Prelude

Amy, now 33, tells her side of the story. The sexual abuse started when she was only 7 years old. She was staying at her stepfather's house. The abuse started by her stepfather exposing himself to her and him wanting her to be silent and not to tell a soul. She did feel strange and somehow knew that what was happening was out of place. The abuse gradually escalated into more serious sexual acts and continued for several months. Since being molested, Amy had images of a sexual nature in her mind and started acting out sexual behaviours at school. Her school progress gradually declined and she started showing signs of low self-esteem and depression. She also suffered from nightmares, enuresis, headaches, stomach aches and vaginal infections.

CHAPTER 1: INTRODUCTION

The majority of individuals experience some or other traumatic event in their lifetime, but only a subset of these individuals develops psychiatric disorders. In the United States 60 % of men and 50 % of women experience a traumatic event in their lifetime, but only 8 % of males and 20 % of females develop post traumatic stress disorder (PTSD) (Kessler et al., 1995). Nearly half of PTSD sufferers have major depression or other anxiety disorders (Schnurr et al., 1997).

These statistics, as well as the story of Amy and so many other cases of early life adverse events, in addition to the limited efficacy of current treatments, are reason enough to try and unravel the molecular mechanisms involved in trauma and stress.

1.1 What is stress?

In order to define stress, a variety of concepts need to be considered, in formulating a clear definition. Two useful categories, namely physiological stress and psychological stress do however form part of the definition of stress. Physiological stress can be defined as any non-specific response of the body to any demand made upon it, according to Hans Selye (1956). The stress response includes three stages, an alerting response, resistance response and an exhaustion response. This Selye termed the general adaptation syndrome. The adrenal hormones play a central role in mediating the responses to stress. Psychological stress is even more complicated to define. According to McGrath (1970), psychological stress can be defined as an imbalance between what an individual perceives the environmental demand to be and the measure of capabilities to respond to the environmental demand. If an imbalance is perceived, it is only stressful to the individual if coping with the situation is important to the individual. Kaplan (1983) defined psychosocial stress as an individual's inability to always perceive a situation as inauspicious. Hobfoll (1988), combined McGrath's and Kaplan's definitions

to formulate a more comprehensive definition for stress: ”*Stress is the state in which individuals judge their response capabilities as unable to meet the threat to the loss of desirable experiental states - states that are dictated by their values and expectations.”* More recently stress has been conceptualised as being elicited by numerous stimuli, including environmental, social or pathological conditions that occur during an individual’s life span and elicit changes in the nervous, endocrine and immune systems (Aloe et al., 2002).

1.2 ‘Two-hit’ model / nature or nurture?

The ‘two-hit’ model refers to two factors, genetic and environmental, that determine how an individual reacts toward social and environmental stimuli. This response determines how adult behaviour is shaped, hence the importance of early life experiences in determining adult behaviour. Research has confirmed the role early life experiences play, especially maternal care, in the development of neurons, the endocrine systems and behaviour (Cirulli et al., 1992; Suchecki et al., 1993). Primate studies have shown that rearing infant monkeys in different social situations, from partial isolation to maternal separation and with no foster care, produce a variety of behavioural abnormalities ranging from depression to advanced psychosis (Harlow et al., 1959).

The importance of environmental stimuli comes to the foreground in the influence on social behaviour. Rats exposed to enriched environments show enhanced memory function (Frick et al., 2003), indications of increased synaptogenesis (Nithianantharajah et al., 2004), and neurogenesis in the brain (Altman et al., 1965). Enrichment also up-regulates the transcription of genes encoding for proteins important for plasticity, learning and memory (Rampon et al., 2000). Also, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) protein levels are elevated in rat hippocampus tissue following enrichment (Mohammed et al., 1990; Falkenberg et al., 1992; Pham et al., 1999).

It is important to emphasize that there is a relationship between genes and the environment in that the influence of the environment on an individual depends on the genotype of the individual. Alternatively the differential effects of genotype can be explained by different environmental inputs (McClearn et al., 2004). Research by Kendler et al. (1995), illustrates how genes and stressful life events work together in the development of depression in twins. Genetic risk for depression in one of a monozygotic twin pair is the highest if the twin partner has depression. The importance of both the genetic influences and the environmental influences on behaviour is therefore evident from these experiments.

1.3 Stress-related disorders

Affective disorders are disorders that display pathological mood and affect, such as the mood and anxiety disorders. Patients suffering from anxiety disorders, as the name indicates, experience anxiety or fear in some or other form in reaction to an event/experience. This event or experience can be perceived as an internal stressor or can be a real threat (external). Anxiety disorders are of the most common psychiatric disorders and include panic disorder, social phobias, obsessive-compulsive disorder, post traumatic stress disorder and generalized anxiety disorder. The aetiology of PTSD is of relevance to this particular study. PTSD is defined by the fourth edition of *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV) as a disorder that develops after an individual has witnessed or was involved in a life-threatening traumatic event. These events can include natural disasters, war, intrusive events e.g. rape, or motor vehicle accidents. The clinical features of PTSD include the following:

(a) Re-experiencing the traumatic event by means of memories, nightmares, and flashbacks or by an individual feeling as though they are reliving the trauma.

(b) Extreme avoidance of any reminders of the trauma, in the form of places, people and thoughts associated with the trauma.

(c) Hyper-arousal, individual having difficulties controlling impulses resulting in aggressive outbursts, difficulty falling asleep or concentrating.

(d) Symptoms that are experienced for at least one month.

(e) Symptoms that cause impairment of day–to–day functioning whether in the workplace, socially or other areas.

The most important determinant of PTSD is the nature and severity of traumatic experience the individual has been exposed to. This, together with the character of the individual, age at time of trauma, gender and race (Brewin et al., 2000) determines whether the prognosis of the outcome is favourable or not (American Psychiatric Association, 1994).

Mood disorders are those disorders that affect mood. Pathological mood may be elevated or depressed and the disorder is characterised by individuals feeling a loss of control, resulting in extreme distress being experienced because of this loss of control. The two most important mood disorders are major depressive disorder and bipolar I disorder. With major depressive disorder just depressive episodes are experienced and with bipolar I disorder a combination of depressive and manic episodes can be experienced as well as just manic episodes. There are other depressive mood disorders that do not reach the severity to be classified as major depression. They include recurrent brief depressive episodes and depressive disorder not otherwise specified. Bipolar II disorder, dysthymic and cyclothymic disorder also form part of mood disorders all with different diagnostic criteria (American Psychiatric Association, 1994). The aetiology of mood disorders is still unclear, hence the importance of research in the underlying molecular mechanisms involved in these affective disorders. The relevance of discussing major depression together with PTSD is that 88 % of men and 79 % of women with PTSD have a co-morbid psychiatric disorder, of which nearly half of PTSD patients suffer from major depression (Kessler et al., 1995; Schnurr et al., 1997). The criteria to be met in order to be diagnosed with having a major depressive episode are as follows:

(a) Having a depressed mood most of the day, nearly every day,

(b) Anhedonia- loss of interest and pleasure in almost all activities,

(c) Weight loss or weight gain,

(d) Insomnia or hypersomnia,

(e) Restlessness,

(f) Fatigue,

(g) Feelings of worthlessness or guilt,

(h) Inability to concentrate,

(i) Suicidal ideation.

Five or more of the above mentioned symptoms have to be present in a two week period and at least one of the symptoms has to be either (a) or (b). These symptoms must cause impaired functioning in different areas, where social and occupational functioning are the most important (American Psychiatric Association, 1994).

1.4 Hypothalamic-Pituitary-Adrenal (HPA) axis

1.4.1 *General activation of HPA activity*

The hypothalamic-pituitary-adrenal (HPA) axis is activated in response to stress. This activation of endocrine hormones can be caused by psychological or a physical stress. The hormones of the HPA axis are often called the 'stress' hormones. These stress hormones are corticotropin-releasing hormone/factor (CRH/CRF), adrenocorticotrophic hormone (ACTH) and cortisol (humans and other primates) or corticosterone (rodents e.g. rats and mice). Shortly after exposure to a stressor, CRH is increased and secreted from the hypothalamus. This in turn stimulates the pituitary gland to secrete ACTH, which then acts on the adrenal cortex to cause the secretion of cortisol/corticosterone (Sapolsky et al., 2000) (see Figure 1). Maximal secretion of corticosteroids is approximately 15 to 30 min after exposure to a brief stressor and reaches baseline levels 60 min after exposure (de Kloet et al., 2004). Circulating cortisol is bound to protein called corticosteroid binding globulin (CBG) and only the unbound cortisol form binds to receptors. Unbound cortisol binds to two receptors, the first is called type I or mineralocorticoid receptors (MR) and the second receptor is called type II or glucocorticoid receptors (GR). MR has the highest affinity for cortisol and after MRs are almost completely saturated, then only does the unbound cortisol bind to the GRs (Varghese et al., 2001).

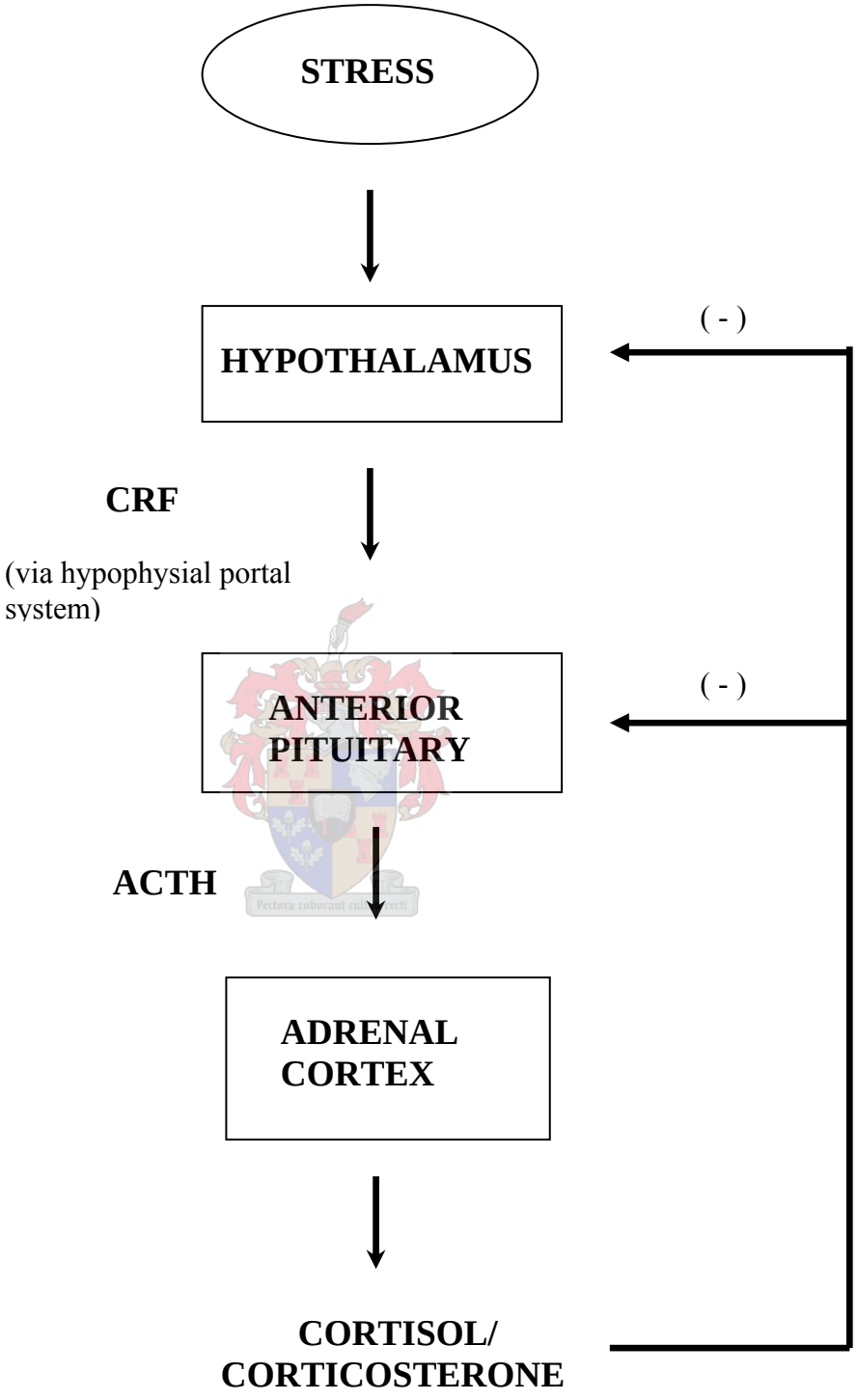


Figure 1.1 Representation of the HPA axis system.

1.4.2 *Neuronal pathways*

It appears as though different types of stressors are directed through different neural pathways in order to stimulate the release of CRH. Systemic stressors, those stressors that are a direct threat to the body, are probably channelled directly to the paraventricular nucleus (PVN) via brainstem catecholaminergic projections. Stressors that require cognitive processing and interpretation appear to be relayed through limbic forebrain structures (Herman et al., 1997). During development it is hypothesised that as different neural pathways mature, these regulate the stress response of the neonate to differential stressors (Sawchenko, 1991; Rosenfeld et al., 1992; Kovacs et al., 1996). In addition to these neuronal pathways other brain pathways have been proposed to converge at the PVN in order to stimulate the secretion of CRH (Sawchenko, 1991).

1.4.3 *Negative feedback*

It is important to note that not only under stress but also under conditions of non-stress, the HPA hormones maintain homeostasis and well being of the brain and the body (Sapolsky et al., 2000). They facilitate information processing and adaptation so that the body is prepared if the same stressor is encountered again (de Kloet, 1991; de Kloet et al., 1998; Sapolsky et al., 2000). Thus, these adaptive processes help the individual to respond to the ever changing environment (McEwen, 2000). However, when exposed to prolonged stressors for extended periods of time, an individual may develop pathological consequences (Sapolsky, 1992), since excess glucocorticoids can have damaging effects (Sapolsky et al., 1986a).

Tight regulation of corticoid secretion is achieved by negative feedback inhibition of the HPA axis. Negative feedback occurs when corticosterone / cortisol inhibit the further secretion of ACTH and CRH at the pituitary and the hypothalamus respectively. The hippocampus and the efferent pathway linking it to the PVN of the hypothalamus also play an important role in the regulation of negative feedback (Cullinan et al., 1993). The feedback inhibition occurs via multiple feedback loops – fast/rate sensitive, intermediate and slow/delayed feedback (Liberzon et al., 1997). Fast feedback occurs within seconds

to minutes during the elevation of corticosterone. The latter is dependent on the absolute concentration of cortisol and on the rate of change in concentration. This involves the interaction of receptors in the hypothalamus and hippocampus (Dallman et al., 1969; Hillhouse et al., 1976; Jones et al., 1976; Young et al., 1991). Fast feedback seems to be mediated by neuronal inputs (e.g. from hippocampus) and supra-pituitary elements of the HPA system (Liberzon et al., 1997). Intermediate feedback occurs over a period of 2-10 hours and is receptor mediated. Slow feedback occurs in states of chronically elevated corticosterone exposure (Suchecki et al., 1995). Mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) determine whether the actions of corticosterone will occur in the fast or slow mode (de Kloet et al., 1998). GR inhibits the stress response and promotes recovery, while MR regulates the sensitivity of the response to stress (de Kloet et al., 2004). The GR receptors are thought to mediate fast feedback via the hippocampus, since its down regulation impairs fast feedback (Young et al., 1990). MRs have a high affinity for endogenous glucocorticoids, whilst GR's bind more readily with synthetic glucocorticoids. When hormone levels are low, it is suggested that MRs regulate the effects of glucocorticoids; however in conditions of rising hormone levels, in response to stress or diurnal rhythm of the hormone, the MRs reach saturation and glucocorticoid activity is mediated primarily by GRs (de Kloet et al., 1998; Pariante et al., 2001).

1.4.4 *The role negative feedback plays in depression and PTSD*

Negative feedback plays a prominent role in the pathophysiology of depression and PTSD. Depression is characterised by a hyposensitive feedback inhibition (Rubin et al., 1987), hence the non-suppression of exogenous dexamethasone (DEX, a synthetic glucocorticoid) (Carroll, 1982; Yehuda et al., 1993a) and the high basal cortisol levels in patients diagnosed with major depression (Sachar, 1975). Conversely PTSD is charaterised by an enhanced negative feedback, causing hypocortisolemia and exaggerated cortisol suppression after dexamethasone administration (Yehuda et al., 1993a). It is important to keep in mind that in PTSD, high corticosteroid binding globulin has been observed, and this may also contribute to the low cortisol levels

(Kanter et al., 2001). It therefore seems that the HPA axis is differently affected in mood and anxiety disorders. This is interesting considering the high rates of co-existence.

1.4.5 *Imbalances in the glucocorticoid system lead to stress-related disorders*

The balance of the two corticoid receptors in the stress response is very important, since once an imbalance of GR/MR occurs, the individual's ability to cope with an adverse event is compromised and homeostasis is disrupted (de Kloet et al., 2003). Allostasis is the process involved in maintaining homeostasis (McEwen et al., 2002). An imbalance between activation and termination of the stress response combined with its recovery is thought to implicate ill health (de Kloet et al., 1998; de Kloet et al., 1999; Holsboer 2001).

Corticosteroids influence mood and cognitive ability and excess or deficiencies cause atrophy and bring a halt to neurogenerative processes in certain brain regions. Corticosterone has two modes of action in the brain. The MRs mediate steroid regulated behavioural responses (de Kloet et al., 1998; de Kloet et al., 1999). According to Oitzl et al. (1992) MR mediated effects are especially important in mediating responses to new experiences. They also retrieve information from this experience to prepare an appropriate behavioural response to deal with other new experiences. Hippocampal GRs promote information storage to facilitate behavioural adaptation and modulate future behavioural responses to stress (de Kloet et al., 1998; de Kloet et al., 1999; Oitzl et al., 2001). The MRs operate proactively to maintain homeostasis, whilst GRs operate reactively to promote homeostasis following stress (Oitzl et al., 1992). Once an imbalance is present, neuroendocrine dysregulation occurs which leads to impaired behavioural adaptation; which in turn may feasibly precipitate the onset of stress-related disorders (de Kloet et al., 1998).

Certain circumstances affect the balance of corticoid receptors and add to the allostatic load of homeostasis. For example 24 hour maternal deprivation causes a decrease in the expression of GR and MR in the hippocampus. Reinstating certain maternal behaviours,

such as stroking or feeding of the pups restores MR and GR mRNA levels in the the brain region (Van Oers et al., 1998b). Interestingly, the down regulation of GR expression following maternal deprivation is independent of circulating corticosterone levels (Van Oers et al., 1999). Early handling may be responsible for the increased hippocampal GR receptor density, but does not influence MR density (Levine, 2000). Other stressors e.g. time-dependent sensitisation (TDS) stress can also increase GR and decrease MR expression in the hippocampus (Liberzon et al., 1999).

