The Effect of Voluntary Exercise on Adult Hippocampal Neurogenesis in Maternally Separated Rats

By
Natasha Sema Hardcastle
HRDNAT003

SUBMITTED TO THE UNIVERSITY OF CAPE TOWN
In fulfillment of the requirements for the degree MSc (Med) Neuroscience

Faculty of Health Sciences
UNIVERSITY OF CAPE TOWN

University of Cape Town

Date of Submission: 15 February 2016
Supervisors: Prof VA Russell, Prof DM Lang and Dr L Marais
Department of Human Biology

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

Declaration

I, *Natasha Sema Hardcastle*, 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.

I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signature: Signed by candidate

Date: 15/02/16…………….

Acknowledgments

I would like to thank UCT and the NRF for funding, Prof VA Russell for her supervision throughout the project, Dr Lelanie Marais for her supervision during the early stages of the project and for her comments on the dissertation, Prof Dirk Lang for his aid with the confocal microscope and supervision, Charles Harris for supplying the equipment, the Human Biology animal unit staff Nuraan Ismail and Abdul Karim Samuels for aiding in the superb care of the study's rats, Taahira Goolam Hoosen from the UCT writing center for her input towards improving my writing style, the neuroscience lab for their encouragement, my family for their support throughout my masters, my grandmother and Yusuf Khan for the use of their cars to get to campus, Ouma for her positivity and chocolates on bad days and Aunty R for believing.

Table of Contents

Declaration ii

List of Abbreviations vi

List of Figures vii

Abstract xii

Introduction 1

1.1) Depression 1
1.2) Neurogenesis in Rodents 5
1.3) The importance of Glia in Neurogenesis 7
1.4) Animal Models for Depression and Behavioral Tests 8
1.5) Rodent Studies linking depression and neurogenesis 11
1.6) Rodent Studies linking exercise and neurogenesis 12
1.7) Tools to study neurogenesis 13
1.8) Challenges with BrdU 14
1.9) Novel Marker EdU 15
1.10) EdU as compared to BrdU 16

Methods 19

2.1) Pilot Study 19
2.1.1) Aim *19*
2.1.2) Animals *19*
2.1.3) Exercise and ethynyl-2'-deoxyuridine (EdU) Injection 19
2.1.4) Transcardial Perfusions 21
2.1.4) Brain Sectioning 22
2.1.6) Staining Wells 22
2.1.7) Immunohistochemistry 22
2.1.8) Quantification 23
2.1.9) Statistics 26
2.2) Experiment 27
2.2.1) Experimental Animals 27
2.2.2) Maternal Separation (MS) 27
2.2.3) Voluntary Exercise 28
2.2.4) Injections 28
2.2.5) Transcardial Perfusions 29
2.2.6) Sectioning 29
2.2.7) Staining Vials & Wells 30
2.2.8) Immunohistochemistry 30
2.2.9) Quantification 34
2.2.10) Statistics 34

Results 36

3.1) Characterisation Data 36
Characterisation of EdU stain in conjunction with cell-type specific markers *43*
3.2) Experimental Results 44
MS higher Weights at PND 60 relative to NMS *44*
No Effect of maternal separation and exercise on the number of EdU-labelled mature neurons and astrocytes in the dentate gyrus of the hippocampus. 45

Number of Surviving EdU-labelled Neurons compared to the number of EdU-labelled astrocytes. 46
No Effect of maternal separation and exercise on the number of surviving immature EdU-labelled neurons in the dentate gyrus of the hippocampus. 48
Neuronal proliferation reduced in maternally separated (MS) rats 49

Discussion 54
4.1.) Pilot Study: Characterising the mitotic marker, EdU 54
No Discernable effect of exercise in PND 74 injected rat cohort 54
EdU-labelled neuronal (EdU/DCX+EdU/NeuN) cell survival was higher in PND 60 injected and exercised rats in the dentate gyrus 55
Differences between cell counts for EdU/NeuN, EdU/DCX and EdU/GFAP undetected in pilot study exercised rats 56
Pooled EdU/DCX+EdU/NeuN cell survival was higher in PND 60 injected and exercised rats than PND 96 injected rats 57
4.2) Experiment: Measuring cell survival and neurogenesis using EdU 58
Weight was increased in MS rats 58
Distance Run by rats was lower in MS rats 59
The Survival of NeuN, GFAP and DCX labelled cells was not affected by MS 59
Voluntary running no detectable effect on Edu Labelled cells 61
Correlation Analysis found a relationship between Neurons (EdU/NeuN) and Astrocytes (EdU/GFAP) 62
Neuronal proliferation (Ki-67/DCX) was reduced in MS rats 63
Exercise significantly affects cell proliferation (Ki-67/DCX) in the Dorsal Hippocampus 64
Dorsal and Ventral hippocampi affected by MS 66
Dorsal Hippocampus affected by Exercise 66
4.3) Conclusion & Future considerations 66

References 69

Appendix 76

List of Abbreviations

5HT- serotonin
ACTH -Adrenocorticotropic hormone
AVP- arginine vasopressin
BDNF- brain-derived neurotrophic factor
BrdU- 5′-bromo-2′-deoxyuridine
BSA- Bovine Serum Albumin
CMS- chronic mild stress model
CRH -corticotropin releasing hormone
DCX -doublecortin
EdU- 5-ethynyl-2′-deoxyuridine
EPM- elevated plus maze
F-ara-EdU- (2′S)-2′-deoxy-2′-fluoro- 5-ethynyluridine
fMRI -functional magnetic resonance imaging
FST- Forced Swim Test
GFAP- Glial Fibrillary Acidic Protein
GL- granular layer
GR- glucocorticoid receptors
HIV/AIDs- Human immunodeficiency virus/ Acquired immune deficiency syndrome
HPA -hypothalamic–pituitary–adrenal
IFN-γ- interferon gamma
IGF- Insulin Growth Factor
IL-1-B- interleukin-1-beta
IL-6- interleukin-6
LTP- long term potentiation
MAO- monoamine oxidase
MDD- Major Depressive Disorder
ML- molecular layer
MR- mineralocorticoid receptors
MS -Maternal Separation
NE -norepinephrine
NMS- non-maternally-separated
NPC- Neural progenitor cells
NR- non-run
NSC -Neural stem Cells
OFT- Open Field Test
PBS- phosphate buffered saline
PFA- Paraformaldehyde
PND- post-natal day
R -run
SGZ- sub-granular zone
SSRI- selective serotonin reuptake inhibitors
TNF- tumor necrosis factor
VEGF- vascular endothelial growth factor

List of Figures

Figure 1. This Diagram shows the hierarchical control of the stress response in the HPA axis (image adapted from Lupien *et al*., 2009). When a stressor is present the brain processes the stress signals and the hypothalamus releases CRH and AVP. These trigger the release ACTH from the pituitary gland stimulating the production of glucocorticoids from the adrenal cortex in addition to the catecholamines such as adrenaline and nor-adrenaline. The response is arrested by glucocorticoids that bind to GR and MR receptors on the hippocampus, hypothalamus, anterior pituitary and frontal cortex and arrest the stress response. 2

Figure 2. Overview of factors that influence depression and treatment plans. Early life trauma can lead to HPA dysfunction and influence neurogenesis. These changes can lead to the development of depression. This is then initially treated using SSRIs and resulting in either the amelioration of depression or treatment resistance. Exercise has been proposed as an additional method to ameliorate depressive-like symptoms as it has been shown to influence HPA axis function and neurogenesis. However, this interaction merits further exploration as little is known about cell survival or genesis in models for depression. 4

Figure 3.Maturation of a young neuron in the dentate gyrus of the hippocampus. Section a) represents the stage of neurogenesis the section b) indicates cell-type markers used to identify the stage of development. Information adapted from Ming & Song. 2002. 6

Figure 4.Comparison of the molecular structure of BrdU and EdU 14

Figure 5. Diagram of treatments each rat group underwent. Adult rats were housed in cages with running wheels that were either locked (NR) or not (R) as indicated by the diagrammatic wheels. The locked wheels are indicated by a red cross. EdU injections were administered at one of three time points (PND 60, 96 or 74). Rats were returned to home cages and perfused either four hours later (Group 1 and 3) or two weeks after injection (Group 2) in order to establish the optimal window of time to study neurogenesis *in vivo.* 20

Figure 6. Co-localisation scatterplots displaying the stain intensity of the pixels selected in the green circle in A and B. Region 1 represents only pixels with EdU stain, region 2 represents only pixels with stain for NeuN (A) and GFAP (B) and region 3 represents the number of pixels which overlap in fluorescence intensity of the respective stains. The majority of pixels overlap in region three indicating that the two stains co-localized in the same structure. The tile scans on the right show the NeuN positive neurons (pink), GFAP positive Astrocytes (Red) and Hoechst stained nuclei (blue). Areas of extreme co-localisation appear white. 25

Figure 7. Graph shows the intensity profile of region indicated by the red arrow. Green line represents EdU stained nucleus, red line indicates GFAP profile and blue line indicates Hoechst. Likewise, on the right the labelled astrocyte cytoskeleton (red) and EdU positive nucleus (green) show the morphological star-shape of glial cells. This pattern of the cytoskeletal GFAP stain cupping the nuclear EdU is a positive indication that the labelled cell is a glial cell. 26

Figure 8. Displaying each treatment group (MS+R, MS+NR, NMS+R, NMS+NR). The rat pups were treated with MS or NMS. These rats were then permitted to reach adulthood and housed with running wheels on PND 54. Wheels were locked or left unlocked allowing some rats voluntary exercise and others not. EdU injections were

administered on PND 60 to measure mitotic activity in the dentate gyri of runners and non-runners. Rats were immediately returned to their home cages with running wheels until PND 74. They were then perfused and brains collected for immunohistochemistry. 28

Figure 9. A representation of the processing of each rat brain collected. After the perfusion adult rat brains were collected and the ventral and dorsal hippocampi were sectioned separately. Five sections of each region of the hippocampus were kept for each molecular label combination. Three molecular label combinations were used to study neurogenesis in the dentate gyrus: EdU/NeuN/GFAP, EdU/DCX and Ki-67/DCX. EdU was used to quantify neuronal or astrocytic survival while Ki-67 Label was used as a pulse. 30

Figure 10. Cell-type specific marker. Combination 1 shows GFAP (red), EdU (green), NeuN (magenta) and Hoechst (blue) and a composite image allowing identification of the EdU labelled cell. 33

Figure 11. Cell-type specific marker combination 2 shows DCX (red), EdU (green) and Hoechst (blue) and a composite image. 33

Figure 12. Cell-type specific marker combination 3 displays DCX (red), Hoechst (blue) and KI-67 (yellow) and composite image. 34

Figure 13. Total number of cells co-labelled with EdU/GFAP, EdU/NeuN or EdU/DCX in the dentate gyrus of the dorsal hippocampus of Sprague Dawley rats injected with EdU at (a) PND 60 or (b) PND 94/95 and perfused on PND 74 or 94/95. Please note that this study's "total" refers to the total cells counted and summed per rat. The above data were tested using the Shapiro-Wilk normality test and found to be non-parametrically distributed. The data were therefore analyzed using the Kruskal-Wallis test. Data points represent the number of counted cells labelled with EdU/GFAP, EdU/NeuN or EdU/DCX in the dentate gyrus summed per section (40 µm) of the rat brain (2-6 sections per rat). Results are expressed as individual data points with bars representing median and inter-quartile range. There were no significant differences in the number of EdU co-labelled cells between the different cell types. 37

Figure 14. Columns represent the median and bars the upper-quartile range of the average number of EdU-labelled cells found in each exercised rat per injection group (n=5). Injections administered on PND 60 had a significantly higher median (6.16 cells) than PND 96 injections (1.1 cells). 38

Figure 15. Average number of cells per rat co-labelled with EdU/GFAP, EdU/NeuN or EdU/DCX in the dorsal hippocampus of Sprague Dawley rats injected with EdU at (a) PND 60 or (b) PND 96. The above data were tested using the Shapiro-Wilk test and found to be parametrically distributed (15a) and non-parametrically distributed (15b). The data were therefore tested for differences between rats injected with EdU at (a) PND 60 using a one-way analysis of variance and (b) PND 96 using the Kruskal-Wallis test. Data points represent the average number of cells labelled with EdU/GFAP, EdU/NeuN or EdU/DCX in the dentate gyrus summed (per 40 µm section of the rat brain 2-6 sections were averaged per rat). Results are expressed as individual data points with bars representing median and inter-quartile range. There were no significant differences in the number of EdU co-labelled cells between the different cell type markers. The data were also tested for differences between rats injected with EdU at (a) PND 60 and (b) PND 96 using the Mann-Whitney U test. There were no significant

differences in the number of EdU co-labelled cells between PND 60 and PND 96 for the different cell type markers. 39

Figure 16. Total (a) and average (b) number of EdU/NeuN plus EdU/DCX co-labelled cells in the dentate gyrus of rats injected with EdU at PND 60 and those injected on PND 96. Data points (a) represent the number of cells labelled with EdU/Neun and EdU/DCX in the dentate gyrus summed per 40-µm section of the rat brain (2-6 sections per rat). Data points (b) represent the average number of EdU/NeuN and EdU/DCX co-labelled cells in 2-6 x 40-µm sections of the dentate gyrus of the dorsal hippocampus of PND 60 and PND 96 EdU-injected rats. The data were tested using the Shapiro-Wilk test and found to be non-parametrically distributed. The Mann-Whitney U test revealed that the dentate gyrus of the dorsal hippocampus of the PND 60 EdU-injected rats had a greater number of EdU/NeuN and EdU/DCX co-labelled cells than PND 96 EdU-injected rats (a,b). Results are expressed as median and interquartile range. *Significant difference between NeuN + DCX 60 and NeuN + DCX 96 median values. 40

Figure 17. Total number of EdU/GFAP, EdU/NeuN and EdU/DCX co-labelled cells in 4-6 sections of dentate gyrus in the dorsal hippocampus of rats that were housed in cages with attached running wheels that were freely moving (a) or immobilized (b) for a period of 20 days and then injected with EdU at PND74, 4 hours prior to perfusion and collection of brains for immunohistochemistry. Data points represent the summed number of EdU-co-labelled cells in 2-6 x 40-µm sections of the dentate gyrus of 3 exercised rats permitted to run for 20 days (a) and 3 non-exercised rats (b). The bars represent the median and interquartile range. 41

Figure 18. Average number of EdU/GFAP, EdU/NeuN and EdU/DCX co-labelled cells in 2-6 sections of dentate gyrus in dorsal hippocampus of rats that were housed in cages with attached running wheels that were freely moving (a) or immobilized (b) for a period of 20 days and then injected with EdU at PND74, 4 hours prior to perfusion and collection of brains for immunohistochemistry. Data points represent the average number of EdU-co-labelled cells in 40-µm sections of the dentate gyrus (4-6 x 40-µm sections of the brain) of 3 exercised rats permitted to run for 20 days (a) and 3 non-exercised rats (b). The bars represent the median and interquartile range. 42

Figure 19. Ki67 and a pulse of EdU labelled the same cells in the dentate gyrus of the hippocampus of a rat injected with EdU on PND 60 (A) Ki-67 labelled cells, (B) EdU labelled cells, (C) Hoechst nuclear label, and (D) merged image (20x). 43

Figure 20. Graph showing the weights of maternally separated runners (MS+R), maternally separated non-runners (MS+NR), non-separated runners (NMS+R) and non-separated non-runners. A Kruskal- Wallis test followed by Dunn's multiple comparison Test showed that MS+NR rats had a higher median then NMS+R rats. 44

Figure 21.Distance run from PND 54-74. The data were found to be non-parametric. The line graph showing the median and interquartile range of the distances run by MS and NMS rats over 20 days of voluntary wheel running. A Wilcoxon matched-pairs signed rank test revealed that NMS+R had a significantly higher median than MS+R (W= 128.0; p= 0.0153). 45

Figure 22. The number of co-labelled EdU/NeuN cells (ai, aii) and EdU/GFAP cells (bi, bii) in the dentate gyrus of the dorsal hippocampus (5 x 40-µm sections) (ai, bi) and the ventral hippocampus (5 x 50-µm sections) (aii, bii) of maternally separated runners (MS+R, n = 4), maternally separated non-runners (MS+NR, n = 5), non-separated runners (NMS+R, n = 5) and non-separated non-runners (NMS+NR, n = 5). The data

were tested using the Shapiro-Wilk test and found to be non-parametrically distributed. The Kruskal-Wallis test revealed no significant differences between groups. Bars represent the median and inter-quartile range. 46
Figure 23. The number of neurons relative to astrocytes in the a) dorsal and b) ventral hippocampus. The data were tested using the Shapiro-Wilk test and found to be non-parametrically distributed. The Mann-Whitney U test revealed no significant difference between the number of EdU/NeuN and EdU/GFAP co-labelled cells in the dentate gyrus of the dorsal and ventral hippocampus. Data points represent the summed number of NeuN and EdU co-labelled cells and GFAP and EdU co-labelled cells counted in 5 sections of dentate gyrus in the dorsal (40-µm sections) (a) and ventral (50-µm sections) (b) dentate gyrus of the hippocampus of each rat (n= 18). Bars show median and inter-quartile range. 47
Figure 24. Correlation analysis of the number of neurons relative to astrocytes in the a) dorsal and b) ventral hippocampus. There was no significant correlation between EdU/GFAP co-labelled astrocytes and EdU/NeuN co-labelled neurons in the dentate gyrus of the dorsal hippocampus of all rat groups (a). There was a significant positive correlation between EdU/GFAP co-labelled astrocytes and EdU/NeuN co-labelled neurons in the dentate gyrus of the ventral hippocampus of rats allowed to exercise for 20 days as well as rats that were housed in cages with immobilized running wheels, 14 days after injection of EdU (Spearman r correlation 0.9006; n= 18). 48
Figure 25. Effect of maternal separation and exercise on the number of surviving immature EdU-labelled neurons in the dentate gyrus of the hippocampus. The data points represent the number of co-labelled EdU/DCX cells in the dorsal (40-µm sections) a) and the ventral (50-µm sections) b) dentate gyrus (total number of EdU/DCX co-labelled cells in 5 sections of the left and right hippocampus of rats in the four treatment groups). The data were tested using the Shapiro-Wilk test and found to be non-parametrically distributed. The Kruskal-Wallis test revealed that there were no significant differences between the treatment groups. Bars represent the median and inter-quartile range of each data set. 49
Figure 26. Confocal tile scan (10 X) showing the dentate gyrus of the hippocampus of a NMS+R rat (A) and a MS+R rat (B). The arrows indicate yellow fluorescence representing Ki-67 labelled cells. There are more Ki-67 labelled cells in the NMS rat than MS rats that underwent the same exercise regime. Scan C (20 X) red (II) representing the dorsal hippocampus shows DCX-labelled immature neurons, blue represents a Hoechst (I) and yellow (III) is Ki-67 marker while D (40 x) displays the yellow nuclear stain surrounded by cytoplasmic DCX stain giving an example of the typical unipolar neuronal structure. The above data were tested using the Shapiro-Wilk test and a) was found to be parametric. The data were tested using a one-way ANOVA and a Tukey post-hoc test to identify group differences. The data from b) were found to be non-parametrically distributed and were tested for differences using the Kruskal-Wallis test. Data points in (a) and (b) represent the summed number of Ki-67/DCX labelled cells in 5 sections of the dorsal (40-µm sections) (a) and ventral (50-µm sections) (b) dentate gyrus. The bars in (a) represent the mean and standard deviations of each group and (b) medians and interquartile ranges. A one-way ANOVA (a) revealed a significant difference between groups, and a Tukey post-hoc test showed that proliferating immature neurons in the dorsal hippocampus are significantly decreased in four MS+R rats compared to five NMS+R rats. The same difference was

found in the ventral hippocampus (by using the Kruskal-Wallis test and Dunn's multiple comparison Test. The NMS+R group in (a) had significantly higher number of proliferating neurons relative to the NMS+NR group (MS+R n=4, MS+NR n=4, NMS+R n=5, NMS+NR n=5)..52

Abstract

THE EFFECT OF VOLUNTARY EXERCISE ON ADULT HIPPOCAMPAL NEUROGENESIS IN MATERNALLY SEPARATED RATS

Maternal separation (MS) has been shown to produce depression-like symptoms in male Sprague Dawley rats. The underlying mechanisms responsible for the development of these depression-like behaviors are unknown. However, a growing body of evidence suggests that a reduction in neurogenesis may be a key-mediating factor.

Voluntary wheel running is a form of exercise that increases neurogenesis and decreases depression-like behaviour in rats. However, the exact molecular role of neurogenesis in exercise-induced antidepressant effects still remains unanswered. This requires new tools to explore the interaction between exercise and neurogenesis *in vivo*. To this end, the novel mitotic-marker, 5-ethynyl-2'-deoxyuridine (EdU), and Ki-67, an endogenous marker of cell proliferation, was characterised in order to study neurogenesis in an MS rat model of depression. Furthermore, this study aimed to provide insight into the effect of voluntary exercise on cell genesis and survival.

To characterise EdU labelling of cells *in vivo,* male Sprague Dawley rats (Characterisation rats n =13) were injected with 50 mg/kg EdU as noted in the literature. The optimal time point to inject the EdU label to measure mitotic activity was found to be post-natal day (PND) 60. MS or non-maternal separation (NMS) was conducted from PND 2 - 14 on experimental rats (n=39). From PND 54–74, experimental rats were housed in cages with attached running wheels (R) or locked running wheels (NR). All experimental rats were injected with 50 mg/kg EdU on PND 60 and transcardially perfused on PND 74 using Phosphate Buffered Saline (PBS) followed by fresh 4% paraformaldehyde. Whole brains were then removed from the skull and placed in 4% paraformaldehyde for three hours. The brains were transferred to a 30% sucrose solution, stored in sucrose for 3 - 5 days and thereafter mounted in optimal cutting medium (OCT) and sectioned using a cryostat. Brain sections of 40 µm from 6.96 to 5.52 mm anterior to the inter-aural line were taken as dorsal and 50 µm sections from 3.84 to 2.76 mm were analyzed as ventral. The marker, EdU was detected in rat brains using the Click-iT EdU Alexa Fluor 488 detection kit. Three molecular marker combinations were used to detect different factors for both dorsal and ventral hippocampi: (1) EdU/GFAP/NeuN, to establish how many EdU labelled cells survive to become neurons or astrocytes (2) EdU/DCX to determine how many EdU labelled cells that have survived for 14 days are immature neurons and (3) Ki-67/DCX to indicate how many mitotically active cells are immature neurons on PND 74. Brain sections were then scanned using a confocal microscope whereby EdU stained nuclei were manually counted and cell phenotypes identified.

The molecular marker combination one and two revealed no differences between treatment groups in the number of EdU-labelled cells in the dorsal and ventral hippocampi. However, a significant correlation was found between EdU/GFAP positive cells and EdU/NeuN positive cells in the ventral hippocampus when all treatment groups were pooled ($r = 0.82$, $n=18$, $p < 0.0001$). The third molecular marker combination revealed significant differences in neurogenesis between groups. The MS+R group had fewer dorsal hippocampal Ki-67/DCX cells relative to NMS+R and NMS+R had significantly higher Ki-67/DCX cell count relative to NMS+NR rats. In the ventral hippocampus MS+R rats had fewer Ki-67/DCX cells compared to NMS+R rats.

The link between neurons and astrocytes in the ventral hippocampi corresponds with reports that an increase in neurons is linked to the presence of astrocytes. However it may also be due to unavoidable variation in the intensity of the stain. The third molecular marker combination (Ki-67/DCX) revealed the most significant finding of this study. It showed that voluntary wheel running significantly increased the number of Ki-67/DCX co-labelled neurons in the dorsal hippocampus of NMS+R rats relative to NMS+NR which is in agreement with the literature that suggests exercise increases neurogenesis. The literature also reports that stress decreases neurogenesis and interestingly MS+R rats had a lower cell count than NMS+R rats. This may indicate an interaction between early life stress and exercise-induced neurogenesis. This finding further suggests that MS alters neurogenesis in adult life and attenuates the effect of exercise on the ventral hippocampus.

Chapter 1

Introduction

1.1) Depression

Depression is a psychiatric condition d by a depressed mood, difficulties at work, loss of interest in hobbies, agitation and in some cases suicide (Hamilton *et al.*, 1960). It is the international leading cause of disability (Marcus *et al.,* 2012) and contributes largely to world economic burden affecting up to 350 million individuals worldwide (Marcus *et al.,* 2012). Depression claims the ranking of 10th leading cause of disability in Southern Sub-Saharan Africa (Murray *et al.,* 2012) with a 9.7% prevalence rate of depression in the absence of any other diseases in South Africa (Tomlison *et al.,* 2009). This has been shown to manifest at a societal level in an association between unemployment, disability and major depressive disorder (Rizvi *et al.,* 2015). In South Africa the alarming 30% unemployment rate presents a particularly significant problem (Klasen & Woodlark, 2009). Therefore understanding the etiology of depression is paramount to effectively treating it in order to reduce the negative impact it has at an individual and societal level.

Adverse childhood experiences have been directly linked to an increased predisposition to development of depression throughout one's life (Chapman *et al.,* 2004). The role of the mother and the maternal hypothalamic–pituitary–adrenal (HPA) axis on offspring is important in childhood development (Goodman, 2014; Shea *et al.,* 2007). Early life trauma reportedly increases long term HPA axis activity, decreases the volume of limbic regions involved in emotional regulation such as the amygdala (Dannlowski *et al*., 2012) and the hippocampus (Duman *et al.,* 2002; Pruessner *et al.,* 2015), decreases connectivity to cognitive areas of the brain such as the prefrontal cortex (Grant *et al*., 2014) and subsequently increases an individual's predisposition to depression (Pariante & Lightman, 2008). It was also found that exposure to high levels of stress hormones called glucocorticoids in early childhood increases the HPA axis activity in adulthood. This increases the stress response as indicated by higher saliva cortisol and adrenocorticotropic hormone (ACTH) present in response to a psycho-social stressor in humans (Hohne *et al.,* 2014). Contrastingly, a decrease in HPA activity was reported in females. Nevertheless, early life trauma clearly resulting in dysregulation of the HPA axis is noted (Kempke *et al.,* 2014). It has recently been suggested that gene expression is altered by exposure to early life stress and that epigenetics may play a role in the receptor density (Romens *et al.,* 2015). Reduced receptor density results in a less efficient regulation of the HPA axis stress response. While not every individual faced with a stressor will develop depression (Nasca *et al.,* 2015) early life trauma influences the function of the HPA axis.

The stress response is regulated by the HPA axis (Figure 1) that is comprised of the hypothalamus, anterior pituitary gland and adrenal cortex and the interactions between them. Functionally the axis encapsulates a mergence of endocrine and neuronal structures. The hypothalamus releases corticotropin releasing hormone (CRH) and arginine vasopressin (AVP). These trigger the release of ACTH from the pituitary gland which in turn stimulates the production of glucocorticoids from the adrenal cortex in addition to the catecholamines such as adrenaline and nor-adrenaline. The ability to regulate this system relies heavily on the ability of the glucocorticoids to bind to mineral corticoid receptors (MR) and glucocorticoid receptors (GR) located in the frontal cortex, hippocampus and hypothalamus (Lupien *et al.,* 2009) (Figure 1). The HPA axis is largely regulated by the hippocampus since it expresses a very high density of GR that respond to the release of the glucocorticoid hormone corticosterone (in rodents) and cortisol (in humans). These hormones are released in response to stress and play a crucial role in the negative feedback loop that acts at many levels, including the hippocampus, to arrest the stress response when a stressor is no longer present. The development of this system is the foundation of one's ability to cope with chronic stress in later adult life (Lupien *et al*., 2009). The interaction between the HPA axis and hippocampus is suggested to contribute to the development of an increased susceptibility to depression.

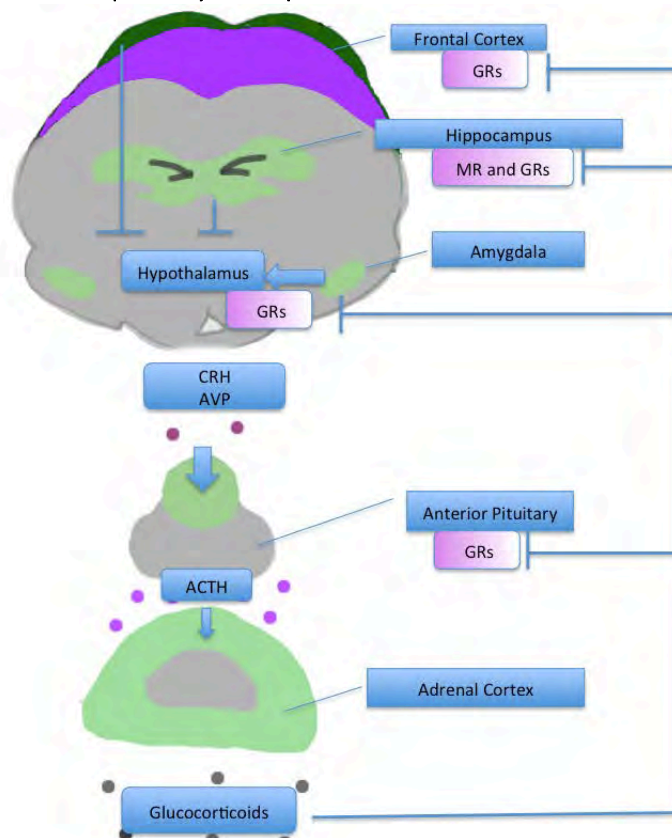


Figure 1. This Diagram shows the hierarchical control of the stress response in the HPA axis (image adapted from Lupien *et al*., 2009). **When a stressor is present the brain processes the stress signals and the hypothalamus releases CRH and AVP. These trigger the release ACTH from the pituitary gland stimulating the production of glucocorticoids from the adrenal cortex in addition to the catecholamines such as adrenaline and nor-adrenaline. The response is arrested by glucocorticoids that bind to GR and MR receptors on the hippocampus, hypothalamus, anterior pituitary and frontal cortex and arrest the stress response.**

The hippocampus matures post-natally and early life stress has been shown to alter HPA activity and reduce GR and MR density on the hippocampus (Lupien *et al.,* 2009) and predispose individuals to depression. As mentioned previously, these receptors are important as they mediate a feedback loop that regulates the stress response (Figure 1) and early life stress is thought to impair the glucocorticoid-mediated feedback loop (Pariante & Lightman, 2008). This may pre-dispose an individual to developing depression later in life and requires effective treatment.

Up to 20% of sufferers of depression do not respond to currently available treatments (Mayberg *et al.,* 2005) that target the monoamine system. Most pharmacological treatments are aimed at increasing monoamine transmission using selective serotonin (5-HT) reuptake inhibitors (SSRIs), norepinephrine (NE) reuptake inhibitors (SNRI) and monoamine oxidase (MAO) inhibitors (Dale *et al.,* 2015). SSRIs are the first line of defense and block serotonin transporters to increase serotonin neurotransmission (Codplan *et al.,* 2014) but reportedly, less than half of all patients respond to them. Long term less than half of all the major depressive disorder (MDD) sufferers achieve remission with currently available treatments (Bennabi *et al.,* 2015; Kemp *et al.,* 2008 ; Rush *et al.,* 2006). In addition, patients can become unresponsive to two or more treatments termed treatment resistant. Targeting points of intervention to treat resistant patients is essential to finding novel, homogenous and effective treatment. There are many postulated causes of depression however, this study primarily discusses maternal separation (MS) stress and the HPA axis and explores exercise-induced neurogenesis as the targeted point of intervention.

Novel treatments for depression using exercise have been explored. Exercise has been used in combination with antidepressant drugs to treat human depression (Blumenthal *et al.,* 1999). Twelve weeks of aerobic exercise in adults suffering from depression who participated in treadmill running five days weekly displayed decreased depressive symptoms as measured by the Hamilton Rating Scale for depression (Dunn *et al.,* 2005). Voluntary exercise has also been shown to result in an array of holistic health benefits including reduced risk of heart disease, hypertension, diabetes and certain cancers (Strohle *et al.,* 2008). Additionally exercise results in a series of cognitive improvements including improved memory and learning (Weuve *et al.,* 2004). The mechanisms behind these benefits are unclear as *in vivo* studies in human subjects are limited by the available technology. A factor that is up regulated by exercise and stimulates neurogenesis is brain derived neurotrophic factor (BDNF). This is a growth factor found in the brain involved in the stress response that is decreased in plasma serum of humans suffering from depression and in cortical serum of post-mortem studies (Schuch *et al.,* 2013; Sen *et al.,* 2008; Shimizu *et al.*, 2003). It also regulates neurogenesis, neurite outgrowth and synaptogenesis, synaptic function and cell survival (Mattson *et al*., 2004). Directly studying exercise-induced neurogenesis and depression is difficult in humans due to the limited imaging technology and since most neurogenic information is correlative or *in vitro* rather than a direct observation of neurogenesis *in vivo*.

Adult neurogenesis occurs in humans (Eriksson *et al.,* 1998) with an estimated cell turn over of 700 new hippocampal neurons per day (Borsini *et al.,* 2015). A clinical imaging study found that individuals suffering from major depressive disorder had significantly smaller posterior hippocampal volumes compared to healthy controls using magnetic resonance imaging (Neumeister *et al.,* 2005). Depressive behavior has been linked to a decrease in hippocampal volume (Krogh *et al*., 2014) with cellular studies indicating that cortisol release and MR/GR receptors causal to the link (Nasca *et al.,* 2015; Anacker *et al.,* 2013). Changes in receptor density influence the HPA axis's ability to control negative feedback (David *et al.,* 2009) affecting neurogenesis. Although not all studies agree that depression is linked to neurogenesis, a study by Reif *et al.,* (2006) found no link between human cell proliferation and depression. On the other hand, immortalized human hippocampal cell cultures have shown that GR are essential in stimulating neurogenesis and increased cortisol levels arrest neurogenesis (Anacker *et al*., 2013). There is likely more than one system and multiple factors interacting and culminating in a depressive phenotype, with neurogenesis featuring as a paramount factor (David *et al.,* 2009) (Figure 2). Neurogenesis has been suggested as a possible factor that may contribute to the anti-depressant action of exercise and cognitive improvements seen in humans. It has been more extensively studied in animals than humans and furthermore, while these processes are considered homologous, variability between species must be noted

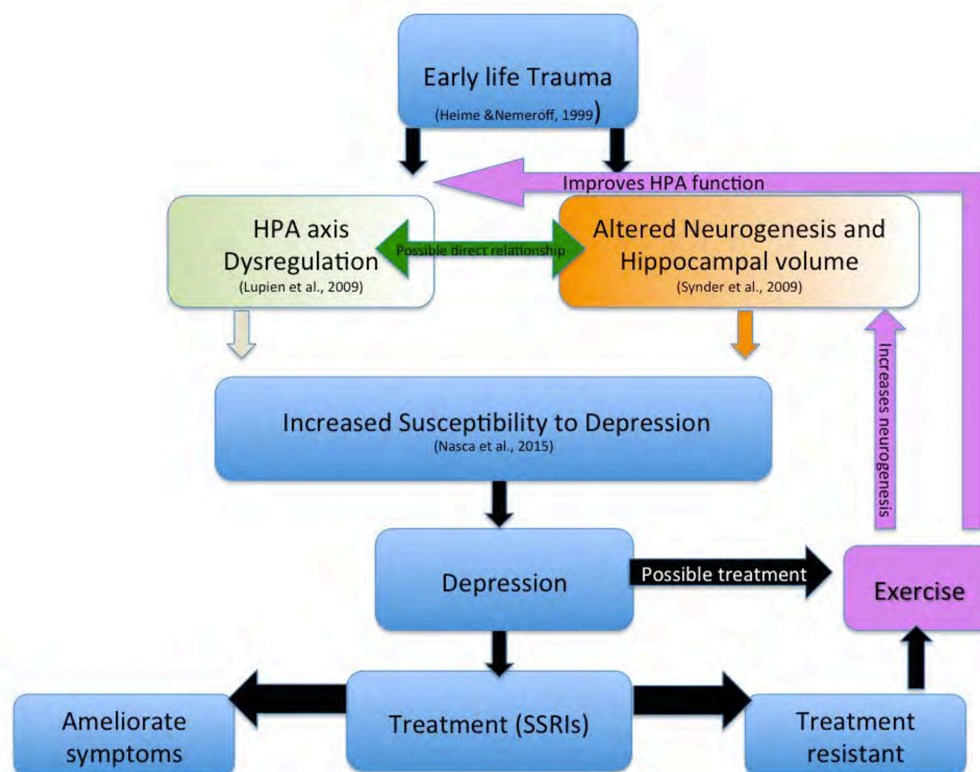


Figure 2. Overview of factors that influence depression and treatment plans. **Early life trauma can lead to HPA dysfunction and influence neurogenesis. These changes can lead to the development of depression. This is then initially treated using SSRIs and resulting in either the amelioration of depression or treatment resistance. Exercise has been proposed as an additional method to ameliorate depressive-like symptoms as it has been shown to influence HPA axis function and neurogenesis. However, this interaction merits further exploration as little is known about cell survival or genesis in models for depression.**

1.2) Neurogenesis in Rodents

The process of adult neurogenesis was discovered in rats during the twentieth century (Altman & Das, 1965). It can be defined as the generation and maturation of functional neurons from Neural Stem Cells (NSC)/ neural progenitor cells (NPC) (Ming & Song, 2011). Reportedly, there are populations of cells that divide symmetrically to give rise to NPC (Ge *et al.,* 2007; Carpenter *et al.,* 1999). These are thought to self renew, migrate and differentiate and integrate into the neuronal circuitry (Seirra *et al.,* 2015; Ge *et al.,* 2007; Fricker *et al.,* 1999). The migration of neurons is regulated by guidance cues and growth factors (Imayoshi *et al.,* 2010; Carpenter *et al.,* 1999; Fricker *et al.,* 1999). Neurogenesis occurs in the adult mammalian brain in a number of regions including the prefrontal, posterior, parietal, and inferior temporal cortex (Ohira *et al.,* 2013; Gould *et al.,* 1999). Cell turnover in the brain is very tightly controlled to prevent tumour formation with the production of stem cells modulated via microglia-mediated apoptosis (Tremblay *et al.,* 2011; Das & Basu, 2008). There is a continuous and modulated turnover of new neurons and in rats there is an estimated cell turnover of 5000 daily (Kent, 2015). The modulation in the basal turnover of cells has been linked to stimuli such as stress, exercise and enrichment. Inhibiting neurogenesis has behavioural and molecular implications that have been linked to depression in mouse and rat models. Only a few specific neurogenic niches that have been thoroughly explored by a variety of studies, more specifically the sub-ventricular zone (SVZ), olfactory bulb and hippocampus (Ming & Song 2011).

