The role of Corticotropin-Releasing Factor in Anxiety disorders

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B.Sc. (Hons.) (U.S.)

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DECLARATION:

I, the undersigned, declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature: ___________________________ Date: ___________________________
SUMMARY

SEPARATION STUDY

Traumatic experiences during childhood can have a negative impact on behaviour later in life. Kendler et al. (1992) found that the loss of a parent during childhood increased the risk to develop major anxiety disorders and could also lead to depressive-like behaviour (Furukawa et al., 1999).

Methods:

We subjected rat pups to maternal separation and determined the effects thereof on adult behaviour. We removed rat pups from their mothers for 3 hours daily from postnatal day 2 to 14. On day 60, the behaviours of the rats were tested using the elevated plus-maze and the open field test. Controls were reared normally.

Behaviours: Amount of time spent and the number of entries into the arms of the maze were noted on the elevated plus-maze, while the total time spent in each zone (inner versus outer) and the number of zone crossings were noted for each rat on the open field arena. The latency to move from the initial placement in the outer zone to the inner zone as well as the number of quadrant crossings was also determined. Defecation, freezing, rearing and grooming behaviours were also noted.

Neurotransmitter levels: Noradrenaline, serotonin and their metabolites were evaluated in maternally separated rats and compared to controls. Their concentrations at basal level, immediately after restraint stress and 15 minutes after restraint stress, were also determined. A HPLC method was followed in these determinations.
ACTH Determinations: All rats were subjected to restraint stress for a 10-minute period. Trunk blood was collected for basal, as well as 15 and 60 minutes post-restraint stress for ACTH determinations.

Results:

Behaviours: The amount of entries was significantly reduced in the separated animals, indicating decreased locomotion. They spent significantly more time in the closed maze arms. A significant increase in defecation frequency and rearing behaviour was noted. These observations are typical of anxious behaviour. In the open field test, the behavioural results were less convincing. Only a significant increase in defecation frequency and a significant decrease in rearing behaviour in separated animals, were observed.

Neurotransmitter levels: No significant differences were noted between separated animals and controls with respect to basal monoamine levels. However, noradrenaline levels were significantly decreased in the frontal cortex 15 minutes after restraint stress and immediately after restraint stress in the hypothalamus and hippocampus in separated animals. MHPG levels were significantly decreased in the frontal cortex immediately after restraint stress. No significant differences were found with respect to serotonin levels. However, significant increases were found in 5HIAA levels in the frontal cortex and hippocampus of separated rats, 15 minutes after restraint stress.

The basal turnover ratios of serotonin (5HIAA/5HT) and noradrenaline (MHPG/NA) did not yield significant results. However, immediately after restraint stress, a significant increase was found in serotonin turnover in the hypothalamus of separated
rats when compared to controls. This turnover rate was also increased in separated rats, 15 minutes after restraint stress in the frontal cortex and hypothalamus.

ACTH Determinations: Basal ACTH levels were significantly higher in separated animals. At 15 minutes post-restraint stress, the levels were significantly lower than controls, indicating a blunted stress response.

Our results therefore showed that maternal separation could lead to anxious behaviours in adult life. These behavioural abnormalities were associated with alterations in the central nervous and neuroendocrinological systems, particularly in response to stressful situations.

CRF STUDY

The maternal separation study indicated that elevated CRF levels could possibly be causally related to abnormalities observed in the anxious animals. We therefore hypothesised that adverse development factors, such as maternal separation, predisposes individuals to develop psychopathologies later in life and that this process was driven by a presence of high CRF levels.

Methods:
Cannulas were implanted into the left lateral ventricles of normal rats, making use of stereotaxic procedures. CRF (3 μg/μl) was injected into the ventricles daily for 5 days. Saline controls were handled similarly, but only injected with saline for the
same time period. Both groups of animals were then compared to naïve controls. Histology was performed to determine the correct placement of the cannulas.

**Behaviours:** The Elevated Plus-maze was employed to determine whether their behaviours were anxious. The number of entries into the various arms of the maze as well as the amount of time spent in the open and closed arms was accumulated. Rearing, freezing, defecation and grooming were also noted.

**ACTH Determinations:** The ACTH levels of CRF-injected, saline-injected and naïve rats were determined 15 minutes after restraint stress.

**Results:**

**Behaviours:** A decrease in the number of entries into the closed arms of the maze was noted in the CRF-injected rats when compared to naïve controls. No significant differences were found between the groups with respect to the amount of time spent in the various arms and the behaviours noted during the experiment.

**ACTH Determinations:** A decrease in ACTH levels was noted in CRF-injected rats 15 minutes after restraint stress when compared to naïve controls. Therefore, although the CRF injections did not alter the behaviour of the rat, they did exhibit a blunted stress response to the stressor.

**Conclusion:**

Our experiments led us to conclude that early adverse experiences, such as maternal separation, can lead to the development of psychopathologies later in life. CRF,
however, is not pivotal in the development of these abnormalities; rather it seems that the neurochemical abnormalities (serotonin and noradrenaline) play a more important role in the development of these mental disturbances. Finally, we hypothesise that combination drug therapy that targets both the noradrenergic and serotonergic neurotransmitter systems could be preferred above those aimed at rectifying the individual neurotransmitter systems in the treatment of psychopathologies, such as anxiety disorders.
OPSOMMING

MOEDERLIKE SKEIDINGS STUDIE

Traumatiese gebeurtenisse wat gedurende kinderjare ervaar word, kan 'n negatiewe impak op die gedrag van dieselfde individue hê, as hulle volwassenheid bereik het. Kendler et al. (1992) het waargeneem dat die verlies van 'n ouer tydens die kinderjare, die risiko om angssteurnisse te ontwikkel, dramaties verhoog en kan ook lei tot 'n depressiewe gemoedtoestand (Furukawa et al., 1999).

Metodes:
Ons het neonatale rotte aan moederlike skeiding blootgestel en die effekte daarvan op gedrag tydens hul volwasse lewe beoordeel. Ons het daagliks die moeders vir 3 ure van die kleintjies af weggeneem, vanaf postnatale dag 2 tot 14. Op dag 60, het ons die gedrag van die diere op die "elevated plus-maze" en die "open field test" getoets. Kontrole rotte het onder normale omstandighede opgegroei.

Gedrag parameters: Die hoeveelheid tyd en aantal kere wat die rotte in die verskillende arms van die "elevated plus-maze" gespandeer het, was waargeneem. Die totale tyd in die "open field" toets se binneste of buitenste sones, die hoeveelheid kruisings tussen die twee sones, die tyd wat dit neem om beweging in die binneste sone te inisiëer, sowel as die hoeveelheid kwadrante wat gekruis was, is genoteleer. Defekasie, botstilstande, steiering, en versorgingsgedragte was ook waargeneem terwyl die rotte in die doolhowe was.
Neurochemiese oordragstowwe: Die hippokampus, hipotalamus en frontale korteks van moederlik-geskeide rotte en kontroles, was uit hul brein gedissekteer om die vlakke van noradrenalien, serotonien en hul metaboliete daarin te bepaal. Basale vlakke sowel as hul konsentrasies onmiddelik na stres en 15 minute na stres, was gedetermineer. ’n HPLC metode was gebruik vir hierdie bepalings.

ACTH bepalings: Rotte, moederlik-geskei en kontroles, was onderwerp aan beperkingstres vir ’n tydsduur van 10 minute. Bloed was op die volgende tydsintervalle gekollekteer vir die bepaling van ACTH vlakke, naamlik basaal, 15 minute en 60 minute na die einde van stresperiode.

Resultate:

Gedrag: Op die “elevated plus-maze” was moederlik-geskeide rotte minder beweeglik omdat hul aanmerklik minder die arms van die doolhowe binne gegaan het. Hulle het ook baie meer tyd in die geslote arms gespandeer. Verder het die eksperimentele rotte meer defekasie bolusse uitgeskei en was die aantal steieringe uitgevoer, ook aanmerklik verhoog. Hierdie patroon van gedrag is tipies dié van angstigheid.

Neurochemiese oordragstowwe: Daar was geen betekenisvolle verskil tussen die basale neurotransmitter vlakke van moederlik-geskeide rotte en hul kontroles. Daarenteen was die vlakke van noradrenalien in die frontale korteks dramaties verhoog by die 15 minute tydsinterval na die stres, asook onmiddelik na die stres in die hipotalamus en hippocampus. MHPG vlakke was egter aanmerklik verlaag in die frontale korteks onmiddelik na die stres. Terwyl daar geen noemenswaardige verskil
in serotonien vlakke waargeneem is nie, was die vlakke van 5HIAA betekenisvol verhoog in die frontale korteks en hippocampus van moederlik-geskeide rotte, 15 minute na die beperkingstres. Geen verskil in die omsettingsverhoudinge van basale serotonien (5HIAA/5HT) of noradrenalien (MHPG/NA) vlakke is gevind nie. Daar was egter 'n betekenisvolle verhoging in die serotonien omset in die hipotalamus van moerdlik-geskeide rotte, onmiddellik na beperkingstres. Hierdie verskil het ook voorgekom 15 minute na die stresperiode in die hipotalamus, sowel as in die frontale korteks.

*ACTH bepalings:* Rotte wat onderwerp was aan moederlike skeiding het verhoogde basale konsentrasies van ACTH getoon. Die ACTH vlakke was egter aanmerklik laer 15 minute na stres toe dit met kontrole groepe vergelyk is.

Ons resultate toon dus dat moederlike-skeiding wel tot angstige gedrag tydens die volwasse lewe kan lei. Hierdie afwyking in gedrag was geassosieër met abnormaliteite in die sentrale senuwee sisteem sowel as die neuroendokrienologiese sisteem van die dier, veral onder toestande van stres.

Na gelang van ons bevindinge in die moederlike skeidingstudie, het dit geblyk dat CRF 'n belangrike rol speel tot daarstelling van angstige gedrag. Daarom het ons in die tweede deel van ons studie gaan kyk of verhoogde vlakke van CRF in die brein moontlik die gedrag van die rot kon verander.
CRF STUDIE

Metodes:
Kannules was in die linker ventrikel van die breine van normale rotte geïmplanteer deur gebruik te maak van stereotaktiese prosedures. CRF (3 μg/μl) was daagliks vir 5 dae aan die rotte toegedien. Rotte wat presies dieselfde gehanteer was 'n fisiologiese soutoplossing ontvang. Hierdie rotte was met naëewe rotte vergelyk. Die korrekte plasing van kannules was met histologiese metodes bevestig.

Gedrag: Die “elevated plus-maze” was gebruik om te bepaal of angstige gedragte by behandelde rotte ontlok was. Die aantal kere wat ‘n rot die verskillende arms van die doolhof binne gaan, sowel as die tyd wat die dier op elke arm deurbring was genotuleer. Die aantal steierings, botstilstande, defekasies en versorgingsbewegings was weereens waargeneem.

ACTH bepalings: Die vlakke van ACTH was bepaal in al die rotgroepe, 15 minute nadat hulle aan 10 minute beperkingstres onderwerp was.

Resultate:
Gedrag: Rotte wat met CRF toegedien was, het op minder geleenthede die toe arms van die “elevated plus-maze” binne gegaan toe hulle met die naëewe groep rotte vergelyk was. Hierdie verskil was betekenisvol. Daar was geen ander noemenswaardige verskille ten opsigte van die ander gedragsparameter nie.
ACTH bepalings: Daar was 'n afname in die ACTH vlakke, 15 minute na die stres toegedien was in rotte wat CRF ontvang het, in vergelyking tot die naïewe kontrole groep.

Hierdie resultate dui daarop dat die toediening van CRF in die brein nie die rot se gedrag, maar wel die dier se respons op stres, beïnvloed het.

Gevolgtrekking:

In die lig van die voorafgaande resultate verky, blyk dit dat moederlike-skeiding tydens die vroeë kinderjare wel kan aanleiding gee tot angstige gedrag tydens volwassenheid. Ons studies dui ook aan dat CRF nie die primêre bron van hierdie gedrags afwykings is nie, maar dat abnormaliteite in die neurochemiese oordragstowwe (serotonien en noradrenalien) eerder die bepalende faktore is. Ten slotte, ons beveel aan dat geneesmiddels wat geskoei is om die serotonerge sowel as die noradrenerge sisteme aan te spreek, voordeel moet geniet in die behandeling van gedragstoornisse, soos angs.
I wish to express my sincere appreciation to the following:

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The Heavenly Father, who deserves all the credit.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>α</td>
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<td>β</td>
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<td>μg</td>
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<td>μl</td>
<td>microlitre</td>
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<td>μm</td>
<td>microns</td>
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<tr>
<td>5HIAA</td>
<td>5-hydroxyindoleamineacetic acid</td>
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<td>5HT</td>
<td>Serotonin</td>
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<tr>
<td>AAO</td>
<td>Acidified Aluminum Oxide</td>
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<tr>
<td>Abm</td>
<td>Antibody, monoclonal</td>
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<td>Abp</td>
<td>Antibody, polyclonal</td>
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<td>Ach</td>
<td>Acetylcholine</td>
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<td>ACTH</td>
<td>Adrenocorticotrophic Hormone</td>
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<td>cAMP</td>
<td>cyclic Adenosine Mono-phosphate</td>
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<td>Concentration</td>
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<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>CRF</td>
<td>Corticotropin-Releasing Factor</td>
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<td>CS</td>
<td>Central Superior Raphe Nucleus</td>
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<td>D</td>
<td>Dorsal Raphe Nucleus</td>
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<tr>
<td>DHBA</td>
<td>3,4-Dihydroxy-benzylamine</td>
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<td>DMNV</td>
<td>Dorsal Motor nucleus of Vagus</td>
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<td>Abbreviation</td>
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<td>DR</td>
<td>Dorsal Raphe nucleus</td>
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<td>DSM</td>
<td>Diagnostical and Statistical Manual of Mental Disorders, 4th Ed.</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>FC</td>
<td>Frontal Cortex</td>
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<td>gram</td>
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<td>GABA</td>
<td>γ-amino-butyric-acid</td>
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<td>HC</td>
<td>Hippocampus</td>
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<td>HPA</td>
<td>Hypothalamic-Pituitary-Adrenal</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<td>Hyp</td>
<td>Hypothalamus</td>
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<td>I$^{125}$</td>
<td>Radioactive Iodine</td>
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<td>Molar</td>
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<td>MFB</td>
<td>Medial Forebrain Bundle</td>
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<td>mg</td>
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<td>MHPG</td>
<td>3-Methoxy-4-hydroxy-phenylglycol</td>
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<td>mm</td>
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<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
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<td>n</td>
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<tr>
<td>NA</td>
<td>Noradrenaline</td>
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<td>NaH2PO4</td>
<td>Sodium Dihydrogen Phosphate</td>
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<td>NTS</td>
<td>Nucleus Tractus Solitarii</td>
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<td>OlfB</td>
<td>Olfactory Bulb</td>
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<td>pg</td>
<td>picogram</td>
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<td>PTSD</td>
<td>Posttraumatic Stress Disorder</td>
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<td>rpm</td>
<td>revolutions per minute</td>
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<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>SSRI</td>
<td>Selective Serotonin Reuptake Inhibitor</td>
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LITERATURE REVIEW

Title: The role of corticotropin releasing factor in anxiety disorders.