According to the glucocorticoid cascade hypothesis, glucocorticoid overproduction is driven indefinitely by chronic stressors. Overproduction of glucocorticoids has been suggested to cause HPA axis hyperactivity, brain abnormalities that include volume reductions in the hippocampus (Sapolsky et al., 1986a). However, insufficient signalling of glucocorticoids has also been implicated in the pathology of stress-related disorders. Decreased hormone bioavailability (hypocortisolemia) and decreased hormone responsivity/receptor sensitivity are suggested to be the cause of insufficient glucocorticoid signalling. The relevance of insufficient glucocorticoid signalling is that even in a state of glucocorticoid hypersecretion, insufficiency can exist. A decrease in hormone availability may be due to deficient production or secretion of cortisol or reduced levels of upstream secretagogues (ACTH and CRH) (Raison et al., 2003). Decreased receptor-mediated signal transduction/receptor sensitivity can be caused by incongruence of receptor numbers, binding affinity or functional capacity. This concerns receptors at all levels of the HPA axis: GRs, MRs, CRH and ACTH receptors.

MDD patients display high cortisol levels following DEX indicating insufficient glucocorticoid signalling in this disorder (Maes et al., 1990). New treatments of stress-related disorders are therefore focussed on enhancing glucocorticoid signalling.

1.4.6 *Stress system buffers*

A number of previous studies report on the positive effect social interactions have on health and well-being (Cohen, 1988). Social support or affiliation appears to dampen the HPA stress response (Kirschbaum et al., 1995; Thorsteinsson et al., 1999). The rodent stress system is buffered against mild early life stressors, but is disturbed by severe trauma, injection, neglect and abuse (de Kloet et al., 2003). This social protective mechanism of the stress response is referred to as social buffering and is probably restricted to species which naturally live in pairs. Certain behavioural and physiological constructs have been proposed as indicators of beneficial social buffering against stress. Physical contact is the primary role player in social buffering to attenuate the stress response. Grooming and stroking behaviours are also important mediators of stress inhibition (Van Oers et al., 1998b; Rosenfeld et al., 1993).

Social buffering of the stress response has been studied intensively in mother-infant attachment relationships (De Vries, 2002). Regarding the infant, social support does appear to buffer the hormonal stress response of the HPA axis, since the presence of familiar social companions lowers the behavioural and hormonal responses at the time of weaning (Levine, 2000). Social buffering is also exhibited in the social bonds that exist between adults. Adult squirrel monkeys were visually exposed to a live snake, either in their social group (two males and two females) or individually caged. When monkeys were tested as a group, the visual exposure to the snake did not elicit increased cortisol concentrations (Vogt et al., 1981) in contrast to when they were singly housed. Social buffering therefore only occurred when monkeys had multiple social partners. Despite its apparent importance little is known about the precise physiological mechanism involved in positive social interactions buffering the stress response (Cohen, 1988; Uchino et al., 1996).

1.5 Neurotrophins

1.5.1 *Synthesis, secretion and function of neurotrophins*

Neurotrophins are homodimeric proteins and are secreted by a variety of cells, including neurons. Neurotrophins play a role in neuronal survival, growth, differentiation, synaptic plasticity and efficacy (Lo, 1996). During development neurotrophins also prevent the death of embryonic neurons (Barde, 1994). Neurotrophins are expressed in almost all neuronal populations in the central and peripheral nervous systems. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are especially important because of their regulation of neuronal survival and differentiation. Other neurotrophins include NT- 4/5 and NT-6 and NT-7 (NT-6 and -7 have only been identified in fish) (Gotz et al., 1994).

Neurotrophins also function to protect neurons against excitotoxic, hypoxic and hypoglycaemic insults (Lessmann et al., 2003). Neurotrophins bind to tropomyosin related kinase (Trk) receptors: NGF binds to TrkA, BDNF binds to TrkB and NT-3 to TrkC. Neurotrophins have a common receptor to which they bind non-specifically with low affinity ($p75^{NTR}$ molecule (LNGFR)). The $p75^{NTR}$ receptor forms part of the tumour necrosis factor-receptor superfamily (Barbacid, 1995).

The trophic effects of neurotrophins are mediated by Trk receptor signalling or by the joint activation of p75 and Trk receptors (Barbacid, 1994). Neurotrophin mRNA, Trk B and C are expressed in high concentrations in the hippocampus. Trk A and p75 molecules are expressed in high concentrations in the basal forebrain cholinergic neurons. The latter neurons project to the hippocampus, thereby influencing hippocampal function. However, the p75 receptor is absent in neurons residing in the hippocampus and neocortex (Leßmann, 1998). Perinatally, NT-3 predominates and is expressed primarily in the hippocampus and cerebellum (Zhou et al., 1994). During development and in adulthood, BDNF becomes the most widespread neurotrophin in the mammalian brain. Its expression is low at birth but increases tremendously during the

first few weeks of life. BDNF expression is found in the hippocampus, neocortex, amygdala and cerebellum (Lessmann et al., 2003).

Neurotrophins influence survival of a variety of neurons during development. Before birth, NT-3 promotes survival and differentiation of sensory neurons. During postnatal development many of these neurons rely on NGF (Lewin et al., 1996). BDNF is a survival factor for certain dorsal root ganglia and a variety of sensory neurons in the PNS (Fritzsch et al., 1997). However, during postnatal development of the PNS, neurotrophins promote differentiation in favour of survival (Lewin et al., 1996).

Neurotrophins are synthesised intracellularly as pre-pro-neurotrophin precursors. The pre-mRNA sequence directs (signal peptide) the rest of the protein to the ribosomes of the endoplasmic reticulum (ER) where the neurotrophins' mRNA is translated and sequestered in the ER (Halban et al., 1994). After sequestration the signal peptide is cleaved off. The remaining pro-neurotrophins in the ER are transported to the Golgi apparatus via transport vesicles (Rothman et al., 1992) and subsequently to the trans-golgi network (TGN). The TGN contains protein convertases that cleave off the pro-sequence. The TGN is also a sorting station, where secretory vesicles are produced and filled with one or a mixture of immature neuropeptides (Merighi, 2002). Small secretory vesicles are transported to the cell periphery. The contents reach maturity en route to the plasma membrane with which it now fuses and is incorporated into the constitutive pathway. The content of small vesicles may be released without any specific trigger (Halban et al., 1994) and is not dependent on increased intracellular calcium levels.

Large vesicles make use of the regulated pathway. Fusion with the plasma membrane is calcium-dependent and only occurs at specific cholesterol rich release sites on the plasma membrane (Lang et al., 2001). Following secretion of neurotrophins from neurons (can also be secreted from skin, muscle or glands), they bind to their respective axonal receptors or $p75^{NTR}$ and are endocytosed and retrogradely transported to the cell body of the neuron where cellular survival signalling cascades are activated

(Oppenheim, 1991). However in the CNS, neurotrophins not only modulate neuronal survival but also differentiation and neuronal function (Murer et al., 2001).

Neurotrophin secretion occurs via a number of release mechanisms. Firstly, secretion of neurotrophins from neurons is an activity/depolarisation-dependent. Depolarisation occurs when the membrane permeability to sodium ions increases almost a hundred fold. Influx of positively charged sodium ions is accompanied by the efflux of positively charged potassium ions. The flow of ions is permitted through various membrane-bound receptors, which include: N-methyl-D-aspartate (NMDA), the non-NMDA (amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/ kainiate), voltage-gated calcium channel (VGCC) and gamma-aminobutyric acid (GABA) receptors (Vander et al., 1994). NGF release is dependent on the generation of action potentials and intracellular release of calcium (Hoener, 2000). Depolarisation-induced Trk phosphorylation in terminal mossy fibres and noradrenergic fibres has indirectly demonstrated activity-dependent secretion of BDNF (Aloyz et al., 1999). Direct evidence of depolarisation-induced BDNF secretion came from BDNF extracellular medium accumulation using Western blot analysis of cultured hippocampal neurons (Goodman et al., 1996).

Secondly, it has also been suggested that secretion of BDNF and NGF are mediated by glutamate, binding to AMPA and metabotrophic glutamate receptors (Canossa et al., 2001). Glutamate release has been correlated with increased BDNF and TrkB mRNA levels in the CA1 region of the rat hippocampus in a concentration-dependent manner (Falkenberg et al., 1996).

Lastly, neurotrophin secretion can be induced by neurotrophins themselves. Stimulation of TrkB and TrkC receptors by using NT-4/5 or NT-3 causes the secretion of endogenous BDNF from hippocampal slices (Canossa et al., 1997). NT-3 secretion has also been reported following spontaneous firing of action potentials from single hippocampal pyramidal neurons that were dependent on the activation of voltage-gated calcium channels (Boukhaddaoui et al., 2001).

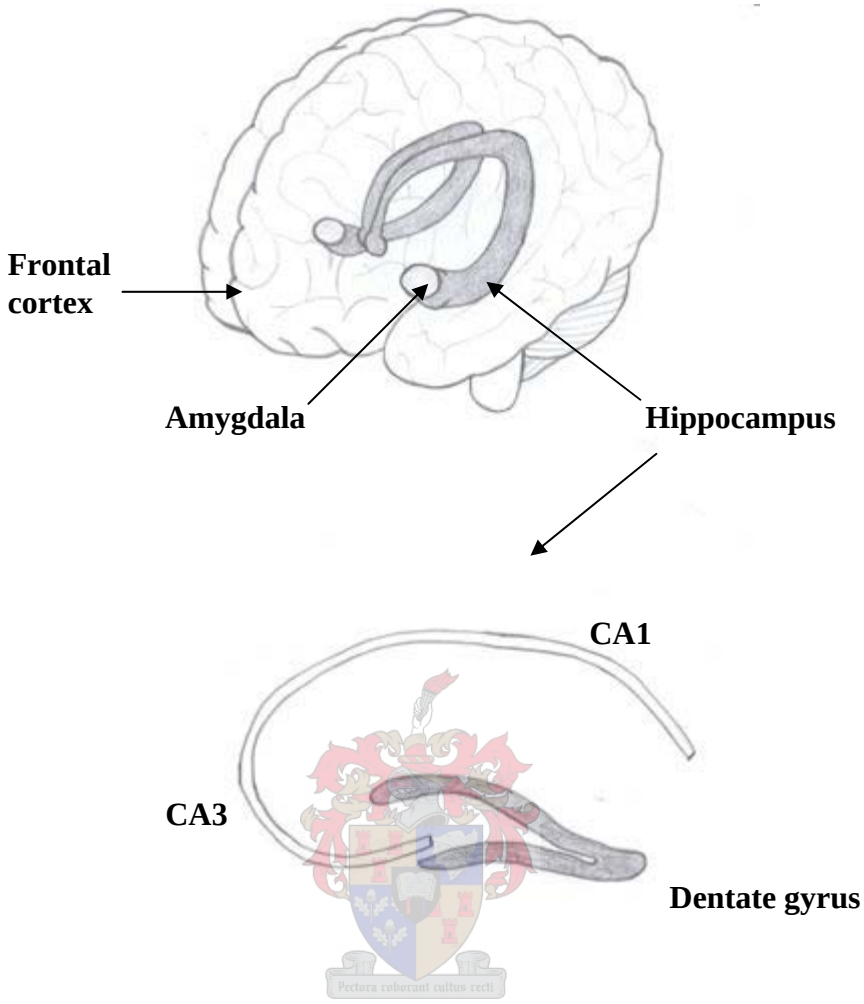


Figure 1.2 Representation of the different subfields of the hippocampus.

1.5.2 *Role of neurotrophins in synaptic activities*

The shaping and formation of neural connections during brain development is known as synaptogenesis. Neurotrophins play a critical role during early developmental stages, particularly in the hippocampus, since almost all granule cells of the dentate gyrus are formed postnatally (Cirulli et al., 2000). Changes in neurotrophin expression during critical developmental periods can therefore alter cell numbers and subsequently affect neuronal function and efficacy (Crain et al., 1973). Since neurotrophins are synthesised in an activity-dependent manner and released upon depolarisation in the CNS, they tend

to influence the electrical neuronal activity, thereby regulating the efficacy of synaptic transmission (Leßmann, 1998).

Neutrophins may take on a neuroprotective role following several types of brain insults. Often these insults are accompanied by extreme excitatory synaptic stimulation (Lindvall et al., 1994). Physiological levels of excitatory synaptic activity, such as sensory stimulation of cortical afferents (Castren et al., 1992), as well as induced long-term potentiation (LTP) via tetanic stimulation of excitatory pathways (Patterson et al., 1992), have been demonstrated to increase BDNF. In this instance BDNF may be regarded as a feedback regulator of excitatory synaptic transmission, by modulating glutamatergic synaptic transmission (Leßmann et al., 1994; Levine et al., 1995; Kang et al., 1995; Scharfman, 1997). This synaptic modulation is mediated via TrkB receptor activation and not the low-affinity p75 receptor (Leßmann, 1998). The observed modulation of glutamate release induced by BDNF is presynaptic in origin (Leßmann et al., 1994) but postsynaptic modification may also take place in parallel via NMDA receptor mediated synaptic transmission (Levine et al., 1998). Interestingly, it has been shown that NT-3 inhibits GABA-ergic synaptic transmission in cortical neurons, which subsequently leads to increased glutamatergic activity (Kim et al., 1994). NGF indirectly influences glutamatergic synaptic activity in the hippocampus and neocortex. However, this effect is more or less restricted to cholinergic neurons which project from the basal forebrain to cortical structures expressing NGF, TrkA and p75 receptors.

In summary, neurotrophins are synthesised and secreted from CNS neurons in an activity dependent manner and can enhance glutamatergic synaptic transmission as well as being feedback modulators in strengthening activated synapses (Leßmann, 1998).

1.5.3 *Learning and stress exert opposing modulation on neurotrophins*

The hippocampus is the brain region involved in learning and memory processes. Neurotrophins, especially BDNF are highly expressed in this region (Hofer et al., 1990) and studies have implicated hippocampal BDNF expression in learning processes.

Contextual and spatial learning are associated with increased BDNF mRNA in the hippocampus (Hall et al., 2000; Mizuno et al., 2000). Interestingly many learning tasks lead to increased corticosterone levels which are known to reduce BDNF mRNA (Schaaf et al., 1998; Schaaf et al., 1999). To address how learning and stress contribute to BDNF levels, rats were subjected to a learning paradigm, in which two groups of rats received the same amount of stress, but one group learned how to avoid the stressor (electric shock) (Scaccianoce et al., 2003). The results from this study indicated that the learning paradigm caused increased hippocampal BDNF expression even in the presence of high corticosterone levels. The researchers concluded that while learning and stress exert opposing effects on BDNF levels in the hippocampus, the positive effect of learning overshadowed the negative impact of stress on BDNF levels (Scaccianoce et al., 2003).

Other studies have implicated BDNF secretion during LTP which is an important electrophysiological substrate for associative learning (Gartner et al., 2002). LTP occurs when certain synapses of afferent neurons are stimulated by high frequencies and results in long term enhancement of glutamatergic synaptic transmission (Bliss et al., 1993). A relationship therefore exists between BDNF, learning and neurogenesis in the hippocampus (Scaccianoce et al., 2003) in that learning is associated with increased neurogenesis (Grassi Zucconi et al., 2002) and also leads to increased BDNF expression in the hippocampus. In addition it has been shown that neurogenesis is regulated by BDNF (Cameron et al., 1998). Learning deficits in rats can be abolished by the intraventricular infusion of BDNF and NGF (Fischer et al., 1994), but the direct causal connection between neurotrophin-dependent synaptic plasticity and learning and memory are still to be established (Leßmann, 1998).

Studies have shown that maternal deprivation in primates and rodents has a negative impact on cognitive development (Suomi, 1997). The same is observed in humans, where parental neglect and maltreatment have profound effects on cognitive development (Ammerman et al., 1986; Trickett et al., 1995). Using a cross-fostering study, a relationship has been established between variations in maternal behaviour and

hippocampal development (Liu et al., 2000a). It was suggested that variations in maternal behaviour (licking, grooming and arched-back nursing) were linked to differential expression of genes encoding for subunits for NMDA receptors, which in turn enhances glutamate sensitivity of hippocampal neurons. BDNF gene expression is also increased (Marini et al., 1998) which leads to improved hippocampal synaptogenesis, resulting in enhanced spatial learning in adulthood (Liu et al., 2000a).

1.5.4 *Up- and down-regulation of neurotrophins*

Several studies have shown that neurotrophin levels in the brain change when subjected to stress. The effects a stressor has on neurotrophins and their receptors (mRNA and protein levels) are stress-specific, depending on the stress paradigm and duration of stress employed. Immobilization stress (2 hours), which results in high corticosterone levels, have been shown to decrease BDNF mRNA levels in the dentate gyrus of the hippocampus, whilst NT-3 mRNA levels surprisingly increase after exposure to immobolization stress (Smith et al., 1995). Longer duration of immobilization stress however (after 8 hours from the onset) reduces all neurotrophin expression levels together with their Trk receptors (Ueyama et al., 1997). Interestingly the communal receptor, p75 mRNA levels remain unchanged. Restraint stress (5 or 15 min) results in reduced NGF levels in the basal forebrain, but hippocampal NGF levels are not significantly affected. Hippocampal NGF protein concentrations remain unchanged when subjected to cold stress, whilst NGF levels in the basal forebrain increase significantly after 1 hour exposure to cold stress on the first day, but no change on day 3 or 5. Rotary stress results in reduced hippocampal NGF levels, whilst this type of stressor does not elicit NGF level changes in the basal forebrain. The same result is also seen when animals are subjected to foot-shock stress. In these different stress paradigms no direct relationship is observed between plasma corticosterone and brain NGF concentration changes. It is suggested that changes in neurotrophins are not a direct consequence of increased corticosterone levels (Scaccianoce et al., 2000). Supporting evidence in line of this observation comes from human experiments done on a

parachutist's first jumping experience. It was observed that plasma NGF levels were increased in those soldiers selected for jumping even before the increase in ACTH and cortisol caused by stress-induced parachute jumping (Aloe et al., 1994a).

