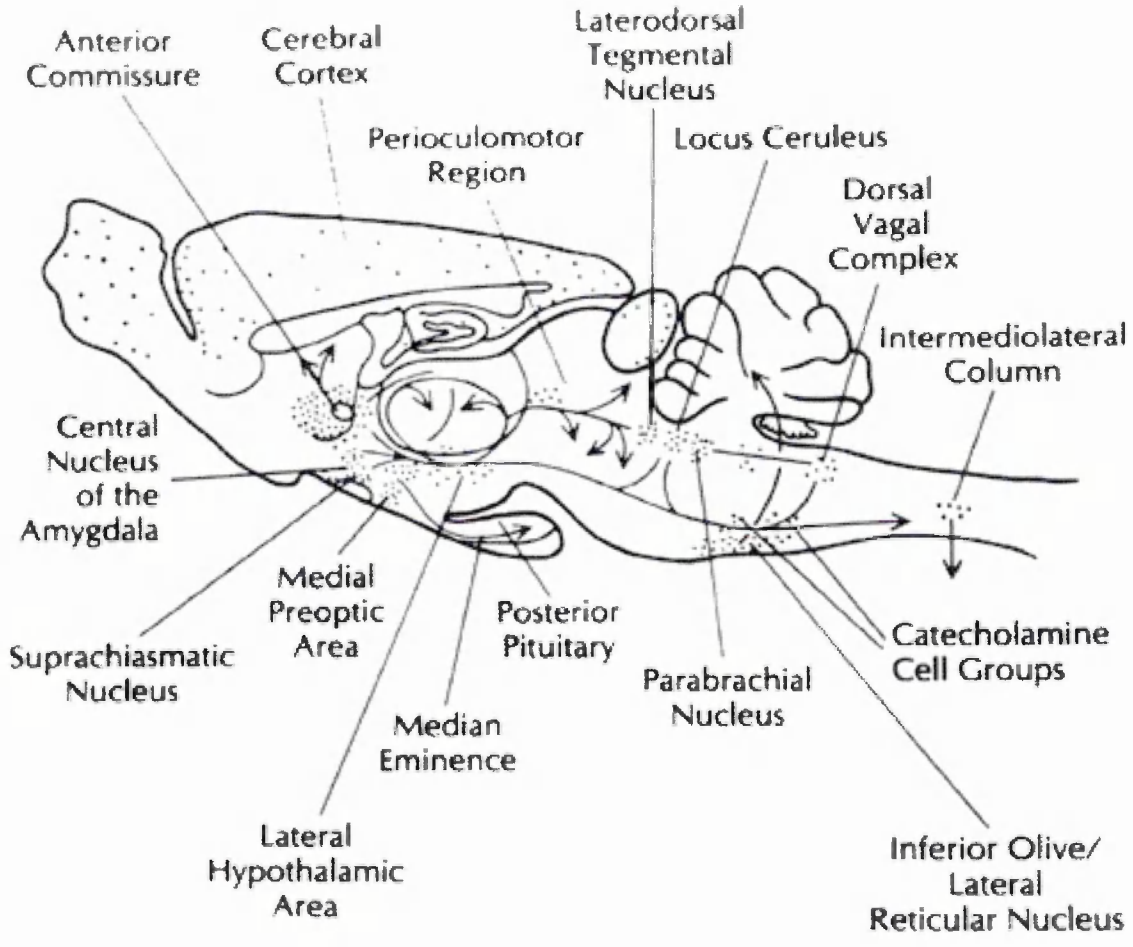


Figure.1.3. Major groups of CRH-producing neuronal perikarya (dots) and their fiber systems (arrows) are shown in a sagittal view of the rat brain (Adapted from Sawchenko and Swanson, 1990).

The actions of CRH are mediated by specific high-affinity receptor sites located in the plasma membrane of the corticotroph (Wynn *et al*, 1983). These receptors have been identified in the pituitary and in several brain areas, including the cerebral and cerebella cortices, and structures related to the limbic system and the control of the autonomic nervous system (Wynn *et al*, 1983; De Souza *et al*, 1985). Many of these limbic structures containing CRH receptors have direct or indirect connections with the PVN of the hypothalamus (Swanson & Sawchenko, 1983). Consequently, modulation of neuronal activity by CRH-receptor interactions within these areas could influence anterior pituitary secretion by altering CRH release into the portal circulation. Administration of relatively

high doses of dexamethasone has been shown to decrease CRH-receptors in the rat pituitary gland (Wynn *et al*, 1985), suggesting that loss or down-regulation of pituitary CRH receptors may partially mediate the inhibitory effect of glucocorticoids on ACTH secretion.

In the hypothalamus of rats, it has been shown that serotonin acting through a cholinergic interneurone causes a dose-dependent release of CRH. Noradrenaline has been found to block the release of CRH induced by serotonin and this was caused by a direct action on the CRH neurones (Jones *et al*, 1976).

Molecular cloning studies have enabled the elucidation of receptor subtypes for the CRH system (Chen *et al*, 1993; Perrin *et al*, 1993); CRH_1 and CRH_2, both of which activate adenylate cyclase cascades (Owens & Nemeroff, 1991; Grigoriadis *et* al, 1996). The CRH_1 receptors are found in high density within the pituitary, as well as the brain, particularly in the neocortex. CRH_2 receptors are more abundant in the periphery, but are also found in some brain areas such as the septum, ventromedial hypothalamus and dorsal raphé nucleus (Potter *et al*, 1994). The CRH_2 subtype exists in two different isoforms in both rat and human; these have been termed $CRH_{2\alpha}$ and $CRH_{2\beta}$ (Chalmers *et al*, 1996), each having a different distribution (Lovenberg *et al*, 1995).

Although quite similar in their central distribution, the CRH_1 receptor mRNA is undetectable in several regions associated with CRH mRNA (Potter *et al*, 1994). For example, within hypothalamic regions known to have high basal CRH mRNA levels such as the PVN, very low and diffuse levels of the CRH_1 receptor mRNA have been measured (Potter *et al*, 1994). Interestingly however, is the fact that either an immune challenge or an immobilisation stress could induce a very selective and strong activation of the CRH_1 receptor mRNA within hypothalamic nuclei involved in the regulation of neuroendocrine functions, particularly the parvocellular division of the PVN (Rivest *et al*, 1995). This fact, taken together with CRH's ability to activate its own gene expression selectively within the PVN (Parkes *et al*, 1993), supports the existence of an ultra-short loop positive feedback

mechanism through which CRH may modulate its own biosynthesis. Excessive CRH production is prevented by multiple levels of negative feedback, that are present throughout the HPA axis, and mediated by corticosterone, ACTH, CRH binding protein and also CRH itself.

1. Stress and the role of Arginine Vasopressin (AVP)

In response to stress there is an increase in the synthesis of CRH mRNA in parvocellular cells in the PVN (Lightman & Young, 1988), an increase in CRH message in the median eminence and an increased release of CRH into portal blood. The extent and time course of changes in CRH in the PVN and median eminence of the hypothalamus following stress are highly dependent on the nature of the stressor as well as the state of the animal.

In patients with major depression, elevated CRH immunoreactivity was found in the CSF (Nemeroff *et al*, 1984). It was therefore hypothesised that the prolonged exposure of target cells to increased concentrations of CRH should result in a ligand-induced downregulation of receptors for the peptide. Indeed, in the anterior pituitary, CRH receptor number decreased after a long-lasting elevation of hypothalamic peptide release into the portal system (Hauger *et al*, 1988). However, in the brain, CRH binding sites seemed not to be affected by experimental conditions, such as chronic manipulations of the HPA activity, during which the neuronal release of CRH is increased (Hauger *et al*, 1988; Hauger & Aguilera, 1992). Based on these findings, it was concluded that different mechanisms exist which regulate the interactions of CRH with its receptors in the pituitary and in the CNS.

Nemeroff *et al* (1988) reported a marked reduction in the number of CRH binding sites in the frontal cortex of suicide victims, and Anderson *et al* (1993) demonstrated a significant loss of CRH binding sites, in the hypothalamus, after severe chronic immobilisation stress in rats, and a downregulation of amygdala CRH binding sites was observed by Hauger *et al* (1993), as a result of high intracerebral CRH concentrations.

CRH_1 and $CRH_{2\alpha}$ receptors differ in their regulation in response to a variety of stressors. Various stressors, for example an immune challenge or immobilisation, have been shown to upregulate CRH_1 mRNA in the PVN of the hypothalamus (Lacroix & Rivest, 1996; Lee & Rivier, 1997), suggesting that this receptor subtype might primarily mediate the effect of stress on the HPA axis.

Two reports of mice lacking the CRH_1 receptor have confirmed a role for this receptor in anxiety-related behaviour (Timpl *et al*, 1998; Smith *et al*, 1998). CRH_1 receptor-deficient mice display decreased anxiety-like behaviour in the dark-light emergence task and the elevated plus-maze, both behavioural paradigms thought to measure anxiety in rodents. Both studies concluded that CRH mediates the behavioural responses to stressors by means of the CRH type 1 receptor (Timpl *et al*, 1998; Smith *et al*, 1998).

CRH systems in the brain have a role in mediating not only the neuroendocrine, but also the autonomic and behavioural responses to stress. For example, CNS administration of CRH to laboratory animals produces physiological and behavioural changes almost identical to those observed in response to stress, including increased heart rate and mean arterial blood pressure due to alterations in the autonomic nervous system, suppression of exploratory behaviour in an unfamiliar environment, induction of grooming behaviour, increased conflict behaviour, and decreased food intake and sexual behaviour (Dunn & Berridge, 1990; Koob *et al*, 1993). The behavioural effects of centrally administered CRH can be reversed by CRH receptor antagonists and are independent of activation of the HPA axis. Furthermore, CRH receptor antagonists alone attenuate many

of the behavioural consequences of stress, under-scoring the role of endogenous CRH in mediating many stress-induced behaviours (Heinrichs *et al*, 1995).

Although AVP is only a weak ACTH secretagogue on its own, it acts synergistically with CRH and is believed to play an important role in sustaining pituitary responsiveness during chronic stress (Gilles *et al*, 1982; Aguilera, 1998). There are two populations of CRH neurones in the PVN, one in which only CRH can be detected and another in which both CRH and AVP coexist (Whitnall *et al*, 1987). Studies based on the levels of immunoreactive peptide and mRNA for CRH and AVP have suggested that differential regulation of these peptides in the PVN plays an important role in determining the responsiveness of the HPA axis during chronic stress (Gilles *et al*, 1982; Aguilera, 1998). Differences in the regulation of AVP and CRH transcription are emphasised by studies which have demonstrated that the response of the AVP gene in CRH neurones to corticosterone is more sensitive than that of the CRH gene (Makino *et al*, 1995b; Ma *et al*, 1997), and that the glucocorticoid receptors in the PVN are downregulated in response to repeated stress (Makino *et al*, 1995a; Herman *et al*, 1995). This suggests a complex regulatory mechanism by which repeated stress may temporarily impair glucocorticoid feedback and thus facilitate AVP gene expression in the CRH neurons. More recently, the development of *in situ* hybridisation techniques with probes directed against introns has allowed a more detailed analysis of the regulation of CRH and AVP gene expression in the PVN (Fremeau *et al*, 1986; Ma & Aguilera, 1999).

The different time courses of CRH and AVP transcript responses clearly imply different regulatory mechanisms for the transcription of these genes, as has been suggested in previous studies (Herman *et al*, 1990; Ma & Aguilera, 1999). Differential sensitivity to corticosterone feedback (Kovacs & Sawchenko, 1996), second messengers, and transcription factors (Kovacs *et al*, 1998) may all be involved.

2. Clinical Relevance

The hypothalamic paraventricular CRH neurones play a key role in the HPA response to stress. These CRH neurones have been hypothesised to be involved in the pathophysiological response of the HPA axis in various stress-related human pathologies, e.g. depression (Holsboer *et al*, 1992). This hypothesis is largely based on reports showing that in depressed patients: **(*a*)** 24-hour cortisol excretion was elevated (Sacher *et al*, 1973); **(*b*)** there was decreased CRH binding in the frontal cortex (Nemeroff *et al*, 1988); **(*c*)** the HPA axis was often unable to respond appropriately to exogenous corticosteroids, as measured by the dexamethasone suppression test (Carroll *et al*, 1981); **(*d*)** ACTH responses to test doses of ovine CRH (Holsboer *et al*, 1985) or human CRH (Holsboer *et al*, 1987) were blunted, which may be due to a down-regulation of anterior pituitary CRH receptors (Holsboer *et al*, 1985; Gold *et al*, 1988a,b), and **(*e*)** elevated CRH concentrations have been reported in the cerebrospinal fluid (CSF) of patients with depression (Nemeroff *et al*, 1984).

According to Raadsheer and colleagues (1994), these blunted responses in depressed patients have been interpreted as an index of CRH hyperexposure because rats that were chronically exposed to CRH develop a reduced CRH receptor efficacy, resulting in blunted ACTH responses to a CRH challenge (Aguilera *et al*, 1990). These findings are believed to reflect desensitised CRH receptors at corticotrophic cells or restricted secretory response of ACTH to CRH, or both, caused by increased basal levels of cortisol. The restricted secretory response is supported by normalised net ACTH output in metyrapone-pretreated depressive patients (Lisansky *et al*, 1989).

1.2.2. Pro-Opiomelanocortin (POMC)

The POMC gene is predominantly expressed in the anterior and intermediate lobes of the pituitary, although lower levels of expression are detected in other tissues, for example the immune system (Bateman *et al*, 1989). The primary protein product of the POMC gene is a 285 amino acid precursor that can undergo differential processing to yield at least eight hormones (**Figure.1.4.**), dependent upon the location of synthesis and the stimulus leading to their production (Smith & Funder, 1988).

CRH induces rapid secretion of ACTH (also called corticotropin) and a variety of other peptides from corticotropes of the anterior pituitary. ACTH is the main physiologically active product of CRH activity, and is derived by post-translational modification of POMC. Longer-term responses of corticotropes to CRH include a marked increase in POMC mRNA (Droin *et al*, 1987).

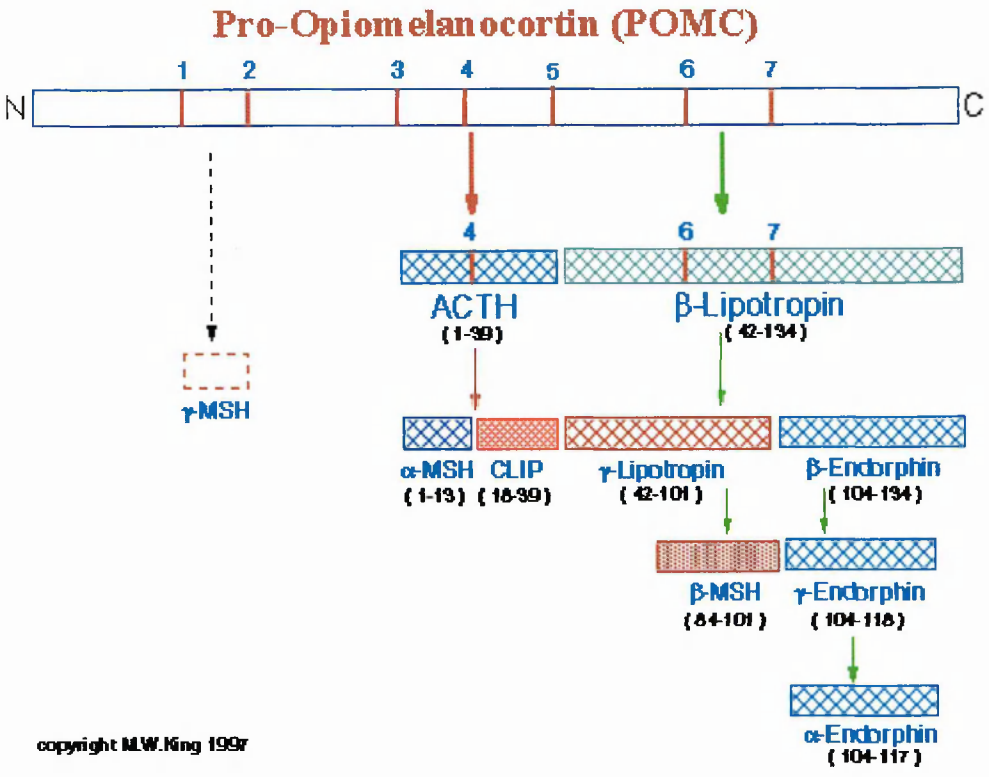


Figure.1.4. Processing of the POMC precursor protein. Cleavage sites are indicated by the numbers 1 to 7 and consist of the sequences, Arg-Lys, Lys-Arg or Lys-Lys. Adrenocorticotrophic hormone (ACTH) and β-lipotropin are products generated in the corticotrophic cells of the anterior pituitary under the control of corticotropin releasing hormone (CRH). Alpha-melanocyte stimulating hormone (α-MSH), corticotropin-like intermediary peptide (CLIP), γ-lipotropin and β-endorphin are products generated in the intermediary pituitary under the control of dopamine. The numbers in parentheses below each hormone indicate the amino acids of POMC present in each. The N-terminus of ACTH is given as amino acid number 1. The presence and function of γ-MSH is unclear in humans, hence the dotted lines, although it is well understood in rodents. (http://web.indstate.edu/thcme/mwking/peptide-hormones.htm)

1.2.3. Structure and Function of the Adrenal Gland

The adrenal cortex is responsible for the production of three major classes of steroid hormones: glucocorticoids, mineralocorticoids, and androgens. It is composed of three main tissue regions: zona glomerulosa, zona fasciculata, and zona reticularis. Although the pathway to pregnenolone synthesis is the same in all zones of the cortex, the zones are histologically and enzymatically distinct, with the exact steroid hormone product depending on the enzymes present in the cells of each zone. The zona glomerulosa is mainly responsible for the conversion of cholesterol to corticosterone and aldosterone. Whereas, cells of the zona fasciculata and zona reticularis can make corticosteroids and the adrenal androgens (dehydroepiandosterone and androstenedione), but not aldosterone.

ACTH regulates the hormone production of the zona fasciculata and zona reticularis. ACTH receptors in the plasma membrane activate adenylate cyclase with production of cAMP. The effect of ACTH on the production of corticosterone is particularly important, with the result that a classical feedback loop is prominent in regulating the circulating levels of CRH, ACTH, and corticosterone.

Substantial evidence indicates a role for central serotonergic neurones in the regulation of the secretion of ACTH, corticosterone, OT, growth hormone (GH), renin and AVP (Chaouloff, 1993). Of the many 5-HT receptor subtypes, only a few have been characterised with respect to their role in neuroendocrine function, for example 5-HT_{1A} and 5-HT_{2A} receptor involvement with ACTH (Hemrick-Luecke & Fuller, 1996).

1.2.4. Corticosteroid receptors

Two types of receptors for adrenal steroids have been identified in the brain (Reul & De Kloet, 1985). The type I corticosteroid receptor, also referred to as the corticosterone-preferring site or MR, has similar, if not equal, apparent affinity *in vitro* for both the endogenous mineralocorticoid (aldosterone) and glucocorticoid (corticosterone in rat and cortisol in humans; De Kloet *et al*, 1975; Sheppard & Funder, 1987). However, *in*

vivo the apparent affinity of these steroids for the MR differs depending on the presence of tissue-modifying factors (Sheppard and Funder, 1986; Funder *et* al, 1988). The type II corticosteroid receptor (GR; classical dexamethasone-binding site) has higher affinity for glucocorticoids than mineralocorticoids (Funder *et al*, 1973; Sheppard & Funder, 1987).

GR is present in nearly all mammalian tissues and has a multitude of physiological functions. In both the brain (in particular the hippocampus) and the anterior pituitary, this receptor is activated in response to stress and is thought to be important in terminating the stress response (Sapolsky *et al*, 1981), through the negative feedback effects of glucocorticoids on the HPA system (Feldman & Conforti, 1980; Dallman *et al*, 1989; **Figure.1.2.**).

MRs are almost exclusively septo-hippocampal in distribution, whereas GRs are found in the anterior pituitary, the ventromedial nucleus, and brain regions thought to be involved in the stress response encompassing the paraventricular, supraoptic, and arcuate nuclei of the hypothalamus, the median eminence, hindbrain noradrenergic and serotonergic cell bodies (Harfstrand *et al*, 1986; Reul *et al*, 1987b). This dual system may enable correct functioning with corticosteroid concentrations ranging from 0.5nM to 50nM, during the diurnal cycle, and up to 100nM or more in response to stress (Barden *et al*, 1995).

Functional differences between MR and GR in mediating corticosteroid negative feedback have been determined in part by the utilisation of selective MR and GR antagonists, for example RU40555 and RU38486 (Ratka *et al*, 1989; Weidenfeld & Feldman, 1993). These studies and others support the idea that MR maintains low basal activity of the HPA axis during the circadian trough (Dallman *et al*, 1987; DeKloet *et al*, 1993) and that GR, with facilitation by MR, constrains the increased HPA axis activity during the circadian peak and during acute stress (Sapolsky *et al*, 1981; Spencer *et al*, 1998). The respective roles of MR and GR in negative feedback regulation are not yet fully elucidated. Electrophysiological and behavioural studies suggest that GR and MR might

exert different, and even functionally antagonistic, effects (DeKloet *et al*, 1993). On the other hand, their effects on the HPA axis regulation appear to be synergistic (Dallman *et al*, 1989; Ratka *et* al, 1989). Understanding of the specific roles of GR and MR with respect to negative feedback regulation is clearly needed for a better understanding of the conditions associated with abnormal negative feedback.

The cellular response to glucocorticoids is dependent on both the availability of steroid and the presence and cellular concentration of functional receptor (Bloom *et al*, 1980; Vanderbilt *et al*, 1987). Ligand-induced down-regulation of GR and its increase in response to the removal of glucocorticoids by surgical ADX have been shown by steroid binding assays (McEwen *et* al, 1974; Reul *et al*, 1987a).

1. Gene expression

The nuclear receptors, like GR and MR, are characterised by a central DNA-binding domain (DBD), which targets the receptor to specific DNA sequences known as hormone responsive elements (HREs). The DBD is composed of two highly conserved zinc fingers that set the nuclear receptors apart from other DNA-binding proteins (Berg, 1989; Klug & Schwabe, 1995). The C-terminal half of the receptor encompasses the ligand-binding domain (LBD), which possesses the essential property of hormone recognition and ensures both specificity and selectivity of the physiological response.

In the absence of ligand, GR are predominantly maintained in the cytoplasm as an inactive multi-protein complex. This complex consists of two Hsp90 molecules plus immunophillins (e.g. Hsp56), p59 and calreticulin (reviewed by Pratt, 1993). Entry of glucocorticoids into the cell and subsequent binding to the LBD of GR leads to a conformational change in the receptor called transformation (Yamamto, 1985). This causes dissociation of the multi-protein complex and allows nuclear translocation of GR by virtue of the nuclear localisation sequence within the DBD. Once within the nucleus, the transformed hormone-receptor complex then binds with increased avidity to specific DNA

sequences termed GREs (for a review see Yamamto, 1985) to activate transcription of responsive genes (referred to as transactivation; Jantzen *et al*, 1987; Beato *et al*, 1996). Such interactions then regulate the transcription of selected genes (Yamamoto & Alberts, 1976; Ringold, 1985). It is also known that the protein products of glucocorticoid responsive genes may themselves regulate the expression of other genes such that glucocorticoids could indirectly activate the expression of entire gene networks (Yamamto, 1985).

2. Stress

Stressful stimuli are known to affect GR and GR mRNA concentrations in the rat brain (Kitraki *et al*, 1999; Calvo & Volosin, 2001). Stress stimulates the HPA axis and one of the prime neuronal targets for corticosteroid action is the hippocampal formation (Herman *et al*, 1993; Hassan *et al*, 1999). This region is known to possess the greatest density of MR and GR binding and mRNA expression in the brain (Reul & DeKloet, 1985; Herman, 1993), indicating a capacity for this brain region to respond to a wide range of corticosteroid concentrations.

The importance of GRs in regulation of hippocampal function and cell viability mandates a keen understanding of mechanisms controlling cellular GR biosynthesis. Studies to date indicate that the hippocampal GR is upregulated at both the binding and mRNA level by adrenalectomy, suggesting corticosteroid autoregulation (Tornello *et al*, 1982; Reul *et al*, 1989). Stress and high-dose corticosteroid administration appear to downregulate hippocampal GR binding, also consistent with autoregulation (Sapolsky *et al*, 1984; Sapolsky & McEwen, 1985). However, effects at the mRNA level have been inconclusive (Herman *et al*, 1989a; Reul *et al*, 1989), suggesting the potential for dissociation between mRNA expression and functional receptor expression. In depth *in vivo* analysis of GR protein regulation has yet to be definitively explored. Furthermore,

although corticosteroids are capable of influencing GR expression, there is no definitive understanding of the respective roles played by MR and GR in this action.

3. Clinical Relevance

Corticosteroids seem to play a key role in the triggering of depression, because: ***(a)*** GRs are present in limbic brain regions, which are of relevance to depression, including the noradrenergic and serotonergic projections to the forebrain (Harfstrand *et al*, 1986); ***(b)*** Corticosteroid receptors are intracellular and, when bound with corticosteroids, are translocated into the cell nucleus, where they bind to DNA and activate the transcription of mRNA (Beato, 1989). Such a genomic action of corticosteroids could provide a neurobiological basis for the known interaction between the genetic predisposition to depression and the effects of life events; ***(c)*** Diminished corticosteroid-receptor concentrations caused by a malfunctioning of systems involved in the regulation of corticosteroid-receptor gene expression (*Late-delayed feedback inhibition*) could be a causative factor in the defective feedback action of cortisol seen in patients suffering from severe depression, and could thus explain their altered HPA function.; ***(d)*** GRs are not fully occupied under resting conditions and hence are sensitive to stress-induced hypercortisolaemia. They are known to be involved in the neuroendocrine effects of stress (Ratka *et al*, 1989), and may also be involved more generally in the neurobiology of stress; ***(e)*** The activation of GRs is required for the development of certain animal models of depression (Veldhuis *et al*, 1985); ***(f)*** There is considerable overlap between the degree of hypercortisolaemia seen in depression and in Cushing's Syndrome. Consequently, if hypercortisolaemia affects mood in Cushing's Syndrome, then it may also do so in depression.

1.2.5. Neurotrophins

Neurotrophic factors (**Table.1.1.**), a subclass of growth factors, are endogenous proteins that regulate the development, maintenance and survival of neurones. They contain at least three distinct families: the neurotrophins (e.g., NGF, BDNF, NT-3 and NT-4/5; Barden *et al*, 1982; Ip *et al*, 1992); the neuropoietic cytokines (e.g., CNTF, IL-6, leukemia inhibitory factor or LIF); and the fibroblast growth factors (e.g., acidic and basic FGF). The members of these families of neutrophic factors demonstrate significant homology (50% or more) and have varying affinities for particular classes of receptors. For example, neurotrophins bind to a low affinity receptor (p75) and to a family of closely related high affinity glycoproteins, tyrosine receptor kinases (Trk).

Table 1.1. Examples of Proteins Reported to Have Neurotrophic Properties.

Proteins with well-documented neutrophic activity	**Proteins with putative Neurotrophic activity**
Acidic fibroblast factor (aFGF)	Cholinergic neuronal differentiation factor (CDF)
Basic fibroblast growth factor (bFGF)	Epidermal growth factor (EGF)
Brain-derived neutrophic factor (BDNF)	Heparin binding Neurotrophic factor (HBNF)
Ciliary Neurotrophic factor (CNTF)	Insulin
Interleukin 1, 3 and 6 (IL-1,3,6)	Insulin like growth factors (IGFs)
Neurotrophin-3 (NT-3)	Protease nexin 1 and 11
Neutrophin-4/5 (NT-4/5)	Transforming growth factor alpha (TGFα)
Nerve growth factor (NGF)	
Glial-derived neurotrophic factor (GDNF)	

The neurotrophins are secreted by cells and act in target-derived, autocrine or paracrine fashion on cell surface receptors. While these growth factors have many effects on neurones, the two most profound effects are promotion of differentiation and survival during development. It is now recognised that both of these actions also may be maintained in adult brains, implicating neurotrophins in plasticity and the response to injury in adult and young animals (Lindsay *et* al, 1994).

Within the CNS, BDNF mRNA displays a much wider distribution than that of central NGF mRNA. Regions producing the mRNA encoding for BDNF include the hippocampus, dentate gyrus, amygdala, projection areas of the olfactory system, inner and outer pyramidal layers of the neocortex, claustrum, cerebellum, and superior colliculus (Hofer *et* al, 1990). It has been shown that BDNF and NT-3 are able to elevate intracellular calcium in hippocampal neurones (Berninger *et al*, 1993). This finding supports the view that neurotrophins might have autocrine and paracrine actions within this brain region.

The known receptors for the neurotrophins include three different glycoprotein tyrosine kinases (TrkA, TrkB, and TrkC; **Figure.1.5**; for a review see Chao, 1992), which are similar in nature to the receptors for the growth factors FGF and EGF. The different neurotrophins bind to these receptors with high affinity ($K_d \cong 10^{-11}$) and with specificity: NGF binds to TrkA, BDNF and NT-4/5 bind to TrkB and NT-3 binds to TrkC. A low affinity receptor, p75, binds NGF and the other neurotrophins with an approximate K_d of $5x10^{-9}$, thus p75 has been referred to as a pan-neurotrophin.

Not only are the receptor mechanisms comparable for the various neurotrophic factors, but also the intracellular signalling pathways are very similar, even for such diverse molecules as NGF, CNTF and FGF (Boulton *et al*, 1994). Thus, a large group of diverse factors act on distinct receptors, leading to similar intracellular molecular changes, which can profoundly impact cell development and survival.

Following the binding of homodimeric neurotrophins to Trk receptors and resultant receptor dimerization, downstream neurotrophin actions occur in two intracellular stages (Schlessinger & Ullrich, 1992). In many ways, these events resemble the actions of other tyrosine kinase-coupled growth factors.

1 The first cytosolic events are initiated by autophosphorylation of the tyrosine receptor kinase.

2 The second stage of the signalling process is gene transcription within the nucleus. This is mediated by mitogen-activated protein (MAP) kinase, S6 kinase II and possibly other activated regulatory proteins, which stimulate response elements, thereby inducing transcription of early genes such as c-*fos*, c-*jun* and c-*myc* (Marutu & Burgess, 1994). This stage of the signalling process is generally thought to account for the most profound effects of the neurotrophins, such as neuronal survival and neurite outgrowth.

It seems that at least in the intact rat brain the expression of the neurotrophins are confined largely to neurones. In addition, the levels of neuronal NGF and BDNF mRNAs are increased by enhanced neuronal activity following glutamate (Zafra *et al*, 1990) and muscarinic receptor stimulation (Berzaghi *et al*, 1993). The activation of glutamate receptors enhances the synthesis of BDNF and NGF in hippocampal neurones both *in vitro* and *in vivo*, whereas stimulation of the GABAergic system decreases their mRNA levels (Zafra *et al*, 1991). Therefore the functional state of the hippocampal neurone seems to determine the levels of BDNF and NGF mRNA. Moreover, depending on the conditions, activation of both major types of glutamate receptors, NMDA and non-NMDA receptors, are able to up-regulate neuronal BDNF and NGF mRNA levels.

BDNF enhances survival, transmitter uptake, and total protein content in central GABAergic neurones (Knusel *et al*, 1991). Additionally, studies verified the augmented survival and uptake of dopamine from mesencephalic substantia nigra neurones in culture, in response to BDNF (Dal Toso *et al*, 1988; Hyman *et al*, 1991).

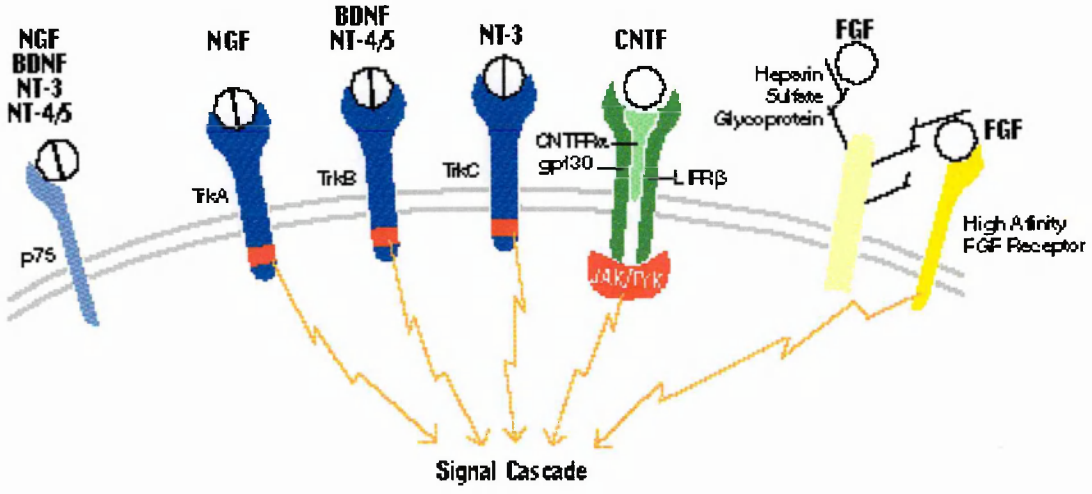


Figure.1.5. Neurotrophic factors and their receptors. Neurotrophin receptors (blue): All neurotrophins bind with low affinity to p75, which has no defined role in neurotrophin signalling. The neurotrophins selectively bind to high affinity Trk receptors and cause the Trk receptor to form homodimers and autophosphorylate, thereby triggering the intracellular signalling cascade. **Neuropoietic cytokine receptors** (green): CNTF binds with low affinity to the specific CNTF alpha-receptor moiety (CNTFR-alpha), which lacks a transmembrane domain. The CNTF:CNTFR-alpha complex interacts with two membrane spanning signalling molecules, gp130 and LIFR-beta, thereby activating an associated tyrosine kinase (JAK/TYK), which transduces the initial intracellular signal. **Fibroblast growth factor receptors** (yellow): FGF binds with low affinity to a heparin sulfate proteoglycan FGF receptor which then facilitates binding of FGF monomers to the high affinity FGF receptor. This interaction leads to receptor autophosphorylation and signal transduction.

Note: Only principal ligand:receptor interactions are shown. High affinity receptors for a specific class of neurotrophic factors are represented with a dark colour, while the low affinity receptor has a lighter shade of that colour. Tyrosine kinase domains are shown in red. (Lipton & Kalil, 1995)

1. Stress

Many types of brain insults induce modifications in neurotrophin mRNA expression in the CNS (Lindvall *et al*, 1994). Single or repeated immobilisation stress application or exogenously applied corticosterone markedly reduce BDNF mRNA and protein levels in the hippocampus (Barbany & Persson, 1992; Schaaf *et al*, 1998). Inversely, adrenalectomy (ADX) results in an increase in BDNF mRNA levels in this structure (Barbany & Persson, 1992; Chao *et al*, 1998).

2. Clinical Relevance

There are several lines of evidence that suggest BDNF is involved in the action of antidepressant treatment and in the stress-induced hippocampal adaptation and pathogenesis of depression (Duman *et al*, 1997a) in the adult animal: ***(a)*** Direct infusion of BDNF protein into the midbrain exerts antidepressant effects in two models of depression, i.e. the forced swim and learned helplessness models (Siuciak *et al*, 1996); ***(b)*** Chronic administration of various classes of antidepressant drugs increases hippocampal BDNF mRNA with the induction following the time-course observed for the therapeutic effects of antidepressant treatments, and that antidepressant pre-treatment ameliorates stress-induced reduction of BDNF mRNA in this brain region (Nibuya *et al*, 1997); ***(c)*** BDNF exerts a strong trophic effect on serotonergic and noradrenergic neurones regulating morphology, neurotransmitter metabolism and firing patterns of these neuronal populations (Mamounas *et al*, 1995); ***(d)*** Chronic stress is known to result in neuronal damage and death. Decreased BDNF expression as a consequence of stress may play a role in stress-induced neuronal damage.

Finally, BDNF mRNA expression is regulated by CREB (Tao *et al*, 1998) whose activity could thereby underlie some of the long-term effects of antidepressant treatment (Nibuya *et al*, 1996). Regions exhibiting an up-regulation of BDNF in response to antidepressant administration overlap closely with the regions that show an up-regulation

of CREB. This spatial correlation suggests that CREB may contribute to the antidepressant-induced increase in hippocampal BDNF expression. A role for the cAMP system in mediating the antidepressant-induced increase in BDNF expression is supported by studies with the phosphodiesterase (PDE) inhibitors papaverine and rolipram (Nibuya *et al*, 1996).

1.2.6. Regulation

Three integrated regulatory forces are imposed on the HPA axis to co-ordinate adrenal secretions through times of inactivity or stress: **(1)** a circadian rhythm of basal activity derived from the suprachiasmatic nucleus (Cascio *et al*, 1987), **(2)** stress-induced responses of the HPA system are far more complex and involve afferent inputs from numerous brain regions, including noradrenergic innervation from the brain stem A1 and A2 cell groups, locus coeruleus (Szafarczyk *et al*, 1985), amygdala (Beaulieu *et al*, 1989), cerebral cortex and hippocampus (Jacobson & Sapolsky, 1991), and **(3)** feedback inhibitory actions of adrenal steroids exerted through corticosteroid receptors.

1. Circadian Rhythms

It is well established that the HPA axis exhibits a circadian rhythm in humans, rats, and other mammals, and basal steroid concentrations that oscillate in a circadian fashion cause this. The concentrations are correlated with the rest-activity cycle of the animal, rather than the light cycle. Thus, in man, steroids begin to rise in the early morning hours, peak around awakening, and then fall throughout the day. In nocturnal animals such as rats, the converse pattern is seen, whereby concentrations peak in late evening and are at a nadir in early morning. Connections between this cycle and rest-activity, sleep, and feeding behaviour have been made. The circadian rhythm in basal activity of the HPA axis results in total circulating corticosteroid concentrations that may be <10 nM at the nadir of the rhythm and about 700 nM at the peak of the rhythm in both man and rats. Of the total

corticosteroid concentration, 99-95% is tightly bound in the circulation to transcortin and is unavailable for diffusion to brain sites. The amount of steroid bound depends on the concentrations of both steroid and transcortin in the circulation. Furthermore, at all times of the day, the system can be stimulated by stressors to cause corticosteroid concentrations that may exceed 1000 nM (for a review see DeKloet *et al*, 1993).

The effects of stress are superimposed on the basal rhythmicity, and there is evidence that stress responsiveness and the effectiveness of negative feedback may also oscillate across the cycle. Thus, at the trough of the rhythm, animals appear to be more sensitive to both stress activation and inhibition by glucocorticoids, suggesting that at this time, the axis is exquisitely responsive. The drive to the axis prior to awakening appears to be initiated by the SCN (Dallman, 1984), leading to enhanced tone of CRH, and resulting in increased activity throughout the HPA axis. However, other neuroregulators such as AVP, OT, and adrenaline are also known to possess ACTH-releasing activity, and immunoneutralisation of CRH does not completely abolish circadian rhythms of plasma ACTH (Carnes *et al*, 1990).

Occupancy of GR occurs in parallel with the stress-induced rise, subsequent fall, and the circadian variation of plasma corticosterone, which illustrates that GR mediate the feedback action of corticosterone on stress-activated brain processes (Munck *et al*, 1984; De Kloet & Reul, 1987). This view is supported by the presence of GR in neurones involved in the regulation of the stress responses, i.e., neurones of the paraventricular and supraoptic nuclei, the ascending aminergic neurons and cortical and limbic neurones (Reul & DeKloet, 1985; Kiss *et al*, 1988).

2. Neurotransmitters

Glucocorticoids have complex and widespread interactions with neurotransmitter and neuropeptide systems. Most major neurotransmitter systems influence CRH. Acetylcholine, serotonin, angiotensin, endogenous cytokines, neuropeptide Y, noradrenaline, and dopamine stimulate CRH secretion, whereas glucocorticoids, glutamate, opiod-derived peptides, GABA, aldosterone and, in some circumstances, noradrenaline inhibit CRH release (Krishnan *et al*, 1991). Evidence for direct serotonergic, dopaminergic (Liposits & Paul, 1989), and GABAergic (Meister *et al*, 1988) innervation of the CRH perikarya of the PVN has also been provided.

Serotonergic neurones projecting from the midbrain raphé nuclei form synapses with CRH-containing neurones in the PVN of the hypothalamus that control pituitary-adrenocortical function (Fuller & Snoddy, 1990). Drugs that enhance serotonergic function such as direct-acting 5-HT agonists, 5-HT precursors, uptake blockers and releasing agents have been shown to increase the release of CRH, ACTH and corticosterone *in vivo* (Fuller & Snoddy, 1990; Van de Karl, 1991). From among the multiple subtypes of serotonergic receptors in the brain, the 5-HT_{1A} and 5-$HT_{2A/2C}$ subtypes appear to mediate activation of HPA function (Fuller & Snoddy, 1990). 8-OH-DPAT, an agonist selective for the 5-HT_{1A} subtype, has been shown to increase corticosterone levels. This effect can be blocked by 5-HT_{1A}-selective antagonists but not by non-5-HT_{1A} antagonists (Fuller & Snoddy, 1990). The 5-HT_{1A} subtype of serotonergic receptors is an important target in the treatment of neuropsychiatric disorders such as anxiety and depressive illness and 5-HT_{1A}-selective compounds have been shown to be clinically effective antidepressants and anxiolytics (Blier *et al*, 1990).

Stress has also been shown to alter levels of 5-HT_{1A} receptors in rats. However, different stressors appear to produce variable effects on 5-HT_{1A} receptors. For instance, restraint stress has been shown to produce an increase in 5-HT_{1A} receptors in the CA4 and the infrapyramidal dentate gyrus (Mendelson & McEwen, 1992). This is surprising since

adrenalectomy also produces an increase in 5-HT_{1A} receptors and exogenous corticosterone administration prevents this increase (Mendelson & McEwen, 1992; Tejani-Butt & Labow, 1994). However, decreases in 5-HT_{1A} binding in the hippocampus have been reported following other stressors such as two weeks of chronic unpredictable stress (Tejani-Butt & Labow, 1994) or chronic social stress (McKittrick *et al*, 1995). It must be noted that these stress paradigms are qualitatively very different and involve different durations of exposure to stressful stimuli. Different stressors produce variable effects on corticosterone as well as 5-HT release.

The interaction between the serotonergic system and the HPA axis is bi-directional in that glucocorticoids also affect the state of the serotonin receptor system. Corticosteroids can alter several aspects of serotonergic neurotransmission including 5-HT metabolism, turnover and release (Curzon, 1972). In addition, adrenal steroids can also directly regulate the expression of 5-HT receptors (Biegon *et al*, 1985; De Kloet *et al*, 1986). With specific regard to the 5-HT_{1A} receptor system, reduction in corticosteroid level following adrenalectomy is associated with an increase in 5-HT_{1A} receptor expression (Mendelson & McEwen, 1992; Tejani-Butt & Labow, 1994) and 5-HT_{1A} receptor mRNA in rat hippocampus (López *et al*, 1993). These increases are prevented by replacement treatment with corticosterone (Chalmers *et al*, 1993). Changes in 5-HT_{1A} receptor density and 5-HT concentrations following adrenalectomy parallel the activity and responsiveness of the HPA axis (Burnet *et al*, 1992).

3. Feedback

The secretion of hypothalamic, pituitary, and target tissue hormones is under tight regulatory control by a series of feedback and feedforward loops. These modes of negative feedback mediated by corticosterone can be distinguished (Keller-Wood & Dallman, 1984) into fast feedback, intermediate or delayed feedback mechanism, and genomically mediated feedback.

(a) **Fast feedback,** which includes the actions (within 10min) of corticosterone on the multisynaptic control of ACTH secretagogues released in the median eminence. It does not involve protein synthesis and may occur presumably at the level of the cell membrane (Dallman & Yates, 1969; Jones, 1979). Evidence for this feedback comes from the following experiments: **(i)** Lesions to the hippocampus or to its projections to the PVN result in impairment of fast feedback and consequently in prolongation of the stress response (Herman *et al*, 1989b; Jacobson & Sapolsky, 1991); and **(ii)** Fast feedback can be investigated in man by measurement of the suppression of ACTH and/or β-endorphin following an infusion of hydrocortisone (Young *et* al, 1991). Changes in excitability via membrane receptor complexes such as the $GABA_A$ receptor may underlie fast feedback action and rapid changes in excitability of antidromically identified CRH neurones, following glucocorticoid iontophoresis, may be involved as well (Saphier & Feldman, 1988). Fast feedback is also desensitised upon chronic exposure to stress (Young *et al*, 1990).

(b) **Intermediate or delayed feedback mechanism**, which involves gene-mediated steroid effects on stimulus secretion coupling, excitability, and intracellular signal transduction pathways. This operates between one and 24 hours after plasma corticosteroid concentrations have risen. The degree of this inhibition is proportional to the integrated release of corticosteroids over time and the delay from this release. In this feedback inhibition, synthesis of CRH and ACTH are inhibited, rather than their release. It is seen in the presence of pathologically high corticosteroid concentrations or following treatment with synthetic glucocorticoids such as dexamethasone. Intermediate or delayed-feedback can be tested by the suppression of HPA function following dexamethasone administration, a test that involves glucocorticoid receptors in the pituitary (Miller *et al*, 1992). The delayed-feedback inhibition is responsible for changes in gene transcription involved in the stress response. These changes in gene transcription include decreased CRH expression in the hypothalamus (Imaki *et al*, 1991; Kovacs & Mezey, 1987) and decreased POMC

(Eberwine & Roberts, 1984) and CRH type 1 receptor expression in the pituitary (Sakai *et al*, 1996).

(c) **Genomically mediated feedback**, has a much slower time course, whereby glucocorticoid receptors negatively control gene expression, decreasing rates of transcription of critical genes such as POMC. Indeed, the negative regulation starts immediately upon receptor activation by steroids, but its consequences on the cell, in terms of mRNA and peptide levels take hours or even days to manifest, because of their intrinsic kinetics and the presence of large reserves. It takes place exclusively at the level of the gene. It includes the blockade of stress-induced CRH (and AVP) gene expression in parvocellular paraventricular nucleus and POMC gene expression in anterior pituitary corticotrophs. GRs may control peptide gene expression directly or in interaction with other transcription factors (Akerblom *et al*, 1988). In late-delayed feedback, there is reduced transcription of RNA for POMC and reduced translation of POMC from mRNA (see **Figure.1.2.**).

In a living organism, all these mechanisms are probably activated simultaneously, but they come into play in different time domains; fast feedback is likely to set the magnitude and duration of each response, whereas genomic feedback sets the range of stress responsiveness of an organism. In addition, these feedback mechanisms can be seen as different lines of defence, with fast feedback being more sensitive, rapid and brain mediated, and genomic feedback being slower but having more profound effects at multiple levels of the axis.

The secretion of CRH and AVP appears to be under tight corticosteroid feedback control during the basal circadian trough and peak as well as during CRH/AVP responses to stressors. Although ACTH-secreting cells in the anterior pituitary respond to corticosteroids *in vitro*, with inhibition of ACTH synthesis and secretion, it seems likely that this effect is primarily a positive feedback that is involved during high level corticosteroid secretion (Levin *et al*, 1988). Interestingly, the basal levels of steroids at the

peak are thought to be sufficiently high to occupy a majority of MR (estimates vary from 60% to 90%) but only occupy a small proportion of GR (~10%). Thus, there may be elements of the axis (e.g., AVP in mpPVN) that are particularly sensitive to circadian drive and are modulated by MR, whereas others are particularly stress responsive and only modulated by GR or both receptors.

1.2.7. Adaptation

Depending on the stress paradigm, repeated stress can result in adaptation or desensitisation of the HPA axis to the homotypic stressor (Kant *et al*, 1987; Ma & Lightman, 1998). However, exposure of repeatedly stressed animals to a novel stress usually results in a greater ACTH response than that seen in naïve control animals (Hashimoto *et al*, 1988; Bhatnagar & Dallman, 1998).

There is good evidence that when adaptation to a repeated stressor occurs there is preservation or even sensitisation of the hormonal response to a heterotypic stress (Vernikos *et al*, 1982; Aguilera, 1998). This suggests that the hypothalamus, and other brain regions, are able to differentiate between different types of stress and modify their response in the light of previous experience.

It is possible that adaptation to the repeated homotypic stress is due to desensitisation of the afferent pathways to the PVN at the synaptic levels or at the parvocellular neurone itself, and that the novel stress uses different pathways and neurotransmitters. However, despite the habituation, there is little evidence of desensitisation, as AVP responses to the repeated stress are preserved (Ma *et al*, 1997), and microdialysis experiments have shown that noradrenaline turnover to the PVN is increased, rather than decreased, during repeated immobilisation (Pacak *et al*, 1992).

Different types of stressors (physical vs. psychological, painful vs. non-painful) are likely to engage the system from different starting points. A major stumbling block to assess the differences has been the need to develop a strategy for detecting stress activation in the CNS. This is a prerequisite to carrying out lesion studies to disrupt the pathway and begin to delineate its components. One approach is to measure peripheral indices of stress – corticosterone and ACTH increases in plasma. However, the ability to monitor CNS correlates of acute activation by various stressors is critical to discerning the existence of unique pathways as well as common elements. The measure has to be rapid enough to detect the activation before negative steroid feedback dampens or even reverses the response. Several studies (Watanabe *et al*, 1994; Lee *et al*, 2001) have been undertaken in an effort to map brain regions activated by various stressors, using IEGs such as *c-fos*, *c-jun* and zif/268 as markers for each neuronal activity.

Stimulatory stressful information is conveyed to the PVN through ascending catecholaminergic projections from the brain stem (Swanson & Simmonds, 1989; Palkovits *et al*, 1992). In addition, studies based on intermediate early gene expression, retrograde tracer, and lesioning of selective pathways have revealed that somatosensory/nociceptive stressors, such as restraint, activate limbic areas in the brain, including the hippocampus, neocortical areas, amygdala, bed nucleus of the stria terminalis, and some hypothalamic and thalamic nuclei (Cullinan *et al*, 1993; Li *et al*, 1997). The limbic structures activated by somatosensory stressors all have direct or indirect connections to the PVN and contain GABA neurones known to inhibit the HPA axis (Herman & Cullinan, 1997). Lesions of the prefrontal cortex, hippocampus, and central amygdala have been reported to potentiate HPA axis responses to somatosensory stressors, supporting the idea that responses to this type of stressor can be modulated by limbic inhibitory circuitry (Herman & Cullinan, 1997). One possible pathway involved in the increased responsiveness to the heterotypic stressor is the parabrachial-posterior-paraventricular-thalamus-amygdala-parvocellular-paraventricular-hypothalamus pathway,

as lesions of the posterior PVN of the thalamus increase ACTH responses to restraint in previously chronically stressed animals, but not naive animals (Bhatnagar & Dallman, 1998). Influences from hypothalamic nuclei, such as the arcuate, ventromedial hypothalamic, and medial preoptic area, may also affect parvocellular neuronal activity during stress. In addition, other neurotransmitters and neuropeptides, including CRH itself, could modulate PVN activity through these pathways. Thus, processing and integration of somatosensory/nociceptive stimuli in the limbic system could activate or suppress inhibitory pathways to the PVN, enhancing or inhibiting parvocellular neurone responses depending on previous experiences and the type of stimulus.

1.3. Antidepressants

1.3.1. Mode of Action

There is a high degree of variation among people with depression in terms of symptoms, course of illness, and response to treatment, indicating that depression may have a number of complex and interacting causes. This variability poses a major challenge to researchers attempting to understand and treat the disorder. However, recent advances in research technology are bringing scientists closer than ever before to characterising the biology and physiology of depression in its different forms and to the possibility of identifying effective treatments for individuals based on symptom presentation.

Many of the biological theories of depression have been intimately related to the chemical effects of antidepressant medications discovered in the late 1950s. Three main biochemical theories of depressive illness have been postulated and are summarised in **Table.1.2.**

Table.1.2. Main Biochemical Theories Of Depressive Illness (Tyrer & Marsden, 1995):-

HYPOTHESIS	CHIEF TENET	EVIDENCE IN SUPPORT
Amine-deficiency hypothesis	Central deficiency of 5-HT and noradrenaline	Reserpine-induced depression. MAOIs and amine-reuptake drugs are antidepressants
Adrenergic supersensitivity hypothesis	α and ß central receptors are supersensitive	Most antidepressants down-regulate receptors after chronic dosage. Treatment effects only shown after chronic dosage.
Cholinergic hypothesis	Excess cholinergic compared with adrenergic activity	Many antidepressants are anticholinergic, cholinergic drugs may induce depression.

These theories have been developed from evidence available from first generation antidepressants, TCAs and MAOIs, and the efficacy and models of action of newly introduced atypical compounds need to be considered. New antidepressants have a variety of pharmacological actions and although these include several properties possessed by TCAs and MAOIs, they show important differences as well. Although there is no common pharmacological action possessed by all those compounds demonstrated to have antidepressant efficacy, the down-regulation of ß-receptors is found with most of the drugs.

Existing antidepressant drugs, except SSRIs, are known to influence the functioning of certain neurotransmitters in the brain, primarily the monoamines, serotonin and noradrenaline. Previous medications – TCAs and MAOIs – affect the activity of these neurotransmitters simultaneously. Their disadvantage is that they can be difficult to tolerate due to side effects or, in the case of MAOIs, dietary restrictions. Recent medications, such as SSRIs, have fewer side effects than the older drugs, making it easier for patients to adhere to treatment. Both generations of medications are effective in relieving depression, although some people will respond to one type of drug, but not another.

The "serotonin/noradrenaline/glucocorticoid link" hypothesis of affective disorders and the action of antidepressants, postulated by Pryor and Sulser (1991), has integrated the glucocorticoid receptor system into an amine hypothesis of affective disorders. A functional link between aminergic and endocrine signalling (corticosteroids) beyond the receptors has been demonstrated in C6 glioma cells at the level of preproenkephalin gene expression (Yoshikawa & Sabol, 1986). *In vivo*, the regulation of preproenkephalin gene expression depends on serotonin, as chronic fluoxetine enhanced the expression of preproenkephalin mRNA in the rat amygdala, and this effect disappeared in rats with depleted concentrations of brain serotonin (Rossby *et al*, 1996).

Effective antidepressant drug therapy normalises the plasma cortisol levels, but this takes at least 2-3 weeks. This suggests that while facilitation of monoaminergic neurotransmission may be the initial mechanism of action of most antidepressants, their beneficial effects are likely due to adaptive processes beyond the acute effects of the drug. These may be mediated by alterations in neuronal gene expression.

Antidepressant medications take several weeks to be clinically effective even though they begin to alter brain chemistry with the very first dose. Research now indicates that antidepressant effects result from slow-onset adaptive changes within brain cells, or neurones. Further, it appears that activation of chemical messenger pathways within neurones, and changes in the way that genes in brain cells are expressed, are the critical events underlying long-term adaptations in neuronal function relevant to antidepressant drug action. A current challenge is to understand the mechanisms that mediate, within cells, the long-term changes in neuronal function produced by antidepressants and other psychotropic drugs and to understand how these mechanisms are altered in the presence of depression.

At least three observations suggest that blockade of monoamine reuptake and inhibition of amine metabolism may be insufficient to explain the action of antidepressants; (a) iprindole, trazodone and mianserin are clinically effective antidepressants despite lacking the above properties and are known as "atypical" antidepressants for this reason; (b) reuptake blockade and inhibition of monoamine metabolism occur immediately after instigation of therapy, yet clinical signs of improvement in depression is generally delayed for 2-3 weeks; (c) cocaine is a blocker of amine uptake, but is not an effective antidepressant (Stahl, 1984).

Earlier research was done on the acute effects of these drugs in brain systems, but these acute effects do not appear to fully explain their antidepressant effects. It was later recognised that their chronic effects were quite different and may be better to help understand their therapeutic effects. Long-term studies of drug effects are considered more relevant because most antidepressants show a time lag of between one and three weeks before effects on mood are seen.

Long-term administration of antidepressant influences monoaminergic systems, not only presynaptically but also postsynaptically. Presynaptic and postsynaptic receptors change in a complex manner and to a different extent in different brain areas (Sulser, 1979; Charney *et al*, 1981). Down-regulation is generally involved. A down-regulated postsynaptic receptor should be associated with diminished function while down-regulation of presynaptic receptors should enhance function. Evidence suggests that chronic administration of TCAs is associated with a down-regulation of post-synaptic β-adrenergic receptor sites accompanied by a loss in sensitivity of adenylate cyclase to stimulation by noradrenaline or isoproterenol (Sulser *et al*, 1978). These changes not only occur after treatment with the TCAs and with the MAOIs, but also with atypical antidepressants such as iprindole and mianserin and even with ECT (Charney *et al*, 1981). Enhancement of monoaminergic functions is believed to underlie the therapeutic efficacy of antidepressants.

1.3.2. Effect on the HPA axis

Previous research has shown that there is a neurochemical and a behavioural similarity between the response to antidepressant treatment and the response to stressful stimulation in animals. The neurochemical similarity concerns the regulation of brain adrenergic receptors. Both antidepressants and stress, when administered chronically, have been found to reduce the density of β-adrenoceptors in various regions of the rat brain (Sellinger-Barnette *et al*, 1980; Stone & Platt, 1982). These reductions are accompanied by corresponding decreases in noradrenaline-sensitive adenylate cyclase activity (Vetulani *et al*, 1976; Stone & Platt, 1982). The behavioural similarity between antidepressants and stress concerns changes in the vulnerability of animals to the adverse effects of stress. Both forms of treatment, when given chronically, have been shown to make animals more resistant to behavioural deficits caused by exposure to subsequent inescapable stress (Sherman *et al*, 1979).

Consistent findings in depressed patients are hyperactivity in the HPA axis with high plasma concentrations of ACTH and cortisol. Long-term antidepressant treatments seem to normalise this hyperactivity, suggesting a link between the HPA axis and the action of antidepressant treatments.