Neurogenesis in the dentate gyrus of the hippocampus is of particular interest as it has been linked to cognitive and behavioral changes (Snyder *et al.,* 2011; Levone *et al.,* 2015). The mechanism behind this is poorly understood but a growing number of studies are slowly piecing together this process (Kirby *et al.,* 2015; Pavlovicova *et al.,* 2015; Schloesser *et al.,* 2009). The sub-granular zone (SGZ) of the dentate gyrus is an active site of neurogenesis which arises between the hilus and the granule cell layer (consisting of no more than three rows of nuclei). It is lined with radial-like glial cells or stem cells/neural progenitor cells that become activated and proliferate into the granular cell layer (Nicola *et al.,* 2015). These cells potentially become neuroblasts differentiating into immature doublecortin (DCX) expressing neurons and thereafter generate dendritic extensions to the mossy fiber tracts in the CA3 region of the hippocampus (Ming and Song. 2011) (Figure 3). The mossy fibers project from the CA3 region to pyramidal cells of the CA1 region that send projections to the subiculum of the hippocampus (Figure 2) and thereafter the target region specific to either the ventral or dorsal hippocampus (Kumazawa-Manita *et al.,* 2013). The hippocampus processes stimuli in a number of ways, one being when information enters the hippocampus via the entorhinal cortex which receives the stimuli, stimulates the dentate gyrus which then links to CA3 region which relays to CA1 region which eventually links to the pre-fontal cortex (Vivar & van Praag, 2013). Newly generated neurons reportedly have a lower threshold for stimulation and require less stimulation to induce long term potentiation (LTP) from between 1 - 1.5 months in mice (Ge, *et al.,* 2007). The

properties, function and survival of newly generated neurons are not well understood as structural changes have been the primary line of investigation (Nicola *et al.,* 2015) specifically in the dentate gyrus as this influences both ventral and dorsal hippocampus that are functionally distinct.

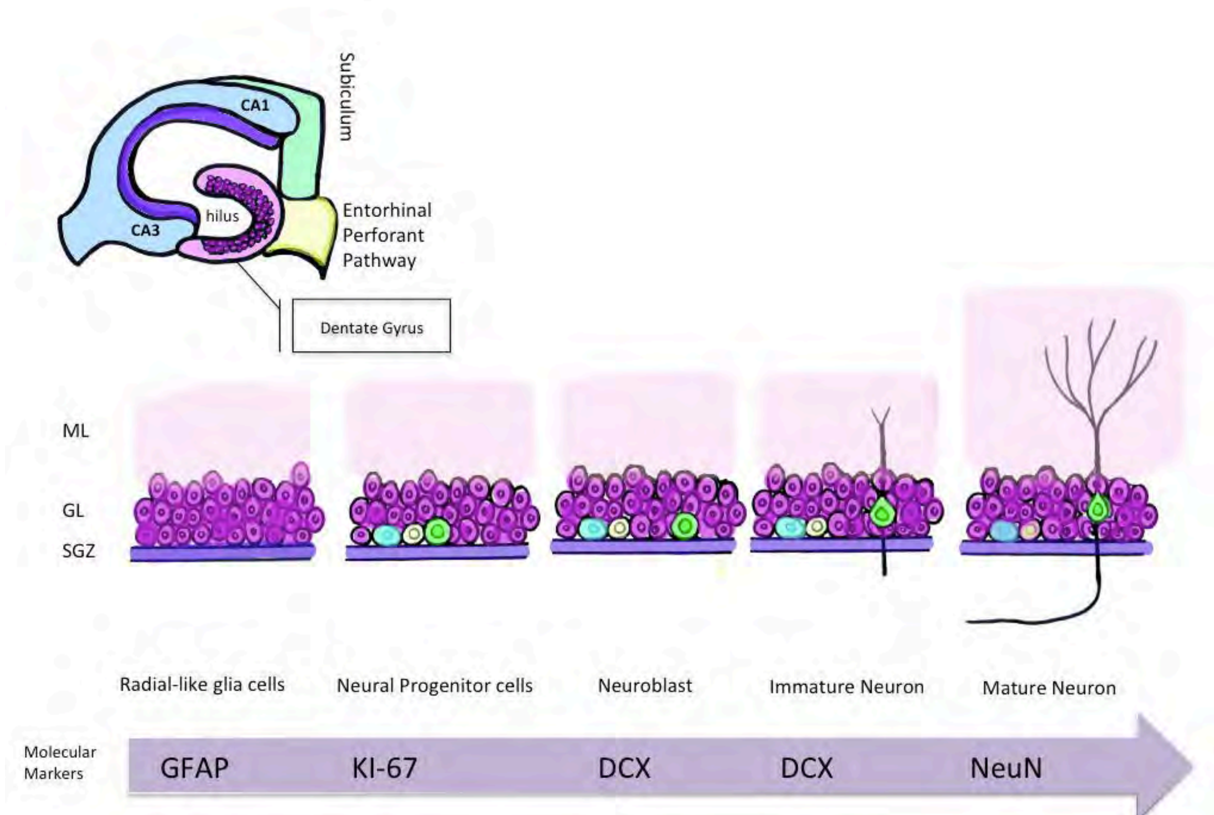


Figure 3.Maturation of a young neuron in the dentate gyrus of the hippocampus. Section a) represents the stage of neurogenesis the section b) indicates cell-type markers used to identify the stage of development. Information adapted from Ming & Song. 2002.

Neurogenesis has different effects on varying regions of the hippocampus. The dorsal dentate gyrus and ventral hippocampus dentate gyrus (DG) are functionally different and their connectivity is also distinctive (Fanselow & Dong, 2010). The dorsal hippocampus sends projections to the cortical regions involved in visuo-spatial information and memory processing and ventral hippocampus sends projections to the prefrontal cortex, amygdala and hypothalamus and areas linked to affective behaviors (Fanselow & Dong, 2010). The dorsal hippocampus controls spatial memory and pattern recognition and the ventral hippocampus controls emotional responses such as anxiety and depression-like behavior (Kheirbek *et al.,* 2013; Spalding *et al.,* 2013). Together the dorsal and ventral hippocampi are both proposed to play an integrated role in contextual memory and learning (Komorowoski *et al.,* 2013). Ablation of neurogenesis or enhancing neurogenesis (pharmacologically) renders alteration to the respective behavioural or cognitive functions mentioned above (Kheirbek *et al.,* 2013).

There have been some deviations to the proposed division of functionalitites. The dorsal and ventral hippocampi have been linked to the stress response. Arresting neurogenesis in the ventral hippocampus did not alter anxiogenic responses to acute restraint stress (Scopinho *et al*., 2013). These results could be due to the model used for depression which was newly modified and the stress employed was acute. It could also be indicative of neurogenesis having a variable role in amelioration of anxiety. However reduced neurogenesis in the ventral hippocampus is thought to mediate the depressive-like behaviour in maternally separated rats (Hulshof *et al.,* 2011) and in addition to this, certain antidepressant drugs have been observed to selectively increase cellular proliferation in the ventral hippocampus of rats (Banasir *et al.,* 2006) despite the tight regulation of cell turnover in the brain.

1.3) The importance of Glia in Neurogenesis

Neuroglia once only considered supportive cells in the brain, also act to release signaling molecules such as cytokines. They do not express the same markers as neurons (DCX and GFAP are not expressed on the same cell). Radial-like glial cells and parenchymal astrocytes are separate cell lineages, however neurons may in fact arise from radial-like glial progenitor cells expressing GFAP (Steiner *et al.,* 2004; Ming & Song, 2005).

The role of astrocytes in physiological conditions and their modulation and relationship to adult neurogenesis in the hippocampus is poorly explored and not well understood. However, several studies have linked them to an increase in neurogenesis (Shigemoto-Mogami *et al.,* 2014; Quesseveur *et al.,* 2013; Lim *et al.,* 1999). Physical insult such as lesions have induced an increase in the production of neurons in the striatum via astrocytes and astrocyte-derived- neuroblasts in mice (Magnusson *et al.,* 2014) suggesting a latent neuronal programming. The mechanism behind this increase is unclear.

In microglia it has been suggested that post-natal stimulation causes the release of cytokines to stimulate neurogenesis (at peak days 4 - 10). Cytokines released by glia such as interleukin-6, interleukin-1-beta, tumor necrosis factor and interferon gamma were shown (at certain concentrations) to stimulate neurogenesis in neurosphere cultures. However, the complexity of the interactions of cytokines *in vivo* may not be accurately reflected in culture and neurons were cultivated from the subventricular zone and thus the results may not hold true for the hippocampus (Shigemoto-Mogami *et al.,* 2014). In contrast, another study of the influence of astroglia on neural stem cell proliferation in the rat hippocampus found that neuronal counts increased by ten-fold when cultured with astrocytes. This study also found that the rate of proliferation and neuronal commitment were increased significantly in cultures where astrocytes were present (Song *et al.,* 2002). A more recent finding is that a reduction in Notch1 protein (a transmembrane ligand-activated receptor involved in cell fate) activity in the brains of mice resulted in an increased neuronal phenotype in mice suggesting another molecular mechanisms for a shift towards neurogenesis (Magnusson *et al.,* 2014).

It would appear that an increase in gliogenesis would be ideal to in turn increase neurogenesis. In support of this, wheel running has been shown to increase the number of astrocytes in the hippocampus (Steiner *et al.,* 2004). Genetic manipulation to up-regulate the expression of BDNF (that is naturally up-regulated via exercise) in astrocytes resulted in an anxiolytic/anti-depressant effect in mice. This provides *in vivo* evidence that cell types other than neurons may play a role in the modulation of neurogenesis and facilitate anxiolytic effects of fluoxetine (Quesseveur *et al.,* 2013). Although no functional link has been made to clearly explain this process at the molecular level in the hippocampus. It does highlight the relevance of astro-gliogenesis in neurogenesis and possibly their unexplored role in the treatment of depression. Improved quality imaging is therefore essential in preserving tissues sufficiently to distinguish between neuronal cell types undergoing neurogenesis.

1.4) Animal Models for Depression and Behavioral Tests

Animal models for depression are widely used and of great importance in understanding the pathophysiology of diseases and have been validated by their use in testing anti-depressants (Sanchez & Meier, 1996). An animal model must encapsulate face-validity, construct validity and predictive validity (Wilner, 1984) in order to be of use in the development of novel drug targets intended for humans. Although more recently their use in the commercial development of novel drugs has reportedly stagnated due to the complexity of depression (Hyman *et al.,* 2014; Belzung, 2014). It has been suggested that models be viewed critically and a broad spectrum of behavioural tests be used with varying sensitivity (Stewart & Kalueff, 2015). The predisposition to developing depression is often not addressed by animal models (Wilner & Belzung, 2014) as issues such as spontaneity and re-occurrence are difficult to reproduce (Kato *et al.,* 2015). However, this does not diminish the role they played in developing SSRI such as Citalopram, Paroxetine, Sertraline, Fluvoxamine and Fluoxetine are used to clinically treat depression (Sanchez & Meier, 1996) which have been validated by improving the reduction of depressive symptomology.

There are a handful of frequently used rodent models for depression namely, MS (Plotsky & Meany 1993), Chronic unpredictable mild stress (CUMS) (Wilner *et al.* 1987), Immune activation (Yirmiya, 1996), Prenatal stress (Mueller & Bale, 2008), Social defeat stress (Henry *et al.,* 1967), Dorsal raphe-specific HTT knockout mice (Lira *et al.,* 2003), Flinders sensitive line rats (Overstreet, 1986), Olfactory bulbectomized rats (Cairncross *et al.,* 1977), and Neonatal clomipramine administration (Mirmiram *et al.,* 1980). Interestingly MS, prenatal stress and Neonatal clomipramine administration are aimed at emulating a predisposition to depression developed via early life stress that is not addressed by the chronic stress paradigms. The MS model specifically has been widely used to explore depression and novel-treatments for depression in animal models (Marais *et al.,* 2009; Daniels *et al*., 2004; Ladd *et al.,* 2004; Aisa *et al.,* 2007). The model has been critically assessed in terms of its validity (Milstein & Holmes, 1997). The quality

and duration of maternal care have been linked to the healthy development of the HPA axis and stress regulation in adulthood (Ricon-cortes & Sulivan, 2015).

MS uses behavioural tests to measure depressive-like behaviors in rodents that are thought to indicate depressive-like behaviours homologous in humans (Cenci *et al.,* 2002). The depressive-like behaviors measured include: time spent immobile in a cylinder of water called the Porsolt Forced Swim Test (FST) (Porsolt *et al.,* 1997) which measures the motivation of the rodent to escape and an increase in time spent immobile is thought to infer depressive-like behavior. However, hypothermia and the stress of swimming are weaknesses of this test. The tail suspension test is another measure used which requires rodents to be suspended by the tail using tape in which "escape-like" behaviours and immobility are measured (Dalvi & Lucki, 1999). This test is very popular as they are thought to measure "behavioural despair" but it has also been challenged as an energy saving mechanism as remaining stationary possibly conserves energy required for later escape (West *et al.,* 1990). The sucrose preference test is used to measure anhedonia (loss of ability to experience pleasure), which is lost in both humans and rodents experiencing depressive-like behaviour (Wilner, 1997). The locomotor activity in the Open Field Test (OFT) can also be measured and analyzed as a measure of depressive-like behavior. This monitors the behavioor of a rat in an enclosed but open environment. The time in the closed arm of an elevated plus maze (EPM) is another indicator of depressive-like behaviour (Daniels *et al.,* 2004; Huot *et al.,* 2001; Grace *et al.,* 2009). Even the frequency of ultrasonic vocalizations emitted can be used as a behavioural measure indicative of depressive-like behaviours. In rats 22 kHz indicates a negative emotional state and 50 kHz a positive state (Knutson *et al.,* 2002).

There is a body of literature on the MS model that has found MS to effectively mimic depressive-like symptoms. It emulates depressive-like behaviours such as increased time spent in the closed arms of the EPM (Daniels *et al.,* 2004; Huot *et al.,* 2001; Grace *et al.,* 2009), decreased sucrose consumption (Huot *et al*., 2001; Asia *et al.,* 2007; Rü̈edi-Bettschen *et al.,* 2005), decreased time in the center of the OFT (Rana *et al.,* 2015; Shu *et al.,* 2015; Asia *et al.,* 2007), decreased mobility in the Porsolt FST (Marais *et al.,* 2009; Maniam & Morris, 2010; Asia *et al.,* 2007; Rana *et al.,* 2015), novelty suppression feeding test (Chee & Manard, 2011) among others. The effects of MS are indicative of increased anxiety and depression-like behaviors in rodents (Daniels *et al.,* 2004; Marais *et al.,* 2009; Grace *et al.,* 2009; Maniam & Morris, 2010; Aisa *et al.,* 2007). MS rats display increased susceptibility to developing depressive-like symptoms after a subsequent trauma in adult life. MS rats exposed to 5 days of restraint stress in adulthood exhibited increased immobility in the FST (Marais *et al*., 2009) an inference of depressive-like behaviour.

The physiological changes caused by MS to the HPA axis include decreased responsiveness to long-term stress and an exaggerated response to acute stressors (e.g. Air-puff startle) (Ladd *et al.,* 2004) and physiological changes such as an increased ACTH response to air-puff startle (Huot *et al.,* 2001). Daniels *et al.,* 2004 reported an increase in basal ACTH in MS rats relative to NMS control rats, and significantly lower levels of ACTH 15 minutes after acute restraint stress proposing a blunted stress response in MS animals. Higher maximal corticosterone levels after restraint stress have been reported

in mice although the rate at which corticosterone returned to base-line was not found to be significantly different to NMS mice (van Heerden *et al.,* 2010). The proposed reasons for this are changes at the level of the HPA axis and in neuronal turnover.

Despite this, MS has been criticized for its ability to reproduce homogenous results in mice within a study (Milstein & Holmes, 1997). The depressive-behavioural phenotype is not consistent and eight strains of MS mice were tested and none displayed a consistent depressive-like phenotype as measured by the forced swim test, elevated plus maze and open field test which do not always agree within a specific treatment group (Milstein & Holmes, 1997). MS also affects mice in a sex dependent manner as MS male mice were seen to be more susceptible to corticosterone and alcohol administration than female mice. Females displayed a significantly higher scoring of locomotor activity relative to male mice in a novelty test (Kawakami *et al.,* 2013). Therefore, only male rats are used in this study to avoid the variability introduced by sex. Another consideration to be noted is individual variability existing in both animals and humans. Thus, the model has not been homogenously reproduced in mice, while rat studies indicate a more reproducible phenotype although results also vary.

A study by Hulshof *et al.,* 2011 using Wistar rats found no increase in anxiety-like behaviour as measured by difference in social interaction scores and exploratory behaviour in a novel environment that is thought to be indicative of Wistar rats being more resistant to MS stress. This finding disagrees with Rana *et al.,* 2015 that showed MS Wistar rats displayed a depressive phenotype while MS WKY rats did not (Grace *et al.,* 2009). No increase was also shown in anxiety-like and depressive-like behaviours in male Sprague Dawley rats as measured by the OFT and EPM. Thus inconsistencies remain in the model's effectiveness to induce a consistent behavioural phenotype in both rats and mice, despite this there are numerous studies that report its effectiveness in producing a depressive-phenotype.

Discrepancies in the model may also be attributed to the loose use of the term "MS" which encapsulates a variety of separation parameters. The model varies in time windows of separation days ranging between PND 1 - 21 and times of separation ranging from 15min - 24h (Schmidt *et al.,* 2004; Aisa *et al.,* 2007; Marais *et al.,* 2009), however the most commonly used method is removing dam (mother) from pups (infants) from PND 2 - 14 for 3 hours daily (Grace *et al.,* 2009; Daniels *et al.,* 2004; Marais *et al.,* 2009; Rana *et al.,* 2015; Shu *et al.,* 2015). The duration of separation and time at which chronic/acute separation is conducted remains variable between studies (Carrera *et al.,* 2009). It would appear that longer periods of separation are more effective at inducing a depressive phenotype (Ladd *et al.,* 2004; Aisia *et al.,* 2007).

The PND 2 - 14 for 3 hours daily MS model is thought to activate the HPA axis and alter the development of the hippocampus as the hippocampus develops post-natally and is rich in GR in the granule cell layer of the hippocampus (Hulshof *et al.,* 2011). The changes to the neurons and regulatory alterations that occur post-natally increase susceptibility to alternations in neuronal functioning and caused enhanced susceptibility of neurons to oxidative stress (Daniels *et al.,* 2011). A more recent study in Sprague Dawley rats showed that MS reduces the capacity for proliferation neuronal

differentiation in the denate gyrus of the hippocampus (Boku *et al*., 2015). It has also been shown to decrease Reelin protein expression (Zhang *et al.,* 2013) that influences migration of radial-like neurons during embryonic development and plays a role in post-natal synaptic strengthening and plasticity in newly generated neurons (Zhang *et al.,* 2013). Additionally MS decreases hippocampal proteins bdnf and creb involved in the process of neurogenesis (Shu *et al.,* 2015). These are just a few of the molecular changes that occur as a result of MS, other changes include decreased MSis c-fos (marker of activated neurons) (Horii-Hayashi *et al.,* 2013) and changes in receptors for glucocorticoids and serotonin (GR and 5HT1A) (Maniam & Morris, 2010; Aisa *et al*., 2007; Ladd *et al*., 2004). These molecular changes caused by MS to the HPA-axis and hippocampus has also been shown to have behavioral implications. Reduced neurogenesis has been suggested to underpin the anxiogenic behaviours.

1.5) Rodent Studies linking depression and neurogenesis

Neurogenesis in the rodent hippocampus is increased by chronic anti-depressants (Nibuya *et al.,* 1995, Nibuya *et al.,* 1996; Duman *et al.,* 2000). Findings like these encouraged further exploration of the link between hippocampal-neurogenesis and depressive-like symptoms (Manij *et al.,* 2003).

In mice inhibiting neurogenesis in the hippocampus through irradiation or administration of exogenous corticosterone (Kong *et al.,* 2014) resulted in a delayed recovery time to normalize corticosterone levels and mice showed an increase in anxiety and depressive-like symptoms (Snyder *et al.,* 2011). The prolonged imbalance in corticosterone release reduces activity of pathways such as TGFb-SMAD2/3 that function to stimulate neurogenesis and hedgehog signaling which stimulates neuronal differentiation. These pathways have been linked to glucocorticoid-based reduction in neurogenesis (Anacker *et al.,* 2013). Contrastingly, Jedynak *et al.,* 2014 found neurogenesis in mutant mice is not linked to depressive-like behavior and refute the notion that there is a meaningful link between them. The earlier literature suggested that the antidepressant fluoxetine acts independently or partly dependent of neurogenesis (David *et al.,* 2007; Santarelli *et al.,* 2003). Another suggestion has been that there is a modulatory and rather than causal relationship between neurogenesis and depressive-like behavior in animals (Vollmayr *et al.,* 2007). Despite the variation in results in mice there has been a body of data in rats supporting a link between neurogenesis and depressive-like behavior and molecular/cellular changes.

Depressive-like behavioural changes were observed when neurogenesis was pharmacologically arrested in Long Evans rats with methylazoxymethanol. Depressive-like behaviour increased as measured by an array of behavioral tests including the forced swim test (Mateus-Pinheiro *et al.,* 2013). Pharmacologically up-regulating neurogenesis reversed these behavioral effects. Adult hippocampal neurogenesis has also been observed in male Sprague Dawley rats. When treated with exogenous corticosterone a reduction in neurogenesis was coupled with an increased immobility time in the Porsolt forced swim test that measures depressive-like behaviour (Yau *et al*.,

2014; Hulshof *et al.,* 2011). Similarly a study using Gravid Sprague Dawley model for depression reported decreased neurogenesis as measured by cell counts in the hippocampus (Lin and Wang, 2014). A link between neurogenesis and depression is further supported by a study by Hushlof *et al*., (2011) that proposed neurogenesis in the ventral hippocampus mediated the depressive-like behavior in maternally separated rats (Hulshof *et al.,* 2011).

Studies observing cellular changes in rat models for stress and depression also report links between neurogenesis and depression. A model for depression using exposure to corticosterones has also been linked to a reduction in the protein reelin in Long Evans rats. Reelin reportedly plays a role in maturation and migration of newly developing neurons and depressive symptoms were coupled with a decrease in this protein and neurogenesis (Fenton *et al.,* 2015). Excessive activation of the HPA axis (which has been linked to depression) via GR causes atrophy and decrease in the size of the hippocampus and decrease in synaptic connectivity with chronic stress decreasing GR density and receptor function in the hippocampus (Isgor *et al.,* 2004). In rats these changes influences neuronal density and morphology (Mateus-Pinheiro *et al.,* 2013). Thus stress has been shown to decrease neurogenesis. Stress has also been linked to levels of synaptophysin that promotes neurogenesis, furthermore depressive-like behaviour was reversed by two weeks of voluntary running via increased levels of synaptophysin promoting hippocampal neurogenesis (Yau *et al.,* 2014).

The suppression of neurogenesis causes molecular changes and reportedly causes a reduction in dendritic spine density (Mateus-Pinheiro *et al.,* 2013). This reduction in dendritic branching was also reported in immature DCX labelled neurons in the granular layer of rats (Fenton *et al.,* 2015) in addition to the decrease in number of neurons present after chronic exposure to corticosterone. This also caused changes in the hippocampal neurogenic niche and decreased the negative feedback receptors required to regulate stress (Mateus-Pinheiro *et al.,* 2013). Thus neurogenesis is thought to be impaired at the molecular and cellular level in rodent models for stress. Targeting and reversing these changes may play a role in decreasing depressive-like behavior in animal models.

1.6) Rodent Studies linking exercise and neurogenesis

Exercise has been studied in a number of rodent models for depression and found to have systemic effects, which alter brain morphology (Stranahan *et al.,* 2007) and metabolism (Speisman *et al.,* 2013; Marais *et al.,* 2010). Exercise has been shown to suppress apoptosis and mitigate autophagy and consequently increase adult hippocampal neurogenesis and improve memory and synaptic plasticity (Kim *et al.,* 2010; Zhang *et al.,* 2013; Bechara & Kelly, 2013).

The question of which type of exercise is optimal and what duration is best for increasing neurogenesis and ameliorating depressive-like symptoms is disputed. Three weeks of forced wheel running of adult Sprague Dawley rats was effective in up-regulating the BDNF-pCREB pathway increasing neurogenesis (Jian-feng *et al.,* 2014),

forced swimming (Rodriguez *et al.,* 2015) has also shown to improve neurogenesis, although intense exercise has been suggested to negate the beneficial effects of exercise (Inou *et al.,* 2015). There is earlier dated literature that suggests that the stress of forced exercise abrogates the positive effect of physical activity by increasing anxiety in mice although both forms of exercise increase neurogenesis (Leasure & Jones, 2008). However, voluntary wheel running has been shown to have beneficial effects on rat memory (Nobel *et al.,* 2014).

Voluntary Wheel running in mice has been shown to increase BDNF levels and neurogenesis including the generation of new cells, normalizing dendritic outgrowths and increasing survival of neurons (Myoung-Hwa *et al.,* 2013). Voluntary wheel running up-regulates neurotrophic growth factors such as vascular endothelial growth factor (VEGF), brain derived neurotrophic factor (BDNF) factors and gene expression of proteins such as Wnt, which have all been implicated in enhanced neuroplasticity (Speisman *et al.,* 2013, Bayod *et al.,* 2014; Gregoire *et al.,* 2014). Running increases the dendritic complexity and density of new neurons generated in the SGZ of the rat dentate gyrus (Stranahan *et al.,* 2007). This increase in neurogenesis improves hippocampal dependent behaviours, improving the ability of rodents to better recognize stressors and subsequently avoid them (Hamilton *et al.,* 2014). Voluntary exercise in rats has been shown to reverse the effects of restraint stress and increase the number of immature neurons and mature functioning neurons present relative to stressed controls (Castilla-Ortega *et al.,* 2014). As such the benefits of this exercise induced up-regulation of neurogenesis is a key area of exploration.

Exercise in the form of voluntary wheel running has been shown to reverse the depressive and anxiety-like behaviour caused by MS (Marais *et al.,* 2009; Grace *et al.,* 2009). It has also has been shown to reverse the molecular changes caused by MS including decreasing the vulnerability of neurons to oxidative stress (Daniels *et al.,* 2011) in addition to reversing the aberrations in molecular expression caused by maternal separation (Maniam & Morris, 2010). This is because exercise is thought to have a compensatory effect and targets the same proteins affected by MS (Dimatelis *et al.,* 2012). Thus this MS model was selected to explore the impact of voluntary wheel running would have on neurogenesis in vivo.

1.7) Tools to study neurogenesis

The gold standard for measuring neurogenesis in vivo and in vitro (Ming & Song, 2005) is the use 5'-bromo-2'-deoxyuridine (BrdU) (Figure 4 A) as an assay. This is a halopyrimidine thymidine analogue that is incorporated into the DNA of cells undergoing mitosis during the S-phase of mitosis. It can be detected using monoclonal antibodies but only after the tissue has been severely denatured in order to permit binding of fluorophores (Gratzner, 1982). This marker gives no indication of the

phenotype of cells undergoing mitosis and subsequently must be used in conjunction with cell-type specific markers in order to identify neurogenesis. There are several commonly used cell type markers including NeuN and calbindin, these label mature neurons. There are also cell-type markers for immature neurons such as DCX or polysialic-acid neural cell adhesion molecule. The use of these markers in conjunction with BrdU allows one to identify neurons undergoing neurogenesis.

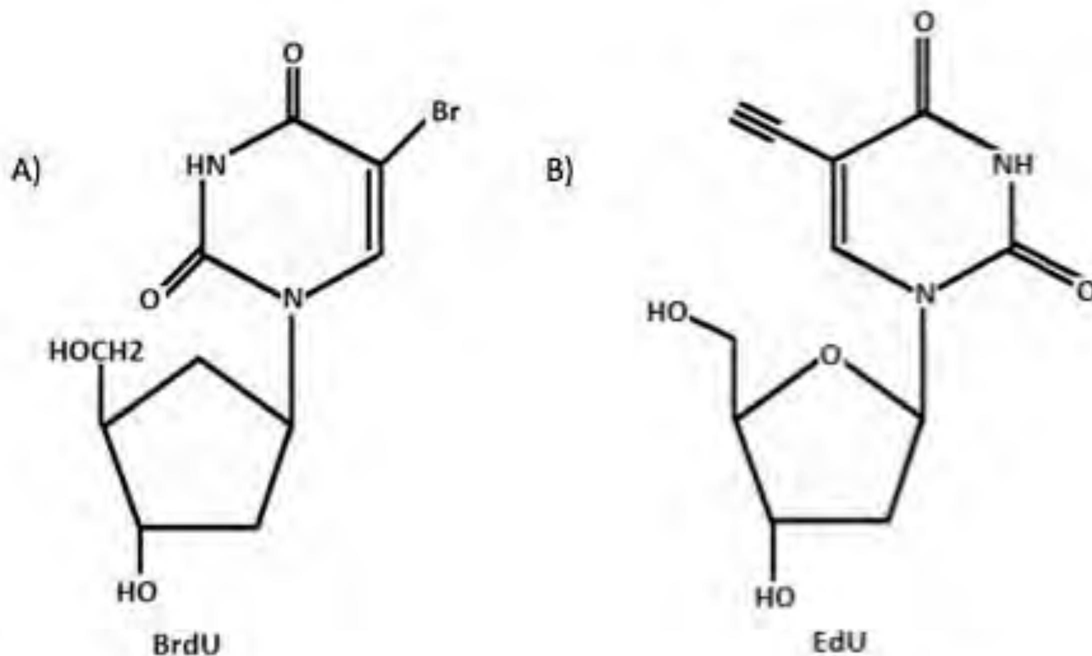


Figure 4.Comparison of the molecular structure of BrdU and EdU

1.8) Challenges with BrdU

BrdU has been used to study neurogenesis in multiple recent rodent studies (He *et al.,* 2014; Gi-Mohapel, 2013; Lehmann *et al.,* 2013). It has also been used to study modulation of neurogenesis in rodents that have been exercised using forced treadmill exercise (Kim *et al.,* 2014), voluntary wheel running (Liu *et al.*, 2013; Rhodes *et al.,* 2003). Despite its prominence and consistent use in many recent studies there has been a body of criticism of the label.

One such issue is that BrdU labelling requires that tissue be exposed to acid and denatured at high temperatures in order for the large fluorophore to bind to the thymidine when it is incorporated into the double helix of the DNA (Wojtowicz & Kee, 2006). This results in the loss of many epitopes that would hinder the use of multiple cell-type labelling as sites for label to bind would be diminished via tissue damage and consequently data could not be used for quantitative analysis (Tang *et al.,* 2008). The process is labor intensive and not compatible with multiple markers. Despite this, many

papers readily use BrdU as a label to study neurogenesis quantitatively (Rhodes *et al.,* 2003; Speisman *et al.,* 2013).

BrdU dosage has also been called into question. The optimal dosage for labelling all cells undergoing neurogenesis at a single time point has been examined. It was found to be 300 mg/kg in rats (Cameron & McKay, 2001), although most studies consistently use the sub-optimal 50mg/kg dose (Ponti *et al.,* 2012; Shruster *et al.,* 2012; Chehrehasa *et al.,* 2009) despite this being shown to label only a fraction of all mitotically active cells. This is possibly because of the perceived toxicity coupled with higher dosages. The most common dosage used is 50 mg/kg (usually in mice) with more than one injection administered over the course of treatment (Lehmann *et al.,* 2013; Gil-Mohapel, 2013; Rhodes *et al.,* 2003). Multiple injections have also been used in labelling rat cells undergoing mitosis (Monje *et al.,* 2003; He *et al.,* 2014; Speisman *et al.,* 2013). Problems arise when trying to quantitatively analyze the total number of cells undergoing mitosis at a single time point that is not possible with sub-optimal labelling or multiple injections which gives an average over longer time periods. Another factor to be considered is the build up of toxicity with a large number of injections.

High levels of BrdU have been found to be toxic specifically when administered during the prenatal stages of development where formation of the cerebellum was altered (Nagoa *et al.,* 1998). However in the context of adult neurogenesis there have been no studies that report any cases of toxicity with high levels of BrdU administration such as 200 mg/kg x 6 injections. No adverse effects were noted after 42 days in mice (Gil-Mohapel *et al.,* 2013). The label was seen to last in the aforementioned study over the period of 42 days however the label (when administered at lower levels) has been found to decay over time (Sauerzweig *et al*., 2009). An experiment using male Sprague Dawley rats found that BrdU leaks into the cytosol after 2-3 weeks and resulted in a punctuated and segmented label in the nucleus of labelled-cells (Sauerzweig *et al.,* 2009). These observations have lead to the development of novel markers for neurogenesis.

1.9) Novel Marker EdU

One such novel marker is 5-ethynyl-2'-deoxyuridine (EdU) a thymidine analogue (Figure 4B) that is detected using a copper catalyzed reaction with a fluorescent azide (Salic & Mitchison, 2008). This reagent is small and does not require denaturation for exposure of EdU binding site and the detection is more sensitive than that of BrdU. It is thought for these reasons it would result in improved in vivo detection of neurogenesis. EdU was reported to be sensitive to low levels of neurogenesis and labels regions of low level of neurogenic turn over such as the mouse brain (96 hours after injection) and small intestines (Salic & Mitchison, 2008). Thus EdU was considered an improvement on BrdU and several studies have undertaken to compare the two labels to illustrate this in an attempt to optimize the assay for neurogenesis (Zeng *et al.,* 2010).

