The potential of exercise to reverse stress induced abnormalities in the rat brain

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DECLARATION

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Abstract

Adverse experiences during early life causes alterations in the development of the central nervous system structures that may result in abnormal functioning of the brain. It is well known that, in humans, adverse early-life experiences such as social separation, deprivation, maternal neglect and abuse increase the risk of developing psychiatric disorders, such as depression, later in life. We used maternal separation in the rat as a model for early life stress to firstly determine how different brain systems are dysregulated by this stressful experience and additional chronic or acute stress during adulthood. Rat pups were separated from their mothers on postnatal day 2-14 for 3 hours per day while control rats were normally reared. The behavior, stress response, neurotrophin, apoptotic marker and serotonin levels in the ventral hippocampus, striatum and frontal cortex were measured during adulthood. A different group of maternally separated rats were allowed chronic voluntary exercise and similar measurements were done to determine whether exercise was able to normalize the deficits caused by early life stress. Differentially expressed cytosolic proteins of the ventral hippocampus of maternally separated rats versus normally reared rats were also identified. Protein expression levels of maternally separated rats that received chronic voluntary exercise or escitalopram treatment were subsequently determined to unravel the mechanism of therapeutic action for these two interventions. We found that maternal separation increased the baseline corticosterone response of rats and induced a blunted adrenocorticotropin hormone after acute restraint stress. Baseline neurotrophin levels were significantly decreased in the ventral hippocampus. Maternal separation followed by chronic restraint stress during adulthood resulted in increased depressive-like behavior compared to control rats. Maternal separation alone or followed by acute restraint

stress during adulthood induced changes in apoptotic marker expression in the striatum and frontal cortex. In rats subjected to maternal separation and chronic restraint stress during adulthood, we found that chronic voluntary exercise decreased their depressive-like behavior and increased brain derived neurotrophin levels in the striatum. Serotonin levels were not affected by maternal separation, but chronic voluntary exercise increased serotonin in the ventral hippocampus of normally reared rats. Maternal separation induced a number of changes in the expression of cytosolic proteins and these stress-induced changes were identified in proteins relating to cytoskeletal structure, neuroplasticity, oxidative stress, energy metabolism, protein metabolism, and cell signaling. Chronic voluntary exercise was able to restore the expression levels of a number of proteins affected by maternal separation that increased the risk for neuronal death. When comparing the efficacy of exercise to that of escitalopram treatment it was evident that, in contrast to exercise, escitalopram targets a different subset of proteins affected by maternal separation, except for a few involved in energy metabolism pathways and neuroprotection. In this study we have shown that chronic voluntary exercise has therapeutic effects in maternally separated rats, decreasing depressive-like behavior, increasing neurotrophin expression and restoring cytosolic protein expression that were dysregulated by early life stress.

Opsomming

Negatiewe stresvolle ervarings gedurende die vroeë stadium van ‘n mens se lewe veroorsaak veranderinge in die ontwikkeling van breinstrukture en het ‘n nadelige uitwerking op die funksionering van die brein. Dit is bekend dat stresvolle ervarings in kinders, byvoorbeeld sosiale afsondering, verwaarlosing en mishandeling, die risiko vir die ontwikkeling van psigiatriese steurings soos depressie gedurende volwassenheid kan verhoog. In hierdie studie gebruik ons moederlike skeiding van neonatale rotte as ‘n model vir vroeë lewensstres om te bepaal hoe dit verskillende sisteme in die brein negatief beinvloed, en dan ook die effek van addisionele kroniese of akute stres gedurende volwassenheid. Die neonatale rotte is weggeneem van hulle moeders af vanaf dag 2 tot 14 vir 3 ure elke dag terwyl kontrole rotte by hulle moeders gebly het. Die gedrag, stres respons, neurotrofiene, apoptotiese merkers en serotonien vlakke is gemeet in die ventrale hippokampus, frontale korteks en striatum gedurende volwassenheid. Rotte wat van hulle moeders geskei is, is dan toegelaat om vir ses weke in wiele te hardloop om te bepaal of kroniese vrywillige oefening die negatiewe effekte wat veroorsaak is deur stres kan ophef. ‘n Bepaling van sitosoliese proteien uitdrukking in die ventrale hippokampus is ook gedoen om die uitwerking van moederlike skeiding op proteienvlak vas te stel. Hierdie protein data is dan vergelyk met die van gestresde rotte wat kroniese oefening of escitalopram behandeling ontvang het om die meganisme van werking van beide behandelings te bepaal. Ons het gevind dat moederlike skeiding die rustende kortikosteroon vlakke van rotte verhoog terwyl dit adrenokortikotropien vlakke na akute stres inhibeer. Moederlike skeiding het ook die neurotrofien vlakke in die ventrale hippokampus verlaag en addisionele kroniese stres gedurende volwassenheid het ‘n verhoging in depressie-agtige gedrag veroorsaak. Moederlike

skeiding alleen, sowel as gevolg deur akute stress gedurende volwassenheid het ook veranderinge in die uitdrukking van apoptotiese merkers in die striatum en frontale korteks veroorsaak. Kroniese vrywillige oefening na moederlike skeiding en addisionele stres gedurende volwassenheid kon depressie-agtige gedrag verlaag en neurotrofienvlakke in die striatum verhoog. Serotonien vlakke was nie beinvloed deur moederlike skeiding nie, maar oefening in kontrole rotte het serotonien verhoog in die ventrale hippokampus. Moederlike skeiding het heelwat veranderinge in die uitdrukking van sitosoliese proteiene van die ventrale hippokampus veroorsaak wat ingedeel kan word in die volgende funksionele kategorieë: sitoskelet, neuroplastisiteit, oksidatiewe stres, energiemetabolisme, proteinmetabolisme en seintransduksie. Oefening kon die uitdrukking van verskeie stres-geïnduseerde veranderinge in proteiene weer herstel terwyl dit wou bleik asof escitalopram se meganisme van werking op ‘n ander vlak geskied. Ons bevindinge bewys dat kroniese vrywillige oefening ‘n goeie behandeling is na vroeë lewenstres en dat dit depressiewe gedrag verminder, neurotrofien vlakke verhoog en sitosoliese proteien vlakke kan herstel.

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Chapter 1

Introduction

Adverse experiences during early life cause alterations in the formation of brain structures that are not matured yet and therefore, the functioning of the brain are also affected negatively (Calabrese et al., 2009). It is well known that, in humans, adverse early-life experiences such as social separation, deprivation, maternal neglect and abuse increase the risk of developing psychiatric disorders later in life (Gilmer & McKinney, 2003). Increased rates of major depression, posttraumatic stress disorder and attention-deficit/hyperactivity disorder in adulthood have been reported for maltreated children (Famularo et al., 1992; Pelcovitz et al., 1994).

One of the characteristic symptoms of patients suffering from anxiety disorders or depression is a dysregulation in the activity of their hypothalamic-pituitary-adrenal (HPA) -axis. Abnormalities such as hyper-secretion of corticotropin-releasing factor (Nemeroff et al., 1984), adrenocorticotropin hormone or cortisol (Servant, 1997; Monteleone et al., 1995; Holsboer et al., 1986) have been associated with these conditions. Animal models are frequently used to investigate the effects of early-life stress on behavior and neurochemistry of rats. Maternal separation has been extensively studied and has been shown to induce abnormalities in the behavior of adult rats that was associated with aberrations in HPA-axis activity similar to that observed in patients with anxiety disorders or depression (Ladd et al., 2000; Daniels et al., 2004; Aisa et al., 2007). These findings proved maternal separation to be an animal model with good face validity to study relevant psychiatric disorders.

The aims of the present study were to firstly determine the effects of maternal separation and subsequent acute or chronic stress later in life on stress hormone levels, behavior and expression of neurotrophin levels and apoptotic markers in regions of the brain that are implicated in depression, namely the hippocampus,

striatum and frontal cortex (Bremner et al., 2000; Drevets et al., 1997; Husain et al., 1991). Furthermore, the effect of maternal separation on cytosolic protein expression of the ventral hippocampus, an area which has been shown to be involved in the regulation of HPA-axis activity and anxiety behavior (Bannerman et al., 2004), was determined using proteomic techniques. Secondly, the benefit of chronic voluntary exercise as treatment was investigated in rats subjected to maternal separation to determine whether exercise was able to reverse changes in specific proteins that were initially altered by early life stress or the combination of early life stress and subsequent chronic stress. Additionally, the effect of a selective serotonin reuptake inhibitor on cytosolic protein expression in the ventral hippocampus of maternally separated rats was also investigated. This enabled the comparison of the mechanism of action of exercise treatment on the brain to that of a pharmacological drug used for the treatment of depression.

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Chapter 2

Literature Review:

The beneficial effects of exercise on central nervous system functioning

Introduction

A large number of studies done in the past few years have shown that exercise has some or other beneficial effect on the body. Evidence for this statement can be seen in the results of both clinical and animal studies, especially work done on animal models, using rats and mice as subjects (Daley et al., 2007; Duman et al., 2008; Radak et al., 2006; Vaynman et al., 2003). Many studies have employed animal models to look at the effects of exercise on behaviour, growth factors, neurotransmitters, stress hormones and the expression of a variety of other signalling proteins in neurons. The literature suggests that exercise overall has positive effects on various aspects of brain functioning, apart from negative effects such as the acute induction of reactive oxygen species after a single bout of exercise (Davies et al., 1982) and surges glucocorticoid levels which may be toxic to neurons at high concentrations (Ploughman et al., 2007; Ploughman et al., 2005).

Clinical studies

A number of clinical studies indicated that exercise has antidepressant effects in humans diagnosed with depression, and is recognized to be a useful treatment option in general populations (Daley et al., 2007). Subsequently, it is has been used as augmentation to pharmacotherapy to treat patients with depression. Recently the question has been raised as to whether exercise as treatment for depression is more effective alone or as an adjunctive treatment, however, since there is currently not enough data available, it is recommended to be used with other treatments (Daley, 2008).

In humans, several aspects of brain functioning can be improved by exercise. Colcombe et al. (2006) found that the a 6 month aerobic exercise intervention 3 times per week increased the brain volume of older adults increased in comparison to controls. These increases were mainly found in the frontal and temporal cortices which are involved in attention and memory processes. It is therefore not surprising that cognitive functioning, as assessed by the Stroop colour and word tests, also improved after exercise (Ferris et al., 2007). Similar results were found in young adults, as an acute bout of moderate exercise increased the consolidation of information for long-term memory formation. Free-recall of items were measured and decreased in controls whilst it was maintained in the exercise group (Coles & Tomporowski, 2008). Aerobic exercise also improves working memory as assessed by the modified Sternberg working memory task. Reaction times were decreased immediately and 30 min after an acute exercise session (Pontifex et al., 2009). These findings supports the notion of exercise being advantageous to memory and cognitive brain functioning.

Clinical trials on patients with depression have shown that chronic exercise holds substantial benefits in the treatment of this disorder. In older adults with minor depression, 16 weeks of exercise reduced depression scores, measured by the Hamilton Depression Rating Scale, and improved several aspects of physical functioning (Brenes et al., 2007). Similarly, in adults with major depression (MD), treadmill walking decreased both clinician-assessed and self-assessed depression scores within 2 weeks, indicating that the antidepressant effect of exercise is achieved much faster than pharmacological treatment (Dimeo et al., 2001). Chronic moderate intensity exercise also decreased depression scores in patients with MD already receiving chronic pharmacological treatment (Craft, 2005). Interestingly,

patients receiving exercise treatment only achieved a higher remission rate than placebo treated controls (Blumenthal et al, 2009).

In general, the quality of life, as measured by the World Health Organization Quality of Life assessment, of patients with MD on pharmacological treatment and exercising, improved significantly compared to patients only receiving pharmacological treatment. This improvement was significant after 8 months of combination treatment (Carta et al., 2008). A modest exercise regime also improved the several aspects of quality of life of postmenopausal women in a dose dependant manner; however this was a randomized controlled trial in non-depressed women (Martin et al., 2009).

Serum brain-derived neurotrophic factor (BDNF) levels increased in healthy young adults after an acute graded exercise test and 30 min of fixed moderate intensity exercise. Serum BDNF levels correlate well with cortical BDNF levels as it is transported readily across the blood-brain barrier (Pan et al., 1998), therefore such an increase in neurotrophins are potentially beneficial to neuronal growth and survival.

The benefits of exercise as treatment are widely accepted. In addition it is recognised as a low cost alternative to pharmacological treatment and this, together with the fact that there are no disabling side-effects with exercise as treatment makes it easy to involve patients in some form of exercise. It is also noteworthy that in many studies it has been found that a mild form of exercise is substantial enough to induce antidepressant effects (Dimeo et al., 2001; Craft, 2005) as well as the other benefits on cognition and memory mentioned before, which means that exercise treatment

should not be a burden to patients, especially those suffering from depression. Interestingly, Radak et al. (2008) showed that the response to physical exercise actually fits an inversed U-shaped curve where moderate exercise increases physiological function and quality of life, while strenuous exercise and overtraining results in a drastic decrease in these parameters, similar to what is achieved with an inactive lifestyle.

One of the limitations of clinical studies is that the data are usually obtained by a variety of assessments or questionnaires performed by a clinician or even self-assessment by the patient. So although it is can be shown that a reduction in depression scores is achieved by regular exercise, little information about the mechanism of action of exercise and its influence on protein expression in the brain can be deduced from these studies. It is therefore necessary to use animal models in which brain and spinal cord tissue, blood and cerebrospinal fluid can be utilized to more specifically determine the effects of exercise on the central nervous system.

Animal studies

Studies done on animals, mainly rats or mice, have provided some insights to understanding the possible mechanisms of action of exercise to achieve its beneficial effects. It is noteworthy that exercise has positive effects in both normal animals and those with conditions related to stress or physical brain insults. A number of these studies made observations on the effect of exercise on more than one brain function and therefore the mechanism of improvement in a certain area, for example behaviour or memory, can be explained by upregulation of specific genes and/or proteins.

Behavior

A number of behavioral tests can be used to assess the beneficial effects of exercise on anxiety- or depressive-like behavior in animals. Decreased anxiety-like behavior has been reported after acute and repeated treadmill training in rats in the social interaction test, measured as increased locomotion and decreased defecation (Chaouloff, 1994). Similarly, environmental enrichment, including free access to running wheels, resulted in reduced anxiety-like behavior in a novelty suppressed feeding protocol where food-deprived enriched mice showed a reduced latency to feed compared to mice kept in standard cages. In this study, it was shown that the decreased anxiety-like behavior was not dependant on hippocampal neurogenesis (Meshi et al., 2006).

Flinders Sensitive Line rats, a genetic animal model for depression, were allowed voluntary wheel running and showed decreased immobility times in the forced swim test, indicating decreased depressive-like behavior (Bjørnebekk et al., 2005). In a rat model of chronic unpredictable stress, anhedonia was increased significantly in a sucrose consumption test, measured as decreased consumption of a sucrose mixture, during and after the stressful period while voluntary wheel running reversed this effect in stressed rats (Zheng et al., 2006). Swimming time in the forced swim test was significantly higher in rats that received a combination of voluntary exercise and antidepressant treatment, indicating decreased depressive-like behavior, while each of the treatments alone did not have a significant effect (Russo-Neustadt et al., 2001). Chronic voluntary running decreased immobility time in the forced swim test while this behavior was reversed when mice were injected with an insulin-like growth

factor 1 (IGF-1) blocker, indicating the importance of IGF-1 on the antidepressant effect of exercise (Duman et al., 2009).

The shuttle box apparatus has two compartments, one which delivers foot shock and another providing a safe environment to which the rat may escape, and the latency to escape is a measure of depressive-like behavior and freezing response a measure of anxiety. Rats were given inescapable foot shock stress and those that were allowed chronic voluntary wheel running had decreased anxiety- and depressive-like behavior in the shuttle-box compared to sedentary rats (Greenwood et al., 2007). Chronic wheel running exercise also protected against an anxiogenic response produced by acute injection of fluoxetine, by reducing escape latency and shock elicited freezing in the shuttle box (Greenwood et al., 2008).

Mice that engaged in chronic voluntary wheel running had decreased depressive-like behavior when tested in the tail suspension test, forced swim test and shuttle-box test. The exercising mice also showed decreased anxiety in the open field, indicated by time spent and distance moved in the centre zone, and elevated plus-maze, indicated by increased entries into and time spent in the open arms of the maze, compared to sedentary mice. The most marked effect was seen 24 hours after the last exercising bout. BDNF deficient mice, unlike wild-type mice, did not have decreased depressive-like behavior after voluntary exercise. This finding also indicates a role of BDNF in depression (Duman et al., 2008).

Learning and Memory

The passive avoidance test is used to study learning and memory. The apparatus has two compartments, and animals have a tendency to go and hide in the dark compartment. The test measures latency to enter a dark compartment one day following electric foot shock to the feet after entering the dark compartment and the animal has to learn to avoid the dark compartment. Chronic exercise increased performance of rats in the passive avoidance test, indicating improved learning and memory function in over-trained rats (Ogonovsky et al., 2005). Using the same test, Radak et al. (2006) demonstrated that 8 weeks of forced swimming increased latency times in the passive avoidance test, indicating better memory, while a subsequent period with no exercise showed that this improvement in memory is temporary and only effective during the period when the animal is exercising.

The Morris water maze (MWM) is also used to assess learning over time and memory during a probe trial, where animal must find an escape platform in a bath, the location which is based on stationary spatial cues. Chronic treadmill running exercise reduced the escape latency during a probe trial after learning in the MWM, indicating improved memory retention (Mello et al., 2008) and restored memory function in maternally deprived rats in comparison to sedentary controls (Mello et al., 2009). Chronic wheel running significantly decreased the time to find the platform in the learning phase over 3 days and increased swimming in the target quadrant of the Morris water maze (MWM) during a probe trial, indicating enhanced learning and memory in these rats. Exercise also reversed the negative effects on learning that were found in rats fed a high fat diet (Molteni et al., 2004). The beneficial effect of exercise on memory function may only be evident after chronic exercise, as aged rats that engaged in life-long voluntary running had improved memory function compared to sedentary rats in the MWM (Stranahan et al., 2008) and chronic

treadmill running, starting in adolescence, also significantly improved learning and memory functioning of rats in the MWM (Uysal et al., 2005).

Chronic maternal deprivation of rat pups disrupted memory measured in the MWM and inhibitory avoidance (IA) task, which also measures latency of rats to step down on a platform after previously receiving foot shocks. Chronic treadmill exercise, however, improved memory function by reversing the deficits observed in long-term memory in the MWM, increasing time spent in the target quadrant of the probe trial, and in the IA test, increasing the step down latency. Offspring of mother rats that were forced to swim during pregnancy was also shown to have increased memory in the step down IA task, with increased latency times compared to control rats (Lee et al., 2006). Forced treadmill running for 10 days improved the short term memory of gerbils after exposure to global ischemia in the brain increasing their step-down latency in the IA task compared to sedentary animals (Sim et al., 2004).

Voluntary exercise in rats increased learning in the MWM and improved memory retention in a probe trial 2 days after learning compared to sedentary rats, however, when BDNF activity in the hippocampus was blocked, the rats showed no improvement in memory function (Gomez-Pinilla et al., 2008; Vaynman et al., 2004). Chronic voluntary wheel running improved learning and memory retention in the MWM while irradiation of the hippocampus significantly decreased neurogenesis and performance in the MWM, indicating the importance of neurogenesis to achieve improvement in memory function (Clark et al., 2008). It has, however, previously been shown that neurogenesis in the adult brain is not necessary for learning in the MWM and novel object recognition test using a cyclin D2 knockout mouse model (Jaholkowski et al., 2009) and it is therefore not known whether the effect of exercise

on learning and memory is achieved through increased neurogenesis. Rats that were allowed access to running wheels also had increased long-term potentiation (LTP) in response to a Θ-patterned stimulus in the hippocampus compared to sedentary rats, while the threshold for induction of LTP was also decreased in exercised rats. This response was attenuated after injection of an N-methyl-D-aspartate (NMDA) antagonist which indicates that LTP is achieved through activation of NMDA receptors (Farmer et al., 2004).

Mechanisms by which exercise may improve brain function

Neuronal proliferation, differentiation and structure

Several studies have indicated that exercise can increase proliferation and differentiation of neurons. Using immunofluorescent staining for 5-bromo-2'-deoxyuridine BrdU (a marker for cell proliferation) and NeuN (a stain for neurons), it was shown that both low intensity treadmill running and voluntary wheel running increased proliferation and differentiation of hippocampal neurons compared to sedentary rats (Uysal et al., 2005; Lou et al., 2008; Farmer et al., 2004), while voluntary running increased hippocampal volume in mice (Clark et al., 2008). In a mouse model for postmenopausal women, ovariectomized mice that engaged in voluntary wheel running had increased neurogenesis in the dentate gyrus of the hippocampus compared to sedentary mice. Running also had the same effect in normal mice that were sham operated compared to sedentary controls, where BrdU and Ki67 (both markers of cell proliferation) positive cells as well as doublecortin (DCX) and calretinin (both markers of cell differentiation) positive cells increased in the dentate gyrus (Jin et al., 2008). Rat pups of pregnant mothers that were

subjected to swimming exercise had increased levels of neurogenesis in the dentate gyrus and CA1-3 regions of the hippocampus (Lee et al., 2006). Chronic wheel running exercise increased proliferation in the dentate gyrus of wild-type mice; however, this increase was blocked in β-endorphin deficient mice which may therefore be involved in running increased cell proliferation (Koehl et al, 2008). Voluntary wheel running increased hippocampal neurogenesis in mice, but increased performance due to a reward did not further increase the amount of cell proliferation, indicating that neurogenesis reaches a ceiling value which is achieved with voluntary performance (Klaus et al., 2009).

Voluntary running for 12 days increased both BrdU and Ki67 positive cells in the dentate gyrus of adult rats (Stranahan et al., 2006; Van Praag et al., 1999), and doubled the amount of newborn surviving cells after a month compared to controls (Van Praag et al., 1999). Chronic treadmill running increased the number and survival of newborn hippocampal neurons while enhancing dendritic lengths (Wu et al., 2008). Adult rats engaging in voluntary running had increased dendritic arborisation and length in the granule cell layer compared to sedentary rats (Redila & Christie, 2006). Gerbils exposed to global ischemia of the brain had increased apoptotic cells and caspase-3 expression in the dentate gyrus compared to controls while 10 days of forced treadmill running significantly decreased the amount of TUNEL-positive and caspase-3 positive cells.

In a rat model for depression, the Flinders Sensitive Line rat, the levels of neuropeptide Y (NPY) mRNA increased in the CA4 and dentate gyrus of the hippocampus and an increase in cell proliferation was observed in the hippocampus after chronic voluntary wheel running. A positive correlation was found between NPY

mRNA and BrdU positive cells in the dentate gyrus, indicating that NPY may be involved in proliferation of neurons (Bjørnebekk et al., 2005; Bjørnebekk et al., 2006). Chronic voluntary wheel running in mice increased the amount of DCX positive cells in the hippocampus of mice with a positive correlation between the amount of running and neurogenesis (Åberg et al., 2008; Fuss et al., 2009) and decreased the number of pyknotic cells, indicating decreased cell death (Fuss et al., 2009). Chronic voluntary wheel running also increased the number of newborn vascular endothelial cells in the hippocampus of rats and this can be an indication that there is more optimal support for the growth of neurons (Ekstrand et al., 2008).

Neurotrophins

Several different neurotrophins are presumably involved in the mechanism by which exercise affects the brain, although BDNF seems to be the most important of them all. A number of studies found that chronic, forced exercise or voluntary wheel running increased BDNF mRNA or BDNF protein levels in the hippocampus of rodents (Cechetti et al., 2008; Fuss et al., 2009; Lou et al., 2008; Duman et al., 2008; Radak et al., 2006; Chen et al., 2006; Farmer et al., 2004; Neeper et al., 1996). Voluntary running distance have also been shown to be positively correlated with increased hippocampal BDNF and BDNF mRNA levels (Johnson et al., 2003; Ying et al., 2005; Ying et al., 2008). Hippocampal levels of BDNF and its tyrosine kinase B (TrkB) receptor were increased after 5 weeks of forced treadmill running in mice (Wu et al., 2008). Similarly, chronic voluntary exercise increased BDNF and TrkB mRNA in the hippocampus of adult rats; however, blocking the action of BDNF abolished this effect (Vaynman et al., 2003; Vaynman et al., 2004). Oliff et al. (1998) found that 6 and 12 hours of voluntary wheel running increased full-length BDNF mRNA

expression with a positive correlation between running distance and mRNA expression in the hippocampus. Exon 1 mRNA was upregulated after 6 hours and maintained at 12 hours, while exon 2 was only significantly upregulated after 12 hours of exercise. Berchtold et al. (2005) found that BDNF protein levels were increased similarly in rats that ran daily or only on alternating days, but the induction of BDNF was much faster in the daily runners. BDNF levels declined faster after ending exercise in the group that only ran on alternating days, whilst BDNF stayed significantly elevated for up to a week in the daily runners.