Stress induced by maternal separation causes significant alterations in NGF levels and behavioural deficits in adult life. For example a short period (45 min) of maternal separation on postnatal day (PND) 3 results in increased NGF expression in the rat hippocampus (Cirulli et al., 1998). Longer periods of maternal separation (3 hours) on PND 9 and PND 16 lead to an even greater increase in NGF expression, in the hippocampus and other brain areas that include the hypothalamus and prefrontal cortex. Interestingly, NGF expression in the dentate gyrus is greater in PND 16 compared to 3 hours maternal separation on PND 9 (Cirulli et al., 2000). Repeated maternal separation (3 hours daily) from PND 2 until PND 14 results in elevated BDNF expression at PND 17. This time period reflects the cumulative effects of repeated maternal separations. As adults, these maternally deprived rats actually show down-regulated BDNF expression in the prefrontal cortex (Roceri et al., 2004). In contrast, the offspring of rat mothers displaying high levels of grooming, licking and arched-back nursing towards their pups actually have higher levels of BDNF mRNA expression in adulthood (Alleva et al., 2001).

These data suggest that a single episode of stress increases neurotrophin expression, resulting in short term protective effects, while prolonged stress decreases neurotrophin expression, ultimately resulting in altered neurplasticity (Molteni et al., 2001; Roceri et al., 2004). Thus, the importance of timing and duration of maternal deprivation or the quality of maternal care, such as poor upbringing conditions may lead to neurobiological changes that can alter normal brain plasticity, culminating in behavioural maladaptations.

1.5.5 *The interaction between corticosterone and neurotrophins*

Corticosterone interacts with neurons in the limbic system and causes not only morphological but also behavioural changes (Roskoden et al., 2004). Morphological changes include the decrease of hippocampal volume after chronic glucocorticoid exposure (Sapolsky et al., 1985), and reduced number of medial septum cholinergic neurons (Tizabi et al., 1989). Stressors that elicit a corticosterone response (e.g. repeated restraint stress for 21 days) cause atrophy of CA3 pyramidal neurons' apical dendrites and a reduction in total apical dendritic length in the rat hippocampus (McEwen, 1999).

Neurotrophins serve as trophic factors for neurons in the basal forebrain, septum and hippocampus (Ebendan, 1989; Ernfors et al., 1990; Wetmore et al., 1990) and appear to play an important role in maintaining the structure of neurons in the hippocampus (Schaaf et al., 1998). Corticosterone is able to exercise a strong influence on the hippocampus, since this brain area is densely populated with corticoid receptors (Gerlach et al., 1972). Neurotrophins and their receptors are possible targets of corticosterone via MRs and GRs. In adult rats, the elimination of corticosterone via adrenalectomy reduces NGF, BDNF and NT-3 mRNA expression in the hippocampus (Roskoden et al., 2004), whilst DEX treatment leads to increased NGF and NT-3 expression (Barbany et al., 1992). During early postnatal development corticosterone treatment (from PND 0-12) results in increased BDNF and NT-3 septal expression during the first postnatal week, as well as increased TrkB mRNA at PND 14 in the septum and the hippocampus and TrkA mRNA in the hippocampus. Hippocampal NGF and NT-4/5 protein levels are also elevated. Corticosterone treatment seems to improve hippocampal connectivity and behavioural capacity later in life (Roskoden et al., 2004), since improved neurotrophin-dependent learning in the navigational task of the Morris water maze has been observed (Gage et al., 1988), as well as improved escape latency (Scaccianoce et al., 2001). The interaction between corticosterone and neurotrophins therefore seems paradoxical in nature, since it leads to both positive and negative outcomes.

Corticosterone treatment dose-dependency was examined in order to explain the aforementioned contradictory observations. It was shown that after a high dose of corticosterone BDNF mRNA levels were decreased 6 hours following injection, whilst a low dose had no effect (Schaaf et al., 1997). Subsequently, corticosterone was administered at a low (30 μg/kg) dose and a high (1 000 μg/kg) dose. The effect corticosterone had on BDNF expression, protein levels and TrkB mRNA levels were measured at 3, 6, 12 and 24 hours. The high dose of corticosterone resulted in a decrease in BDNF mRNA and protein levels in the dentate gyrus and CA1 at the 3 hour time period, and returned to basal levels at the 6 hour time period. After 4 and 6 hours this down-regulation of BDNF mRNA levels was reflected at the protein level. However, between the 6 and the 12 hour time period BDNF mRNA levels in both the dentate gyrus and the CA1 subfield increased meaningfully. Interestingly, mRNA levels in the dentate gyrus returned to basal levels at 12 hours post corticosterone injection, but the CA1 subregion showed even higher basal levels when compared to pre-corticosterone injection. Surprisingly, BDNF mRNA levels once again decreased between 12 and 24 hours after corticosterone administration. The low dose corticosterone administration also resulted in decreased BDNF expression levels at the 3 hour time period, but this effect was not as pronounced as observed with the higher dose. No significant effects could be detected after 6 hours. Twelve and 24 hours after corticosterone administration BDNF mRNA levels increased when compared to 3 hours post-treatment, however once again changes were not as pronounced as the high dose group. Corticosterone had no effect on TrkB mRNA levels. Low doses of corticosterone saturate MR and occupy few GR in the hippocampus (Reul et al., 1987); however a high dose of corticosterone results in a significant increase in GR occupation and decreases in BDNF mRNA levels are likely derived from corticosterone mediated GR activation (Schaaf et al., 1998).

This mechanism of high corticosterone levels suppressing neurotrophins is also able to account for neuron damage associated with elevated corticosterone concentrations. In support of this, almost all hippocampal subfields (CA1, CA3 and the dentate gyrus), BDNF expression is down-regulated when exposed to chronic high levels of

corticosterone (Chao et al., 1994; Smith et al., 1995). Interestingly, the CA3 region only displayed down-regulated BDNF mRNA levels after prolonged exposure to high corticosterone levels, while in the dentate gyrus immediate effects are observed after a single corticosterone treatment. The weak response of the CA3 region towards corticosterone was explained by low GR expression in this region (Van Eekelen et al., 1988). In summary, it appears that a critical level of corticosterone must be maintained for homeostasis to occur, while imbalances can have long-lasting negative consequences involving alterations in neurotrophin expression.

1.5.6 *Neurotrophins and behaviour*

Neurobehavioural disorders in humans, significantly impact NGF plasma concentration when compared to healthy subjects (Aloe et al., 1994b), however these effects vary across disorders and may not be true for all affective disorders. BDNF has also been studied in the pathology of depression (Duman et al., 1997; Schaaf et al., 1998). It has been noted that BDNF is down-regulated in response to stress and elevated corticosterone. Fear and anxiety provoking stimuli have been implicated in increased NGF release in parachutists (Aloe et al., 1994a), while anxiety experienced by alcohol and heroin withdrawal has been observed to up-regulate plasma NGF levels (Aloe et al., 1996).

A number of animal studies aimed at elucidating the mechanisms that trigger the release of neurotrophins under stressful conditions have been conducted. In mice, subordination and defeat experiences result in significantly increased NGF levels when compared to levels in male mice displaying dominant, attacking behaviour (Maestripiere et al., 1990). This result suggests that psychological stimuli are also potent activators of NGF release. It is thought that subordinate animals perceive a fighting situation as uncontrollable, as demonstrated by the increased amount of freezing behaviours of subordinate animals (Frischknecht et al., 1988). Interesting differences exist between social and non-social/physical stress in the physiological coping responses (Axelrod et al., 1984).

Aggressive behaviour has been found to increase NGF serum levels in socially isolated male mice. The NGF serum level was also directly related to the number of fighting episodes (Aloe et al., 1986). Interestingly, NGF-treated mice spend much less time engaged in fighting behaviours than controls (Bigi et al., 1992) and NGF inhibited aggressive grooming behaviour in these animals (Alleva et al., 2001). NGF administration thus causes an inhibitory effect on aggressive behaviour in males. Similarly, lactating females that display aggressive behaviour in order to protect their pups from predators are considered to be showing 'uncontrollable' behaviour. Lack of controllability in these females relates to greater defensive behaviours and higher plasma NGF levels (Alleva et al., 1996).

NGF expression may also be regulated by behavioural activation. It is hypothesised that the increase in brain NGF levels reflects a compensatory mechanism that attempts to maintain brain plasticity in the face of psychosocial stressful events (Alleva et al., 1989).

1.6 Maternal separation model

Maternal separation is considered a form of childhood neglect and abuse, and this in turn leads to an increased vulnerability to developing psychopathologies in adulthood (Heim et al., 1997; Ladd et al., 2000). The maternal separation model is based on an observation by Calhoun (1963) that subordinate dams have to build their nests far away from food and water supplies. This results in the dam being separated from her pups for some time, even for as long as 2-3 hours (Liu et al., 2000b). Thus, the maternal separation model enables the study of the psychobiology of mother-infant relationships.

1.6.1 *Psychobiology of attachment*

Psychobiology can be defined as the study of the effect of the environment and behaviour on biological systems (Levine, 2000). When investigating the psychobiology of attachment, the primate model is a more suitable model than the rodent model, since primates exhibit a better representation of human attachment relationships. Bowlby's

theory of infant attachment states that attachment behaviours are formed during the first months of life to establish a measure of secureness in the relationship between the caring parent and infant (Bowlby, 1969). This trusting relationship encourages the child to explore the physical and social environment with confidence and when faced with stressful situations, the child can be reassured with the safety and comfort embedded in the parent-child relationship. This demonstrates the importance of parent-infant relationships in order for a child to feel secure and also to mediate the development of a child's ability to cope with stress (Lapsley et al., 2000). According to Newport et al. (2002) the parent-child interaction is critical in order to understand the developing human psyche, since a child does not develop in a psychological vacuum.

Studies done with the squirrel monkey (*Saimiri sciureus*) using a maternal separation model show increased plasma cortisol levels in both the mother and the infant monkeys (Levine and Wiener, 1988). However, other studies showed that endocrine responses are dependent on the type of separation model used (Wiener et al., 1987; Wiener et al., 1990) and that the level of elevation of cortisol is influenced by the social support structures present at the time of separation. Activation of the hypothalamic-pituitary-adrenal axis by different separation paradigms has for example, been noted when the infant goes to hospital, or is separated from its mother, or is exposed to a novel environment. Interestingly, the reverse situation also creates another separation paradigm, i.e. when the mother goes to hospital. In this scenario the social structures and familiar environment are still in place for the infant. In the first separation paradigm, where the infant goes to hospital, the most severe elevation in cortisol is observed. This demonstrates that familiarity of the environment does play a role in regulating the HPA axis in response to separation (Levine, 2000).

1.6.2 *Maternal care enhances or suppresses certain biological systems*

Maternal care has been shown to influence certain aspects of development. In subtle ways the mother influences physiological processes such as cardiac response, growth hormone and sleep/wake cycles in the developing infant (Hofer, 1978; Hofer, 1984).

These physiological aspects are referred to as 'hidden regulators' by Hofer (1978). When pups are separated from their mothers for a 24 hour period, pups experience a 40% decline in heart rate. This response has been attributed to the absence of milk during the deprivation period, since normal cardiac response is restored after milk infusion. The reduction in growth hormone after maternal deprivation is due to the absence of tactile stimulation by the mother, since stroking the anogenital region of deprived pups result in normalization of growth hormone secretion. After maternal separation, sleep/wake rhythms are disturbed and are restored by suckling and not feeding (Hofer, 1978; Hofer, 1984). On the other hand, maternal care suppresses HPA-axis activity of the pups. This is referred to as the stress-hyporesponsive period (SHRP) when the HPA-axis is not activated upon exposure to mild stress.

1.6.3 *Maternal deprivation activates stress responses*

Maternally separated pups, deprived of maternal care, exhibit behavioural and neuroendocrine dysregulation when tested in adulthood. Similar neuroendocrine abnormalities are also seen in patients with depression and anxiety disorders (Heit et al., 1997; Ladd et al., 2000). It has been proven that the hypothalamic-pituitary-adrenal axis is activated by the absence of maternal care (de Kloet et al., 2003). Maternal separation on PND 6 until PND 20 of 4 to 6 hours daily, results in an increased ACTH response but normal corticosterone levels in response to stress experienced in adulthood (Ladd et al., 1996). In contrast a 24 hour maternal separation on PND 3 results in a normal ACTH response, but elevated corticosterone levels in response to a stressor on PND 4. Maternal deprivation of 24 hours on PND 11 leads to a normal corticosterone but a blunted ACTH response to stress on PND 12 (Van Oers et al., 1998a). These results indicate the variability of the HPA axis response to different maternal deprivation paradigms. However, it also shows the importance of maternal care in normal maturation as the absence thereof does result in neuroendocrinological alterations that resemble the neurobiological picture of behavioural disorders. For instance, depression is characterised by increased cortisol and a deficient feedback inhibition of the HPA-axis.

PTSD on the other hand is characterised by decreased cortisol and an enhanced negative feedback inhibition (Yehuda, 2001; Kanter et al., 2001).

The neonatal period shortly after birth (PND 4-14), is referred to as a stress non-responsive period, where the HPA system appears non-responsive (SHRP) (Sapolsky et al., 1986a). The SHRP is characterised by low concentrations of corticosterone in response to mild stressors (de Kloet et al., 2004). These low levels of corticosterone appear to be essential for normal brain development, since high corticosterone concentrations decrease mitosis, DNA content and brain size, as well as altered myelination of neurons in rats (Levine, 2001). Also, the absence of corticosterone after adrenalectomy negatively affects CNS development, demonstrating the essential role of of corticosterone in normal neuronal maturation. The hypo-responsive period reflects low adrenal sensitivity, given that administration of exogenous ACTH does not cause significant corticosterone elevation (Levine et al., 1967a). De Kloet et al. (2004), hypothesise that there are specific features that may mediate the hypo-responsiveness of the HPA system of the neonate. For example, afferent neural connections to the PVN are immature and can thus not be operational during the SHRP (Rinaman, 2001; Bouret et al., 2004), hence the HPA-axis cannot be fully activated. Also, the negative feedback inhibiting mechanism blocking the release of ACTH from the anterior pituitary is enhanced (de Kloet et al., 2004). However, the HPA-axis of the neonatal rat can be activated by maternal deprivation, resulting in an adult-like HPA response (Levine et al., 2002). Thus, maternal deprivation produces a stress-responsive HPA system during the developmental period when animals not exposed to maternal deprivation are non-responsive (Levine et al., 2001).

Levine et al. (1958) have shown that early handling of pups accelerates their development. For example handled pups opened their eyes earlier than non-handled pups. These authors argue that handling must affect a number of systems involved in development, including the stress response. Furthermore, the effects of early handling

result only from shorter periods of maternal deprivation, since longer periods would neutralise the beneficial effects of early handling (Levine et al., 2000).

1.6.4 *The influence of maternal behaviours: feeding and stroking*

Maternal behaviour such as feeding inhibits the developing HPA-axis in the SHRP (Levine, 2000). Pups that are fed through a cannula, without the presence of the dam, display similar corticosterone levels to non-deprived pups (Rosenfeld et al., 1993). This suggests that feeding is one aspect of maternal behaviour that suppresses the adrenal secretion of corticosterone. Another maternal feature of behaviour that impacts on HPA activity is stroking. Anogenital stroking by the dam serves to stimulate both urination and defecation (Levine et al., 2000). Suchecki et al. (1995), applied stroking (three times) to the anogenital area during a 24 hour period while pups were artificially fed. These authors report that stroking appears to regulate ACTH secretion/levels, while on the other hand feeding appeared to regulate adrenal sensitivity by modulating the amount of corticosterone released.

These observations underline the importance of maternal care in regulating the neuroendocrine system. Conversely, these observations indicate that when pups are deprived of maternal care detrimental consequences will follow that may affect the processes of normal maturation and behaviour, negatively. It is therefore evident that feeding and stroking are two crucial maternal behaviours for normal development, since artificial reinstatement reverses most of the effects of maternal deprivation (Van Oers et al., 1998b; Van Oers et al., 1999). Subsequent work has shown that a single episode of deprivation lasting 8 to 24 hours is required to sensitise the adrenal gland to respond to ACTH or stress (Levine et al., 2000). This deprivation of maternal care causes increased release of ACTH in response to stress, increased expression of corticotrophin-releasing hormone (CRH) and decreased expression of both MR and GR receptors in the hippocampus. Maternal deprivation also causes a dramatic increase in the expression of the *c-fos* gene in the hypothalamic paraventricular nucleus. Stroking of pups three times during the deprivation period reverses the increase of ACTH and CRH expression as

well as the decline of mineralocorticoid receptors (MR) in the hippocampus. Artificial stroking also rectifies the *c-fos* expression in the hypothalamus caused by deprivation (Van Oers et al., 1998b). Stroking therefore regulates ACTH levels, basal gene expression of CRH, c-fos and MR (Schmidt et al., 2002a).

In contrast to these findings, deprived pups exposed to a saline injection (acute stressor) at PND 6, 12 and 18, display increased levels of both ACTH and corticosterone and lower CRH expression (Kovaks et al., 1996). However, CRH is not the only ACTH secretagogue of the hypothalamus. Arginine vasopressin (AVP) also stimulates the pituitary to secrete ACTH. It has been shown that when adult rats are exposed to a chronic stressor, CRH mRNA returns to basal levels and AVP mRNA expression is increased (Scaccianoce et al., 1991; Bartanusz et al., 1993). Pups deprived for 24 hours of maternal care have shown that the increase in ACTH runs parallel with an increase in AVP gene expression. There seems to be a switch from CRH to AVP-mediated release of ACTH when dealing with acute and chronic stressors. Thus, certain factors of maternal care affect pups even to the level of gene expression, in regulating control of ACTH secretion and neuroendocrine responses (Dent et al., 2000).

1.6.5 *The effects of maternal deprivation are highly dependent on the developmental period*

Age dependent effects of maternal separation on behaviour, correlate with corticosterone activity (Lehmann et al., 2002). Rats that are maternally deprived on PND 4 show reduced active avoidance learning, and rats maternally deprived on PND 9 demonstrate increased active avoidance learning behaviour in a water maze (Lehmann et al., 1999). A single maternal separation of 24 hours during the SHRP period leads to increased ACTH and corticosterone levels in response to novelty, or saline injections (Stanton et al., 1988b; Levine et al., 1992; Suchecki et al., 1995). Maternal separation during early (PND 4-5), mid-late (PND 9-10) and post (PND 18-19) SHRP period demonstrate no consistent differences in basal corticosterone levels. The effects of maternal separation are therefore highly dependent on the time period during development.

