The Effects of Long-Term Exercise and Stopping Long-Term Exercise on Behaviours and Neurobiology.

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Thesis abstract

Background: The immune hypothesis of major depression proposes that depression and associated anxiety and cognitive impairment share a common pathophysiology of neuroinflammation. This might arise from altered levels of tumour necrosis factor alpha (TNF) and changes in TNF signalling via the neurodegenerative TNFR1 and the more neuroprotective TNFR2. The limited efficacy of pharmacotherapies for these conditions necessitates alternative therapies. Preclinical and clinical literature overwhelmingly suggest exercise can reduce depressive symptoms and related anxiety and cognitive impairment, possibly by altering TNF and TNF receptor signalling mediated neuroinflammation. These beneficial effects of exercise suggest that stopping exercise could have adverse effects for depression, anxiety, and cognitive function and associated neurobiology. Our overall hypothesis proposed that exercise would improve anxiety-like, depression-like, and cognition-like behaviours, including in WT and TNFR1$^{-/-}$ exercise mice, whereas exercise would not benefit TNF$^{-/-}$ or TNFR2$^{-/-}$ exercise mice. We also hypothesised the cessation of exercise would have detrimental effects on behaviours and hippocampal neurobiology.

Methods: We utilised mouse models of voluntary wheel running compared to no-exercise and exercise control mice to investigate the effects of long-term exercise and stopping long-term exercise on depression-, anxiety-, and cognition-like behaviours. This work was performed in three sections. Firstly, behaviours were investigated over the lifespan of wild type (WT) mice at four, nine, and fourteen months of age. We then examined cognitive-like and affective-like behaviours in nine month old TNF and TNF receptor deficit WT, TNF$^{-/-}$, TNFR1$^{-/-}$, and TNFR2$^{-/-}$ mice compared to their respective controls. Finally, we investigated the effects of stopping exercise on anxiety-, depression-, and cognition-like behaviours in exercise cessation mice (EC) compared to no-exercise control (CONT) and exercise control mice (EXC) during middle age. This study utilised quantitative polymerase chain reaction (qPCR) and immunohistochemistry to examine the effects of exercise cessation of hippocampal neurobiology.

Results: Exercise reduced overt anxiety-like behaviours over the lifespan of WT mice, but increased freezing behaviours and spatial learning latencies in young female mice compared to control mice. Exercise improved recognition memory and spatial learning in nine month old WT mice, but had no effects on depression-like behaviours at any age or in any strain. Consistent with our hypotheses, exercise reduced anxiety in TNFR2$^{-/-}$ mice. Contrary to expectations, TNF$^{-/-}$ and TNFR1$^{-/-}$ exercise mice had impaired recognition memory and spatial learning, whereas exercise improved spatial learning with no changes in recognition memory in TNFR2$^{-/-}$ mice. Compared to EXC mice, EC mice displayed significant increases in anxiety-like, depression-like, and cognitive-like impairment behaviours. Exercise cessation also altered differential gene expression (10/75 hippocampal genes) including altered neurotrophic (*Ntrk1*), monoaminergic (*Slc6a4*), and immune *(IL10, Gfap*) gene expression.

Discussion: We found noteworthy unanticipated effects of long term exercise suggesting further characterisation of the behavioural effects of long term exercise is warranted. Furthermore, significantly increased anxiety-like, depression-like, and cognition-like impairments and altered hippocampal neurobiology following exercise cessation are suggestive of behavioural and neurobiological changes that may occur in humans after ceasing exercise. These findings need replication and investigation in preclinical and possibly clinical studies to elucidate the adverse behavioural and physiological sequelae of exercise cessation.

Thesis Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Publications

1. Morgan, J.A., et al., *Ceasing exercise induces depression-like, anxiety-like, and impaired cognitive-like behaviours and altered hippocampal gene expression.* Brain Research Bulletin, 2019. **148**: p. 118-130.
2. Morgan, J.A., et al., *The effects of aerobic exercise on depression-like, anxiety-like, and cognition-like behaviours over the healthy adult lifespan of C57BL/6 mice.* Behavioural Brain Research, 2018.
3. Morgan, J.A., et al., *Exercise related anxiety-like behaviours are mediated by TNF receptor signalling, but not depression-like behaviours.* Brain Research, 2018. **1695**: p. 10-17.
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Presentations

1. Poster: Julie Morgan, Andrew Olagunju, Frances Corrigan, Bernhard Baune. “Does ceasing exercise induce depressive symptoms? A systematic review of experimental trials including immunological and neurogenic markers”, European Royal College of Psychiatrists International Congress, Birmingham, June 2018.
2. Poster: Julie Morgan, Gaurav Singhal, Emily Jaehne, Catharine Jawahar, Frances Corrigan, Bernhard Baune. “Ceasing exercise induces depression-like, anxiety-like, and impaired cognition-like behaviours and hippocampal dysfunction”, European Royal College of Psychiatrists International Congress, Birmingham, June 2018.
3. Poster: Julie Morgan, Gaurav Singhal, Emily Jaehne, Catharine Jawahar, Frances Corrigan, Bernhard T. Baune. ‘TNF signalling via the TNFR1 and TNFR2 mediates the effects of exercise on anxiety-like and depression-like behaviours in WT, TNF-/-, TNFR1-/-, and TNFR2-/- mice, Adelaide, AU Florey International Postgraduate Research Conference, Sept 2017.
4. Poster: Julie Morgan, Gaurav Singhal, Emily Jaehne, Catharine Jawahar, Frances Corrigan, Bernhard T. Baune. ‘Does exercise cessation induce depression in middle age?’ Adelaide, AU Florey International Postgraduate Research Conference, Sept 2015.
5. Invited oral presentation: Julie Morgan, Frances Corrigan, Emily Jaehne, Bernhard T. Baune. ‘Chronic physical exercise induced adaptations in the brainstem and hypothalamus: A brief review of exercise effects on stress responses, the circadian clock, and energy balance metabolism’, Adelaide, The Society for Mental Health Research, Dec 2014.
6. Invited oral presentation: Julie Morgan, Frances Corrigan, Emily Jaehne, Bernhard T. Baune. ‘Exercise induced effects on anxiety, cognition, and depression in early adulthood and middle age’, Adelaide, The Society for Mental Health Research, Dec 2014.
7. Poster: Julie Morgan, Emily Jaehne, Gaurav Singhal, Frances Corrigan, Bernhard T. Baune. ‘Exercise induced effects on anxiety, cognition, and depression in early adulthood and middle age’, Adelaide, AU Florey International Postgraduate Research Conference, Sept 2014.
8. Poster: Julie Ayliffe, Frances Corrigan, Emily Jaehne, Bernhard T. Baune. ‘Physical exercise impacts cognitive-like and depression-like behaviours in C57BL/6 mice’, Adelaide, Australasian Neurosciences Society 34th Annual meeting, Jan 2014.
9. Poster: Julie Ayliffe, Frances Corrigan, Emily Jaehne, Bernhard T. Baune. ‘Exercise neurobiology: A review of the effects of exercise in regions of the brain’, Adelaide, UA Faculty of Health Sciences Postgraduate Research Conference, Aug 2013.
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Chapter 1. Introduction.