In an adult rat model for stroke, with induced focal ischemia on one side of the brain, single sessions of both motorized and voluntary wheel running also resulted in increased BDNF levels in the ischemic and intact hippocampus compared to that of sedentary rats (Ploughman et al., 2007). In a rat model of traumatic brain injury, voluntary exercised rats had increased hippocampal BDNF levels compared to sedentary rats (Griesbach et al., 2008). In aged rats, chronic swimming exercise increased BDNF levels in the whole brain of rats and interestingly, it was significant only in over-trained rats compared to controls (Ogonovsky et al., 2005). Chronic voluntary wheel running was also able to reverse the reduction of BDNF in rats that were fed a high fat diet. Increased BDNF positively correlated to phosphorylated cyclic AMP response element binding protein (p-CREB) levels and negatively correlated to latency to find the platform in the learning phase of the MWM (Molteni et al., 2004). Acute voluntary exercise also increased BDNF mRNA in the hippocampus of mice, but this effect was not seen in $CREB^{IR}$ mice in which the activity of CREB is blocked (Chen & Russo-Neustadt, 2009). Acute low intensity treadmill running in adult rats, in contrast to moderate intensity exercise, did not significantly increase plasma corticosterone levels and resulted in increased BDNF mRNA and protein

levels in the hippocampus (Soya et al., 2007). Voluntary running increased hippocampal BDNF mRNA and protein levels, however, when rats were treated with a nitric oxide synthase (NOS) inhibitor, exercise could not induce these changes, indicating that the activity of nitric oxide is essential for the effect of exercise (Chen et al., 2006). This is presumably achieved through increased PI-3 kinase activity, which was also upregulated by exercise, since nitric oxide activates synthesis of PI-3 kinase (Chen et al., 2006).

IGF-1 increased in the hippocampus and blood serum after forced exercise and hippocampal IGF-1 positively correlated with running distance (Ploughman et al., 2005). IGF-1 has been shown to mediate the beneficial effect of exercise in the brain of adult rats engaging in voluntary running for a week. BDNF mRNA and protein levels increased in several regions of the hippocampus in response to exercise, but the effect was reversed when blocking IGF-1 in the brain (Chen & Russo-Neustadt, 2007). Exercise-induced neurogenesis in the adult rat hippocampus is also dependent on peripheral IGF-1 and vascular endothelial growth factor (VEGF), since blocking the uptake of these growth factors into the brain abolished increases in neurogenesis observed after voluntary exercise (Trejo et al., 2001; Fabel et al., 2003).

Nerve growth factor (NGF) mRNA was increased in the hippocampus and caudal cortex of rats engaging in short term wheel running exercise (Neeper et al., 1996). Rats subjected to a focal model for stroke, middle cerebral artery occlusion (MCAO), 12 weeks of treadmill running increased NGF mRNA in the brain compared to sedentary rats. The infarct size after MCAO was significantly lower in the exercised rats indicating that NGF may have protected these rats against neuronal death.

Running also increased NGF and p-75 (the NGF receptor) mRNA levels in the brains of normal rats (Ang et al., 2003).

Adult rats that underwent cervical hemisection had decreased neurotrophin-3 (NT-3) mRNA levels in the spinal cord while voluntary wheel running for 2 weeks managed to increase NT-3 mRNA levels to normal control levels (Ying et al., 2008). In a similar study in spinal cord injured rats, NT-3 mRNA was also upregulated after 28 days of exercise compared to normal controls (Ying et al., 2005).

Chronic voluntary running increased VGF and BDNF protein expression in the hippocampus of adult mice. VGF infusions in these mice have been shown to decrease immobility times in the forced swim and tail suspension tests, and the induction of VGF could therefore be a possible mechanism by which exercise induces its antidepressant effect (Hunsberger et al., 2007).

Neurotransmitters

Another potential benefit of exercise may be its ability to regulate neurotransmitter levels in the brain. Serotonin (5-HT) levels were measured with microdialysis in the ventral hippocampus of rats in response to acute treadmill running and was shown to be increased significantly compared to the baseline values during running as well as in the recovery phase after running and returned to baseline levels after 2 hours (Béquet et al., 2001). Similarly, both 5-HT and its metabolite 5-hydroxyindolacetic acid (5-HIAA) were also shown to be increased in both the ventral hippocampus and the frontal cortex after intensive treadmill running in rats (Gomez-Merino et al., 2001). Chronic exercise also increased the synthesis and metabolism of 5-HT in the

cerebral cortex of rats (Dey et al., 1992) while norepinephrine and 5-HT were increased in the cerebellum, cerebral cortex and midbrain of rats (Brown et al., 1979). The antidepressant effect of exercise are possibly related to its ability to increase 5-HT neurotransmission, as it is presumed that depression is related to decreased availability of 5-HT (Nutt et al., 2007; Hou et al., 2006).

Activation of intracellular signalling pathways

It is evident from animal studies that BDNF signalling is an important factor in exercise induced upregulation of transcription factors and neuroplasticity related proteins. Synapsin-1, which is involved in neurotransmitter vesicle transport, increased in the intact hippocampus and cortex of rats with focal ischemia after a forced walking exercise bout. Increases in this protein could be a downstream effect of BDNF on synaptic plasticity (Ploughman et al., 2005). Short term voluntary wheel running increased synapsin-1, transcription factor p-CREB protein levels and CREB mRNA in the hippocampus of rats while no effect where seen when blocking BDNF activity (Vaynman et al., 2003; Vaynman et al., 2004). Chronic voluntary exercise also increased p-synapsin-1 levels in the hippocampus and reversed the reduction of synapsin-1 mRNA, p-synapsin 1 and p-CREB in rats that were fed a high fat diet (Molteni et al., 2004). Consumption of a high fat diet seems to reduce synaptic plasticity, while exercise reverses this situation by upregulating proteins involved in neurotransmission and increases transcription. In adult rats with spinal cord injury, voluntary wheel running increased the mRNA, protein levels and activation synapsin-1, CREB and growth cone-associated protein (GAP)-43 mRNA, a neuroplasticity related protein involved in axonal growth and dendritic remodeling, compared to

sedentary rats. The upregulation of these proteins did not take place in rats that were injected with a BDNF signalling blocker (Ying et al., 2005; Ying et al., 2008).

Wheel running exercise increased p-TrkB, phosphatidylinositol 3 (PI-3) kinase, p-protein-dependent kinase-1(PDK-1), p-Akt and p-CREB in the hippocampus (Chen & Russo-Neustadt, 2005; Chen & Russo-Neustadt, 2009) which shows that the effect of exercise is achieved through the PI-3 kinase signalling pathway while the activation of CREB is involved in upregulation of BDNF and neuronal survival. Voluntary exercise increased mRNA expression of a number of proteins involved in energy metabolism in the rat hippocampus: AMP-activated protein kinase, ubiquitous mitochondrial creatine kinase, uncoupling protein 2 and ghrelin mRNA were all significantly increased after a week of running. However, when BDNF activity was blocked, these increases in energy metabolism related proteins were not observed (Gomez-Pinilla et al., 2008).

Adult rats subjected to chronic restraint stress had a twofold increase of levels of the Bax oligomer 270, a pro-apoptotic protein, in their motor cortices, but rats that were allowed to engage in voluntary running, did not show and increase in Bax levels in response to stress (Haack et al., 2008). Chronic swimming exercise also reduced the amount of phosphorylated c-jun, an immediate early gene which plays a role in apoptosis, in the cerebellum of rats, suggesting that exercise can reduce the risk for cell death (Toldy et al., 2005). Light intensity treadmill running increased the expression of the immediate early gene c-fos in the hippocampus of adolescent rats. This indicates increased neuronal activity which is essential for learning and memory processes in the hippocampus (Lee et al., 2003a). Increases in c-fos was dependent

on intensity of exercise until the 7th day of exercise, whereafter it starts returning to control levels (Lee et al., 2003b).

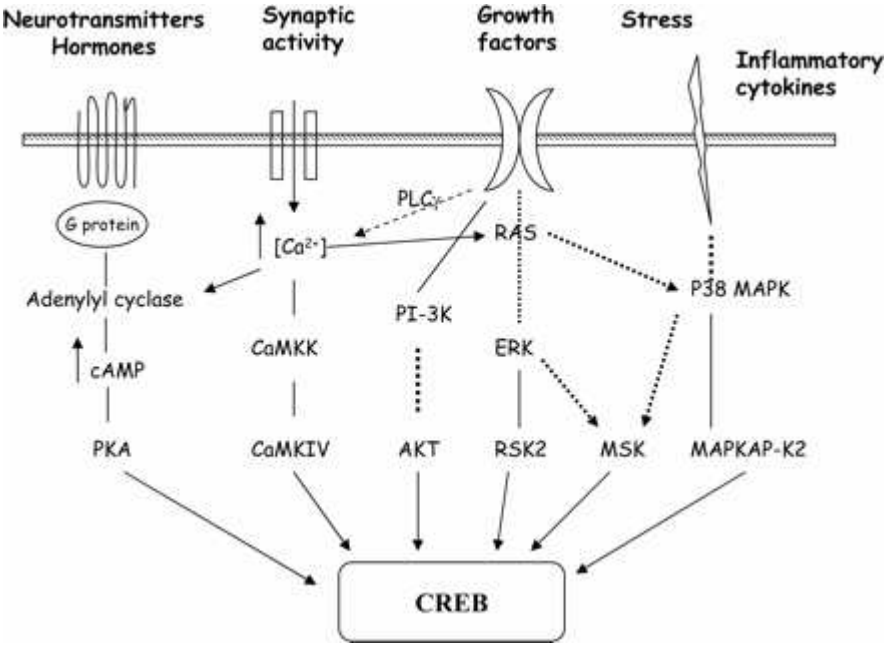

Fig. 1. Schematic representation of signaling pathways modulating CREB activation. CREB activates transcription of target genes in response to a vast array of stimuli, including neurotransmitters, hormones, growth factors, synaptic activity, stressors, and inflammatory cytokines. These stimuli activate a variety of intracellular signaling pathways, leading to activation of a number of protein kinases such as PKA, Ca^{2+}/CaMKIV, and MAPK. (Tardito et al. ,2006).

Reduction in oxidative stress

Chronic exercise has the ability to protect neurons against oxidative stress. Reactive carbonyl derivatives, as markers of oxidative protein damage, were decreased after chronic swimming exercise in rats while the activity of the proteasome complex, which is involved in the repair of damaged proteins, was increased (Ogonovsky et al,

2005). Chronic wheel running reduced the levels of oxidized proteins in rats on regular diet as well as rats on a high fat diet that has been shown to induce oxidative stress (Molteni et al., 2004). Chronic swimming exercise reduced free radical concentration in the cerebellum of adult rats and this effect was still seen 8 weeks after training was discontinued (Radak et al., 2006). Rats subjected to traumatic brain injury, by inducing cortical contusion, and subsequently allowed to engage in voluntary wheel running for a week had significantly reduced protein carbonyl levels, a measure of oxidized proteins, in their hippocampi compared to sedentary rats (Griesbach et al., 2008).

Regulation of the hypothalamic pituitary adrenal-axis (HPA-axis)

A few studies have shown that exercise regulates HPA-axis functioning in response to stressful conditions. In a model for chronic stress, corticosterone was administered to rats for several days to induce hypoactivity of the HPA-axis, while chronic treadmill running normalized the dysregulated activity by increasing CRF mRNA expression and ACTH levels in blood serum (Kim et al., 2008). Chronic voluntary wheel running in adult rats facilitated the habituation of the HPA-axis in response to repeated noise stress. The plasma corticosterone levels measured directly after the stress were significantly lower compared to sedentary controls on all the days measured, except on the first day of stress exposure (Sasse et al., 2008). Chronic voluntary exercise in mice reduced their anxiety response to a novel cage compared to sedentary rats, by reducing adrenocorticotropin hormone (ACTH) with or without a running wheel present in the novel cage and reducing corticosterone only when a running wheel was present in the cages (Droste et al., 2003). Droste et al. (2006) also found that mice that exercised had an attenuated stress response to

novelty and that their hypothalamic CRF mRNA was decreased under basal conditions.

Conclusion

In summary, it is evident from human and animal studies that acute and chronic exercise has positive effects on the brain of adolescent, adult and aged subjects. Exercise has beneficial effects in previously sedentary animals as well as in animal models of a variety of pathological conditions induced by early life stress, chronic stress, ischemia, spinal cord injury and high fat diet. Under these circumstances, exercise has been shown to be effective as pre- or post-treatment to minimize or reverse their detrimental effects.

The positive effects of exercise appear to be related to increased neurotrophin activity, mainly involving BDNF signalling. Neurotrophins increase neurogenesis and neuroplasticity in the hippocampus, resulting in improvement of learning and memory which is regulated by the hippocampus. Exercise has antidepressant and anxiolytic effects in rodents and also reduces their response to stress. The antidepressant effects of exercise are also possibly related to the levels of neurotrophins or neurotransmitters in the brain. Furthermore, regular exercise causes adaptation of signalling pathways to protect neurons against reactive oxygen species, including the upregulation of antioxidants (Radak et al., 2005). Previous studies have shown that both forced and voluntary exercise induce positive effects, although voluntary and low intensity exercise may have an advantage of not inducing high glucocorticoid levels while exercising. Voluntary exercise is also considered a more acceptable form of treatment to human subjects as opposed to forced exercise.

According to the literature, exercise has various beneficial effects on multiple systems in the brain and can potentially be employed as treatment for a number of conditions relating to stress. As early life stress predisposes individuals to a number of psychiatric disorders relating to dysregulated neurotransmitter, neurotrophin and stress hormone systems, we used chronic voluntary exercise to determine whether it is efficient as treatment in a rat model of early life stress in the experiments described in chapters 5 and 7.

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Chapter 3

Maternal separation of rat pups increases the risk of developing depressive-like behavior after subsequent chronic stress by altering corticosterone and neurotrophin levels in the hippocampus

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Abstract

Children that are abused have an increased risk for developing psychiatric disorders later in life, because of the negative effects of stress on the developing brain. We used a maternal separation model in rats to see how neurotrophins, stress hormones, behavior and the antioxidant potential of serum are affected. Rat pups were separated from their mothers for 3 hours/day on days 2-14. Maternal separation causes changes in levels of NGF and NT-3 in the dorsal and ventral hippocampus, increased basal corticosterone levels and decreased ACTH levels after acute restraint stress. The antioxidant potential of the rat serum was significantly lower in the maternal separation group. Depressive-like behavior, measured during a forced swim test, was seen in maternally separated rats after additional chronic stress during adulthood. Maternal separation caused downregulation of neurotrophins in the ventral hippocampus, possibly as an effect of high corticosterone levels, but compensatory mechanisms against cell death may be involved as neurotrophin levels increased in the dorsal hippocampus. Decreased antioxidant potential of serum could have been an effect of downregulated neurotrophin levels.

Keywords: Maternal separation, Stress hormones, Behavior, Neurotrophins, Antioxidant potential, Chronic stress, Depression

Introduction

Children that are subjected to neglect and sexual, physical or emotional abuse, have an increased risk of developing a psychiatric disorder later in life. Increased rates of major depression, posttraumatic stress disorder and attention-deficit/hyperactivity disorder in adulthood have been reported for maltreated children (Heim & Nemeroff, 2001; Nemeroff, 2004; Wals & Verhulst, 2005). One of the characteristic biochemical features of these disorders is the presence of a dysregulated hypothalamic-pituitary-adrenal (HPA) -axis as evidenced by a hypersecretion of corticotropin-releasing factor (CRF) (Nemeroff et al., 1984), as well as abnormalities in the basal secretion of adrenocorticotropin hormone (ACTH) and cortisol (Yehuda et al., 1995; Servant, 1997; Monteleone et al., 1995; Holsboer et al., 1986).

Postnatal separation of rodent pups from their mothers during their early development also results in HPA-axis abnormalities. Basal corticosterone levels and adrenal weights have been reported to be lower in separated than in normally reared rats (Slotten et al., 2006), while elevated levels of ACTH and corticosterone, in conjunction with increased CRF mRNA in the hypothalamus, have been observed in maternally deprived rats when compared to controls (Plotsky & Meany, 1993). In similar studies, alterations in HPA-axis activity have been associated with anxiety-like behaviors in the elevated plus-maze and open field (Ladd et al., 2000; Daniels et al., 2004) or depressive-like behavior during a forced swim test (El Khoury et al., 2006). These basic studies suggest that maternal separation in rats may be a useful animal model to investigate how early life experiences may lead to the development of abnormal behavior later in life.

Apart from the neuroendocrine abnormalities observed in humans, imaging studies on depressed patients show structural changes in the brain that include atrophy of the hippocampus, frontal cortex and striatum (Bremner et al., 2000; Sheline et al., 1999; Coffey et al., 1992; Drevets et al., 1997; Krishnan et al., 1992; Husain et al., 1991). A link was proposed between circulating glucocorticoids and neuronal damage in neuropathological conditions such as depression. A number of studies demonstrated a reduction in hippocampal neuron cell number induced by prolonged exposure to elevated concentrations of corticosterone (Sapolsky, 1985a; Sapolsky, 1985b; Sapolsky et al., 1985). Rat pups that were maternally separated for a 24 hour period had increased cell death in the hippocampus and cerebral cortex (Zhang et al., 2002). Futhermore, decreased neurogenesis has also been found in the hippocampus of adult rats that were subjected to maternal separation (Mirescu et al., 2004), and this may therefore be an additional mechanism that contribute to the neuro-anatomical changes observed in stressed subjects.

Glucocorticoid mediated neuron damage may involve the generation of reactive oxygen species (McIntosh & Sapolsky, 1996). Reactive oxygen species affect proteins, DNA and lipids and therefore inefficient removal or neutralization of these compounds may compromise overall cellular function (Cochrane, 1991). It has been shown that acute swim stress in rats decreased the antioxidant potential of serum 1 hour post stress, while restraint stress induced the same reaction after 24 h (Van Rensburg et al., 2006). Chronically restrained rats displayed lower total antioxidant reactivity and increased lipoperoxidation in the hippocampus (Fontella et al., 2005). It is therefore evident that stressful events pose an oxidative threat with deleterious consequences.

The survival, differentiation and proliferation of neurons involve neurotrophins such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3), (Lessmann et al., 2003; Barnabé-Heider & Miller, 2003; Heldin, 1995). Stress-induced reduction in growth factor levels may result in neuronal death (Smith et al., 1995; Sapolsky, 1992). This decrease may be differentially expressed over time for the various growth factors. In maternally separated animals, BDNF mRNA was decreased in the hippocampus at 16 days of age (Kuma et al., 2004), while NGF levels was decreased already at day 10 (Manni et al., 1998). These results suggest that stress may affect the levels of growth factors negatively, which in turn may have a detrimental impact on neuronal survival. Interestingly, neurotrophic factors can also protect neurons against oxidative stress by modulation of the expression of genes encoding antioxidant enzymes (Mattson et al., 1995).

The present study therefore hypothesizes that adverse events early in life compromise the development of the brain via a mechanism that include malfunctioning of the HPA-axis, as well as alterations in the levels of neurotrophins. Such changes render individuals incapable of coping with subsequent stressors and hence increase their vulnerability to develop behavioral abnormalities. In order to test our hypothesis, rat pups were subjected to maternal separation and their risk to develop depressive behavior following subsequent exposure to stress was assessed. To identify possible mediators of early life stress-induced vulnerability, CRF levels in the hypothalamus and plasma levels of ACTH and corticosterone at baseline, as well as in response to acute restraint stress, were measured. Hippocampal, striatal and frontal cortical tissue were used to determine neurotrophin levels and, in addition, the

antioxidative potential of serum was determined as an indicator of global oxidative stress.

Materials and Methods

This project was approved by the Committee for Experimental Animal Research of the University of Stellenbosch and all procedures were approved on the basis of minimizing pain and discomfort to animals (project number: P04/10/020). Male Sprague-Dawley rats were housed under standard laboratory conditions (12 h/12 h light/dark cycle; lights on at 6:00 am; food and water *ad libitum*) in the Central Research Facility of the University of Stellenbosch.

Maternal Separation

Rat pups were separated from day 2-14 for 3 hours a day in the morning (Ladd et al., 2004; Daniels et al., 2004). For this procedure, the mother was removed from the home cage and pups were placed under infrared lights (30-33 ºC) in an isolated room. Control pups were left undisturbed with their mothers. All pups were weaned on day 21 and male offspring were kept in pairs; 2 control or 2 maternally separated rats. A total number of 20 rats were maternally separated and 20 rats normally reared as controls.

Behavior

In addition to maternal separation, a separate group of rats were chronically stressed during adulthood (control n = 7; control with chronic stress n = 8; maternal separation n = 6; maternal separation with chronic stress n = 7). These rats were restrained for 4 hours on 5 consecutive days (day 60-64) after which depressive-like behavior was

measured. On day 65 the rats were habituated to a forced swim test in a cylinder (20 cm water at 25 ℃) for 15 minutes. 24 Hours later, rats were placed in cylinders again and their behavior recorded for 5 minutes. Immobility time was considered as rats floating passively, making small movements to keep their heads above the water level (El Khoury et al., 2006). Video recordings were rated manually afterwards, while the observer was blind to the treatment groups. Locomotor activity was not measured in these rats before the forced swim test. Data for other maternally separated rats on the open field and elevated plus maze were studied and showed no differences to control rats in terms of locomotor activity (see chapter 4). We therefore established that maternal separation has no effect on locomotor activity which could influence the immobility times in the forced swim test.

HPA-axis

The rats were divided into 2 groups (n=10 for each group) on day 83. The first group was decapitated for baseline values while the second group was restrained for 10 minutes and decapitated 15 min after restraint. Plasma, serum and hypothalamus tissue were collected and stored in liquid nitrogen. CRF levels in the hypothalamus were measured with a CRF ELISA kit from Cosmo Bio. Briefly, the samples were sonicated for 30 seconds in 200 µl buffer and reconstituted for assay with 120 µl of buffer. The rest of the extraction procedure and assay were done according to the specification of the manufacturer. Absorbance was read on a Bio-Tek Synergy HT plate reader. Plasma ACTH was measured using a ^{125}I immunoradiometric assay from Euro-Diagnostica. Plasma was thawed and a volume of 200 µl was assayed in duplicate, incubated overnight and radioactivity measured with a Packard gamma counter. Plasma corticosterone was measured using the ImmuChem ^{125}I

corticosterone radioimmunoassay from MP Biochemicals. 10 µl of plasma were diluted in 2 ml of the steroid diluent and 100 µl of the dilution assayed in duplicate.

Neurotrophins

Immediately after decapitation, the frontal cortex, hippocampus and striatum were dissected from the brain and stored in liquid nitrogen for the determination of baseline NGF and NT-3 levels. Only rats that were used for baseline HPA-axis determinations were used for neurotrophin measurements, since restraint stress could have altered neurotrophin levels. The hippocampus was divided into dorsal and ventral regions. Since there is no clear demarcation between these 2 areas, the whole hippocampus was taken out of the brain and the first third which is situated dorsally was collected for dorsal hippocampus samples. The mid-section was excluded since there is a gradual transition between the dorsal and ventral hippocampus (Bannerman et al., 2004). The last third of the ventral part of the hippocampus was collected as ventral hippocampus. Neurotrophin levels were measured with Promega ELISA kits. The frontal cortex, striatum, dorsal and ventral hippocampus samples were weighed and 300 µl lysis buffer was added to each sample. Samples were sonicated for 15 seconds and centrifuged at 4ºC for 20 min. The supernatant was stored at -20 ºC until analysis. All samples were assayed in duplicate and a 1:2 dilution was used for NT-3 and a 1:4 dilution for NGF. Absorbance was read on a Bio-Tek Synergy HT plate reader.

Anti-oxidative potential of serum

The anti-oxidative potential of serum from maternally separated and control animals were measured in vitro by adding it to a system that produces hydroxyl radicals. The latter causes the formation of highly fluorescent mono-hydroxylated benzoic acid

products. Antioxidants present in serum will inhibit the hydroxylation of benzoic acid. The mixture consisted of 10 mM benzoic acid, 10 % ascorbic acid, phosphate buffered saline, serum and hydrogen peroxide, and was adapted to a micro-method from Van Rensburg et al. (2006). Each sample was done in triplicate. Four assay controls were included, namely (i) PBS and benzoic acid; (ii) PBS, benzoic acid and control serum; (iii) PBS, benzoic acid, control serum and ascorbic acid; and (iv) PBS, benzoic acid and ascorbic acid. Fluorescence was measured with a Perkin-Elmer LS50B luminescence spectrophotometer (excitation 305 nm; emission 440 nm). Readings were taken at 1 h and again at 20 h.