1.1 Introduction

Epidemiological studies have found that anxiety disorders are the most prevalent of the psychiatric disorders. The economic burden of anxiety disorders alone totalled $42.3 billion in 1990 in the USA (Greenberg et al., 1999). This statistic included the cost of psychiatric services as well as prescription drug costs, absenteeism and the decrease in work productivity.

Maladaptive development factors may be a major contributory factor to psychopathology later in life. Maternal separation, a potent naturalistic stressor (Stanton et al., 1988) could also be seen as a maladaptive development factor, and has been associated with the development of affective disorders (Kendler et al., 1993). It is for this reason that we decided to use an animal model to investigate the effect of maternal separation and its role in the development of subsequent anxiety disorders and abnormal stress responses during adulthood. Since corticotropin-releasing factor (CRF) is one of the main hormones involved in the stress response (Habib et al., 2000), we also evaluated its role in this phenomenon.
1.2 Anxiety Disorders

1.2.1 Manifestations, Symptoms and Behaviours

Anxiety has been described as a physiological reaction, and is defined in the DSM-IV (1994) as the unpleasant experience of fear in the absence of an appropriate stimulus. An anxiety disorder, on the other hand, is classified as excessive anxiety, which occurs regularly for at least 6 months (DSM-IV, 1994). The person finds it difficult to control the worry, which is associated with three (or more) of the following six symptoms:

(1) restlessness or feeling keyed up or on edge;

(2) being easily fatigued;

(3) difficulty concentrating or mind going blank;

(4) irritability;

(5) muscle tension;

(6) sleep disturbance (difficulty falling or staying asleep, or restless unsatisfying sleep).

The anxiety, worry, or physical symptoms causes clinically significant distress or impairment in social, occupational, or other important areas of functioning. The disturbance is not due to the direct physiological effects or a general medical condition (DSM-IV, 1994). Recent reviews have noted that this disorder often occurred along with other disorders, such as depression (Enns et al., 2001) and social phobia (Rickels & Rynn, 2001).
In order for Posttraumatic Stress Disorder (PTSD), a specific type of anxiety disorder, to be diagnosed in a patient, the person must have been exposed to a traumatic event in which both of the following were present:

(1) the person experienced, witnessed, or was confronted with an event or events that involved actual or threatened death or serious injury, or a threat to the physical integrity of self or others;
(2) the person's response involved intense fear, helplessness, or horror.

The traumatic event is persistently re-experienced in one (or more) of the following ways:

(1) recurrent and intrusive distressing recollections of the event, including images, thoughts, or perceptions;
(2) recurrent distressing dreams of the event;
(3) acting or feeling as if the traumatic event were recurring (includes a sense of reliving the experience, illusions, hallucinations, and dissociative flashback episodes, including those that occur on awakening or when intoxicated);
(4) intense psychological distress at exposure to internal or external cues that symbolize or resemble an aspect of the traumatic event;
(5) physiological reactivity on exposure to internal or external cues that symbolize or resemble an aspect of the traumatic event.

The duration of the disturbance exceeds 1 month and causes clinically significant distress or impairment in social, occupational, or other important areas of functioning (DSM-IV, 1994).
1.2.2 Treatment

The treatment of anxiety disorders is varied in accordance with the specific anxiety disorder that is diagnosed. The drug treatment is usually given in conjunction with cognitive-behavioural therapy. According to a recent review (Ballenger et al., 2001), most anxiety disorders are treated with antidepressants, including serotonin reuptake inhibitors, serotonin-noradrenaline reuptake inhibitors, and non-sedating tricyclic antidepressants. These treatment paradigms were not effective in all forms of anxiety disorders and therefore the search for new therapeutic strategies continues. We decided to investigate the role of CRF in anxiety disorders in the hope of finding data that would support the inclusion of CRF antagonists in future treatment regimes.

1.3 Stress

Anxiety has been reputed to be an indicator of excess adrenergic activity. It is known that combat veterans, who suffer from PTSD, have heightened sympathetic nervous system activity (Blanchard et al., 1991). There are at least two stress systems, which are interlinked, but are activated differentially in response to a stressor. Gerra et al. (2001) hypothesised that the sympathetic-adrenal medulla system is a generalised system that reacts to any general stress, while the hypothalamic-pituitary-adrenal (HPA) axis is specific for novel-type stressors and the defense reaction.

1.4 Hypothalamic-Pituitary-Adrenal axis
Studies reviewed by Nash & Maickel (1988) and Udelsman & Chrousos (1988) have illustrated that hypothalamus-pituitary-adrenal axis activation is a key component of the stress response, which entails the release of ACTH and cortisol (Smyth et al., 1998). Walker et al. (1991) showed that the rat’s neonatal pituitary adrenocortical system is already fully functional a day after birth, by subjecting 1, 5 and 10 day old rat pups to various stressors.

During stress, CRF synthesis in the paraventricular nucleus is increased and CRF is then released from nerve terminals at the median eminence into the hypothalamic-hypophysial portal vascular system (Review by Hodges, 1984). On reaching the pituitary gland, CRF binds to its receptors. Studies suggest that these receptors are coupled through a guanine nucleotide binding protein to a second messenger system (Review by De Souza, 1995). The second messenger system includes the activation of adenylate cyclase (Abou-Samra et al., 1987), which stimulates cAMP-dependent protein kinase in the anterior pituitary cells, eventually leading to adrenocorticotropic hormone (ACTH) secretion. ACTH then stimulates the release of glucocorticoids including cortisol, in humans, and corticosterone, in rats, from the adrenal cortex (Review by Arborelius et al., 1999) (See figure 1.1).

There are two types of glucocorticoid receptors: the mineralocorticoid receptor and the glucocorticoid receptor. Corticosterone has a higher affinity for the mineralocorticoid receptor, which is found in the limbic areas of the brain. These regulate the basal HPA axis activity. Glucocorticoid receptors are located throughout the brain, including the limbic structures and paraventricular nucleus, where CRF is produced (Review by Arborelius et al., 1999).
Figure 1.1: Diagrammatical representation of the Hypothalamic-Pituitary-Adrenal axis and the pathway involved in the release of cortisol/corticosterone. The regulation of the HPA axis involves the hippocampus and certain neurotransmitters represented here as well as an innate negative feedback mechanism.
A review by De Kloet (2000) indicated that during stress, the amount of corticosterone released is enough so that all the glucocorticoid receptors are occupied, which serves to suppress the hyperactivity of the HPA axis.

1.4.1 Regulation of HPA axis

The HPA axis is regulated by internal (within the axis itself) and external factors. The cortisol released by the adrenal cortex, serves to inhibit the HPA axis by acting on glucocorticoid receptors located at the level of the pituitary, hypothalamus and hippocampus. (Review by De Kloet et al., 1987; Liposits 1987b). Cortisol, therefore, helps terminate the stress response through negative feedback inhibition by acting on the areas that initiate the stress response and the release of cortisol (see figure 1.1).

The hippocampus also participates in the inhibition of corticosterone secretion and the return of the HPA axis to a state of homeostasis after a stress response (Bratt et al., 2001). Efferent connections have been established from the hippocampus to the hypothalamus (Cullinan et al., 1993). Further support for the involvement of this brain area in the regulation of the stress response, came from studies that showed how experimental stressors, such as infusion of the excitotoxin, kainic acid, resulted in morphological changes within the hippocampus (Stein-Behrens et al., 1994) and elevated circulating corticosterone levels (Daniels et al., 1990).

Studies have shown that ACTH secretion is also regulated by a number of other neurohormones outside of the HPA axis, including γ-amino-butyric-acid (GABA), acetylcholine (Ach), serotonin (5HT), noradrenaline (NA) and neuropeptide Y (NPY).
(Delbende et al., 1992), suggesting the participation of more brain areas in the stress response (see figure 1.1).

1.5 Maternal Separation

1.5.1 Early life adverse events and Anxiety disorders

Events that occurred at the critical stages of a child’s development could often lead to diseases that manifest themselves in their adult lives. Studies reviewed showed that individuals subjected to childhood sexual abuse also suffered from adult-onset depression and displayed signs of a dysregulated HPA axis (Review by Weiss et al., 1999). Upon investigating adverse experiences in childhood, Zaidi and Foy (1994) proposed that it left combat veterans more susceptible to develop PTSD, after leaving the army.

Heim et al. (2001) found that women who were abused as children, presented with symptoms of anxiety and depression in adulthood. Women that experienced other childhood adversities such as family violence, parent psychopathology or alcoholism, also had an increased susceptibility to develop depression in their later lives (Hammen et al., 2000).

In her review, Arborelius et al. (1999) described the phenomenon of childhood abuse or neglect as a stressful life event that caused "biological wounds" and increased the individual's vulnerability to develop affective disorders such as depression or anxiety, later in life.
Kendler et al. (1992) found that the loss of a parent during childhood increased the risk to develop major anxiety disorders by 7% to 20.5%. Similarly, Generalised Anxiety Disorder has been associated with parental separation. Others have shown that maternal separation could lead to anxiety and depressive-like behaviour (Kendler et al., 1992; Furukawa et al., 1999). These observations clearly indicate a strong correlation between adverse experiences during childhood and the development of psychiatric disorders in adult life.

1.5.2 Maternal Separation and Stress

A number of animal studies have been performed to characterise the effects of maternal separation on the activity of the HPA axis later in life (Maccari et al., 1995; Graham et al., 1999). Kalin (1989) found that in non-human primates, which have been maternally separated from their mothers, central administration of CRF led to increased pituitary-adrenal activation. In rodents, separation of an infant from its mother resulted in an increase in basal corticosterone levels and a decrease in growth hormone secretion (Kuhn et al., 1990), as well as increased noradrenaline release in the dorsal hippocampus (Matthews et al. 2001).

The effect of maternal separation on the HPA axis activity varies in accordance with different separation paradigms. For instance, if weaned at day 21–30, the HPA axis response was diminished and low levels of corticosterone were exhibited after restraint stress. However, if left in the cage with the mother until day 30–40, when the HPA axis is fully developed, an increased response was observed (Cook, 1999).
Placement of a guinea pig infant into a novel cage environment without its mother also led to increased cortisol release (Hennessy et al., 1995). Interestingly, if a guinea pig infant is exposed to a novel environment with a female other than its mother, the HPA activity was suppressed (Hennessy et al., 2000). This could suggest that the lack of tactile stimulation, rather than a response due to food or temperature alterations, led to the behavioural abnormalities seen in adult guinea pigs that were previously maternally separated. This theory has also been suggested to be the cause of the behavioural abnormalities seen in maternally separated rats (Review by Kuhn and Schanberg, 1998).

Ladd et al. (1996) proposed that the anterior pituitary CRF receptors of rats suffering from emotional trauma, such as parental separation, became more sensitive. Therefore, on suffering stress in their later life, the response was augmented. These authors found an increase in CRF levels in the median eminence, leading to an augmented ACTH response. Interestingly they did not find any significant differences between maternally separated animals and controls with respect to corticosterone concentrations, suggesting that the HPA axis could have been differentially affected. Vazquez et al. (2000) however, found elevated plasma ACTH as well as increased corticosterone levels in maternally separated animals when compared to controls. Similar increases in cortisol concentrations have also been demonstrated in young monkeys immediately following separation from their mother (Laudenslager et al., 1995). The lack of concordance between the above results could possibly be ascribed to the variation in experimental animals used and protocols followed.
The effects of maternal separation on other brain structures have been investigated. Elevated levels of ACTH and noradrenaline in response to a stressor were discovered in the locus coeruleus in adult rats previously subjected to maternal separation as pups (Liu et al., 2000). This suggests that the noradrenaline increase could have been responsible for the increased HPA axis response to restraint stress.

Another study showed that maternal separation led to a decrease in glucocorticoid receptor concentrations in the hippocampus (Maccari et al., 1995). They suggested that these glucocorticoid receptors, due to the early maternal stress, became hypersensitive when subjected to a later stress, resulting in an augmented ACTH response.

Some studies have suggested that the hypocortisolism and not hypercortisolism is induced in maternally separated rats. This review indicated that one of the causes of hypocortisolism may be due to increased negative feedback of cortisol on the HPA axis (Heim et al., 2000). Alternatively, hypersecretion of CRF from the hypothalamus occurring under chronic stress conditions, may have led to the downregulation of CRF receptors on the pituitary and ultimately hyposecretion of cortisol (Review by Heim et al., 2000).

1.6 Biochemical Abnormalities associated with Anxiety disorders and the Stress response

Some of the neurochemical abnormalities associated with anxiety disorders include aberrations within the noradrenergic and serotonergic systems. It has been suggested
that early adverse experiences may permanently alter these neurotransmitter systems (Rosenblum et al., 1994).

1.6.1 Noradrenaline

The locus coeruleus is one of the major nuclei of the brain stem where noradrenergic cell bodies reside. It is located in the brain stem tegmentum of the caudal midbrain and rostral pons (Crossman & Neary, 1995) (see figure 1.2 a, b). It consists of approximately 1500 densely packed cells (Swanson, 1976) with projections leading to the prefrontal cortex and hippocampus (Review by Anand et al., 2000). The locus coeruleus is sensitive to external environmental stimuli and the body's internal homeostasis (Review by Anand et al., 2000). The effects of noradrenaline are mediated by noradrenergic receptors, classified as either alpha (α) or beta (β) (Velley et al., 1991). Alpha-receptors are found on pre- and post-synaptic noradrenergic membranes, while beta-adrenergic receptors occur mostly postsynaptically. The activity of neurons of the locus coeruleus is regulated by mechanisms that include its own negative feedback system (Singewald et al., 1999).

1.6.1.1 Noradrenaline and Affective disorders

The noradrenergic system has long been implicated in the pathophysiology of stress (Tanaka et al., 1983; Tsuda et al., 1986; Owens et al., 1993a). Models of anxiety, such as the fear potentiated startle, led to increased basal noradrenaline and dopamine levels in rat hypothalamus tissue after 24 hours (Sajdyk et al., 1997). This change however was suggested to be due to a decrease in the release of the neurotransmitter
into the extracellular fluid, rather than an increase in synthesis of noradrenaline, as the tyrosine hydroxylase concentration did not change. Antidepressants acting on the central noradrenergic system have been shown to decrease the locus coeruleus firing rate (Valentino et al., 1990; Valentino & Curtis, 1991), illustrating the relationship between affective disorders and noradrenaline release.