Deprived pups from all three different ages showed an increased corticosterone response to restraint stress in adulthood (5 months old) in a study conducted by Lehmann et al. (2002). This is important in demonstrating that the effect of maternal separation on the endocrine system persists into adulthood. These findings are contradictory to the finding of increased basal ACTH and corticosterone levels in adulthood when rats were maternally deprived at PND 3 (Rots et al., 1996). It is possible that PND 3 falls outside the SHRP period, with a different stress response being one explanation for some of the differences in outcome. However, animals maternally deprived at PND 3 demonstrate increased ACTH levels in response to a saline injection at PND 20, whilst deprivation at PND 11 results in an attenuated ACTH response to saline injection at PND 20 (Van Oers et al., 1997). When comparing the latter study to the study of Lehmann et al. (2002) the latter study demonstrates that the endocrine response at PND 20 was not maintained until adulthood.

1.6.6 *Reciprocity in mother-infant relationships*

The mother determines the early environmental circumstances of her pups. The mother provides nutrients, warmth and grooming for the survival of her young, thereby regulating her offspring's physiology and behaviour (Hofer, 1994). However, it is important to remember that mother-infant relationships are reciprocal in nature in that both regulate each other's biological systems. Development of behaviour and endocrine responses to stress of pups is influenced by variations in maternal behaviour (Meaney et al., 1993). It has been demonstrated that the maternal separation stress paradigm may also be a stressor of the dam, since the paradigm caused anxiety-like behaviours in the dams (Kalinichev et al., 2000; Huot et al., 2004). These maternal variations have been referred to as the non-genomic behavioural heritage for individual differences in response to stress (Levine, 2000). The reciprocal nature in mother-infant relationships increases survival of the infant in order to reproduce and rear their own offspring. The way in which the mother cares for her young is conducted in a species specific manner. There are different ways of feeding, grooming and protecting the young among different

species (Rosenblatt et al., 1996). The maternal behaviours displayed by the rat mother include the licking of the pups in the anogenital regions and taking on a nurturing posture over their pups, known as arched back nursing (ABN). The rat pups in turn orient themselves toward their mother in specific ways and even display nipple preferences (Rosenblatt, 1971). Vocalizations and odour signals also evoke maternal responses directed toward her pups. All these reciprocal behaviours ultimately promote normal maturation as well as normal behavioural development of the infant (Alberts et al., 1990; Shettleworth, 1993). Fleming et al. (1999) hypothesised that how the mother responds to pups, partly determines the neurobiological and behavioural changes in the pups. These changes in the pups will eventually determine how these pups will in return respond to their own offspring. This is referred to as intergenerational effects.

A whole range of experiences determines how the mother will respond towards her pups. Pre-pubertal experiences, in the form of siblings often caring for their younger siblings, shapes their nurturing experiences, and absence there of often results in first time mothers rejecting or neglecting their young (Pryce, 1996). Postpartum experiences also influence how the mother responds to her offspring, where the decline of parturition hormones affects maternal behavioural responses. In rats, early postpartum experiences with their offspring mediate maternal responsiveness towards their young (Fleming et al., 1999). Maternal responsiveness is initially mediated through the hormones of parturition (e.g. progesterone, oxytocin and estrogen) but the nurturing maternal behaviour carries on after the decline in these hormones; maternal responsiveness and behaviour are then controlled by neural mechanisms which are activated by parturition hormones (Fleming et al., 1996; Lee et al., 1999). After hormonal effects have cleared, which facilitate the maternal relationship with her offspring; mothers depend on learning mechanisms to respond to their young (Fleming et al., 1994).

Intergenerational effects refers to how one has been cared for by one's own mother subsequently influences the way in which you as a mother in turn, care for your offspring. Not only does the mother learn how to care for her young, but the offspring in

turn gain experience that they mirror in their own behaviour toward their offspring (Fleming et al., 1999). Pups early olfactory and somatosensory experiences (being nursed and licked) demonstrate increased levels of licking and grooming and ABN in their own motherly behaviour towards their offspring (Fleming et al., 1999). Infants that received more stimulation from their mother in turn display more stimulation of their own infants.

The effects of early postnatal handling have been found to alter mother-infant interactions. It is proposed that early handling of pups alter the mother's response towards her pups and that this altered behavioural response of the mother leads to individual differences in the HPA response to stress in adulthood, when compared to mothers of non-handled pups (Liu et al., 1997) - this is according to the 'maternal mediation hypothesis' (Denenberg et al., 1962). Offspring of mothers displaying increased ABN and licking and grooming (LG), demonstrate decreased activity of the HPA axis in response to stress (restraint stress) in adulthood (Liu et al., 1997) as well as reduced fearfulness (Levine, 2000). When offspring of high LG-ABN mothers are treated with a corticosterone injection 3 hours before exposure to restraint stress, the ACTH response is greatly reduced, when compared to low LG-ABN offspring, indicating greater feedback sensitivity in high LG-ABN animals. CRH mRNA in the hypothalamus, which is the neural signal for ACTH release, was also greatly decreased in the high LG-ABN offspring. The above findings of high LG-ABN offspring are thought to be mediated by increased hippocampal GR expression in these animals which in turn is mediated by maternal behaviour (Liu et al., 1997).

1.6.7 *The effects of maternal separation on cognition*

Another aspect of interest is whether variations in maternal care relate to individual differences in cognitive development. Spatial learning, memory and hippocampal synaptogenesis in pups are promoted by mothers displaying high levels of LG-ABN. Using the Morris water maze test, high LG-ABN offspring show shorter swim paths and less searching to locate target platforms when compared to low LG-ABN offspring.

High LG-ABN pups also display increased levels of synaptogenesis and synaptic survival. The effect of cross-fostering on hippocampal development has been investigated, whereby pups that are born to low LG-ABN mothers are reared by high LG-ABN mothers and vice versa. Surprisingly, spatial learning and memory is not affected by the nature of the rearing mother. High LG-ABN pups do however display increased BDNF expression in the hippocampus as well as increased NMDA receptor subunit (NR2A and NR2B) expression at PND 8. These findings indicate that maternal care, which mediates experience-dependent neural development in the offspring, results in increased NMDA receptor levels which in turn leads to increased BDNF expression and ultimately in enhanced hippocampal synaptic development (Liu et al., 2000a).

1.6.8 *Familiarity versus maternal presence*

Other studies suggest that the familiarity of social partners reduces or totally suppresses the cortisol response to stress (Levine, 1993). At the time of weaning, being in the presence of familiar social partners reduces the HPA response of pups when losing their mother. This phenomenon is referred to as social buffering. In monkeys only multiple social partners buffer against the stress response. A predictable and stable environment has also been shown to suppress the HPA response to stress (Levine, 2000). In contrast work on which aspects of the mother-infant interaction regulate the non-responsiveness of the HPA axis during SHRP; the presence of a lactating dam reduces the corticosterone response of maternally deprived pups to novelty or a saline injection (Stanton et al., 1990), while the presence of a non-lactating dam, a littermate or another adult male is unable to do so (Stanton et al., 1988a; Cirulli et al., 1992). However in a separate study, the presence of an anaesthetized dam completely suppressed the corticosterone response. In this particular study, the aim was to determine whether the presence of the mother (although anaesthetized) rather than food, suppressed the corticosterone response to a novelty stressor (Stanton et al., 1987). These findings clearly show the importance of a familiar and stable environment for normal HPA axis development.

1.7 Time-Dependent Sensitisation (TDS) model

Several animal models have been developed to model stress-related disorders in humans for example electric shock (Pynoos et al., 1996), underwater trauma (Richter-Levin, 1998) and the exposure of a rat to a predator (Adamec et al., 1997). All these models aim to inflict intensely stressful experiences, aversive challenges and situational reminders of the traumatic event, which ultimately aim to cause behavioural, autonomic and hormonal responses that reflect the diagnostic criteria of the mental illness. The stress-restress or the time dependent sensitisation (TDS) model has been proposed as a possible animal model for PTSD (Yehuda et al., 1993b). The TDS model involves the exposure of animals to a traumatic stressful event that includes 2 hours restraint stress followed by 20 min forced swim stress and exposure to ether/halothane vapours until loss of consciousness. The traumatic session is followed by a situational reminder (swim re-stress) 7 days later. The re-stress serves the purpose of reminding the animal of the trauma, thus maintaining the stress disorder over time. According to Pynoos et al. (1996), exposure to situational reminders is associated with bidirectional abnormal behavioural patterns, i.e. when exposed to a fear provoking situation the behaviour can either be one of fearfulness or fearlessness (Rachman, 1980). Chronicity of exposure to situational reminders serves to maintain the long-term symptoms of fear related behavioural disturbances as shown by the studies done after the 1988 Armenian earthquake, when heightened arousal symptoms have been observed in individuals chronically exposed to damaged buildings and sites where traumas occurred (Pynoos et al., 1993).

It is important not to overlook the fact that only a subset of individuals exposed to trauma eventually develops disorders such as PTSD (Yehuda et al., 1995). Animals too respond to stress in a heterogeneous manner and it has been proposed that animals be divided into distinct groups, namely a well-adapted and maladapted group, using cut-off behavioural criteria based on exploratory behaviour (Cohen et al., 2003). For instance animals that explore mazes extensively are generally ‘well-adapted’ and those that

display anxious behaviour and low locomotor activity may be classified as maladaptive (Cohen et al., 2003). Maladapted rats often show increased plasma corticosterone and ACTH concentrations when exposed to a predator, indicating a dysfunctional HPA axis; whereas the well-adapted animals do not. This suggests that exposure per se is not a causal factor for developing PTSD-like symptoms. In fact human studies have shown that specific forms of trauma cause different prevalence rates of PTSD. Thirty two percent of female rape victims meet the criteria for lifetime prevalence of PTSD and 12.4 % for current PTSD (Resnick et al., 1993). Thirty percent of Vietnam War veterans meet the criteria for lifetime prevalence and 15 % for a current rate of PTSD (Jordan et al., 1991).

In addition to HPA axis abnormalities, animals exposed to situational reminders also demonstrate unusual aggressive behaviour (Pynoos et al., 1996). This finding is in agreement with the DSM-IV symptom criteria that include the irritability and outbursts of anger in patients with PTSD (American Psychiatric Association, 1994).

Harvey et al. (2003) have investigated the effects of TDS on endocrine, cognitive and hippocampal/cortical $5HT_{1A/2A}$ receptor levels in rats. They reported decreased corticosterone levels 7 days after the prolonged stress compared to the controls. This observation is in accordance with previous studies showing hypersensitive HPA axis negative feedback (Yehuda et al., 2000). The effect of TDS stress on spatial memory performance has been assessed by using the Morris water maze. The traumatised animals show higher escape latency times during the learning period. Memory acquisition is also affected as reflected by less time spent in the target quadrant. Following 7 days post TDS stress, animals demonstrate increased hippocampal $5HT_{1A}$ receptor density but reduced binding affinity compared to the controls. Prefrontal cortex $5HT_{2A}$ receptor density does not differ between stressed and control animals; however the binding affinity of $5HT_{2A}$ receptors are significantly increased in the stressed animals 7 days post TDS stress. Serotonin inhibits hippocampal function via $5HT_{1A}$ receptors

(Bijak et al., 2001). It is suggested that the increase in $5HT_{1A}$ receptors after TDS stress accounts for the decreased memory function (Harvey et al., 2003).

1.8 Measuring animal behaviour

Individuals exposed to early adverse experiences are at increased risk of developing anxiety disorders, depression or even both as evident from various epidemiological studies. Good animal models of anxiety and depression require the study of behaviour as well as the measurement of physiological and endocrine functions (Rodgers et al., 1997). Models that expose animals to unconditioned stimuli have a much higher ecological validity and provide a behavioural repertoire that is more representative than when conditioned models are used (Rodgers et al., 1994).

Physiological and behavioural responses are shaped by environmental/social and genetic factors. Studies can to some degree control for genetic influences by using the same strain of animals, but environmental inputs (experimental conditions) must be manipulated in order to approximately model certain aspects of human conditions, since animal models can not accurately reflect human disorders (Cohen et al., 2003).

1.8.1 *Elevated Plus Maze (EPM)*

The elevated plus maze (EPM) is an animal model for human anxiety. This model makes use of unconditioned or spontaneous behavioural responses (File, 1992; Dawson et al., 1995). Behaviour on the EPM is considered to reflect anxiety based on the animal's fear of height (Pynoos et al., 1996). Later studies have confirmed that height above the floor is not the actual fear-provoking factor (Falter et al., 1992; Treit et al., 1993), but variations in open arm rim height impact on the amount of exploration in this arm. The higher the rim of the open arms the greater the exploratory behaviour (Treit et al., 1993). When animals are exposed to a novel environment (EPM) an internal conflict evolves between its curiosity to explore the maze and avoiding the fear provoking environment (Rodgers et al., 1997). After observing that rats explore the enclosed arms more

frequently and consistently, Montgomery (1955) suggests that the same exploratory drive exists for both open and closed arms of the maze, and concludes that avoidance of the open arms is due to higher levels of fear experienced in the open arms. Since the open arms of the maze result in greater fear generation, it provides the model with face validity (Rodgers et al., 1997). It is important to take into account that animals should display greater fearful behaviour and an increased plasma corticosterone response in open arms than when in closed arms (Pellow et al., 1985). Subsequently, the EPM is often used in the evaluation of anxiolytic agents (Lister, 1987; Treit et al., 1993).

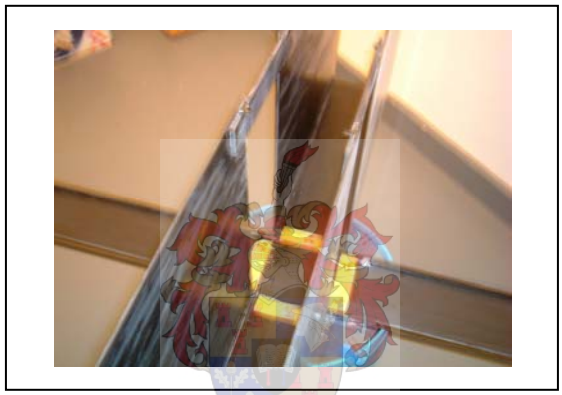


Figure 1.3 The elevated plus maze.

1.8.2 *Open Field*

The open field test is frequently used as an animal model for depression. The social interaction of depressives is described as having a profound lack of interest in the environment (Klein, 1974) and a tremendous avoidance of social activities (Pollard et al., 1975). This is demonstrated by 'emotional' animals that display reduced motor activity, grooming and rearing as well as high defecation levels in the open field paradigm. These animals also avoid the middle of the field, whereas 'non-emotional' animals show high levels of grooming and rearing and low levels of defecation, and exploration particularly occurs in the middle of the open field (Denenberg, 1969; Walsh et al., 1976).

Figure 1.4 The open field.

1.9 Coping mechanisms in response to stress

How an individual copes with a particular stressor will determine in part whether the outcome will have positive or negative consequences. Psychological factors (e.g. control) are important in determining the manner in which an individual will respond to a stress endocrinologically (Levine, 2000). Weiss (1972) has done research in the field of coping mechanisms in response to stress. These coping mechanisms include control, predictability and feedback. For example when an animal is exposed to a shock stressor and the animal is able to regulate the duration or the intensity of the shock; this is referred to as control. When the animal receives information regarding the onset of the shock, a measure of predictability can be exerted. Further, if information is received of the efficacy of the animal's stress response, feedback is gained. The number of coping responses and the feedback of these responses are a measure of the actual amount of stress an animal is experiencing (Weiss, 1972).

A variety of species (rats, dogs and monkeys) have shown that control modulates the HPA response to stress. Active avoidance in rats was used to observe the resultant effect on corticosterone levels (Coover et al., 1973). They found a decreased corticosterone level on the seventh training day compared to the first training day and as training

continued, further decreases in corticosterone were observed. This phenomenon was attributed to the effects of control and predictability of the stress response and is sometimes referred to as habituation. Another study also demonstrated that primates could control their HPA axis reactivity in response to loud aversive noise levels (Hanson et al., 1976). The interaction between control and predictability has since been highlighted, because predictability in the absence of control results in an exacerbated stress response (Davis et al., 1982; Dess et al., 1983). The effects of predictability are the most profound when the control option is also available (Levine, 2000). Loss of control increases stress reactivity. Using an extinction paradigm, animals were trained to push a lever for access to food and water. This association was reinforced on a continuous basis. Withdrawal of reinforcement during the extinction period resulted in increased corticosterone levels. A loss of control had therefore been defined as the removal of reinforcement when it was expected and this led to HPA axis activation (Coover et al., 1977).

According to Levine (2000), the most important deduction to be made in investigating psychological factors activating stress responses is that cognitive processes are involved. These include those processes activated at the onset of the stressful situation as well as the cognitive representations of previous experiences. Arousal is increased when discrepancies occur between these two cognitive processes and the HPA axis is activated as a result (Hennessy et al., 1979). It is evident that psychological factors regulate physiological stress responses (Levine, 2000). In support, other studies also demonstrated that psychosocial stress or the lack of predictability and control in social situations, result in activated stress responses as well as deterioration of health and well-being (Wilkinson, 1999; Repetti et al., 2002).

Interestingly, adverse early life events do not always have negative outcomes; certain individuals have the capacity to thrive in stress situations whilst others inevitably loose the ability to cope with stressful situations and this has been ascribed to genetic individual differences (de Kloet et al., 2004). Resilience is demonstrated by the

individual who experiences traumatic events and still continues to have positive emotional experiences and exhibit minor disruptions in their functional abilities. Maddi and Kobasa (1984) have demonstrated those resilient individuals' exhibit high levels of challenge, commitment and control in their lives and that they have learned to use their problem-solving capabilities effectively.

In summary, adverse events early in life have frequently been associated with psychopathology later in life. Previously we have shown that maternal separation as well as TDS results in the development of behavioural and neuroendocrine abnormalities (Daniels et al., 2004; Uys et al., 2006). Whilst the hormonal changes were pronounced, the behavioural alterations were not entirely convincing.

The present study therefore hypothesised that the combined stressful events of maternal separation and TDS would yield stronger evidence of behavioural abnormalities. This proposal seems acceptable since the human scenario often reflects a combination of stressful conditions. In order to investigate the effects of multiple adverse life events on behaviour during adulthood, we subjected rats to various forms of stress at different time periods throughout the animals' lifespan. To elucidate the underlying mechanisms that may precipitate the proposed behavioural abnormalities, we collected blood for ACTH and corticosterone determinations, and brain tissue (frontal cortex, dorsal and ventral hippocampus) for neurotrophin (BDNF, NGF and NT-3) measurements. Adult behavioural responses were evaluated using the elevated plus maze and the open field.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

All chemicals used were of purest grade and of highest standard available.

2.2 Animals

Male Sprague-Dawley offspring (n = 40) were used in all experiments. Animals were kept in the animal facility at Stellenbosch University. All procedures were approved by the Committee for Experimental Animal Research of the University. Rats were housed (2-3) in 40 x 25 x 20 cm Plexiglas boxes, with a metal grid and sawdust as bedding. Temperature was kept constant at 22 ºC, humidity at 55 % and food and water was available *ad libitum* for the full duration of the experiment.