Statistical analyses

Non-parametric tests were used for statistical analyses and $p<0.05$ considered as significant. A Kruskal-Wallis test followed by Dunn's post hoc test was done to compare chronic stress groups with maternal separation or normally reared controls. Mann-Whitney tests were done to compare control and maternal separation groups. Graphpad Prism (version 4.01) was used for analyses. Values are presented as means ± standard error of the mean.

Results

Behavior

Immobilization time in the forced swim test was used as an indicator of depressive-like behavior. There was no significant difference between control rats with or without subsequent exposure to chronic stress, neither was there a significant difference between normally reared (control groups) and maternally separated animals ($p>0.05$). However, rats that were maternally separated and chronically restrained

during adulthood, showed a significant increase in immobility time compared to the control group ($p<0.05$; Fig. 1).

HPA-axis

The CRF levels in the hypothalamus of maternally separated rats were higher than those of controls at baseline as well as after the acute stress (Table 1). However, this increase did not reach statistical significance ($p>0.05$). Baseline ACTH values were similar in both groups ($p>0.05$). The ACTH concentration increased dramatically after the acute stress in both groups, but this elevation was significantly less in maternally separated rats, when compared to controls ($p<0.05$; Table 1). Baseline corticosterone levels of maternally separated rats were significantly higher than that of control rats ($p<0.05$) and there was no difference after acute stress ($p>0.05$; Table 1).

Neurotrophins

There were no significant differences between maternally separated and control rats with respect to the levels of NGF or NT-3 in the frontal cortex or striatum ($p>0.05$). However, both NGF and NT-3 were significantly decreased in the ventral hippocampus ($p<0.05$; Fig. 2 and 3), while NT-3 was significantly increased in the dorsal hippocampus of maternally separated rats ($p<0.05$; Fig. 4).

Anti-oxidative potential of serum

The difference between the 20 h and 1 h readings was used as a measure of the anti-oxidative potential of serum of control and maternally separated animals. Fluorescence levels were significantly higher in maternally separated rats, which indicated that the serum of maternally separated rats had less anti-oxidant potential.

The ability of control serum to scavenge free radicals was therefore significantly higher ($p<0.05$; Fig. 5).

Discussion

A number of clinical studies indicate that early life stressful experiences such as child neglect and abuse may predispose young individuals to the development of psychiatric disorders later in life (Kessler & Magee, 1993; Mullen et al., 1996). However, our current understanding of the molecular mechanisms involved remains limited. In the present study we used maternal separation as an early life stress, and showed that rats subjected to maternal separation developed depression-like behavior following exposure to subsequent chronic stress. Maternally separated rats also displayed signs of a malfunctioning HPA axis, had altered NGF and NT-3 levels in the hippocampus and their serum had a significantly reduced capacity to scavenge free radicals.

Increased immobility during the forced swim test is considered reflective of depressive-like behavior (El Khoury et al., 2006; Aisa et al., 2007). In our experiments, one group of rats received chronic restraint stress during adulthood in addition to maternal separation. Interestingly, only the rats that received the combination of the 2 stressors, maternal separation together with chronic stress, showed increased immobility in a forced swim test compared to control rats that received no stress. Although maternal separation alone does not evoke depression–like behavior in adult rats, it increases the vulnerability of the rat to develop such behavior when subsequently exposed to chronic stress. The onset of depression in humans has also been linked to stressful life events (Kendler et al., 1999).

It is not clear as to what neurobiological mechanisms early life stress affects to generate a state of psychiatric vulnerability, therefore the effect of maternal separation on the functioning of the HPA-axis was assessed. Basal corticosterone levels were elevated in maternally separated rats, while ACTH showed a blunted response to acute stress and no increase in corticosterone. These neuroendocrine findings were similar to those reported earlier (Daniels et al., 2004) and are in agreement with clinical observations showing decreased cortisol release after CRF administration in depressive patients (Holsboer et al., 1986). Since the hypothalamic CRF levels were the same for both maternal separation and control groups before and after acute stress, it is likely that the synthesis of CRF was similar in both groups. However, it may be possible that the release of CRF following acute stress in the maternally separated rats could have been lower, explaining the blunted ACTH response observed in these animals. Alternatively, portal release of CRF in maternally separated rats could have been persistently high for an extended period of time, leading to a down-regulation of CRF binding sites at the level of the anterior pituitary, and hence the reduced ACTH response after acute stress. Support for this proposal comes from findings showing maternal separation leading to increased CRF mRNA in the paraventricular nucleus of the hypothalamus (Plotsky et al., 2005; Plotsky & Meany, 1993). Sustained release of high levels of CRF may also explain the elevated concentration in basal corticosterone in maternally separated animals. Adrenal gland weights were not measured in our study, but hypersecretion of corticosterone from adrenal glands could cause negative feedback and decreased ACTH release following stress.

There appears to be a close correlation between corticosterone levels and the levels of neurotrophins in rats. Studies by Roskoden et al. (2004) have shown that early postnatal administration of corticosterone leads to increases in neurotrophin mRNA and neurotrophin receptors in the hippocampus. These increases were particularly evident in NGF and NT-3 mRNA and protein levels. It has been proposed that this up-regulation in neurotrophins may reflect a compensatory mechanism of the brain to avoid further cell damage or death (Roskoden et al., 2004; Faure et al., 2006). In our study, where basal corticosterone concentrations were found to be elevated, the levels of neurotrophins were differentially altered in the hippocampus when compared to controls. The hippocampus was divided into dorsal and ventral regions, since it has been shown that these regions have separate functions, respectively memory and learning and aversively motivated behaviors, and different efferent and afferent connections (Bannerman et al., 2004; Trivedi & Coover, 2004). Our previous studies have also shown that there is a difference in neurotrophin levels in the dorsal and ventral hippocampus (Uys et al., 2006). Calfa et al. (2007) has shown that rats displaying social defeat behavior had greater Fos reactivity in the ventral and dorsal hippocampus compared to controls. This effect was strongly inhibited by a glucocorticoid receptor antagonist in the ventral hippocampus, suggesting that glucocorticoids affect the dorsal and ventral hippocampus differently. Maggio & Segal (2007) also showed significant differences in the ability of the ventral and dorsal hippocampus to generate long term potentiation in response to corticosterone.

Our data showed that the concentrations of both NGF and NT-3 were decreased in the ventral hippocampus of the maternally separated rats. This result is similar to earlier data showing maternal separation to reduce NGF levels in the hippocampus of rat pups (Manni et al., 1998), while traumatic events in young rats led to a

decrease in NT-3 and BDNF in the ventral hippocampus (Uys et al., 2006). These findings therefore all support a stress-induced reduction in neurotrophin levels in the ventral hippocampus. The decrease in neurotrophins in the ventral hippocampus as a consequence of stress is probably mediated through increased corticosterone binding to receptors in the hippocampus which could in turn decrease the transcription rate of neurotrophins (Joëls & Vreugdenhil, 1998). In contrast to the ventral hippocampus, we found increased NT-3 levels in the dorsal hippocampus of maternally separated rats. Previously, increases in NGF, NT-3 and BDNF were observed after periods of separation and repetitive stressful events (Faure et al., 2006; Faure et al., 2007; Greisen et al., 2005). Brief separations have also been shown to increase NGF mRNA in the dentate gyrus in rat pups (Cirulli et al., 2000). The upregulation of neurotrophins following prolonged stress have been described as part of a compensatory mechanism of the brain. Evidently, a stressful event downregulates neurotrophins and, after a period of time, neurotrophins may be upregulated in order to counteract neuronal death. Support for such a mechanism derives from the observation that following maternal deprivation, BDNF mRNA in the hippocampus initially decreased at 16 days of age, but significantly increased at 30 and 60 days of age (Kuma et al., 2004). The upregulation of NT-3 in the dorsal hippocampus, in contrast to it being downregulated in the ventral hippocampus, is an indication that certain brain areas are affected differently by stress.

A measurement of the antioxidant potential in the serum of maternally separated rats and control rats showed that maternally separated rats had a lower antioxidant potential. The expression of antioxidants in the hippocampus could be regulated by neurotrophins (Mattson et al., 1995) and, since NGF and NT-3 were decreased in the ventral hippocampus of the maternally separated rats, this could have been a

possibility. Oxidative stress was not measured in brain tissue, since it was used for neurotrophin determinations. Blood and other non-neuronal tissue can be used to detect oxidative stress that occurs in the brain (Gibson & Huang, 2002). Increased corticosterone may also play a role, as previous reports demonstrated, that acute and chronic stress decreased antioxidant potential of serum and brain tissue (Van Rensburg et al., 2006; Fontella et al., 2005).

In summary, maternal separation of rats predisposes them to develop depression-like behavior following subsequent exposure to chronic stress. A possible mechanism for this predisposition involves the dysregulation of the HPA axis resulting in a decrease or an increase in neurotrophin levels and an increase in oxidative stress. The reduction in NGF and NT-3 in the ventral hippocampus, which is involved in aversive behaviors, may therefore reflect the vulnerability of a subject to develop depressive behavior. On the other hand, increases in the level of NT-3 in the dorsal hippocampus that has to do with learning and memory processes (Bannerman et al., 2004; Trivedi & Coover, 2004), may indicate the attempts of the brain to develop new strategies to combat the stressful onslaught. Of note in this regard is that selective serotonin re-uptake inhibitors that are used as antidepressants (Bareggi et al., 2007), also normalize the elevation in corticosterone following trauma (Uys et al., 2006), increase neurotrophin gene expression (Dwivedi et al., 2006) and stimulate the immune-reactivity of antioxidant enzymes (Kim do et al., 2007). It would be simplistic to think that the complex effects of maternal separation follow a linear relationship i.e. excess glucocorticoids, altered neurotrophin levels leading to neuron damage. It may therefore be useful to look at the expression of other proteins, for instance those involved in apoptosis and neurogenesis, to better understand the deleterious effects of maternal separation in the brain.

Acknowledgements

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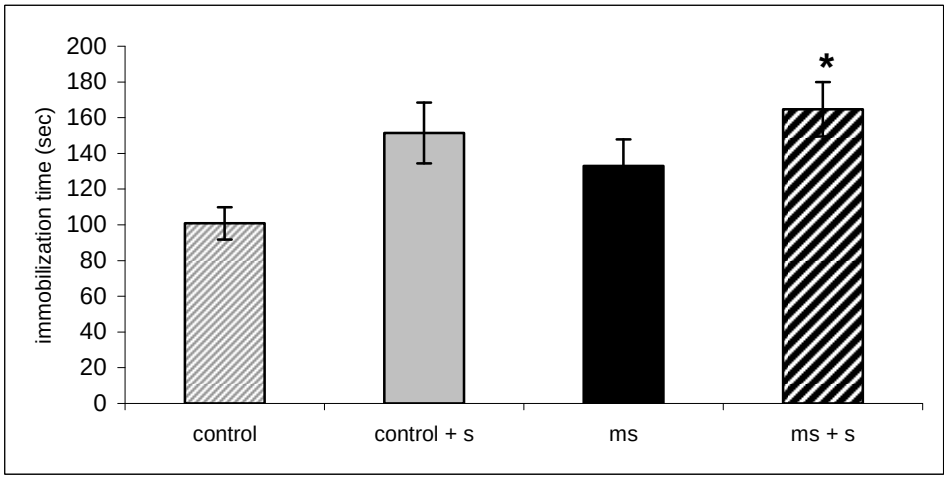

Fig. 1. Immobilization time (seconds) during the forced swim test (n=6-8). H=8.238 ; *p<0.05, compared to control group. s = chronic restraint stress during adulthood.

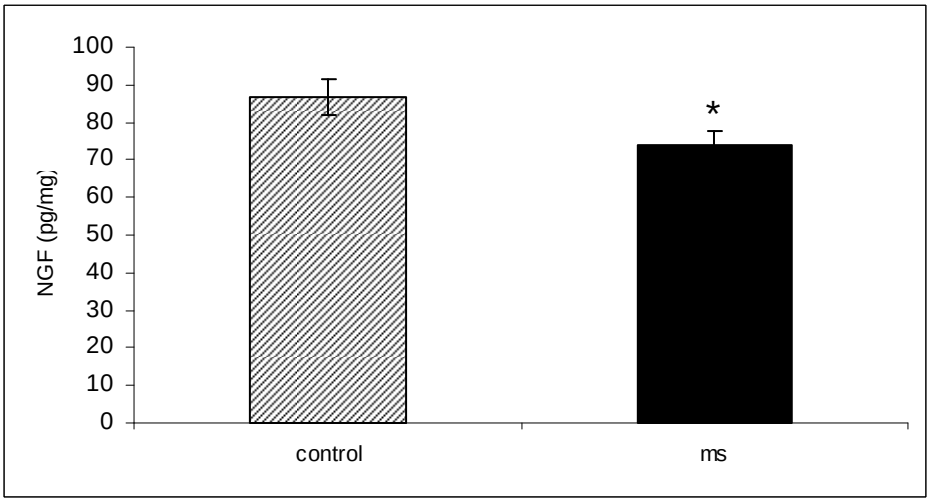

Fig. 2. NGF levels in the ventral hippocampus (pg/mg; n=9-10). * p<0.05 compared to control group.

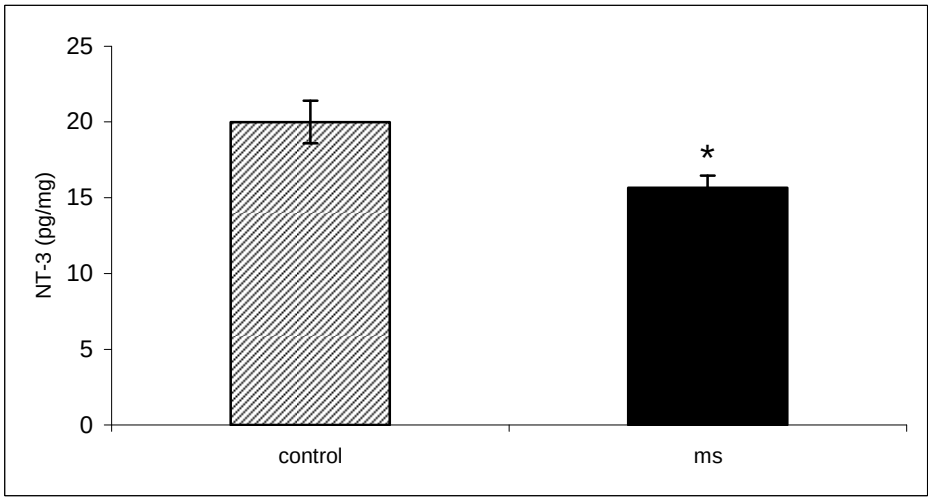

Fig. 3. NT-3 levels in the ventral hippocampus (pg/mg; n=9-10). * p<0.05 compared to control group.

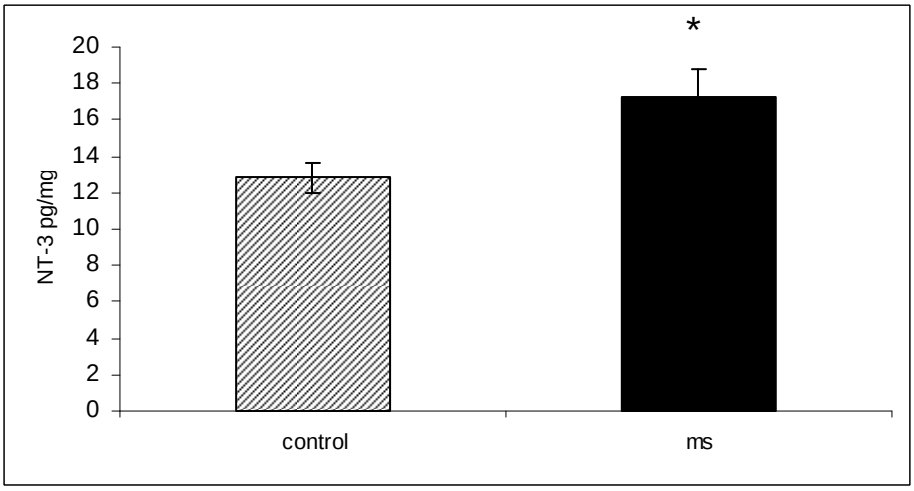

Fig. 4. NT-3 levels in the dorsal hippocampus (pg/mg; n=9-10). * p<0.05 compared to control group.

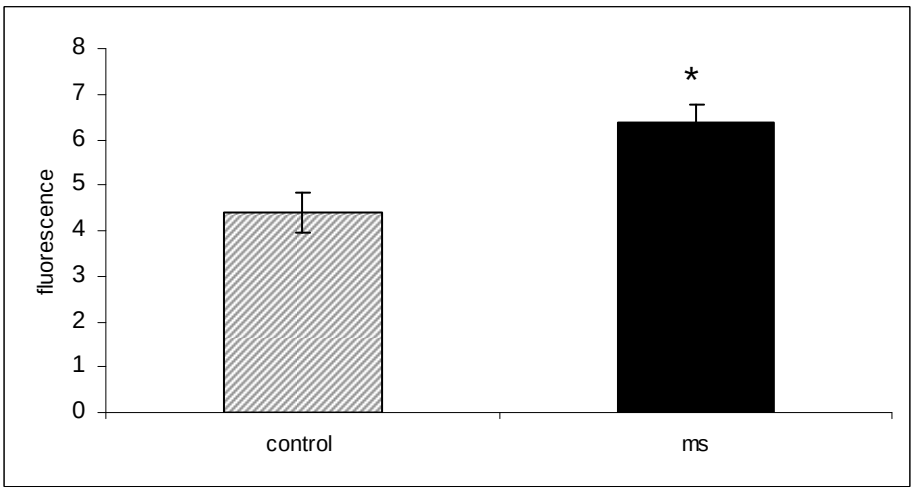

Fig. 5. Fluoresence produced by the hydroxylation of benzoate as a measure of antioxidant potential of serum (n=5-6). *p<0.05 compared to control group.

Table 1: CRF, ACTH and corticosterone levels, baseline and 15 min after restraint stress (n=8-10). *p<0.05 compared to relevant control group.

Group	**CRF (pg/mg)**	**ACTH (pg/ml)**	**Corticosterone (ng/ml)**
Baseline			
Control	208.31± 26.58	40.56 ± 5.34	77.95 ± 18.26
Maternal separation	249.45 ± 34.24	48.23 ± 4.12	144.86 ± 17.82 *
Acute stress			
Control	234.07 ± 46.37	162.78 ± 17.01	377.37 ± 25.79
Maternal separation	279.5 ± 46.37	111.76 ± 9.43 *	391.91 ± 23.77

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Chapter 4

Maternal separation stress alters the expression of apoptotic markers in the frontal cortex and striatum of adult rats

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Abstract

Maternal separation (MS) during the neonatal period is an animal model of early adversity in humans. A number of studies have shown that MS adversely affects neuronal growth particularly in hippocampal regions. The aim of this study was to determine if MS is associated with changes in anxiety-like behavior and markers of apoptosis in the hippocampus, striatum and frontal cortex. Rats were subjected to MS on postnatal day 2-14 for 3 h per day. Subsequent to MS, the rats were subjected to acute restraint stress or no stress and levels of Bcl-2, p-Bad and caspase-3/cleaved caspase-3 was measured by western blots in the hippocampus, striatum and frontal cortex. No differences were found in the hippocampus, but MS or subsequent acute stress changed levels of apoptotic markers in the striatum and frontal cortex, indicating an increased risk for apoptosis in these brain areas. MS did not influence the behavior of the rats in the elevated plus-maze or open field. Early life stress and subsequent stress increases the risk for apoptosis in the striatum and frontal cortex of rats without inducing anxiety-like behavior.

Keywords

Maternal separation; apoptosis, striatum and frontal cortex.

Introduction

Maternal separation (MS) during the neonatal period is frequently used as a rodent model of early adversity in human infants. MS has been shown to cause alterations in behavioral response to a novel environment and rats subjected to repeated MS show increased levels of anxiety-like behavior in adulthood (Romeo et al., 2003; Daniels et al., 2004; Troakes & Ingram, 2009; Lambás-Señas et al., 2009). Similarly, it has been shown that stressful experiences, other than early life stress, during adolescence also induce anxiety- and depressive-like behaviors in rats (Uys et al., 2006; Tsoory et al., 2007). Since early life stress predisposes individuals to the development of psychiatric disorders (Katerndahl et al., 2005, Heim & Nemeroff, 2001; Nemeroff, 2004; Wals & Verhulst, 2005), the behavioral response of MS animals during adulthood may confirm the involvement of early life stress in the development of anxiety disorders or depression.

A number of animal studies have shown that MS, whether applied in a single session or chronically, causes morphological alterations in the brain. These effects were particularly seen in the hippocampal region where the number of synapses in the CA1 and CA3 regions was decreased and the structure of neurons impaired. In the dentate gyrus a decreased number of neurons were observed after 24 hours of MS (Karakas et al., 2009; Fabricius et al., 2008), possibly related to the inhibition of cell proliferation. Cell proliferation in the dentate gyrus was decreased by a single episode or repeated MS (King et al., 2004; Mirescu et al., 2004; Kikusui et al., 2009), while degeneration of neurons was found in the dentate gyrus and Ammon's horn (Llorente et al., 2009). Repeated MS has also led to decreased mRNA concentrations of growth-associated protein-43, a marker for neuronal plasticity, in

the hippocampus (Law et al., 2009). In the rostral migratory system, repeated MS similarly decreased proliferation of neurons and led to neuronal degeneration (Račeková et al., 2009).

Other stressful experiences affect the brain similarly to early life stress. Acute restraint stress of rats during adolescence decreased cell proliferation in the hippocampus (King et al., 2004), while both acute and chronic exposure to a predator decreased the number of hippocampal neurons and increased apoptosis in the chronically stressed rats (Zhao et al., 2007). In MS rats, acute restraint stress during adulthood have been shown to induce a blunted ACTH response while chronic stress induced depressive-like behavior (Marais et al., 2008) and the development of psychiatric disorders in humans may also be subsequent to other life stressors (Kendler et al., 1999).

MS-induced apoptosis or decreased neurogenesis may be mediated by neurotrophins given their role in the proliferation and survival of neurons (Barnabé-Heider & Miller, 2003; Huang & Reichardt, 2003). In the prefrontal cortex, neonatal MS mice had reduced mRNA levels of transforming growth factor-α (Romeo et al., 2004), while MS rats had reduced brain derived neurotrophic factor (BDNF) mRNA levels in the frontal cortex (Roceri et al., 2004), hippocampus and striatum (Lippmann et al., 2007). Nerve growth factor (NGF) and neurotrophin-3 (NT-3) levels are also decreased in the hippocampus of MS rats (Marais et al., 2008), while rats subjected to early life trauma and subsequent stressors had decreased BDNF and NT-3 levels in the hippocampus (Uys et al. 2006). Both these studies found increased basal plasma corticosterone levels in the experimental rats. Studies by Sapolsky (1985a; 1985b) and Sapolsky et al. (1985) have shown that increased levels of

corticosterone, similar to what is seen during stressful periods, may induce neuronal death. It has also been found that stress-induced glucocorticoid levels reduces neurotrophin expression in the rat brain (Adlard & Cotman, 2004) and positively correlates with increased apoptosis in the hippocampus (Zhao et al., 2007). In contrast, Mirescu et al. (2004) showed that neuronal proliferation can be increased in MS rats by reducing their corticosterone levels.

The aim of this study was to determine if MS induces anxiety-like behavior and whether markers for apoptosis are changed, either by MS alone or the combination of MS and acute stress, during adulthood. Clinical brain imaging studies on patients with depression showed atrophy of the hippocampus, frontal cortex and striatum (Bremner et al., 2000; Coffey et al., 1992; Drevets et al., 1997; Husain et al., 1991). Therefore, these 3 brain regions are used in this study to see whether changes in neuronal survival, induced by early life stress and persisting into adulthood, are restricted to a certain area of the brain, as the majority of animal studies found differences in neuronal proliferation or apoptosis only in the hippocampus. We measured the expression of three pivotal members of the apoptotic pathway including Bcl-2, phosphorylated-Bad (p-Bad) and caspase-3 (casp-3), since previous studies have not highlighted the influence of MS on the expression of specific markers of apoptosis.

