The effect of exercise on spatial learning and hippocampal proteins in maternally separated adult rats

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DECLARATION

I, Nokuthula Makena**, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.**

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Abstract

Repeated maternal separation (MS) has been reported to induce changes in hypothalamic-pituitary-adrenal (HPA) axis activity leading to abnormal stress responses later in life. Such alterations have also been linked to poor cognitive function. In contrast, exercise enhances cognitive function. Previously, we reported that MS improved object location memory. However, exercise had no effect on object location memory despite increases in levels of synaptophysin and phospho-extracellular signal-regulated protein kinase (pERK) in the hippocampus of non-separated-exercised rats. In the current study, the same MS technique and three-week voluntary exercise regimen were tested to determine their effect on spatial learning in young adult Sprague-Dawley (SD) rats. A total of 144 rats were either maternally separated from postnatal day 2 to 14 or designated as controls. At postnatal day 50, rats were transferred to cages with attached running wheels. Approximately half of the rats were allowed to exercise voluntarily in the wheels whilst the wheels attached to the cages of the remaining non-exercising rats were immobilised. Rats were divided into 3 cohorts. Cohort 1 provided baseline levels of pERK, mitogen-activated protein kinase phosphatase-1 (MKP-1) and brain derived neurotrophic factor (BDNF) after exercise. Cohorts 2 and 3 were trained in the Morris Water Maze (MWM) 1 and 15 days post-exercise, respectively. Consistent with our previous findings, pERK was increased in non-separated-exercised rats post-exercise. MKP-1, the regulator of pERK, was also increased in the non-separated-exercised group. BDNF was decreased in the MS non-exercised group but augmented by exercise. All groups trained immediately after exercise performed similarly in the MWM but MS rats from cohort 3 had better reversal spatial memory. According to these results, repeated MS decreased neurotrophic factors but did not alter the plasticity-related proteins measured in this study. However, this phenomenon was not associated with performance in the spatial learning and memory task in the MWM. These current observations support our previous findings that MS can cause adaptations that lead to improved learning and memory in adulthood.

List of abbreviations

ACTH	Adenocorticotrophic hormone
ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
CA1	Cornu Amonnis area 1
CA3	Cornu Ammonis area 3
CaMKII	Calcium/calmodulin-dependent protein kinase II
CORT	Cortisol/corticosterone
CREB	cAMP responsive element binding protein
CRH	Corticotrophin-releasing hormone
CUS	Chronic unpredictable stress
DG	Dentate gyrus
dHC	Dorsal hippocampus
DUSP1	Dual specificity phosphatase-1
EPM	Elevated plus maze
ERK	Extracellular signal-regulated protein kinase
HC	Hippocampus
HPA	Hypothalamus-pituitary-adrenal
IGF-1	Insulin-like growth factor-1
MAPK	Mitogen-activated protein kinase
MKP-1	Mitogen-activated protein kinase phosphatase-one
MS	Maternal separation/ maternally separated
MWM	Morris Water Maze
NMDAR	N-methyl D-aspartate receptor
nR	Non-runner
NS	Non-separated
OF	Open field
OFT	Open field test
pERK	Phospho-extracellular signal-regulated protein kinase
pJNK	Phospho-c-Jun N-terminal kinases
PKC	Protein kinase C
pMEK	Phospho-mitogen-activated protein kinase kinase
PP2A	Protein phosphatase 2A
PTSD	Post-traumatic stress disorder
R	Runner
SD	Sprague-Dawley
SEM	Standard error of the mean
TrkB	Tyrosine kinase B

Chapter 1: Introduction

1.1 Stress/ developmental stress

In South Africa, health statistics show that 16.5% of non-communicable disorders in adults are behavioural and mental disorders [1]. These psychological disorders are potentiated by one's early life experience [2]. Indeed, clinical evidence shows that a relationship exists between adverse childhood episodes and increased risk for adult psychiatric disorders [3–5]. These psychiatric disorders may also co-exist with learning and memory impairments [6]. Approximately 12% of men and women with psychiatric conditions in South Africa had reported to have been physically abused as children [1]. Neglect is another form of abuse and in itself makes the victim prone to abusive behaviour or increases the likelihood of being a recipient of abuse in later life [7]. Child neglect can also affect an individual's physical and cognitive development [8].

The hypothalamus-pituitary-adrenal axis (HPA) is the neuroendocrine pathway that is activated upon exposure to stressful stimuli; nerve cells in the hypothalamus secrete corticotrophin-releasing factor (CRF) that stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH) which then results in the release of adrenal hormones [9]. Plasma cortisol (corticosterone in rats) levels have been used to define the level of HPA axis activity which corresponds to the level of stress that is experienced by the individual [10]. The prefrontal cortex, hippocampus and amygdala are key regulators of the HPA axis [7,11]. They regulate the stress response by recognising the level to which the stressor poses a threat and project signals accordingly to the hypothalamus. When the threat has subsided, a negative feedback mechanism exists at each level of the HPA axis to regulate the intensity of the stress response and eventually to return HPA activity to resting levels. Altered HPA axis

activity arises when the negative feedback system is impaired and fails to decrease glucocorticoid secretions resulting in higher levels of cortisol or corticosterone. It is known that early-life stress impacts HPA axis activity causing it to react in an abnormal fashion in adulthood[2].

The effects of perinatal stress, modelled by various perinatal stressors, on the HPA axis at different stages of life have been well summarised before [2,12,13]. Babies, whose mothers reported higher psychological stress, initially behaved similarly to their counterparts but took much longer to recover from a heel-stick stress test [14]. Similarly, when recovery to a heel-stick stress test was correlated with maternal gestational cortisol at gestational week 13, babies from mothers with higher cortisol took longer to recover from the stress test [14]. This provides evidence that prenatally, maternal HPA axis activity has a role in programming infant HPA axis activity possibly due to passage of the maternal glucocorticoids via the placenta to the offspring's blood [4]. In a study examining the relationship between caregiver contact and stress reactivity, adults that experienced prolonged periods of separation from both parents due to civil unrest were found to have increased plasma ACTH and salivary cortisol compared to adults who did not experience separation from their primary caregivers [15]. Moreover, the same adults with increased ACTH displayed higher stress reactivity compared to non-separated adults when tested in the Trier Social Stress Test [15]. This suggests that impaired HPA axis negative feedback also produces behavioural effects. Animal models can be used to explain how maternal stress and cortisol levels can affect offspring. Acute prenatal stress of a rat dam causes a decrease in the offspring's glucocorticoid levels [16]. A mild repeated stressor (short periods of maternal separation) administered postnatally results in increased glucocorticoids while a more severe stressor decreases glucocorticoids (single 24-hour maternal separation) [2]. However, an acute postnatal stressor reduces glucocorticoids

in plasma [16]. Repeated separation of pups from dams for periods of just 3 hours per day from postnatal day (P) 2 to 14 produced long-term effects with increased basal levels of CRF mRNA in the hypothalamus measured in adulthood [17]. Elevated CRF has in turn been shown to lead to down-regulation of glucocorticoid receptors in the hippocampus thereby affecting stress reactivity in later life [18].

Maternal separation (MS) is the experimental paradigm used to mimic the effects of early life stress in laboratory rodents. It involves the separation of the dam from her pups for 3- 6 hours per day during the first 2- 3 weeks of life, a period in which pups are highly dependent on dams for care[17,19]. During this stage of development, the neonate's HPA axis is normally non-responsive to stressful stimuli. However, MS results in HPA activity that is subsequently enhanced in later life [20], leading to a shift in basal HPA axis activity [10,21,22]. Handling of the pups is sometimes used as a positive control and is especially relevant if the MS pups will be handled when separated from the dam. However, handling of pups has been shown to oppose the effects of MS with handled pups displaying less anxiety-like behaviour in the open field test (OFT) and reduced fear conditioning in a learned fear test compared to MS rats[23]. This evidence indicates that the effect of MS is dependent on severity, frequency, and context of the separation period. The most commonly used separation period is one whereby pups are separated from their dams for 3 hours per day from postnatal day 2 to 14 [24,25] with brief daily handling or no handling as control groups [26].

As HPA axis regulators, the amygdala and hippocampus have receptors for glucocorticoids. Abnormal activity of the HPA axis impacts on these structures, which are also involved in learning and memory [10,27,28]. Given their role in emotional regulation and learning, it is

unsurprising that adults that have experienced adversity in their formative years have an increased probability of having learning deficits [4]. Animal experiments suggest that these deficits can potentially be reversed with drug therapy [29]. However, the majority of drug therapies for cognitive impairments are focused on patients with Alzheimer's Disease, dementia and such disorders [30]. Therefore, research into developmental stress-induced cognitive deficits is a necessary and valid line of investigation.

Glucocorticoids act on the hippocampus and play a role in cellular growth and maintenance [28]. Changes in hippocampal neurogenesis and dendritic branching are often associated with MS as it alters glucocorticoid secretion [31]. Stereological analysis of hippocampal tissue of mice subjected to a single MS period revealed that there were fewer neurons in the dentate gyrus, an area of the hippocampus where neurogenesis occurs, than in control mice [32]. The ventral hippocampus is more sensitive to the effect of MS on neurogenesis [33]. The different types of memory and their associated brain areas are listed in Table 1.1.

Table 1.1: Brain areas involved in different types of learning

Type of learning/memory	Brain region	Reference(s)
Sensory memory	Cortex	[34]
Novel object recognition memory	Medial pre-frontal cortex, Peri-rhinal cortex, CA1	[35–37]
Temporal ordering	Medial pre-frontal cortex, Peri-rhinal cortex, CA1	[35,38]
Object location	Medial pre-frontal cortex, Peri-rhinal cortex, CA1, CA3	[35,39]
Object-in-place	Medial pre-frontal cortex, Peri-rhinal cortex, CA1	[35,37]
Spatial memory	Parahipocampal cortex, Striatum, Prefrontal cortex, hippocampus	[38,40]
Working memory	Hippocampus, Frontal cortex	[41,42]
Motor skills	Basal ganglia, Cerebellum	[43]
Emotional memory	Amygdala	[44,45]

CA3=Cornu Ammonis area 3, CA1= Cornu Amonnis area 1, DG= Dentate gyrus

Learning and memory in MS rats tends to be impaired, a deficit that is usually accompanied by changes in the levels of proteins that are required for plasticity and learning [10,29,32,46,47].

Developmental stress-induced reductions in hippocampal volumes, proteins of plasticity and neurotrophins have been observed in MS animals [9]. Clinical observations of patients have also demonstrated the impact that growing up in a financially strained family has on hippocampal volume in later life [48].

MS also causes behavioural changes that can be attributed to altered negative feedback in the HPA axis [18,21,49]. MS rats are tested for anxiety-like behaviours in the Open Field Test (OFT) and elevated plus maze (EPM)[50]. In these tests, anxiety-like behaviour is indicated by increased thigmotaxis in the open field or a significant decreased preference for the open arms of the EPM[50]. MS rats also have an increased likelihood to develop depressive-like behaviours [21], as measured by proxies of helplessness and anhedonia in the forced swim and sucrose preference tests respectively [51,52].

1.2 Exercise

Physical exercise has long been recommended as part of a healthy lifestyle with its health benefits extending to the central nervous system. Exercise acts by stimulating the sympathetic nervous system to eventually increase the secretion of glucocorticoids and catecholamines [53]. The adrenal glands become enlarged after chronic exercise suggesting that there is increased activity of these glands to effect changes that will ensure the required energy supply of the organism is met. The link between exercise and stress has been found experimentally in rats that underwent 4 weeks of regular forced exercise displaying a blunted response to stress thereby leading the authors to suggest that chronic exercise reduces the sensitivity of the HPA axis[54]. In rats, four weeks of voluntary exercise increased adrenal activity indicated by increased plasma corticosterone levels [54]. However, exercise did not alter the expression of CRF in the hypothalamus nor did it cause a decrease in glucocorticoid

receptors in the pituitary gland despite elevated plasma glucocorticoids [55]. From this evidence it can be concluded that exercise modulates the HPA axis [53,55].

The hippocampus, a regulator of the HPA axis, expresses both mineralocorticoid and glucocorticoid receptors and is therefore sensitive to activation of the HPA axis[56]. However, though exercise activates the HPA axis, the resultant effect differs from stress-induced HPA axis activity [57], as exercise does not affect the expression of mineralocorticoid and glucocorticoid receptors at the hippocampal level [58]. Exercise has, however, been found to bring about beneficial effects similar to pharmacological anti-depressant agents with exercised rats exhibiting diminished anxiety- and depressive-like behaviours [59]. Similarly, following exercise, mice had lower acoustic startle amplitude, spent more time in the centre of the OFT and attempted to escape from the OF apparatus fewer times than sedentary mice [60]. In addition, exercised mice have shown less immobility in the forced-swim test, a behavioural assay of depressive-like behaviour in rodents [59]. This is further evidence that exercise modulates the HPA axis to ameliorate abnormal behaviour.

As exercise has been shown to increase HPA axis activity, exercise acts physiologically as a stressor [57]. However, due to the involvement of higher cognitive areas (prefrontal cortex, hippocampus and amygdala) in HPA axis regulation, the effects of exercise are different from those of threatening stressors, i.e. the exercise activity has to be perceived as non-threatening for it to have an overall beneficial effect on the body [57].

Another mechanism by which exercise influences the brain is via locus coeruleus (LC) activation. Exercise stimulates the sympathetic nervous system, which causes the hypothalamus to stimulate neurons in the LC, which produce and release norepinephrine in several brain areas including the prefrontal cortex, hippocampus, amygdala and hypothalamus [61–63]. Norepinephrine release in these brain areas modulates the stress response and plays a role in memory and learning. Treadmill exercise causes an increase in basal norepinephrine levels in the hypothalamus of rats [64]. Dams that were allowed to exercise had offspring that had better spatial memory in the Morris Water Maze (MWM) than offspring of non-exercised dams [65]. Lesioning the noradrenergic neurons of the same pups abolished the effect of exercise on memory [65].

Exercise favours neurogenesis over neurodegeneration in spite of increased corticosterone [66]. The stimulation of neurotrophin release underlies the mechanisms by which exercise exerts positive effects on the central nervous system. Physical exercise increases the release of insulin-like growth factor-1 (IGF-1) which has been identified as a precursor for neurogenesis [67]. Neurogenesis can be measured by counting cells and dendritic branching [33,67]. Exercise increases the number of BrdU, a marker for neurogenesis, positive cells in the hippocampus [67]. Affective disorders such as depression have been linked to decreased neurogenesis in the hippocampus and amygdala and exercise has been shown to alleviate depressive-like behaviours possibly due to the exercise-mediated neurogenesis [59,68]. Additional research has highlighted the neuroprotective effects of exercise. Exercise protected dopaminergic neurons in the substantia nigra from 6-hydroxydopamine-induced lesion in a rat model of Parkinson's Disease by decreasing the rate of cell loss [69]. In a murine model of Alzheimer's disease, exercise decreased the number of plaque deposits in the cortex which also correlated with improved memory [70].

Exercise also plays a role in learning and memory with exercised rats able to discriminate between novel and familiar objects and novel object location better than their sedentary counterparts[71,72]. In addition, treadmill exercise enhanced spatial learning in the MWM task [73]. In a brain injury model , exercise failed to enhance learning in the MWM in sham-lesioned rats [74]. However, in rats that had experienced brain injury, immediate exercise enhanced learning and memory while exercise delayed by 2 weeks had detrimental effects [74]. This research suggests that the timing of the exercise remains crucial in its effect. Equally important is the intensity and level of exercise as evidence shows that low levels of exercise were surprisingly more beneficial for memory than higher levels of exercise. Rats that ran longer distances and sedentary rats had similar discrimination indices for a novel object whereas rats that ran shorter distances had significantly lower discrimination indices [75].

Exercise also improves memory by enhancing biochemical pathways that are involved in consolidation and retrieval of information [76]. Exercise-induced stimulation of the prefrontal cortex, hippocampus, and amygdala resulted in the activation of secondary messenger systems in target cells. Calcium/calmodulin-dependent protein kinase II (CaMKII), a protein required in long-term potentiation (LTP)[77], was increased in rats following voluntary exercise [78]. Exercise increased growth factors such as brain-derived neurotrophic factor (BDNF), IGF-1 and nerve growth factor (NGF) [67,79]. BDNF-deficient mice were resistant to the antidepressant effects of exercise [59]. In an active avoidance test, exercise decreased the immobility and number of escapes similarly to the antidepressant, desipramine [59]. BDNF increases are also dependent on the activity of the mitogen-activated protein kinase (MAPK) pathway, blockade of this pathway rendered exercise ineffective in increasing BDNF levels [59].

There is much controversy as to whether voluntary exercise is as effective as forced treadmill exercise. Both forms of exercise increase cerebral glucose metabolism, though treadmill exercise appears to have a greater effect than voluntary exercise [80]. Forced exercise increases markers of glucose metabolism, glucose transporter proteins 1 and 3, phosphofructokinase, lactate dehydrogenase and monophosphate kinase, to a greater extent than voluntary exercise [80]. On the contrary, voluntary exercise improved motor function recovery in rats with brain ischemia, whereas involuntary exercise (exercise induced by an electric shock) and forced exercise (treadmill running) were ineffective [81]. In the same rats with brain ischemia, hippocampal BDNF levels were also higher in the voluntary exercise group compared to the forced exercise group [81]. This may have been due to the increased corticosterone levels in the forced exercise group [81] as increased levels of corticosterone are associated with decreased neurotrophin levels and diminished neuroprotection [55]. This evidence demonstrates that when exercise is emotionally stressful, as demonstrated by higher levels of glucocorticoids, its beneficial effects are abolished. In summary, exercise stimulates the HPA axis to cause anti-anxiety effects [59]; neuroprotection [69,82] and neurogenesis [66,67].These effects of exercise are influenced by the type and intensity of exercise[80,81].

In clinical studies, the duration and intensity of exercise have been shown to affect hippocampal volume, serum BDNF levels, and spatial memory. In older men, aged 55 – 80 years, aerobic exercise compared to stretching, was associated with increased serum BDNF, and increased hippocampal volumes. Furthermore, the intensity of exercise (as indicated by VO2 max) was correlated with higher serum levels of BDNF[71]. In younger men, aerobic exercise also increased BDNF levels but a correlation between serum BDNF levels and intenstity or duration of exercise was not apparent[83].

For the present study, voluntary exercise was chosen as it was considered more representative of real-life situations whereby people choose when and how much to exercise at a given time.

1.3 Learning and memory

Memory is the process of encoding, storing, and retrieving newly acquired information. Learning is achieved when that new information is used to make behavioural adjustments in response to certain stimuli [84]. Atkinson and Shiffrin were amongst the first psychologists to try to define the process of learning and memory [85]. The first model in 1968 only comprised short- and long-term memory and a possible pathway for amnesia (Figure 1.1) and simply stated that newly acquired information was first stored in the short-term store before being transferred to the long-term storage compartments, failing that, it was forgotten [86]. Later, a sensory memory phase was included which, even today, is regarded as the first stage of memory consolidation [85]. However, the Shiffrin-Atkinson model came under heavy criticism as it assumed information in short-term memory would automatically be transferred to the long-term stores; short-term memory was the only gateway to long-term memory and; short-term memory was working memory[87]. Almost a decade later, after having studied clinical data and noting these inconsistencies, Baddeley and Hitch proposed the working memory model (Figure 1.2) [88,89]. Theirs was a 3-component model comprising a visuo-spatial sketchpad, a phonological loop, and the central executive. The visuo-spatial sketchpad was the temporary storage for visual and spatial information, the central executive was the control centre that was responsible for focussing and dividing attention and allowed for the switching between tasks; while the phonological loop maintained the information through rehearsal [87,89]. These components were so-named due to the types of experiments that were carried out

in those days. Human subjects were usually presented with visual sequences that they would have to later recall under different conditions [89]. One of the first experiments aimed at discriminating working memory from short-term memory involved giving participants a grammatical task while they were required to recall audibly presented number sequences. As the sequences grew longer, performance in the grammatical task slowed down. The result of this experiment suggested to Baddely and Hitch that working memory was not a single store. This is how they proposed the 3-component model [87]. Further investigation into the role of the phonological loop revealed that it was essential for new long-term language acquisition but that the already-stored language could also influence the recall of newly acquired words [87].

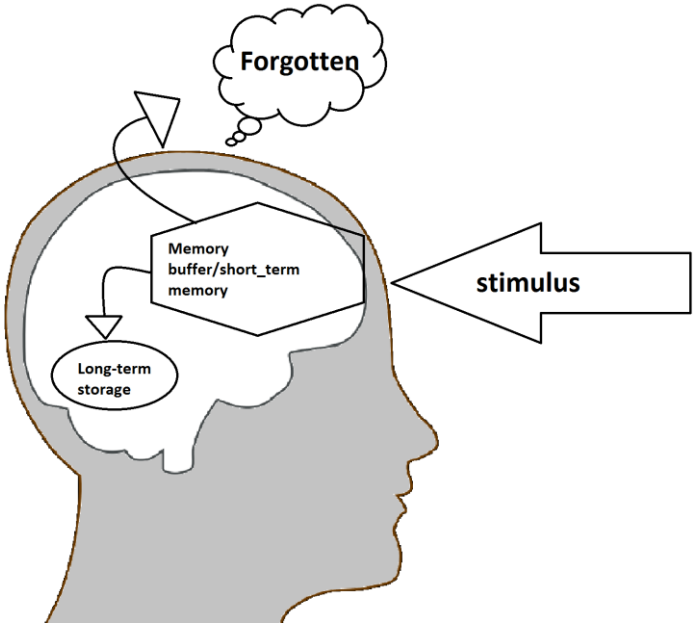

Figure 1.1. First working memory system proposed by Shiffrin and Atkinson in 1968. Outline of head from clipartbest.com

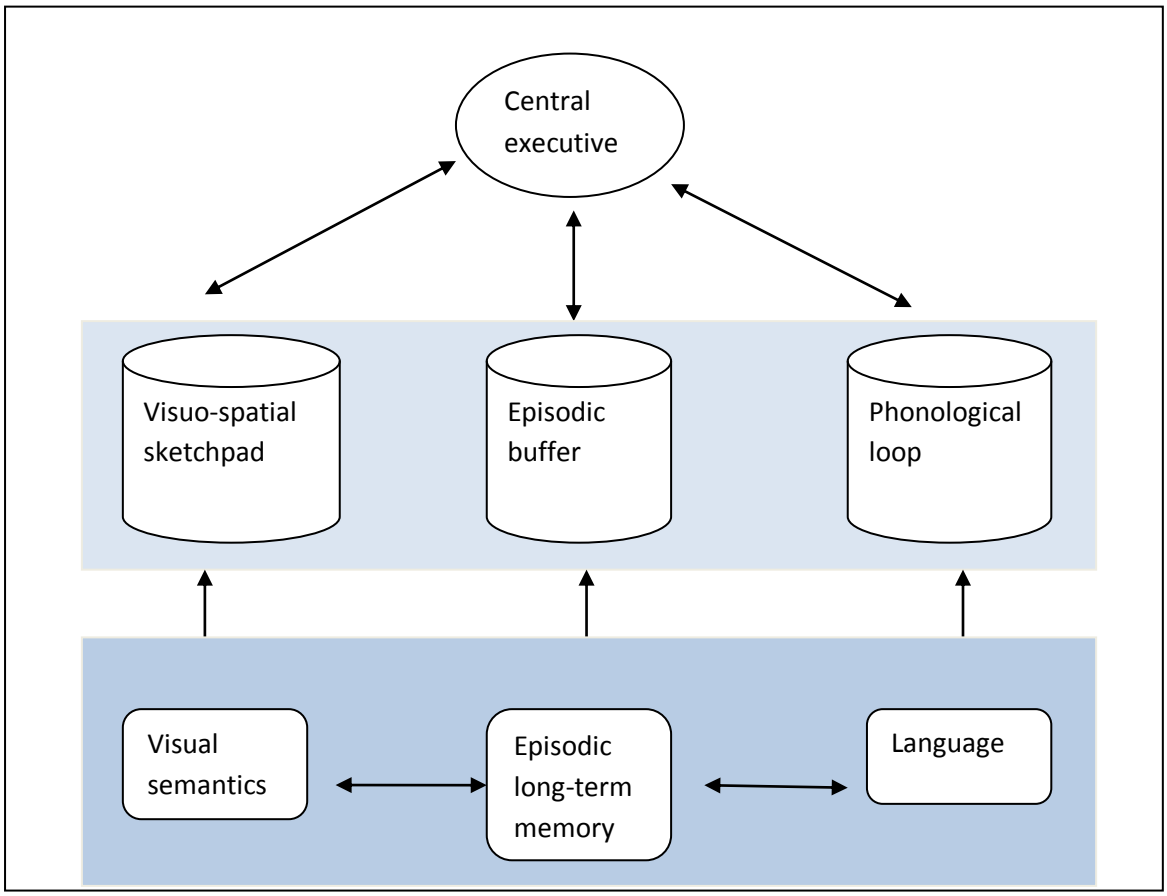

Figure 1.2. Working memory model proposed by Baddeley in 2000.

The visio-spatial sketchpad gave rise to visual semantics. With this new information, a multimodal working memory model was introduced whereby language, visual semantics, and episodic long-term memory exist as the crystalline part of the system and an episodic buffer, together with the visio-spatial sketchpad and phonological loop make up the fluid system. The fluid system is highly influenced by the crystalline system and modulated by the central executive (Figure 1.2) [87]. This model accounts for the complex manipulations, which can be made to remembered information with or without a stimulus.

To date, several types of memory have been described that can be classified under these four main groups: sensory, working memory , short-term, and long-term memory and are further categorised according to the type of information that is being learned and the brain region in which that information is to be stored [90]. The brain has a highly sophisticated organisation, which allows for the different types of memory to be processed in functionally unique regions thereof. As seen in Table 1,1., not a single brain region is solely responsible for only a single type of learning and memory. Brain regions that are known to be involved in learning or memory include the frontal cortex, hippocampus, amygdala, striatum, cerebellum and basal ganglia amongst others [91]. Some types of learning rely more on a certain brain region than the other, for example, imaging studies in humans revealed that temporal ordering memory required more activity in the pre-frontal cortex whereas spatial memory was dependent on para-hippocampal activity. Furthermore, the amygdala is important in emotional conditioning [45].The striatum facilitates procedural and reward-based learning [92] while the frontal cortex is important for executive function [19]. The hippocampus largely mediates recognition, spatial and working memory. Our knowledge of the extent to which the hippocampus is involved in learning and memory became expanded when patient H.M., who suffered anterograde amnesia following bilateral transection of the medial temporal lobe, was studied [93]. Patient

H.M. had suffered from debilitating epileptic seizures. The resection improved his medical condition but he would have to live with the inability to form new memories. He was left with anterograde amnesia and poor working memory [93]. In patient H.M. and others that underwent similar operations, working memory appeared to be fine until they were disrupted, at which point they would suffer from amnesia [93]. It appeared that their working memory capabilities were limited to the fluid system, which was no longer in communication with the crystalline system that had been described by Baddely and Hitch.

The factors that affect memory include, but are not limited to, sleep; exercise [94]; stress [95]; drugs [96] as well as the general environment [97]. Exercised rats had a lower discrimination index for a novel object in the novel object recognition test compared to sedentary rats [94]. Drugs of abuse may negatively affect cognitive function. Rats that were exposed to chronic cocaine had longer mean path lengths to a hidden platform in the MWM task [96]. This showed that the cocaine-treated rats had trouble remembering the shortest path to the platform or that they did not form solid associations with the spatial cues and the platform position. An enriched environment affects learning and memory positively, low maternal care caused a decrease in the discrimination of a novel object and this effect was ameliorated by environmental enrichment [97]. In this current project, exercise was investigated to determine its effect on memory and learning in MS animals. Spatial learning and memory was assessed by means of the MWM.