The process of adaptation to stress has been associated with increases in binding at 5-HT_{1A} receptors in the hippocampus as well as enhancement of sensitivity to 5-HT agonists (Kennet *et al*, 1985b). On the other hand, prolonged exposure to high levels of corticosterone, such as might occur in animals that continue to secrete corticosterone in response to a repeated stressor, has been found to reduce numbers of 5-HT_{1A} receptors in the hippocampus (Mendelson & McEwen, 1992), whereas ACTH treatment increases binding to 5-HT_2 receptors in cerebral cortex. In view of the relationships between stress, serotonin and depression, there is reason to suspect that the mechanisms by which the antidepressant drugs act may be most obvious when evaluated in animals being subjected to stress. Indeed, there is evidence that stress may even enhance the effects of some

antidepressant drugs. For example, Nankai and colleagues (1991) presented evidence that restraint stress enhances the ability of desipramine to down-regulate serotonin transporter-binding sites in prefrontal cortex and hypothalamus. These studies raise the possibility that stress may alter the effects of antidepressant drugs on regional neurochemistry. Moreover, the opposite action of antidepressants, such as imipramine and tianeptine, on serotonin uptake raise the question whether these drugs share common effects on brain chemistry or produce entirely different patterns of response.

Recent work has raised the possibility that among the many long-term targets of antidepressant treatments may be regulation of neurotrophins. A role for BDNF in the action of antidepressant treatments is supported by several lines of evidence. First, electrical (e.g. ECT) or chemical-induced seizures increase the expression of BDNF and its receptor, TrkB, in the brain (Isackson *et al*, 1991). Second, stress decreases the expression of BDNF in the hippocampus and other limbic brain regions (Smith *et al*, 1995a,b), an effect that could contribute to the atrophy of stress-vulnerable neurones in the hippocampus (Sapolsky *et al*, 1985; Stein-Behrens *et al*, 1994). Stress-induced atrophy, and, in extreme cases, cell death, may contribute to the loss of hippocampal control of the HPA axis and hypercortisolism often exhibited in depression (Herman *et al*, 1989b; Young *et al*, 1991).

The different serotonin receptor subtypes are known to selectively couple to and regulate several intracellular signal transduction pathways, including the cAMP, phosphatidylinositol (PI), and calcium pathways. These intracellular pathways would be expected to mediate the actions of drugs that act on the serotonergic system. In addition, the influence of long-term activation of these receptor-coupled second messenger systems in response to chronic drug administration could lead to adaptations that contribute to the therapeutic action of these treatments. Recent studies have begun to examine adaptations of these pathways in response to chronic antidepressant administration (for a review see Duman *et al*, 1997a). One system that has been examined in some detail

is the cAMP second messenger system. It has been demonstrated that chronic administration of SSRIs, as well as other types of antidepressants, results in adaptations of the cAMP second messenger pathway, including upregulation of CREB.

Upregulation of the transcription factor CREB indicates that specific target genes may also be regulated by, and could mediate the action of chronic antidepressant administration. Various classes of antidepressants increase the expression of CREB (Nibuya *et al*, 1996). Among the multiple target genes that could be regulated by CREB is BDNF (Duman, 1998). Chronic administrations of SSRIs, or other types of antidepressants, increase the expression of BDNF and TrkB in hippocampus (Nibuya *et al*, 1996). This may result from adaptations of intracellular pathways. Chronic antidepressant drug treatments, like chronic ECS, increase nuclear levels of cAMP-dependent protein kinase and CREB (Nestler *et al*, 1989). Up-regulation of the cAMP-PKA-CREB cascade by chronic antidepressant drug treatments could underlie the increased expression of BDNF and TrkB mRNA.

The possibility that increased expression of BDNF and TrkB results from upregulation of the cAMP pathway and CREB is supported by both correlative and direct information. First, the time course for both increased expression of CREB and BDNF/TrkB is similar, and the expression of both CREB and BDNF/TrkB is observed in the same layers of hippocampus (i.e., CA1 and CA3 pyramidal and dentate gyrus granule cell layer). Second, infusion of CREB antisense decreases basal and ECS induction of BDNF in hippocampus (Duman *et al*, 1994). Third, chronic administration of inhibitors of phosphodiesterase, the enzyme responsible for cAMP breakdown, increases the expression of CREB and BDNF (Nibuya *et al*, 1996). Fourth, studies in cultured cells demonstrate that activation of the cAMP pathway increases the expression of BDNF and TrkB (Condorelli *et al*, 1994). These results indicate that the expression of BDNF and TrkB are positively regulated by the cAMP pathway and CREB.

Although the hypothesis that antidepressant treatments lead to an increase in neurotrophin concentrations because of activation of CREB (Duman *et al*, 1997b) is attractive, some data is not consistent with it. For example, chronic administration with venlafaxine did not elevate the mean steady-state concentration of CREB mRNA and also significantly reduced the amount of phosphorylated CREB in nuclear lysates of the rat cortex (Rossby *et al*, 1999).

One effect of BDNF, that could be relevant to the therapeutic actions of antidepressants, is regulation of serotonin neurones. Local infusion of BDNF into midbrain or cerebral cortex is reported to increase serotonin levels (Siuciak *et al*, 1994) and to protect serotonin neurones from neurotoxin-induced damage (Mamounas *et al*, 1995).

Repeated stress can result in neuronal atrophy and death of vulnerable neurones, and can increase the vulnerability to other neuronal insults, including excitotoxins, hypoxia-ischaemia, and hypoglycaemia (McEwen & Gould, 1990; Stein-Behrens *et al*, 1994). In addition to increasing the expression of BDNF mRNA, the ability of antidepressant treatments to block the down-regulation of BDNF mRNA by stress could also help reduce the damaging effects of stress. In support of this hypothesis, chronic administration of an atypical antidepressant, tianeptine, is reported to block the atrophy of hippocampal neurones resulting from stress (Watanabe *et al*, 1992).

Future studies will determine if chronic antidepressant treatments have similar neuroprotective effects against stress, as well as other neuronal insults. In addition, elevated expression of BDNF could play a role in the reinstatement of hippocampal feedback inhibition of the HPA axis in response to antidepressant treatments: hypercortisolism in depression is thought to result, at least in part, from loss of hippocampal inhibition (Young *et al*, 1991).

Several antidepressants, such as imipramine, fluoxetine, idazoxan and phenelzine, have been shown to decrease CRH gene expression in the rat PVN (Brady *et al*, 1992). Although long-term administration of tianeptine did not alter CRH mRNA levels, indicating that the reduction of CRH content in the hypothalamus cannot be ascribed to an action at the transcriptional level (Delbende *et al*, 1994). This study, in agreement with other reports (Chappell *et al*, 1986), revealed that chronic administration of tianeptine reversed the effects of stress at different levels of the HPA axis. Acute stress was shown to cause a significant reduction of CRH content in the hypothalamus without affecting ACTH level in the pituitary, and chronic treatment abolished stress-evoked inhibition of CRH. Similarly, tianeptine significantly reduced the increase in plasma ACTH and corticosterone levels induced by restraint stress (Delbende *et al*, 1994). It is believed that tianeptine modulates the activation of the HPA axis through its capacity to reduce the availability of serotonin to its receptors. Since TCAs increase the glucocorticoid receptor level in the hippocampus and hypothalamus (**Figure.1.6**; Peiffer *et al*, 1991), the modulatory activity of tianeptine on the HPA axis might be mediated through restoration of the negative feedback action of glucocorticoid hormone at the hippocampal or hypothalamic level.

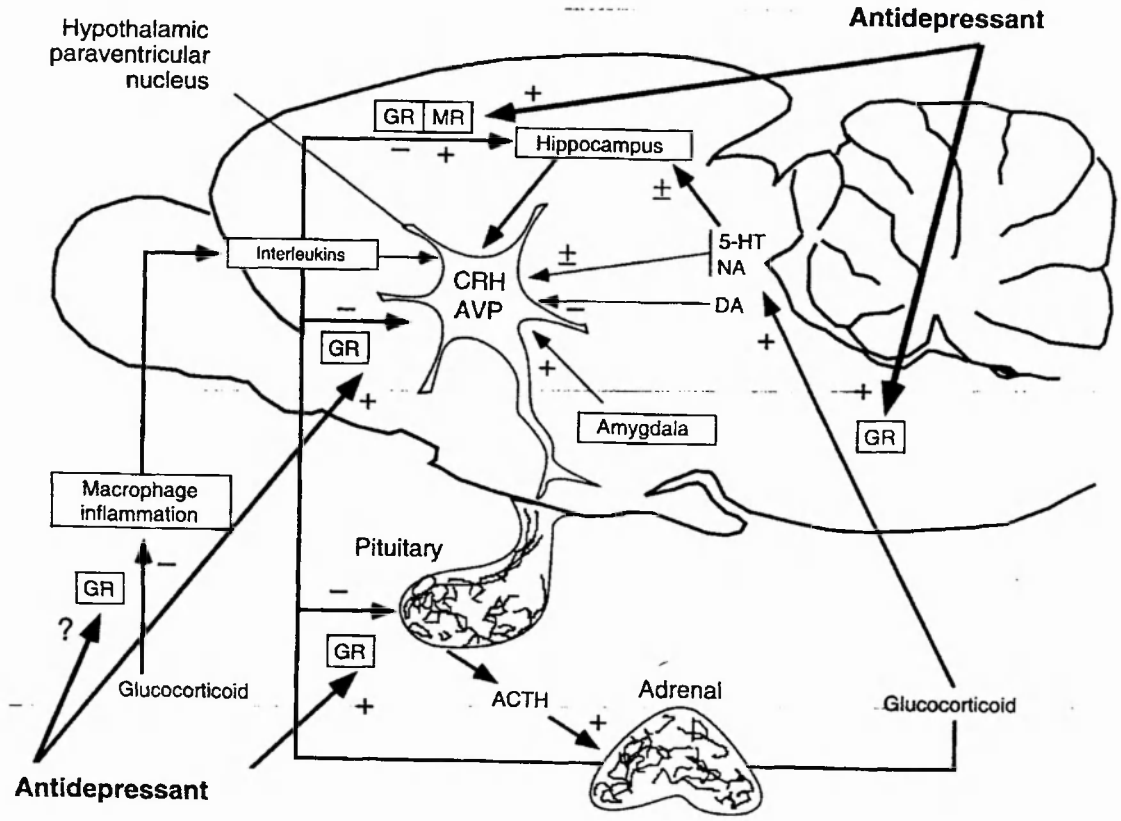


Figure.1.6. A novel mechanism for antidepressants acting on the HPA system. Schematic representation of the antidepressant-induced increases in GR and MR gene expression that suggests a novel mechanism of action for these drugs on the HPA system. Stimulatory (+) and inhibitory (-) actions of neural inputs to brain regions involved in HPA-system regulation, and the sites of corticosteroid regulation are shown. The sites, at which antidepressants have stimulatory actions on GR or MR, or both, are indicated. (Barden *et al*, 1995).

1.4. Aims and Objectives of the Thesis

Several classes of antidepressants have been demonstrated to increase corticosteroid receptor binding and mRNA in various regions of the rat brain over a period similar to the time course in which therapeutic effects are observed in depressed patients. For this reason, it has been suggested that the up-regulation of corticosteroid receptors, thus normalising defective HPA activity, may be a mechanism common to the various classes of antidepressants used in the therapy of depression.

Many studies have focused their investigations on corticosteroid receptor alterations in adrenalectomised or 'naive' animals in animal models of depression. Adrenally-intact animals were used in these studies in order to determine the possible changes in GR in animals that have not undergone any surgical procedures and in the presence of endogenous ligand, thereby allowing for a closer comparison to clinical observations of GR alterations following stress and antidepressant administration.

Restraint stress was used as an established animal model of depression, to define transcriptional and translational mechanisms underlying HPA axis regulation *in vivo*, by examining GR mRNA, CRH type 1 receptor mRNA and BDNF mRNA. The features of restraint stress as a stress paradigm (chapter 5 and 7) suggest that a dysfunctional HPA axis, as reflected by defective negative feedback resulting from stress-induced corticosteroid receptor down-regulation, would be observed. Behavioural and endocrinological investigations (chapter 3 and 4) were conducted on the predator stress model, as it has not been studied or developed as extensively as the restraint stress paradigm has.

The work presented in this thesis is primarily concerned with monitoring HPA axis alterations under various stress situations and following administration of an SSRI, namely paroxetine, using plasma levels of corticosterone and ACTH as indicators of stress. Plasma corticosterone and ACTH were measured using radioimmunoassays, glucocorticoid receptors were quantified using radioligand-binding assays, and GR mRNA, CRH type 1

receptor mRNA and BDNF mRNA were quantified using RT-PCR and agarose gel electrophoresis.

Measurements were also made to compare endocrinological measures with behavioural parameters (elevated plus-maze and/or sucrose preference) as measured in the restrained rat, and a stress-related paradigm encompassing predator stress exposure. Therefore the aims of this work were;

1. To establish a behavioural paradigm based on predator stress, which could be expanded to a potential model for chronic stress/depression.
2. To determine the effects of predator stress on elevated plus-maze measures, sucrose preference, plasma corticosterone and glucocorticoid receptor binding in various brain regions of mice.
3. To examine the effects of acute and chronic restraint stress on anhedonia, as well as GR binding, GR mRNA, CRH type 1 receptor mRNA and BDNF mRNA in various brain regions of rats.
4. To investigate the effects of acute and chronic administration of an SSRI, paroxetine, on anhedonia, plasma corticosterone and ACTH secretion, as well as GR binding, GR mRNA, CRH type 1 receptor mRNA and BDNF mRNA in various brain regions of rats.
5. To investigate the effects of acute and chronic administration of an SSRI, paroxetine, in combination with restraint stress on anhedonia, plasma corticosterone and ACTH secretion, as well as GR binding, GR mRNA, CRH type 1 receptor mRNA and BDNF mRNA in various brain regions of rats.

CHAPTER 2. BIOCHEMICAL METHODS AND MATERIALS

2.1 Introduction

In this initial experimental chapter, the basic methodology employed in this thesis will be described.

2.2 Introduction to radioligand binding studies

Receptor binding studies are possible because of the high affinity that some agonists and antagonists have for their receptor. Consequently, at low concentrations of drug, a high proportion is bound to the receptor compared to the proportion which binds to non-receptor sites. Although only minute amounts of receptor are present in most tissues (typically less than 1pmol/mg protein), the amount of drug bound can be measured by radiolabelling it and measuring the amount of radioactivity bound to the tissue. It is essential to separate the bound drug from that which is free in solution, and this can be achieved by filtration (drug bound to the tissue is retained on the filter paper, but unbound drug passes through) or by centrifugation (in which the bound drug is trapped in the tissue pellet).

Prior to the widespread use of *in vitro* binding assays, the properties of receptors were inferred from the measurement of biological responses. The utilisation of radioligand binding assays to characterise receptors provides an enormous amount of information and also a direct approach to the study of drug-receptor interactions.

Ligand binding techniques are a direct means of studying drug or neurotransmitter-receptor interactions. They use the capacity of highly radiolabelled compounds to interact with specific receptors in a given tissue. Ligands are chosen for their high affinity for a specific type of receptor and tissue containing a high density of that particular receptor is usually studied. However, this is not always the case, especially in the brain. Frequently a particular ligand will label more than one receptor and a given tissue will invariably

contain different receptors. This implies that careful consideration must be given to the methodology employed and to the definition of specific receptor.

2.2.1. Receptor binding assay procedure

Tissue preparations containing the receptor of interest are incubated to equilibrium with one or more concentrations of the appropriate radioligand. Separation of the bound from free ligand is dependent on the equilibrium dissociation constant (K_d) of the radioligand for the binding site. For ligands with *K*d values of 10^{-8}M or less, rapid vacuum filtration through glass fibre filters is generally the most appropriate method whereas for ligands with higher K_d values, separation by centrifugation, column chromatography and precipitation of ligand-receptor complex and adsorption of free ligand techniques are applied (Bylund, 1992).

In the case of vacuum filtration, the radioactivity bound to the filter is termed the **total binding**. This consists of:

i) **Specific receptor binding** - radioligand bound to its binding site on the receptor of interest and;

ii) **Non-specific binding** - radioligand bound to other non-receptor components in the tissue preparation (e.g. membrane proteins and lipids, glass fibre filters).

The non-specific component of this binding can be measured by the inclusion of parallel samples of a non-radioactive compound that is known to interact with the receptor system under investigation at a concentration sufficient to totally displace the radioligand from specific binding sites.

There are two types of experimental protocols that have been used during this thesis:

1. Saturation binding experiments measure the equilibrium binding of a range of concentrations of the radioactive ligand (^{3}H-dexamethasone). The relationship between binding and ligand concentration can be analysed to determine the number of sites, B_{max}, and the ligand affinity, K_d of certain receptors (glucocorticoid) within particular brain regions (cortex and hippocampus).

2. Competitive binding experiments measure equilibrium binding of a single concentration of radioligand at various concentrations of an unlabelled competitor. This data can be analysed to determine the affinity of the competitor for the receptor.

2.3 Corticosteroid receptor binding assay using rat and mouse brain tissues

2.3.1. Animal husbandry

Experiments were carried out using male Wistar rats (body weight 200 – 250 g) bred in the Biological Research Facility at St. George's Hospital Medical School or male BALB/c mice (body weight 20-25 g) from the Nescot breeding colony. Animals were group-housed and maintained in a temperature (19-22°C) and light (light period 07.00-21.00 h) controlled room. A standard laboratory diet and drinking water were available *ad libitum.*

2.3.2.Dissection of rat and mouse brain regions

Male Wistar rats and male BALB/c mice were killed by cervical dislocation and decapitation. The brain was rapidly removed onto ice and the cortex and hippocampi dissected as follows. The cortices were separated and dissected on either side of the mid-line with curved iris forceps. The hippocampi, which were clearly visible, were removed by gently teasing them out from under the cerebellum and away from the cortices with the iris forceps, and placing them into ice-cold vials. The striata, which were clearly visible, were removed by pinching them with the iris forceps, care being taken not to remove any underlying tissue. The cortices were then carefully cut away from the rest of the brain, and

cut into quarters (rat brain) or left as halves (mouse brain) and placed into separate ice-cold vials.

2.3.3. ^{3}H-Dexamethasone Radioactive Ligand

^{3}H-Dexamethasone (specific activity 81 – 89 Ci/mmol) was obtained from Amersham International Plc. The ligand was kept at -20°C and diluted to the required concentrations with incubation buffer on the day of the experiment.

2.3.4.Buffers

For list of drugs and chemicals (and suppliers), see section 2.6.3. All buffers for the receptor binding assays were made with distilled water. The required pH at 20°C was achieved by using 6 M HCl. The buffers were kept at 4°C or on ice throughout the experiments.

i) **Incubation buffer (TEDGM) (pH 7.4):**

Component (final assay concentration)

10 mM	Tris
1 mM	EDTA (disodium salt)
35 mM	Sodium molybdate
1 mM	DTT
10 %	Glycerol

ii) **Wash buffer (pH 7.4):**

10 mM	Tris-HCl buffer

2.3.5. Tissue Preparation

Rat or mouse cortex and hippocampi were homogenised in 20 or 30 volumes (w/v), respectively, of ice-cold incubation (TEDGM) buffer (pH 7.4), using a motor driven teflon pestle and glass homogeniser (homogeniser speed 1400 rpm x 10 up/down strokes). The resulting homogenate was centrifuged at 105,000 x *g* for 1 h 10 min at 4°C using an Optima L70 Ultracentrifuge fitted with a 70.1 Ti rotor (Beckman Instruments Inc). The resulting supernatant was decanted immediately and stored on ice for the assay.

2.3.6. GR binding assay incubation procedure (general)

Assays were performed in 10 mL borosilicate test tubes (16mm x 16mm x 100mm), which were maintained on ice throughout the assay procedure. The total incubation volume for the assay was 250µL consisting of:

i. 100 µL of tissue cytosolic preparation (equivalent to 5 mg wet weight of original tissue).
ii. 100 µL of ^{3}H-dexamethasone solution (0.625-20 nM final assay concentration)
iii. 50 µL displacing compound (at a range of concentrations) or 10% EtOH (the diluent for the displacing compound)

Total binding containing the tissue preparation, ^{3}H-dexamethasone and 10% EtOH was determined in duplicate.

Non-specific binding containing the tissue preparation, ^{3}H-dexamethasone and the displacing agent was determined singly or in duplicate.

The assay was initiated by the addition of tissue supernatant following which the tubes were thoroughly mixed and placed in a refrigerator, at 4°C, for approximately 24 h (20-26 h). Tissue supernatant was also stored at -70°C for subsequent protein determination using the method of Lowry and colleagues (1951).

2.3.7.Separation of bound and free radioligand

The assay was terminated by rapid vacuum filtration (600 mm Hg) through Whatman glass fibre GF/F filter strips (Whatman International Ltd, U.K.), pre-treated for approximately 24 h with 0.3% PEI, using a Brandel Cell Harvester (Semat Technical Ltd, U.K.). The filters were quickly washed with 16 mL of ice-cold 10mM Tris-HCl wash buffer (pH 7.4). This minimised the amount of non-specific binding bound to the glass fibre filters. The procedure of filtration and washing was conducted over approximately 15 sec.

2.3.8.Determination of radioactivity and scintillation counting

The glass fibre filters were cut out of the strips and placed into individual 10 mL plastic scintillation vials. 6 mL of scintillation fluid (Optiphase Safe, Wallac, U.K) was added and the vials capped and shaken for 1-2 h before being transferred to the liquid scintillation counter (2200CA Tri-Carb LSC, Packard Instruments, U.K.) for the determination of radioactivity.

2.3.9.^{3}H-dexamethasone standards

The amount of radioligand added to the assay on each experimental day was determined by the inclusion of standards. These consisted of 100 µL aliquots of each concentration of ^{3}H-dexamethasone used in the assay and were used for subsequent calculations. They were prepared, in duplicate, on each experimental day and counted to determine the total assay concentration of radioligand.

2.3.10. Calculations and analysis of results

Specific binding was defined as the difference between total binding and that occurring in the presence of a maximum concentration of displacing agent (non-specific binding). This was calculated as the difference of the mean values of replicate determinations of total and non-specific binding. The blank (radioactive counts determined by only 6ml of the scintillation cocktail) were subtracted from all the standards as well as the total and non-specific values. The precise method of calculation will be given in greater detail in each experimental section.

2.3.11. Determination of the saturability of ^{3}H-dexamethasone binding to rat cortical cytosolic preparations

1. Introduction

Saturation experiments investigate the specific binding of ligand to the receptor at various concentrations of radioligand to obtain estimates for B_{max} (a measure of the receptor concentration in the sample) and K_d (the equilibrium dissociation constant). One of the most important and relevant uses of B_{max} and K_d determinations by saturation binding studies is the estimation of changes in receptor concentration and affinity in disease or following pharmacological or other experimental interventions.

This section aims to demonstrate that the GR assay method used in the present experiments generates reliable saturation binding data relating to the specific binding of ^{3}H-dexamethasone to rat cytosolic tissue preparations.

2 Methods

Assay procedures were carried out as described in section 2.3. Binding was determined at a range of ^{3}H-dexamethasone concentrations (0.625-20nM). Specific binding was defined in the presence of 0.5μM RU 28362.

3. Calculations and analysis of results

Radioactivity from saturation experiments (in dpm) were converted to the appropriate units (^{3}H-dexamethasone standards to nM, and TB and NSB incubations to fmoles/assay). Mean values were obtained from the replicate determinations of TB and NSB following which specific ^{3}H-dexamethasone binding was calculated by subtracting NSB values from TB values.

The converted data was subjected to non-linear regression analysis (GraphPad Prism v2.1) to give estimates of B_{max} and K_d following which, B_{max} values were converted to fmoles/mg protein using protein values determined for each of the samples. For the preliminary saturation experiments, the data were also displayed as Scatchard plots with subsequent linear regression analysis, visual inspection of which provided some indication as to whether the ligand-receptor interaction being studied was described adequately by a simple bimolecular reaction. Hill plots of the saturation data enabled the quantitation of deviation of the ligand-receptor interaction from the law of mass action. Differences in binding constants between groups were determined using Students t-test or ANOVA with a significance level set at $p<0.05$.

4. Results

Figure 2.1(A) shows a typical saturation plot of ^{3}H-dexamethasone binding. The non-specific binding of ^{3}H-dexamethasone in rat cortical preparations increased linearly over the ligand concentrations 0.625-20 nM. The total and specific binding (defined using 0.5 μM RU 28362) of ^{3}H-dexamethasone appeared to saturate at the higher ligand concentrations.

Non-linear regression analysis yielded a B_{max} value of 23.6 fmol/assay, equivalent to a B_{max} of 171 fmol/mg protein. A K_d value of 2.41 nM was obtained from this plot.

Figure 2.1(B) shows a Scatchard plot of the data in Figure 2.4.1(A) with subsequent linear regression analysis resulting in a B_{max} of 23.5 fmol/assay equivalent to a B_{max} of 170 fmol/mg protein and a K_d of 2.17 nM.

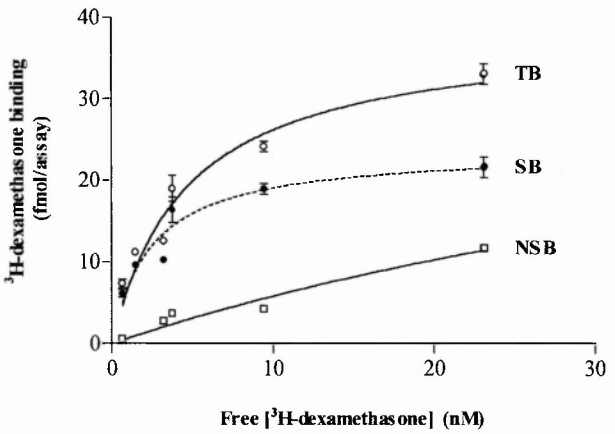
Figure 2.1(C) shows a Hill plot of the saturation data from Figures 2.4.1(A) and 2.4.1(B). A Hill coefficient (n_H) of 1.23 ± 0.21 was obtained from this plot.

The results of several saturation assays are shown in a representative plot (**Figure 2.1.**). Non-linear regression analysis using a one-site binding model resulted in similar values for binding parameters as Scatchard analysis. Statistical analysis of the data, using t-tests showed no significant differences between B_{max} values estimated by non-linear regression and those estimated by Scatchard analysis (whether expressed in fmol/mg tissue or fmol/mg protein). One–way analysis of variance of the K_d data showed no significant differences in K_d values when calculated using non-linear regression, Scatchard or Hill analysis.

The mean B_{max} value calculated from these experiments (using non-linear regression analysis) was 139 ± 12 fmol/mg protein and the mean K_d value calculated was 2.15 ± 0.38 nM.

Figure 2.1.

(A)

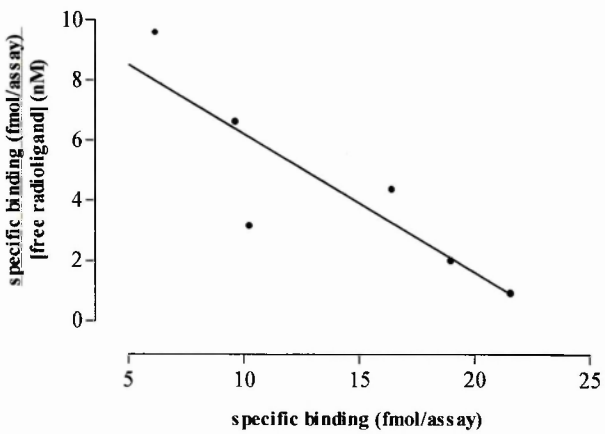


(A) Representative saturation plot of ^{3}H- dexamethasone binding to GR in rat cortical cytosolic preparations.

GR binding assays were carried out as described in section 2.3. Data represent the mean (± standard deviation) for total binding and non-specific binding determinations in duplicate. At a ligand concentration of 3nM, mean (± SD) values for bound dpm were;
Total binding = 2348 ± 52
Non-specific binding = 533 ± 0
Specific binding = 1815 ± 52
% specific binding = 77.3%

B_{max} = 23.6 fmol/assay
Equivalent to 171 fmol/mg protein
K_d = 2.41 nM

(B)

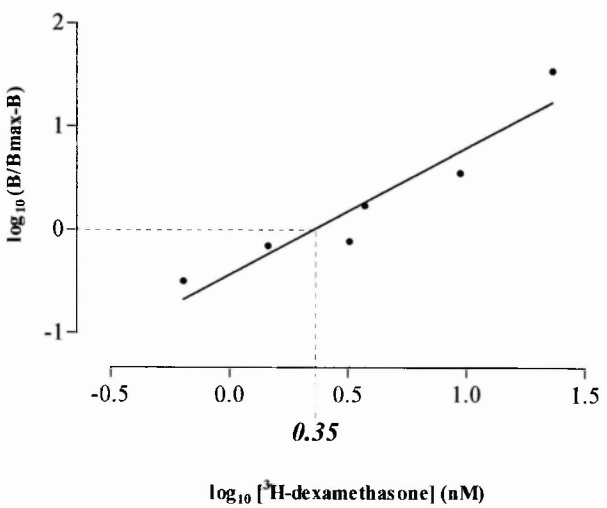


(B) Scatchard plot of ^{3}H-dexamethasone binding to GR in a rat cortical cytosolic preparation (using data shown in Figure 2.1A)

Binding parameters (B_{max} and K_D) were calculated using linear regression analysis. Values obtained were;

B_{max} = 23.5 fmol/assay
(converted using protein value=170 fmol/mg protein)
K_d = 2.17 nM

(C)



(C) Hill plot of ^{3}H-dexamethasone binding to GR in a rat cortical cytosolic preparation (using data shown in Figure 2.1A).

y-axis = *Log10 ($B/(B_{max}$-$B)$) where;*
B = specific ^{3}H-dexamethasone binding at particular ligand concentration.
B_{max} = B_{max} values using Scatchard analysis.

Linear regression analysis was used to calculate the slope of the plot, which corresponds to the Hill coefficient.
Hill coefficient (nH) = 1.23± 0.21
The Hill binding constant (K_d) of ^{3}H-dexamethasone is calculated from the x-axis where log10= ($B/(B_{max})$) = 0

2.3.12. Selective displacement of ^{3}H-dexamethasone binding to rat cytosolic preparations using competition binding assays

1. Introduction

Competition experiments are important in defining the pharmacology of the binding between the ligand and the receptor. Given a single radioligand whose affinity for a particular receptor subtype has been established, radioligand competition assays provide a means for determining the binding affinity of any other unlabelled compound for the same receptor. Applications of the radioligand competition experiment include the validation of assays, screening for identification of ligands for a particular receptor, investigation of the interactions of various compounds with receptors and the determination of receptor density and affinity by use of the same compound as the labelled and unlabelled ligand. Competition experiments measure the binding of a single concentration of radiolabelled ligand in the presence of various concentrations of unlabelled ligands. The generation of an inhibitory constant, IC_{50} or K_i is generally the purpose of performing displacement binding assays.

2. Methods

Assay procedures were carried out as described in section 2.3. Competition studies were carried out at a single concentration of 3nM ^{3}H-dexamethasone. Compounds tested for competition were added in concentrations ranging from $5x10^{-12}$ to $5x10^{-3}$M. Specific binding was defined using 0.5μM RU 28362. The compounds tested for displacement of ^{3}H-dexamethasone binding to corticosteroid receptors were RU28362, dexamethasone, corticosterone, hydrocortisone, aldosterone and ethanol.

3. Calculations and analysis of results

Mean values were calculated from the replicates and converted to % specific binding. 100% and 0% specific binding were equivalent to the amount of ^{3}H-dexamethasone binding in the absence and presence of 5μM hydrocortisone, respectively. Competing drugs were tested over a range of concentrations for their capacity to compete for this specific binding. The data was plotted on a semilogarithmic plot resulting in a displacement curve and an IC_{50} value was determined by non-linear regression analysis (GraphPad Prism v3.0). This was subsequently used to calculate the K_i for each unlabelled compound using the equation of Cheng and Prusoff (1973). K_i values are expressed in Table 2.1. as geometric means of several determinations as, due to the log-normal distribution of drug affinities, these appear to be best described in this manner rather than by using arithmetic means.

4. Results

Table 2.1. summarises the competition experiments for each compound tested while **Figure 2.2.** displays representative competition experiments for each compound. Comparison of one-site vs two-site competitive binding was determined using Graphpad Prism v3.0, using an F-test (**Table 2.2**). All of the steroids tested competed for specific ^{3}H-dexamethasone binding to rat cytosolic fractions in a concentration-dependent manner. The affinity of competitors for ^{3}H-dexamethasone binding to GR ranged from 0.018 – 6.03nM with the following rank order of potency; RU28362 > dexamethasone > corticosterone > hydrocortisone > aldosterone, with no displacement of specific binding by EtOH. These results from the competition binding studies have also demonstrated a glucocorticoid-like pattern of receptor binding (Vedder *et al*, 1993).

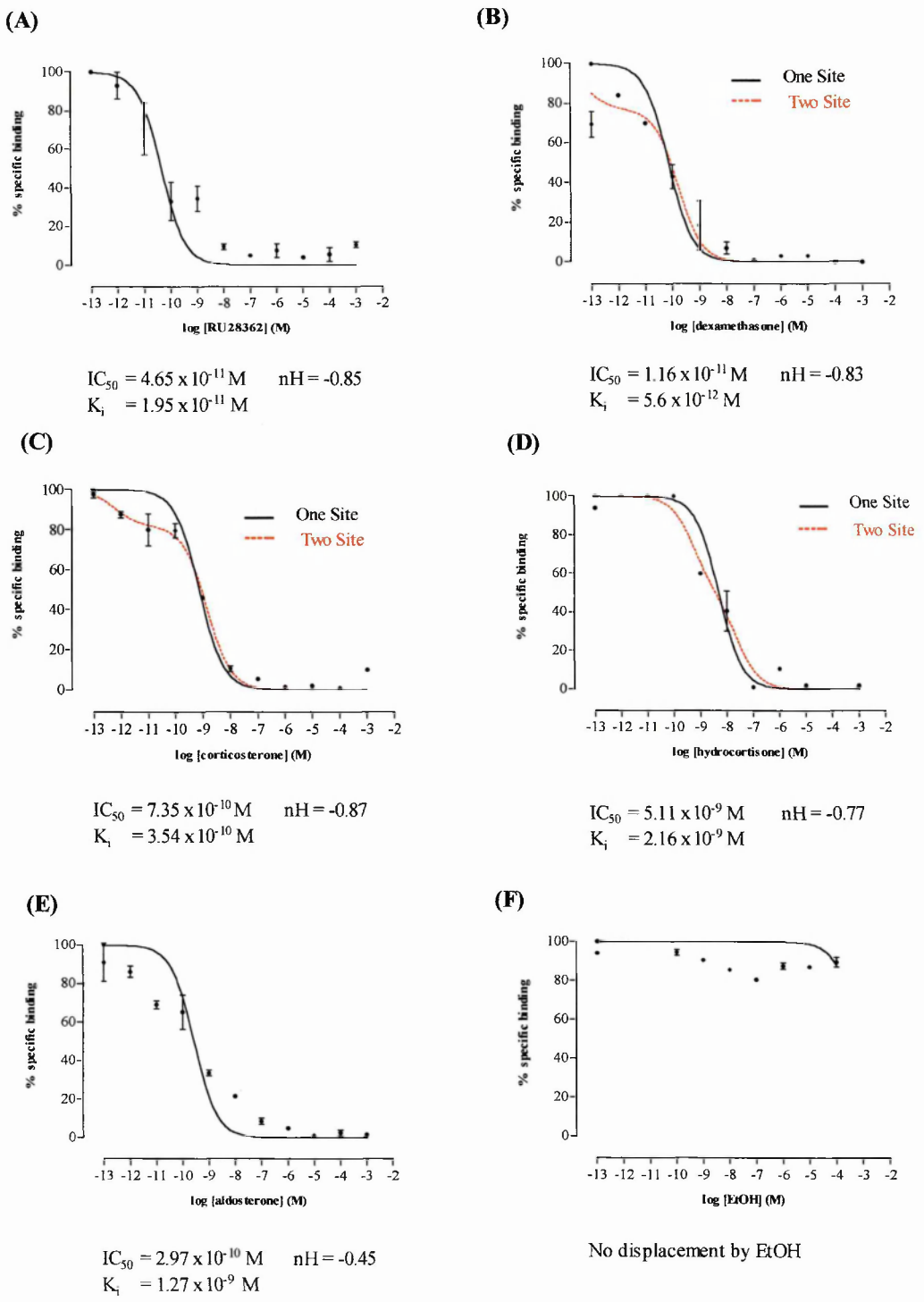
Table 2.1.

Displacement of specific ^{3}H-dexamethasone binding to GR in rat cytosolic preparations by RU 28362, dexamethasone, corticosterone, hydrocortisone, aldosterone and ethanol.

COMPOUND TESTED FOR DISPLACEMENT	K_I (nM)
RU 28362	**0.018** (0.0068 – 0.049)
Dexamethasone	**0.87** (0.48 – 1.58)
Corticosterone	**3.16** (1.15 –8.69)
Hydrocortisone	**4.83** (2.06 – 11.32)
Aldosterone	**6.03** (3.23 – 11.2)
EtOH	—

Summary of values obtained from experiments testing the displacement of specific ^{3}H-dexamethasone binding to GR in rat cytosolic preparations by various compounds. Data from four independent experiments conducted on separate occasions were fitted to one-site competition curve resulting in estimations of IC_{50} (concentration of unlabelled compound which causes 50% displacement of maximal binding) from which K_I (affinity of the competing compound for the receptor) was calculated using the Cheng-Prusoff equation for each compound. The K_d value of ^{3}H-dexamethasone binding obtained in parallel saturation experiments used in these calculations was 2.5nM. K_I is expressed as a geometric mean with numbers in parentheses indicating the range for each value.

Figure.2.2.



Displacement of specific ^{3}H-dexamethasone binding from GR in rat cytosolic preparations by RU28362 (**A**), dexamethasone (**B**), corticosterone (**C**), hydrocortisone (**D**), aldosterone (**E**) and EtOH (F). Rat cytosolic fractions were prepared as described in section 2.3.5. Data shown is from a single experiment and fitted to a one-site and/or a two-site competition curve.

Table 2.2.

Comparison of two-site vs one-site analysis of the competion binding data.

RU 28362	
Dfn , Dfd	4 , 17
F	1.948
P value	0.07
Best Fit Equation	One-Site
DEXAMETHASONE	
Dfn , Dfd	2 , 19
F	8.384
P value	0.0025
Best Fit Equation	Two-Site
CORTICOSTERONE	
Dfn , Dfd	2 , 21
F	26.06
P value	<0.0001
Best Fit Equation	Two-Site
HYDROCORTISONE	
Dfn , Dfd	2 , 12
F	4.771
P value	0.0299
Best Fit Equation	Two-Site
ALDOSTERONE	
Dfn , Dfd	2 , 21
F	2.57
P value	0.06
Best Fit Equation	One-Site

Data from the individual experiments from **Figure2.2** were fitted to one-site and two-site competition curves to determine the comparison of fits.

2.3.13. Statistical analysis

Statistical evaluations were carried out using one- and two-way analysis of variance, according to the data, as well as the Student's t-test. The level of significance was chosen as $p < 0.05$. A more detailed account of data compilation and subsequent analysis will be given in each experimental section.

2.3.14. Protein determination using the Lowry Method

1. Introduction

Protein content in our tissue samples was estimated using the colorimetric method of Lowry *et al* (1951). This method utilises hydrolytic reactions to break the protein down into its constitutive amino acids. A coloured complex results from interactions between an alkaline copper–phenol reagent and tyrosine and tryptophan residues in the protein. The protein content of the sample is estimated via a spectrophotometric reading of absorbance.

2. Methods

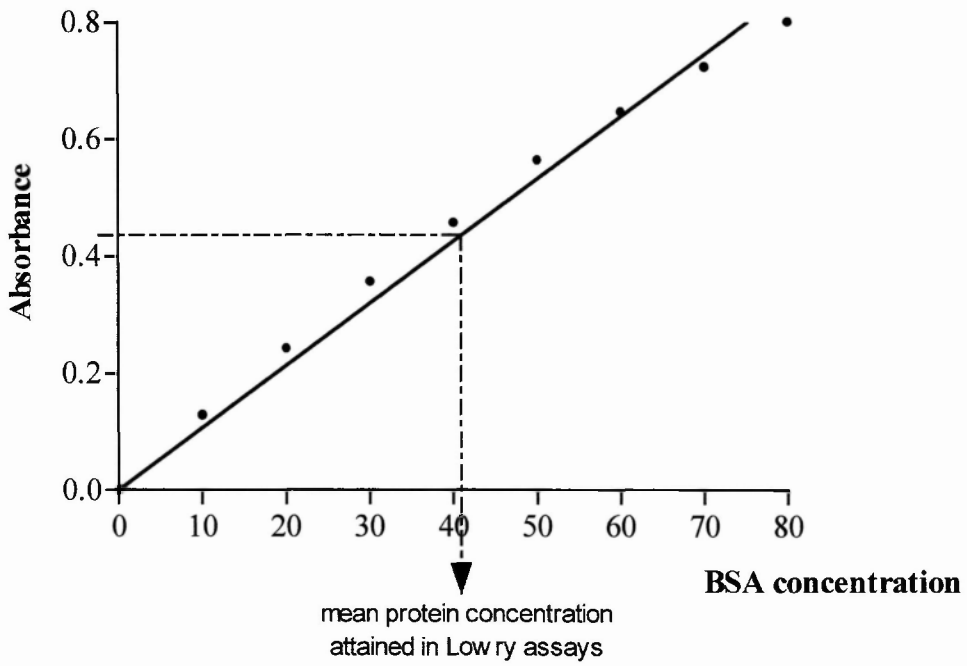
The Lowry protein assay was adapted from the standard protocol (Lowry *et al,* 1951). The presence of Tris and glycerol as components of the incubation buffer used in the CR assay, posed a problem. These two compounds are found to interfere with the measurement of protein by the Lowry method by contributing blank colour and/or decreasing chromophore development with protein (Rej *et al,* 1974). This distortion was overcome by diluting the tissue samples with distilled water (1:4) therefore effectively diluting out the interfering components. Incorporation of the same amounts of these compounds in the standards also overcame the problems we encountered. The standard curve was therefore constructed in incubation buffer that had been diluted with distilled water by the same factor as the samples for the protein assay.

3. Results

Standard curves obtained in the Lowry protein assay were linear over the range of 10-80μg BSA (see **Figure 2.3.**).

Figure 2.3.

Protein determination using the Lowry method



Standard curve and mean protein concentration obtained using the Lowry assay. Standard curves were linear over the range of 10-80μg BSA. Average values in our studies were 41μg/100μL volume (160 μg/assay when multiplied by 4, the dilution factor for the tissue samples).

4. Protein determination using Lowry method

Protein values obtained using this method were very consistent. Dilution of the samples and construction of the standard curve in diluted incubation buffer overcame the problem of interfering compounds such as Tris and glycerol.

2.4 Radioimmunoassay (RIA)

2.4.1. Introduction

The gamma-B corticosterone assay kit (Immunodiagnostic Systems Ltd (IDS), Tyne and Wear) utilised ^{125}I-labelled corticosterone and a specific anti-corticosterone antiserum, the ACTH assay kit (Diagnostic Systems Limited (DSL), USA) utilised ^{125}I-labelled ACTH and a specific anti-ACTH antiserum to determine the levels of corticosterone and ACTH in EDTA-plasma samples by the double antibody technique.

Radioimmunoassay follows the basic principle that there is competition between a radioactive and non-radioactive antigen for a fixed number of antibody binding sites. The percentage BOUND radiolabelled antigen decreases as a function of the increasing concentration of unlabelled antigen in the test sample. Separation of the BOUND and FREE radiolabelled antigen is necessary in order to determine the quantity of unlabelled antigen. This is accomplished by the addition of a second antibody directed towards the immunoglobulin present in the original antiserum. The quantity of unlabelled antigen in an unknown sample is then determined by comparing the radioactivity of the precipitate, after centrifugation and decanting, with values established using known standards in the same assay system.

2.4.2. Preparation of Samples

For Chapter 4, trunk blood was collected in chilled, 50ml centrifuge tubes. The blood was stored at -20°C for 1 h and then left at room temperature for 20 min. The blood clot was dislodged and the supernatant transferred, by careful pipetting, to a clean centrifuge tube. The supernatant was then centrifuged in a Centaur centrifuge at room temperature (2000 x *g* for 10 min). Plasma was stored in plastic tubes at -70°C.

For Chapters 5-7, trunk blood was collected in ice-chilled, 10ml EDTA-coated tubes. Samples were immediately centrifuged in a Centaur centrifuge at room temperature (2,500 x *g* for 20 min), and plasma aliquots were stored at -70°C until analysis of corticosterone and ACTH concentrations. The sensitivity of the corticosterone assay was 0.39ng/ml, and the ACTH assay was 3.5pg/ml.

Due to the high concentrations of corticosterone and ACTH within the plasma, samples were diluted with either corticosterone buffer (1:10 dilution) or ACTH buffer (1:2 dilution).

Tubes were prepared in duplicate for TC, NSB, Bo, corticosterone standards and samples as described in the RIA kit protocol. The amount of radioactivity in the tubes was counted for at least 1 minute in a gamma counter.

2.4.3. Specificity

According to data supplied by the manufacturer of the kit, the cross-reactivity of the ACTH antiserum has been measured against various compounds (**Table. 2.3.**). The percent cross-reactivity is expressed as the ratio of the ACTH concentration to the concentration of the reacting compound at 50% binding of the 0 pg/ml ACTH Standard.

Table 2.3.

The cross-reactivity of the ACTH antiserum in the commercial kit against various compounds.

COMPOUND	% CROSS-REACTIVITY
ACTH (1-39)	100
ACTH (1-24)	100
ACTH (22-39)	0.03
α-Melanotropin	0.09
ACTH (1-10)	ND
ACTH (4-11)	ND
ACTH (34-39)	ND
β-Melanotropin	ND
Γ-Melanotropin	ND
Corticotropin-Like Intermediate Peptide	ND

ND = Non-Detectable (<0.01)

2.5. RNA extraction with reverse-transcriptase polymerase-chain reaction (RT-PCR) and Agarose gel electrophoresis.

2.5.1. RNA Extraction

1. Materials

Glassware, materials and solutions were treated with DEPC to inactivate RNases. Glassware, Eppendorf tubes and tips were soaked in distilled water with 1% (v/v) DEPC, incubated at 37°C overnight, and then autoclaved.

2. Homogenisation

Frozen rat cortex (0.1-0.2g) was weighed and transferred to the glass-teflon homogeniser, on ice, and a 10 x weight of cortex, of RNA isolator™ was added. The tissue was homogenised at 1400 revolutions per minute (6 up and down strokes), and was kept on ice throughout. The RNA Isolator™ contains chaotropic agents, which rapidly denature and inactivate cellular RNases, but homogenisation was also performed rapidly in order to immediately inactivate the RNases. The tissue homogenate was divided into equal proportions and transferred to Eppendorf tubes and incubated in a fume-cupboard for 7 min at room temperature to permit complete dissociation of nucleoprotein complexes.

3. Phase separation

Phase separation was achieved by adding 0.2 ml of chloroform per 1 ml of RNA Isolator™. The tubes were capped and mixed by gentle inversion until completely emulsified, resulting in a pale-pink cloudy solution. They were incubated at room temperature, in the fume-cupboard, for 15 min and centrifuged in a JOUAN A14 microcentrifuge, at 12500 x *g* for 15 min, at 4°C. The contents of the tubes separate into 3 distinct layers: a lower red, phenol-chloroform phase; a white opaque interphase; and a colourless, upper aqueous phase. The low pH of the phenol allows the RNA to selectively partition into the aqueous phase, free from DNA and protein, which remain in the organic

phase. The volume of the top aqueous layer is about 60% of the volume of RNA isolator™ used for homogenisation.

4. RNA precipitation

The top, aqueous phase was carefully pipetted into an Eppendorf tube, therefore still resulting in two Eppendorfs per sample. The isolated RNA was concentrated by precipitation with isopropanol.

The precipitation of DNA and RNA, which is allowed to form in the presence of moderate concentrations of monovalent cations, is recovered by centrifugation and re-dissolved in an appropriate buffer at the desired concentration. This technique is rapid and is quantitative even with picogram amounts of DNA and RNA. The three major variables are:

1. The temperature in which the precipitate is allowed to form.
2. The type and concentration of monovalent cations used in the precipitation mixture.
3. The time and speed of centrifugation.

Therefore 1 ml of isopropanol was added to each tube, and the contents mixed by gentle inversion. 400 µl was transferred to an Eppendorf tube, resulting in 3 tubes each containing ~800 µl of solution. Five hundred microlitres of isopropanol was added to each tube, and the contents mixed by gentle inversion. This resulted in a cloudy solution, suggesting the precipitation of RNA. The tubes were incubated for 15 min, at room temperature, to allow full RNA precipitation to occur. The tubes were centrifuged in a JOUAN A14 microcentrifuge, at 8700 x *g* for 15 min, at 4°C and the supernatant was decanted without disturbing the gel-like pellet, containing RNA.

5. RNA wash

The pellet was washed once by adding 1 ml of 75% ethanol and mixed by gentle inversion, to remove any contaminating salts that may inhibit enzymatic reactions. Therefore, using at least 1 ml of 75% ethanol per 1 ml of RNA Isolator™ used for the initial homogenisation. The tubes were centrifuged in a JOUAN A14 microcentrifuge, at 5600 x *g* for 5 min, at 4°C. The ethanol was decanted without disturbing the pellet, and the tubes were dried on absorbent tissue in a fume-cupboard for 5 min, with the vent on. Complete evaporation of the 75% ethanol wash is required when the RNA samples are to be used for RT-PCR, as any residual ethanol may affect enzyme activity. After drying, all remaining ethanol will have evaporated leaving only the pellet and possibly water in the tubes. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility.

The pellets in two of the tubes, were then re-suspended in 20 µl, and mixed by gentle pipetting and the contents were then transferred to the third tube, and mixed by gentle pipetting (1 tube containing all the isolated RNA from one tissue sample, suspended in 40 µl of DEPC-treated, sterilised water). The isolated RNA suspended in water was stored at -70°C for up to 3 months.

6. Determination of absorbance

Wavelength scanning of the absorbance of the RNA isolation, was performed on a Philips PU8700 Series UV/Vis Spectrophotometer (© Pye Unicam Ltd, Cambridge).

7. Calculation of RNA recovery from isolation methods

(a) ***Spectrophotometric Determination of the Amount of RNA or DNA:***

For quantitating the amount of RNA or DNA, absorbance was measured at wavelengths of 260 nm and 280 nm. The reading at 260nm allows calculation of the concentration of DNA in the sample. An OD of 1 corresponds to approximately 50 μg/ml for double-stranded DNA, 40 μg/ml for single-stranded DNA and RNA, and ~20 μg/ml for single-stranded oligonucleotides. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 and 2.0 respectively. A higher ratio indicates that there is DNA contamination in the sample, whereas a lower ratio indicates protein or phenol contamination of the sample.

(b) ***RNA concentration (μg):***

One A260 nM unit equals 40 μg of RNA/ml of suspension. Therefore:

- A260 nM value *40 = μg RNA / μl of sample pipetted into the curvette (i.e., 5 μl)
- μg RNA ÷ 5 = μg RNA / μl
- If 40μl of suspension isolated from a set amount of tissue (mg), then 40 *(μg RNA/ μl) = μg RNA / 40 μl or μg RNA / amount of tissue (i.e., 110 mg)
- Then μg RNA ÷110mg = μg RNA / mg tissue

  The yield from cortex was 1 – 1.5 μg RNA / mg tissue.

2.5.2. DNase treatment

1. Solutions

(a) RQ1 RNase-Free DNase, 1unit/μl

(b) Restriction Endonuclease (RE) Multicore Buffer (1x)

Contains 25 mM Tris-Acetate (pH 7.5 at 37°C), 100 mM potassium acetate, 10 mM magnesium acetate and 1 mM DL-dithiothreitol (DTT).

(c) 1% (v/v) DEPC-treated sterilised, deionised water.

(d) Chloroform AnalaR grade (BDH).

(e) Isoamylalcohol AnalaR grade (BDH).

(f) Phenol (Sigma).

(g) Absolute 99.9% Ethanol AnalaR grade (BDH).

2. Phenol-chloroform-isoamylalcohol extraction

Isolated RNA samples were removed from -70°C, and the RE multicore buffer and RQ1 RNase-free DNase were removed from -20°C to defrost at room temperature and then kept on ice throughout the procedure. In separate Eppendorf tubes for each sample, the following reaction was mixed using gentle pipetting:

2 µl of RE multicore buffer

1 µl of RQ1 RNase-free DNase

18 µl of isolated RNA

The tubes were centrifuged up to 800 x *g* to quickly mix the contents, at room temperature using the JOUAN A14 microcentrifuge and incubated in a 37°C waterbath for 1 h. During incubation the Phenol-Chloroform-Isoamylalcohol (PCI) solution was prepared, in a fume-cupboard, using the following ratios:

Phenol	Chloroform	Isoamylalcohol
25	24	1

Making sure that the solutions were mixed in the correct order, i.e., isoamylalcohol then chloroform then phenol. The chloroform denatures proteins and facilitates the separation of the aqueous and organic phase, and the isoamylalcohol reduces foaming during extraction.

After incubation, the tubes were centrifuged briefly up to 1394 x *g*, at room temperature, using the JOUAN A14 microcentrifuge. 20 µl PCI solution was added to each tube containing the digested sample, and vortexed for 60 sec to mix the contents. The tubes were incubated on ice for 2 min. Following centrifugation, the contents of the tubes separated into 2 layers: a clear phenol-chloroform phase and a colourless, upper aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA partitions to the lower phase.

The top, aqueous phase was carefully pipetted into an Eppendorf tube, so not to disturb the DNA in the lower phase. 2.5-fold amount of absolute alcohol was added to each tube (therefore, if 20 µl of the aqueous layer was removed, then 50 µl of absolute alcohol was added), and the contents mixed by gentle inversion. The tubes were incubated at -20°C for 1 h and centrifuged in a JOUAN A14 microcentrifuge, at 12500 x *g* for 10 min, at room temperature. The supernatant was removed by pipetting, without disturbing the gel-like pellet containing RNA, on the side and bottom of the tube.

The pellet was washed once by adding 500 µl of 75% ethanol and mixed by gentle inversion, to remove any salts present. The tubes were centrifuged in a JOUAN A14 microcentrifuge, at 5600 x *g* for 4 min, at room temperature. The ethanol was removed by pipetting, without disturbing the pellet, and the tubes were dried on absorbent tissue in a fume-cupboard for 5 min. Complete evaporation of the 75% ethanol wash is required when the samples are to be used for RT-PCR, as any residual ethanol may affect enzyme activity. After drying, all remaining ethanol will have evaporated leaving only the pellet and possibly water in the tubes. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. The pellet in each tube, was then re-suspended in 20 µl of DEPC-treated, sterilised water, and mixed by gentle pipetting. This was stored at -70°C for up to 3 months.

3. Determination of absorbance

Refer to section 2.5.3.7.

4. Calculation of RNA recovery after DNase treatment

Refer to Section 2.5.3.8.

2.5.3. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

1. Introduction

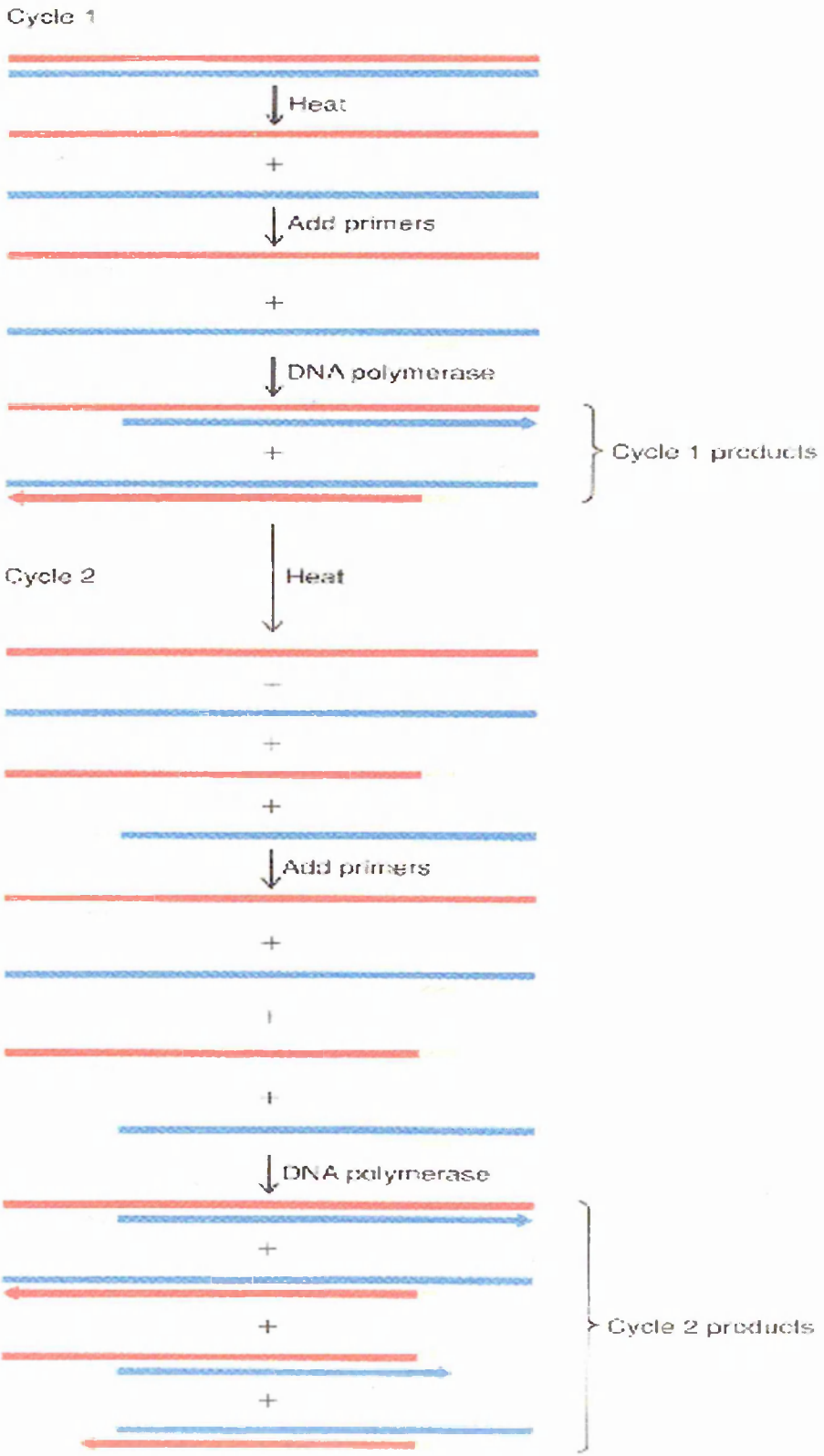
Numerous techniques have been developed to measure gene expression in tissues and cells. These include Northern Blots, coupled reverse transcription and PCR amplification (RT-PCR), RNase protection assays, *in situ* hybridisation, dot blots and S1 nuclease assays. Of these methods, RT-PCR is the most sensitive and versatile.