It has been suggested that depression is caused by a decrease in noradrenaline levels resulting from the prolonged stimulation of NA release during chronic stress, thereby depleting the system (Review by Anand et al., 2000). A study on rats conducted by Stone & Quatermain (1999), concluded that impaired brain alpha-1 noradrenergic neurotransmission contributed to the symptoms manifested in depression.

1.6.1.2 Noradrenaline and the Stress response

There is a strong link between chronic stress and psychopathology (Review by Rudolph et al., 2000; Melia and Duman, 1991). In humans, it has been found that noradrenaline and adrenaline increased after a stress response (Gerra et al., 2001). This response however did not change when the stress was repeated, while the ACTH and cortisol levels, biological markers of the stress response, were diminished. This indicated that noradrenaline was involved in the stress response, but not necessarily with habituation to that stress.

Increases in MHPG (3-methoxy, 4-hydroxy phenylglycol), a noradrenaline metabolite (see figure 1.3), have been found in the hypothalamus of rats exposed to a psychological stressor (Tsuda et al., 1986). According to Tanaka et al. (1983) this
increase in noradrenaline breakdown after a stressor, occurs within the first hour after the stress had been applied. Although this elevation was delayed in the hippocampus and cerebral cortex, a significant increase was still produced. Thus, the authors concluded that the brain did not react uniformly to a stressor, but rather that those particular brain areas responded differentially depending on the nature and time course of the stressor.

1.6.1.3 Noradrenaline and Serotonin

In addition to its own feedback system, the locus coeruleus also receives a number of inputs from other neurotransmitters, particularly serotonin and CRF. On the other hand, noradrenergic alpha-receptors are present on serotonergic neurons e.g. in the hippocampus where they exert a tonic inhibitory action on the serotonergic neurons (Mongeau & Blier, 1993). These two neurotransmitter systems are therefore functionally closely linked.

1.6.2 Serotonin

Serotonin was first described as a vasoconstrictor in the blood. It is synthesised from tryptophan and functions as a neurotransmitter in the brain. Its biochemical name is 5-hydroxytryptamine (5HT). It is catabolised into 5HIAA (5-hydroxyindoleacetic acid) by monoamine oxidase (see figure 1.4), and therefore one can use the concentration of this metabolite as an indicator of serotonin metabolism (Review by Petty et al., 1996). It is produced in the brain stem neurons, which innervate virtually all the areas of the central nervous system (see figure 1.2 a, c). The serotonergic neurons are mostly
Figure 1.2a): Schematic illustration of the major noradrenaline (NA) and serotonin (5HT) pathways in the medial forebrain bundle (MFB) originating from the locus coeruleus (LC) and the dorsal raphe nucleus (DR), and their connection to the paraventricular nucleus (PVH) of the hypothalamus. The hippocampus (HC) is also depicted here. Adapted from Swanson et al. (1983).
Figure 1.2 b): The 2 main noradrenergic pathways, the locus coeruleus and lateral tegmentum pathways in the rat brain. (Abbreviations: DMNV, Dorsal Motor Nucleus of Vagus; NTS, Nucleus Tractus Solitarii; Olf. B, Olfactory bulb; ST, striatum; Thal, Thalamus). Adapted from Ganong, 1989).
Figure 1.2 c): A close up view of the main serotonergic pathways originating from the raphe nucleus in the rat brain. (Abbreviations: CS, D, M: Central Superior, Dorsal, Medial Raphe Nucleus; ST, Striatum). Adapted from Ganong, 1989).
Figure 1.3: Noradrenaline catabolism and its metabolite, MHPG. Adapted from Ganong (1989).
Figure 1.4: Serotonin synthesis and breakdown into its metabolite, 5HIAA. Adapted from Ganong (1989).
associated with the raphe nuclei within the brain stem. The ascending fibres from the raphe nucleus to the forebrain structures are mostly involved in the neural sleep mechanisms (Crossman & Neary, 1995) and in the regulation of complex intelligent adaptive behavior (Review by Baumgarten and Grozdanovic, 1995). The descending fibres modulate nociceptive function (Crossman & Neary, 1995).

1.6.2.1 Serotonin and Affective disorders

The serotonergic-system has strongly been implicated in the neurochemistry of anxiety disorders (Reviews by Boyer, 2000; Jetty et al., 2001). It is very important in controlling mood (Ramboz et al., 1998), and therefore malfunctioning of this system could lead to the development of affective disorders. Serotonin receptor abnormalities have been associated with the development of anxiety disorders and depression (Review by Baumgarten & Grozdanovic, 1995). Extracellular serotonin has been noted to increase in rats as anxiety levels increased with exposure to a novel stressor (Wright et al., 1992). When treated with anxiolytics, these rats displayed a decrease in anxious behaviour and extracellular serotonin levels. Altered serotonin activity have been found in combat veterans suffering from PTSD (Southwick et al., 1997) and in patients with obsessive compulsive disorders (Cath et al., 2001). Genetic deletions of serotonin receptors have increased the vulnerability of knock-out mice to display anxious behaviours, aggression, and drug abuse (Gingrich & Hen, 2001).

1.6.2.2 Serotonin and the Stress response
Serotonin has been described as the homeostatic setpoint for neurotransmitters in the brain, after a stressor occurred (Review by Petty et al., 1996). This theory described serotonin as equilibrating the common neurotransmitters involved in mental illness, such as GABA, dopamine and noradrenaline, back to its normal behavioural chemistry after a disruption, such as a stressor.

Therefore it is not surprising that increased amounts of serotonin was released from the dorsal hippocampus of rats after a stressor (Matsuo et al., 1996). Shimizu et al. (1992) also found that immobilisation stress increased both extracellular serotonin and 5HIAA secretion in the hypothalamus of rats. Kawahara et al. (1993) made a similar discovery, illustrating increased extracellular serotonin levels in the prefrontal cortex and amygdaloid nuclei of the rat, which had suffered a psychological stress. Others have also found that serotonergic pathways were very susceptible to immobilisation/restraint stress, particularly in the cerebral cortex (Morgan et al., 1975).

Restraint stress increased the binding of serotonin to 5HT-1a receptors in the hippocampus (Mendelson and McEwen, 1991) as well as elevating 5HT turnover in the anterior hypothalamus (De Souza and Van Lõon, 1986). A review by Fuller (1992) suggested that the 5HT-1a, 5HT-2 or 5HT-2c receptors may be involved in the activation of pituitary-adrenocortical function in response to stress and that serotonin may play a role in controlling the circadian rhythmicity of adrenocortical secretion.

1.6.2.3 Serotonin and Noradrenaline
It therefore appears that both of these neuronal systems (serotonin and noradrenaline) participate in the stress response and are involved in the development of affective disorders. According to Dinan (1996), neurotransmitter abnormalities seen in patients with affective disorders were actually due to abnormalities in the stress response. These authors theorised that the rise in cortisol levels after a stressor, altered the central noradrenaline and serotonin levels and not \textit{visa versa}.

\textbf{1.7 Corticotropin-Releasing Factor}

Corticotropin-releasing factor (CRF) is a 41-residue peptide synthesised in a large pro-hormone form and released in its active state via a calcium-dependent mechanism (Review by Nemeroff, 1992). Specific binding sites were identified in the anterior and intermediate lobes of the pituitary that serves primarily to regulate ACTH secretion from the anterior pituitary gland (see figure 1.1). Other CRF receptors have been identified in the brain and spleen (Review by De Souza, 1995). In the central nervous system these receptors seem to be heterogeneously distributed (Owens \textit{et al.}, 1993a). Subsequently, CRF immunoreactive neurons have been identified in the raphe nucleus and the locus coeruleus (Cummings \textit{et al.}, 1983). In particular, the brain regions that were involved in autonomic function, limbic areas and olfaction reflected the highest concentrations of CRF-binding sites (Olschowka \textit{et al.}, 1982; De Souza and Kuhar, 1986). There are two CRF receptors, which vary in length: CRF\(_1\), a 415 amino acid protein and CRF\(_2\), a 411 amino acid protein (Review by De Souza, 1995). CRF\(_1\) receptors are localised in the pituitary and the brain, while CRF\(_2\) receptors are more concentrated in the periphery, but have also been identified in the septum, hypothalamus and dorsal raphe nucleus (Review by Arborelius \textit{et al.}, 1999). The
CRF$_2$ receptor is further subdivided into two isoforms: CRF$_{2a}$ and CRF$_{2b}$ (Review by Chalmers, 1996).

1.7.1 Noradrenaline

1.7.1.1 CRF affects Noradrenaline

Evidence have indicated that there is direct synaptic contact between the CRF terminal and the dendrites of the locus coeruleus noradrenaline cells (Chappell et al., 1986). A study by Van Bockstaele et al. (1996) showed that the majority of CRF synapses led to excitation of the locus coeruleus. CRF could also indirectly affect the locus coeruleus discharge via presynaptic actions onto other afferent axon terminals. It could therefore modulate the effect of other co-localised neurotransmitters.

Melia and Duman (1991) showed that CRF was necessary for the induction of tyrosine hydroxylase, the rate limiting noradrenergic biosynthetic enzyme and that CRF increased the expression of this enzyme. It was suggested that CRF could regulate tyrosine hydroxylase via second messengers (cAMP) or via activation of such intracellular pathways.

It has been shown that intracerebroventricular administration of CRF increased the activity of noradrenergic neurons in the locus coeruleus (Valentino et al., 1983; Valentino & Foote, 1988; Curtis et al., 1997). It also led to increased cortical and hippocampal release of noradrenaline (Palamarchouk et al., 2000) and increased stress-induced locomotor activity (Lowry & Moore, 1991). These responses (elicited
via central administration of CRF) were hypothesised to originate from the CRF-locus coeruleus connection. Kawahara et al. (2000) found that the CRF receptor antagonist, CP-154,526 suppressed the stimulation of noradrenaline during stress. Emoto et al. (1993) demonstrated that acute injections of CRF increased MHPG levels in various brain areas including the hypothalamus, amygdala and locus coeruleus. These findings clearly indicate that CRF-noradrenaline interactions may be of importance in the animal’s normal response to stress.

1.7.1.2 Effects of Noradrenaline on CRF

Lavicky and Dunn (1993) found that CRF neurons could in turn be activated by noradrenergic systems. A vast amount of input to the paraventricular nucleus where CRF is produced originated from the noradrenergic neurons located in the brainstem. These neurons signalled onto the paraventricular nucleus via α-1 adrenoreceptors (Liu et al., 2000). Electrical stimulation of noradrenergic fibres from the ventral noradrenergic ascending bundle, increased the CRF concentration in the hypophysial portal system (Plotsky, 1987). A similar effect of noradrenaline-stimulated CRF release has been hypothesised in a review by Koob (1999) to occur in the amygdala, forebrain, hypothalamus and the bed nucleus of the stria terminalis.

1.7.2 Serotonin

1.7.2.1 CRF’s effects on serotonin
The dorsal raphe nucleus is innervated by nerve fibres from the hypothalamus containing corticotropin-releasing factor, and expresses CRF receptors (Price et al., 1998). CRF was found to affect serotonin and its metabolites as well as producing behavioural activation (Lavicky & Dunn, 1993; Song et al., 1995). A study conducted by Kirby et al. (2000) suggested that a stressor led to decreased extracellular serotonin levels in the dorsal raphe nucleus, which was similar in magnitude and time course to the CRF-induced decrease in serotonin. Intraraphe administration of CRF inhibited the serotonergic discharge rate in the lateral septum (Price et al., 1998), confirming the negative effect of CRF on serotonergic neurons.

Kirby et al. (2000) discovered that CRF produced its inhibitory effects on the dorsal raphe nucleus discharge rate at lower doses, while producing an excitatory effect at higher doses. These effects were 300 times more potent when administered directly to the dorsal raphe nucleus than intracerebroventricularly. Their findings strengthened the evidence suggesting a connection between CRF and the serotonergic system. They also implicated the CRF$_1$ receptor subtype in these interactions, which could possibly mediate the inhibitory stimuli.

**1.7.2.2 Serotonin’s effects on CRF**

Serotonin on its own has been shown to have a profound effect on CRF regulation. A study by Liposits et al. (1987a) showed that serotonin-containing terminals formed axo-dendritic and axo-somatic synapses with CRF-immunoreactive neurons in the paraventricular nucleus. This pathway suggests that the serotonergic neural system could influence the HPA axis via direct action on the CRF synthesising neurons. In a
review, Fuller (1992) describes how CRF-containing neurons receive projections from the serotonin neurons located in the midbrain raphe nuclei. The serotonin-induced CRF release was found to be via a mechanism involving both protein kinase C and protein kinase A (Hu et al., 1992).

Serotonin has also been found to increase ACTH secretion (Gibbs & Vale, 1983) and stimulate the release of CRF in rat hypothalami through the 5HT-2 receptor (Calogero et al., 1989; Hillhouse & Milton, 1989). However, recent studies indicate that chronic treatment with SSRI's leads to a decrease in Pro-opiomelanocortin (POMC) mRNA (a precursor of ACTH) and HPA axis desensitization (Jensen et al., 1999).

1.7.3 CRF and the Stress response

CRF has been described as the central co-ordinator of the endocrinological, autonomic, immunologic and behavioural stress responses (Review by Heim & Nemeroff, 1999; Habib et al., 2000). It is the main regulator of ACTH release during stress (see figure 1.1). A review of clinical studies has shown that CRF may be the main neuro-hormone mediating the effects of early life stress on subsequent pathology (Review by Heim et al., 1997).

When CRF is administered intracerebroventricularly, it induced behavioural and other abnormalities typical of a stress response, such as increased motor activation (Britton et al., 1986; Song et al. 1995) and a reduction in exploratory behaviour in rats in response to a stressor (Koob et al., 1993). These behavioural effects were reversible with CRF antagonists suggesting that CRF was indeed involved in these stress-mediated abnormal behavioural responses.
In a separate study, daily intracerebroventricular injections of CRF did not lead to habituation of body temperature, heart rate and motor function (all physiological responses to stress) over a ten-day period (Buwalda et al., 1998). They demonstrated that 1 μg of CRF was too little to mimic chronic behavioural stressors.

Chronic stress has been shown to lead to a reduction in CRF receptor number and decreased CRF binding to its receptor in the anterior pituitary gland (Anderson et al., 1993). Increased CRF production was found in the paraventricular nucleus after the organism was exposed to stressful stimuli (Suda et al., 1988). Chronic infusion of CRF, led to increased levels of ACTH secretion (Hotta et al., 1991), which could be due to the oversensitisation and therefore hyperactivity of the CRF receptors on the pituitary gland.

1.7.4 CRF and Affective disorders

-CRF not only enhances behavioural responses to stress, but has also been shown to lead to behavioural effects characteristic of anxiogenic compounds, and therefore might be related to anxiety disorders (Britton et al., 1982; Koob et al., 1993).