1.3.1 A test of spatial memory: the Morris Water Maze

The MWM is used routinely to measure spatial learning and memory in rats. It simply consists of a tank filled with water in which a platform is submerged. Rats are placed in the tank at different positions and have to learn the location of the hidden platform within a

predetermined period. With daily training, learning will be observed in normal rats as a decrease in the mean latency to find the platform. After 3-5 days, the platform is removed from the tank and the memory for the platform position is tested in a probe trial. Rats with intact memory will spend more time searching for the platform in the region that they expect to find it. Reversal memory can be tested by simply changing the location of the platform and retraining rats to find it. Many other MWM test paradigms exist and each tests a different type of memory, summarized by Vorhees and Williams (2006) [98]. The protocol described above is the most widely employed to test spatial learning and memory. Other specialised and more complex spatial memory tests exist such as the radial arm maze which can be performed in or out of a water tank [99].

Spatial information is encoded in the dorsal hippocampus [100]. The cells that are responsible for this function are the pyramidal cells of the CA1 region [101]. Not all cells of the CA1 region have the same function in spatial encoding. It is thought that cells that make up firing fields that relate to the position of the animal are place cells [102]. Cells that map the external environment are called grid cells and are found in the entorhinal cortex [103]. Recently, place cells have also been identified in the ventral hippocampus but they differ in the scaling of the represented environment compared to those located in the dorsal hippocampus [104]. Neurons that encode place do not work independently but form part of a neuronal network of 4-6 neurons [105]. The network synchronicity is dependent on the size of the network and inversely proportional to the distance between the neurons [105]. In the CA1 region, there is no anatomical clustering of place cells unlike in the CA3 region [106] and the spatial firing field is biased towards the centre of the environment [106] but could also be involved in decision making [107]. It was found that place cells had increased activity when the rat was at the start position of a double-Y maze. This led to the suggestion that place cells are involved in

encoding the intended destination of the rat [107]. Recently, the relationship between grid cell and place cell firing was investigated in live rodents placed in a novel environment. The place cells and grid cells work in a synergistic fashion to recognise spatial novelty signals and aid in decision making [108].

1.3.2 Molecular substrates of learning and memory

The molecular basis of learning and memory is LTP, a process by which synaptic connections are enhanced in response to stimulus [109]. The receptors known to be essential for LTP are the glutamate receptors. N-methyl-D-aspartate (NMDA) receptors, especially those found in the CA1/CA3 pyramidal cells of the hippocampus, are the most researched but AMPA receptors also fall into the class of glutamate receptors. When glutamate binds to NMDA receptors or AMPA receptors, sodium ions move rapidly into the cell with minimal loss of potassium ions causing depolarisation at the membrane [110]. The resistance of these receptors are differentially controlled. AMPA receptors will produce a strong linear current-voltage relationship when partnered with an abundance of GluA2 subunits whereas NMDA receptors cause a calcium ion influx as well as a magnesium efflux [110]. Activation of glutamate receptors can result in either LTP or long-term depression (LTD). The former occurs when both pre- and post-synaptic receptors are activated but long-term depression occurs with repeated pre-synaptic activity without post-synaptic activity [110]. Memory consolidation is usually associated with LTP while amnesia is associated with LTD.

Normal stimulation of presynaptic neurons results in the release of neurotransmitters and neurotrophins into the synaptic cleft [109,111]. Tetanic stimulation (50 – 100Hz/second) induces

LTP either via tyrosine kinase B (TrkB) signalling or glutamate-dependent pathways [111,112]. In the early phase of LTP, protein subunits in the cytoplasm are assembled and chaperoned to their place of need but late LTP is reached when proteins are manufactured *de novo* by a process that is mainly mediated by the phosphorylation of cyclic-AMP responsive binding element (CREB) which is a transcription factor for most proteins required in memory consolidation [109,112]. CREB is dependent on CaMKII to activate it in the glutamate-dependent pathway [113] but protein kinase C (PKC) plays a role in the activation of CREB in the BDNF-dependent pathway [114]. Calcium is a potent secondary messenger and plays a very important role in the events that lead to the activation of CREB and consolidation of memory either by activating CaMKII or PKC [113,114]. Because in both LTP and LTD there is influx of calcium ions, the cells needs to know which process should occur. CaMK II requires higher numbers of calcium ions, therefore calcium is also important in determining whether LTP or LTD will occur [110].

1.3.2.1 Brain-derived neurotrophic factor

Neurotrophins play a critical role in normal central nervous system functions from axonal growth to synaptogenesis to cell differentiation and survival. BDNF is the most characterised of the neurotrophins and is well regulated at multiple phases of production (reviewed in Greenberg et al, 2009) [115]. BDNF is encoded by the *Bdnf* gene found on chromosome 11. The gene contains multiple reading frames and at least 18 identical transcripts can be produced, which all encode identical protein products, preproBDNF. The distribution of BDNF in the neuron is determined by mRNA. mRNA with long 3'tails were found to be clustered in the cell body while the short 3'end mRNA were trafficked to the dendrites. The differentiation of the mRNA ensures that translation of the protein can occur closest to its site of function [115].

BDNF exists in two forms: pro-BDNF and mature BDNF or mBDNF that is the functional form. Once BDNF has been translated into the inactive proBDNF, it gets cleaved in the Golgi apparatus into the mature form, which then becomes active in cellular development as well as LTP [115].

BDNF is involved in a number of neuronal processes and it has been implicated in many neuropathologies [116,117]. Structural functions of BDNF include normal and therapeutic axon regeneration [74,118]. BDNF is also required in many forms of learning and memory [119,120]. When levels of this neurotrophin are decreased, LTD as a result of decreased neurogenesis may occur [116].

BDNF released into the synaptic cleft binds to TrkB receptors on the membrane of the pre- or postsynaptic cells [121]. TrkB receptor activation will stimulate a number of other messenger pathways such as the mitogen-activated protein kinase/ extracellular signal-regulated protein kinase (MAPK/ERK) pathway to bring about protein synthesis; and the protein kinase B (AKT) pathway that suppresses apoptosis [112,122]. Other pathways that are activated by BDNF-TrkB complex are phosphatidylinositol-3-OH kinase, and phospholipase Cγ cascades. Together, activation of these signalling pathways will favour cell proliferation and survival [118], processes which are required for plasticity to form the biological basis of learning and memory [120,122–124].

BDNF-mediated synaptogenesis can occur via the already mentioned pathways or by glutamate-dependent pathways. After eyeblink conditioning in turtles, immunohistochemical

analysis of tissue showed high localisation of synaptophysin and AMPA receptor subunits in conditioned groups but not the control group [122]. Exercise increases BDNF however there is much controversy about the effect of MS on neurotrophin levels [21,79,117]. As discussed earlier, MS and exercise increase circulating glucocorticoids, which in turn regulate BDNF. BDNF mRNA expression in the CA2 region is similarly affected by MS and corticosterone stimulation individually, however, corticosterone administration exaggerated the effect of MS on BDNF expression in the hippocampus[125]. This provides further evidence for differential control of BDNF due to either MS or increased corticosterone. Similarly, adrenalectomised rats treated with corticosterone show an initial increase in BDNF, which quickly decreases after 2 hours and normalises again after 24 hours [126]. Upon further investigation, exons IV and V transcripts were found to be decreased in corticosterone-treated adrenolectomised animals, showing that corticosterone-mediated changes in BDNF expression were controlled by exon IV and V in maternally deprived rats [126]

1.3.2.2 Extracellular signal-regulated kinase

ERK forms part of the MAPK family and is involved in many intracellular processes. Some of these processes result in changes in the cytoskeleton, growth factor and protein synthesis and subsequently affect learning, synaptogenesis and the general development of cells. Certainly, many cell growth and survival pathways and tissue proliferation processes require MAPK/ERK signalling [127,128]. Three activators of ERK have been identified and they act under different conditions: (i) stress-induced MAPK kinase1 or MEK1, (ii) c-Mos (a proto-oncogene protein kinase) in the reproductive system and, (iii) protooncogene TPL2 (tumour progression locus 2 protein) (reviewed in Shaul and Seger, 2007) [129]. Since the scope of this

project is focussed on developmental stress, only the stress-induced cascade will be discussed.

ERK is activated by phospho-mitogen-activated protein kinase kinase (pMEK) and deactivated by protein phosphatase-2 (PP2A) and mitogen-activated protein kinase phosphatase-1 (MKP-1) [129,130]. Phosphorylated ERK (pERK) is translocated into the nucleus where it activates a number of transcription factors [131]. The most researched of these transcription factors is cyclic-AMP responsive binding element or CREB [132]. CREB's role has been linked to the consolidation of memory [109].

In the hippocampus, pERK activity is essential in some forms of learning and memory; particularly fear conditioning although, electrophysiological studies show that it is also essential for LTP [133,134]. Since pERK has been associated with stress, its role in memory and learning has been highly focussed on learning in emotional contexts such as odour aversion and passive avoidance tasks [135,136]. Phosphorylation of ERK increased following a noxious stimulus allowing for rapid consolidation and stabilisation of the experience [136]. High levels of glucorcotocoids, which are associated with stress, may also be involved in the activation ERK and the deactivation of pERK. Acute treatment of mast cells with glucocorticoids inhibited the activation of ERK for up to 24 hours due to increased MKP-1 levels[137]. MKP-1 is a known regulator of pERK.

The effect of exercise on pERK levels has not been well investigated. One study showed that 7 days of exercise caused a decrease in pERK levels in the hippocampus [138]. Preliminary studies done in this laboratory showed that MS abolishes the increase in pERK which was

caused by exercise [139]. This suggests that acute (7 days) and chronic (3 weeks) exercise affect pERK differentially. In this current study, the temporal extent to which exercise increases ERK activation was investigated by examining pERK levels in rats immediately, 10 days, and 25 days after exercise. The latter groups were also trained in the MWM, which allowed us to investigate the influence of pERK levels on memory and learning at these two time points.

1.3.2.3 MKP-1

MKP-1 is a member of the dual-specificity phosphatase group of phosphatases [140]. As their name suggests, these phosphatases dephosphorylate kinases at the tyrosine and threonine residues in order to inactivate the kinase protein [128]. MKP-1 is activated by glucocorticoids and cytokines in response to stress [137,141]. Substrates of MKP-1 that have been identified include pERK, pMEK and pJNK [138,142]. Inducing pERK up-regulation with cytokines resulted in the up-regulation of MKP-1 within 30 minutes, by 60 minutes, MKP-1 was at its peak and pERK levels had started to decrease [141]. Glucocorticoid stimulation of MKP-1 also heightened MKP-1 expression after 1 hour and normalisation of levels occurred 24 hours later [137]. Moreover, glucocorticoids decreased the rate at which MKP-1 was degraded via proteosome-mediated mechanisms which are slow and correspond with the sluggish desphosphorylation of pERK in the presence of glucocorticoids [137].

The role of MKP-1 has been well researched in cancer studies [143,144] and depression [142]. In both pathologies, MKP-1 is up-regulated to quench aberrant MAPK signalling in an attempt

to restore normal cellular function. Cytokines also stimulate MKP-1 activity but in a manner that differs from the glucocorticoid-mediated pathway[137].

One of the roles of pERK is to consolidate fear conditioning yet, the function of MKP-1, a regulator of pERK, has not yet been characterised in learning and memory. Logically, it could be speculated that higher levels of MKP-1 may have a negative impact on cognitive function as it is a negative regulator of pERK. This is because higher levels of MKP-1 have been correlated to higher levels of circulating glucocorticoids and has been implicated in the presentation of depression-like features [137,142]. One of the common features that accompany depression is poorer cognitive function. Previous research in our laboratory failed to find a correlation between MKP-1 levels and cognitive function [139]. It may be that MKP-1 has a homeostatic role in that it regulates pERK and returns increased pERK to basal levels without having an impact on cognitive function. There is also evidence that suggests that MKP-1 is highly inducible by BDNF and has a role in axonal growth but not when MKP-1 levels are pathologically high [145].

Very few studies have investigated the effect of exercise on hippocampal MKP-1 levels. Acute exercise increased MKP-1 levels in the hippocampus corresponding with the decreased pERK levels [138]. This is consistent with earlier studies that determined the regulatory nature of MKP-1 effects on the MAPK pathway. However, it does not agree with studies that show that increased glucocorticoids have a slower activation effect on MKP-1 and ERK [137]. This suggests that acute exercise employs a different mechanism to regulate the MAPK pathway. In our previous study, MS and exercise tended to increase MKP-1 levels but the findings were not significan[139]. It was hypothesized that by examining the levels of MKP-1 under

different circumstances, in this project would further elucidate the function of this protein in developmental stress and learning and memory.

1.4 Aims

At present, more and more evidence suggests that developmental stress has detrimental effects on learning and memory [29,95,125,146]. However, there are data that show the contrary to be true as well [16,139]. In fact, in our laboratory, early studies showed that there was no effect of MS on memory and learning while exercise improved it in juvenile rats [72]. In a follow-up study, MS seemed to enhance object location memory in adult rats while exercise had no effect [139]. Both these studies suggest that the age of the rats tested determined the efficacy of exercise in promoting learning in MS rats. In both experiments, the rats had been subjected to a series of memory tests. Therefore it could not be said for certain whether the changes in protein levels were a result of the MS and exercise interaction or whether it was dependent on the different memory tests [147]. Therefore, in this next experiment the effect of MS and exercise were investigated in only one learning and memory paradigm, the MWM which tests spatial learning and memory [98]. In a previous study in our laboratory, similar effects of MS and exercise were observed in both dorsal and ventral hippocampus [139]. It has now also been demonstrated that protein lateralisation, which relates to hippocampal function, exists [148,149], therefore, protein analysis will be done in left and right dorsal hippocampi separately to determine whether the effects of MS and exercise are lateralised in the dorsal hippocampus. Male rats were chosen especially for this study because others have shown that males (human) have a higher reactivity to stress than females [15].

1. Therefore this study includes the following aims: To determine the effect of MS and exercise on hippocampal protein levels including as ERK, pERK, BDNF and MKP-1 in adult rats post-exercise.
2. To determine the effect of MS and or exercise on memory and learning in a spatial learning task
    i. 1 day post exercise
    ii. 15 days post exercise
3. To determine the effect of MWM training on hippocampal proteins in MS rats post-exercise. It is believed that spatial encoding occurs in the dorsal hippocampus, hence the selection of that particular brain region [100,150].

It is hypothesised that MS would down-regulate pERK via increased MKP-1 expression which will subsequently result in decreased BDNF. Exercise will result in increased BDNF turnover, thus increasing pERK signalling, perhaps to an extent to which increased MKP-1 cannot regulate pERK levels. The mechanism by which we hypothesised this would happen is detailed in Figure 1.3.

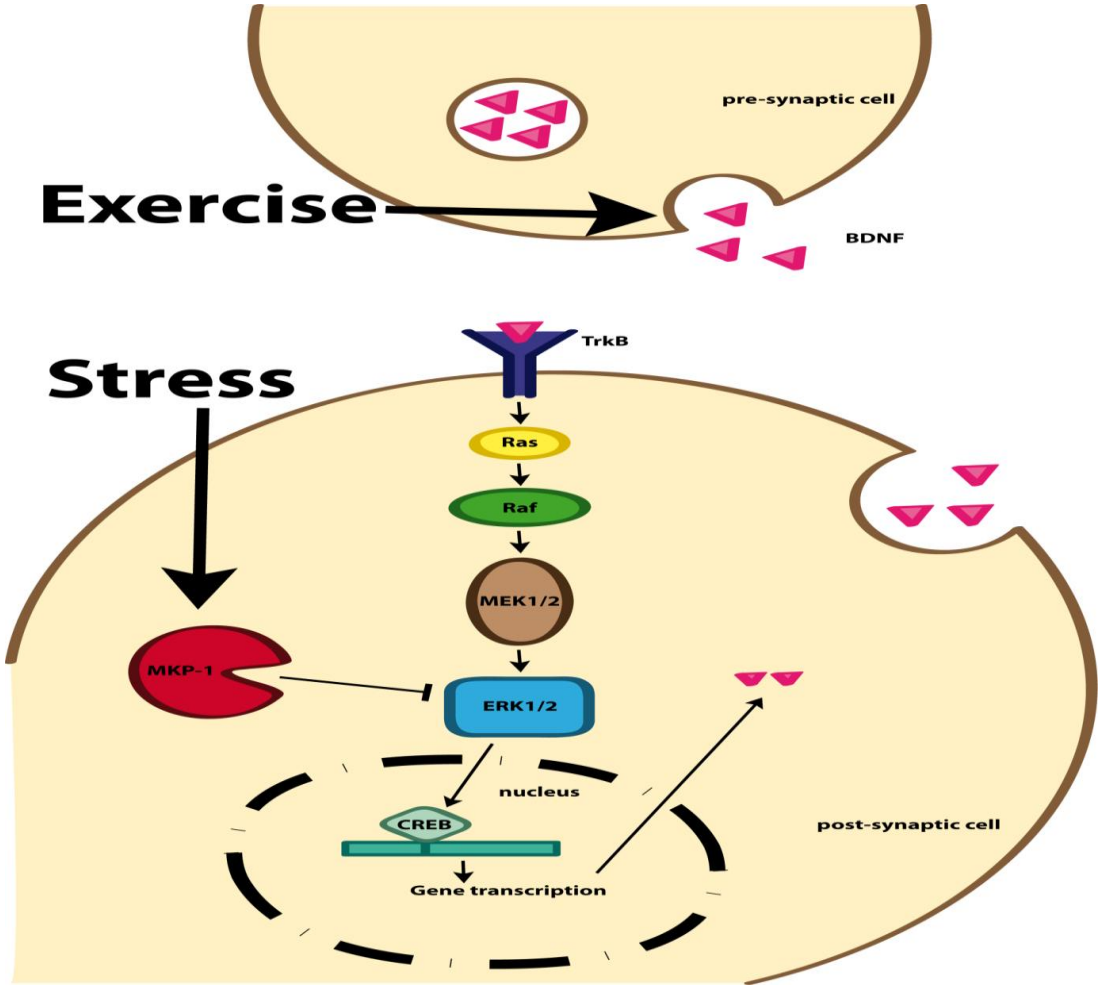

Figure 1.3. Hypothesized effect of MS and exercise on hippocampal BDNF/ERK signalling.

Chapter 2: Effect of maternal separation on anxiety-like behaviour, exercise, and hippocampal protein levels

2.1. Introduction

MS effects on stress reactivity can be determined both behaviourally and by using biochemical analyses of tissue of the affected animals [21]. Tests such as the OFT and EPM allow for detection of changes in behavioural responses to stress while serum levels of HPA axis-associated hormones provide a broader view of the underlying physiological response [50].

Previously, it was reported that MS caused anxiety-like behaviours in 28-day old pups when tested in the open field [139] but pups were not tested in the elevated plus maze. Some researchers have also used the open field not to determine anxiety-like effects but to observe changes in locomotor activity [151].

In the current study, 28-day old pups were exposed to both the elevated plus maze and open field to assess whether anxiety-like behaviours could be detected using both tests and to determine whether MS may have altered locomotor activity within these two apparatuses. Additionally, voluntary exercise activity was investigated to determine whether MS would alter it.

MS has been found to stunt hippocampal development and lead to lower levels of plasticity-related proteins [32]. Previously, in our laboratory, the levels of certain proteins involved in plasticity were measured in the hippocampus after MS and exercise. Incidentally, MS had no effect on hippocampal protein levels but exercise increased synaptophysin, CaMKII and pERK [78,139]. In the earlier study by Hescham et al (2009), the increased plasticity markers were consistent with improved memory of the exercised groups while in the latter study by

Makena et al (2012), increased markers of plasticity failed to correlate with efficient learning and memory. In both instances, proteins were analysed in rats that had undergone a battery of memory tests on consecutive days. In this experiment, the confounding effects of serial memory testing were eliminated by assigning a cohort of rats to be used in protein assays at the end of the exercise regimen. This cohort will be referred to as the 'Naïve group' as they were not tested in the Morris Water Maze. The aim of this experiment was to determine the post-exercise protein levels in MS rats.

2.2 Methods

2.2.1 Animals

Adult Sprague-Dawley (SD) rats were obtained from the University of Cape Town Animal Unit and were housed under standard laboratory conditions in a 12h: 12h light/dark cycle with lights on at 06:00. The temperature was maintained at 21°C ±2°C and humidity at ~66%. The rats had free access to water and standard rat chow. All protocols were authorised by the Faculty of Health Sciences Animal Ethics Committee of the University of Cape Town (009/007; 011/028). The study was carried out according to international guidelines (South African National Standard: The Care and Use of Rats for Scientific Purposes, 2008).

A total of 43 rats were used to assess the effect of MS and exercise on hippocampal protein levels immediately after the voluntary exercise regimen. Rats were randomly chosen after exercise to be a part of the Naive cohort while the remainder were used for the MWM experiments.

2.2.2 Maternal separation

Male and female rats were paired for mating and the studs were removed from the cage 2 weeks after the pairing. The date of birth was designated postnatal day zero or P0. On P2, litters were culled to 8 pups, with preference for male pups. Females in the litters were kept at a maximum of 4. Whole litters were assigned to the MS group or the non-separated group. The MS litters were separated from the dams for three hours each day between 08:30 and 13:00 from P2-P14 [152]. The pups were taken to a different room and placed under infrared lamps (temperature= 32 ±1°C) to prevent hypothermia. The MS period of 180 minutes per day in the first 2 weeks of life was chosen due to its ability to reliably induce changes in neurochemistry and behaviour [13,19]. Non-separated pups were left undisturbed with the dam [17]. From P15 the pups and dams were left undisturbed except for regular cleaning of cages [153] until weaning at P21.

2.2.3 Behavioural measures of anxiety

Rats were taken to the testing room with a lux of 48, at least 1 hour prior to the commencement of tests to allow them to habituate to the testing room. Rats were first placed into the EPM for 5 minutes and then transferred to the OFT for a further 5 minutes. Testing took place between 8h00 and 11h00 at P28. Video footage was analysed using Ethovision software, Noldus (Netherlands).

2.2.3.1 Elevated plus maze

The EPM was used to assess the level of anxiety-like behaviour in the rats [154]. The apparatus consisted of a black plus shaped maze raised 50cm above the floor (Figure 2.2). The maze

was divided into open and closed arms (measuring 10 cm x 45 cm) with a central 10 cm x 10 cm area. The walls of the closed arms were 40 cm high. Rats were placed in the centre square facing an open arm and allowed to explore the maze for a 5-minute period. The apparatus was cleaned with soap and sprayed with 70% ethanol prior to the start of each trial. Parameters that were measured were time spent in the open and closed arms as well as frequency of transitions between the open and closed arms. Rats were classified as exhibiting anxiety-like behaviours when they spent significantly more time in the closed arms compared to others [154].

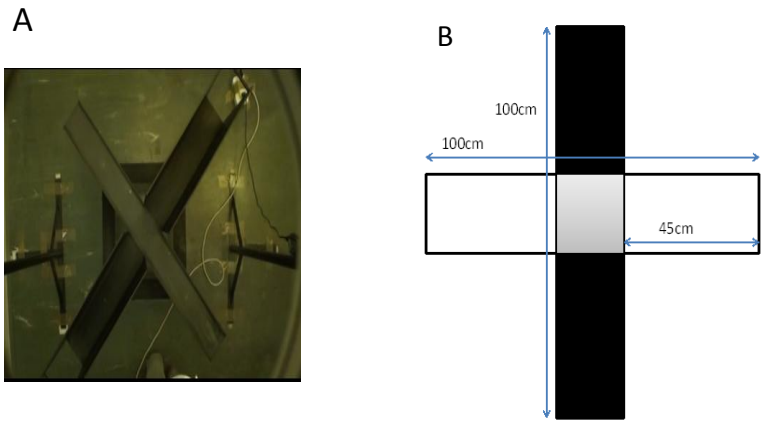

Figure 2.2. Elevated plus maze apparatus. (A) The maze as seen through the lens of the camera. (B) Schematic representation indicating the open and closed arms and dimensions thereof.

2.2.3.2 Open field test

The open field arena comprised a wooden box (100 cm x 100 cm x 60 cm) with a black interior. An imaginary line (marked only at the corners) was used to demarcate the inner and outer zones on the floor of the arena. The inner zone measured 70 cm x 70 cm and was 15 cm from the walls of the box (Figure 2.3). Each rat was placed in the arena facing the right-hand corner and allowed to explore the arena freely for a 5-minute period. The apparatus was cleaned with soap and sprayed with 70% ethanol prior to the start of each trial. The parameters measured were mean velocity, total distance travelled, time spent in the outer zone, duration in the inner zone, and frequency of transitions to the inner and outer zones. Rats that had spent significantly less time in the inner zone than others were considered to be displaying anxiety-like behaviours [50].

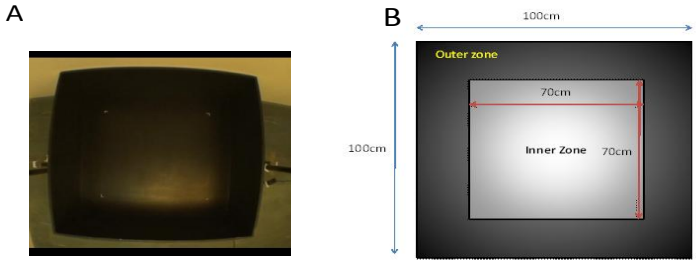

Figure 2.3. Open field apparatus. (A) The open field as seen through the camera lens. The inner and outer zones were demarcated with an imaginary line using the white marks at the corners of the boundary, which was clearly marked in the schematic representation (B).

2.2.4 Exercise

At P50, rats were transferred to the exercise room where they were housed singly in running-wheel cages for the duration of the 3-week voluntary exercise regimen. The cages were composed of stainless steel with attached running wheels, which were in turn connected to a computer that recorded the number of revolutions. The circumference of the running-wheel was 1m therefore one wheel revolution was equivalent to 1 meter. The level of activity was measured by the number of revolutions recorded in a 24-hour period and for this reason, voluntary exercise activity is reported as distance covered. Mechanical clocks connected to the wheels were used to check that rats were running but were not used for analysis. The wheels of the cages of non-exercised rats were immobilised to prevent exercise and to maintain similar environmental conditions. At P70, the rats were removed from the running-wheel cages and housed communally in groups of 3-5 rats per cage.

2.2.5 Tissue collection and preparation

Following the end of the voluntary exercise period, rats were removed from the running-wheel cages and housed communally in standard cages. One hour after removal from running-wheel cages, rats were killed and brain tissue harvested for protein analysis.

Rats were deeply anaesthetised with halothane and decapitated, after which, the head was plunged into liquid nitrogen for 5-6 seconds before removal of the brain from the skull and rapid dissection of specific regions. This method was reported to reduce phospho-protein degradation in harvested tissue samples [155]. The left and right dorsal hippocampus were dissected out on ice, snap-frozen in liquid nitrogen and stored at -80°C.