RT-PCR employs AMV or MMLV (or MuLV) reverse transcriptases for first strand cDNA synthesis. Second strand cDNA synthesis and subsequent PCR amplification is performed with thermostable DNA polymerases (e.g., *Thermus flavus (Tfl)* DNA polymerase).

The PCR process amplifies short (approximately 100-500bp) segments of a longer DNA molecule. A typical RT-PCR reaction includes the sample of target RNA, reverse transcriptase, a thermostable DNA polymerase, two oligonucleotide primers, dNTPs, reaction buffer and magnesium. The components of the reaction are mixed and placed in a thermal cycler, which is an automated instrument that takes the reaction through a series of different temperatures for varying amounts of time. This series of temperature and time adjustments is referred to as one cycle of amplification. Each RT-PCR cycle theoretically doubles the amount of targeted template sequence (amplicon) in the reaction. Ten cycles theoretically multiply the amplicon by a factor of about one thousand; 20 cycles, by a factor of more than a million in a matter of hours (**Figure 2.4.**).

Prior to the amplification cycles an initial step is required in RT-PCR, where target RNA is converted to cDNA using 3′ primers and the enzyme reverse transcriptase. Following the first-strand cDNA synthesis, the reverse transcriptase is inactivated and the RNA/cDNA hybrid is then denatured by a 2 min incubation at 94°C. This step leads directly into the second-strand cDNA synthesis and PCR amplification phase of the procedure. After this initial stage, each cycle of PCR amplification consists of a number of steps; denaturation, annealing and polymerisation. Each step denatures the template producing two oligonucleotide-primed single-stranded DNA templates, sets up the polymerisation reaction, and synthesises a copy of each strand of the template being targeted. These steps are optimised for each template and primer pair combination. The initial step in a cycle denatures the target DNA by heating it to 95°C or higher for 15 sec to 2 min. In the denaturation process, the two intertwined strands of DNA separate from one another, producing the necessary single-stranded DNA template for the thermostable polymerase.

Figure 2.4.



Amplifying DNA by the polymerase chain reaction (PCR; Weaver & Hedrick, 1992)

The next step of a cycle reduces the temperature to approximately 40-60°C. At this temperature, the oligonucleotide primers can form stable associations (anneal) with the separated target DNA strands and serve as primers for DNA synthesis by a thermostable DNA polymerase. This step lasts approximately 30-60 sec.

Finally, the synthesis of new DNA begins when the reaction temperature is raised to the optimum for the thermostable DNA polymerase. For most thermostable DNA polymerases this temperature is approximately 74°C. Extension of the primer by the thermostable polymerase lasts approximately 1-2 min. This step completes one cycle, and the next cycle begins with a return to 95°C for denaturation. After 20-40 cycles, the amplified nucleic acid may be analysed for size, quantity, sequence, etc., by gel electrophoresis, or used in further experimental procedures (e.g., cloning).

2. Solutions

(a) Avian Myeloblastosis Virus (AMV) Reverse Transcriptase, 20 units / μl (Promega)

One unit is defined as the amount of enzyme which catalyses the incorporation of 1 nmol of dTTP into acid-insoluble form in 10 min at 37°C in 50 mM Tris-HCL (pH 8.3), 40 mM KCL, 8.75 mM $MgCl_2$, 10 mM DTT, 0.1 mg/ml BSA, 1.0 mM radiolabelled dTTP, 0.25 mM poly(A)$_{400}$ and 0.25 mM oligo(dT)$_{50}$.

(b) Tfl DNA Polymerase, 5 units / μl (Promega)

This is a thermostable enzyme with a molecular weight of approximately 94,000 daltons isolated from *Thermus flavus*.

(c) dNTP Mix, 10 nM (Promega)

The dNTPs used were greater than 98% triphosphates content, and were provided at a concentration of 100 nM in water at pH 7.5. To create the 10 nM dNTP mix, 100µl of each of dATP, dCTP, dGTP and dTTP were mixed together in a sterile DEPC-treated Eppendorf tube and 600µl of nuclease-free water was added (1:10 dilution). The Eppendorf tube was vortexed to thoroughly mix the contents, and stored at –20°C.

(d) AMV / Tfl 10x Reaction Buffer (Promega)

It contains 200 mM Tris-Acetate (pH 8.9 at 25°C), 100 mM ammonium sulphate, 750 mM potassium acetate, and 0.5% Tween.

(e) $MgSO_4$, 25 mM (Promega)

(f) Reverse Oligonucleotide Primer (Go-Oli-Go ™, Pharmacia Biotech)

I. ß-Actin

The β-actin primer pairs used in this study were designed with the aid of a Mac GeneJockey program from published β-actin cDNA sequences (Nudel *et al*, 1983) to amplify sequences within the coding region of β-actin (nucleotides 262-542).

Sequence (5'-3'): **TAC AAC CTC CTT GCA GCT CC**

MW = 5810; Tm = 64.4; µg/OD = 31.4

II. Brain-Derived Neutrophic Factor

The BDNF primer pairs used in this study were designed with the aid of a Mac GeneJockey program from published BDNF cDNA sequences (Maisonpierre *et al*, 1991) to amplify sequences within the coding region of BDNF (nucleotides 286-835).

$RBDNF_2$ Sequence (5'-3'): **TCT ATC CTT ATG AAC CGC CAG C**

MW = 6609; 65.9 nmol; Tm = 66.0; µg/OD = 32.8

III. Corticotrophin Releasing Factor-Receptor 1

No. 563 Sequence (5'-3'): **AAG CCG AGA TGA GGT TCC AGT GG**

The CRH-R1 primer pairs used in this study were designed with the aid of a Mac GeneJockey program from published CRH-R1 cDNA sequences (Perrin *et al*, 1993) to amplify sequences within the coding region of CRH-R1 (nucleotides 221-562).

IV. Glucocorticoid Receptor

The GR primer pairs used in this study were designed with the aid of a Mac GeneJockey program from published GR cDNA sequences (Miesfeld *et al*, 1986) to amplify sequences within the coding region of GR (nucleotides 225-676).

No. 225 Sequence (5'-3'): **TCT CAG GCA GAT TCC AAG CAG C**

MW = 6704.3; Tm = 69.9; µg/OD = 32.0; nmol = 64.6

(g) Forward Oligonucleotide Primer (Go-Oli-Go™, Pharmacia Biotech)

I. ß-Actin

Sequence (5'-3'): **ACA ATG CCG TGT TCA ATG G**

MW = 5954; Tm = 64.2; µg/OD = 34.1

II. Brain-Derived Neutrophic Factor

$FBDNF_1$ Sequence (5'-3'): **CGA GAG GTC TGA CGA CGA CG**

MW = 6173; 43.7nmol; Tm = 68.8; µg/OD = 31.0

III. Corticotrophin-Releasing Factor

No. 221 Sequence (5'-3'): **TGG ACC TCA TTG GCA CCT GCT GG**

IV. Glucocorticoid Receptor

No. 676 Sequence (5'-3'): **TT CAC ACT GCC TCC GTT GGT GC**

MW = 6653.2; Tm = 73.6; µg/OD = 35.0; nmol = 54.0

(h) ***1% (v/v) DEPC-treated, sterilised water***

(i) 100bp DNA ladder (Promega)

The ladder consists of 11 double-stranded DNA fragments with sizes of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 1500 bp. The 500 bp is present at triple the

intensity of the other fragments and serves as a reference indicator. All other fragments appear with equal intensity on the gel.

The 100 bp ladder is supplied in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and was mixed well prior to use. Five microlitres (650 ng) of the ladder contains approximately 150 ng of the 500 bp DNA fragment and 50 ng of each of the other ten DNA fragments. It was stored at -20°C.

Blue/Orange 6X Loading Dye: This dye is used for loading DNA samples into gel electrophoresis wells and tracking migration during electrophoresis. It was composed of 15% Ficoll® 400, 0.03% Bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 10 mM Tris-HCL (pH 7.5) and 50 mM EDTA. The concentration used in the subsequent experiments was one part loading dye for every five parts 100 bp DNA ladder.

3. RT-PCR Protocol

(a) Mastermixes

I. ß-Actin:

Reagent	µl / RNA sample
1% (v/v) DEPC-treated, sterilised water	12.55
AMV-*Tfl* (10x) Reaction Buffer	3.10
dNTP mix	2.60
Forward Primer	1.25
Reverse Primer	1.25
25mM $MgSO_4$	2.50
AMV Reverse Transcriptase (20units/µl)	0.25
Tfl DNA Polymerase (5 units/µl)	0.50
RNA sample	1.00
Total Volume for RT-PCR Reaction =	25µl/RNA sample

II. BDNF mRNA:

1% (v/v) DEPC-treated, sterilised water		14.75
AMV-*Tfl* (10x) Reaction Buffer		3.00
dNTP mix		1.00
Forward Primer ($FBDNF_1$)		1.25
Reverse Primer ($RBDNF_2$)		1.25
25mM $MgSO_4$		2.00
AMV Reverse Transcriptase (20units/µl)		0.25
Tfl DNA Polymerase (5 units/µl)		0.50
RNA sample		1.00
Total Volume for RT-PCR Reaction	=	25µl/RNA sample

III. CRH-R1 mRNA:

1% (v/v) DEPC-treated, sterilised water		12.00
AMV-*Tfl* (10x) Reaction Buffer		3.00
dNTP mix		3.25
Forward Primer (221)		1.00
Reverse Primer (563)		1.00
25mM $MgSO_4$		2.50
AMV Reverse Transcriptase (20units/µl)		0.25
Tfl DNA Polymerase (5 units/µl)		1.00
RNA sample		1.00
Total Volume for RT-PCR Reaction	=	25µl/RNA sample

IV. GR mRNA:

Reagent		µl / RNA sample
1% (v/v) DEPC-treated, sterilised water		12.35
AMV-*Tfl* (10x) Reaction Buffer		3.10
dNTP mix		2.60
Forward Primer (676)		1.20
Reverse Primer (225)		1.50
25mM $MgSO_4$		2.50
AMV Reverse Transcriptase (20units/µl)		0.25
Tfl DNA Polymerase (5 units/µl)		0.50
RNA sample		1.00
Total Volume for RT-PCR Reaction	=	25µl/RNA sample

The mastermix (prior to the addition of the RNA sample) was dispensed in the above order, in one Eppendorf tube (therefore, if there were 6 samples then 6 x each volume of the components of the mastermix were dispensed into one Eppendorf tube). All the components were kept on ice throughout. They were vortexed before being added into the mastermix, but the completed mastermix was mixed by gentle pipetting before being split into the required Eppendorf tubes (24 µl per Eppendorf). Then 1 µl of RNA sample was added to each Eppendorf tube, containing the mastermix, and then centrifuged at a pulse up to 800 x g, at room temperature, using the JOUAN A14 microcentrifuge. The eppendorf tubes went through a pre-heat program before the RT-PCR was initialised. This was to prevent the primers attaching to unwanted sequences within the RNA sample, before RT-PCR.

(b) Pre-heat Method:

52°C for 3 min.

(c) OMN-E Thermal Cycler (Hybaid) Method:

I. ß-Actin:

42°C for 50 min (RNA converted to cDNA) - one cycle

94°C for 5 min (AMV-RT is inactivated and the RNA/cDNA hybrid denatured) – one cycle

94°C for 60 sec (DENATURATION)
55°C for 60 sec (ANNEALING)
72°C for 60 sec (POLYMERISATION)
} 35 cycles

II. BDNF mRNA:

48°C for 45 min (RNA converted to cDNA) - one cycle

94°C for 2 min (AMV-RT is inactivated and the RNA/cDNA hybrid denatured) – one cycle

94°C for 30 sec (DENATURATION)
61.9°C for 60 sec (ANNEALING)
72°C for 120 sec (POLYMERISATION)
} 40 cycles

III. CRH-R1 mRNA:

48°C for 45 min (RNA converted to cDNA) - one cycle

94°C for 2 min (AMV-RT is inactivated and the RNA/cDNA hybrid denatured) – one cycle

94°C for 60 sec (DENATURATION)
69.5°C for 60 sec (ANNEALING)
72°C for 120 sec (POLYMERISATION)
} 40 cycles

IV. GR mRNA:

48°C for 45 min (RNA converted to cDNA) - one cycle

94°C for 2 min (AMV-RT is inactivated and the RNA/cDNA hybrid denatured) – one cycle

94°C for 30 sec (DENATURATION)
69°C for 60 sec (ANNEALING)
70°C for 60 sec (POLYMERISATION)
} 40 cycles

2.5.4. 2% Agarose gel electrophoresis

1. Introduction

Factors affecting the rate of DNA migration in agarose gels (**Figure.2.5.**), include:

(a) *Molecular size of the DNA.* Molecules of linear, double-stranded DNA, which tend to become orientated in an electric field in an end-on position, migrate through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs. Larger molecules migrate more slowly because of greater frictional drag and because they worm their way through the pores of the gel less efficiently than smaller molecules.

(b) *Agarose concentration.* A linear DNA fragment of a given size migrates at different rates through gels containing different concentrations of agarose. There is a linear relationship between the logarithm of the electrophoretic mobility of DNA (μ) and the gel concentration (τ), which is described by the equation: $\log \mu = \log \mu_0 - K_r\tau$ where μ_0 is the free electrophoretic mobility of DNA and K_r is the retardation coefficient, a constant that is related to the properties of the gel and the size and shape of the migrating molecules. Thus, by using gels of different concentrations it is possible to resolve a wide size range of DNA molecules (**Table 2.4.**).

Table 2.4

Range of separation in gels containing different amounts of agarose.

Amount of agarose in gel (g)	Efficient range of separation of Linear DNA molecules (kb)
0.3	5 - 60
0.6	1 - 20
0.7	0.8 - 10
0.9	0.5 - 7
1.2	0.4 - 6
1.5	0.2 - 8
2.0	0.1 - 2

(c) *Conformation of the DNA.* Superhelical circular (form I), nicked circular (form II), and linear (form III) DNAs of the same molecular weight migrate through agarose gels at different rates.

(d) *Applied Voltage.* At low voltage, the rate of migration of linear DNA fragments is proportional to the voltage applied. However, as the electric field strength is raised, the mobility of high-molecular-weight fragments of DNA increases differentially. Thus, the effective range of separation in agarose gels decreases as the voltage is increased.

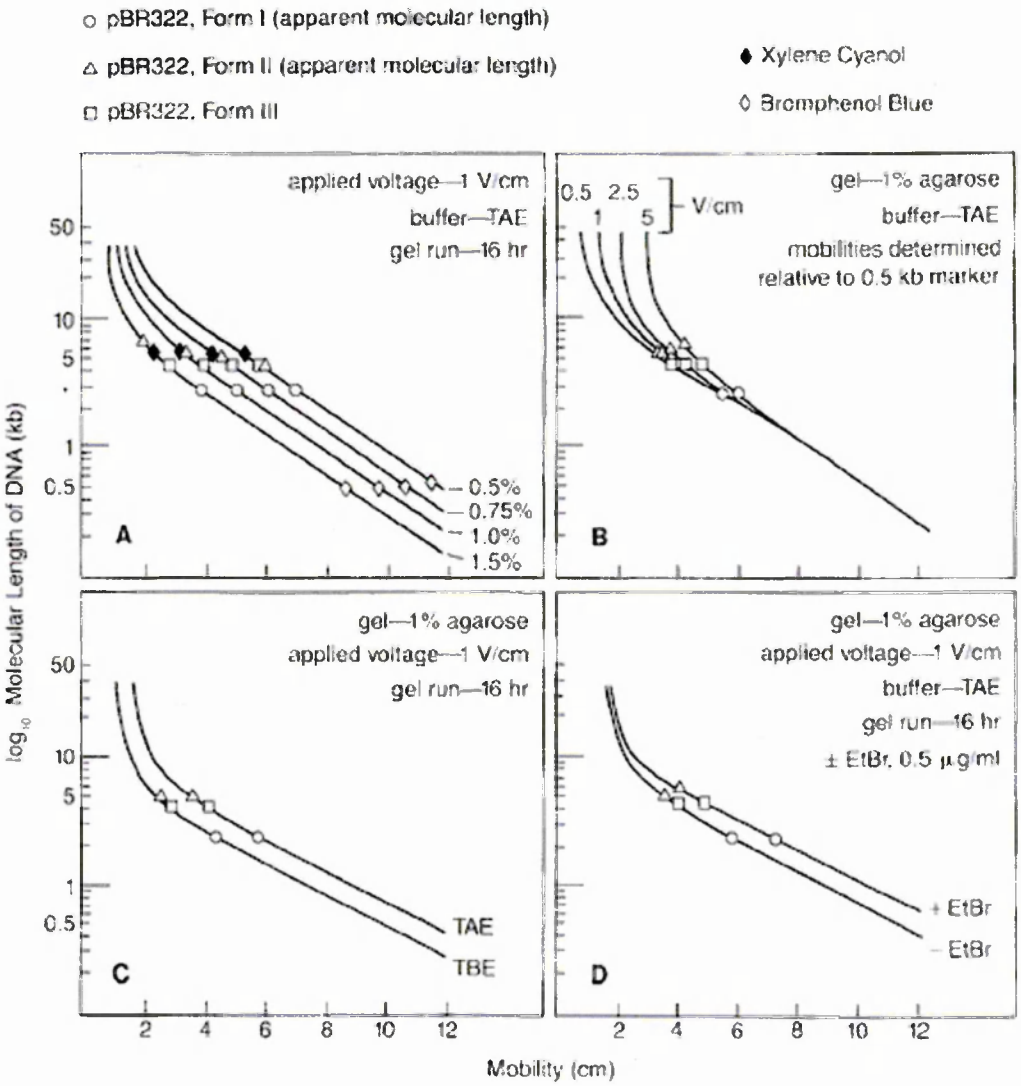
(e) *Direction of the Electric Field.* DNA molecules larger than 50-100kb in length migrate through agarose gels at the same rate if the direction of the electric field remains constant.

(f) *Base Composition and Temperature.* Either the base composition of the DNA or the temperature at which the gel is run does not significantly affect the electrophoretic behaviour of DNA in agarose gels. Thus, in agarose gels, the relative electrophoretic mobilities of DNA fragments of different sizes do not change between 4°C and 30°C. In general, agarose gels are run at room temperature.

(g) *Presence of Intercalating Dyes.* Ethidium bromide, a fluorescent dye that is used to detect DNA in agarose gels, reduces the electrophoretic mobility of linear DNA by about 15%. The dye intercalates between stacked base pairs, extending the length of linear and nicked circular DNA molecules and making them more rigid.

(h) *Composition of the Electrophoresis Buffer.* The electrophoretic mobility of DNA is affected by the composition and ionic strength of the electrophoresis buffer. In the absence of ions (e.g., if electrophoresis buffer is omitted from the gel by mistake), electrical conductance is minimal and DNA migrates very slowly, if at all. In buffers of high ionic strength (e.g., if 10 x electrophoresis buffer is used by mistake), electrical conductance is very efficient and significant amounts of heat are generated. Several different buffers are available for electrophoresis of native double-stranded DNA. These contain EDTA (pH 8.0) and TAE, TBE, or TPE.

Figure 2.5.



The effect of (**A**) agarose concentration, (**B**) applied voltage, (**C**) electrophoresis buffer, and (**D**) ethidium bromide on migration of DNA through Agarose gels (Ausubel *et* al, 1987).

2. Materials

(a) Fast-Trak Gel System (Hybaid):

Mini Apparatus: 1, 6.5 x 10.0 x 1.5cm UV transparent gel tray.

1 x main chamber assembly with built-in level and levelling feet.

1 x interlocking safety cover with leads.

1 x comb holder.

1 x 10-well comb.

Midi Apparatus: 1, 10.0 x 20.0 x 1.5cm UV transparent gel tray.

1 x main chamber assembly with built-in level and levelling feet.

1 x interlocking safety cover with leads.

2 x comb holders.

2 x 20-well combs.

(b) Camera (Polaroid CU-5 88-46, Genetic Research Instrumentation Ltd)

Hood size: 13.97 x 19.05 cm.

Area photographed: 10.80 x 13.65 cm.

Magnification: 0.65 X

Lens: 12.70 cm.

(c) Microwave (Toshiba)

(d) Polaroid Film (Polaroid Type 667, coaterless black and white, instant film 8.26 x 10.80 cm.)

Lens Aperture/exposure time: f/8, 2 sec

Recommended exposure meter setting: ISO 3000/36°

Spectral sensitivity: Panchromatic

Contrast: Medium

Resolution: 12-14 line pairs/mm

Image size: 7.30 x 9.53 cm.

Processing time: 60 seconds

(e) Power pack (Vodam® SAE 2761, Shandon Southern)

(f) UV illuminator

3. Chemicals

(a) Agarose Electrophoresis Grade (GIBCO BRL, Life Technologies, Scotland).

(b) Bromophenol blue (pH 2.8-4.6; Hopkin & Williams, Searle Company).

(c) 95% Ethidium Bromide (Sigma).

(d) Ficoll (Sigma)

4. Solutions

(a) ***10 x TBE Buffer***

109.03g Tris-base, 55.65g Boric acid and 9.31g of EDTA dissolved in 800ml of sterilised, deionised water. The volume was increased to 1litre, adjusting the pH to 8.2 using 6M NaOH and then stored at room temperature or 4°C.

(b) ***Agarose***

2% agarose is used in this gel electrophoresis.

(c) ***Loading Buffer***

25% (w/v) Ficoll

0.25% (w/v) Bromophenol Blue

- Dissolved in sterilised, deionised water

(d) ***Ethidium Bromide Solution (2,7-Diamino-10-ethyl-9-phenyl-phenanthidinium bromide; 10mg/ml stock solution)***

2.5.5. Agarose Gel Electrophoresis Protocol:

1x TBE buffer was made up on the day of the gel electrophoresis, i.e., 35 ml of 10x TBE (refer to Section 2.5.5.2a) made up to 350 ml with sterilised, deionised water for a 10-well gel electrophoresis experiment. This allowed 30 ml of 1 x TBE buffer to be used to dissolve 0.6 g of pure agarose (2% agarose gel), which was then gently heated for approximately 2 min on full power in a microwave, stirring the gel mixture throughout. This was then cooled before pouring into the gel apparatus, and inserting the comb for the wells. It was left for approximately 30 min to allow the gel to set, before gently removing the comb and pouring the remaining 1 x TBE buffer (approximately 320 mls) over the gel and immersing it for approximately 30 min, prior to the experiment.

RT-PCR samples containing loading buffer (5 µl/sample) were removed from -70° and defrosted at room temperature, and kept on ice before loading the sample onto the agarose gel, β-actin was used as an internal control and included on each gel. 15 µl of each sample was loaded into a well of the horizontal gel and 145V was applied between the ends of the gel for approximately 75 min, generating an electric field. Electrophoresis was carried out in pH 8.2, determined by the 1 x TBE buffer. Since the DNA molecules are negatively charged they move towards the anode, the size of the molecule being the major factor that determines the rate of movement.

The DNA can be visualised under UV light after staining with 0.5-1 µg/ml ethidium bromide. Ethidium bromide is often included in the gel, but alters the mobility of linear molecules. Therefore, at the end of the electrophoresis, the agarose gel was removed and immersed in 0.5-1 µg/ml ethidium bromide for 20 min, allowing time for the nucleic acids to be sufficiently stained. The ethidium bromide was removed and the gel immersed in sterilised, deionised water for approximately 40 min to wash any of the excess dye from the gel. The gel was visualised on a UV transilluminator, and a Polaroid photograph was taken, before discarding the gel. Sum intensity of the bands calculated using 1D Image Analysis Software from Kodak Scientific Imaging Systems.

2.6 Drugs and chemicals

2.6.1 Kits

Kit	Supplier
Gamma-B Corticosterone Assay Kit	(IDS, UK)
ACTH RIA Kit	(DSL, USA)

2.6.2 Drugs

Drug	Molecular Weight	Supplier
Aldosterone (d-aldosterone)	360.4	Sigma
Dexamethasone	392.5	Sigma
Hydrocortisone	362.5	Sigma
Paroxetine HCl	374.8	GlaxoSmithKline
RU 28362 (11β, 17β-dihydroxy-6-methyl-17α(1-propynyl)-androsta-1, 4, 6-trione-3-one)	352.45	Pfizer

2.6.3 Chemicals

Chemical	Supplier
Agarose (electrophoresis grade)	GIBCO BRL / Life Technologies
AMV Reverse Transcriptase (20 units/µl)	Promega UK, Ltd.
AMV/ *Tfl* (10x) Reaction Buffer	Promega UK, Ltd.
Boric Acid	Sigma
Bovine Serum Albumin (BSA)	Sigma
Bromophenol Blue (pH 2.8 – 4.6)	Hopkin & Williams / Searle Co.
Chloroform (AnalaR grade)	BDH
Copper Sulphate. $5H_2O$ (Analytic reagent)	May & Baker Ltd.
1,2,4,6,7 [^{3}H]-dexamethasone	Amersham
Diethylpyrocarbonate (DEPC)	Sigma
Dithiothreitol (DTT)	Sigma
100 bp DNA ladder	Promega UK, Ltd.
100 mM dNTPs (dATP, dCTP, dGTP, dTTP)	Promega UK, Ltd.
Absolute Ethanol (AnalaR grade)	BDH
Ethidium Bromide (95%)	Sigma
Ethylenediamine-Tetraacetic Acid dihydrate (EDTA)	Sigma
Ficoll	Sigma
Folin & Ciocalteu's Phenol Reagent	Sigma
Glycerol (Glycerine minimum 99%)	Sigma
Isoamylalcohol (AnalaR grade)	BDH
Isopropanol alcohol (AnalaR grade)	BDH
$MgSO_4$ (25mM)	Promega UK, Ltd.
Optiphase Safe	Wallac

Phenol	Sigma
Polyethylenimine (50% in water; PEI)	Aldrich
Potassium Chloride	Fisons Ltd.
Restriction Endonuclease Multicore Buffer (1x)	Promega UK, Ltd.
RNA Isolator™	Genosys Biotechnology Inc.
RQ1 RNase-free DNase (1 unit/µl)	Promega UK, Ltd.
Sodium Carbonate anhydrous (GPR grade)	BDH
Sodium Chloride (AnalaR grade)	BDH
Sodium Hydroxide	Prolabo
Sodium Molybdate Dihydrate (ACS Reagent)	Sigma
Tfl DNA Polymerase (5 units/µl)	Promega UK, Ltd.
Tris-hydroxymethylaminomethane hydrochloride	Prolabo
Trisodium Citrate dihydrate	Sigma

CHAPTER 3. PREDATOR STRESS STUDY: Development and Optimisation of Behavioural Testing Protocols

3.1 Introduction

3.1.1. Elevated-Plus Maze

The elevated plus-maze is claimed to be an "ethologically valid" animal model of anxiety because it uses "natural stimuli" that can induce anxiety in humans. It is assumed that the open arms of the maze combine the fear of a novel, brightly lit open space and the fear of balancing on a relatively narrow, raised platform. By contrast, the closed arms have high walls forming a narrow alley that affords good protection from potential predators (it is possible that these fears may be similar to agoraphobia, vertigo and xenophobia, respectively; Dawson & Trickelbank, 1995). When a rat or mouse is allowed to freely explore the elevated plus-maze for a fixed time, it spends only 20-25% of its time exploring the open arms, suggesting that these assumptions are correct.

The utility of the plus-maze is dependent upon rate-dependency factors, with low anxiety baselines less than optimal for detecting anti-anxiety effects and high baselines virtually useless for detecting anxiety enhancement (Rodgers & Cole, 1993).

Although such problems can be partially surmounted by the selection of appropriate genetic strains and optimal test conditions, a number of authors (Cruz *et al*, 1994; Rodgers & Johnson, 1995; Espejo, 1997) have argued that test sensitivity, reliability, and ecological validity may be improved by focussing upon what the animals actually do in the maze, as well as their physical location. In this context, and stemming from the work of the Blanchards' on antipredator defence in rodents (Blanchard *et* al, 1994), several research groups have begun to routinely score aspects of the actual behavioural acts and postures displayed by animals in the maze as well as the more usual spatiotemporal measures.

Whereas diverse behaviours were observed (e.g., rearing, grooming, head-dipping), there were high levels of risk assessment (i.e., stretch-attend postures) shown by mice in this test. This finding was not only consistent with the view that risk assessment is a dominant response in potentially dangerous situations, but also with subsequent studies confirming that mice generally display higher levels of this behaviour than rats. Furthermore, it was also apparent that several behaviours (including risk assessment) were predominantly exhibited from relatively protected/safe areas of the maze (closed arms and/or centre platform), a finding that not only confirmed the importance of thigmotactic cues in maze exploration but also suggested the potential utility of differentiating these behaviours as a function of where on the maze they occurred ("percentage protected" measures).

Mice that are exposed to the scent of an aggressive conspecific show a comparable increase in risk assessment in the absence of consistent alterations in the classical measures of anxiety. The latter findings imply that enhanced anxiety may not necessarily be accompanied by significant changes in open arm entries or time spent on the open arms (Rodgers & Cole, 1993).

Physiological confirmation of the aversive or stressful effects of plus-maze exposure is provided by evidence of post-test elevations in pain latencies (Rodgers & Cole, 1994) and skin conductance levels (Haller *et* al, 1998). Furthermore, consistent with the effects of various physical and psychosocial challenges (for a review see Haller *et* al, 1998), exposure to the plus-maze produces a significant increase in plasma corticosterone (Pellow *et* al, 1985; Holmes *et* al, 1998).

Previous studies have shown that a broad range of prior stress has an anxiogenic effect on rats and mice tested in the plus-maze, although some negative results were also found. For example, prior immobilisation was found to be anxiogenic (Martijena *et* al, 1997) or to have no effect (McBlane & Handley, 1994). Furthermore, it

was shown in rats that prior stress (habituation to a novel environment and footshock) decreased the percentage of time spent in the open arms (Da Cunha *et* al, 1992).

A major problem with the elevated plus-maze is that numerous variables are known to influence maze results, with the result that several contradictory findings are reported in the literature (Trullas & Skolnick, 1993; Rodgers & Johnson, 1995).

The principal organismic variables of interest are species, strain, age and gender, all of which have been shown to affect behavioural baselines in the maze (Rodgers & Cole, 1994). For example, significant strain differences have been reported for rats (Costall *et al*, 1989) and, particularly, mice (Cole *et al*, 1995; Trullas & Skolnick, 1993).

Housing conditions, lighting levels, time of testing and test duration, pretesting, prior handling, prior stress and prior maze experience have also been shown to radically affect basal anxiety levels in the plus-maze (for a review see Rodgers & Cole, 1994). For example, individual housing increases anxiety in rats but decreases it in mice, a difference that may relate to species variation in social organisation (Rodgers & Dalvi, 1997).

In addition to the above factors, which have been more or less systematically studied for their impact on plus-maze behaviour, a range of other less well-researched variables can be identified. Although not often mentioned in research reports, they may nevertheless have a significant bearing upon inter-laboratory variability in pharmacological profiles, and include: light/dark cycle and lighting levels in the holding facility; duration of adaptation to laboratory conditions prior to testing; presence of experimenter during testing; elevation of the maze; material from which maze is constructed; height of maze walls; transparency/opacity of walls; open arm flooring; definition of an arm entry; and so forth (Rodgers & Dalvi, 1997).

Repeated testing is one of the procedural variables where contradictory results have been reported. Some laboratories have found stable test-retest profiles (Lister, 1987; File, 1992), although the majority have reported reduced open arm exploration (File, 1990; Rodgers & Johnson, 1995). The literature is based on inter-test intervals of days and weeks. It is well known that repeated exposure to a novel environment leads to habituation of the behavioural responses (Claus & Bindra, 1960), emerging inactivity or stationary patterns over time (Kaesermann, 1986), a fact that deserves further investigation concerning the elevated plus-maze.

3.1.2. Sucrose Preference

Chronic sequential exposure to a variety of mild unpredictable stressors (CMUS) causes performance deficits in behavioural paradigms that measure responsiveness to rewards in a variety of different ways. For example, CMUS depresses the consumption of, and preference for, palatable sweet solutions (i.e., sucrose and saccharin). These behavioural changes may be maintained for several months, but normal behaviour has been reported to be restored by chronic administration (3-4 weeks) of tricyclic or atypical antidepressants. This attenuation of sucrose preference has been interpreted as anhedonia, a loss of reinforcing capacity, a core symptom in the diagnosis of depression (DSM-IV, 1994).

Results from a study by Calvo-Torrent *et al* (1999) show that predatory stress attenuates the level of sucrose intake after 2-3 weeks of chronic exposure, similar to that observed with a variety of other stressors in rats (Katz, 1982; Papp *et* al, 1996) and mice (Monleon *et* al, 1995). Thus, this study by Calvo-Torrent *et al* (1999), showed that the sucrose intake test sensitively detects changes in mice after exposure to predatory stress.

3.2 Methods

3.2.1. Elevated Plus-Maze (EPM)

1. Animals

Forty-three adult male BALB/c mice weighing about 30g were singly housed and maintained in a controlled temperature (24°C), constant humidity (~55%) and a 12h light:dark cycle (lights on from 7:00h to 19:00h). Mice were bred in our laboratory at Nescot, and were left for at least three weeks with food and tap water available *ad libitum*, to habituate to the single housing. The use of these animals in the following scientific procedures were regulated by the Animals (Scientific Procedures) Act 1986.

2. Method

An elevated plus-maze (50cm off the ground) made from white, opaque plexiglass was used. It comprised of two opposing "open" arms (30 x 5 x 0.25cm) and two opposing "closed" arms (30 x 5 x 15cm) with a 5cm square central area. A muslin cloth attached to a wooden frame (128 x 110cm) enclosed the entire maze, but the top part of the frame was covered in a mesh material, so it did not obscure the observer's view of the mice. Attached to the top of the wooden frame, centrally directed over the maze (70cm above the maze), was a 60W bulb (approximately 1500 Lux) directly illuminating the entire maze, but not close enough to heat it up. During the test periods, only the spotlight over the maze provided illumination within the test room.

For the plus-maze tests, the mice were moved to the test room, in their home cages, immediately prior to being tested, to prevent adaptation (**Figure. 3.1.**). Each mouse was placed in the central square, facing an enclosed arm, and allowed to freely explore the maze for 5 min. Both spatiotemporal and ethological measures (**Table.3.1.**) were recorded for every minute of the total time (0-1 min, 1-2 min, 2-3 min, 3-4 min & 4-5 min), to give an indication of the change in behaviours over the total time the mouse was exposed to the maze. In view of the importance of thigmotactic cues in plus-maze exploration

(Treit *et al*, 1993), stretch-attend postures, scanning and sniffing (denoted with * in **Table.3.1.**.) were further differentiated as "protected" (i.e., occurring on/from the relative security of the closed arms/centre platform) or "unprotected" (i.e., occurring on/from open arms). Data for the latter measures are, therefore, given both as total scores and "percent protected" scores [(protected/total) x 100]. After the test, the animal was returned to its home cage. The plus-maze apparatus was carefully mopped after each test using soapy water to remove any residual odour due to urine or faeces from the mice themselves.

3. Statistical Analysis

A statistical approach was used to identify patterns of behavioural change within and between trials whereby the entire dataset, i.e., standard measures and all ethological measures from Trial 1, were analysed by factor analysis using a principal components solution with orthogonal rotation (varimax) of the factor matrix: this method ensures that the extracted factors are independent of one another. Factor pattern matrices were identified using a combination of the Kaiser criterion (factors must have eigenvalues < 1) and the Cattell Scree test (on a simple line plot, the point at which the smooth decrease in eigenvalues levels off to the right; Hendrie, 1991). The factor loading of each behavioural item indicates how well that item correlates with the factor(s); (range –1.0 to +1.0) and, in accordance with previous studies (Montgomery, 1955; Hendrie *et al*, 1996), only factor loadings of >0.4 are reported.

Table.3.1.

Categories of behaviour analysed on the EPM.

Total Entries	Number of entries to any arm of the maze. An entry was defined as all four paws crossing the line into the arms.
Open Entries	Number of entries to an open arm.
Closed Entries	Number of entries to a closed arm.
% Open Arm Entries	The number of open arm entries, expressed as a % of the total arm entries.
% Closed Arm Entries	The number of closed arm entries, expressed as a % of the total arm entries.
Open Arm	Time the mouse spent on the open arms.
Closed Arm	Time the mouse spent on the closed arms.
% Open Arm	The time spent in the open arms, expressed as a % of the duration of the session.
% Closed Arm	The time spent in the closed arms, expressed as a % of the duration of the session.
% Centre	The time spent in the centre, expressed as a % of the duration of the session.
Non exploratory Behaviours:	
Freezing / Immobility	Number of squats or cringes.
Self-Grooming	A species-typical sequence beginning with the snout, progressing to the ears and ending with a whole body groom.
Risk Assessment Behaviours:	
Closed Arm Returns	Exiting from a closed arm with only the forepaws and then returning into the same arm.
End-Exploring	The number of times the mouse reached the end of an arm.
Sniffing*	Olfactory exploration of the maze floor and walls, and occasional air sampling
Scanning*	Where the mouse investigates its surroundings, often accompanied with body stretches and including head-dipping. This is the exploratory movement of the head/shoulders directed over the sides of the maze.
Stretch-Attend Posture*	Forward elongation of head and shoulders followed by retraction to original position.
Rearing	The number of times that the mouse rises onto its hind-limbs.
Defecation	Total number of faecal boli that the mouse left in the different sections of the plus-maze after the duration of the session.

4. Results of the Factor Analysis of Trial 1

The behavioural measures comprised of some 15 items (**Table 3.2.**), and the correlations between the different behavioural measures in Trial 1 of the EPM is shown in **Table 3.3A** and **B**, Kaiser and Scree plot analyses identified 4 "factors" or components, which together accounted for 71.3% of the total variance in this dataset (**Table 3.4.**). The estimation of significant "factors" is determined by examination of the eigenvalues and their relative magnitudes in the correlation matrix.

Factor 1 showed high loadings for closed arm entries, % open arm entries (negative loading), % closed arm entries, % time on open arms (negative loading), % time on closed arms, total end-exploring, total head-dipping/scanning (negative loading) and total rearing. Total stretch-attend postures (negative loading) loaded only moderately on this factor.

Total arm entries, open arm entries, and closed arm entries loaded heavily on Factor 2, whilst total end-exploring loaded only moderately on this factor. Total sniffing loaded heavily on Factor 3. Total closed arm returns and total defecation loaded heavily on Factor 4. All loadings are positive unless otherwise stated, and Factor 1 correlates to anxiety, Factor 2 to locomotor activity, Factor 3 with risk assessment and Factor 4 with decision-making and approach/avoid behaviours.

Table 3.2.

Spatiotemporal and ethological description of behaviour displayed by control male BALB/c mice (n = 43), in a five minute EPM test.

Behaviour	Mean ± sem
Total number of arm entries	3.2 ± 0.37
Number of open arm entries	1.7 ± 0.20
Number of closed arm entries	1.5 ± 0.27
Number of closed arm returns	1.0 ± 0.30
% Open arm entries	61.0 ± 5.57
% Closed arm entries	39.0 ± 5.57
% Time on open arms	45.0 ± 5.35
% Time on closed arms	32.0 ± 5.45
Total counts of sniffing	59.3 ± 2.72
Total counts of grooming	0.6 ± 0.12
Total counts of end-exploring	2.9 ± 0.46
Total counts of head-dipping (HD)/scanning	27.1 ± 1.84
Total counts of stretch-attend postures (SAP)	27.5 ± 2.23
Total counts of rearing	1.5 ± 0.37
Total counts of defecation	5.3 ± 0.44
% Protected sniffing	57.0 ± 0.14
% Protected head-dipping (HD)/scanning	50.0 ± 0.14
% Protected stretch-attend postures (SAP)	55.0 ± 0.14

This table summarises the data set upon which the present factor analyses were performed. This analysis included all spatiotemporal and behavioural measures recorded using a video-camera in Trial 1.

Table 3.3. A and B: Correlation Matrix.

A

	Total Arm Entries	Open Arm Entries	Closed Arm Entries	Closed Arm Returns	% Open Arms	% Closed Arms	% Time on Open Arms	% Time on Closed Arms
Total Arm Entries	1	0.687**	0.850**	-0.129	-0.289	0.289	-0.322*	0.313*
Open Arm Entries	0.687**	1	0.202	-0.298	0.355*	-0.355*	0.227	-0.283
Closed Arm Entries	0.850**	0.202	1	0.042	-0.647**	0.647**	0.599**	0.627**
Closed Arm Returns	-0.129	-0.298	0.042	1	-0.519**	0.519**	-0.464**	0.500**
% Open Arm Entries	-0.289	0.355*	-0.647**	-0.519**	1	-1**	0.813**	-0.909**
% Closed Arm Entries	0.289	-0.355*	0.647**	0.519**	-1**	1	-0.813**	0.909**
% Time on Open Arms	-0.322*	0.227	-0.599**	-0.464**	0.813**	-0.813**	1	-0.838**
% Time on Closed Arms	0.313*	-0.283	0.627**	0.500**	-0.909**	0.909**	-0.838**	1
Total Sniffing	-0.267	-0.054	-0.321*	-0.061	0.151	-0.151	0.141	-0.269
Total Grooming	0.058	-0.261	0.267	0.225	-0.440**	0.440**	-0.357*	0.547**
Total End-exploring	0.651**	0.168	0.755**	0.122	-0.597**	0.597**	-0.489**	0.654**
Total HD/Scanning	-0.158	0.285	-0.420**	-0.086	0.596**	-0.596**	0.529**	-0.592**
Total SAP	-0.364*	0.089	-0.555**	-0.260	0.530**	-0.530**	0.568**	-0.645**
Total Rearing	0.344*	-0.163	0.582**	0.210	-0.667**	0.667**	-0.580**	0.727**
Total Defecation	0.040	-0.019	0.068	0.189	-0.199	0.199	-0.109	0.153
% Protected Sniffing	0.341*	-0.234	0.629**	0.435**	-0.818**	0.818**	-0.990**	0.826**
% Protected HD/Scanning	0.129	-0.347*	0.425**	0.480**	-0.761**	0.761**	-0.953**	0.763**
% Protected SAP	0.291	-0.226	0.556**	0.445**	-0.789**	0.789**	-0.972**	0.801**

B

	Total Sniffing	Total Grooming	Total End-exploring	Total HD	Total SAP	Total Rearing	Total Defecation	% Protected Sniffing	% Protected HD/Scanning	% Protected SAP
Total Arm Entries	-0.267	0.058	0.651**	-0.158	-0.364*	0.344*	0.040	0.341*	0.129	0.291
Open Arm Entries	-0.054	-0.261	0.168	0.285	0.089	-0.163	-0.019	-0.234	-0.347*	-0.226
Closed Arm Entries	-0.321*	0.267	0.755**	-0.420**	-0.555**	0.582**	0.068	0.629**	0.425**	0.556**
Closed Arm Returns	-0.061	0.225	0.122	-0.086	-0.260	0.210	0.189	0.435**	0.480**	0.445**
% Open Arm Entries	0.151	-0.440**	-0.597**	0.596**	0.530**	-0.667**	-0.199	-0.818**	-0.761**	-0.789**
% Closed Arm Entries	—	—	—	—	-0.530**	0.667**	0.199	0.818**	0.761**	0.789**
% Time on Open Arms	0.141	-0.357*	-0.489**	0.529**	0.568**	-0.580**	-0.109	-0.990**	-0.953**	-0.972**
% Time on Closed Arms	-0.269	0.547**	0.654**	-0.592**	-0.645**	0.727**	0.153	0.826**	0.763**	0.801**
Total Sniffing	1	-0.290	-0.283	-0.105	0.691**	-0.380*	-0.113	-0.117	-0.045	-0.080
Total Grooming	-0.290	1	0.192	-0.293	-0.379*	0.267	0.328*	0.342*	0.318*	0.311*
Total End-exploring	-0.283	0.192	1	-0.445**	-0.487**	0.717**	0.082	0.512**	0.361*	0.470**
Total HD/Scanning	-0.105	-0.293	-0.445**	1	0.266	-0.500**	0.146	-0.556**	-0.501**	-0.484**
Total SAP	0.691**	-0.379*	-0.487**	0.266	1	-0.503**	-0.081	-0.573**	-0.462**	-0.491**
Total Rearing	-0.380*	0.267	0.717**	-0.500**	-0.503**	1	0.193	0.592**	0.519**	0.547**
Total Defecation	-0.113	0.328*	0.082	0.146	-0.081	0.193	1	0.086	0.053	0.110
% Protected Sniffing	-0.117	0.342*	0.512**	-0.556**	-0.573**	0.592**	0.086	1	0.940**	0.965**
% Protected HD/Scanning	-0.045	0.318*	0.361*	-0.501**	-0.462**	0.519**	0.053	0.940**	1	0.950**
% Protected SAP	-0.08	0.311*	0.470**	-0.484**	-0.491**	0.547**	0.110	0.965**	0.950**	1

The correlations between the different behavioural measures in Trial 1 of the EPM. Statistical significant differences are denoted by * = $p<0.05$ and ** = $p<0.01$.

HD = Head-Dipping; **SAP** = Stretch-Attend Postures

Table 3.4.

Orthogonal factor loadings for standard plus-maze measures, plus all ethological measures.

Variable	Factor 1	Factor 2	Factor 3	Factor 4
Total Arm Entries	—	0.908	—	—
Open Arm Entries	—	0.785	—	—
Closed Arm Entries	0.646	0.606	—	—
Closed Arm Returns	—	—	—	0.559
% Open Arm Entries	-0.901	—	—	—
% Closed Arm Entries	0.901	—	—	—
% Time on Open Arms	-0.789	—	—	—
% Time on Closed Arms	0.859	—	—	—
Total Sniffing	—	—	0.844	—
Total grooming	—	—	—	—
Total end-exploring	0.647	0.477	—	—
Total head-dipping (HD)/scanning	-0.761	—	—	—
Total stretch-attend postures (SAP)	-0.434	—	—	—
Total rearing	0.6878	—	—	—
Total defecation	—	—	—	0.500

Factor loadings of < 0.4 are not included. The four factors account for 71.3% of the total variance.

5. Discussion

The factor analysis incorporated all plus-maze parameters analysed in Trial 1, including the various ethological measures related to murine defensive behaviour. Four factors emerged from this investigation of the data (**Table 3.4.**), which confirmed previous investigations in this area (Rodgers & Johnson, 1995). Factor 1 (anxiety) retained very high loadings on traditional spatiotemporal measures. Importantly, several ethological measures (end-exploring, head-dipping/scanning, SAP and rearing), previously interpreted as indicative of anxiety (Rodgers *et* al, 1994), were also found to load moderately/highly on this factor. These latter items are probably related by virtue of reflecting a reluctance to

leave the security of the closed arms. Factor 2 (locomotor activity) included total arm entries as well as closed arm entries. Total sniffing/olfactory investigation was the only measure to load highly on Factor 3 (risk assessment), which is arguably a very important means of information gathering in a macrosmatic species and so represents risk assessment. Factor 4 seems homologous with decision-making, approach/avoid behaviours. The moderate loadings of closed arm returns and defecation tend to confirm its relationship with decision making, as the former occurs exclusively in closed arms while the latter almost always occurs in secure areas of the maze.

In conclusion, the factor analysis confirms that this elevated plus-maze allows the observation of primary spatiotemporal indices of anxiety as well as a range of defensive behaviours. Not only does the behavioural profile comprise factors related to anxiety and locomotor activity as previously thought, but also includes dimensions that appear to be related to vertical activity, exploration, risk assessment, and decision-making. So this EPM was chosen to be used in the following predator stress study.

3.2.2 Sucrose Consumption and Sucrose Preference

The purpose of this experiment was to ascertain the difference between a one-bottle sucrose consumption test and a two-bottle sucrose preference test on group-housed male BALB/c mice.

1. Animals

Twenty-four male BALB/c mice (Nescot breeding colony) weighing from 20-26g were housed in groups of 6 animals per cage and maintained in a controlled temperature (24°C), constant humidity (~55%) and a 12h light:dark cycle. Mice were housed as above for at least 7 days with free access to food and water before the experiment. The use of

these animals in the following scientific procedures were regulated by the Animals (Scientific Procedures) Act 1986.

2. Methods

Two cages of 6 mice were water and food deprived for 4 h (08:00 to 12:00), and then given a pre-weighed bottle containing a sucrose solution (0-4% w/v) for 4 h (12:00 to 16:00). Two other cages of six mice were also food and water deprived for 4 h (08:00 to 12:00), and given a pre-weighed bottle containing a sucrose solution and a pre-weighed bottle of tap water for 4 h (08:00 to 12:00). After the sucrose tests the mice were given free access to food and water overnight (16:00 to 08:00). Therefore each cage of six mice had access to two different sucrose solutions over two days.

The bottles were weighed before and after the 4 h test (weight of bottle plus liquid (before) – weight of bottle plus liquid (after) = weight / volume of liquid consumed in 4 h per cage). The amount of water and sucrose consumed in the two-bottle test was calculated as a percentage of the total amount of liquid consumed per cage, and divided by the number of mice per cage (n=6), to give the average amount of water and sucrose consumed per mouse. Body weights were monitored daily, and compared against their weights prior to the experiment. Six empty cages containing a bottle of water and a bottle containing a 1% sucrose solution were assessed for leakage over the 4 h test periods. These bottles were weighed before and after the tests.

3. Statistical Analysis

Statistical evaluations were made with paired or unpaired Student's *t* test, as indicated, or analysis of variance.

4. Results

In the two-bottle test, sucrose was preferred over water at all the sucrose concentrations, with maximum preference of 62% observed at 2% sucrose (F=1.0, P=0.0145), due to a 174% increase in sucrose intake compared to the other concentrations (**Table.3.5.**).

Table 3.5.

Two-bottle sucrose preference test in group-housed male BALB/c mice

Sucrose Concentration (% w/v)	Sucrose Intake/cage (g)	Sucrose Preference / cage (% w/v)
0.5	4.7	52
1.0	4.2	57
2.0	11.5	62
4.0	3.8	52

Intake of water and different concentrations of sucrose (0.5%, 1%, 2%, and 4% w/v) over 4 h using the two-bottle preference test in BALB/c mice. Results are expressed as mean (whereby the amount shown was consumed by 6 mice for each concentration).

In the one-bottle test, significantly more of the 0.5% sucrose concentration was consumed over the other concentrations by 70% (**Table 3.6.**.)

Table 3.6.

One-bottle sucrose consumption test in group-housed male BALB/c mice.

Sucrose Concentration (% w/v)	Sucrose Intake/cage (g)
0.5	8.0
1.0	3.4
2.0	5.3
4.0	5.4

Intake of different concentrations of sucrose (0.5%, 1%, 2%, and 4% w/v) over 4 h using the one-bottle consumption test in BALB/c mice. Results are expressed as mean (whereby the amount shown was consumed by 6 mice for each concentration).

Therefore, the results are different between the one- and two-bottle tests, which indicates that the type of test used determines the sucrose concentration preferred as a reward by group-housed male mice. This can only be an assumption, as for each sucrose

concentration, only one cage was used (therefore the sample size is too small for statistical analysis). There was no difference in body weights between the cages, suggesting the consumption from two bottles instead of one does not significantly affect the body weight. The small amount (0.5-1g) consumed per mouse (a similar amount to the leakage produced per bottle), suggesting that 4 h food and water deprivation followed by 4 h consumption is not sufficiently long to produce a quantifiable amount of consumed liquid. This may be due to the fact that mice are more active at night, and this test was conducted during the day when the mice were effectively asleep.

3.2.3. Sucrose Preference and Food Intake over 24h

Based on the results from the previous experiment, the food and water deprivation was increased to 6 h, as well as increasing the sucrose preference test period to 24h (so as to include the period when the mice are most active, at night). Leakage from these bottles was assessed over 24 h, in parallel with this experiment. The sample size was increased to five, whereby each cage acted as its own control and was tested with all of the sucrose concentrations (0-4% w/v), on separate occasions. Food consumption over the test period was also assessed.

1. Animals

Thirty male BALB/c mice as per Section 3.2.2.1.

2. Method

Mice were given free access to food and two bottles of water, counterbalanced across the feeding compartment, for 48 h prior to the experiment. Then the mice were water and food deprived for 6 h (08:00 to 14:00), and given a pre-weighed bottle containing a sucrose solution (0-4% w/v), a pre-weighed bottle of tap water, and a set

amount of food pellets for 24 h (14:00 to 14:00). After the sucrose tests the mice were given free access to food and water for 18 h (14:00 to 08:00). Therefore each cage had access to all the sucrose solutions, therefore acting as their own control, over 5 days.

The bottles were weighed before and after the 24 h tests (weight of bottle plus liquid (before) – weight of bottle plus liquid (after) = weight / volume of liquid consumed in 24 h per cage). The food pellets were also weighed before and after the 24 h tests (weight of food pellets (before) – weight of food pellets (after) = weight / volume of food pellets consumed in 24 h per cage).

The amount of water and sucrose consumed in the two-bottle tests were calculated as a percentage preference per cage (% Sucrose Preference = [(Sucrose intake/Total liquid intake)*100]), and divided by the number of mice per cage (n=6), to give the average amount of water and sucrose consumed per mouse, over 24 h. Body weights were monitored daily, and compared against weights prior to the experiment. Six empty cages containing a bottle of water and a bottle containing a 1% sucrose solution were assessed for leakage over the 24 h test periods, and were weighed before and after the tests.

3. Statistical Analysis

Statistical evaluations were made with paired or unpaired Student's *t* test, as indicated, or analysis of variance.

4. Results

Expressed as a percentage preference, sucrose was slightly preferred over water, but there seems to be no significant variation between the different sucrose concentrations (**Table 3.7.**). Yet, the amount of fluid intake over the test period appears to be no different

from the previous experiment. There appears to be the same amount of food consumed irrespective of the sucrose concentration.

Table 3.7.

Food, water and sucrose intake from group-housed male BALB/c mice

Sucrose Concentration (% w/v)	Sucrose Preference (% w/v)	Total Fluid Intake (g)	Food Intake (g)
0	N/A	30.9 ± 2.4	32.0 ± 3.6
0.5	53.8 ± 5.4	34.3 ± 4.7	38.4 ± 5.2
1.0	58.4 ± 6.4	30.2 ± 2.7	34.9 ± 2.8
2.0	55.6 ± 2.2	31.6 ± 2.5	31.4 ± 3.2
4.0	59.4 ± 11.5	28.0 ± 2.2	36.8 ± 3.8

Intake of sucrose (0-4% w/v), tap water and food intake from group-housed male BALB/c mice over 24 h, after 6 h food and water deprivation. Results are expressed as mean ± sem (n=5 per concentration).

3.2.4. Single-housing

The purpose of this experiment was to construct a sucrose concentration curve in singly-housed male BALB/c mice. Single-housing was chosen as a more reliable way to calculate the sucrose preference for each individual mouse to be tested.

1. Animals

Forty-eight male BALB/c mice weighing from 20-26g were singly housed and maintained in a controlled temperature (24°C), constant humidity (~55%) and a 12h light:dark cycle. These mice were bred in our laboratory. They were housed under these conditions for at least seven days with free access to food and two-bottles of water, counterbalanced across the cage, before the experiment. These mice were monitored daily for any significant changes in appearance, i.e. loss of weight, lack of movement, etc., due to the stress of being singly housed. The use of these animals in the following scientific procedures were regulated by the Animals (Scientific Procedures) Act 1986.

2. Method

Mice were given free access to food and two bottles of water, counterbalanced across the feeding compartment, for 48 h prior to the experiment. All the bottles to be used in the tests were labelled with their respective bungs, and tested for leakage over 24 h prior to the experiment; half containing tap water and the other half containing 4% w/v sucrose. Mice were then water and food deprived for 6 h (08:30 to 14:30), and given a pre-weighed

bottle containing a sucrose solution (0-32% w/v), a pre-weighed bottle of tap water, and a set amount of food pellets for 24 h (14:30 to 14:30). For each sucrose solution, half the mice (n=3) had the sucrose bottle on the left side of the cage, and the other half had the sucrose bottle on the right side of the cage.

After the sucrose tests the mice were given free access to food and water for approximately 48 h. The test was repeated with the bottles switched to the other side of the cages, i.e. if the sucrose bottle was on the left side in the first test then it was changed to the right side in the second test.

3. Statistical Analysis

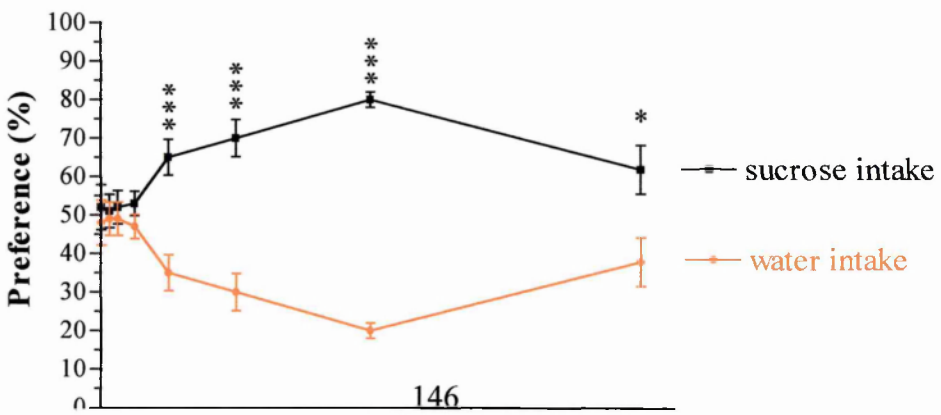
Statistical evaluations were made with paired or unpaired Student's *t* test, as indicated, or analysis of variance.

4. Results

From 0-2% sucrose there was no preference for sucrose over water, but the preference for sucrose significantly increased to around 80% (at a sucrose concentration of 16%; $F=5.368$, $P<0.0001$) and decreased slightly to about 65% preference at a concentration of 32% (**Figure 3.1.**). Food consumption seemed to mimic this with a gradual increase from just below 8g to 14g in association with 2-32% sucrose concentration ($F=3.743$, $P=0.0014$). A concentration of 8% w/v sucrose solution was chosen to be used in the following experiments, as it was significantly preferred by 223% over water, allowing for changes due to stress to be monitored accurately.

Figure 3.1.