Depression is characterised by increased CRF release and increased discharge rates of the locus coeruleus. Curtis et al. (1999) showed that swim stress, an animal model of depression, functionally alters the CRF receptors that have an impact on locus coeruleus activity. These studies clearly demonstrate the intricate relationship between the locus coeruleus, the HPA axis and psychiatric disorders.
Increased anxiety levels measured behaviourally were noted after administration of CRF into the locus coeruleus (Review by Weiss et al., 1994). Southwick et al. (1997) found clinical evidence associating chronic alterations in noradrenergic, serotonergic and glucocorticoid systems in people suffering from PTSD, even up to 25 years after the stress had occurred. An increase in CRF receptor binding in the raphe nucleus was found in anxious rats that were previously maternally separated. This result suggested an upregulation of CRF receptors in this area (Ladd et al., 1996). In a review by De Souza (1995), the author suggested that there might be a process occurring above the hypothalamus, which led to the excess secretion of endogenous CRF, resulting in the development of psychopathology.

The review by Heim et al. (1997) reported how increased activation of CRF neuronal systems was found in patients with Major Depressive Disorder and PTSD. In a follow-up review, Heim et al. (2000) indicated that PTSD manifested itself neuroendocrinologically, which included low baseline cortisol secretion, supersuppression of cortisol by dexamethasone, blunted ACTH response to CRF and increased cerebral spinal fluid CRF concentrations. Owens and Nemeroff (1993b) also reviewed clinical studies, which showed that drug-free depressed patients exhibited HPA axis hyperactivity, increased cerebral spinal fluid CRF concentrations, a blunted ACTH release in response to CRF, reduced density of CRF receptors in the frontal cortex, and pituitary and adrenal gland hypertrophy. These results illustrated the role of increased CRF levels in depressed patients.

A blunted ACTH response to CRF administration has also been observed in patients with Panic disorder (Roy-Byrne et al., 1986). In patients with PTSD and Obsessive
Compulsive Disorder (OCD), elevated cerebral spinal fluid CRF levels as well as a blunted ACTH response have been found. However, in contrast to depression, these patients exhibited hypocortisolism (Review by Yehuda, 1997).

Yehuda et al. (Review, 1991)-described a possible mechanism as to why the ACTH response to CRF is blunted in anxiety disorders as seen in PTSD. She proposed that the pituitary gland could be hyperresponsive to cortisol, due to an increased concentration of glucocorticoid receptors on the pituitary gland. Nutt et al. (2000) therefore suggested that there was a stronger negative feedback inhibition and this resulted in a decreased cortisol concentration and ACTH baseline. Another study also found a lower ACTH response in PTSD patients (Smith et al., 1989). Central administration of CRF produced many signs of anxiety disorders and increased CRF neuronal activity has been observed in PTSD patients (Review by Heim et al., 1997).

We therefore suggest that elevated CRF levels could be causally related to the increased negative feedback and therefore blunted ACTH response, exhibited by patients with anxiety disorders such as PTSD. We also propose that adverse development factors, such as maternal separation, predisposes individuals to develop psychopathologies later in life and functionally alters the neurotransmitter systems involved in the development of these psychopathologies.

1.8 Previous Studies

In our attempts to study maladaptive factors during development, we have previously investigated the effects of overcrowded rearing conditions on behaviour during
adulthood. This study showed altered serotonergic receptor function in the hippocampus and abnormal behavioural responses when tested on the elevated plus-maze (Daniels et al., 2000). The data indicated that developmental factors do indeed contribute deterministically to adult behaviour in terms of anxiety and may do so by disturbing the neurotransmitter homeostasis. We have therefore decided to investigate another factor in the development process i.e. maternal separation, and its consequences on adult behaviour and the stress response. We also wanted to establish whether an increase in CRF content of the brain alone, could lead to abnormal and stress responses and behaviours indicative of psychopathologies.

1.9 Aims of present study

We decided to use a Sprague-Dawley rat model in order to mimic the behavioural abnormalities found in clinical studies investigating early adverse conditions.

Therefore, the aims of our study were:

1) To determine the effect of maternal separation on rats in their later life by:

   i) measuring anxiety levels behaviourally making use of the Elevated Plus-maze and Open Field tests;

   ii) determining whether there are changes in release of Noradrenaline and Serotonin responses after restraint stress;

   iii) evaluating the HPA axis response in terms of ACTH secretion after restraint stress.
2) To elucidate the role of CRF in anxious behaviour, often related to maternal separation by:

i) injecting CRF chronically intracerebroventricularly;

ii) measuring the behaviour of rats subjected to these chronic CRF injections;

iii) determining the HPA axis activity of rats that received CRF.
CHAPTER 2

METHODOLOGICAL PROCEDURES

2.1 Introduction

Described below are the methodologies that were employed in the separation (Chapter 3) and corticotropin-releasing factor (Chapter 6) studies. Sprague-Dawley rats weighing between 200g to 350g were used in all experiments. The animals were held in the animal facility of the University of Stellenbosch, where the temperature was kept constant at 22 °C, humidity at 55% and food and water was available ad lib for the duration of the experiments.

2.2 Chemicals and Equipment used

All chemicals and reagents used were of the purest grade commercially available. A full list of the chemicals, reagents and equipment appears in appendices A, B and C.

2.3 Behavioural Studies

2.3.1 Elevated Plus-maze

The Elevated Plus-maze is an apparatus used to examine the behaviour of rats (see figure 2.1). It is in the form of a cross with its north-south axis being closed and its west-east axis, open. Both axes are elevated half a meter from the ground.
Figure 2.1: The Elevated Plus-maze.

Figure 2.2: The Open Field Test arena.
The maze has been validated as a measure of anxiety by various authors (Pellow et al., 1985; Rodgers and Dalvi, 1997). The maze uses stimuli that would usually lead to anxiety in their natural environment. The open arms signify open spaces and evoke a stronger fear reaction than the closed arms, which serve to provide shelter for anxious rats (Rodgers and Dalvi, 1997). The time spent in the different arms of the maze was therefore noted. The more anxious the rat, the more it would avoid the open arms and remain in the closed arms of the maze. The number of entries into the various arms of the maze was also determined and served as a measure of the rat's locomotor activity.

2.3.1.1 Procedure

The rats spent a total of 5 minutes in the maze. This time interval was chosen because it was shown that avoidance behaviour decreases and fatigue increases after 5 to 10 minutes on the maze (Pellow et al., 1985). The rats were placed in the centre of the maze and then allowed to run either into the open or closed arms of the maze. The time spent in the enclosed or open arms of the maze was recorded using stopwatches and the number of entries was noted manually. All experiments were recorded with a video camera to facilitate scoring at a later opportunity.

2.3.1.2 Scoring

In order to determine the amount of time spent and number of entries in each arm, certain demarcations were applied. Two paws had to be inside the line indicating the entrance to the various arms, which signalled the start of the time spent in the specific arm. The stopwatch was stopped when all four paws were over the line again. At this point, the rat accumulated 1 entry into the specific arm. The time spent and number of
entries in a particular area were accumulated. The maze was cleaned between every test with 70% alcohol to ensure that the rat’s behaviour was not affected by the detection of another rat’s scent.

A number of specific behaviours were also recorded while the animal was on the elevated plus-maze. Freezing and defecation were noted as indicators of anxious behaviour, while grooming and rearing were considered representative of relaxed behaviour. Freezing was denoted as the absolute stillness for approximately 1 second, of the rat. Defecation was represented as the number of times the animal defecated in a session. Grooming and rearing were also noted. A minimum of 6 independent observers recorded the behaviours. Gloves were used throughout the experiment.

2.3.2 Open Field

The Open Field test is another technique designed to measure anxious and relaxed behaviours in rats (see figure 2.2). It is constructed out of opaque Plexiglass (Lucite), with white walls and a light gray floor. The dimensions of our arena were 100cm x 100cm (floor) x 50cm (walls). The quadrants and zones on the floor were marked with electrical tape. The peripheral or outer zone was specified at 15cm from the outer walls. The remaining area in the center served as the center or inner zone. Quadrants of 20cm x 20 cm’s were demarcated throughout the arena and served to evaluate the animal’s locomotor activity. The arena was placed in the center of a quiet room with even lighting. By making use of a lamp aimed at the centre of the maze, the light level was brightened, while the outer zone remained dimmer. The brightened centre served as anxiogenic stimuli, as rats would usually avoid bright, open spaces. More time spent in the inner zone, indicated increased levels of exploratory and therefore relaxed
behaviour (Personal communication, Plotsky). The number of small blocks crossed and crossings between the inner and outer zone, provided us an indication of the rat's locomotor activity. Increased locomotor activity showed a desire to explore the maze and therefore was considered representative of relaxed behaviour. Latency to move from initial placement into the inner zone of the maze was another parameter of the level of anxiety in a rat. The more anxious the rat, the longer the delay in crossing into the inner zone.

2.3.2.1 Procedure

Each rat was tested in a single trial of 10 minutes. The rat was placed in one quadrant of the arena in the outer zone. A camera mounted on a high tripod was used to record the behaviour of the animals on the maze. This enabled the 8 trained volunteers who were blinded to the treatment conditions of the animals, to score the behaviors at their leisure. Between animals, the arena was cleaned with a 70% alcohol solution.

2.3.2.2 Scoring

The total time spent in each zone (inner versus outer) and the number of zone crossings were noted for each rat. The latency to move from the initial placement in the outer zone to the inner zone as well as the number of quadrant crossings was also noted. All 4 paws had to be over the demarcated lines to constitute a crossing. Other behaviours measured included rearing (relaxed behaviour) and defecation frequency (anxious behaviour).
2.4 Restraint Stress

The rats designated to undergo restraint stress, were placed in the laboratory an hour beforehand so as to allow acclimatisation and to eliminate the novel stress elicited by an unfamiliar environment. Each rat was then individually subjected to restraint stress, by placing it in a confined holder. It was equipped with areas for breathing and defecation. They were subjected to 10 minutes of restraint stress. Trunk blood was collected at various time intervals (15 and 60 minutes post restraint stress) in the morning for ACTH determinations. Basal ACTH levels were also noted in a separate group of rats that were not subjected to restraint stress. Trunk blood was collected in EDTA tubes and kept on ice to minimise proteolysis of ACTH. The tubes were centrifuged in a Sorvall RC-5B Refrigerated Superspeed Centrifuge for ten minutes at 3000 rpm. One millilitre of plasma was collected and stored in liquid nitrogen for later assaying.

2.5 ACTH Determinations

This assay was used to evaluate the response of the HPA axis to the restraint stress. ACTH was used as an indicator of HPA axis activity. A radioactive kit purchased from Nichols Institute Diagnostics (California, USA) was used to determine the ACTH levels of the rats.

2.5.1 Principle of ACTH assay
The ACTH radioisotopic assay made use of a monoclonal antibody (Abm), which was radioactively labelled with $^{125}$I, and a polyclonal antibody (Abp), which also had an affinity for biotin and avidin. Both have a high affinity and specificity for ACTH. The polyclonal antibody has an affinity for the C-terminal region of ACTH, while the monoclonal antibody binds to the N-terminal. They worked synergistically to form a molecular sandwich. The radioactivity served to detect the concentration of ACTH present in the sample. An avidin-coated bead was added to the assay, which bound to the polyclonal antibody and therefore served to precipitate the molecular sandwich (see figure 2.3). The amount of ACTH bound to the bead was then represented as the amount of radioactivity present. A LKB Wallac 1261 Multigamma counter determined the amount of radioactivity present.

2.5.2 ACTH assay procedure

A total of 63 polypropylene tubes were used during the course of an assay. The tubes were labelled according to the table 2.1 below:

<table>
<thead>
<tr>
<th>Tubes</th>
<th>Number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>Duplicate</td>
<td>Total amount of radioactivity</td>
</tr>
<tr>
<td>Blank</td>
<td>Duplicate</td>
<td>Serves as the 0 value for the counter</td>
</tr>
<tr>
<td>Std 1-6</td>
<td>Duplicate</td>
<td>Standards to draw the dose response curve. Amounts vary form 5-1550 pg/ml</td>
</tr>
</tbody>
</table>
Figure 2.3: Schematic representation of the ACTH sandwich, which contains the ACTH molecule, polyclonal (Abp) and monoclonal (Abm) antibodies, as well as radioactive iodine ($^{125}\text{I}$).
The samples were kept on ice for the duration of the experiment. The blank tubes were prepared by adding 4 ml distilled water to each of the tubes. Two millilitres of distilled water was added to the standards and external controls. Two hundred microlitre aliquots were transferred into the allocated tubes. The same amount was extracted from the samples collected and the internal standards. The radioactive marker, $^{125}\text{I}$ (100 μl) and the antibody solution consisting of both antibodies (100 μl) was then added to all the tubes. The tubes were vortexed and placed at an angle of 30° in a specified rack. A single avidin bead was added to each of the tubes, which were then covered with parafilm and allowed to incubate at room temperature for 22 hours. Distilled water (550 ml) was added to the washing solution (50 ml) to serve as the washing buffer. Two milliliters of the solution was added to the tubes, except the total-counts tubes, after the incubation period, to wash off the unbound antibodies. This was repeated and the bead was left to dry, whilst still in the tubes. The tubes

<table>
<thead>
<tr>
<th></th>
<th>ACTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_j$</td>
<td>Duplicate</td>
</tr>
<tr>
<td></td>
<td>External control</td>
</tr>
<tr>
<td></td>
<td>Values between 27-41 pg/ml. Lower boundary for counter.</td>
</tr>
<tr>
<td>$K_k$</td>
<td>Duplicate</td>
</tr>
<tr>
<td></td>
<td>External control.</td>
</tr>
<tr>
<td></td>
<td>Values between 280-421 pg/ml. Upper boundary for counter.</td>
</tr>
<tr>
<td>MK1-3</td>
<td>Single</td>
</tr>
<tr>
<td></td>
<td>Internal controls.</td>
</tr>
<tr>
<td>Samples 1-40</td>
<td>Single</td>
</tr>
<tr>
<td></td>
<td>Samples collected.</td>
</tr>
</tbody>
</table>
were then placed in a LKB Wallac 1261 Multigamma counter for a minute, to produce the amount of counts per minute (cpm), which was equivalent to the concentration of ACTH molecules. A standard curve was drawn up making use of the Allegro computer package, and the values from the standards provided in the kit. The concentration of the samples was then determined using this graph. The intra and interassay coefficient of variance obtained were within the acceptable limits, as they were 3% and 7.8% respectively.