Studies were undertaken to compare quantitative measurement of neurogenesis by EdU and BrdU. A study by Zeng *et al*., (2010) sought to directly compare the use of EdU and

BrdU in mice having undergone voluntary exercise and restraint stress. Mice were injected with 200 mg/kg EdU once off and an equivalent amount of BrdU and perfused 4 hours later. Counts of the cells in the dentate gyrus revealed no significant difference between BrdU labelled cells and EdU labelled cell numbers although both labels revealed that mice that exercised had significantly higher number of labelled cells relative to control mice. Thus the study highlights the novel marker EdU as a useful replacement for BrdU in quantitative studies where preserving antigenicity of tissue is preferable. The study also showed that there was no interaction between BrdU and EdU labels allowing for the use of both labels in the same animal.

1.10) EdU as compared to BrdU

EdU has been used in conjunction with BrdU in a number of studies exploring the mechanisms of neurogenesis (Bonaguidi *et al.,* 2008; Guo *et al.,* 2009; Nagashima *et al.,* 2013; Worlitzer *et al.,* 2013; Vadodaria *et al*., 2013). It has been used largely as a short-term label with BrdU being used as a long-term label (Shuster *et al.,* 2012). This is in line with the discovery that at high levels EdU is toxic in mice and alters nuclear structure (Ponti *et al*., 2012). This was found in mice that were administered 16 injections of 50 mg/kg (one every 2 hours) so it requires a high dosage before any side effects are noted *in vivo*. Another criticism of EdU *in vivo* was that it altered metabolic activity and decreased cell proliferation in vivo and alternative improved marker (2'S)-2'-deoxy-2'-fluoro- 5-ethynyluridine (F-ara-EdU) was considered a superior label in terms of sensitivity and minimally interfering with genomic expression (Neef &Luedtke, 2011). This study did not compare tissues from *in vivo* rodent models and subsequently may not accurately represent metabolism in a living rodent model.

However, EdU has been shown to be a superior marker relative to BrdU that has been shown to render inconclusive results due to poor tissue quality (Worlitzer *et al.,* 2013). Qualitative analysis has shown that EdU preserved tissue quality and improved the epitope binding of multiple labels such as NeuN (Chehrehasa *et al.,* 2009). This has led to EdU being used instead of its counterpart BrdU in several studies (Culley *et al*., 2011; Becker *et al.,* 2012; Vadodaria *et al*., 2013; Bhansali *et al.,* 2014; Azzarelli *et al.,* 2014). It should be noted that many studies still use BrdU as the first option for labelling.

Another marker to take note of which is frequently used as a tool to study neurogenesis is Ki-67. This is a large nuclear protein with a molecular mass of 395 kDa, which is endogenous and variably expressed during the different stages of mitosis. The obvious benefit is that it is already present in the cells and thus no stressful injections are required (Wojtowicz & Kee, 2006; Shingemoto-Mogami *et al.,* 2014; Gregoire *et al.,* 2014). Surprisingly little is known about the function of this molecule considering its popularity but it would appear to have a primary role in cell proliferation and is widely used as a medical diagnostic tool to diagnose proliferation of malignant cells such as cancer cells (Brown & Gatter, 2002).

Thus using the above mentioned novel marker EdU and Ki-67 this study attempted to explore the physiological process of adult neurogenesis in the hippocampus of rats

subjected to MS and then later exercised using voluntary-wheel running in an attempt to further elucidate the relationship between depression and neurogenesis, and explore the hypothesis that neurogenesis is increased in MS rats as is cellular proliferation and neuronal survival. These markers would improve the quality of data attained from the hippocampus and explores neurogenesis in an attempt to target novel drug targets for depression.

Purpose of the Study

We hypothesized that neurogenesis in MS rats would be decreased and then increased by voluntary exercise as it would be in NMS rats, as would cellular proliferation and cell survival. Exercise has been reported to ameliorate the behavioral and molecular effects of MS. This study aimed to study exercise-induced hippocampal neurogenesis. It also attempted to accurately capture this quantitatively using imagining tools based on the current literature.

Aims

1. To characterise the use of thymidine analogue EdU in conjunction with co-labels. In order to accurately identify, count and quantify cell-types undergoing neurogenesis.

2. Determine whether MS blocks the effect of exercise on cell genesis and/or survival in the hippocampus and/or prevents these proliferating cells from becoming mature neurons or glia.

3. Explore whether or not voluntary exercise affects cell genesis and survival in the dentate gyrus of the hippocampus specifically in MS rats.

Objectives

- Characterise EdU and co-labels GFAP, NeuN, DCX and Ki-67 optimally, specifically focusing in the adult rat hippocampus.
- Using stain combinations EdU/GFAP/NeuN, EdU/DCX and KI67/DCX to compare cell genesis and survival in the hippocampi MS rats.
- To establish the effect of voluntary wheel running on MS-rats by housing them with running wheels

Chapter 2

Methods

2.1) Pilot Study

2.1.1) Aim

The first aim of the pilot study was to characterise EdU labelling of hippocampal cells. The optimal volume and concentration for EdU labelling of cells would then be used to study neurogenesis in the dentate gyrus of MS rats and NMS rats that had been housed in cages with locked or unlocked running wheels. EdU was used to compare the survival of cells that underwent cell genesis after EdU injections were administered, additionally the marker Ki-67 was used as a pulse to measure mitotic activity at the time of perfusion. The optimization of these labels was necessary to use them to identify group differences in the experimental rats.

2.1.2) Animals

Ten male Sprague-Dawley rats were obtained from the Animal Facility of the University of Cape Town. An additional five Sprague-Dawley rats were purchased from The University of Stellenbosch. Rats were obtained at PND 30 whereby, two rats were used to optimize EdU-label and the remaining thirteen rats were used for the pilot study.

Rats were housed in the Satellite Animal Facility in the Department of Human Biology under standard conditions 12 h: 12 h light/dark cycle with lights on at 6:00. The temperature was set at 22 ± 2°C. Rats had access to food and water *ad libitum*. Rats were fed Nutroscience rat chow (See Addendum). All procedures were authorised by the Health Sciences Faculty Animal Ethics Committee of the University of Cape Town (protocol no. 012/061). The Study was carried out in accordance with the South African National Standard: The care and use of animals for scientific purposes 10386:2008.

2.1.3) Exercise and ethynyl-2'-deoxyuridine (EdU) Injection

Rat treatment included voluntary exercise (in order to stimulate neurogenesis in the brain) in the form of voluntary wheel running. Distance travelled was measured manually using daily wheel rotations (one rotation was equivalent to one meter). On postnatal day (PND) 54/55 rats were placed in cages with adjoined running wheels in a separate wheel running room in the satellite animal facility in the Human Biology

Department. Rats had free access to Nutroscience rat chow and water and had an additional option of powdered food.

All rats were intraperitoneally (IP) injected with the mitotic marker EdU (50 mg/kg, 6.66 mg/ml) on either PND 60/61 or PND 74/75 or PND 94/95. This was done to label and monitor cells undergoing mitosis.

Rats were divided into three groups. Group one consisted of five rats that were allowed to run from PND 54 - 96 with EdU injections administered to rats on PND 96 four hours prior to perfusion. Group two consisted of five rats that were exercised from PND 54 - 74 and a third group of three rats was housed in locked wheels, which acted as a control group. EdU injections were administered on PND 74 four hours prior to perfusion (Figure 5).

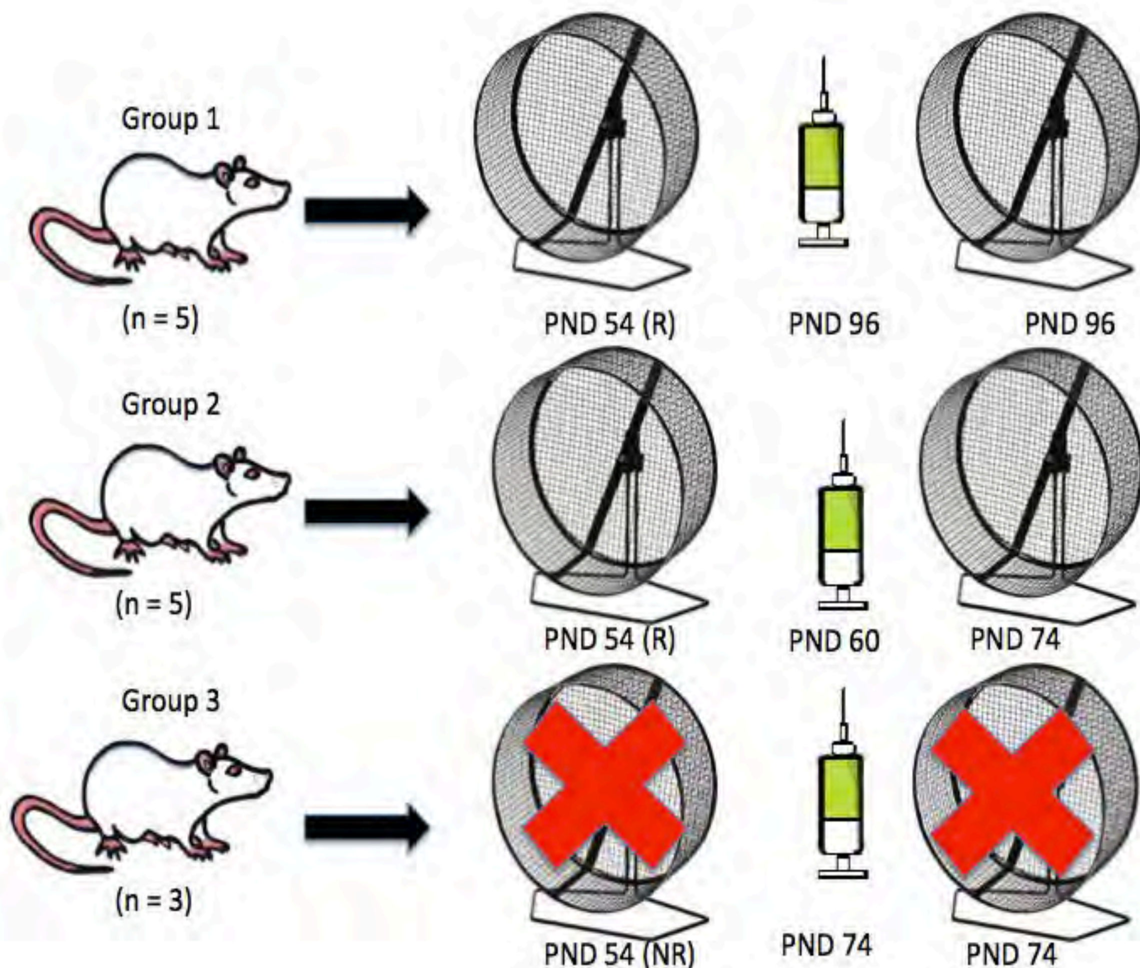


Figure 5. Diagram of treatments each rat group underwent. Adult rats were housed in cages with running wheels that were either locked (NR) or not (R) as indicated by the diagrammatic wheels. The locked wheels are indicated by a red cross. EdU injections were administered at one of three time points (PND 60, 96 or 74). Rats were returned to home cages and perfused either four hours later (Group 1 and 3) or two weeks after injection (Group 2) in order to establish the optimal window of time to study neurogenesis *in vivo.*

2.1.4) Transcardial Perfusions

Paraformaldehyde (PFA, 4%) was prepared in the morning of perfusion using fresh 0.01 M phosphate buffered saline (PBS) (see addendum for full composition). Paraformaldehyde powder (Sigma-Aldridge) was weighed out and stirred on a hot plate until the solution reached 66 °C. At this temperature, a saturated Sodium Hydroxide solution was added in a drop-wise fashion until the precipitate cleared to adjust the pH to approximately 6.9. Thereafter the solution was permitted to cool to room temperature. This solution was prepared in a fume hood

A Gilson mini-pulse 2 pump was used to emulate the pumping motion of the heart and to pump solution through the vascular system. The perfusion tubes were flushed with ethanol to sterilize tubes and then PBS to ensure no ethanol or contaminants remained. These tubes consisted of a valve that allowed one to switch from PBS flow to PFA without losing pressure.

Rats were habituated (in perspex cages containing wood shavings and had access to food and water *ad libitum*) for one hour prior to procedure. Animals were deeply anesthetized using halothane (Safeline Pharmaceuticals).

The deeply anaesthetized rats were secured to the operating surface and a lateral incision was made through the integument and abdominal wall below the ribcage. An incision was made along the length of the ribcage to expose the pleural cavity and the sternum was lifted away to expose the heart. The connective tissue around the heart was carefully trimmed away and the perfusion tube needle was inserted into the left ventricle and a small incision was made in the posterior of the right atrium to release pressure. The rat was then exsanguinated with 50 ml - 100 ml 0.01 M PBS (pH 7.4) solution. The valve was then switched from buffer to 4 % PFA 300 - 400 ml. Then PFA was pumped through the body of the rats taking note of whole body fasciculation (which indicated cross-linking of proteins in muscles). The perfusion process took on average 1 hour per rat. It was noted that 100 ml 0.01 M PBS and 400 ml 4 % PFA were optimal for rats ranging between 350-400g.

Thereafter, the rats were decapitated using a guillotine and the brain removed immediately and placed in 4 % PFA for three hours. The brain was transferred to 30 % sucrose solution and stored in sucrose at 4°C until it sank (3 - 5 days).

2.1.4) Brain Sectioning

Brains were extracted and stored using three methods. Six brains were stored in 30 % sucrose and processed immediately upon sinking, two brains were frozen in OCT medium and kept in the -20 °C freezer for 4/5 days after sinking before being sectioned. Two brains were frozen in 30 % sucrose and stored for four days prior to sectioning. The tissue quality varied. Processing brains immediately rendered optimal tissue quality. The final three rats were sectioned immediately upon sinking in 30% sucrose with no more than 1 day between sinking and sectioning. Whole brains were frozen in OCT medium and cryo-sectioned coronally using a cryostat (Leica cryostat CM1850) at a thickness of 40 µm. Every 3rd section of dorsal hippocampus was collected (total 6 sections per rat per stain combination) in 3 % Triton X-100 (Merck) in PBS.

2.1.6) Staining Wells

Sections were stored in Greiner well plates (6 x 4) containing either the appropriate wash or antibody incubation medium. A fresh well plate was used for each immunohistochemistry step (e.g. blocking, washing and antibody incubations) and each brain section was transferred manually using a fine bristled paintbrush.

2.1.7) Immunohistochemistry

Brain sections were collected using wells containing 0.03 % Triton-X-100 in PBS. These were incubated in the fridge for an hour to permeabilise cell membranes to allow entry and binding of fluorescent antibodies to epitopes on brain sections. EdU was the first molecular marker to be detected.

The EdU detection was performed prior to incubation with additional antibodies. The Invitrogen Imaging kit (C10337) Click-iT reaction cocktail was used. The method was modified for fixed free-floating tissue with endogenous EdU. The click-iT reagent cocktail was prepared with volumes listed in the Invitrogen product information sheet. The kit comprised of 4 components: a click-iT reaction buffer, $CuSO_4$, Alexa-Fluor and a reaction buffer additive. Brain sections were incubated in 1000 µl click-iT reagent; 860 µl 1x click-iT buffer, 40 µl CuSo4, 2.5 µl Alexa Fluor-Azide and 100 µl of reaction buffer. Adding the reagents in this order ensured a specifically buffered environment for the reaction to take place. The cocktail was prepared in the dark as the reaction is light sensitive. The reaction is a copper-catalyzed covalent reaction between an azide (fluorophore) and an alkyne (EdU) and required 30 minutes for the

reagents to react optimally binding the fluorophore to the EdU, which was already integrated into the DNA of the fixed brain tissue.

Tissue was blocked in 3 % Bovine Serum Albumin (BSA) (Roche Diagnostics, USA) for 2 x 10 min to prevent non-specific binding of antibodies. Tissue was thereafter incubated in primary antibody (prepared in a blocking solution; 20 ml 0.01M PBS, 200 mg BSA & 0.1 ml 100 % Triton-X-100) for 16 hours at ambient room temperature. Brain sections were washed in 3 % Triton X-100 in PBS for 3 x 10 min and then incubated in secondary antibody (which was made up in the aforementioned blocking solution) for 5 h. The sections were manually transferred into wells containing 3 % Triton X-100 in PBS for 3 x 10 min washes prior to a 15 min Hoechst incubation followed by a 10 min PBS wash. Sections were then free floated in PBS, placed on slides in anti-fade and cover slipped. Two staining combinations were used for characterisation. The first was EdU and the immature neuronal marker Doublecortin (DCX), and the second, EdU in conjunction with a cell-type specific marker for mature neurons (NeuN) and Glial Fibrillary Acidic Protein (GFAP), which labels astrocytes (Table 3 and Table 4).

2.1.8) Quantification

The confocal microscope (LSM 510 Zeiss) was used to scan brain sections and Zen 2009 software was used to identify cell-type specific markers using co-localisation and profiling (See Table 1, 2 for parameters).

Table 1. Tile-scan Settings (20x) for overview scan of hippocampus

Laser	Excitation Wavelength (nm)	Power (%)	Emission Filters
Mai Tai	750	0.5	390-465 (blue)
Argon 488	488	5	500-550 (green)

Table 2. Detail Scan Settings (10x) for dentate gyrus

Laser	Excitation Wavelength (nm)	Power (%)	Emission Filters
Mai Tai	750	10	390-465 (blue)
Argon 488	488	5	500-550 (green)
Diode powered solid state (DPSS)	561	10	575-630 (red)
HeNe633	633	10	650-710 (purple)

Confocal Lasers scanned each pixel of the specimen gathering data about the intensity of each marker's fluorescence. The co-localisation function was used to identify areas of overlap between fluorescent markers. These fluorescent signal intensities were used to estimate the correspondence between the two or more markers that spatially overlapped. This confirmed whether two markers were associated with the same structure (Figure 6).

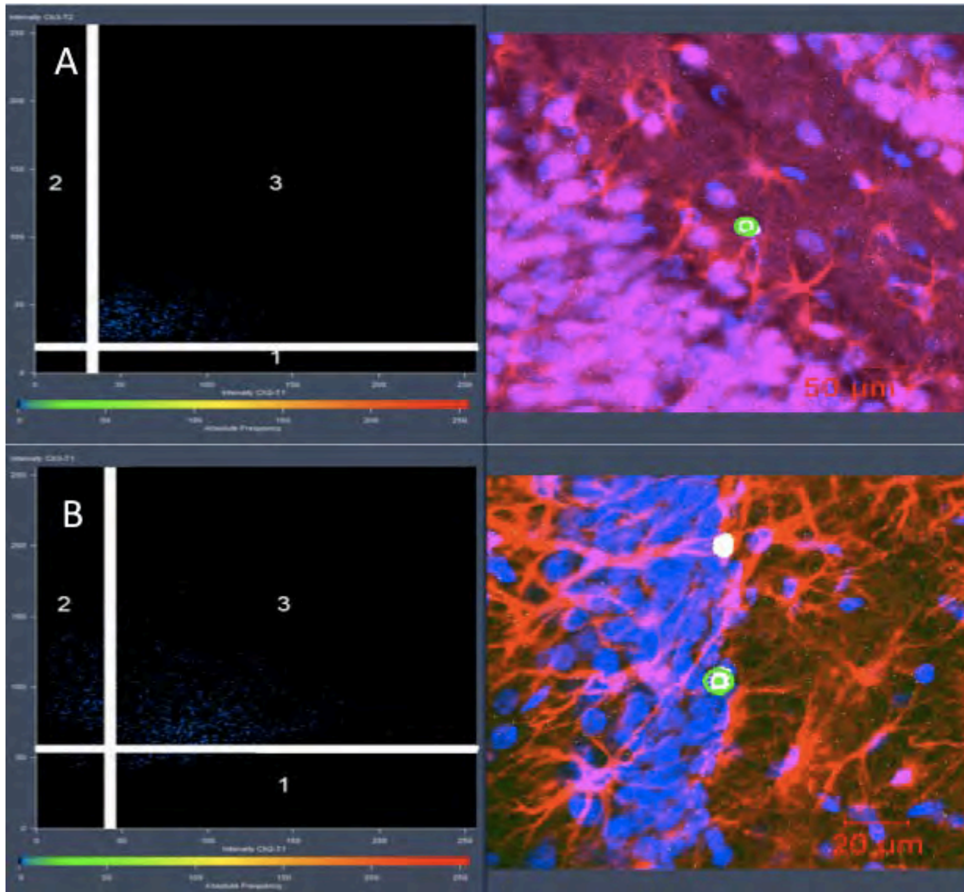


Figure 6. Co-localisation scatterplots displaying the stain intensity of the pixels selected in the green circle in A and B. Region 1 represents only pixels with EdU stain, region 2 represents only pixels with stain for NeuN (A) and GFAP (B) and region 3 represents the number of pixels which overlap in fluorescence intensity of the respective stains. The majority of pixels overlap in region three indicating that the two stains co-localized in the same structure. The tile scans on the right show the NeuN positive neurons (pink), GFAP positive Astrocytes (Red) and Hoechst stained nuclei (blue). Areas of extreme co-localisation appear white.

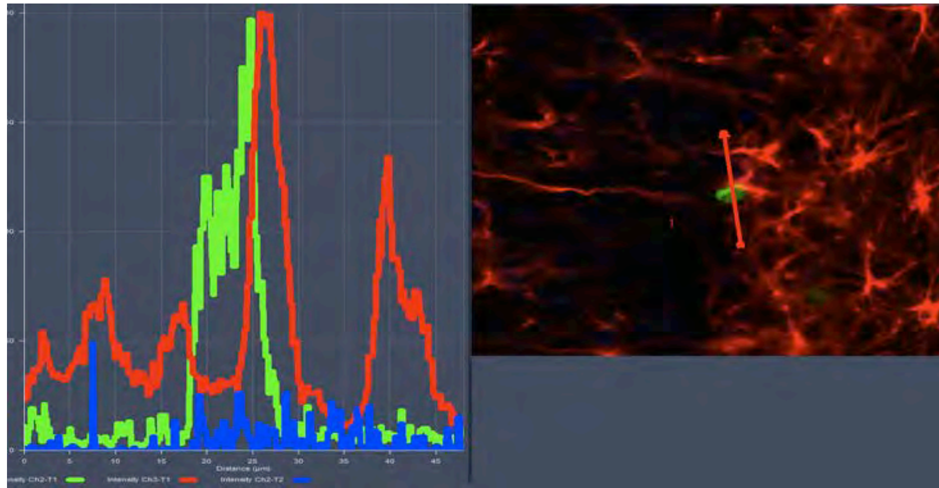


Figure 7. Graph shows the intensity profile of region indicated by the red arrow. Green line represents EdU stained nucleus, red line indicates GFAP profile and blue line indicates Hoechst. Likewise, on the right the labelled astrocyte cytoskeleton (red) and EdU positive nucleus (green) show the morphological star-shape of glial cells. This pattern of the cytoskeletal GFAP stain cupping the nuclear EdU is a positive indication that the labelled cell is a glial cell.

The Intensity profiling function was used in conjunction with the co-localisation function. This provided a visual representation of the pattern of florescence intensities emitted by markers over a selected area of interest. Profile analysis separated channels and represented each pixel's various channels' florescence intensities. This allowed for further identification of EdU co-labelled cells.

The co-localisation (Figure 6) and intensity profiling (Figure 7) functions were used to identify EdU co-labelled cells expressing combinations of EdU and DCX, EdU and NeuN (6 A) or EdU with GFAP (6 B). Using this method the study was able to identify neurons due to overlap in pixel intensity (6 A) and glia via the cupped pattern in intensity profiling peaks and troughs (Figures 6 B and 7). This was supported by the co-localisation function.

2.1.9) Statistics

All statistics were carried out using Statistica V.12 (StatSoft 2012) and Prism V.5 (GraphPad Software 2010). Characterisation data distribution was identified as being normal or non-parametric using the Shapiro-Wilk test. Thereafter either a One-Way Analysis of Variance (ANOVA) with a Tukey post hoc test or a Kruskal-Wallis test coupled with Dunn's multiple comparison tests were performed as appropriate. These tests were used in order to identify differences between groups and where specific differences occurred between mean/median values, respectively. Data were reported as mean and standard deviation or median and inter-quartile range.

2.2) Experiment

2.2.1) Experimental Animals

Once the mitotic marker (EdU) had been characterised, the effect of maternal separation (MS) on the hippocampus was investigated. Additionally the effect of voluntary wheel running on the hippocampus was studied in MS rats in order to explore the influence of exercise on neurogenesis in the MS rat-model of depression.

Thirty-nine Sprague Dawley rats were obtained from the University of Cape Town Animal Unit. Thirty-nine animals were perfused and their brains sectioned for immunohistochemistry. Eighteen animals were lost to the study because of poor EdU staining. The remaining 21 brains were used to test for differences between treatment groups. Animals were bred and housed in the University of Cape Town Animal Unit under standard conditions 12 h: 12 h light/dark cycle (lights on at 6:00 A.M.) with a temperature of 23 ± 2°C. Rats had access to food and water *ad libitum*. All procedures were authorized by the Faculty of Health Sciences Animal Ethics Committee of the University of Cape Town (012/061). The Study was carried out in accordance with the South African National Standard: The care and use of animals for scientific purposes 10386:2008. Rats were fed Tekland Equifeed rat chow (see appendix for details of composition).

2.2.2) Maternal Separation (MS)

Rats were bred over three months and a maximum of four male rats were taken from each litter. Studs and Dams were housed together until litters were born at which time the stud was removed. On PND 2, litters were culled to eight pups (with a minimum of two females and two males per litter).

Litters were randomly assigned to MS (n=19) or NMS (n=20) groups. Five dams were used to breed MS litters with one female being re-used and contributing two litters to the study. Five dams were used to breed NMS litters. A different stud was used for each litter. On PND 2 - 14 pups were separated from dams between 9:00 and 12:00 and transported to a different room in the animal unit to prevent vocal communication between mother and pups. The dam was removed from the home cage and kept in a single cage within the rat room. Pups were placed under an infrared light (temperature = 31 ± 1°C). This lamp was attached to an adjustable stand and raised to 26 cm above the pups. NMS litters were left undisturbed.

Pups and dams were housed together until PND 21 at which time pups were weaned and housed with 3 - 4 littermates. Nineteen rats were MS and twenty rats were NMS however, due to variability in the staining of brain sections only 10 MS rats and 11 NMS rats were used for statistical analysis.

2.2.3) Voluntary Exercise

Rats were housed in single cages with attached running wheels from PND 54 - 74. Twenty rats were housed in cages with free running wheels (n=10) and could exercise at will and twenty rats were housed in cages with locked wheels (n=10). Of those rats only 11 were used in the statistical analysis due to variability in early brain staining. The method was the same as described in section 2.1.2. The daily rotations (distance travelled) were manually recorded.

2.2.4) Injections

All rats received a single IP EdU (50 mg/kg) injection on PND 60. Rats were weighed on the day of injection and EdU (1 mg/0.15 ml or 6.66 mg/ml) was dissolved in 2.5x mM PBS at room temperature (Figure 8).

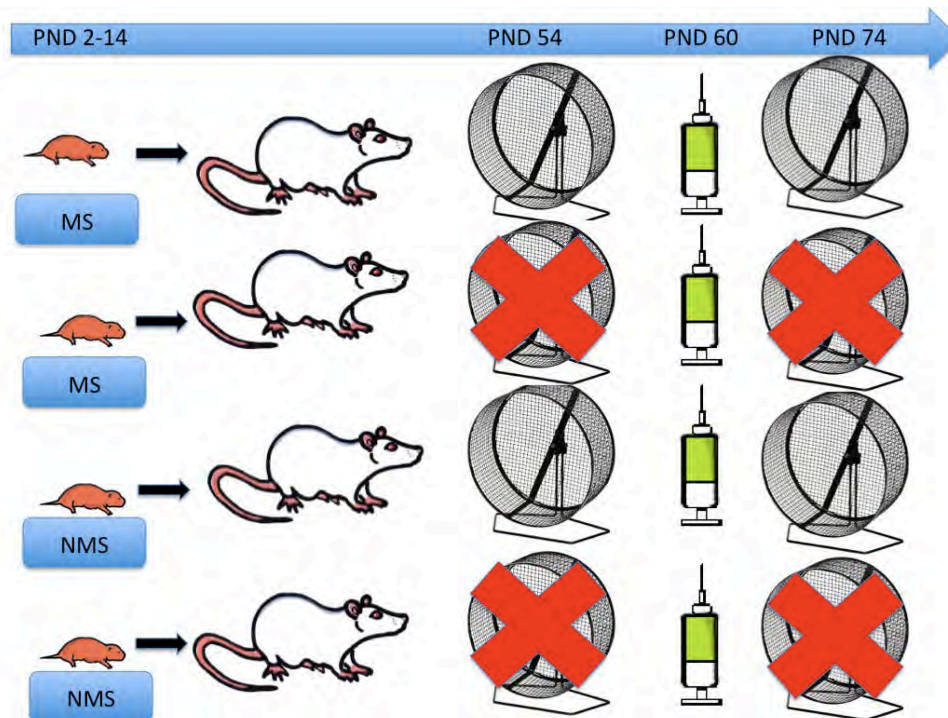


Figure 8. Displaying each treatment group (MS+R, MS+NR, NMS+R, NMS+NR). The rat pups were treated with MS or NMS. These rats were then permitted to reach adulthood and housed with running wheels on PND 54. Wheels were locked or left unlocked allowing some rats voluntary exercise and others not. EdU injections were administered on PND 60 to measure mitotic activity in the dentate gyri of runners and non-runners. Rats were immediately returned to their home cages

with running wheels until PND 74. They were then perfused and brains collected for immunohistochemistry.

2.2.5) Transcardial Perfusions

Thirty-nine rats were perfused in the afternoon between 13:00 - 17:00. Perfusions took place on PND 74 according to the method previously described in chapter 2.1.3. The whole brain of each rat was removed from the skull, placed in 4 % PFA for three hours and then dehydrated in 30 % sucrose solution or 3 - 5 days until the brain sank. Thereafter brains were sectioned with no more than two days between sinking and sectioning.

2.2.6) Sectioning

Brain sections were frozen using OCT to prevent ice-crystal formation (which would damage the tissue). Brains were mounted upon a chuck using OCT and sectioned using a cryostat microtome. The coronal sections containing the dorsal hippocampus (40 µm sections from 6.96 to 5.52 mm anterior to the inter-aural line) and the ventral hippocampus (50 µm sections from 3.84 to 2.76 mm anterior to the inter-aural line) were collected. The ventral hippocampus was larger in surface area and broke more easily and therefore 50 µm was found to be the optimal sectioning thickness. Every 5thsection was collected for the dorsal and ventral hippocampus (Figure 9). Sections were submerged in 3 % Triton X-100 in PBS for 1 hour prior to immunohistochemistry.

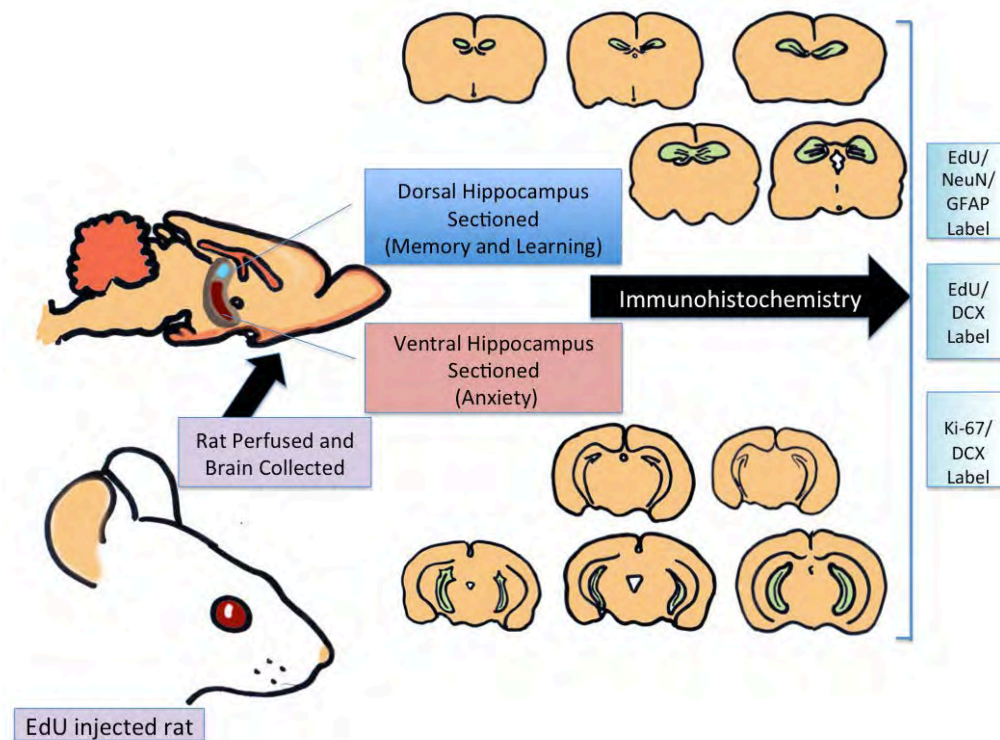


Figure 9. A representation of the processing of each rat brain collected. After the perfusion adult rat brains were collected and the ventral and dorsal hippocampi were sectioned separately. Five sections of each region of the hippocampus were kept for each molecular label combination. Three molecular label combinations were used to study neurogenesis in the dentate gyrus: EdU/NeuN/GFAP, EdU/DCX and Ki-67/DCX. EdU was used to quantify neuronal or astrocytic survival while Ki-67 Label was used as a pulse.

2.2.7) Staining Vials & Wells

To increase the number of brain sections stained per session, individual drainable staining-vials were constructed.

These plastic drainable vials were placed in separate Greiner 24-well plates containing primary antibodies, secondary antibodies, washes and Hoechst stain. The staining-vials were allowed to drain and were transferred between plates for each step of the immunohistochemistry procedure.

2.2.8) Immunohistochemistry

Staining was performed according to the methods outlined in section 2.1.6. There were three staining combinations used per rat brain. The first used NeuN and GFAP in conjunction with the EdU label to ascertain how many progenitor cells survived cellular integration and the fate of the specific cell types (Figure 9). The second stain combination was DCX and EdU and this stain was used to

determine how many EdU labelled immature Neurons were present after 20 days of exercise (Figure 9). The third stain comprised Ki-67 and DCX and was a pulse label used to determine the number of mitotically active cells that were destined to become neurons at the end of the exercise period (Figures 10-12). The amount of EdU click-iT reagent used for the first 18 brains stained sub-optimally and thus only 21 EdU labelled brains were processed for counting.