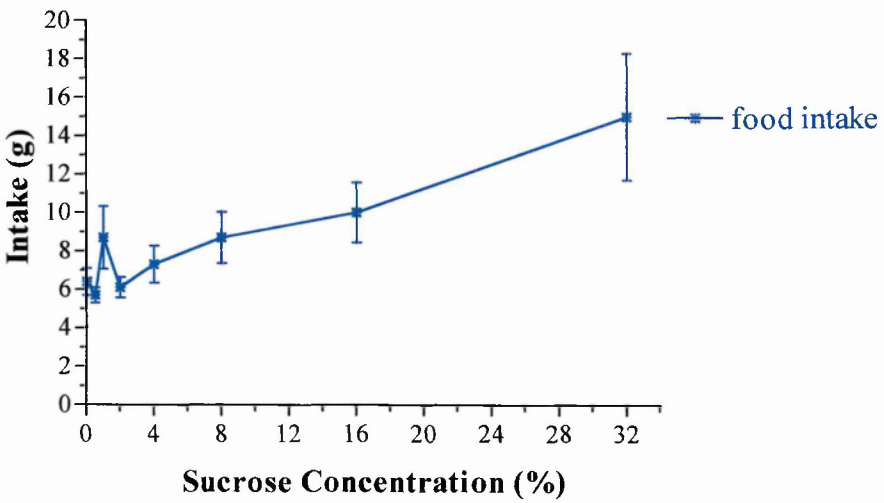
Sucrose preference (% of total fluid intake) and food intake (g) in singly housed male BALB/c mice.



(A)

(B)

Consumption of different concentrations of sucrose (0%, 0.5%, 1%, 2%, 4%, 8%, 16% and 32% w/v) over 24 h using the two-bottle preference test in singly housed male BALB/c mice (n = 6 per concentration). Results are expressed as mean (± sem). **(A)** Intake of water and different concentrations of sucrose (0-32%), and **(B)** Intake of food, from singly housed male BALB/c mice, after 6 h food and water deprivation. Unpaired t-test analysis, where * = p <0.05, *** = p<0.001.



3.2.5. With or without food and water deprivation

The purpose of this experiment was to ascertain the difference between prior food and water deprivation and no prior deprivation on the sucrose preference test in singly housed male BALB/c mice.

1. Animals

Fourteen male BALB/c mice as per Section 3.2.4.1.

2. Method

Mice were given free access to food, one bottle of tap water and one bottle containing an 8% w/v sucrose solution, counterbalanced across the feeding compartment, for 48 h prior to the experiment. This served as a training period. The same bottles were used from the previous pilot study, therefore their leakage was known. Seven mice were then water and food deprived for 6 h (08:00 to 14:00), and given a pre-weighed bottle containing an 8% sucrose solution, a pre-weighed bottle of tap water, and a set amount of food pellets for 24 h (14:00 to 14:00). The other seven mice were not food and water deprived, but were given a pre-weighed bottle containing an 8% w/v sucrose solution, a pre-weighed bottle of tap water, and a set amount of food pellets for 24 h (14:00 to 14:00). Half of the mice, that were deprived and not deprived, had the sucrose bottle on the left side of the cage, and the other half had the sucrose bottle on the right side of the cage. After the sucrose tests the cages were given free access to food and water.

3. Statistical Analysis

Statistical evaluations were made with paired or unpaired Student's *t* test, as indicated, or analysis of variance.

4. Results

Percentage preference for sucrose remained ~86% with or without deprivation, and the amount of liquid consumed did not differ significantly (**Table 3.8.**). Therefore, the use

of deprivation prior to the sucrose preference test does not appear to increase the volume of liquid consumed, or change the preference for sucrose. So to limit the number of stressors applied to the mice (i.e. single housing, odour and presence of rats, elevated plus-maze), deprivation was not used prior to the sucrose preference tests in subsequent experiments.

Table 3.8.

With or without 6 h food & water deprivation.

	MEAN +/-SEM	N
After 6 h food & water deprivation	85 ± 3.1	7
Without 6 h food and water deprivation	88 ± 4.0	7

Sucrose preference (% w/v) of single-housed male BALB/c mice, after 24 h access to food, water and 8% sucrose solution, using the two-bottle preference test.

5. Conclusion

A two-bottle sucrose preference test was chosen, using singly-housed male BALB/c mice. This test was to be measured overnight. No food and water deprivation was to be carried out prior to the test and food intake would be included as part of the measurements.

CHAPTER 4. PREDATOR STRESS STUDY

4.1. Introduction

Several studies have shown that there are physiological and behavioural changes resulting from exposure to predatory stress. They include opiate and non-opiate-dependent analgesia (Hendrie & Neill, 1991; Kavaliers, 1988), increase immobility

(Hendrie *et* al, 1996) and increase of heart and breathing rate (Weiner, 1992). Moreover, if the exposure to a predator is prolonged, hyperthyroidism and a decrease of body weight are induced (Weiner, 1992). Additionally, there are decreases in food intake (Blanchard *et* al, 1993a; Hendrie & Neill, 1991) and inhibition of sexual behaviour immediately after the exposure to a predator (Blanchard & Blanchard, 1989). It is interesting to note that the predator stress in such studies is based on the threat of predation rather than on physical attack, indicating that the sensory stimuli (i.e., odours and calls) from a predator specifically induce changes (Hendrie & Neill, 1991; Zangrossi & File, 1992b).

The work of the Blanchards (Blanchard & Blanchard, 1989; Blanchard *et* al, 1998) provides evidence that the behaviours exhibited in the presence of a predator relate to fear, whereas those evoked by the odour of a predator indicate anxiety. However, there is also evidence to suggest that the nature of the anxiety induced by exposure to novelty and to predator odours may differ. There are also pharmacological differences; low doses of benzodiazepines have an anxiolytic effect in rat tests employing novelty, whereas only high doses change behaviour during exposure to cat odour. Behaviourally it has been shown that rats showing strong or weak avoidance of cat odour did not differ in other tests of anxiety. Interestingly, although on the first exposure there is a strong correlation between behavioural and corticosterone responses to cat odour, these measures dissociate with repeated exposures, with the corticosterone, but not the behavioural, response habituating.

Animals are capable of displaying diverse defensive reactions in response to external threats, e.g., a predator. In mammals (e.g., rats) such behaviours classically compromise freezing, flight, defensive threat/attack and even death-feigning, and are dependent upon imminent threat and escape opportunity (Blanchard *et al*, 1990). However,

research has shown that the rodent defensive repertoire is more elaborate than suggested by this classical description. In potentially dangerous situations (for example, when a predator has been seen but is no longer present), laboratory rats (Blanchard & Blanchard, 1989) and mice (Blanchard *et* al, 1995b) have been reported to engage in a cluster of behaviours collectively referred to as risk assessment. These responses, originally identified in specially constructed visible burrow systems, are characterised by cautious approaches to a surface area where a predator (cat) has briefly been presented and include; (i) scanning the danger area from tunnel openings, (ii) stretch attend, or flatback, postures directed towards the danger area and (iii) stretched, or flatback, locomotion upon initial re-entry into the danger area. Very similar behavioural patterns have been observed in the rat defensive burying paradigm (Molewijk *et* al, 1995) and in mice exposed to conspecific odours (Kaesermann, 1986), supporting the contention that their function is to inform behavioural strategy in potentially dangerous situations (Blanchard *et* al, 1993b). As many animal models of anxiety are based upon exploration of novel (and hence, potentially dangerous) environments, it would be assumed that these situations should also elicit risk assessment.

More recently, Dielenberg *et al* (1999) demonstrated that rats confronted with a cat-odour impregnated collar displayed robust avoidance responses towards this stimulus. Such exposure also resulted in anxiogenic responses in the social interaction and elevated plus-maze tests (Zangrossi & File, 1992a), that is, when animals are no longer in the presence of the stressful predator odour.

Laboratory animals demonstrate a variety of endocrine changes in response to predator exposure. Groups of rats exposed to cat odour for the first time displayed significant increases in plasma corticosterone concentrations. However, this effect was

found to habituate following five exposures (File *et al,* 1993) thus demonstrating a dissociation of behavioural and corticosterone responses to predator odour exposure.

Increased ACTH and plasma corticosterone concentrations were reported in rats five minutes after cat exposure as compared to non-stressed controls (Adamec & Shallow, 1993). This study also measured CRH, AVP and bombesin concentrations in specific regions of HPA circuitry. CRH levels were increased in the anterior hypothalamus and decreased in the dorsomedial hypothalamus. AVP concentrations were found to be reduced in the lateral hypothalamus.

These changes indicate that the status of the HPA axis is significantly altered following predator (odour) exposure. These data also verify the predator exposure procedure as being a stressful stimulus to many animals (i.e fish, marmosets, and squirrel monkeys) as elevations of ACTH and the concomitant release of corticosterone are hallmarks of the stress response (Weiner & Levine, 1992; Barros *et al*, 2001; Kagawa & Mugiya, 2002).

4.2. Aims

To determine if the stress induced by predatory stimuli, as indicated by the plasma concentrations of corticosterone, would affect:

1. The specific binding of ^{3}H-dexamethasone to cortical GR. We were particularly interested to see if any reductions in brain GR would occur following chronic predator stress which would be consistent with a GR down-regulation hypothesis in depression (refer to section. 1.3.3).
2. Their behaviour in two tests – namely the sucrose preference test, which has been used as an indicator of "depression" (Willner *et* al, 1992; Papp *et* al, 1996) and the EPM test, which has been used as an indicator of "anxiety" (Pellow *et* al, 1985; Hogg, 1996).

4.3. Chronic Stress Study

As described in the previous chapter (Chapter 3), both sucrose preference and the EPM tests had been optimised in order to be used in this study. In the present study, predator stress was induced by exposing male BALB/c mice to the visual presence and odour of wild male Brown Norway rats housed in the same room. We decided to use BALB/c mice in our studies, as these are known to display high reactivity to stress (Lu *et al*, 1998; Karen Mellowdew, Institute of Psychiatry, personal communication) - with Brown Norwegian rats as the predator species (as these are not kept in our animal facility and would possess a novel odour). Using the idea of predator odour as a chronic stressor and the visual presence of a predator as an acute stressor we aimed to produce a pattern of intense behavioural and endocrine responses that would be very slow to habituate. Rats have been observed, in nature as well as in the laboratory, to kill and consume mice (Karli, 1956; O'Boyle, 1975). Preliminary behavioural investigations by Griebel *et al* (1995), have clearly demonstrated the importance of the presence of a rat to elicit the full range of specific fearful/defensive behaviours in the mouse.

4.3.1. Method

1. Animals

Adult male BALB/c mice from the Nescot breeding colony, weighing about 30g were singly housed and maintained in a controlled temperature (24°C), constant humidity (~55%) and a 12h light:dark cycle (lights on from 7:00h to 19:00h). Mice were left for at least three weeks with food and tap water available *ad libitum*, to habituate to the single housing. The use of these animals in the following scientific procedures were regulated by the Animals (Scientific Procedures) Act 1986.

2. Predator Stress Procedure

Due to the results from the previous chapter, a two-bottle sucrose preference test was chosen, using singly-housed male BALB/c mice. This test was to be measured overnight. No food and water deprivation was to be carried out prior to the test and food intake would be included as part of the measurements.

Mice were given 48h pre-exposure to a two-bottle test; one containing an 8% sucrose solution and one containing tap water, counterbalanced across the cage. The animals were given free access to food pellets during this training period. Mice were tested weekly, on the plus-maze and the two-bottle sucrose preference test for three weeks to achieve a stabilised baseline.

After the baseline of sucrose preference had stabilised, approximately three weeks, the mice that had not significantly preferred sucrose to the tap water (achieved >60% preference for sucrose) were excluded before the mice were placed into relevant groups. These groups were:

(1) Mice sacrificed after 4 weeks of the stressors (n =14),

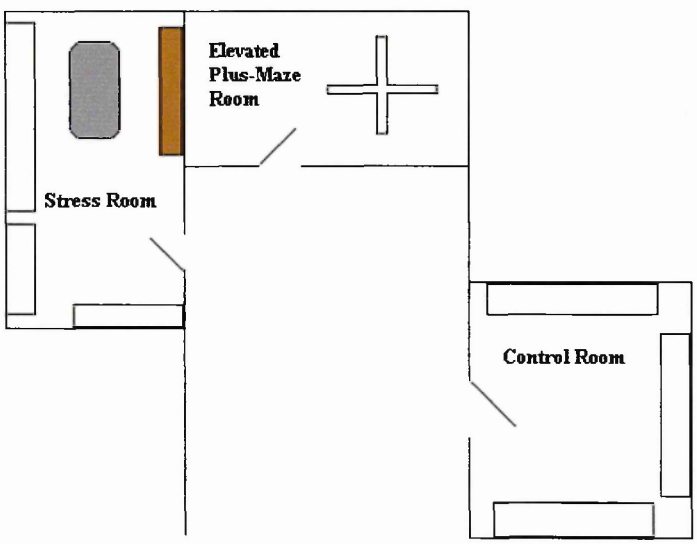
(2) Mice sacrificed after 8 weeks of the stressors (n =14),

(3) Mice sacrificed after 8 weeks of the stressors + 2 weeks after the stress was removed (n =14), and

(4) Mice sacrificed after 8 weeks of the stressors, and then tested for the first time on the elevated plus-maze (n =14).

Half of these groups (*stressed group*) were placed in a separate room and subjected to the predator stressor, whilst the others remained in their home room (*control group*; **Figure.4.1.**). The predator stressor involved continuous exposure to the odour of male Brown Norway rats, in cages within the room and the intermittent exposure of the mice to the visual presence of the male Brown Norway rats. This involved placing the mice, in their home cages, in a playpen (100 x 68 x 65cm) filled with foam packing (this was provided as a distraction for the rats as well as to support the cages within the playpen). Then placing 1/ 2 / 3 rats (the number of rats was random) into the playpen to move freely around for about 3 min. This stress procedure was conducted randomly in the morning or the afternoon, and at least once a day for up to 8 weeks.

Figure.4.1



MICE RATS PLAYPEN

Allocation of animals during the different phases of the experimental procedure.

All of the mice (except group 4) were tested weekly on the elevated plus-maze and the two-bottle sucrose preference test. The mice in the "stress" room were tested after their last acute stressor, and the controls were tested at the same time. Each time we took care to change clothes when entering and leaving the "stress" room, to avoid the transfer of odour etc, from the rats to the control animals.

3. Elevated Plus-Maze (EPM)

As described in Section 3.2.1. The effects of repeated testing ("trials") and "stress" on gross plus-maze profiles were initially analysed by two-factor repeated measures ANOVA (trials x stress), followed by Newman-Keuls comparisons. One further statistical approach was used to identify patterns of behavioural change within and between trials. The data for each 5-min trial were broken down into 1 min time bins and subjected to a three-factor repeated measures ANOVA (trial x stress x time bin), followed by Newman-Keuls comparisons.

The data was subjected to a two-factor repeated measures ANOVA analysis, unless otherwise stated. In addition, ANOVAs of separate groups were used and post hoc comparisons were carried out with the Newman-Keuls test. Student's *t*-test was applied when necessary. In all cases, the level of significance used was $\rho < 0.05$.

4. Sucrose Preference Tests

For the two-bottle sucrose preference test, each mouse was given a pre-weighed bottle containing an 8% sucrose solution and a pre-weighed bottle containing tap water, counterbalanced across their home cage, for 17 h (16:00 to 09:00). Half of the mice were given the sucrose bottle on the left side of the feeding compartment, and the other half were given the bottle on the right side of the feeding compartment. The bottles were alternated for each test, to prevent placement preference. Food consumption was also determined, over the 17 h test period, by re-weighing a set amount of food pellets. This involved collecting the remains of the pellets within the cage, to prevent these from being calculated as consumed. All tests were carried out in the home cage to prevent unnecessary stress and to avoid any extraneous effects attributable to a novel environment (**Figure. 4.1.**).

The effects of repeated testing ("trials") and "stress" on gross plus-maze profiles were analysed by two-factor repeated measures ANOVA (trials x stress), followed by Newman-Keuls comparisons.

5. Radioimmunoassay of plasma corticosterone concentrations

Plasma corticosterone concentrations were measured by using gamma-B I^{125} - Corticosterone radioimmunassay commercial kits (refer to Section 2.4.).

The effects of repeated testing ("trials") and "stress" on gross plus-maze profiles were analysed by two-factor completely randomised ANOVA (trials x stress), followed by Newman-Keuls comparisons.

6. Specific binding of ^{3}H-dexamethasone to mouse cytosolic corticosteroid receptors

Mice, from the "stress" room, were sacrificed 24 h after the last introduction of the acute stressor, and the controls were sacrificed at the same time. All sacrifice and dissection procedures were conducted as described in section 2.3.2. Preparation of mouse cytosolic fractions was carried out essentially as described in section 2.3.5. Subsequent procedures and analysis were carried out as described in section 2.3.

The effects of repeated testing ("trials") and "stress" on gross plus-maze profiles were analysed by two-factor completely randomised ANOVA (trials x stress), followed by Newman-Keuls comparisons.

7. Protein measurement

Protein content was measured using Lowry's method with bovine albumin as standard (Lowry *et al*, 1951).

4.4. Results

4.4.1. Corticosterone Radioimmunoassay

Statistical analysis indicated that time alone significantly affected the plasma corticosterone levels ($F_{2,36}$=25.36; $p<0.0001$), and so did stress alone ($F_{1,36}$=8.124; p=0.0072). The combined effect of time and stress was also highly significant ($F_{2,36}$=5.351; p=0.0092).

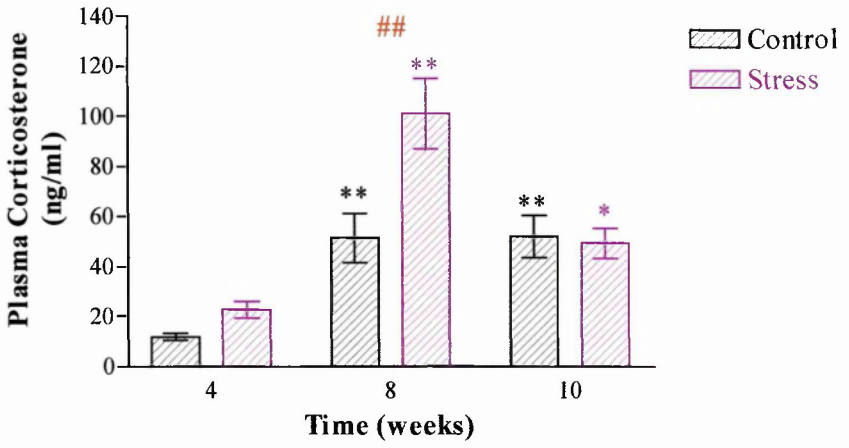
Plasma corticosterone concentration did not differ from controls in mice exposed to predator stress for 4 weeks, but was significantly higher (by 97%) in those exposed to predator stress for 8 weeks. Following 10 weeks of predator stress, plasma corticosterone did not differ from controls (**Figure 4.2A**). Control values significantly varied between 10 and 50 ng/ml.

When Group 2 (8 weeks of stress or non-stress with weekly EPM tests) was compared to Group 4 (8 weeks of stress or non-stress followed by the first EPM test) they were not significantly different, suggesting that the EPM test alone had no effect upon the plasma corticosterone levels (**Figure 4.2B**).

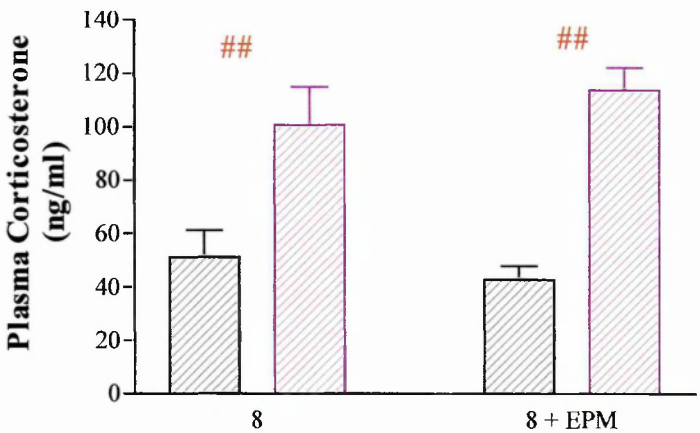
Figure. 4.2.

Plasma corticosterone concentration following exposure to predator stress.

(A)



(B)



Plasma corticosterone concentration in singly-housed male BALB/c mice (n = 7 per group), were measured in samples taken immediately following exposure to predator stress. Data are expressed as mean ± sem. **(A)** Comparison between control mice left in their holding room (control group) and mice exposed to predator stress for 4 weeks, 8 weeks or 10 weeks before they were sacrificed (stress group). **(B)** Comparison between mice exposed to 8 weeks of EPM in combination with predator stress (8 + EPM) and mice exposed to a single EPM following 8 weeks of predator stress (8). ##, $p<0.01$ compared to control; **, $p<0.01$ compared to 4 weeks of control; **, $p<0.01$ compared to 4 weeks exposure to predator stress.

4.4.2. Glucocorticoid Receptor (GR) Binding

There was no significant difference in either B_{max} or K_d values, between stressed and control mice (**Table 4.1.**). Although, control B_{max} values steadily increased from 110.8 to 135.5 fmol/mg protein.

Table 4.1.

Glucocorticoid receptor B_{max} and K_d values in cortical cytosolic fractions, following exposure to predator stress.

GROUPS	N	B_{max} (fmol/mg protein)	K_d (nM)
4 weeks "control."	6	110.8 +/- 9.7	3.5 +/- 0.9
4 weeks "stress"	6	148.0 +/- 14.0	2.6 +/- 0.4
8 weeks "control"	6	127.1 +/- 16.8	2.7 +/- 0.5
8 weeks "stress"	6	122.3 +/- 10.9	2.7 +/- 0.4
10 weeks "control"	6	135.5 +/- 10.6	2.2 +/- 0.3
8 weeks "stress" + 2 weeks after the removal of "stress"	6	114.4 +/- 6.5	2.6 +/- 0.5
8 weeks "control" followed by the first EPM test.	5	133.9 +/- 10.3	2.8 +/- 0.7
8 weeks "stress" followed by the first EPM test.	5	132.3 +/- 13.9	4.0 +/- 1.2

Specific ^{3}H-dexamethasone binding to cortical GR (taken from singly-housed male BALB/c mice) was measured following exposure to predator stress. Samples were taken immediately after exposure to predator stress. Data are expressed as mean ± sem. No statistically significant differences were found between control and stress groups.

4.4.3. Sucrose Preference

Sucrose preference remained high throughout (~ 90%) and did not differ between the groups (**Figure 4.3.**). Food intake gradually decreased by 40% in the stressed mice (**Figure 4.4.**), from 5-3g, compared to the baseline, although it did not differ between the groups. No significant differences were found between and within groups in terms of body weights (**Table.4.2.**).

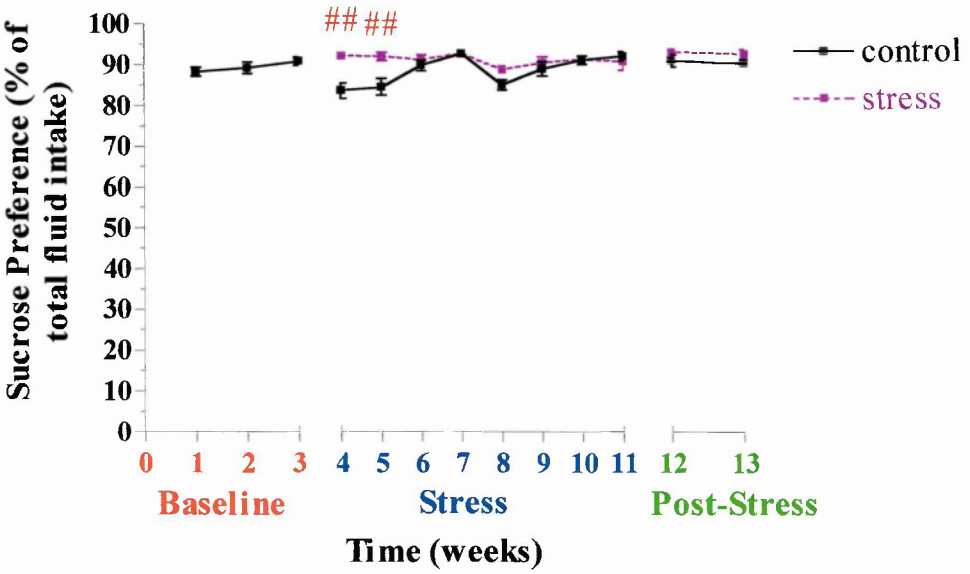
Table.4.2.

Body weights (g) of control and stress singly housed male BALB/c mice.

Session	Trial (weeks)	Groups			
		Control		Stressed	
		Mean +/- SEM	N	Mean +/- SEM	N
BASELINE	1	29.9 +/- 0.03	55		
	2	29.9 +/- 0.03	55		
	3	29.9 +/- 0.03	55		
STRESS	1	29.5 +/- 0.44	21	30.3 +/- 0.66	21
	2	29.8 +/- 0.42	21	30.2 +/- 0.71	21
	3	30.8 +/- 0.53	21	30.2 +/- 0.79	21
	4	29.9 +/- 0.53	21	30.5 +/- 0.81	21
	5	30.2 +/- 0.73	14	29.9 +/- 1.15	14
	6	30.5 +/- 0.76	14	30.3 +/- 1.14	14
	7	30.5 +/- 0.82	14	30.2 +/- 1.15	14
	8	30.4 +/- 0.87	14	30.8 +/- 1.17	14
REMOVAL	1	30.7 +/- 1.04	7	29.7 +/- 1.00	7
OF STRESS	2	31.3 +/- 0.96	7	30.0 +/- 0.98	7

Figure. 4.3.

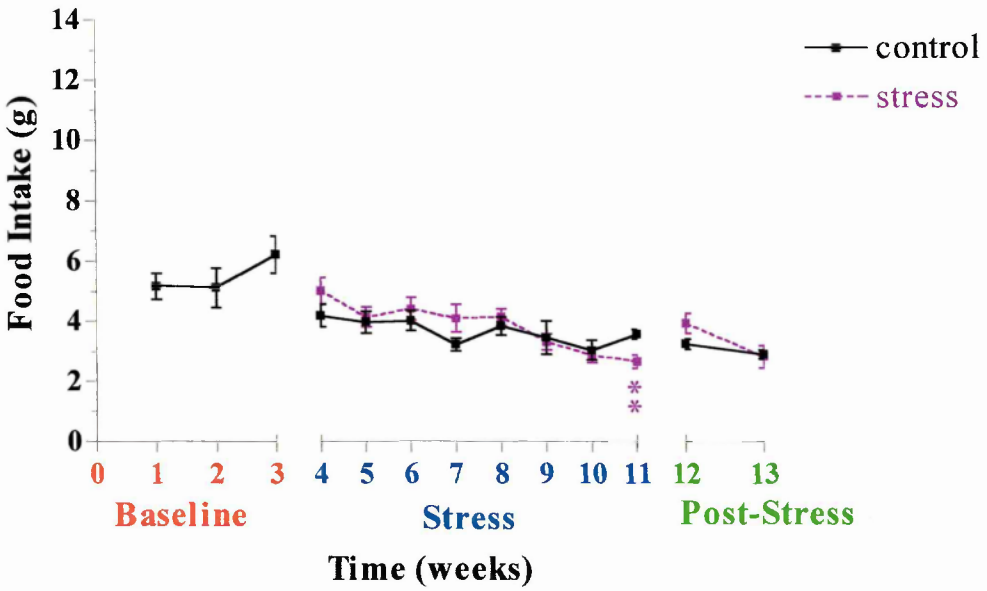
Sucrose preference (% of total fluid intake) following predator stress exposure.



Sucrose preference (% of total fluid intake) measured over 22 h, from singly-housed male BALB/c mice (n = 10 per group), immediately after exposure to predator stress. Data are expressed as mean ± sem. Comparison between control mice left in their holding room (control group) and mice exposed to predator stress (stress group), before they were sacrificed. Baseline values calculated prior to allocation to either of the groups. ##, $p<0.01$ compared to control.

Figure. 4.4.

Food intake (g) following predator stress exposure.



Food intake (g) measured over 22 h, from singly-housed male BALB/c mice (n = 10 per group), immediately after exposure to predator stress. Data are expressed as mean ± sem. Comparison between control mice left in their holding room (control group) and mice exposed to predator stress (stress group), before they were sacrificed. Baseline values calculated prior to allocation to either of the groups. **, $p<0.01$ compared to baseline.

4.4.4. Elevated Plus-Maze (EPM)

A) Effect of Repeated Test Experience and "Stress":

i. Arm Entries

After just one attempt on the elevated plus-maze, mice seemed to be significantly stressed, as they made almost no open arm entries by Trial 2 (**Figure.4.5A and B**).

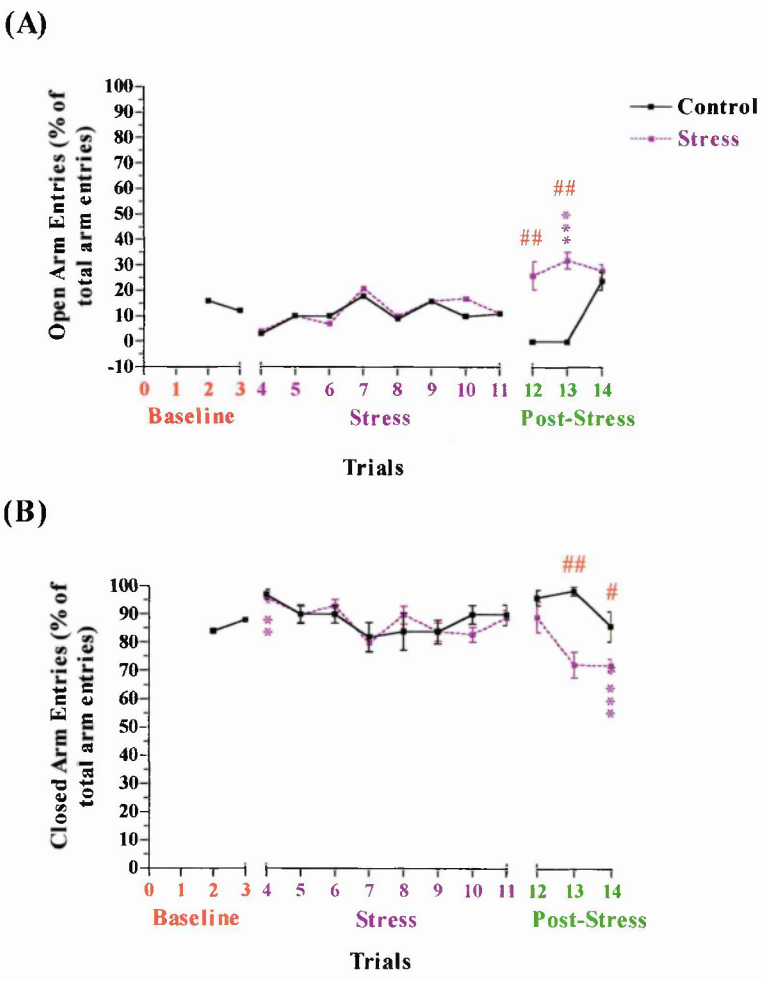
Throughout the "stress" period (Trial 4-11) statistical analysis indicated that stress significantly affected the percentage of open arm entries ($F_{1,264}$=115.9; $p<0.0001$), as well as repeated trials ($F_{7,264}$=1097; $p<0.0001$). Yet, only repeated trials significantly affected the percentage of closed arm entries ($F_{7,264}$=3.687; $p=0.0008$). Statistical analysis also indicated that stress ($F_{1,264}$=6.676; $p=0.0103$) and repeated trials ($F_{7,264}$=3.736; $p=0.0007$) significantly affected the total arm entries, including an 81% increase in the response by the stress mice compared to the controls in Trial 10 (**Figure.4.6.**), suggesting an increase in locomotion by the stressed mice.

As soon as the stress was removed, statistical analysis indicated that stress ($F_{1,21}$=76.7; $p<0.0001$) and repeated trials ($F_{2,21}$=11.09; $p=0.0005$) significantly affected the percentage of open arm entries as well as the percentage of closed arm entries ($F_{1,30}$=21.74; $p<0.0001$ and $F_{2,30}$=5.454; $p=0.0095$, respectively). The stress group increased their percentage of open arm entries whilst the control response was decreased towards baseline values (**Figure.4.5B**). Therefore, the first two weeks after the removal of stress provoked an increase in open arm entries in the stress group and a decrease in the

control group. This was emphasised by the significant decrease in the total arm entries ($F_{1,26}$=4.35; p=0.0470) by the control mice, during this period (**Figure.4.5A**).

Figure.4.5.

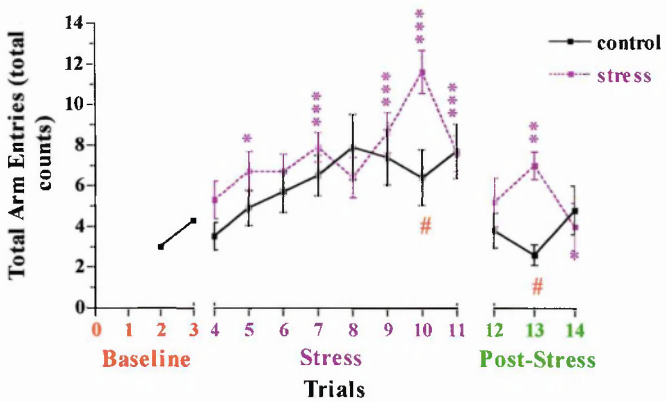
Percentage Arm entries on the EPM following exposure to predator stress.



BALB/c mice (n=8 per group) were subjected to elevated plus-maze (EPM) tests immediately following predator stress exposure. Data are expressed as mean ± sem. **(A)** % open arm entries and **(B)** % closed arm entries during a 5 min trial per week (n = 21 for baseline and weeks 1-4 of the stress, n = 14 for weeks 5-8 of the stress, and n = 7 for the 3 weeks after the stress was removed). Mice were given free access to food and water throughout. Baseline values are the means prior to allocation to either of the groups. #, p<0.05; # #, p<0.01 compared to control; **, p<0.01; ***, p<0.001 compared to baseline, within the stress group.

Figure.4.6.

Total arm entries on the EPM following exposure to predator stress

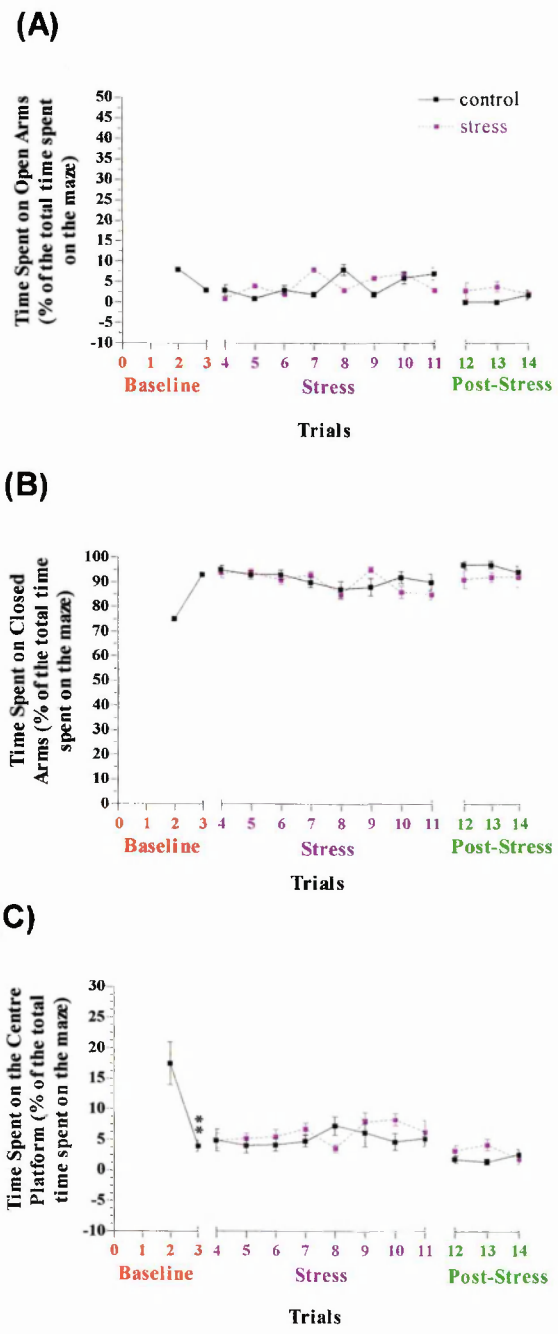


BALB/c mice (n=8 per group) were subjected to elevated plus-maze (EPM) tests immediately following predator stress exposure. Data are expressed as mean ± sem. Total arm entries (total counts of open + closed arm entries), during a 5 min trial per week (n = 21 for baseline and weeks 1-4 of the stress, n = 14 for weeks 5-8 of the stress, and n = 7 for the 3 weeks after the stress

was removed). Mice were given free access to food and water throughout. Baseline values are the means prior to allocation to either of the groups. #, $p<0.05$ compared to control; *, $p<0.05$; **, $p<0.01$; **, $p<0.001$ compared to baseline, within the stress group.

Figure.4.7.

Time spent in the different sections of the EPM following exposure to predator stress.



BALB/c mice (n=8 per group) were subjected to elevated plus-maze (EPM) tests immediately following exposure to predator stress. Data are expressed as mean ± sem. **(A)** Time shown as seconds spent on the open arms (% of the total time spent on the maze), **(B)** Time shown as seconds spent on the closed arms (% of the total time spent on the maze) , and **(C)** Time shown as seconds spent on the centre platform (% of the total time spent on the maze), during a 5 min trial per week (n = 21 for baseline and weeks 1-4 of the stress, n = 14 for weeks 5-8 of the stress, and n = 7 for the 3 weeks after the stress was removed). Mice were given free access to food and water throughout. Baseline values are the means prior to allocation to either of the groups. **, $p<0.01$ compared to baseline, within the controls.

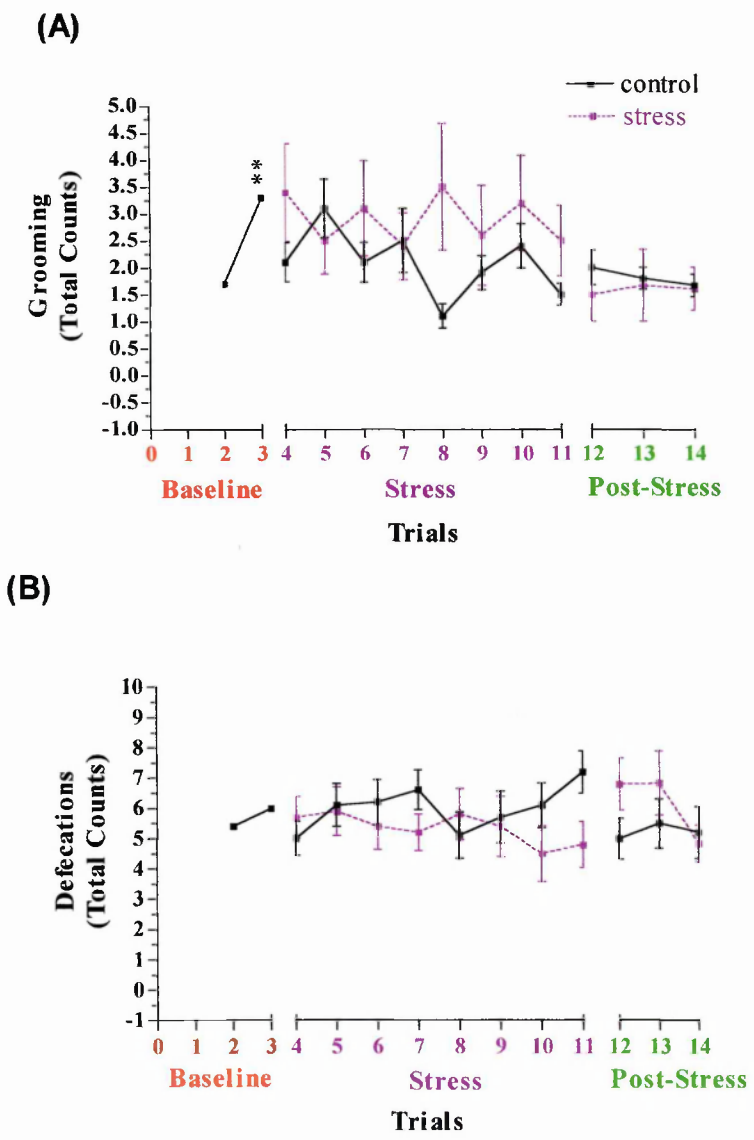
Throughout the "stress" period (Trial 4-11) statistical analysis indicated that only repeated trials significantly affected the percentage of time spent on the open arms ($F_{7,264}$=8.831; p<0.0001) and the closed arms ($F_{7,264}$=3.660; p=0.0009). As soon as the stress was removed, statistical analysis indicated that stress ($F_{1,25}$=5.687; p=0.0250) was only significant upon the time spent on the open arms. Time spent on the central platform was not statistically significant during the "stress" and "post-stress" trials, between the groups (**Figure.4.7 A-C**). Therefore, the mice, controls or stress, spent most if not all of their time in the closed arms.

ii. Non-exploratory Behaviour and Defecation

After 3 weeks of baseline measurements, grooming scores increased by 700%. During the "stress" period (Trial 4-11) statistical analysis indicated that stress significantly increased the grooming scores ($F_{1,264}$=5.656; p=0.0181), but had no significant effect after the removal of the stressors (**Figure.4.8A**). No freezing/immobility was scored for any of the mice, throughout the study. Defecation scores were not statistically significant during the "stress" and "post-stress" trials, between the groups (**Figure.4.8B**).

Figure.4.8.

Non-exploratory behaviour and defecation on the EPM following exposure to predator stress.



BALB/c mice (n = 8 per group) were subjected to elevated plus-maze (EPM) tests immediately following exposure to predator stress. Data are expressed as mean ± sem. **(A)** Total counts of grooming and **(B)** total counts of defecation, during a 5 min trial per week (n = 21 for baseline and weeks 1-4 of the stress, n = 14 for weeks 5-8 of the stress, and n = 7 for the 3 weeks after the stress was removed). Mice were given free access to food and water throughout.

iii. Risk Assessment Behaviour

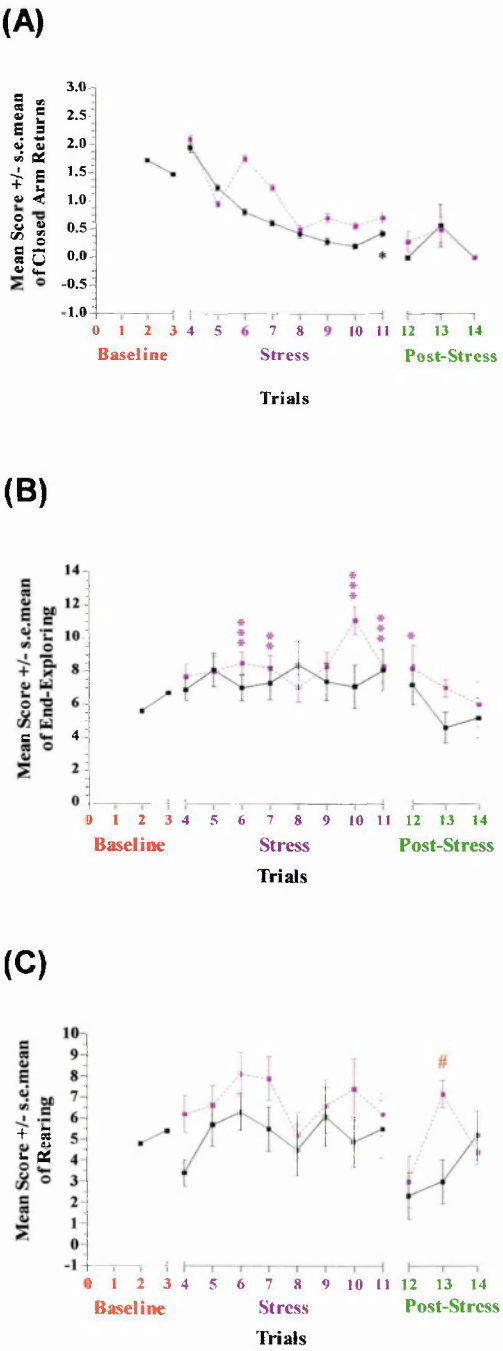
Throughout the "stress" period (Trial 4-11) statistical analysis indicated that stress ($F_{1,264}$=94.25; p<0.0001) and repeated trials ($F_{7,264}$=142.7; p<0.0001) significantly affected the number of returns into the closed arms (**Figure.4.9A**), as well as the responses of scanning with head-dipping (**Figure.4.10B**) by the mice ($F_{1,264}$=5.983; p=0.0151 and $F_{7,264}$=7.391; p<0.0001, respectively). Sniffing responses and the number of stretch-attend postures (SAP) exhibited by the mice (**Figure.4.10A and C**), were only significantly affected by the repeated trials ($F_{7,264}$=7.001; p<0.0001 and $F_{7,264}$=3.609; p=0.0010, respectively), whereas the amount of rearing by the mice was significantly affected by stress ($F_{1,264}$=7.904; p=0.0053). The amount of exploring of the ends of the arms, by the mice, was not statistically significant between the two groups during this period.

As soon as the stress was removed, statistical analysis indicated that scanning with head-dipping was significantly affected by repeated trials ($F_{2,26}$=3.73; p=0.0377), the amount of rearing by the mice was significantly affected by repeated trials ($F_{2,26}$=3.526; p=0.0442), and the number of SAPs were significantly affected by stress ($F_{1,27}$=9.041;; p=0.0057). Both sniffing and end-exploring responses were not statistically significant between the two groups after the removal of the stressors. Control values for all these behaviours were variable throughout the study.

Overall, the risk assessment behaviour was increased in the stress mice during the introduction of stress (especially end-exploring and scanning with head-dipping), but there was no significant difference between stressed mice and controls during these trials. Once the stress was removed, previously stressed mice gradually decreased in all the behaviours whereas the controls remained stable or gradually increased. This is more pronounced in the scanning with head-dipping, but there were also no significant differences between stressed and controls in this period of the study.

Figure.4.9.

Risk assessment behaviours exhibited on the EPM following exposure to predator stress.

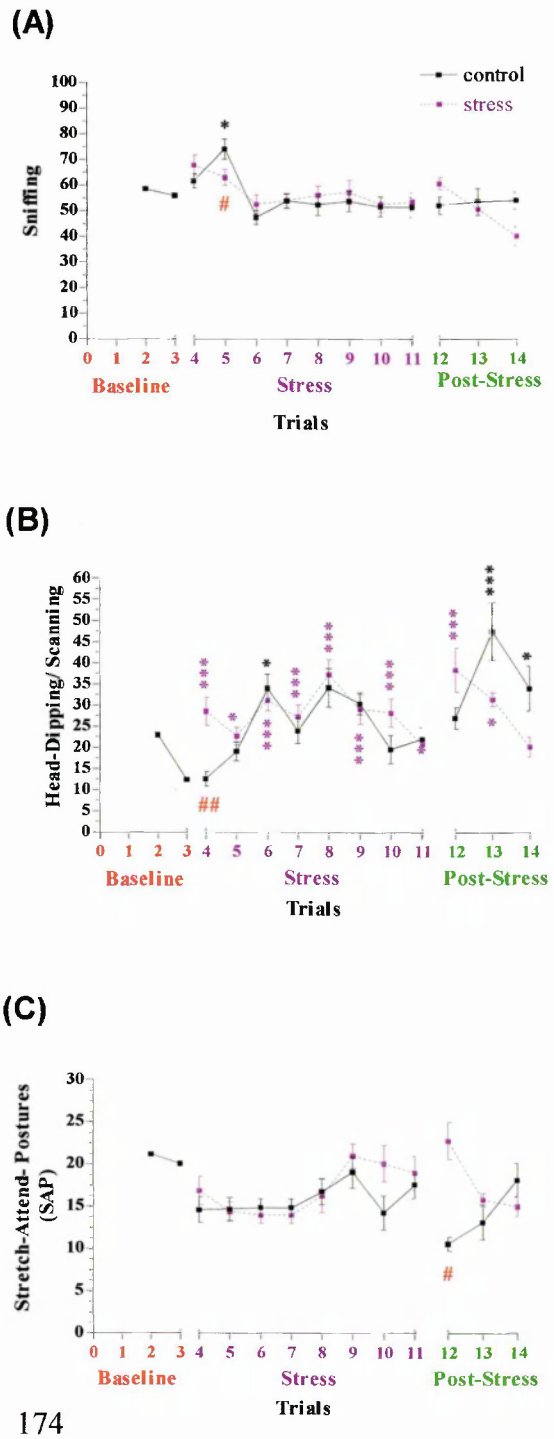


BALB/c mice (n=8 per group) were subjected to elevated plus-maze (EPM) tests immediately following exposure to predator stress. Data are expressed as mean ± sem. **(A)** Total counts of closed arm returns, **(B)** total counts of end-exploring of the arms , and **(C)** total counts of rearing, during a 5 min trial per week (n = 21 for baseline and weeks 1-4 of the stress, n = 14 for weeks 5-8 of the stress, and n = 7 for the 3 weeks after the stress was removed). Mice were given free access to food and water throughout. Baseline values are the means prior to allocation to either of the groups. #, $p<0.05$ compared to control; *, $p<0.05$ compared to baseline, within the control group; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ compared to baseline, within the stress group.

Figure.4.10.

Risk assessment behaviours exhibited on the EPM following exposure to predator stress.

BALB/c mice (n=8 per group) were subjected to elevated plus-maze (EPM) tests immediately following exposure to predator stress. Data are expressed as mean ± sem. **(A)** Total counts of sniffing, **(B)** total counts of head-dipping /scanning , and **(C)** total counts of stretch-attend postures (SAP), during a 5 min trial per week (n = 21 for baseline and weeks 1-4 of the stress, n = 14 for weeks 5-8 of the stress, and n = 7 for the 3 weeks after the stress was removed). Mice were given free access to food and water throughout. Baseline values are the means prior to allocation to either of the groups. #, $p<0.05$, ##, $p<0.01$ compared to control; *, $p<0.05$ compared to baseline, within the control group; *, $p<0.05$; ***, $p<0.001$ compared to baseline, within the stress group.



4.5 Discussion

Plasma corticosterone concentrations were significantly elevated by chronic predatory stress, compared to controls, and this response was abolished 2 weeks after the removal of the stressors. According to previous investigations, exposing mice to a rat reliably increases plasma corticosterone concentrations and may affect immune functioning (Lu *et al*, 1999).

Previous findings (Pellow *et al*, 1985; Holmes *et* al, 1998) have shown that repeated exposure to the plus-maze results in significant elevated plasma corticosterone concentrations. The current results show there was no difference between the group of mice that had been tested on the plus-maze for the first time after 8 weeks and the group that had been tested weekly for 8 weeks, suggesting that repeated exposure to the plus-maze alone had no significant effect upon the plasma corticosterone levels in this current study.

There was no significant difference (**Table.4.2.**) in B_{max} or K_d values of GR, between stressed and control mice throughout the study. There was a trend in Bmax towards an increase in the stressed mice at 4 weeks, compared to their controls, but this did not reach statistical significance, suggesting a possible upregulation of glucocorticoid receptors after 4 weeks of stress.

It would have been beneficial if the current study had examined GR mRNA as well as GR, as stressful stimuli are known to affect GR mRNA levels in the rat brain. A recent study by Paskitti and colleagues (2000) examined the effect of acute and chronic stress on the regulation of the expression of the GR and MR genes in the hippocampal formation. They concluded that acute stress decreases the availability of GR mRNA without demonstrably affecting transcription, suggesting reduced GR mRNA stability. Therefore acute stress decreases GR mRNA expression by largely post-transcriptional mechanisms. However, elevations in basal corticosterone secretion seen following chronic stress are not

sufficient to markedly down-regulate GR/MR expression in a long-term fashion. Kitraki *et al* (1999), showed a statistically significant down-regulation of GR mRNA both in the hippocampus and in the cerebellum of rats stressed for 8, 10 and 14 days. The same degree of down-regulation was also detected in the same brain areas of rats stressed for 14 days and left undisturbed for 48h or 8 days after stress.

Transgenic mice expressing antisense RNA against GR have shown that impairment of GR evolves in disturbed neuroendocrine regulation and certain behavioural responses to stress. This life-long glucocorticoid receptor impairment has profound consequences for neuroendocrine regulation (unaltered corticosterone concentrations) and certain behavioural responses (increased grooming behaviour) to stress (Linthorst *et* al, 2000).

The results obtained in this study showed that sucrose preference remained very high at about 80-90% (**Figure 4.3.**). Thus, this study did not show that the sucrose preference test detected any changes in the mice after exposure to predator stress. This contrasts with the results obtained in a similar study by Calvo-Torrent et al (1999), who showed that predatory stress attenuated the level of sucrose intake after 2-3 weeks of chronic exposure, similar to that observed with a variety of other stressors in rats (Katz, 1982; Papp *et* al, 1996) and mice (Monleon *et al*, 1995). There are a number of differences between these two experiments: the animals used (they use CD1 mice, whereas we use BALB/c mice), test procedures differ (we handled our mice daily, theirs appeared unhandled; they tested during the dark phase, we tested during the light; they had food and water deprivation prior to the sucrose tests, we had food and water freely available throughout; our trials on the elevated plus-maze were 5 min, whereas theirs were 10 min), predatory stress procedures differed (their stress room consisted of male rats paired with females, offspring with their mothers as well as young group-housed male and female rats, whereas ours only consisted of group-housed male Brown Norway rats; their stressed mice

could only smell and hear the rats, whereas ours also had the physical presence of the rats). Both the sucrose preference and elevated plus-maze data are very sensitive to methodological factors, any one (or combination) of these variables could account for the differences observed.

Food consumption followed a similar trend in both groups and gradually decreased throughout the study. No significant differences were found between groups and within groups in term of their body weights (**Table.4.3.**). So although the mice appeared to reduce their food intake after exposure to predator stress, this did not appear to have an impact upon their body weights.

Despite previous studies showing that individual housing can increase food consumption and body weight in rats, and that social isolation is associated with pain inhibition and increased measures of anxiety, Moles and Cooper (1995) did not find any difference in the sucrose intake in individually housed male mice in comparison with male mice housed in pairs. This suggests that, unlike male rats, male mice do not consume a higher amount of palatable food when housed in social isolation. Differences in the social organisation of the two species may account for the diverse impact of individual housing on isolation-induced food intake. In fact, as territorial animals, male mice tend, in natural situations, to be "behaviourally isolated" in that they do not tolerate mature conspecifics in their territory.

Muscat and Willner (1992) demonstrated that the reduction of sucrose consumption by chronic mild stress is independent of housing conditions. In particular, the effect does not depend upon the imposition of isolation stress on the control group. Therefore, this current study has not shown the widely hypothesised theory that there should be a significant reduction in sucrose consumption, and preference, by the stressed mice. It has also been suggested by Muscat and Willner (1992), that stressed animals actually tend to

eat more than controls, rather than less, an effect also observed in other studies. This was not evident in the current study.

After a week's exposure to the predator, the stress mice showed an increase in closed arm entries and total arm entries compared to their controls (**Figure.4.5. and Figure.4.6.**). There were also increases in non-exploratory behaviour, i.e. grooming (**Figure.4.8A**), as well as risk assessment behaviours, i.e., end-exploring, rearing, sniffing and scanning (**Figure.4.9. and Figure.4.10.**). After 8 weeks of chronic predatory stress, stressed mice visited the open and closed arms more frequently, although the time spent in them was unchanged from their controls. No differences were found between groups in terms of risk assessment or emotionality, as expressed by the amount of faecal boli present on the maze (**Figure.4.8B**).

The first two weeks after the removal of stress provoked an increase in open arm entries in the stress group and a decrease in the control group. This suggests that control mice were more stressed and did not want to enter the open arms and remained in the closed arms, and the stress mice become bolder and wanted to enter the open arms more frequently than before and stayed away from the closed arms. This is reflected in the total arm entries, whereby there is a 170% increase by the stress mice, compared to the controls, in Trial 13 (**Figure.4.6.**).

In an extensive investigation of the plus-maze performance of 16 inbred mouse strains, Trullas and Skolnick (1993) found that over 70% of the variance in open-arm activity measures could be attributed to genetic factors. Using a derived index of plus-maze responsivity, they were able to categorise strains into four distinct groups: nonreactive (e.g., BALB/c), intermediate-low reactive (e.g., C3H.SW/SnJ), intermediate-high reactive (e.g., DBA/2J), and high reactive (e.g., C57BL/6J).

Despite these strain differences in initial response patterns, present findings confirm that prior maze experience produces major changes in the way in which rodents react to this test (Lee & Rodgers, 1990; Gonzalez & File, 1997). Ethological analysis demonstrates that changes occur in many of the behaviours and not just the conventional plus-maze indices. Measures that were displayed consistently between trials were entry latency (decrease), centre time (decrease), head dipping (decrease), percent open entries (decrease), closed-arm entries (increase), and closed time (increase). Thus, on retest, mice move more rapidly from the centre platform into a closed arm, spend more time in the closed arms and less time on the centre platform, and show less exploratory head-dipping. The present results from the control mice confirm this, although they showed an increase in exploratory head-dipping.

According to Calvo-Torrent et al (1999), after being exposed to chronic predatory stress, stressed mice showed a higher level of anxiety (spent less time on the open arms and showed a lower frequency of unprotected head-dipping) than controls, although no differences in the level of emotionality were observed.

There is a relationship between preference for the closed arms and the total number of arm entries. An animal that has a marked aversion to the open arms is restricted to exploring only two (closed) arms, in contrast to the animal that does venture out on to the open arms. It is suggested that it would therefore habituate more rapidly to the two closed arms, thereby making fewer total entries than the animal that explores all four arms. The present results showed that the mice, in both groups, remained almost exclusively in the closed arms, but the stressed mice significantly enter more arms than the control mice. So although they appeared to be limited to only two closed arms, the stressed mice seemed more active and preferred to move rapidly between the two arms.

The influence of the central square on the behaviours detected in the plus-maze is largely unknown, although it has been suggested that the exploratory behaviours seen in

this area of the maze may relate to some kind of assessment and/or decision-making process. In this current study, time spent on the central platform was not statistically significant between the groups. Although, two factors relating to central square activity were identified in the factor analysis performed in the Fernandes and File study (1996), and these factors were considered to separately reflect assessment and/or decision-making related to the openness and to the height of the maze viewed from the protected areas of the maze. Previous factor analyses have also identified factors related to central square activity, and this area may, thus, play an integral role in determining the avoidance of the open arms of the plus-maze.