Background

This chapter begins with a brief outline of the scale of the challenge posed by depression and related anxiety and cognitive impairment. It then continues with an outline of the theoretical underpinnings of depression research, including the predominant theory of depression pathophysiology for the much of the 20th century – the catecholamine theory of depression. The more recent hypotheses about the involvement of the immune system in depression including the macrophage theory of depression and the immune hypothesis of depression are then outlined. The role of pro-inflammatory cytokines in depression-, anxiety-, and cognition-like impairment behaviours are then described, and this is followed by an in-depth introduction to tumour necrosis factor alpha (TNF) and TNF receptor signalling in depression and cognitive function. This section then continues with an introduction to the therapeutic effects of exercise for depression and related anxiety and cognitive function. The chapter concludes with the overall research question and hypothesis for the thesis.

The scale of the challenge of major depressive disorder

Major Depressive Disorder (MDD) with the associated anxiety and cognitive impairment are serious and debilitating mental conditions that often affect patients' function and participation in life and work. The Australian Bureau of Statistics National Survey of Health 2017-2018 observed that 20.1% or 4.8 million Australians experienced a mental condition such as depression or anxiety (ABS 2018). This was increased from 4.0 million in the previous National Health Survey of Mental Health and Well-being conducted in 2007 (ABS 2018). There are equally concerning figures about depression world-wide. The World Health Organisation (2018) reports depression is common globally, with over 300 million people currently experiencing depression, and this contributes to around 800,000 deaths by suicide annually (World Health Organisation 2018). Depression and the associated anxiety and cognitive impairment are clearly extensive and serious public health issues that need comprehensive and in-depth research to improve our understanding of the aetiology and pathophysiology of these conditions, and to develop treatments with improved efficacy.

Theoretical underpinnings for research into major depressive disorder

The major theoretical basis for depression research until the 1990s was the "catecholamine hypothesis of affective disorders" (Schildkraut 1965, p. 509). Schildkraut proposed that some, if not all depressions were associated with a reduction or deficiency of catecholamines, and of norepinephrine, dopamine, and serotonin availability in particular (Schildkraut 1965). It was suggested that in the absence of methods to confirm or reject the hypothesis, the hypothesis had heuristic value as a frame of reference for integrating research findings suggesting the actions of the antidepressant medications were mediated by catecholamines (Schildkraut 1965). Several decades later in 1991, it was suggested by Smith that depression research had been largely unsuccessful for a century, and that may have arisen from the assumption that the research focus ought to be on the brain. Smith suggested this fundamental assumption may have been flawed and proposed a persuasive theory about the involvement of elevated peripheral levels of pro-inflammatory macrophage factors interleukin 1 (IL1), interferon alpha (INFα), and tumour necrosis factor alpha (TNF) in the aetiology of depression. This was the first postulated involvement of the immune system in depression pathophysiology and was entitled *The Macrophage Theory of Depression*.

The macrophage theory of depression was based on seven observations (Smith 1991). Firstly,

participants given INFα therapy for six days developed symptoms that met the criteria for a major depressive episode. These included severe fatigue, anorexia, psychomotor slowing, confusion, and/or inability to concentrate. Second, macrophage expression of corticotropin-releasing factor (CRF) and adrenocorticotropin (ACTH) accounted for endocrine abnormalities in depression - including elevated cortisol. Third, populations with conditions involving macrophage activation such as rheumatoid arthritis and atherosclerosis showed a higher incidence of depression than in the general population. Fourth, microglia residing in the brain express pro-inflammatory cytokines. Fifth, oestrogen increases macrophage expression of IL1, and this explained the consistently higher incidence of depression in females than in males. Sixth, eicospaentanoic acid supresses macrophages whereas linoleic acid activates them. Seven, a notable body of epidemiological observations showed that increases in the prevalence of depression over time corresponded with a higher dietary intake of linoleic acid and reduced intake of eicospaentanoic acid that were consistent with the macrophage hypothesis of depression. Smith proposed that patients with depression ought to be assessed for sources of infection, diet, and damaged or dying tissues that might stimulate an inflammatory response and contribute to depression. Overall, Smith asserted the macrophage theory of depression integrated a considerable body of previously unexplained epidemiology and physiology, and provided a testable theory that could be applied in clinical practice (Smith 1991). Furthermore, the theory constituted a fresh theoretical basis for depression research.