Previously it was shown that maternal separation and exercise affected protein levels in the dorsal and ventral hippocampi similarly [139]. In this study, it was to be determined whether hemispheric differences exist in the dorsal hippocampus due to maternal separation and/or exercise. Brain tissue samples were randomly pooled from two rats in the same group to yield one sample for biochemical analysis (n=5-6). Tissue was sonicated in lysis buffer (137 mM sodium chloride (NaCl), 20 mM Tris-hydrochloride, 1% nonyl phenoxypolyethoxylethanol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/mL aprotinin, 1 µg/mL leupeptin, 0.5 mM sodium vanadate) for 10 seconds, mixed on a vortex stirrer and centrifuged at 12 000g for 15 minutes at 4°C. The supernatant was collected and boiled for 5 minutes. Protein concentration in each sample was determined by bicinconinic acid (BCA) assay (Pierce Thermo Scientific) according to manufacturer's instructions. Five bovine serum albumin (BSA) standards ranging between 0 and 200 µg/mL were prepared in lysis buffer. 150 µL of BSA standards or protein samples were pipetted in duplicate into a 96-well microplate. 150 µL of working reagent was added to each well and the plate was gently shaken, covered and incubated for 30 minutes at 37°C. An absorbance reading was taken at 562 nm on a plate reader, standard curve was computed, and protein concentrations determined using Excel (Microsoft, USA). Tissue was pooled from two rats to make a single sample for protein analysis using ELISA for BDNF and Western Blot for pERK, ERK, MKP-1 and p38.

2.2.6 Western Blot analysis of tissue levels

Proteins were separated by SDS-PAGE for 1.5 hours at 150V and transferred to a nitrocellulose membrane at 100 V for 1 hour. Membranes were blocked with 5% bovine serum albumin in Tris-buffered saline with tween (TBS-T: 20 mM Tris, 150 mM NaCl, 0.05% Tween-20). The primary antibodies used were rabbit-anti-pERK (1:2000), rabbit-anti-ERK (1:2000, Cell Signaling, USA), rabbit-anti-MKP-1 (1:500, Santa-Cruz) and rabbit-anti-

p38 (1:5000, Abcam). The secondary antibody was goat-anti-rabbit (1:2000, Santa Cruz). Proteins were detected by chemiluminescence (Pierce Thermo Scientific) on X-ray film (AGFA). The West Pico chemiluminescence variant was used to detect ERK, MKP-1 and p38 while West Dura was used to detect pERK as its increased sensitivity was required to detect very small amounts of phospho- protein. A result for MKP-1 in the right dorsal hippocampus was not obtained due to very low signal response.

2.2.7 Enzyme-linked immunosorbent assay

BDNF quantification was achieved by enzyme-linked immunosorbent assay according to manufacturer's instructions (Promega, US). The tissue was not acid-treated; therefore, only mature soluble BDNF was quantified. Left and right hippocampi were analysed on different 96-well microplates. The microplates were coated with anti-BDNF monoclonal antibody in carbonate coating buffer at 4°C overnight. After a gentle wash, the plates were blocked with 1X Block and Sample buffer for an hour at room temperature. The standard curve for BDNF was prepared by serially diluting BDNF in 1X Block and Sample buffer so that concentrations ranged from 0 pg/mL to 500 pg/mL. BDNF standards and samples were added to the microplates and incubated for 2 hours at room temperature with shaking. Standards and samples were then incubated with Anti-human BDNF polyclononal antibody (1:500) for 2 hours at room temperature with shaking. Then, anti-Ig-Y horseradish peroxidise conjugate (1:200) was added to standards and samples and incubated for 1 hour at room temperature with shaking. TMB (3,3',5,5'-tetramethylbenzidine) One Solution was then added to the plates for 10 minutes with shaking and the reaction was stopped with 1N HCl. Absorbance was recorded at 450 nm on a plate reader within 30 minutes of having stopped the reaction. In between each step, the microplates were washed gently with tris-buffered saline with tween.

2.2.8 Statistical analysis

Statistica 10 software was used to analyse data (StaSoft, USA). All data were checked for normality using the Shapiro-Wilk test. Where data were found to be normally distributed, parametric tests were employed (ANOVA, student's t-test) otherwise, non-parametric tests (MannWhitney U test) were employed. Significance was set at $p<0.05$ and trends at $p<0.075$.

2.3 Results

2.3.1 Elevated plus maze

A Shapiro-Wilk test revealed that all EPM data were non-parametrically distributed. Therefore, a Mann-Whitney U test was used to detect any MS-induced differences in behaviour. Maternal separation did not produce any significant differences in closed arm duration, open arm duration, number of entries into the closed arms or the total distance covered (Table 2.1).

2.3.2 Open Field Test

The total distance covered, time spent in the inner zone, time spent in the outer zone and zone transitions were measured in the OFT. All parameters were found to be not normally distributed using the Shapiro-Wilk's test; therefore, the Mann-Whitney U test was used to determine significant differences in behaviour between MS and non-separated groups (NS). No significant differences were found between MS and NS groups in any of the measurements (Table 2.1).

2.3.3 Running wheel activity

The Shapiro-Wilk test for normality showed the exercise data to be non-parametric. A Mann-Whitney U test revealed that there was no significant difference in the distance run by MS rats and non-separated rats. The detail of daily running activity is included in Figure 2.4.

2.3.4 Western Blot analysis of protein levels

For the protein data, which were normally distributed, outliers were identified and discarded from the data set when they were found to be greater or less than two standard deviations from the mean.

2.3.4.1 Phospho-ERK

In the left dorsal hippocampus, two-way ANOVA revealed a Separation*Exercise interaction ($F_{(1,15)}$=4.499, $p<0.05$) in pERK levels. Duncan's post-hoc test showed that exercise increased pERK by almost 43% in non-separated rats ($p<0.05$, non-separated non-runners compared to non-separated runners) (Figure 2.5A).

In the right dorsal hippocampus, there were no effects of separation ($F_{(1, 15)}$=0.278, $p>0.05$) and no effect of exercise ($F_{(1,15)}$=0.323, $p>0.05$) (Figure 2.5B).

2.3.4.2 MKP-1

In the left dorsal hippocampus, two way ANOVA revealed that there was no main effect of either separation or exercise on the level of MKP-1 in the left dorsal hippocampus. However,

Duncan's post-hoc test revealed that MKP-1 was markedly increased in the non-separated runners group compared to non-separated non-runners ($p<0.05$) (Figure 2.6.). Data for the right dorsal hippocampus could not be attained due to very poor signal response.

2.3.5 BDNF ELISA

The distribution of the complete data set for BDNF was found to be non-parametric therefore a hemispheric effect on BDNF was revealed by the Mann-Whitney u test ($p= 0.029$). However, the data sets for the individual hemispheres were parametric and therefore ANOVA was employed. In the left dorsal hippocampus, two-way ANOVA revealed a tendency towards a separation*exercise interaction ($F_{(1,16)}=4.17, p=0.054$). Duncan's post-hoc test revealed that MS decreased BDNF levels ($p=0.032$, MS non-runners compared to NS non-runners) while exercise normalised BDNF levels in MS rats ($p=0.030$, MS non-runners compared to MS runners) (Figure 2.7A).

In the right dorsal hippocampus, there was no effects of separation and exercise but a tendency towards a separation*exercise effect ($F(1,15)=3.965$, $p=0.0610$) was revealed by two-way ANOVA.

Table 2.1. Maternal separation did not affect motor function or induce anxiety-like behaviours in 28-day old rats

		Non-separated	Maternally separated
EPM	Duration in open arms (s)	30.6 ±19.4	32.5 ±17.7
	Duration in closed arms (s)	246.5 ±26.7	252.4 ± 26.7
	Entries into closed arms	5 ± 2.5	5.4± 2.0
	Total distance (cm)	1379.3 ± 334.8	1471.3 ± 266.2
OF	Duration in outer zone (s)	270.3 ±18.7	269.9 ±20.6
	Duration in inner zone (s)	13.0 ±11.7	10.0 ±9.8
	Entries into inner zone	5.8 ±4.45	5.2 ±4.0
	Total distance (cm)	3059.4 ±834.1	2976.9 ±883.6

EPM = elevated plus maze; OF= open field. Data presented as mean ± standard deviation. n =34

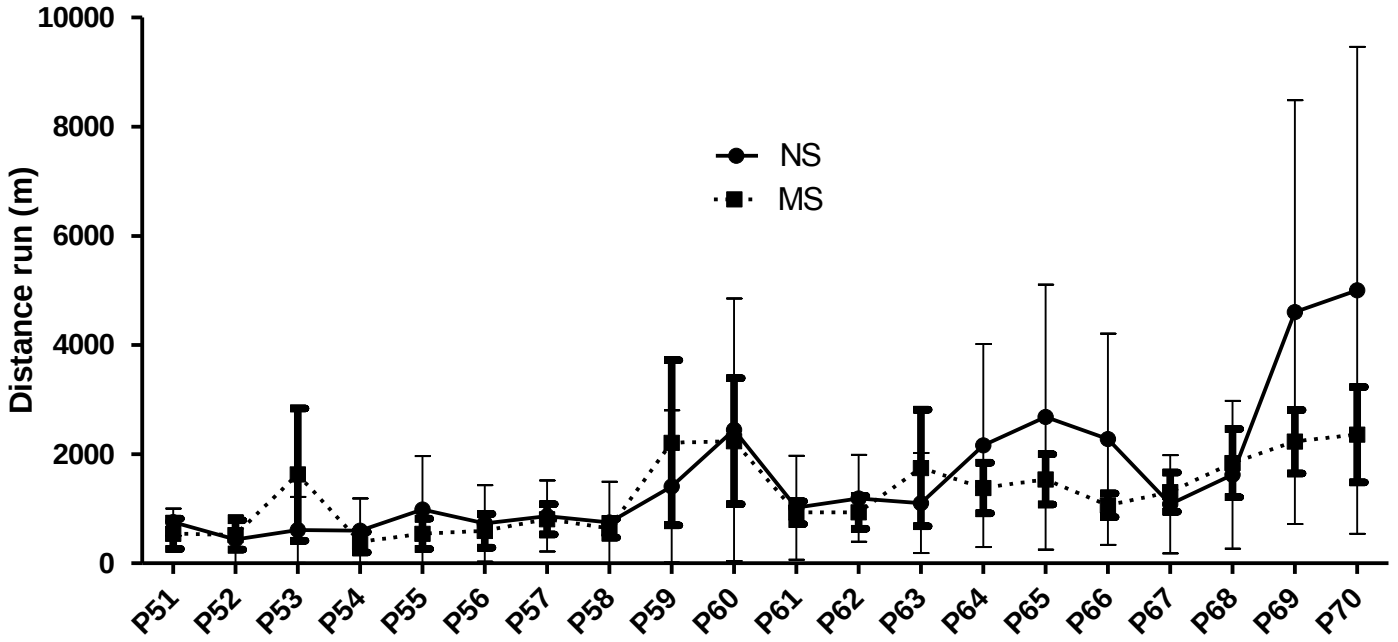

Figure 2.4. Running distance covered from postnatal day 50 (P50) to P70. Distance run was calculated from the number of wheel revolutions clocked by the computer (one revolution =1m). Readings were taken at 17:00 each day (an hour before the dark cycle). Data is presented as mean ±SEM. (NS= non-separated; MS= maternally separated), n=34-36

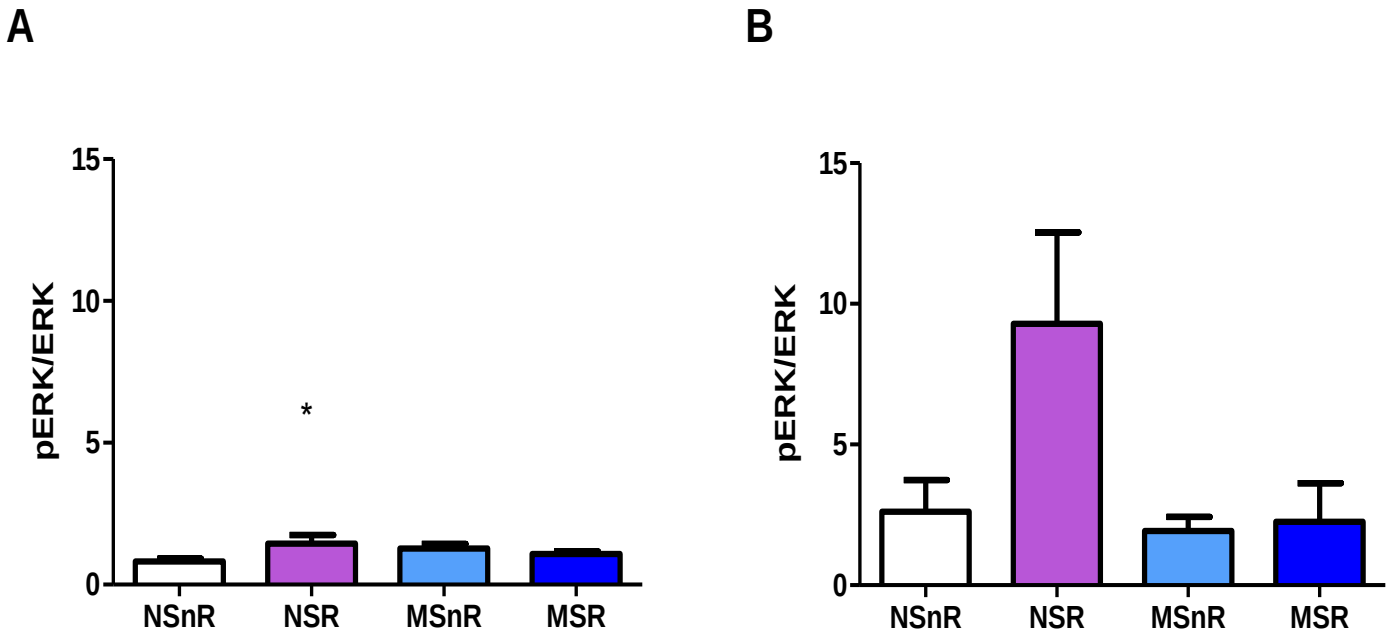

Figure 2.5. Relative densitometry of pERK in the left (A) and right (B) dorsal hippocampus. *vs. NSnR ($p<0.05$). Data are presented as mean ±SEM, (NSnR= non-separated non-runners; NSR= non-separated runners; MSnR= maternally separated non-runners; MSR= maternally separated runners), n=6. Reference protein was p38.

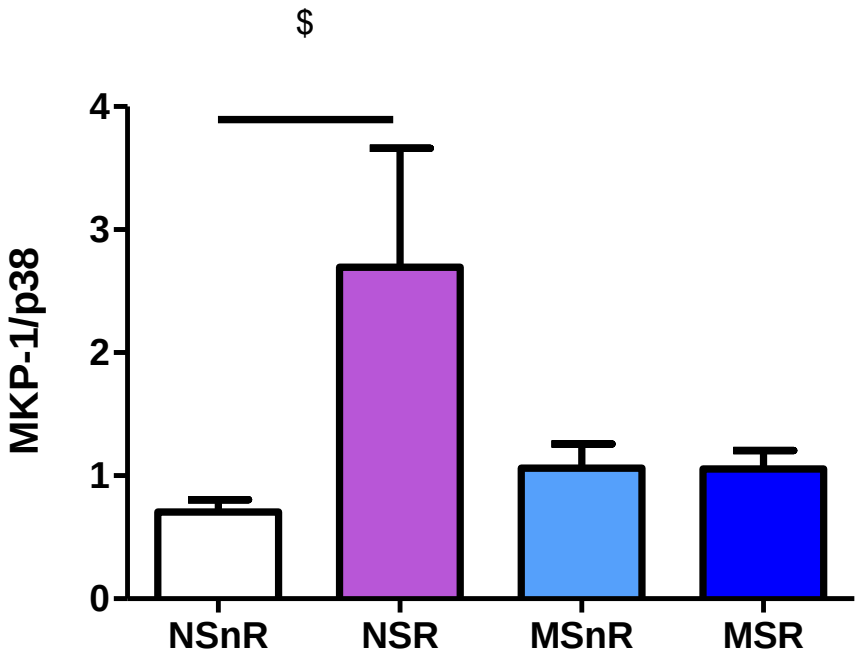

Figure 2.6. Relative MKP-1 densitometry in the left dorsal hippocampus. Exercise increased MKP-1 in non-separated rats. $ NSnR vs. NSR ($p<0.05$). Results for the right dorsal hippocampus could not be obtained. Data are presented as mean ±SEM, (NSnR= non-separated non-runners; NSR= non-separated runners; MSnR= maternally separated non-runners; MSR= maternally separated runners; n=6)

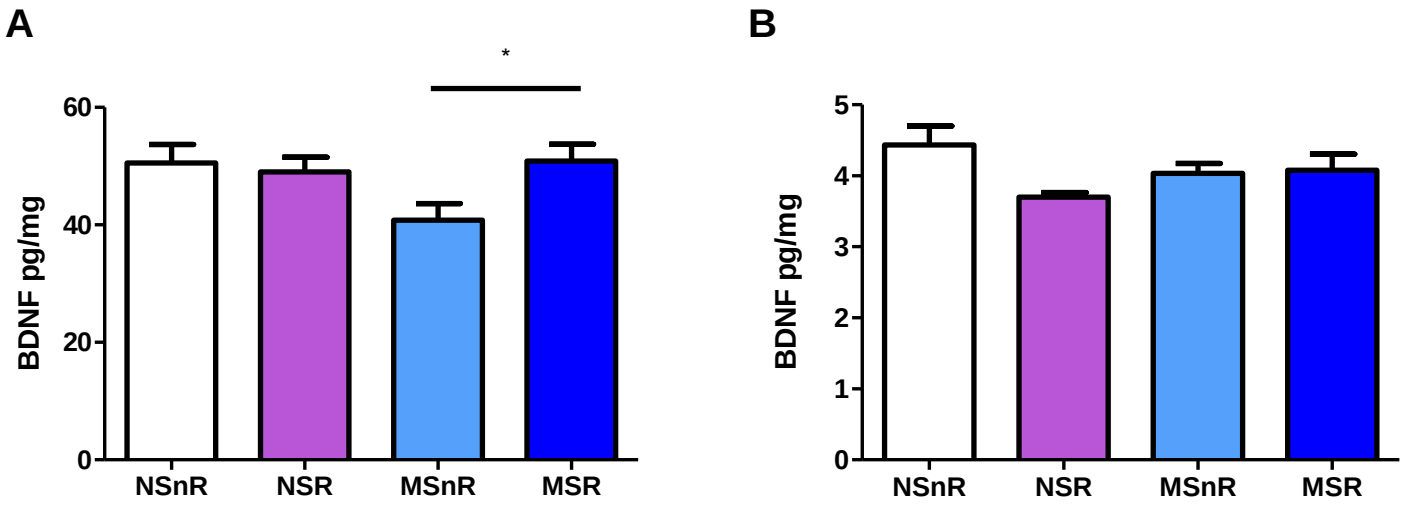

Figure 2.7. BDNF levels in left and right dorsal hippocampus. (A) Left dorsal hippocampus, (B) right dorsal hippocampus. Data are mean ±SEM, (NSnR= non-separated non-runners; NSR= non-separated runners; MSnR= maternally separated non-runners; MSR= maternally separated runners), $p<0.05$, n=6

2.4 Discussion

In the current study, there were no differences between MS and NS rats in their behaviour in the OFT and EPM, suggesting that MS did not induce anxiety-like behaviours. This observation did not agree with previous findings where it was reported that MS produced anxiety-like behaviours in 28-day-old and 75-day-old SD rats [139]. However, the older rats showed behavioural deficits due to MS when tested in the EPM but not the OFT [139]. The discrepancy in these results may be due to deviation in the behavioural testing protocol. Previously, rats were allowed a period of 1 hour between tests [139]. However, in the present study, rats were subjected to tests without a break in between. It is therefore possible that the lack of differences in behaviour were due to a lack of sufficient rest periods between tests. Despite these obvious differences in testing protocols in the above-mentioned studies, several studies have similarly failed to find MS-induced behavioural differences in the OFT and EPM in adult Wistar rats [23,94,156].

In the first 9 days of exercise, most rats showed an initial low level of running, amounting to less than 2 km per day. This was consistent with habituation to the running-wheel cage environment which had been noted in previous experiments [72,157]. At P59-60, a slight increase in distance travelled by both groups was observed and two more sharp increases in running distance were observed at P65 and P70 in the non-separated group only. However, at no point did these groups differ in running activity. Therefore, it appears that MS had no effect on the total distance run by rats for the duration of the voluntary exercise regimen. This finding agrees with other published reports from this and other laboratories [72,139,158].

Similar levels of pERK were observed in both left and right dorsal hippocampus. High variability in pERK levels was evident in the right dorsal hippocampus of the NS rats and

may be the reason for lack of differences in protein levels between the NSnR and NSR groups. In the left dorsal hippocampus, exercise increased pERK in non-separated rats but failed to raise the levels in MS rats. This recent finding was consistent with previously published work from this laboratory [139].

MKP-1 data was only obtained for the left dorsal hippocampus and not the right as with other proteins. This was due to very poor detection signal from the protein even though a well-characterised protocol had been employed. A few combinations of buffers and blocking agents were used to try to increase the signal without success. Even a more sensitive chemiluminescent solution (West Dura, Pierce Thermo Scientific) was used for detection with no subsequent improvement in signal. In the left dorsal hippocampus exercise increased MKP-1 in non-separated rats relative to sedentary non-separated rats. This observation was in agreement with work by Hu et al (2009) who showed that a 7-day exercise program increases MKP-1 in the hippocampus [138].

Taking into consideration the inverse relationship between MKP-1 and pERK, it would be expected that when either protein is increased the other will decrease [140,142]. However, it was found that both proteins were increased in the same group. This current observation contradicted earlier reports that these two proteins were inversely proportional to each other [138,142]. However, earlier work in the laboratory did show that after 3 weeks of voluntary exercise, pERK and MKP-1 had increased in the same group [139].

BDNF levels differed according to the hemisphere. BDNF was higher in the left dorsal hippocampus compared to the right hippocampus. In the left dorsal hippocampus, MS decreased BDNF and exercise normalised BDNF levels in MS rats. Exercise did not have an effect in non-separated rats. In the right dorsal hippocampus, exercise reduced BDNF in non-

separated rats and had no effect in MS rats. These data provide evidence for laterality-based effects of both MS and exercise on neurotrophin levels. Furthermore, differential interactions between MS and exercise are asymmetrical. BDNF can also cross the blood brain barrier and enhance neurotransmitter release during exercise [159]. This was also in agreement with a clinical study that found that the intensity of exercise was correlated to increased levels of BDNF in the left and right anterior hippocampus but not in the left and right posterior hippocampus [71]. The posterior hippocampus in humans corresponds anatomically with the dorsal hippocampus of the rat.

In the left dorsal hippocampi of naïve rats, no change in BDNF was found in non-separated rats regardless of exercise. However, running did ameliorate the decrease in BDNF caused by MS. The lack of differences in BDNF levels between the exercised and non-exercised rats from the non-separated groups was not expected as previous studies have shown an exercise-induced increase in BDNF in normal rats [79,160]. Griesbach et al (2004) reported that exercise increased hippocampal BDNF in sham-lesioned rats and restored BDNF levels to normal in rats that had experienced brain injury by fluid-percussion at a location corresponding to the dorsal hippocampus [74]. However, in another study, exercise failed to increase the levels of BDNF mRNA in the medial CA3 region of the rat hippocampus [161].

In Chapter 1, it was hypothesised that MS would decrease pERK levels due to increased MKP-1 and that BDNF, which is synthesised in response to increased ERK activation would also be decreased. As per previous work, pERK was increased by exercise but not affected by MS. However, BDNF was decreased by MS only in the left dorsal hippocampus while exercise had the same effect in the right hippocampus. Chapter 3 will investigate the short-term effects of exercise on spatial learning in the MWM. It would be interesting to see

whether hippocampal protein levels post-exercise might predict the outcome of the MWM task when animals are trained a day after the cessation of exercise. According to previously published work, it is expected that the exercised group will not exhibit improved memory due to the increased pERK in the hippocampus[139].

Chapter 3: Short-term effect of exercise on spatial learning and hippocampal proteins in maternally separated rats

3.1. Introduction

Previous reports have shown that MS impairs cognitive function in rats of varying ages [72] due to reduced hippocampal volumes and neurons in the hippocampus [32]. The aim of the following experiment was to determine whether post-exercise protein levels would predict the outcome of spatial learning in the MWM. A direct relationship between BDNF and intact memory has been demonstrated previously whereby knocking out genes that encode for BDNF resulted in impaired memory and conversely, increased levels of the neurotrophin resulted in improved memory [119,162]. The same was also true for pERK [163,164]. The previous experiment in this study revealed that BDNF was reduced by MS in the left dorsal hippocampus and reduced by exercise in the right dorsal hippocampus in non-separated rats (Figure 2.7). Consistent with previously reported findings, pERK was only increased by exercise in non-separated rats (Figure 2.5). Taken together, these results suggest that learning would be impaired in the MS non-running rats.

3.2. Methods

Animals were housed in the University of Cape Town Department of Human Biology Satellite Animal facility under the conditions described in Chapter 2 Section 2.1. Litters were designated as either MS or NS and exposed to the separation paradigm described in Section 2.2 of Chapter 2. Rats were again divided into running and non-running groups based on their access to a running wheel as described in Section 2.4 of Chapter 2.

3.2.1 Morris Water Maze

The MWM was used to test for spatial and reversed spatial learning and memory. The water maze was placed in the centre of a room with different spatial cues on the walls. The circular tank had a diameter of 173 cm and a height of 63 cm and was raised 30 cm off the floor (Figure 3.1). The tank was filled with water, which was maintained at a temperature of 21° ±1°C.

A B

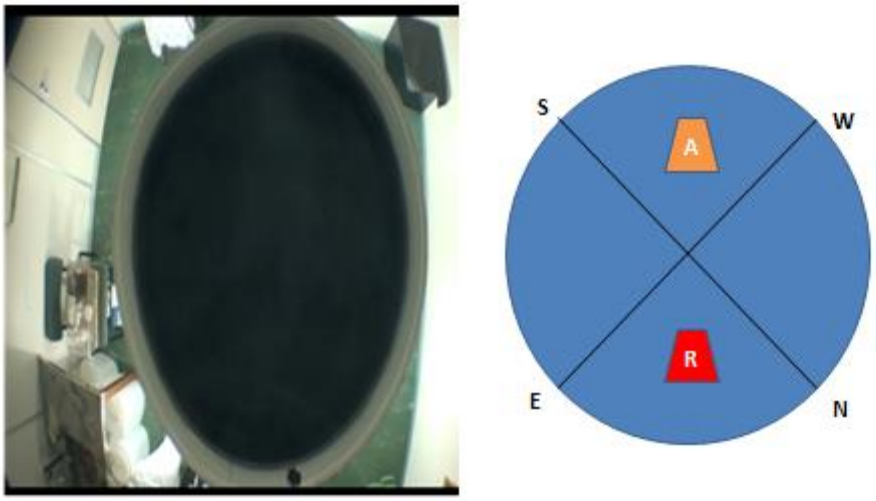

Figure 3.1. MWM. (A) The tank as seen through the lens of the camera. The black box in which rats were kept to dry after swimming can be seen situated in the top right corner. (B) Schematic representation of the tank depicting the imaginary quadrants that were used to mark starting points for different trials. N,E,S,W, represented the imaginary cardinal points used to mark starting points for training trials. The trapezia indicate the position of the platform during acquisitional training 'A' and reversal training 'R'.

A clear Perspex platform with a diameter of 15 cm, was submerged 1 cm below the water surface, and placed 25 cm from the wall of the tank in southwest (acquisition) or northeast

(reversal) quadrants of the tank (Figure 3.1B).. Black tempera powder paint was used to dye the water in order to mask the submerged platform and to allow accurate tracking of the animal (white against black background) on Ethovision software (Noldus, Waginegen, the Netherlands). Navigational cues in the room consisted of black-and-white pictures printed on A3 paper stuck up high on the wall; an extraction fan on blackened windows on the north-eastern wall; the door to the room on the south-eastern side as well as a fire hydrant sign on the north-western wall.