Table 3. Data on Primary antibodies used

Primary Antibody	Manufacturer	Lot Number	Concentration	Dilution
Mouse Monoclonal anti-NeuN	Millipore	219341	Unknown	1:500
Goat Polyclonal anti-DCX	Santa Cruz	C2413	200 μg/ml	1:200
Polyclonal Rabbit Anti-Glial Fibrillary Acidic Protein	DAKO	Z0334 00094739	2.9mg/ml	1:500
Rabbit Polyclonal anti-Ki-67	Millipore	2147027	Unknown	1:250

Table 4.Data on Secondary antibodies used

Secondary Antibody	Manufacturer	Lot Number	Concentration	Dilution
Cy5 Donkey Anti-Mouse IgG	Jackson ImmunoResearch	108624	1.4mg/ml	1:500
Cy3 Donkey anti-rabbit IgG	Jackson ImmunoResearch	109771	1.5mg/ml	1:500
Cy5 Affini-pure Donkey Anti-Rabbit IgG	Jackson ImmunoResearch	112031	1.5mg/ml	1:500
Cy3 Donkey Anti-goat IgG	Jackson ImmunoResearch	Unknown	1.5mg/ml	1:500

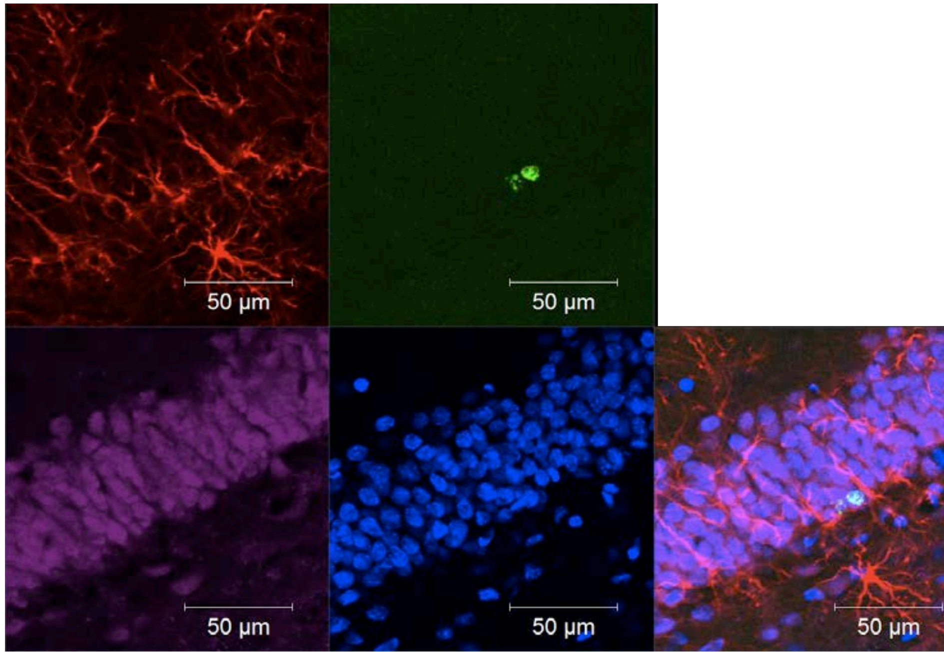


Figure 10. Cell-type specific marker**. Combination 1 shows GFAP (red), EdU (green), NeuN (magenta) and Hoechst (blue) and a composite image allowing identification of the EdU labelled cell.**

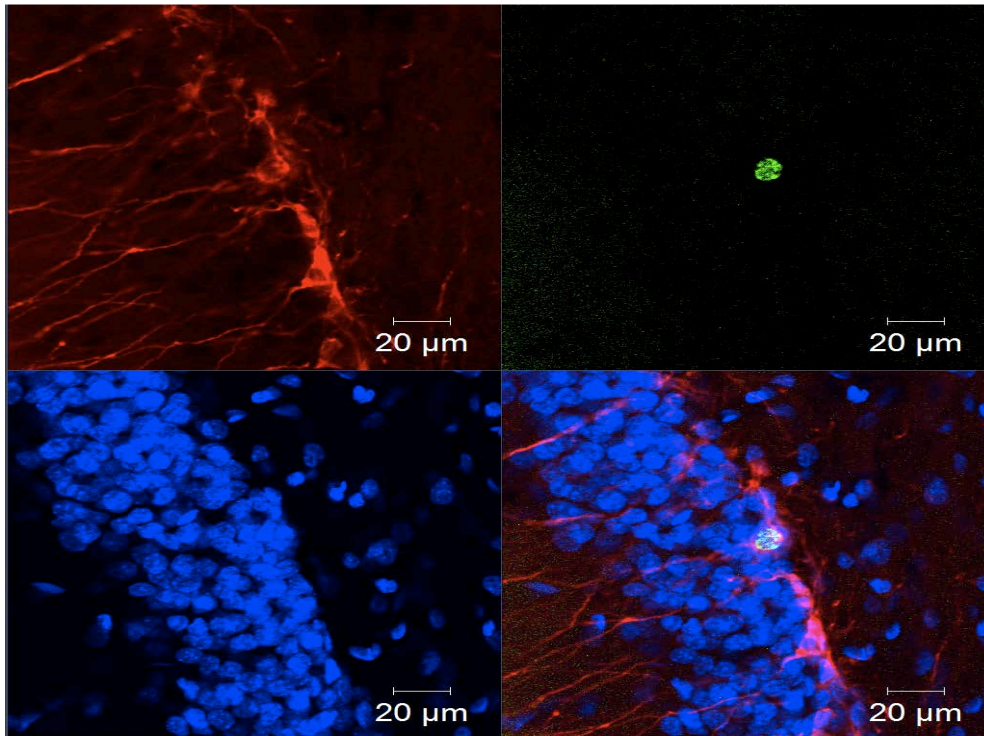


Figure 11. Cell-type specific marker **combination 2 shows DCX (red), EdU (green) and Hoechst (blue) and a composite image.**

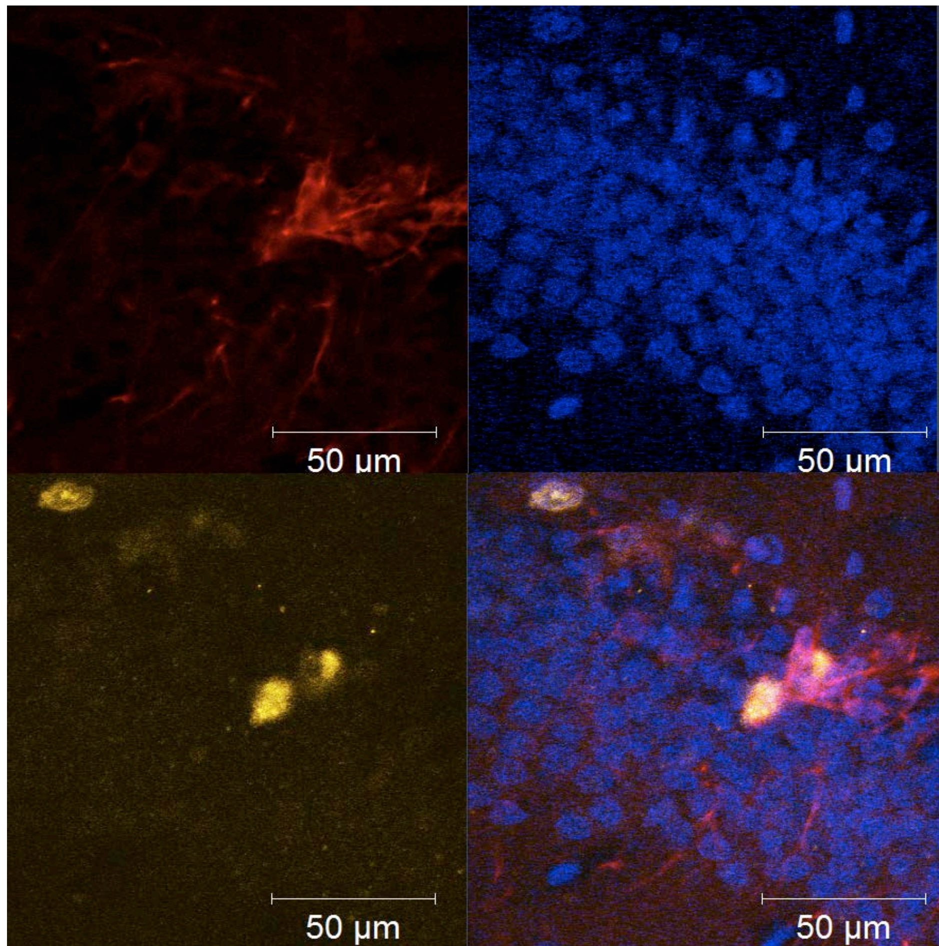


Figure 12. Cell-type specific marker combination 3 displays DCX (red), Hoechst (blue) and KI-67 (yellow) and composite image.

2.2.9) Quantification

The process of quantification was the same as that described in section 2.1.7. Thus EdU co-labelling with NeuN and GFAP was established using co-localisation and profile analysis for stain 1, co-labelling of EdU and DCX was established for stain 2 using the same methods and co-localisation of Ki-67 and DCX for stain combination 3 was also established. The specific cell-type counts were averaged separately for each rat brain area and analyzed according to treatment groups.

2.2.10) Statistics

The distance travelled by each rat was measured in meters travelled per day. The Shapiro-Wilk Test revealed that the data were not normally distributed. The repeated measures Wilcoxon Test (Wilcoxon signed-rank test) was applied to the running data to determine whether there was any difference between MS and NMS rats in terms of distance travelled. The weight data were found to be non-parametric and a Kruskal-Wallis Test coupled with Dunn's multiple comparison Test was conducted. The Shapiro-Wilk test was used to establish

whether the data for each stain combination 1, 2 and 3 were normally distributed. Thereafter either a one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test or a Kruskal-Wallis Test coupled with Dunn's multiple comparison test were performed as appropriate.

A non-parametric Spearman's rank correlation coefficient was determined for the data of stain 1 followed by a linear regression and testing for goodness of fit.

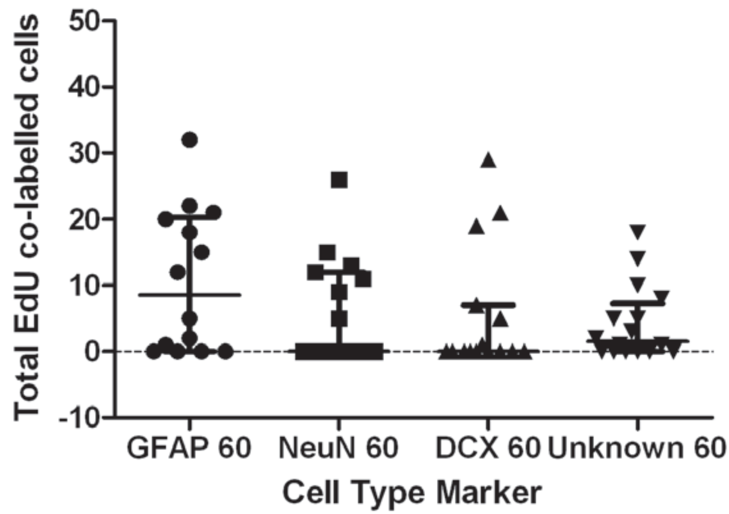
Chapter 3

Results

3.1) Characterisation Data

The EdU label was characterised in an *in vivo* animal model using male Sprague Dawley rats. EdU-labelled cells in the dentate gyrus of the hippocampus were counted in 2 - 6 x 40 μm sections of the rat brain. The data were found to be non-parametrically distributed. The Kruskal-Wallis test revealed there were no significant differences in the number of cell–type specific labelled cells of the 5 rats injected on PND 60 or the 5 rats injected on PND 96 (Figure 13).

a)



b)

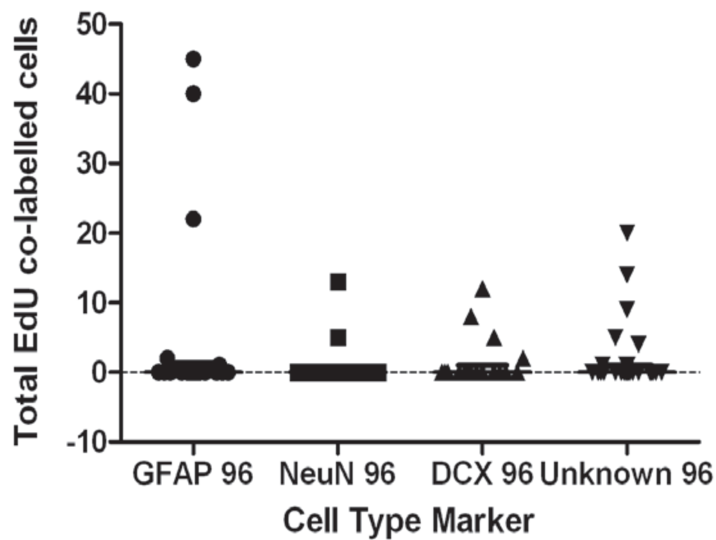


Figure 13. Total number of cells co-labelled with EdU/GFAP, EdU/NeuN or EdU/DCX in the dentate gyrus of the dorsal hippocampus of Sprague Dawley rats injected with EdU at (a) PND 60 or (b) PND 94/95 and perfused on PND 74 or 94/95. Please note that this study's "total" refers to the total cells counted and summed per rat. The above data were tested using the Shapiro-Wilk normality test and found to be non-parametrically distributed. The data were therefore analyzed using the Kruskal-Wallis test. Data points represent the number of counted cells labelled with EdU/GFAP, EdU/NeuN or EdU/DCX in the dentate gyrus summed per section (40 μm) of the rat brain (2-6 sections per rat). Results are expressed as individual data points with bars representing median and inter-quartile range. There were no significant differences in the number of EdU co-labelled cells between the different cell types.

The average number of co-labelled cells (averages of 2 - 6 sections of dentate gyrus per rat) was calculated. Examining only the EdU label the two injection time points were compared using a Mann-Whitney U test. The median of PND 60 injections was higher than PND 96 injections. There was a difference between EdU-labelled cell counts 14 days after injection of EdU compared to 4 hours after injection (Figure 14). This suggested that injections of EdU given four hours prior to perfusion labelled fewer cells than those rats injected 2 weeks prior to perfusion and collection of the brains for immunohistochemistry.

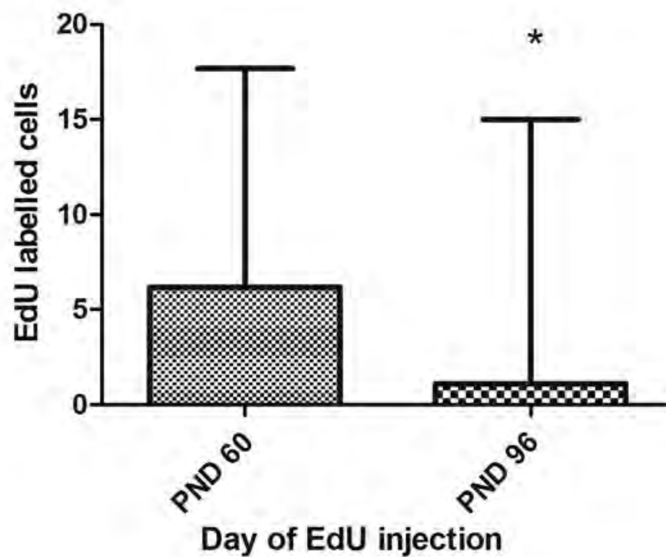
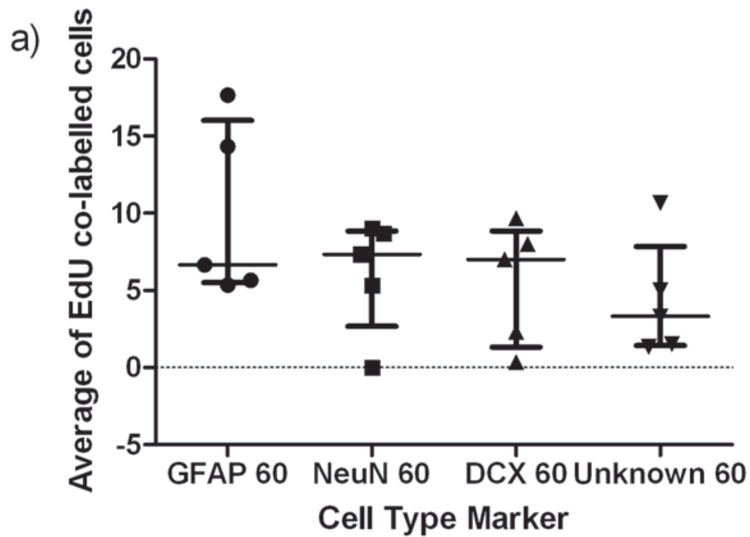


Figure 14. Columns represent the median and bars the upper-quartile range of the average number of EdU-labelled cells found in each exercised rat per injection group (n=5). Injections administered on PND 60 had a significantly higher median (6.16 cells) than PND 96 injections (1.1 cells).

The average number of counted co-labelled cells (averages of 2-6 sections of dentate gyrus per rat) was used to compare cell phenotypes using a one-way ANOVA and Kruskal-Wallis test. No significant differences were observed (Figure 15). The two injection time points were compared using this data. The average number of co-labelled cells per cell-type specific marker was compared using a Mann-Whitney U test. The Medians per rat that received an injection on PND 60 were GFAP 7; NeuN 7; DCX 7 and unknown cell types 3 (Figure 3.1.3a). The Medians of rats injected on PND 96 were GFAP 0; NeuN 0; DCX 2 and unknown cell types 1.There was no difference between EdU-labelled cell types 14 days after injection of EdU compared to 4 hours after injection (Figure 15).



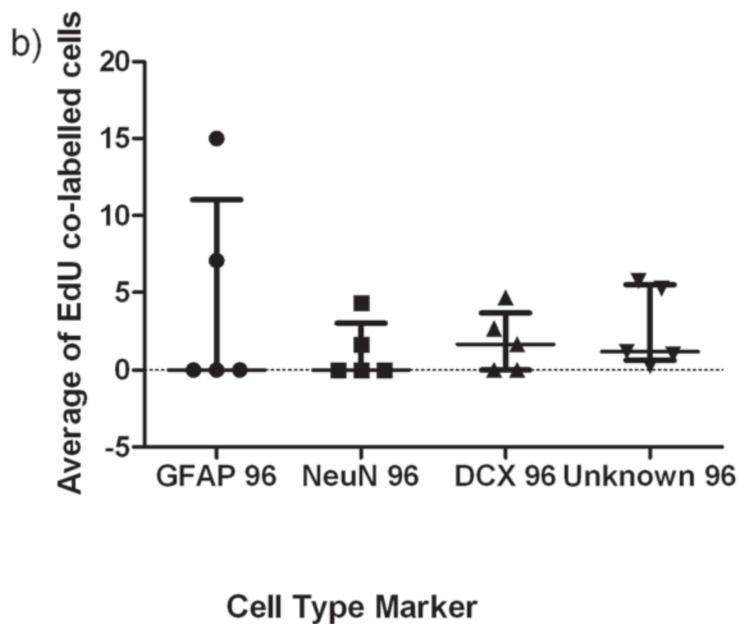


Figure 15. Average number of cells per rat co-labelled with EdU/GFAP, EdU/NeuN or EdU/DCX in the dorsal hippocampus of Sprague Dawley rats injected with EdU at (a) PND 60 or (b) PND 96. The above data were tested using the Shapiro-Wilk test and found to be parametrically distributed (15a) and non-parametrically distributed (15b). The data were therefore tested for differences between rats injected with EdU at (a) PND 60 using a one-way analysis of variance and (b) PND 96 using the Kruskal-Wallis test. Data points represent the average number of cells labelled with EdU/GFAP, EdU/NeuN or EdU/DCX in the dentate gyrus summed (per 40 µm section of the rat brain 2-6 sections were averaged per rat). Results are expressed as individual data points with bars representing median and inter-quartile range. There were no significant differences in the number of EdU co-labelled cells between the different cell type markers. The data were also tested for differences between rats injected with EdU at (a) PND 60 and (b) PND 96 using the Mann-Whitney U test. There were no significant differences in the number of EdU co-labelled cells between PND 60 and PND 96 for the different cell type markers.

The study sought to identify differences in neurogenesis thus NeuN and DCX co-labelled cells were pooled in rats injected on PND 60 and those injected on PND 96 (Figure 16). There was a marked difference in the total and average number of EdU/NeuN and EdU/DCX co-labelled cells in the dentate gyrus of rats injected with EdU at PND 60 and those injected on PND 96 (Mann-Whitney U test). This suggested that injections of EdU given four hours prior to perfusion labelled fewer neurons than those rats injected 2 weeks prior to perfusion and collection of the brains for immunohistochemistry.

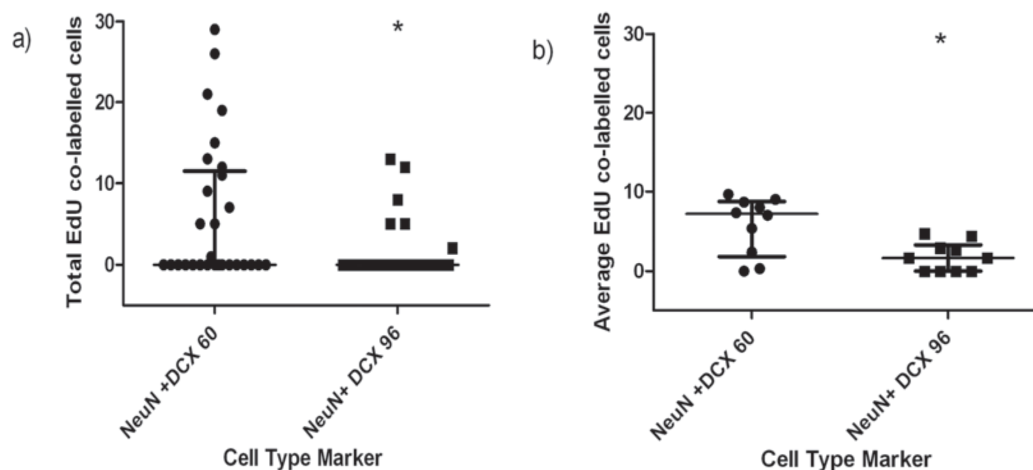


Figure 16. Total (a) and average (b) number of EdU/NeuN plus EdU/DCX co-labelled cells in the dentate gyrus of rats injected with EdU at PND 60 and those injected on PND 96. Data points (a) represent the number of cells labelled with EdU/NeuN and EdU/DCX in the dentate gyrus summed per 40-µm section of the rat brain (2-6 sections per rat). Data points (b) represent the average number of EdU/NeuN and EdU/DCX co-labelled cells in 2-6 x 40-µm sections of the dentate gyrus of the dorsal hippocampus of PND 60 and PND 96 EdU-injected rats. The data were tested using the Shapiro-Wilk test and found to be non-parametrically distributed. The Mann-Whitney U test revealed that the dentate gyrus of the dorsal hippocampus of the PND 60 EdU-injected rats had a greater number of EdU/NeuN and EdU/DCX co-labelled cells than PND 96 EdU-injected rats (a,b). Results are expressed as median and interquartile range. *Significant difference between NeuN + DCX 60 and NeuN + DCX 96 median values.

The results obtained from the rats injected with EdU on PND 74, four hours prior to transcardial perfusion showed no significant difference between exercised and non-exercised rats in any of the cell-type specific markers (Mann-Whitney U test, Figure a low level of EdU labelling was observed in both exercised and non-exercised groups with no significant difference between the different cell types within each group (Kruskal-Wallis test, Figure 17). Despite voluntary wheel running for 20 days the total number of EdU/GFAP, EdU/NeuN and EdU/DCX co-labelled cells in 2 - 6 sections of

dorsal hippocampus per exercised rat had a median value of 0 when injected with EdU 4 hours prior to perfusion.

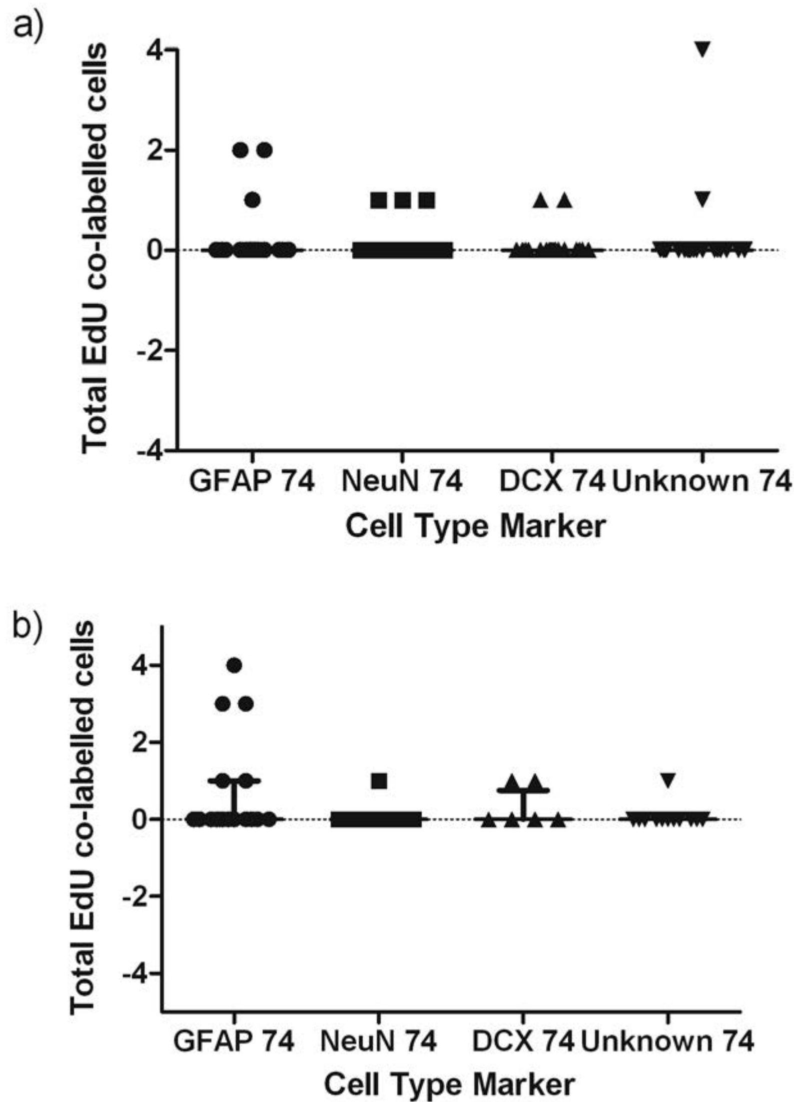


Figure 17. Total number of EdU/GFAP, EdU/NeuN and EdU/DCX co-labelled cells in 4-6 sections of dentate gyrus in the dorsal hippocampus of rats that were housed in cages with attached running wheels that were freely moving (a) or immobilized (b) for a period of 20 days and then injected with EdU at PND74, 4 hours prior to perfusion and collection of brains for immunohistochemistry. Data points represent the summed number of EdU-co-labelled cells in 2-6 x 40-µm sections of the dentate gyrus of 3 exercised rats permitted to run for 20 days (a) and 3 non-exercised rats (b). The bars represent the median and interquartile range.

Similarly, a Mann-Whitney U test comparing the average number of EdU/GFAP, EdU/NeuN and EdU/DCX co-labelled cells in 2-6 sections of dentate gyrus in dorsal hippocampus revealed no significant difference between exercised and non-exercised rats in any of the cell-type specific markers (Figure 18). The Kruskal-Wallis test revealed no significant difference between the different cell types within each group (Figure 18).

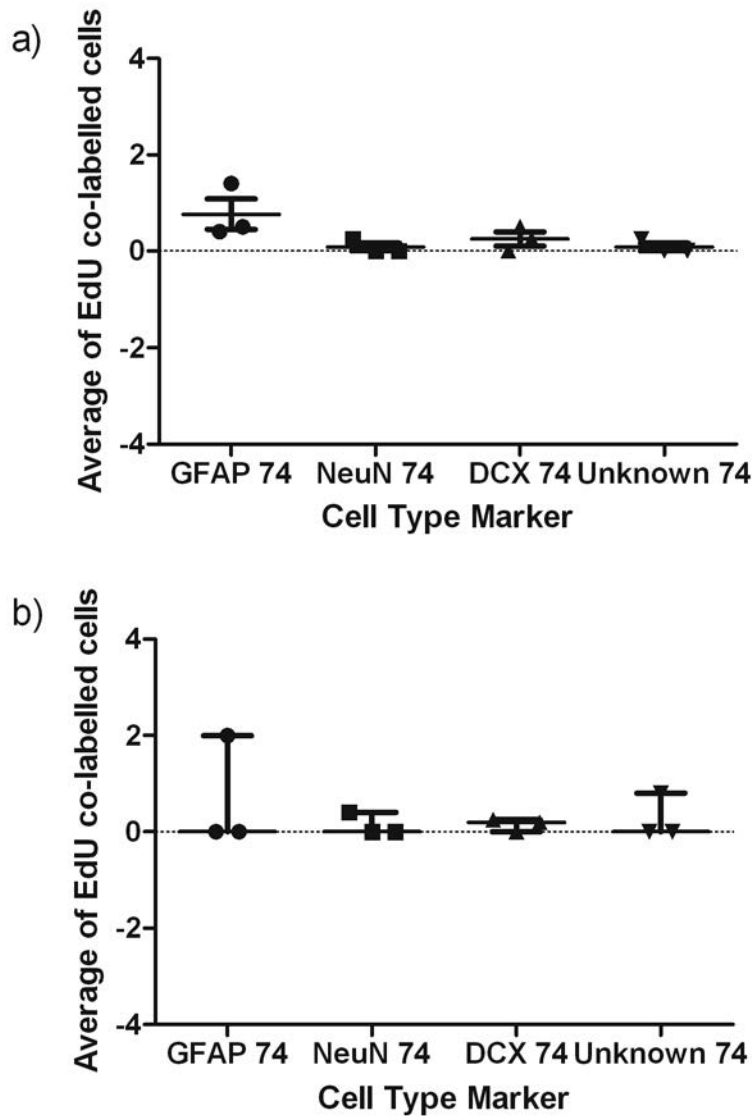


Figure 18. Average number of EdU/GFAP, EdU/NeuN and EdU/DCX co-labelled cells in 2-6 sections of dentate gyrus in dorsal hippocampus of rats that were housed in cages with attached running wheels that were freely moving (a) or immobilized (b) for a period of 20 days and then injected with EdU at PND74, 4 hours prior to perfusion and collection of brains for immunohistochemistry. Data points represent the average number of EdU-co-labelled cells in 40-µm sections of the dentate gyrus (4-6 x 40-µm sections of the brain) of 3 exercised rats permitted to run for 20 days (a) and 3 non-exercised rats (b). The bars represent the median and interquartile range.

Characterisation of EdU stain in conjunction with cell-type specific markers

Three sections taken from one experimental rat showed that Ki-67 labelled EdU stained cells throughout the dentate gyrus (Figure 19). These sections were also used to determine concentrations of reagents required for optimal EdU, DCX, NeuN and GFAP staining (See methods Table 2,3 for further details). It was shown that Ki-67 labelled the same cells as an EdU pulse. It did not address the question of neuronal survival, but could be used to effectively label mitotically active cells at the time of perfusion. Thus EdU was used to measure neuronal survival after 14 days and Ki-67 at the end point of the experiment at the time of perfusion. This would provide insight into how many neurons were generated and how many survived to maturation.

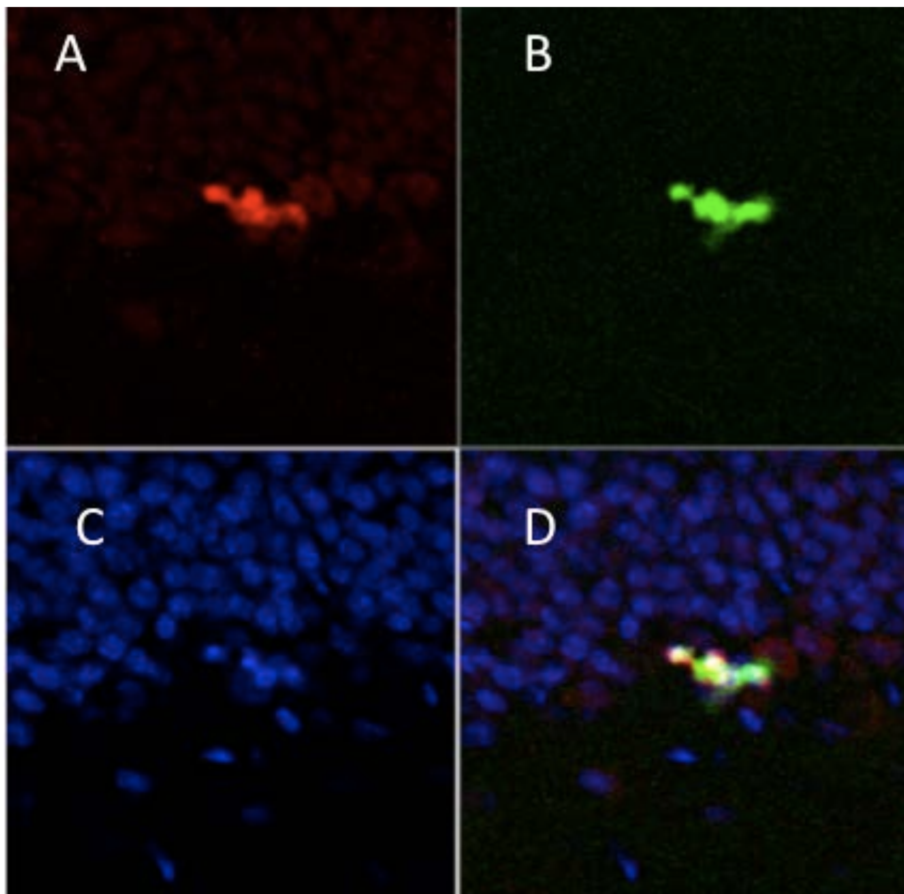


Figure 19. Ki67 and a pulse of EdU labelled the same cells in the dentate gyrus of the hippocampus of a rat injected with EdU on PND 60 (A) Ki-67 labelled cells, (B) EdU labelled cells, (C) Hoechst nuclear label, and (D) merged image (20x).

3.2) Experimental Results

Once the novel marker EdU was characterised with co-labels the rats were separated into four experimental groups. Two groups were used investigate the effect of MS relative to NMS rats and the other two groups sought to explore the effect of exercise in the hippocampus MS+R and NMS+R.

MS higher Weights at PND 60 relative to NMS

Weights of each treatment group were measured and the data's distribution was found to be non-parametric. A Kruskal-Wallis test showed that the medians of the body weights were different and a Dunn's multiple comparison test showed MS+NR rats had a higher weight (340g) than NMS+R (256g) rats (Figure 20).

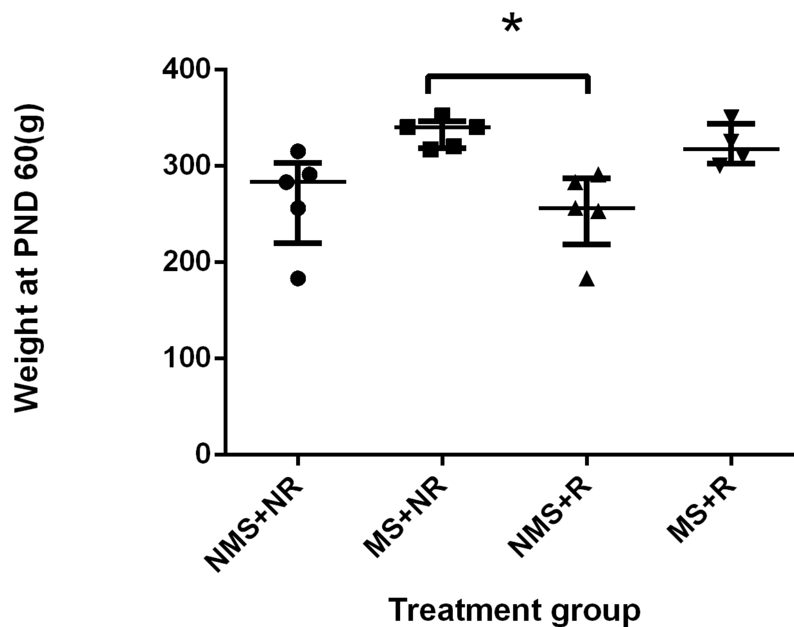


Figure 20. Graph showing the weights of maternally separated runners (MS+R), maternally separated non-runners (MS+NR), non-separated runners (NMS+R) and non-separated non-runners. A Kruskal- Wallis test followed by Dunn's multiple comparison Test showed that MS+NR rats had a higher median then NMS+R rats.

A Wilcoxon matched-pairs signed rank test showed a significant difference between the medians of distances travelled by MS and NMS rat groups per day. The MS rats showed a significantly lower median than NMS. The non-runners data is not represented on the graph as these rats did not exercise (Figure 21).