2.6 High Performance Liquid Chromatography

2.6.1 Principles of the method

The neurotransmitter levels of some animals were determined to ascertain the effect of maternal separation on the central nervous system. A high performance liquid chromatography (HPLC) method adapted from Russell et al. (1985)-was followed. The method is based on the principle of reverse-phase, ion-pair liquid chromatography. The concentration of noradrenaline, serotonin and their metabolites, MHPG and 5HIAA, was determined in the hippocampus, frontal cortex and hypothalamus of the rats (see figure 2.6).

Reverse-phase liquid chromatography was employed to quantitate the amount of monoamines present in our samples. This type of chromatography indicates that the mobile phase has polarity and the stationary phase is non-polar, unlike normal phase chromatography, where the mobile phase is non-polar and the stationary phase, polar. The mobile phase consists of an aqueous buffer (phosphate) with an organic solvent
(methanol). Counter-ions are also present in the mobile phase, which are opposite in charge to the sample molecule, which we are interested in. These molecules then form an ion-pair that is insoluble in the mobile phase and soluble in the stationary phase, the silica-packed column. Differential migration (see figure 2.4) is the key to separating molecules. The equilibrium of the particles between the mobile and stationary phases determines their retention time. The time the molecules take to move through the column silica packing, through a process known as eddy diffusion, (see figure 2.5) also determines the band width and retention times. Eventually, the particles emerge from the column, separated, and are identified by the detector, which then records their retention times and concentrations on a chromatograph (Snyder & Kirkland, 1979) (see figure 2.7).

2.6.2 Equipment Specifications

The BAS (Bioanalytical Systems Inc.) High Performance Liquid Chromatography machine was used to analyse these samples. The column employed was a Phenomenex Luna 5 micron C18 (2), 250 x 4.6 mm silica column with a Phenomenex Security Guard, guard column. The electrode was set at 0.70V, the temperature was kept at 27 °C and the flow rate of the sample through the system was 1ml/min. The sensitivity of the readings was set at 2 nA. This method was optimised in the laboratory specifically for our conditions.

2.6.3 Preparation of HPLC
Figure 2.4: Differential migration. Compounds $A$ and $C$ at equilibrium are either mostly in the stationary phase, $C$, and therefore move slowly or they are mostly in the mobile phase, $A$, and therefore move faster through the column. This is the basis of separation in liquid chromatography. Adapted from Snyder and Kirkland (1979).
Figure 2.5: Molecular spreading. If particles move through the wider gaps between the silica particles of the column, they will move faster (red arrows) and therefore elute first. Moving through the smaller gaps (blue pointer), retards their movement. This phenomenon is known as eddy diffusion. Adapted from Snyder and Kirkland (1979).
Figure 2.6: The frontal cortex, hypothalamus and hippocampus of a rat. Adapted from Swanson et al. (1983).
Figure 2.7: A representative chromatogram illustrating the various retention times of the monoamines.
The electrode was polished on the first day of every week with polishing alumina. The reference electrode was removed and kept in 3M NaCl for the duration of the polishing process. The acidified aluminum oxide was activated at 200 °C on that same day. It was established that it remains activated for at least a further 7 days.

2.6.4 Buffer

A Sodium Dihydrogen Phosphate, pH 3, buffer containing EDTA, Heptasulphonic acid and methanol was used as the polar mobile phase.

The following reagent concentrations were present in the buffer:

- NaH$_2$PO$_4$ 5.52g/l
- EDTA 50mg/l
- Heptasulphonic acid 50mg/l
- Methanol 15%

The chemicals were added to 2 litres of highly polished water, obtained from a Milli-Q reagent water system. After the reagents had dissolved, the pH of the buffer was adjusted to pH 3 using phosphoric acid. It was then degassed and filtered twice using a Millipore filter system with 0.22 micron filters. This prevented any particles or air bubbles damaging the column or producing a false reading.

2.6.5 Monoamines extracted directly from tissue (Direct method)
The dissected brain tissue i.e. the hippocampus, frontal cortex and hypothalamus of each rat, was weighed with a Sartorius electronic balance and then suspended in 30 volumes of ice-cold 0.1M Perchloric acid buffer, containing 0.3mM EDTA and 0.5mM sodium metabisulphate. The samples were homogenised with 6 strokes of the pestle, then sonicated for 20 seconds, in order to maximise the release of the catecholamines from the vesicles. The samples were allowed to stand for 20 minutes on ice to accommodate the extraction process, and then centrifuged at 13000 rpm (21000 x gravity) for 20 minutes (Russell et al., 1985) in a Sorvall centrifuge. Centrifugation allowed the larger proteins to precipitate, forming a pellet, while the supernatant, containing the desired monoamines, was used for the duration of the experiment. To enable the noradrenaline (NA) peak to be better separated from the solvent frontal peak, of which a large portion is ascorbic acid, ascorbate oxidase was added to the supernatant (McKay et al., 1984). It was necessary to adjust the supernatant to pH 5 with 10M potassium acetate (5μl/200μl) before the addition of this enzyme. 5μl of Ascorbate oxidase (1mg/ml), which was dissolved in ammonium acetate buffer (0.1M ammonium acetate adjusted to pH 5.0 with glacial acetic acid) was added to 200μl of the extract. The samples were allowed to stand for 10 minutes on ice and then filtered for 2 minutes through a 0.2μm microfilter prior to injection onto the column.

Standards (1 mg/10 ml) (serotonin, 5HT; 5-hydroxyindoleacetic Acid, 5HIAA; 3-Methoxy-4-Hydroxy-phenylglycol, MHPG; noradrenaline, NA) were made up in 0.1M acetic acid containing 0.1% Sodium Metabisulphate. The standards were then added to 0.1M perchloric acid buffer (5μl: 5ml) containing the same concentrations of potassium acetate and ascorbate oxidase in order to quantitate the unknowns.
according to standard peak heights. The results were expressed in ng/g wet weight of the tissue using the following formula:

\[
\text{Conc} = \frac{\text{Sample PH (mV)} \times [\text{STD}] \times \text{Sample Vol (\mu l)}}{\text{STD PH (mV)}} \times \text{Inject Vol (\mu l)} \times \frac{1000}{\text{tissue wt (mg)}}
\]

Where “Conc” is the concentration in ng/g wet weight, “[STD]” is the concentration of standard in ng/20\(\mu\)l and “Inject Vol” is the volume of supernatant injected (20 \(\mu\)l in our experiments).

For determination of MHPG and 5HT, 0.805 and 0.45 was multiplied respectively to the total amount detected, since the standards took on the form of salts and these corrections were therefore necessary.

2.6.6 Optimisation of Direct method

It was discovered that the frontal peak possibly resulting from the catabolism of the tissue samples by the perchloric acid added, partially masked the noradrenaline peak height even though ascorbate oxidase was added to decrease this front. The frontal peak lasted for 4.34 minutes. By adjusting the methanol concentration in the mobile phase, we could determine when the various monoamines would be eluted. We therefore adjusted the methanol concentration from 15.2% to 9.9% to elute the noradrenergic peak later than 3.41 minutes. We tested each (NA, MHPG, 5HT and 5HIAA) molecule separately, making use of standards, and found that the noradrenaline peak appeared at 4.12 minutes. The frontal peak, however, still interfered with the readings. The run time was subsequently extended from 30
minutes to 45 minutes in order to elute 5HIAA, which occurred at 39.22 minutes and let the remainder of the other molecules still present in the sample, to be completely eluted. This step was essential, as the remaining molecules tended to cause a build up on the column that interfered with subsequent sample readings.

We decreased the organic solvent further to 5% methanol buffer. This resulted in NA eluting at 5.65 minutes and 5HIAA, which appears last, at 78.13 minutes increasing the run time to almost 2 hours. The decrease in methanol concentration also led to a widening of the 5HT and 5HIAA peaks and a decrease in the concentration detected, which rendered this method as unsatisfactory.

We subsequently decided to determine the levels of MHPG, 5HT and 5HIAA using the original tissue extraction method prior to adjustments, while the acidified aluminum oxide extraction method was employed to determine the concentration of NA.

2.6.7 Characterisation of Acidified Aluminum Oxide Method

To determine whether the extraction of NA using the acidified aluminum oxide was suitable, we set up the following experiment. This method was based on the protocol of Anton and Sayre (1962), but adjusted and characterised to suit the needs of our experiments.

Acidified Aluminum Oxide (AAO) was activated at 200 °C for 2 hours, prior to experimentation. AAO (35mg) was added to each of 3 glass conical extraction vials, of which one served as the internal standard. Five tubes were set up:
1) "Sample 1" contained 5ml 0.1M Perchloric acid buffer (0.3mM EDTA and 0.5mM sodium metabisulphate, pH 3) and 5 µl NA.

2) 1:10 Dilution of DHBA, containing 90 µl buffer and 10 µl DHBA

3) "Sample 6 " contained 2 ml polished water, 40 µl 0.13M sodium metabisulphate and 4 µl of tube 5.

4) The Standard tube contained 5 µl NA and DHBA made up to 5 ml in buffer.

5) Standards diluted 1:10 i.e. 80 µl buffer, 10µl DHBA and 10µl NA

The samples already contained 0.1M Perchloric acid buffer (0.3mM EDTA and 0.5mM sodium metabisulphate, pH 3) from the previous tissue extraction method and we therefore only had to add sodium metabisulphate to the internal standard: “sample 6”.

The 3 glass-extraction vials represented samples of supernatant extracted from tissue.

1) Glass vial 1 contained 1.8 ml polished water + 200 µl tube 1 + 4µl tube 2.

2) Glass vial 2 contained 1.6 ml polished water + 400 µl tube 1 + 4µl tube 2.

3) Glass vial 3 contained 2ml of tube 3.

Glass vial 1 and 2 served as samples containing 1 ng/ 20 µl sample injected and 2ng/20µl sample injected respectively. Glass vial 3 served as the internal standard.
(“sample 6”), which was used to calculate percentage recovery of NA from the sample extraction.

One millilitre of Tris buffer (1.5M, pH 8.7) was added to each vial to neutralise the 0.1M perchloric acid buffer and to provide an alkaline medium for noradrenaline to sufficiently bind to the Acidified Aluminum Oxide (AAO). The vials were then placed on a rotary mixer for 10 minutes and thereafter allowed to settle on ice. The supernatant was then aspirated with a glass pasteur pipette and 1 ml 0.83M Tris (pH 8.6) buffer was added to each vial. The contents were mixed and allowed to stand before the supernatant was collected. This step was repeated and the final suspension transferred into plastic collection tubes (see figure 2.8). These tubes were sealed with parafilm and centrifuged for 1 minute. The AAO, which was bound to the NA, remained on the filter and was washed with polished water (300 μl). The tubes were then vortexed and spun again for 1 minute to allow the water to filter through. The bottom half of the collecting tube ensemble was then replaced with a fresh collecting tube. In order to extract the bound NA from the AAO, we added 400 μl of the 0.1M Perchloric acid buffer and mixed the tubes using the vortex machine. The tubes were spun for 1 minute, the bottom half representing the supernatant with monoamines was removed, capped, and placed on ice. The entire method described above was performed on ice to reduce enzyme activity and the autocatabolism of NA.

Homovanillic acid standard (5 μl/5ml 0.1M Perchloric acid buffer) was also run to determine the length of the run period, as it is usually the last metabolite to be eluted from the sample.

Samples (20μl) were then injected into a BAS High Performance Liquid Chromatograph machine, which was connected to a Varian 4270 integrator. The standards (20μl) prepared in tube 4 were also injected to determine their
Figure 2.8: A diagrammatical representation of the collection tubes used in the HPLC method.
concentrations. These values were used to calculate the concentration of NA present in our "samples" and the time that it eluted from the sample.

The chromatogram indicated that this method (see calculation 1) had an 84.7% recovery rate and the emerging concentration of the extracted catecholamines was almost equal to the amount injected. This process was repeated to ensure its accuracy. The second trial had an 81.8% recovery rate. Sample 1 revealed that 0.99 ng/20 μl was detected, compared to the 1 ng/20μl injected and sample 2 detected 1.98 ng/20 μl, in comparison to the 2 ng/20μl injected.

Calculation 1:

% Recovery:

\[
\text{peak height DHBA in glass vial 3} \times 100 \\
\text{peak height STD}
\]

Concentration of NA (5HT/MHPG/5HIAA)

\[
\text{NA PH S1 (2)} \times [\text{NA}]_{\text{STD}} \\
\text{DHBA PH S1 (2)} \\
\text{NA PH S6} \\
\text{DHBA PH S6}
\]
Where "NA" is Noradrenaline, "PH" is peak height, "s" is sample, "DHBA" is 3,4-Dihydroxy-benzylamine, "STD" refers to the Standard. The concentration is measured in ng/20 µl.

We therefore concluded that a combination of the two methods could be used to determine the NA, 5HT, MHPG, 5HIAA in the various brain areas. In order to detect the amount of NA present, the tissue was prepared making use of the tissue extraction method. The supernatant was then used in the direct tissue and acidified aluminum oxide extraction method. Together with the addition of polished water to the glass vials to make up 2ml, the supernatant served as the "plasma samples" for the acidified aluminum oxide method. It was decided that 200 µl of sample would be used. However, should the amount of supernatant be insufficient, the amount of polished water would be adjusted accordingly.

A few experiments were performed to compare the direct tissue extraction method with the acidified aluminum oxide procedure. We found that there was a significant discrepancy between the NA values of the two methods, while the standards yielded comparative results (see calculation 2). We concluded that even though there was some contribution to the NA values from the frontal peak using the direct method, this procedure was chosen as we assumed that the extraction conditions were similar for all samples. We therefore employed the tissue extraction method for all subsequent monoamine determinations.

Calculation 2:
Example of Noradrenaline peak heights discrepancies in the same tissue sample:
AAO: 1459
Direct tissue extraction method: 11240
MATERNAL SEPARATION

STUDY
3.1 Introduction

Adverse events that occur at crucial stages of a child's development often lead to psychological abnormalities that manifest themselves in their adult lives. Studies reviewed have shown that individuals subjected to childhood sexual abuse suffered from adult-onset depression and displayed signs of a dysregulated HPA axis (Weiss et al., 1999). Similarly, Kendler et al. (1992) found that the loss of a parent during childhood increased the risk to develop major anxiety disorders and could also lead to depressive-like behaviour (Furukawa et al., 1999).

Animal studies have shown that centrally administered CRF in maternally separated non-human primates, led to increased pituitary-adrenal activation (Kalin, 1989). In rodents, maternal separation of an infant resulted in an increase in basal corticosterone levels and a decrease in growth hormone secretion (Kuhn et al., 1990), as well as increased noradrenaline release in the dorsal hippocampus (Matthews et al., 2001).

These findings led us to hypothesise that maternal separation predisposes individuals to develop stress-related psychopathologies later in life and functionally alters the central neurotransmitter systems involved in the development of these psychopathologies. We also proposed that the efficacy of the HPA axis to respond to a stressor in adulthood would be severely compromised due to this adverse development factor. We have therefore devised the following experiments in order to evaluate the HPA axis’ response to a stressor during adulthood and to see whether maternally separated animals displayed anxious behaviours later in their lives.
also investigated whether the behavioural abnormalities were associated with alterations in the neurotransmitter systems of their brains.