Either forced or voluntary passage onto the open arms of the EPM has been associated with elevated plasma corticosterone concentrations, increased freezing, and production of faecal boli, hormonal and behavioural changes that are indicative of increased anxiety. Expression of the open arm data as % of the total number of arm entries (% open entries) or total time spent (% total time) on either the open or closed arms corrects for overall changes in exploration of the maze and helps reduce activity-induced artefacts. Locomotor activity was assessed by monitoring the total or closed arm entries, the latter being a purer measure as it changes independently of % open entries and % total time.

The behaviour of the mice on the EPM did not appear to change with repeated testing according to this present data, although contradictory results have been described in the literature. Treit *et al* (1993) not only reported that rats increase their avoidance of open arms on retest, but also found no evidence of habituation after 18 daily trials. Furthermore, a single forced exposure to the open arms greatly reduced 24 h retest escape latencies from an open arm and markedly increased time spent in the enclosed arms. This evidence suggests that rodents retain a strong memory for the threat posed by the open arms, and it is consistent with an experimentally induced sensitisation of fear.

The EPM data in this study appears badly flawed, perhaps due to the set-up alone being too "stressful," i.e., maybe the 60W spotlight was too bright or the experiment should have been conducted under less intense red light, the mice may have needed time to habituate to the test room before being placed onto the maze, using opaque perspex made the maze more anxiolytic with the light, maybe we should have tested during the dark phase etc. These factors may have contributed to an anxiogenic effect caused by the plus-maze alone in this study, attributing to the mice remaining primarily in the closed arms and therefore proving impossible to detect a change with the added predator stressor.

Falter *et al* (1992) showed that none of the environmental changes, including light intensity, form or height of the maze, or a stressful experience prior to testing, such as immobilisation or electric shock, was able to alter the pattern of exploration in the plus-maze. The lack of effect of such a wide range of stressors suggests that the anxiety modelled in the plus-maze in that particular rat population was rather a situational-bound response, elicited in rodents introduced into that particular environment.

A similar resistance in the baseline exploration level in control animals, to modification by external factors, has previously been reported by Pellow (1986) who found that neither habituation, increased illumination, nor isolation, induced significant changes in the exploration of the control animals. Nevertheless, under certain extreme conditions, it is possible to decrease significantly the open arm visits in control animals. For instance, by increasing the light intensity on the plus-maze from 20 lux up to 1200 lux, Morato and Castrechini (1989) succeeded in reducing significantly the exploration of the open arms. Long-term social isolation from the day of weaning constitutes another extreme condition. Isolated animals are aggressive and neophobic as well as show an anxiety-like profile in the elevated plus-maze test and increased locomotor activity (Hilakivi *et al*, 1989).

One important inter-laboratory variation in plus-maze methodology is the presence of open arm ledges. Ledges have been added to the open arms to both encourage open arm

exploration and to prevent animals falling off the maze following drug administration. However, the results of Fernandes and File's (1996) study have shown that the inclusion of ledges on the open arms is not a trivial alteration in plus-maze construction. Comparison of the factor analyses on the behaviours measured in the plus-mazes, with and without ledges, found clear distinctions between the two mazes.

The presence of ledges not only reduced the value of the number of closed arm entries, as a measure of activity in the maze, again stressing the need for caution when interpreting activity in this test, but on trial 1 also shifted the loading of unprotected head-dipping from the factor reflecting anxiety to a separate factor. It is possible that there is a reduction in the nature/extent of the anxiety/fear presented by the open arms with ledges, and as a consequence, head-dipping behaviour no longer relates to anxiety but to a directed exploratory behaviour assessing the height of the maze.

4.6 Conclusions

These results indicated that exposure to the presence and odour of a potential predator (rat) increased the plasma corticosterone concentrations and that this response was abolished 2 weeks after the removal of the predator, indicating that this natural threat was a source of stress in these male mice.

DEVELOPMENT AND USE OF "STRESS" ANIMAL MODELS OF DEPRESSION TO STUDY THE MODE OF ACTION OF ANTIDEPRESSANT DRUG TREATMENTS

Volume II of II

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A thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy

2003

North East Surrey College of Technology (Nescot)

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CHAPTER 5. THE EFFECT OF ACUTE AND CHRONIC RESTRAINT STRESS IN RATS UPON THE HPA AXIS

5.1. Introduction

5.1.1. Restraint stress as a model for stress / depression

The ability to respond to stress is a fundamental mechanism in mammals, one that involves the activation of the HPA axis (Sapolsky & Meaney, 1986), culminating in the release of corticosterone from the adrenal cortex.

In 1936, Selye reported that certain physiological changes occurred in experimental animals exposed to a wide variety of stressors. These changes represented some of the classic signs of the stress syndrome – adrenal hypertrophy and thymicolymphatic involution. Selye observed that immobilising or restraining the rat led to the manifestation of his stress syndrome (Selye, 1936). Many different restraint procedures have subsequently been used. Historically, the restraint procedure was used as an experimental procedure for producing gastric lesions in the rat. In the 1960's, Bonfils and Lampling (1963) popularised the restraint procedure, and the technique was intensely studied in North America by Brodie and his co-workers (for a review see Brodie, 1971). Since then, many effective procedures have been developed for immobilising animals and the data generated has expanded to include central neurochemical consequences of stress, as well as drug effects upon these responses. Immobilisation is now used as a standard stress procedure for the purpose of observing the various physiological and biochemical responses to stress.

5.1.2. Endocrinological changes following restraint stress

Exposure of rats to stressful stimuli results in a marked elevation of plasma ACTH and corticosterone concentrations (Cook *et al*, 1973; Suemaru *et al* 1985). It is generally recognised that corticosteroid hormones exert a negative feedback signal, on both the pituitary and the hypothalamus, capable of inhibiting HPA axis activity.

Repeated exposure to stress often leads to adaptation within stress response systems that is evident by a change in the magnitude of subsequent elicited stress responses. In some cases repeated stress leads to an enhanced or sensitised stress response and in other cases it leads to blunted or habituated stress response (Pitman *et al*, 1990; McCarty & Gold, 1996).

Repeated homotypic stress can result in habituation of corticosterone responses, and this has been described for restraint stress (Armario *et al*, 1988; Hashimoto *et al*, 1988). The desensitisation of corticosterone responsiveness to repeated restraint may in part be associated with the desensitisation of the pituitary ACTH response and down-regulation of the anterior pituitary CRH receptors, but exposure of chronically stressed rats to a different (heterotypic) stressor induces greater and more rapid increases in plasma ACTH and corticosterone concentration (Hashimoto *et al*, 1988). This suggests that a centrally mediated mechanism can selectively modulate the response to different incoming signals. Studies of animals exposed to stress have demonstrated that differential regulation of negative feedback is possible, depending on the particular variables of stress exposure (Young *et al*, 1990; Liberzon *et al*, 1997).

Cook *et al* (1973) reported that there was no decrease in the ability to maintain an elevated ACTH concentration during 2 h of ether anaesthesia and assumed that the magnitude and duration of the increase in plasma ACTH concentration was related to the duration and intensity of the ACTH-releasing stimulus. On the other hand, it has been reported that adaptation of the pituitary-adrenal response occurs during more prolonged stress, e.g. continuous sound or immobilisation stress (Henkin & Knigge, 1963; Bohus, 1969). Rivier and Vale (1987) reported that rats exposed to electroshocks for 3-5 h showed a marked increase in plasma ACTH concentrations 10 min after the beginning of the stress, followed by a decline despite continuous exposure to the stimulus, and they concluded that the inability to maintain elevated plasma ACTH concentrations appeared to be mediated through both the temporary decrease in a readily releasable pituitary ACTH pool and the

negative feedback exerted by corticosterone. On the other hand, Sakellaris and Vernikos-Dannellis (1975) reported that the pituitary-adrenal system was not inhibited by the circulating steroid level but actually sensitised to other stimuli. They and others (Dallman & Jones 1973; Armario *et al*, 1985) have postulated that repeated chronic stress, i.e., cold and restraint, might cause an "increased drive" to the ACTH-secreting mechanism, which compensates or overrides the corticosteroid feedback.

Although the importance of stress in life appears to be significant, the cellular and molecular mechanisms involved in the pathophysiology of stress remain largely unknown. Corticosteroids are known to mediate stress-related endocrine, behavioural, and autonomic responses. However, hardly any systematic work has studied the regulation of corticosteroid action during prolonged repeated stress. Immobilisation/restraint stress is the most commonly used paradigm to study the stress-related biological, biochemical, and physiological responses (Alexandrovà, 1994). The available data on plasma concentrations of corticosteroids are mostly confined to a single acute or 7 day chronic immobilisation/restraint stress in rats (Meaney *et al*, 1991; Aguilera *et al*, 1996). It is reported that circulating corticosteroids reach a ceiling after a single acute immobilisation/restraint stress (Omrani *et al*, 1980; Meaney *et al*, 1991). However, it is not clear whether prolonged repeated stress results in further elevations in circulating corticosteroid concentrations or if there is some kind of adaptation in the release of corticosteroids by rats to chronic stress. It is now abundantly clear that cell- and tissue-specific glucocorticoid actions are mediated through receptors (GR and MR), and there is good correlation between cellular concentrations of these receptors and biological responses (Vanderbilt *et al*, 1987). Unfortunately, at the present time, our understanding of the regulation of these receptors during acute and repeated stress remains limited.

Several chronic stress paradigms have been shown to reduce MR and GR mRNA concentration (Herman & Watson, 1995; Makino *et al*, 1995b) or receptor binding densities in the rat brain (Sapolsky *et al*, 1984). Following a chronic variable stress

paradigm, GR mRNA expression has been shown to be negatively correlated with PVN CRH mRNA expression, suggesting a relationship between elevated CRH gene expression and down-regulation of GR at the level of the PVN (Herman *et al*, 1995). Many of these effects are dependent upon the sustained high concentrations of corticosterone that are produced during the stressor.

In the past five years, there have been important advances in understanding the physiology of the CRH system and its response to stress. The role of CRH receptor subtypes in emotional processes and its relation to depression and anxiety behaviours has been recently reviewed (Holsboer, 1999; Steckler & Holsboer, 1999).

Stress is known on the one hand to activate hypothalamic CRH pathways, which are involved in the regulation of the endocrine responses of the pituitary, and on the other hand to modulate the activity of extrahypothalamic CRH neuronal networks, which are responsible for the coordination of behavioural and autonomic reactions to stress (Fisher & Brown, 1983; Dallman, 1993). For example, chronic immobilisation stress has been shown to increase CRH immunoreactivity in the anterior hypothalamus, the paraventricular hypothalamus, and the locus coeruleus, and to decrease CRH immunoreactivity in the medial preoptic area and the dorsal vagal complex of the rat brain (Chappell *et al*, 1986).

An immune challenge or an immobilisation stress can induce a very selective and strong activation of the CRH type 1 receptor mRNA within hypothalamic nuclei involved in the regulation of neuroendocrine functions, particularly the parvocellular division of the PVN (Rivest *et al*, 1995). This fact, taken together with the ability of CRH to activate its own gene expression selectively within the PVN (Parkes *et al*, 1993), supports the existence of an ultra-short loop positive feedback mechanism through which CRH may modulate its own biosynthesis.

There are several lines of evidence suggesting that neurotrophin function is altered in stress-related affective disorders, and that increased BDNF could be involved in the aetiology and treatment of these illnesses. First, chronic stress is reported to cause atrophy and, in some severe cases, death of vulnerable CA3 neurones in the hippocampus (Wooley *et al*, 1990). Atrophy of hippocampal neurones has also been observed after repeated chronic stress in rodents, and in response to psychosocial stress in nonhuman primates. Second, stress is reported to decrease the expression of BDNF in CA3 pyramidal and dentate gyrus granule cell layers in the hippocampus (Smith *et al*, 1995b). Down-regulation of BDNF could contribute to the atrophy of CA3 neurones, or render these neurones more susceptible to other factors, such as corticosteroids, that are induced in response to repeated stress. Third, brain imaging studies have shown that there is a small, but significant reduction in the volume of the hippocampus in patients with depression or posttraumatic stress disorder (Sheline *et al*, 1996; Sapolsky, 1996). Atrophy and decreased function of the hippocampus could explain the loss, in depressed patients, of negative feedback control that this brain region exerts on the HPA axis (Young *et al*, 1991).

Sweet solutions, such as saccharin and sucrose, are palatable to rodents and are consumed in preference to water (Collier & Novell, 1967). In the CMUS model, responsivity of rats to reward is measured by preference for a palatable sucrose solution over tap water and by absolute sucrose consumption (Willner *et al*, 1987). The first publication describing the model (Willner *et al*, 1987) reported a reduction in sucrose preference after 3 weeks of CMUS, and later publications reported a deficit in consumption apparent after 2 or 3 weeks of CMUS (Papp *et al*, 1991; Willner *et al*, 1994). In these latter reports, preference data were either not obtained or not reported.

The relationship between glucocorticoids and consumption of sweet drinks is unknown. Bhatnagar and colleagues (2000) showed that the voluntary intake of sweet saccharin (2 mM) is strongly and persistently determined by the circulating concentrations of corticosterone, suggesting that corticosterone strongly affects the reinforcing properties of pleasurable experiences.

Płaźnik *et al* (1989) used a model of acute restraint stress (1 h), whereby they produced a short-term, but significant decrease of saccharin preference in a two-bottle test. As far as we are aware, sucrose preference and therefore anhedonia has not been tested after chronic restraint-stress in rats.

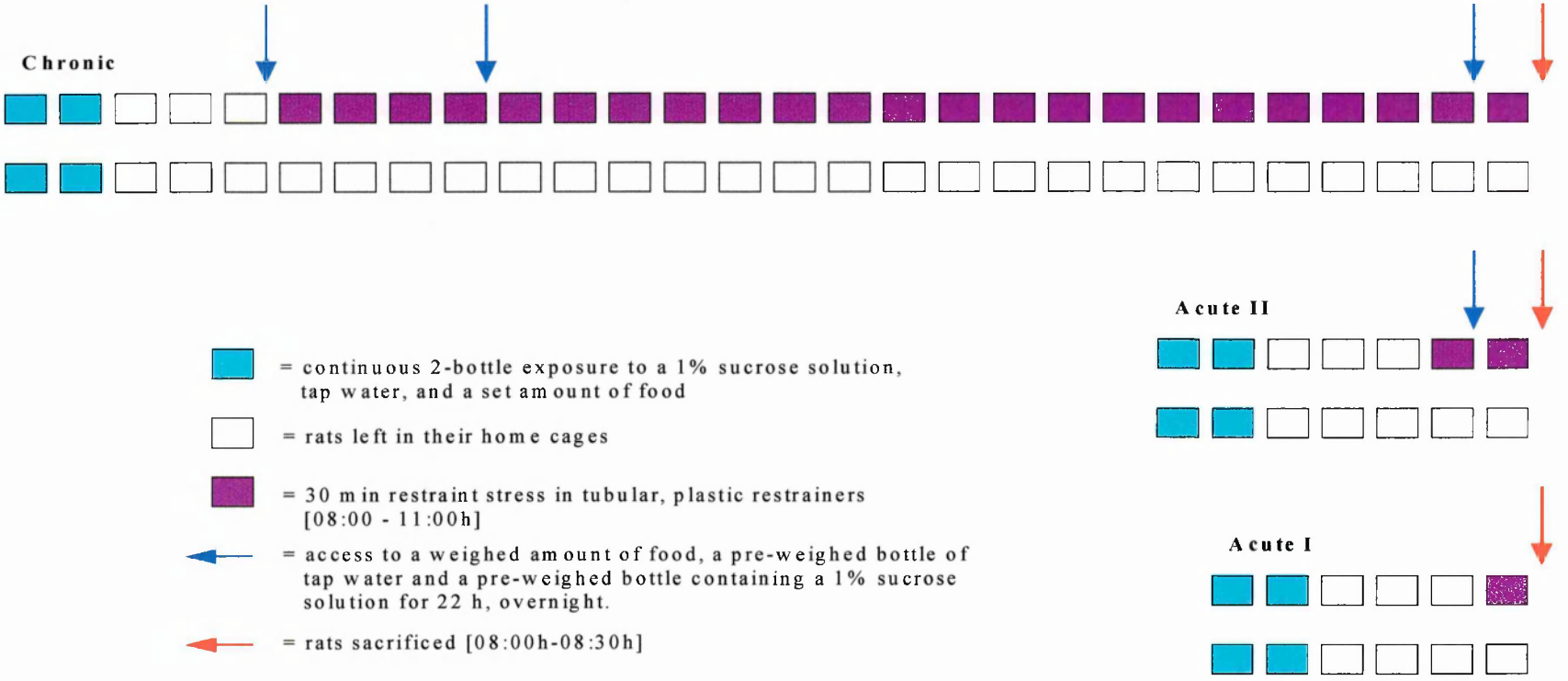
5.2. Aims

To study a stress paradigm in which rats are repeatedly exposed to restraint stress, which is considered an example of a psychological or processive stressor in which the triggering of a stress response results from the situation (of being trapped in the tube) rather than a result of direct noxious stimuli (Herman *et al*, 1996). The present chapter focused on the HPA axis as a stress response system to monitor (Dhabbar *et al*, 1997; Kalman *et al*, 1997), using plasma concentrations of corticosterone and ACTH as indicators of stress. Specifically examining the effect of restraint stress on GR density, GR mRNA, CRH type 1 receptor mRNA and BDNF mRNA concentrations in the brain. Sucrose preference was used as a behavioural measure of anhedonia.

5.3. Study Design

The following chapters (Chapters 5, 6 and 7) are part of one study that has been separated in order to approach and discuss each particular aspect of the study. Refer to Figure. 5.1. for a description of the study design for this specific chapter.

Figure.5.1.



A schematic diagram of the study design, refer to sections 5.4.2. and 5.4.3. Each square represents 1 day.

5.4. Methods

5.4.1. Animals

Adult male Wistar rats weighing 250-300 g were housed in pairs and maintained in a controlled temperature (20-22°C), constant humidity (~ 60%) and a 12 hr light:dark cycle (lights on from 7:00 h to 19:00 h). Rats were bred in the Biological Research Facility at St. George's Hospital Medical School. Food and tap water were available *ad libitum*. Rats were weighed weekly for the duration of the investigation. The use of these animals in the following scientific procedures were regulated by the Animals (Scientific Procedures) Act 1986.

5.4.2. Restraint Stress Procedure

Rats were handled twice daily up until the time of testing in order to minimise procedural stress (approximately 7-10 days prior to experimentation). To minimise variation in plasma corticosterone and ACTH concentrations during the day, the restraint stress procedure was carried out between 8:00 and 11:00 h. As reviewed in the literature (Reul and DeKloet, 1985; Spencer *et al*, 1990), it is well established that the plasma corticosterone concentration is lowest during morning hours and, thus, the maximum concentration of corticosterone responses in animals should be provoked during the morning.

Restraint stress was conducted on a tabletop in a room adjacent to the holding room, in well-ventilated adjustable length cylindrical plexiglass tubes (6.3 cm diameter and 15.5 ± 2.5 cm length). **Acute II** and **Chronic** groups of rats also received 1ml/kg p.o. of distilled water, 30 min prior to the first restraint stress.

All stress procedures were carried out in compliance with National Animal Welfare Laws and Home Office Guidelines of the Care & Use of Laboratory Animals.

5.4.3. Sucrose Preference Tests

Sucrose preference tests were conducted before restraint stress and after day 1, day 4 and day 22 of restraint stress (**Figure. 5.1.**). On these days, during restraint stress, rats were released from the restrainers after 30 min and placed back into their home cages, in their holding room. There they had access to a weighed amount of food, a pre-weighed bottle of tap water and a pre-weighed bottle containing a 1% sucrose solution for 22 h, overnight. At the end of the sucrose preference test, the sucrose, water and food were removed and weighed. Control rats were also subjected to a sucrose preference test at the same times.

5.4.4. Radioimmunoassay of plasma corticosterone and ACTH concentrations

Plasma corticosterone and ACTH concentrations were measured by using radioimmunassay commercial kits (refer to Section 2.4.).

5.4.5. Other Assays

All sacrifice and dissection procedures were conducted as described in section 2.3.2, and took place in a room separate from both the holding room and the room in which the animals were stressed. Preparation of rat cytosolic fractions was carried out as described in section 2.3.5. Subsequent procedures and analysis were carried out as described in section 2.3.7.

Protein content was measured using Lowry's method with bovine albumin as standard (Lowry *et al*, 1951).

Preparation of rat tissue was carried out essentially as described in section 2.5.1.2. Subsequent RNA isolation, RT-PCR and agarose gel electrophoresis and analysis were carried out as described in section 2.5.

5.5. Statistical Analysis

The data is presented as the arithmetic mean +/- s.e.mean. The effects of time ("days") and restraint-stress on the biochemical and behavioural measures were analysed by two-factor completely randomised ANOVA (restraint-stress x time), followed by Newman-Keuls post-hoc comparisons. Statistical analyses were performed using the software package, "GBStat" v6.5 .

5.6. Results

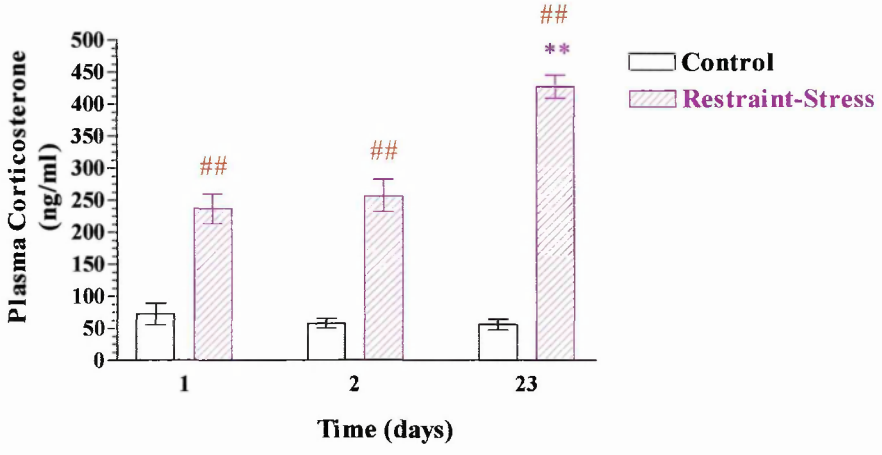
5.6.1. Plasma ACTH and Corticosterone Concentrations.

1. Corticosterone

Statistical analysis indicated that restraint stress exposure alone ($F_{1,29}$=272.5; p<0.0001), time alone ($F_{2,29}$=14.34; p<0.0001) and the interaction between restraint stress and the length of exposure ($F_{2,29}$=18.14; p<0.0001) significantly affected the plasma corticosterone concentration (**Figure.5.2.**). A single, 30 min restraint stress exposure increased plasma corticosterone concentration by just over 300% compared to controls. A second exposure produced a similar increase. Repeated exposure to restraint stress increased plasma corticosterone concentration by almost 700% compared to control.

Figure.5.2.

Plasma corticosterone concentration following exposure to restraint-stress.



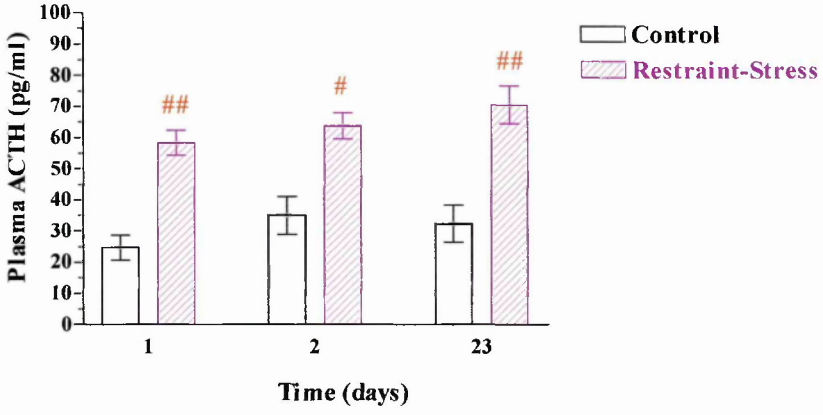
Plasma corticosterone concentration in male Wistar rats, housed in pairs (n=8 per group), were measured in samples taken immediately following exposure to restraint stress. Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group) and rats restrained for 30 mins/day (restraint-stress group) for 1 day, 2 days or 23 days before they were sacrificed. ##, p<0.01 compared to control; **, p<0.01 compared to one 30 min exposures to restraint stress.

2. Adrenocorticotrophic Hormone (ACTH)

Statistical analysis indicated that restraint stress alone significantly affected ($F_{1,27}$=63.63; p<0.0001) the plasma ACTH concentration (**Figure.5.3.**). A single, 30 min restraint stress exposure increased plasma ACTH concentration by 136% compared to controls. A second restraint stress produced similar results. Repeated exposure to restraint stress increased plasma ACTH concentration by just over 100% compared to control.

Figure.5.3.

Plasma ACTH concentration following exposure to restraint-stress.



Plasma ACTH concentration in male Wistar rats, housed in pairs(n=8 per group), were measured in samples taken immediately following exposure to repeated daily restraint stress. Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group) and rats restrained for 30 mins/day (restraint-stress group) for 1 day, 2 days or 23 days before they were scarificed. #, p<0.05; ##, p<0.01 compared to control.

5.6.2. Glucocorticoid Receptor (GR) Binding

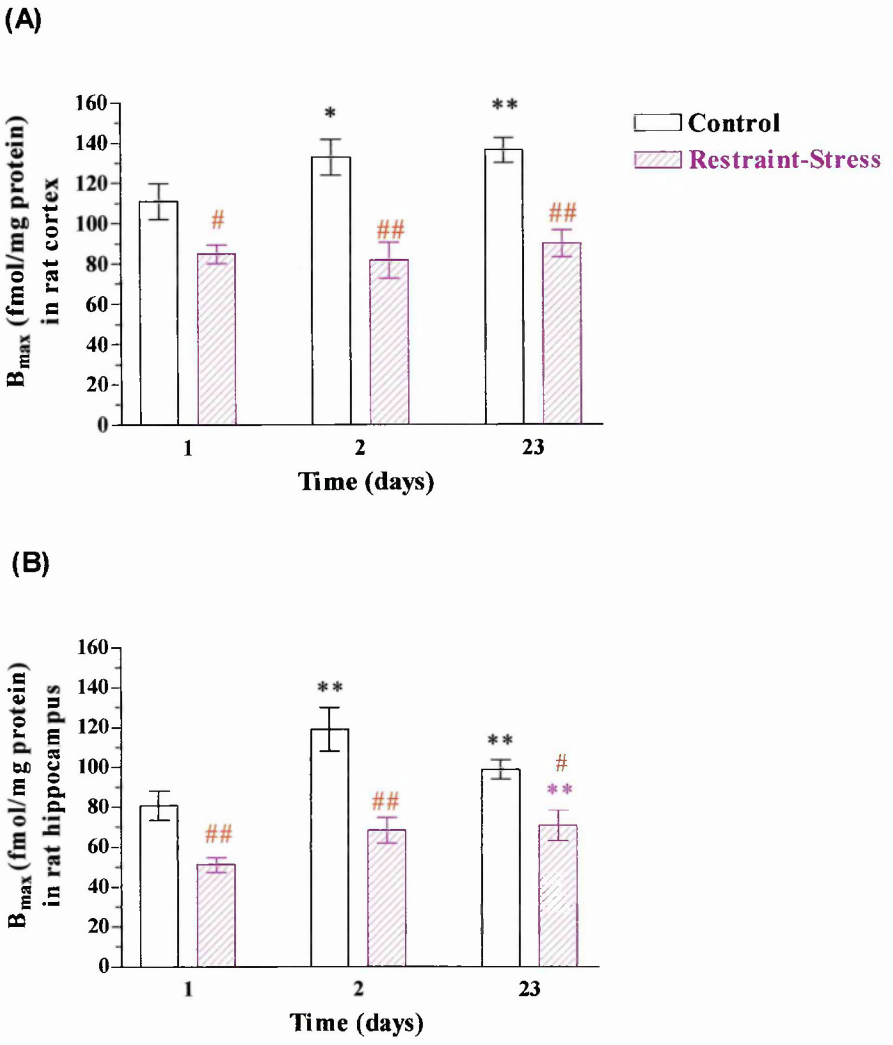
1. B_{max}

Statistical analysis indicated that restraint stress alone significantly affected ($F_{1,31}$ = 67.0; p<0.0001) the B_{max} values from glucocorticoid receptor binding within the cortex (**Figure.5.4A**). A single, 30 min restraint stress exposure decreased cortical B_{max} values by 24% compared to controls, which remained the same following a second exposure. Repeated exposure to restraint stress decreased cortical B_{max} values by 34% compared to control. Control values varied between 110 and 140 fmol/mg protein.

In the hippocampus, statistical analysis indicated that restraint stress alone ($F_{1,30}$ = 54.4; p<0.0001), length of time of the experiment ($F_{2,30}$= 12.14; p=0.0001), and the interaction of restraint stress with the length of exposure ($F_{2,30}$ = 12.10; p=0.0001) significantly affected the B_{max} values from glucocorticoid receptor binding (**Figure.5.4B**). A single 30 min restraint stress exposure decreased B_{max} levels by 40% compared to controls. Repeated exposure to restraint stress decreased hippocampal B_{max} levels by 30% compared to control. Control values were more variable than in the cortex (ranging from 80 and 120 fmol/mg protein).

Figures.5.4.

Glucocorticoid Receptor B_{max} values from cortical (A) and hippocampal (B) cytosolic fractions following exposure to restraint stress



GR B_{max} values in cortex and hippocampus (taken from male Wistar rats, housed in pairs (n = 8 per group)) were measured in samples taken immediately following exposure to daily restraint stress. Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group) and rats restrained for 30 min/day (restraint-stress group) for either 1 day, 2 days or 23 days before they were scarificed, **(A)** B_{max} in cortical tissue **(B)** B_{max} in hippocampal tissue. #, $p<0.05$ and ##, $p<0.01$ compared to control; *, $p<0.05$and **, $p<0.01$ compared to control after 1 day; **, $p<0.01$ compared to one or two 30 min exposures ro restraint stress.

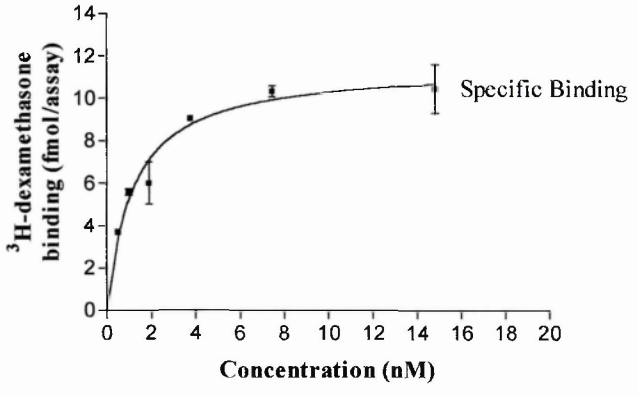
2. K_d

Statistical analysis indicated that restraint stress alone ($F_{1,37}$ = 66.04; p<0.0001) and the length of time of the experiment ($F_{2,37}$ = 5.078; p=0.0113) significantly affected the K_d levels from glucocorticoid receptor binding within the cortex (**Figure.5.6A**). A single, 30 min restraint stress exposure increased cortical K_d values by 180% compared to controls, which increased by a further 40% following a second exposure. Repeated exposure to restraint stress increased cortical K_d levels by 140% compared to control. An example of a saturation curve is represented in **Figure. 5.5**.

In the hippocampus, statistical analysis indicated that restraint stress alone ($F_{1,33}$ = 5.125; p=0.0303) and the length of time of the experiment ($F_{2,33}$ = 10.97; p=0.0002) significantly affected the K_d levels from glucocorticoid receptor binding (**Figure.5.6B**). A single 30 min restraint stress exposure increased K_d levels by 64% compared to controls. A second restraint stress produced similar results. Repeated exposure to restraint stress increased hippocampal K_d levels by 30% compared to controls. Control values were more variable than in the cortex (ranging from 1.00 and 1.88 nM).

Figure 5.5.

Example of a saturation plot of ^{3}H-dexamethasone binding to GR in a rat cortical cytosolic fraction following exposure to restraint stress for 1 day

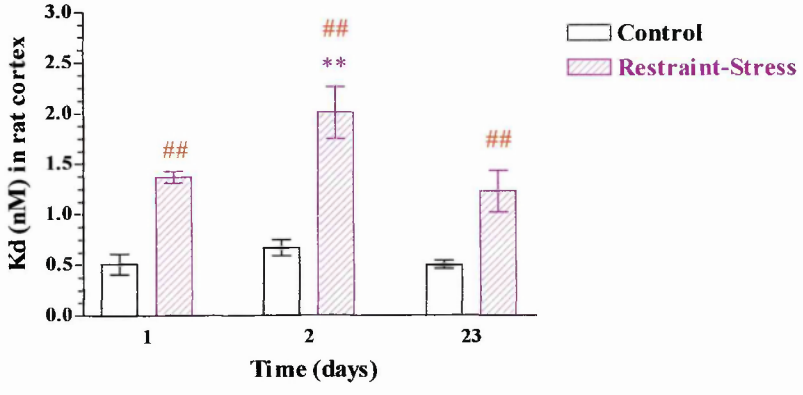


B_{max} = 11.49 fmol/assay (equivalent to 349.2 fmol/mg protein) and K_d = 1.17 nM

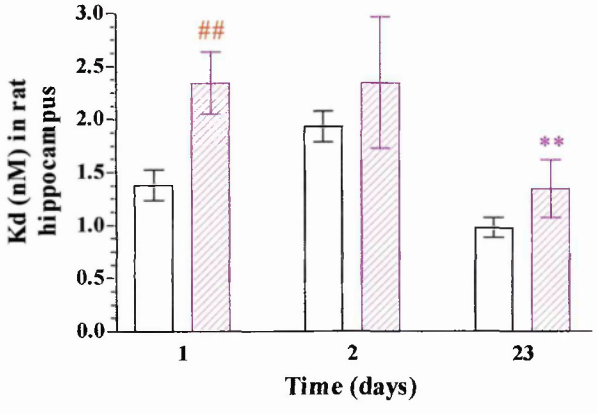
Figures.5.6.

Glucocorticoid Receptor K_d values in cortical (A) and hippocampal (B) cytosolic fractions following exposure to restraint stress.

(A)



(B)



GR K_d values in cortex and hippocampus (taken from male Wistar rats, housed in pairs (n = 8 per group) were measured in samples taken immediately following exposure to daily restraint stress. Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group) and rats restrained for 30 min/day (restraint-stress group) for either 1 day, 2 days or 23 days before they were sacrificed, (**A**) K_d in cortex (**B**) K_d in hippocampus. ##, $p<0.01$ compared to control; **, $p<0.01$ compared to one or two 30 min exposures to restraint stress.

5.6.3. Glucocorticoid Receptor (GR) mRNA

Cortical GR mRNA levels did not change following a second exposure of restraint stress, compared to control. Repeated exposure to restraint stress had no effect compared to control (**Figure.5.7.**). Control values varied between 0.6 and 1.1 (arbitrary units).

5.6.4. Brain-Derived Neutrophic Factor (BDNF) mRNA

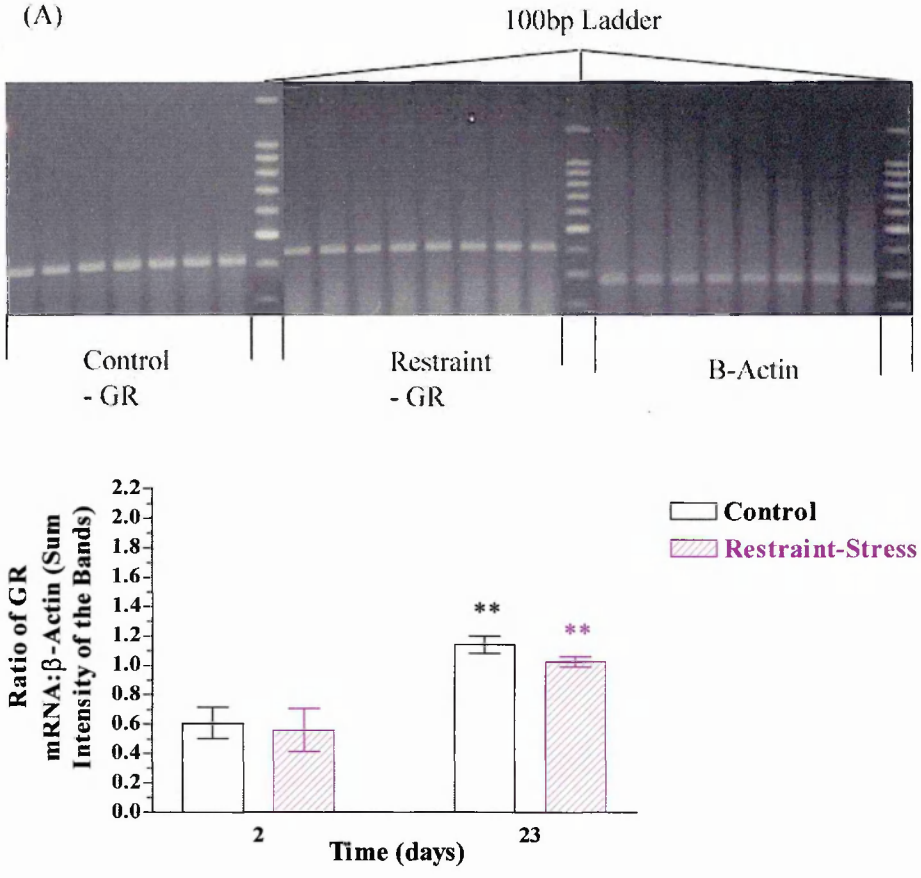
Cortical BDNF mRNA levels increased by 30% following a second exposure of restraint stress, compared to the control. Repeated exposure to restraint stress decreased cortical BDNF mRNA levels by 36% compared to control (**Figure.5.8.**). Control values varied between 1.1 and 1.4.

5.6.5. Corticotropin-Releasing Hormone (CRH) Type 1 Receptor mRNA

Cortical CRH-R1 mRNA levels decreased by 30% following a second exposure of restraint stress, compared to the control, although this was not statistically significant (**Figure.5.9.**).

Figure. 5.7.

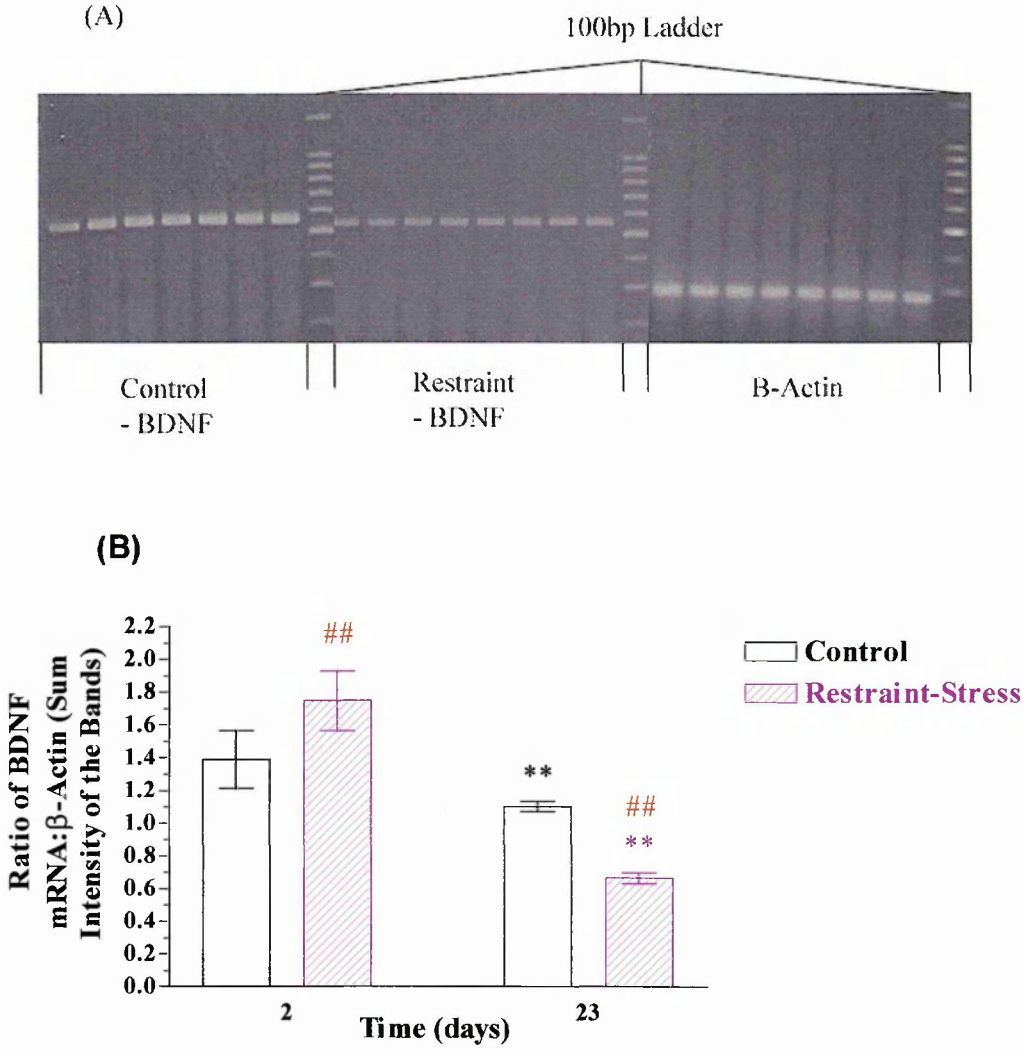
Cortical GR mRNA and β-Actin mRNA following exposure to restraint stress.



Cortical GRmRNA and β-Actin mRNA from male Wistar rats, housed in pairs (n=8 per group), were measured in samples taken immediately following exposure to restraint stress. (**A**) RT-PCR and agarose gel electrophoresis analysis of GR mRNA expression. β-Actin mRNA expression was used as an internal control. Control samples were run on a gel with β-Actin mRNA and a 100bp ladder, the restraint samples were run on a separate gel with β-Actin mRNA and a 100bp ladder. (**B**) Ratio of cortical GR mRNA: β-Actin mRNA (sum intensity of the bands). Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group) and rats restrained for 30 min/day (restraint-stress group) for either 2 days or 23 days before they were sacrificed. **, $p<0.01$ compared to controls after one or two days; **, $p<0.01$ compared to one or two 30 min exposures to restraint stress.

Figure. 5.8.

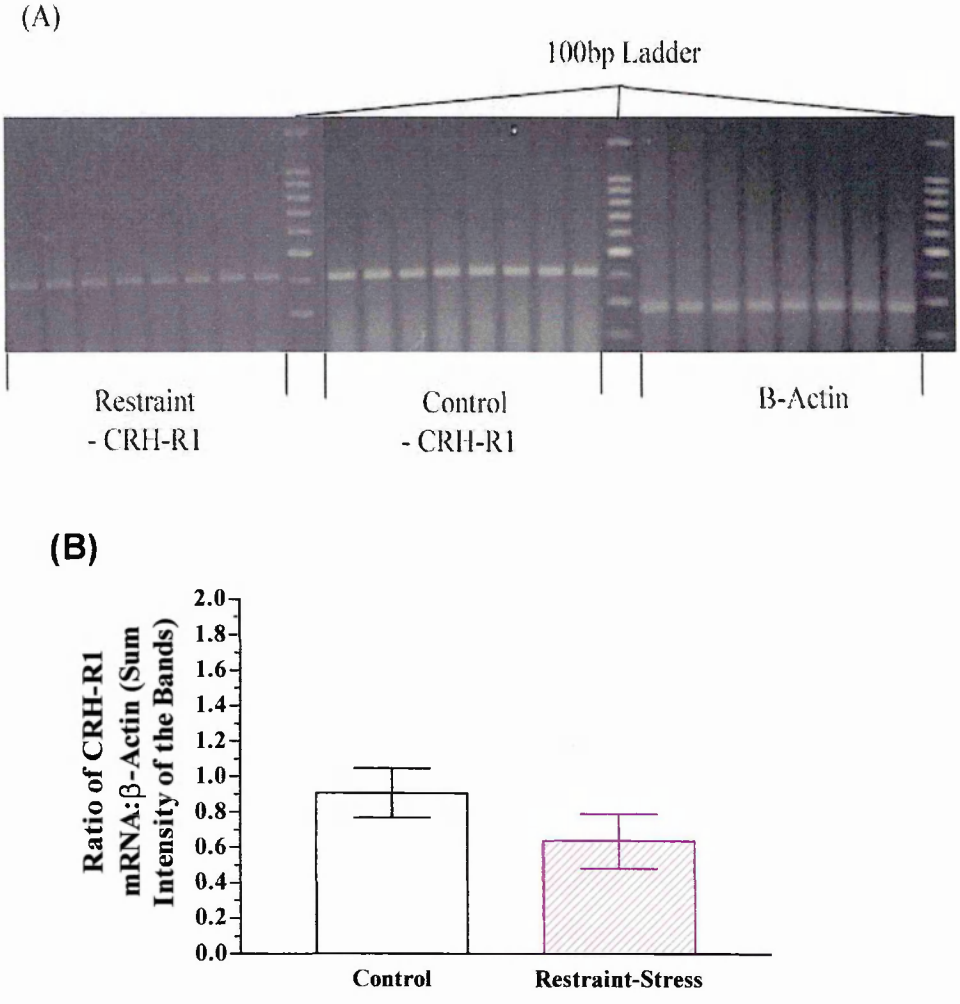
Cortical BDNF mRNA and β-Actin mRNA following exposure to restraint stress.



Cortical BDNF mRNA and β-Actin mRNA from male Wistar rats, housed in pairs (n=8 per group), were measured in samples taken immediately following exposure to restraint stress. (**A**) RT-PCR and agarose gel electrophoresis analysis of BDNF mRNA expression. β-Actin mRNA expression was used as an internal control. Control samples and restraint samples were run on 1 gel with 100bp ladders and β-Actin mRNA was run on a separate gel with a 100bp ladder. (**B**) Ratio of cortical BDNF mRNA: β-Actin mRNA (sum intensity of the bands). Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group) or restrained for 30 min/day (restraint-stress group) for either 2 days or 23 days before they were sacrificed. ##, $p<0.01$ compared to control; **, $p<0.01$ compared to one or two days of control; **, $p<0.01$ compared to one or two 30 min exposures to restraint stress.

Figure.5.9.

Cortical CRH type 1 receptor mRNA and β-Actin mRNA following restraint stress exposure for 2 days.



Cortical CRH-R1 mRNA and β-Actin mRNA from male Wistar rats, housed in pairs (n=8 per group), were measured in samples taken immediately following exposure to restraint stress. **(A)** RT-PCR and agarose gel electrophoresis analysis of CRH-R1 mRNA expression. β-Actin mRNA expression was used as an internal control. Control samples were run on a gel with β-Actin m RNA and a 100bp ladder, restraint samples were run on a separate gel with β-Actin mRNA and a 100bp ladder. **(B)** Ratio of cortical CRH-R1 mRNA: β-Actin mRNA (sum intensity of the bands). Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group) and rats exposed to two 30 min exposures to restraint stress (restraint-stress group) before they were scarificed. There were no significant differences between the groups.

5.6.6. Behavioural Measurements

1. Sucrose Preference

Restraint-stress alone had no significant effect upon the sucrose preference after 1, 4, or 22 days. (**Table.5.1.**).

Table. 5.1.

Sucrose preference (% of total fluid intake), following exposure to restraint stress.

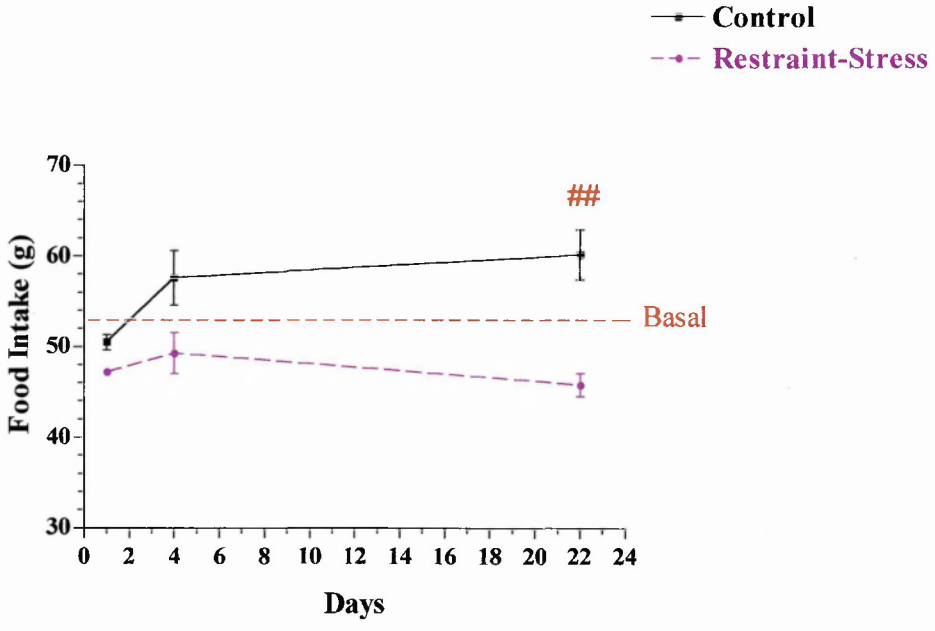
GROUPS	Mean ± sem	N
BASAL	96.5 ± 0.7	13
ACUTE RESTRAINT-STRESS (1 x 30-minutes)		
Control + 1ml/kg p.o. distilled water (VEHICLE)	95.3 ± 2.6	4
Restraint-Stress + 1ml/kg p.o. distilled water	96.1 ± 0.7	4
ACUTE RESTRAINT-STRESS (4 x 30-minutes)		
Control + 1ml/kg p.o. distilled water (VEHICLE)	89.8 ± 6.1	3
Restraint-Stress + 1ml/kg p.o. distilled water	96.2 ± 1.7	3
CHRONIC RESTRAINT-STRESS (22 x 30-minutes)		
Control + 1ml/kg p.o. distilled water (VEHICLE)	91.9 ± 5.5	3
Restraint-Stress + 1ml/kg p.o. distilled water	97.3 ± 0.5	3

2. Food Intake

Statistical analysis indicated that restraint stress alone significantly affected ($F_{1,18}$ = 6.952; p=0.0168) the food intake over 22 h (**Figure.5.10.**). A single 30 min restraint stress exposure had no effect compared to basal levels or control, and this remained the same following a fourth exposure. Repeated exposure to restraint stress decreased food intake by 24% compared to control.

Figure.5.10.

Food intake following exposure to restraint stress



Food intake (g) by male Wistar rats, housed in pairs (n = 4 per group), measured over 22 h. Each pair of rats within a cage were placed in the same treatment group. All cages were kept on the same rack on one side of the holding-room to minimise variation. Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group) and rats restrained for 30 min/day (restraint-stress group) for either 1 day, 4 days or 22 days. ##, p<0.01 compared to control.

5.7. Discussion

The main findings of the present experiments can be summarised as follows: **(1)** acute and chronic restraint increased both corticosteroid and ACTH concentrations and decreased GR in the cortex and hippocampus; **(2)** acute restraint increased BDNF gene expression in the cortex, whereas chronic restraint decreased it within the same brain region; **(3)** chronic restraint decreased food intake.

The marked increase in the plasma concentrations of ACTH and corticosterone (**Figure.5.2. and Figure.5.3.**) confirm the activation of the HPA axis during restraint stress and indicates habituation has not occurred after 23 days of a repeated homotypic stress.

Several researchers studying HPA axis reactivity to a stress applied after a first stimulus have reported that prior stimulation does not cause any blunting of the response to the second superimposed stress and, in some cases, hyperresponsiveness has been noted (Dallman & Jones, 1973; Hauger *et al*, 1988). This has been seen in the plasma concentrations of both corticosterone and ACTH, in the present study. Suggesting that the repeated exposure to restraint stress was sufficient to override the glucocorticoid negative feedback.

Disproportional elevations in resting plasma corticosteroid concentrations with respect to ACTH are usually observed in chronically stressed rats, (Dallman *et al*, 1992; Aguilera, 1994) as was shown in the current results, and clinical conditions such as depression (Gold *et al*, 1988a,b). Study by Aguilera *et al* (1996) demonstrated that the mechanism responsible for increased plasma corticosterone concentrations included both increased steroidogenic capacity of the zona fasciculate of the adrenal, and decreased corticosterone metabolism.

The responsiveness of the adrenal cortex to regulatory hormones is critical in the control of plasma steroid concentrations during chronic stress. Episodic increases of plasma ACTH are largely responsible for the changes in steroidogenic enzymes in both

zones of the adrenal cortex during repeated stress. On the other hand, regulation of glucocorticoid clearance is determined by factors other than ACTH.

It is well documented that exposure to stress for several days does not appear to cause any loss of pituitary response or pituitary ACTH content (Sakellaris & Vernikos-Danellis, 1975; Young & Akil, 1985) and on the contrary, results in a state of sustained activation of the mechanisms that mediate the pituitary response to stress. This suggests that rats exposed to stress may first undergo a period of transient loss of pituitary responsiveness which might protect the organism against excessive circulating amounts of ACTH; however, if the stress persists beyond this adaptation phase, pituitary sensitivity is restored, and ACTH stores increase again, thus preventing the animal from staying in a prolonged period of (possibly detrimental) inability to adequately release ACTH. It is possible that CRH, which exerts a trophic effect on ACTH synthesis (Vale *et al*, 1983), represents a major mediator of this recovery phenomenon.

Stress alters the secretion of one or more of the hypothalamic factors, which interact at the pituitary to increase the secretion of ACTH. AVP and OT have been shown to modulate the effect of CRH on ACTH secretion and appear to play a key role in mediating the ACTH response to stress. Although AVP is a relatively weak secretagogue for ACTH, it markedly potentiates the activity of CRH both *in vitro* and *in vivo*. The role of OT is more complex. The type of stressor appears to determine the relative importance of these secretagogues in ACTH response. After restraint, ACTH release is primarily mediated by the active increase of OT, and AVP does not appear to play a role. When restraint is associated with moderate levels of physical components and during immobilisation, both secretagogues are involved in the ACTH response (Scantamburlo *et al*, 2001).

AVP plays an important role in the control of ACTH secretion during stress, mainly by modulating the regulatory effect of CRH in the pituitary corticotroph (Gillies & Lowry, 1979; DeGoeij *et al*, 1992). In control non-stressed rats, approximately 50% of CRH-containing neurones in the parvocellular area of the PVN of the hypothalamus coexpress AVP, which is released into the pituitary portal circulation from nerve terminals located in the external zone of the median eminence (Whitnall *et al*, 1987; Plotsky, 1991). A number of studies have shown that the activity of the parvocellular vasopressinergic system is enhanced after adrenalectomy or chronic stress (Sawchenko *et al*, 1984; Harbuz & Lightman, 1992). Therefore examining AVP and OT levels during this study could have given us an insight into their role in the control of ACTH secretion during repeated stress.

Possible mechanisms to be considered for the failure of glucocorticoids to inhibit ACTH secretion during chronic stress are a decrease in GR in the pituitary or brain and changes in the functional activity of the GR. In the current study acute and chronic restraint stress led to a reduction in the density of GR in both cortex and hippocampus (**Figure.5.4.**). This data is compatible with previous findings that stress down-regulates hippocampal GRs (Sapolsky *et al*, 1984; Herman *et al*, 1995).

Decreased hippocampal GR concentrations are associated with reduced glucocorticoid negative feedback on ACTH release following the termination of stress (Sapolsky *et al*, 1986), which is consistent with the marked increase in the plasma concentrations of corticosterone and ACTH following the exposure to restraint stress, shown in section 5.6.1.

Studies have shown that exogenous administration of large doses of corticosteroids reduces cytosolic GR number in the hippocampus (Tornello *et al*, 1982). This finding suggests the possibility that chronic secretion of glucocorticoids stimulated by chronic stress may down-regulate GR in the brain. The principal finding of the study by Sapolsky

et al (1984) was that repeated stress reduced the number of GR in the brain, a finding confirmed in the present experiment, and it seems likely that this is mediated by elevated corticosterone secretion during stress. They found that repeated stress caused a reduction in total available receptor number, rather than a simple redistribution, which is in agreement with the observations of Cidlowski and Cidlowski (1981). Mechanisms underlying these examples of down-regulation could include decreased synthesis of new receptors, increased degradation or preferential sequestering of receptors in inactive pools, or destruction of receptor-containing neurones.

Accumulating evidence suggests that GR activation suppresses the hippocampal output (Joels & DeKloet, 1992), theoretically resulting in the disinhibition of the HPA axis. Van Haarst *et al* (1997) demonstrated that an icv injection of GR antagonist increased plasma ACTH and corticosterone concentrations at the diurnal peak, whereas the intrahippocampal injection of GR antagonist produced an opposite, inhibitory effect, indicating a positive glucocorticoid feedback influence on the HPA axis through hippocampal GR. They proposed the importance of GR in the PVN itself in the glucocorticoid-mediated restraint of the activity of PVN CRH and AVP neurones, based on previous reports showing suppression of CRH biosynthesis by local administration of glucocorticoids (Kovacs & Mezey, 1987), and suggested that feedback inhibition through GR in the PVN may override a positive feedback effect through hippocampal GR. In this context, decreased GR mRNA levels in the PVN rather than in other brain regions are important for the attenuation of glucocorticoid-induced negative feedback on the activity of PVN CRH and AVP neurones during repeated restraint stress.

The current study indicates that a single 30 min restraint stress exposure and repeated exposure had no affect on GR mRNA in the cortex compared to control (**Figure.5.7.**). This is in contrast with the above results (Section 5.8.2.), showing a reduction in the density of GRs in both cortex and hippocampus. This contradiction could

be due to increased receptor breakdown, which is quicker than the production of the mRNA.

Chronic exposure to stress has previously decreased GR mRNA in the hippocampus, which is in conflict with the current results (Sapolsky *et* al, 1984; Kitraki *et al*, 1999). There is a possibility that the stress-induced GR down-regulation was due to its increased receptor occupancy and internalisation rather than to a decrease in receptor synthesis. The increased secretion of corticosterone could also have mediated the delayed up-regulatory effect of restraint stress upon GR mRNA and could emphasise the region-specific sensitivity of GR mRNA to corticosterone. The majority of previous studies investigating the effects of chronic stress upon GR mRNA have been conducted in the hippocampus and very little is known about GR mRNA within other brain areas, therefore these effects could be region-specific.