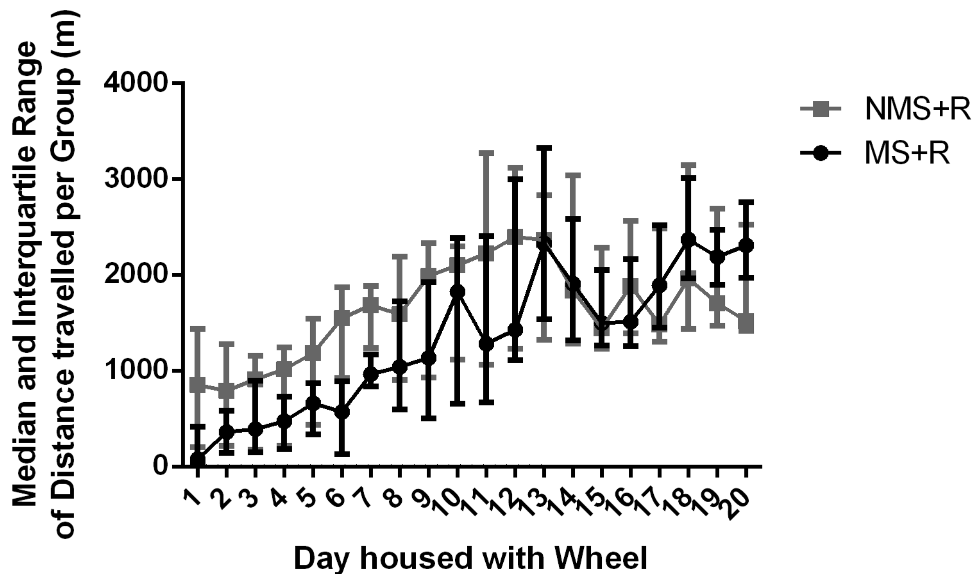


Figure 21.Distance run from PND 54-74. The data were found to be non-parametric. The line graph showing the median and interquartile range of the distances run by MS and NMS rats over 20 days of voluntary wheel running. A Wilcoxon matched-pairs signed rank test revealed that NMS+R had a significantly higher median than MS+R (W= 128.0; p= 0.0153).

No Effect of maternal separation and exercise on the number of EdU-labelled mature neurons and astrocytes in the dentate gyrus of the hippocampus.

The number of co-labelled EdU/NeuN and EdU/GFAP cells in the dentate gyrus of MS+R, MS+NR, NMS+R and NMS+NR were compared (Figure 22). The Kruskal-Wallis test revealed that there were no significant differences between treatment groups in either the dorsal or the ventral hippocampus.

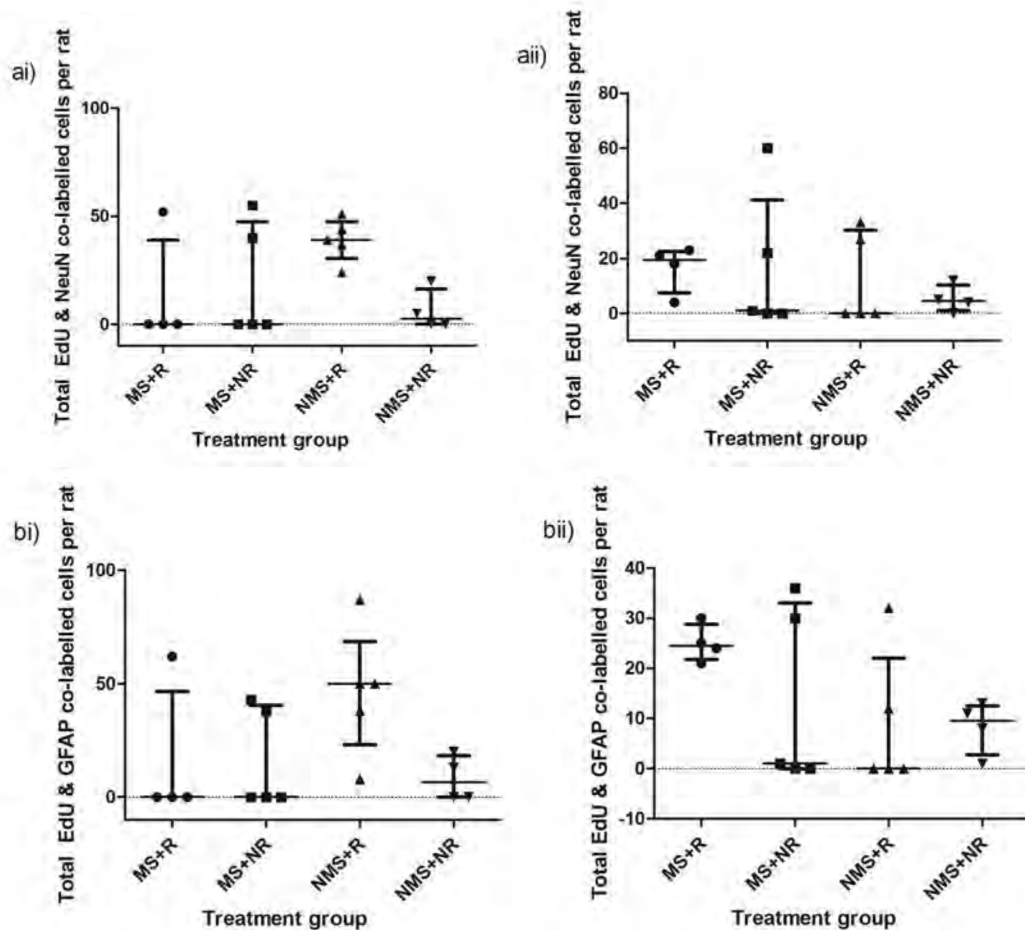


Figure 22. The number of co-labelled EdU/NeuN cells (ai, aii) and EdU/GFAP cells (bi, bii) in the dentate gyrus of the dorsal hippocampus (5 x 40-µm sections) (ai, bi) and the ventral hippocampus (5 x 50-µm sections) (aii, bii) of maternally separated runners (MS+R, n = 4), maternally separated non-runners (MS+NR, n = 5), non-separated runners (NMS+R, n = 5) and non-separated non-runners (NMS+NR, n = 5). The data were tested using the Shapiro-Wilk test and found to be non-parametrically distributed. The Kruskal-Wallis test revealed no significant differences between groups. Bars represent the median and inter-quartile range.

Number of Surviving EdU-labelled Neurons compared to the number of EdU-labelled astrocytes.

The data obtained from both exercised and non-exercised rats were pooled and the number of surviving astrocytes was compared to the number of neurons that survived 14 days after injection of EdU, to establish if there was a dominant phenotype expressed by newly generated cells. There was no significant difference between the

number of neurons and astrocytes in the dorsal (a) or ventral (b) dentate gyrus of the hippocampus as shown by the Mann-Whitney U non-parametric test (Figure 23).

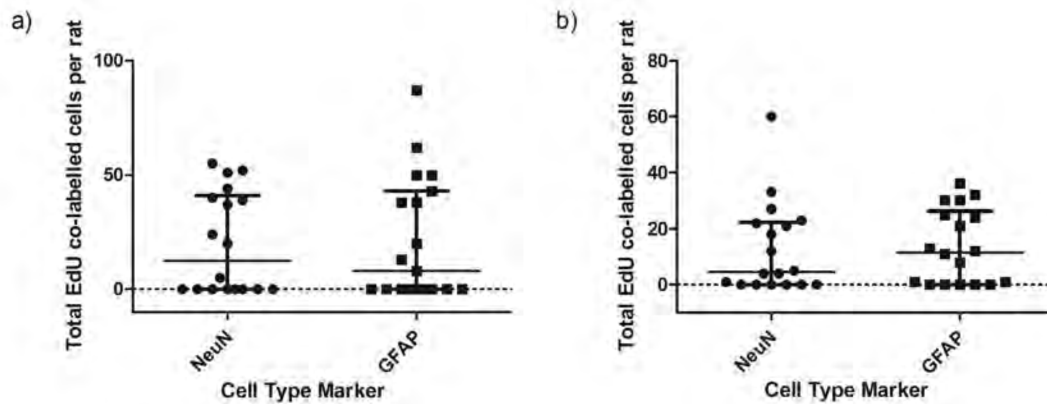


Figure 23. The number of neurons relative to astrocytes in the a) dorsal and b) ventral hippocampus. The data were tested using the Shapiro-Wilk test and found to be non-parametrically distributed. The Mann-Whitney U test revealed no significant difference between the number of EdU/NeuN and EdU/GFAP co-labelled cells in the dentate gyrus of the dorsal and ventral hippocampus. Data points represent the summed number of NeuN and EdU co-labelled cells and GFAP and EdU co-labelled cells counted in 5 sections of dentate gyrus in the dorsal (40-µm sections) (a) and ventral (50-µm sections) (b) dentate gyrus of the hippocampus of each rat (n= 18). Bars show median and inter-quartile range.

The dorsal and ventral GFAP and NeuN data was found to be non-parametric. There was a significant correlation between the number of EdU/NeuN co-labelled cells (neurons) and EdU/GFAP co-labelled cells (astrocytes) that survived 14 days after injection of EdU (Spearman r correlation) in the ventral hippocampus but not the dorsal hippocampus

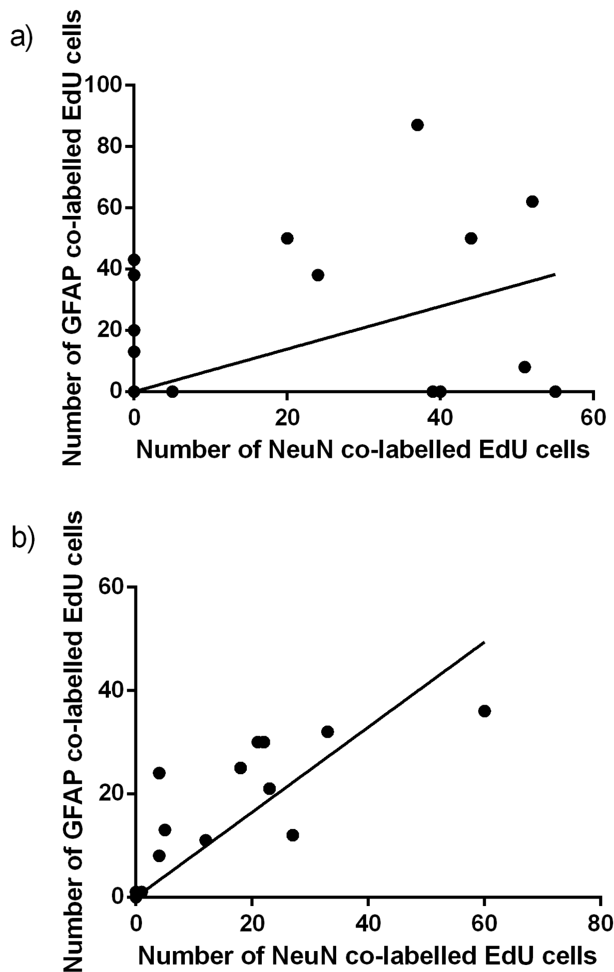


Figure 24. Correlation analysis of the number of neurons relative to astrocytes in the a) dorsal and b) ventral hippocampus. There was no significant correlation between EdU/GFAP co-labelled astrocytes and EdU/NeuN co-labelled neurons in the dentate gyrus of the dorsal hippocampus of all rat groups (a). There was a significant positive correlation between EdU/GFAP co-labelled astrocytes and EdU/NeuN co-labelled neurons in the dentate gyrus of the ventral hippocampus of rats allowed to exercise for 20 days as well as rats that were housed in cages with immobilized running wheels, 14 days after injection of EdU (Spearman r correlation 0.9006; n= 18).

No Effect of maternal separation and exercise on the number of surviving immature EdU-labelled neurons in the dentate gyrus of the hippocampus.

There was no significant difference in the number of EdU/DCX co-labelled (immature) neurons between treatment groups (maternally separated and non-separated rats that were allowed to exercise or not). Data were analyzed using a Kruskal-Wallis test and no significant differences were found. There was a low level of labelling and many rat brain sections displayed no EdU/DCX co-labelled cells (Figure 25).

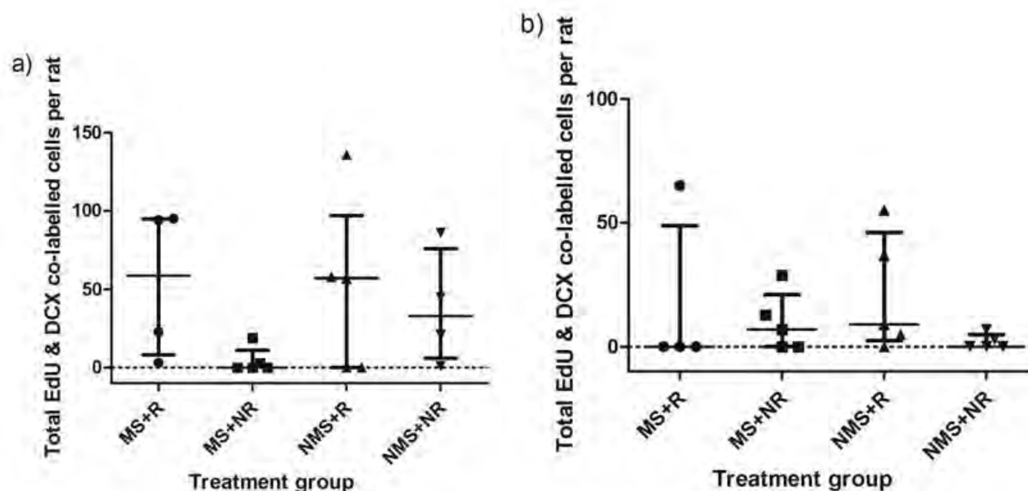
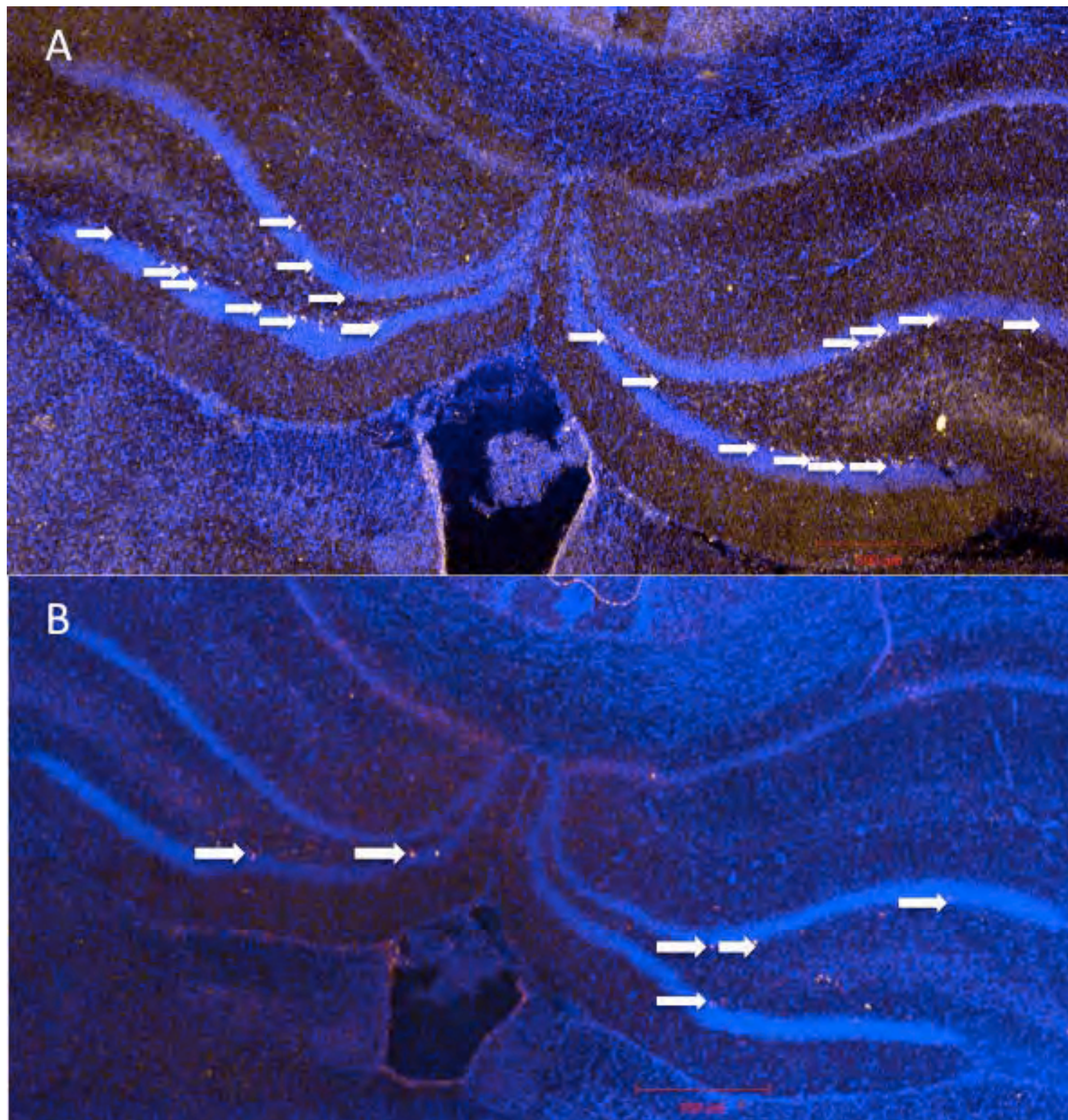


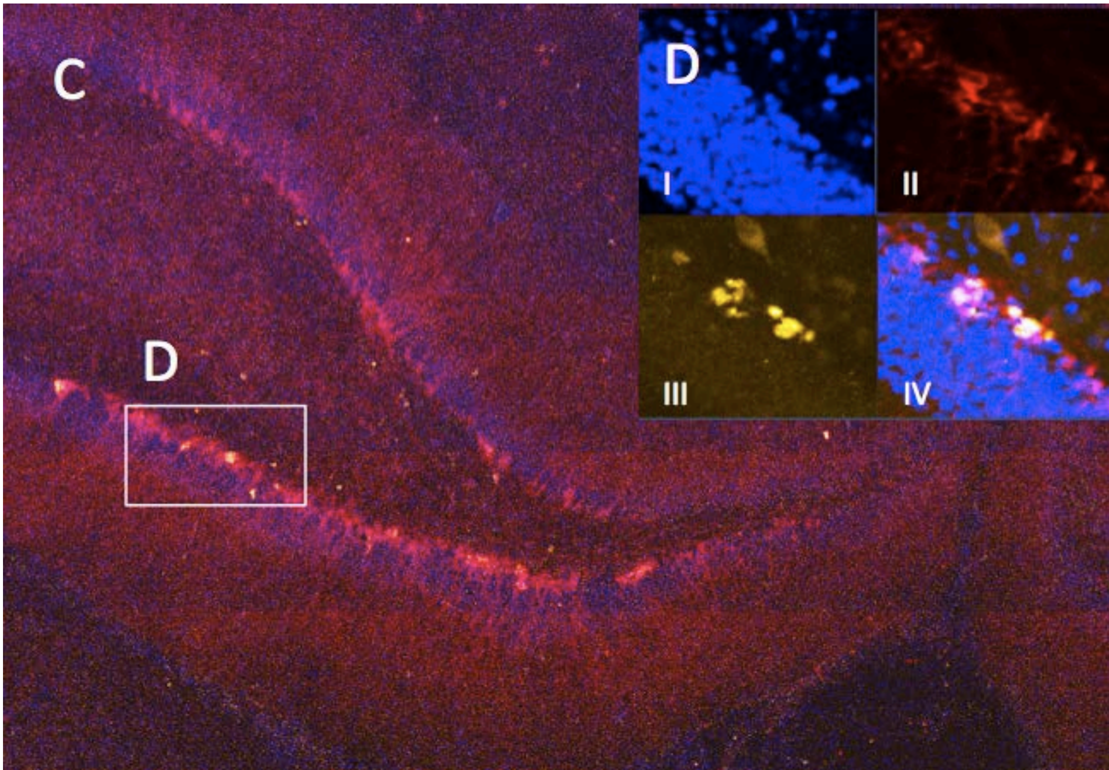
Figure 25. Effect of maternal separation and exercise on the number of surviving immature EdU-labelled neurons in the dentate gyrus of the hippocampus. The data points represent the number of co-labelled EdU/DCX cells in the dorsal (40-µm sections) a) and the ventral (50-µm sections) b) dentate gyrus (total number of EdU/DCX co-labelled cells in 5 sections of the left and right hippocampus of rats in the four treatment groups). The data were tested using the Shapiro-Wilk test and found to be non-parametrically distributed. The Kruskal-Wallis test revealed that there were no significant differences between the treatment groups. Bars represent the median and inter-quartile range of each data set.

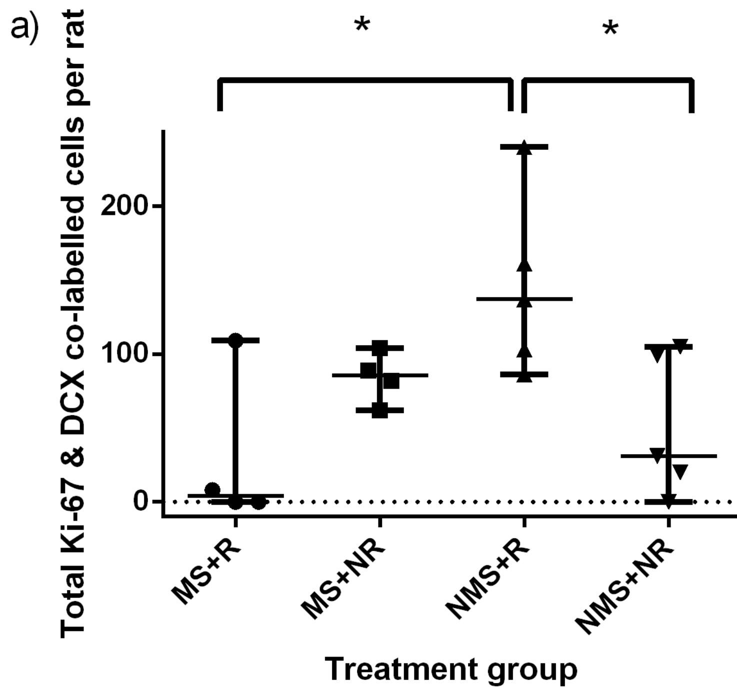
Neuronal proliferation reduced in maternally separated (MS) rats

Confocal microscope tile scans (10 X) of the dentate gyrus (Scan A) showed that there were more proliferating neurons in NMS+R rats (Scan A) than MS+R rats (Scan B) as indicated by the arrows indicating yellow fluorescence representing Ki-67 labelled cells (Kruskal-Wallis test, $P < 0.05$, Figure 26). The qualitative Scan C (20 X) shows a sample section of half the dentate gyrus. The red represents DCX-labelled immature neurons, blue represents Hoechst stained nuclei and yellow is the Ki-67 marker. These markers were used to identify immature neurons and count them to quantify and compare rat treatment group. The data were tested using the Shapiro-Wilk test and was found to be parametric (Figure 26a) and data were tested using a one way ANOVA and a Tukey post-hoc test to identify group differences. The data from Figure 26b were non-parametrically distributed and tested for differences using the Kruskal-Wallis test and Dunn's post-hoc test for group differences. The ANOVA revealed that there was a significant difference between the number of non-EdU labeled cells and counted Ki-67/DCX co-labelled cells in the different treatment groups. The Tukey post-hoc test revealed that the number of Ki-67/DCX co-labelled cells was significantly decreased in the MS+R rats compared to the NMS+R group of rats. The NMS+R group had more proliferating neurons than the NMS+NR group of rats (Figure 26a, 26b). The ventral hippocampus followed the same pattern with the MS+R group having significantly

lower median number of Ki-67/DCX co-labelled cells than the NMS+R group (Kruskal-Wallis test followed by Dunn's multiple comparison test).




C
D
D
I
II
III
IV



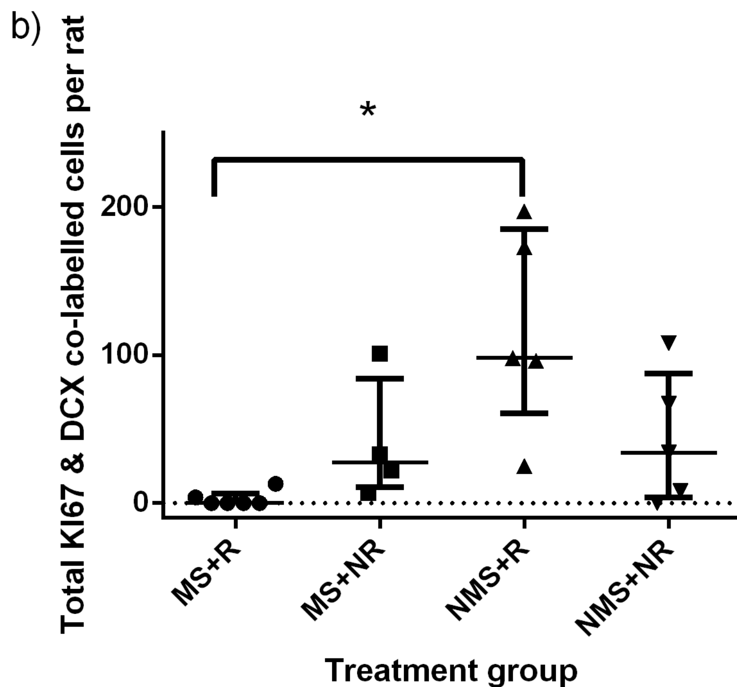


Figure 26. Confocal tile scan (10 X) showing the dentate gyrus of the hippocampus of a NMS+R rat (A) and a MS+R rat (B). The arrows indicate yellow fluorescence representing Ki-67 labelled cells. There are more Ki-67 labelled cells in the NMS rat than MS rats that underwent the same exercise regime. Scan C (20 X) red (II) representing the dorsal hippocampus shows DCX-labelled immature neurons, blue represents a Hoechst (I) and yellow (III) is Ki-67 marker while D (40 x) displays the yellow nuclear stain surrounded by cytoplasmic DCX stain giving an example of the typical unipolar neuronal structure. The above data were tested using the Shapiro-Wilk test and a) was found to be parametric. The data were tested using a one-way ANOVA and a Tukey post-hoc test to identify group differences. The data from b) were found to be non-parametrically distributed and were tested for differences using the Kruskal-Wallis test. Data points in (a) and (b) represent the summed number of Ki-67/DCX labelled cells in 5 sections of the dorsal (40-µm sections) (a) and ventral

(50-µm sections) (b) dentate gyrus. The bars in (a) represent the mean and standard deviations of each group and (b) medians and interquartile ranges. A one-way ANOVA (a) revealed a significant difference between groups, and a Tukey post-hoc test showed that proliferating immature neurons in the dorsal hippocampus are significantly decreased in four MS+R rats compared to five NMS+R rats. The same difference was found in the ventral hippocampus (by using the Kruskal-Wallis test and Dunn's multiple comparison Test. The NMS+R group in (a) had significantly higher number of proliferating neurons relative to the NMS+NR group (MS+R n=4, MS+NR n=4, NMS+R n=5, NMS+NR n=5).

Chapter 4

Discussion

4.1.) Pilot Study: Characterising the mitotic marker, EdU

The purpose of the pilot study was to characterise the use of the thymidine analogue, EdU to measure neurogenesis in Sprague Dawley rat brains. This novel mitotic marker is reportedly superior to its counter-part BrdU as it is a more sensitive marker with lower toxicity in animals. This makes it the ideal marker to be used *in vivo* to quantify neurogenesis in a rat model for depression. Therefore our study undertook to establish the novel technique to measure neurogenesis in an MS model for depression primarily by optimizing EdU immunohistochemistry and thereafter establishing a method to quantify this with the co-labels Ki-67, NeuN, DCX and GFAP. It should be noted the effect of exercise was not analyzed as a behavioural treatment in this aspect of the pilot study. Exercise was used to up-regulate neurogenesis and enhance the study's ability to identify mitotic activity. Therefore all rats in this objective of this study were housed with running wheels excluding three rats involved in a validation experiment comparing run and non-run rats which were housed with locked wheels.

No Discernable effect of exercise in PND 74 injected rat cohort

A small study to compare the effect of exercise in PND 74 injected (and perfused) rats was conducted (Chapter 3 Figure 17 and 18). There was no discernable difference between rats that were exercised and sedentary rats. All group medians were lower than 2 cells per 40µm section. Exercise is reported to up-regulate mitotic activity in neurons and glia in the dentate gyrus of rodents (van Praag *et al.,* 2002; Pusic *et al.,* 2014) and thus our rats were housed in cages with running wheels for two weeks (PND 74) or in cages with locked wheels.

The literature reports that mice housed with adjoined running wheels have a range of mitotically active hippocampal cells between approximately 600 - 1600 (as measured with EdU) (Zeng *et al.,* 2010). Castilla Ortega (2014) found that approximately 220 BrdU-positive cells were present in mice after 24 days of exercise. Gregorie *et al.,* (2014) found approximately 1600 mitotically active cells present in the dentate gyrus of mice after four weeks of wheel running. An older study by Steiner *et al.* (2004) showed that wheel running increased neurogenesis significantly increasing both BrdU/NeuN (1000 vs. 3000 cells in the DG) and BrdU/GFAP cells (150 vs. 450 cells in the DG). Our study was conducted in rats, and the literature reports a similar effect of exercise as seen in mice. Long Evans rats housed with running wheels for 6 - 11 weeks had 1600 DCX cells per DG in runners and 1225 in non-runners (Winocur *et al.,* 2014). In agreement with

the above literature Speisman *et al.,* (2013) found that non-runner rats had significantly fewer BrdU labelled cells per dentate gyrus (1600) as compared to runners (2300 BrdU positive cells). Thus their finding was that voluntary exercise increased neurogenesis in rats. We did not find this, possibly due to low levels of label detection.

EdU-labelled neuronal (EdU/DCX+EdU/NeuN) cell survival was higher in PND 60 injected and exercised rats in the dentate gyrus

After just under three weeks of exercise, there were significantly more EdU-labelled cells present in the dentate gyrus of rats when injected with EdU at PND 60 (Figure 14) compared to those cells present in the dentate gyrus of rats injected 4 hours prior to perfusion on PND 96. Studies have reported EdU-labelled cell survival at separate time points but few directly compared different injection time points in rat or mice models (Zeng *et al*., 2010; Steiner *et al*., 2004).

When to inject EdU for detection of EdU in exercised mice has been previously explored and addressed by Zeng *et al*. (2010). Zeng and his colleagues found that a delay of 4 hours rendered a significantly higher EdU labelling of dentate gyrus cells (1600 EdU labelled cells per mouse brain) compared to a 100 mg/kg injection administered 30 days prior to perfusion (mean of 632 ± 64 EdU labelled cells in the entire hippocampus). The reason for EdU-labelled cell values being lower than those seen in the literature can partly be attributed to the study being conducted in mice. A similar study conducted in mice by Castella-Ortega *et al*. (2014) showed that in the entire hippocampus of an exercised mouse there were approximately 200 cells labelled by BrdU (which is comparable to EdU). The higher values are further supported by a study examining adult mice exercised in a wheel for 4 weeks that estimated there were 6000 immature NeuroD labelled-neuoblasts per dentate gyrus. Mice are notably smaller than rats and subsequently may require less EdU to label all mitotically active cells. In contrast when using the common BrdU injection at the same dosage (50mg/kg) in mice, Steiner *et al*. (2004) showed that a lower number of labelled cells (100 BrdU positive cells per mouse dentate gyrus) were rendered upon injection after 4 hours relative to an injection and subsequent brain harvesting three days later (700 BrdU positive per mouse DG). In mice it was found that utilizing as little as one day between injection and perfusion is effective (Chehrehasa *et al*., 2009). A more recent study investigated the injection of pregnant dams with EdU on the same day as tissue collection and found this to be effective (Bhansali *et al*., 2014). This demonstrates the potent labelling ability of EdU over shorter periods of time in mice. This contrasts with our study's findings that did not find four hours to be optimal in rats. In our study, we found that an EdU injection 4 hours prior to perfusion rendered a lower level of labelled cells than 14 days prior to perfusion (Figure 14). This finding, though supported by Zeng *et al*., (2010), warrants further exploration since the survival time of EdU *in vivo* is not currently well explored in the literature involving rats. This would be an ideal future avenue to explore in terms of optimization.

In the current study the reason for such low level labelling on PND 96 may be due to the insufficiency of a single EdU pulse to detect surviving neurons optimally. This could

also suggest that BrdU and EdU take longer to cross the blood brain barrier in rats than in mice. It is also possible that the dosage of EdU was not optimal for immediate detection, however, 50 mg/kg appeared to be a functional dosage to permit the quantification of cells. This aspect requires further research in order to fully optimise the methodology.

Another suggested method for optimisation is to increase the number of injections. This was also explored in mice given three injections of 50 mg/kg of EdU. This was found to effectively label mitotically active cells (Bonaguidi *et al*., 2008). Zeng at al. (2010) used one 200mg/kg injection to quantify the number of mitotically active cells in mice that underwent voluntary wheel running, despite their standard curve indicating that 50 mg/kg was optimal for mice. The majority of studies using rat-models also used multiple injections or a higher dosage of mitotic marker with as many as five rounds of 50 mg/kg BrdU (which is thought to be comparable to EdU) being administered per rat (Speisman *et al*., 2013). In the brains of male Sprague Dawley rats forced to run for approximately 1 hour daily for 3 weeks there were 2225 BrdU/NeuN labelled cells in the dentate gyrus after four days of 50mg/kg twice daily (Jian-feng *et al*., 2014). A study by Chehrehasa *et al*. (2009) used only one 50 -mg/kg injection of EdU in a rat-model but did not quantify cells and analyzed sections qualitatively. Thus in order to better quantify labelled neurons more injections would potentially be beneficial in order to optimally quantify the various cell types identified.

Differences between cell counts for EdU/NeuN, EdU/DCX and EdU/GFAP undetected in pilot study exercised rats

The ability to distinguish between cell phenotypes is essential to identifying neurons and astrocytes enabling us to gain a better understanding of their interactions and functions. All rats were exercised and while many studies explore the effects of exercise on neurogenesis/mitotic activity (Bayod *et al*., 2014; Castilla-Ortega *et al*., 2014, Fuss *et al*., 2014; Gregoire *et al*., 2014) relatively few explore phenotypic expression of the newly generated cells or the influence of exercise on phenotype expression (Mateus-Pinheiro *et al*., 2013; Speisman *et al*., 2013; Steiner *et al*., 2004; van Praag *et al*., 2002). This study assessed exercised rodents and compared the phenotype expression between all labelled cells using molecular markers GFAP for astrocytes, NeuN for mature neurons and DCX for immature neurons. Ki-67 was also utilized as an additional stain in order to detect pulses of mitotic activity. We found (Figure 13 and Figure 15) no detectable differences between cell phenotypes in any of the explored injection times i.e. PND 60, PND 96 or PND 74 (Figure 18) which is further discussed below.

Our findings using the GFAP stain (Figure 13, Figure 18) are different to those seen in the literature but agree with a study that explored neurogenesis in non-exercised mice (Steiner *et al*., 2004). When mice injected with BrdU (4 hours prior to perfusion) there was no difference between GFAP-labelled cells and neurons (20 BrdU-labelled cells per dentate gyrus) (Steiner *et al*., 2004). A study conducted by Mateus Pinheiro *et al*.,

(2013) compared the phenotypes in male Wistar non-exercised controls and exercised rats. This was conducted after neurogenesis was pharmacologically blocked and then unblocked. The study found a lower number of BrdU/GFAP cells (3×10^{-3} cells per 100 µm dentate gyrus) than BrdU/NeuN cells (7×10^{-3} per 100 µm dentate gyrus) (Mateus-Pinheiro *et al*., 2013). Thus despite agreeing with findings in non-exercised mice it would appear further research is needed in exercised rats.