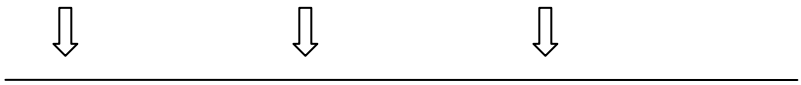
2.3 Experimental Groups

- Control

PND:	**1**	**21**	**28**	**35**	**36**	**42**	**60**	**61**	**67**	**68**
Exp Con:		**W+H**	**H**	**H**	**H**	**H**	**H**	**H**	**B**	**D**

Figure 2.1 Study design of control group. W, weaned, H, handled, B, behaviour, and D, decapitation.

- Maternal separation and re-stress



PND:	**2**		**14**	**21**		**28**	**35**	**36**	**42**	**60**	**61**	**67** **68**
Exp Con:		**MS**		**W+H**		**H**	**RS**	**H**	**H**	**RS**	**H**	**B** **D**

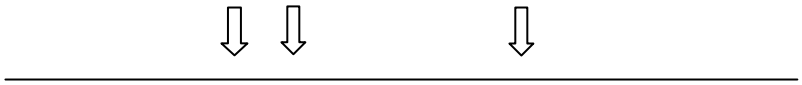
Figure 2.2 Study design of maternal separation group. MS, maternal separation, RS, re-stress.

- Maternal separation with TDS and re-stress

PND:	**2**		**14**	**21**		**28**	**35**	**36**	**42**	**60**	**61**	**67** **68**
Exp Con:		**MS**		**W+H**		**TDS**	**RS**	**H**	**H**	**RS**	**H**	**B** **D**

Figure 2.3 Study design of maternal separation with TDS group. TDS, time-dependent sensitisation.

- TDS and re-stress



PND:	**1**			**21**		**28**	**35**	**36**	**42**	**60**	**61**	**67** **68**
Exp Con:				**W+H**		**TDS**	**RS**	**H**	**H**	**RS**	**H**	**B** **D**

Figure 2.4 Study design of the TDS group.

2.4 Maternal separation and re-stress

Birth was designated as post natal day (PND) 1. Maternal separation occurred from PND 2 until PND 14 for 180 minutes between 08h30-13h00 each day. Maternal separation involved removing the dam from the home cage containing the pups in a heated deprivation room (room temperature was kept between 31 ºC and 33 ºC). The deprivation room was kept under the same lighting conditions as the colony room. The dam was returned to the colony room during the deprivation period. After the deprivation period the dam was placed back with the pups in the heated deprivation room and the whole cage containing the dam and pups were returned to the colony room.

The maternally separated animals also underwent re-stress swimming sessions on PND 35 and PND 60. This served as additional stresses in adolescence and adulthood. Other researchers in the field using animal models of maternal separation did not re-stress after the postnatal period. This was therefore the first study that focused on the effects of repeated stress on the behaviour and neurochemistry of adult rats. For the remainder of the time, rats were left undisturbed except for handling on PND 42 and 61.

2.5 Trauma and re-stress

The time dependent sensitisation (TDS) model is based on the exposure of 3 different stressors of increasing intensity, followed by a single exposure (re-stress session) 7 days after the last stressor (Yehuda et al., 1993b; Liberzon et al., 1997; Harvey et al., 2003). The TDS model is a useful animal model for studying post traumatic stress disorder (PTSD), an anxiety disorder that develops after a person has experienced a traumatic event and is characterised by the person re-experiencing the trauma either by memories of the event in the form of flashbacks or actually reliving the whole experience emotionally. The re-stress sessions of the TDS model therefore serve as situational reminders of the traumatic event, which in turn maintain behavioural disturbances. We employed the TDS model to control rats as well as maternally separated rats to

investigate whether the effects of a traumatic event was exacerbated by prior exposure to early life adverse events.

2.5.1 TDS Procedure

The TDS procedure involved a triple stressor on PND 28 followed by a re-stress 7 days later (PND 35 = adolescence) and a second re-stress 25 days later (PND 60 = adulthood).

Rats were placed in a plexiglass restrainer for 2 hours with the tail gate adjusted to ensure restraint without impairing circulation to the limbs. Immediately after restraint stress, rats were subjected to a 20 min forced swim stress, where rats were placed individually in 18 cm deep ambient water (25 ºC) in a perspex swim tank. After the swim stress, rats were towel dried and returned to their cages and left undisturbed for 30 min. Thereafter the rats were exposed to ether vapours until loss of consciousness, upon which they were returned to their home cages and left undisturbed until the re-stress sessions.

A separate group of animals that were subjected to TDS, were also re-stressed on PND 35 and 60. Once again the re-stress involved a 20 min forced swim in 18 cm ambient water (25 ºC) in a perspex swim tank. For the remainder of the time animals were left undisturbed except for handling on PND 42 and 61.

2.6 Behavioural Assessment

Behavioural assessment occurred on PND 67. Behaviour was analysed using the open field and elevated plus maze. Behaviour was scored manually by at least 2 independent observers that were blind to the experimental protocol.

2.6.1 Open field

Behavioural responses were recorded using a video camera (Sony Digital Handycam, DCR-TRV 130E, Japan) during a 5 min observational period in the open field. The open field is a 1 m x 1 m plexiglass apparatus 50 cm in height. The field is divided into an outer and inner zone. The field was cleaned with 70 % alcohol after each observation period, to prevent the effect of scent on animal behaviour. The number of squares crossed in the open field was counted and served as a measure of activity. Rearing (rising on the hind limbs and a measure of exploratory behaviour) was used as an additional measure of activity.

2.6.2 Elevated plus maze

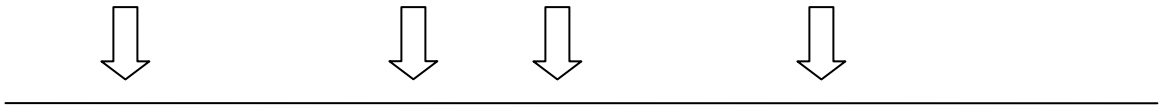
The elevated plus maze test is based on the fact that a novel environment elicits both curiosity and fear in the rat, since the rat is anxious and wants to avoid the novel environment but at the same time is curious and wants to explore the new environment (Rodgers et al., 1997). Each rat spent 5 min on the elevated plus maze. The rats were placed in the centre of the maze and allowed to move into either the open or the closed arms. The number of entries was counted into both the open and closed arms and the time spent in the closed and open arms were recorded with stopwatches. The 'ratio time' was calculated as the time spent in the open arms divided by the total time spent on the maze and served as an indication of fear and anxiety. Rearing behaviour was indicative of relaxed behaviour, especially if this occurred in the open arms. The maze was cleaned with 70 % alcohol after each behavioural recording to avoid scent affecting other animals' behavioural responses.

2.7 Decapitation

Animals were decapitated in a separate room on PND 68 and trunk blood collected in pre-cooled ethylenediamine tetraacetate (EDTA) test tubes. The blood samples were

then centrifuged at 3 000 rpm for 10 min at 4 ºC to separate the plasma, whereafter the samples were frozen at -80 ºC until assayed.

Brains were dissected into frontal cortex and dorsal and ventral hippocampus. The brain tissue samples were frozen in liquid nitrogen and stored at -80 ºC for neurotrophin determinations at a later stage.



PND:	2	14	21	28	35	42	60	61	67	68
Exp Con:	MS		W+H	TDS	RS	H	RS	H	B	D

Figure 2.5 Representation of study design on a time line.

2.8 Sample Analysis / Biochemical assays

2.8.1 Adrenocorticotrophic hormone (ACTH) determination

Plasma ACTH concentrations were determined using a solid phase immunoradiometric (IRMA) assay (EURIA-ACTH c.t., Euro-diagnostica, Medeon, Sweden), which is commercially available. An IRMA is different from a RIA in that the radio-labelled antibody is used in excess and does not compete with the unlabelled antibody.

Two polyclonal antibodies (Ab) were used in excess that bound to different epitopes on ACTH. The kit supplied test tubes coated with primary sheep anti-rabbit anti-ACTH. This antibody bound non-competitively to the C-terminal region of ACTH. The radio-iodinated sheep IgG (^{125}I ACTH-antibody) antibody bound to the amino terminal region of ACTH, forming a sandwich type complex. The excess radioactive tracer was removed by aspiration and washing. The remaining radioactivity in the test tubes was measured

using a gamma counter and was directly proportional to the concentration of ACTH in the sample.

2.8.2 Corticosterone determination

Plasma corticosterone concentrations were determined using commercially available radioimmunoassay (RIA) kits (ImmuChem^{TM} Double Antibody Corticosterone ^{125}I RIA kit, MP Biomedicals, Orangeburg, NY). The RIA is a competitive-binding immunoassay in which a known concentration of radio-labelled hormone competes with the unknown concentration of unlabelled hormone in the binding of a known amount of antibody. As the fraction of unlabelled hormone bound to the antibody increases, the bound fraction of radio-labelled hormone decreases and vice versa. Known concentrations of unlabelled hormone were included in the kit to construct a dose-response (standard) curve and the unknown concentration of hormone was extrapolated from the data. Radioactivity was measured by using the gamma counter.

2.8.3 Neurotrophin level determination

Neurotrophin levels were measured using an enzyme-linked immunosorbent assay (ELISA). The ELISA is based on the same principles of a RIA and IRMA, but detection is non-radioactive. A sandwich formation is formed in the assay. First, plates are coated with a monoclonal antibody (mAb) to bind soluble neurotrophins. Thereafter the bound neurotrophin binds to the primary or polyclonal Ab. The excess unbound pAb is washed off and the bound pAb binds to the secondary Ab, which is conjugated to horseradish peroxidase (HRP). This catalytic reaction of the enzyme linked to the secondary antibody leads to the colour production and enables quantification of neurotrophin concentration, since the colour intensity is proportional to the amount of neurotrophin present.

Dorsal and ventral hippocampus and frontal cortex brain tissue samples were weighed and suspended in 400 µl lysis buffer (137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 %

Nonidet P-40, 10 % glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 1 µg/ml leupeptin and 0.5 mM sodium vanadate). The samples were then sonicated for 30 s in the lysis buffer and vortexed and centrifuged at 12 000 rpm at 4 ºC for 30 min and the supernatants were aliquoted into test tubes.

BDNF, NGF and NT-3 concentrations were determined using the Emax ImmunoAssay system (Promega, Madison, USA). Flat bottom 96 well Greiner ELISA plates were first coated with a mix containing carbonate coating buffer (pH 9.7) and either anti-BDNF mAb or anti-NGF pAb or Anti-Human NT-3 pAb for an overnight incubation at 4 ºC. The following day, the plates were blocked with 1 x block & sample buffer for a 1 h incubation period at room temperature to prevent non-specific binding. Next, a linear standard curve was generated for each plate by performing serial dilutions of known concentrations of BDNF, NGF and NT-3 in duplicate. The linear range for BDNF and NGF extended from 0-500 pg/ml and for NT-3 from 0-300 pg/ml. Sample dilutions (100 µl) were made and added in duplicate to the rest of the wells and incubated for 2 h (BDNF) or for 6 h (NGF and NT-3) with shaking at room temperature. The plates were then incubated with Anti-Human BDNF pAb (for 2 h at room temperature with shaking), Anti-NGF mAb and Anti-NT-3 mAb (overnight at 4 ºC). Following this, plates were incubated with Anti-IgY HRP Conjugate for 1 h at room temperature (BDNF), Anti-Rat IgG HRP Conjugate (NGF) and Anti-Mouse IgG HRP Conjugate (NT-3) for 2.5 h at room temperature. Peroxidase activity was colorimetrically detected by adding TMB solution to wells for an incubation period of 10 min (BDNF and NGF) and 15 min (NT-3) with shaking at room temperature. The enzymatic reaction was stopped by adding 1 M HCl to the wells. Within 30 min after stopping the reaction, the optical density (absorbance) of each well was read at 450 nM on a Bio-Rad benchmark microplate reader utilising the Microplate Manager version 5.2 software. Quantification of neurotrophins is expressed as pg/mg wet weight.

Experimental Groups (n=10)	PND 2-14	PND 21	PND 28	PND 35	PND 42	PND 60	PND 61	PND 67	PND 68
Control	-	W+H	H	H	H	H	H	B	D
MS	MS	W+H	H	RS	H	RS	H	B	D
MS + TDS	MS	W+H	TDS	RS	H	RS	H	B	D
TDS	-	W+H	TDS	RS	H	RS	H	B	D

Table 2.1 Summary of study design. H, handled; W, weaned; TDS, triple stressor; RS, re-stress; B, behaviour; D, decapitation.

2.9 Statistical Analysis

The non-parametric equivalent of the analysis of variance (ANOVA), the Kruskal-Wallis test was used to analyse behaviour, ACTH, CORT and neurotrophin data. Statistical significance of the χ^2-statistic was followed by non-parametric independent groups Mann-Whitney U post hoc tests. Non-parametric tests were used because of small sample sizes per group and an $n < 10$ cannot be shown to follow a Gaussian distribution. Statistical tests were all done with the aid of the SPSS (version 13.0) statistical programme. The level of significance was accepted as $p<0.05$.

CHAPTER 3: RESULTS

The effects of different stress paradigms on the rats' behaviour and neurochemistry were investigated, yielding a number of interesting observations. Comparisons between the control group and the various stressed groups, as well as between the different stress groups were made and are reported in this chapter.

Abbreviations: MS + TDS = animals subjected to both maternal separation and time-dependent sensitisation stress.
MS = maternal separation followed by two additional stressors
TDS = time-dependent sensitisation stress group.

3.1 Behavioural analysis in the elevated plus maze (EPM) and the open field

On the elevated plus maze it was noted that the number of entries into the open arms of the MS + TDS group tended to be about half that of the other groups (1.7 ± 0.63 number of entries into the open arms of the MS + TDS group versus 4.3 ± 0.87 in the MS group). However, none of the findings on the EPM reached statistical significance (Table 3.1).

Kruskal-Wallis non-parametric analysis equivalent to the ANOVA was again performed on the parameters of the open field. Whilst the number of blocks crossed was at least 20 % lower in the MS + TDS group compared to all the other groups, this difference was not significant. Similarly, a 35 % decrease in time spent in the inner zone in the MS and MS + TDS groups compared to TDS and controls, did not reach significance. Therefore none of the measurements reached meaningful differences between groups (Table 3.2).

Table 3.1 Behavioural parameters measured on the elevated plus maze. Values presented as mean ± SEM. Statistical analysis: Kruskal-Wallis (non-parametric ANOVA), * $p < 0.05$. Control: n = 10, MS: n = 10, TDS; n = 10, MS + TDS: n = 10.

Group		Nr. Entries Open Arm	Nr. Entries Closed Arm	Time / Total Time	Rearing
MS + TDS	Mean ± SEM	1.7 ± 0.63	11.4 ± 1.47	0.1 ± 0.02	6.5 ± 1.46
MS	Mean ± SEM	4.3 ± 0.87	11.8 ± 1.03	0.2 ± 0.04	3.7 ± 0.80
TDS	Mean ± SEM	3.4 ± 0.69	11.1 ± 1.14	0.2 ± 0.06	5.6 ± 1.70
Control	Mean ± SEM	3.2 ± 0.61	12.0 ± 1.32	0.2 ± 0.05	7.4 ± 1.38

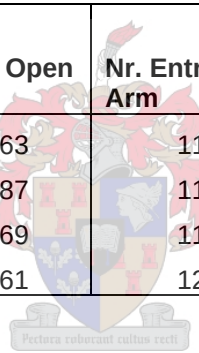


Table 3.2 Behavioural parameters measured on the open field. Values presented as mean ± SEM. Statistical analysis: Kruskal-Wallis (non-parametric ANOVA), * $p < 0.05$. Control: n = 10, MS: n = 10, TDS; n = 10, MS + TDS: n = 10.

Group		Nr. Blocks	Nr. Crossings	Time Inner Zone	Time Outer Zone	Rearing
MS + TDS	Mean ± SEM	80.3 ± 12.28	8.8 ± 2.67	10.3 ± 3.92	289.8 ± 3.92	12.3 ± 3.79
MS	Mean ± SEM	120.9 ± 9.32	9.0 ± 2.31	10.1 ± 2.98	289.9 ± 2.98	11.3 ± 1.71
TDS	Mean ± SEM	106.0 ± 12.92	8.4 ± 2.42	16.0 ± 5.75	284.0 ± 5.75	14.1 ± 2.05
Control	Mean ± SEM	108.8 ± 8.37	11.0 ± 2.28	17.0 ± 4.26	283.0 ± 4.26	12.7 ± 3.39

3.2 The effects of repeated stress on basal plasma adrenocorticotrophin (ACTH) and corticosterone levels in control and stressed groups

Animals subjected to maternal deprivation showed significant decreased basal ACTH levels when compared to the control rats (p=0.049), as well as to the TDS group (p=0.016). Maternally deprived animals that were also exposed to TDS, displayed significantly reduced ACTH concentrations when compared to TDS group only (p=0.049). Interestingly, control and TDS rats had similar ACTH levels, whilst MS and MS + TDS animals showed comparably lower ACTH concentrations (Figure 3.1).

The plasma corticosterone concentrations of all the groups showed a pattern that mirrored the ACTH results. Animals that underwent maternal deprivation, demonstrated decreased corticosterone levels when compared to controls (p=0.049) as well as to rats subjected to the TDS (p=0.004). Contrary to the ACTH data, animals subjected to a combination of MS + TDS did not differ significantly from any of the groups (Figure 3.2).

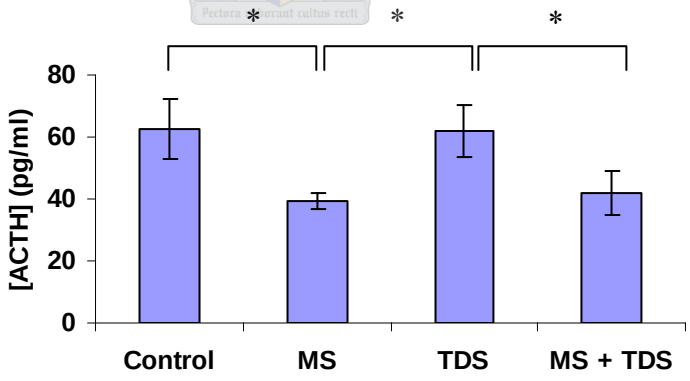


Figure 3.1 ACTH concentrations in pg/ml in the control and stressed groups. Values are given as mean ± SEM. Statistical analysis: Kruskal-Wallis (non-parametric ANOVA) ($\chi^2_{.05}(3) = 8.747$, p=0.033) followed by Mann-Whitney U (independent groups, non-parametric) post hoc test, * $p<0.05$, n = 10 per group.