The training schedule was carried out according to Vorhees and Williams (2006) [98]. Briefly, rats were habituated to the testing room for at least 1 hour before testing. Acquisitional training took place on days 1 to 5. On day 6, the rat's memory was tested in the probe trial. Reversal training took place from day 7 to 9 with day 10 being the reversal probe trial. Each training trial lasted no more than 2 minutes while the probe trials were a fixed 90 seconds. In between trials, rats were kept in a black box lined with towelling to facilitate drying. Rats were trained and tested between 11:00 and 14:00. The reversal-training schedule was, reduced to 3 days as rats tend to perform better in the reversal stage of training [98]. After each trial, the rats were dried with paper towels then returned to the home cage.

No rats were excluded from the MWM analysis as they had managed to locate the platform, unassisted, by the end of training sessions on day 3 as failure to locate the platform by the end of the 3rd day of training was considered as an inability to learn [72].

One hour after the end of the reversal probe on P80, rats were killed and tissue was harvested for Western Blot and ELISA detection of proteins as indicated in Section 2.5, 2.6 and 2.7 of Chapter 2. Tissue samples from two rats per experimental group were pooled to create a single sample for the ELISA and Western Blot analyses of BDNF, pERK, MKP-1.

3.3 Results

3.3.1 Morris Water Maze

The latency to reach the platform during the acquisition phase of the task was normally distributed and was therefore analysed by factorial ANOVA for stress and exercise effects. A main effect of day of training and a Day*Separation interaction were observed, ($F_{(4,156)}$ =130.21, $p<0.005$) and ($F_{(4,156)}$=3.01, $p<0.05$) respectively, with no main effect of separation or exercise on the escape latency. Duncan's post-hoc tests revealed no intergroup differences on the different days of training.

The latency to reach the platform during the reversal learning phase of the task was also normally distributed. During reversal training, an effect of training day was revealed ($F_{(2,18)}$=13,33, $p<0.0001$). A Separation*Day interaction and Day*Separation*Exercise interaction were also observed ($F_{(2,18)}$=3,59, $p<0.05$) and ($F_{(2,18)}$=4.08, $p<0.05$, respectively). Duncan's post-hoc test revealed that the latency to reach the platform decreased for all groups significantly on day 2 and 3 of reversal training ($p= 0.0001$, compared to day 1). Duncan's post-hoc test revealed that MS rats had lower latencies on days 1 and 2 but not day 3. Duncan's post-hoc test revealed that on day 1 non-separated non-runners had lower latencies

to reach the platform compared to all other groups (p=0.005 vs. NSR, p=0.046 vs. MSnR, p=0.0005 vs. MSR). (Figure 3.2A).

The total distance of the swim path to the platform was normally distributed during both the acquisition and reversal phases of the MWM. Repeated measures ANOVA revealed a day effect ($F_{(4,156)}$=134.75, p<0.0001) but not an effect of either separation or exercise on the total distance to platform. A Day*Separation interaction was also significant ($F_{(4,156)}$=4.07, p<0.005). Duncan's post-hoc test revealed that all groups had significantly lower day-to-day swim paths but there were no intergroup differences in the total distance covered to the platform. During reversal training, a day effect was revealed ($F_{(2,18)}$=12.49, p<0.0001) and a Exercise*Day interaction ($F_{(2,18)}$=4,47, p<0.005). Duncan's post-hoc test revealed that on day 1, non-separated non-runners had covered a shorter distance to get to the platform compared to non-separated runners (p=0.018) (Figure 3.2B).

Behaviour in the MWM was also analysed for thigmotaxy and was found to be normally distributed during the acquisition and reversal phases of the task. Similar to distance to platform, a main effect of day of training was observed ($F_{(4,156)}$=129.06) and a day*Separation interaction ($F_{(4,156)}$=4.17), p<0.005). Duncan's post-hoc test revealed that MS non-runners exhibited less thigmotaxy than non-separated non-runners (p=0.025). During reversal training, a day effect was observed ($F_{(2,18)}$=12,10, p<0001). Duncan's post-hoc revealed that on Day 1 of reversal training, non-separated non-runners exhibited significantly lower thigmotactic behaviour than non-separated runners (p=0.001), MS non-runners (p=0.018) (Figure 3.2C).

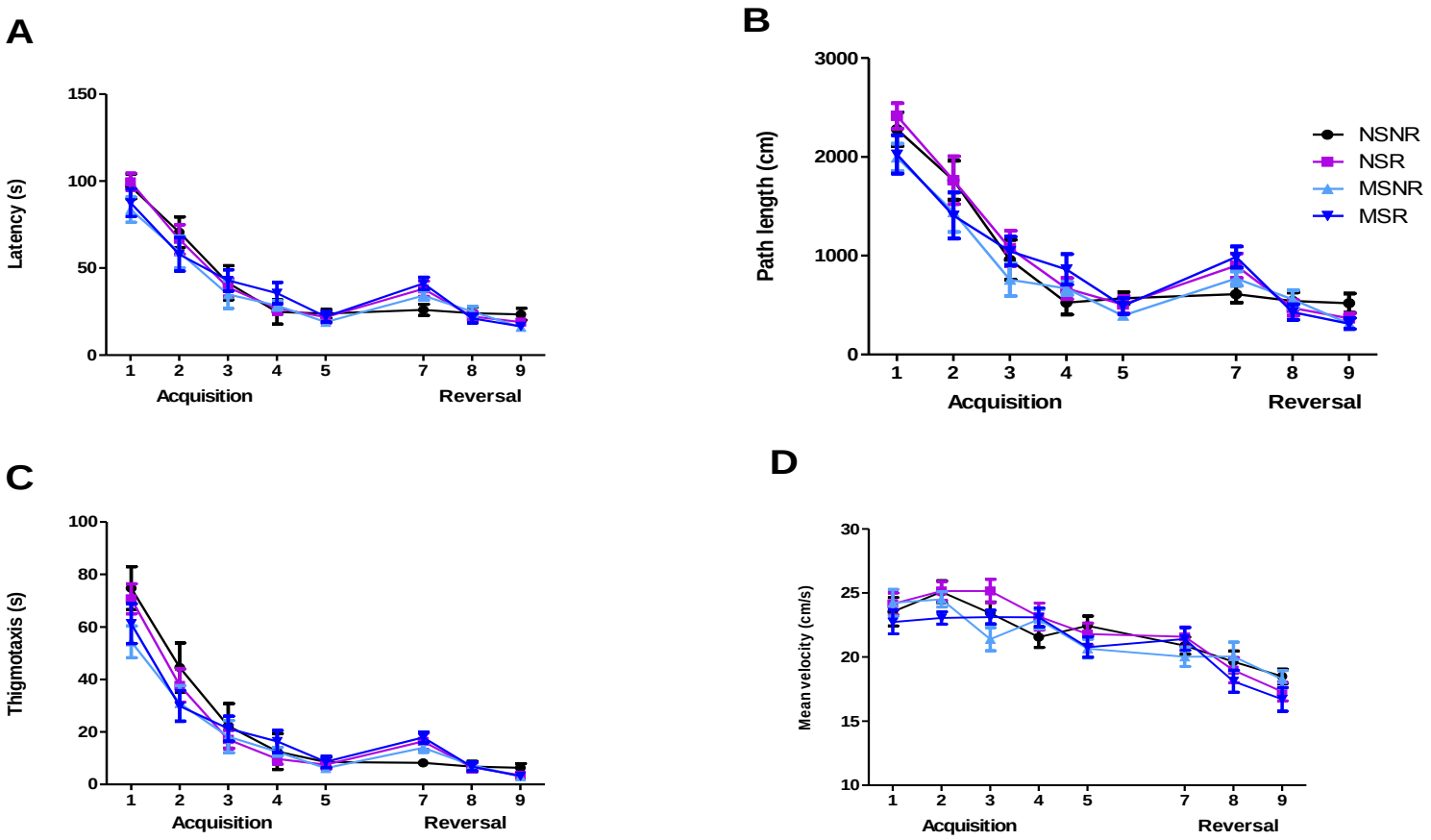

Figure 3.2. Maternal separation and exercise had no effect on spatial learning and memory in rats trained 1 day post-exercise. Acquisition training took place on Days 1-5. On day 6 rats were subjected to a probe trial followed by reversal training from day 7- 9 with the reversal probe trial on day 10. (NSnR= non-separated non-runners; NSR= non-separated runners; MSnR= maternally separated non-runners; MSR= maternally separated runners)

The mean velocity during acquisition and reversal tasks was also normally distributed. During acquisitional training, repeated-measures ANOVA revealed a day effect ($F_{(4,156)}$=10.68, p<0.0001) and a Day*Separation interaction ($F_{(4,156)}$=2.8, p=0.027). Duncan's post-hoc test revealed no intergroup differences in velocity. During reversal training, repeated measures ANOVA revealed an effect of day ($F_{(2,18)}$=12,82, p<0.0001); a Separation*Day interaction ($F_{(2,18)}$=3.59,p<0.05) as well as a Exercise*Day interaction ($F_{(2,18)}$=4,08), p<0.05). Duncan's post-hoc test showed that on Day 1, non-separated non-runners had a lower mean velocity compared to non-separated runners (p=0.001) and MS non-runners (p= 0.018) (Figure 3.2D).

Spatial learning and memory were assessed by swim pattern during probe trials conducted after both the acquisition and reversal phases of the task. The time spent in the target quadrant was normally distributed in both probe trials. During the acquisition probe trial, two-way ANOVA revealed no effect of either separation ($F_{(1,40)}$= 0.06, p=0.81) or exercise ($F_{(1,40)}$= 0.16, p=0.69) on the amount of time spent in the target quadrant (Figure 3.3A). During the reversal probe trial, two-way ANOVA revealed no effect of either separation ($F_{(1,40)}$= 0.46, p=0.50) or exercise ($F_{(1,40)}$= 1.89, p=0.18) on the amount of time spent in the target quadrant (Figure 3.3B).

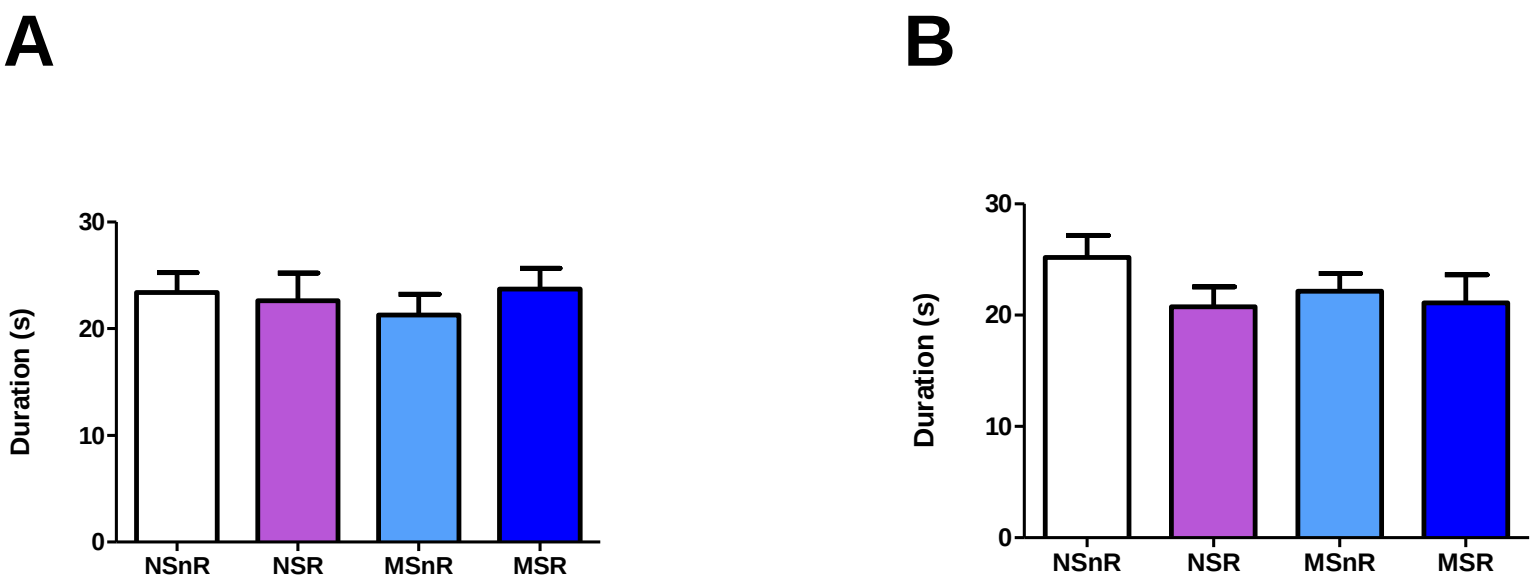

Figure 3.3. Maternal separation and exercise did not affect spatial memory. (A) Acquisition probe: time spent in the target quadrant, (B) reversal probe: time spent in the reversal quadrant. (NSnR= non-separated non-runners; NSR= non-separated runners; MSnR= maternally separated non-runners; MSR= maternally separated runners), n=12

3.3.2 Western Blot analysis of protein levels

For the protein data, which were normally distributed, outliers were identified and discarded from the data set when they were found to be greater or less than two standard deviations from the mean.

3.3.2.1 Phospho-ERK

Two-way ANOVA revealed a Separation*Exercise interaction on pERK levels in the left dorsal hippocampus ($F_{(1,20)}$= 7.86, $p<0.05$). Though appearing to be increased in the nMSR group, pERK levels were not significantly different to the NSNR group. In fact, pERK was highest in the MSnR group ($p<0.05$) (Figure 3.4). There were no significant effects in the right dorsal hippocampus.

3.3.2.1 MKP-1

Two-way ANOVA revealed a significant effect of Exercise ($F_{(1,55)}$=4.05, $p<0.05$) in the left dorsal hippocampus. MS had no significant effect on MKP-1 levels in the left dorsal hippocampus

3.3.3 BDNF measurement

Mann-Whitney U test showed that there was a significant difference between left and right BDNF values $p<0.001$. In the right dorsal hippocampus, Two-way ANOVA revealed a significant effect of exercise on BDNF levels ($F_{(1,20)}$=4.89), $p <0.05$. Two-way ANOVA revealed that in the left dorsal hippocampus there was a tendency towards a Separation effect

($F_{(1,12)}$= 3,93, p=0.06). Duncan's post-hoc test showed that were no intergroup differences (Figure 3.6).

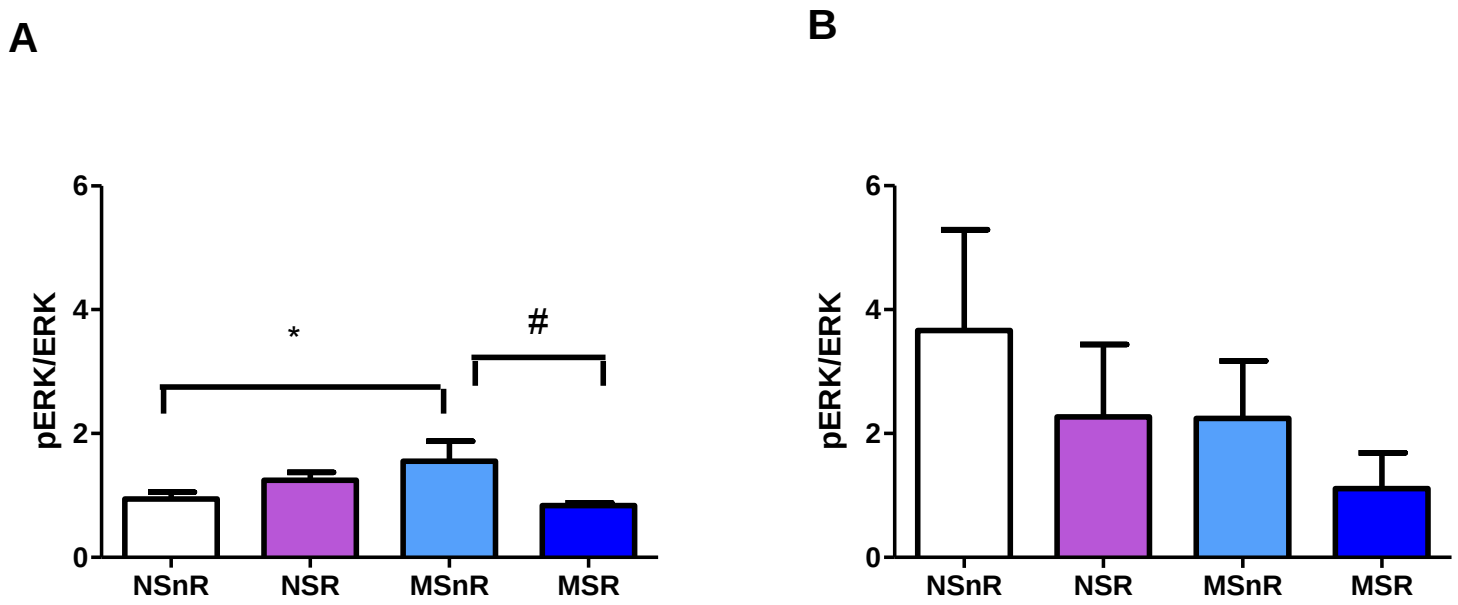

Figure 3.4. Relative densitometry of pERK in the left and right hippocampus. (A) Maternal separation increased pERK but exercise normalised the levels in maternally separated rats (*MSnR vs. NSnR, #MSnR vs. MSR, $p<0.05$). (B) There was no effect of separation or exercise in the right dorsal hippocampus. (NSnR= non-separated non-runners; NSR= non-separated runners; MSnR= maternally separated non-runners; MSR= maternally separated runners)

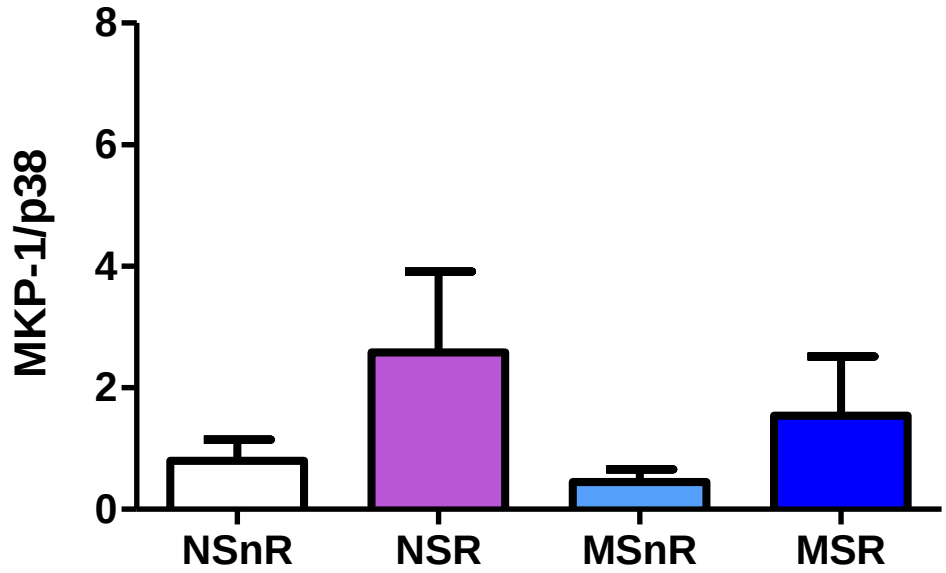

Figure 3.5. Relative densitometry of MKP-1 in left dorsal hippocampus. (NSnR= non-separated non-runners; NSR= non-separated runners; MSnR= maternally separated non-runners; MSR= maternally separated runners) n= 6

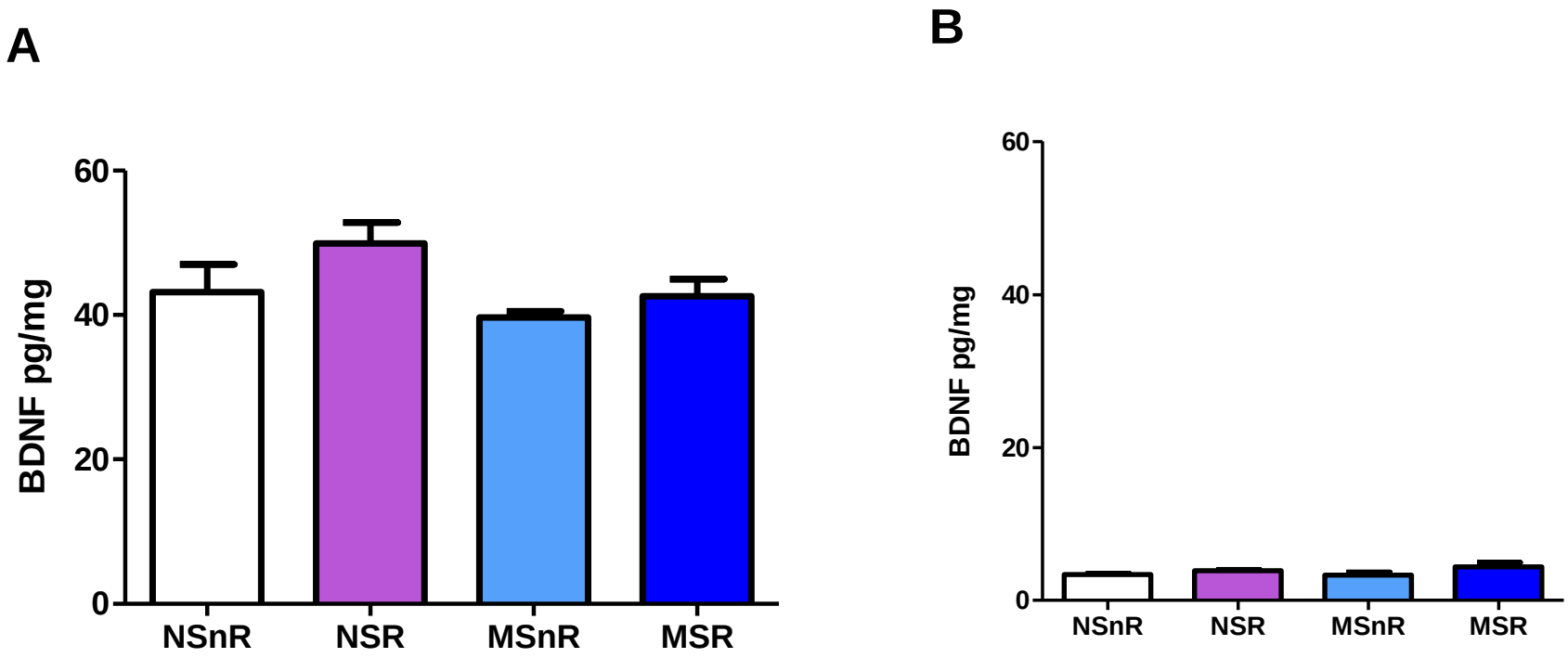

Figure 3.6. BDNF in the left (A) and right (A) dorsal hippocampus amounts remained unchanged after Morris Water Maze training. Data presented as means ±SEM, (NSnR= non-separated non-runners; NSR= non-separated runners; MSnR= maternally separated non-runners; MSR= maternally separated runners, n=6

3.4. Discussion

During the acquisitional phase of the MWM, the latency to find the platform was high on Day 1 of training at an average of 82.6 seconds and gradually decreased to an average of 22.1 seconds by Day 5 of training. During reversal training, the latency on the first day was not as high as it was on the first day of acquisitional training, but was nearly equal to the average latency of Day 3 of acquisitional training (34.9 seconds compared to 43 seconds in acquisitional phase). By Day 3 of reversal training, the latency had decreased to 18.7 seconds. A similar reduction over time was seen with the path length and thigmotaxy. Mean velocity, on the other hand was unchanged during the acquisition phase though it was decreased during the reversal trials. Of note was that the non-separated non-runners had reached their full learning potential during reversal training, as there was no day-to-day change in the latency, path length, and thigmotaxy.

The probe trials revealed that all groups had similar memory for the platform location as they all spent similar amounts of time in the target quadrants. Approximately 13% (below chance) of the probe trial was spent in the target quadrant in both the acquisition and reversal phases. Non-separated non-runners spent slightly more time in the reversal target quadrant. Although this did not reach statistical significance, it was however, consistent with the reversal training learning curve that had a near horizontal slope.

The lack of intergroup differences in spatial learning was supported by the work of O'Callaghan et al [165]. They reported that forced exercise did not alter learning and memory in the MWM although they found that it had a beneficial effect on LTP and object recognition

memory [165]. Droste et al (2007) also suggested that, exercised rats that are exposed to physical stressors such as swimming (forced swim test), displayed a marked stress response compared to mild psychological stressors [53]. Although the MWM and forced swim test are used to measure different behavioural parameters, it can be agreed that they both induce physical stress due to the forced swimming. Therefore, it is possible that the stress caused by swimming had a role in diminishing the intergroup differences in behaviour in the MWM task. Such effects, in future, can be tested by habituating the animals to swimming before the actual commencement of the MWM task.

Additionally, Grace et al (2009) reported no effect of either MS or exercise on spatial learning and memory in the MWM test, although on Day 5 of acquisition training, exercise decreased the latency to reach the platform [72]. In contrast, a single 24-hour MS produced no differences in spatial acquisitional learning in the Barnes maze compared to non-separated rats but reversal learning was impaired by MS which could have been due to the decreased number of neurons in the dentate gyrus [32]. Even after a short voluntary exercise regimen, exercised rats were reported to have spent more time in the target quadrant than sedentary rats, showing that they had memory for the region in which the platform was placed [162]. This goes to show that the varied MS and exercise paradigms chosen will have different effects on the different types of learning and memory.

In the left dorsal hippocampus, MS caused an increase in pERK levels that were normalised by exercise. Exercise had no effect in the non-separated groups. The significant MS-exercise interaction demonstrated once more that exercise tended to increase pERK in non-separated rats but normalised the increase induced by MS. This interaction was limited to the left dorsal hippocampus after MWM training. In the right dorsal hippocampus, both MS and exercise

decreased (although not significantly) pERK. Compared to the naive group, pERK was most increased in the MS non-runners group as opposed to the non-separated runners. This suggested that further stimulation of the HPA axis by repeated brief swimming activity in the MWM test changed the sensitivity of the MAPK/ERK pathway in MS rats but not in NS runners.

Maternal separation did not alter MKP-1 levels in the left dorsal hippocampus. Exercise increased MKP-1 levels in the left dorsal hippocampus of both non-separated and MS rats. This finding was consistent with work by Hu et al (2009), who showed that MKP-1 levels increased in response to exercise [138].

In the MS groups, an inverse relationship between pERK and MKP-1 levels was observed (r= -0.213) which was consistent with the regulatory role that MKP-1 was reported to have on pERK [142,145]. Within the non-separated groups, the relationship between these two proteins was found to be positive (r=0.599). This may have been due to the unchanged pERK levels in the non-separated runners. The mechanism by which exercise was able to regulate pERK via MKP-1 in MS rats may have been through the increase of glucocorticoids which stimulate MKP-1expression to eventually decrease pERK to basal levels [137,138].

Overall, the protein levels post MWM training suggest that pERK and MKP-1 levels in the left dorsal hippocampus did not have an impact on learning. BDNF, however, was a better predictor of learning outcomes as it remained unchanged in all groups and thus corresponded with the lack of differences in the spatial learning task.

BDNF levels in the left dorsal hippocampus were greater than in the right dorsal hippocampus for all groups. This may have been due to inter-plate differences as the left and right samples of the dorsal hippocampus were analysed on different plates or it could be due to functional differences of the left and right dorsal hippocampus. There were no intergroup differences in the left and right BDNF levels after MWM training.

In summary, MS and exercise did not affect spatial learning in the MWM task when trained 1 day post-chronic voluntary exercise. Training in the MWM caused a marked increase in pERK only in MS non-runners while exercise normalised pERK levels only in the left dorsal hippocampus.