Another study by van Praag *et al*. (2002) examined neuronal phenotype in exercised mice injected with the mitotic marker, green fluorescent protein (GFP). These mice were housed in cages with running wheels for 4 weeks and brains processed 48 hours after injection. This is closer in time frame to the current study that exercised rats for approximately 3 weeks and injected them 2 weeks prior to perfusion. It was found that 50 % of the cells were unidentifiable. There were no mature neurons labelled, 63 % of the 260 GFP-labelled cells (163 cells) were immature neurons and 4.6 % (11.96 cells) were GFAP labelled. These results suggest that exercise up-regulated the neuronal phenotype specifically. However, these are mice injected with the GFAP transgene, which may alter gene expression and phenotype (van Praag *et al*., 2002). This may in part be due to the increase in anti-inflammatory cytokine, IL-1, released from astrocytes, which promotes neuronal activity (Pusic *et al*., 2014) and possibly astrocyte activity too. This reasoning does not explain the increase seen in GFAP-labelled cells in exercised non-mutant mice by Steiner *et al*. (2004). Thus this finding could be a reflective only of those cells that were identified in the van Praag Study (noting that 50 % were unidentifiable), which disagrees with the findings of this study that found no differences.

In exercised rats Speisman *et al*. (2013) found that conditioned running for 18 weeks (rats housed with running-wheels and rewarded with food pellets) resulted in the major phenotype being neuronal. It was shown that there were more BrdU/NeuN (70% of 2300 BrdU-positive cells in the whole dentate gyrus) relative to BrdU/DCX (5% of 2300 BrdU-positive cells in the whole dentate gyrus) and BrdU/GFAP (10% of 2300 BrdU-positive cells in the whole dentate gyrus) positive cells. These results were obtained 3 weeks after injections possibly explaining the increase in mature neuronal phenotype, as neurons take up to 21 days to mature. Therefore it should be noted that our study might have required earlier injection time points to detect mature neuronal differences. Perhaps more injections at an earlier time point would render higher levels of mature neuronal labelling but does not explain the lack of differences seen in immature neurons.

Pooled EdU/DCX+EdU/NeuN cell survival was higher in PND 60 injected and exercised rats than PND 96 injected rats

After just less than three weeks of exercise, there were significantly more labelled neurons detected by injecting EdU on PND 60 than 4 hours prior to perfusion on PND 96 (Figure 16). In an attempt to detect differences in neurogenesis, optimal injection time point and identify phenotypes of mitotically active cells the NeuN and DCX data

were pooled. The total and average number of EdU/NeuN + EdU/DCX showed that significantly more cells expressed the EdU marker 2 weeks after injection of EdU (Figure 16). This finding is in agreement with Steiner *et al*. (2004) who pooled BrdU/NeuN- and BrdU/DCX-labelled cells and found that injections 4 hours prior to perfusion rendered significantly lower cell counts than perfusions 1 week after BrdU injections (10 cells after 4 h vs. 150 after 3 days). There is no exact replication of this in the literature, but it suggests that a larger sample size could possibly reveal differences in phenotype or injection time points may be an influential factor.

In summary, the pilot study was able to successfully confirm the use of EdU to detect mitotically active cells in the DG of rats. EdU was further d with co-labels GFAP, NeuN, DCX and Ki-67 for the detection of phentotypical differences. There were no detectible effects shown of exercise and analysis of cell phenotypes separately did not reveal differences in neurogenesis. By combining DCX- and NeuN-labelled cells it appeared that EdU injections at PND 60, 14 days before perfusion, rendered better labelling than pulse injections on PND 74, 4 hours prior to perfusion. The mitotic marker, Ki-67, appeared to be a better pulse-label of proliferating cells at the end of 20 days of running (Figure 19).

4.2) Experiment: Measuring cell survival and neurogenesis using EdU

After characterisation of EdU, it was found that EdU would be effective in measuring cell survival and Ki-67 was used as a pulse to measure neurogenesis at the time of sacrifice after 20 days being housed with running wheels. Therefore using the novel tool we measured neurogenesis to determine two objectives. Firstly we were interested in determining whether MS blocks the effect of exercise on cell genesis and/or survival in the hippocampus and/or prevents these proliferating cells from becoming mature neurons or glia. Secondly we hoped to determine whether or not voluntary exercise affected cell genesis and survival.

Weight was increased in MS rats

Weights were captured on the day of injection for each rat (PND 60) in order to determine a behavioral measure of food consumption (Figure 20). In our cohort of rats, the MS+NR rats weighed more than the NMS+R rats on PND 60. This finding is similar to findings by Hamilton *et al*. (2010) who found the weights of age matched sham-injected male Long Evans rats to be approximately 374.2g in MS and 354.2g NMS on PND 72. This confirmed our rats were in the correct weight range. Exercise and weight have been linked and when wheel running was introduced to rats it was found to increase weight (Noble *et al*., 2014) and MS has been shown to decrease (Huot *et al*., 2001) weight or have no effect (Lee *et al*., 2013). MS rats had significantly increased weights compared to NMS rats. It is possible that MS altered the biochemistry of the brains of rats linked to an increase in weight gain (Daniels *et al*., 2011) or possibly MS rats consumed more rat chow on a daily basis.

Distance Run by rats was lower in MS rats

Voluntary wheel running is a measure of exercise in rodents and increases over time as it is considered rewarding as shown by conditioned place preference (CPP) testing (Greenwood *et al*., 2011). It was necessary to ensure that all animals ran similar distances to confirm that the level of exercise was homologous between MS and NMS groups. However, the Wilcoxon matched-pairs signed rank test showed that medians were different between the 20 days of running between MS and NMS rat groups (Figure 21). Our findings are similar to Greenwood *et al*., 2011 who used Fischer rats and found that they ran 0.5 km/day - 2.5 km/day over 39 days. We saw an effect of MS on running distance with MS having lower daily medians than NMS. Stress is reported to decrease running distance and non-stressed-run mice ran significantly more than previously stressed and run mice (Castilla-Ortega *et al*., 2014). This may be considered plausible in understanding the behavioral effects MS has on the rats.

The Survival of NeuN, GFAP and DCX labelled cells was not affected by MS

After establishing that both MS and NMS ran sufficient daily-distances the number of surviving neurons (EdU/NeuN), immature neurons (EdU/DCX) and astrocytes (EdU/GFAP) were compared between groups. There was no detectable effect on neuronal survival in MS or voluntary exercise groups (Figure 22 and Figure 25).

The effect of MS and exercise on EdU-labelled neuronal survival was explored (Figure 22a). There were no significant differences on any of the treatment groups in the dorsal or the ventral hippocampus. This study found no effect of MS stress on mature neurons contrasting with present literature. Stress has been shown to decrease later neuronal survival in rats. Temporarily blocking neurogenesis in rats has shown to later alter the level of neurogenesis once the block is removed, the BrdU/NeuN labelled cells in the dentate gyrus of adult Wistar rats have been decreased in rats that experienced stress (Mateus-Pinheiro *et al*., 2013). Unpredictable chronic mild stress treatments also alters mature neuronal number Lajud *et al*. (2012) found that fifteen-day-old MS Sprague Dawley rats had a decrease in BrdU/NeuN labelled cells (which localized with DCX). MS specifically, reportedly decreases the number of mature surviving neurons in rats. It was shown that 35 % of the BrdU cells were NeuN with an estimate of 700 BrdU/NeuN cells in MS rats (Lajud *et al*., 2012). This study's results are possibly due to the dosage of the EdU being insufficient to detect differences between groups. Alternatively the sample size (n=4/5 depending on stain) was relatively small to identify group differences between the NMS and MS groups. Upon pooling the dorsal and ventral hippocampi we still found no significant differences using a Mann Whitney U test (n=10/9) so it is more likely that the dosage 50mg/kg was too low in adult rats. The novel EdU marker did not label sufficient cells for significant quantification of differences at this dosage. It should be noted this factor might have influenced all EdU results.

This study also examined astrogliogenesis using the marker GFAP. Similar to the finding above, there was no detectable difference between the surviving EdU/GFAP cells present (Figure 22b). Our findings in astrocytes agrees with an earlier study by Mirescu *et al*. (2004) that showed astrocyte counts were not different between MS and NMS rats. Despite not being significantly different the astrocyte number was low relative to neurons (7% of approximately 3000 BrdU labelled cells thus 210 GFAP per DG). However, chronic stress in adult rats reduced the number of microglia (including astrocytes) expressed in the DG with 90 cells per mm^2 DG labelled in CUS rodents and 120 cells per mm^2 DG in controls. Thus adult stress has been shown to decrease microglia (Kriesel *et al*., 2013). Mateus-Pinheiro *et al*., (2013) found pharmacologically arresting mitotic activity decreased astrocytes 3 fold (with 3×10^{-3} per 100 µm DG in controls and 1×10^{-3} per 100 µm DG). Similarly stress is thought to decrease the number of surviving astrocytes. Kriesel *et al*. (2013) found a causal relationship between stress and a decrease in microglia (including astrocytes), and linked neurogenesis to depressive-like behavior. There was also decrease in markers for glial activity. Stressors such as chronic stress and forced exercise decrease astrogliogenesis possibly due to exposure to glucocorticoids (Roman *et al*., 2005; Mandyam *et al*., 2007; Nune *et al*., 2011). Therefore it is likely MS effects astrocytes similarly to neurons decreasing number. This study was unable to detect any changes due to afore mentioned reasons.

There was no significant difference found between treatment groups co-labelled with EdU/DCX (Figure 25). This marker combination compares the number of surviving immature neurons (Shruster *et al*., 2012; Monje *et al*., 2003). There were no detectable differences seen between immature neurons. These findings agree with Hulshof *et al*., 2011. The study reported that there was no difference in neuronal differentiation between MS Wistar rats relative to NMS controls labelled with DCX (approximately 2000 cells per dorsal hippocampus and 2500 per ventral). Our findings and those of Hulshof *et al*. (2011) are not in agreement with the current literature, which have reported a decrease of neuronal differentiation and immature neurons in rodents treated with early developmental stress and specifically MS. A study by Zhao *et al*. (2014) used maternal Sleep Deprivation (a pre-natal developmental stressor) and measured 45000 BrdU/DCX cells per DG and sleep controls vs. 3000 BrdU/DCX cells in pups born to sleep deprived dams. The above mentioned study by Lajud *et al*. (2012) also showed that MS stress decreased the number of BrdU/DCX labelled neurons in the dentate gyrus of PND 15 Sprague Dawley rats (9600 BrdU/DCX cells/DG vs. 3300 BrdU/DCX /DG). Mirescue *et al*. (2004) found a decrease in immature neuronal DG cells in MS rats. BrdU/Tuj1 (a marker for immature neurons) positive cells showed a decrease in BrdU cells labelled a week after 200mg/kg BrdU injections (4000 in MS vs. 6000 NMS controls). Thus pre-natal stress and early post-natal stress are consistently reported to decrease immature neuron number in rodents.

Variability in results among studies could possibly be due to animal age, strain and different MS methods. It has been suggested that immature neurons influence behavior as they have been shown to extend dendrites as soon as one week after

differentiation (Cameron & McKay, 2001) and are able to establish new functional connections within four weeks in mice (Kee *et al*., 2007), establish LTP at a lower thresholds than mature neurons (Ge, *et al*., 2007) and have been linked to behavioral changes in mice (Sanatrelli *et al*., 2003). Thus presumably they are more susceptible to MS stress. Despite this the effect of MS on neuronal differentiation has not been widely explored within the literature and as such our study indicates that MS alone is not sufficient to alter neuronal differentiation.

Voluntary running no detectable effect on Edu Labelled cells

The voluntary running also had no detectable effect in any of the EdU labelled treatment groups (Mature neurons, astrocytes or immature neurons (Figure 22 and Figure 25). This finding does not support current literature in both mice and rats. In mice Rhodes *et al*. (2003) permitted voluntary wheel running. They found runners had 800 BrdU positive cells and non-wheel runners had 200 BrdU positive cells. The 97% BrdU cells in runners were neuronal and 88% in non-runners. As little as 7 days of wheel running in mice was shown to up-regulate the number of surviving neurons labelled with NeuN (Rhodes *et al*., 2003). A study by van Praag *et al*., 2005 showed that 3 month old mice that had been housed with running wheels for 45 days had 81.3 % BrdU/NeuN labelled cells (2355 cells counted of which 1914 were NeuN co-labelled) relative to the 49.9 % BrdU/NeuN cells (613 cells counted of which 300 NeuN cells) non-runners. Thus exercise is shown to up-regulate mature neuronal survival in mice. Stress combined with exercise reportedly also influences neuronal survival. The marker BrdU/CB (Calbindin a marker for mature neurons) showed that restraint stress combined with voluntary exercise generated 150 neurons in the DG and approximately 50 in purely exercised mice relative to approximately 10 neurons in controls (Castilla-Ortega *et al*., 2014). This was 24 days after injections were administered.

In rats Speisman *et al*. (2013) showed that there was no difference in mature neuron numbers between voluntary runners and non-runners. Their study reported 1610 BrdU/NeuN cells for runners (70% of 1600) and 1120 for non-runner controls (70% of 2300) (Speisman *et al*., 2013). A study by Roman *et al*. (2008) used forced activity on adult Wistar rats and found that it did not significantly affect BrdU labelled cells relative to sedentary controls. After 8 days of forced locomotor activity in a rotating barrel 780 cells (22.2% of 4200 cells) were neuronal in runners and 958 BrdU/NeuN cells (13.5 % of 6000 cells) in controls. The effect of forced exercise may be the reason for this finding, unlike the current study that explored voluntary exercise. There are studies that have explored the neuro-protective effects of exercise in MS rats and found voluntary wheel running reduced proteins involved in apoptosis in the ventral hippocampus (Daniels *et al*., 2011) it has also been shown than 6 weeks of voluntary wheel-running increases levels of BDNF in the rodent brain (Marais *et al*., 2009). Thus exercise alters the biochemistry of the brain possibly influencing neuronal survival and genesis in MS rats, although further research is needed. It has been shown that running up-regulates the markers of neurogenesis for longer periods of time and thus an increasing neurogenesis due to a decrease in plasma corticosterone (Gregorie *et al*., 2014).

There was also no detectable effect of voluntary exercise on astrogliogenesis, which agrees with the findings of van Praag *et al*., 2005 who also found that exercise had no affect on astrogliogenesis with (non-runners 48 BrdU/GFAP cells in the DG and 35,25 in mice runners) a similar finding was confirmed by Rhodes *et al*. (2003). This is further supported in rats by a study by Speisman *et al*. (2013) reporting that found no difference between conditioned runners and non runners labelled with BrdU/GFAP and 10% of all BrdU cells were labelled with GFAP (160 cells in controls and 240 in runners per DG).

A decrease in stress-induced hormones is thought to modulate neurogenesis and neuronal differentiation. Contrasting to this hormones such as glucocorticoids are released during exercise stress, and have been shown to activate receptors in hippocampal slices that cause progenitor cells to become astrocytes in tissue culture (Anacker *et al*., 2013). Thus the relationship and molecular mechanisms linking stress, exercise and astrogliogenesis remains unclear. This contradicts findings that exercise induced an increase in neurogenesis is linked to BDNF levels, which do not appear to affect astrogliogenesis significantly (Rhodes *et al*., 2003). Forced exercise and other forms of stress decrease astrocytic proliferation (Roman *et al*., 2005).

We did not see an effect of exercise on any of the treatment group double labelled with DCX/EdU. The exercised rats had a wider range of data points than non-runners. This could be because EdU has also been found to alter metabolic activity and decrease cellular proliferation (Neef & Luedtke, 2011) and possibly early neuronal differentiation. However, our finding agrees with a study using BrdU by Gregorie *et al*., 2014 that found three weeks of running had no effect on the number of DCX/BrdU labelled cells in the dorsal hippocampus of mice. This disagrees with Castilla-Ortega *et al*., 2014 who found that 23 days of voluntary wheel running was sufficient to induce significant up-regulation of DCX/BrdU labelled cells (approximately 100 present in the DG after 24 days) and also reversed the effects of chronic restraint stress normalizing the number of immature neurons labelled relative to non-stressed control mice.

Correlation Analysis found a relationship between Neurons (EdU/NeuN) and Astrocytes (EdU/GFAP)

To further assess the relationship between neurons and astrocytes the data from the two stains were pooled. We compared the survival of the two phenotypes (EdU/GFAP vs. EdU/NeuN). The EdU/NeuN co-labelled cells from all groups were pooled and EdU/GFAP co-labelled cells were pooled separately and compared using a Mann Whitney U test. This was permissible as there was no significant difference between the treatment groups (Figure 24). There was also no significant difference between the total number of co-labelled EdU/GFAP cells and EdU/NeuN cells in the dorsal or ventral hippocampi (Figure 23). Our findings are in agreement with Roman *et al*., 2005.

Using double labelled BrdU/NeuN/GFAP cells they reported no significant differences between BrdU/NeuN and BrdU/GFAP labelled cells in exercised or non-exercised or rats stressed with sleep deprivation. Mateus-Pinheiro *et al*., 2013 also reported no significant difference in basal BrdU/GFAP labelled cells relative to BrdU/NeuN labelled cells. Despite this finding of no difference between neurons and astrocytes survival a correlation was performed to determine if there was a link between the neurons and astrocytes (Figure 24). Upon pooling the data from all treatment groups our study revealed no significant correlation between neurons and astrocytes in the dorsal hippocampus but there was a significant correlation between neurons and astrocytes in the ventral hippocampus (Figure24b).

There are no other studies (to our knowledge) that have used EdU/NeuN and EdU/GFAP to identify a correlation between the two cell types. Haggeman *et al*., 2013 found that mutant mice unable to express GFAP had a significant decrease in mature neurons cells (20 NeuN in mutants vs. 100 labelled cells in controls). The reduction in GFAP was correlated with the depletion in neurons in mutant mice. Correlation analysis was not conducted with GFAP but analysis of EdU/NeuN and EdU/S100β were conducted and compared. The correlation between astrocytes and neurons has been attributed to astrocytes playing a direct role in promoting neurogenesis (Haggeman *et al*., 2014). Astrocytes strengthening synapses in the hippocampus is possibly the most likely reason for the correlation between the two neuronal cell types proliferation. Astrocytes increase synaptic strength and increase synaptic transmission, neuronal activity (Kirby *et al* 2013) and in turn enhance neuroplasticity (Nagler *et al*., 2001; Ullian *et al*., 2001). Disruption in microglial signaling can result in perturbations in the regulation of neuronal excitability and activity, which is essential to the integration of newly generated neurons (Pusic *et al*., 2014). A decrease in astrocytes has been linked to depressive behavior (Kriesel *et al*., 2014). This is hypothesized to occur due to a down-regulation in neurogenesis. This was shown by pharmacological stimulation reversing these behavioral changes and up regulating BrdU-labelled cells. This correlation may also only occur in the ventral hippocampus as it regulates the stress response and anxiety-like behavior (Bannerman *et al*., 2004) as this system is particularly vulnerable to stress.

Neuronal proliferation (Ki-67/DCX) was reduced in MS rats

The marker, Ki-67 was used as a mitotic pulse measurement of proliferation and the DCX co-label was used to distinguish how many of those cells were committing to the neuronal lineage (Figure 26). It was used to measure the number of mitotically active neuroblasts that were present at the time of perfusion. This was in conjunction with the EdU marker that measured survival from injection time point until perfusion. We found that there were significantly fewer Ki-67/DCX positive cells in MS + R rats relative to NMS + R rats in both the dorsal and ventral dentate gyrus (Figure 26).

Many studies report that MS (Mirescue *et al*., 2004; Lelsie *et al*., 2011; Lajud *et al* 2012) and Maternal Deprivation (MD) (Oomen *et al*., 2010) decrease cellular proliferation in the DG of rats and mice alike. As previously discussed MS and MD are

thought to decrease neurogenesis, and four weeks after pharmacologically arresting neurogenesis in Wistar rats there was still lower number of proliferating Ki-67 positive cells in the DG relative to controls (Mateus-Pinheiro *et al*., 2013). Not all studies agree with this finding and Suri *et al*. (2013) found no effect of MS in Sprague Dawley rats on cellular proliferation (700 - 800 BrdU positive cells in the sub-granular zone and granule cell layers of both groups with approximately 130 DCX positive cells in MS rats). Despite the variability in the literature this study did find MS+R rats to have lower cell counts. It is thought that an increase in plasma corticosterone in MS rats is responsible for the reduction in neuronal proliferation (Lajud *et al*., 2012). This is linked to immature neurons being sensitive to the increased presence of glucocorticoids such as corticosterone (Mirescu *et al*., 2004; Lupien *et al*., 2009). Thus the early life stress of MS causes alterations in the functioning of the HPA axis, which regulates stress. The most prominent theory is that maternal separation and exercise influence levels of BDNF or its downstream targets that influence neurogenesis and depressive-like behavior. Increases or decreases in immature proliferating neurons can be explained via an increase or decrease in BDNF.

Maternal separation has been shown to decrease levels of BDNF in the hippocampus. The effects of a reduction in BDNF were seen to last for several weeks (Lippmann *et al*., 2007) and exercise has been shown to increase the levels of BDNF in the striatum of MS rats that were exposed to chronic voluntary wheel running (Marais *et al*., 2009). This adds to the findings of a study conducted by Makena *et al*., 2012 who found that MS altered the effects of exercise and rats subject to wheel running and MS had significantly lower levels of synaptophysin, phospho-extracellular signal related kinase (p-ERK) in the hippocampus. This could indicate that MS blocks the effects of increased BDNF via acting on ERK that stimulates neurotrophic factors and enhances neuronal growth and neurogenesis (Hao *et al*., 2004). The effect of maternal deprivation also alters cell morphology and decreases the number of proliferating neurons (Oomen *et al*., 2010). Immature granule neurons are thought to be involved in anxiety regulation the decrease in neurons and are required for several behavioral functions (Mirescu *et al*., 2004)

BDNF may not be the only factor influencing early neurogenesis. One MS study found elevated BDNF in the hippocampus resulted in no increase in neurogenesis (Greisen *et al*. 2005) and a study by Yau *et al*., 2014 found increased neurogenesis linked to an increase in synaptophysin and it receptor PSD95 and not BDNF. Thus it is possible the changes seen are not solely BDNF dependent. The activation of Erk via phosphorylation was shown to enhance survival and neurogenesis as shown via BrdU/NeuN co-labelling of neurons in the hippocampus of male rats (Hao *et al*., 2004). Therefore our findings support the possibility of MS influencing Erk signaling and possibly exacerbating a reduction in neuronal proliferation caused by voluntary wheel running.

Exercise significantly affects cell proliferation (Ki-67/DCX) in the Dorsal Hippocampus

In the dorsal hippocampus the NMS+R rats Ki-67/DCX counts are higher than NMS+NR (Figure 26). Our results show that exercise specifically influences neuronal proliferation and differentiation at the earliest stages of cell differentiation. These results agree with previous literature (Castilla-Ortega *et al*., 2014; Nobel *et al*., 2014) that states voluntary exercise in rodents has increases neurogenesis. Our results are similar to those seen in forced-run mice for 23 days 170 BrdU/DCX positive cells and non-run had 70 BrdU/DCX positive cells as estimated for the entire dentate gyrus (as multiplied by 8 due to sampling) and an increase in PCNA-labelled proliferating cells relative to controls (Castilla-Ortega *et al*., 2014). However, forced locomotor activity has also been shown to decrease cell proliferation in mice (Roman *et al*., 2005). In male Sprague Dawley rats forced wheel running for 3 weeks also resulted in 1400 DCX positive cells in the dentate gyrus with controls having 9000 DCX positive cells (Jiang Feng *et al*., 2014). The effects of exercise are not always homogenous depending on the kind of exercise (Roman *et al*., 2005).

Voluntary exercise has specifically shown to increases neurogenesis in rats (Nobel *et al*., 2014). Voluntary wheel running in Sprague Dawley rats increases cellular proliferation in CA3 region of the hippocampus (Nobel *et al*., 2014) and the DG. As previously mentioned the dentate gyrus has been better explored as it functions have been linked to anxiety and depressive-like behaviors relevant to this study (Matheus-Pinero *et al*., 2014). Voluntary wheel running in adult rats (for 11 weeks) has been shown to increase neuronal differentiation (16000 DCX cells/DG vs. 13000 DCX cells/DG with no mitotic marker) (Winocur *et al*., 2014). This is supported by Speisman *et al*. (2013) who also reported an increase in BrdU/DCX labelled cells in the DG of run rats. Twelve days of voluntary exercise followed by enrichment did not increase Ki-67 labelled cells in Male Long-Evans (approximately 4800 positive cells in the DG) (Hamilton *et al*., 2014). Runners administered exogenous corticosterone had a higher number of proliferating DG cells relative to non-runners (Yau *et al*., 2014). Therefore even with stress hormones present running increases proliferation and neuronal differentiation. Exercise increases proteins in signaling pathways linked to its release such as Akt, ERK, CaMKII and CREB, BDNF/TrkB. This supports findings that indicate exercise activates the BDNF-CreB pathways that enhance neuronal plasticity (Jian-Feng *et al*., 2014).

Our findings differ to the above studies and this is in part due to studies sampling and multiplying out errors in counts. Estimations vary based on brain sampling are not always the same; every 16th section (Hamilton *et al*., 2014) and 8 sections from -1.22 and -2.54 mm from bregma (Castilla-Oretega. *Et al*., 2014), BrdU was quantified on every 6th section between Bregma 21.06 mm and 22.98 mm of the hippocampus (8 sections total/marker/animal) (Gregorie *et al*., 2014). The study by Jiang-Feng (2014) does not specify co-ordinates and states every 6th section of dentate gyrus was used for estimations with 10 sections taken per animal. Thus identifying areas in the DG where more or less neurogenesis occurs would be beneficial to homing in on which areas are most affected and for homogenous comparisons. The number of injections and dosage of mitotic markers varied between experiments also.

Dorsal and Ventral hippocampi affected by MS

Our study sought to separately analyze the dorsal and ventral hippocampus because they have shown to functionally differ (Bannerman *et al*., 2004). MS rats had a significantly reduced number of Ki-67/DCX labelled immature neurons relative to NMS runners (MS+R vs. NMS+R) in both brain regions. However, there was no significant increase in NMS+R relative to NMS +NR in the ventral hippocampus.

Most studies have focused on the dorsal hippocampus, but a paper by Hulshof *et al*., 2011 explored MS in both the ventral and dorsal hippocampus and found there was only a decrease in Ki-67 measured proliferation in the ventral hippocampus after MS+R vs. NMS. This is not in agreement with our findings that saw MS+ NR decrease counts in both dorsal and ventral regions in run rats. The treatment groups look at the combined effect of MS and exercise rather than MS singularly which may account for this difference.

Dorsal Hippocampus affected by Exercise

The effect of exercise was found to be region specific in this study and was also able to show that NMS + R had a higher mean than NMS +NR in the dorsal hippocampus. The findings in the dorsal hippocampus show that running for 20 days increases proliferation of neurons only on the dorsal hippocampus of NMS rats. This is different from Lui *et al*., 2013 whose research showed running increased cellular proliferation in the dorsal and ventral hippocampus. This study however was conducted in mice and could be the reasons for the difference.

The ventral hippocampus regulates the stress response and anxiety-like behavior while the dorsal hippocampus is involved in spatio-temporal memory (Bannerman *et al*., 2004). Makena *et al*. (2012) found the effects of voluntary-wheel running exercise to be observed only in the dorsal hippocampus of MS rats with increases in synaptophysin and p-ERK. This study found that MS+R groups had lower cells counts than NMS+R groups in both ventral and dorsal hippocampus. The findings regarding neurogenesis in the dorsal and ventral hippocampus require more exploration before any conclusions may be drawn.

4.3) Conclusion & Future considerations

Depression is a widespread psychiatric condition disease (Marcus *et al*., 2012) for which current treatments have variable efficacy. It has been linked to adult

neurogenesis in humans (Eriksson *et al*., 1998). As such novel tools to study neurogenesis are needed to further explore neurogenesis and its link with depressive-like behaviors (Mateus-Pinheiro *et al*., 2013). The MS rat model for depression is an ideal model in which to do so as it has been firmly explored in terms of inducing depressive-like behavior and altering brain biochemistry analogous to human depression.

We investigated the effect of MS and exercise (using the novel EdU mitotic marker) and found was no detectable difference on neuron, astrocyte or immature neuron survival. The study did however propose a link between newly generated astrocytes and neurons in the ventral hippocampus suggesting there could be a biological link between the two phenotypes cell fates. This is the first time this has been done using EdU. This resulted in improved epitope preservation and tissue quality for quantifying cell counts more accurately in an MS model.

We further explored the impact of MS and voluntary exercise on neuronal proliferation (as measured by pulse of Ki-67 and DCX). MS+R groups displayed a decrease in the number of proliferating cells in both ventral and dorsal hippocampus relative to NMS+R groups. This suggests that MS possibly diminishes the beneficial effects of exercise. The dorsal hippocampus also showed an increase in labelled cells in NMS+R relative to NMS+NR. Thus exercise was shown to increase the number of immature neurons generated in the DG. The current literature suggests that, BDNF is a plausible link as it has been linked to the anxiolytic effects of exercise in the MS in rat model. There is limited understanding in this area and it would prove useful further establish the mechanism causing the decrease in neuronal proliferation.

In order to further explore the relationship between Maternal Separation and neurogenesis it is necessary to improve quantification of EdU labelled cells. While one 50mg/kg injection in rats was effective in detecting neurogenesis, a higher dosage or increase in the number of injections could be explored to optimize staining levels.

In an attempt to detect differences in neurogenesis at different injection time points and then identify phenotypes of mitotically active cells the NeuN and DCX data were pooled. The total and average number of EdU/NeuN + EdU/DCX showed that significantly more cells expressed the EdU marker 2 weeks after injection of EdU (Figure 26). This finding is in agreement with Steiner *et al*. (2004) who pooled BrdU/NeuN- and BrdU/DCX-labelled cells and found that injections 4 hours prior to perfusion rendered significantly lower cell counts than perfusions 1 week after BrdU injections (10 cells after 4 h vs. 150 after 3 days). There is no exact replication of this in the literature, but it suggests that a larger sample size could possibly reveal differences in phenotype. This avenue merits further research.

This study is the first (to our knowledge) to effectively use EdU in an MS rat model for depression and as such further elucidates the link between depression and neurogenesis and exercise. It provides new insights to the effect of stress and exercise on proliferating neurons in the adult rat hippocampus.

References

1. Aisa, B., Tordera, R., Lasheras, B., Del Río, J. & Ramírez, M. J. Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats. *Psychoneuroendocrinology* **32,** 256–66 (2007).

2. Altman, J. & Das, G. D. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.* **124,** 319–335 (1965).

3. Anacker, C. *et al.* Glucocorticoid-related molecular signaling pathways regulating hippocampal neurogenesis. *Neuropsychopharmacology* **38,** 872–83 (2013).

4. Arbor, A. Delayed Effects of Chronic Variable Stress During Peripubertal- Juvenile Period on Hippocampal Morphology and on Cognitive and Stress Axis Functions in Rats. **648,** 636–648 (2004).

5. Azzarelli, R. *et al.* An antagonistic interaction between PlexinB2 and Rnd3 controls RhoA activity and cortical neuron migration. *Nat. Commun.* **5,** 3405 (2014).

6. Banasr, M., Soumier, A., Hery, M., Mocaër, E. & Daszuta, A. Agomelatine, a New Antidepressant, Induces Regional Changes in Hippocampal Neurogenesis. *Biol. Psychiatry* **59,** 1087–1096 (2006).

7. Bayod, S. *et al.* Wnt pathway regulation by long-term moderate exercise in rat hippocampus. *Brain Res.* **1543,** 38–48 (2014).

8. Bechara, R. G. & Kelly, Á. M. Exercise improves object recognition memory and induces BDNF expression and cell proliferation in cognitively enriched rats. *Behav. Brain Res.* **245,** 96–100 (2013).

9. Becker, L., Kulkarni, S., Tiwari, G., Micci, M.-A. & Pasricha, P. J. Divergent fate and origin of neurosphere-like bodies from different layers of the gut. *Am. J. Physiol. Gastrointest. Liver Physiol.* **302,** G958–65 (2012).

10. Belzung, C. Innovative drugs to treat depression: did animal models fail to be predictive or did clinical trials fail to detect effects? *Neuropsychopharmacology* **39,** 1041–51 (2014).

11. Bennabi, D. *et al.* Risk factors for treatment resistance in unipolar depression: a systematic review. *J. Affect. Disord.* **171,** 137–41 (2015).

12. Bhansali, P., Rayport, I., Rebsam, A. & Mason, C. Delayed neurogenesis leads to altered specification of ventrotemporal retinal ganglion cells in albino mice. *Neural Dev.* **9,** 11 (2014).

13. Boku, S. *et al.* Neonatal maternal separation alters the capacity of adult neural precursor cells to differentiate into neurons via methylation of retinoic acid receptor gene promoter. *Biol. Psychiatry* **77,** 335–344 (2015).

14. Bonaguidi, M. a *et al.* Noggin expands neural stem cells in the adult hippocampus. *J. Neurosci.* **28,** 9194–204 (2008).

15. Booth, D. G. *et al.* Ki-67 is a PP1-interacting protein that organises the mitotic chromosome periphery. 1–22 (2014). doi:10.7554/eLife.01641

16. Borsini, A., Zunszain, P. A., Thuret, S. & Pariante, C. M. The role of inflammatory cytokines as key modulators of neurogenesis. *Trends Neurosci.* **38,** 145–157 (2015).

17. Brewer, M., Pickel, J., Cameron, H. A. & Snyder, J. S. Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. 3–7 (2011). doi:10.1038/nature10287

18. Cameron, H. A. & Mckay, R. D. G. Adult Neurogenesis Produces a Large Pool of New Granule Cells in the Dentate Gyrus. **417,** 406–417 (2001).

19. Carpenter, M. K. *et al.* In vitro expansion of a multipotent population of human neural progenitor cells. *Exp. Neurol.* **158,** 265–278 (1999).

20. Castilla-Ortega, E. *et al.* Voluntary exercise followed by chronic stress strikingly increases mature adult-born hippocampal neurons and prevents stress-induced deficits in "what-when-where" memory. *Neurobiol. Learn. Mem.* **109,** 62–73 (2014).

21. Chapman, D. P. *et al.* Adverse childhood experiences and the risk of depressive disorders in adulthood. *J. Affect. Disord.* **82,** 217–25 (2004).

22. Chehrehasa, F., Meedeniya, A. C. B., Dwyer, P., Abrahamsen, G. & Mackay-Sim, A. EdU, a new thymidine analogue for labelling proliferating cells in the nervous system. *J. Neurosci. Methods* **177,** 122–30 (2009).

23. Dannlowski, U. *et al.* Limbic scars: long-term consequences of childhood maltreatment revealed by functional and structural magnetic resonance imaging. *Biol. Psychiatry* **71,** 286–93 (2012).

24. Das, S. & Basu, A. Inflammation: a new candidate in modulating adult neurogenesis. *J. Neurosci. Res.* **86,** 1199–208 (2008).

25. Doetsch, F., Caille, I., Lim, D. A., Garcı, J. M. & Alvarez-buylla, A. Subventricular Zone Astrocytes Are Neural Stem Cells in the Adult Mammalian Brain. **97,** 703–716 (1999).

26. Dunn, A. L., Trivedi, M. H., Kampert, J. B., Clark, C. G. & Chambliss, H. O. Exercise treatment for depression: efficacy and dose response. *Am. J. Prev. Med.* **28,** 1–8 (2005).

27. Fanselow, M. S. & Dong, H. Review Are the Dorsal and Ventral Hippocampus Functionally Distinct Structures ? *Neuron* **65,** 7–19 (2010).

28. Fricker, R. A. *et al.* Site-Specific Migration and Neuronal Differentiation of Human Neural Progenitor Cells after Transplantation in the Adult Rat Brain. **19,** 5990–6005 (1999).

29. Fuss, J. *et al.* Exercise boosts hippocampal volume by preventing early age-related gray matter loss. *Hippocampus* **24,** 131–4 (2014).