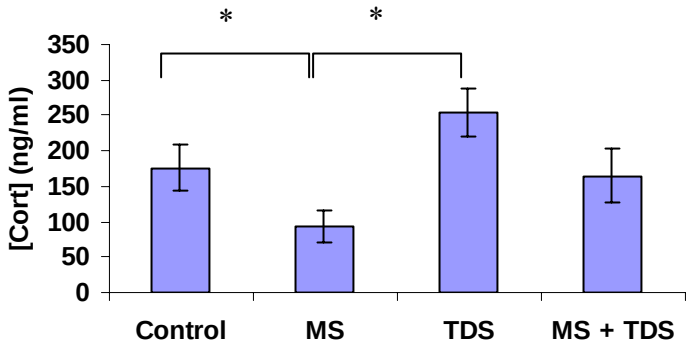


Figure 3.2 Corticosterone concentrations in ng/ml in the control and stressed groups (MS, TDS and MS + TDS). Data is presented as means ± SEM. Statistical analysis: Kruskal-Wallis (non-parametric ANOVA) ($\chi^2_{.05}(3) = 9.43$, p=0.024) followed by Mann-Whitney U post hoc test, * $p<0.05$, n = 10 per group.

3.3 The effects of repeated stress on brain-derived neurotrophic factor (BDNF) protein levels in both the dorsal and ventral hippocampus and the frontal cortex

Animals subjected to TDS stress displayed decreased brain-derived neurotrophic factor (BDNF) levels in the dorsal hippocampus when compared to control rats (p=0.019), as well as to the MS + TDS group (p=0.022). The maternally separated animals exhibited BDNF levels similar to controls, that were significantly higher than those of the levels of the TDS stress animals (p=0.026) (Figure 3.3).

There was a significant increase in BDNF protein levels of the ventral hippocampus when the MS + TDS group were compared to the control (p=0.014), maternal deprivation (p=0.034) and the TDS stress (p=0.011) groups (Figure 3.4).

The various stress paradigms employed resulted in no significant effect on BDNF protein concentrations in the frontal cortex. All the stressed groups had similar BDNF protein levels compared with controls (Figure 3.5).

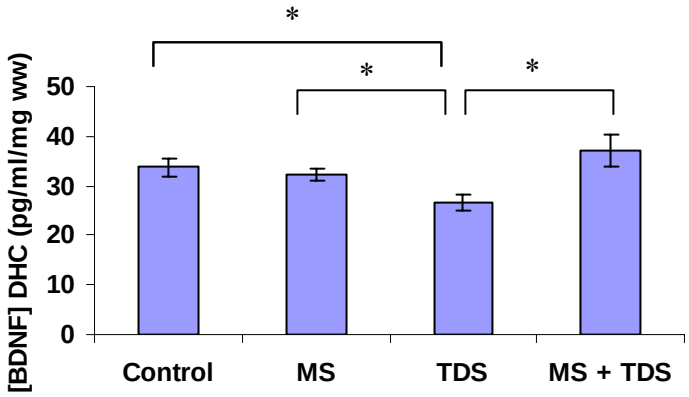


Figure 3.3 Brain-derived neurotrophic factor (BDNF) concentration in pg/ml/mg ww in the dorsal hippocampus (DHC). Data is presented as mean ± SEM. Statistical analysis: Kruskal-Wallis (non-parametric equivalent of ANOVA) ($\chi^2_{.05}(3) = 9.641$, p=0.022) followed by Mann-Whitney U post hoc test, * p<0.05. Control: n = 10, MS : n = 8, TDS: n = 10, MS+ TDS: n = 9.

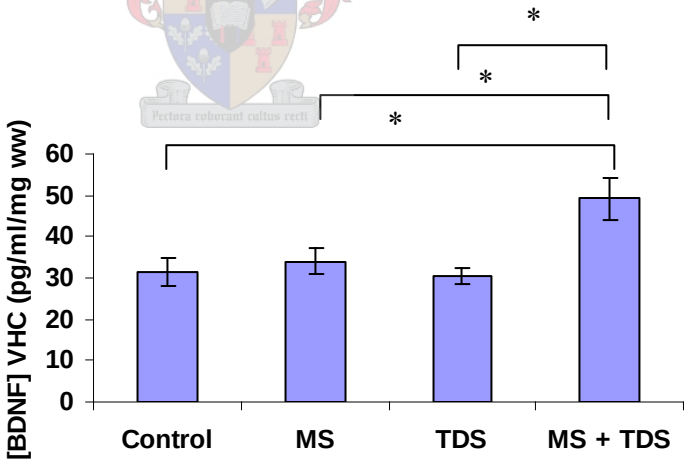


Figure 3.4 Brain-derived neurotrophic factor (BDNF) concentration in pg/ml/mg ww in the ventral hippocampus (VHC). Values are presented as mean ± SEM. Statistical analysis: Kruskal-Wallis (non-parametric ANOVA) ($\chi^2_{.05}(3) = 9.139$, p=0.027) followed by Mann-Whitney U post hoc test, * p<0.05. Control: n = 9, MS: n = 9, TDS: n = 9, MS + TDS: n = 10.

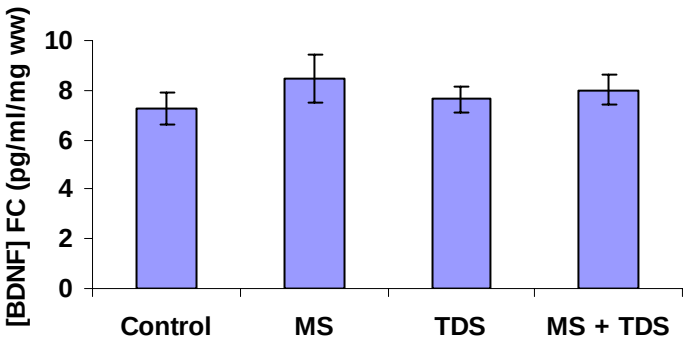


Figure 3.5 Brain-derived neurotrophic factor (BDNF) concentration in pg/ml/mg ww in the frontal cortex (FC). Data is presented as mean ± SEM. Statistical analysis: Kruskal-Wallis (non-parametric ANOVA) ($\chi^2_{.05}(3) = 2.304$, p=0.512), * $p<0.05$. Control: n = 10, MS: n = 10, TDS: n = 10, MS + TDS: n = 9.

3.4 The effect of repeated stress on nerve growth factor (NGF) concentration in the dorsal and ventral hippocampus

Maternally separated animals exhibited significantly higher nerve growth factor (NGF) protein levels in the dorsal hippocampus when compared to the non-stressed control animals (p=0.0001). The same was observed in the combined stress group, consisting of both maternal deprivation and TDS stress, having meaningful increased NGF protein levels compared to the control animals (p=0.007). The dorsal hippocampus NGF levels of the maternally deprived group (p=0.002) and the combined group (MS + TDS) (p=0.007) were also significantly higher when compared to the TDS stress animals (Figure 3.6).

Maternally deprived animals showed increased NGF levels in the ventral hippocampus when compared to the control group (p=0.005). NGF levels in the ventral hippocampus were significantly increased in the animals subjected to maternal separation and TDS stress (MS + TDS) when compared to control animals (p=0.006) and the TDS stress group (p=0.003) (Figure 3.7).

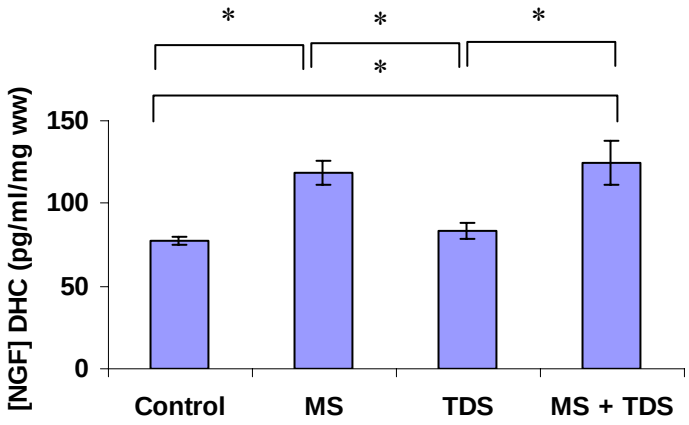


Figure 3.6 Nerve growth factor (NGF) protein levels in pg/ml/mg ww in the dorsal hippocampus (DHC). Values are given as mean ± SEM. Statistical analysis: Kruskal-Wallis (non-parametric ANOVA) ($\chi^2_{.05}(3) = 18.684$, p=0.0001) followed by Mann-Whitney U post hoc test, * p<0.05. Control: n = 9, MS: n = 9, TDS: n = 10, MS + TDS: n = 9.

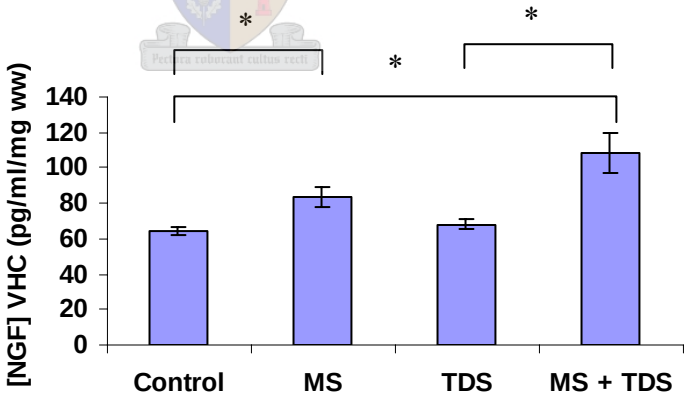


Figure 3.7 Nerve growth factor (NGF) protein levels in pg/ml/mg ww in the ventral hippocampus (VHC). Values are given as mean ± SEM. Statistical analysis: Kruskal-Wallis (non-parametric ANOVA) ($\chi^2_{.05}(3) = 15.714$, p=0.001) followed by Mann-Whitney U post hoc test, * p<0.05. Control: n = 8, MS: n = 9, TDS: n = 10, MS + TDS: n = 10.

3.5 The effect of repeated stress on neurotrophin-3 (NT-3) concentrations in the dorsal and ventral hippocampus and the frontal cortex

Neurotrophin-3 (NT-3) protein levels in the dorsal hippocampus were significantly higher in the maternally deprived animals (p=0.01) and combined stress group (MS + TDS) (p=0.05) when compared to the non-stressed control group. When animals of the maternally separated group were compared with the TDS stress group, a meaningful increase in NT-3 protein levels in the MS group was observed in the dorsal hippocampus (p=0.0001). The TDS stress animals also showed significantly lower NT-3 protein levels in the dorsal hippocampus when comparing these rats to the group exposed to both maternal deprivation and TDS stress (p=0.011) (Figure 3.8).

Significantly higher NT-3 levels in the ventral hippocampus were found in the combined stressor group (MS + TDS) when compared to both the maternal deprivation (p=0.001) and the TDS stress group (p=0.0001), as well as to the control animals (p=0.001) (Figure 3.9).

A similar effect was observed in the frontal cortex regarding NT-3 protein levels. The combined stress animals (MS + TDS) exhibited increased NT-3 levels when compared to other stress paradigms employed, TDS (p=0.001) and MS (p=0.016), including the control group (p=0.001), this being significantly so (Figure 3.10).

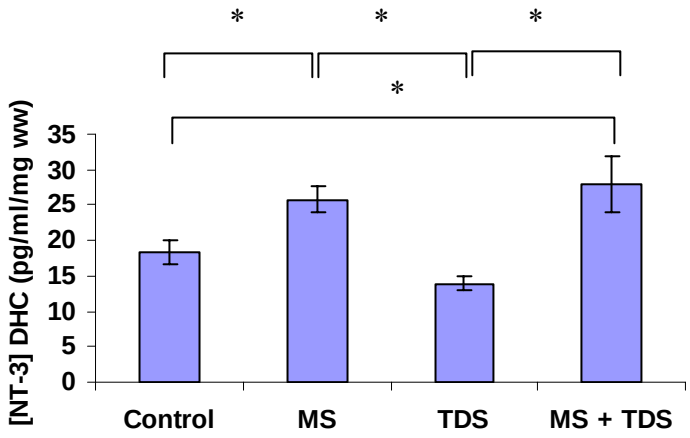


Figure 3.8 Neurotrophin-3 (NT-3) protein levels in pg/ml/mg ww in the dorsal hippocampus (DHC). Data is presented as mean ± SEM. Statistical analysis: Kruskal-Wallis (non-parametric ANOVA) ($\chi^2_{.05}(3) = 17.230$, $p=0.001$) followed by Mann-Whitney U post hoc test, * $p<0.05$. Control: n = 10, MS: n = 10, TDS: n = 10, MS + TDS: n = 9.

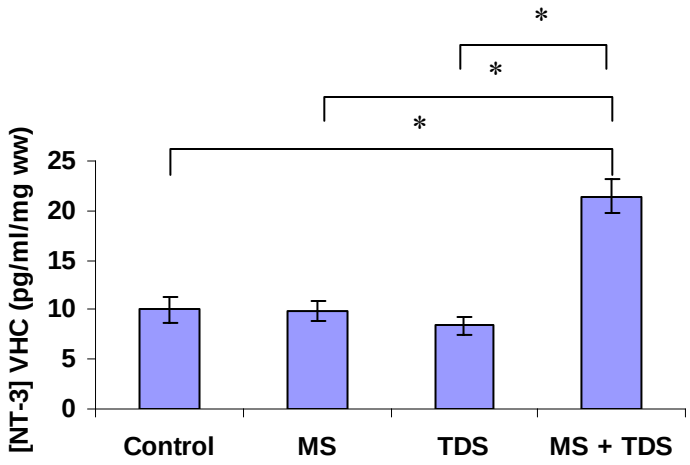


Figure 3.9 Neurotrophin-3 (NT-3) protein levels in pg/ml/mg ww in the ventral hippocampus (VHC). Data is presented as mean ± SEM. Statistical analysis: Kruskal-Wallis (non-parametric ANOVA) ($\chi^2_{.05}(3) = 20.633$, p=0.0001) followed by Mann-Whitney U post hoc test, * p<0.05. Control: n = 8, MS: n = 8, TDS: n = 10, MS + TDS: n = 10.

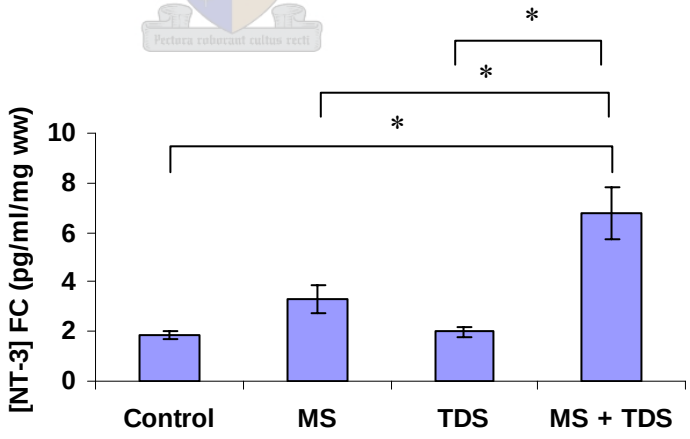


Figure 3.10 Neurotrophin-3 (NT-3) protein levels in pg/ml/mg ww in the frontal cortex (FC). Data is presented as mean ± SEM. Statistical analysis: Kruskal-Wallis (non-parametric ANOVA) ($\chi^2_{.05}(3) = 16.348$, p=0.001) followed by Mann-Whitney U post hoc test, * p<0.05. Control: n = 9, MS: n = 10, TDS: n = 10, MS + TDS: n = 8.

CHAPTER 4: DISCUSSION

When individuals are subjected to traumatic adverse events early in life, a possibility exists that they may develop psychopathologies later in life. We have previously demonstrated that maternal separation and TDS stress on their own have the ability to elicit behavioural and neurochemical abnormalities that persisted into adulthood. Whilst the neurochemical changes were dramatic, the behavioural alterations were more moderate (Daniels et al., 2004; Uys et al., 2006). In the present study, animals were exposed to repeated stressful events that included maternal separation combined with TDS, as well as another swim stress session on PND 60. These stressors represent adverse experiences at various periods in the life cycle of the rat. The aim therefore was to determine whether repetitive stress – as a better representation of what a person would experience during a lifetime - would result in cumulative behavioural and/or neurochemical changes.

4.1 Behaviour

The behaviour of stressed and non-stressed animals was assessed on the elevated plus maze and open field test. Our data from both these tests yielded no significant changes in any of the parameters measured. However, tendencies for decreased locomotor activity were observed in both tests for the MS + TDS group. Animals subjected to maternal separation also tended to spent less time in the inner zone of the open field. Whilst these decreases were between 20 and 35 %, the inability to demonstrate statistical significance for this difference is likely to be a result of inadequate power (n = 10) and the degree of individual variability within the measured parameter (Tabel 3.1 and Tabel 3.2).

4.1.1 *The effects of maternal separation on adult behaviour*

A number of studies have demonstrated that maternal separation has long-term effects on brain development which may lead to anxiety related behaviours in adulthood (Huot

et al., 2002; Roceri et al., 2004; Daniels et al., 2004). It was therefore surprising not to observe any significant changes within our group of animals that were subjected to maternal separation, this despite rigorous attention to experimental conditions as in the previous study by Daniels et al. (2004). These included the same rat strain, the same time of day on the day of separation, as well as sticking to the same separation methodology i.e. removal of the dam from the pups without handling of pups. When compared to our previous experiments (Daniels et al., 2004), one difference that may explain the inconsistency of our results was the place where the experiments were actually performed. The present study was conducted at the Animal Facility of the Faculty of Natural Sciences, University of Stellenbosch at Stellenbosch, whereas the earlier study was done at another site in the Animal Facility of the Faculty of Health Sciences on the Tygerberg Campus of the University. These facilities are 30 km apart. It may therefore be possible that the environmental conditions of the housing facilities at the Stellenbosch site were of such a nature that it neutralised the development of affective behaviours. It has been shown that more enriching environment can improve brain development, learning and memory and neurobiological plasticity (Mohammed et al., 2002; Fernández-Teruel et al., 2002). An additional and perhaps more important difference was the change in protocol. Although animals were subjected to a similar maternal separation paradigm, additional stress sessions on PND 35 and 60 were added to the experimental protocol in the present study. It may therefore be possible that these stress sessions could have dampened possible behavioural changes. Alternatively the handling that was associated with the extra stress sessions could also have contributed to the present results as handling per se has been shown to reduce anxiety levels in rats (Meaney et al., 1991).