Differential regulation of GR gene expression by different stressors has previously been reported (Watanabe *et al*, 1992; Herman & Spencer, 1998), and can be attributed to the nature, intensity and duration of a stressful stimulus, as well as to the time point after the stress at which GR mRNA concentrations are estimated. This is consistent with the present results and show that GR mRNA concentrations are not altered in the cortex shortly after an acute exposure to restraint, which is probably a result of the relatively short time interval between the stress procedure and the sacrifice of the animal and the nature of this stressor (Kitraki *et al*, 1999). Even in cells, the time of appearance of GR down-regulation can vary considerably (Dong *et al*, 1988; Vig *et al*, 1994).

The mechanism of down-regulation has been referred to be corticosterone dependent (Makino *et al*, 1995b) and is exerted primarily at the level of transcription of the GR gene (Rosewicz *et al*, 1988; Hoeck et al, 1989), although post-transcriptional actions cannot be excluded (Dong *et al*, 1988).

Previous studies document varying degrees of GR mRNA down-regulation following stress. The chronic intermittent stress paradigm usually produces minimal decreases in GR mRNA concentrations (Herman *et al*, 1995; Herman & Spencer, 1998), consistent with previous studies using other stress paradigms (Mamalaki *et al*, 1993; Herman *et al*, 1995). In contrast, chronic immobilisation can produce larger (40-50%) decreases in hippocampal GR expression, suggesting that the stability of GR expression may be modulated by stressor intensity or duration (Makino *et al*, 1995b).

This current study indicated that a single 30 min restraint stress exposure significantly increased BDNF mRNA concentrations within the cortex, but repeated restraint stress caused a significant reduction in BDNF mRNA concentrations within this brain region (**Figure.5.8.**). Previous studies have shown a down-regulation of BDNF mRNA concentration, mainly in the hippocampus, in response to acute and chronic stress (Zafra *et al*, 1991; Vaidya *et al*, 1999).

The role of corticosterone in mediating BDNF is not clear. According to Schaaf *et al* (2000), corticosterone suppresses the BDNF expression at the mRNA and protein level in a subfield-specific way. They propose a model for the mechanism of action, in which activated MR and GR repress transcriptional activity of the BDNF promoter site-specifically via interaction with other transcription factors (Schaaf *et al*, 2000). This could possibly be extended to other brain regions.

The stress-induced changes in BDNF expression have been found to be largely opposite to those produced by seizures and ischaemia. For instance, BDNF mRNA concentrations are increased throughout the brain up to 40 fold, by various seizure paradigms (Isackson *et al*, 1991; Rocamora *et al*, 1992). Likewise, ischaemia induces a transient increase in BDNF mRNA concentrations in the dentate gyrus (Takeda *et al*, 1993). The direction of these observed changes in BDNF mRNA concentrations induced by seizures or ischaemia is consistent with the notion that

glutamate, which is released during seizures and ischaemia, positively regulates BDNF expression (Zafra *et al*, 1991). The fact that stress induced effects are opposite to those caused by seizures and ischaemia might imply that the underlying mechanisms driving growth factor expression are opposite too. Yet, restraint stress, like seizures and ischaemia, has been reported to increase glutamate turnover (Gilad *et al*, 1990; Moghaddam, 1993). Therefore, it is possible that our acute restraint stress positively regulated BDNF expression through increased glutamate or other neurotransmitters, such as norepinephrine, 5-HT, or GABA, but then the effects of these factors on BDNF expression decreased after chronic restraint stress, suggesting a desensitisation to or clearance of these factors over this time period.

Smith and colleagues (1995a) have shown that acute (2 h) or repeated immobilisation stress increases BDNF mRNA concentrations in the PVN, lateral hypothalamus, anterior and neurointermediate lobes of the pituitary. This is in distinct contrast to stress-induced decreases in extrahypothalamic areas, including basolateral amygdala, claustrum, and cingulated cortex as well as the hippocampus. Therefore, this current study confirms that acute stress increased BDNF mRNA concentrations in the cortex, an effect that may also enhance the BDNF-responsiveness of this brain region, and that BDNF-mediated responses may be either decreased or increased by chronic stress, depending on the brain region involved. In addition, these concentrations of BDNF mRNA in cortex are not regulated by chronic restraint stress, which could result from habituation to the same stress on repeated exposure.

While corticosterone generally appears to decrease BDNF in previous studies, stress has been shown to decrease BDNF in the absence of corticosteroids, at least in the dentate gyrus (Smith *et al*, 1995b). This suggests that corticosterone feedback is not the only part of the stress response contributing to the observed decrease in BDNF mRNA concentrations. However, because the most robust and consistent decreases in BDNF occur

in stressed animals which are adrenally intact, it is likely that basal concentrations of glucocorticoids are necessary for maximal inhibition of BDNF mRNA by stress, just as glucocorticoids are required for maximal stimulation of BDNF by kainic acid (Barbany & Persson, 1993).

Consequently, BDNF is a stress-responsive intercellular messenger that may be an important component of the stress-response. Therefore, changes in the expression of neutrophic factors are part of the brain's response to stress. Whether changes in neutrophic factor availability actually contribute to the damage in the brain caused by stress or are an adaptive value for facing future stressors remains to be explored.

In the current study acute restraint stress did not cause any significant changes in CRH type 1 receptor mRNA within the cortex (**Figure.5.9.**), although, there was a trend towards a decrease compared to control.

Numerous studies have examined the regulation of CRH; however, little is known about the mechanisms controlling the expression of CRH receptors in the brain. Some studies have found no alterations in levels of CRH receptor ligand binding in the brain by chronic stress or corticosterone administration (Wynn *et al*, 1984; Hauger *et al*, 1988), whereas others have demonstrated that levels of CRH receptors in the brain are decreased by repeated stress (Anderson *et al*, 1993) or intracisternal injection of CRH (Hauger *et al*, 1993).

Robust transcriptional activation of the gene encoding the CRH type 1 receptor (but not the type 2) has been previously observed in the rat endocrine hypothalamus, after different types of neurogenic and systemic stressors. A selective and transient increase of CRH type 1 receptor mRNA expression was found in the parvocellular PVN of acutely challenged rats (Rivest *et al*, 1995; Bonaz & Rivest, 1998), whereas little notable changes were detected in other regions of the brain. This suggests that sophisticated mechanisms

take place in a site-dependent manner to regulate the CRH type 1 receptor in stressed animals.

Acute stress has been shown to cause biphasic changes in CRH receptor mRNA expression with an early decrease followed by an increase, which is consistent with these present results. However, in the absence of corticosteroids in adrenalectomised rats, stress results in prolonged CRH receptor mRNA loss, suggesting that interactions between corticosteroids and hypothalamic factors are critical for regulation of CRH receptor mRNA. The data from the study by Ochedalski and colleagues (1998) shows that interaction between CRH and glucocorticoids counteracts individual inhibitory effects of these regulators alone, and that such effects are likely to contribute to the regulatory pattern of pituitary CRH receptors during acute stress.

High concentrations of corticosterone or CRH have been shown to synergistically decrease CRH receptor mRNA concentrations in the anterior pituitary, which was shown by our decrease in CRH type 1 receptor mRNA in the cortex, although looking at the CRH concentrations could have confirmed this, and that high corticosterone has an inhibitory effect on PVN CRH receptor mRNA levels (Makino *et al*, 1995a). The extrahypothalamic regions, such as amygdala, bed nucleus of the stria terminalis and the cortex may have different sensitivities to corticosterone or CRH for the regulation of CRH receptor mRNA. Although stress stimulation of CRH mRNA in the PVN is glucocorticoid independent, basal levels are likely to be under dual, transcriptional and post-transcriptional, control by glucocorticoids (Luo *et al*, 1995).

There was no significant change in sucrose preference throughout restraint-stress exposure (**Table.5.1.**). Plaźnik *et al* (1989) showed that a single 1 h immobilisation stress produced a short-term reduction of saccharin preference in rats. The effect was stable and lasted for 1-2 days, though there was some variability in the control group preference across all the experiments.

Possible reasons for the current lack of a reduction in sucrose preference after acute and chronic restraint-stress may be due to a number of differences in our study compared to previous studies:

1. Rats were housed in pairs, not single-housed, to limit any extraneous stress other than restraint;
2. They were not food or water deprived, although decreases in sucrose drinking can be seen in both food-deprived and non-deprived animals (Muscat & Willner, 1992), as well as in studies in which the CMUS procedure excluded periods of food and water deprivation (Muscat & Willner, 1992; Bertrand *et al*, 1997);
3. A two-bottle preference test was used, although the effects of CMUS have been reported in both single-bottle tests and in two-bottle preference tests (Willner *et al*, 1987; D'Aquila *et al*, 1997);
4. Sucrose preference was measured over 22 h rather than 1 h as used in previous studies. This was mainly to include the dark phase when the rats are most active, but also because previous tests conducted over 1 h produced small volumes to weigh and it proved difficult to ascertain any differences;
5. Restraint-stress was used rather than CMUS, which as far as we are aware has not been done before;
6. Sucrose was used rather saccharin, although the calorie content of the sucrose appeared to be unimportant as the bodyweights remained unchanged and there was only a slight decrease in the intake of plain water;
7. The control and stressed rats were handled daily, and previous CMUS studies have not reported if the animals were handled throughout their experiments;
8. Reversed lighting has previously been used, but we refrained from doing this to cause as little disruption as possible to the animals and to therefore make the study easier to conduct within the animal house facilities. There is a diurnal variation in sensitivity to

chronic mild stress, at least in Wistar rats, which, according to D'Aquila *et al* (1997), show little or no response to chronic mild stress when tested during the light phase of the light-dark cycle, but show typical decreases in sucrose consumption and preference when tested at the start of the dark phase. Therefore, although we tried to counteract this by testing the Wistars over a 22 hr period, which encompassed the dark phase, it also included part of the light phase and so could have compromised the results;

9. It has been shown that sensitivity to CMUS varies between strains (Griffiths *et al*, 1992; Pucilowski *et al*, 1993); therefore, although this procedure has been shown to be effective a variety of strains of rat, it might not be effective in Wistars;

10. Just as there are strain differences in sensitivity to inducing anhedonia, differences in sensitivity may also exist between rats of the same strain from different suppliers; such differences could arise either from genetic drift or from differences in rearing procedures. Our animals mainly came from the Biological Research Facility at SGHMS but some were obtained from suppliers. It has been observed that there is both strain and supplier differences in sensitivity to sucrose (Lush, 1989). According to Willner (1997), the 1% sucrose concentration that is routinely used in PVG or Lister hooded rats during his CMUS experiments, is only marginally preferred to water in some batches of Wistar rats, leading to unstable patterns of consumption in repeated tests, even in control animals. This trend is shown in the present study; therefore choosing a different concentration of sucrose could produce a more stable pattern of intake and therefore preference.

Food intake decreased after repeated exposures to restraint-stress (**Figure.5.10.**), suggesting that stress slightly suppressed food intake in the current study, but this was further enhanced by the gradual increase in the controls. This measure was primarily used to establish changes in sucrose drinking were not due to a change in appetite, and that the animals preferred the sucrose without affecting the amount of food eaten. There was no change in the body weights of the animals in this present study. As sucrose preference was unchanged, we decided to look at this data to determine if eating behaviour had been modified by single and repeated exposures to restraint stress.

A study by Ely *et al* (1997) suggests that the severity and duration of exposure to stressors are capable of modifying eating behaviour. A chronic moderate stress, i.e., restraint, does not alter normal food consumption, but leads to changes in specific appetites, i.e., sweet food ingestion.

Exposure to stress causes a wide range of behavioural and physiological alterations in organisms, but only a few of them are sensitive to particular characteristics of the stressor, such as intensity and duration (Martí & Armario, 1998). Food intake is one of the variables sensitive to stress. Whereas exposure to short-term and mild stressors has been reported to transiently increase food intake (Morley *et al*, 1983), exposure to stressors of certain severity, including surgery, experimentally-induced inflammation, endotoxins, foot shock, crowding, and various types of restraint, always reduces food intake in the hours after stress (Stone & Platt, 1982; Berton *et al*, 1998), as shown in the current results.

Stress-induced reduction in food intake has been previously demonstrated both as a maintained decrease in 24 h food intake during and after repeated daily restraint stress (Kennett *et al*, 1986; Krahn *et al*, 1990), and as an acute response in the hours immediately after a single stress (Krahn *et al*, 1990).

Central mechanisms involved in the stress-induced inhibition of food intake have not been fully elucidated but certain peptides and neurotransmitters are thought to be involved in the response. It is well established that monoamines (Kennett *et al*, 1987) and CRH (Krahn *et al*, 1990) influence feeding behaviour and mediate behavioural and physiological responses to stress (Sutton *et al*, 1982; Krahn *et al*, 1988). Both CRH (Krahn *et al*, 1986) and/or serotonin (Kennett *et al*, 1986) are elevated in response to stress in a number of brain areas, including those that are involved in the regulation of feeding behaviour (Makino *et al*, 1995b). Intracerebroventricular administration of CRH produces behaviours typical of stress, including depression of appetite (Sutton *et al*, 1982; Krahn *et al*, 1986).

5.8 Conclusions

This series of experiments demonstrated that a repeated restraint stress paradigm significantly decreased the glucocorticoid negative feedback response of the HPA axis, for over 3 weeks. Emphasising that failure to adapt to a repeated daily restraint stress schedule is a useful animal model for depression, and this model has been shown to respond appropriately to antidepressant administration (Curzon, 1989).

CHAPTER 6. THE EFFECT OF ACUTE AND CHRONIC PAROXETINE ADMINISTRATION IN RATS UPON THE HPA AXIS.

6.1. Introduction

Numerous anatomical, biochemical, and behavioural investigations have established the existence of functional interactions between central serotonin systems and the HPA axis. Serotonin activates all levels of the HPA axis (Delbende *et al,* 1992; Fuller, 1996). For example, serotonin stimulates the release of CRH from hypothalamic slices. Corticosterone in turn influences serotonin synthesis, turnover and receptors via MR and GR (Chaouloff, 1993; Meijer & DeKloet, 1998). Serotonergic neurones are also involved in stress-induced activation of the HPA axis (De Souza & Van Loon, 1986) and in the circadian variations of ACTH and corticosteroid secretion (Szafarczyk *et al*, 1985). Furthermore, various types of drugs that increase serotonergic transmission (such as serotonin agonists, serotonin uptake inhibitors and serotonin releasers) stimulate the activity of the HPA axis (Bruni *et al*, 1982; Fuller & Snoddy, 1990).

Paroxetine is an SSRI (Tulloch & Johnson, 1992), a class of structurally unrelated drugs that enhance serotonergic transmission by blocking the presynaptic active membrane transport mechanism for the reuptake of serotonin (Frazer, 1997) and consequently increase serotonergic activity at the postsynaptic receptor (Johnson, 1992). The affinity of paroxetine for the serotonin reuptake site is 2 to 3 orders of magnitude greater than serotonin and as with the other members of the class, it effectively increases the concentration of endogenous serotonin in the synaptic cleft (Richelson, 1999). Paroxetine is the most potent inhibitor of serotonin reuptake of currently available antidepressants. It is a weak inhibitor of norepinephrine uptake but it is still more potent at this site than the other SSRIs and this may contribute to its clinical efficacy at higher doses (Tulloch & Johnson, 1992). The selectivity of paroxetine, i.e., the ratio of inhibition of

uptake of noradrenaline to serotonin (NA/5-HT) is amongst the highest of the SSRIs (Hyttel, 1994). It has little affinity for catecholaminergic, dopaminergic or histaminergic receptors and by comparison with TCAs, has therefore, a reduced propensity to cause central and autonomic side effects (Tulloch & Johnson, 1992). Paroxetine exhibits some affinity for the muscarinic cholinergic receptor but much less than the TCAs (Tulloch & Johnson, 1992).

In addition, the secondary adaptive changes of somatodendritic ($5\text{-}HT_{1A}$) and terminal ($5\text{-}HT_{1B/1D}$) autoreceptors observed with paroxetine are different to those observed with TCAs. Long-term administration of paroxetine in rats (over 2-3 weeks) decreases the responsiveness of these receptors, leading to greater serotonin release with each action potential, in contrast to the sensitisation of postsynaptic $5\text{-}HT_{1A}$ receptors, which occurs, with TCAs (Blier *et al*, 1990). Maximal antidepressant effects are observed only after weeks or even months of repeated treatment, suggesting that in addition to the inhibition of serotonin reuptake, these adaptive changes in synaptic serotonergic receptors are likely to be important for the therapeutic efficacy of paroxetine. Most long-term studies of antidepressants have focused on their effects on autoreceptors or postsynaptic receptors and the responses they elicit (Blier & Bouchard, 1994; Auerbach & Hjorth, 1995).

It is well established that noradrenergic neurones modulate the 5-HT system. Dorsal raphé 5-HT neurones receive noradrenergic projections from the locus coeruleus (Loizou, 1969; Baraban & Aghajanian, 1980), a nucleus that gives rise to more than 40-45% of noradrenergic innervation of the brain. The noradrenergic neurones located in the locus coeruleus modulate the activity of 5-HT neurones in the dorsal raphe nucleus via excitatory α_1-adrenoceptors (Baraban & Aghajanian, 1980). In turn, noradrenergic neurones of the locus coeruleus receive dense 5-HT projections, which have been shown to have an inhibitory effect (Léger & Descarriers, 1978; Vertes & Kocsis, 1994). Alterations in noradrenergic and serotonergic function have long been implicated in depression

because clinically effective antidepressants directly interact with these systems and produce observable changes in depressive symptomatology (Schildkraut, 1965a; Owens & Nemeroff, 1994). The emergence of SSRIs as the treatment of choice for depression has shifted the recent emphasis of the monoamine theory toward serotonergic mechanisms rather than noradrenergic dysfunction (Baldwin & Rudge, 1995; Stanford, 1996).

Several pieces of data support the idea that serotonergic afferents modulate noradrenergic neuronal activity in the locus coeruleus. Serotonergic immunoreactive fibers provide a dense innervation of the locus coeruleus area (Palkovits *et al*, 1974; Léger & Descarriers, 1978). Serotonin attenuates sensory-evoked responses of locus coeruleus neurones (Segal, 1979) and decreases glutamate-induced excitation of these cells when applied locally (Bobker & Williams, 1989; Aston-Jones *et al*, 1991).

Previous studies have demonstrated that acute SSRIs have no effect on the spontaneous firing activity of locus coeruleus noradrenergic neurones (Béïque *et al*, 1999). A recent study indicates that the long-term (21 days), but not short-term (2 days) administration of paroxetine greatly reduced the spontaneous firing rate of the locus coeruleus noradrenergic neurones (Szabo *et al*, 1999). In contrast, the acute administration of an SSRI reduced the firing rate of 5-HT neurones of the dorsal raphé nucleus in the rodent brain (De Montigny *et al*, 1981; Quinaux *et al*, 1982). However, these neurones regain their normal firing rate after long-term administration (Blier & DeMontigny, 1983). This has been shown to be due to desensitisation of the somatodendritic 5-HT_{1A} autoreceptor, which modulates their firing activity (Blier & DeMontigny, 1983). The terminal 5-HT autoreceptor controlling 5-HT release also desensitises following long-term SSRI administration (Blier *et al*, 1988). These two modifications, in the presence of sustained 5-HT reuptake blockade, result in an increased amount of 5-HT released in the forebrain, per action potential.

The efficacy of SSRIs, like paroxetine, are normally attributed to their ability to increase brain 5-HT function, although recent preclinical findings have shown that paroxetine also increases extracellular concentrations of noradrenaline, following repeated administration (Hajós-Korcsok *et al*, 2000; Owens *et al*, 2000). The recent study by Hajós-Korcsok *et al* (2000) found that repeated but not acute administration of paroxetine causes an increase in extracellular noradrenaline (and serotonin) concentration in the rat hippocampus. This facilitatory effect of paroxetine on noradrenaline may reflect a serotonin-noradrenaline interaction, in which case other SSRIs may have a similar effect. An increase in noradrenaline function may contribute to the antidepressant effect of paroxetine, and possibly other SSRIs.

6.1.1. Endocrinological changes following antidepressant administration

By virtue of their ability to inhibit the reuptake of 5-HT, SSRIs elevate the extracellular concentrations of 5-HT in the synapse. Consequently, the activation of post-synaptic 5-HT receptors in the hypothalamus stimulates the secretion of several hormones. Therefore, increases in serum corticosterone, CRH and ACTH have been described with SSRIs in rats (Bourin *et al,* 2001). Indeed, several studies using rats have indicated that a single injection of fluoxetine increases plasma concentrations of ACTH and corticosterone (Fuller *et al*, 1976). Acute administration of fluoxetine and paroxetine also increase plasma cortisol concentration in humans (Reist *et al*, 1996). SSRIs are substantially less efficacious in increasing plasma concentrations of hormones than 5-HT releasing drugs such as d-fenfluramine (Lucey *et al*, 1992; Coccaro *et al*, 1996). One explanation for this weak neuroendocrine response to an acute administration of SSRIs is that by blocking 5-HT reuptake in the cell body region in the raphé, they subsequently activate somatodendritic 5-HT_{1A} autoreceptors. These 5-HT_{1A} autoreceptors in the raphe nuclei provide negative feedback inhibition of serotonergic firing. Therefore, the tendency of

SSRIs to increase the concentrations of 5-HT in the synapse are negated by activation of the negative feedback, leading to reduced release of 5-HT. Consequently, less activation of post-synaptic 5-HT receptors occurs and a less robust neuroendocrine response to acute administration of SSRIs is observed.

A number of studies have described that the function of GR is reduced in depressed patients (GR resistance) and that antidepressants act by reversing these putative GR changes (Pariante & Miller, 2001). Specifically, studies in depressed patients, animals, and cellular models have demonstrated that antidepressants increase GR expression, enhance GR function and promote GR nuclear translocation; this, in turn, is associated with enhanced GR-mediated negative feedback by endogenous corticosteroids, and thus with reduced resting and stimulated HPA axis activity (Pariante & Miller, 2001).

Previous investigations first performed on primary cultures of rat brain (Pepin *et al*, 1989) and later in different brain regions and the pituitary of the rat *in vivo* (Brady *et al*, 1991; Reul *et al*, 1994), have demonstrated that antidepressants have varying affects on GR mRNA concentrations. They have shown a decrease and increase in anterior pituitary (Brady *et al*, 1991; Reul *et al*, 1994), increase in the hypothalamus (Peiffer *et al*, 1991; Reul *et al*, 1994), increase or not altered in the hippocampus (Peiffer *et al*, 1991; Reul *et al*, 1994), and not altered in the neocortex and amygdala (Reul *et al*, 1994). Other studies have demonstrated that administration of antidepressants increase the activity of the GR-gene promoter (Pepin *et al*, 1992) and induce the steroid-independent translocation of the GR in mouse fibroblast cells (Pariante *et al*, 1997). The hypothesis of a primary effect of antidepressants on steroid receptor function were further corroborated by the findings that the antidepressant administration in rats resulted in changes of various brain HPA parameters such as increases in steroid receptor mRNA concentrations and protein concentrations as well as a decrease of HPA hormone secretion (Brady *et al*, 1991; Reul *et al*, 1994). These observations led to the assumption that the

common mechanism of action of the different classes of antidepressants is the restoration of neuroendocrine feedback functions due to their action on GR function and GR-mediated cellular effects (Holsboer & Barden, 1996). It was concluded that this mode of action finally causes a normalisation of HPA-regulated hormone secretion with subsequent clinical improvement (Reul *et al*, 1994; Holsboer & Barden, 1996).

A number of animal studies have shown that long-term (10-28 days) administration of antidepressants increases GR and/or MR binding (Reul *et al*, 1993; Budziszewska *et al*, 1994) and GR and/or MR mRNA expression (Peiffer *et al*, 1991; Seckl & Fink, 1992) in the hippocampus, and GR binding in the hypothalamus (Reul *et al*, 1993). Such time courses coincide closely with those of antidepressant therapy: long-term (10 days-several weeks) administration of antidepressants is necessary to see the first signs of clinical efficacy. Thus, the up-regulation of brain GR and/or MR appears to be pivotal for the therapeutic action of antidepressants.

The hypothesis that antidepressants exert their clinical effects through direct modulation of the GR is one of the most striking and innovative models of the mechanism of action of this class of drugs (Barden, 1999; Pariante & Miller, 2001).

It has been previously shown that mixed 5-HT/noradrenaline antidepressant drugs could increase the number of GR and thereby normalise the feedback inhibition of corticosterone secretion (Seckl & Fink, 1992; Reul *et al*, 1993). It is therefore hypothesised that an increase in serotonergic transmission might regulate the HPA axis functioning through a regulation of corticosteroid receptors. The effects of a short-term increase in serotonin transmission on corticosteroid receptors are not yet clear. Conflicting results might come from **(i)** the use of drugs that have differential short-term effects on 5-HT metabolism (Seckl & Fink, 1992; Yau *et al,* 1997), and **(ii)** the difficulty to correlate the effects on MR and GR mRNA concentrations and the effects on binding sites.

Antidepressant administration influences the expression of genes encoding for hypothalamic-pituitary-adrenal axis constituents. Thus, chronic treatment with some tricyclic antidepressants downregulates the CRH mRNA concentration in the PVN (Brady *et al*, 1991) and upregulates the hippocampal MR (Brady *et al*, 1991) and the GR mRNA concentration (Peiffer *et al*, 1991; Rossby *et al*, 1995). However, the latter effect is not a common mechanism of antidepressants, as fluoxetine does not cause such an effect (Rossby *et al*, 1995). Therefore, the molecular mechanism underlying the effects of antidepressants on GR function remains unclear.

One mechanism through which CRH may modulate a broad spectrum of physiological and behavioural responses is via actions on ascending neuromodulatory systems, such as serotonergic systems. Several lines of evidence support the hypothesis that CRH plays a role in regulating serotonergic neurotransmission. First, moderate to high densities of CRH-immunoreactive neuronal cell bodies and fibers are associated with serotonergic neurones in brainstem raphe structures (Cummings *et al*, 1983; Ruggiero *et al*, 1999). Second, CRH_1 and CRH_2 receptor binding sites, receptor mRNA expression, and CRH_1 receptor-immunoreactive neurones have been identified in raphé nuclei (De Souza *et al*, 1985; Chen *et al*, 2000), raising the possibility that CRH or CRH-like peptides may have direct receptor-mediated actions on serotonergic neurones. Third, exogenous CRH or CRH-like peptides alter serotonin metabolism or neurotransmission in studies using *ex vivo* tryptophan hydroxylase activity assays and *in vivo* microdialysis (Singh *et al*, 1992).

According to a recent study by Isogawa *et al* (2000), the CRH type 1 receptor antagonist, CP-154,526, produced a decrease in dialysate concentration of noradrenaline and serotonin, but not dopamine, in the hippocampus of rats. This suggests that the CRH type 1 receptor is linked with the release of noradrenaline and 5-HT in the hippocampus.

The possible existence of an interaction between the central serotonin and BDNF systems has recently attracted wide interest. BDNF, for example, has been shown to influence the survival and function of serotonin neurones in the rat brain (Mamounas *et al*, 1995). Previous studies have also shown that the chronic administration of SSRIs increases BDNF mRNA expression in the hippocampus, suggesting that increased serotonergic function influences BDNF gene expression (Zetterström *et al*, 1998). In contrast, a more recent study has shown that acute 5-HT_2 receptor stimulation results in decreased BDNF mRNA in the rat dentate gyrus, while there was an increase in neocortical areas (Vaidya *et al*, 1997).

The possibility that BDNF is involved in the actions of antidepressant treatment is also supported by results from behavioural, neurochemical, and morphological studies. Chronic infusion of BDNF is reported to have antidepressant effects in two behavioural models of depression, the forced swim and learned helplessness paradigms (Siuciak *et al*, 1996). BDNF has been demonstrated to have potent neutrophic effects on serotonin neurones when infused into midbrain (Siuciak *et al*, 1994). In addition, infusion of BDNF into forebrain results in a dramatic elevation of serotonin neuronal fiber density, and protection of neurones from neurotoxic damage (Mamounas *et al*, 1995). Upregulation of BDNF in response to antidepressant administration could have similar behavioural effects, and could enhance serotonergic neurotransmitter function. These findings also indicate that there is a positive, reciprocal interaction between 5-HT and BDNF; chronic SSRI administration increases levels of BDNF, and upregulated BDNF would be expected to increase serotonin neuronal function.

6.2. Aims

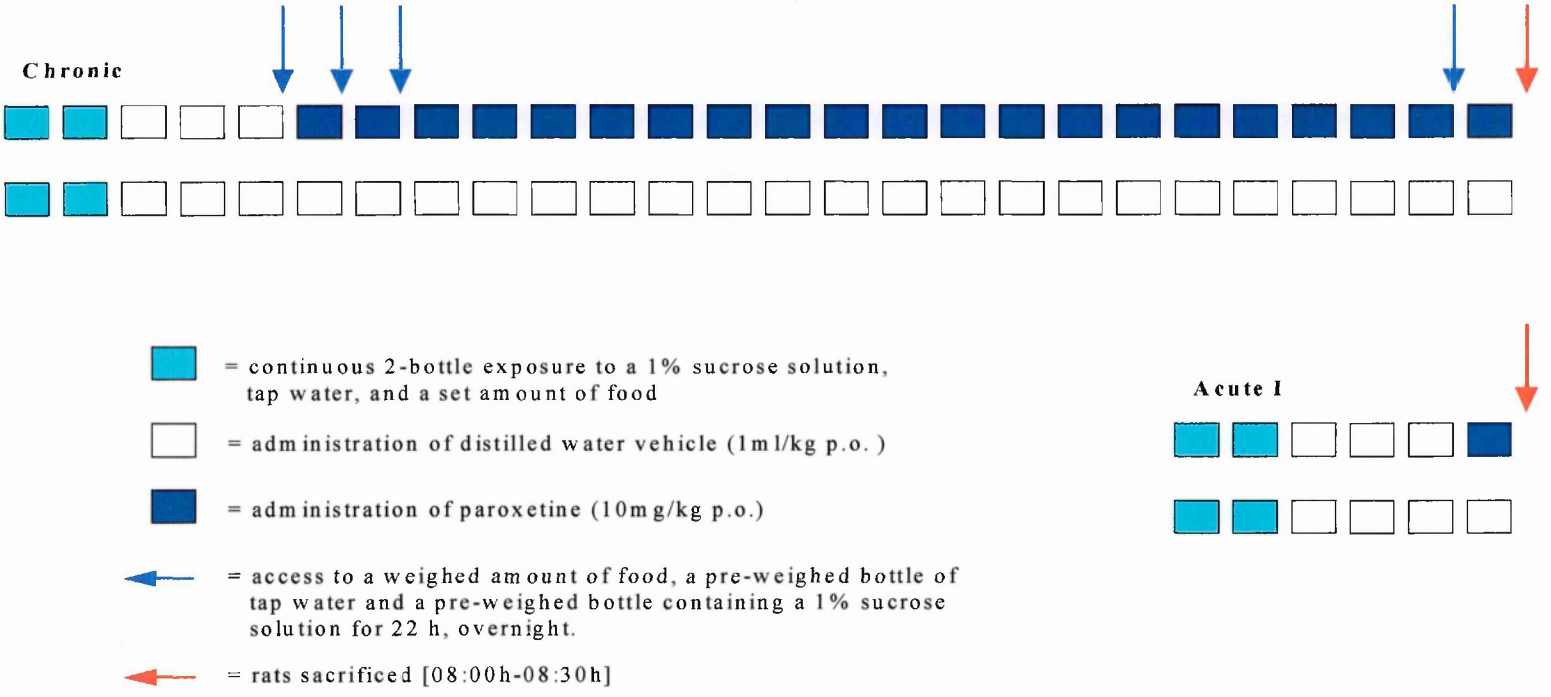
To study the effects of acute and chronic antidepressant administration on the HPA axis. Paroxetine was selected for several reasons: **(i)** it is the most potent inhibitor of the 5-HT transporter currently available, **(ii)** in the rat it has a long half-life (8h; Owens *et al*, 2000) which makes it particularly useful for repeated administration.

Animal studies have shown that long-term *in vivo* administration of antidepressants up-regulates hippocampal GR and/or MR (Brady *et al*, 1991; Budziszewska *et al*, 1994), but it is not clear whether this up-regulation is evoked through a direct action of antidepressants on these receptors. Therefore the direct effects of short- and long-term paroxetine administration on GR density, GR mRNA, CRH type 1 receptor mRNA and BDNF mRNA concentrations were examined in the rat brain. Sucrose preference was used as a behavioural measure of anhedonia.

6.3. Study Design

This chapter is part of one study, alongside Chapters 5 and 7. Refer to Figure. 6.1. for a description of the study design for this specific chapter. Rats were handled twice daily up until the time of testing in order to minimise procedural stress (approximately 7-10 days prior to experimentation), and during this time they were given two bottles of water per cage, to minimise place preference (refer to Section.5.3.2.).

Figure. 6.1



A schematic diagram of the study design, refer to sections 6.4.2. and 6.4.3. Each square represents 1 day.

6.4. Methods

6.4.1. Animals

Adult male Wistar rats – as per Section 5.4.1. The use of these animals in the following scientific procedures were regulated by the Animals (Scientific Procedures) Act 1986.

6.4.2. Paroxetine Administration

Previous experiments in our laboratory used 5mg/kg p.o. paroxetine but this had no effect when administered with restraint stress, whereas preliminary experiments had shown an effect with 10mg/kg p.o. Also other investigations used oral doses of 10mg/kg p.o. Therefore this dose was chosed for the following studies. Rats received daily administration of paroxetine (10mg/kg p.o.) or distilled water (1ml/kg p.o.). The later were the same control animals (**Acute II and Chronic**) that were studied in the previous chapter (refer to section.5.3).

6.4.3. Sucrose Preference Test

The sucrose preference tests were conducted at the same time each day, as described in section 5.4.3.

6.4.4. Radioimmunoassay of plasma corticosterone and ACTH concentrations

Plasma corticosterone and ACTH concentrations were measured by using radioimmunassay commercial kits (refer to Section 2.4.).

6.4.5. Other Assays

GR binding, protein measurements and mRNA were carried out as in section 5.4.5

6.5. Statistical Analysis

The data is presented as the arithmetic mean +/- s.e.mean. The effects of time ("days") and paroxetine on all the biochemical and behavioural measures were analysed by two-factor completely randomised ANOVA (paroxetine x time), followed by Newman-Keuls post-hoc comparisons. All statistical analyses were performed using the software package, "GBStat" v6.5.

6.6. Results

6.6.1 Plasma Corticosterone and ACTH Concentrations.

1. Corticosterone

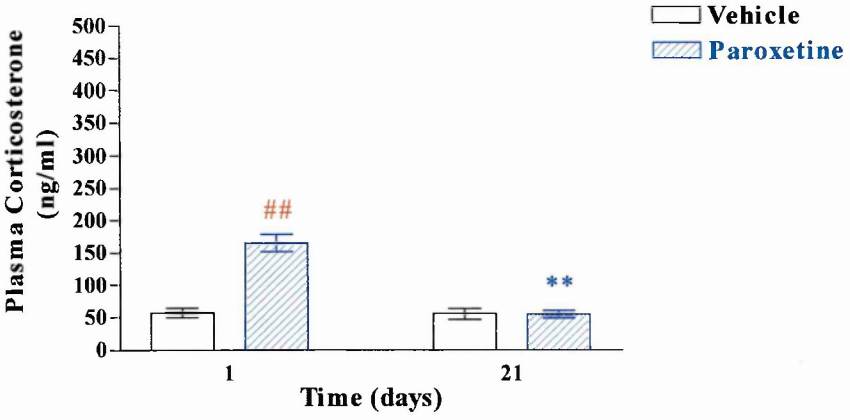
Statistical analysis indicated that paroxetine adminstration alone ($F_{1,21}$=40.47; $p<0.0001$), time alone ($F_{1,21}$=44.41; $p<0.0001$) and the interaction between paroxetine and the length of administration ($F_{1,21}$=41.44; $p<0.0001$) significantly affected the plasma corticosterone concentrations (**Figure.6.2.**). A single paroxetine administration increased plasma corticosterone concentrations by 166%, compared to vehicle. Plasma corticosterone concentrations in rats given paroxetine for 21 days were the same as in vehicle-treated rats.

2. Adrenocorticotrophic Hormone (ACTH)

A single paroxetine administration had no significant effect upon plasma ACTH concentrations compared to vehicle. Plasma ACTH concentrations in rats given paroxetine for 21 days were not significantly different from the vehicle-treated rats (**Figure.6.3.**).

Figure.6.2.

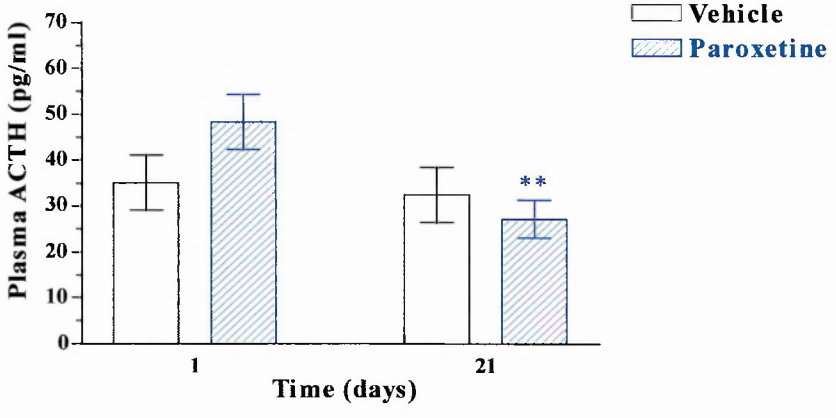
Plasma Corticosterone Concentration following Paroxetine Administration.



Plasma corticosterone concentrations in male Wistar rats, housed in pairs (n=8 per group), were measured in samples taken following administration of paroxetine. Data are expressed as mean ± sem. Comparison between vehicle-treated rats (vehicle group) and paroxetine-treated rats (paroxetine group) left in their homecages for 1 day or 21 days before they were scarificed. ##, $p<0.01$ compared to vehicle; **, $p<0.01$ compared to one administration of paroxetine.

Figure.6.3.

Plasma ACTH concentration following paroxetine administration.



Plasma ACTH concentrations in male Wistar rats, housed in pairs (n=8 per group), were measured in samples taken following administration of paroxetine. Data are expressed as mean ± sem. Comparison between vehicle-treated rats (vehicle group) and paroxetine-treated rats (paroxetine group) left in their homecages for 1 day or 21 days before they were scarificed. **, $p<0.01$ compared to one administration of paroxetine.

6.6.2. Glucocorticoid Receptor Binding

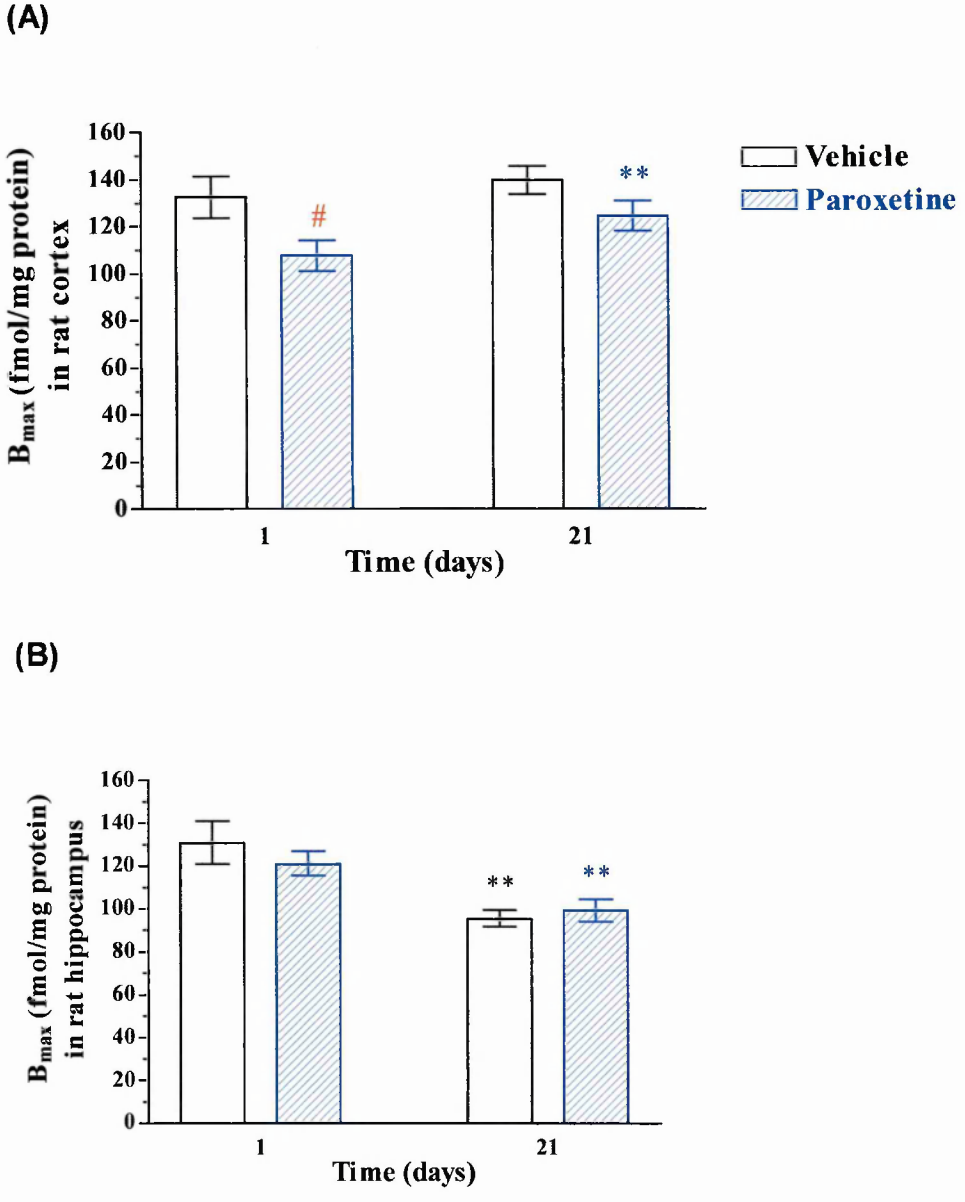
1. B_{max}

Statistical analysis indicated that paroxetine alone ($F_{1,23}$=4.613; p=0.0409) and the length of time of the experiment ($F_{1,23}$=5.846; p=0.0226) significantly affected the B_{max} values from glucocorticoid receptor binding within the cortex (**Figure.6.4A**). A single paroxetine administration decreased cortical GR B_{max} by 20% compared to vehicle. Repeated paroxetine administration had no significant effect on cortical GR B_{max} compared to vehicle.

In the hippocampus, statistical analysis indicated that the length of time of the experiment significantly affected ($F_{1,23}$=31.02; p<0.0001) the B_{max} values from glucocorticoid receptor binding (**Figure.6.4B**), as shown by the control values varying between 95 and 130 fmol/mg protein. A single paroxetine administration or repeated paroxetine administrations had no significant effect on hippocampal GR B_{max} compared to vehicle.

Figure.6.4.

Glucocorticoid Receptor Bmax values from cortical (A) and hippocampal (B) cytosolic fractions following administration of paroxetine.



GR Bmax values in cortex and hippocampus (taken from male Wistar rats, housed in pairs (n = 8 per group)) were measured in samples taken following administration of paroxetine. Data are expressed as mean ± sem. Comparison between vehicle-treated rats (vehicle group) and rats administered with paroxetine 10mg/kg p.o. per day (paroxetine group) for either 1 day or 21 days before they were sacrificed, **(A)** Bmax in cortical tissue **(B)** Bmax in hippocampal tissue. #, $p<0.05$ compared to vehicle; **, $p<0.01$ compared to vehicle after 1 day; **, $p<0.01$ compared to one administration of paroxetine.

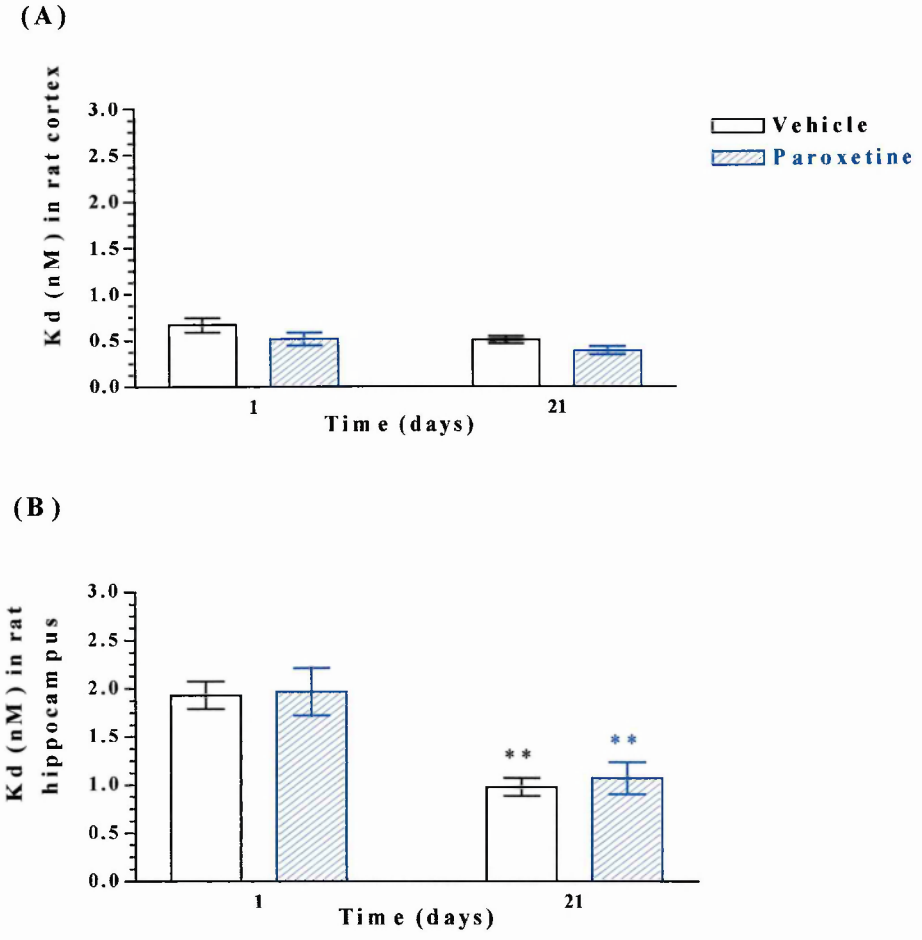
2. K_d

In the cortex, statistical analysis indicated that length of time of the experiment significantly affected ($F_{1,27}$=8.412; p=0.0081) the K_d values from glucocorticoid receptor binding (**Figure.6.5A**). A single paroxetine administration or repeated paroxetine administrations had no significant effect upon cortical GR K_d compared to vehicle.

In the hippocampus, statistical analysis indicated that length of time of the experiment significantly affected ($F_{1,26}$=20.19; p=0.0002) the K_d values from glucocorticoid receptor binding (**Figure.6.5B**), as shown by the control values varying between 1.00 and 1.88 nM. A single paroxetine administration or repeated paroxetine administrations had no significant effect upon hippocampal GR K_d compared to vehicle.

Figure.6.5.

Glucocorticoid Receptor Kd values from cortical (A) and hippocampal (B) cytosolic fractions following administration of paroxetine.



GR Kd values in cortex and hippocampus, taken from male Wistar rats, housed in pairs (n = 8 per group) were measured in samples taken following administration of paroxetine. Data are expressed as mean ± sem. Comparison between vehicle-treated rats (vehicle group) and paroxetine-treated rats (paroxetine group) left in their homecages for either 1 day or 21 days before they were sacrificed, (**A**) Kd in cortical tissue (**B**) Kd in hippocampal tissue.**, $p<0.01$ compared to 1 day of vehicle; **, $p<0.01$ compared to one administration of paroxetine.

6.6.3. Glucocorticoid Receptor mRNA

Statistical analysis indicated that paroxetine administration ($F_{1,23}$=11.8; p = 0.0022) and length of time of the experiment ($F_{1,23}$=15.37; p = 0.0007) significantly affected the GR mRNA concentrations in the cortex (**Figure.6.6.**). A single paroxetine administration had no significant effect upon cortical GR mRNA concentrations compared to vehicle. Repeated paroxetine administrations decreased cortical GR mRNA concentrations by 45% compared to vehicle but this is appears to be due to an increase in controls rather than an effect of drug administration. Control values varied between 0.6 and 1.2 arbitrary units.

6.6.4. Brain-Derived Neutrophic Factor (BDNF) mRNA:

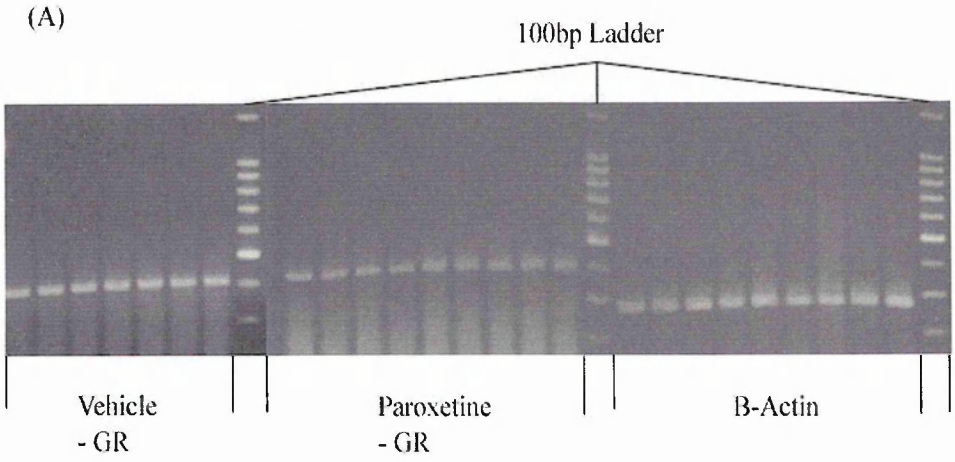
Statistical analysis indicated that paroxetine administration ($F_{1,25}$=32.74; p<0.0001) and length of time of the experiment ($F_{1,25}$=57.43; p<0.0001) significantly affected the BDNF mRNA concentrations in the cortex (**Figure.6.7.**). A single paroxetine administration had no significant effect upon cortical BDNF mRNA concentrations compared to vehicle. Repeated paroxetine administration decreased cortical BDNF mRNA concentrations by 70% compared to vehicle. Control values varied between 1.1 and 1.4 arbitrary units.

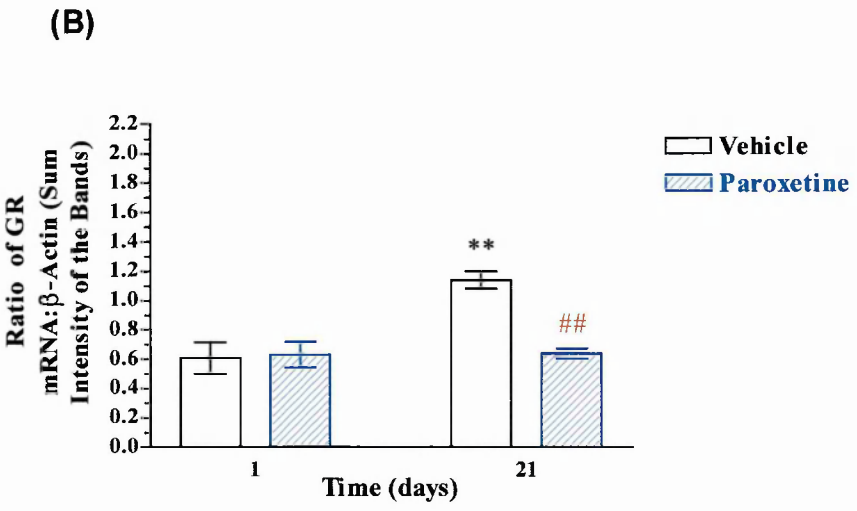
6.6.5. Corticotropin-Releasing Hormone (CRH) Type 1 Receptor mRNA

Statistical analysis indicated that a single paroxetine administration had no significant effect upon the CRH type 1 receptor mRNA after 2 days (**Figure.6.8.**).

Figure. 6.6.

Cortical GR mRNA and β-Actin mRNA following paroxetine administration.

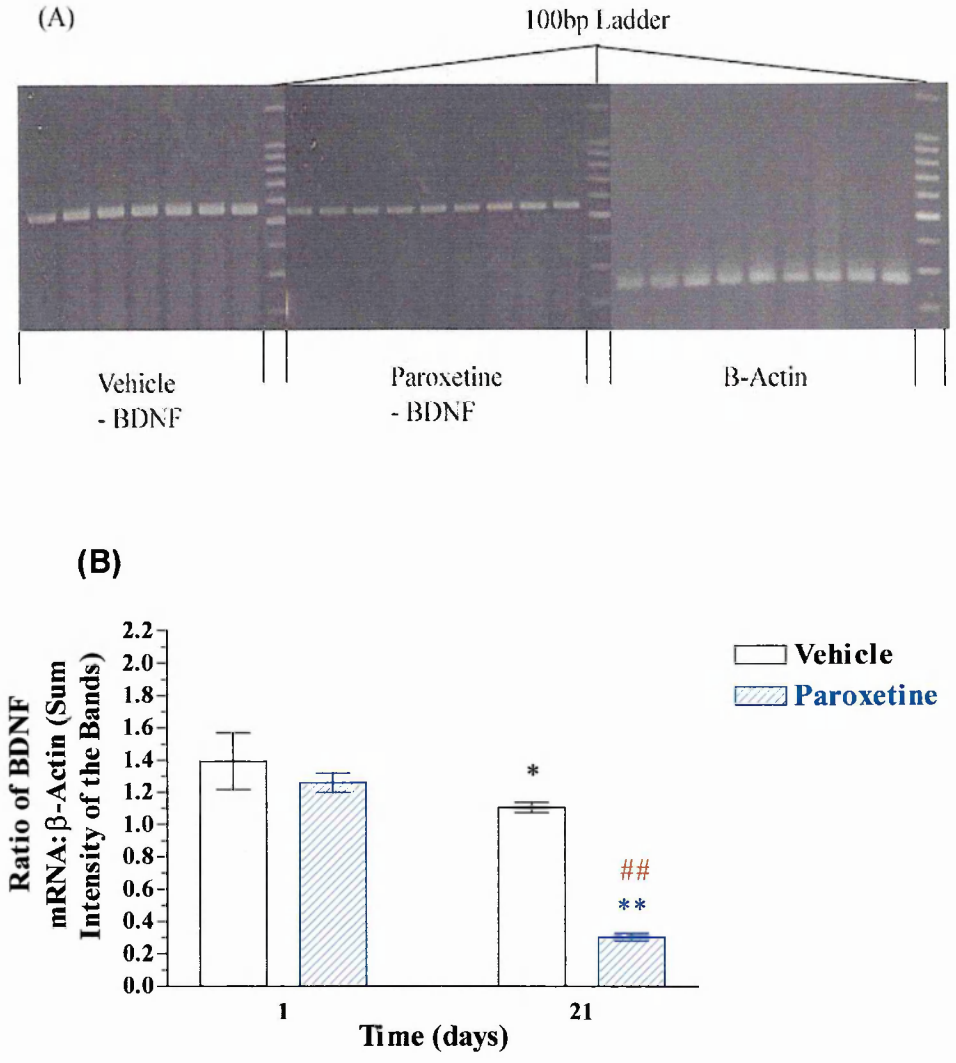




Cortical GR mRNA and β-Actin mRNA concentrations from male Wistar rats, housed in pairs (n=8 per group). (**A**) RT-PCR and agarose gel electrophoresis analysis of GR mRNA expression. β-Actin mRNA expression was used as an internal control. Vehicle samples were run on a gel with β-Actin mRNA and a 100bp ladder, paroxetine samples were run on a separate gel with β-Actin mRNA and a 100bp ladder. (**B**) Ratio of cortical GR mRNA: β-Actin mRNA (sum intensity of the bands). Data are expressed as mean ± sem. Comparison between vehicle-treated rats (vehicle group) and paroxetine-treated rats (paroxetine group) left in their homecages for either 1 day or 21 days before they were sacrificed. ##, $p<0.01$ compared to vehicle; **, $p<0.01$ compared to 1 day of vehicle.

Figure. 6.7.

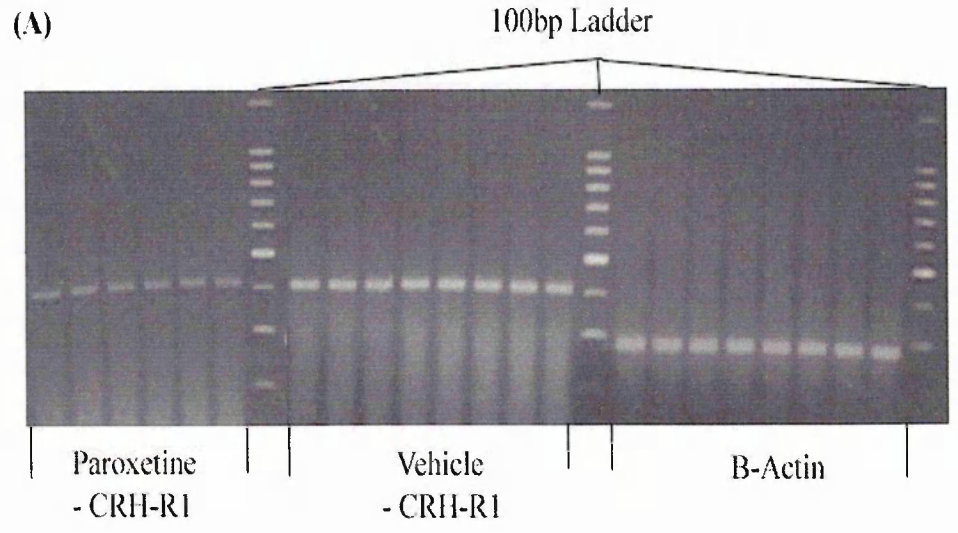
Cortical BDNF mRNA and β-Actin mRNA following paroxetine administration.