**METHODS**

**3.2 Separation Paradigm**

Male-female pairs were housed under normal conditions, to allow mating. Immediately after the rat pups were born, the adult males were removed, while the mothers remained with the pups. From post-natal day 2 to day 14, the rat pups were separated everyday from their mothers for a period of 3 hours a day. The separations occurred between 09:00 and 13:00. Great care was taken not to handle the pups in any way, to prevent our scent being transferred onto them with subsequent rejection by the mother. During the separation period, the pups were housed in a separate room, to disallow communication with their mothers by use of ultra-sound vocalisations (Hofer et al., 1994). After the 3 hours, the pups were rejoined with their mothers. After day 14, they were returned to normal housing until they were weaned on day 22 (see figure 3.1). The rats were then grouped according to gender and were housed as adults under normal conditions with the exception that they were handled every day. This served to familiarise them with being handled, so that this would not become a novel stress when the experiment was being performed.

On day 60, the rats were classified as adults and the behaviour tests were subsequently performed. The behaviour measurements were taken in the same room as they were housed so as not to introduce any form of novel stress, that may
Figure 3.1: Schematic representation of our separation paradigm.
influence the readings. Rats that were treated similarly, but were not separated between postnatal day 2 and 14, served as controls.

3.3 Behavioural Studies

The procedure as described in section 2.3 was employed to evaluate whether the animals were anxious. Separated rats (n=32) and controls (n=25) were evaluated either on the elevated plus-maze and/or the open field maze to determine the nature of their behaviour. These tests were conducted in the afternoons.

3.4 Neurotransmitter Levels

After being subjected to the behavioural tests, the same rats were decapitated the next morning and the hippocampus, hypothalamus and frontal cortex dissected for neurotransmitter detection. The neurotransmitter levels of noradrenaline (NA), serotonin (5HT) and their metabolites (MHPG, 5HIAA respectively) of separated animals (n=32) versus controls (n=25), were established using high performance liquid chromatography as described in section 2.6. Neurotransmitter levels were also evaluated in another group of previously separated rats that were subjected to a subsequent stressor. This was done to determine whether there were any alterations in the neurochemical response following a stressor. Brain tissue was collected at basal (n=37), immediately (n=10) and 15 minutes (n=10) post-restraint stress. The restraint stress procedure as described in section 2.4 was employed.
3.5 ACTH Determinations

The HPA axis’ response to stress was also evaluated in some of the animals subjected to behavioural testing, using the restraint stress paradigm described in section 2.4. Trunk blood was collected in the morning at 3 time intervals, namely basal (n=17) as well as 15- (n=10) and 60-minutes (n=10) post-restraint stress for the determination of plasma ACTH levels (section 2.5). Rats not conforming to the standard experimental protocol were excluded from the study. These included rats that fell off the maze or had their heads stuck in the restraining apparatus.
RESULTS

4.1 Behavioural Studies

4.1.1 Elevated Plus-maze

An independent t-test was applied to the data, and revealed significant differences between maternally separated (n=20) and control rats (n=14) with respect to the number of entries into the open (p<0.001) and the closed (p<0.05) arms of the maze (see figure 4.1). The separated rats entered the arms of the elevated plus-maze significantly less than controls indicating a decrease in locomotion (p<0.05).

The amount of time spent in the closed arms of the maze was also significantly increased (p<0.05) in maternally separated animals (see figure 4.2).

With regards to the behaviours noted during each session on the elevated plus-maze, an independent t-test yielded significant increases in defecation frequency and rearing behaviours in maternally separated animals (p<0.05) (see table 4.1).

4.1.2 Open Field Test

When the independent t-test was applied to the data of the open field test, no significant differences were found between separated animals (n=20) and controls (n=16) with respect to the time latency to enter the inner zone (see figure 4.3), the number of blocks crossed (see figure 4.4), the amount of time spent in the inner or...
Figure 4.1: Significant differences were found between maternally separated (n=20) and control (n=14) animals with respect to the number of entries into the open arms (p< 0.001) and closed arms (p< 0.05) on the elevated plus-maze. All values represented here are the means ± SEM.
Figure 4.2: Significant differences were found between maternally separated (n=20) and control (n=14) animals with respect to the amount of time spent in the closed arms (p< 0.05) of the elevated plus-maze. All values represented here are the means ± SEM.
Table 4.1: Behaviours noted on the elevated plus-maze are expressed here as means ± SEM. Significant differences were found between maternally separated (n=20) and control (n=14) animals in their defecation frequency (feces) and rearing behaviour (p<0.05).

<table>
<thead>
<tr>
<th>Elevated plus-maze behaviours: separated vs. control (means ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Separated</td>
</tr>
<tr>
<td>Control</td>
</tr>
</tbody>
</table>

* p < 0.05
Time latency to move into inner zone of the open field arena
(separated vs. control)

Figure 4.3: No significant differences were found between separated (n=20) and control (n=16) rats in the time latency to move to the centre of the arena. All values are expressed in means ± SEM.
Figure 4.4: In the open field test, no significant differences were found in maternally separated rats with respect to the number of blocks that were crossed during the experiment. Values are expressed as means ± SEM.
Figure 4.5: Maternally separated (n=20) rats did not differ significantly from controls (n=16) in the amount of time spent in the inner zone of the open field test. Values represented here are the means ± SEM.
Amount of time spent in the outer zone of the open field arena  
(separated vs. control)

Figure 4.6: Maternally separated (n=20) rats did not differ significantly from controls (n=16) in the amount of time spent in the outer zone of the open field test. Values represented here are the means ± SEM.
Number of crossings between inner and outer zones of the open field arena
(separated vs. control)

Figure 4.7: Maternally separated (n=20) rats did not differ significantly from controls (n=16) in the number of crossings between the inner and outer zones of the open field test. Values represented here are the means ± SEM.
Table 4.2: Rearing and freezing behaviours noted on the open field test were significantly different between separated (n=20) and control (n=16) rats (p<0.05). All values are expressed as means ± SEM.

<table>
<thead>
<tr>
<th>Behaviours on the open field arena (means ± SEM)</th>
<th>Rear*</th>
<th>Feces*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Separated</td>
<td>36.6 ± 2.0</td>
<td>0.75 ± 0.23</td>
</tr>
<tr>
<td>Control</td>
<td>46.6 ± 4.2</td>
<td>0.06 ± 0.06</td>
</tr>
</tbody>
</table>

* p < 0.05
outer zones (see figure 4.5 and 4.6) or the number of crossings between the inner and outer zones of the open field arena (see figure 4.7).

Rearing and freezing behaviours that were noted during each session, did however yield significant results (see table 4.2). Maternally separated animals displayed decreased rearing behaviours and an increase in defecation frequency, when compared to controls (p<0.05).

4.2 Neurotransmitter Levels

The independent t-test was also applied to the neurotransmitter data of maternally separated (n=32) and control (n=25) rats. No significant differences were found with respect to basal NA, 5HT, 5HIAA and MHPG levels in the frontal cortex, hypothalamus or hippocampus (see figure 4.8).

Noradrenaline levels were however, significantly decreased in the frontal cortex (see 4.10 and 4.11) (p<0.01) 15 minutes after restraint stress and immediately after restraint stress in the hypothalamus (p<0.05) (see figure 4.9 and 4.12) and hippocampus (see figure 4.9 and 4.13) (p<0.01). MHPG levels were significantly decreased in the frontal cortex (p<0.05) immediately after restraint stress (see figures 4.9 and 4.11).

No significant differences were found with respect to serotonin levels. However, significant increases were found in 5HIAA levels in the frontal cortex (p<0.05) (see figure 4.11) and hippocampus (p<0.01) (see figure 4.13) of separated rats, 15 minutes after restraint stress (see figure 4.10).
Figure 4.8: Basal monoamine concentrations in the frontal cortex, hypothalamus and hippocampus of maternally separated rats (n=22) and their controls (n=15). There were no significant differences between separated and control rats. All values are represented as means ± SEM.
Figure 4.9: Monoamine concentrations in the frontal cortex, hypothalamus and hippocampus of maternally separated rats (n=5) vs. controls (n=5) immediately after restraint stress was applied, is represented on this graph. Significant differences were found between separated and control rats with respect to MHPG in the frontal cortex (p<0.05) and noradrenaline in the hypothalamus (p<0.05) and hippocampus (p<0.01). All values are represented as means ± SEM.
Figure 4.10: Monoamine concentrations in the frontal cortex, hypothalamus and hippocampus of maternally separated rats (n=5) and controls (n=5), 15 minutes after restraint stress was applied. Significant differences were found in the frontal cortex with respect to noradrenaline (p<0.01) and 5HIAA (p<0.05). 5HIAA also differed significantly between separated and control rats in the hippocampus (p<0.01). All values are represented as means ± SEM.
Figure 4.11: In the frontal cortex, significant differences were found between maternally separated animals and controls immediately after restraint stress with respect to MHPG ($p<0.05$) levels. Significant differences were also found in 5HIAA ($p<0.05$) and NA ($p<0.01$) levels, 15 minutes after restraint stress in this area. All values are represented as means ± SEM.
Monoamine concentrations in the hypothalamus before and after restraint stress (separated vs. controls)

Figure 4.12: In the hypothalamus, only NA was significantly different between maternally separated and control animals, immediately after restraint stress ($p<0.05$). All values are represented here as means ± SEM.
Figure 4.13: Significant differences were found between maternally separated animals and control groups immediately after stress with respect to NA levels ($p<0.01$). Highly significant differences ($p<0.01$) were also found 15 minutes after restraint stress with respect to 5HIAA. All values are represented here as means ± SEM.
Table 4.3: Noradrenaline and serotonin turnover ratios expressed as means ± SEM, before restraint stress (Basal) in separated (n=22) vs. control (n=15) animals. No significant differences were found.

<table>
<thead>
<tr>
<th>Ration</th>
<th>Separated</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC</td>
<td>0.14 ± 0.05</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>Hyp</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>HC</td>
<td>0.16 ± 0.06</td>
<td>0.14 ± 0.1</td>
</tr>
</tbody>
</table>

Turnover ratios: Basal (Means ± SEM)

<table>
<thead>
<tr>
<th>Ratios</th>
<th>MHPG/NA</th>
<th>5HIAA/5HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Separated</td>
<td>1.02 ± 0.05</td>
<td>1.75 ± 0.12</td>
</tr>
<tr>
<td>Control</td>
<td>0.92 ± 0.05</td>
<td>2.05 ± 0.11</td>
</tr>
</tbody>
</table>
Table 4.4: Noradrenaline and serotonin turnover expressed as means ± SEM immediately after restraint stress in separated (n=5) vs. control (n=5) animals. Highly significant differences were found with respect to serotonin turnover in the hypothalamus (p<0.01).

<table>
<thead>
<tr>
<th>Ratios</th>
<th>Turnover ratios: 0 minutes post-stress (Means ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Separated</td>
<td></td>
</tr>
<tr>
<td>FC</td>
<td>MHPG/NA</td>
</tr>
<tr>
<td></td>
<td>5HIAA/5HT</td>
</tr>
<tr>
<td>Hyp</td>
<td>MHPG/NA</td>
</tr>
<tr>
<td></td>
<td>5HIAA/5HT</td>
</tr>
<tr>
<td>HC</td>
<td>MHPG/NA</td>
</tr>
<tr>
<td></td>
<td>5HIAA/5HT</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>FC</td>
<td>MHPG/NA</td>
</tr>
<tr>
<td></td>
<td>5HIAA/5HT</td>
</tr>
<tr>
<td>Hyp</td>
<td>MHPG/NA</td>
</tr>
<tr>
<td></td>
<td>5HIAA/5HT</td>
</tr>
<tr>
<td>HC</td>
<td>MHPG/NA</td>
</tr>
<tr>
<td></td>
<td>5HIAA/5HT</td>
</tr>
</tbody>
</table>

** p < 0.01

Table 4.5: Noradrenaline and serotonin turnover ratios in separated (n=5) vs. control (n=5) animals, 15 minutes after restraint stress, expressed as means ± SEM. Significant differences were noted in the frontal cortex (p<0.01) and hypothalamus (p<0.05) with respect to serotonin turnover.

<table>
<thead>
<tr>
<th>Ratios</th>
<th>Turnover ratios: 15 minutes post-stress (Means ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Separated</td>
<td></td>
</tr>
<tr>
<td>FC</td>
<td>MHPG/NA</td>
</tr>
<tr>
<td></td>
<td>5HIAA/5HT</td>
</tr>
<tr>
<td>Hyp</td>
<td>MHPG/NA</td>
</tr>
<tr>
<td></td>
<td>5HIAA/5HT</td>
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<tr>
<td>HC</td>
<td>MHPG/NA</td>
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<tr>
<td></td>
<td>5HIAA/5HT</td>
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<tr>
<td>Control</td>
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<tr>
<td>FC</td>
<td>MHPG/NA</td>
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<td></td>
<td>5HIAA/5HT</td>
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<tr>
<td>Hyp</td>
<td>MHPG/NA</td>
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<td></td>
<td>5HIAA/5HT</td>
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<tr>
<td>HC</td>
<td>MHPG/NA</td>
</tr>
<tr>
<td></td>
<td>5HIAA/5HT</td>
</tr>
</tbody>
</table>

* p < 0.05
** p < 0.01
The turnover ratios of serotonin (5HIAA/5HT) and noradrenaline (MHPG/NA) did not yield significant results when the independent t-test was applied to the basal neurotransmitter levels (n=37) data (see table 4.3). However, immediately after restraint stress, a significant increase was found in serotonin turnover in the hypothalamus of separated rats when compared to controls (p<0.01) (see table 4.4). This turnover rate was also increased in separated rats, 15 minutes after restraint stress in the frontal cortex (p<0.01) and hypothalamus (p<0.05) (see table 4.5).