Mechanisms involved in the macrophage theory of depression were tested over the next five years largely by Maes et al. in work examining aspects of endocrinology and adaptive immunity in depression (including but not limited to Maes, Bosmans et al. 1991, Maes, Bosmans et al. 1991, Maes, Minner et al. 1991, Maes, Claes et al. 1992, Maes, Scharpe et al. 1992, Maes, Stevens et al. 1992, Maes, Stevens et al. 1992, Maes, Bosmans et al. 1993, Maes, Meltzer et al. 1993, Maes, Meltzer et al. 1993, Maes, Scharpé et al. 1993, Maes 1994, Maes, Lambrechts et al. 1994, Maes, Meltzer et al. 1994). This work was outlined in the next seminal work 'Evidence for an immune response in major depression: A review and hypothesis' (Maes 1995), and formed the basis for much research about immunity in depression over the next twenty-five years.

More recent literature outlines the immune associated mechanisms of depression pathophysiology and describes the sequelae of altered cell-mediated immunity (Leonard and Maes 2012). Research supporting immune involvement in the pathophysiology of depression includes: a) altered cell-mediated immunity with immune cell activation and inflammation with increased pro-inflammatory cytokines including those from T cells; b) the anti-inflammatory effects of antidepressants on inflammation and cell-mediated immunity; c) pro-inflammatory cytokine related changes in the metabolism of serotonin; and d) increases in inflammation that appear associated with oxidative and nitrosative stress. The concept of neuro-progression or cognitive deficits in depression was also described around this time, and was reported to involve a combination of neuronal apoptosis and neurodegeneration, with reductions in neurogenesis and neuroplasticity and associated neuroanatomical dysfunction (Berk, Kapczinski et al. 2011, Leonard and Maes 2012).

Pro-inflammatory cytokines in major depressive disorder

Research subsequent to the postulated inflammatory hypothesis of major depressive disorder has shown that altered expression of pro-inflammatory cytokines including IL1, IL6, and TNF are associated with depression and anxiety behaviours in mouse models and in humans (Howren, Lamkin et al. 2009, Dowlati, Herrmann et al. 2010, Liu, Ho et al. 2012, Köhler, Freitas

et al. 2017). Psychological stress in humans can involve significant increases in circulating IL1, IL6, and TNF (Black 2002, Steptoe, Hamer et al. 2007, Goshen and Yirmiya 2009, Marsland, Walsh et al. 2017). It is thought these cytokines could cross the blood-brain-barrier (BBB) at regions where the BBB is absent such as the organum vasculosum laminae terminalis and the median eminence, where the regional integrity of the BBB is disrupted, or via active transport by specific carrier proteins (Schiepers, Wichers et al. 2005, Miller and Raison 2016). Once in the brain, pro-inflammatory cytokines can act via receptors on astrocytes and microglial cells, thus contributing to the activation of these cells that in turn increases their expression of pro-inflammatory cytokines, and contributes to inflammatory conditions in the brain (Breder, Dinarello et al. 1988, McGeer and McGeer 1995, Schiepers, Wichers et al. 2005) that appear to involve depression and related anxiety and cognitive impairment behaviours.

Whilst there are exceptions (Ovaskainen, Koponen et al. 2009), meta-analyses of clinical studies examining the association between pro-inflammatory cytokines and depression have overwhelmingly shown elevated IL6 and TNF are positively associated with depression (Dowlati, Herrmann et al. 2010, Strawbridge, Arnone et al. 2015, Goldsmith, Rapaport et al. 2016, Kohler, Freitas et al. 2017). Elevated levels of IL6 and TNF are also associated with depression severity, treatment non-response, and completed suicide (Maes, Bosmans et al. 1997, Yoshimura, Hori et al. 2009, Serafini, Pompili et al. 2013, Valkanova, Ebmeier et al. 2013, Black and Miller 2015, Strawbridge, Arnone et al. 2015, Gananca, Oquendo et al. 2016). Moreover, meta-analyses of anti-cytokine treatment in patients with chronic inflammatory conditions and secondary depression (n=1309) has shown anti-cytokine therapy reduced depressive symptoms compared to placebo (Kappelmann, Lewis et al. 2018), suggesting pro-inflammatory cytokines may have a key role in the pathogenesis and pathophysiology of depression characterised by elevated inflammation (Kappelmann, Lewis et al. 2018). Collectively these findings suggest that targeting altered pro-inflammatory cytokine levels could be a worthwhile therapeutic strategy for the treatment of depression.

Preclinical research investigating the effects of elevated levels of pro-inflammatory cytokines in the periphery and brain are largely consistent with the findings in clinical studies. Elevated cytokines including IL1, IL6, and TNF are associated with sickness-like behaviours in adult mice and rats in a dose and time-dependent manner to produce reductions in motor activity and food and water intake, and altered cognition that resemble aspects of human depressive symptoms (Dantzer, O'Connor et al. 2008). For instance, adult wild type (WT) mice subjected to chronic stress have displayed increased depressive-like symptoms including decreased preference for sucrose and reductions in social exploration that were associated with increases in brain IL1, and these symptoms were not evident in $IL1^{-/-}$ mice (Goshen, Kreisel et al. 2007). IL1 is also associated with depression related anxiety-like behaviours. Compared to WT mice, $IL1^{-/-}$ mice displayed an absence of chronic stress related increases in anxiety-like behaviours in the elevated plus maze, light-dark box, and novelty induced hypophagia (Koo and Duman 2009). Conversely, intracerebroventricular administration of IL1β resulted in increases in anxiety-like behaviours in the elevated plus maze (Song, Horrobin et al. 2006, Leonard and Maes 2012). IL1 thus appears to be a contributing factor to the pathogenesis and pathophysiology of depression- and related anxiety-like behaviours.