Chapter 4: Long-term effects of exercise on spatial learning and hippocampal protein in maternally separated rats

4.1. Introduction

The effects of MS are known to be long lasting as they occur at a time when the brain is still undergoing development [28]. The outcome of maternal separation in later life can be mitigated by environmental enrichment and exercise [166,167]. Exercise was chosen in this study to ameliorate the potentially deleterious effects of MS. In the investigation described in Chapter 3, MS and/or exercise did not alter learning and memory in the MWM when rats were trained 1 day after the cessation of the voluntary exercise regimen. However, it was also found that exercise could prevent MS-induced pERK increases in the left dorsal hippocampus after MWM training.

The effects of exercise depend on the frequency, duration and intensity of the chosen mode of exercise [168,169]. Exercise has been reported to enhance spatial memory via BDNF-dependent pathways [170]. BDNF levels were shown to be highest in exercised rats after 3 weeks of voluntary running and that spatial memory was more efficient in rats trained 1-week post-exercise compared to 2 weeks post-exercise. The data in Chapter 2 and 3 contradict their findings. The potential reasons for the discrepancies have been discussed in Chapter 3.

In this chapter, the effects of MS and exercise were investigated in a group of rats that were allowed to rest 15 days after the end of the voluntary exercise regimen to determine whether a delay in MWM training would yield dissimilar results to those in Chapter 3.

4.2. Materials and Methods

Forty-eight male Sprague-Dawley rats were used to determine the long-term effects of exercise in MS and NS groups on spatial learning and memory. As described in Chapter 2, whole litters were either MS or left undisturbed (NS) from postnatal day 2 to 14. At P50, rats were allowed voluntary exercise until P70 after which they were group-housed. Rats were tested for spatial learning in the MWM from P85 -94 (15 days post-exercise). The MWM protocol is described in Chapter 3. One hour after completing the reversal probe trial on P94, rats were killed and brain tissues harvested for biochemical analyses of BDNF, pERK and MKP-1 levels, as described in Chapter 2.

4.2.1 Statistical analysis

The Shapiro-Wilk's test was used to confirm that the data was normally distributed. Then, the repeated measures two-way analysis of variance was used to determine main effects of either separation and/or exercise with day of training being the within group factor. Two-way analysis of variance was used to determine main effects of Western Blot and ELISA data. Where necessary, Duncan's post-hoc test was used to compute inter-group differences.

4.3. Results

4.3.1 Morris Water Maze

The latency to reach the platform was normally distributed during both acquisition and reversal phases of the task. A repeated-measures ANOVA revealed a main effect of training day for acquisitional training ($F_{(4,176)}$= 148.32, $p<0.0001$) as well as a Day*Separation*Exercise interaction ($F_{(4,176)}$=2.49, $p<0.04$). Duncan's post-hoc tests revealed no intergroup differences on the different days of training (Figure 4.1A The latency to reach the platform during the reversal learning phase of the task was also normally distributed. During reversal training, an effect of training day was revealed ($F_{(2,40)}$=13,33, $p<0.0001$). Day*Separation*Exercise interaction was also observed ($F_{(2,40)}$=3.38, $p<0.05$). Duncan's post-hoc test revealed that all groups had lower latencies on Day 2 and Day 3 compared to Day 1 ($p<0.05$) (Figure 4.1A).

The total distance covered to reach the platform was normally distributed in both the acquisition and reversal phases of the task. During acquisitional training, there was no effect of exercise or separation. An effect of training day was observed ($F_{(4,176)}$=156.38, $p<0.0001$. Duncan's post hoc test revealed that all groups covered shorter distances to reach the platform with day-to-day training ($p<0.05$) and on Day 3, MS non-runners covered a greater distance than non-separated non-runners ($p=0.037$). During reversal training, a day effect was revealed ($F_{(2,40)}$=13,28, $p<0.0001$) and a Separation*Exercise*Day interaction ($F_{(2,40)}$=3,66, $p<0.005$). Duncan's post-hoc test revealed that all groups had covered a shorter distance to reach the platform with continued training ($p<0.05$) however MS runners had covered a shorter distance than non-separated non-runners (Figure 4.1B).

Thigmotaxic behaviour in the MWM was analysed during the MWM and found to be normally distributed. During acquisitional training, repeated measures ANOVA revealed the day of training as a main effect ($F_{(4,176)}$=148.32, p<0.0001) and a day*separation*exercise interaction was also observed ($F_{(4,176)}$=4.00, p=0.004). Duncan's post-hoc test revealed that all groups exhibited decreased thigmotactic behaviour with increasing training (p<0.05); on Days 2 and 3, non-separated non-runners displayed less thigmotaxy than MS non-runners (p=0.007 and p=0.016, respectively During reversal training, a day effect was observed ($F_{(2,40)}$=14,56, p<0001). Duncan's post hoc test revealed that all groups displayed significantly lower day-to-day thigmotaxy (p<0.05) (Figure 4.1C).

The mean swim velocity was normally distributed. During acquisition training, repeated-measures ANOVA revealed a day effect ($F_{(4,176)}$=10.01, P<0.0001) and a tendency towards a separation effect ($F_{(4,176)}$=3.96, p<0.053). Duncan's' post hoc test revealed that non-separated non-runners had decreased velocity on Day 5 compared to Day 1 (p<0.05) and MS runners had lower mean velocities on Day 4 compared to Day 1 (p<0.05). During reversal training, repeated measures ANOVA revealed an effect of day ($F_{(2,40)}$=6,52, p<0.0001) in the mean velocity. Duncan's post hoc test revealed that non-separated non-runners had decreased mean velocity on day 3 compared to day 1 (p<0.05); non-separated non-runners had decreased velocity on Day 3 compared to Day 1 (p<0.01); MS non-runners had decreased mean velocity on Day 3 compared to Day 1 (p<0.05) and, MS runners had decreased velocity on Days 2 and 3 compared to Day 1 (p< 0.05 and p<0.05, respectively) (Figure 4.1D).

Memory for the platform location was tested in probe trials. Data from the probe trials after acquisition and reversal learning were normally distributed. During acquisition, two-way ANOVA revealed no effect of MS ($F_{(1,40)}$=0.01, p=0.91) and exercise ($F_{(1,40)}$= 1.77, p=0.19) on the amount of time spent swimming in the target quadrant (Figure 4.2A). During reversal training, a separation effect was revealed in time spent in the reversal quadrant after reversal training ($F_{(1,40)}$=23.88, p<0.0001). Duncan's post hoc test revealed that MS non-runners spent more time in the target quadrant compared to non-separated non-runners (p<0.0001) while MS runners also spent more time in the target quadrant than non-separated runners (p<0.00001) (Figure 4.2B).

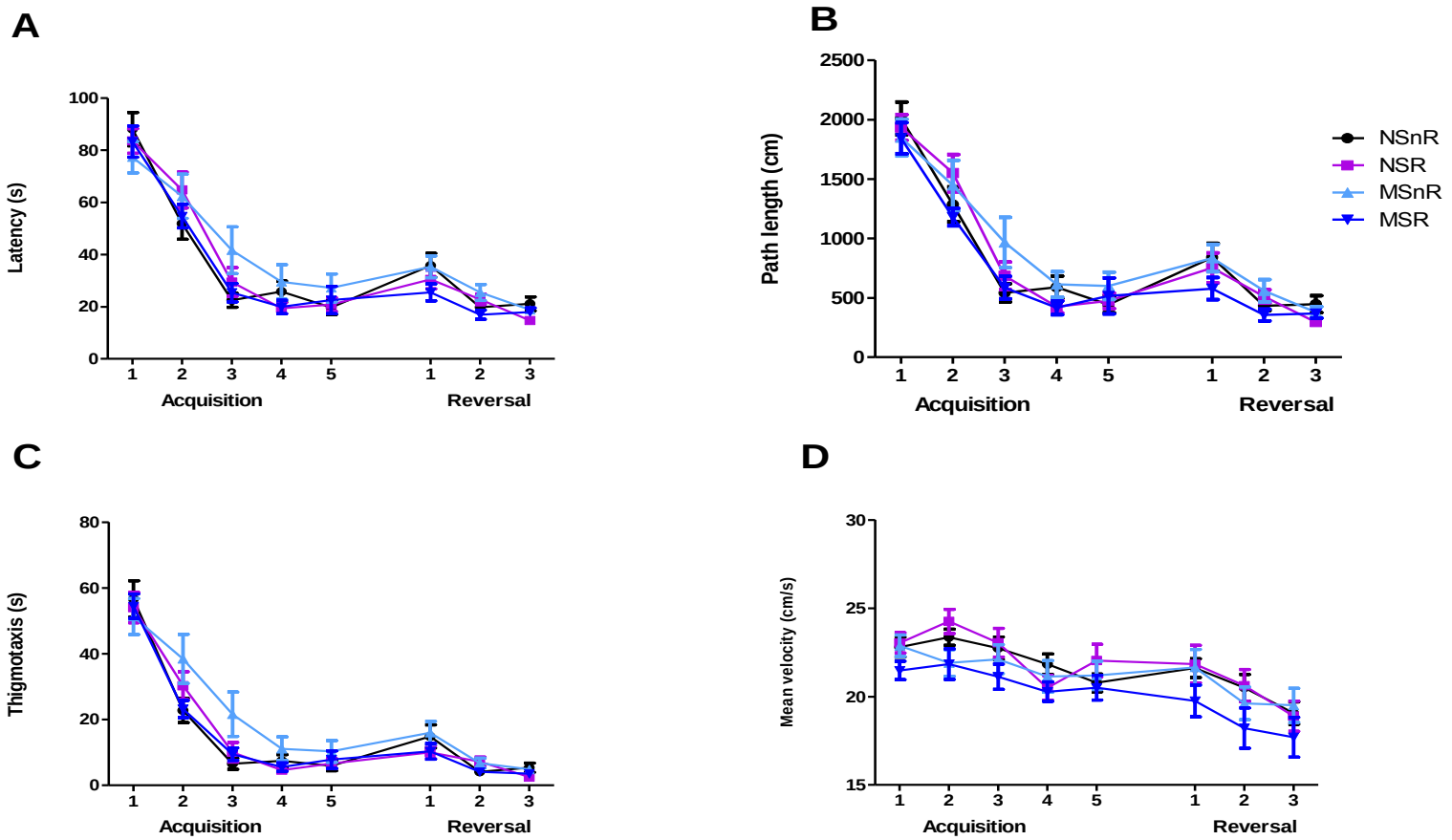

Figure 4.1. Maternal separation and exercise do not affect spatial learning in the Long-term effects of exercise group of rats. (A) Latency to platform; (B) Path length; (C) Thigmotaxis; (D) mean velocity. Data is presented as mean ±SEM, n=12 (NSnR= non-separated non-runners; NSR= non-separated runners; MSnR= maternally separated non-runners; MSR= maternally separated runners)

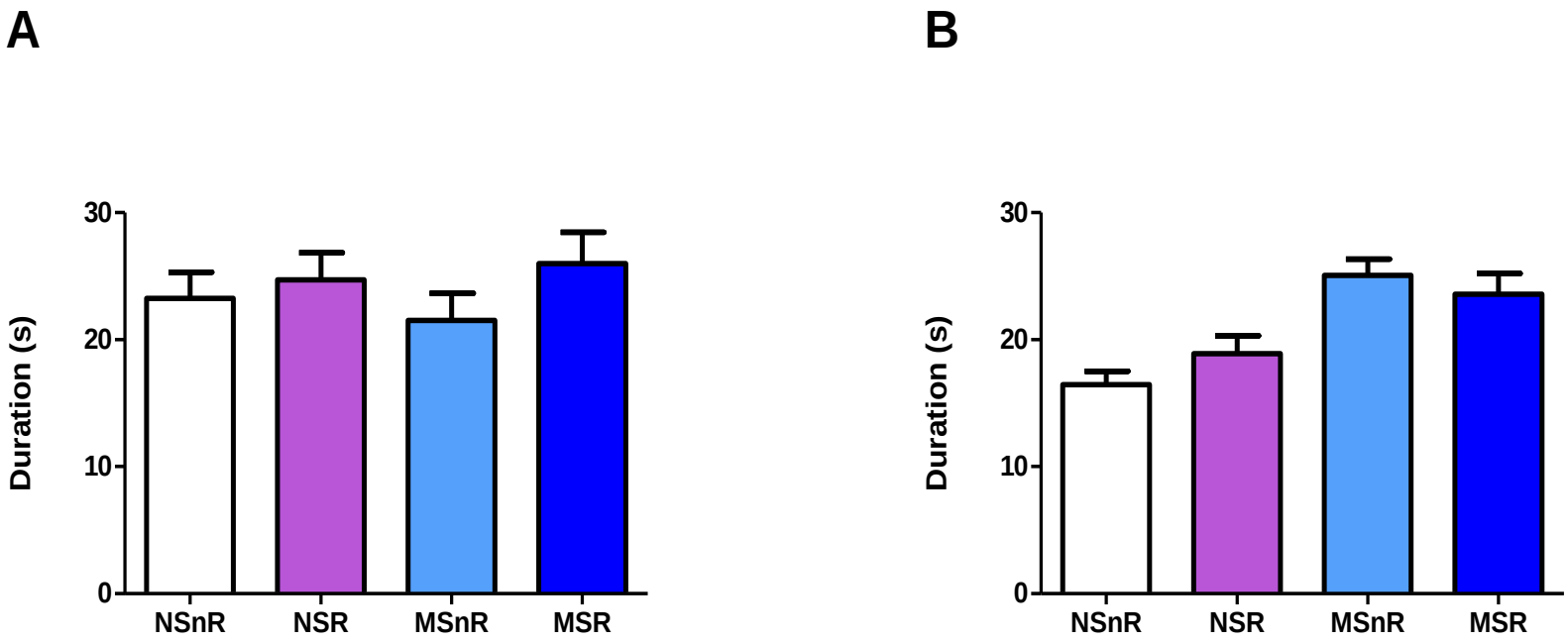

Figure 4.2. Probe trials for Cohort 3. (A) Acquisition probe trial. (B) Reversal probe trial. N=12 (NSnR= non-separated non-runners; NSR= non-separated runners; MSnR= maternally separated non-runners; MSR= maternally separated runners)

4.3.2 Western Blot analysis of proteins

4.3.2.1 pERK

In the right dorsal hippocampus, two-way ANOVA revealed that there was no effect of MS ($F_{(2,641)}$=32.69, p=0.21) or exercise ($F_{(64,1)}$= 71.76, p=0.48) on pERK levels (Figure 4.3).

4.3.2.2 MKP-1

A two-way ANOVA found no differences in MKP-1 levels between groups.

4.3.3 ELISA measurement of BDNF

BDNF levels were normally distributed and so analysed by factorial ANOVA. Maternal separation significantly reduced BDNF levels in non-exercised rats ($F_{(1,16)}$=6.12, p<0.05).

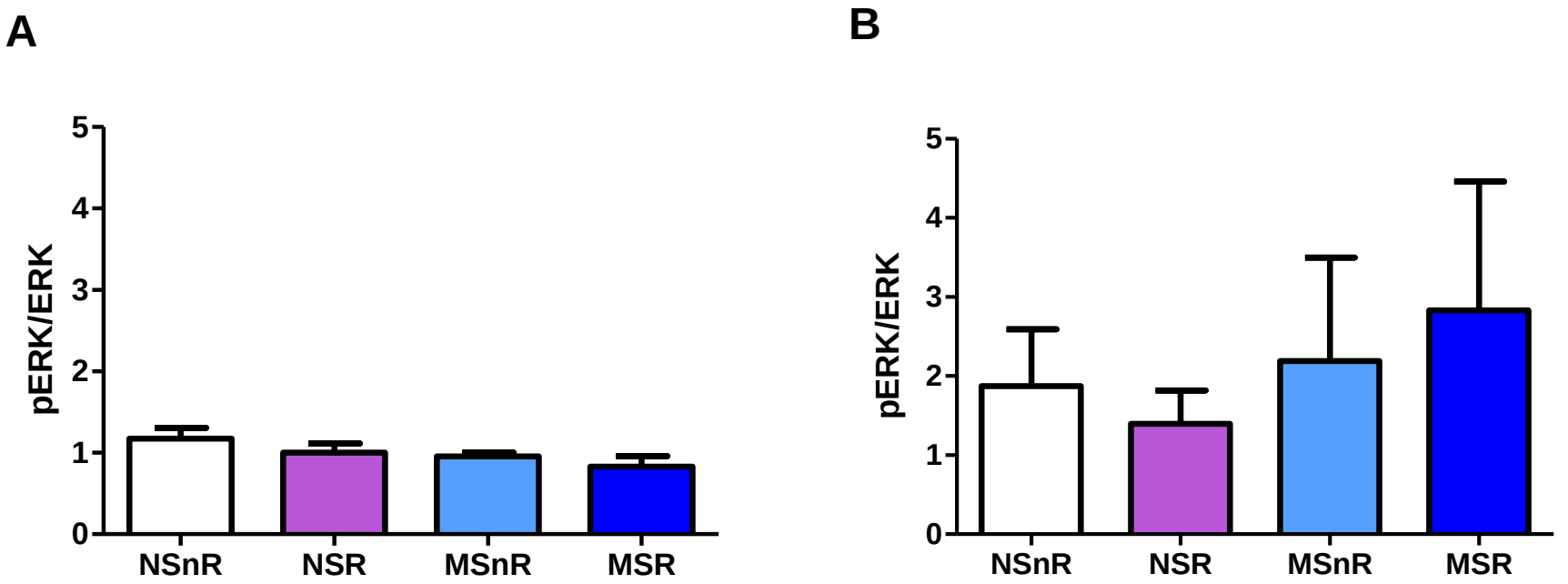

Figure 4.3. Relative pERK levels in left (A) and right (B) dorsal hippocampus of short-term effects groups. N=6, Data presented as means ±SEM (NSnR= non-separated non-runners; NSR= non-separated runners; MSnR= maternally separated non-runners; MSR= maternally separated runners).

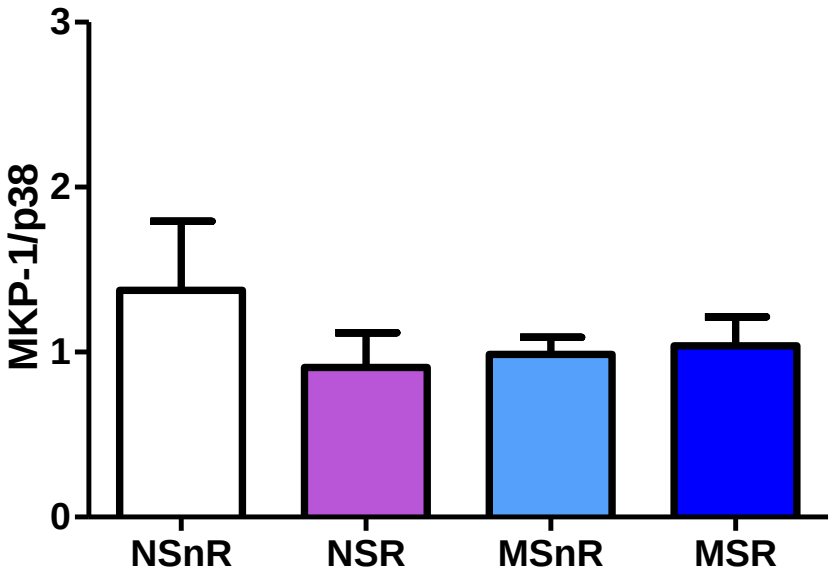

Figure 4.4. Relative densitometry of MKP-1 in the left dorsal hippocampus of short-term effects group. Data presented as means ±SEM, (NSnR= non-separated non-runners; NSR= non-separated runners; MSnR= maternally separated non-runners; MSR= maternally separated runners, n=6)

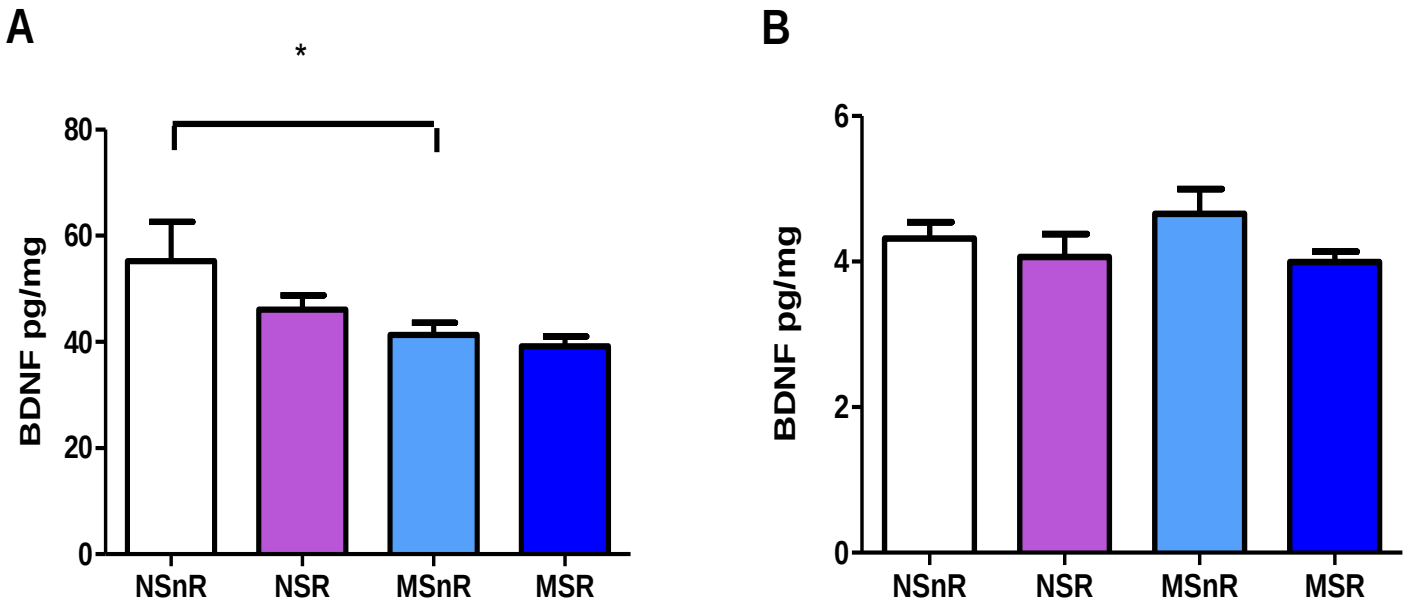

Figure 4.5. BDNF concentration in left (A) and right (B) dorsal hippocampus of rats that were tested in the Morris Water Maze, a day after the cessation of exercise (P71). Maternal separation decreased BDNF (*$p<0.05$, MSnR compared to NSnR). Data is presented as means +SEM. n=6, (NSnR= non-separated non-runners; NSR= non-separated runners; MSnR= maternally separated non-runners; MSR= maternally separated runners)

Discussion

As with the cohort of rats trained immediately after exercise, the latency to reach the platform, path length, and thigmotaxy were high on the first day of acquisition training and gradually decreased as the animals learned to locate the hidden platform in the MWM (Figure 4.1). The same trend was also observed in the reversal training (Figure 4.1). This revealed that all animals had the same propensity to locate the hidden platform. Moreover, the probe trial revealed that MS and exercise did not affect memory for the platform location in the acquisition probe. However, in the reversal probe, MS rats spent more time in the reversal quadrant than the non-separated rats. This suggested that MS rats were more efficient in relearning the new location of the platform.

Early literature suggested that MS rats would have unaltered or impaired memory in the MWM which is opposite to our findings in the current study. O'Callaghan (2007) found no effect of exercise on spatial learning and memory while Grace et al (2009) found that MS had no effect but exercise greatly reduced the latency to reach the platform on the last day of training but with no differences in the probe trial [72,165]. However, it would be beneficial to note that the studies mentioned had not investigated the effect of MS or exercise on reversal learning in the MWM. This suggests that there may be different mechanisms involved in acquisition and reversal learning in the hippocampus. Furthermore, these mechanisms may be more responsive to the effects of MS as opposed to exercise.

It is known that exercise enhances memory by BDNF-dependent pathways [170], however, previous data revealed that immediately after exercise, BDNF was not increased in the

exercised groups corresponding to unaltered spatial memory (Chapter 3). It has been established that efficient learning in the MWM is associated with LTP, for which activation of NMDA receptors is required. In the MWM, it has been shown that NMDA-associated LTP was required for acquisition training and reversal training [171]. This was demonstrated by blockade of NMDA receptors during the different phases of MWM training. Rats with impaired NMDA function had attenuated learning and memory in the different phases of the MWM [171].

In contrast, efficient reversal learning has been associated with long-term depression or LTD. LTD is characterised by the pruning of synaptic bouttons and decreased neurogenesis. Such events are conducive for amnesia, which may be essential for relearning or updating older, irrelevant information. As MS is known to be associated with LTD, it is therefore not surprising that MS rats were more efficient in recalling the platform location after the reversal training in the MWM.

MS rats had improved reversal spatial memory in the Morris Water Maze task but decreased BDNF in the left dorsal hippocampus. These data suggested that delaying spatial learning after exercise had no beneficial effects in non-separated rats while MS, regardless of their activity state, improved reversal learning.

No differences in pERK levels in this experiment were found which is in contrast with the previous MS- and exercise-induced increase in pERK seen in MWM training immediately after exercise. The effect of exercise may have been diminished by the delay between the

cessation of running and the start of the spatial learning task. This observation may be explained by the work of Widenfalk et al (1999). Histological assessment of hippocampal BDNF in spontaneously-hypertensive rats revealed that deprivation of habitual exercise causes a decrease in BDNF levels detectable as early as 1 hour post-wheel locking[161]. Decreased levels in BDNF have been correlated to poor spatial learning[119]. The data of this study revealed that only MS maintains its negative effect on BDNF levels in the longer term while exercise-induced BDNF reduction is short-lived. The disparity between results from this current study and that of Widenfalk et al (1999) may be due to the different strains of rats used to assess the effect that rest may have on protein levels after voluntary exercise regimen.

Vaynmann et al (2004) proposed that exercise increases BDNF release from pre-synaptic terminals and activates TrkB receptors on both pre- and post-synaptic membranes. In the presynaptic cell, activation of CaMKII and the MAPK pathway will stimulate vesicular release of glutamate into the synapse. Increased glutamate will have the resultant effect of CREB activation and even more BDNF release into the synapse [162]. Therefore, blockade of any of these steps could result in impairment of memory. However, this was not entirely consistent with the results of this study. Immediate post-exercise data showed that MS was associated with a decrease in BDNF in the left hippocampus but pERK levels remained unchanged (Figures 2.1A and 2.2A). Furthermore, in the left hippocampus, BDNF was decreased but pERK increased in the non-separated runners. Moreover, it was expected that maternally separated sedentary rats would exhibit impaired memory; however, they had improved reversal memory. Therefore, the immediate post-exercise protein levels were not a good predictor for MWM task outcomes.

Chapter 5: General discussion and conclusion

This study sought to address two issues that arose from previous work namely,

1. To establish whether a 3-week voluntary exercise regimen would have differential effects on spatial learning and memory at two different time points after the cessation of exercise in MS rats, and
2. To establish the effect of a 3-week voluntary exercise regimen on protein levels in the left and right dorsal hippocampus in MS.