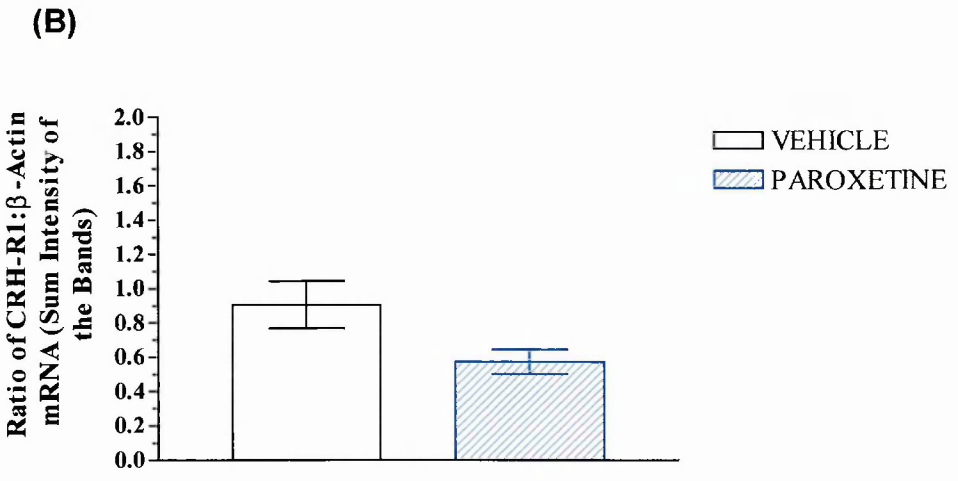


Cortical BDNF mRNA and β-Actin mRNA from male Wistar rats, housed in pairs (n=8 per group), were measured in samples taken immediately following paroxetine administration. (**A**) RT-PCR and agarose gel electrophoresis analysis of BDNF mRNA expression. β-Actin mRNA expression was used as an internal control. Vehicle samples were run on a gel with a 100bp ladder, the paroxetine samples were run on another gel with a 100bp ladder, and the β-Actin mRNA was run on a separate gel with a 100bp ladder. (**B**) Ratio of cortical BDNF mRNA: β-Actin mRNA (sum intensity of the bands). Data are expressed as mean ± sem. Comparison between vehicle-treated rats (vehicle group) and paroxetine-treated rats (paroxetine group) left in their homecages for either 1 day or 21 days before they were sacrificed. ##, $p<0.01$ compared to vehicle; *, $p<0.05$ compared to 1 day of vehicle; **, $p<0.01$ compared to one administration of paroxetine.

Figure.6.8.

Cortical CRH type 1 receptor mRNA and β-Actin mRNA following a single paroxetine administration.





Cortical CRH type 1 receptor:β-Actin mRNA from male Wistar rats, housed in pairs (n = 8 per group), were measured in samples taken immediately following paroxetine administration. **(A)** RT-PCR and agarose gel electrophoresis analysis of CRH type 1 receptor mRNA expression. β-Actin mRNA expression was used as an internal control. Vehicle samples and paroxetine samples were run on 1 gel with 100bp ladders and β-Actin mRNA was run on a separate gel with a 100bp ladder. **(B)** Ratio of cortical CRH type 1 receptor:β-Actin mRNA (sum intensity of the bands). Data are expressed as mean ± sem. Comparison between vehicle-treated rats (vehicle group) and paroxetine-treated rats (paroxetine group) left in their homecages for 1 day before they were sacrificed.

6.6.6. Behavioural Measurements

1. Sucrose Preference

Single or repeated paroxetine administration had no significant effect upon sucrose preference compared to vehicle or basal levels. (**Table.6.1.**).

Table.6.1.

Sucrose preference (% of total fluid intake) following paroxetine administration.

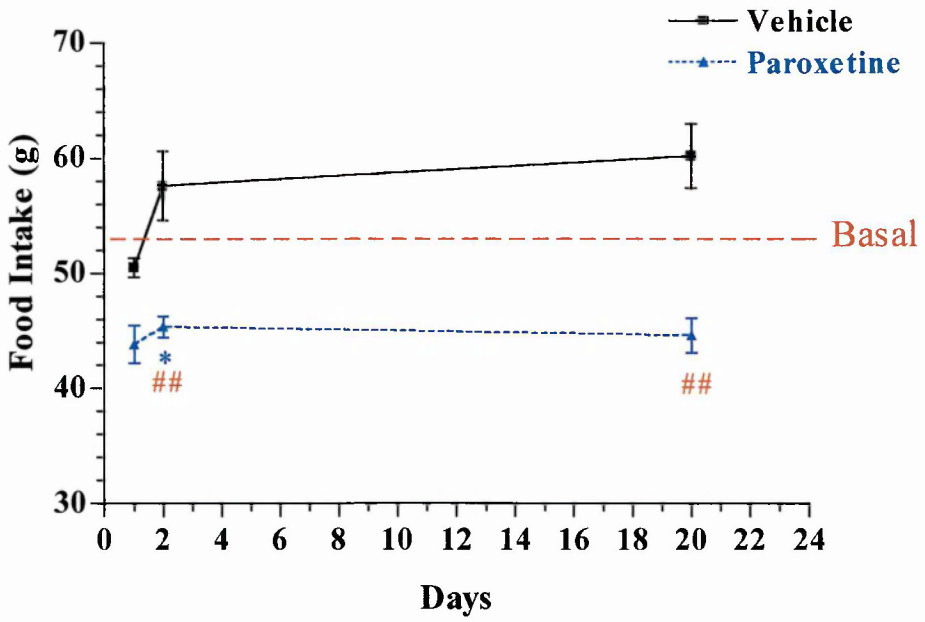
GROUPS	**Mean ±**	**N**
BASAL	96.5 ± 0.7	13
1 DAY		
1ml/kg p.o. distilled water	95.3 ± 2.6	4
10mg/kg p.o. Paroxetine	96.1 ± 0.7	4
2 DAYS		
1ml/kg p.o. distilled water	89.8 ± 6.1	3
10mg/kg p.o. Paroxetine	95.1 ± 0.6	3
20 DAYS		
1ml/kg p.o. distilled water	91.9 ± 5.5	3
10mg/kg p.o. Paroxetine	95.1 ± 0.7	3

2. Food Intake

Statistical analysis indicated that paroxetine significantly affected ($F_{1,18}$ = 12.77; p=0.0022) the food intake over 22 h (**Figure.6.9.**). A single paroxetine administration decreased food intake by about 20% compared to basal levels but this was not significantly different compared to vehicle. A second paroxetine administration (day 2) significantly decreased food intake by about 25% compared to vehicle and repeated paroxetine administration significantly decreased food intake by about 30% compared to vehicle.

Figure.6.9.

Food intake following administration of paroxetine.



Food intake (g) from male Wistar rats, housed in pairs (n = 4 per group), measured over 22 h. Data are expressed as mean ± sem. Comparison between vehicle-treated rats (vehicle group) and paroxetine-treated rats (paroxetine group) left in their home cages for either 1 day, 2 days or 20 days. ##, $p<0.01$ compared to vehicle; *, $p<0.05$ compared to one administration of paroxetine.

6.7. Discussion

The main findings of the present experiments can be summarised as follows: **(1)** acute paroxetine increased plasma corticosterone but had no effect upon plasma ACTH concentration; **(2)** acute paroxetine decreased GR in the cortex; **(3)** chronic paroxetine decreased BDNF gene expression in the cortex; **(4)** acute and chronic paroxetine decreased food intake.

The current data indicated that plasma corticosterone concentrations were significantly elevated by acute paroxetine administration, but this decreased after chronic administration (**Figure.6.2.**), suggesting that there was a time-lag in the effectiveness of paroxetine in reducing plasma corticosterone concentrations. Acute administration had no significant effect on plasma ACTH concentrations but after chronic administration of paroxetine ACTH concentration decreased below the controls (**Figure.6.3.**). These results confirm previous investigations that have shown long-term antidepressant administration (>2 weeks) decreases plasma corticosterone concentrations and attenuate HPA activity in comparison to acute administration (Reul *et al*, 1993; Rowe *et al*, 1997).

Reports in the literature have stated that acute administration of SSRIs increases the secretion of several hormones, i.e. ACTH, corticosterone and cortisol, GH, prolactin and AVP, but chronic administration with SSRIs does not alter basal blood concentrations of hormones, a finding confirmed by the present results. However, adaptive changes are induced by long-term administration with SSRIs in serotonergic, noradrenergic and peptidergic neural function. Neuroendocrine challenge tests both in experimental animals and in humans indicate that chronic SSRIs produce an increase in serotonergic terminal function, accompanied by desensitisation of post-synaptic 5-HT_{1A} receptor-mediated cortisol, GH and OT responses, and by supersensitivity of post-synaptic 5-HT_{2A} (and/or 5-HT_{2C}) receptor-mediated secretion of hormones. Chronic exposure to SSRIs does not alter the neuroendocrine stress-response and produces inconsistent changes in α_2

adrenoceptor-mediated GH secretion. Overall, the effects of SSRIs on neuroendocrine function are dependent on adaptive changes in specific neurotransmitter systems that regulate the secretion of specific hormones (for a review see Raap & Van de Kar, 1999).

These findings are interesting when taken in the context of the time course needed for SSRIs to exert their therapeutic efficacy in major depression. The increase in 5-HT release resulting from long-term SSRI treatment would theoretically lead to an increased activation of 5-HT_{2A} receptors on noradrenergic locus coeruleus neurones (Haddjeri *et al*, 1997). This would yield an increased inhibitory response and ultimately a decrease in firing activity of locus coeruleus noradrenergic neurones, as observed by Szabo *et al* (1999). SSRIs thus decrease the locus coeruleus firing rate, which may ultimately also attenuate noradrenaline release in projection areas. This in turn may have a profound impact on the α_2-adrenergic heteroreceptors on the 5-HT terminals, thus diminishing the inhibitory influence of these noradrenergic receptors and contributing to the increase of 5-HT neurotransmission by the SSRI (Szabo *et al,* 1999). Therefore examining serotonin and noradrenaline concentrations during this study could have given us an insight into their role in the control of the HPA axis.

The current study indicated that the effect of paroxetine on the densities of GR differed according to the brain region. In the cortex, there was a 20% reduction in receptor number after acute administration (**Figure.6.4A.**). In the hippocampus, there was no change in GR binding (**Figure.6.4B**). Previous studies in our laboratory have shown paroxetine induced decreases in corticosteroid receptors in the cortex and thymus following 14 and 28 days of administration, respectively (Maurya M, 2001).

All previous investigations into the effects of chronic antidepressant administration on brain corticosteroid receptor concentration have been carried out in animals that were adrenalectomised prior to the measurement of corticosteroid receptor binding activity. This procedure has the effect of removing residual endogenous ligand from binding sites thus enabling the measurement of changes in (available) corticosteroid receptor concentration under conditions of minimal nuclear occupancy. However;

i) the procedure of adrenalectomy itself is found to interfere with the regulation of corticosteroid receptors (Reul *et al*, 1989; Karst *et al*, 1997).

ii) in depression, corticosteroid receptor regulation by stress and/or antidepressant treatment occurs in the presence of the endogenous ligand.

It is essential therefore to investigate corticosteroid receptor binding activity in the presence of endogenous ligand in laboratory animals to determine whether the up-regulation of corticosteroid receptors observed following long-term antidepressant administration is an effect observed in the adrenally-intact animal. This study has shown that this effect is not observed in the cortex or hippocampus of adrenally-intact rats after 21 days of paroxetine administration, suggesting that the presence of endogenous ligand does alter the corticosteroid receptor binding activity following long-term antidepressant administration.

The neuroanatomical specificity of these effects also supports the hypothesis of altered corticosteroid receptor plasticity following stress and antidepressant administration. Many changes in corticosteroid receptor concentrations are observed in the hippocampus (the location of most brain MR and some GR) and the hypothalamus, both regions that are heavily involved with corticosteroid receptor regulation. Since many of the changes in receptor plasticity are observed in GR (which are more widely distributed), the

investigation of these receptors in various other brain regions (and peripheral tissues) would also be of interest.

A study by Sémont *et al* (2000) showed that the expression of hippocampal and hypothalamic corticosteroid receptors were regulated by short-term stimulation of serotonin neurotransmission through a corticosterone-independent mechanism. However, the regulation of corticosteroid receptors by serotonin were different according to the method used to stimulate serotonin neurotransmission, i.e. stimulation of serotonin synthesis and release by 5-HTP, or inhibition of serotonin reuptake by an SSRI. Antidepressant drugs have also been shown to directly increase GR binding sites in primary hippocampal neurones, which are devoid of pre-synaptic serotonergic innervations (Hery *et al*, 2000). This suggests that the effects of antidepressants on hippocampal corticosteroid receptor mRNA expression could be independent of its actions on serotonin.

In this present study, acute administration of paroxetine had no effect on GR mRNA concentrations within the cortex after acute paroxetine administration, and this remained during chronic administration (**Figure.6.6.**). Therefore, in the cortex the densities of GR binding sites did not correlate well with their respective mRNA. A possible explanation for the lack of change in cortical GR mRNA expression after paroxetine administration, would be that paroxetine might have produced regionally selective changes, which were not detected using the methods in this study. Indeed, Yau *et al* (1997) pointed out the subregional specificity of the effects of 5-HT upon hippocampal corticosteroid receptors. Nevertheless, the involvement of a post-transcriptional effect of serotonin on GR binding sites cannot be excluded. The phosphorylation status of GR may play an important role in the regulation of GR densities (Webster *et al,* 1997); and, since serotonergic binding to most of its receptors activates kinases, serotonin could regulate GR densities directly, by acting on their phosphorylation status. In the cortex, the discrepant

findings between the decrease in GR protein and no change in the mRNA expression could be due to the different half lives of GR mRNA and protein (Dong *et al*, 1988).

Several groups have shown increased MR and GR mRNA expression in the hippocampus following long-tem antidepressant administration (Brady *et al*, 1991; Yau *et al*, 1995), and therefore reduce HPA axis activity. However, very little has been reported using SSRI antidepressants upon corticosteroid receptor mRNA expression. Brady *et al* (1992) reported no change in hippocampal MR and GR mRNA expression following 2-weeks of fluoxetine administration while another study showed no change in hippocampal GR mRNA concentrations after 10-days of fluoxetine administration (Rossby *et al*, 1995).

Some antidepressants, e.g., desipramine, also induce increases in GR gene promoter activity, GR mRNA concentrations (Pepin et *al*, 1992a; Rossby *et al*, 1995), and in the receptor density (Przegalinski & Budziszewska, 1993). However, other antidepressants, such as oxaprotiline, citalopram and mianserin do not affect the receptor density (Budziszewska *et al,* 1994), suggesting that the effect upon GR mRNA and binding varies between antidepressant drugs.

The hippocampus is densely innervated by serotonergic fibers originating from the raphe nuclei. Serotonin is a potent regulator of hippocampal MR and GR, with neurotoxic lesions to central serotonergic innervations decreasing mRNA concentrations (Yau *et al*, 1994) and serotonin directly increasing GR mRNA expression in hippocampal neurones in culture (Mitchell *et al*, 1990). However, less is known about the effects of antidepressants on cortical corticosteroid receptor mRNA expression.

In a recent study, fluoxetine administered to rats for 7 days reduced the mean firing rate of serotonin neurones in the dorsal raphe (Czachura & Rasmussen, 2000), thus limiting the amount of serotonin released into the synapse. Hence a delayed substantial increase in extracellular serotonin concentrations in the hippocampus after fluoxetine

administration may in part explain the delayed therapeutic effects of antidepressants on corticosteroid receptors and thus normalisation of the HPA axis.

GR is a hormone-activated transcription factor, which binds to a specific DNA sequence (GRE) and acts as a regulator of gene expression. The GR-mediated gene transcription can be modulated by cAMP/PKA-PLC/PKC- and CAM-mediated signal transduction pathways (Maroderm *et al*, 1993; Ning & Sanchez, 1995) whose activities are affected by antidepressant drugs (Silver *et al*, 1986; Nibuya *et al*, 1996). To date, only the effect of desipramine on GR-mediated transcription has been determined, and this is dependent on experimental conditions, i.e., on the concentration and time of its and dexamethasone presence in the medium, and on the presence/absence of steroids in the serum added to an incubation medium (Pariante *et al*, 1997; Pepin *et al*, 1992a). Pariante *et al* (1997) found that desipramine induced GR translocation from the cytoplasm to the nucleus in the absence of steroids (with no effect alone on GR-mediated gene transcription) and potentiated dexamethasone-induced GR translocation and dexamethasone-induced GR-mediated gene transcription. They suggested that one important aspect of the effects of antidepressants *in vivo* may be to facilitate GR-mediated feedback inhibition on the HPA axis, by facilitating GR translocation and function, and thereby reverse glucocorticoid hypersecretion in depression.

A wide variety of neurotransmitters, which are regulated differentially by distinct stressors, may be involved in the regulation of hippocampal or cortical GR mRNA and/or binding (Herman, 1993). A recent study has shown the regulation of hippocampal GR or GR mRNA by catecholamines (through a β-adrenergic receptor), NMDA, or GABA-A receptors (Tritos *et al*, 1999).

After acute administration of paroxetine, there was a slight decrease in BDNF expression in the cortex, but after chronic administration this was further decreased **(Figure.6.7.)**.

Very few investigations have been conducted on the effects of antidepressant drugs on BDNF mRNA concentrations, yet they all indicate that antidepressants increase BDNF mRNA, which is opposite to the current results. For example, according to Nibuya *et al* (1996), chronic, but not acute, administration of several different antidepressant drugs including tranylcypromine, desipramine, sertraline, and mianserin, significantly increased BDNF mRNA concentrations in the hippocampus, but only tranylcypromine significantly increased expression of BDNF in frontal cortex.

Zetterström *et al* (1999) demonstrated that paroxetine reduced BDNF mRNA concentrations in the dentate gyrus but was without effect in the frontal cortex in rats, suggesting that serotonin modulated BDNF mRNA concentrations in a different way according to the brain region. This is compatible with microdialysis studies demonstrating that acute paroxetine administration increased extracellular serotonin concentration in hippocampus (Sharp, 1992) but lacked a significant effect in frontal cortex (Sharp *et al*, 1997).

The present results indicated that a single paroxetine administration had no significant effect upon CRH type 1 receptor expression in cortical tissue (**Figure.6.8.**), although there was a trend towards a decrease.

There is very little literature on the effects of antidepressants, particularly SSRIs, upon CRH receptor expression in the brain. Fluoxetine administration (10mg/kg i.p.) has been shown to cause a significant increase in CRH–R1 receptor mRNA levels in the PVN but with a slow synthesis of the receptor mRNA, as high concentrations were only discernible 360 minutes after fluoxetine administration (Torres *et al*, 1998). As this was conducted in the PVN with fluoxetine, there is a possibility that differences may occur due to the brain region examined as well as the antidepressant used.

In this present study, acute and chronic paroxetine administrations had no significant effect upon sucrose preference (**Table.6.1.**), but suppressed food intake (**Figure.6.9.**), suggesting that this antidepressant does not have an effect upon sucrose preference, in this current study.

Chronic treatments with tricyclic or atypical antidepressants have been shown to reverse chronic mild stress-induced anhedonia, but at doses which have no effect on rewarded behaviours in non-stressed animals (for a review see Willner, 1995). Both 5-HTP and fluoxetine, as well as other SSRIs, are well known to reduce food intake in rats (for a review see Dourish, 1992).

6.8 Conclusions

This series of experiments demonstrated that paroxetine significantly affected components of the HPA axis under baseline conditions, resulting in similar responses elicited by stress (**Table.6.2.**).

Table.6.2.

Similar responses elicited by either 30 min restraint stress or 10mg/kg p.o. paroxetine administration.

Measurement	Treatment	1 day	21/23 days
Corticosterone (ng/ml)	10mg/kg p.o. Paroxetine	166% ↑	-
	30 min Restraint Stress	200% ↑	-
Cortical GR Bmax (fmol/mg protein)	10mg/kg p.o. Paroxetine	20% ↓	-
	30 min Restraint Stress	24% ↓	-
Cortical GR mRNA (sum intensity of the bands)	10mg/kg p.o. Paroxetine	No Change	-
	30 min Restraint Stress	No Change	-
Cortical BDNF mRNA (sum intensity of the bands)	10mg/kg p.o. Paroxetine	-	70% ↓
	30 min Restraint Stress	-	36% ↓
Cortical CRH-R1 mRNA (sum intensity of the bands)	10mg/kg p.o. Paroxetine	No Change	-
	30 min Restraint Stress	No Change	-
Food Intake (g) in 22h	10mg/kg p.o. Paroxetine	-	25% ↓
	30 min Restraint Stress	-	24% ↓

Refer to Sections 5.6.1.1., 5.6.2., 5.6.3., 5.6.4., 5.6.5., and 5.6.6.2. respectively, for the restraint stress data.

CHAPTER 7. THE EFFECT OF PAROXETINE ADMINISTRATION, IN RATS, ON THE STRESS-INDUCED RESPONSES OF THE HPA AXIS.

7.1. Introduction

The normalisation of the hyperactive HPA axis and the mood-stabilising effects seem to occur simultaneously during antidepressant administration, indicating that the two effects are either directly or indirectly interdependent (Holsboer *et al*, 1982; Holsboer & Barden, 1996). Thus, the effects of long-term antidepressant administration on the HPA axis and the feedback inhibition of glucocorticoids are therefore important in understanding the mechanisms by which antidepressants exert their therapeutic effects.

Stress-related behavioural paradigms, particularly those associated with increased anxiety or conditioned fear, may activate topographically organised mesolimbocortical serotonergic systems. For example, behavioural paradigms associated with increased anxiety or conditioned fear increase serotonin metabolism or release in the medial prefrontal cortex (Adell *et al*, 1997; Dunn, 1988), cingulated cortex (Palkovits *et al*, 1976), entorhinal cortex (Blanchard *et al*, 1991; Ge *et al*, 1997), nucleus accumbens (Inoue *et al*, 1993; Ge *et al*, 1997), amygdala (Blanchard *et al*, 1991; Amat *et al*, 1998), and dorsal hippocampus (Joseph & Kennett, 1983; Ge *et al*, 1997). This topographically selective activation of serotonergic neurotransmission suggests that the serotonergic neurones activated by these stress-related stimuli may reside in the median raphé nucleus (Vertes & Martin, 1988; Vertes *et al*, 1999) and ventral and interfascicular regions of the caudal dorsal raphé nucleus (Pierce *et al*, 1976).

Previous exposure to stressful stimuli has been shown to result in an upregulation of tryptophan hydroxylase mRNA concentrations (coding for the rate limiting enzyme in serotonin synthesis) in the dorsal and median raphé nuclei (Chamas *et al*, 1999) and

enhance the responsiveness of mesolimbocortical serotonergic neurotransmission to a subsequent stress (De Souza & Van Loon, 1986; Adell *et al*, 1988a). Intense psychophysical stress is believed to sensitise the animal so that subsequent behavioural responses to stress (including behavioural anxiety and fear) are exaggerated 24 or 48 h later (Graeff *et al*, 1996). This behavioural sensitisation is believed to be a result of prolonged, enhanced sensitivity of serotonergic neurones located in the caudal portion of the dorsal raphé nucleus, possibly involving a functional desensitisation of somatodendritic 5-HT_{1A} receptors (Laaris *et al*, 1997; Grahn *et al*, 1999).

In addition to the behavioural and neurochemical consequences of exposure to stress there is an abundance of literature dealing with the attenuating effect of antidepressant treatments on stress-induced HPA axis activation (Reul *et al*, 1993; Holsboer & Barden, 1996). While it has been reported that both TCAs and MAOIs attenuate stress-related increases in HPA axis activity (Reul *et al*, 1994), less attention has been given to the effect of SSRIs on stressor-induced HPA axis activation.

In spite of a large number of studies on the neurochemical changes in stress, an equivocal case is yet to be made for the role of a specific neurotransmitter in this important neurobiological disorder. The difficulty arises from the fact that there is no single neurotransmitter system that appears to be responsible for the stress-induced damage to the hippocampal neurones. A recent study by Sunanda *et al* (2000) evaluated the effect of restraint stress on the alterations in the concentrations of biogenic amines, amino acids and acetylcholinesterase activity in the hippocampus of male Wistar rats. They showed a significant decrease in the concentrations of noradrenaline, dopamine, 5-HT and acetylcholinesterase activity in the stressed rats compared to controls. However, concentrations of glutamate were significantly increased in stressed rats. These results indicate that chronic restraint stress (6 h per day for 21 days) decreases aminergic and

cholinergic neurotransmission, and increases the glutamatergic transmission in the hippocampus.

As with other effective antidepressants, although the primary pharmacological action is understood, little is known regarding the ultimate mechanism(s) of action of the SSRIs that serve to confer therapeutic efficacy. One possibility is that affective disorders are the result of a disruption of some appropriate level of balance among central monoaminergic systems (Schatzberg & Schildkraut, 1995).

Failure to adapt to a daily restraint stress schedule repeated over 5-7 days has been used as an animal model for depression, and this model has been shown to respond appropriately to antidepressant pre-treatment (Curzon, 1989).

7.1.1. Endocrinological changes following exposure to restraint stress with antidepressant administration.

While SSRIs are prescribed for anxiety and panic disorders, little information is available regarding their effects on stress-induced release of hormones. Daily injections of rats with fluoxetine (5mg/kg/day) for 21 days did not alter the corticosterone response to forced swim stress (Duncan *et al*, 1998). Daily injections of fluoxetine (10mg/kg/day) to rats for 14 days did not alter the effect of conditioned fear stress on the secretion of ACTH, corticosterone, OT, prolactin or renin. These data suggest that the neuroendocrine stress responses are not altered by chronic SSRI treatment. The importance of hormones such as corticosterone/cortisol and the enzyme renin for survival probably requires multiple neurotransmitter mechanisms to mediate the effects of stress on their secretion. It is unlikely that all these neural mechanisms would be altered in a similar manner by SSRIs.

In addition to the behavioural and neurochemical consequences of exposure to stress there is an abundance of literature dealing with the attenuating effect of antidepressant treatments on stress-induced HPA axis activation (Reul *et al*, 1993; Holsboer & Barden, 1996).

Farisse *et al* (1999) have shown that there is a tonic activation of serotonin turnover by corticosterone through GR in the mouse hippocampus, and that stress-induced stimulation of serotonin metabolism in the brain stem and hippocampus (but not the cortex, striatum or hypothalamus) appeared to be delayed in transgenic mice deficient in GR compared to control mice. These results are particularly relevant for mood disorders where alterations of serotonergic transmission might be secondary to an impairment of GR functions. Recent studies have also indicated that life-long GR impairment has profound consequences for behavioural and neuroendocrine responses to a psychological stressor, and that long-term impaired functioning of GR evolves in hyper-responsiveness of the raphe-hippocampal serotonergic system (Linthorst *et al*, 2000).

López *et al* (1998) studied the effect of CMUS and antidepressant treatment on 5-HT_{1A}, GR and MR densities in rat hippocampus. They found that rats subjected to CMUS showed a significant elevation of basal plasma corticosterone, a decrease in 5-HT_{1A} mRNA and binding, as well as alterations in the MR/GR ratio. These were prevented by imipramine or desipramine (but not zimelidine) administration, suggesting that alterations in 5-HT_{1A} receptor levels and in the MR/GR balance may be one of the mechanisms by which stress may trigger and/or maintain depressive episodes.

Therefore very little work has been conducted on the effect of stress, particularly restraint, and antidepressant treatment on GR mRNA and binding in the rat brain.

Major depression is a condition that has been associated with a predisposing influence of major stressors, particularly early in life, and with neurochemical and neuroendocrine findings of CRH hypersecretion (Arborelius *et al*, 1999). On the basis of

these observations, it has been hypothesised that antidepressants may act in part by reducing CRH synthesis or secretion, either tonically or in response to stress (Stout *et al*, 2002).

Stress-related stimuli, particularly behavioural paradigms associated with increased anxiety or conditioned fear (Pezzone *et al*, 1993; Chung *et al*, 2000) including opiate withdrawal (Chieng *et al*, 1995; Chahl *et al*, 1996) and intracerebroventricular infusion of CRH or CRH-like peptides (Vaughan *et al*, 1995; Bittencourt & Sawchenko, 2000), activate immediate-early gene expression within the dorsal raphe nucleus. Based on these findings and evidence that stress-related stimuli increase serotonergic neurotransmission in the MRN and DRN (Adell *et al*, 1997; Maswood *et al*, 1998) and limbic forebrain regions, especially in response to intense, uncontrollable, or unpredictable stimuli (Adell *et al*, 1988b; Amat *et al*, 1998), one hypothesis is that stress increases serotonergic neurotransmission via the actions of CRH on subpopulations of serotonergic neurones that contribute to the mesolimbocortical serotonergic innervation of the forebrain.

7.1.2. Neurotrophin changes following exposure to restraint-stress with antidepressant administration.

The finding that stress decreases the expression of BDNF within the hippocampus suggests that regulation of neurotrophins could also contribute to the effects of stress on neuronal survival and function (Smith *et al*, 1995b). In adrenalectomised rats the influence of stress on the down-regulation of BDNF concentration was not significantly altered suggesting that glucocorticoids alone could not fully explain the regulation of BDNF by stress (Smith *et al*, 1995b). There are several neurotransmitter systems, including monoamine systems, that are influenced by stress and that could regulate the expression of BDNF (Kutchel, 1991; Chaouloff, 1993). One of these is the serotonergic system, which is profoundly influenced by stress (Joseph & Kennett, 1983; Vahabzadeh & Fillenz, 1994).

Moreover, recent work has shown that activation of 5-HT_{2A} receptors decreases concentrations of BDNF mRNA in the hippocampus in a manner similar to that observed after stress. This finding raises the possibility that the stress-induced down-regulation of BDNF concentrations may be mediated by release of 5-HT and activation of 5-HT_{2A} receptors.

7.1.3. Behavioural changes following exposure to restraint-stress with antidepressant administration.

A series of previous studies showed that the CMUS-induced anhedonia could be effectively reversed by chronic administration of antidepressant drugs including tricyclics, atypical antidepressants and monoamine oxidase inhibitors, but not with drugs devoid of an antidepressant activity (Willner *et al*, 1987; Papp *et al*, 1996).

A growing number of studies have revealed that like the antidepressive effect on depressed patients, prolonged but not acute administration of antidepressant reverses stress-induced behavioural disturbances. Noradrenergic and serotonergic projections have long been thought to be involved in the pathophysiology of mood disorders and in the mechanism of action of antidepressant drugs. However, and in addition to these neurotransmitters, a role for dopamine as part of the biochemical basis of depression has also been suggested (Willner, 1983; Kapur & Mann, 1992). Moreover, and despite the fact that antidepressant drugs have traditionally been reported to exert their primary action on noradrenergic and serotonergic neurones, a role for dopaminergic processes in the central effects of such drugs has also been suggested. For instance, acute and chronic antidepressants can influence dopaminergic activity on frontocortical areas (Tanda *et al*, 1996).

D'Aquila *et al* (1994) have previously demonstrated that acute administration of dopamine receptor agonists reduces the consumption of a sweet solution in chronically stressed rats successfully administered with tricyclic or atypical antidepressants, at doses that did not reduce consumption in non-stressed animals or in untreated stressed animals. These data suggest that an increase in dopamine receptor responsiveness is responsible for the action of antidepressant drugs in this model (Muscat *et al*, 1990; Cheeta *et al*, 1994).

Central mechanisms involved in the stress-induced inhibition of food intake have not been fully elucidated, but certain peptides and neurotransmitters are thought to be involved in the response. It is well established that monoamines (Kennett *et al*, 1987) and CRH (Krahn *et al*, 1990) influence feeding behaviour and mediate behavioural and physiological responses to stress (Sutton *et al*, 1982; Krahn *et al*, 1988). Several investigators have attributed stress-induced anorexia to activation of CRH (Krahn *et al*, 1986) and/or serotonin pathways (Kennett *et al*, 1986; Shimizu *et al*, 1989). Both of these transmitters are elevated in response to stress in a number of brain areas, including those that are involved in the regulation of feeding behaviour (Shimizu *et al*, 1992; Makino *et al*, 1995b). Intracerebroventricular administration of CRH produces behaviours typical of stress, including depression of appetite (Sutton *et al*, 1982; Krahn *et al*, 1986).

7.2. Aims

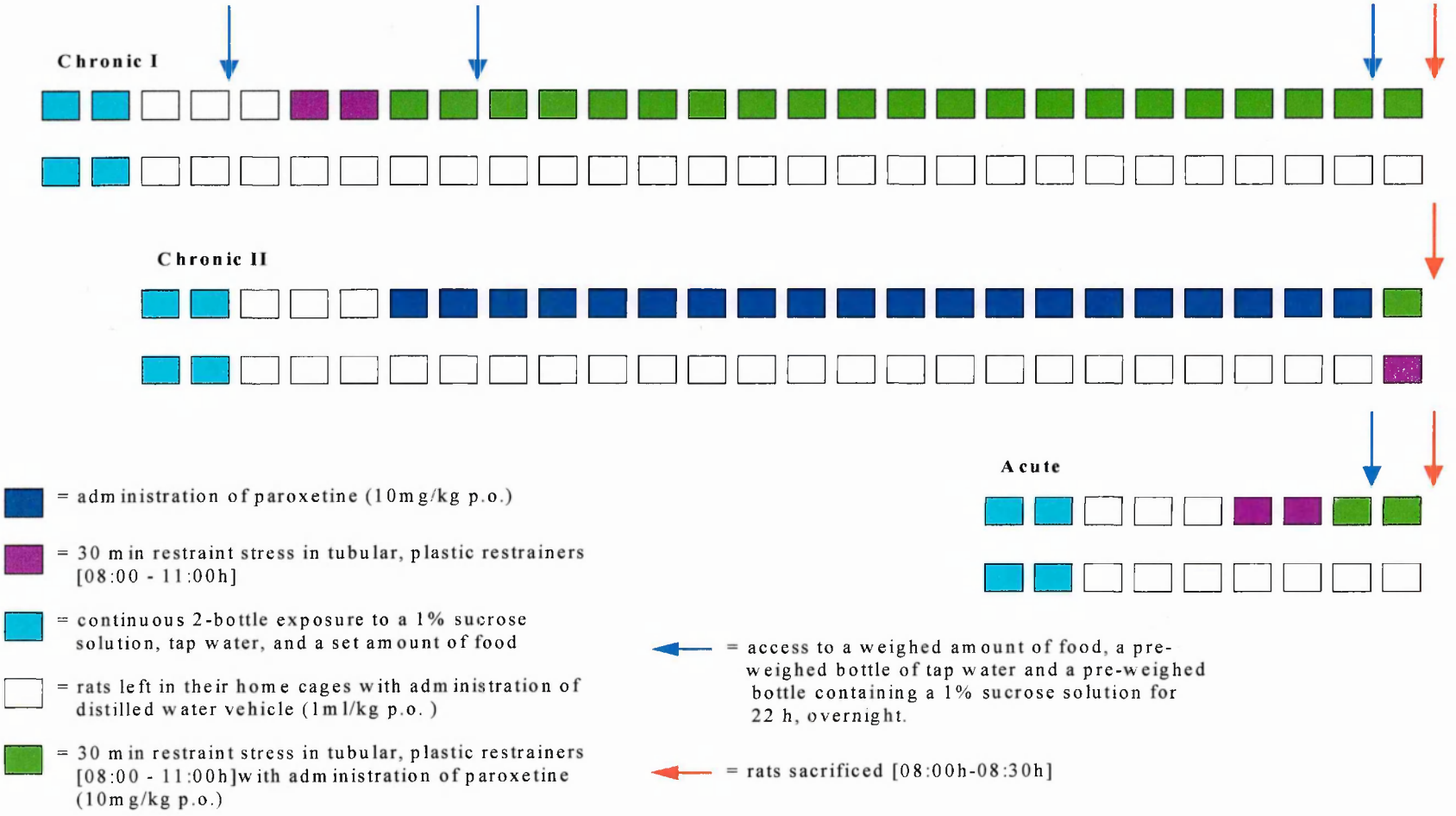
To study a stress paradigm in which rats are repeatedly exposed to restraint stress in combination with acute and chronic administration of the SSRI, paroxetine. Furthermore, to examine if chronic paroxetine alone can affect an acute restraint, and if this differs from the acute results. The present studies focused on the HPA axis as a stress response system to monitor (Dhabbar *et al*, 1997; Kalman *et al*, 1997), using plasma concentrations of corticosterone and ACTH as indicators of stress. Specifically to examine the effect of restraint stress, in combination with acute and chronic administrations of paroxetine, on corticosterone and ACTH as indicators of stress. Specifically to examine the effect on GR density, GR mRNA, CRH type 1 receptor mRNA and BDNF mRNA concentrations in the brain. Sucrose preference was used as a behavioural measure of anhedonia.

7.3. Study design

This chapter is part of one study, alongside Chapters 5 and 6. Refer to Figure. 7.1. for a description of the study design for this specific chapter. In Chapters 5 and 6 restraint stress or paroxetine administration, respectively, were considered on their own, upon the HPA axis. Therefore data from these have a direct relevance upon this chapter, and the results from "stress" animals in Chapter 5 have been included as a direct comparison within the results section 7.6.

Rats were handled twice daily up until the time of testing in order to minimise procedural stress (approximately 7-10 days prior to experimentation), and during this time they were given two bottles of water per cage, to minimise place preference (refer to Section.5.4.3.).

Figure. 7.1



A schematic diagram of the study design, refer to sections 7.4.2, 7.4.3 and 7.4.4. Each square represents one day

7.4. Methods

7.4.1. Animals

Adult male Wistar rats – as per Section 5.4.1. The use of these animals in the following scientific procedures were regulated by the Animals (Scientific Procedures) Act 1986.

7.4.2. Restraint Stress Procedure

This was conducted as described in section 5.4.2. In the **Chronic I** group, the *stress* rats were subjected to 30 min/day restraint stress exposure, for 2 days (refer to **Figure. 7.1.**). This was to achieve a stress-response (as established in the previous section 5.7) before the administration of drugs.

7.4.3. Paroxetine Administration

Rats were handled twice daily up until the time of testing in order to minimise procedural stress (approximately 7-10 days prior to experimentation), and during this time they were given two bottles of water per cage, to minimise place preference (refer to Section.5.4.3.). Previous experiments in our laboratory used 5mg/kg p.o. paroxetine but this had no effect when administered with restraint stress, whereas preliminary experiments had shown an effect with 10mg/kg p.o. Also other investigations used oral doses of 10mg/kg p.o. Therefore this dose was chosen for the following studies. Rats received daily administrations of paroxetine (10mg/kg p.o.) or distilled water (1ml/kg p.o.). The latter were the same control animals (**Acute II** and **Chronic**) that were studied in the previous chapters (refer to sections.5.4 and 6.4.).

7.4.4. Sucrose Preference Test

The sucrose preference tests were conducted at the same time each day, as described in section 5.4.3.

7.4.5. Radioimmunoassay of plasma corticosterone and ACTH concentrations

Plasma corticosterone and ACTH concentrations were measured by using radioimmunassay commercial kits (refer to Section 2.4.).

7.4.6. Other Assays

GR binding, protein measurements and mRNA, were carried out as in section 5.4.5.

7.5. Statistical Analysis

The data is presented as the arithmetic mean +/- s.e.mean. The effects of time ("days"), restraint-stress and paroxetine on biochemical and behavioural measures were analysed by three-factor completely randomised ANOVA (restraint-stress x paroxetine x time), followed by Newman-Keuls post-hoc comparisons. All statistical analyses were performed using the software package, "GBStat" v6.5 .

7.6. Results

7.6.1 Plasma Corticosterone and ACTH Concentration.

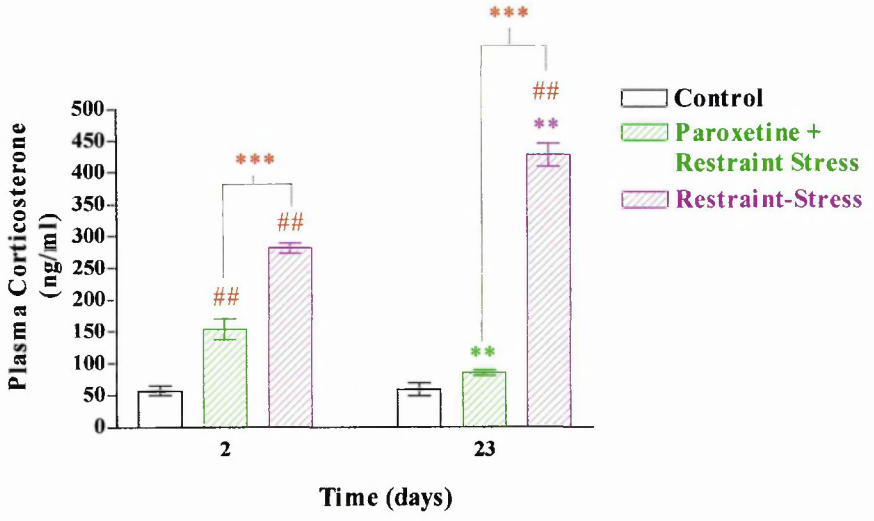
1. Corticosterone

Statistical analysis indicated that restraint stress exposure with paroxetine administration ($F_{1,21}$=41.38; $p<0.0001$), length of time of the experiment ($F_{1,21}$=13.20; $p=0.0016$) and the interaction between restraint stress exposure with paroxetine administration and the length of time ($F_{1,21}$=11.82; $p=0.0025$) significantly affected the plasma corticosterone concentration (**Figure.7.2.**).

Two days of paroxetine administration decreased stress-induced plasma corticosterone concentrations by 45%, which was still an increase of 166% compared to controls. Repeated paroxetine administration decreased stress-induced plasma corticosterone concentrations by 80%, which was still an increase of 45% compared to controls.

Figure.7.2.

Plasma corticosterone concentrations following exposure to restraint stress with or without paroxetine administration.



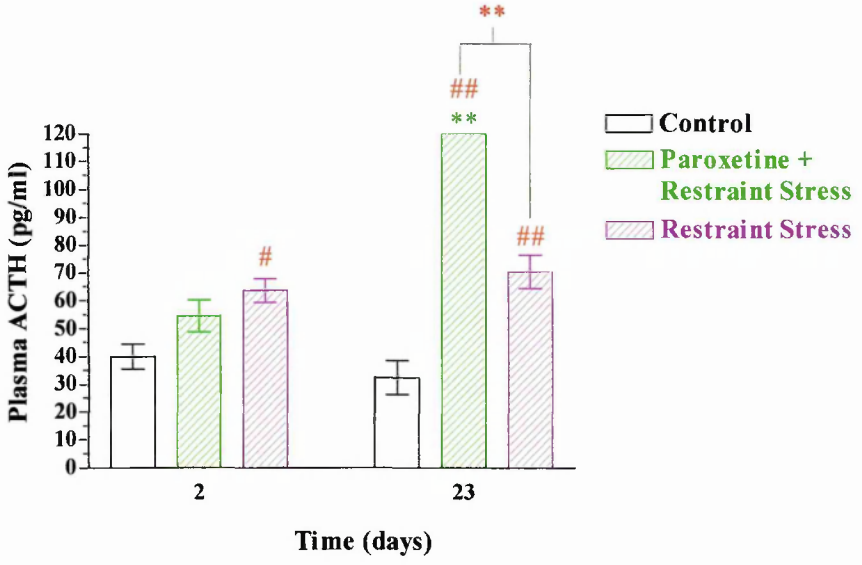
Plasma corticosterone concentrations in male Wistar rats, housed in pairs (n=8 per group), were measured in samples taken immediately following exposure to restraint stress. Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group), rats dosed with 10mg/kg p.o paroxetine and restrained for 30 min/day (paroxetine + restraint stress) and rats restrained for 30 min/day (restraint stress from Section 5.6.1.1) for 2 days or 21 days before they were sacrificed. ##, $p<0.01$ compared to control; **, $p<0.01$ compared to 2 days of exposures to restraint stress with paroxetine administration ; **, $p<0.01$ compared to 2 days of exposures to restraint stress; ***, $p<0.001$ effect of paroxetine on restraint stress.

2. Adrenocorticotrophic Hormone (ACTH)

Statistical analysis indicated that restraint stress exposure with paroxetine administration ($F_{1,18}$=54.73; p<0.0001), time alone ($F_{1,18}$=22.82; p=0.0002) and the interaction between restraint stress exposure with paroxetine administration and the length of time ($F_{1,18}$=28.56; p<0.0001) significantly affected the plasma ACTH concentrations (**Figure.7.3.**). Two days of paroxetine had no significant effect upon the stress-induced increase in plasma ACTH, although this was still a 36% increase compared to controls. Repeated paroxetine administration enhanced stress-induced increase in plasma ACTH concentrations by 78%, and by 240% compared to control.

Figure.7.3.

Plasma ACTH concentrations following exposure to restraint stress with or without paroxetine administration.



Plasma ACTH concentrations in male Wistar rats, housed in pairs (n=8 per group), were measured in samples taken immediately following exposure to restraint stress. Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group), rats dosed with 10mg/kg p.o paroxetine and restrained for 30 min/day (paroxetine + restraint stress) and rats restrained for 30 min/day (restraint stress from Section 5.6.1.2.) for 2 days or 23 days before they were sacrificed. #, p<0.05 and ##, p<0.01 compared to control; **, p<0.01 compared to 2 days of exposures to restraint stress with paroxetine administration; **, p<0.01 effect of paroxetine on restraint stress.

7.6.2. Glucocorticoid Receptor Binding

1. B_{max}

Statistical analysis indicated that administration of paroxetine with restraint stress exposure ($F_{1,21}$=17.86; p=0.0004) significantly effected the B_{max} values from GR binding within the cortex (**Figure.7.4A**). Two days of paroxetine administration reduced the stress-induced decrease of cortical B_{max} values by 34%, and similarly compared to control. Repeated paroxetine administration had no significant effect upon the stress-induced decrease of cortical B_{max} values, although this was decreased by nearly 30% compared to control.

Statistical analysis indicated that administration of paroxetine with restraint stress exposure ($F_{1,22}$=65.17; p<0.0001) and the interaction between the paroxetine administration with restraint stress exposure and the length of time ($F_{1,22}$=19.84; p=0.0002), but not the length of time of the experiment alone significantly effected the B_{max} values from GR binding within the cortex (**Figure.7.4B**). Two days of paroxetine administration had no significant effect upon the stress-induced decrease of hippocampal B_{max} values, although this was still a decrease of just over 50% compared to controls. Repeated paroxetine administrations had no significant effect upon the stress-induced decrease of hippocampal B_{max} values, although this was still a decrease of just over 20% compared to control. Control values varied between 90 and 130 fmol/mg protein.

2. K_d

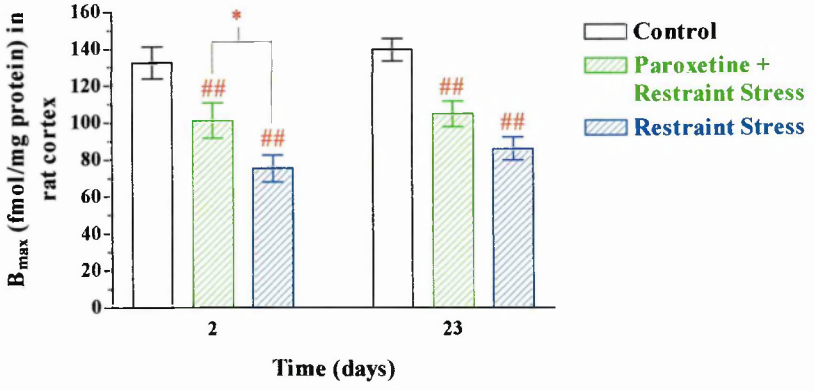
Statistical analysis indicated that administration of paroxetine with restraint stress exposure ($F_{1,25}$=83.31; p<0.0001), length of time of the experiment ($F_{1,25}$=31.09; p<0.0001) , and the interaction between the paroxetine administration with restraint stress exposure and the length of time ($F_{1,25}$=13.57; p=0.0011) significantly effected the K_d values from GR binding within the cortex (**Figure.7.5A**). A single administration of paroxetine with two 30 min exposures of restraint stress, increased cortical K_d values by nearly 160% compared to controls. Repeated exposure to restraint stress in combination with paroxetine administrations, increased cortical K_d values by 50% compared to control but with no significant effect in comparison to restraint stress alone.

Statistical analysis indicated that the length of time of the experiment alone ($F_{1,24}$=39.07; p<0.0001) significantly effected the K_d values from GR binding within the hippocampus (**Figure.7.5B**). Acute or chronic administration of paroxetine with restraint stress had no significant effect upon the K_d values from GR binding within the hippocampus, in comparison to restraint stress alone. Control values from the hippocampus varied between 1.00 and 1.88 nM.

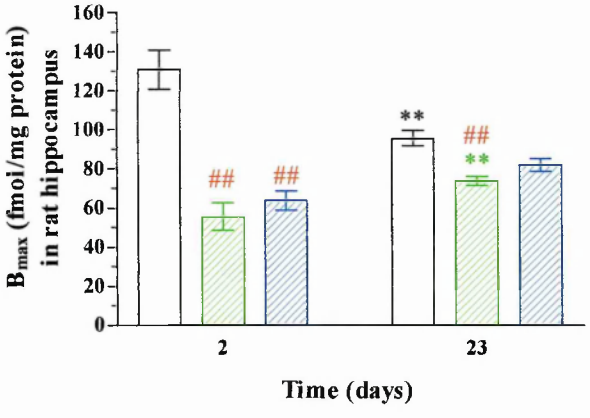
Figure.7.4.

Glucocorticoid receptor B_{max} values in cortical (A) and hippocampal (B) cytosolic fractions following exposure to restraint stress with or without paroxetine administration.

(A)



(B)

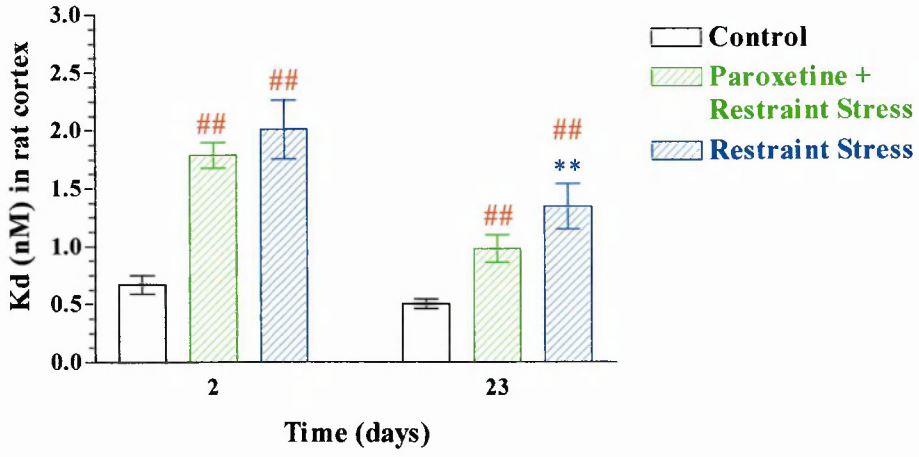


GR B_{max} values in cortex and hippocampus, taken from male Wistar rats, housed in pairs (n = 8 per group), were measured in samples taken immediately following exposure to restraint stress. Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group), rats dosed with 10mg/kg p.o paroxetine and restrained for 30 min/day (paroxetine + restraint stress) and rats restrained for 30 min/day (restraint stress from Section 5.6.2.1.) for 2 days or 23 days before they were sacrificed, (A) B_{max} in cortical tissue (B) B_{max} in hippocampal tissue. #, $p<0.05$; ##, $p<0.01$ compared to control; **, $p<0.01$ compared to 2 days of exposure to restraint stress with paroxetine administration; **, $p<0.01$ compared to 2 days of exposure to restraint stress; *, $p<0.05$ effect of paroxetine on restraint stress.

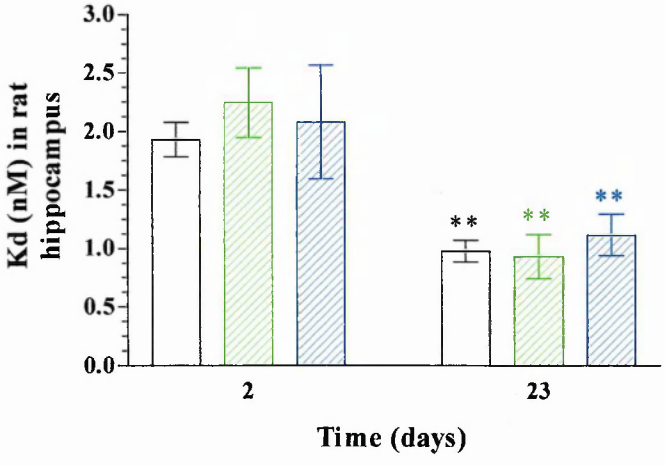
Figure.7.5.

Glucocorticoid receptor K_d values in cortical (A) and hippocampal (B) cytosolic fractions following exposure to restraint stress with or without paroxetine administration.

(A)



(B)



GR K_d values in cortex and hippocampus, taken from male Wistar rats housed in pairs (n = 8 per group), were measured in samples taken immediately following exposure to daily restraint stress. Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group), rats dosed with 10mg/kg p.o paroxetine and restrained for 30 min/day (paroxetine + restraint stress) and rats restrained for 30 min/day (restraint stress from Section 5.6.2b) for 2 days or 23 days before they were sacrificed, (A) K_d in cortical tissue (B) K_d in hippocampal tissue. ##, $p<0.01$ compared to control; **, $p<0.01$ compared to 2 days of control; **, $p<0.01$ compared to 2 days exposure of restraint stress with a single paroxetine administration; **, $p<0.01$ compared to 2 days exposure of restraint stress

7.6.3. Glucocorticoid Receptor mRNA

Statistical analysis indicated that administration of paroxetine with restraint stress exposure ($F_{1,21}$=7.731; p=0.0112), and length of time of the experiment alone ($F_{1,21}$=18.15; p=0.0003) significantly effected cortical GR mRNA concentrations (**Figure.7.6.**). Two days of paroxetine administration had no significant effect upon cortical GR mRNA concentrations compared to controls or restraint stress. Repeated paroxetine administration enhanced stress-induced decrease in cortical GR mRNA concentrations by 58%, and decreased by nearly 40% compared to control.

7.6.4. Brain-Derived Neutrophic Factor (BDNF) mRNA:

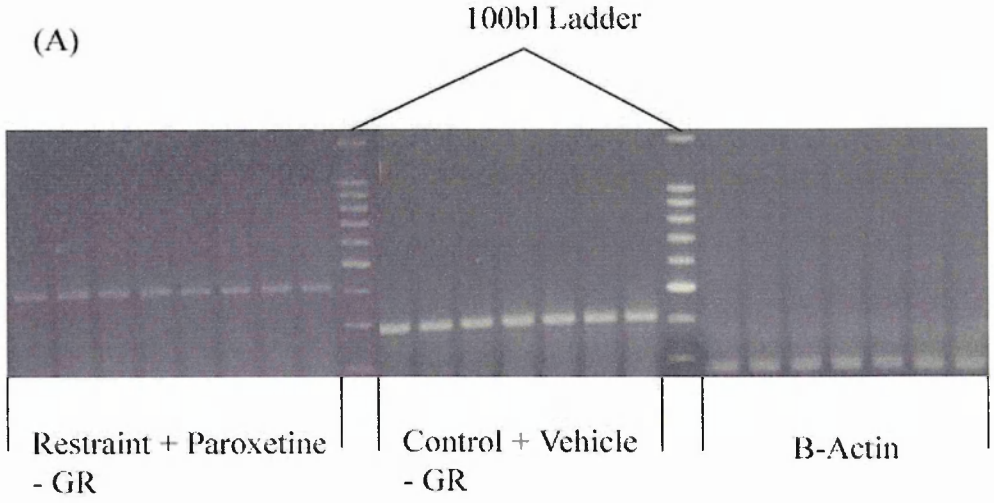
Statistical analysis indicated that length of time of the experiment alone ($F_{1,23}$=14.38; p=0.0009) significantly effected cortical BDNF mRNA concentrations (**Figure.7.7.**). Two days of paroxetine administration reversed the stress-induced increase of cortical BDNF mRNA concentrations by over 30% to controls levels. Repeated paroxetine administration had no significant effect upon stress-induced decrease in cortical BDNF mRNA, but was reduced by nearly 30% compared to control.

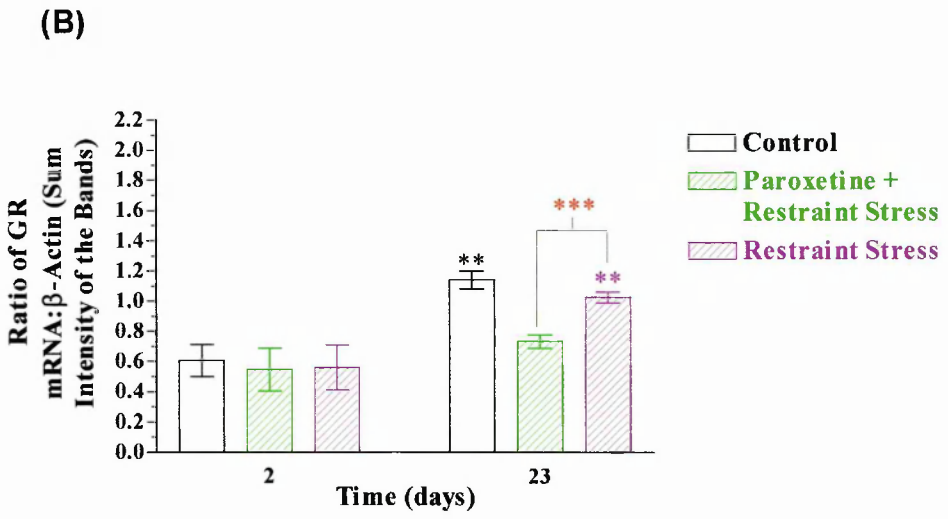
7.6.5 Corticotrophin-Releasing Hormone (CRH) Type 1 Receptor mRNA

Acute administration of paroxetine with restraint stress had no significant effect upon the stress-induced decrease of cortical CRH-R1 mRNA concentration (**Figure.7.8.**), but it was decreased by just over 20% compared to control.

Figure. 7.6.

Cortical GR mRNA and β-Actin mRNA following exposure to restraint stress with or without paroxetine administration.