Research into the behavioural effects of IL6 have found similar results. The administration of IL6 in the hippocampus or amygdala increased immobility time in the forced swim test (FST) (Wu and Lin 2008). However, adult $IL6^{-/-}$ mice have demonstrated reduced stress induced depression-like behaviours of learned helplessness, immobility time in the FST and reduced sucrose preference (Chourbaji, Urani et al. 2006). Similarly, repeated social stress in adult wild type (WT) and $IL6^{-/-}$ mice induced anxiety-like behaviours in WT mice that were not evident

in IL6$^{-/-}$ mice (Niraula, Witcher et al. 2019). Moreover, elevated levels of IL6 were apparent in rats with high anxiety compared to rats with lower levels of anxiety (Leonard and Maes 2012). Similar behaviours seem to arise as a result of altered TNF in the CNS, however these will be introduced in detail below. Considered together, the preclinical literature about the effects of the pro-inflammatory cytokines IL1 and IL6 in the brain suggest these cytokines have noteworthy roles in the pathogenesis and pathophysiology of depression- and related anxiety-like behaviours.

Pro-inflammatory cytokines and neuro-progression in major depressive disorder

Neuro-progression in major depressive disorder is characterised by an increase in the number, duration, and severity of depressive episodes, with higher rates of recurrent episodes, recurrent episodes arising from a lower threshold, and progressive cognitive dysfunction (Kessing 2015). It is thought that most patients with depression do not return to premorbid levels of cognitive function during remission (Trivedi, Rush et al. 2006), thus presenting a focus for the treatment of cognitive dysfunction during both episodes of depression and remission.

Neuro-progression is thought to involve elevated expression of pro-inflammatory cytokines in the brain. However, IL1, IL6, and TNF are essential for normal cognitive function in physiological (un-activated) conditions (McAfoose and Baune 2009, Baune, Camara et al. 2012), and are involved in the molecular mechanisms that underpin synaptic plasticity to form memories via Hebbian plasticity (Hebb 1949). Hebbian plasticity involves the strengthening or weakening of interconnected synapses arising from synaptic stimulation – long-term potentiation (LTP) and long-term depression (LTD) respectively. IL1β has been shown to modulate synaptic transmission in the hippocampus, inhibit LTP, and maintain LTP to contribute to memory consolidation (McAfoose and Baune 2009). However, IL1β involvement in hippocampal dependent memory follows an inverted 'U' shaped pattern, with physiological levels of IL1β contributing to the normal development of memories, but elevations or reductions in levels impairing memory (Avital, Goshen et al. 2003, Goshen, Kreisel et al. 2008).

Elevated brain IL6 (expressed by microglial and astrocytic cells) may exert detrimental effects on cognitive function via its effects on hippocampal neurogenesis. Transgenic adult mice over expressing astrocytic IL6 have displayed a 63% reduction in neurogenesis in the dentate gyrus of the hippocampus, whereas mice not expressing IL6 displayed improvements in learning in the radial arm maze and passive avoidance testing (Monje, Toda et al. 2003, Braida, Sacerdote et al. 2004, Hryniewicz, Bialuk et al. 2007, McAfoose and Baune 2009). However, IL6 deficit has also impaired the exploration of novel objects, suggesting some aspects of hippocampal neurogenesis dependent learning and memory may be affected by reductions in IL6 although others may not. Thus, IL6 may also exert its effects in an inverted 'U' shaped pattern with both elevated and reduced levels of IL6 disrupting aspects of cognitive-like functioning.

TNF is also intimately involved in depression, cognitive function, and neuro-progression in depression. Elevated expression of TNF has been shown to disrupt the BBB and increase BBB permeability (Cheng, Desse et al. 2018). TNF is therefore thought to contribute to the entry of peripheral pro-inflammatory cytokines into the brain to activate microglia and astrocytes that can result in neuro-inflammation, depression, and related cognitive dysfunction. However, there appears to be extensive involvement of TNF in multiple aspects of the pathogenesis and pathophysiology of depression and related anxiety and cognitive function, and thus TNF signalling via the TNF receptors will be discussed in detail below.

TNF signalling via the TNF receptors

Endogenous TNF plays major roles in the aetiology and pathophysiology of neuroinflammation associated with depression and related anxiety and cognitive dysfunction, so understanding and effectively intervening in altered TNF signalling are important research aims. This section will therefore introduce TNF signalling via the TNF receptors TNFR1 and TNFR2, and the effects of TNF signalling on depression, related behaviours, and neuro-progression.

TNF is a transmembrane 26KDa protein (pro-TNF) that is expressed by peripheral cells such as endothelial cells, fibroblasts, and immune cells including microglia (Tsai, Yie et al. 1996, Falvo, Tsytsykova et al. 2010). Transmembrane TNF is cleaved of the membrane component by the metalloprotease TNF-converting enzyme (TACE/ADAM) to form soluble TNF (sTNF) that circulates in bloods around the body (Sedger and McDermott 2014, Yang, Wang et al. 2018). Both sTNF and TNF bind to the two membrane bound receptors TNFR1 and TNFR2 to confer the effects of TNF throughout the body and in the brain. TNFR1 or p55/p60 is expressed on most cells in the body, whereas TNFR2 (p75/p80) is only found on the surface of endothelial cells, cells of haematopoietic lineage including T cells, and on microglia, oligodendrocytes, and neuronal subtypes in the brain (Grell, Douni et al. 1995, Sedger and McDermott 2014). Most cells therefore express levels of TNFR1, but only some cells express detectable levels of TNFR2 (Carpentier, Coornaert et al. 2004), suggesting that TNF might confer its actions largely by signalling via the TNFR1. As with the cleavage of TNF to sTNF, the membrane bound receptors TNFR1 and TNFR2 can be cleaved into their soluble forms soluble TNFR1 (sTNFR1) and soluble TNFR2 (sTNFR2) by TACE enzymes, and both can bind with pro-TNF and (mature) TNF (Yang, Wang et al. 2018). TNF levels are considered to increase with ageing (Fagiolo, Cossarizza et al. 1993, Han, Hosokawa et al. 1995).