Materials and Methods

This project was approved by the Committee for Experimental Animal Research of the University of Stellenbosch (project number: P04/10/020). The experiments were performed in the Central Research Facility of the University of Stellenbosch. Male

Sprague-Dawley rats were used for the experiments. Rats were housed under standard laboratory conditions (12h/12h light/dark cycle; lights on at 6:00am; food and water *ad libitum*).

Maternal Separation

Rat pups were separated from postnatal day 2-14 for 3 hours a day in the morning before 13h00 (Marais et al., 2008). For this procedure, the mother was removed from the home cage, but remained in the housing room. The pups were transferred to an isolated room where they were placed under infrared lights to maintain the ambient temperature at 30-33ºC. Control pups were left undisturbed with their mothers. All pups were weaned on postnatal day 21 and male offspring were kept in pairs; 2 controls or 2 MS rats.

Behavior

To test whether MS affects anxiety-like and locomotor behavior of the rats during adulthood, they were subjected to the open field and elevated plus-maze tests on postnatal day 82. These two tests were done immediately after each other and rats were scored for 5 min. in each of the tests. The behavioral tests were performed in the morning between 9:00 and 12:00, recorded and then analyzed with Noldus Ethovision version 3.1. Parameters measured in the open field included total distance moved, time spent in the inner and outer zones, entries into the inner and outer zones. Parameters measured in the elevated plus-maze included total distance moved, time spent in the open and closed arms, number of entries into the open and closed arms as well as number of entries and time spent in the center

zone. Rearing and grooming behaviors were scored manually for both tests, and the number of bouts was recorded in each case. The number of rats used for behavioral measurements were n=35 for controls and n=36 for MS.

Acute stress response

In addition to measuring baseline levels of apoptotic markers in MS rats, some of the rats were also subjected to acute restraint stress on postnatal day 83 to assess how the expression of proteins are influenced by subsequent acute stress during adulthood. The acutely stressed group of rats were placed in restrainers for 10 minutes, put back into their home cages and decapitated 15 minutes post-stress (n=10). Rats in the non-stressed group were taken from the home cage and rapidly decapitated (n=10). Rat brains were dissected on ice and tissue samples (hippocampus, frontal cortex and striatum) were snap frozen and stored in liquid nitrogen until the analyses were done.

Western blots

The baseline and 15 min post acute restraint stress levels of Bcl-2, p-Bad and caspase-3/cleaved caspase-3 levels in the frontal cortex, striatum and hippocampus were measured with western blots. The number of rats used in each group were n=5. These were rats taken from the bigger group that all underwent the same behavioral tests.

Sample preparation:

Samples were homogenized on ice (2 X 5sec) in 1 ml of lysis buffer containing 50 mM TRIS (pH 7.4), 1% sodium deoxycholate, 50 mM NaF, 20µM ZnCl, 1 mM $NaVO_3$, 0.5 mM PMSF, 2 µg/ml aprotinin and leupeptin and 0.5% NP40. Determination of the protein concentration in each sample was done according to Bradford (1976). Aliquots of 100 µl were prepared by adding 25 µl of sample buffer, 5 µl of reducing agent (Bio-Rad), Millipore water and sample to obtain a concentration of 2 µg/µl.

Western blotting:

SDS-gel electrophoresis was done using 4-12% precast gels (Bio-Rad Criterion XT). Samples were boiled for 5 min before 40µg of protein for each sample and 5µl of protein standard were loaded onto each gel (Bio-Rad Kaleidoscope). XT MOPS running buffer (Bio-Rad) were used for Bcl-2 (26 kDa) and p-Bad (23 kDa) protein determinations, while XT MES running buffer (Bio-Rad) were used for caspase-3 and cleaved caspase-3 determinations (36 and 18 kDa). Separated proteins were transferred to PVDF membrane (Immobilon-P; Millipore) that was treated with 100% ethanol and rinsed with Millipore water. Membranes were blocked overnight at 4 ℃ in fat free milk (5% in PBS/tween). The next day the membranes were washed with PBS/tween and incubated with primary antibody (1:1000) in PBS/tween for 2 hours at room temperature. Anti-Bcl-2 mouse monoclonal, anti-phospho Bad (Ser112) rabbit polyclonal and anti-caspase-3 rabbit polyclonal (Stressgen) were used to identify the different proteins. Membranes were washed and incubated for 1h with a secondary antibody (anti-mouse IgG HRP 1:25000; anti-rabbit IgG HRP 1:100000; Amersham Biosciences) in 5% milk in PBS/tween. Membranes were washed and visualized with LumiGLO Reserve (KPL). Densitometry was done using UN-SCAN-IT software (Slikscience) and each sample normalized with β-tubulin levels. For this purpose,

membranes were stripped and incubated with β-tubulin primary antibody (1:1000; Cell Signaling) and anti-rabbit IgG secondary antibody (Amersham Biosciences).

Statistical analyses

Student'sT-tests were employed for behavioral data analyses since large group numbers were used (n>30). Non-parametric Mann-Whitney tests were used for western blot analyses since smaller group numbers were used (n=5). In all statistical tests, $p<0.05$ was considered as significant. GraphPad Prism version 4.02 was used for analyses. Values are presented as means ± standard error of the mean.

Results

Behavior

There were no significant differences between the MS and control groups in any of the parameters measured in the elevated plus-maze or open field tests ($p>0.05$) . MS did not induce anxiety-like behavior or influence locomotor behavior (Table 1.).

Western blots

There were no significant differences in the hippocampal levels of Bcl-2, p-Bad or casp-3 between the groups ($p>0.05$). In the frontal cortex, there was a significant increase in Bcl-2 levels in acutely stressed MS rats (Fig. 1; $p<0.05$) and no other significant differences were observed in this area. In the striatum, there was a significant increase in Bcl-2 levels (Fig. 2; $p<0.05$) and a decrease in p–Bad levels

(Fig. 3.) in non-stressed MS rats and a significant increase in p-Bad in acutely stressed MS rats (Fig. 4; $p<0.05$). There was also a significant increase in casp-3 and cleaved casp-3 levels in acutely stressed MS rats (Fig. 5; $p<0.05$).

Discussion

Psychiatric disorders are complex disorders involving the interplay between many neurobiological systems. Despite numerous studies investigating the etiology of its associated neuroendocrine-behavioral symptomology, the pathophysiology of these disorders is still not entirely clear.

In this study, rats subjected to MS showed differences in the levels of Bcl-2, p-Bad, caspase-3 and cleaved caspase-3 in the frontal cortex and mainly in the striatum, but not in the hippocampus. There were no differences in the behavior measured in MS rats compared to control rats.

The adult MS rats showed no increases in anxiety-like behavior or changes in locomotor activity when compared to control rats. Similar results were found in other studies where the open field and elevated plus-maze were used (Roman et al., 2006; Slotten et al., 2006). Early life stress predisposes humans to the development of a number of psychiatric disorders, and therefore do not necessarily induce anxiety in adults. Rat studies also confirm this as MS also induces depressive-like behavior in the forced swim test (El Khoury et al., 2006; Aisa et al., 2007; Lee et al., 2007; Lambás-Señas et al., 2009).

In our study, there were no differences in expression of the apoptotic markers in the hippocampus between MS and control rats. Previous studies have extensively focused on the involvement of the hippocampus in psychiatric disorders, for instance, the hippocampus has been shown to be decreased in volume in patients with anxiety disorders and depression (Gueze et al., 2005) and studies on animal models have shown increased apoptosis and decreased neurogenesis in the hippocampus of rodents subjected to MS (King et al., 2004; Mirescu et al., 2004; Kikusui et al., 2009; Llorente et al., 2009). We have previously shown that MS differentially affects certain areas of the hippocampus where neurotrophins are downregulated in the ventral hippocampus and upregulated in the dorsal hippocampus (Marais et al., 2008). A possible explanation for not finding any significant differences in protein expression in the present study could be the fact that we have used whole hippocampi and was unable to detect regional differences. Acute restraint stress induced significant elevations in plasma corticosterone levels in the rats (Marais et al., 2008), but its effect of altering transcription (Hayashi et al., 2004) in the hippocampus possibly requires more than the overall time of 25 min. that we used in this experiment.

In contrast to the hippocampus, significant changes were observed in the frontal cortex and striatum of MS rats. Bcl-2 levels were significantly increased in both these brain areas, but in the frontal cortex only after acute stress. Bcl-2 is typically an anti-apoptotic protein that inhibits cell death (Veis et al., 1993). Bcl-2 prevents increases in mitochondrial membrane permeability, thereby blocking the release of pro-apoptotic cytochrome-c from mitochondria. This in turn inhibits the activation of caspases and the initiation of apoptosis (Deshager & Martinou, 2000). These effects have been confirmed with *in vitro* experiments where over-expression of Bcl-2 has been shown to prevent the activation of downstream executioners of apoptosis and

cell death (Chinnaiyan et al., 1996). Increased Bcl-2 levels in the frontal cortex and striatum of MS rats may possibly be indicative of a compensatory mechanism for neuronal protection.

Bad is a pro-apoptotic protein that binds to anti-apoptotic proteins of the Bcl-2 family to inhibit their function (Yang et al., 1995). However, when Bad is phosphorylated (p-Bad), it cannot bind to these proteins and thereby inhibits apoptosis (Zha et al., 1996). We found that p-Bad is decreased in the striatum of MS rats consistent with risk for apoptosis. Normally, neurotrophins activate survival pathways that disable pro-apoptotic proteins, such as Bad, via phosphorylation (Yao & Cooper, 1995; Datta et al., 2000; McKernan et al., 2009). A number of previous studies reported neurotrophins to be decreased after early life stress (Roceri et al., 2004; Lippmann et al, 2007; Marais et al., 2008), possibly increasing the vulnerability of neurons to apoptosis. Interestingly, in our MS rats, subsequent acute stress resulted in increased levels of p-Bad in the striatum. This finding indicates that a compensatory mechanism to protect neurons in the striatum after stress has been maintained, similar to the increase in Bcl-2 levels in the striatum and frontal cortex.

Casp-3 and the activation thereof, measured as cleaved casp-3, is a marker of the execution phase of apoptotic cell death, the enzyme is responsible for proteolysis of a number of protein substrates such as poly- (ADP-ribose) polymerase, which functions in repairing deoxyribonucleic acid (DNA). These substrate proteins are important for DNA and cytoskeletal structure and their cleavage leads to the inhibition of their function (Cohen, 1997). Whilst there were no significant differences in the baseline levels of caspase-3 in any brain region studied, a significant increase in casp-3 and cleaved casp-3 was observed in the striatum of MS rats after acute

restraint stress. The upregulation of these enzymes also support the finding that p-bad was decreased in the striatum at baseline level which could lead to the induction of apoptosis.

Acute stress in MS rats during adulthood seems to have a marked effect on certain markers of apoptosis, which is not detected at baseline level. Clinical studies also prove that early life stress predisposes individuals, but the manifestation of psychiatric disorders during adulthood is subsequent to exposure of other acute or chronic stressors (Heim & Nemeroff, 2001).

The MS rats used in this study also had a dysregulated HPA-axis as reflected by increased baseline levels of plasma corticosterone compared to control rats (Marais et al., 2008). Acute restraint stress induced significant elevations in plasma corticosterone in MS and control rats (Marais et al., 2008; see chapter 3). Du et al. (2009) showed that chronic glucocorticoid treatment reduced mitochondrial Bcl-2 levels in the frontal cortex and that translocation of glucocorticoid receptors to the mitochondria regulates its membrane function. Chronic, high doses of corticosterone reduce mitochondrial membrane potential and calcium holding capacity, increasing the risk for apoptosis. Therefore, the risk for neuronal apoptosis may have been influenced by elevation of glucocorticoid levels in our MS rats.

Conclusion

The present study showed that certain markers for apoptosis, Bcl-2, p-Bad and casp-3, changed in response to early life stress and subsequent acute stress during adulthood. These markers were apparent in the striatum and frontal cortex, but not

in the hippocampus. This was surprising given that a number of previous reports have focused on hippocampal neuronal degeneration. Previous studies have shown that MS affects neurotrophins and future studies should therefore focus on the combined effect of early life stress and subsequent stressors during adulthood on the expression of proteins involved in neurotrophin-activated pathways together with apoptotic markers, specifically in the striatum and frontal cortex.

Acknowledgements

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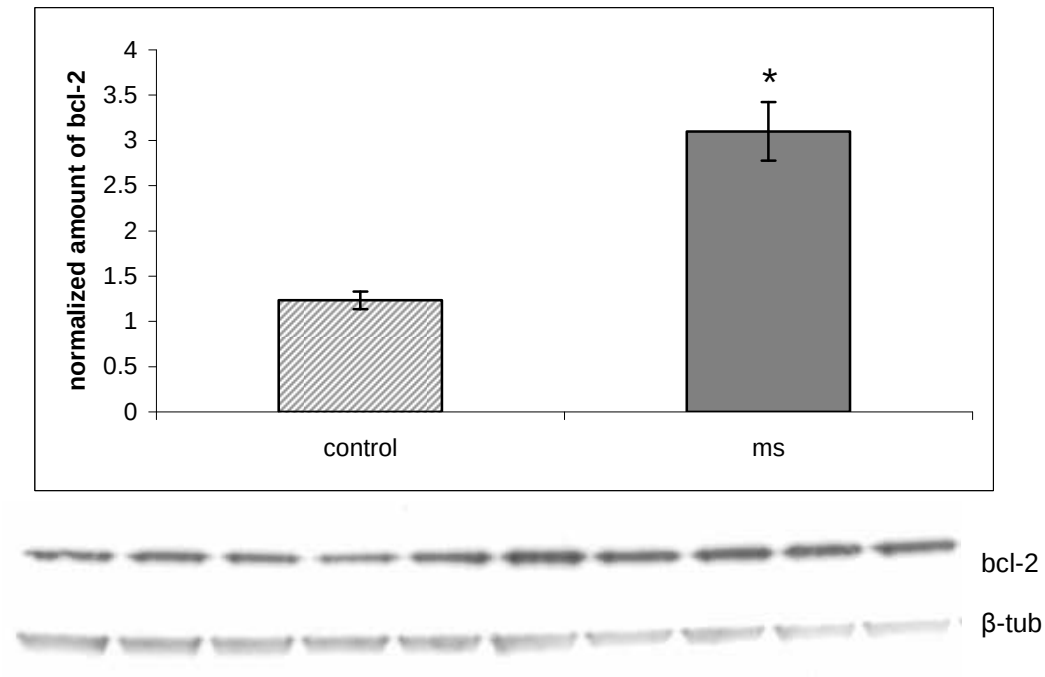

Fig. 1. Bcl-2 levels in the frontal cortex after acute stress. * p<0.05.

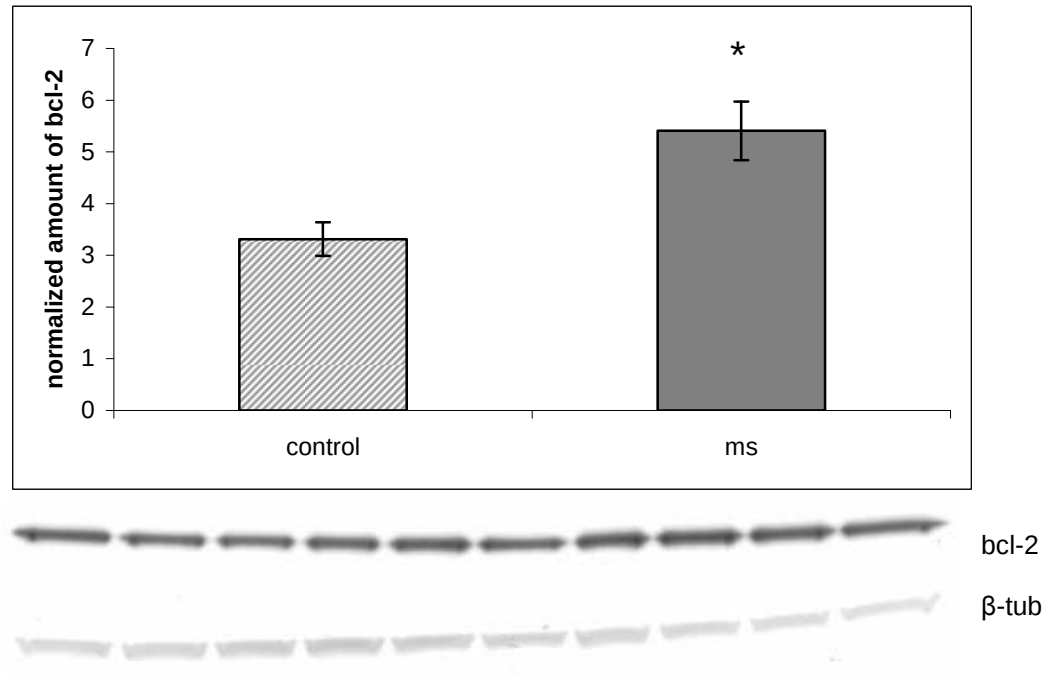

Fig. 2. Baseline bcl-2 levels in the striatum. * p<0.05.

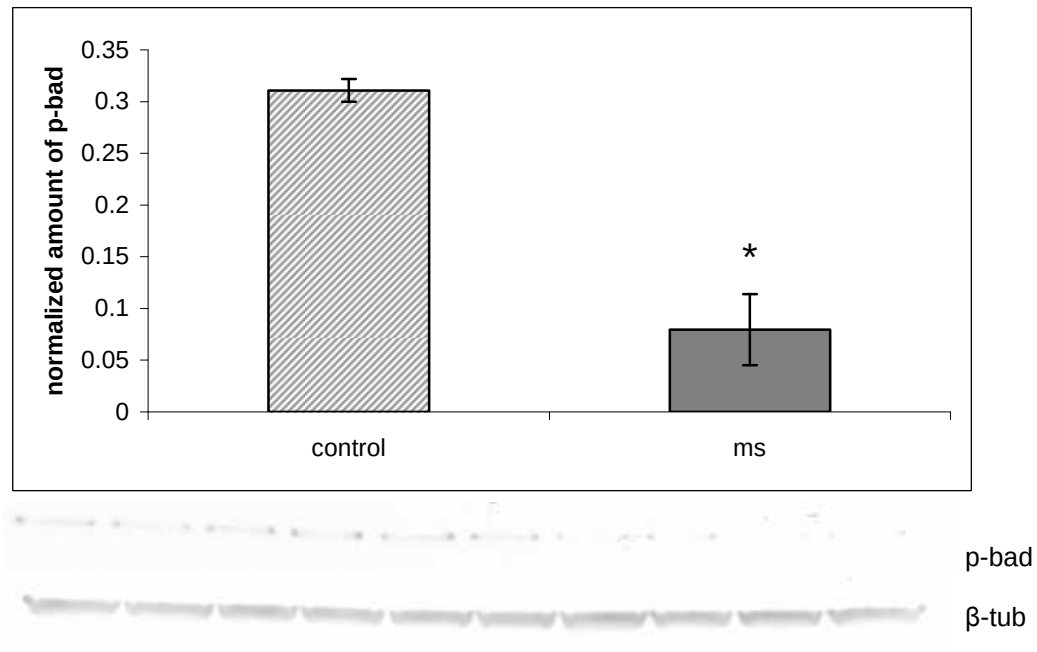

Fig. 3. Baseline p-bad levels in the striatum. * p<0.05.

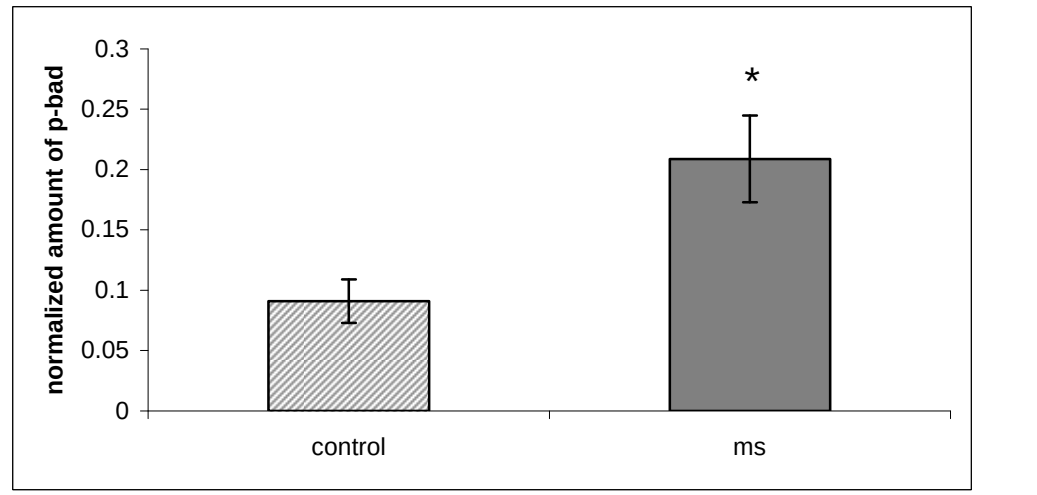

Fig. 4. P-bad levels in the striatum after acute stress. * p<0.05.

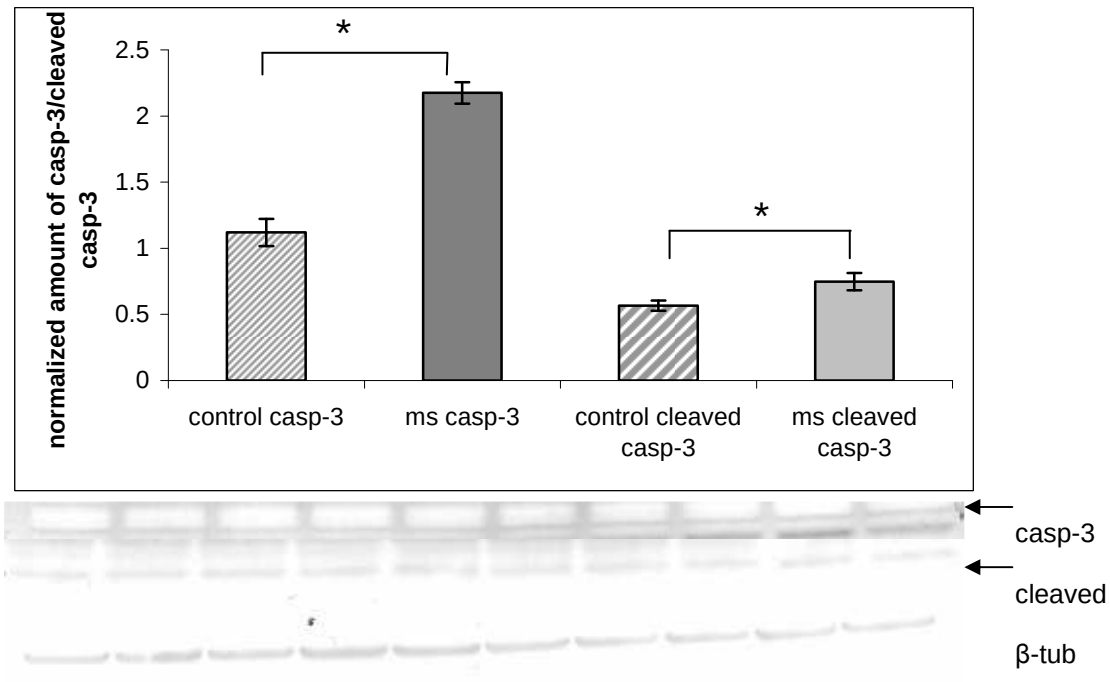

Fig. 5. Casp-3 and cleaved casp-3 levels in the striatum after acute stress. * $p<0.05$.

Table 1. Behavioral data for the open field and elevated plus-maze. Values presented as means ± standard error of the mean (SEM). Time spent was measured in seconds and distance moved in cm. Rearing and grooming were measured as number of bouts.