5.1. Maternal separation enhances spatial memory in later stages of training

The escape latency, path length, mean velocity, and thigmotaxis were measured to assess acquisition and reversal of spatial learning and memory and the results of these analyses are summarised in table 5.1. There were no differences in the measured parameters which indicated that all groups in both cohorts learned similarly well during both acquisition and reversal phases of the MWM. However, when reference memory was tested using the probe trial, it was found that MS rats from the cohort 3, the 'long-term effects of exercise group,' had improved reversal memory. The current finding corresponded with previous work, whereby MS rats had improved memory in the tasks that were presented to rats much later (in the training schedule) after a 3-week voluntary exercise regimen [139]. Of note, was that the object location test required some element of spatial mapping as with the MWM. However, it could be argued that the former required the use of proximal cues in addition to spatial mapping as the objects that are sampled were in the near environment of the rats versus cues on the wall which are not in the tank of the MWM [172].

5.2. The beneficial effects of exercise may be weakened by inappropriate housing conditions

The failure of exercise to improve memory was consistent with previously reported work [139]. Stanahan et al (2006) described how the beneficial effects of exercise could be influenced by housing conditions; for example, social isolation during voluntary exercise delayed the rate of neurogenesis and attenuated the stress response to cold swim stress test compared to group-housed rats [66]. Social housing conditions in the current experiment may have caused the diminished effect of exercise on behavioural parameters. However, had the exercise regimen been started post-weaning, the beneficial effects of exercise may have been more pronounced as social isolation post- weaning confers resilience to stress in later life [173]. Therefore, the timing at which the exercise is started should be well considered in future for the outcomes of the experiment.

5.3. Stimulation of the MEPK/ERK pathway is differentially affected by maternal separation and exercise

Previous work from our laboratory revealed that exercise and MS stimulate the MAPK/ERK pathway[139]. New evidence arising from the current study suggests that the exercise- and MS-induced changes in protein are abolished by training in the MWM task (Table 5.2). Moreover, the differential effect of the two interventions on BDNF in the naive group suggested that exercise and MS affect the MAPK pathway by different means. Rearing conditions affected the effect which exercise had on protein levels. BDNF levels were restored to normal in MS runners but no change was observed in non-separated rats. Phospho-ERK was increased by exercise in non-separated rats but not in MS runners.

Exercise is known to stimulate the MAPK pathway by increasing BDNF release [174] however, the mechanism by which MS impacted pERK is yet to be established.

It was first thought that MS decreased pERK through up-regulated MKP-1 which was activated by glucocorticoids [137], however, in the previous study, evidence to support this hypothesis was not found [139]. Another mechanism may have been through limiting BDNF-induced pERK activation. Data from this study also did not support that alternative hypothesis, as BDNF was not decreased in MS non-runners prior to being tested in the MWM indicating that the effects of MS and exercise on BDNF-pERK-MKP-1 interactions could have been complicated by MWM training.

MKP-1 was altered by exercise only in non-separated rats. It took another 10 days of brief forced exercise to elicit a response in MS rats but a 15-day rest after voluntary exercise before brief repeated forced exercise abolished MKP-1 response to exercise. In previous work, MKP-1 was slightly increased by exercise and MS, but the effect failed to reach significance due to high sample variability[139]. These results were consistent with work by others, whereby sub-acute voluntary exercise regimen resulted in increased MKP-1 in the hippocampus of adult rats [138].

5.4. Maternal separation alone did not affect MKP-1 levels

Maternal separation, a primer for depressive-like behaviours, had no effect on MKP-1 at any time point. Previous research using a chronic unpredictable stress (CUS) model of depression caused an increase in MKP-1 in the dentate gyrus, CA1 and CA3 regions of the hippocampus

while the deletion of the MKP-1 gene resulted in an increase in pERK2 [142]. MS has been used to increase susceptibility to depression [10] but on its own was not enough to elicit MKP-1 elevation even behavioural manifestations were absent [21]. Repeated MS differs from CUS in that MS was a predictable stressor. Therefore MS rats quickly adapted to respond to the impending isolation stress whereas CUS rats learned to become helpless, one of the features of depression [173,175]. This data together with the work of Duric et al (2010) and Hu et al (2009), also suggested that the MKP-1 response was immediate and quickly decayed after the removal of a stressor or stimulus but may stay elevated under pathological conditions such as depression [138,139,142] perhaps in an attempt to prevent JNK-induced apoptosis [138,144].

A significant difference in hemispheric BDNF levels was observed across all groups at all time points (Table 5.2). Left hippocampal BDNF levels were nearly 10-fold higher than right hippocampus. Such major differences in BDNF have not yet been reported but an approximate 14% decrease in right hippocampal BDNF compared to left hippocampus was observed in sham-lesioned sedentary rats with values of BDNF ranging between 100 and 250 pg/mg wet weight[74]. Others have reported BDNF levels as high as 800 pg/mg in whole hippocampus [176]; while some reported values less than 26 pg/mg in left hippocampus [116]. Possible reasons for the wide range of BDNF quantities in the hippocampus may be attributed to differences in tissue preparation as acid treatment of samples yields higher BDNF quantities as it allows for the quantification of pro-BDNF and mature BDNF [116]. In this study, the left and right dorsal hippocampal tissue samples were prepared on different days but the ELISA was carried out on the same day although measured on different plates. A more probable reason could be linked to the type of treatment or interventions, such as antidepressants or environmental enrichment, used to affect BDNF levels [153,177].

5.5. Hemispheric differences in BDNF quantities exist and may be dependent on experimental interventions

Analysis of hippocampal protein levels established that MS decreased BDNF in the left dorsal hippocampus while exercise decreased BDNF but increased pERK and MKP-1 in the right dorsal hippocampus (Table 5.2). As increased BDNF was reported to be essential for improved memory, it was expected that MS rats would also have increased BDNF. It has also been reported that BDNF levels decrease gradually after the cessation of exercise [161] suggesting that signalling events downstream of BDNF may have played a role in learning long after the neurotrophin levels decrease. Indeed, microarray data revealed that BDNF was required to activate the pERK signalling pathway, a reaction that only occurred with chronic exercise [174].

New evidence suggests that BDNF may also be regulated by MKP-1 [178]. Knocking out the MKP-1 gene in mice resulted in the inability of exogenous BDNF to stimulate axonal branching [178]. Therefore, under normal circumstances MKP-1 functioned to regulate trophic events in the brain [145]. Sustained increases in MKP-1 may be related to a state of disease such as depression [142] and cancer [144].

Table 5.1. Summary of MWM results

	Short-term effects of exercise group				Long-term effects of exercise group			
	Acquisition learning	Acquisition Probe	Reversal learning	Reversal probe	Acquisition learning	Acquisition Probe	Reversal learning	Reversal probe
Maternal separation	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**+**
Exercise	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

0= no effect, + = improvement

Table 5.2. Summary results of protein levels

	Naive group			Short-term effects of exercise group			Long-term effects of exercise group		
	BDNF	pERK	MKP-1	BDNF	pERK	MKP-1	BDNF	pERK	MKP-1
Maternal separation	**-(L)**	**0**	**0**	**0**	**+(L)**	**0**	**-(L)**	**0**	**0**
Exercise	**-(R)**	**+(L)**	**+(L)**	**0**	**0/-**	**0**	**0**	**0**	**0**

-= decrease; 0= no change; += increase. L= left dorsal hippocampus; R= right dorsal hippocampus

5.6. The effect of maternal separation and/or exercise across the left and right hippocampus has to be further studied

The data in this current study strongly suggested that the effect of exercise on hippocampal proteins was largely targeted towards the left dorsal hippocampus rather than the right dorsal hippocampus (Table 5.2). In the left dorsal hippocampus, pERK and MKP-1 were increased while in the right dorsal hippocampus BDNF was decreased. The functional significance of this finding is yet to be determined as, at present, very few studies exist that dissect the functional roles for the left and right dorsal hippocampus in spatial learning and memory. A group of researchers inactivated the left and right dorsal hippocampi of rats with lidocaine and then trained the rats in the MWM [149]. It was found that inactivation of the left dorsal hippocampus before the probe trial did not affect memory but bilateral or right side infusions of lidocaine, impaired memory for the platform. Interestingly, when lidocaine was infused before acquisition, learning was not impaired in either group but rats with left dorsal hippocampal infusions experienced impaired memory [149]. These findings provided evidence that the right dorsal hippocampus played a role in retrieval processes while the left is involved in consolidation of new information [149]. This observation was consistent with early clinical data whereby patients with unilateral lobectomies resulted in poorer memory performance which was most severe in patients with right temporal lobectomy [179]. These earlier works still do not explain the current data from this study as pERK was increased in the left dorsal hippocampus of non-separated runners however, this group did not exhibit improved memory at either time point when tested in the MWM, suggesting that a more efficient pERK signalling pathway in hippocampus was not the only requirement for improved spatial memory.

5.7. Alternative accounts of results not explored experimentally

5.7.1. Maternal care

Although not measured in this current study, it is worth noting that other aspects of maternal care play a role in the development and subsequent behaviours of rats [97,180,181]. High licking and grooming as well as arched-back nursing have been described as maternal behaviours that affect pups just as much as environmental enrichment [97]. Increased licking and grooming behaviour from the dam was correlated with improved brain plasticity; long-lasting survival of neuronal cells and increases in basic fibroblastic growth factor were observed in adult rats [182]. This is especially important because this study involved MS, which has previously been found to alter maternal care [180]. However, it is unlikely that MS rats in this current study had improved memory due to high maternal care because brief MS (15 minutes), not the 3-hour separation, induced increased licking grooming by the dam [182].

5.7.2. Attentional abilities

Another possible explanation for improved learning in MS rats could be due to the increased focus and attention which was required for optimal learning to take place [183]. Attention-based learning requires that cues be relevant to the task thus making the learning of a task easier [183,184]. Attention was a requirement for the assimilation of environmental cues necessary to learn the position of the spatial cues in relation to the platform. In this study, there were no intentionally distracting cues involved. Even in reversal training, all spatial cues remained the same as in the acquisition trials, thus the static environment may have facilitated reversal learning especially in MS rats, which had improved memory. It has been demonstrated that MS enhanced attentional abilities in an active avoidance task [184]. Therefore, it was possible that the MS rats in this current study had improved reversal learning memory due to

enhanced attention to the task. However, the pathways involved in spatial learning and active avoidance are slightly different. Thus, the possibility exists that the MS-induced attention was unique to the active avoidance pathway.

In contrast, exercise has been reported to have age-dependent effects on attentional abilities [185]. Attention was not altered in young adults but was improved in older adults [185]. Although, the above-mentioned study was conducted in humans, it is possible that exercise may have very little effect on attention in young adult rats too. There is limited literature examining the effects of exercise on attention. In the only study found, exercise improved learning in the same manner as methylphenidate, a treatment for ADHD, which improves attention in individuals with ADHD [186]. However, it is not known whether this improvement was a result of improved attentional abilities [186].

5.8. Study limitations

Several limitations impact the findings of this study, including the non-handling of non-separated pups. Handling of the pups would have created a greater difference in anxiety-like behaviours and may have been a better suited control as the MS rats were handled during the MS paradigm [187]. However, the rats in this current study were not handled in the interests of methodological consistency, so as to be able to compare the results to previous work in the laboratory. The absence of a communally-housed control group for non-exercised rats may have also confounded the results as social isolation creates stress and therefore the baseline for behaviour may not have been a true representation [66]. However, to keep all experimental conditions the same, the control group had to be placed in immobilised running-wheel cages.

These data showed that MS and exercise had differential effects on left and right dorsal hippocampal proteins. Future studies should be directed at determining whether these effects also extend to other brain regions known to be involved in spatial learning such as the pre-frontal cortex, the ventral hippocampus, and possibly, the striatum. As improved attentional abilities were suggested to have played a role in the improvement of reversal memory in MS rats, studies that investigate attention could also be done to determine the effect of exercise on attention in young adult rats.

5.9.Conclusion

New evidence arising from this study has highlighted that maternal separation enhanced reversal spatial memory when the MWM task is carried out 2 weeks after the end of voluntary exercise program. Exercise during the juvenile stage of rats did not change the outcomes of MWM task immediately nor 15 days after the cessation of exercise.

Protein analysis of the left dorsal hippocampus, suggested that exercise and MS act through different pre-cursor pathways to elevate pERK levels in naive rats. The decrease in BDNF due to MS and exercise were restricted to the left and right dorsal hippocampus, respectively.

Increases in pERK were also maintained for at least 10 days post voluntary exercise possibly due to brief repeated swimming activity in the MWM task. This is evidenced by the decayed pERK signal in Cohort 3 that was deprived of any form of exercise for 15 days post-voluntary exercise regimen.

The lack of significant correlations in behaviour and protein levels also suggests that the left hippocampus has very little to do with learning of a spatial task but may be more indicative

of HPA axis activity as the left hippocampus is known to be involved in mediating stress responses.

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Appendix A: Research outputs

- Poster presentation at the Neurological Association of South Africa, 2013

  Title: *Elevated plasticity-related protein levels post-exercise do not predict the outcome of spatial learning and memory in the Morris Water Maze task in maternally separated rats*

- HVS Image SFN 2013 Travel Award winner for oral presentation at Society for Neuroscience's Neuroscience Meeting 2013

  Title: *The differential effects of maternal separation and exercise on spatial learning and memory and plasticity proteins in the hippocampus*

Appendix B: Animals used

Table A1: Animal grouping and codes

COHORT 1			COHORT 2			COHORT 3		
Experimental group	**Litter code**	**Rat number**	**Experimental group**	**Litter code**	**Rat number**	**Experimental group**	**Litter code**	**Rat number**
NSnR	X	122	NSnR	K	62	NSnR	M	75
NSnR	X	123	NSnR	M	73	NSnR	W	117
NSnR	AK	192	NSnR	M	74	NSnR	W	118
NSnR	AK	190	NSnR	Q	92	NSnR	W	119
NSnR	AK	189	NSnR	Q	93	NSnR	W	120
NSnR	AJ	183	NSnR	Q	94	NSnR	W	121
NSnR	AJ	184	NSnR	AB	143	NSnR	X	124
NSnR	AH	185	NSnR	AB	144	NSnR	X	125
NSnR	P	89	NSnR	AC	145	NSnR	V	114
NSnR	P	90	NSnR	AC	146	NSnR	V	115
NSR	K	60	NSnR	AH	174	NSnR	AG	170
NSR	M	71	NSnR	AH	175	NSnR	AG	171
NSR	AK	191	NSR	K	62	NSR	M	69
NSR	AH	172	NSR	M	70	NSR	M	72
NSR	AJ	186	NSR	Q	95	NSR	Z	136
NSR	AJ	187	NSR	Q	96	NSR	Z	137
NSR	AG	188	NSR	Q	97	NSR	Z	138
NSR	AH	166	NSR	Z	133	NSR	V	116
NSR	/	173	NSR	Z	134	NSR	V	116B
NSR	P	88	NSR	Z	135	NSR	AC	149
MSnR	P	91	NSR	AC	147	NSR	AC	150
MSnR	L	67	NSR	AC	148	NSR	AG	167

MSnR	N	80	NSR	AH	176	NSR	AG	168
COHORT 1			**COHORT 2**			**COHORT 3**		
Experimental group	**Litter code**	**Rat number**	**Experimental group**	**Litter code**	**Rat number**	**Experimental group**	**Litter code**	**Rat number**
MSnR	O	86	NSR	AK	189	NSR	AG	169
MSnR	AE	158	MSnR	L	66	MSnR	L	68
MSnR	AE	159	MSnR	N	81	MSnR	N	82
MSnR	AE	160	MSnR	N	82	MSnR	O	87
MSnR	AF	161	MSnR	R	99	MSnR	T	108
MSnR	AF	162	MSnR	R	100	MSnR	T	107
MSnR	AF	163	MSnR	S	102B	MSnR	AA	139
MSnR	AF	164	MSnR	S	103	MSnR	AA	140
MSnR	P	98	MSnR	U	109	MSnR	AA	141
MSR	L	63	MSnR	U	110	MSnR	AA	142
MSR	N	76	MSnR	U	111	MSnR	AD	151
MSR	O	84	MSnR	AE	154	MSnR	AD	152
MSR	AI	178	MSnR	AE	155	MSnR	AD	153
MSR	AI	179	MSR	L	64	MSR	L	65
MSR	AI	180	MSR	N	78	MSR	N	77
MSR	AI	181	MSR	R	101	MSR	N	79
MSR	AI	182	MSR	R	102	MSR	O	85
MSR	AF	165	MSR	Q	112	MSR	T	104
MSR	P	101A	MSR	Q	113	MSR	T	105
			MSR	Y	126	MSR	T	106
			MSR	Y	127	MSR	Y	130
			MSR	Y	128	MSR	Y	131
			MSR	Y	129	MSR	Y	132
			MSR	AE	156	MSR	AD	194
			MSR	AE	157	MSR	AD	195

Table A2: Summary of experimental groups

	Naive group (cohort 1)				**Short-term effects of exercise group (cohort 2)**				**Long-term effects of exercise group (cohort 3)**			
Separation	Non-separated		Maternally separated		Non-separated		Maternally separated		Non-separated		Maternally separated	
Exercise	Non-exercised	Exercised	Non-exercised	Exercised	Non-exercised	Exercised	Non-exercised	Exercised	Non-exercised	Exercised	Non-exercised	Exercised
Group	NSnR N=11	NSR N=11	MSnR N=11	MSR N=10	NSnR N=12	NSR N=12	MSnR N=12	MSR N=12	NSnR N=12	NSR N=12	MSnR N=12	MSR N=12

Cohort 1 or the Naive group only underwent maternal separation and exercise without being trained in the Morris water maze. Cohorts 2 and 3 were trained in the MWM 1 and 15 days post-exercise, respectively. NSnR= non-separated non-runner; NSR= non-separated runner; MSnR= maternally separated non-runner; MSR= maternally separated runners.

Appendix B: Statistical analyses

Morris water maze (short-term)

Variable	Descriptive Statistics (ST Distance in MWM acq 30 April)								
	Valid N	Mean	Median	Minimum	Maximum	Lower Quartile	Upper Quartile	Std.Dev.	Standard Error
Day 1	43	2199.82	2227.95	1142.66	3301.43	1734.38	2607.13	536.826	81.865
Day 2	43	1613.63	1527.87	386.35	2942.08	970.96	2302.36	722.482	110.177
Day 3	43	970.40	738.93	124.07	2556.60	515.90	1361.71	576.434	87.905
Day 4	48	681.41	595.84	157.35	1796.78	387.26	864.04	408.109	58.905
Day 5	48	490.16	438.68	103.73	1014.30	316.35	587.11	240.147	34.662

Effect	Repeated Measures Analysis of Variance (ST Distance in MWM acq Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	29939752	1	29939752	384.603	0.00000
Separation	86910	1	86910	1.116	0.29718
Exercise	45146	1	45146	0.579	0.45091
Separation*Exercise	3658	1	3658	0.047	0.82950
Error	3035986	39	77845		
DAY	8071596	4	2017899	134.751	0.00000
DAY*Separation	243800	4	60950	4.070	0.00363
DAY*Exercise	31333	4	7833	0.523	0.71889
DAY*Separation*Exercise	20412	4	5103	0.340	0.85010
Error	2336095	156	14975		

Duncan test; variable DV_1 (ST Distance in MWM acq 30 April)
Approximate Probabilities for Post Hoc Tests
Error: Between; Within; Pooled MS = 2755E2, df = 106.37

Cell No.	Separation	Exercise	DAY	{1} 2281.8	{2} 1764.8	{3} 958.99	{4} 525.67	{5} 569.17	{6} 2415.9	{7} 1763.9	{8} 1076.3	{9} 668.35	{10} 506.12
1	NS	nR	Day 1		0.00380	0.00000	0.00000	0.00000	0.55789	0.04348	0.00001	0.00001	0.0000
2	NS	nR	Day 2	0.00380		0.00000	0.00000	0.00000	0.01032	0.99719	0.00656	0.00004	0.0000
3	NS	nR	Day 3	0.00000	0.00000		0.02353	0.04050	0.00001	0.00161	0.63194	0.26469	0.0967
4	NS	nR	Day 4	0.00000	0.00000	0.02353		0.79591	0.00001	0.00001	0.04147	0.56018	0.9370
5	NS	nR	Day 5	0.00000	0.00000	0.04050	0.79591		0.00001	0.00002	0.05898	0.66475	0.8056
6	NS	R	Day 1	0.55789	0.01032	0.00001	0.00001	0.00001		0.00029	0.00000	0.00000	0.0000
7	NS	R	Day 2	0.04348	0.99719	0.00161	0.00001	0.00002	0.00029		0.00008	0.00000	0.0000
8	NS	R	Day 3	0.00001	0.00656	0.63194	0.04147	0.05898	0.00000	0.00008		0.03404	0.0029
9	NS	R	Day 4	0.00001	0.00004	0.26469	0.56018	0.66475	0.00000	0.00000	0.03404		0.4007
10	NS	R	Day 5	0.00001	0.00001	0.09677	0.93706	0.80562	0.00000	0.00000	0.00294	0.40077	
11	MS	nR	Day 1	0.24197	0.31296	0.00006	0.00001	0.00001	0.09568	0.34230	0.00030	0.00001	0.0000
12	MS	nR	Day 2	0.00098	0.18574	0.06036	0.00051	0.00090	0.00015	0.16117	0.13335	0.00332	0.0004
13	MS	nR	Day 3	0.00001	0.00012	0.43142	0.35964	0.43873	0.00001	0.00011	0.23118	0.69313	0.3429

Repeated Measures Analysis of Variance (ST Thigmotaxis in MWM ac
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	171333.	1	171333.	154.479	0.00000
Separation	923.4	1	923.4	0.832	0.36713
Exercise	8.6	1	8.6	0.007	0.93016
Separation*Exercise	674.6	1	674.6	0.608	0.44016
Error	43255.	39	1109.		
DAY	90248.	4	22562.	129.059	0.00000
DAY*Separation	2917.	4	729.3	4.171	0.00308
DAY*Exercise	215.0	4	53.8	0.307	0.87265
DAY*Separation*Exercise	65.6	4	16.4	0.093	0.98431
Error	27271.	156	174.8		

Duncan test; variable DV_1 (ST Thigmotaxis in MWM acq 30 April)
Approximate Probabilities for Post Hoc Tests
Error: Between; Within; Pooled MS = 361.68, df = 94.309

Cell No.	Separation	Exercise	DAY	{1} 74.754	{2} 44.554	{3} 22.296	{4} 12.492	{5} 8.5000	{6} 70.700	{7} 37.658	{8} 17.133	{9} 9.6833	{10} 7.5542
1	NS	nR	Day 1		0.00000	0.00000	0.00000	0.00000	0.62504	0.00008	0.00001	0.00001	0.00000
2	NS	nR	Day 2	0.00000		0.00025	0.00000	0.00000	0.00374	0.40636	0.00426	0.00029	0.00014
3	NS	nR	Day 3	0.00000	0.00025		0.14594	0.04275	0.00001	0.09266	0.58672	0.20072	0.14315
4	NS	nR	Day 4	0.00000	0.00000	0.14594		0.53458	0.00001	0.00972	0.60105	0.73491	0.60325
5	NS	nR	Day 5	0.00000	0.00000	0.04275	0.53458		0.00001	0.00293	0.37103	0.89439	0.90923
6	NS	R	Day 1	0.62504	0.00374	0.00001	0.00001	0.00001		0.00000	0.00000	0.00000	0.00000
7	NS	R	Day 2	0.00008	0.40636	0.09266	0.00972	0.00293	0.00000		0.00122	0.00000	0.00000
8	NS	R	Day 3	0.00001	0.00426	0.58672	0.60105	0.37103	0.00000	0.00122		0.24240	0.15588
9	NS	R	Day 4	0.00001	0.00029	0.20072	0.73491	0.89439	0.00000	0.00000	0.24240		0.74110
10	NS	R	Day 5	0.00000	0.00014	0.14315	0.60325	0.90923	0.00000	0.00000	0.15588	0.74110	
11	MS	nR	Day 1	0.02463	0.23796	0.00053	0.00002	0.00001	0.06406	0.05796	0.00009	0.00001	0.00001
12	MS	nR	Day 2	0.00002	0.12317	0.32930	0.06074	0.02413	0.00004	0.41843	0.15552	0.03087	0.01912
13	MS	nR	Day 3	0.00001	0.00524	0.64549	0.54683	0.32650	0.00001	0.03984	0.90385	0.37696	0.28557

Repeated Measures Analysis of Variance (ST Latency in MWM acq 30 April
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	518480.	1	518480.	379.189	0.00000
Separation	594.0	1	594.0	0.434	0.51369
Exercise	170.8	1	170.8	0.124	0.72563
Separation*Exercise	353.8	1	353.8	0.258	0.61385
Error	53326.	39	1367.		
DAY	136881.	4	34220.	130.212	0.00000
DAY*Separation	3167.2	4	791.8	3.012	0.01987
DAY*Exercise	294.8	4	73.7	0.280	0.89029
DAY*Separation*Exercise	155.8	4	39.0	0.148	0.96354
Error	40997.	156	262.8		

Duncan test; variable DV_1 (ST Latency in MWM acq 30 April)
Approximate Probabilities for Post Hoc Tests
Error: Between; Within; Pooled MS = 483.71, df = 106.31

Cell No.	Separation	Exercise	DAY	{1} 96.996	{2} 70.592	{3} 41.572	{4} 24.787	{5} 23.804	{6} 99.696	{7} 66.579	{8} 38.900	{9} 26.833	{10} 21.800
1	NS	nR	Day 1		0.00034	0.00000	0.00000	0.00000	0.77824	0.00407	0.00001	0.00001	0.00001
2	NS	nR	Day 2	0.00034		0.00011	0.00000	0.00000	0.00607	0.67563	0.00349	0.00007	0.00001
3	NS	nR	Day 3	0.00000	0.00011		0.03770	0.02897	0.00001	0.01921	0.78047	0.18398	0.08570
4	NS	nR	Day 4	0.00000	0.00000	0.03770		0.88901	0.00001	0.00015	0.20378	0.83104	0.78066
5	NS	nR	Day 5	0.00000	0.00000	0.02897	0.88901		0.00001	0.00011	0.17989	0.76820	0.84551
6	NS	R	Day 1	0.77824	0.00607	0.00001	0.00001	0.00001		0.00001	0.00000	0.00000	0.00000
7	NS	R	Day 2	0.00407	0.67563	0.01921	0.00015	0.00011	0.00001		0.00024	0.00000	0.00000
8	NS	R	Day 3	0.00001	0.00349	0.78047	0.20378	0.17989	0.00000	0.00024		0.12960	0.03841
9	NS	R	Day 4	0.00001	0.00007	0.18398	0.83104	0.76820	0.00000	0.00000	0.12960		0.53511
10	NS	R	Day 5	0.00001	0.00001	0.08570	0.78066	0.84551	0.00000	0.00000	0.03841	0.53511	
11	MS	nR	Day 1	0.19056	0.17597	0.00010	0.00001	0.00001	0.12857	0.09473	0.00004	0.00001	0.00001
12	MS	nR	Day 2	0.00040	0.26778	0.09402	0.00185	0.00143	0.00017	0.44505	0.05892	0.00332	0.00081
13	MS	nR	Day 3	0.00001	0.00109	0.52683	0.34682	0.31410	0.00001	0.00375	0.69051	0.43653	0.24924

Repeated Measures Analysis of Variance (ST Velocity in MWM acq 30 A
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	113118.	1	113118.	5818.17	0.00000
Separation	38.6	1	38.6	1.987	0.16656
Exercise	3.2	1	3.2	0.164	0.68785
Separation*Exercise	9.7	1	9.7	0.500	0.48366
Error	758.2	39	19.4		
DAY	220.8	4	55.2	10.683	0.00000
DAY*Separation	58.4	4	14.6	2.827	0.02671
DAY*Exercise	38.9	4	9.7	1.879	0.11671
DAY*Separation*Exercise	24.6	4	6.2	1.191	0.31701
Error	806.2	156	5.2		