The TNF receptors TNFR1 and TNFR2 are thought to have quite different functions although there does seem to be some overlap in roles. TNFR1 is involved in cellular apoptosis two via two sequential signalling cascades, but can also activate the transcription of NF-KB to promote cellular survival (Micheau and Tschopp 2003). Apoptosis cascades via TNFR1 signalling commence with the binding of TNF to TNFR1 and the release of the silencer of death domains (SODD) protein. The TNF receptor–associated death domain (TRADD) then recruits the adaptor protein receptor-interacting protein (RIP), TNF-R–associated factor 2 (TRAF2), and the Fas-associated death domain (FADD) that recruits caspase 8 to catalyse apoptosis (Chen and Goeddel 2002). In contrast, TNF signalling via the limited expression of TNFR2 is thought to promote cellular proliferation, migration, and survival, and to be protective from glutamate related excitotoxicity (Yang, Wang et al. 2018). TNF binds with TNFR2 and recruits cytoplasmic TRAF-2–cIAP-1–cIAP-2 complexes that can inhibit apoptosis and initiate NF-KB activation (Sun and Ley 2008, Naudé, den Boer et al. 2011, Yang, Wang et al. 2018) giving rise to its pro-survival functions. The apoptotic signalling of TNFR1 and the pro-survival functions of TNFR2 signalling appear increasingly relevant to depression pathogenesis and pathophysiology because it is thought that a shift towards TNF signalling via the TNFR1 and the associated cellular apoptosis could be a mechanism that contributes to both symptoms and neuro-progression in depression (Baune, Camara et al. 2012).

TNF signalling in depression

Indeed there is now a body of clinical research analysed by meta-analyses that overwhelmingly suggests TNF is elevated in at least a subset of patients with depression (Dowlati, Herrmann et al. 2010, Liu, Ho et al. 2012, Strawbridge, Arnone et al. 2015, Köhler, Freitas et al. 2017) and several studies have also reported changes in TNF receptor levels

(Brunoni, Machado-Vieira et al. 2015, Haroon, Daguanno et al. 2018). Only around 30% of patients experience full remission during the first treatment for depression (Gaynes, Warden et al. 2009) and elevated TNF at baseline has been shown to predict treatment non-response (Strawbridge, Arnone et al. 2015). Of note, is that treatment resistance and/or repeated depressive episodes often involves cumulative cognitive deficits (Strawbridge, Young et al. 2018) that appear to mediate functional impairment during depression (F Carvalho, K Miskowiak et al. 2014). These include impairments in attention, psychomotor speed, processing speed, visual learning and memory, verbal memory, and executive function (McDermott and Ebmeier 2009, Bora, Harrison et al. 2012, Lee, Hermens et al. 2012, Rock, Roiser et al. 2014, Trivedi and Greer 2014). However, such deficits commonly persist beyond the remission of affective symptoms to contribute to neuro-progression with repeated episodes (Bortolato, F Carvalho et al. 2014). There therefore remains an unmet need for effective treatments to reduce cognitive impairment during depression and remission (Bortolato, Miskowiak et al. 2016), and the modulation of TNF and TNF signalling via the TNF receptors might be one approach that could reduce neuroinflammation with benefits for these behaviours.

TNF signalling in cognition and neuro-progression in depression

Preclinical research investigating the effects of TNF and TNF receptor signalling have shed some light on potential effects of the modulation of TNF and its receptors on depression and related cognitive function and neuro-progression. TNFR1 and TNFR2 deficit in adult mice has resulted in reductions of immobility time in the FST (Simen, Duman et al. 2006), suggesting that TNF signalling via the TNFR1 and TNFR2 are indeed involved in the pathogenesis and pathophysiology of depression-like behaviours in adult mice, and possibly in adult humans. TNF is also involved in the molecular processes of LTP and memory consolidation in mice (Lynch 2000).

Consistent with a role for TNF in LTP, TNF deficiency in adult $TNF^{-/-}$ mice has resulted in poorer learning and memory in the novel object recognition test, and impaired spatial learning and learning efficacy in the Barnes maze compared to adult WT mice with intact TNF signalling (Baune, Wiede et al. 2008). However, pathologically elevated levels of TNF inhibited long-term potentiation in the dentate gyrus of adult rats (Butler, O'connor et al. 2004), suggestive of disrupted synaptic plasticity and memory formation in the context of TNF associated neuroinflammation. Interestingly, the inhibition of LTP by TNF arises from TNF signalling via TNFR1 activation (Albensi and Mattson 2000, McAfoose and Baune 2009). Moreover, the intracerebroventricular administration of TNF in adult mice increased depressive-like behaviours in the FST and tail suspension test, and involved reductions in the consumption of chocolate milk, whereas TNF receptor 1 (TNFR1) knockout mice displayed reduced depression-like behaviours in these tests compared to WT mice (Brebner, Hayley et al. 2000, Kaster, Gadotti et al. 2012). Moreover, the deficit of either TNFR1 or TNF receptor 2 (TNFR2) has involved reductions in immobility time in the FST and greater consumption of sucrose in the sucrose preference test compared to their WT littermates (Simen, Duman et al. 2006). Collectively preclinical research about the effects of TNF and the TNF receptors on depression-like behaviours suggests that reduced expression TNF or the TNF receptors might reduce depression-like behaviours, but the blockade of TNF signalling could have adverse effects. Nevertheless, the safe and effective modulation of elevated TNF seems a highly worthwhile pursuit because it might reduce neuroinflammation and the related depressive-like and cognitive-like impairment behaviours.

Exercise for depression and related cognitive dysfunction

The aim to modulate TNF in depression has seen the development of TNF antagonists or inhibitors, including infliximab and etanercept amongst others (Bortolato, Carvalho et al. 2015). However, treatments aiming to reduce TNF can involve serious adverse effects, including demyelination of myelinated cells in the CNS and hepatotoxicity (French, Bonacini et al. 2016, Kemanetzoglou and Andreadou 2017), so the safe modulation of TNF in the CNS remains an important therapeutic aim for research efforts. Physical exercise is an intervention that has minimal (and quite manageable) risks or adverse effects, has demonstrated efficacy for reducing depression, and has a net anti-inflammatory effect. Exercise may therefore be effective for reducing TNF mediated inflammation in depression and related anxiety and cognitive impairment in adults and will thus be introduced in detail below.