	Control		**Maternal separation**	
	Mean	**SEM**	**Mean**	**SEM**
Open Field				
Total distance moved	3006.52	65.31	2793.35	87.33
Time in inner zone	44.87	4.13	39.48	4.62
Time in outer zone	254.93	4.12	260.42	4.61
Entries into inner zone	17.34	1.27	14.36	1.04
Entries into outer zone	18.17	1.26	15.11	1.04
Rearing	38.94	1.86	37.56	2.04
Grooming	0.89	0.20	1.06	0.22
Elevated plus-maze				
Total distance moved	1383.03	50.17	1461.42	139.49
Time in open arms	41.09	4.12	50.75	5.8
Time in closed arms	222.79	5.48	210.06	6.59
Time in center zone	32.46	2.46	33.31	3.6
Entries into open arms	4.8	0.54	7.14	1.68
Entries into closed arms	13.03	0.87	12.58	1.28
Entries into center zone	17.31	1.03	16.39	1.02
Rearing	5.40	0.17	6.92	0.72
Grooming	1.51	0.30	1.14	0.19

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Chapter 5

Exercise increases BDNF levels in the striatum and decreases depressive-like behavior in chronically stressed rats

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Abstract

Early life stress in humans can affect the development of neurons and neurotransmitter systems and predispose an individual to the subsequent development of depression. Similarly, in rats, maternal separation causes anxiety- and depressive-like behavior and decreased corticosterone levels. Patients receiving pharmacological treatment for depression often experience negative side-effects or do not respond optimally and therefore the use of exercise as alternative antidepressant treatment is investigated. The aim of the study was to see whether rats subjected to both early life stress and chronic stress later in life show differences in depressive-like behavior, neurotrophin levels, stress hormone levels and antioxidant capacity of serum after chronic voluntary exercise as treatment. Rat pups were maternally separated and one group were allowed access to running wheels for 6 weeks while control rats were also handled and put in cages without running wheels. All rats were subjected to chronic restraint stress for 5 days, 3 hours per day, during adulthood. A forced swim test was done to test for depressive-like behavior. Neurotrophins were measured in the ventral hippocampus and striatum; baseline stress hormones were measured in blood plasma as well as the anti-oxidative potential of serum. Compared to controls, rats that exercised had no difference in baseline stress hormones, but had decreased immobility times in the forced swim test, increased brain derived neurotrophic factor (BDNF) levels in the striatum and decreased anti-oxidative potential of their serum. The mechanism by which depressive-like behavior was improved may have been mediated through increased striatal BDNF levels, resulting in increased neuroplasticity and the prevention of neuronal death.

Key Words

Maternal separation, Animal models of depression, Brain derived neurotrophic factor, Exercise.

Introduction

Patients on antidepressant treatment often experience one or more adverse side-effects that decrease their quality of life and prevent remission and because of this, treatment is sometimes discontinued (Kelly et al., 2008; Hu et al., 2004). Apart from side-effects, it has also been reported that a high rate of patients do not respond to (19-34%) or only partially respond (29-46%) to antidepressant treatment (Fava & Davidson, 1996). Evidently, there is a need for alternative treatment options in addition to current pharmacotherapy. A recent review on clinical studies have shown that exercise is effective as antidepressant treatment; it can be also be used in combination with other treatment and that the side-effects are minimal (Daley, 2008).

In humans, early life stress (Gilmer & McKinney, 2003) and other stressful life events (Paykel, 2001; Kendler et al., 1999) have been associated with the development of depression. Increased depressive-like behavior was also observed in rats subjected to both early life stress and subsequent chronic stress during adulthood, but not in rats subjected to only one of these stressors (Marais et al., 2008). These results suggest that early life stress can predispose an individual to the development of depression, which is then precipitated by a subsequent stressor. Imaging studies have shown that the volume of the hippocampus, striatum and frontal cortex is decreased in patients with depression (Bremner et. al., 2000; Sheline et. al., 1999; Drevets et al., 1997; Krishnan et al., 1992; Husain et al., 1991). In the hippocampus,

such a decrease in volume was attributed to a marked reduction in neurogenesis or increased neuronal atrophy resulting from high circulating glucocorticoid levels after stressful events (Gould et al., 2000; McEwen, 2000).

Maternal separation in rodents may be a particularly useful model of early adversity and depression and the mechanism may involve alterations in neurotrophins (Marais et al., 2008; Kuma et al., 2004; Manni et al., 1998). A decreased number of neurons, decreased proliferation and increased apoptosis of hippocampal neurons have also been observed in maternally separated rodents (Fabricius et al, 2008; Mirescu et al, 2004; Lee et al., 2001). Thus, animal studies indicate that maternal separation stress leads to alterations in neurotrophins, with decreased growth and survival of neurons.

Exercise, like antidepressant administration, increased neurotransmitter availability as well as neurotrophin expression in clinical and rodent studies (Blier et al., 1987; Duman, 2002; Engesser-Cesar et al., 2007; Min et al., 2003; Russo-Neustadt et al, 2000). Both brain-derived neurotrophic factor (BDNF) and serotonin (5-HT) activate signaling pathways and activate transcription factors that influence the expression of proteins to regulate neural plasticity, stress resistance and cell survival. An increase in 5-HT can therefore indirectly increase neurotrophin expression through activation of transcription factors (Mattson et al., 2004). Other studies that pointed towards an interaction between antidepressants, exercise and neurotrophin expression found the upregulation of BDNF mRNA after both antidepressant treatment and voluntary exercise (Russo-Neustadt et al., 2001), the increase in BDNF levels in the hippocampus after acute treadmill running (Soya et al., 2007) and increased BDNF in the hippocampus following chronic voluntary exercise after traumatic brain injury (Griesbach et al., 2008). Vaynman et al. (2003) also found that voluntary exercise

not only increased mRNA levels of BDNF, but also that of its primary receptor tropomyosin related kinase receptor B (TrkB) and the transcription factor c-AMP response element binding (CREB) in the hippocampus. Similarly, in humans, serum BDNF levels were increased together with the improvement in cognitive function after acute cycling exercise (Ferris et al., 2007).

Various forms of exercise have been used in animal studies, such as treadmill running, swimming and wheel running. Forced exercise coupled with stressors, for example electric shocks during treadmill exercise (Brown et al., 2007) or putting the rats in water (Hall et al, 2001), may increase corticosterone levels and subsequently minimize the beneficial effect of the exercise on neuron structure and function . For instance, significantly higher plasma corticosterone levels were seen in rats after a single forced swim test (Hall et al, 2001) while moderate intensity but not low intensity treadmill running also increased plasma corticosterone for up to 60 min after running (Soya et al., 2007). In our experiments, we used voluntary wheel running with rats having free access to running wheels only during their active phase in order to minimize the induction of stress while exercising. Apart from the effects of increased corticosterone, oxidative stress may also be induced by exercise as an effect of aerobic metabolism (Davies et al., 1982). The increase in free radical formation after exercise can induce damage to proteins and therefore it is important to measure oxidative status after exercise.

The aim of the present study was to further elucidate the current understanding of the mechanism by which exercise exerts its beneficial effects, using a rat model for depression. Our model of early adversity to induce depression was maternal separation, with a subsequent chronic stressor during adulthood. We wanted to

establish what the effect of chronic exercise is on behavior, hypothalamic pituitary adrenal-axis activity, neurotrophin levels and the antioxidant potential of the serum of stressed rats.

Materials and Methods

This project was approved by the Committee for Experimental Animal Research of the University of Stellenbosch (project number: P04/10/020). The experiments were performed in the Central Research Facility of the University of Stellenbosch. Male Sprague-Dawley rats were used for experiments. Rats were housed under standard laboratory conditions (12h/12h light/dark cycle; lights on at 6:00am; food and water supplied *ad libitum*).

Experimental Design

Maternal separation as an early life stressor

All rat pups were separated from their mothers on day 2-14 for 3 hours per day in the morning (Marais et al., 2008). For this procedure, the pups were removed from their mother and placed under infrared lights keeping the ambient temperature at 30-33ºC in an isolated room. Pups were weaned on day 21 and male rats were kept together in standard cages: 2 or 3 sedentary controls (n = 11) or 2 exercised rats (n=12).

Exercise

A randomly selected group of maternally separated rats were subjected to chronic voluntary exercise as a treatment. These rats were placed in cages equipped with running wheels during their active phase (corresponding to the dark cycle from 6:00

am to 6:00 pm) for a period of 6 weeks, on day 40-82 for 5 days of the week. Experimental rats were kept in pairs in the exercise cages, each having access to their own running wheel so that we could monitor whether they ran or not. The two animals in a cage were kept apart with a perforated Perspex separator between them that facilitated visual and oral communication between the rats. The control group of maternally separated rats were similarly handled but had no access to exercise wheels.

Chronic stress during adulthood

All rats were chronically restrained for 5 consecutive days during adulthood, from day 76 to 80. Rats were placed in Perspex restrainers for 3 hours each day during the morning and then put back in their home cages. The rats were allowed to continue exercising during their active phase.

Blood and tissue collections

Rats were decapitated for blood and tissue collection on day 83 at 9:00 in the morning. Trunk blood was collected immediately after decapitation for stress hormone level determinations (in EDTA tubes to collect plasma) as well as for the antioxidant assay (in polypropylene tubes to collect serum). The brain was dissected on a cooled Perspex sheet to collect the ventral hippocampus and striatum for neurotrophin level determinations.

<u>Parameters measured</u>

Behavior

On day 81 the rats were habituated for 15 minutes to a forced swim test in a cylinder with a height of 32 cm, diameter of 32 cm, water depth of 28 cm (to ensure that the adult rats could not reach the bottom with their tails to keep their noses above the water) and temperature of 25 ℃. 24 Hours later, r ats were placed in the cylinders again and their behavior recorded for 5 minutes. Immobility time was considered as rats floating passively, making small movements to keep their heads above the water level (El Khoury et al., 2006; Marais et al., 2008). Total immobility time (seconds) was measured during the 5 min trial on day 82 as a measure of depressive-like behavior. The forced swim test was done in the morning to allow the rats to keep exercising during their active phase. Locomotor activity was not measured prior to the forced swim test.

HPA-axis

Trunk blood was collected after decapitation in EDTA tubes, centrifuged for 10 min. at 4℃ and plasma stored in liquid nitrogen until a nalysis. Adrenocorticotrophic hormone (ACTH) was measured using a ^{125}I immunoradiometric assay from Euro-Diagnostica. Plasma was thawed and a volume of 200 µl was assayed in duplicate, with overnight incubation. Corticosterone was measured using the ImmuChem ^{125}I corticosterone radioimmunoassay (MP Biochemicals). 10 µl of plasma were diluted in 2 ml of the steroid diluent and 100 µl of the dilution assayed in duplicate. Radioactivity was measured with a Packard gamma counter.

Neurotrophins

Following decapitation, the ventral hippocampus and striatum were dissected from the brain and stored in liquid nitrogen for the determination of BDNF, nerve growth factor (NGF) and neurotrophin-3 (NT-3) levels. These were measured with Promega

ELISA kits. Samples were weighed and 300 µl lysis buffer added to each sample. Samples were sonicated for 30 seconds and centrifuged at 4 ºC for 20 min. The supernatant was stored at -20 ºC until analysis. All samples were assayed in duplicate and a 1:2 dilution was used for NT-3 and a 1:4 dilution for BDNF and NGF. Absorbance was read on an ELISA plate reader (Bio-Tek Synergy HT) and the concentration of each sample was calculated by the computer by plotting the absorbance values on standard curve with known concentrations generated by the assay.

Anti-oxidative potential of serum

Trunk blood was collected after decapitation in clean tubes, centrifuged for 10 min. at 4℃ and serum stored in liquid nitrogen until analy sis. The anti-oxidative potential of serum was measured in vitro by adding it to a system that produces hydroxyl radicals. The latter causes the formation of highly fluorescent mono-hydroxylated benzoic acid products. Anti-oxidants present in serum inhibit the hydroxylation of benzoic acid. The mixture consisted of 10mM benzoic acid, 10% ascorbic acid, phosphate buffered saline, serum and hydrogen peroxide, and was adapted to a micro-method of Van Rensburg et al. (2006). The assay was done in a black 96-well plate and each sample was assayed in triplicate. Four assay controls were included, namely (i) PBS and benzoic acid; (ii) PBS, benzoic acid and control serum; (iii) PBS, benzoic acid, control serum and ascorbic acid; and (iv) PBS, benzoic acid and ascorbic acid. Fluorescence was measured with a Perkin-Elmer LS50B luminescence spectrophotometer (excitation 305nm; emission 440nm). Readings were taken at baseline, 3h, and again at 20 h after all the reagents were added. This was done to ensure that the reaction took place before the first reading and then allowed to run overnight with the second reading at 20 h.

Statistical analysis

Statistical analysis was done using GraphPad Prism 4 software. Sedentary rats were compared with exercised rats using Mann-Whitney tests. A non-parametric test was used because of relatively small group numbers. The significance level was considered as $p<0.05$.

Results

Immobility time of exercised rats was significantly less than that of sedentary rats in the forced swim test (Fig. 1.) There was no significant difference in the baseline plasma ACTH and corticosterone levels between exercised and sedentary rats (data not shown). There was also no significant difference in the neurotrophin levels of the ventral hippocampus between the 2 groups (data not shown). Rats that exercised had increased BDNF levels in the striatum compared to sedentary rats (Fig. 2.), but no difference was found in NGF or NT-3 levels (data not shown). Exercised rats had significantly lower serum anti-oxidative potential measured at both 3h (data not shown) and 20h (Fig. 3.), with the baseline value subtracted, after the start of the assay. The increase in relative fluorescence is an indication that there are less anti-oxidants in the serum.

Discussion

Rats receiving exercise treatment after being maternally separated as pups and again chronically stressed as adults, showed less immobility in a forced swim test

than rats that did not exercise. Therefore, chronic voluntary exercise was effective in reducing depressive-like behavior in stressed rats. Our results were in accordance with the findings of previous studies showing that chronic wheel running in mice produces antidepressant-like effects in various tests including the forced swim test, tail suspension test and learned helplessness test (Duman et al., 2008; Duman et al., 2009). Although the decreased immobility scores in the forced swim test cannot be directly extrapolated to depressive behaviour in humans, it is noteworthy that clinical studies also reported reduced symptoms of depression in patients receiving augmentation with chronic exercise treatment compared to patients that did not exercise (Dimeo et al., 2001; Craft, 2005; Trivedi et al., 2006).

The 6 weeks of exercise resulted in a significant increase in striatal BDNF. Binding of neurotrophins to Trk receptors stimulates the growth, plasticity and survival of neurons (Huang & Reichardt, 2003), and therefore the observed raised levels in BDNF should have a positive effect on the functioning of striatal neurons in exercised rats. BDNF has been shown to be neuroprotective and can prevent decline in brain function associated with neurodegeneration (Henningan et al., 2007). Min et al. (2003) found that exercise increases 5-HT synthesis in the dorsal raphe nucleus. Increased BDNF in the striatum could therefore be a result of increased release of 5-HT from neurons originating in the raphe nucleus. This is plausible since the expression of BDNF may be mediated through increased signaling of 5-HT that activates transcription factors such as CREB (Mattson et al., 2004). In support of this suggestion is data showing that wheel running in rats increased mRNA of CREB and synapsin I, a synaptic protein involved in neurotransmitter release (Vaynman et al. 2003), activated the phosphatidylinositol 3-kinase pathway via Trk receptors and increased p-CREB in the hippocampus (Chen & Russo-Neustadt, 2005).

In spite of existing data strongly supporting our proposal that the increase in BDNF in the striatum may be related to increased 5-HT levels, it is unknown whether it was the case in our rats, because neurotransmitter levels were not measured in the present study. Exercise has also been shown to increase 5-HT in the frontal cortex and ventral hippocampus of rats (Béquet et al., 2001; Gomez-Merino et al., 2001) and therefore should upregulate BDNF expression in these brain areas. Our study, however, showed no differences in BDNF levels in the ventral hippocampus of rats that exercised compared to sedentary rats. Our findings further suggest that the effect of exercise may be BDNF specific, since exercise did not upregulate NGF or NT-3 levels in the ventral hippocampus or striatum. However, these results are compatible with earlier findings indicating that mainly BDNF is involved in antidepressant effects (Duman et al., 2008; Shirayama et al., 2000; Siuciak et al., 1996). This view is also supported by a report of Johnson et al. (2003) who found BDNF, but not NT-3, to be increased in the hippocampus of mice that ran in wheels while a positive correlation was made between BDNF levels and running distance. Our results and previous studies therefore agree that mainly BDNF and not any other neurotrophins are likely to be involved in the beneficial effect of exercise.

We chose to use voluntary exercise with free access to running wheels so that the rats would experience minimal stress when exercising, because high levels of corticosterone can have a toxic effect on the brain and increase cell death (Sapolsky, 1985a; Sapolsky, 1985b; Sapolsky et al., 1985). The average baseline corticosterone or ACTH levels did not differ between the two groups, so although maternal separation may lead to increased plasma corticosterone levels as previously seen (Marais et al., 2008), exercise did not alter the basal secretion of

corticosterone in our maternally separated rats. We did not measure corticosterone levels during or directly after the end of an exercise session, so we cannot say whether exercise itself induced a stress response or not. Blood was only collected about 3 hours after the last active cycle in which the rats were allowed to exercise. Previous studies found increases in corticosterone levels after acute forced exercise but not after low intensity running (Hall et al, 2001; Soya et al., 2007) and even chronic voluntary running in hamsters induced corticosterone release (Borer et al., 1992). Low intensity running increased BDNF mRNA and neurogenesis in the rat hippocampus, while high intensity running did not have the same beneficial effects (Lou et al., 2008). These observations are important as they suggest that differences in the duration and intensity of exercise can determine its beneficial effects.

An acute effect of exercise seen in this study is that it decreased the anti-oxidant potential of rat serum. This is consistent with previous studies showing that both acute swimming and restraint stress in rats similarly reduced the anti-oxidant potential of serum and that depletion of anti-oxidants occurs when free radical concentration increases (Van Rensburg et al., 2006). Aerobic exercise increases the production of free radicals and this leads to oxidative stress (Davies et al., 1982). Nevertheless, chronic exercise did not increase free radical accumulation or oxidative protein damage in rat brain tissue (Toldy et al., 2005; Ogonovsky et al., 2005; Radak et al., 2006). It is thought that adaptation occurs during chronic exercise after initial increases in reactive oxygen species. This apparently occurs by altering signaling pathways that lead to the upregulation of anti-oxidants and other pro-survival genes (Radak et al., 2005). Neurotrophins also protect against oxidative stress by upregulating anti-oxidants (Mattson et al., 1995) and in our study, increased levels of BDNF could therefore be beneficial in this process.

Conclusions

Chronic voluntary exercise was beneficial to rats that were subjected to early life stress and a subsequent stressor during adulthood. It reduced depressive-like behavior measured during a forced swim test and increased levels of BDNF in the striatum. Our results are consistent with a range of data suggesting that exercise has neuroplastic effects, and that this may be mediated by BDNF. Further work on this rat model is needed to establish the effect of exercise on neurotransmitter synthesis and release and the expression of neuroplasticity related proteins.

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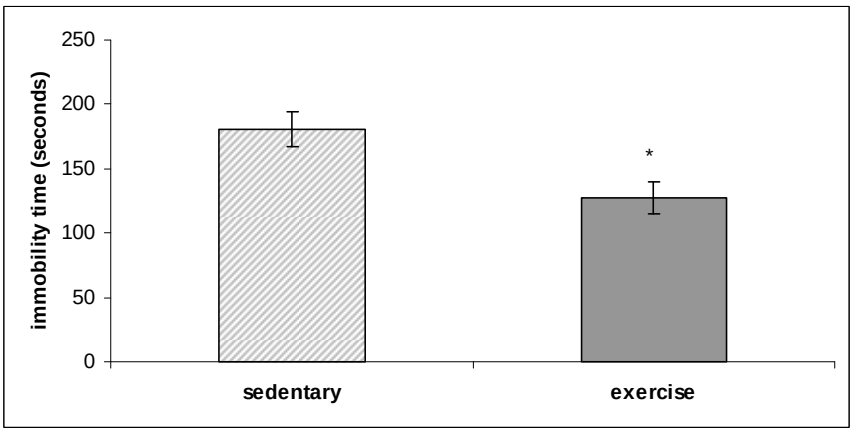

Fig. 1. Immobility time in the forced swim test was lower in rats that exercised (n=12) compared to sedentary rats (n=11) subjected to maternal separation and chronic restraint stress. *p<0.05. Values expressed as means ± standard error of the mean.

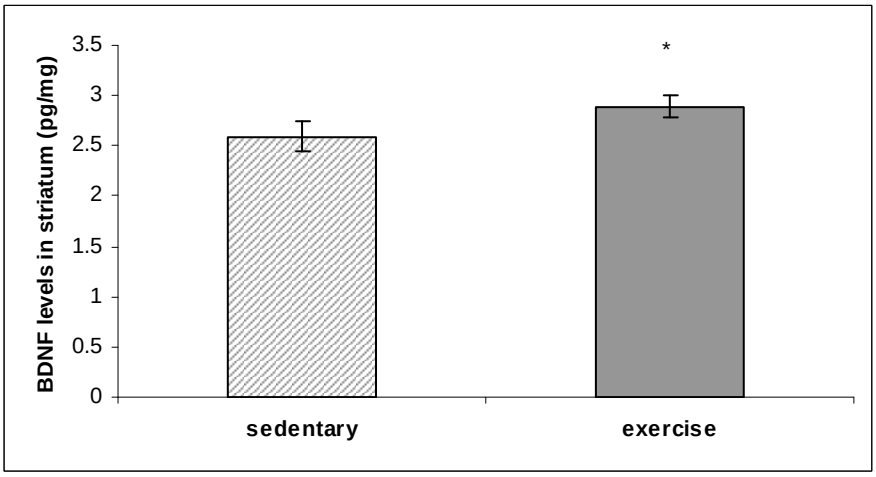

Fig. 2. BDNF levels in the striatum were increased in rats that exercised (n=10) compared to sedentary rats (n=11) subjected maternal separation and chronic restraint stress. *p<0.05. Values expressed as means ± standard error of the mean.

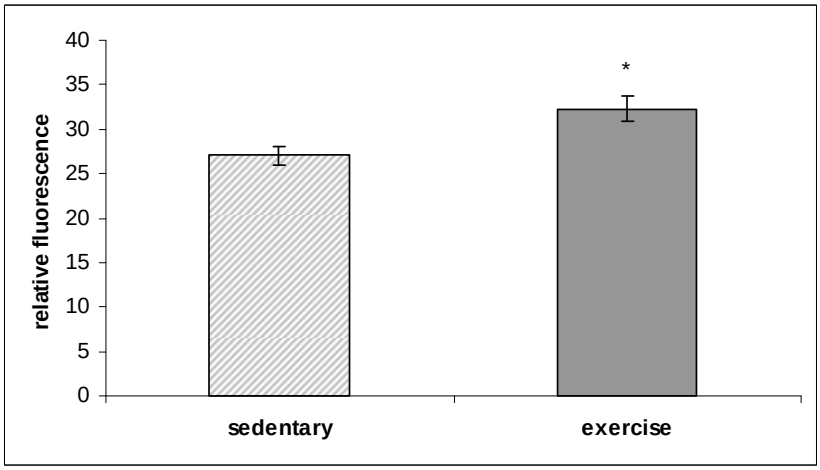

Fig. 3. The anti-oxidative potential of the serum of exercised rats (n=11) was lower than that of sedentary rats (n=11) subjected to maternal separation and chronic restraint stress at the 20h time interval. *$p<0.05$. Values expressed as means ± standard error of the mean.

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Chapter 6

A proteomic analysis of the ventral hippocampus of rats subjected to maternal separation and escitalopram treatment

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Abstract

Early life stress is known to predispose humans to the development of depression. Developmental stress has been shown to cause various changes in neurotransmitter systems, neurotrophin expression and the hypothalamic pituitary adrenal-axis in the rat brain. The aim of this study was to identify which cytosolic proteins are altered by maternal separation, as a model for depression, as well as by chronic antidepressant treatment. Rats were maternally separated from postnatal day 2-14 for 3 hours per day while control rats were normally reared. Both groups were divided and received either escitalopram or saline injections for 6 weeks starting from postnatal day 40. The ventral hippocampal tissue was fractionated and the cytosolic fraction used for 2-D-gel electrophoresis and liquid chromatography coupled to mass spectrometry analyses to identify peptides. Mascot database searches were done to identify proteins that were differentially expressed between the groups. Proteins that were significantly changed by maternal separation included amongst others: molecular chaperones and proteins related to energy metabolism; neuroplasticity; oxidative stress regulation; and protein metabolism. Treatment with escitalopram, a selective-serotonin reuptake inhibitor, induced changes in a different group of proteins, except for a few involved in energy metabolism and neuroprotective pathways. The results indicate which cytosolic proteins are changed by early life stress and may therefore be involved in the development of depression.

Keywords

Proteomics, Early life stress, Maternal separation, Rat model for depression.

Introduction

It is well known that children subjected to early life stress, such as physical or sexual abuse, or neglect, are predisposed to developing depression or anxiety disorders during adolescence or adulthood (Kessler & Magee, 1993; Pelcovitz et al, 1994; Gilmer & McKinney, 2003). Stressful encounters during this critical developmental stage of the brain may induce various changes in the hypothalamic-pituitary adrenal axis (HPA-axis), neurotransmitter and neurotrophin levels.