Duncan test; variable DV_1 (ST Velocity in MWM acq 30 April)
Approximate Probabilities for Post Hoc Tests
Error: Between; Within; Pooled MS = 8.0228, df = 129.44

Cell No.	Separation	Exercise	DAY	{1} 23.527	{2} 25.081	{3} 23.421	{4} 21.550	{5} 22.433	{6} 24.142	{7} 25.147	{8} 25.148	{9} 23.150	{10} 21.793
1	NS	nR	Day 1		0.17453	0.91438	0.09622	0.35849	0.64162	0.26696	0.27312	0.77510	0.24552
2	NS	nR	Day 2	0.17453		0.15249	0.00197	0.02509	0.49501	0.95725	0.95941	0.18887	0.02662
3	NS	nR	Day 3	0.91438	0.15249		0.11325	0.40146	0.60088	0.24236	0.24737	0.82545	0.27113
4	NS	nR	Day 4	0.09622	0.00197	0.11325		0.40358	0.08384	0.01507	0.01550	0.27956	0.84292
5	NS	nR	Day 5	0.35849	0.02509	0.40146	0.40358		0.25699	0.07283	0.07424	0.62123	0.60368
6	NS	R	Day 1	0.64162	0.49501	0.60088	0.08384	0.25699		0.37522	0.38493	0.38118	0.04687
7	NS	R	Day 2	0.26696	0.95725	0.24236	0.01507	0.07283	0.37522		0.99890	0.08957	0.00362
8	NS	R	Day 3	0.27312	0.95941	0.24737	0.01550	0.07424	0.38493	0.99890		0.09250	0.00376
9	NS	R	Day 4	0.77510	0.18887	0.82545	0.27956	0.62123	0.38118	0.08957	0.09250		0.24623
10	NS	R	Day 5	0.24552	0.02662	0.27113	0.84292	0.60368	0.04687	0.00362	0.00376	0.24623	
11	MS	nR	Day 1	0.61202	0.51719	0.57026	0.07545	0.23742	0.94537	0.50355	0.51541	0.45648	0.10634
12	MS	nR	Day 2	0.49504	0.63340	0.45821	0.04992	0.17531	0.79020	0.62097	0.63508	0.35905	0.07271
13	MS	nR	Day 3	0.15734	0.01245	0.17649	0.90069	0.45080	0.06759	0.01110	0.01142	0.23998	0.76363

Repeated Measures Analysis of Variance (Analysis in MWM Rev
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	17652685	1	17652685	188.2944	0.000000
Separation	8611	1	8611	0.0919	0.762117
Exercise	170310	1	170310	1.8166	0.179071
Day	2511986	2	1255993	13.3972	0.000003
Separation*Exercise	134397	1	134397	1.4336	0.232445
Separation*Day	328706	2	164353	1.7531	0.175595
Exercise*Day	334974	2	167487	1.7865	0.169911
Separation*Exercise*Day	685115	2	342558	3.6539	0.027437
Error	21093853	225	93750		

Effect	Univariate Tests of Significance for Distance to platform (ST Ave in MWM Rev Sigma-restricted parameterization Effective hypothesis decomposition SS	Degr. of Freedom	MS	F	p
Intercept	7429236	1	7429236	133.6007	0.000000
Separation	64183	1	64183	1.1542	0.296863
Exercise	4170	1	4170	0.0750	0.787325
Day	1389317	2	694658	12.4921	0.000396
Separation*Exercise	41335	1	41335	0.7433	0.399938
Separation*Day	299025	2	149512	2.6887	0.095124
Exercise*Day	497253	2	248627	4.4711	0.026519
Separation*Exercise*Day	53691	2	26845	0.4828	0.624840
Error	1000940	18	55608		

Cell No.	Duncan test; variable Distance to platform (ST Ave in MWM Rev 18 Oct) Approximate Probabilities for Post Hoc Tests Error: Between MS = 55608., df = 18.000 Separation	Exercise	Day	{1} 512.13	{2} 619.50	{3} 327.99	{4} 729.06	{5} 246.64	{6} 328.81	{7} 737.45	{8} 462.01	{9} 316.11	{10} 1268.8	{11} 316.83	{12} 229.41
1	NS	nR	1		0.624135	0.443198	0.353212	0.290153	0.431426	0.349730	0.818633	0.428019	0.004645	0.424711	0.263367
2	NS	nR	2	0.624135		0.237952	0.617099	0.145930	0.230679	0.611299	0.498353	0.229149	0.011529	0.226576	0.130979
3	NS	nR	3	0.443198	0.237952		0.113956	0.733682	0.997109	0.110210	0.563942	0.959961	0.001024	0.959343	0.685443
4	NS	R	1	0.353212	0.617099	0.113956		0.066272	0.110073	0.969500	0.269621	0.109685	0.027756	0.107973	0.059022
5	NS	R	2	0.290153	0.145930	0.733682	0.066272		0.735977	0.063198	0.384752	0.750769	0.000569	0.762005	0.937192
6	NS	R	3	0.431426	0.230679	0.997109	0.110073	0.735977		0.107689	0.543967	0.958857	0.000955	0.959632	0.686569
7	MS	nR	1	0.349730	0.611299	0.110210	0.969500	0.063198	0.107689		0.263961	0.104803	0.023960	0.103705	0.056114
8	MS	nR	2	0.818633	0.498353	0.563942	0.269621	0.384752	0.543967	0.263961		0.549519	0.003167	0.544460	0.352081
9	MS	nR	3	0.428019	0.229149	0.959961	0.109685	0.750769	0.958857	0.104803	0.549519		0.001036	0.997465	0.708412
10	MS	R	1	0.004645	0.011529	0.001024	0.027756	0.000569	0.000955	0.023960	0.003167	0.001036		0.000984	0.000504
11	MS	R	2	0.424711	0.226576	0.959343	0.107973	0.762005	0.959632	0.103705	0.544460	0.997465	0.000984		0.714612
12	MS	R	3	0.263367	0.130979	0.685443	0.059022	0.937192	0.686569	0.056114	0.352081	0.708412	0.000504	0.714612	

Univariate Tests of Significance for Duration in periphery (ST Ave in MWM Rev
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	2185.444	1	2185.444	53.97132	0.000001
Separation	57.800	1	57.800	1.42742	0.247692
Exercise	1.964	1	1.964	0.04849	0.828187
Day	980.325	2	490.162	12.10496	0.000466
Separation*Exercise	23.328	1	23.328	0.57610	0.457668
Separation*Day	158.490	2	79.245	1.95702	0.170196
Exercise*Day	245.857	2	122.928	3.03582	0.073097
Separation*Exercise*Day	10.162	2	5.081	0.12548	0.882835
Error	728.868	18	40.493		

Cell No.	Duncan test; variable Duration in periphery (ST Ave in MWM Rev 18 Oct) Approximate Probabilities for Post Hoc Tests Error: Between MS = 40.493, df = 18.000														
	Separation	Exercise	Day	{1} 9.5167	{2} 11.733	{3} 4.1167	{4} 14.600	{5} 1.9000	{6} 1.9000	{7} 16.750	{8} 7.5333	{9} 4.1833	{10} 26.200	{11} 3.7500	{12} 2.3500
1	NS	nR	1		0.707361	0.405243	0.419020	0.261847	0.264122	0.267885	0.736869	0.396864	0.017291	0.383160	0.286467
2	NS	nR	2	0.707361		0.252677	0.627772	0.154717	0.156391	0.425056	0.503350	0.248078	0.032961	0.236814	0.170865
3	NS	nR	3	0.405243	0.252677		0.125054	0.731117	0.736059	0.070434	0.585522	0.991088	0.003313	0.950451	0.777559
4	NS	R	1	0.419020	0.627772	0.125054		0.072560	0.073735	0.715741	0.278768	0.122590	0.073577	0.116345	0.080776
5	NS	R	2	0.261847	0.154717	0.731117	0.072560		1.000000	0.039464	0.399196	0.728353	0.001785	0.767368	0.939202
6	NS	R	3	0.264122	0.156391	0.736059	0.073735	1.000000		0.040226	0.402309	0.731415	0.001861	0.774315	0.943211
7	MS	nR	1	0.267885	0.425056	0.070434	0.715741	0.039464	0.040226		0.169343	0.069161	0.121314	0.065156	0.044184
8	MS	nR	2	0.736869	0.503350	0.585522	0.278768	0.399196	0.402309	0.169343		0.571422	0.009521	0.558246	0.432274
9	MS	nR	3	0.396864	0.248078	0.991088	0.122590	0.728353	0.731415	0.069161	0.571422		0.003162	0.945311	0.776289
10	MS	R	1	0.017291	0.032961	0.003313	0.073577	0.001785	0.001861	0.121314	0.009521	0.003162		0.003089	0.001991
11	MS	R	2	0.383160	0.236814	0.950451	0.116345	0.767368	0.774315	0.065156	0.558246	0.945311	0.003089		0.812397
12	MS	R	3	0.286467	0.170865	0.777559	0.080776	0.939202	0.943211	0.044184	0.432274	0.776289	0.001991	0.812397	

Effect	Univariate Tests of Significance for Latency to platform (ST Ave in MWM Rev Sigma-restricted parameterization Effective hypothesis decomposition				
	SS	Degr. of Freedom	MS	F	p
Intercept	17016.81	1	17016.81	183.8890	0.000000
Separation	285.39	1	285.39	3.0840	0.096065
Exercise	3.98	1	3.98	0.0430	0.838129
Day	2374.23	2	1187.11	12.8283	0.000344
Separation*Exercise	55.61	1	55.61	0.6010	0.448280
Separation*Day	665.16	2	332.58	3.5940	0.048609
Exercise*Day	756.85	2	378.43	4.0894	0.034348
Separation*Exercise*Day	45.60	2	22.80	0.2464	0.784230
Error	1665.69	18	92.54		

Cell No.	Duncan test; variable Latency to platform (ST Ave in MWM Rev 18 Oct) Approximate Probabilities for Post Hoc Tests Error: Between MS = 92.538, df = 18.000														
	Separation	Exercise	Day	{1} 21.883	{2} 27.717	{3} 16.933	{4} 31.925	{5} 13.100	{6} 15.400	{7} 37.250	{8} 23.217	{9} 16.617	{10} 56.300	{11} 17.825	{12} 13.525
1	NS	nR	1		0.538350	0.601132	0.307896	0.388053	0.514775	0.131388	0.881114	0.589610	0.002214	0.649655	0.407811
2	NS	nR	2	0.538350		0.283110	0.637684	0.162977	0.231313	0.318014	0.614719	0.275177	0.007027	0.315007	0.173117
3	NS	nR	3	0.601132	0.283110		0.145550	0.699499	0.871279	0.055504	0.520445	0.971757	0.000813	0.920361	0.726712
4	NS	R	1	0.307896	0.637684	0.145550		0.078989	0.116131	0.551971	0.360785	0.141097	0.016109	0.164513	0.084181
5	NS	R	2	0.388053	0.162977	0.699499	0.078989		0.807801	0.028619	0.324092	0.718386	0.000420	0.637965	0.962052
6	NS	R	3	0.514775	0.231313	0.871279	0.116131	0.807801		0.043458	0.438300	0.891475	0.000646	0.803819	0.833447
7	MS	nR	1	0.131388	0.318014	0.055504	0.551971	0.028619	0.043458		0.158688	0.053847	0.043825	0.063574	0.030522
8	MS	nR	2	0.881114	0.614719	0.520445	0.360785	0.324092	0.438300	0.158688		0.507328	0.002738	0.569297	0.342153
9	MS	nR	3	0.589610	0.275177	0.971757	0.141097	0.718386	0.891475	0.053847	0.507328		0.000808	0.898546	0.743677
10	MS	R	1	0.002214	0.007027	0.000813	0.016109	0.000420	0.000646	0.043825	0.002738	0.000808		0.000927	0.000445
11	MS	R	2	0.649655	0.315007	0.920361	0.164513	0.637965	0.803819	0.063574	0.569297	0.898546	0.000927		0.665025
12	MS	R	3	0.407811	0.173117	0.726712	0.084181	0.962052	0.833447	0.030522	0.342153	0.743677	0.000445	0.665025	

Effect	Univariate Tests of Significance for Mean velocity (ST Ave in MWM Rev Sigma-restricted parameterization Effective hypothesis decomposition SS	Degr. of Freedom	MS	F	p
Intercept	9736.945	1	9736.945	2245.388	0.000000
Separation	17.595	1	17.595	4.058	0.059166
Exercise	0.708	1	0.708	0.163	0.690898
Day	38.684	2	19.342	4.460	0.026709
Separation*Exercise	2.006	1	2.006	0.463	0.505062
Separation*Day	7.533	2	3.766	0.869	0.436409
Exercise*Day	18.719	2	9.360	2.158	0.144468
Separation*Exercise*Day	7.313	2	3.656	0.843	0.446654
Error	78.056	18	4.336		

Cell No.	Duncan test; variable Mean velocity (ST Ave in MWM Rev 18 Oct) Approximate Probabilities for Post Hoc Tests Error: Between MS = 4.3364, df = 18.000 Separation	Exercise	Day	{1} 21.388	{2} 19.766	{3} 17.614	{4} 21.468	{5} 17.291	{6} 17.485	{7} 16.771	{8} 17.755	{9} 17.969	{10} 20.418	{11} 16.249	{12} 16.471
1	NS	nR	1		0.430454	0.093374	0.967014	0.074819	0.086242	0.048197	0.101005	0.114703	0.615964	0.031090	0.037426
2	NS	nR	2	0.430454		0.312696	0.422414	0.261540	0.293851	0.181134	0.330199	0.357237	0.735845	0.123663	0.145676
3	NS	nR	3	0.093374	0.312696		0.090020	0.874725	0.946473	0.689619	0.941977	0.862407	0.200105	0.530566	0.595118
4	NS	R	1	0.967014	0.422414	0.090020		0.071249	0.082568	0.045785	0.098401	0.113480	0.608203	0.029380	0.035457
5	NS	R	2	0.074819	0.261540	0.874725	0.071249		0.920238	0.787668	0.826609	0.752674	0.163631	0.621804	0.688734
6	NS	R	3	0.086242	0.293851	0.946473	0.082568	0.920238		0.727463	0.895226	0.818647	0.186213	0.565940	0.631381
7	MS	nR	1	0.048197	0.181134	0.689619	0.045785	0.787668	0.727463		0.647289	0.581618	0.109713	0.798636	0.876434
8	MS	nR	2	0.101005	0.330199	0.941977	0.098401	0.826609	0.895226	0.647289		0.911532	0.214272	0.492294	0.555193
9	MS	nR	3	0.114703	0.357237	0.862407	0.113480	0.752674	0.818647	0.581618	0.911532		0.238295	0.435493	0.494439
10	MS	R	1	0.615964	0.735845	0.200105	0.608203	0.163631	0.186213	0.109713	0.214272	0.238295		0.072953	0.086828
11	MS	R	2	0.031090	0.123663	0.530566	0.029380	0.621804	0.565940	0.798636	0.492294	0.435493	0.072953		0.908423
12	MS	R	3	0.037426	0.145676	0.595118	0.035457	0.688734	0.631381	0.876434	0.555193	0.494439	0.086828	0.908423	

Reversal training

Distance to platform

Effect	Multivariate Tests of Significance (Analysis Output-Thuli MWM1 reversal(3)(1)) Sigma-restricted parameterization Effective hypothesis decomposition					
	Test	Value	F	Effect df	Error df	p
Intercept	Wilks	0.096338	81.29393	3	26	0.000000
Separation	Wilks	0.958736	0.37302	3	26	0.773140
Exercise	Wilks	0.881248	1.16788	3	26	0.340965
Separation*Exercise	Wilks	0.864390	1.35967	3	26	0.277000

Cell No.	LSD test; variable Day 1 (Analysis Output-Thuli MWM1 reversal(3)(1)) Probabilities for Post Hoc Tests Error: Between MS = 1534E2, df = 28.000					
	Separation	Exercise	{1} 650.31	{2} 846.96	{3} 1031.4	{4} 739.74
1	MS	R		0.317073	0.086590	0.613723
2	MS	nR	0.317073		0.428163	0.583003
3	NS	R	0.086590	0.428163		0.184907
4	NS	nR	0.613723	0.583003	0.184907	

LSD test; variable Day 2 (Analysis Output-Thuli MWM1 reversal(3)(1))
Probabilities for Post Hoc Tests
Error: Between MS = 62895., df = 28.000

Cell No.	Separation	Exercise	{1} 340.92	{2} 571.97	{3} 497.04	{4} 538.33
1	MS	R		0.072049	0.265370	0.089309
2	MS	nR	0.072049		0.613861	0.787474
3	NS	R	0.265370	0.613861		0.765936
4	NS	nR	0.089309	0.787474	0.765936	

LSD test; variable Day 3 (Analysis Output-Thuli MWM1 reversal(3)(1))
Probabilities for Post Hoc Tests
Error: Between MS = 28818., df = 28.000

Cell No.	Separation	Exercise	{1} 359.28	{2} 360.56	{3} 384.64	{4} 397.85
1	MS	R		0.987949	0.787064	0.615401
2	MS	nR	0.987949		0.810323	0.659167
3	NS	R	0.787064	0.810323		0.888027
4	NS	nR	0.615401	0.659167	0.888027	

Morris water maze (long-term)

Effect	Repeated Measures Analysis of Variance (LT Distance in MWM acq 30 Ap Sigma-restricted parameterization Effective hypothesis decomposition SS	Degr. of Freedom	MS	F	p
Intercept	23929042	1	23929042	509.545	0.00000
Separation	3287	1	3287	0.007	0.93370
Exercise	32250	1	32250	0.686	0.41174
Separation*Exercise	77383	1	77383	1.647	0.20597
Error	2066308	44	46961		
DAY	7373690	4	1843422	156.380	0.00000
DAY*Separation	75519	4	18879	1.601	0.17594
DAY*Exercise	26305	4	6576	0.557	0.69352
DAY*Separation*Exercise	95593	4	23898	2.027	0.09257
Error	2074700	176	11788		

Duncan test; variable DV_1 (LT Distance in MWM acq 30 April)
Approximate Probabilities for Post Hoc Tests
Error: Between; Within; Pooled MS = 1882E2, df = 141.14

Cell No.	Separation	Exercise	DAY	{1} 2011.5	{2} 1286.3	{3} 542.33	{4} 589.58	{5} 443.83	{6} 1936.1	{7} 1551.2	{8} 685.54	{9} 427.03	{10} 474.92
1	NS	nR	Day 1		0.00000	0.00000	0.00000	0.00000	0.67040	0.01812	0.00000	0.00000	0.0000
2	NS	nR	Day 2	0.00000		0.00000	0.00000	0.00000	0.00066	0.15920	0.00129	0.00000	0.0000
3	NS	nR	Day 3	0.00000	0.00000		0.75358	0.52977	0.00000	0.00000	0.49170	0.57222	0.7230
4	NS	nR	Day 4	0.00000	0.00000	0.75358		0.37483	0.00000	0.00000	0.62861	0.44218	0.5743
5	NS	nR	Day 5	0.00000	0.00000	0.52977	0.37483		0.00000	0.00000	0.26099	0.92445	0.8606
6	NS	R	Day 1	0.67040	0.00066	0.00000	0.00000	0.00000		0.01034	0.00000	0.00000	0.0000
7	NS	R	Day 2	0.01812	0.15920	0.00000	0.00000	0.00000	0.01034		0.00000	0.00000	0.0000
8	NS	R	Day 3	0.00000	0.00129	0.49170	0.62861	0.26099	0.00000	0.00000		0.12740	0.2090
9	NS	R	Day 4	0.00000	0.00000	0.57222	0.44218	0.92445	0.00000	0.00000	0.12740		0.7503
10	NS	R	Day 5	0.00000	0.00002	0.72300	0.57437	0.86064	0.00000	0.00000	0.20907	0.75035	
11	MS	nR	Day 1	0.39460	0.00318	0.00000	0.00000	0.00000	0.62757	0.11102	0.00000	0.00000	0.0000
12	MS	nR	Day 2	0.00364	0.36387	0.00000	0.00000	0.00000	0.01161	0.55690	0.00004	0.00000	0.0000
13	MS	nR	Day 3	0.00000	0.08785	0.03656	0.05657	0.01064	0.00000	0.00215	0.11254	0.00860	0.0161

Repeated Measures Analysis of Variance (LT Thigmotaxy in MWM acq 30 Apri
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	115256.	1	115256.	234.588	0.00000
Separation	480.3	1	480.3	0.977	0.32818
Exercise	404.2	1	404.2	0.822	0.36932
Separation*Exercise	903.9	1	903.9	1.839	0.18189
Error	21617.9	44	491.3		
DAY	76907.2	4	19226.8	197.884	0.00000
DAY*Separation	595.3	4	148.8	1.531	0.19496
DAY*Exercise	220.4	4	55.1	0.567	0.68687
DAY*Separation*Exercise	1550.4	4	387.6	3.989	0.00400
Error	17100.5	176	97.2		

Duncan test; variable DV_1 (LT Thigmotaxy in MWM acq 30 April)
Approximate Probabilities for Post Hoc Tests
Error: Between; Within; Pooled MS = 175.99, df = 122.05

Cell No.	Separation	Exercise	DAY	{1} 56.629	{2} 22.750	{3} 6.5417	{4} 7.4458	{5} 5.9167	{6} 54.050	{7} 30.304	{8} 10.067	{9} 4.6208	{10} 6.6708
1	NS	nR	Day 1		0.00000	0.00000	0.00000	0.00000	0.65725	0.00000	0.00000	0.00000	0.00000
2	NS	nR	Day 2	0.00000		0.00025	0.00051	0.00014	0.00000	0.18981	0.03471	0.00381	0.00947
3	NS	nR	Day 3	0.00000	0.00025		0.83436	0.87658	0.00000	0.00006	0.58064	0.75182	0.98098
4	NS	nR	Day 4	0.00000	0.00051	0.83436		0.73470	0.00000	0.00011	0.66586	0.65836	0.88622
5	NS	nR	Day 5	0.00000	0.00014	0.87658	0.73470		0.00000	0.00004	0.52198	0.82378	0.89690
6	NS	R	Day 1	0.65725	0.00000	0.00000	0.00000	0.00000		0.00000	0.00000	0.00000	0.00000
7	NS	R	Day 2	0.00000	0.18981	0.00006	0.00011	0.00004	0.00000		0.00000	0.00000	0.00000
8	NS	R	Day 3	0.00000	0.03471	0.58064	0.66586	0.52198	0.00000	0.00000		0.26513	0.46306
9	NS	R	Day 4	0.00000	0.00381	0.75182	0.65836	0.82378	0.00000	0.00000	0.26513		0.65893
10	NS	R	Day 5	0.00000	0.00947	0.98098	0.88622	0.89690	0.00000	0.00000	0.46306	0.65893	
11	MS	nR	Day 1	0.37962	0.00000	0.00000	0.00000	0.00000	0.61486	0.00016	0.00000	0.00000	0.00000
12	MS	nR	Day 2	0.00179	0.00650	0.00000	0.00000	0.00000	0.00562	0.13158	0.00000	0.00000	0.00000

Repeated Measures Analysis of Variance (LT Latency in MWM acq 30 April
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	454237.	1	454237.	536.945	0.00000
Separation	190.7	1	190.7	0.225	0.63731
Exercise	296.8	1	296.8	0.350	0.55668
Separation*Exercise	1063.8	1	1063.8	1.257	0.26820
Error	37222.5	44	846.0		
DAY	134902.	4	33725.0	148.324	0.00000
DAY*Separation	1162.3	4	290.6	1.278	0.28044
DAY*Exercise	859.5	4	214.9	0.945	0.43930
DAY*Separation*Exercise	2268.5	4	567.1	2.494	0.04469
Error	40018.3	176	227.4		

Duncan test; variable DV_1 (LT Latency in MWM acq 30 April)
Approximate Probabilities for Post Hoc Tests
Error: Between; Within; Pooled MS = 351.09, df = 146.99

Cell No.	Separation	Exercise	DAY	{1} 88.117	{2} 51.921	{3} 22.492	{4} 25.825	{5} 19.746	{6} 83.567	{7} 64.754	{8} 29.475	{9} 19.408	{10} 20.829
1	NS	nR	Day 1		0.00000	0.00000	0.00000	0.00000	0.55197	0.00482	0.00000	0.00000	0.0000
2	NS	nR	Day 2	0.00000		0.00000	0.00006	0.00000	0.00011	0.12737	0.00590	0.00012	0.0002
3	NS	nR	Day 3	0.00000	0.00000		0.62880	0.69068	0.00000	0.00000	0.43686	0.72712	0.8279
4	NS	nR	Day 4	0.00000	0.00006	0.62880		0.40775	0.00000	0.00000	0.65662	0.48890	0.5709
5	NS	nR	Day 5	0.00000	0.00000	0.69068	0.40775		0.00000	0.00000	0.29584	0.96481	0.8951
6	NS	R	Day 1	0.55197	0.00011	0.00000	0.00000	0.00000		0.00402	0.00000	0.00000	0.0000
7	NS	R	Day 2	0.00482	0.12737	0.00000	0.00000	0.00000	0.00402		0.00000	0.00000	0.0000
8	NS	R	Day 3	0.00000	0.00590	0.43686	0.65662	0.29584	0.00000	0.00000		0.17923	0.2350
9	NS	R	Day 4	0.00000	0.00012	0.72712	0.48890	0.96481	0.00000	0.00000	0.17923		0.8370
10	NS	R	Day 5	0.00000	0.00022	0.82796	0.57097	0.89516	0.00000	0.00000	0.23509	0.83704	
11	MS	nR	Day 1	0.20488	0.00195	0.00000	0.00000	0.00000	0.45033	0.09875	0.00000	0.00000	0.0000
12	MS	nR	Day 2	0.00193	0.20238	0.00000	0.00001	0.00000	0.01099	0.74835	0.00005	0.00000	0.0000
13	MS	nR	Day 3	0.00000	0.18062	0.02994	0.06386	0.01423	0.00000	0.00543	0.13258	0.01327	0.0184

Repeated Measures Analysis of Variance (LT Velocity in MWM acq 30 Apri
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	115554.	1	115554.	7719.27	0.00000
Separation	59.3	1	59.3	3.961	0.05280
Exercise	4.2	1	4.2	0.279	0.60019
Separation*Exercise	17.3	1	17.3	1.156	0.28819
Error	658.7	44	15.0		
DAY	141.4	4	35.4	10.01	0.00000
DAY*Separation	18.1	4	4.5	1.283	0.27857
DAY*Exercise	18.8	4	4.7	1.331	0.26016
DAY*Separation*Exercise	10.9	4	2.7	0.769	0.54685
Error	621.6	176	3.5		