Clinical research about exercise for depression

There is now an extensive body of clinical research examined by meta-analyses that have investigated the efficacy of exercise for depression in adults and older adults. Exercise has been found to be effective for reducing primary (and secondary) depression in adults (Josefsson, Lindwall et al. 2014, Adamson, Ensari et al. 2015, Kelley, Kelley et al. 2015, Kvam, Kleppe et al. 2016, Schuch, Vancampfort et al. 2016, Krogh, Hjorthoj et al. 2017, Kelley and Kelley 2018) as a stand-alone treatment, or in conjunction with antidepressant therapy (Danielsson, Noras et al. 2013). Supervised aerobic exercise programs undertaken three times a week at moderate intensity for at least nine weeks have been reported as effective for reducing depression in adults (Stanton and Reaburn 2014). Consistent with findings in adults, meta-analyses of exercise for depression in older adults have found exercise also reduces depression in older people (Bridle, Spanjers et al. 2012, Schuch, Vancampfort et al. 2016, Perez-Lopez, Martinez-Dominguez et al. 2017). Whilst earlier meta-analyses reported relatively modest effects of exercise for depression in older people, a later review has suggested these may have been underestimated due to publication bias (Schuch, Vancampfort et al. 2016). This study further suggested that the effectiveness of exercise for treating depression in older people was such that exercise ought to be a routine intervention included in the management of depression in older patients (Schuch, Vancampfort et al. 2016).

There is now sufficient clinical research to enable meta-analysis of resistance exercise interventions for treating depression, and Gordon et al (2018) have recently shown that resistance exercise reduces depression in adults. Resistance exercise was effective for reducing depressive symptoms regardless of health status, the volume of resistance exercise, or the significance of improvements in strength (Gordon, McDowell et al. 2018). There is also meta-analytic evidence emerging about the mechanisms involved in conferring the effects of exercise for depression. Interestingly, whilst reductions in depressive symptoms do not appear to involve increases in BDNF (Dinoff, Herrmann et al. 2018, Kurebayashi and Otaki 2018), reductions in cortisol are evident (Beserra, Kameda et al. 2018), suggesting that adaptivity of the stress-response systems are involved with mediating the effects of exercise for depression. This further suggests that cross-talk between the neuroendocrine system and immune systems could occur and is suggestive that exercise related changes in the immune system - such as in pro-inflammatory cytokines - could also be involved.

Clinical research about exercise for cognition and neuro-progression in depression

Cognitive ability has been closely linked with functional recovery in patients with depression (Baune, Malhi et al. 2018), and depression increases the risk for the development of mild cognitive impairment in patients aged over 50 (OR = 2.79; 95% CI = 1.70, 4.59) (Lara, Koyanagi et al. 2017). The treatment of cognitive impairment in depression is therefore a priority concern. Few studies to date have investigated the effects of exercise on cognitive function or neuro-progression in depression. However, current research (n=2604) suggests that moderate to vigorous activity modifies the relationship between depression and cognitive impairment to preserve cognitive function in older adults (Hu, Smith et al. 2019). Other research suggests mixed findings, with some studies supporting an effect of exercise for cognition in depression, and others showing no benefit (Greer, Furman et al. 2017). A randomised controlled trial (RCT) study by Greer et al (2015) examining the effects of a low dose (4 k/cal per kilogram of body weight per week (KKW)) or a high dose (16KKW) of exercise found both doses improved processing speed, attention, visual memory, and spatial planning in patients with a partial response to serotonin reuptake inhibitors (SSRIs). Another RCT examined the effects of aerobic exercise on cognitive control in depression, and found the eight week moderate intensity intervention improved indices of conflict monitoring (Olson, Brush et al. 2017) However, a recent systematic review of controlled and uncontrolled trials investigating exercise for cognitive impairment in depression reported no effect of exercise on global cognition or on individual cognitive domains (Sun, Lanctot et al. 2018). The authors of this review noted however, that low intensity interventions with compliance rates of over 70% significantly improved global cognition. Overall, the effects of exercise for cognitive function and neuro-progression in depression remain unclear, and further high-quality investigations are needed.

Preclinical research about exercise for depression- and related anxiety-like behaviours

Preclinical research investigating the effects of exercise for depression- and related anxiety and cognition-like impairment behaviours in adult mice and rats have largely suggested that exercise can reduce these behaviours although there are several exceptions. However, a cautionary note on exercise models in preclinical research is warranted (Morgan, Corrigan et al. 2015). Preclinical models of exercise utilise either voluntary wheel running or forced exercise such as treadmills that are set to regulate specific speeds of running. Settings can be for light, moderate, vigorous or varied running. In contrast, mice running voluntarily on wheels tend to run in short bursts at a preferred cruising speed (De Bono, Adlam et al. 2006). Forced exercise is thought to involve emotional distress arising from coercion of the animal that may confound outcomes (Dishman, Berthoud et al. 2006, Lin, Chen et al. 2012, Morgan, Corrigan et al. 2015). It is also problematic to generalise forced exercise protocols to humans. The following outline of the effects of exercise on depression- and related anxiety- and cognition-like behaviours in preclinical research will therefore only include studies utilising voluntary wheel running methods.