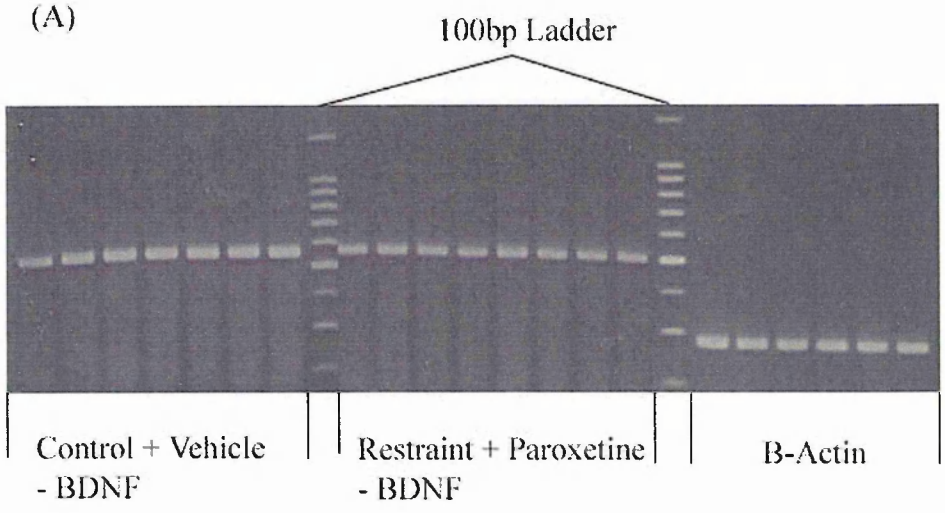




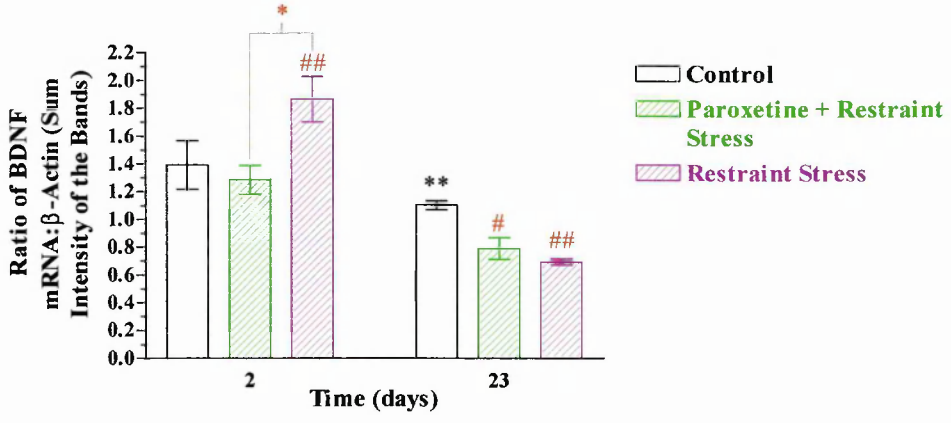
Cortical GR mRNA and β-Actin mRNA from male Wistar rats, housed in pairs (n=8 per group), were measured in samples taken immediately following exposure to restraint stress. (**A**) RT-PCR and agrose gel electrophoresis analysis of GR mRNA expression. β-Actin mRNA expression was used as an internal control. C ontrol samples with β-Actin mRNA and a 100bp ladder were run on a gel, the paroxetine + restraint stress samples were run on a separate gel with β-Actin mRNA and a 100bp ladder. (**B**) Ratio of cortical GR mRNA: β-Actin mRNA (sum intensity of the bands). Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group), rats dosed with 10mg/kg p.o paroxetine and restrained for 30 min/day (paroxetine + restraint stress) and rats restrained for 30 min/day (restraint stress from Section 5.6.3) for 2 days or 23 days before they were sacrificed.**, $p<0.01$ compared to 2 days of control; **, $p<0.01$ compared to 2 days exposure of restraint stress; ***, $p<0.001$ effect of paroxetine on restraint stress.

Figure. 7.7.

Cortical BDNF mRNA and β-Actin mRNA following exposure to restraint stress with or without paroxetine administration.



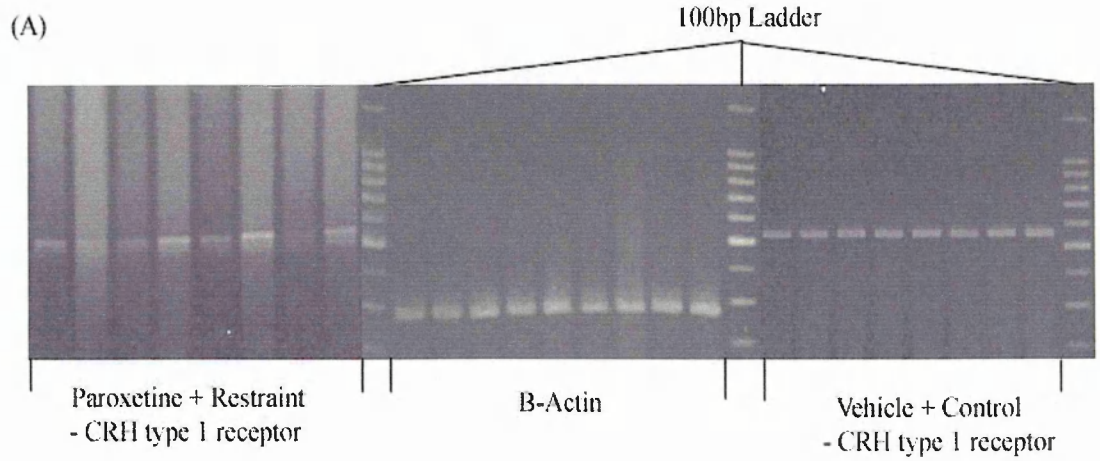
(B)



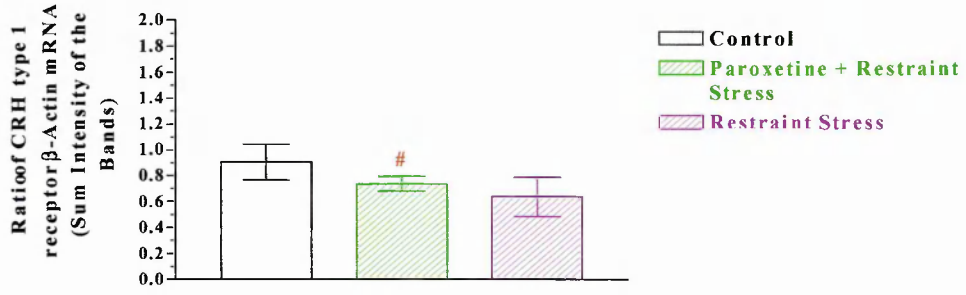
Cortical BDNF mRNA and β-Actin from male Wistar rats housed in pairs (n=8 per group), were measured in samples taken immediately following exposure to daily restraint stress. (**A**) RT-PCR and agarose gel electrophoresis analysis of BDNF mRNA expression. β-Actin mRNA expression was used as an internal control. Control samples and paroxetine + restraint sampleswere run on a gel with a 100bp ladder and β-Actin mRNA was run on a separate gel with a 100bp ladder. (**B**) Ratio of cortical BDNF mRNA: β-Actin mRNA (sum intensity of the bands). Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group), rats dosed with 10mg/kg p.o paroxetine and restrained for 30 min/day (paroxetine + restraint-stress) and rats restrained for 30 min/day (restraint stress from Section 5.6.4) for 2 days or 23 days before they were sacrificed. #, $p<0.05$ and ##, $p<0.01$ compared to control; **, $p<0.01$ compared to 2 days of control; *, $p<0.05$ effect of paroxetine on restraint stress.

Figure. 7.8.

Cortical CRH type 1 receptor and β-Actin mRNA following two 30 min exposures to restraint stress with or without a single paroxetine administration.



(B)



Cortical CRH type 1 receptor : β-Actin mRNA from male Wistar rats, housed in pairs (n=8 per group), were measured in samples taken immediately following exposure to restraint stress. **(A)** RT-PCR and agarose gel electrophoresis analysis of CRH type 1 receptor mRNA expression. β-Actin mRNA expression was used as an internal control. Control samples were run on a gel with a 100bp ladder, paroxetine + restraint stress samples were run on a separate gel with a 100bp ladder, and β-Actin mRNA samples were run on another gel with a 100bp ladder. **(B)** Ratio of cortical CRH type 1 receptor : β-Actin mRNA (sum intensity of the bands). Data are expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group), rats dosed with 10mg/kg p.o paroxetine and restrained for 30 min/day (paroxetine + restraint-stress) and rats restrained for 30 min/day (restraint stress from Section 5.6.5) for 2 days before they were sacrificed.. #, $p<0.05$ compared to control.

7.6.6. Behavioural Measurements

1. Sucrose Preference

Single or repeated exposure to restraint stress with paroxetine administration had no significant effect upon sucrose preference compared to vehicle, basal or restraint stress alone (**Table.7.1.**).

Table.7.1.

Sucrose preference (% of total fluid intake) following exposure to restraint stress with or without paroxetine administration.

GROUPS	Mean ± s.e.mean	N
BASAL	96.5 ± 0.7	13
ACUTE RESTRAINT-STRESS (1 x 30 min) + PAROXETINE (1 x 10mg/kg p.o.)		
Control + 1ml/kg p.o. distilled water (VEHICLE)	95.3 ± 2.6	4
Restraint Stress+ 1ml/kg p.o. distilled water (VEHICLE)	96.1 ± 0.7	4
Restraint Stress + 10mg/kg p.o. paroxetine	95.6 ± 0.9	4
ACUTE RESTRAINT-STRESS (4 x 30 min) + PAROXETINE (2 x 10mg/kg p.o.)		
Control + 1ml/kg p.o. distilled water (VEHICLE)	89.8 ± 6.1	3
Restraint Stress+ 1ml/kg p.o. distilled water (VEHICLE)	96.2 ± 1.7	3
Restraint Stress + 10mg/kg p.o. paroxetine	95.2 ± 0.7	3
CHRONIC RESTRAINT-STRESS (22 x 30 min) + PAROXETINE (21 x 10mg/kg p.o.)		
Control + 1ml/kg p.o. distilled water (VEHICLE)	91.9 ± 5.5	3
Restraint Stress+ 1ml/kg p.o. distilled water (VEHICLE)	97.3 ± 0.5	3
Restraint Stress + 10mg/kg p.o. paroxetine	95.2 ± 1.7	3

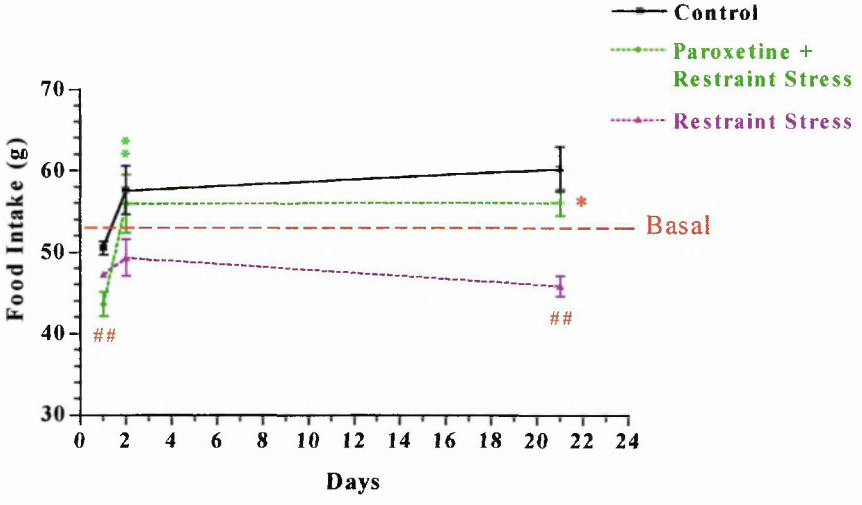
Restraint Stress with Vehicle data from Section 5.6.6.1.

2. Food Intake

Statistical analysis indicated that length of time of the experiment alone ($F_{2,17}$=3.754; p=0.0446) significantly effected food intake (**Figure.7.9.**). A single administration of paroxetine had no significant effect upon the stress-induced decrease in food intake, but this was decreased by nearly 25% compared to basal levels, and by 20% compared to control. Two days of paroxetine administration had no significant effect upon the stress-induced decrease in food intake, as well as basal and control levels. Repeated paroxetine administration reversed the stress-induced decrease in food intake by 22% with no significant effect compared to control.

Figure.7.9.

Food intake following exposure to restraint stress with or without paroxetine administration.



Food intake (g) measured over 22 h, from male Wistar rats, housed in pairs. Data expressed as mean ± sem. Comparison between vehicle-treated rats left in their home cages (control group), rats dosed with 10mg/kg p.o paroxetine and restrained for 30 min/day (paroxetine + restraint-stress) and rats restrained for 30 min/day (restraint stress from Section 5.6.6.2.) for 1 day, 2 days or 23 days before they were sacrificed. ##, $p<0.01$ compared to control; **, $p<0.01$ compared to 2 days of exposure to restraint stress with paroxetine administration; *, $p<0.05$ effect of paroxetine on restraint stress.

7.7.Chronic Paroxetine Administration Followed by a Single 30min Restraint Stress Exposure

7.7.1. Plasma Corticosterone

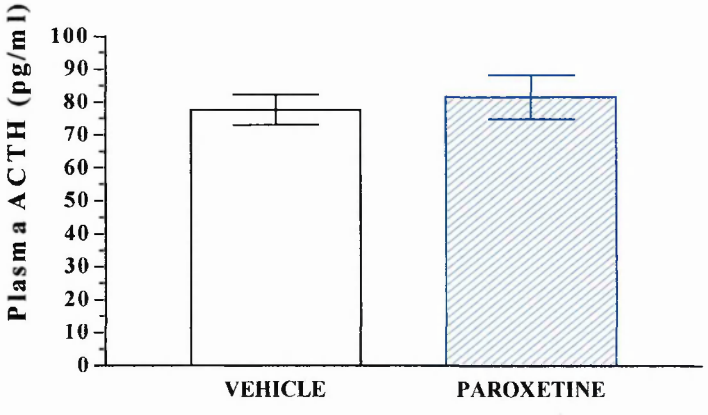
Unfortunately, these samples were compromised due to a problem with the RIA and it was unable to be repeated due to insufficient remaining plasma.

7.7.2. Plasma ACTH

Twenty-one days of paroxetine administration, followed by a single 30 min restraint stress exposure, had no significant effect upon plasma ACTH concentration compared to the vehicle-treated rats (**Figure.7.10.**).

Figure.7.10.

Plasma ACTH concentrations after chronic paroxetine administrations followed by a single 30 min restraint stress exposure.



Plasma ACTH concentrations in male Wistar rats, housed in pairs (n = 8 per group), were measured in samples taken immediately following restraint stress exposure. Data are expressed as mean ± sem. Comparison between vehicle-treated rats (vehicle) and rats dosed daily with 10mg/kg p.o. paroxetine for 21 days (paroxetine group), followed by a single 30 min restraint stress exposure before they were sacrificed. There were no statistically significant differences between the groups.

7.7.3. Glucocorticoid Receptor (GR) Binding

1. B_{max}

Statistical analysis indicated that chronic paroxetine administration, followed by a single 30 min restraint stress exposure significantly affected ($F_{5,9} = 1.862$; $p = 0.0482$) the cortical GR B_{max} levels. Twenty-one days of paroxetine administration, followed by a single 30 min restraint stress exposure, increased cortical GR B_{max} values by 40% compared to the vehicle-treated rats (**Figure.7.11A**).

Twenty-one days of paroxetine administration, followed by a single 30 min restraint stress exposure, had no significant effect upon hippocampal GR B_{max} levels compared to the vehicle-treated rats (**Figure. 7.11B**).

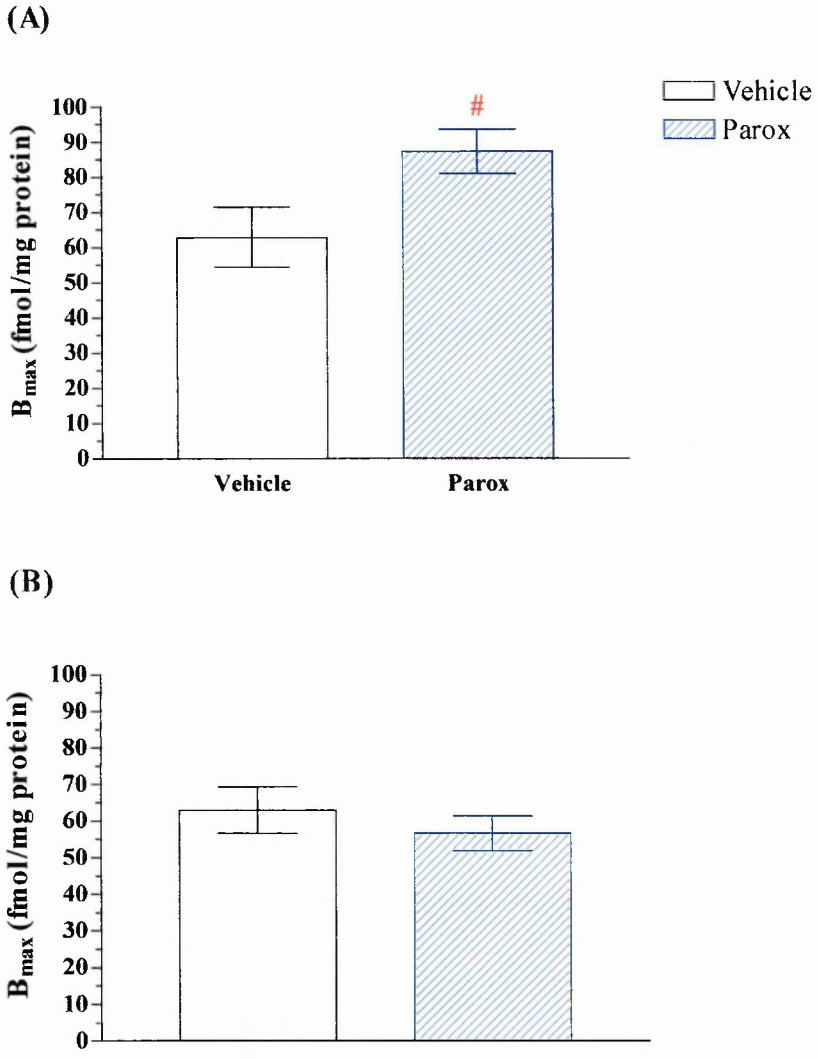
2. K_d

Twenty-one days of paroxetine administration, followed by a single 30 min restraint stress exposure, increased cortical GR K_d values by nearly 50% compared to the vehicle-treated rats (**Figure.7.12A**).

Twenty-one days of paroxetine administration, followed by a single 30 min restraint stress exposure, had no significant effect upon hippocampal GR K_d values compared to the vehicle-treated rats (**Figure.7.12B**).

Figure.7.11.

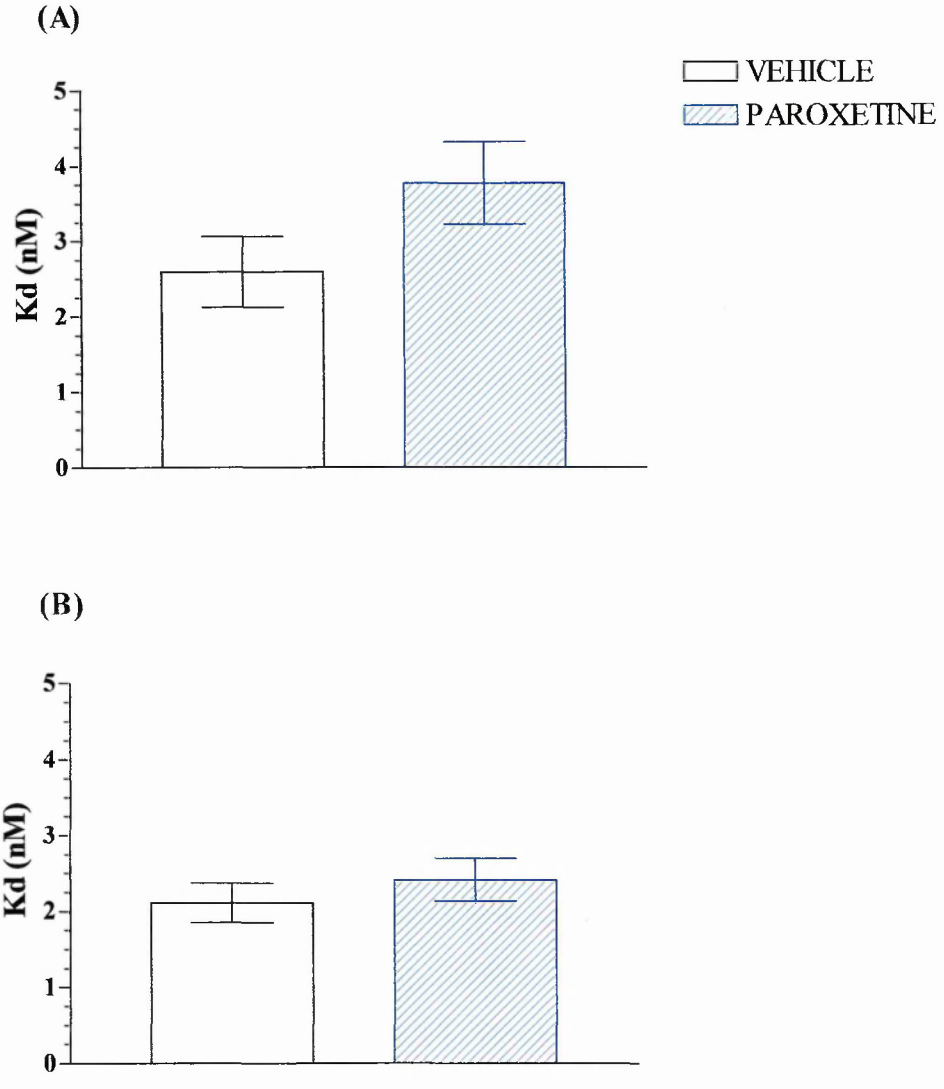
Glucocorticoid receptor B_{max} values in cortical (A) and hippocampal (B) cytosolic fractions, after chronic paroxetine administration followed by a single 30 min restraint stress exposure.



GR B_{max} values in cortex and hippocampus, from male Wistar rats housed in pairs (n = 8 per group, were measured in samples taken immediately following restraint stress exposure. Data are expressed as mean ± sem. Comparison between vehicle-treated rats (vehicle group) and rats dosed daily with 10mg/kg p.o. paroxetine for 21 days (paroxetine group), followed by a single 30 min restraint stress exposure before they were sacrificed.**(A)** B_{max} from cortical tissue, **(B)** B_{max} from hippocampal tissue. #, $p<0.05$ compared to vehicle.

Figure.7.12.

Glucocorticoid receptor K_d values in cortex (A) and hippocampus (B) cytosolic fractions, after chronic paroxetine administration followed by a single 30 min restraint stress exposure.



GR K_d values in cortex and hippocampus, from male Wistar rats, housed in pairs (n = 8 per group), were measured in samples taken immediately following restraint stress exposure. Data are expressed as mean ± sem. Comparison between vehicle-treated rats (vehicle group) and rats dosed daily with 10mg/kg p.o. paroxetine for 21 days (paroxetine group), followed by a single 30 min restraint stress exposure before they were sacrificed. **(A)** K_d from cortical tissue, **(B)** K_d from hippocampal tissue. There were no statistically significant differences between the groups.

7.7.4. GR mRNA

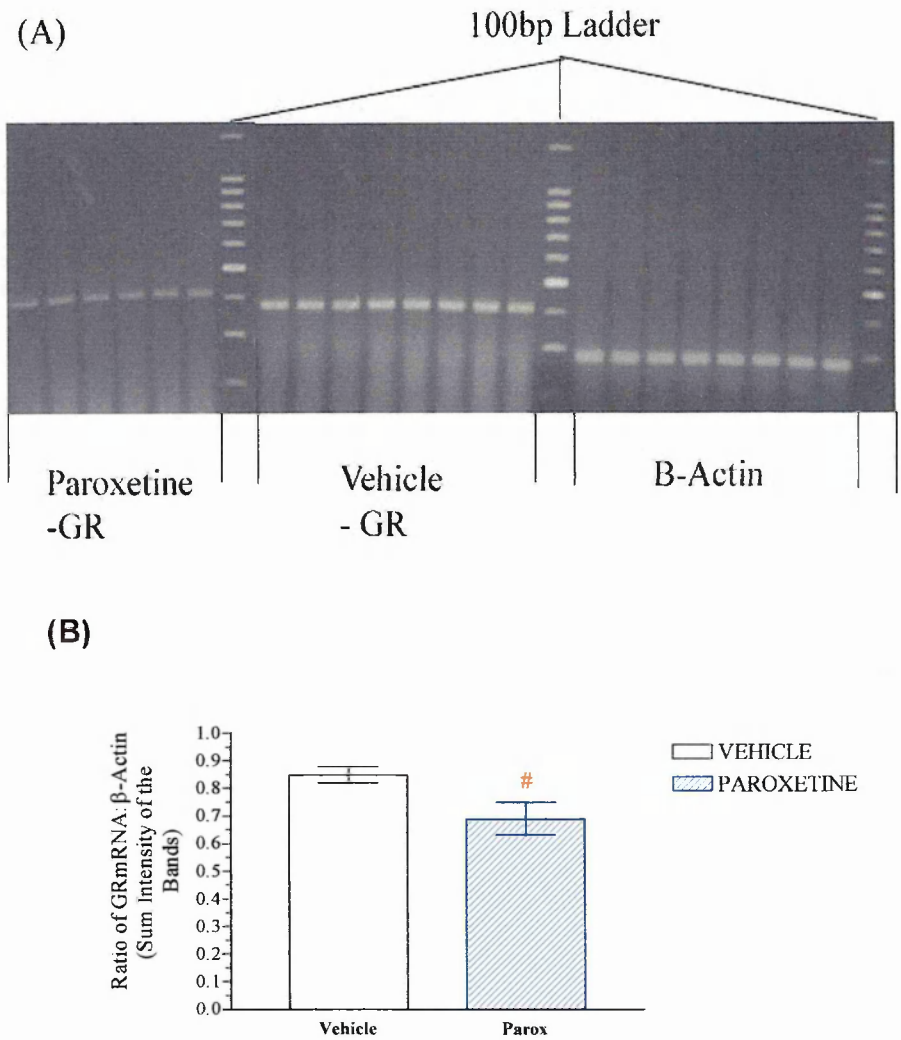
Statistical analysis indicated that chronic paroxetine administration, followed by a single 30-min restraint stress exposure significantly affected ($F_{5,7}$ = 3.686; p=0.0441) cortical GR mRNA concentration. Twenty-one days of paroxetine administration, followed by a single 30 min restraint stress exposure, decreased cortical GR mRNA concentrations by nearly 20% compared to the vehicle-treated rats (**Figure.7.13.**).

7.7.5. BDNF mRNA

Statistical analysis indicated that chronic paroxetine administration, followed by a single 30-min restraint stress exposure significantly affected ($F_{4,7}$ = 16.62; p=0.0001) cortical BDNF mRNA concentration. Twenty-one days of paroxetine administration, followed by a single 30 min restraint stress exposure, increased cortical BDNF mRNA concentrations by 733% compared to the vehicle-treated rats (**Figure.7.14.**).

Figure. 7.13.

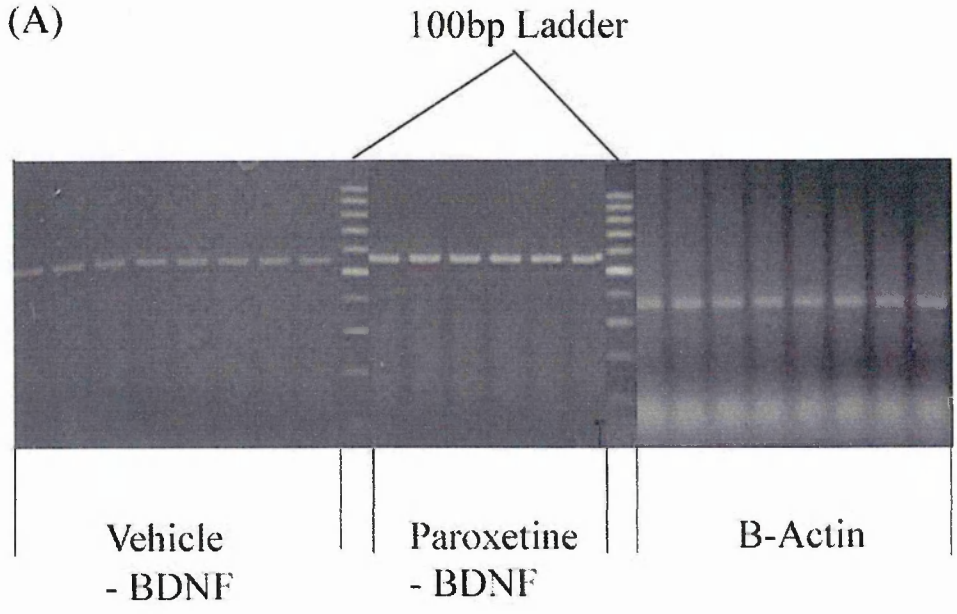
Cortical GRmRNA: β-Actin mRNA after chronic paroxetine administration followed by a single 30 min restraint stress exposure.

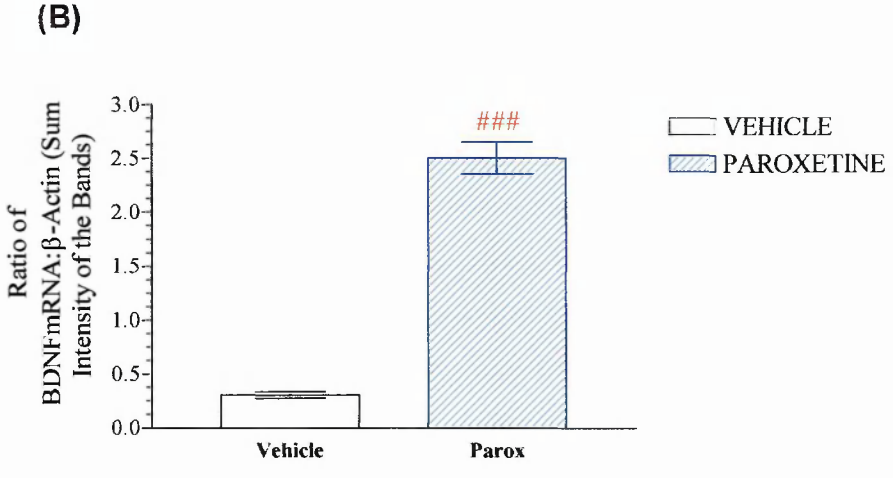


Cortical GR mRNA and β-Actin mRNA in male Wistar rats, housed in pairs (n=8 per group), were measured in samples taken immediately following restraint stress exposure. (**A**) RT-PCR and agarose gel electrophoresis analysis of GR mRNA expression. β-Actin mRNA expression was used as an internal control. Vehicle samples were run on a gel with with β-Actin mRNA and a 100bp ladder, paroxetine samples were run on a separate gel with β-Actin mRNA and a 100bp ladder. (**B**) Ratio of cortical GR mRNA:β-Actin mRNA (sum intensity of the bands). Data are expressed as mean ± sem. Comparison between vehicle-treated rats (vehicle group) and rats dosed daily with 10mg/kg p.o. paroxetine for 21 days (paroxetine group), followed by a single 30 min restraint stress exposure before they were sacrificed. #, $p<0.05$ compared to vehicle.

Figure.7.14.

Cortical BDNF mRNA and β-Actin mRNA after chronic paroxetine administration followed by a single 30 min restraint stress exposure.





Cortical BDNF mRNA and β-Actin mRNA from male Wistar rats, housed in pairs (n = 8 per group), were measured in samples taken immediately following restraint stress exposure. (**A**) RT-PCR and agarose gel electrophoresis analysis of BDNF mRNA expression. β-Actin mRNA expression was used as an internal control. Vehicle samples and paroxetine samples were run on a gel with a 100bp ladder and β-Actin mRNA was run on a separate gel with a 100bp ladder. (**B**) Ratio of cortical BDNF mRNA: β-Actin (sum intensity of the bands). Data are expressed as mean ± sem. Comparison between vehicle-treated rats (vehicle group) and rats dosed daily with 10mg/kg p.o. paroxetine for 21 days (paroxetine group), followed by a single 30 min restraint stress exposure before they were sacrificed. ###, $p<0.001$ compared to vehicle.

7.8. Discussion

The main findings of the present experiments can be summarised as follows: **(1)** paroxetine reversed the stress-induced increase in plasma corticosterone but enhanced plasma ACTH concentration; **(2)** chronic paroxetine administered prior to a single restraint stress did not prevent the ACTH response to stress; **(3)** paroxetine reduced the stress-induced downregulation of GR in the cortex but not the hippocampus; **(4)** chronic paroxetine administered prior to a single restraint stress enhanced GR levels in the cortex but not the hippocampus; **(5)** chronic paroxetine with restraint stress enhanced the stress-induced downregulation of GR gene expression in the cortex; **(6)** chronic paroxetine administered prior to a single restraint stress had no effect upon GR gene expression in the cortex; **(7)** paroxetine reversed the stress-induced upregulation of BDNF gene expression in the cortex; **(8)** chronic paroxetine administered prior to a single restraint stress enhanced BDNF gene expression in the cortex; **(9)** acute paroxetine had no effect upon stress-induced downregulation of CRH type 1 receptor gene expression in the cortex; **(10)** chronic paroxetine reversed the stress-induced decrease in food intake.

The complete reversal of the stress-induced increase of plasma corticosterone by paroxetine, after 21 days (**Figure.7.2.**), suggests that feedback inhibition by corticosterone was facilitated by treatment with this antidepressant, which acts predominantly on the serotonergic system. Specific SSRIs (zimelidine and fluoxetine) have previously been unable to prevent the stress-induced rise in plasma corticosterone (López *et al*, 1998), and this failure to block the rise in corticosterone was associated with an inability to fully prevent down regulation of 5-HT_{1A} receptor gene expression. The discrepancy between studies may be due to the different stressor used (CMUS), the length of the study (28 days), as well as the route of administration of the drugs (i.p. vs p.o.).

Although the measurement of plasma ACTH concentration by RIA in general accurately reflects the physiological status, it is clear that the RIA may in some circumstances give values different from those determined by bioassay. For example, bioactive ACTH disappears from the circulation faster than immunoreactive ACTH (Besser *et al*, 1971).

Purification of pituitary peptides and identification by RIAs has detected several peptides with ACTH immunoreactivity. Characterisation of peptides with ACTH immunoreactivity have been most extensively performed in a mouse cell line (AtT-20) derived from an ACTH-secreting tumour of the anterior pituitary and subsequently characterised in other species including rats and man (Yalow, 1976; Orth & Nicholson, 1977). These peptides were classified according to their molecular weights and referred to as 'big' ACTH which includes both 31K and 22K ACTH, 'intermediate' ACTH (13K ACTH) and 'little' ACTH (4.5K ACTH). The different forms of ACTH are thought to represent different steps in the processing of POMC. Thus, 'little' ACTH is authentic $ACTH_{1\text{-}39}$, 'intermediate' ACTH is a glycosylated form of $ACTH_{1\text{-}39}$ while 'big' ACTH presumably represents POMC and the 22K biosynthetic intermediate (Eipper & Mains, 1980). While all forms of ACTH may be detected by immunoassays, they do not all possess full biological activity. 'Intermediate' ACTH is equipotent with authentic ACTH, while 'big' ACTH has less than 1% the potency of $ACTH_{1\text{-}39}$ in terms of steroidogenic activity (Gasson, 1979). In normal rat pituitary tissue only 1% of the POMC precursor is secreted into the general circulation, the majority being proteolytically processed before release (Eipper & Mains, 1980). Therefore, the commercial RIA kit used to determine the concentrations of ACTH could have detected forms of ACTH that were non-biologically active as well as biologically active. These different forms of ACTH could have reacted with the paroxetine to give an enhanced response. Therefore, to ensure that only

biologically-active ACTH would be detected in any future studies, an immunoradiometric assay (IRMA) kit would replace the RIA used here.

Paroxetine administered for 21 days prior to a restraint stress had no effect upon plasma ACTH concentration (**Figure.7.3.**), suggesting that this particular antidepressant did not appear to have a protective effect, in this study, on the HPA axis in response to an acute psychological stressor. It would have been helpful if this could have been confirmed by assessing the plasma corticosterone concentrations under these conditions.

Previously, rats treated daily for 24 days with paroxetine (7.5mg/kg i.p.) did not alter the stress associated elevation in serum corticosterone provoked by the FST, and therefore failed to alter the HPA axis response to this stress (Connor *et al*, 2000). The variability in results may be attributable in part to the dose used and route of drug administration (7.5mg/kg i.p. rather than 10mg/kg p.o.), as well as that paroxetine may have a different effect according to the stressor used (FST instead of restraint stress).

According to the literature, glucocorticoid hormones have major effects on behaviour, hippocampal morphology, and serotonergic neurotransmission (Nausieda *et al*, 1982; Mendelson & McEwen, 1992), downregulating hippocampal 5-HT_{1A} receptors at the level of receptor mRNA expression (Chalmers *et al*, 1993). This latter effect has also been observed after chronic unpredictable stress and prevented by concomitant imipramine administration.

In another study, however, Papp *et al* (1994) found that chronic exposure to mild stress increased 5-HT_{1A} binding in the hippocampus, an effect also observed after chronic imipramine administration. In addition, a neuroendocrine functional study showed the development of a supersensitivity of 5-HT_{1A} receptors in animals submitted to stressful situations where escape responses could not be accomplished (Korte *et al*, 1992). Therefore, adrenal steroids may have biphasic effects on serotonin systems. By facilitating 5-HT_{1A} –mediated neurotransmission that may be necessary for development of stress

tolerance, chronic exposure to high concentrations of corticosteroids, however, may lead to a downregulation of 5-HT_{1A} receptors and consequent failure of the system (Chalmers *et al*, 1994).

According to Joseph and Kennett (1983), at least part of the corticosteroid response to restraint stress is mediated by an increase in serotonergic activity that is dependent on an increased supply of the precursor, tryptophan, and that this can be antagonised by other amino acids that compete with tryptophan for access to the brain. After repeated restraint-stress, the concentrations of brain noradrenaline have also been shown to decrease in rats (Pericic *et al*, 1987).

Harbuz *et al* (1993), showed that p-chlorophenyl-alanine (PCPA) pre-treatment, which resulted in a 95% depletion in hypothalamic serotonin, had no effect on basal concentrations of ACTH or the increase in response to a physical stress. Plasma ACTH concentrations were also not affected by serotonin depletion in response to the predominantly psychological stress of restraint. Both basal and restraint stress-induced circulating corticosterone concentrations were however further stimulated in the PCPA-pre-treated rats suggesting a possible inhibitory serotonergic tone at the adrenal level.

Jorgensen *et al* (1998) concluded that 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors, but not 5-HT_3 receptor were involved in the stress-induced ACTH secretion following restraint-, ether-, cold swim or endotoxins in rats. Further that serotonergic neurones in the raphe nuclei were activated during restraint stress, and that these neurones and neurones in the PVN of the hypothalamus, were important for the mediation of the restraint stress-induced ACTH response.

Paroxetine partially reversed the stress-induced downregulation of cortical but not hippocampal GR levels (**Figure.7.4A. and B.**), and this was reflected when paroxetine was administered for 21 days prior to a restraint stress (**Figure.7.11A. and B**), suggesting that paroxetine has a protective and regulatory role in GR levels within the brain, but that this appears to be region-specific.

The precise cellular mechanisms underlying the desensitisation in the corticotroph and the brain regions affected are at present unclear. Regarding the brain regions affected, one possibility is that GRs increase in several noradrenergic and serotonergic nuclei following long-term antidepressant treatment as reported by Kitayama *et al* (1988). This would increase the negative feedback inhibition of the HPA axis and reduce the stress response. Another possibility is a desensitisation of 5-HT_{1A} receptors in the PVN (Li *et al*, 1997), but this effect cannot account for all antidepressants, since chronic treatment with desipramine, a noradrenaline reuptake inhibitor, did not attenuate 8-OH-DPAT induced hormone release, indicating that other mediators must contribute to the action (Li *et al*, 1997).

Chronic stress, using the CUMS model, has been shown to be associated with an alteration in the MR/GR ratio in the hippocampus; the changes in MR/GR ratio were prevented if chronically stressed rats were treated with desipramine or zimelidine (López *et al*, 1998). Therefore, it would be beneficial to examine MR under these conditions, to assess their influence on GR.

The present data shows that chronic paroxetine further decreased the stress-induced downregulation of GR gene expression in the cortex (**Figure.7.5.**), but that chronic paroxetine administered before a restraint stress increased it (**Figure.7.13.**).

Some studies have found increases in MR and /or GR mRNA after 2 weeks of antidepressant treatment (Peiffer *et al*, 1991; Seckl & Fink, 1992), but others have not found changes in this time frame (Brady *et al*, 1991). Lopez and colleagues (1998) found no changes in GR and MR mRNA in hippocampus after 4 weeks of desipramine treatment; however, they found that desipramine administration restored the abnormal MR/GR ratio in stressed animals to control levels. This may represent one of the multiple mechanisms by which antidepressants may enhance feedback and maintain low corticosterone concentrations, even in the presence of stress. Zimelidine had some effect on MR/GR ratio, but not enough to offset the "drive" of the HPA axis, at least within 4 weeks.

The present data shows that acute paroxetine administration reversed the stress-induced increase in cortical BDNF mRNA levels. Chronic paroxetine administration with restraint stress partially reversed the stress-induced decrease in cortical BDNF mRNA levels, although the latter was not significant (**Figure.7.6.**). Yet, chronic paroxetine administered before a restraint stress dramatically increased the stress-induced cortical BDNF mRNA levels (**Figure.7.14.**).

According to Nibuya *et al* (1996), chronic (21 days), but not acute (1 day), administration of several antidepressant drugs including tranylcypromine, desipramine, sertraline, and mianserin completely blocked down-regulation of BDNF mRNA concentrations in the hippocampus in response to restraint stress, in agreement with the present results, although there was not a complete blockade, on chronic exposure. Discrepancies between studies may be attributable to the different brain regions examined (i.e. hippocampus, cortex), the use of different antidepressants, the methods and/or sensitivity of mRNA analyses used (i.e. *in situ*, RT-PCR), as well as species differences (i.e. mice, rat).

Recently, the cAMP signalling pathway has been implicated in antidepressant action after chronic treatment. CRE-mediated gene transcription is upregulated in the cortex and hippocampus after chronic antidepressant administration in the rat (Thome *et al*, 2000). CREB expression and function are upregulated by chronic antidepressant treatment in both rodents and humans (Nibuya *et al*, 1996; Dowlatshahi *et al*, 1998). CREB upregulation may activate downstream targets such as BDNF after antidepressant treatment by binding to CRE elements located in the promoter region of the BDNF gene (Nibuya *et al*, 1995; Tao *et al*, 1998). Therefore, temporal and regional upregulation of BDNF mRNA and its receptor, TrkB, parallels CREB mRNA activation after chronic antidepressant administration (Nibuya *et al*, 1996).

With the use of selective serotonin receptor antagonists, Vaidya *et al* (1999) determined that serotonin release during stress and activation of the 5-HT_{2A} receptor may be one of the mechanisms via which stress influences BDNF expression. They showed only partial blockade of the stress response by the 5-HT_{2A} receptor, which implies that other neurotransmitter systems are involved. The noradrenergic neuronal pathways are powerfully influenced by stress and noradrenaline is reported to regulate the expression of BDNF (Thoenen *et al*, 1991; Vahabzadeh & Fillenz, 1994). Although in their study, pre-treatment with prazosin, an α_1-adrenergic receptor antagonist, or propanolol, a $\beta_{1/2}$-adrenergic receptor antagonist, did not influence the stress regulation of BDNF expression (Vaidya *et al*, 1999). Also the CRH neuropeptide system and the CRH type 1 receptor subtype are known to mediate many central effects of stress (Herman & Cullinan, 1997). Yet pre-treatment with the CRH type 1 receptor antagonist, CP 154,526, did not block the stress-induced decrease in BDNF mRNA concentrations, indicating that this receptor subtype is not involved in the stress effect under their conditions (Vaidya *et al*, 1999). It is possible that the CRH type 2 receptor subtype could play a role in the regulation of BDNF

concentrations by stress, although there are currently no selective antagonists for this receptor.

Serotonergic inputs from the DRN are thought to exert a global control over the hippocampus, via modulation of local inhibitory interneurones (Freund *et al*, 1990). Serotonin released into the hippocampus during immobilisation stress could activate 5-HT_{2A} receptors expressed on GABAergic interneurones and increase GABA release. Activation of 5-HT_{2A} receptors is reported to increase the firing of GABAergic neurones and to thereby induce IPSPs in granule cells (Piguet & Galvan, 1994). Such an effect on the firing rate of granule cells could explain the finding that GABA decreases BDNF expression in the hippocampus (Zafra *et al*, 1991).

The present data shows that acute paroxetine had no effect upon the stress-induced downregulation of CRH type 1 receptor mRNA levels in the cortex (**Figure.7.7.**). It would have been constructive to have considered chronic effects upon this particular aspect of the HPA axis in order to determine whether this antidepressant would have had an opposing effect on the stress-induced response.

According to Lowry *et al* (2000) serotonergic responses to CRH were enhanced after exposure of rats to isolation housing and repeated restraint stress for 5 days. They concluded that these observations suggested that CRH actions on serotonergic neurones might play an important role in behavioural responses associated with anxiety and conditioned fear, extending previous hypothetical models for the complex neurobiological mechanisms underlying these behavioural states (Gray, 1982; Davis, 1998).

The study by Lowry *et al* (2000) supports the hypothesis that CRH acts on a topographically organised subpopulation of serotonergic neurones to activate mesolimbocortical serotonergic pathways during intense, prolonged, uncontrollable or unpredictable stress. They also showed that mesolimbocortical and mesostriatal serotonergic systems are differentially regulated by CRH; this in turn may contribute to the

dissociation of mesolimbocortical and mesostriatal serotonergic activity during stress (Clement *et al*, 1998).

Exposure of rats to chronic, unpredictable, and diverse stressors were found to reduce rat preference for saccharin and sucrose for more than 2 weeks after termination of the stress regime: the effect being attenuated by chronic pre-treatment of rats with antidepressant drugs (Katz, 1982; Willner *et al*, 1987). Nevertheless, the current data showed no stress-induced reductions and therefore the administration of paroxetine had little effect upon sucrose preference compared to controls (**Table.7.3.**).

This current study demonstrated that chronic paroxetine administration reversed the stress-induced decrease in food intake (**Figure.7.9.**). A study by Harleem and Parveen (1994) showed that a single 2 h episode of restraint stress decreased food intake and the growth rate of rats, as confirmed by the stress-induced reduction shown in section 5.6.6.2. although the body weights remained unchanged They also demonstrated that an acute challenge with 2 h restraint increased serotonin synthesis in the cortex, hypothalamus, midbrain and hindbrain of previously unrestrained rats, but not those adapted to 5 days of 2 h daily restraint.

7.9. Conclusion

A considerable body of literature supports an inhibitory influence of brain serotonin on the HPA axis response to stress. This has been confirmed by the use of paroxetine, subsequently increasing brain serotonin, within these experiments. However, the effects of chronic administration of paroxetine prior to restraint stress need to be investigated further, due to the inconclusive results obtained.

CHAPTER 8. GENERAL DISCUSSION

The overall aim of the studies described in this thesis was to investigate the response of the HPA axis to different stressors, namely predator stress and restraint stress, and the subsequent adaptations following administration of paroxetine. Particular emphasis was placed on chronic stress and its impact on corticosteroids and their interaction with various receptors within the brain; despite extensive literature on the HPA axis and its link with stress and depression, most animal studies investigating antidepressant effects are performed under baseline conditions; however, in humans with depression, antidepressants are given to them under an "altered" (one could argue "stressed") condition. Therefore, investigating the effect of antidepressants on the HPA axis under conditions of chronic stress may be closer to the clinical situation, as a significant number of patients with depression show evidence of HPA overactivity. Two studies were carried out and the findings of each have been presented and discussed in detail within each of the separate chapters. This chapter will summarise the major findings, consider their biological significance, and discuss some of their implications.

The initial study (Chapters 3 and 4) involved the development and use of a predator stress paradigm in mice. Plasma corticosterone concentrations showed that a stressful response had been achieved using the presence and odour of rats, over 8 weeks, and that this effect was decreased when the mice were removed from the "stress" room. This was partially substantiated by the significant decrease in food consumption but this stressful procedure did not appear to affect sucrose preference or cortical GR binding. The EPM data is questionable due to the overwhelmingly high presence of the mice in the closed arms throughout the study, suggesting that the apparatus and experimental conditions were too "stressful", and any anxiogenic effects by the stress procedures were masked by this. So this data cannot be relied upon, in this study.

Therefore, this model was shown to be stressful, through increased plasma corticosterone concentration, but the behavioural tests used (sucrose preference and EPM) need more preliminary experiments to substantiate them in our laboratory. Results from these behavioural tests were conflicting with previous experiments, suggesting that they just complicated the issue rather than corroborated the plasma corticosterone concentrations (the basic indicator of stress). So this model could work as a natural predator model of stress, but the behavioural tests used to verify this need careful consideration and must be able to stand alone in the laboratory before being involved with this model.

Several of the classic alterations associated with animal models of depression have not yet been observed in the predator stress paradigm. Investigations being conducted in this area are still in the early stages and the hypothesis that this behavioural paradigm may constitute to some degree, an animal model of stress/depression/anxiety is recent. Overall responses of rats and mice to predator exposure indicate that this stimulus is a stressful one (see section 4.1). Responses to this stressor also indicate high levels of anxiety-like behaviour in animals exposed to predator stress. Some of these changes observed in this paradigm can be compared to those observed in human stress situations and also in a number of various other putative models of depression and anxiety.

The second study was separated into three distinct chapters (Chapters 5, 6 and 7) in order to approach and discuss each particular aspect of the study. The first of these chapters (Chapter 5) looked at acute and chronic restraint stress in rats, and assessed the HPA axis under these conditions. This section of the study demonstrated that this particular stressor provoked HPA axis responses for 3 weeks without adaptation or habituation, through increased plasma corticosteroid concentration, decreased GR in both cortex and hippocampus, decreased BDNF mRNA within the cortex, as well as a decrease in food intake. Such findings endorse this as a chronic animal model of depression.

The second chapter of this study (Chapter 6) looked at acute and chronic paroxetine administration in rats, on the baseline conditions of the HPA axis. This section of the study demonstrated that paroxetine elicited "stress-like" responses of the HPA axis under normal conditions. For instance, acutely administered paroxetine increased plasma corticosterone concentration which were reduced after chronic administration, but it decreased GR, GR mRNA and BDNF mRNA in the cortex, as well as decreased food intake even after chronic administration. These confirm previous results in the literature (apart from GR mRNA whereby we have shown a decrease when others have shown an increase). Chronic administration of antidepressant drugs on GR and GR mRNA have been shown to vary between different antidepressants and brain regions assessed.

Previous studies have reported increases in hippocampal GR/GR mRNA following long-term administration of TCAs or moclobemide – these studies were conducted in adrenalectomised animals thus possibly rendering GR in this region more sensitive to fluctuations induced by antidepressant administration. The observed neuroanatomical specificities of the antidepressant effects in the current studies could possibly also relate to differing monoaminergic innervations of theses areas which may indirectly be affecting GR in these regions. In the current studies, changes have been observed primarily in the cortex and not in the hippocampus, suggesting a different mechanism may be active in the presence of endogenous ligand. The data obtained may have been confounded by the presence of endogenous corticosterone in the system at the time of assay as previous investigations used adrenalectomised animals only. However, the procedure of adrenalectomy itself can also interfere with the status of the HPA axis and though, in these studies, we may have been measuring only ~70-90% of the total GR population, this was sufficient to enable reflections of any changes in numbers of receptors following various procedures.

The final chapter of this study (Chapter 7) examined acute and chronic paroxetine administration on the HPA axis under the influence of restraint stress, as well as being administered chronically prior to an acute restraint stress. This section of the study demonstrated that this particular antidepressant had a variable impact on the HPA axis under chronic stress, whether given prior to restraint or during restraint. Paroxetine reversed the stress-induced effects upon plasma corticosterone concentration, GR and BDNF mRNA in the cortex, as well as food intake, confirming that part of its action is to attenuate the HPA axis under long-term stressful conditions. Whereas, when given for 3 weeks prior to acute restraint, paroxetine failed to alter the ACTH response to this stress and cortical GR mRNA, but protected GR in the cortex, and enhanced BDNF mRNA in the cortex.

The GR binding assay may be subject to the criticism that it is a rather sensitive assay that has the potential to be affected by numerous external factors. In our laboratory however, this assay has proven to be reliable over the years and the results of the studies conducted over the duration of this thesis have not been subject to large fluctuations.

Our data was generated using adrenally-intact animals, which contained endogenous circulating corticosterone at the time of death. Therefore, many precautions were taken in order to ensure that basal corticosterone concentrations remained within the normal range with minimal fluctuations.

Previous investigations have not always used the same radioligand to measure corticosteroid receptors; Budziszweska *et al* (1994) used ^{3}H-corticosterone whereas Reul *et al* (1994) have used ^{3}H-dexamethasone and ^{3}H-aldosterone in their experiments – these variations in corticosteroid receptor measurement methods may also contribute to observed differences in results.

The lack of a consistent up- or down regulation of GR in our studies may also be attributed to the doses of antidepressants used as these differed between previous studies and our own, as did the route/method of administration. Future experiments may benefit from establishing dose-response effects of antidepressants and also the measurement of MR in these samples.

In order to obtain valid data pertaining to plasma corticosterone and ACTH concentrations, it was important to use controlled conditions throughout. Any deviations from normal light/dark cycles, noise, light intensity and temperature may have generated the possibility of altering basal hormone concentrations, as well as inducing shifts in normal circadian rhythms. The circadian pattern of corticosteroid secretion also required samples to be collected at the same time each day. Animals were minimally disturbed at all times to reduce the risk of stress-induced corticosteroid secretion, unless otherwise stated. The levels of circulating corticosterone at the time of sacrifice corresponded with a 70-90% occupation of MR indicating that most of the receptors being measured in our investigations were GR. This conclusion was supported by the demonstration of a GR-like competition profile and similar binding parameters were obtained in saturation experiments using the selective GR agonist RU28362 to define specific binding.

The subjective nature of these and the lack of standardised behavioural testing procedures across laboratories makes it difficult to compare data from various studies. Optimisation of testing procedures (as described in Chapter 3) for our own studies ensured that protocols were consistently observed. The species of animals used also appears to greatly impact any behavioural measurements (Capeless & Whitney, 1995). As the use of behavioural models and transgenic animals in the development of psychiatric disorders increases so does the importance of choosing animals with the appropriate genetic background, again, an issue raised by the lack of standardisation of protocols.

Discrepancies between studies may be attributed to numerous possible variations in animal species used, the testing protocols and the receptor binding assays.

Depressed patients exhibit an altered timing of rest/activity. It may be useful, in future studies, to constantly monitor activity in animal model investigations instead of obtaining 'snapshot measures', as animals with HPA disorders are more likely to display altered circadian rhythmicity.

Based on previous data (López *et al*, 1998), it is apparent that specific 5HT receptors may be directly regulated in response to alterations of corticosteroid concentrations, which can result from repeated stress. Although it is clear that corticosteroids can regulate 5-HT receptors, it is also important to remember that regulation can exist in the other direction. Acute administration of 5-HT1A and 5-HT2 agonists can cause release of ACTH and corticosterone, and destruction of central 5-HT neurones decreases hippocampal GR and MR gene expression (Seckl & Fink, 1992). Therefore the relationship between corticosteroids and 5-HT in the brain is complex and tightly controlled. As has been previously shown, stress-induced corticosteroid release affects 5-HT receptor function (López *et al*, 1998) but stress can also affect other receptors through non-corticosteroid-mediated pathways (Post, 1992). The interplay of these factors may lead to the emergence, or maintenance, of affective symptoms. Similarly, antidepressants can counteract this phenomenon by affecting 5-HT receptor function directly (Welner *et al*, 1989; Blier & de Montigny, 1994) and by simultaneously regulating stress-induced corticosteroid secretion. This does not exclude the possibility that steroids can be acting simultaneously through other systems (e.g. noradrenaline), thereby synergistically affecting mood and behaviour.

The HPA response to a severe chronic stressor is likely to involve several neurotransmitter systems. Therefore, a compound that modulates a single neurotransmitter may not be able to prevent, or may even potentiate, the HPA axis activation in response to a chronic stress. The failure of an antidepressant to restore peripheral corticosteroids to baseline concentrations may therefore impair its ability to correct a "central" monoaminergic deficit.

In conclusion, the studies in this thesis have centred on the HPA axis and attempted to break down the effects of chronic stress to try and provide a fuller picture of the effect of stress in relation to depression, and to elucidate how antidepressants alleviate these symptoms through this axis. Overall, these studies show that basal activity of the HPA axis in the male mouse and rat undergoes marked change in response to chronic stress, depending on the stressor used. Also, it illustrates the general principle that the efficacy of antidepressant treatment may be dictated, in part, by its ability to alter the activity of the HPA axis (López *et al*, 1998; Holsboer & Barden, 1996).

8.1. Future Work

The Predator Stress model requires more thorough preliminary investigations to attribute the neurochemical and behavioural changes to the nature of the stimulus, by examining rat odour (acute and chronic exposure) and the visual presence of the rats (acute and chronic exposure) separately, as well as the contribution of novelty and any handling stress. Assessments also need to be made to study the effects of rat exposure on general activity and location with respect to the rat (proxemic avoidance) and the behavioural response to rat odour. Also to give a more complete analysis of the long-term effect of this model, neurochemical and endocrine assays need to be conducted at more regular intervals, as well as looking at the effects of acute exposure.

Factors worthy of investigation in the second study (Chapters 5-7) include: (a) endocrine assays of corticosterone and ACTH conducted at more regular intervals to give a more comlete analysis of the long-term effects; (b) examination of GR mRNA within other brain regions; (c) examination of BDNF mRNA within other brain regions, as well as its receptor, TrkB, under chronic as well as acute conditions. The neurotrophins have far-reaching effects on cell function and all known cellular effects mediated by these factors result from the tyrosine kinase activities of the receptors; (d) examination of CRH concentration, possibly through microdialysis, as well as CRH type 1 receptor mRNA within other brain regions, under chronic as well as acute conditions. Numerous studies have examined the regulation of CRH; however, little is known about the mechanism controlling the expression of CRH receptors in the brain under basal or stress conditions; (e) examination of serotonin concentration, possibly through microdialysis, to try and understand the effects of paroxetine upon the HPA axis under basal and stress conditions.

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