Cell No.	Separation	Exercise	DAY	{1} 22.797	{2} 23.363	{3} 22.741	{4} 21.838	{5} 20.776	{6} 23.042	{7} 24.255	{8} 23.050	{9} 20.493	{10} 22.041
Duncan test; variable DV_1 (LT Velocity in MWM acq 30 April) Approximate Probabilities for Post Hoc Tests Error: Between; Within; Pooled MS = 5.8194, df = 135.96													
1	NS	nR	Day 1		0.52213	0.94173	0.29204	0.02700	0.81738	0.20223	0.81907	0.05565	0.49224
2	NS	nR	Day 2	0.52213		0.49084	0.10166	0.00406	0.76140	0.36480	0.75084	0.01635	0.26389
3	NS	nR	Day 3	0.94173	0.49084		0.31369	0.03085	0.78568	0.19220	0.78611	0.06091	0.50726
4	NS	nR	Day 4	0.29204	0.10166	0.31369		0.23423	0.31552	0.04098	0.31673	0.25528	0.85384
5	NS	nR	Day 5	0.02700	0.00406	0.03085	0.23423		0.06027	0.00249	0.06064	0.78919	0.29072
6	NS	R	Day 1	0.81738	0.76140	0.78568	0.31552	0.06027		0.15142	0.99123	0.00476	0.26340
7	NS	R	Day 2	0.20223	0.36480	0.19220	0.04098	0.00249	0.15142		0.13877	0.00001	0.01200
8	NS	R	Day 3	0.81907	0.75084	0.78611	0.31673	0.06064	0.99123	0.13877		0.00478	0.26735
9	NS	R	Day 4	0.05565	0.01635	0.06091	0.25528	0.78919	0.00476	0.00001	0.00478		0.09616
10	NS	R	Day 5	0.49224	0.26389	0.50726	0.85384	0.29072	0.26340	0.01200	0.26735	0.09616	
11	MS	nR	Day 1	0.93428	0.66079	0.89678	0.38164	0.08137	0.86846	0.21953	0.87123	0.04802	0.45976
12	MS	nR	Day 2	0.42901	0.22063	0.44558	0.95130	0.34283	0.33071	0.04387	0.33370	0.24232	0.88780

Morris Water maze Reversal (Long-term)

Multivariate Tests of Significance (LT Ave in MWM Rev 18 Oct)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	Test	Value	F	Effect df	Error df	p
Intercept	Wilks	0.001122	2492.821	5	14	0.000000
Separation	Wilks	0.834617	0.555	5	14	0.732659
Exercise	Wilks	0.635604	1.605	5	14	0.222650
Day	Wilks	0.310221	2.227	10	28	0.046504
Separation*Exercise	Wilks	0.679777	1.319	5	14	0.311860
Separation*Day	Wilks	0.360380	1.864	10	28	0.094622
Exercise*Day	Wilks	0.560587	0.940	10	28	0.513598
Separation*Exercise*Day	Wilks	0.567689	0.916	10	28	0.532420

Univariate Tests of Significance for Distance to platform (Analysis in MWM re
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	58319303	1	58319303	177.4614	0.000000
Separation	29587	1	29587	0.0900	0.764415
Exercise	583718	1	583718	1.7762	0.183963
Day	8731178	2	4365589	13.2842	0.000004
Separation*Exercise	477111	1	477111	1.4518	0.229503
Separation*Day	1149464	2	574732	1.7489	0.176327
Exercise*Day	1187700	2	593850	1.8070	0.166513
Separation*Exercise*Day	2407979	2	1203990	3.6637	0.027180
Error	73941938	225	328631		

Cell No.	Duncan test; variable Distance to platform (Analysis in MWM rev stats 2 19Oct.stw) Approximate Probabilities for Post Hoc Tests Error: Between MS = 3286E2, df = 225.00														
	Separation	Exercise	Day	{1} 623.93	{2} 583.63	{3} 289.67	{4} 709.98	{5} 372.34	{6} 385.80	{7} 1211.5	{8} 350.74	{9} 277.71	{10} 559.32	{11} 379.56	{12} 323.18
1	NS	nR	1		0.828435	0.134076	0.643533	0.245246	0.248320	0.002254	0.213463	0.123585	0.746242	0.249268	0.175014
2	NS	nR	2	0.828435		0.185400	0.526136	0.320844	0.319226	0.001367	0.283144	0.172264	0.895963	0.323992	0.236383
3	NS	nR	3	0.134076	0.185400		0.057866	0.691635	0.661206	0.000005	0.759760	0.948704	0.219693	0.675379	0.856997
4	NS	R	1	0.643533	0.526136	0.057866		0.120512	0.122543	0.007005	0.101872	0.052469	0.468258	0.122834	0.080037
5	NS	R	2	0.245246	0.320844	0.691635	0.120512		0.946301	0.000028	0.907510	0.659240	0.366836	0.969026	0.805582
6	NS	R	3	0.248320	0.319226	0.661206	0.122543	0.946301		0.000029	0.866560	0.627847	0.350752	0.973229	0.770708
7	MS	nR	1	0.002254	0.001367	0.000005	0.007005	0.000028	0.000029		0.000020	0.000004	0.001039	0.000029	0.000010
8	MS	nR	2	0.213463	0.283144	0.759760	0.101872	0.907510	0.866560	0.000020		0.726139	0.327221	0.885303	0.882201
9	MS	nR	3	0.123585	0.172264	0.948704	0.052469	0.659240	0.627847	0.000004	0.726139		0.205366	0.642314	0.819946

10	MS	R	1	0.746242	0.895963	0.219693	0.468258	0.366836	0.350752	0.001039	0.327221	0.205366		0.365938	0.276326
11	MS	R	2	0.249268	0.323992	0.675379	0.122834	0.969026	0.973229	0.000029	0.885303	0.642314	0.365938		0.786937
12	MS	R	3	0.175014	0.236383	0.856997	0.080037	0.805582	0.770708	0.000010	0.882201	0.819946	0.276326	0.786937	

Univariate Tests of Significance for Duration in periphery (Analysis in MWM r
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	15743.10	1	15743.10	67.63508	0.000000
Separation	59.18	1	59.18	0.25423	0.614606
Exercise	807.51	1	807.51	3.46919	0.063825
Day	6782.61	2	3391.30	14.56962	0.000001
Separation*Exercise	218.28	1	218.28	0.93775	0.333897
Separation*Day	824.40	2	412.20	1.77089	0.172545
Exercise*Day	651.84	2	325.92	1.40020	0.248687
Separation*Exercise*Day	1303.91	2	651.95	2.80091	0.062875
Error	52372.20	225	232.77		

Cell No.	Duncan test; variable Duration in periphery (Analysis in MWM rev stats 2 19Oct.stw) Approximate Probabilities for Post Hoc Tests Error: Between MS = 232.77, df = 225.00														
	Separation	Exercise	Day	{1} 12.741	{2} 10.162	{3} 3.2000	{4} 12.813	{5} 4.7250	{6} 3.1467	{7} 27.330	{8} 4.3417	{9} 3.3565	{10} 10.500	{11} 4.6750	{12} 2.6960
1	NS	nR	1		0.627220	0.102620	0.988510	0.141515	0.104509	0.004498	0.141437	0.104011	0.650628	0.150023	0.091326
2	NS	nR	2	0.627220		0.226365	0.632612	0.271857	0.230540	0.001192	0.289864	0.227325	0.945631	0.298957	0.207137
3	NS	nR	3	0.102620	0.226365		0.103818	0.789742	0.991408	0.000006	0.829990	0.974787	0.211555	0.790479	0.924507
4	NS	R	1	0.988510	0.632612	0.103818		0.148904	0.105165	0.003352	0.144547	0.105927	0.663280	0.154880	0.091626
5	NS	R	2	0.141515	0.271857	0.789742	0.148904		0.787171	0.000018	0.942548	0.805313	0.274032	0.991945	0.732793
6	NS	R	3	0.104509	0.230540	0.991408	0.105165	0.787171		0.000007	0.829611	0.968531	0.214374	0.789292	0.927445
7	MS	nR	1	0.004498	0.001192	0.000006	0.003352	0.000018	0.000007		0.000017	0.000007	0.001254	0.000019	0.000005
8	MS	nR	2	0.141437	0.289864	0.829990	0.144547	0.942548	0.829611	0.000017		0.842218	0.275965	0.946301	0.773513
9	MS	nR	3	0.104011	0.227325	0.974787	0.105927	0.805313	0.968531	0.000007	0.842218		0.214125	0.804104	0.905349
10	MS	R	1	0.650628	0.945631	0.211555	0.663280	0.274032	0.214374	0.001254	0.275965	0.214125		0.289513	0.191601
11	MS	R	2	0.150023	0.298957	0.790479	0.154880	0.991945	0.789292	0.000019	0.946301	0.804104	0.289513		0.734841
12	MS	R	3	0.091326	0.207137	0.924507	0.091626	0.732793	0.927445	0.000005	0.773513	0.905349	0.191601	0.734841	

Effect	Univariate Tests of Significance for Latency to platform (Analysis in MWM re Sigma-restricted parameterization Effective hypothesis decomposition SS	Degr. of Freedom	MS	F	p
Intercept	126894.7	1	126894.7	257.3941	0.000000
Separation	251.8	1	251.8	0.5107	0.475595
Exercise	761.7	1	761.7	1.5451	0.215159
Day	13147.1	2	6573.5	13.3338	0.000003
Separation*Exercise	758.0	1	758.0	1.5375	0.216283
Separation*Day	2028.3	2	1014.1	2.0571	0.130223
Exercise*Day	1344.1	2	672.0	1.3632	0.257948
Separation*Exercise*Day	3338.0	2	1669.0	3.3854	0.035599
Error	110924.4	225	493.0		

Cell No.	All Groups Duncan test; variable Latency to platform (Analysis in MWM rev stats 2 19Oct.stw) Approximate Probabilities for Post Hoc Tests Error: Between MS = 493.00, df = 225.00 Separation	Exercise	Day	{1} 26.729	{2} 25.588	{3} 15.300	{4} 31.113	{5} 18.438	{6} 18.040	{7} 51.226	{8} 18.292	{9} 15.339	{10} 27.318	{11} 18.750	{12} 16.888
1	NS	nR	1		0.874029	0.188681	0.570372	0.300396	0.301641	0.001252	0.305735	0.185211	0.934850	0.299370	0.248404
2	NS	nR	2	0.874029		0.233313	0.492466	0.353020	0.360639	0.000858	0.363221	0.228752	0.823022	0.342413	0.301066
3	NS	nR	3	0.188681	0.233313		0.068448	0.712087	0.734400	0.000005	0.719099	0.995668	0.170085	0.689821	0.837409
4	NS	R	1	0.570372	0.492466	0.068448		0.126651	0.125755	0.005241	0.128382	0.066848	0.598298	0.128683	0.097759
5	NS	R	2	0.300396	0.353020	0.712087	0.126651		0.959048	0.000021	0.983857	0.709498	0.280477	0.965393	0.847964
6	NS	R	3	0.301641	0.360639	0.734400	0.125755	0.959048		0.000021	0.972127	0.726892	0.277365	0.930022	0.872926
7	MS	nR	1	0.001252	0.000858	0.000005	0.005241	0.000021	0.000021		0.000022	0.000005	0.001302	0.000022	0.000010
8	MS	nR	2	0.305735	0.363221	0.719099	0.128382	0.983857	0.972127	0.000022		0.714618	0.282811	0.952787	0.856073
9	MS	nR	3	0.185211	0.228752	0.995668	0.066848	0.709498	0.726892	0.000005	0.714618		0.167382	0.688179	0.829732
10	MS	R	1	0.934850	0.823022	0.170085	0.598298	0.280477	0.277365	0.001302	0.282811	0.167382		0.284273	0.226667
11	MS	R	2	0.299370	0.342413	0.689821	0.128683	0.965393	0.930022	0.000022	0.952787	0.688179	0.284273		0.823027
12	MS	R	3	0.248404	0.301066	0.837409	0.097759	0.847964	0.872926	0.000010	0.856073	0.829732	0.226667	0.823027	

Effect	Univariate Tests of Significance for Mean velocity (Analysis in MWM rev Sigma-restricted parameterization Effective hypothesis decomposition SS	Degr. of Freedom	MS	F	p
Intercept	81007.31	1	81007.31	3260.306	0.000000
Separation	99.52	1	99.52	4.006	0.046553
Exercise	28.53	1	28.53	1.148	0.285088
Day	324.03	2	162.02	6.521	0.001767
Separation*Exercise	0.03	1	0.03	0.001	0.973681
Separation*Day	39.01	2	19.51	0.785	0.457330
Exercise*Day	44.26	2	22.13	0.891	0.411785
Separation*Exercise*Day	101.04	2	50.52	2.033	0.133306
Error	5590.47	225	24.85		

Cell No.	Duncan test; variable Mean velocity (Analysis in MWM rev stats 2 19Oct.stw) Approximate Probabilities for Post Hoc Tests Error: Between MS = 24.847, df = 225.00 Separation	Exercise	Day	{1} 21.086	{2} 20.916	{3} 17.540	{4} 21.280	{5} 18.196	{6} 18.010	{7} 21.350	{8} 17.073	{9} 17.222	{10} 18.172	{11} 17.478	{12} 17.807
1	NS	nR	1		0.916473	0.057536	0.904155	0.091007	0.090535	0.879111	0.035551	0.041789	0.100542	0.056162	0.075947
2	NS	nR	2	0.916473		0.067116	0.833907	0.092454	0.101399	0.810979	0.043007	0.050014	0.109081	0.066177	0.086922
3	NS	nR	3	0.057536	0.067116		0.047000	0.725633	0.786904	0.045045	0.796633	0.854517	0.727377	0.969360	0.869006
4	NS	R	1	0.904155	0.833907	0.047000		0.081292	0.076720	0.965683	0.027996	0.033282	0.087036	0.045508	0.063218
5	NS	R	2	0.091007	0.092454	0.725633	0.081292		0.914796	0.082186	0.567376	0.615375	0.988480	0.706951	0.830353
6	NS	R	3	0.090535	0.101399	0.786904	0.076720	0.914796		0.074879	0.623074	0.672864	0.919921	0.769439	0.900198
7	MS	nR	1	0.879111	0.810979	0.045045	0.965683	0.082186	0.074879		0.026315	0.031465	0.086066	0.043324	0.061099
8	MS	nR	2	0.035551	0.043007	0.796633	0.027996	0.567376	0.623074	0.026315		0.926635	0.570279	0.815495	0.694141
9	MS	nR	3	0.041789	0.050014	0.854517	0.033282	0.615375	0.672864	0.031465	0.926635		0.617984	0.873982	0.746878
10	MS	R	1	0.100542	0.109081	0.727377	0.087036	0.988480	0.919921	0.086066	0.570279	0.617984		0.710079	0.833433
11	MS	R	2	0.056162	0.066177	0.969360	0.045508	0.706951	0.769439	0.043324	0.815495	0.873982	0.710079		0.849903
12	MS	R	3	0.075947	0.086922	0.869006	0.063218	0.830353	0.900198	0.061099	0.694141	0.746878	0.833433	0.849903	

BDNF

All Groups
Univariate Tests of Significance for New BDNF R (ELISA bdr
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	1184.90	1	1184.90	1665.42	0.00000
Separation	0.790	1	0.790	1.110	0.29584
Exercise	0.137	1	0.137	0.193	0.66171
Separation*Exercise	0.112	1	0.112	0.157	0.69332
Error	48.380	68	0.711		

Training=N
Univariate Tests of Significance for New BDNF R (ELISA bdn
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	421.587	1	421.587	700.518	0.00000
Separation	0.368	1	0.368	0.611	0.44335
Exercise	2.160	1	2.160	3.589	0.07270
Separation*Exercise	0.105	1	0.105	0.175	0.67987
Error	12.036	20	0.601		

Training=S
Univariate Tests of Significance for New BDNF R (ELISA bdn
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	332.585	1	332.585	425.202	0.00000
Separation	0.363	1	0.363	0.464	0.50332
Exercise	3.823	1	3.823	4.888	0.03885
Separation*Exercise	0.562	1	0.562	0.718	0.40658
Error	15.643	20	0.782		

Training=L
Univariate Tests of Significance for New BDNF R (ELISA bdnf
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	434.797	1	434.797	1004.18	0.00000
Separation	0.108	1	0.108	0.251	0.62187
Exercise	1.271	1	1.271	2.937	0.10202
Separation*Exercise	0.246	1	0.246	0.568	0.45970
Error	8.659	20	0.433		

All Groups
Duncan test; variable New BDNF R (ELISA bdnf)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = .71147, df = 68.000

Cell No.	Separation	Exercise	{1} 4.0350	{2} 3.8689	{3} 4.1658	{4} 4.1571
1	NS	nR		0.55674	0.66529	0.66566
2	NS	R	0.55674		0.34381	0.33961
3	MS	nR	0.66529	0.34381		0.97568
4	MS	R	0.66566	0.33961	0.97568	

Training=N
Duncan test; variable New BDNF R (ELISA bdnf)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = .60182, df = 20.000

Cell No.	Separation	Exercise	{1} 4.4337	{2} 3.7010	{3} 4.5487	{4} 4.0813
1	NS	nR		0.13652	0.79999	0.44087
2	NS	R	0.13652		0.09658	0.40607
3	MS	nR	0.79999	0.09658		0.33593
4	MS	R	0.44087	0.40607	0.33593	

Training=S
Duncan test; variable New BDNF R (ELISA bdnf)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = .78218, df = 20.000

Cell No.	Separation	Exercise	{1} 3.3535	{2} 3.8456	{3} 3.2935	{4} 4.3978
1	NS	nR		0.34676	0.90771	0.06585
2	NS	R	0.34676		0.31901	0.29251
3	MS	nR	0.90771	0.31901		0.05949
4	MS	R	0.06585	0.29251	0.05949	

Training=L
Duncan test; variable New BDNF R (ELISA bdnf)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = .43299, df = 20.000

Cell No.	Separation	Exercise	{1} 4.3180	{2} 4.0601	{3} 4.6551	{4} 3.9922
1	NS	nR		0.50521	0.38564	0.42772
2	NS	R	0.50521		0.15327	0.85998
3	MS	nR	0.38564	0.15327		0.12410
4	MS	R	0.42772	0.85998	0.12410	

All Groups
Univariate Tests of Significance for New BDNF L (ELISA b
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	150369.(	1	150369.(	2077.37	0.00000
Separation	778.9	1	778.9	10.76	0.00163
Exercise	24.9	1	24.9	0.344	0.55942
Separation*Exercis	108.4	1	108.4	1.498	0.22518
Error	4922.	68	72.4		

Training=N
Univariate Tests of Significance for New BDNF L (ELISA bd
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	54850.0	1	54850.0	1133.68	0.00000
Separation	91.83	1	91.83	1.898	0.18352
Exercise	110.5	1	110.5	2.284	0.14635
Separation*Exercis	201.69	1	201.69	4.169	0.05458
Error	967.64	20	48.38		

Training=S
Univariate Tests of Significance for New BDNF L (ELISA bdn
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	46140.2	1	46140.2	1041.00	0.00000
Separation	174.23	1	174.23	3.931	0.06131
Exercise	141.66	1	141.66	3.196	0.08898
Separation*Exercis	21.51	1	21.51	0.485	0.49400
Error	886.46	20	44.32		

Training=L
Univariate Tests of Significance for New BDNF L (ELISA bdnf
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	49569.1	1	49569.1	464.611	0.00000
Separation	653.23	1	653.23	6.1227	0.02242
Exercise	189.61	1	189.61	1.7773	0.19747
Separation*Exercise	71.80	1	71.80	0.673(	0.42168
Error	2133.7	20	106.69		

All Groups
Duncan test; variable New BDNF L (ELISA bdnf)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = 72.384, df = 68.000

Cell No.	Separation	Exercise	{1} 49.628	{2} 48.350	{3} 40.595	{4} 44.226
1	NS	nR		0.65375	0.00386	0.07544
2	NS	R	0.65375		0.01083	0.15061
3	MS	nR	0.00386	0.01083		0.20489
4	MS	R	0.07544	0.15061	0.20489	

Training=N
Duncan test; variable New BDNF L (ELISA bdnf)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = 48.382, df = 20.000

Cell No.	Separation	Exercise	{1} 50.515	{2} 49.009	{3} 40.805	{4} 50.895
1	NS	nR		0.71167	0.03187	0.92578
2	NS	R	0.71167		0.05461	0.66281
3	MS	nR	0.03187	0.05461		0.03040
4	MS	R	0.92578	0.66281	0.03040	

Training=S
Duncan test; variable New BDNF L (ELISA bdnf)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = 44.323, df = 20.000

Cell No.	Separation	Exercise	{1} 43.165	{2} 49.917	{3} 39.669	{4} 42.635
1	NS	nR		0.09440	0.40077	0.89189
2	NS	R	0.09440		0.02232	0.08692
3	MS	nR	0.40077	0.02232		0.44961
4	MS	R	0.89189	0.08692	0.44961	

Training=L
Duncan test; variable New BDNF L (ELISA bdnf)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = 106.69, df = 20.000

Cell No.	Separation	Exercise	{1} 55.204	{2} 46.123	{3} 41.311	{4} 39.148
1	NS	nR		0.14362	0.03802	0.02116
2	NS	R	0.14362		0.42932	0.28193
3	MS	nR	0.03802	0.42932		0.72083
4	MS	R	0.02116	0.28193	0.72083	

pERK

Effect	SS	Degr. of Freedom	MS	F	p
Univariate Tests of Significance for L pERK (Sheet1 in Pro Sigma-restricted parameterization Effective hypothesis decomposition					
Intercept	81.3010	1	81.3010	505.424	0.00000
Separation	0.0043	1	0.0043	0.027	0.86945
Exercise	0.0370	1	0.0370	0.230	0.63332
Time	0.3668	2	0.1834	1.140	0.32705
Separation*Exercise	1.4858	1	1.4858	9.236	0.00360
Separation*Time	0.2709	2	0.1354	0.842	0.43615
Exercise*Time	0.5820	2	0.2910	1.809	0.17321
Separation*Exercise*Time	0.8794	2	0.4397	2.733	0.07366
Error	9.0080	56	0.1608		

Cell No.	Separation	Exercise	{1} .97827	{2} 1.2305	{3} 1.2793	{4} .92263
Duncan test; variable L pERK (Sheet1 in Proteins June 2012) Approximate Probabilities for Post Hoc Tests Error: Between MS = .16086, df = 56.000						
1	NS	nR		0.07240	0.04212	0.68776
2	NS	R	0.07240		0.72463	0.03768
3	MS	nR	0.04212	0.72463		0.01954
4	MS	R	0.68776	0.03768	0.01954	

Effect	SS	Degr. of Freedom	MS	F	p
All Groups Univariate Tests of Significance for L pERK (Sheet1 in Proteins June 2 Sigma-restricted parameterization Effective hypothesis decomposition					
Intercept	82.5353	1	82.5353	475.079	0.00000
Separation	0.0002	1	0.0002	0.001	0.97299
Exercise	0.0462	1	0.0462	0.266	0.60762
Separation*Exercise	1.5727	1	1.5727	9.052	0.00374
Error	11.1186	64	0.1737		

Time=N
Univariate Tests of Significance for L pERK (Spreadsheet in Proteins June
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	30.4636	1	30.4636	146.601	0.00000
Separation	0.0124	1	0.0124	0.059	0.80938
Exercise	0.2545	1	0.2545	1.225	0.28216
Separation*Exercise	0.9350	1	0.9350	4.499	0.04727
Error	3.9481	19	0.2078		

Time=S
Univariate Tests of Significance for L pERK (Spreadsheet in Proteins June
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	31.5590	1	31.5590	158.372	0.00000
Separation	0.0605	1	0.0605	0.303	0.58762
Exercise	0.2520	1	0.2520	1.265	0.27401
Separation*Exercise	1.5659	1	1.5659	7.858	0.01097
Error	3.9854	20	0.1992		

Time=L
Univariate Tests of Significance for L pERK (Spreadsheet in Proteins J
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	20.5062	1	20.5062	324.466	0.00000
Separation	0.1993	1	0.1993	3.153	0.09366
Exercise	0.1121	1	0.1121	1.774	0.20045
Separation*Exercise	0.0033	1	0.0033	0.052	0.82157
Error	1.0744	17	0.0632		

All Groups
Duncan test; variable L pERK (Sheet1 in Proteins June 2012)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = .17373, df = 64.000

Cell No.	Separation	Exercise	{1} .97827	{2} 1.2305	{3} 1.2793	{4} .92263
1	NS	nR		0.08286	0.04985	0.69876
2	NS	R	0.08286		0.73447	0.04486
3	MS	nR	0.04985	0.73447		0.02418
4	MS	R	0.69876	0.04486	0.02418	

Time=N
Duncan test; variable L pERK (Spreadsheet in Proteins June 2012.
Approximate Probabilities for Post Hoc Tests
Error: Between MS = .20780, df = 19.000

Cell No.	Separation	Exercise	{1} .82334	{2} 1.4389	{3} 1.2745	{4} 1.0811
1	NS	nR		0.04814	0.12890	0.35142
2	NS	R	0.04814		0.54941	0.22421
3	MS	nR	0.12890	0.54941		0.48208
4	MS	R	0.35142	0.22421	0.48208	

Time=S
Duncan test; variable L pERK (Spreadsheet in Proteins June 2012.xls
Approximate Probabilities for Post Hoc Tests
Error: Between MS = .19927, df = 20.000

Cell No.	Separation	Exercise	{1} .94354	{2} 1.2494	{3} 1.5549	{4} .83901
1	NS	nR		0.24928	0.03496	0.68949
2	NS	R	0.24928		0.25000	0.14672
3	MS	nR	0.03496	0.25000		0.01775
4	MS	R	0.68949	0.14672	0.01775	

Time=L
Duncan test; variable L pERK (Spreadsheet in Proteins June 2012.xlsx
Approximate Probabilities for Post Hoc Tests
Error: Between MS = .06320, df = 17.000

Cell No.	Separation	Exercise	{1} 1.1749	{2} 1.0031	{3} .95423	{4} .83284
1	NS	nR		0.28519	0.19652	0.05791
2	NS	R	0.28519		0.75757	0.31498
3	MS	nR	0.19652	0.75757		0.44635
4	MS	R	0.05791	0.31498	0.44635	

All Groups
Univariate Tests of Significance for R pERK (Sheet1 in Proteins
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	135.385	1	135.385	49.8469	0.00000
Separation	6.904	1	6.904	2.5420	0.11800
Exercise	0.561	1	0.561	0.2068	0.65146
Separation*Exercise	7.700	1	7.700	2.8353	0.09929
Error	119.504	44	2.716		

Time=N
Univariate Tests of Significance for R pERK (Spreadsheet in Proteins June
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	63.631	1	63.631	26.3784	0.00005
Separation	1.790	1	1.790	0.7421	0.39920
Exercise	1.068	1	1.068	0.4428	0.51337
Separation*Exercise	8.565	1	8.565	3.5508	0.07413
Error	48.244	20	2.412		

Effect	Time=L Univariate Tests of Significance for R pERK (Spreadsheet in Proteins June Sigma-restricted parameterization Effective hypothesis decomposition SS	Degr. of Freedom	MS	F	p
Intercept	71.8797	1	71.8797	21.0709	0.00017
Separation	5.6550	1	5.6550	1.6577	0.21261
Exercise	0.0007	1	0.0007	0.0002	0.98866
Separation*Exercise	0.9957	1	0.9957	0.2918	0.59498
Error	68.2264	20	3.4113		

MKP-1

Effect	Univariate Tests of Significance for L MKP-1 (Sheet1 in Proteins Jun Sigma-restricted parameterization Effective hypothesis decomposition SS	Degr. of Freedom	MS	F	p
Intercept	106.527	1	106.527	47.5348	0.00000
Separation	3.9662	1	3.9662	1.7697	0.18889
Exercise	9.078(	1	9.078(	4.0508(	0.04905
Time	1.187	2	0.593	0.2649	0.76822
Separation*Exercise	2.152	1	2.152	0.9604(	0.33136
Separation*Time	1.078	2	0.539(	0.2405	0.78703
Exercise*Time	8.018(	2	4.009(	1.7889(	0.17673
Separation*Exercise*Time	4.315	2	2.157	0.9628	0.38813
Error	123.256	55	2.241(		

Cell No.	Duncan test; variable L MKP-1 (Sheet1 in Proteins June 2012 Approximate Probabilities for Post Hoc Tests Error: Between MS = 2.2410, df = 55.000 Exercise	{1} .90843	{2} 1.6527
1	nR		0.04701
2	R	0.04701	