A major factor in the efficacy of wheel running for reducing depression-, anxiety-, and cognition-like impairment behaviours appears to be the duration of exercise. Adult female rats given ad libitum access to running wheels for one or three days were not protected by exercise from subsequent restraint stress induced anxiety-like behaviours (Jones, Gupton et al. 2016). However, wheel running for two to four weeks in adult male C57BL/6 mice increased the time spent in the central anxiogenic regions of the open field, with increases in rearing and crossings in these regions that are suggestive of reductions in anxiety-like behaviours

(Binder, Droste et al. 2004, Duman, Schlesinger et al. 2008, Salam, Fox et al. 2009). Furthermore, male adult mice undertaking ad libitum wheel running for two weeks did not display the anticipated reductions in depression-like behaviours of immobility time in the FST, whereas mice that ran for three to six weeks demonstrated reduced immobility time in both the FST and tail suspension test (Duman, Schlesinger et al. 2008, Trejo, Llorens-Martin et al. 2008, Cunha, Oliveira et al. 2013, Huang, Dong et al. 2017). Whilst species differences between mice and rats may have contributed to the variation in outcomes, overall this research suggests that wheel running for two or more weeks is needed to alter anxiety-like behaviours, but a minimum of four weeks running is needed to reduce depression-like behaviours in adult mice. This further suggests that exercise may need to be maintained for ongoing benefits. However, there is a lack of research examining the effects of long-term or lifelong exercise on depression- and related anxiety-like behaviours, and the effects of stopping long-term exercise remain unknown.

Preclinical research about exercise for cognition-like behaviours

Preclinical research about exercise for cognition-like behaviours has shown similar results. For instance, shorter durations of five days of running in male adult Sprague Dawley rats did not change latencies in the passive avoidance test (Yau, Lau et al. 2012). However, longer durations of four to seven weeks of running resulted in significant increases in passive avoidance latencies that are suggestive of an improvement in passive avoidance learning (Chen, Lin et al. 2008, Liu, Chen et al. 2009). These studies suggest that chronic exercise is needed to change cognitive-like behaviours in adult mice. There are few studies examining the effects of longer-term running on cognition-like behaviours in older mice. However, one seminal investigation has shown that 45 days of voluntary running from 18 months of age in male mice significantly improved learning and retention in the Morris water maze compared to age-matched controls (van Praag, Shubert et al. 2005). Similarly, six months exercise from 9 months of age in female C57BL/6 mice significantly improved spatial memory latencies in the Morris water maze (Marlatt, Potter et al. 2012). Nevertheless, there is research showing longer durations of exercise have no effects or apparently detrimental effects of exercise on depression- and anxiety-like behaviours. Interestingly, three to four weeks of wheel running in male adult mice reduced exploration of the centre regions of the open field, increased latencies to leave the protected and dark regions of the elevated plus maze and light/dark box, and did not change in immobility time in the FST (Fuss, Ben Abdallah et al. 2010, Onksen, Briand et al. 2012). Also interesting was that these behaviours were correlated with increases in hippocampal neurogenesis, suggesting that exercise associated neurogenesis could contribute to increases in anxiety-like behaviours (Fuss, Ben Abdallah et al. 2010). Another study by Binder et al (2004) showed that four weeks of wheel running resulted in a reluctance of mice to enter the centre regions of the open field, suggestive of an increase in anxiety-like behaviours. Speculatively speaking, from an evolutionary perspective it could be that increased anxiety might be associated with increased vigilance to environmental stimuli that could increase awareness of predators. Whilst these studies suggest adverse or no effects of exercise on anxiety- and depression-like behaviours, research examining exercise in preclinical models has largely shown exercise can reduce such behaviours. Interestingly however, whilst the role of neuroinflammation is increasingly considered to play a pivotal role in the aetiology and pathophysiology of depression and related cognitive impairment behaviours, there remains limited research investigating whether exercise confers its beneficial effects on these behaviours via possible modulatory effects on neuroinflammation.

Limitations of the research and the current work

There are however several limitations of the preclinical research to date. First, the majority of preclinical research investigating exercise for depression-like and related behaviours are conducted in young adult animals (Binder, Droste et al. 2004, Duman, Schlesinger et al. 2008, Salam, Fox et al. 2009) with few studies in older animals (Pietrelli, Lopez-Costa et al. 2012). This has resulted in a paucity of information about the effects of exercise in older and ageing animals. Second, research involving exercise in preclinical models are largely confined to male animals (Binder, Droste et al. 2004, Duman, Schlesinger et al. 2008, Salam, Fox et al. 2009, Fuss, Ben Abdallah et al. 2010) so the effects of exercise in female adult mice and rats remain less well understood. This is concerning given the prevalence of depression is commonly higher in women than in men (World Health Organisation 2016, World Health Organisation 2018). Third, a better understanding of the effects of voluntary wheel running on behaviours is needed. Fourth, whilst research to date commonly investigate durations of wheel running of three to six weeks (Duman, Schlesinger et al. 2008, Trejo, Llorens-Martin et al. 2008, Liu, Chen et al. 2009, Cunha, Oliveira et al. 2013, Huang, Dong et al. 2017), there are few studies examining the effects of long-term or lifelong voluntary exercise on depression- and related anxiety- and cognitive impairment behaviours (Marlatt, Potter et al. 2012). Fifth, there remains no investigation into whether exercise confers its effects on depression and related cognitive impairment behaviours via neuro-inflammatory related mechanisms, and TNF and TNF receptor signalling in particular. Sixth, whilst there is some research examining the effects of exercise in models of stress, restraint, or pathology induced affective and cognition-like behaviours (Nichol, Parachikova et al. 2007, Parachikova, Nichol et al. 2008, Nichol, Deeny et al. 2009, Aguiar, Lopes et al. 2016, Jones, Gupton et al. 2016), there remain few studies examining the effects of exercise on these behaviours in normal healthy mice. Given these limitations of the research to date, this thesis addresses the overall question:

"What are the effects of lifelong voluntary exercise and stopping lifelong exercise on depression- and related anxiety- and cognitive-like behaviours and associated TNF signalling in healthy ageing male and female mice?"

We hypothesise that

a) lifelong exercise will prevent the aetiology of depression-, anxiety-, and cognition-like behaviours whereas stopping exercise will increase these behaviours
b) lifelong exercise associated TNF signalling will improve depression-, anxiety-, and cognition-like behaviours in WT and TNFR1$^{-/-}$ mice, but not in TNFR2$^{-/-}$ or TNF$^{-/-}$ mice.