A rat model for early life stress, maternal separation (ms), has been used in various laboratories to study the effects of stressful experiences during childhood on the brain. We have also successfully used this rat model to study dysregulation of the HPA system (Daniels et al., 2004; Marais et al., 2008), which may be relevant to understanding depression in humans in terms of changes in the neurobiological systems. For instance, HPA-axis activity is dysregulated in patients with depression, as evidenced by increases in basal cortisol levels (Heim & Nemeroff, 1999), and a blunted ACTH response following intravenous injection of corticotrophin releasing factor (Holsboer et al, 1986). Wong et al. (2000) observed increased basal plasma cortisol levels but no difference in basal ACTH levels during a 30 h period in patients with depression compared to normal controls. Our maternally separated rats showed similar endocrine changes with elevated baseline corticosterone levels and a blunted ACTH response after acute restraint stress was induced (Marais et al., 2008; Daniels et al., 2004). Increased cortisol levels can affect aerobic energy metabolism pathways as the binding of cortisol to the glucocorticoid receptor (GR) regulates transcription of proteins including BAX (Bcl-2 associated X protein), which binds to the mitochondrial membrane. The GR-complex also binds to the membrane and

influences the membrane potential, which can lead to increased cytochrome c release and apoptosis (Iijima, 2006; Zhang et al., 2006).

Dysregulation in the serotonergic system also plays a role in the development of depression and selective serotonin reuptake inhibitors (SSRI's) achieve their therapeutic effect by increasing synaptic serotonin levels (Blier et al., 1987). Clinical studies have shown low cerebrospinal fluid serotonin levels in female patients with depression and in suicidal patients with depression (Hou et al., 2006) and a decreased number of serotonin $(5\text{-HT})_1$ receptors in the hippocampus of depressive patients (Cheetham et al., 1990). In addition, the prolactin response to intravenous citalopram, an SSRI, injection was shown to be blunted in depressive patients indicating decreased availability of serotonin in the brain (Bhagwagar et al., 2002). Similarly, ms rats had decreased serotonin levels in the dorsal hippocampus and medial pre-frontal cortex (Matthews et al., 2001) and the administration of SSRI's (citalopram, escitalopram and fluoxetine) to normally reared or ms rats exhibited anti-depressant effects (Kuśmider et al., 2007; El-Khoury et al., 2006; Leventopoulos et al., 2009). Quantitative autoradiography showed that rats subjected to early life stress had decreased 5-HT_{1A} receptor binding in their brains and which was increased by chronic fluoxetine treatment (Leventopoulos et al., 2009) and 5-HT_{1A} receptor agonists have antidepressant effects since it decreased the immobility time of rats in the forced swim test (Detke et al., 1995).

Neurotrophins, which are important for cell survival and neuroplasticity (Hennigan et al., 2007), are also affected by early life stress, since decreased levels of nerve growth factor (NGF) and neurotrophin-3 (NT-3) were measured in the ventral hippocampus of ms rats (Marais et al., 2008) and brain derived neurotrophic factor

(BDNF) mRNA decreased in whole hippocampi (Kuma et al., 2004). These reductions in neurotrophin levels can cause decreased proliferation or increased neuronal death of hippocampal neurons, such as the reduction in hippocampal volume as seen patients with depression (Bremner et. al., 2000; Sheline et. al., 1999). Indeed, it was found that serum or plasma BDNF levels were significantly lower in patients with depression than in controls (Karege et al., 2002; Lee et al., 2007).

It is evident from previous studies that the development of depression after a stressful event is not caused by one specific alteration in the brain, but rather by many included in downstream pathways of neurotransmitter or neurotrophin binding that in turn affect the expression of signalling proteins (Duman et al., 1997). The differential expression of cytosolic proteins may also participate in the pathogenesis of depression. To test this hypothesis, we subjected rats to ms and compared the expression profile of proteins in their ventral hippocampi to that of normally reared animals. The ventral hippocampus was selected because our previous work showed ms to induce significant reductions in neurotrophin levels in this brain region (Marais et al., 2008).

Using proteomic techniques (2-Dimensional gel electrophoresis coupled to mass spectrometry) we wanted to identify which proteins were upregulated or downregulated by early life stress, suggesting their involvement in the development of depression. Furthermore, a comparison was made between rats that have been treated with escitalopram and appropriate controls to establish whether proteins that are changed by ms were in fact targeted by an SSRI.

Materials and Methods

This project was approved by the Committee for Experimental Animal Research of the University of Stellenbosch (project number: P04/10/020). The experiments were performed in the Central Research Facility of the University of Stellenbosch. Male Sprague-Dawley rats were used for experiments. Rats were housed under standard laboratory conditions (12h/12h light/dark cycle; lights on at 6:00am; food and water *ad libitum*).

Maternal separation

Rat pups in the ms group (n=9) were separated from their dams between postnatal days 2-14 for 3 hours per day in the morning (Marais et al., 2008). For this procedure, the respective dams was removed from the home cage and pups carried to an isolated room. During separation, the pups were placed under infrared lights that maintained the ambient temperature at 30-33 º C. Another group of rats (n=9) were normally reared (nr) with their mothers and served as controls. The cages were all cleaned twice a week and no culling was performed on the litters. All pups were weaned on postnatal day 21, after which the males were kept in pairs for further experimentation.

Escitalopram treatment

Both ms and nr rats were divided into 2 groups: escitalopram treated (n=3 for each group) and saline treated (n=6 for each group). Escitalopram dissolved in saline (5 mg/kg/day; Uys et al., 2006) or saline only was administered via intra-peritoneal injection for 6 weeks from day 40-82. Rats were killed on postnatal day 83 and ventral hippocampal tissue collected (the bottom1/3 of both hippocampi) and stored

in liquid nitrogen until analysis. The ventral hippocampus was used since we have previously found significant alterations in the neurotrophin levels of maternally separated rats in this brain region (Marais et al., 2008).

2-Dimensional (2-D) gel electrophoresis

Ventral hippocampi were fractionated using a Calbiochem Proteoextract subcellular proteome extraction kit (Merck). The protein concentrations of each of the cytosolic fractions were then determined with a Bradford assay (Bradford, 1976). A ReadyPrep 2-D clean-up kit (Bio-Rad) was used to remove substances from the sample that are known to interfere with Isoelectric focusing (IEF)/2-D-gel electrophoresis. The precipitated sample was resuspended in 2-D sample/rehydration buffer (Bio-Rad) and protein concentrations determined with a RC/DC protein assay (Bio-Rad). Iso-electric focusing was done on an 11 cm pH 5-8 IEF strip (Bio-Rad) with 150µg of protein in a volume of 200 µl. These strips and the protein concentration were chosen based on previous work done in our laboratory (Uys et al., 2008). Strips were rehydrated for 12 h (2 ml mineral oil added on top after an hour) and focusing done for 40 000 Vh in a Protean IEF cell (Bio-Rad). Mineral oil was removed using blotting paper and strips were then incubated for 15 min with equilibration buffer I and II (Bio-Rad) with 2.5 % w/v iodoacetamide (Sigma) added to buffer II. Strips were run on pre-cast Bis-Tris 4-12 % Criterion XT gels (Bio-Rad) and these were fixed in a 40 % methanol and 7 % acetic acid solution for an hour. Three rats from each experimental group were run in triplicate against their respective control groups. Three comparisons were done namely: (1) ms rats were compared with nr rats (control group); (2) ms rats injected with escitalopram were compared to ms rats injected with saline (control group) and (3) nr rats injected with escitalopram were compared with nr rats injected with saline (control group). Gels

were stained overnight in Coomassie Colloidal Blue (Sigma) and destained in 25 % methanol for 2.5 hours. Gels were scanned on a GS-800 densitometer (Bio-Rad) and differentially expressed protein spots ($p<0.05$; t-test) were identified using the PD Quest Advanced, version 8.0.1 software package (Bio-Rad). Gels were stored in a 25% ammonium sulphate solution until spots were manually excised into a 96-well microtitre plate. Spots from all 6 gels were cut out for each protein and placed in the same well to increase the concentration of the protein.

Destaining, digestion and peptide extraction

Spots were destained twice using 50 % acetonitrile in 100 mM ammonium bicarbonate, rinsed with acetonitrile and allowed to air dry for 10 minutes. The spots were the reduced with 10 mM dithiothreitol in 100 mM ammonium bicarbonate for 30 minutes followed by alkylation with 55 mM iodoacetamide in 100 mM ammonium bicarbonate. The gel spots were then rinsed with acetonitrile and 100 mM ammonium bicarbonate followed by acetonitrile for a further 3 washes. A 25 µl aliquot of 6 ng/µl trypsin was added to each sample and allowed to incubate at 37 ^{o}C for 4.5 hours. The resulting peptides were initially extracted using 30 µl of an aqueous solution containing 2% acetonitrile and 1% formic acid. A second extraction using 15 µl of an aqueous solution containing 51% acetonitrile and 0.5% formic acid was then performed and combined with the first extraction in a cooled second 96-well plate. At this stage, and if necessary, the extractions were stored at – 80 ^{o}C prior to analysis by mass spectrometry.

Peptide separation by in-line liquid chromatography (LC) and electrospray ionisation mass spectrometry (ESI-MS)

The extracted tryptic peptides were resolved using an in-line NanoAcquity LC and autosampler system. LC solvents were supplied by Mallinckrodt Baker, Inc. A 4.9 □l aliquot of each sample was injected onto a nanoACQUITY UPLC™ trapping column 10kpsi Symmetry C18 180 µm x 20 mm 5 µm (Waters) equilibrated in 3% aqueous acetonitrile containing 0.1% formic acid and the column flushed with 1% aqueous acetonitrile/0.1% formic acid at 15 $\mu L min^{-1}$ for 1 minute. The peptides were then eluted onto a nanoACQUITY UPLC BEH C18 Column, 1.7 µm, 100 µm x 100 mm, 10K psi (Waters) at 1.2 $\mu L min^{-1}$ using a linear gradient of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in acetonitrile) and run over 20 min. The eluted peptides were analysed on a Micromass Q-Tof Global Ultima mass spectrometer fitted with a nano-LC emitter (New Objective) with an applied capillary voltage of 3-4 kV. The instrument was calibrated against a collisionally induced decomposition (CID) spectrum of the doubly charged precursor ion of [glu^1]-fibrinopeptide B (GFP – Sigma-Aldrich F3261). A calibration was accepted when the average error obtained on a subsequent acquisition was <10 ppm. Sensitivity was assessed by an injection of 50 fmol of a phosphorylase B tryptic digest giving a base peak intensity >1000 counts per sec in MS mode on the most intense peptide. The instrument was operated in data dependent acquisition (DDA) mode over the mass/charge (*m/z*) range of 50-2000. During the DDA analysis, both MS and tandem mass spectrometry (CID) were performed on the three most intense peptides as they eluted from the column. The uninterpreted MS/MS data were processed using the Waters ProteinLynx Global Server v2.3 software package (smoothed, background subtracted, centred and deisotoped) then mass corrected against the doubly charged GFP peptide. A peak list file was created and subjected to Mascot using MS/MS Ion

search and the SwissProt database to identify proteins (www.matrixscience.com). Search parameters specified were: fixed modifications of carbamidomethyl (C); variable modifications of oxidation (M) and phosphorylation (ST, Y); 1 missed trypsin cleavage was allowed for; peptide tolerance was set at 0.2 Da and MS/MS tolerance was 0.5 Da and peptide charge was +2 and +3. Mono-isotopic mass was used and proteins identified were significant ($p<0.05$) according to the probability-based MOWSE scores.

Results

The expression of a number of cytosolic proteins in the ventral hippocampus was affected by maternal separation or escitalopram treatment. Table 1 and 2 lists the proteins and their functions upregulated and downregulated respectively in ms rats compared to nr rats (control group). Table 3 and 4 lists the proteins and their functions that are upregulated and downregulated respectively in escitalopram ms rats vs. saline treated ms rats (control group). Table 5 and 6 lists the proteins and their functions that are upregulated and downregulated respectively in escitalopram nr vs. saline treated nr rats (control group). Proteins that were identified in more than one experimental analysis, i.e. in more than one rat of the same group, are indicated with an asterisk in the results tables.

Discussion

In the present study, the effects of ms on cytosolic protein levels in ventral hippocampal tissue were assessed with 2D-gel electrophoresis and mass-spectrometry techniques. Previous studies have mainly focused on the effect of ms

on neurotransmitters (Matthews et al., 2001; Daniels et al., 2004) or neurotrophins (Marais et al., 2008), which subsequently alter signalling pathways. We wanted to acquire more information about the effect of early life stress on the expression of cytosolic proteins to identify specific proteins that may be involved in the development of depression.

Maternal separation

Proteins that increased in response to ms, include several heat shock proteins or molecular chaperones that are known to be induced by stress. These are heat shock proteins 60, 70, 71 and stress-induced phosphoprotein 1, a co-chaperone that links heat shock proteins 70 and 90. These molecular chaperones usually function in transport of proteins within the cell and prevent misfolding and aggregation of old and new proteins under stressful conditions (Walter & Buchner, 2002).

Maternal separation increased a number of proteins that appear to have beneficial effects in neurons, for example dihidropyrimidase-related protein (DRP)-2 which is involved in neuroplasticity and specifically axonal outgrowth and regeneration (Minturn et al., 1995; Inagaki et al., 2001), and aminoacylase-1, which is known to influence the activity and cellular location of sphingosine kinase type 1, a promoter of cell growth and inhibitor of apoptosis (Maceyka et al., 2004; Xia et al., 2002). These proteins are possibly upregulated in response to stress as a compensatory mechanism of the brain to protect against the adverse effects of ms.

Additionally, proteins related to the protection of neurons to oxidative stress were increased. Increased ferritin heavy chain suggests protection of neurons by sequestering iron in ferrous form (Theil, 1987), resulting in a reduction in the

formation of superoxide free radicals via the fenton reaction (Kitahara et al., 1995). In a similar, protective way, increased levels of carbonic anhydrase 2 maintain the pH balance of cells by catalyzing the reaction of carbon dioxide (CO_2) hydration, reducing intracellular CO_2 levels (Pocker & Sarkanen, 1978). A high amount of CO_2 increase acidity and induce oxidative stress in cells (Bentez de Souza et al., 2004).

A number of proteins upregulated by ms are involved in amino acid or protein metabolism. D-3-phosphoglycerate dehydrogenase is involved in the metabolic pathway of the biosynthesis of L-serine, and is therefore essential for neuronal cell proliferation (De Koning et al., 2003) and dendritic and axonal growth in neuronal cell cultures (Savoca et al., 1995). Protein disulfide-isomerase catalyzes the formation of disulfide bonds in proteins and is involved in reactivation of denatured proteins (Yao et al., 1997). Upregulation of these proteins may therefore reflect the attempts of the brain to restore damaged neurons and proteins in ms rats.

Several of the upregulated proteins are involved in energy metabolism pathways, for example the subunits of adenosine triphosphate (ATP)-synthase enzyme complex and dihidrolipoyl dehydrogenase that forms part of the pyruvate dehydrogenase complex that decarboxylates pyruvate into acetyl-CoA (Lissens et al., 2000). The increased levels of these proteins indicate that ms induced an increase in aerobic metabolism and production of ATP. This suggestion is supported by the increase in nucleoside diphosphate kinase B since this enzyme is involved in the synthesis of nucleoside triphosphates including ATP. Interestingly, nucleoside diphosphate kinase B also regulates transcription and deoxyribonucleic acid (DNA) binding (Kimura, 2003). On the contrary, nucleoside diphosphate kinase A levels were decreased after ms.

Other proteins involved in glycolysis and the citric acid cycle were also decreased indicating a reduction in the activity of these pathways. Triosephosphate isomerase is involved in glycolysis where it interconverts dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). Decreased levels of this enzyme increase DHAP which is spontaneously converted to methylglyoxal. Methylglyoxal in turn modifies DNA and increases neuronal death, and it is therefore indicated in neurodegenerative diseases (Gnerer et al., 2006; Oláh et al., 2005). Glyoxalase domain-containing protein 4, part of the glyoxalase 1 family, contributes to the detoxification of methylglyoxal (Thornally, 2003), and the reduction of this protein may lead to increased cell death. Interestingly, glyoxalase 1 mRNA has been found to be decreased in patients with major depressive disorder (Fujimoto et al., 2008); suggesting that decreased levels of this protein can be used as a biomarker for depression.

Inositol monophosphatase is another enzyme with significant clinical relevance. Decreased levels of this enzyme were been observed in our ms rats as well in the brains of patients with depression (Coupland et al., 2005), while treatment with myo-inositol has been shown to decrease depression scores in patients (Levine et al., 1995). The reduction in inositol monophosphatase will cause decreased production of myo-inositol, which is part of the phosphatidylinositol signaling pathway. This pathway is activated by monoamine receptor binding, which serves to increase Ca^{2+} release and entry into the cell, and replenishes phosphatidylinositol biphosphate in the cell membrane (Harvey et al., 2002; Berridge & Irvine, 1984). Alternatively, since this pathway is closely linked to intracellular Ca^{2+} levels, an alteration in its function may lead to dysregulation of Ca^{2+} homeostasis. Interestingly, ms also decreased

neurogranin, which is implicated in neuroplasticity of dendritic spines and axons, by regulating calmodulin availability and Ca^{2+} immobilization in response to neurotransmitter receptor binding (Gerendasy & Sutcliffe, 1997). Calmodulin-dependent kinase II is involved in the modulation of neurotransmission and synaptic plasticity (Braun & Schulman, 1995). Another protein relating to neurotransmission is Amphiphysin 1, which is important for synaptic vesicle endocytosis and neurotransmitter recycling (Di Paolo et al., 2002) and was found to be downregulated in ms rats. In addition, DJ-1 mutations are normally found in patients with Parkinson's disease and the function of this protein is to reduce reactive oxygen species formed by dopamine catabolism and is therefore neuroprotective (Hedrich et al., 2004; Lev et al., 2009). It may therefore also be possible that decreased levels of DJ-1 found in our ms rats reflect a disturbance in the dopaminergic system.

Escitalopram treatment

Escitalopram treatment affected the expression of a number of different proteins in ms rats as opposed to nr rats. Only 4 of the proteins related to energy metabolism were similarly upregulated in both ms and nr rats chronically treated with escitalopram in comparison to their saline injected controls. These were dihydrolipoyllysine-residue acetyltransferase component of the pyruvate dehydrogenase complex, pyruvate dehydrogenase E1 component subunit beta, isocitrate dehydrogenase [NAD] subunit alpha, and triosephosphate isomerase. As previously discussed, these proteins all function in aerobic energy metabolism pathways, the pyruvate dehydrogenase complex, the citric acid cycle and glycolysis to produce ATP.

Rats chronically treated with fluoxetine or venlafaxine showed similar upregulation of hippocampal proteins as our escitalopram treated groups (Khawaja et al., 2004). These proteins were alpha-enolase and pyruvate dehydrogenase E1 component subunit beta involved in energy metabolism and ATP production. In addition, dimethylarginine dimethylaminohydrolase 1 (DDAH1), which regulates nitric oxide synthase (NOS) activity by hydrolyzing asymmetrically methylated arginine residues, which are competitive inhibitors of NOS, was upregulated (Ogawa et al., 1989; Tran et al., 2000).

Chronic escitalopram treatment increased the expression of some of the cytosolic proteins in the ventral hippocampus that were downregulated after ms. The increase in triosephosphate isomerase is important due to its function in reducing the concentration of methylglyoxal in neurons. Glyoxalase 1 was also upregulated in ms rats treated with escitalopram, which also functions in detoxification of methylglyoxal (Thornalley et al., 2006). DDAH1 and isocitrate dehydrogenase [NAD] subunit alpha were also upregulated after escitalopram treatment.

The expression of a number of proteins that were not affected by ms was also significantly altered in the treatment groups. In nr rats, treatment increased levels of neuronal protein 22, which is related to neuronal morphology, which binds to the cytoskeleton, and is also implicated in neuroplasticity since increased levels have been found during synaptogenesis (Mori et al., 2004; De la Heras et al., 2007; Depaz and Wilce, 2006). However, the reduction of DRP-2, 4 and 5, which are involved in axonal growth (Minturn et al., 1995; Inagaki et al., 2001) was observed after treatment in ms rats. A previous study also indicated that DRP-2 is related to escitalopram resistance in a chronic mild stress model for depression, since it was

differentially regulated between responders and non-responders to escitalopram treatment (Bisgaard et al., 2007).

Escitalopram increased complexin in ms rats, a protein which facilitates Ca^{2+}-triggered neurotransmitter release at synapses as observed in complexin-knockout mice where the synaptic activity of neurons was decreased (Xue et al., 2008). The β-subunits of guanine nucleotide-binding protein (G-protein) were increased in nr rats treated with escitalopram. When neurotransmitters bind to their G-protein coupled receptors, various intracellular signalling pathways are activated (Gilman, 1987). Usually the β-subunit of G-proteins specifically activates phospholipase C and adenylyl cyclase 2 (Boyer et al., 1992; Chen et al., 1997), but it has also been shown to be incorporated into microtubules and is thought to play a regulatory role in cytoskeletal structure (Wu et al., 1998).

Conclusions

Maternal separation predisposes rat pups to develop depressive-like behaviour (Marais et al., 2008). The results of the present study indicate that the development of this behavioural abnormality may be associated with the alteration in the expression of a large number of cytosolic proteins in the ventral hippocampus. Chronic treatment with escitalopram only affects the expression of a few of the proteins that were altered by ms and it therefore is likely that escitalopram targets another group of cytosolic proteins to achieve its therapeutic effect. A limitation of this study is that it does not confirm the observations with another method such as western blotting, and this could be considered in future studies. The current data obtained with ms as a rat model for depression may be important as it indicates

pathways and specific proteins that are potentially involved in the development of depression, thereby providing greater insight into the pathogenesis of the disorder.

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Table 1: Proteins upregulated in the ventral hippocampus of maternally separated rats compared to normally reared rats (control). n=3; * proteins identified in more than one experimental analysis of the same groups.

Protein	Accession no.	Mass (Da)	Ratio to control	Mascot score	Sequence coverage (%)	Isoelectric point (pI)	Function
60 kDa heat shock protein, mitochondrial	P63039	61088	1.48	129	12	5.91	Molecular chaperone
Aminoacylase-1A	Q6AYS7	46060	1.17	185	30	6.03	Differentiation of neurons
ATP synthase subunit alpha, mitochondrial	P15999	59831	3.26	55	4	9.22	ATP synthesis
ATP synthase subunit d, mitochondrial	P31399	18809	1.24	100	38	6.17	ATP synthesis
Carbonic anhydrase 2	P27139	29267	1.68	112	19	6.89	Calcium regulation
D-3-phosphoglycerate dehydrogenase	O08651	57256	1.87	370	23	6.28	L-serine synthesis
Dihydrolipoyl dehydrogenase, mitochondrial	Q6P6R2	54574	1.62	142	16	7.96	Pyruvate carboxylation
Dihydropyrimidinase-related protein 2	P47942	62638	1.89	64	1	5.95	Neuroplasticity
Ferritin heavy chain	P19132	21113	1.14	87	22	5.85	Protects against oxidative stress
Glutamate dehydrogenase 1, mitochondrial	P10860	61719	2.66	174	26	8.05	Glutamate metabolism
Heat shock 70 kDa protein 4	O88600	94795	1.23	251	16	5.13	Molecular chaperone
Heat shock cognate 71 kDa protein	P63018	71055	1.62	343	24	5.37	Molecular chaperone
Nucleoside diphosphate kinase B	P19804	17386	1.6	201	50	6.92	nucleoside triphosphate synthesis
Phosphatidylethanolamine-binding protein 1	P31044	20788	2.1	156	22	5.48	Serine protease inhibitor
Proteasome subunit beta type-7*	Q9JHW0	30250	1.38	104	12	8.13	Protein catabolism
Protein disulfide-isomerase A3	P11598	57044	1.44	298	36	5.88	Formation of disulfide bonds in proteins
Stress-induced-phosphoprotein 1	O35814	63158	1.67	203	18	6.4	Co-chaperone linking Hsp-70/Hsp-90
Transitional endoplasmic reticulum ATPase	P46462	89977	1.21	398	31	5.14	Vesicle formation in endoplasmic reticulum

Table 2: Proteins downregulated in the ventral hippocampus of maternally separated rats compared to normally reared rats (control). n=3; * proteins identified in more than one experimental analysis of the same groups.