In an analogous study Kalinichev et al. (2002) investigated the behavioural responses of maternally separated male and female rodents using a similar maternal separation paradigm as in our study i.e. 3 hours daily from PND 2 until 14. These authors compared their separated animals to non-handled controls on the elevated plus maze in dim and normal light conditions, and reported that the percentage of maternally separated animals entering the open arms of the elevated plus maze decreased by at least 60%

under normal lighting conditions when compared to dim lighting conditions. In addition, these animals entered the closed arms of the elevated plus maze substantially more than their non-handled controls. These findings highlighted the impact of lighting conditions on behavioural parameters and emphasised the importance of keeping lighting conditions constant between experiments. As we never measured the lighting of our two facilities, this factor may also have contributed to the difference in results obtained between the two studies.

Using the same maternal deprivation procedure (3 hours from PND 2 until 14), Huot et al. (2004) measured anxiety-like behaviours using the elevated plus maze and compared their 3 hour maternally separated animals to a control group that was briefly separated for 15 min. In this study no differences between groups regarding entries and time spent on both the open and closed arms of the maze were found. These results were comparable to the observations obtained in the present study even though their control group was subjected to brief handling.

The findings of the above-mentioned studies clearly show that the effects of maternal separation on behaviour are complex and can be confounded by many factors. In this regard it is interesting to note that differential behavioural results are seen as factors of the timing and duration of maternal separation. A maternal separation period shorter than 3 hours between PND 3 and 10 resulted in increased anxiety levels on the elevated plus maze (Wigger et al., 1999). On the other hand a longer period of maternal separation (3 hour deprivation period over the first three weeks of life) resulted in attenuated levels of anxiety (McIntosh et al., 1999).

Animals separated between PND 2 and 10 for 6 hours daily, showed no meaningful differences in behaviour compared to control rats on open field activity when tested in adulthood (Rhees et al., 2001). A reduction in activity levels was observed in the open field when animals were subjected to a 4.5 hour maternal separation daily for the first 3 weeks of life (Ogawa et al., 1994). From this it is clear how different maternal separation paradigms can lead to contrasting results, and therefore standardisation of the

duration and timing of maternal deprivation is necessary to allow more accurate comparison of data.

It is also evident that there is great variability in terms of the control groups used in the different maternal separation studies. According to Matthews et al. (2003) a non-handled control group ought to be described as animal facility reared animals, as these animals are subjected to a measure of handling or brief periods of separation with normal husbandry practices. In view of this, some studies included another control group – one where the rats were exposed to brief handling. This was considered important as early handling paradigms had been shown to reduce the levels of fear in adulthood as tested on the elevated plus maze and the open field test (Levine et al., 1967b; Bodnoff et al., 1987). Since our control groups were not subjected to brief handling during the separation period, it may therefore be possible that this aspect of the experimental design could have contributed to the resulting discrepancies in observations – neuroendocrine parameters and neurotrophin levels.

In addition, the maternal separation paradigm itself may bring about changes in maternal behaviour upon reuniting the dam with her pups, thus changing the dynamics of mother-pup interactions. It was found that mothers licked and groomed their pups more often when pups were subjected to handling when compared to non-handled animals. The handling also resulted in the dam having more frequent nest bouts (Liu et al., 1997). The change in mother-pup interactions may therefore mediate long-lasting changes within the neuroendocrine system and mediate behavioural responses of individuals to stressors. This had been referred to by Denenberg et al. (1999) as the 'maternal mediation hypothesis', or the non-genomic behavioural mode of transmission of maternal behaviour on the stress response of the pups (Francis et al., 1999). Adult rats who receive high levels of licking, grooming and arched-back nursing postnatally, display fewer fear-related behaviours and a moderate HPA response to subsequent stress (Francis et al., 1999). Interestingly, cross fostering pups of low LG-ABN reared by high LG-ABN mothers, resulted in offspring that were less anxious when exposed to novel environments. These animals spent significantly more time exploring the inner zone of

the open field, when compared to pups of high LG-ABN mothers reared by low LG-ABN dams (Francis et al., 1999). In the present study we did not separate dams based on high and low LG-ABN behaviours, and as such variances in mother-pup interactions within the separated group of animals too, could have masked differences between the maternally separated group of rats and their respective controls.

It therefore seems that maternal mediation is an essential component in the development of behavioural and endocrine responses that impact the animal's ability to cope with future stressors, and not to predispose the animal to possible pathologies later in life. It may be expected that the removal of maternal mediation by means of the maternal separation paradigm may lead to deleterious effects later in life, similar to when children are exposed to neglect and abuse and go on to develop psychopathologies in adulthood (Heim et al., 1997; Ladd et al., 2000). However the vast number of variations in the currently used maternal separation protocols in different laboratories makes it difficult to draw firm conclusions as to the exact nature of the resultant behavioural abnormalities.

4.1.2 *The effects of the time-dependent sensitisation stress model on adult behaviour*

Various animal models have been developed to demonstrate that traumatic experiences or stressful situations result in long term behavioural and endocrinological abnormalities. The time-dependent sensitisation stress or the stress-restress model is based on such a traumatic experience followed by situational reminders (Liberzon et al., 1997). These situational reminders serve the purpose of preventing desensitisation of the traumatic event. Pynoos et al. (1996) used a modified TDS model, in which animals received a 10 second electric shock trauma, followed by a 1 minute electric shock once a week over a 6 week study period, to serve the purpose of sensitisation to a prior traumatic experience. Behavioural assessment occurred via exploratory behaviour in a neutral environment (clear plastic cage) and a fear-provoking elevated plus maze at 3 time points (24 hrs, 3 weeks, 6 weeks) during the experimental period after the shock trauma. The animals exposed to electrical shock, were further separated into groups with or without situational reminders. Locomotor activity in the neutral environment

significantly increased in the animals that received the shock treatment at the 24 hour time period. However, this effect in activity was ascribed to the shock treatment itself, and not to the shock received as situational reminder, as locomotor activity returned to normal levels by the end of the 3 week time point. It has been suggested that the increased locomotor activity can possibly be ascribed to non-habituation. Interestingly, on the elevated plus maze, abnormal behavioural patterns were observed both in animals that were subjected to situational reminders and those that received only the initial shock treatment. These animals displayed a reduced number of entries into the open arms of the maze, and the degree of this reduction depended on whether the animals were exposed to situational reminders or not. Seventy percent of animals subjected to situational reminders displayed abnormal behaviour as opposed to 50 % of animals that were not. These findings indicated that situational reminders maintain behavioural disturbances to a greater extent, over time.

Importantly, the concept of 'context specificity' comes to mind, in that the purpose of situational reminders is to remind the individual of trauma experienced in the past. In the study of Pynoos et al. (1996), this was achieved by giving additional shock treatments as reminders of the initial shock trauma the animals had received. In our study, using the TDS stress paradigm, the traumatic experience consisted of 3 differential stressors (restraint stress, swim stress and ether exposure) and the reminder was one single stressor that was originally included in the trauma (i.e. swim stress). One could therefore speculate on whether the absence of significant behavioural abnormalities observed in our TDS rats could have resulted from a diminished impact of the situational reminder as its context specificity could have been dampened by the presence of two other stressors during the trauma period. However, a study by Uys et al. (2006) demonstrated that animals subjected to the same TDS protocol displayed excessive rearing behaviour in both the open field and the elevated plus maze when compared to the non-stressed controls. It was suggested that this excessive rearing behaviour reflected high levels of anxiety experienced by the animals that could be associated with the hyper-arousal or hyper-alertness hypothesis. Hyperarousal is linked to the fact that trauma survivors with

PTSD often have higher levels of catecholamines, e.g. adrenaline, under normal resting levels and in response of being reminded of their trauma (Keane et al., 2001).

Another model based on an intense stressful experience is the exposure of a rat to its predator (Adamec et al., 1997). Cohen et al. (2003) used this model, whereby rats were exposed to a cat for a 10 minute period. Only 25.3 % of rats exposed to the predator displayed fear-related behavioural disturbances. This result was considered plausible as only a subset of individuals exposed to trauma develop PTSD (Yehuda et al., 1995). In addition it also underlined the importance of utilising cut-off behavioural criteria. It had been proposed that animals ought to be categorised in two groups; those that are maladapted and exhibit behavioural abnormalities as a result of the trauma and those that are well adapted and do not develop any disturbances after experiencing intense stress. The use of such behavioural criteria would probably result in a better representation of the human condition as animals, similar to humans, appear to respond to stress in different ways, representing the heterogeneity of stress responses. As we did not categorise our TDS group, this may explain why our data on the stressed animals is not significantly different to their controls.

4.1.3 *Behavioural consequences as a result of the combined effects of maternal separation and time-dependent sensitisation stress model*

As far as is known, the effects of the combination of maternal separation and TDS stress on behaviour and neurochemistry on rats has not been investigated before. We have investigated whether TDS stress would enhance the behavioural effects of maternal separation. Surprisingly, no behavioural changes were found to be significant in animals subjected to both maternal separation and TDS stress in our experiments, although tendencies for decreased locomotor activity and increased anxiety levels have been noted. Since no behavioural disturbances have been observed in either stress paradigms when tested independently, it seemed plausible that the additive effect of both, should also not cause any significant behavioural abnormalities (no effect + no effect = no effect). However we and others have convincingly shown that maternal separation can

alter anxiety levels (Wigger et al., 1999; McIntosh et al., 1999; Daniels et al., 2004) and TDS stress can lead to excessive rearing behaviour on the elevated plus maze in adulthood (Uys et al., 2006). We speculate that the exposure to TDS during the adolescent period did not exacerbate the effects of maternal separation, and that these two stress paradigms exerted antagonistic effects on the brain.

Zimmerberg et al. (2002) subjected pre-natally alcohol exposed rats to early weaning, akin to maternal deprivation (negative experience), in order to determine whether this would generate worse behavioural outcomes than in rats that were only exposed to alcohol. They reported that all subjects exposed to alcohol showed poor performance on the Morris water maze, and that early weaning exacerbated this learning impairment. In addition, the early weaned alcohol exposed rats also showed the greatest reduction in growth, but interestingly there was no synergistic effect between alcohol exposure and early weaning with respect to activity levels. These findings indicated that some aspects of behaviour may be exacerbated by repetitive stress (eg. learning), while others are not (eg. motor activity). It may therefore be possible that the parameters used in the present study were not sensitive enough to detect possible influences of MS + TDS on the animals' behaviour. Positive experiences (e.g. handling and environmental enrichment) can neutralise the damaging effects of alcohol exposure on behavioural outcomes. For instance, environmental enrichment increased performance on the Morris water maze of prenatal alcohol exposed rats to the same degree as that of the control animals (Hannigan et al., 1993). Also, 3 minutes of handling daily from PND 2 until 15 resulted in a reversal of the deleterious effects of alcohol exposure. The positive effects included improved growth, thermoregulatory and corticosterone responses to stress in adulthood (Weinberg et al., 1995).

Along the same theme, Lehmann et al. (2000) investigated the long-term effects of maternal separation on emotional and attentional processes, with a background of pre-natal stress. In these experiments, the open field test, active avoidance test (pre-pulse inhibition) and conditioned emotional response paradigms were used to assess the animals' behaviour. The authors were interested in the long-term consequences of

maternal separation in combination with negative pre-natal experiences (e.g. pregnant dams being exposed to restraint stress). The data showed that pre-natal stress decreased open field locomotor activity and delayed active avoidance, which were indicative of impaired emotionality. Maternal separation, on the other hand, led to enhanced pre-pulse inhibition and active avoidance. When combined, the maternal separation neutralised the negative effects of the prenatal stress. It is therefore possible that stressors may interact with each other in such a way that the combined behavioural consequences can not always be explained by the separate effects of individual stressors. It is known that electric shock is an effective treatment for severe major depression (electroconvulsive therapy, ECT) when rapid remission is needed (Coffey et al., 1990). ECT changes physiological stress systems to exhibit behaviour in the direct opposite spectrum of mood and affect, in other words, a shift towards manic behaviour. This means that a stress-related condition is successfully treated by imposing another physical stress upon the patient. We feel that a similar situation may exist in our experiment where rats were subjected to maternal separation and the later exposure to TDS partially cancelled some of its negative effects. Taken together it appears as if the cumulative consequences of dissimilar stressors on behaviour may be varied i.e. synergistic or antagonistic, and seems difficult to predict.

4.2 Hypothalamic-Pituitary-Adrenal axis

Stress stimuli are processed by a number of neuronal pathways leading to the activation of the HPA-axis. The hypothalamus, and in particular the paraventricular nucleus, receives neuronal inputs to regulate the release of CRH and AVP, whichare mediators of the physiological stress response (Sapolsky et al., 1986b; McEwen, 1999). Corticosteroids are terminal secretagogues of the HPA-axis and are secreted from the adrenal glands. Corticosteroids exert their influence on areas expressing mineralocorticoid (MR) and glucocorticoid (GR) receptors. Brain areas expressing corticosteroid receptors include the hypothalamus and pituitary glands and especially the hippocampus where these receptors are highly expressed. Here they play an important role in negative feedback inhibition of the axis. Other limbic areas influenced by

corticosterone include the amygdala and the frontal cortex. These limbic areas mediate cognitive and emotional aspects of the stress response (de Kloet et al., 2004).

4.2.1 *The effects of maternal separation on the HPA-axis*

Dysregulation of the HPA axis is a common feature of many stress-related disorders (Kanter et al., 2001; Raison et al., 2003; Yehuda et al., 2004). Previously we have shown that early life adverse events such as maternal separation result in long lasting effects on HPA axis regulation as demonstrated by elevated baseline ACTH levels as well as a blunting of the ACTH response following restraint stress (Daniels et al., 2004). Our findings confirm abnormal HPA axis activity, similar to a previous study employing a comparable protocol. Interestingly, instead of increased baseline ACTH levels, we observed a reduction in the basal ACTH concentration in the maternal separation group and a corresponding decrease in corticosterone levels (Figure 3.1 and Figure 3.2). The result in the first study i.e. increased basal ACTH following maternal deprivation, was explained by prolonged hypersecretion of CRH. Since the rats were subjected to additional stressors in the present study, it may be possible that the release of CRH from the hypothalamus was even greater causing desensitisation of CRH receptors at the levels of the anterior pituitary, thus leading to diminished stimulation of ACTH release and corticosterone secretion. This seemed plausible as a comparable maternal separation paradigm had been shown to lead to decreased CRF1 receptor binding in cortical areas - frontal cortex, cingulate cortex and parietal cortex. This binding was associated with reduced pituitary-adrenal reactivity upon subsequent stress (Ladd et al., 2005). Therefore it may be possible for similar decreases in CRF1 binding to occur at the level of the pituitary.

Our findings supported the work of others, suggesting that maternal deprivation during periods in which pups require parental attention, results in long lasting neurochemical changes that included changes in the HPA axis (Plotsky et al., 1993; Ladd et al., 1996). However these effects seemed to be variable and depended on the timing as well as the day/s of separation (Newport et al., 2002). For instance, maternal separation from PND 6

until 20 for a 4 to 6 hour period increases ACTH, with normal corticosterone responses to subsequent stressor in adulthood (Ladd et al., 1996). In contrast, a single 24 hour separation on PND 3 resulted in a normal ACTH and increased corticosterone levels in response to aversive stimuli (Workel et al., 1997). While these reports demonstrate variability in responsivity of the HPA-axis when deprivation occurs during various developmental phases, some degree of HPA axis dysregulation is consistent. This however, is not surprising as maternal separation is considered a very severe form of stress, since a single episode of 24 hour maternal deprivation during the SHRP period, in which a mild stressor (e.g. exposure to novelty or saline injection) does not elicit a stress response, has been shown to activate the HPA-axis (Suchecki et al., 1995; Schmidt et al., 2002a,b). When the HPA axis response was investigated more closely, it was found that both ACTH and corticosterone were increased during the first 8 hours of the 24 hour deprivation period. Following the first 8 hour period, corticosterone levels remained elevated, while ACTH levels were dampened by the impact of negative feedback mechanisms of the HPA-axis (Schmidt et al., 2004). These discordant results were ascribed to increased sensitivity of the adrenal glands to ACTH at the later stages of the 24 hour deprivation period (Stanton et al., 1988b; Okimoto et al., 2002). This study therefore suggested that changing a receptor's sensitivity to its ligand, is an important and rapid mechanism through which the HPA axis facilitates the individual's ability to cope with or adapt to stressful conditions.

Elevations in corticosterone may also result in reduced MR and/or GR number in the hippocampus and paraventricular nucleus of the hypothalamus (de Kloet et al., 2004). Since glucocorticoid receptors partly regulate negative feedback of the HPA-axis, a decrease in these receptors in the CNS may cause impaired negative feedback inhibition (Jacobson et al., 1991), thereby maintaining high circulating corticosterone levels. It is therefore evident that maternal care must be an important external factor responsible for the development of normal HPA axis function, since removal of maternal care may lead to increased basal levels of both ACTH and corticosterone (Suchecki et al., 1995), and alterations in the HPA axis response to subsequent stressors (Stanton et al., 1988b).

In the present study, ACTH and corticosterone basal levels of MS rats were significantly decreased in comparison to the control animals. These reduced hormone levels could have resulted from pituitary-adrenal exhaustion, because of the additional stress sessions on PND 35 and 60. The pituitary and adrenal glands may have been hypofunctioning after multiple stress exposure. Alternatively, inhibitory elements of the HPA-axis may have been enhanced resulting in more pronounced negative feedback inhibition. A similar mechanism has been proposed for the reduced cortisol levels observed in patients suffering from PTSD (Yehuda et al., 2004).

In contrast to our finding of a hyporesponsive HPA axis following repeated stress, are the results of Huot et al. (2004). These authors subjected animals to maternal deprivation for a 3 hour period daily from PND 2 until 14 and exposed them to an air-puff startle paradigm during adulthood (PND 60-90) in order to investigate HPA responsiveness. In addition to the maternal deprivation paradigm, some litters were also fostered with different mothers during the deprivation period. Results demonstrated that the maternally deprived animals exhibited meaningfully higher ACTH levels when compared to a 15 min deprived group and the fostered maternally deprived group. The fostered maternally deprived group did not differ significantly from the 15 min deprived and the 15 min fostered groups in terms of ACTH secretion in response to the air-puff startle paradigm. However, corticosterone release was exaggerated in the maternally separated group in response to the stressor, when compared to the control or fostered groups. These data suggest a hyper-responsiveness of the HPA axis in maternally deprived animals. This was ascribed to impaired glucocorticoid feedback inhibition, while down-regulation of glucocorticoid receptors in maternally deprived rats have been observed in the hippocampus in another study (Sutanto et al., 1996). The fostered animals and the 15 min handled group reacted similarly to the stressor regarding HPA-axis responsivity, emphasising the impact of early life maternal care.