4.3 ACTH Determinations

When the independent t-test was applied to the ACTH data of separated (n=20) and control (n=20) rats, a significant increase in basal ACTH concentration in separated rats (p<0.05) was obtained. Markedly decreased ACTH concentrations was found in maternally separated rats 15 minutes after restraint stress (p<0.05) (see figure 4.14). Interestingly, at the 60 minute post restraint stress time-interval, no significant differences were observed between the ACTH levels of separated and control rats.
ACTH Concentrations before and after restraint stress:
(separated vs. control)

Figure 4.14: Significant differences were found between maternally separated (n=20) and control (n=20) rats in basal as well as 15 minutes post-restraint stress ACTH levels (p<0.05). All values are represented here as means ±SEM.
5.1 Behavioural Studies

5.1.1 Elevated plus-maze

The elevated plus-maze is extensively employed in studies using rodent models of anxiety. It is therefore also useful to test the psychological and the neurochemical effects of anxiolytic and anxiogenic agents (Dawson *et al.*, 1995). The maze has been validated and used as a measure of anxiety by various authors (Pellow *et al.* 1985; Rodgers *et al.* 1997). It complies with ethical standards because it uses stimuli that lead to anxiety in the natural environment of the animals. For example, Pellow *et al.* (1985) validated the maze behaviourally, pharmacologically and physiologically. She showed that neither novelty, nor illumination altered the rats’ behaviour on the maze and concludes that their aversion to heights and open spaces was a critical determinant for the behaviours elicited on the maze. The pharmacological studies illustrated that clinically effective anxiolytics increased the exploration of the open arms, while a decrease in exploration of these arms was noted with anxiogenics.

Adverse rearing conditions, such as maternal separation, often manifest in behavioural abnormalities. It is therefore not surprising that our results reflect these findings. Our behavioural results indicate that maternally separated animals were indeed more anxious than their controls. A decrease in the number of entries into the various arms
of the maze (see figure 4.1) and therefore a decrease in locomotor activity were noted in separated rats. An increase in the amount of time spent in the closed arms of the maze (see figure 4.2), as well as the increase in defecation frequency (see table 4.1) in separated rats illustrates anxious behaviour. We cannot explain the increased rearing behaviour seen in the separated animals, as rearing is usually seen as relaxed behaviour. Perhaps it reflects that the degree of anxiety is not severe enough to affect all parameters significantly.

Our results, however, are in concordance with studies following a similar separation paradigm (Ladd et al., 2000), where maternally separated rats for instance, spent less time in the open arms of the maze demonstrating increased anxious behaviour.

5.1.2 Open Field Test

The Open Field test is another behavioural method designed to measure anxiety in rats. The maze has been shown to be effective in evaluating the behaviours of rats when injected with anxiolytic compounds (Crawley, 1985).

Our results indicate that there were limited differences in maternally separated animals with respect to controls. These results therefore do not fully support our elevated plus-maze behavioural data. The behaviours displayed while on the open field arena only agreed with those on the elevated plus-maze, with respect to the increase in defecation frequency (see table 4.2). Decreased rearing behaviour was also noted. Both are representative of anxious behaviours.
We initially employed both mazes to consolidate our behavioural findings. Perhaps the innate differences in the protocols of the two tests contributed to their inconsistency. The rats were allowed 5 minutes on the elevated plus-maze and 10 minutes on the open field test. Five minutes was chosen for the elevated plus-maze, as avoidance behaviour decreased and fatigue increased after this time period (Pellow et al., 1985). Perhaps then, 10 minutes was too long a time to spend on the open field test and the extra time masked the actual effects of our separation paradigm.

Other studies have also indicated discrepancies between the results of the elevated plus-maze and the open field test (Schmitt & Hiemke, 1998; Paterson et al., 2001). One study evaluated the effects of benzodiazipine, a known anxiolytic agent, in rats and found that it increased the amount of time spent in the open arms, as well as the number of entries, but failed to have an effect on the open field test (Osborn et al., 1998). Chaouloff et al. (1994) suggested that the elevated plus-maze was a more adequate tool to measure anxiety, while the open field test was more suited to measure locomotor activity. We therefore suggest that this protocol be applied in future studies.

These results indicate the impact of rearing conditions on subsequent adult behaviour. It is known that depletion of noradrenaline and serotonin levels are associated with anxiety disorders (van Praag et al., 1990). Studies by Rodgers et al. (1997) showed a clear relationship between serotonin and noradrenaline re-uptake inhibitors and subsequent anxiolytic behaviours displayed by rats on the elevated plus-maze. These neurotransmitter levels were therefore investigated to further understand the effects of maternal separation on adult behaviour.
5.2 Neurotransmitter Levels

Some of the neurochemical abnormalities associated with anxiety disorders include aberrations within the noradrenergic and serotonergic systems. It has been suggested that early adverse experiences may permanently alter these neurotransmitter systems (Rosenblum et al., 1994).

There is a strong link between chronic stress and psychopathology (Review by Rudolph et al., 2000; Melia and Duman, 1991). In humans, it has been found that noradrenaline (NA) and adrenaline increased after a stress response (Gerra et al., 2001). It has also been suggested that depression is caused by a decrease in noradrenaline resulting from the prolonged stimulation of NA release during chronic stress, thereby depleting the system (Review by Anand et al., 2000).

The serotonergic-(5HT)-system has strongly been implicated in the neurochemistry of anxiety disorders (Reviews by Boyer, 2000; Jetty et al., 2001). It is very important in controlling mood (Ramboz et al., 1998), and therefore malfunctioning of this system could lead to the development of affective disorders. Genetic deletions of serotonin receptors have increased the vulnerability of knock-out mice to anxiety disorders, aggression, and drug abuse (Gingrich & Hen, 2001).

To investigate the neurochemistry of maternally separated rats, we measured NA, 5HT and their metabolites, MHPG and 5HIAA respectively in the frontal cortex, hypothalamus and hippocampus.
The results showed that our paradigm did not affect the basal monoamine concentrations (see figure 4.8). We also investigated their response to a subsequent stress and found that immediately after stress (see figure 4.9), NA was decreased in the hypothalamus and hippocampus, while MHPG was significantly lower in the frontal cortex (see figure 4.11). Fifteen minutes after a subsequent stress (see figure 4.10), NA levels decreased in the frontal cortex, while 5HIAA increased in the frontal cortex and the hippocampus. If one wants to investigate the results over a time scale in the various brain areas, we see that NA content was significantly decreased immediately after the subsequent restraint stress in the hypothalamus and the hippocampus (see figures 4.12 and 4.13). NA levels were also significantly lower, 15 minutes after restraint stress in the frontal cortex (see figure 4.11). Noradrenaline’s metabolite, MHPG, was also significantly lower immediately after stress in the frontal cortex (see figure 4.11).

The fact that there was no significant differences in baseline neurotransmitter levels, indicate that maternal separation did not have an effect on the systems. Alternatively, it may reflect neurotransmitter levels after compensation has occurred following the initial insult during childhood and therefore no significant differences were noted later in life. However, aberrations in the stress response manifested in the neurotransmitter systems. Our results show that the NA system was actually compromised and therefore the compensation mechanism previously mentioned was inadequate in a stress situation. The hippocampus and hypothalamus was seen to be more severely affected, as a quick depletion of NA tissue content was noted in these areas. The frontal cortex NA systems were only affected 15 minutes after the stress was removed and therefore was slightly less affected than the aforementioned brain areas.
It has previously been suggested that depression is caused by a decrease in noradrenaline resulting from the prolonged stimulation of NA release during chronic stress, thereby depleting the system (Review by Anand et al., 2000). A study on rats conducted by Stone and Quatermain (1999), concluded that impaired brain alpha-1 noradrenergic neurotransmission contributed to the symptoms manifested in depression. A conclusion can therefore be drawn that if this noradrenaline scenario is seen in depressed patients, it could also manifest similarly in other psychopathologies originating from chronic stress, such as anxiety disorders. Perhaps then, our data substantiates this conclusion, in that a decrease in NA tissue content, as well as behavioural abnormalities characteristic of anxiety disorders, were noted.

Serotonin has been described as the homeostatic setpoint for neurotransmitters in the brain, after a stressor occurred (Review by Petty et al., 1996). This theory described serotonin as equilibrating the common neurotransmitters involved in mental illness, such as noradrenaline, back to its normal behavioural chemistry after a disruption (see figure 5.1).

In our experiments, serotonin’s metabolite, 5HIAA, was significantly increased in maternally separated rats, 15 minutes after the subsequent stress in the frontal cortex and the hippocampus (see figure 4.11 and 4.13). The serotonergic turnover ratios immediately after stress were significantly increased in the hypothalamus (see table 4.4). Fifteen minutes later, the turnover rate was increased in separated rats with respect to controls in the frontal cortex and hypothalamus (see table 4.5).
Figure 5.1: An illustration depicting the serotonergic homeostatic setpoint theory. Adapted from Petty et al. (1996).
Increased amounts of serotonin released from the dorsal hippocampus of rats after a stressor, have been previously reported (Matsuo et al., 1996). Shimizu et al. (1992) also found that immobilisation stress increased both extracellular serotonin and 5HIAA secretion in the hypothalamus of rats. Kawahara et al. (1993) made a similar discovery, illustrating increased extracellular serotonin levels in the prefrontal cortex and amygdaloid nuclei of the rat, which had suffered a psychological stress.

Our results therefore tie in with these findings, as increased amounts of 5HIAA was found in the frontal cortex and hippocampus, as well as increased serotonin turnover rate in the frontal cortex and hypothalamus after stress. These increases in serotonin turnover and 5HIAA, corresponds with the decrease seen in NA directly and 15 minutes after restraint stress. We therefore suggest that this mechanism helps to restore the low NA levels in maternally separated rats to normal levels. As NA levels decreased more in separated rats than controls, significantly more serotonin was catabolised to counteract this effect (see figure 5.1). Therefore, the increased amount of serotonin turnover in the separated rats needed to compensate for the decrease in NA levels further illustrates the complex interaction between these 2 neurotransmitter systems.

5.3 ACTH Determinations

Increased CRF production has been found in the paraventricular nucleus after an organism was exposed to stressful stimuli (Suda et al., 1988). Chronic infusion of CRF, led to increased levels of ACTH secretion (Hotta et al., 1991), which could be
due to the oversensitisation and therefore hyperactivity of the CRF receptors on the pituitary gland.

In order to determine the efficacy of the HPA axis in separated animals in response to a subsequent stress, these animals and their controls, were subjected to 10 minutes of restraint stress and ACTH concentrations were measured 15 and 60 minutes after this stress.

Our results show a blunted ACTH response to a stressor in maternally separated rats (see figure 4.14). Since baseline levels were increased in these rats, it suggested either increased CRF release and/or impairment in the feedback inhibition of the HPA axis. We would like to propose that our maternal separation paradigm acted as a chronic stressor, leading to prolonged increase in the release of CRF. This could cause CRF receptors to be downregulated at the level of the anterior pituitary, and therefore the blunted response to a subsequent stress. This desensitivitiy at the pituitary level could furthermore impact negatively on the feedback inhibitory mechanisms by ACTH or even corticosterone, and hence the observed elevated baseline levels (see figure 5.2).

Another suggestion is that the blunted ACTH response to CRF as seen in PTSD, can stem from a hyper-responsive pituitary gland to cortisol (Review by Yehuda et al. 1991). The mechanism involved an increase in the concentration of glucocorticoid receptors on the pituitary gland causing a stronger negative feedback inhibition (Nutt et al., 2000). Therefore the HPA axis under these circumstances was overly inhibited, resulting in the blunted ACTH responses (see figure 5.3).
Figure 5.2: Our theory of the blunted ACTH response in maternally separated rats after a subsequent stressor: receptor downregulation.
Figure 5.3: Another theory depicting the blunted ACTH response in maternally separated rats due to a hyperresponsive pituitary gland. Adapted from Nutt et al. (2000).
We would like to suggest that elevated CRF levels could be causally related to the increased negative feedback and therefore blunted ACTH response, exhibited by patients with anxiety disorders such as PTSD. We further propose that adverse development factors, such as maternal separation, predisposes individuals to develop psychopathologies later in life and functionally alters the neurotransmitter systems involved in the development of these psychopathologies.
CORTICOTROPIN-RELEASING FACTOR

STUDY
CHAPTER 6

6.1 Introduction

Many patients suffering from anxiety disorders often present with a dysregulated HPA axis as evidenced by either a hypercortisolism, hypocortisolism and/or a blunted ACTH response to stress (Roy-Byrne et al., 1986; Review by Yehuda, 1997). CRF is primarily responsible for ACTH release from the anterior pituitary. Therefore prolonged elevated CRF levels could be causally related to the neuroendocrine abnormalities associated with anxiety disorders. We proposed that the blunted response seen in our maternally separated animals was also due to elevated CRF levels and could therefore be related to the anxiogenic behaviours elicited by these animals. Since CRF projections from the paraventricular nucleus to other brain areas have already been established (Chappell et al., 1986; Van Bockstaele et al., 1996), it would appear that CRF is able to influence the function and activity-of these neurons. We decided to investigate the effects of increased central CRF levels, by injecting CRF intracerebroventricularly into the brains of normal rats and subsequently determining their behaviours.

METHODS

Male Sprague-Dawley rats weighing between 250g to 300g was used in these experiments. Prior to injections, the animals were housed individually for handling purposes.
6.2 Stereotaxis

Stereotaxic techniques were used to implant indwelling cannulas into the ventricles of rats (n=15). The co-ordinates (Anterior-posterior: -1.2mm from interaural line, Medial-lateral: -1.8mm from mid-suture, Dorsal-Ventral: -3.4mm (from the surface of the skull)) were followed from the atlas of Paxinos and Watson (1986). The interaural line as well as lambda and bregma were noted for each individual rat. The zero line was taken as the inter-aural line and the incisor bar was adjusted accordingly.

In order to inject the CRF solution into the lateral ventricle, the stereotaxis apparatus was employed to position a cannula into the desired area (see figure 6.1). The rats were anesthetised with equithesin (1.5 - 2 ml/300g body weight) (Appendix C), thereafter the hair on the upper part of the head was shaved and the area sterilised with a 70% alcohol solution. An incision of approximately 20mm long was made above the midline. The subcutaneous tissue was removed in order to expose the cranium.

An electric dental drill was employed to drill 2 holes of approximately 1mm in order to insert 2 screws. These served to anchor and secure the cranioplastic cement to the skull. A hole above the left ventricle, as determined by the previously mentioned co-ordinates, was drilled to expose the dura of the brain. The dura was then pierced with a needle, after the excess bone was carefully removed with forceps. The guide cannula was carefully inserted according to the predetermined depth. Surgicell was placed around the cannula to protect the brain from toxins. Cranioplastic cement was now applied in order to secure the cannula in its position. A dummy cannula was implanted in the guide cannula to maintain its viability over the experimental period.
Figure 6.1: Stereotaxic apparatus

Figure 6.2: Injection procedure of CRF into the ventricles.
After allowing a 3-day recovery period, 3μg of CRF (Sigma) dissolved in 1μl of physiological saline was administered-intracerebroventricularly into the left lateral ventricle (n=5). A 10μl Hamilton syringe was used to inject the CRF solution over a 1 minute period while the animals were moving freely in their cages (see figure 6.2). The animals were injected daily in the morning between 9:00 and 13:00, over a 5-day period.

These animals were compared to saline-injected animals (n=5) and naïve (n=6) controls. Saline-injected rats received the same treatment as the experimental rats. This was done to eliminate the possibility that the results obtained were due to the experimental procedure and not due to the substance injected. Naïve rats were housed individually and handled for 5 days. They were not subjected to injections or placement of cannulas.