Protein	Accession no.	Mass (Da)	Ratio to control	Mascot score	Sequence coverage (%)	Isoelectric point (pI)	Function
[Protein ADP-ribosylarginine] hydrolase	Q02589	40220	0.4	91	13	5.62	Catalyzes de-ADP-ribosylation of proteins
Aldehyde dehydrogenase, mitochondrial	P11884	54813	0.83	196	23	5.83	Converts aldehydes to acids
Amphiphysin 1	O08839	64493	0.48	119	15	4.95	Neurotransmitter recycling
Annexin-5	P14668	35779	0.3	226	30	4.93	Apoptosis
COP9 signalosome complex subunit 4	Q68FS2	46546	0.48	366	21	5.6	Ubiquitin-dependant protein degradation
Elongation factor Tu, mitochondrial	P85834	49890	0.66	245	32	7.23	Protein synthesis
Glutathione S-transferase P	P10299	23652	0.64	83	13	6.89	Conjugates glutathione to targets, reduces oxidative stress
Glyceraldehyde-3-phosphate dehydrogenase	P04797	35805	0.89	121	9	8.14	Glycolysis
Glyoxalase domain-containing protein 4	Q5I0D1	33532	0.63	145	31	5.11	Removes methylglyoxal from mitochondria
Inositol monophosphatase	P97697	30834	0.56	211	21	5.17	Generation of myo-inositol
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	Q99NA5	40044	0.75	171	19	6.47	Citric acid cycle
N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	O08557	31805	0.79	228	23	5.75	Hydrolyses NOS inhibitors
Neurogranin	Q04940	7720	0.86	179	26	6.54	Neuroplasticity
Nucleoside diphosphate kinase A	Q05982	17296	0.84	169	51	5.96	Nucleoside triphosphate synthesis
Phosphoglycerate mutase 1	P25113	28928	0.88	304	47	6.67	Glycolysis
Protein DJ-1	O88767	20190	0.74	131	19	6.32	Protection against oxidative stress
Stress-70 protein, mitochondrial	P48721	74097	0.84	545	37	5.97	Molecular chaperone
Triosephosphate isomerase*	P48500	27345	0.78	197	20	6.89	Glycolysis

Table 3: Proteins upregulated in the ventral hippocampus of maternally separated rats after treatment with escitalopram vs. saline treated maternally separated rats (control). n=3; * proteins identified in more than one experimental analysis of the same groups.

Protein	Accession no.	Mass (Da)	Ratio to control	Mascot score	Sequence coverage (%)	Isoelec-tric point (pI)	Function
Alpha-enolase	P04764	47.128	1.43	82	8	6.16	Glycolysis
Complexin-1/Complexin-2	P63041/ P84087	15.12/ 15.394	1.29/ 1.62	51	8	4.93/ 5.06	Neurotransmitter release
Cytosol aminopeptidase	Q68FS4	56.15	1.08	201	7	6.77	Protein catabolism
D-3-phosphoglycerate dehydrogenase	O08651	56.493	1.43	112	4	6.28	Amino-acid synthesis
Dihydrolipoyllysine-residue acetyltransferase, mitochondrial	P08461	67.166	1.22	82	3	8.76	Pyruvate decarboxylation
Dihydrolipoyllysine-residue succinyltransferase, mitochondrial	Q01205	48.925	1.32	203	8	8.89	Pyruvate decarboxylation
EF-hand domain-containing protein D2	Q4FZY0	26.759	1.22	76	23	5.01	Calcium regulation
Glyoxalase 1	Q6P7Q4	20.82	1.51	91	14	5.12	Detoxification of methylglyoxal
Heat shock protein 105 kDa	Q66HA8	96.419	1.32	313	10	5.4	Molecular chaperone
Hypoxanthine-guanine phosphoribosyltransferase	P27605	24.477	1.54	88	5	6.07	Purine salvage pathway
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	Q99NA5	39.614	1.39	150	8	6.47	Citric acid cycle
Malate dehydrogenase, cytoplasmic	O88989	36.483	1.47	141	9	6.16	Citric acid cycle
N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	O08557	31.426	1.24	142	11	5.75	Hydrolyses NOS inhibitors
Prohibitin	P67779	29.82	1.23	121	12	5.57	Inhibits DNA synthesis
Protein-L-isoaspartate(D-aspartate) O-methyltransferase	P22062	24.641	1.2	143	12	7.14	Protein repair
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	P49432	38.982	1.28	146	8	6.2	Pyruvate decarboxylation
Thioredoxin-dependent peroxide reductase, mitochondrial	Q9Z0V6	28.295	1.56	94	8	7.14	Redox regulation
Triosephosphate isomerase *	P48500	26.849	5.57	261	25	6.89	Glycolysis
Ubiquitin carboxyl-terminal hydrolase isozyme L1	Q00981	24.838	1.29	155	33	5.14	Protein catabolism
Voltage-dependent anion-selective channel protein 2	P81155	31.746	1.27	157	13	7.44	Apoptosis

Table 4: Proteins downregulated in the ventral hippocampus of maternally separated rats after treatment with escitalopram vs. saline treated maternally separated rats (control). n=3; * proteins identified in more than one experimental analysis of the same groups.

Protein	**Accession no.**	**Mass (Da)**	**Ratio to control**	**Mascot score**	**Sequence coverage (%)**	**Isoelec-tric point (pI)**	**Function**
Aconitate hydratase, mitochondrial	Q9ER34	85.433	0.32	245	5	7.87	Citric acid cycle
Alcohol dehydrogenase [NADP+]	P51635	36.506	0.66	124	19	6.84	Converts alcohols to aldehydes
Aminoacylase-1A	Q6AYS7	45.804	0.5	163	7	6.03	Differentiation of neurons
Annexin A5	P14668	35.745	0.61	80	4	4.93	Apoptosis
Carbonic anhydrase 2	P27139	29.114	0.23	69	6	6.89	Calcium regulation
Cytosolic acyl coenzyme A thioester hydrolase	Q64559	42.735	0.46	121	6	8.8	Fatty acid synthesis
Dihydropyrimidinase-related protein 2 *	P47942	62.278	0.65	367	18	5.95	Neuroplasticity
Dihydropyrimidinase-related protein 4 *	Q62951	61.086	0.49	263	14	6.3	Neuroplasticity
Dihydropyrimidinase-related protein 5 *	Q9JHU0	61.54	0.67	348	13	6.6	Neuroplasticity
Dynactin subunit 2	Q6AYH5	44.148	0.71	51	5	5.14	Mitosis
Fructose-bisphosphate aldolase C	P09117	39.284	0.43	228	15	6.67	Glycolysis
Glutamate dehydrogenase 1, mitochondrial	P10860	61.416	0.74	103	4	8.05	Glutamate metabolism
GTP-binding nuclear protein Ran	P62828	24.423	0.42	118	12	7.01	Protein transport
Phosphoglycerate mutase 1	P25113	28.832	0.28	59	11	6.67	Glycolysis
Synapsin-2	Q63537	63.457	0.41	79	5	8.73	Neurotransmitter release

Table 5: Proteins upregulated in the ventral hippocampus of normally reared rats after treatment with escitalopram vs. saline treated rats (control). n=3; * proteins identified in more than one experimental analysis of the same groups.

Protein	Accession no.	Mass (Da)	Ratio to control	Mascot score	Sequence coverage (%)	Isolelectric point (PI)	Function
ATP synthase subunit d, mitochondrial	P31399	18.763	1.91	105	30	6.17	ATP synthesis
Bcl-2-like protein 11	O88498	22.056	3.35	35	6	6.1	Apoptosis
Creatine kinase B-type	P07335	42.725	1.43	293	20	5.39	Phosphate transfer
Dihydrolipoyllysine-residue acetyltransferase, mitochondrial	P08461	67.166	1.32	254	12	8.76	Pyruvate decarboxylation
Endoplasmic reticulum protein ERp29	P52555	28.575	1.47	217	13	6.23	Protein transport
Eukaryotic translation initiation factor 4H	Q5XI72	27.324	1.49	74	5	6.67	Translation
Fructose-bisphosphate aldolase C	P09117	39.284	1.29	517	33	6.67	Glycolysis
Glutamine synthetase *	P09606	42.268	2.31	198	18	6.64	Protein catabolism
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1/beta-2	P54311/ P54313	37.377/ 37.331	1.49	115/124	13	5.6	Initiates signal transduction pathways
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	Q99NA5	39.614	2.16	343	20	6.47	Citric acid cycle
Neuronal protein 22	P37805	22.501	1.44	217	32	6.84	Microtubule structure
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial *	P49432	38.982	1.32	177	9	6.2	Pyruvate decarboxylation
Triosephosphate isomerase	P48500	26.849	1.64	403	51	6.89	Glycolysis

Table 6: Proteins downregulated in the ventral hippocampus of normally reared rats after treatment with escitalopram vs. saline treated rats (control). n=3.

Protein	Accession no.	Mass (Da)	Ratio to control	Mascot score	Sequence coverage (%)	Isoelectric point (PI)	Function
78 kDa glucose-regulated protein	P06761	72.347	0.47	234	12	5.07	Molecular chaperone
Alpha-enolase	P04764	47.128	0.43	324	20	6.16	Glycolysis
Fascin	P85845	21.781	0.72	97	15	5.86	Microtubule structure
Glutathione S-transferase P	P10299	23.901	0.67	105	13	6.89	Conjugates glutathione to targets, reduces oxidative stress
Glyceraldehyde-3-phosphate dehydrogenase	P04797	35.828	0.63	96	26	8.14	Glycolysis
Malate dehydrogenase, cytoplasmic	O88989	36.483	0.53	85	12	6.16	Citric acid cycle
Phosphoglycerate kinase 1	P16617	44.538	0.64	314	25	8.02	Glycolysis
Proteasome subunit beta type-4	P34067	29.197	0.71	155	12	6.45	Protein catabolism
Protein-L-isoaspartate(D-aspartate) O-methyltransferase	P22062	24.641	0.74	99	7	7.14	Protein repair or degradation
Pyridoxine-5'-phosphate oxidase	O88794	30.184	0.56	174	9	8.66	Vit. B6 synthesis
Pyruvate kinase isozymes M1/M2	P11980	57.818	0.64	106	5	6.63	Glycolysis
Tubulin alpha-1A chain	P68370	50.136	0.34	189	13	4.94	Microtubule structure
UMP-CMP kinase	Q4KM73	22.169	0.75	158	22	5.66	Phosphate transfer

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Chapter 7

Chronic exercise in rats subjected to early life stress – a quantitative proteomic analysis of the ventral hippocampus and determination of serotonin availability in selected brain areas

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Abstract

Exercise is well known to have beneficial effects on the brain by inducing neurotrophins and serotonin. Maternal separation is used as a rat model for depression and chronic voluntary exercise as subsequent treatment to establish if exercise exerts a beneficial effect on the brain after early life stress and specifically which cytosolic proteins are involved in its mechanism of action. Rats were subjected to maternal separation from day 2-14 for 3h/day or normally reared. All rats were either subjected to chronic voluntary wheel running for 6 weeks or kept as sedentary controls. Baseline serotonin was measured in the ventral hippocampus, frontal cortex and striatum while quantitative proteomics was done on the ventral hippocampus. Maternal separation did not change serotonin levels in the brain, but exercise increased serotonin in the ventral hippocampus of normally reared rats compared to maternally separated exercised rats. The proteomic analysis identified several proteins that were changed by maternal separation stress, some of which could be restored by subsequent chronic exercise. Exercise also induced changes in cytosolic proteins in normally reared rats, and was shown to have a beneficial effect on the brain.

Keywords

Maternal separation; exercise; quantitative proteomics; serotonin

Introduction

Exercise has previously been shown to have antidepressant effects in humans (Daley, 2008) as well as in animals used as models for depression (Duman et al., 2008; Bjørnebekk et al., 2005). Rats subjected to chronic exercise showed

decreased depressive-like behaviour in the forced swim test and decreased anhedonia in the sucrose consumption test (Bjørnebekk et al., 2005; Zheng et al., 2006). Despite the beneficial effect of exercise as antidepressant, the underlying molecular mechanism by which exercise exerts its effects in the brain is not entirely understood.

Early life stress, or maternal separation (MS), is used as a model for depression in rats and may predispose rats to develop increased depressive-like behavior during adulthood (Lee et al., 2007; Marais et al., 2008). MS also decreases neurotrophin levels and neurogenesis in the hippocampus (Marais et al., 2008; Manni et al., 1998; Fabricius et al., 2008; Mirescu et al., 2004). Interestingly, both acute and chronic exercise treatments have been shown to be beneficial, an this effect was suggested to result from increased neurotrophin gene expression and neuronal proliferation, particularly in the hippocampus (Lou et al., 2008; Farmer et al., 2004), while exercise induced neurotrophin levels positively correlated with running distances (Johnson et al., 2003; Ploughman et al., 2005). Therefore, the mechanism by which exercise induces neuronal proliferation probably involves the activation of neurotrophin activated pathways. It has furthermore been shown that chronic wheel running in adult rats increased hippocampal BDNF, phosphatidylinositol 3 (PI-3) kinase and activated levels of the transcription factor cyclic AMP response element binding protein (CREB), while BDNF levels positively correlated with p-CREB (Chen & Russo-Neustadt, 2005; Molteni et al., 2004).

Stressful experiences in the early neonatal period, when development of many neural systems takes place, may also affect the development of neurotransmitter systems and including the serotonergic system (Matthews et al., 2001; Papaioannou et al.,

2002) and has been shown to induce depressive-like behaviors in adulthood (El Khoury et al., 2006; Aisa et al., 2007; Lee et al., 2007; Lambás-Señas et al., 2009). Exercise has antidepressant effects, while it can also regulate the synthesis and availability of serotonin (5-HT) in the brain (Béquet et al., 2001; Gomez-Merino et al., 2001; Dey et al., 1992).

The ventral hippocampus is innervated by serotonergic neurons from the raphe nucleus (Azmitia & Segal, 1978) and could therefore be an important area in the brain when investigating the development of depression, a disorder which is thought to be related to decreased 5-HT neurotransmission (Nutt et al., 2007; Hou et al., 2006). It is also known that the brain's 5-HT system regulates behavior, hormone function and mood (Kalia, 2005; Ruhé et al., 2007). In patients with major depression, serotonergic neurotransmission dysfunction is evident as a blunted prolactin response to a 5-HT agonist or selective 5-HT reuptake inhibitor (Mann et al., 1995; Bhagwagar et al., 2002).

In this study, the ventral hippocampus was used for proteomic analysis since it is implicated in the development of depression and our previous results showed that maternal separation decreased neurotrophin expression in this area (Marais et al., 2008). While it is known that exercise increases neurotrophin expression, the aim of this study was to determine which other cytosolic proteins are changed by chronic voluntary exercise treatment in the ventral hippocampus of MS rats. These specific changes were then related to the potential beneficial effect of exercise in a model for early life stress. In addition, to determine whether MS and chronic voluntary exercise affects serotonergic neurotransmission, 5-HT levels were measured in the striatum, frontal cortex and ventral hippocampus, since clinical studies have shown that

atrophy of these specific brain areas occurs in patients diagnosed with depression (Bremner et al., 2000; Coffey et al., 1992; Drevets et al., 1997; Husain et al., 1991).

Materials and Methods

This project was approved by the Committee for Experimental Animal Research of the University of Stellenbosch (project number: P04/10/020). The experiments were performed in the Central Research Facility of the University of Stellenbosch. Male Sprague-Dawley rats were used for experiments. Rats were housed under standard laboratory conditions (12h/12h light/dark cycle; lights on at 6:00 am; food and water *ad libitum*).

Maternal separation

A group of rat pups were separated from their mothers on day 2-14 for 3 hours per day in the morning (Marais et al., 2008). For this procedure, the pups were removed from their mother and placed under infrared lights keeping the ambient temperature at 30-33 º C in an isolated room. A second group of rats was normally reared with their mothers. All pups were weaned on day 21. The rats were kept together in standard cages: 2 or 3 controls or 2 exercised rats per cage.

Exercise

Both maternally separated (MS) and normally reared (NR) rats were randomly divided into 2 groups: exercise or control. The exercise groups were allowed to do chronic voluntary exercise and were placed in cages equipped with running wheels during their active phase (corresponding to the dark cycle) for a period of 6 weeks, on day 40-82, for 5 days of the week. Experimental rats were kept in pairs in the

exercise cages, each having access to their own running wheel so that we could monitor whether they ran or not. The two animals in a cage were kept apart with a perforated Perspex separator between them that facilitated visual and oral communication between the rats. The sedentary groups were similarly handled but had no access to exercise wheels.

Brain tissue collection

Rats were decapitated on day 83 around 10:00 in the morning and the frontal cortex, striatum and ventral hippocampus were dissected from the brain, snap frozen and stored in liquid nitrogen until further analyses.

Serotonin levels

Baseline serotonin levels were measured in the frontal cortex, striatum and ventral hippocampus using enzyme-linked immunosorbent assay (ELISA) kits (IBL international). Tissue lysates were prepared using the following method: Tissue was weighed and a volume of 20 µl cooled buffer (25 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1 mM EGTA) was added to each 1 mg of tissue. Samples were homogenized, vortexed and centrifuged at 15000 g for 15 min at 4 º C. The supernatant was frozen and stored at - 80 º C. On the day of the assay, samples were centrifuged again at 12 000 g for 10 min at 4 º C. The assay was then performed according to the manufacturer's instructions. Absorbance was read on a Bio-Tek Synergy HT plate reader. Serotonin concentration (ng/ml) was determined using a standard curve generated from the assay.

Proteomics

Ventral hippocampi, n = 3 in each group, were fractionated using a Calbiochem Proteoextract subcellular proteome extraction kit (Merck). The protein concentrations of the cytosolic fractions were determined with a Bradford assay before lipids, salts and detergents were removed using a ReadyPrep 2D clean-up kit (Bio-Rad). Samples were then resuspended in 100mM ammonium bicarbonate. All samples were freeze dried and stored at 4 º C before analyses.

Each sample was re-suspended in 50µl 1% PPS silent surfactant (Protein Discovery) in 500mM triethylammonium bicarbonate (TEAB) buffer and the insoluble matter removed by centrifugation. Protein concentration was determined using a nanodrop spectrophotometer and the samples from each group combined using equal amounts of protein from each individual sample. Samples were digested overnight in trypsin (1:20, enzyme:protein ratio) and the resultant digestion evaluated using both mass spectrometric and liquid chromatography.

iTRAQ mass tag labeling was done using a 4-plex labeling kit from Applied Biosystems. The samples were dried down in a Labconco centrivap concentrator and resuspended (1mg/ml) in iTRAQ dissolution buffer and 40 µg of protein from each sample used for the analysis. The samples were dried down again and resuspended in 20 µl dissolution buffer. iTRAQ mass tag labeling with isobaric tags was done on tryptic peptides for 2 hours at room temperature. The groups were labeled as follows: MS control – reporter signal 114, NR control – reporter signal 115, MS exercise – reporter signal 116 and NR exercise – reporter signal 117. An aliquot of each sample was mixed for confirmation of labeling with mass spectrometry and MS/MS. The data indicated that all 4 samples were modified with iTRAQ tags. After mixing the samples from the 4 groups, the iTRAQ reagents were removed from

the mixture using a strong cation exchange (SCX) solid phase extraction (SPE) device. The sample was re-suspended in SCX equilibration buffer (5mM KH2PO4 (Sigma), 25 % acetonitrile (ACN) (ROMIL)) and applied to a pre-equilibrated SCX SPE device (Supelco SupelClean). The peptides were eluted from the device with 300 µl elution buffer (1M HCO2NH4/25 % acetonitrile (Sigma)). The sample volume was reduced to 25 µl using a roto-evaporator (Eppendorf) and the sample adjusted to 90 µl with LC loading buffer. The samples were desalted using ZipTip C18 SPE devices (Millipore).

The sample mixture was then submitted to 1-dimensional liquid chromatography (Dionex Ultimate 3000 nano-LC) using a Pepmap C_{18} reverse phase column (75 µm x 15 cm, LCPackings) with gradient elution. The solvent systems were A: 2 % ACN/H2O, 0.1 % trifluoroacetic acid (TFA); B: 80 % ACN/H2O, 0.08 % TFA. The sample was loaded onto the column using Solvent A. Peptides were eluted in 5 % B over 5 min, 5 – 15 % B over 5 min, 15 % - 45 % B over 70 min and 45 % - 60 % B over 10 min with a flow rate of 200 nl/min. The eluted peptides were spotted onto a MALDI source plate using a Probot (LCPackings) with continuous matrix addition at 600 nl/min. The matrix was 7.5 mg/ml α-cyano-4-hydroxycinmamic acid (Fluka) with 10 mM $NH_4H_2PO_4$ (Fluka) in 66 % acetonitrile, 0.1 % TFA. PepMix4 (LaserBiolabs) 5 point calibration mixture was spiked into the sample at an average of 10 fmol/µL (final quantity of 6.6 fmol total peptide/spot). Fractions were collected every 12 seconds and the collection started 16 min after sample injection.

Tandem mass spectrometry was performed using an Applied Biosystems 4800 MALDI ToF/ToF. Parent ion spectra were recorded in linear positive ion mode with 500 shots/spectrum and laser intensity of 3800 arbitrary units. The grid voltage was

set to 16 kV. The spectra were processed using the PepMix4 internal calibration points. MS/MS spectra were recorded in positive mode with 1 kV deceleration voltage and a total of 600 laser shots/spectrum with the laser set to 5000 arbitrary units. Mass spectral data was analyzed using Peak Explorer software and data analysis was performed with ProteinPilot™ software using the *Rattus rattus* database. The iTRAQ quantitation was performed with default settings and relative abundance expressed in terms of reporter signal 115 (NR control group).

Statistics

Data for the 5-HT assays were analyzed with non-parametric Kruskal Wallis tests and Dunn's post hoc tests using GraphPad Prism 4 software. Quantitative proteomics data were analysed with Student's t-tests using ProteinPilot™ software. The significance level was considered as $p < 0.05$.

Results

Serotonin

There were no significant differences in 5-HT levels between the groups in the frontal cortex or striatum (n = 8-10 in each group). There were no significant differences in 5-HT levels of NR and MS rats in the ventral hippocampus, however the NR exercise group had significantly higher levels of 5-HT compared to the MS exercise group (n = 5-7; Fig. 1).

Proteomics

A number of cytosolic proteins in the ventral hippocampus were identified and quantified from proteomic analyses. The expression of 33 of these proteins (listed in

Table 1) was significantly changed by MS or exercise compared to the NR control group (Table 2). These proteins included, amongst others, proteins functioning in cytoskeletal structure, energy metabolism, calcium (Ca^{2+}) regulation, synaptic plasticity, neuronal survival and protection against oxidative stress. MS stress induced changes in a variety of proteins, some of which were normalized by exercise, while exercise alone also induced changes in the expression of proteins not affected by MS.

Discussion

Serotonin

The baseline serotonin levels did not differ between the NR and MS rats in any of the 3 brain regions investigated. It therefore seems as if MS had no effect on 5-HT availability, which was surprising since previous studies have found that 5-HT levels are decreased (Matthews et al., 2001) or increased (Papaioannou et al., 2002) by MS. Matthews et al. (2001) found increased 5-HT was however found in postmortem brain tissue which could explain the discrepancy between our results and previous findings. Papaioannou et al. (2002) found increased 5-HT in the brain of prepubertal rats, but, similar to our results, no differences between controls and MS rats during adulthood. Exercise in NR or MS rats did not induce any changes in 5-HT levels in the striatum or frontal cortex, however, it significantly increased 5-HT levels in the ventral hippocampus of NR rats, but only compared to MS exercised rats and not to NR control rats. Previous literature reported chronic exercise to increase the synthesis and metabolism of 5-HT in the cerebral cortex and midbrain of rats (Dey et al., 1992; Brown et al., 1979). Stimulation of 5-HT metabolism may however only be

an acute effect as exercise-induced increases in 5-HT have been shown to return to baseline after 2 hours (Béquet et al., 2001). We therefore also assumed that our measurements depicted baseline levels since it was measured at least 3 hours after the last exercise session.

Although it has previously been shown that early life stress decreases 5-HT levels in the rat brain (Lee et al., 2007; Arborelius & Eklund; 2007) we did not observe any changes in serotonin levels in clinically relevant brain areas. The mechanism by which MS or exercise induce changes in cytosolic protein levels could however be independent of serotonin neurotransmitter levels. Chronic early life stress can also induce changes in neurotrophin expression levels (Marais et al., 2008) and thereby affect the activation or transcription of other signaling proteins while exercise may also exert its therapeutic effect in MS rats through upregulation of neurotrophins (Marais et al., 2009a). The proteomics data shows that exercise affects various signalling pathways and protects against oxidative stress. Its effect may involve the activation of other neurotransmitter systems such as noradrenaline.