30. Ge, S., Yang, C.-H., Hsu, K.-S., Ming, G.-L. & Song, H. A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. *Neuron* **54,** 559–66 (2007).

31. Goodman, S. Commentary: the multifaceted nature of maternal depression as a risk factor for child psychopathology--reflections on Sellers et al. (2014). *J. Child Psychol. Psychiatry.* **55,** 121–3 (2014).

32. Grace, L., Hescham, S., Kellaway, L. a, Bugarith, K. & Russell, V. a. Effect of exercise on learning and memory in a rat model of developmental stress. *Metab. Brain Dis.* **24,** 643–57 (2009).

33. Grant, M. M. *et al.* Early Life Trauma and Directional Brain Connectivity Within Major Depression. **4826,** 4815–4826 (2014).

34. Grégoire, C.-A., Bonenfant, D., Le Nguyen, A., Aumont, A. & Fernandes, K. J. L. Untangling the influences of voluntary running, environmental complexity, social housing and stress on adult hippocampal neurogenesis. *PLoS One* **9,** e86237 (2014).

35. Greisen, M. H., Altar, C. A., Bolwig, T. G., Whitehead, R. & Wörtwein, G. Increased adult hippocampal brain-derived neurotrophic factor and normal levels of neurogenesis in maternal separation rats. *J. Neurosci. Res.* **79,** 772–8 (2005).

36. Guo, F., Ma, J., McCauley, E., Bannerman, P. & Pleasure, D. Early postnatal proteolipid promoter-expressing progenitors produce multilineage cells in vivo. *J. Neurosci.* **29,** 7256–70 (2009).

37. Hamilton, G. F. *et al.* Exercise and environment as an intervention for neonatal alcohol effects on hippocampal adult neurogenesis and learning. *Neuroscience* **265,** 274–90 (2014).

38. He, F., Zou, J.-T., Zhou, Q.-F., Niu, D.-L. & Jia, W.-H. Glatiramer acetate reverses cognitive deficits from cranial-irradiated rat by inducing hippocampal neurogenesis. *J. Neuroimmunol.* **271,** 1–7 (2014).

39. Hospital, J. R., Brown, D. C. & Gatter, K. C. Ki67 protein : the immaculate deception ? 2–11 (2002).

40. Hulshof, H. J. *et al.* Maternal separation decreases adult hippocampal cell proliferation and impairs cognitive performance but has little effect on stress sensitivity and anxiety in adult Wistar rats. *Behav. Brain Res.* **216,** 552–60 (2011).

41. Huot, R. L., Thrivikraman, K. V, Meaney, M. J. & Plotsky, P. M. Development of adult ethanol preference and anxiety as a consequence of neonatal maternal separation in Long Evans rats and reversal with antidepressant treatment. *Psychopharmacology (Berl).* **158,** 366–73 (2001).

42. Inoue, K., Okamoto, M., Shibato, J., Lee, M. C. & Matsui, T. Correction : Long-Term Mild , rather than Intense , Exercise Enhances Adult Hippocampal Neurogenesis and Greatly Changes the Transcriptomic Profile of the Hippocampus. 2–3 (2015). doi:10.1371/journal.pone.0133089

43. Kato, T., Kasahara, T., Kubota-Sakashita, M., Kato, T. M. & Nakajima, K. Animal models of recurrent or bipolar depression. *Neuroscience* (2015). doi:10.1016/j.neuroscience.2015.08.016

44. Kawakami, S. E., Quadros, I. M. H., Machado, R. B. & Suchecki, D. Sex-dependent effects of maternal separation on plasma corticosterone and brain monoamines in response to chronic ethanol administration. *Neuroscience* **253,** 55–66 (2013).

45. Kempermann, G., Jessberger, S., Steiner, B. & Kronenberg, G. Milestones of neuronal development in the adult hippocampus. *Trends Neurosci.* **27,** 447–52 (2004).

46. Kempke, S. *et al.* Effects of early childhood trauma on hypothalamic-pituitary-adrenal (HPA) axis function in patients with Chronic Fatigue Syndrome. *Psychoneuroendocrinology* **52,** 14–21 (2015).

47. Kent, B. A., Oomen, C. A., Bekinschtein, P., Bussey, T. J. & Saksida, L. M. ScienceDirect Cognitive enhancing effects of voluntary exercise , caloric restriction and environmental enrichment : a role for adult hippocampal neurogenesis and pattern separation ? *Curr. Opin. Behav. Sci.* **4,** 179–185 (2015).

48. Kheirbek, M. a *et al.* Differential control of learning and anxiety along the dorsoventral axis of the dentate gyrus. *Neuron* **77,** 955–68 (2013).

49. Kim, B. *et al.* Treadmill exercise improves short-term memory by enhancing neurogenesis in amyloid beta-induced Alzheimer disease rats. **10,** 2–8 (2014).

50. Kirby, E. D., Kuwahara, A. A., Messer, R. L. & Wyss-coray, T. Adult hippocampal neural stem and progenitor cells regulate the neurogenic niche by secreting VEGF. **112,** (2015).

51. Komorowski, R. W. *et al.* Ventral Hippocampal Neurons Are Shaped by Experience to Represent Behaviorally Relevant Contexts. **33,** 8079–8087 (2013).

52. Kumazawa-Manita, N., Hama, H., Miyawaki, A. & Iriki, A. Tool use specific adult neurogenesis and synaptogenesis in rodent (Octodon degus) hippocampus. *PLoS One* **8,** e58649 (2013).

53. Ladd, C. O., Huot, R. L., Thrivikraman, K. V, Nemeroff, C. B. & Plotsky, P. M. Long-term adaptations in glucocorticoid receptor and mineralocorticoid receptor mRNA and negative feedback on the hypothalamo-pituitary-adrenal axis following neonatal maternal separation. *Biol. Psychiatry* **55,** 367–75 (2004).

54. Leasure, J. L. & Jones, M. Forced and voluntary exercise differentially affect brain and behavior. *Neuroscience* **156,** 456–65 (2008).

55. Lee, M.-H. *et al.* Impaired neurogenesis and neurite outgrowth in an HIV-gp120 transgenic model is reversed by exercise via BDNF production and Cdk5 regulation. *J. Neurovirol.* **19,** 418–31 (2013).

56. Lehmann, M. L., Brachman, R. a, Martinowich, K., Schloesser, R. J. & Herkenham, M. Glucocorticoids orchestrate divergent effects on mood through adult neurogenesis. *J. Neurosci.* **33,** 2961–72 (2013).

57. Levone, B. R., Cryan, J. F. & Leary, O. F. O. Neurobiology of Stress Role of adult hippocampal neurogenesis in stress resilience. *Neurobiol. Stress* **1,** 147–155 (2015).

58. Lippmann, M., Bress, A., Nemeroff, C. B., Plotsky, P. M. & Monteggia, L. M. Long-term behavioural and molecular alterations associated with maternal separation in rats. *Eur. J. Neurosci.* **25,** 3091–8 (2007).

59. Lupien, S. J., McEwen, B. S., Gunnar, M. R. & Heim, C. Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat. Rev. Neurosci.* **10,** 434–45 (2009).

60. Magnusson, J. P. *et al.* A latent neurogenic program in astrocytes regulated by Notch signaling in the mouse. *Science (80-.).* **346,** 237–241 (2014).

61. Maniam, J. & Morris, M. J. Voluntary exercise and palatable high-fat diet both improve behavioural profile and stress responses in male rats exposed to early life stress: role of hippocampus. *Psychoneuroendocrinology* **35,** 1553–64 (2010).

62. Marais, L., Stein, D. J. & Daniels, W. M. U. Exercise increases BDNF levels in the striatum and decreases depressive-like behavior in chronically stressed rats. *Metab. Brain Dis.* **24,** 587–97 (2009).

63. Marcus, M. & Taghi, Y. DEPRESSION. 6–8 (2012).

64. Mateus-Pinheiro, a *et al.* Sustained remission from depressive-like behavior depends on hippocampal neurogenesis. *Transl. Psychiatry* **3,** e210 (2013).

65. Mayberg, H. S. *et al.* Deep brain stimulation for treatment-resistant depression. *Neuron* **45,** 651–60 (2005).

66. Michael, A. & Kalueff, A. V. Developing better and more valid animal models of brain disorders. *Behav. Brain Res.* **276,** 28–31 (2015).

67. Ming, G. & Song, H. Adult neurogenesis in the mammalian central nervous system. *Annu. Rev. Neurosci.* **28,** 223–50 (2005).

68. Mirescu, C., Peters, J. D. & Gould, E. Early life experience alters response of adult neurogenesis to stress. *Nat. Neurosci.* **7,** 841–6 (2004).

69. Monje, M. L., Toda, H. & Palmer, T. D. Inflammatory blockade restores adult hippocampal neurogenesis. *Science* **302,** 1760–5 (2003).

70. Murray, C. J. L. *et al.* Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* **380,** 2197–223 (2012).

71. Nagashima, M., Barthel, L. K. & Raymond, P. a. A self-renewing division of zebrafish Müller glial cells generates neuronal progenitors that require N-cadherin to regenerate retinal neurons. *Development* **140,** 4510–21 (2013).

72. Nasca, C., Bigio, B., Zelli, D., Nicoletti, F. & McEwen, B. S. Mind the gap: glucocorticoids modulate hippocampal glutamate tone underlying individual differences in stress susceptibility. *Mol. Psychiatry* **20,** 755–63 (2015).

73. Neumeister, A. *et al.* Reduced hippocampal volume in unmedicated, remitted patients with major depression versus control subjects. *Biol. Psychiatry* **57,** 935–7 (2005).

74. Nicola, Z., Fabel, K. & Kempermann, G. Development of the adult neurogenic niche in the hippocampus of mice. **9,** 1–13 (2015).

75. Nishi, M., Horii-Hayashi, N., Sasagawa, T. & Matsunaga, W. Effects of early life stress on brain activity: implications from maternal separation model in rodents. *Gen. Comp. Endocrinol.* **181,** 306–9 (2013).

76. Noble, E. E. *et al.* Exercise reduces diet-induced cognitive decline and increases hippocampal brain-derived neurotrophic factor in CA3 neurons. *Neurobiol. Learn. Mem.* **114,** 40–50 (2014).

77. Ohira, K., Takeuchi, R., Shoji, H. & Miyakawa, T. Fluoxetine-induced cortical adult neurogenesis. *Neuropsychopharmacology* **38,** 909–20 (2013).

78. Page, M. E., Detke, M. J., Dalvi, a., Kirby, L. G. & Lucki, I. Serotonergic mediation of the effects of fluoxetine, but not desipramine, in the rat forced swimming test. *Psychopharmacology (Berl).* **147,** 162–167 (1999).

79. Ponti, G. *et al.* Cell cycle and lineage progression of neural progenitors in the ventricular-subventricular zones of adult mice. *Proc. Natl. Acad. Sci. U. S. A.* **110,** E1045–54 (2013).

80. Porsolt, R. D. & Willner, P. Historical perspective on CMS model. 363–364 (1997).

81. Praag, H. Van *et al.* Functional neurogenesis in the adult hippocampus. **415,** 1030–1034 (2002).

82. Pruessner, M. *et al.* Reduced hippocampal volume and hypothalamus-pituitary-adrenal axis function in first episode psychosis: evidence for sex differences. *NeuroImage. Clin.* **7,** 195–202 (2015).

83. Quesseveur, G. *et al.* BDNF overexpression in mouse hippocampal astrocytes promotes local neurogenesis and elicits anxiolytic-like activities. *Transl. Psychiatry* **3,** e253 (2013).

84. Rana, S., Pugh, P. C., Jackson, N., Clinton, S. M. & Kerman, I. A. Neuroscience Letters Inborn stress reactivity shapes adult behavioral consequences of early-life maternal separation stress. *Neurosci. Lett.* **584,** 146–150 (2015).

85. Rating, A. Scale for depression. 56–62 (1960).

86. Rhodes, J. S. *et al.* Exercise increases hippocampal neurogenesis to high levels but does not improve spatial learning in mice bred for increased voluntary wheel running. *Behav. Neurosci.* **117,** 1006–16 (2003).

87. Rizvi, S. J. *et al.* Depression and Employment Status in Primary and Tertiary Care Settings. **60,** 2015 (2015).

88. Romens, S. E., McDonald, J., Svaren, J. & Pollak, S. D. Associations between early life stress and gene methylation in children. *Child Dev.* **86,** 303–9 (2015).

89. Salic, A. & Mitchison, T. J. A chemical method for fast and sensitive detection of DNA synthesis in vivo. (2007).

90. Santarelli, L. *et al.* Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* **301,** 805–9 (2003).

91. Sauerzweig, S., Baldauf, K., Braun, H. & Reymann, K. G. Time-dependent segmentation of BrdU-signal leads to late detection problems in studies using BrdU as cell label or proliferation marker. *J. Neurosci. Methods* **177,** 149–59 (2009).

92. Savage, E., Pawluski, J. L., Csa, E., Steinbusch, H. W. M. & Hove, D. V. A. N. D. E. N. EFFECTS OF STRESS EARLY IN GESTATION ON HIPPOCAMPAL NEUROGENESIS AND GLUCOCORTICOID RECEPTOR DENSITY. **290,** 379–388 (2015).

93. Schloesser, R. J. *et al.* Antidepressant-like Effects of Electroconvulsive Seizures Require Adult Neurogenesis in a Neuroendocrine Model of Depression. *Brain Stimul.* **8,** 862–7 (2015).

94. Schmidt, M., Enthoven, L., Woezik, J. H. G. Van, Levine, S. & Oitzl, M. S. The Dynamics of the Hypothalamic-Pituitary-Adrenal Axis During Maternal Deprivation. **16,** 52–57 (2004).

95. Schuch, F. B. *et al.* The effects of exercise on oxidative stress (TBARS) and BDNF in severely depressed inpatients. *Eur. Arch. Psychiatry Clin. Neurosci.* **264,** 605–13 (2014).

96. Shea, A. K. *et al.* The effect of depression, anxiety and early life trauma on the cortisol awakening response during pregnancy: preliminary results. *Psychoneuroendocrinology* **32,** 1013–20 (2007).

97. Shigemoto-Mogami, Y., Hoshikawa, K., Goldman, J. E., Sekino, Y. & Sato, K. Microglia enhance neurogenesis and oligodendrogenesis in the early postnatal subventricular zone. *J. Neurosci.* **34,** 2231–43 (2014).

98. Shruster, A., Ben-Zur, T., Melamed, E. & Offen, D. Wnt signaling enhances neurogenesis and improves neurological function after focal ischemic injury. *PLoS One* **7,** e40843 (2012).

99. Shu, C. *et al.* Blunted Behavioral and Molecular Responses to Chronic Mild Stress in Adult Rats with Experience of Infancy Maternal Separation. 81–87 (2015). doi:10.1620/tjem.235.81.Correspondence

100. Song, H., Stevens, C. F. & Gage, F. H. Astroglia induce neurogenesis from adult neural stem cells. 39–44 (2002).

101. Spalding, K. L. *et al.* Dynamics of hippocampal neurogenesis in adult humans. *Cell* **153,** 1219–27 (2013).

102. Speisman, R. B., Kumar, A., Rani, A., Foster, T. C. & Ormerod, B. K. Daily exercise improves memory, stimulates hippocampal neurogenesis and modulates immune and neuroimmune cytokines in aging rats. *Brain. Behav. Immun.* **28,** 25–43 (2013).

103. Tang, X., Falls, D. L., Li, X., Lane, T. & Luskin, M. B. Antigen-retrieval procedure for bromodeoxyuridine immunolabeling with concurrent labeling of nuclear DNA and antigens damaged by HCl pretreatment. *J. Neurosci.* **27,** 5837–44 (2007).

104. Tomlinson, M., Grimsrud, A. T., Stein, D. J., Williams, D. R. & Myer, L. O RIGINAL A RTICLES The epidemiology of major depression in South Africa : Results from the South African Stress and Health study. **99,** 368–373 (2009).

105. Tremblay, M.-E. *et al.* The Role of Microglia in the Healthy Brain. *J. Neurosci.* **31,** 16064–16069 (2011).

106. Vadodaria, K. C., Brakebusch, C., Suter, U. & Jessberger, S. Stage-specific functions of the small Rho GTPases Cdc42 and Rac1 for adult hippocampal neurogenesis. *J. Neurosci.* **33,** 1179–89 (2013).

107. Vollmayr, B., Mahlstedt, M. M. & Henn, F. a. Neurogenesis and depression: what animal models tell us about the link. *Eur. Arch. Psychiatry Clin. Neurosci.* **257,** 300–3 (2007).

108. Willner, P. Psychopharmacology The validity of animal models of depression. 1–16 (1984).

109. Wojtowicz, J. M. & Kee, N. BrdU assay for neurogenesis in rodents. *Nat. Protoc.* **1,** 1399–405 (2006).

110. Worlitzer, M. M. a, Viel, T., Jacobs, A. H. & Schwamborn, J. C. The majority of newly generated cells in the adult mouse substantia nigra express low levels of Doublecortin, but their proliferation is unaffected by 6-OHDA-induced nigral lesion or Minocycline-mediated inhibition of neuroinflammation. *Eur. J. Neurosci.* **38,** 2684–92 (2013).

111. Yang, Z., Ming, G.-L. & Song, H. Genetically targeting new neurons in the adult hippocampus. *Cell Res.* **21,** 220–2 (2011).

112. Yau, S. & So, K. Review Adult Neurogenesis and Dendritic Remodeling in Hippocampal Plasticity : Which One Is More Important ? **23,** 471–479 (2014).

113. Zeng, C. *et al.* nervous system. 21–32 (2011). doi:10.1016/j.brainres.2009.12.092.Evaluation

114. Zhang, J., Qin, L. & Zhao, H. Early repeated maternal separation induces alterations of hippocampus reelin expression in rats. *J. Biosci.* **38,** 27–33 (2012).

115. van heerden et al., 2010.pdf.

116. Cellular and Molecular Mechanisms Underlying the Treatment of Depression: Focusing on Hippocampal G-Protein-Coupled Receptors and Voltage-Dependent Calcium Channels. 1–44

Appendix

Appendix

Figure 13 a

GFAP 60	NeuN 60	DCX 60	Unknown 60
18.	12.	5.	10.
21.	26.	7.	18.
32.	9.	29.	5.
20.	11.	21.	1.
12.	0.	1.	8.
15.	15.	19.	2.
2.	0.	0.	3.
22.	13.	0.	1.
1.	5.	0.	14.
5.	0.	0.	0.
0.	0.	0.	5.
0.	0.	0.	0.
0.	0.	0.	0.
0.	0.	0.	0.
	0.	0.	0.
			0.

Number of values	60
Shapiro-Wilk normality test	
W	0.7684
P value	< 0.0001
Passed normality test (alpha=0.05)?	No
P value summary	****
Sum	388.0

Table Analyzed	Day 60 injection exercised (NP)
Kruskal-Wallis test	
P value	0.3020
Exact or approximate P value?	Approximate
P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	4
Kruskal-Wallis statistic	3.648
Data summary	
Number of treatments (columns)	4
Number of values (total)	60

	GFAP 60	NeuN 60	DCX 60	Unknown 60
Number of values	14	15	15	16
Minimum	0.0	0.0	0.0	0.0
25% Percentile	0.0	0.0	0.0	0.0
Median	8.500	0.0	0.0	1.500
75% Percentile	20.25	12.00	7.000	7.250
Maximum	32.00	26.00	29.00	18.00
Mean	10.57	6.067	5.467	4.188
Std. Deviation	10.77	7.968	9.523	5.588
Std. Error of Mean	2.878	2.057	2.459	1.397
Lower 95% CI of mean	4.354	1.654	0.1927	1.210
Upper 95% CI of mean	16.79	10.48	10.74	7.165

Figure 13 b

GFAP 96	NeuN 96	DCX 96	Unknown 96
0.	0.	0.	0.
1.	0.	0.	0.
0.	0.	2.	0.
22.	0.	5.	9.
0.	0.	8.	0.
40.	13.	0.	20.
2.	0.	0.	1.
0.	0.	12.	1.
45.	5.	0.	14.
0.	0.	0.	0.
0.	0.	0.	1.
0.	0.	0.	0.
0.	0.	0.	4.
0.	0.	0.	0.
0.	0.	0.	5.
0.	0.	0.	0.
0.	0.	0.	0.
0.	0.		0.
	0.		0.
	0.		0.
			0.
			0.
			0.
			0.
			0.
			0.

Shapiro-Wilk normality test	
W	0.3898
P value	< 0.0001
Passed normality test (alpha=0.05)?	No
P value summary	****
Sum	210.0

Table Analyzed	Day 96 injection exercised(NP)		
Kruskal-Wallis test			
P value	0.4413		
Exact or approximate P value?	Gaussian Approximation		
P value summary	ns		
Do the medians vary signif. (P < 0.05)	No		
Number of groups	4		
Kruskal-Wallis statistic	2.693		
Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
GFAP 96 vs NeuN 96	7.717	No	ns
GFAP 96 vs DCX 96	2.475	No	ns
GFAP 96 vs Unknown 96	-0.02564	No	ns
NeuN 96 vs DCX 96	-5.241	No	ns
NeuN 96 vs Unknown 96	-7.742	No	ns
DCX 96 vs Unknown 96	-2.501	No	ns

	GFAP 96	NeuN 96	DCX 96	Unknown 96	
Number of values	18	20	17	26	81
Minimum	0.0	0.0	0.0	0.0	0.0
25% Percentile	0.0	0.0	0.0	0.0	0.0
Median	0.0	0.0	0.0	0.0	0.0
75% Percentile	1.250	0.0	1.000	1.000	0.0
Maximum	45.00	13.00	12.00	20.00	45.00
Mean	6.111	0.9000	1.588	2.115	2.593
Std. Deviation	14.23	3.059	3.483	4.918	7.682
Std. Error of Mean	3.354	0.6840	0.8448	0.9645	0.8536
Lower 95% CI of mean	-0.9647	-0.5317	-0.2026	0.1290	0.8939
Upper 95% CI of mean	13.19	2.332	3.379	4.102	4.291

Figure 14

PND 60	PND 96

6.666667	7.083333
14.333330	0.000000
5.666667	15.000000
17.666670	0.000000
5.333333	0.000000
9.000000	4.333333
8.666667	0.000000
7.333333	0.000000
5.333333	1.666667
0.000000	0.000000
8.000000	0.000000
2.333333	0.000000
6.666667	7.083333
14.333330	0.000000
5.666667	15.000000
17.666670	0.000000
5.333333	0.000000
9.000000	4.333333
8.666667	0.000000
7.333333	0.000000
5.333333	1.666667
0.000000	0.000000
8.000000	0.000000
2.333333	0.000000
9.666667	4.666667
7.000000	1.666667
0.3333333	2.666667
5.000000	0.250000
10.666670	1.200000
1.500000	5.750000
1.333333	1.000000
3.333333	5.250000

Shapiro-Wilk normality test	
W	0.8649
P value	0.0002
Passed normality test (alpha=0.05)?	No
P value summary	***

Table Analyzed	EdU average comparison PND60 VS 96
Column A	PND 60
vs.	vs.
Column B	PND 96
Mann Whitney test	
P value	< 0.0001
Exact or approximate P value?	Exact
P value summary	****
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	1362 , 718.0
Mann-Whitney U	190.0
Difference between medians	
Median of column A	6.667
Median of column B	0.1250
Difference: Actual	6.542
Difference: Hodges-Lehmann	5.208

Figure 15a

GFAP 60	NeuN 60	DCX 60	Unknown 60
6.666667	9.000000	8.000000	5.000000
14.333330	8.666667	2.333333	10.666670
5.666667	7.333333	9.666667	1.500000
17.666670	5.333333	7.000000	1.333333
5.333333	0.000000	0.3333333	3.333333

Shapiro-Wilk normality test

W	0.9510
P value	0.3827
Passed normality test (alpha=0.05)?	Yes
P value summary	Ns

Anova

Table Analyzed	Copy of Day 60 Injection Average redone Oct (NP)	
ANOVA summary		
F	1.104	
P value	0.3703	
P value summary	ns	
Are differences among means statistically significant? (P < 0.05)	No	
R square	0.1120	
Brown-Forsythe test		
F (DFn, DFd)	0.2789 (4, 35)	
P value	0.8897	
P value summary	ns	
Significantly different standard deviations? (P < 0.05)	No	
Bartlett's test		
Bartlett's statistic (corrected)	1.017	
P value	0.9071	
P value summary	ns	
Significantly different standard deviations? (P < 0.05)	No	
ANOVA table	SS	DF
Treatment (between columns)	87.94	4
Residual (within columns)	697.3	35
Total	785.2	39
Data summary		
Number of treatments (columns)	5	
Number of values (total)	40	

Table Analyzed	Copy of Day 60 Injection Average redone Oct (NP)
Kruskal-Wallis test	
P value	0.6032
Exact or approximate P value?	Approximate
P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	5
Kruskal-Wallis statistic	2.734
Data summary	
Number of treatments (columns)	5
Number of values (total)	40

ANOVA summary		
F	1.539	
P value	0.2428	
P value summary	ns	
Are differences among means statistically significant? (P < 0.05)	No	
R square	0.2240	
Brown-Forsythe test		
F (DFn, DFd)	0.2714 (3, 16)	
P value	0.8451	
P value summary	ns	
Significantly different standard deviations? (P < 0.05)	No	
Bartlett's test		
Bartlett's statistic (corrected)	0.9739	
P value	0.8076	
P value summary	ns	
Significantly different standard deviations? (P < 0.05)	No	
ANOVA table	SS	DF
Treatment (between columns)	87.94	3
Residual (within columns)	304.7	16
Total	392.6	19
Data summary		
Number of treatments (columns)	4	
Number of values (total)	20	

DF	MS	F (DFn, DFd)	P value
3	29.31	F (3, 16) = 1.539	P = 0.2428
16	19.04		
19			

Figure 15b

GFAP 96	NeuN 96	DCX 96	Unknown 96
7.083333	4.333333	0.000000	0.25
0.000000	0.000000	0.000000	1.20
15.000000	0.000000	4.666667	5.75
0.000000	1.666667	1.666667	1.00
0.000000	0.000000	2.666667	5.25

Shapiro-Wilk normality test	
W	0.7177
P value	< 0.0001
Passed normality test (alpha=0.05)?	No
P value summary	****
Sum	50.53

Table Analyzed	Copy of Copy of Day 96 Injection Average redone Oct (NP)
Kruskal-Wallis test	
P value	0.7700
Exact or approximate P value?	Approximate
P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	5
Kruskal-Wallis statistic	1.814
Data summary	
Number of treatments (columns)	5
Number of values (total)	40

Figure 16a

NeuN +DCX 60	NeuN+ DCX 96
12.	0.
15.	13.
0.	0.
26.	0.
0.	0.
0.	0.
9.	0.
13.	0.
0.	0.
11.	0.
5.	5.
0.	0.
0.	0.
0.	0.
5.	0.
19.	0.
0.	0.
7.	0.
0.	0.
0.	0.
29.	0.
0.	0.
0.	2.
21.	12.
0.	0.
0.	5.
1.	0.
0.	0.
0.	8.
	0.

0.

Shapiro-Wilk normality test	
W	0.5442
P value	< 0.0001
Passed normality test (alpha=0.05)?	No
P value summary	****

Table Analyzed	Comparison Totals 2 injections(NP)
Column A	NeuN +DCX 60
Vs	vs
Column B	NeuN+ DCX 96
Mann Whitney test	
P value	0.0198
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	1015 , 815
Mann-Whitney U	319.0

Figure 16b Averages

NeuN +DCX 60	NeuN+ DCX 96
9.000000	4.333333
8.666667	0.000000
7.333333	0.000000
5.333333	1.666667
0.000000	2.888889
8.000000	0.000000
2.333333	0.000000
9.666667	4.666667
7.000000	1.666667
0.3333333	2.666667

Shapiro-Wilk normality test	
W	0.8847
P value	0.0215
Passed normality test (alpha=0.05)?	No
P value summary	*

Table Analyzed	Comparison Totals 2 injections(NP) averages
Column A	NeuN +DCX 60
vs	vs
Column B	NeuN+ DCX 96
Mann Whitney test	
P value	0.0164
Exact or approximate P value?	Gaussian Approximation
P value summary	*
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	137 , 73
Mann-Whitney U	18.00

Figure 17a

GFAP 74	NeuN 74	DCX 74	Unknown 74
0.	0.		0.
0.	0.	0.	0.
0.	1.	0.	1.
0.	0.	0.	0.
1.	0.	0.	0.
0.	0.	0.	0.
6.	1.	0.	4.
2.	0.	0.	0.
0.	1.	0.	0.

2.	0.	0.	0.
0.	0.	0.	0.
0.	0.	0.	0.
0.	0.	0.	0.
0.	0.	0.	0.
0.	0.	1.	0.
0.	0.	0.	0.
0.	0.	0.	0.
0.	0.	0.	0.
0.	0.	1.	0.
0.	0.	0.	0.
0.	0.		
0.			

Shapiro-Wilk normality test	
W	0.3307
P value	< 0.0001
Passed normality test (alpha=0.05)?	No
P value summary	****
Sum	21.00

Table Analyzed	Day 74 injections non-exercised(NP)
Kruskal-Wallis test	
P value	0.9161
Exact or approximate P value?	Approximate
P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	5
Kruskal-Wallis statistic	0.9577
Data summary	
Number of treatments (columns)	5
Number of values (total)	164

Figure 17 b

GFAP 74	NeuN 74	DCX 74	Unknown 74
0.	0.		0.
0.	0.	0.	1.
0.	0.	1.	0.
4.	0.	0.	0.
0.	0.	0.	0.
0.	0.	0.	0.
0.	0.	1.	0.
0.	0.	0.	0.
1.	0.	0.	0.
3.	1.		0.
0.	0.		0.
0.	0.		0.
0.	0.		
3.			
1.			
0.			
0.			

Shapiro-Wilk normality test	
W	0.4366
P value	< 0.0001
Passed normality test (alpha=0.05)?	No
P value summary	****
Sum	16.00

Kruskal-Wallis test	
P value	0.4273
Exact or approximate P value?	Approximate

P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	5
Kruskal-Wallis statistic	3.846
Data summary	
Number of treatments (columns)	5
Number of values (total)	100

Figure 18a

GFAP 74	NeuN 74	DCX 74	Unknown 74
0.00	0.0		0.0
0.00	0.0	0.00	0.0
0.00	1.0	0.00	1.0
0.00	0.0	0.00	0.0
1.00	0.0	0.00	0.0
0.00	0.0	0.00	0.0
2.00	0.4	0.20	0.8
0.00	0.0	0.00	0.0
0.00	0.0	0.25	0.0

Shapiro-Wilk normality test	
W	0.5037
P value	< 0.0001
Passed normality test (alpha=0.05)?	No
P value summary	****
Sum	6.650

Kruskal-Wallis test	
P value	0.9996
Exact or approximate P value?	Approximate
P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	5
Kruskal-Wallis statistic	0.05388
Data summary	
Number of treatments (columns)	5
Number of values (total)	70

Figure 18b

GFAP 74	NeuN 74	DCX 74	Unknown 74
0.0	0.00		0.00
1.4	0.00	0.25	0.25
0.4	0.00	0.50	0.00
0.5	0.25	0.00	0.00

Shapiro-Wilk normality test	
W	0.6755
P value	0.0001
Passed normality test (alpha=0.05)?	No
P value summary	***

Kruskal-Wallis test	
P value	0.2719
Exact or approximate P value?	Approximate
P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	5
Kruskal-Wallis statistic	5.154
Data summary	
Number of treatments (columns)	5
Number of values (total)	30

Figure 20
weights

MS	NMS
310.	315.

300.	183.
325.	283.
350.	291.
352.	256.
320.	315.
317.	183.
340.	283.
340.	291.
	256.

Shapiro-Wilk normality test	
W	0.8630
P value	0.0110
Passed normality test (alpha=0.05)?	No
P value summary	*

Table Analyzed	Copy of Weights of treatment groups
Column A	MS
vs	vs
Column B	NMS
Mann Whitney test	
P value	0.0009
Exact or approximate P value?	Gaussian Approximation
P value summary	***
Are medians signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	131 , 59
Mann-Whitney U	4.000

Figure 20 Redone weights by group

MS+R	MS+NR	NMS+R	NMS+NR
310.	352.	315.	315.
300.	320.	183.	183.
325.	317.	283.	283.
350.	340.	291.	291.
	340.	256.	256.

Shapiro-Wilk normality test	
W	0.8630
P value	0.0110
Passed normality test (alpha=0.05)?	No
P value summary	*

Table Analyzed	Weights of treatment groups
Kruskal-Wallis test	
P value	0.0046
Exact or approximate P value?	Approximate
P value summary	**
Do the medians vary signif. (P < 0.05)	Yes
Number of groups	4
Kruskal-Wallis statistic	13.01
Data summary	
Number of treatments (columns)	4
Number of values (total)	19

Number of families	1		
Number of comparisons per family	6		
Alpha	0.05		
Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
NMS+NR vs. MS+NR	-9.200	No	ns
NMS+NR vs. NMS+R	1.800	No	ns
NMS+NR vs. MS+R	-6.900	No	ns
MS+NR vs. NMS+R	11.00	Yes	*
MS+NR vs. MS+R	2.300	No	ns

NMS+R vs. MS+R	-8.700	No	ns		
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1	n2
NMS+NR vs. MS+NR	6.600	15.80	-9.200	5	5
NMS+NR vs. NMS+R	6.600	4.800	1.800	5	5
NMS+NR vs. MS+R	6.600	13.50	-6.900	5	4
MS+NR vs. NMS+R	15.80	4.800	11.00	5	5
MS+NR vs. MS+R	15.80	13.50	2.300	5	4
NMS+R vs. MS+R	4.800	13.50	-8.700	5	4

Figure 21 Running

DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	DAY10	DAY11	DAY 12
265.	401.	532.	458.	613.	549.	976.	1275.	1307.	1681.	1467.	1921.
846.	809.	770.	837.	1027.	1410.	1523.	1511.	1698.	1851.	2204.	2282.

DAY 13	DAY 14	DAY 15	DAY 16	DAY 17	DAY 18	DAY 19	DAY 20
2388.	1957.	1642.	1694.	1956.	2461.	2198.	2341.
2189.	2084.	1669.	1917.	1788.	2182.	1974.	1862.