6.3 Histology

Histology was performed on each rat to determine whether the cannula was placed correctly. Only the results of those with correct cannula placement were taken into consideration (see figure 6.3). The Cresyl Fast Violet colouring method was employed to determine whether our co-ordinates for the left lateral ventricle were accurate. Brain slices were frozen to −20°C using the Leica CM 1850 cryostat machine. Slices of 20 microns thick were then cut with this machine by moving the knife angled at 30° with respect to the tissue surface. The sections were collected directly onto the slide and placed in Xylool for 5minutes. Placing them for 1 minute in 100%, 96% and then into 70% alcohol hydrated the
Figure 6.3: A histological photo illustrating the tract of the cannula placed into the lateral ventricle.
slices. They were then differentiated and stained by placing them in Filtered Cresyl Violet for 10 minutes. Placing them in 70%, 96% and then 100% alcohol dehydrated the sections. The slides were finally mounted using D.P.X. and evaluated under a light microscope (Leitz).

6.4 Behavioural Studies

The behaviours of the CRF-injected rats (n=5) versus saline-injected (n=4) and naïve (n=6) rats were evaluated on the elevated plus-maze, in the afternoon following the last injection, as described in 2.3.1 above. We chose to only use this maze, as it rendered the best results in the separation study.

6.5 ACTH Determinations

The efficacy of the HPA axis response to stress of CRF injected rats (n=5) versus saline controls (n=5) and naïve rats (n=5) was determined by making use of the restraint stress paradigm described in 2.4 above. All trunk blood samples were collected in the morning following the behavioural tests. The previous results in the separation study (see chapter 4) indicated a dramatic blunted ACTH response after 15 minutes post-restraint stress. We decided therefore to only determine the ACTH levels (section 2.5) at this time point.
CHAPTER 7

RESULTS

7.1 Behavioural Studies

7.1.1 Elevated Plus-maze

As the number of animals used in these experiments was relatively small and the data was not normally distributed, it was considered to be appropriate to make use of a non-parametric test. The Kruskal-Wallis analysis of variance was applied to the behavioural data between CRF-injected (n=5), saline-injected (n=4) and naïve (n=6) rats. Significant differences were found between the groups with respect to the number of entries into the closed arms of the elevated plus-maze (p<0.05) (see figure 7.1). A Mann-Whitney U test yielded a significant decrease in the number of entries into the closed arms in CRF-injected rats when compared to naïve controls (p<0.05). A Kruskal-Wallis ANOVA test showed no significant differences between the groups with respect to the time spent in the arms of the elevated plus-maze (see figure 7.2). No significant differences were found between the groups with respect to the other behaviours noted during each session (see table 7.1).

7.2 ACTH Determinations

The Kruskal-Wallis analysis of variance test did not yield significant results between CRF-injected (n=5), saline-injected (n=5) and naïve (n=5) rats. However, when a
Number of entries in the Open and Closed arms of the elevated plus-maze in naïve, saline-injected and CRF-injected rats

<table>
<thead>
<tr>
<th></th>
<th>Open</th>
<th>Closed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>Saline</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>CRF</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure 7.1: No significant differences were found between CRF-injected rats, saline-injected rats and naïve rats with respect to the number of entries into the open arms of the elevated plus-maze. However, significant differences were found between naïve (n=6) and CRF-injected (n=5) rats with respect to the number of entries into the closed arms (p<0.05). All values represented here as medians ± SEM.
Amount of time spent in the various arms of the elevated plus-maze in naive, saline-injected and CRF-injected rats

Figure 7.2: No significant differences were found between CRF-injected rats, saline-injected rats and naïve rats with respect to the amount of time spent in the open or closed arms of the elevated plus-maze. All values represented here as medians ± SEM.
Table 7.1: No significant differences were found in the 4 behaviours noted between CRF-injected (n=5), saline-injected (n=4) and naïve (n=6) rats. Results shown are the medians ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Feces</th>
<th>Grooming</th>
<th>Rearing</th>
<th>Freezing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>0 ± 0</td>
<td>0 ± 0.08</td>
<td>11 ± 0.72</td>
<td>2 ± 0.6</td>
</tr>
<tr>
<td>Saline</td>
<td>0 ± 0.25</td>
<td>0.5 ± 0.38</td>
<td>7 ± 2</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>CRF</td>
<td>0 ± 0</td>
<td>0 ± 0.12</td>
<td>5.5 ± 2.5</td>
<td>0.5 ± 0.65</td>
</tr>
</tbody>
</table>
Mann-Whitney U test was applied to the data, a decrease in ACTH concentrations was found in CRF-injected rats when compared to naïve controls (p<0.05) (see figure 7.3).
Figure 7.3: No significant differences were found in ACTH levels 15 minutes after restraint stress between CRF-injected (n=5) and saline-injected (n=5) rats. Significant differences were however found between CRF-injected and naïve rats (n=5; p<0.05). All values are represented here as medians ± SEM.
CHAPTER 8

DISCUSSION

8.1 Behavioural Studies

8.1.1 Elevated plus-maze

CRF has been described as the central coordinator of the endocrinological, autonomic, immunologic and behavioural stress responses (Review by Heim & Nemeroff, 1999; Habib et al., 2000). It is the main regulator of ACTH release during stress.

In previous studies, when CRF was administered intracerebroventricularly, it induced behavioural and other abnormalities typical of a stress response, such as increased motor activation (Britton et al., 1986; Song et al., 1995) and a reduction in exploratory behaviour in rats in response to a stressor (Koob et al., 1993). CRF antagonists reversed these behavioural effects, suggesting that CRF is involved in these stress-mediated abnormal behavioural responses. A review of clinical studies has shown that CRF may be the main neuro-hormone mediating the effects of early life stress on subsequent pathology (Review by Heim et al., 1997).

In studies by Britton et al. (1982) in which the authors reported CRF to elicit anxious behaviours, they administered acute injections of CRF ranging from 1.5 to 150 pmol. At 150 pmol, there was a significant decrease in rearing and food approach behaviours, and an increase in grooming frequency. On the other hand, Song et al.
(1995) administered CRF intracerebroventricularly with concentrations ranging from 0.1 to 1 μg over 5 days and found this regime to cause increased locomotor and anxiogenic behaviours on the open field and elevated plus-maze tests. In our case, we injected 3 μg CRF into the ventricles of adult Sprague-Dawley rats for 5 days and found a decrease in the number of entries into the closed arms of the maze (see figure 7.1), indicating a decrease in locomotor activity. Our results are therefore in contrast to the findings of others. The differences in outcomes may perhaps be the result of differences in methodological approaches.

It may be that our injection paradigm, although chronic, was more representative of an episodic regime. The 24-hour period between the last injection and behavioural tests could also have been too long. Perhaps if CRF was administered throughout the day and the behaviours evaluated immediately after the last injection, it would be more representative of a chronic stress and would therefore have elicited more significant results. The dilution factor i.e. 1 μl CRF solution into a larger ventricular volume, could also have contributed and diminished the effects of CRF.

8.2 ACTH Determinations

While we observed little behavioural abnormalities following the CRF administration, we did however find a tendency for a blunted ACTH response 15 minutes after restraint stress (see figure 7.3). It was felt that if the respective experimental groups were to increase, the difference between the saline-treated and CRF-injected rats would become significant. The result however, was interesting as it suggested that it is a more sensitive parameter to elevated CRF in the brain than the neurotransmitter
systems present. This may possibly explain the lack of any significant behavioural abnormalities in CRF-treated animals. Perhaps, a more aggressive treatment regime is needed to elicit any behavioural abnormalities. These experiments therefore suggest that if behavioural abnormalities are noted, then the insult or neurochemical aberration causing the abnormalities, must be of a certain magnitude. It furthermore suggests that the disturbance resides mostly in the central nervous system. It is for this reason why pharmaceutical manipulation of the neurotransmitter systems of the brain is beneficial for certain anxiety disorders. However, since not all outcomes are favourable following drug therapy, it illustrates the complexity of behavioural manifestations seen in mental illnesses as it represents a conglomerate of many brain areas and neurotransmitter systems.
CHAPTER 9

CONCLUSION

The present study was undertaken to investigate the effects of early adverse conditions on the behaviour of adults in later life. In order to execute this investigation, rat pups were subjected to maternal separation and their behaviours examined at adulthood. Using the elevated plus-maze and open field tests, we found maternal separation to elicit behavioural abnormalities, representative of anxiety disorders. We can therefore conclude that early adverse experiences can lead to anxious behaviours.

Although there was no significant differences in the basal levels of noradrenaline, serotonin and their metabolites of maternally separated animals compared to controls, alterations in noradrenergic and serotonergic neurotransmitter systems were however noted when the rats were exposed to a subsequent stressor. These findings therefore suggested that the rat compensated for the initial disturbances in the central nervous system caused by maternal separation. This compensation was however insufficient to enable a normal stress response.

Upon evaluating the effect of maternal separation on a neuroendocrinological parameter, we found that not only was the basal ACTH levels higher, but also that the ACTH levels were blunted in response to a subsequent stress. These results suggest that either the CRF levels of maternally separated rats were chronically elevated leading to the desensitisation of the HPA axis at the level of the anterior pituitary, or that the HPA axis was overly inhibited by chronically high levels of circulating
corticosterone. It therefore appeared that CRF could indeed possibly play a role in the abnormalities seen in maternally separated rats.

In order to investigate further this possibility, we evaluated the effect of centrally administered CRF on the behavioural and stress response of normal rats. Treating freely moving rats for 5 days with CRF (3 μg / μl) hardly produced any significant behavioural abnormalities. However, a blunted ACTH response was noted 15 minutes after restraint stress in CRF-injected rats. These observations suggest that increasing the levels of CRF in the brain was inadequate to induce behavioural abnormalities, and yet it appeared to desensitise the HPA axis response to a stressor.

Our experiments led us to conclude that early adverse experiences, such as maternal separation, could lead to the development of abnormal behaviours representative of anxiety disorders, later in life. CRF, however, is not pivotal in the development of these abnormalities; rather it seems that the neurochemical abnormalities (serotonin and noradrenaline) play a more important role. Since CRF release is also under the control of noradrenaline and serotonin, it may facilitate the effects of these neurotransmitters on the HPA axis (see figure 9.1).

Our behavioural data noted on the elevated plus-maze and the open field test was inconsistent. Future studies should rather employ the elevated plus-maze as a measure of anxious behaviour, and the open field test as a measure of the locomotor activity of the animals. I would also suggest that one reduce the time period from 10 to 5 minutes on the open field test, to avoid fatigue becoming a confounding variable.
Figure 9.1: Our hypothesis of the effects of maternal separation on neurotransmitters, behaviour and the stress response.
With regards to the CRF study, future investigations should employ a more vigorous treatment regime with multiple CRF injections throughout the day. The lack of significant differences between the naïve and saline-injected groups can be partially attributed to the variation in ACTH levels obtained in the naïve group. Increasing the number of rats in each group would decrease this variation and a more accurate reflection could be generated.

Finally our data suggests that combination drug therapy that targets both the noradrenergic and serotonergic neurotransmitter systems should perhaps be considered above those aimed at rectifying the individual neurotransmitter systems in the treatment of psychopathologies, such as anxiety disorders.
CHAPTER 10

REFERENCES


58. M Hotta, T Shibasaki, N Yamauchi et al. The effects of chronic central administration of corticotropin-releasing factor on food intake, body weight, and


111. U Schmitt, C Hiemke. Combination of open field and elevated plus-maze: a suitable test battery to assess strain as well as treatment differences in rat behaviour. Progress in Neuropsychopharmacology and Biological Psychiatry 1998 22(7): 1197-1215.


Appendix A

Companies:

AFROX: South Africa

BAS: Bioanalytical Systems Inc. West La Fayette, Indiana, USA

BDH: England

Hamilton: Reno, Nevada, USA

MERCK: Darmstadt, Germany

SIGMA: St Louis, Missouri, USA

Chemicals and reagents used:

AFROX: Helium

Liquid Nitrogen

BAS: Acidified Aluminum Oxide

Polishing Alumina

BDH: 1-Heptane Sulphonic Acid, Sodium salt

MERCK: Sodium dihydrogen Phosphate

Methanol, HPLC grade

Phosphoric Acid

Glacial Acetic Acid

Potassium Acetate
Sodium Metabisulphate, dry GR
Perchloric Acid
Sodium Chloride

SIGMA: Ethylenediaminetetraacetic Acid
Ascorbate Oxidase
Noradrenaline free base
Serotonin, Creatine Sulphate complex
5-Hydroxyindoleamineacetic acid
4-Hydroxy-3-methoxy-phenylglycol, Hemipiperazine salt
3,4-Dihydroxy-benzylamine, Hydrobromide
Tris (Hydroxymethyl) aminomethane
Corticotropin Releasing-factor
Homovanillic acid

Other:
ACTH radioactive kit, Nichols Institute Diagnostics, California, USA
Ammonium Acetate
Equithesin (see appendix C)
Cresyl Fast Violet
Xylol
DPX
Appendix B

Equipment Used:

ACTH Determinations

LKB Wallac 1261 Multigamma radioactive counter

Histology

Leica CM 1850 cryostat machine
Leitz laborlux K light microscope
Cranioplastic cement
Surgicell

HPLC

BAS: Liquid Chromatograph with Varian 4270 Integrator
0.2 micron filters
Plastic Collection tubes
Glass conical vials
Microfiltration Centrifuge

Phenomenex: Luna 5 micron C18, 250x4.6mm silica column
Security Guard, guard column
Other: Sartorius Electronic balance scale
Millipore filter system with 0.22 micron filters
Beckman 34 pH meter
Polished Water, Milli-Q reagent water system
Magnetic Stirrer, Chemlab SS3, England
Sorvall RC-5B Refrigerated superspeed centrifuge
MSE sonicator
Heidolph homogeniser
Hamilton 100 µl syringe

*Stereotaxis*

Cannulas were purchased from Plastics one, inc., Virginia, USA.
Silastic brand silicone tubing were purchase from Hospital Products (S.A.).
David Kopf stereotaxic apparatus was used for all stereotaxic work.
10 µl Hamilton syringe

Electric Dental Drill
Appendix C

Equithesin (Anaesthetic agent):

1. 81ml Sodium Pentobarbital (60mg/ml) 39mM
2. 21g Chloral Hydrate 0.25M
3. 10.6g MgSO₄ 0.086M
4. 198ml Propylene Glycol 39.6%
5. 50ml Absolute alcohol 10%

The above reagents were combined in a beaker and stirred until all components were dissolved. The resulting cocktail was made up to 500ml with distilled water. The equithesin was stored in a dark area to prevent auto-oxidation.