In an earlier study the same authors showed that repeated maternal separation caused significant increases in corticosterone secretion as early as on PND 7 (Huot et al., 2002) in the normally stress hypo-responsive period of the animals. In this study the basal

corticosterone levels did not differ between groups when measured at adulthood, but the maternally separated group exhibited higher ACTH and corticosterone levels when exposed to the air-puff startle stressor. These discrepancies with our findings seem best accounted for by the different stress paradigms employed. Handling effects may also have contributed to the differences, as the maternal separation paradigm of Huot et al. (2002) involved removing the pups from the dam, whilst in our study the dam was removed from the pups, minimising any additional handling complexities during the separation period. Daily handling of neonatal rats has been shown to result in decreased HPA-axis responsiveness (Meaney et al., 1991), and this may explain why there was no difference in basal corticosterone concentrations in the Huot study. The use of a different strain of rat (Long-Evans hooded vs Sprague-Dawley rats), could also have accounted for the difference in neuroendocrine outcomes and indicates the importance of genetic make–up in determining the impact of stress on an individual.

In another study, Roceri et al. (2004) subjected 3 month old animals to a chronic stress paradigm in adulthood following a period of early life maternal separation. The chronic stress involved 2 weeks of forced swimming twice a day. The aim of this study was to investigate whether maternal deprivation would alter the responsiveness of the brain to a prolonged stressor. Blood samples were taken before (basal levels) and 30 min after the swim stress on day 1 and 13 of the swim stress paradigm. Corticosterone levels increased tremendously after swim stress in both control and maternal deprivation groups when compared to basal corticosterone levels. No significant effect between rearing condition and the stressor was found. These results contrasted with the previous findings of others (Workel et al., 1997; Van Oers et al., 1998a; Huot et al., 2004), where both control and maternally separated animals displayed similar HPA axis responsiveness after swim stress exposure.

Some investigators found maternal deprivation to increase HPA axis responsiveness to stressors in adulthood (Van Oers et al., 1998a; Huot et al., 2004). Our study suggests that maternal deprivation and additional stressors both in adolescence and adulthood, decrease basal activity of the HPA axis. However, Roceri et al. (2004) observed similar

basal activity of the HPA axis between maternally deprived and control animals. These differences in HPA axis activity indicate that variations in stress paradigms may affect an individual's neuroendocrine system in dissimilar ways. The responsivity of the HPA-axis also appears to be extremely variable when responding to different stressful stimuli. The long-term consequences of subjecting animals to various aversive stimuli on the maturation of their HPA axis, therefore remains complex.

4.2.2 *The effect of time-dependent sensitisation on the HPA axis*

Studies focussing on the effects of TDS on HPA axis activity are limited. In the present study we found plasma ACTH and corticosterone levels to be similar in both the non-stressed control and TDS group (Figure 3.1 and Figure 3.2). This was a surprising result as another parallel study in our laboratory employing the TDS model, resulted in ACTH levels similar to control rats, but a meaningful increase in corticosterone levels. This observation was attributed to the desensitisation of the adrenal glands to ACTH concentrations resulting in the dysregulation of negative feedback inhibition of the HPA stress system (Uys et al., 2006). Harvey et al. (2003) used a similar TDS stress paradigm and determined plasma corticosterone levels 7 days post stress. In these experiments stressed animals exhibited significantly lower corticosterone levels when compared to the non-stressed control group. This observation was explained by hypercortisolemia during and after TDS stress that caused sensitisation of the inhibitory elements of the negative feedback inhibition of the HPA-axis over time, resulting in the decreased corticosterone concentrations 7 days post re-stress.

Liberzon et al. (1997) employed the TDS stress paradigm to investigate feedback regulation of the HPA-axis. They reasoned that the regulation of corticoid secretion was necessary to avoid the potential damaging effect of excessive amounts of this steroid (Sapolsky et al., 1986). Animals were subsequently pre-treated with either corticosterone or saline before the first triple stressor and the re-stress exposure seven days later. However, restraint stress during the re-stress period of TDS resulted in ACTH and corticosterone responses that were comparable in both the corticosterone-treated and

saline-treated groups. Thus, pre-treating animals with corticosterone before the triple stressor did not prevent fast feedback sensitisation 7 days later during re-stress, indicating that of the impact of the traumatic triple stressor, as well as the early presence of this impairment (dysregulated feedback inhibition) persists.

From the above described studies as in the case of maternal deprivation, different TDS stress protocols may exert different influences on the HPA axis. For instance, older rats (250 g) subjected to restraint stress (Liberzon et al., 1997) were used, as opposed to younger adults (150-180 g) that were subjected to swim re-stress (Uys et al., 2006). Overall current data suggest that the effects of TDS remain unclear and require more investigation.

4.2.3 *The effect of both maternal separation and TDS stress on HPA-axis activity*

In this, the first study to investigate the cumulative effects of maternal separation and TDS on HPA axis activity in adulthood, we found firstly, the combination of maternal separation and TDS resulted in decreased basal ACTH levels similar to the rats subjected to maternal separation alone. Secondly, the maternal separation and TDS group displayed corticosterone levels similar to controls (Figure 3.1 and Figure 3.2). The latter results were surprising as it was expected that the additive effects of maternal deprivation plus TDS plus a further swim stress session on PND 60 would present the animal with an extreme stressful condition. It was anticipated that the magnitude of this allostatic load would present a great challenge to the individual to maintain neuroendocrine homeostasis. The fact that the ACTH levels were equally decreased in the two groups (MS and MS + TDS), suggests that the impact of maternal deprivation on pituitary function was possibly maximal and that a minimum of ACTH secretion was necessary for the overall survival of the animal. Decreased ACTH concentrations can possibly result from a stress system exposed to chronic onslaughts resulting in an exhaustion of and/or a desensitised pituitary gland. Animals exposed to both maternal separation and TDS stress did not differ from any of the other groups regarding corticosterone concentrations, and displayed similar levels to the control animals. It

therefore appeared that subjecting rats to TDS resulted in a normalising effect, leading to a partial restoration of endocrine irregularities imposed by maternal separation, a finding that would concur with the absence of behavioural changes in the combined group.

4.3 Neurotrophins

4.3.1 *The impact of maternal separation on brain neurotrophin levels*

In the present study, animals subjected to maternal deprivation displayed increased NGF levels in both the dorsal and ventral hippocampus, as well as significantly elevated NT-3 protein concentrations in the dorsal hippocampus when compared to non-stressed control rats. No significant differences were observed in BDNF levels in any of the brain areas investigated (Figure 3.3 – Figure 3.10).

The effects of maternal separation on neurotrophin levels in the brain are only described in a few studies. Cirulli et al. (2000) reported increased NGF levels in the brain following maternal separation in the frontal cortex, dentate gyrus and paraventricular nucleus of the hypothalamus. They noted that the increase in NGF was greater when the period of maternal separation was longer (3 hours vs 1 hour), suggesting a close association between the degree of stress and the magnitude of the NGF response. Also that a short maternal separation (45 minutes) stimulated NGF mRNA expression in the dentate gyrus as early as PND 3, despite unchanged protein levels at that time (Cirulli et al., 1998). Increased cell death was evident in these animals, it was proposed that the enhancement of NGF reflected a compensatory response by the brain to combat the deleterious effects of the deprivation stress. The fact that we obtained raised NGF levels in rats at adulthood suggests that alterations in NGF expression can be long-lasting and confirmed its important role in the survival of neurons and the maintenance of normal brain function.

The fact that changes in NGF concentrations have been observed in the paraventricular nucleus of the hypothalamus suggests that this growth factor may also be involved in

neuroendocrine regulation (Gould et al., 1990; Scaccianoce et al., 1993). It has been shown that stress associated with increased corticosterone levels also led to decreased neurotrophic levels (Schaaf et al., 1998). On the other hand, our data demonstrate that repeated maternal separation was associated with decreased corticosterone and increased NGF levels. These findings suggested that an inverse relationship may exist between these two parameters under conditions of stress. Interestingly, in an acute experiment, administration of corticosterone (20 mg/kg) resulted in the elevation of NGF expression levels in the hippocampus after 3 hours (Barbany et al., 1992). More support for direct links between NGF and corticosterone came from studies which showed that NGF stimulates HPA axis activity by enhancing the secretion of ACTH and glucocorticoids from the pituitary and adrenal glands respectively (Otten et al., 1979; Scaccianoce et al., 1993; Smith, 1996). The enhancement of ACTH and glucocorticoid secretion via NGF stimulation occurred via NGF-induced increase and release of hypothalamic arginine-vasopressin (AVP), since in vitro hypothalamic cell cultures produced an increase in AVP and not CRH in the presence of NGF-containing incubation medium (Scaccianoce et al., 1993). A 24 hour maternal separation was also able to cause a switch from CRH to AVP-mediated release of ACTH from the pituitary (Scaccianoce et al., 1991; Bartanusz et al., 1993).

It therefore seems as if NGF responses are extremely sensitive to fluctuations in corticosterone concentrations on the one hand, while on the other, NGF has the ability to regulate the activity of the HPA axis. The hippocampus with its high presence of GR and MR receptors may be of particular importance in this two-way communication system – a system that may be one of the crucial elements of the strategies employed by the brain to protect itself against corticosterone toxicity and to preserve neuron function for normal HPA axis activity and perhaps normal behavioural patterns.

Roceri et al. (2004), employing a similar maternal separation paradigm, found, as we did, that there was no change in BDNF levels when measured at adulthood. They demonstrated a transient increase in BDNF expression in the hippocampus and prefrontal cortex when measured at PND 17, that disappeared by PND 35. When

maternally deprived adult animals were exposed to a chronic swim paradigm, BDNF levels did not undergo any meaningful changes, while control animals displayed attenuated BDNF expression levels in the prefrontal cortex but reduced protein levels in the striatum (Roceri et al., 2004). These findings suggested that BDNF responses to stress may not be as long-lasting as that of NGF. Furthermore, the study by Roceri et al. (2004) also suggested that neurotrophin responses to stressful situations may occur also in other brain areas and that these responses may be varied in nature.

The importance of normal neurotrophin concentrations in the brain was accentuated by high levels of maternal care (increased levels of licking, grooming and arched back nursing) resulted in offspring with increased BDNF expression, cholinergic innervation of the hippocampus, and improved spatial learning and memory (Liu et al., 2000a). This implies that withdrawal of maternal care or neglect would also have detrimental repercussions on cognitive development of the young (Suomi, 1997).

The elevation in NT-3 levels observed in our maternal separated animals was in agreement with experiments showing that corticosterone administration in early postnatal life (first week postnatally) causes significant increases in NT-3 mRNA levels as well as NT-3 receptors in the septum and hippocampus (Roskoden et al., 2004). Since, high corticosterone concentrations had been shown to lead to dendritic atrophy and neuronal loss (Chao et al., 1994; Smith et al., 1995), the up-regulation of the NT-3 system may also be part of the brain's compensatory mechanisms to neutralise the damaging effects of the stress. This could contribute to the prevention of further loss of neurons in the hippocampus (Roskoden et al., 2004).

In summary it seems that maternal separation associated with initial raised corticosterone concentrations also results in increased NGF and NT-3 levels as part of a compensatory action to minimise the consequences of stress. Since control and maternally separated animals did not differ in behaviour, it may be possible that this lack of difference in behaviour may in fact indicate the degree of compensation that has

occurred and that the differences in neurochemical parameters merely reflects the status of the mediators of this compensation.

4.3.2 *The effects of TDS on brain neurotrophin levels*

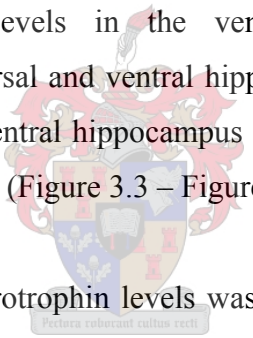
In contrast to maternal separation, the TDS paradigm resulted in decreased BDNF levels in the dorsal hippocampus whereas no significant changes in NGF or NT-3 concentrations were observed in any of the brain areas studied (Figure 3.3 – Figure 3.10). This result was similar to the findings of Uys et al. (2006) who found reduced BDNF levels in the dorsal hippocampus. This data suggests that TDS stress exerted a strong influence on BDNF levels, particularly in the dorsal hippocampus. As the hippocampus is known to be a targeted brain area during stress, and more importantly, the dorsal and ventral hippocampus are differentially affected by stress and have preferential roles in brain processes (Trivedi et al., 2004; Bannerman et al., 2004), variations in growth factor responses in either dorsal or ventral regions are not surprising. It appeared as if regional differences in neurotrophin involvement were dependent on the time, as well as the type of stress applied. When TDS stress is compared to our maternal separation paradigm, a significant difference may therefore be the time interval at which the stressor was applied: maternal separation occurred shortly after birth, whilst TDS trauma was administered in peri-adolescence. Regarding the actual nature of the stress, maternal deprivation basically entailed separation stress; whilst for TDS three independent modes of stress were applied (restraint, swim and ether vapour). Dissimilarities such as these may therefore underlie the observation that maternal separation led to increased neurotrophin levels and TDS resulted in reduced neurotrophin levels or concentrations similar to control animals.

In contrast to our findings, Uys et al. (2006) reported decreased levels of NT-3 in the dorsal and ventral hippocampus and the frontal cortex. It may be possible that trauma experienced in adolescent life followed by adulthood stressors, may result in partial restoration of neurotrophin concentrations. Such a response may reflect a protective reaction of the brain to maintain normal structure and function, especially under

conditions of raised glucocorticoid levels resulting form repetitive stress exposure. This is plausible if the observed decrease in corticosterone concentrations in the present study resulted from prolonged hypersecretion of CRH and eventual pituitary-adrenal exhaustion.

4.3.3 *The combined effects of maternal deprivation and TDS stress on neurotrophin levels in the brain*

Studies focusing on repetitive stress and neurotrophins are very limited and no current literature exists on the neurochemical effects of maternal deprivation followed by adolescent trauma and later adult stressors. We showed that animals exposed to the combined stressors of maternal separation, TDS, and swim re-stress, resulted in increased BDNF protein levels in the ventral hippocampus, increased NGF concentrations in both the dorsal and ventral hippocampus and elevated levels of NT-3 in the dorsal hippocampus, ventral hippocampus and the frontal cortex when compared to controls and the TDS group (Figure 3.3 – Figure 3.10).



As the effect of TDS on neurotrophin levels was limited to decreased BDNF levels in the dorsal hippocampus, the absence of significant changes in the dorsal hippocampus of animals that were subjected to both maternal separation and TDS suggested that the prior exposure to maternal separation buffered the impact of TDS on the levels of this neurotrophin. Interestingly, animals subjected to a combination of the stressors yielded increased BDNF concentrations in the ventral hippocampus that indicated an exaggerated response. A similar picture emerged for NT-3 levels of the MS + TDS group in the ventral hippocampus and frontal cortex. However, the increases in NT-3 in dorsal hippocampus and NGF in both the dorsal and ventral hippocampus were equivalent to that of rats subjected to maternal separation alone. These results showed a cumulative effect on neurotrophin levels between maternal separation and TDS in some brain areas (e.g. NT-3 in the ventral hippocampus and frontal cortex), but in other neurotrophin systems (BDNF and NGF in the dorsal hippocampus) the impact of

maternal separation appeared to have been maximal as exposure to the combination of stressors did not result in any further changes in neurotrophin levels.

The above mentioned data once again confirm the regional nature of hippocampal function and suggest that the dorsal hippocampus may be specifically responsive to maternal deprivation. Increases in BDNF, NGF and NT-3 would be consistent with studies showing that the dorsal hippocampus was vulnerable to insults such as glucocorticoid toxicity (Uno et al., 1994; McIntosh et al., 1996) and therefore required dramatic compensatory responses to protect it from malfunctioning.

The increased neurotrophin levels as a consequence of maternal deprivation and the direct opposite decrease in growth factors when subjected to TDS stress is difficult to explain. It could be speculated that stress experienced postnatally is reacted upon more aggressively because the brain structures are still developing, whilst when stress is applied later in life, these structures are already fully developed and may be more resilient to the onslaughts of raised glucocorticoid levels. In those brain areas where the combination of maternal deprivation followed by TDS stress resulted in even higher neurotrophin levels, it may be possible that this greater increase was part of an adaptive compensatory mechanism that became re-kindled during the adolescence stress and persisted throughout adult life. Thus, when maternal separation was applied postnatally, these compensatory systems were primed so that stress onslaughts later in life led to responses reflecting an overly protective mechanism. This was plausible as corticosterone administration in early postnatal life, as would be the case with maternal separation, has been shown to cause increased NT-3 and BDNF expression and receptor levels in the septum and hippocampus (Roskoden et al., 2004).

The general picture that emerged was one that showed that the maternal separation resulted in increased neurotrophin levels and this increase was further enhanced by TDS trauma in adolescence followed by adulthood re-stress. This advanced compensatory neurotrophin response was part of the brain's strategy to protect the hippocampus and

the frontal cortex against the deleterious effects of high levels of adrenal corticoids associated with the stress.

CHAPTER 5: CONCLUSION

Amy, being a victim of child abuse, like so many other individuals exposed to early adversity, has an increased susceptibility of developing psychiatric illnesses later in adult life.

The purpose of this study was to investigate the effect of multiple stressful situations during the course of a lifespan, on the behaviour and neurochemistry of rats. We established that the combination of maternal deprivation and TDS resulted in no significant behavioural abnormalities, and that these two stress paradigms may exert antagonistic effects on some of the parameters measured, while on others, a synergistic effect was noted.

Decreased ACTH levels were observed in the combination stressor group. This finding was ascribed to the severe impact maternal separation had on pituitary-adrenal function. Similar corticosterone concentrations were observed between stressed and control animals and it appeared as if TDS partially normalised the endocrine irregularities imposed by maternal deprivation.

Neurochemically we observed a substantial up-regulation of neurotrophic factors in some brain areas of the combined stressor group possibly reflecting compensatory mechanisms of the brain aimed at neutralising the damaging effects of repetitive stressors. The lack of behavioural abnormalities in this group of animals may perhaps mirror this compensation.

Our study again highlights the complexity of repeated stress exposure. Whilst our experiments provide some insight into the possible role of neurotrophins in the development of and/or the predisposed susceptibility to behavioural abnormalities, more studies are necessary to clearly delineate the interactions between the various physiological systems involved.

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