Shapiro-Wilk normality test	
W	0.9354
P value	0.0242
Passed normality test (alpha=0.05)?	No
P value summary	*

Table Analyzed	EACH DAY DIFFRENT
Friedman test	
P value	0.0134
Exact or approximate P value?	Approximate
P value summary	*
Are means signif. different? (P < 0.05)	Yes
Number of groups	20
Friedman statistic	35.14
Data summary	
Number of treatments (columns)	20
Number of subjects (rows)	2

Number of families	1		
Number of comparisons per family	190		
Alpha	0.05		
Dunn's multiple comparisons test	Rank sum diff.	Significant?	Summary
DAY 1 vs. DAY 2	1.000	No	ns
DAY 1 vs. DAY 3	0.0	No	ns
DAY 1 vs. DAY 4	-1.000	No	ns
DAY 1 vs. DAY 5	-6.000	No	ns
DAY 1 vs. DAY 6	-6.000	No	ns
DAY 1 vs. DAY 7	-10.00	No	ns
DAY 1 vs. DAY 8	-10.00	No	ns
DAY 1 vs. DAY 9	-14.00	No	ns
DAY 1 vs. DAY10	-19.00	No	ns
DAY 1 vs. DAY11	-24.00	No	ns
DAY 1 vs. DAY 12	-29.00	No	ns
DAY 1 vs. DAY 13	-32.00	No	ns
DAY 1 vs. DAY 14	-27.00	No	ns
DAY 1 vs. DAY 15	-15.00	No	ns
DAY 1 vs. DAY 16	-22.00	No	ns
DAY 1 vs. DAY 17	-21.00	No	ns
DAY 1 vs. DAY 18	-32.00	No	ns
DAY 1 vs. DAY 19	-27.00	No	ns
DAY 1 vs. DAY 20	-26.00	No	ns
DAY 2 vs. DAY 3	-1.000	No	ns

DAY 2 vs. DAY 4	-2.000	No	ns
DAY 2 vs. DAY 5	-7.000	No	ns
DAY 2 vs. DAY 6	-7.000	No	ns
DAY 2 vs. DAY 7	-11.00	No	ns
DAY 2 vs. DAY 8	-11.00	No	ns
DAY 2 vs. DAY 9	-15.00	No	ns
DAY 2 vs. DAY10	-20.00	No	ns
DAY 2 vs. DAY11	-25.00	No	ns
DAY 2 vs. DAY 12	-30.00	No	ns
DAY 2 vs. DAY 13	-33.00	No	ns
DAY 2 vs. DAY 14	-28.00	No	ns
DAY 2 vs. DAY 15	-16.00	No	ns
DAY 2 vs. DAY 16	-23.00	No	ns
DAY 2 vs. DAY 17	-22.00	No	ns
DAY 2 vs. DAY 18	-33.00	No	ns
DAY 2 vs. DAY 19	-28.00	No	ns
DAY 2 vs. DAY 20	-27.00	No	ns
DAY 3 vs. DAY 4	-1.000	No	ns
DAY 3 vs. DAY 5	-6.000	No	ns
DAY 3 vs. DAY 6	-6.000	No	ns
DAY 3 vs. DAY 7	-10.00	No	ns
DAY 3 vs. DAY 8	-10.00	No	ns
DAY 3 vs. DAY 9	-14.00	No	ns
DAY 3 vs. DAY10	-19.00	No	ns
DAY 3 vs. DAY11	-24.00	No	ns
DAY 3 vs. DAY 12	-29.00	No	ns
DAY 3 vs. DAY 13	-32.00	No	ns
DAY 3 vs. DAY 14	-27.00	No	ns
DAY 3 vs. DAY 15	-15.00	No	ns
DAY 3 vs. DAY 16	-22.00	No	ns
DAY 3 vs. DAY 17	-21.00	No	ns
DAY 3 vs. DAY 18	-32.00	No	ns
DAY 3 vs. DAY 19	-27.00	No	ns
DAY 3 vs. DAY 20	-26.00	No	ns
DAY 4 vs. DAY 5	-5.000	No	ns
DAY 4 vs. DAY 6	-5.000	No	ns
DAY 4 vs. DAY 7	-9.000	No	ns
DAY 4 vs. DAY 8	-9.000	No	ns
DAY 4 vs. DAY 9	-13.00	No	ns
DAY 4 vs. DAY10	-18.00	No	ns
DAY 4 vs. DAY11	-23.00	No	ns
DAY 4 vs. DAY 12	-28.00	No	ns
DAY 4 vs. DAY 13	-31.00	No	ns
DAY 4 vs. DAY 14	-26.00	No	ns
DAY 4 vs. DAY 15	-14.00	No	ns
DAY 4 vs. DAY 16	-21.00	No	ns
DAY 4 vs. DAY 17	-20.00	No	ns
DAY 4 vs. DAY 18	-31.00	No	ns
DAY 4 vs. DAY 19	-26.00	No	ns
DAY 4 vs. DAY 20	-25.00	No	ns
DAY 5 vs. DAY 6	0.0	No	ns
DAY 5 vs. DAY 7	-4.000	No	ns
DAY 5 vs. DAY 8	-4.000	No	ns
DAY 5 vs. DAY 9	-8.000	No	ns
DAY 5 vs. DAY10	-13.00	No	ns
DAY 5 vs. DAY11	-18.00	No	ns
DAY 5 vs. DAY 12	-23.00	No	ns
DAY 5 vs. DAY 13	-26.00	No	ns
DAY 5 vs. DAY 14	-21.00	No	ns
DAY 5 vs. DAY 15	-9.000	No	ns
DAY 5 vs. DAY 16	-16.00	No	ns
DAY 5 vs. DAY 17	-15.00	No	ns
DAY 5 vs. DAY 18	-26.00	No	ns
DAY 5 vs. DAY 19	-21.00	No	ns
DAY 5 vs. DAY 20	-20.00	No	ns
DAY 6 vs. DAY 7	-4.000	No	ns
DAY 6 vs. DAY 8	-4.000	No	ns
DAY 6 vs. DAY 9	-8.000	No	ns
DAY 6 vs. DAY10	-13.00	No	ns
DAY 6 vs. DAY11	-18.00	No	ns

DAY 6 vs. DAY 12	-23.00	No	ns
DAY 6 vs. DAY 13	-26.00	No	ns
DAY 6 vs. DAY 14	-21.00	No	ns
DAY 6 vs. DAY 15	-9.000	No	ns
DAY 6 vs. DAY 16	-16.00	No	ns
DAY 6 vs. DAY 17	-15.00	No	ns
DAY 6 vs. DAY 18	-26.00	No	ns
DAY 6 vs. DAY 19	-21.00	No	ns
DAY 6 vs. DAY 20	-20.00	No	ns
DAY 7 vs. DAY 8	0.0	No	ns
DAY 7 vs. DAY 9	-4.000	No	ns
DAY 7 vs. DAY10	-9.000	No	ns
DAY 7 vs. DAY11	-14.00	No	ns
DAY 7 vs. DAY 12	-19.00	No	ns
DAY 7 vs. DAY 13	-22.00	No	ns
DAY 7 vs. DAY 14	-17.00	No	ns
DAY 7 vs. DAY 15	-5.000	No	ns
DAY 7 vs. DAY 16	-12.00	No	ns
DAY 7 vs. DAY 17	-11.00	No	ns
DAY 7 vs. DAY 18	-22.00	No	ns
DAY 7 vs. DAY 19	-17.00	No	ns
DAY 7 vs. DAY 20	-16.00	No	ns
DAY 8 vs. DAY 9	-4.000	No	ns
DAY 8 vs. DAY10	-9.000	No	ns
DAY 8 vs. DAY11	-14.00	No	ns
DAY 8 vs. DAY 12	-19.00	No	ns
DAY 8 vs. DAY 13	-22.00	No	ns
DAY 8 vs. DAY 14	-17.00	No	ns
DAY 8 vs. DAY 15	-5.000	No	ns
DAY 8 vs. DAY 16	-12.00	No	ns
DAY 8 vs. DAY 17	-11.00	No	ns
DAY 8 vs. DAY 18	-22.00	No	ns
DAY 8 vs. DAY 19	-17.00	No	ns
DAY 8 vs. DAY 20	-16.00	No	ns
DAY 9 vs. DAY10	-5.000	No	ns
DAY 9 vs. DAY11	-10.00	No	ns
DAY 9 vs. DAY 12	-15.00	No	ns
DAY 9 vs. DAY 13	-18.00	No	ns
DAY 9 vs. DAY 14	-13.00	No	ns
DAY 9 vs. DAY 15	-1.000	No	ns
DAY 9 vs. DAY 16	-8.000	No	ns
DAY 9 vs. DAY 17	-7.000	No	ns
DAY 9 vs. DAY 18	-18.00	No	ns
DAY 9 vs. DAY 19	-13.00	No	ns
DAY 9 vs. DAY 20	-12.00	No	ns
DAY10 vs. DAY11	-5.000	No	ns
DAY10 vs. DAY 12	-10.00	No	ns
DAY10 vs. DAY 13	-13.00	No	ns
DAY10 vs. DAY 14	-8.000	No	ns
DAY10 vs. DAY 15	4.000	No	ns
DAY10 vs. DAY 16	-3.000	No	ns
DAY10 vs. DAY 17	-2.000	No	ns
DAY10 vs. DAY 18	-13.00	No	ns
DAY10 vs. DAY 19	-8.000	No	ns
DAY10 vs. DAY 20	-7.000	No	ns
DAY11 vs. DAY 12	-5.000	No	ns
DAY11 vs. DAY 13	-8.000	No	ns
DAY11 vs. DAY 14	-3.000	No	ns
DAY11 vs. DAY 15	9.000	No	ns
DAY11 vs. DAY 16	2.000	No	ns
DAY11 vs. DAY 17	3.000	No	ns
DAY11 vs. DAY 18	-8.000	No	ns
DAY11 vs. DAY 19	-3.000	No	ns
DAY11 vs. DAY 20	-2.000	No	ns
DAY 12 vs. DAY 13	-3.000	No	ns
DAY 12 vs. DAY 14	2.000	No	ns
DAY 12 vs. DAY 15	14.00	No	ns
DAY 12 vs. DAY 16	7.000	No	ns
DAY 12 vs. DAY 17	8.000	No	ns
DAY 12 vs. DAY 18	-3.000	No	ns

DAY 12 vs. DAY 19	2.000	No	ns		
DAY 12 vs. DAY 20	3.000	No	ns		
DAY 13 vs. DAY 14	5.000	No	ns		
DAY 13 vs. DAY 15	17.00	No	ns		
DAY 13 vs. DAY 16	10.00	No	ns		
DAY 13 vs. DAY 17	11.00	No	ns		
DAY 13 vs. DAY 18	0.0	No	ns		
DAY 13 vs. DAY 19	5.000	No	ns		
DAY 13 vs. DAY 20	6.000	No	ns		
DAY 14 vs. DAY 15	12.00	No	ns		
DAY 14 vs. DAY 16	5.000	No	ns		
DAY 14 vs. DAY 17	6.000	No	ns		
DAY 14 vs. DAY 18	-5.000	No	ns		
DAY 14 vs. DAY 19	0.0	No	ns		
DAY 14 vs. DAY 20	1.000	No	ns		
DAY 15 vs. DAY 16	-7.000	No	ns		
DAY 15 vs. DAY 17	-6.000	No	ns		
DAY 15 vs. DAY 18	-17.00	No	ns		
DAY 15 vs. DAY 19	-12.00	No	ns		
DAY 15 vs. DAY 20	-11.00	No	ns		
DAY 16 vs. DAY 17	1.000	No	ns		
DAY 16 vs. DAY 18	-10.00	No	ns		
DAY 16 vs. DAY 19	-5.000	No	ns		
DAY 16 vs. DAY 20	-4.000	No	ns		
DAY 17 vs. DAY 18	-11.00	No	ns		
DAY 17 vs. DAY 19	-6.000	No	ns		
DAY 17 vs. DAY 20	-5.000	No	ns		
DAY 18 vs. DAY 19	5.000	No	ns		
DAY 18 vs. DAY 20	6.000	No	ns		
DAY 19 vs. DAY 20	1.000	No	ns		
Test details	Rank sum 1	Rank sum 2	Rank sum diff.	n1	n2
DAY 1 vs. DAY 2	5.000	4.000	1.000	2	2
DAY 1 vs. DAY 3	5.000	5.000	0.0	2	2
DAY 1 vs. DAY 4	5.000	6.000	-1.000	2	2
DAY 1 vs. DAY 5	5.000	11.00	-6.000	2	2
DAY 1 vs. DAY 6	5.000	11.00	-6.000	2	2
DAY 1 vs. DAY 7	5.000	15.00	-10.00	2	2
DAY 1 vs. DAY 8	5.000	15.00	-10.00	2	2
DAY 1 vs. DAY 9	5.000	19.00	-14.00	2	2
DAY 1 vs. DAY10	5.000	24.00	-19.00	2	2
DAY 1 vs. DAY11	5.000	29.00	-24.00	2	2
DAY 1 vs. DAY 12	5.000	34.00	-29.00	2	2
DAY 1 vs. DAY 13	5.000	37.00	-32.00	2	2
DAY 1 vs. DAY 14	5.000	32.00	-27.00	2	2
DAY 1 vs. DAY 15	5.000	20.00	-15.00	2	2
DAY 1 vs. DAY 16	5.000	27.00	-22.00	2	2
DAY 1 vs. DAY 17	5.000	26.00	-21.00	2	2
DAY 1 vs. DAY 18	5.000	37.00	-32.00	2	2
DAY 1 vs. DAY 19	5.000	32.00	-27.00	2	2
DAY 1 vs. DAY 20	5.000	31.00	-26.00	2	2
DAY 2 vs. DAY 3	4.000	5.000	-1.000	2	2
DAY 2 vs. DAY 4	4.000	6.000	-2.000	2	2
DAY 2 vs. DAY 5	4.000	11.00	-7.000	2	2
DAY 2 vs. DAY 6	4.000	11.00	-7.000	2	2
DAY 2 vs. DAY 7	4.000	15.00	-11.00	2	2
DAY 2 vs. DAY 8	4.000	15.00	-11.00	2	2
DAY 2 vs. DAY 9	4.000	19.00	-15.00	2	2
DAY 2 vs. DAY10	4.000	24.00	-20.00	2	2
DAY 2 vs. DAY11	4.000	29.00	-25.00	2	2
DAY 2 vs. DAY 12	4.000	34.00	-30.00	2	2
DAY 2 vs. DAY 13	4.000	37.00	-33.00	2	2
DAY 2 vs. DAY 14	4.000	32.00	-28.00	2	2
DAY 2 vs. DAY 15	4.000	20.00	-16.00	2	2
DAY 2 vs. DAY 16	4.000	27.00	-23.00	2	2
DAY 2 vs. DAY 17	4.000	26.00	-22.00	2	2
DAY 2 vs. DAY 18	4.000	37.00	-33.00	2	2
DAY 2 vs. DAY 19	4.000	32.00	-28.00	2	2
DAY 2 vs. DAY 20	4.000	31.00	-27.00	2	2
DAY 3 vs. DAY 4	5.000	6.000	-1.000	2	2

DAY 3 vs. DAY 5	5.000	11.00	-6.000	2	2
DAY 3 vs. DAY 6	5.000	11.00	-6.000	2	2
DAY 3 vs. DAY 7	5.000	15.00	-10.00	2	2
DAY 3 vs. DAY 8	5.000	15.00	-10.00	2	2
DAY 3 vs. DAY 9	5.000	19.00	-14.00	2	2
DAY 3 vs. DAY10	5.000	24.00	-19.00	2	2
DAY 3 vs. DAY11	5.000	29.00	-24.00	2	2
DAY 3 vs. DAY 12	5.000	34.00	-29.00	2	2
DAY 3 vs. DAY 13	5.000	37.00	-32.00	2	2
DAY 3 vs. DAY 14	5.000	32.00	-27.00	2	2
DAY 3 vs. DAY 15	5.000	20.00	-15.00	2	2
DAY 3 vs. DAY 16	5.000	27.00	-22.00	2	2
DAY 3 vs. DAY 17	5.000	26.00	-21.00	2	2
DAY 3 vs. DAY 18	5.000	37.00	-32.00	2	2
DAY 3 vs. DAY 19	5.000	32.00	-27.00	2	2
DAY 3 vs. DAY 20	5.000	31.00	-26.00	2	2
DAY 4 vs. DAY 5	6.000	11.00	-5.000	2	2
DAY 4 vs. DAY 6	6.000	11.00	-5.000	2	2
DAY 4 vs. DAY 7	6.000	15.00	-9.000	2	2
DAY 4 vs. DAY 8	6.000	15.00	-9.000	2	2
DAY 4 vs. DAY 9	6.000	19.00	-13.00	2	2
DAY 4 vs. DAY10	6.000	24.00	-18.00	2	2
DAY 4 vs. DAY11	6.000	29.00	-23.00	2	2
DAY 4 vs. DAY 12	6.000	34.00	-28.00	2	2
DAY 4 vs. DAY 13	6.000	37.00	-31.00	2	2
DAY 4 vs. DAY 14	6.000	32.00	-26.00	2	2
DAY 4 vs. DAY 15	6.000	20.00	-14.00	2	2
DAY 4 vs. DAY 16	6.000	27.00	-21.00	2	2
DAY 4 vs. DAY 17	6.000	26.00	-20.00	2	2
DAY 4 vs. DAY 18	6.000	37.00	-31.00	2	2
DAY 4 vs. DAY 19	6.000	32.00	-26.00	2	2
DAY 4 vs. DAY 20	6.000	31.00	-25.00	2	2
DAY 5 vs. DAY 6	11.00	11.00	0.0	2	2
DAY 5 vs. DAY 7	11.00	15.00	-4.000	2	2
DAY 5 vs. DAY 8	11.00	15.00	-4.000	2	2
DAY 5 vs. DAY 9	11.00	19.00	-8.000	2	2
DAY 5 vs. DAY10	11.00	24.00	-13.00	2	2
DAY 5 vs. DAY11	11.00	29.00	-18.00	2	2
DAY 5 vs. DAY 12	11.00	34.00	-23.00	2	2
DAY 5 vs. DAY 13	11.00	37.00	-26.00	2	2
DAY 5 vs. DAY 14	11.00	32.00	-21.00	2	2
DAY 5 vs. DAY 15	11.00	20.00	-9.000	2	2
DAY 5 vs. DAY 16	11.00	27.00	-16.00	2	2
DAY 5 vs. DAY 17	11.00	26.00	-15.00	2	2
DAY 5 vs. DAY 18	11.00	37.00	-26.00	2	2
DAY 5 vs. DAY 19	11.00	32.00	-21.00	2	2
DAY 5 vs. DAY 20	11.00	31.00	-20.00	2	2
DAY 6 vs. DAY 7	11.00	15.00	-4.000	2	2
DAY 6 vs. DAY 8	11.00	15.00	-4.000	2	2
DAY 6 vs. DAY 9	11.00	19.00	-8.000	2	2
DAY 6 vs. DAY10	11.00	24.00	-13.00	2	2
DAY 6 vs. DAY11	11.00	29.00	-18.00	2	2
DAY 6 vs. DAY 12	11.00	34.00	-23.00	2	2
DAY 6 vs. DAY 13	11.00	37.00	-26.00	2	2
DAY 6 vs. DAY 14	11.00	32.00	-21.00	2	2
DAY 6 vs. DAY 15	11.00	20.00	-9.000	2	2
DAY 6 vs. DAY 16	11.00	27.00	-16.00	2	2
DAY 6 vs. DAY 17	11.00	26.00	-15.00	2	2
DAY 6 vs. DAY 18	11.00	37.00	-26.00	2	2
DAY 6 vs. DAY 19	11.00	32.00	-21.00	2	2
DAY 6 vs. DAY 20	11.00	31.00	-20.00	2	2
DAY 7 vs. DAY 8	15.00	15.00	0.0	2	2
DAY 7 vs. DAY 9	15.00	19.00	-4.000	2	2
DAY 7 vs. DAY10	15.00	24.00	-9.000	2	2
DAY 7 vs. DAY11	15.00	29.00	-14.00	2	2
DAY 7 vs. DAY 12	15.00	34.00	-19.00	2	2
DAY 7 vs. DAY 13	15.00	37.00	-22.00	2	2
DAY 7 vs. DAY 14	15.00	32.00	-17.00	2	2
DAY 7 vs. DAY 15	15.00	20.00	-5.000	2	2
DAY 7 vs. DAY 16	15.00	27.00	-12.00	2	2

DAY 7 vs. DAY 17	15.00	26.00	-11.00	2	2
DAY 7 vs. DAY 18	15.00	37.00	-22.00	2	2
DAY 7 vs. DAY 19	15.00	32.00	-17.00	2	2
DAY 7 vs. DAY 20	15.00	31.00	-16.00	2	2
DAY 8 vs. DAY 9	15.00	19.00	-4.000	2	2
DAY 8 vs. DAY10	15.00	24.00	-9.000	2	2
DAY 8 vs. DAY11	15.00	29.00	-14.00	2	2
DAY 8 vs. DAY 12	15.00	34.00	-19.00	2	2
DAY 8 vs. DAY 13	15.00	37.00	-22.00	2	2
DAY 8 vs. DAY 14	15.00	32.00	-17.00	2	2
DAY 8 vs. DAY 15	15.00	20.00	-5.000	2	2
DAY 8 vs. DAY 16	15.00	27.00	-12.00	2	2
DAY 8 vs. DAY 17	15.00	26.00	-11.00	2	2
DAY 8 vs. DAY 18	15.00	37.00	-22.00	2	2
DAY 8 vs. DAY 19	15.00	32.00	-17.00	2	2
DAY 8 vs. DAY 20	15.00	31.00	-16.00	2	2
DAY 9 vs. DAY10	19.00	24.00	-5.000	2	2
DAY 9 vs. DAY11	19.00	29.00	-10.00	2	2
DAY 9 vs. DAY 12	19.00	34.00	-15.00	2	2
DAY 9 vs. DAY 13	19.00	37.00	-18.00	2	2
DAY 9 vs. DAY 14	19.00	32.00	-13.00	2	2
DAY 9 vs. DAY 15	19.00	20.00	-1.000	2	2
DAY 9 vs. DAY 16	19.00	27.00	-8.000	2	2
DAY 9 vs. DAY 17	19.00	26.00	-7.000	2	2
DAY 9 vs. DAY 18	19.00	37.00	-18.00	2	2
DAY 9 vs. DAY 19	19.00	32.00	-13.00	2	2
DAY 9 vs. DAY 20	19.00	31.00	-12.00	2	2
DAY10 vs. DAY11	24.00	29.00	-5.000	2	2
DAY10 vs. DAY 12	24.00	34.00	-10.00	2	2
DAY10 vs. DAY 13	24.00	37.00	-13.00	2	2
DAY10 vs. DAY 14	24.00	32.00	-8.000	2	2
DAY10 vs. DAY 15	24.00	20.00	4.000	2	2
DAY10 vs. DAY 16	24.00	27.00	-3.000	2	2
DAY10 vs. DAY 17	24.00	26.00	-2.000	2	2
DAY10 vs. DAY 18	24.00	37.00	-13.00	2	2
DAY10 vs. DAY 19	24.00	32.00	-8.000	2	2
DAY10 vs. DAY 20	24.00	31.00	-7.000	2	2
DAY11 vs. DAY 12	29.00	34.00	-5.000	2	2
DAY11 vs. DAY 13	29.00	37.00	-8.000	2	2
DAY11 vs. DAY 14	29.00	32.00	-3.000	2	2
DAY11 vs. DAY 15	29.00	20.00	9.000	2	2
DAY11 vs. DAY 16	29.00	27.00	2.000	2	2
DAY11 vs. DAY 17	29.00	26.00	3.000	2	2
DAY11 vs. DAY 18	29.00	37.00	-8.000	2	2
DAY11 vs. DAY 19	29.00	32.00	-3.000	2	2
DAY11 vs. DAY 20	29.00	31.00	-2.000	2	2
DAY 12 vs. DAY 13	34.00	37.00	-3.000	2	2
DAY 12 vs. DAY 14	34.00	32.00	2.000	2	2
DAY 12 vs. DAY 15	34.00	20.00	14.00	2	2
DAY 12 vs. DAY 16	34.00	27.00	7.000	2	2
DAY 12 vs. DAY 17	34.00	26.00	8.000	2	2
DAY 12 vs. DAY 18	34.00	37.00	-3.000	2	2
DAY 12 vs. DAY 19	34.00	32.00	2.000	2	2
DAY 12 vs. DAY 20	34.00	31.00	3.000	2	2
DAY 13 vs. DAY 14	37.00	32.00	5.000	2	2
DAY 13 vs. DAY 15	37.00	20.00	17.00	2	2
DAY 13 vs. DAY 16	37.00	27.00	10.00	2	2
DAY 13 vs. DAY 17	37.00	26.00	11.00	2	2
DAY 13 vs. DAY 18	37.00	37.00	0.0	2	2
DAY 13 vs. DAY 19	37.00	32.00	5.000	2	2
DAY 13 vs. DAY 20	37.00	31.00	6.000	2	2
DAY 14 vs. DAY 15	32.00	20.00	12.00	2	2
DAY 14 vs. DAY 16	32.00	27.00	5.000	2	2
DAY 14 vs. DAY 17	32.00	26.00	6.000	2	2
DAY 14 vs. DAY 18	32.00	37.00	-5.000	2	2
DAY 14 vs. DAY 19	32.00	32.00	0.0	2	2
DAY 14 vs. DAY 20	32.00	31.00	1.000	2	2
DAY 15 vs. DAY 16	20.00	27.00	-7.000	2	2
DAY 15 vs. DAY 17	20.00	26.00	-6.000	2	2
DAY 15 vs. DAY 18	20.00	37.00	-17.00	2	2

DAY 15 vs. DAY 19	20.00	32.00	-12.00	2	2
DAY 15 vs. DAY 20	20.00	31.00	-11.00	2	2
DAY 16 vs. DAY 17	27.00	26.00	1.000	2	2
DAY 16 vs. DAY 18	27.00	37.00	-10.00	2	2
DAY 16 vs. DAY 19	27.00	32.00	-5.000	2	2
DAY 16 vs. DAY 20	27.00	31.00	-4.000	2	2
DAY 17 vs. DAY 18	26.00	37.00	-11.00	2	2
DAY 17 vs. DAY 19	26.00	32.00	-6.000	2	2
DAY 17 vs. DAY 20	26.00	31.00	-5.000	2	2
DAY 18 vs. DAY 19	37.00	32.00	5.000	2	2
DAY 18 vs. DAY 20	37.00	31.00	6.000	2	2
DAY 19 vs. DAY 20	32.00	31.00	1.000	2	2

Table Analyzed	Running stats
Column A	MS+R
vs	vs
Column B	NMS+R
Mann Whitney test	
P value	0.3648
Exact or approximate P value?	Gaussian Approximation
P value summary	ns
Are medians signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	376 , 444
Mann-Whitney U	166.0

Figure 22

**********check neurons d

MS+R	MS+NR	NMS+R	NMS+NR
0.	40.	39.	20.
52.	0.	24.	0.
0.	0.	51.	5.
0.	55.	44.	0.
	0.	37.	

Shapiro-Wilk normality test	
W	0.7983
P value	0.0014
Passed normality test (alpha=0.05)?	No
P value summary	**

Kruskal-Wallis test	
P value	0.3327
Exact or approximate P value?	Approximate
P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	5
Kruskal-Wallis statistic	4.584
Data summary	
Number of treatments (columns)	5
Number of values (total)	36

Ventral neurons

MS+R	MS+NR	NMS+R	NMS+NR
30.	30.	32.	11.
25.	36.	0.	13.
24.	0.	12.	1.
21.	0.	0.	8.
	1.	0.	

Shapiro-Wilk normality test	
W	0.8596
P value	0.0121
Passed normality test (alpha=0.05)?	No
P value summary	*

Table Analyzed	Glia V no restain
Kruskal-Wallis test	

P value	0.4594
Exact or approximate P value?	Approximate
P value summary	ns
Do the medians vary signif. (P < 0.05)	No
Number of groups	5
Kruskal-Wallis statistic	3.623
Data summary	
Number of treatments (columns)	5
Number of values (total)	36

22b

MS+R	MS+NR	NMS+R	NMS+NR
0.	43.	38.	20.
62.	0.	8.	0.
0.	0.	50.	13.
0.	38.	87.	0.
	0.	50.	

Shapiro-Wilk normality test	
W	0.8201
P value	0.0030
Passed normality test (alpha=0.05)?	No
P value summary	**

Table Analyzed	Glia D no restain		
Kruskal-Wallis test			
P value	0.1291		
Exact or approximate P value?	Gaussian Approximation		
P value summary	ns		
Do the medians vary signif. (P < 0.05)	No		
Number of groups	4		
Kruskal-Wallis statistic	5.665		
Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
MS+R vs MS+NR	-0.3750	No	ns
MS+R vs NMS+R	-6.475	No	ns
MS+NR vs NMS+NR	0.5000	No	ns
NMS+R vs NMS+NR	6.600	No	ns

Ventral

MS+R	MS+NR	NMS+R	NMS+NR
30.	30.	32.	11.
25.	36.	0.	13.
24.	0.	12.	1.
21.	0.	0.	8.
	1.	0.	

Shapiro-Wilk normality test	
W	0.8596
P value	0.0121
Passed normality test (alpha=0.05)?	No
P value summary	*

Table Analyzed	Glia V no restain		
Kruskal-Wallis test			
P value	0.3183		
Exact or approximate P value?	Gaussian Approximation		
P value summary	ns		
Do the medians vary signif. (P < 0.05)	No		
Number of groups	4		
Kruskal-Wallis statistic	3.519		
Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
MS+R vs MS+NR	4.425	No	ns
MS+R vs NMS+R	6.425	No	ns
MS+NR vs NMS+NR	0.5750	No	ns
NMS+R vs NMS+NR	-1.425	No	ns

Figure 24

ventral correlation

Shapiro-Wilk normality test	
W	0.8417
P value	0.0001
Passed normality test (alpha=0.05)?	No
P value summary	***

Number of XY Pairs	18
Spearman r	0.9006
95% confidence interval	0.7416 to 0.9638
P value (two-tailed)	P<0.0001
P value summary	***
Exact or approximate P value?	Gaussian Approximation
Is the correlation significant? (alpha=0.05)	Yes

dorsal

Shapiro-Wilk normality test	
W	0.8203
P value	P<0.0001
Passed normality test (alpha=0.05)?	No
P value summary	***
Sum	776.0

Number of XY Pairs	18
Spearman r	0.1974
95% confidence interval	-0.3105 to 0.6176
P value (two-tailed)	0.4324
P value summary	ns
Exact or approximate P value?	Gaussian Approximation
Is the correlation significant? (alpha=0.05)	No

Figure 25

DORSAL DCX

MS+R	MS+NR	NMS+R	NMS+NR
94.	19.	0.	1.
3.	0.	57.	86.
23.	3.	0.	45.
95.	0.	58.	21.
	0.	136.	

Shapiro-Wilk normality test	
W	0.8200
P value	0.0029
Passed normality test (alpha=0.05)?	No
P value summary	**

VENTRAL

MS+R	MS+NR	NMS+R	NMS+NR
65.	7.	5.	7.
0.	0.	37.	0.
0.	13.	55.	0.
0.	0.	9.	0.
	29.	0.	3.

Shapiro-Wilk normality test	
W	0.6742
P value	< 0.0001
Passed normality test (alpha=0.05)?	No
P value summary	****

Figure 26 Ki-67 dorsal

MS+R	MS+NR	NMS+R	NMS+NR
0.	82.	103.	31.
0.	89.	86.	105.
8.	62.	240.	0.
109.	104.	137.	99.
		161.	20.

Shapiro-Wilk normality test	
W	0.9153
P value	0.1066
Passed normality test (alpha=0.05)?	Yes
P value summary	ns

Table Analyzed	Ki-67/DCX Dorsal			
ANOVA summary				
F	5.054			
P value	0.0140			
P value summary	*			
Are differences among means statistically significant? (P < 0.05)	Yes			
R square	0.5199			
Brown-Forsythe test				
F (DFn, DFd)	0.5691 (3, 14)			
P value	0.6445			
P value summary	ns			
Significantly different standard deviations? (P < 0.05)	No			
Bartlett's test				
Bartlett's statistic (corrected)	3.592			
P value	0.3090			
P value summary	ns			
Significantly different standard deviations? (P < 0.05)	No			
ANOVA table	SS	DF	MS	P valu
Treatment (between columns)	35964	3	11988	P = 0.01
Residual (within columns)	33207	14	2372	
Total	69171	17		
Data summary				
Number of treatments (columns)	4			
Number of values (total)	18			

Number of families	1					
Number of comparisons per family	6					
Alpha	0.05					
Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary		
MS+R vs. MS+NR	-55.00	-155.1 to 45.10	No	ns		
MS+R vs. NMS+R	-116.2	-211.1 to -21.19	Yes	*		
MS+R vs. NMS+NR	-21.75	-116.7 to 73.21	No	ns		
MS+NR vs. NMS+R	-61.15	-156.1 to 33.81	No	ns		
MS+NR vs. NMS+NR	33.25	-61.71 to 128.2	No	ns		
NMS+R vs. NMS+NR	94.40	4.872 to 183.9	Yes	*		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	q
MS+R vs. MS+NR	29.25	84.25	-55.00	34.44	4	2.259
MS+R vs. NMS+R	29.25	145.4	-116.2	32.67	4	5.028
MS+R vs. NMS+NR	29.25	51.00	-21.75	32.67	4	0.9415
MS+NR vs. NMS+R	84.25	145.4	-61.15	32.67	4	2.647
MS+NR vs. NMS+NR	84.25	51.00	33.25	32.67	4	1.439
NMS+R vs. NMS+NR	145.4	51.00	94.40	30.80	5	4.334

Ventral

MS+R	MS+NR	NMS+R	NMS+NR
0.	101.	96.	8.
4.	22.	173.	67.
13.	7.	98.	0.
0.	33.	25.	108.
0.		197.	34.
0.			

Shapiro-Wilk normality test	
W	0.8023
P value	0.0009
Passed normality test (alpha=0.05)?	No
P value summary	***

Table Analyzed	Ki-67/DCX Ventral
Kruskal-Wallis test	
P value	0.0109
Exact or approximate P value?	Approximate
P value summary	*
Do the medians vary signif. (P < 0.05)	Yes
Number of groups	4
Kruskal-Wallis statistic	11.17
Data summary	
Number of treatments (columns)	4
Number of values (total)	20

Number of families	1		
Number of comparisons per family	6		
Alpha	0.05		
Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
MS+R vs. MS+NR	-7.000	No	ns
MS+R vs. NMS+R	-11.70	Yes	**
MS+R vs. NMS+NR	-6.700	No	ns
MS+NR vs. NMS+R	-4.700	No	ns
MS+NR vs. NMS+NR	0.3000	No	ns
NMS+R vs. NMS+NR	5.000	No	ns

Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1	n2
MS+R vs. MS+NR	4.500	11.50	-7.000	6	4
MS+R vs. NMS+R	4.500	16.20	-11.70	6	5
MS+R vs. NMS+NR	4.500	11.20	-6.700	6	5
MS+NR vs. NMS+R	11.50	16.20	-4.700	4	5
MS+NR vs. NMS+NR	11.50	11.20	0.3000	4	5
NMS+R vs. NMS+NR	16.20	11.20	5.000	5	5

PBS (10X)

80g NaCl
2g KCl
14.4g Na_2HPO_4
2.4 KH_2PO4
Top this up to 1 Litre of double distilled water.

BSA block

20 ML pbs
200mg BSA
0.1 ml 3 % Triton

Composition rat chows

Nutrient content (dry matter)	Unit	Equifeeds (pre-autoclave)	Equifeeds (post-autoclave)	Nutroscience (pre-autoclave)	Nutroscience (post-autoclave)
Protein	g/100g	21,47	21,36	19,73	19,62
Fibre	g/100g	6,65	7,13	4,17	4,48
Fat	g/100g	5,87	5,13	6,19	5,92
Starch	g/100g	26,03	25,98	38,02	38,96
Gross energy (GE)	MJ/kg	18,12	18,32	18,55	18,55
Calcium (Ca)	g/100g	1,06	1,09	1,36	1,35
Phosphorous (P)	g/100g	0,90	0,86	0,82	0,82
Ca:P ratio	N/A	1,18	1,26	1,67	1,66
Amino acids					
Alanine	g/100g	0,76	0,99	1,16	1,24
Arginine	g/100g	1,99	1,52	1,55	1,28
Aspartic acid	g/100g	0,72	2,01	0,99	1,74
Cysteine	g/100g	0,09	0,06	0,12	0,14
Glutamic acid	g/100g	1,88	3,53	2,39	2,80
Glycine	g/100g	1,03	0,94	1,11	1,17
Histidine	g/100g	0,88	0,88	1,64	1,31
HO-Proline	g/100g	0,04	0,04	0,14	0,10
Isoleucine	g/100g	0,95	0,94	0,79	0,77
Leucine	g/100g	1,53	1,57	1,62	1,54
Lysine	g/100g	1,56	1,18	1,39	1,17
Methionine	g/100g	0,36	0,28	0,43	0,38
Phenylalanine	g/100g	1,14	1,03	0,93	0,84
Proline	g/100g	1,10	1,06	1,12	1,08
Serine	g/100g	1,15	1,11	1,28	1,51
Threonine	g/100g	0,81	0,81	0,80	0,85
Tryptophan	g/100g	0,44	0,42	0,32	0,31
Tyrosine	g/100g	0,54	0,71	0,50	0,72
Valine	g/100g	0,96	0,97	0,86	0,87

N/A; not applicable
ND; Not determined
Reference for minimal nutrient requirements (highlighted in green): *Nutrient Requirements of Laboratory Animals*