Proteomics

Quantitative proteomics was done to establish what effect maternal separation and subsequent exercise have on cytosolic protein expression of the ventral hippocampus. Exercise generally increased the expression of structural proteins, eg. microtubule-associated proteins tau, 1A and 1B in NR and MS rats. These proteins are responsible for the stability and arrangement of microtubules (Maccioni & Cambiazo, 1995). On the other hand, maternally separated exercised rats had decreased levels of microtubule associated protein tau. MS reduced the amount of

actin and tubulin α-1B in the ventral hippocampus, but it was increased again after exercise, also indicative of increased neuronal plasticity of the cytoskeletal structure (Kueh and Mitchison, 2009). Increased levels of cytoskeletal proteins indicates that exercise is beneficial to neurons since structural defects that impact on neuronal functioning, such as those observed after early life stress (Llorente et al. 2009), may be avoided. Reports of voluntary exercise improving dendritic structure (Redila & Christie, 2006) and increasing β-tubulin in the adult rat hippocampus (Ding et al., 2006), supported our interpretation of exercise neutralising the detrimental effects of maternal separation on neuronal structure. Interestingly, MS resulted in increased tropomyosin 5 levels, which binds to actin filaments and not only contributes to neuronal structure, but also enhances axoplasmic transport, synaptic rearrangement and vesicle movement (Dufour et al., 1998; Lees-Miller et al., 1990). It therefore appeared as if tropomyosin was increased in response to early life stress to facilitate neurotransmission.

A number of energy metabolism proteins were changed by MS or exercise. Creatine kinase B, involved in phosphate transfer from creatine phosphate to ADP, resulting in ATP production (Jacobus & Lehninger, 1973; Rango et al., 1997), was decreased after MS, but subsequent exercise significantly increased its expression relative to control levels. Ding et al. (2006) also showed that mitochondrial creatine kinase increased in the hippocampus of rats after voluntary exercise. Creatine kinase generally increases in response to exercise in skeletal muscle and other organs (Amelink et al., 1988) and this increase is considered necessary for high energy demands in various tissues. LRRGT00046 is an acyl-CoA binding protein and is therefore involved in fatty-acid metabolism (Knudsen et al., 1989). This protein was increased in MS rats, indicating enhanced energy metabolism, but was not affected

by exercise. Exercise decreased the expression of nucleoside diphosphate kinase A in NR rats but no changes were observed in the MS groups. This protein is involved in synthesis of adenosine tri-phosphate (ATP; Kimura, 2003) and the specific reason for its decreased expression in response to exercise is therefore difficult to explain. Proteins involved in glycolysis and the citric acid cycle were also decreased by MS or exercise in the ventral hippocampus, indicating reduced efficiency in the production of ATP. Glyceraldehyde-3-phosphate dehydrogenase was decreased in MS rats, similar to what we have previously found (Marais et al., 2009b). Malate dehydrogenase functions in the citric acid cycle and catalyzes the conversion of malate into oxaloacetate and was decreased by MS, but restored to control levels after exercise. Kirchner et al. (2008) found a similar increase in mitochondrial malate dehydrogenase after treadmill exercise. In summary, it seems as if MS stress induced strain on the energy production machinery while exercise alleviated this burden.

Maternal separation and exercise also affected the expression of proteins involved in the protection against oxidative stress. Heat shock cognate 71 kDa protein decreased in the ventral hippocampus after maternal separation and this is in contrast to what we have previously observed (Marais et al., 2009b). These molecular chaperones usually function in transport of proteins within the cell and prevent misfolding and aggregation of proteins under stressful conditions (Walter & Buchner, 2002). Decreased levels of heat shock proteins after early life stress may therefore indicate that neurons are at risk for oxidative stress and cell death, given their role in neuroprotection (Latchman, 2004). Heat shock cognate levels in separated exercised rats were similar to control levels, once again pointing towards a restorative role for exercise. MS and exercise on their own increased superoxide

dismutase Cu/Zn (SOD), an antioxidant protein that protects neurons against oxidative stress. Upregulation of antioxidant proteins have been observed after chronic stress for example, SOD was also shown to be increased after chronic exercise in a transgenic mouse model for Alzheimer's disease (Leem et al., 2009). Acute exercise is also known to induce oxidative stress by increasing reactive oxygen species (Davies et al., 1982) and the upregulation of SOD may be a protective mechanism in response to such an increase in free radicals. Surprisingly, this enzyme was not upregulated in rats that were subjected to both MS and exercise. Polyubiquitin is involved in binding, degradation or repair of abnormally folded proteins that are induced by stress (Ciechanover, 1994; Fornace et al., 1989). Similar to SOD, this protein was also upregulated by MS and exercise in NR rats. This result is partially in agreement with previous reports of stress and exercise-induced increases in oxidative stress (Sonneborn and Barbee, 1998; Davies et al., 1982). However, protein levels in MS exercised rats were similar to NR rats indicating that exercise was able to reduce stress-induced proteins to control levels. In contrast, exercise decreased the expression of peroxiredoxin-6 in NR rats. This antioxidant protein scavenges free radicals, thereby limiting oxidative damage to various proteins (Wang et al., 2003) and has been shown to inhibit Ca^{2+} influx protecting cells against glutamate toxicity (Fatma et al., 2008). In terms of the effects of MS and exercise on oxidative stress, our results suggest that exercise may be able to protect neurons against stress-induced oxidative stress.

Proteins related to synaptic plasticity was also influenced by both MS and exercise. Secretogranin-1 is involved in the release of neurotransmitters from vesicles and is found in dendrites, axon terminals and secretory granules of neuroendocrine cells (Munoz et al., 1990; Nolan et al., 1985). It plays a role in collection of secretory

products from the Golgi apparatus and the subsequent formation of secretory granules such as synaptic neurotransmitter vesicles (Ozawa & Takata, 1995). Exercise increased secretogranin-1 levels in NR rats indicating its positive effect on neurotransmission. The clathrin light chain is another protein involved in the formation of endocytic vesicles by mediating binding of clathrin to actin filaments to produce the force for invagination of membranes (Newpher et al., 2006). Clathrin light chain expression was independently increased by both MS and exercise. Interestingly, this effect was not additive as rat subjected to MS and exercise displayed protein levels comparable to that of the exercise group. It seems as if the observed stress-induced increase may have been maximal, serving as a compensatory mechanism to increase neurotransmitter release and recycling.

The survival of neurons also seems to be compromised by early life stress. MS decreased γ-enolase, a protein that increases the survival of neurons, particularly under hypoxic conditions (Hattori et al., 1995). Decreased levels of γ-enolase after MS could have increased cell death in the ventral hippocampus. However, separated rats that exercised showed normalised levels of this protein, indicating that exercise abolished the stress-induced reduction of γ-enolase. Similarly, acidic (leucine-rich) nuclear phosphoprotein 32 family member A also protects neurons against apoptotic cell death by inhibiting caspase-3 (Sun et al., 2006). This protein was upregulated in MS rats, while exercise normalised its expression to control levels. In the same manner, high mobility group box 1 (HMGB1) was increased after MS and decreased in separated exercised rats when compared to control levels. This protein is actively secreted by dendritic cells (Dimitriu et al., 2005) and has been shown to increase neurite outgrowth in central neurons (Rauvala & Pihlaskari, 1987). MS may have increased HMGB1 to induce neuronal plasticity in response to stress.

Jiang et al. (2007) have shown that HMGB1 increases in apoptotic cells and this may support the earlier finding of MS inducing apoptosis in ventral hippocampal neurons. Interestingly, the significant reduction in HMGB1 as observed in MS exercised rats may imply that exercise has anti-apoptotic properties.

The expression of a number of proteins involved in cell signalling and Ca^{2+} regulation were also altered by MS and exercise. MS increased 14-3-3 protein zeta/delta levels, a protein that is part of complex responsible for the activation of Raf-1 by protein kinase C-ζ (Acs et al., 1995; Van der Hoeven et al., 2000). Elevations in 14-3-3 protein zeta/delta concentrations may be indicative of enhanced signalling of the mitogen-activated protein kinase (MAPK) pathway. This protein was however not significantly affected by exercise in our rats. Peptidyl-prolyl cis-trans isomerase (PPIase) FKBP1A and A were both upregulated by MS, and these proteins are involved in the regulation of apoptotic signalling pathways. For example, PPIase can inhibit calcineurin activity which is involved in apoptosis (Liu et al., 1991; Shibasaki & McKeon, 1995) as well as mediate the antitumor effects of rapamycin via inhibition of mTOR signalling (Hidalgo & Rowinsky, 2000). Interestingly, PPIase A also acts as a free radical scavenger, protecting cells from oxidative stress (Yu-song et al., 2009; Doyle et al., 1999). Since exercise reduced the separation-induced upregulation of this protein, it may be possible that this enzyme is part of another mechanism by which oxidative stress was reduced in MS rats after chronic exercise. Calmodulin is an endogenous Ca^{2+} buffer in neurons (Baimbridge et al, 1992). Increases in Ca^{2+} concentrations activates Ca^{2+}/calmodulin dependent pathways that may lead to cell death, while blocking calmodulin has been shown to inhibit brain injury after ischemia (Shirasaki et al., 2006). Calmodulin expression increased significantly in MS rats, reflecting a greater risk for cellular damage. Its expression in MS exercised rats was

however similar to controls, demonstrating the normalising effect of exercise at this level. Neuron-specific protein PEP-19 binds to calmodulin (Slemmon et al., 1996) and was also increased in MS rats. It has been shown that overexpression of PEP-19 inhibits calmodulin activity and protects against Ca^{2+} mediated neuronal death (Kanazawa et al., 2008). Whether increased PEP-19 levels reflect the animal's neurochemical response to protect the brain after early life stress remains to be established. The B-subunit regulates the binding of Ca^{2+} to calcineurin which is a Ca^{2+}/calmodulin-dependent phosphatase (Perrino et al., 1995; Klee et al., 1979). Calcineurin subunit B decreased in both MS groups, but was not affected by exercise. Contrary to expectation another Ca^{2+} binding protein, hippocalcin, was decreased only in NR exercised rats. This indicated a negative effect of exercise, since hippocalcin plays a role in the activation of slow afterhyperpolarization which prevents runaway neuronal activity (Tzingounis et al., 2007) and activation of the transcription factor CREB (Kobayashi et al., 2005).

In the category of protein metabolism, MS increased transmembrane protease serine 13 levels. The enzymatic properties of this protein suggest that it may be involved in proteolytic processing of growth factor precursors (Kido & Okumura, 2008) in which case it is important for the activation of neurotrophins. This suggestion fits previous observations of increased neurotrophin expression after early life stress (Faure et al., 2006). Phosphatidylethanolamine-binding protein 1 was also upregulated in MS rats, but downregulated in MS exercised rats. This protein is proposed to play a role in the activation of gene transcription since it binds to various nucleotides (Bucquoy et al., 1994). Plasminogen activator inhibitor 1 RNA-binding protein was upregulated in rats that were subjected to exercise alone. This protein binds to type-1 plasminogen

activator inhibitor, a serine protease inhibitor, and plays a role in mRNA stability (Heaton et al., 2001).

Conclusion

MS did not induce any changes in 5-HT in the ventral hippocampus, striatum or frontal cortex. Exercise increased 5-HT relative to MS exercised rats but not NR control rats, therefore it is possible that chronic voluntary exercise can increase baseline 5-HT levels. The effect of exercise on 5-HT may however only be an acute effect as previously reported (Béquet et al., 2001).

Quantitative proteomics of the ventral hippocampus revealed that MS induced several changes in cytosolic protein expression that may put these neurons at risk for cell death. Our experiments further showed that many of these changes could be reversed by exercise, thereby protecting neurons against potential stress-induced damage. Exercise in NR rats also had beneficial effects by increasing cytoskeletal- and plasticity-related proteins. In some cases, however, separation-induced dysregulation in protein expression could not be restored by subsequent exercise and further studies should be done to pinpoint specific protein targets of exercise after early life stress.

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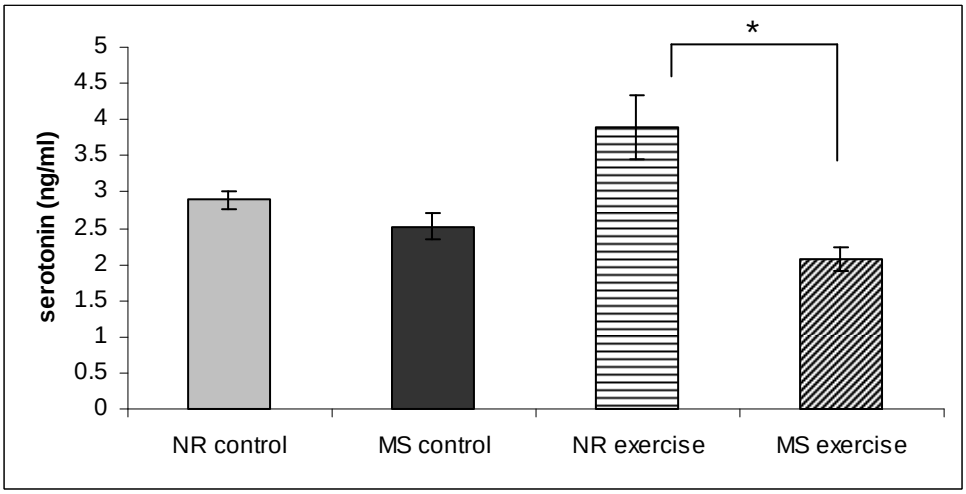

Fig 1. Serotonin levels in the ventral hippocampus of rats. * = $p<0.01$

Table 1. Alphabetical list of differentially expressed proteins and their functions identified from iTRAQ analysis.

Protein	% Cove-rage	Accession no.	Function
14-3-3 protein zeta/delta	21.22	P63102	Adapter in signal transduction complexes
Acidic (Leucine-rich) nuclear phosphoprotein 32 family, member A	8.10	Q5PPH9	Inhibits apoptosis
Actin, cytoplasmic 1	35.20	P60711	Microtubule structure
Calcineurin subunit B type 1	30.59	P63100	Ca^{2+} binding regulation
Calmodulin	71.14	P62161	Ca^{2+} regulation
Clathrin light chain A	14.92	P08081	Coats synaptic vesicles
Creatine kinase B-type	38.06	P07335	Phosphate transfer
Fructose-bisphosphate aldolase A	17.58	P05065	Glycolysis
Gamma-enolase	24.19	P07323	Neuroprotection
Glyceraldehyde-3-phosphate dehydrogenase	14.11	P04797	Glycolysis
Heat shock cognate 71 kDa protein	15.48	P63018	Molecular chaperone
High mobility group box 1	25.58	B4F758	Dendritic cell maturation
LRRGT00046	7.67	Q6TXF3	Acyl-CoA binding
Malate dehydrogenase, cytoplasmic	14.37	O88989	Citric acid cycle
Malate dehydrogenase, mitochondrial	7.40	P04636	Citric acid cycle
Microtubule-associated protein 1A	7.28	P34926	Microtubule structure
Microtubule-associated protein 1B	6.99	P15205	Microtubule structure
Microtubule-associated protein tau	10.64	P19332	Microtubule structure
Neuron-specific calcium-binding protein hippocalcin	38.34	P84076	Ca^{2+} regulation
Neuron-specific protein PEP-19	51.06	Q8CHN7	Calmodulin inhibitor
Nucleoside diphosphate kinase A	30.26	Q05982	Nucleoside triphosphate synthesis
Peptidyl-prolyl cis-trans isomerase A	26.83	P10111	Protein folding
Peptidyl-prolyl cis-trans isomerase FKBP1A	22.22	Q62658	Protein folding
Peroxiredoxin-6	11.16	O35244	Protects against oxidative stress
Phosphatidylethanolamine-binding protein 1	41.71	P31044	Serine protease inhibitor
Plasminogen activator inhibitor 1 RNA-binding protein	15.72	Q6AXS5	Serine protease inhibitor
Polyubiquitin	79.00	Q63654	Protein binding
Pyruvate kinase isozymes M1/M2	14.31	P11980	Glycolysis
Secretogranin-1	2.96	O35314	Regulates secretion of neurotransmitters
Superoxide dismutase [Cu-Zn]	31.90	Q6PEC5	Protects against oxidative stress
Transmembrane protease, serine 13	2.23	B2RYJ5	Serine protease
Tropomyosin 5	27.82	P97726	Microtubule structure
Tubulin alpha-1B chain	22.62	Q6P9V9	Microtubule structure

Table 2. Quantitative results for the expression of cytosolic proteins in the ventral hippocampus. Significantly different p-values indicated in bold ($p < 0.05$).

	Fold change relative to NR control group (reporter 115)					
	MS control		**MS exercise**		**NR exercise**	
Protein	**114:115**	**p-value**	**116:115**	**p-value**	**117:115**	**p-value**
Creatine kinase B-type	0.825	**0.034**	1.535	**0.006**	1.035	0.656
Actin, cytoplasmic 1	0.596	**0.001**	2.487	**0.022**	0.815	**0.013**
Tubulin alpha-1B chain	0.315	**0.003**	2.405	0.285	0.763	**0.002**
Gamma-enolase	0.696	**0.025**	1.205	0.427	0.846	0.062
Heat shock cognate 71 kDa protein	0.534	**0.000**	0.709	0.314	0.683	0.195
Glyceraldehyde-3-phosphate dehydrogenase	0.737	**0.048**	0.590	0.384	0.654	0.064
Calcineurin subunit B type 1	0.764	**0.008**	0.528	**0.001**	0.552	0.153
Malate dehydrogenase, cytoplasmic	0.682	**0.002**	1.061	0.606	0.837	**0.031**
Malate dehydrogenase, mitochondrial	0.470	**0.001**	0.758	0.611	0.928	0.066
Calmodulin	1.758	**0.000**	0.806	0.377	0.963	0.909
Phosphatidylethanolamine-binding protein 1	1.973	**0.002**	0.542	**0.015**	1.108	0.544
14-3-3 protein zeta/delta	1.368	**0.022**	1.776	0.056	0.765	0.343
Tropomyosin 5	6.874	**0.000**	0.966	0.911	0.987	0.868
Superoxide dismutase [Cu-Zn]	1.486	**0.016**	0.554	0.050	1.952	**0.002**
Polyubiquitin	3.226	**0.000**	0.671	0.069	1.321	**0.006**
Peptidyl-prolyl cis-trans isomerase A	1.587	**0.014**	0.558	**0.010**	0.865	0.312
High mobility group box 1	1.585	**0.007**	0.432	**0.034**	1.770	0.174
Neuron-specific protein PEP-19	7.341	**0.044**	1.101	0.711	1.034	0.856
Clathrin light chain A	2.737	**0.007**	1.564	0.055	1.481	**0.005**
LRRGT00046	9.086	**0.001**	0.420	0.222	0.848	0.448
Acidic (Leucine-rich) nuclear phosphoprotein 32 family, member A	1.205	**0.030**	0.884	0.284	0.774	0.235
Peptidyl-prolyl cis-trans isomerase FKBP1A	1.918	**0.032**	0.857	0.605	1.227	0.528
Transmembrane protease, serine 13	2.322	**0.010**	3.029	0.083	1.381	0.108
Peroxiredoxin-6	0.860	0.131	0.986	0.920	0.626	**0.001**
Neuron-specific calcium-binding protein hippocalcin	0.971	0.732	0.822	0.116	0.550	**0.000**
Microtubule-associated protein tau	1.907	0.097	0.413	**0.038**	2.630	**0.021**
Pyruvate kinase isozymes M1/M2	0.737	0.592	3.821	0.102	0.773	**0.036**
Nucleoside diphosphate kinase A	0.906	0.274	0.979	0.724	0.559	**0.020**
Fructose-bisphosphate aldolase A	0.621	0.067	0.489	**0.018**	0.847	0.595
Secretogranin-1	1.469	0.074	1.676	0.137	2.064	**0.028**
Microtubule-associated protein 1A	1.366	0.166	2.138	**0.006**	2.038	**0.002**
Plasminogen activator inhibitor 1 RNA-binding protein	0.896	0.051	0.646	0.251	2.487	**0.004**
Microtubule-associated protein 1B	0.746	0.168	1.235	0.629	2.958	**0.004**

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Chapter 8

General Conclusions

Early life stress in children is known to predispose them to the development of psychiatric disorders that include depression and anxiety disorders. In this study, we wanted to determine whether exercise would be beneficial to rats exposed to early life stress. We used maternal separation as a model for early life stress with additional exposures to stress during adulthood to generate a rat model for depression. Firstly we characterized the overall consequences of maternal separation on brain functioning by determining it effects on behavior, aspects of the hypothalamic-pituitary-adrenal-axis, neurotrophin levels and markers of apoptosis, in brain regions related to clinical depression. We also evaluated changes in the expression of cytosolic proteins in the ventral hippocampus of maternally separated rats. Secondly, in subsequent experiments, maternally separated rats were allowed chronic voluntary exercise to assess whether it has beneficial effects on the systems that were negatively affected by early life stress.

Maternal separation followed by chronic restraint stress during adulthood resulted in increased depressive-like behavior compared to control rats. With baseline measurements we observed no differences in anxiety-like behavior when maternally separated rats were compared to controls. Our neurochemical data indicated that maternally separated animals had increased baseline corticosterone concentrations and blunted adrenocorticotropin hormone response after acute restraint stress. These rats had significantly lower neurotrophin levels in the ventral hippocampus but not in the striatum or frontal cortex, when compared to normally reared controls. Maternal separation alone and followed by acute restraint stress during adulthood induced changes in apoptotic marker expression in the striatum and frontal cortex, which were more pronounced in the striatum, indicating that these neurons may be at risk for apoptosis.

In rats subjected to maternal separation and chronic restraint stress during adulthood, we found that 6 weeks of wheel running exercise decreased their depressive-like behavior. This change in behavior was associated with increased brain derived neurotrophin levels in the striatum. Interestingly, neurotrophins did not increase in the ventral hippocampus where it was reduced after maternal separation. Furthermore, exercise did not significantly change baseline corticosterone and adrenocorticotropin hormone levels. Serotonin levels, which were not significantly altered by maternal separation, were increased by chronic voluntary exercise in normally reared rats. This suggested that exercise has the potential of regulating neurotransmitter concentrations.

Proteomic analysis of the ventral hippocampus revealed that maternal separation induced a number of significant changes in the expression of the cytosolic proteins. These proteins were related to cytoskeletal structure, neuroplasticity, oxidative stress, energy metabolism, protein metabolism, and cell signaling. Chronic voluntary exercise was able to restore the expression levels of a number of the proteins affected by maternal separation, especially those that increased the risk for neuronal death. The proteomic results showed that the mechanism of action by which exercise improves brain functioning after early life stress was related to proteins in various functional categories. Exercise in normally reared rats also induced a beneficial effect by increasing the expression of cytoskeletal and neuroplasticity proteins. When comparing the efficacy of exercise to that of escitalopram, an antidepressant drug, it was evident that, in contrast to exercise, escitalopram targets a different subset of proteins to those that were changed by maternal separation, except for a few involved in energy metabolism pathways and neuroprotection.

In summary, we have shown that chronic voluntary exercise has beneficial effects in maternally separated rats, decreasing depressive-like behavior, increasing neurotrophin expression and restoring cytosolic protein expression that were dysregulated by early life stress.

These research experiments can potentially benefit by addressing some of the shortcomings to the work already presented here. These shortcomings may be taken into account for future studies. Maternal separation is used as a model for early life stress, and can be employed as a model to study the development of depression since we have seen endocrine and neurotrophic changes in the rat brain corresponding to clinical observations in patients with depression. However, it seems as if changes in behavior only appear after an additional repetitive stressor during adulthood, and this needs to be standardized in the rat model. The behavioral tests performed on the adult rats can also be done in more detail, for example a number of other tests are available for the determination of depressive-like behavior: the tail-suspension test and sucrose consumption test for anhedonia being well described in literature. There are also additional tests available for anxiety-like behavior for example the light/dark box. Locomotor activity should also be tested for in conjunction with the forced-swim test to see whether differences locomotor behavior, which may have resulted from maternal separation stress or exercise, influences swimming performance. It would also be useful to measure oxidative stress in the brain tissue that was used for protein determinations, providing that there are larger group numbers to be able to yield enough brain tissue, since the peripheral measurement of oxidative status may not optimally reflect the condition in the brain. Blood plasma could effectively be used to determine corticosterone levels

at several time-points in the exercised rats (before, during and afterwards), to in fact establish whether the type of exercise used induces a significant stress response that could interfere with its beneficial effect on the brain. In this way, different exercise regimens, for example long term swimming or treadmill running can be compared with voluntary running to assess which is a better model to study. Other measurements can also be made to ensure that the parameters that we measure are more clinically relevant. Clinical studies focus more on peripheral measurements, and use blood plasma to determine glucocorticoids, neurotrophins and prolactin for diagnostic purposes. Since exercise is clinically used as augmentation to pharmacotherapy, the effect of different forms of exercise together with antidepressant drug treatment in a rat model can also generate valuable data.