The aim of this thesis is thus: To investigate the effects of lifelong voluntary exercise and stopping lifelong exercise on depression- and related anxiety- and cognitive-like behaviours and associated TNF signalling in healthy ageing male and female mice.

Chapter 2. The effects of aerobic exercise on depression-like, anxiety-like, and cognition-like behaviours over the healthy adult lifespan of C57BL/6 mice

Statement of Authorship

Title of Paper	The effects of aerobic exercise on depression-like, anxiety-like, and cognition-like behaviours over the healthy adult lifespan of C57BL/6 mice.		
Publication Details	Morgan, J. A., G. Singhal, F. Corrigan, E. J. Jaehne, M. C. Jawahar and B. T. Baune (2018). "The effects of aerobic exercise on depression-like, anxiety-like, and cognition-like behaviours over the healthy adult lifespan of C57BL/6 mice." Behavioural Brain Research.		
Name of Lead Author (Candidate)	Julie A. Morgan		
Contribution to the Paper	Data collection utilising mouse models and wet lab techniques, data analyses, interpretation, writing and redrafting manuscripts. Overall percentage: 80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	28th November, 2018
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Contribution to the Paper	Conception of the project, design of methodology or experimental protocol, guidance about manuscript drafts.		
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By Signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

"The effects of aerobic exercise on depression-like, anxiety-like, and cognition-like behaviours over the healthy adult lifespan of C57BL/6 mice."[1]

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Research report

The effects of aerobic exercise on depression-like, anxiety-like, and cognition-like behaviours over the healthy adult lifespan of C57BL/6 mice

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ABSTRACT

Preclinical studies have demonstrated exercise improves various types of behaviours such as anxiety-like, depression-like, and cognition-like behaviours. However, these findings were largely conducted in studies utilising short-term exercise protocols, and the effects of lifetime exercise on these behaviours remain unknown. This study investigates the behavioural effects of lifetime exercise in normal healthy ageing C57BL/6 mice over the adult lifespan. 12 week-old C57BL/6 mice were randomly assigned to voluntary wheel running or non-exercise (control) groups. Exercise commenced at aged 3 months and behaviours were assessed in young adult (Y), early middle age (M), and old (O) mice (n = 11–17/group). The open field and elevated zero maze examined anxiety-like behaviours, depression-like behaviours were quantified with the forced swim test, and the Y maze and Barnes maze investigated cognition-like behaviours. The effects of lifetime exercise were not simply an extension of the effects of chronic exercise on anxiety-like, depression-like, and cognition-like behaviours. Exercise tended to reduce overt anxiety-like behaviours with ageing, and improved recognition memory and spatial learning in M mice as was expected. However, exercise also increased anxiety behaviours including greater freezing behaviour that extended spatial learning latencies in Y female mice in particular, while reduced distances travelled contributed to longer spatial memory and cognitive flexibility latencies in Y and O mice. Lifetime exercise may increase neurogenesis-associated anxiety. This could be an evolutionary conserved adaptation that nevertheless has adverse impacts on cognition-like function, with particularly pronounced effects in Y female mice with intact sex hormones. These issues require careful investigation in future rodent studies.

[1] This chapter has been published and the text has been included here with minimal changes to the published article.

Research report preamble

Chapter one has described that preclinical research to date have demonstrated that chronic exercise can contribute to improving anxiety-like, depression-like, and impaired cognition-like behaviours. However, such research has largely been conducted in adult male animals utilising exercise protocols of up to one month, and there remains a lack of understanding of the effects of lifetime voluntary exercise in male and female mice on anxiety-like, depression-like, and cognition-like behaviours.

RESEARCH QUESTION: The research reported in this chapter addresses the question *"What are the effects of lifetime voluntary exercise on anxiety-like, depression-like, and cognition-like behaviours over the lifespan in young adult (Y), early middle age (M), and old (O) mice?"*

STUDY AIM: The aim of this research was therefore to investigate the effects of lifetime voluntary exercise on anxiety-like, depression-like, and cognition-like behaviours in normal healthy aging C57BL/6 mice over the adult lifespan in Y, M, and O mice.

Twelve week-old C57BL/6 mice were randomly assigned to voluntary wheel running or non-exercise (control) groups. Exercise commenced at 3 months of age, and behaviours were assessed at four, nine, and fourteen months of age in Y, M, and old O mice respectively (n=11-17/group). The open field and elevated zero maze examined anxiety-like behaviours, depression-like behaviours were quantified with the forced swim test, and the Y maze and Barnes maze investigated cognition-like behaviours.

This research report describes that the effects of lifetime exercise were not simply an extension of the effects of chronic exercise on anxiety-like, depression-like, and cognition-like behaviours. Exercise tended to reduce overt anxiety-like behaviours with ageing, and improved recognition memory in M mice as was expected. Interestingly however, exercise increased anxiety-like freezing behaviours that extended spatial learning latencies in Y female mice in particular, and reduced distances travelled contributed to longer spatial memory and cognitive flexibility latencies in Y and O mice.

Introduction

Depression, anxiety, and cognitive impairment are some of the most prevalent neuropsychiatric conditions currently requiring therapeutic solutions. World Health Organisation reports indicate that depression is currently the leading cause of disability worldwide (World Health Organisation 2018). The prevalence of depression is highest in young adulthood with reductions in middle age and increases in older age, and is twice as common in women (Sutin, Terracciano et al. 2013, World Health Organisation 2016). However there is considerable overlap between anxiety, depression, and cognitive impairment, and around 90% of patients with depression experience comorbid anxiety (Gorman 1996). Depression also shows features of cognitive impairment with impairments to spatial and verbal memory and processing speed (Snyder 2013), and interestingly, recent work has found that mild cognitive impairment also increases the risk for depression and anxiety (Mirza, Ikram et al. 2017). Furthermore, although it has been widely suggested that depression may increase the risk for dementia (Saczynski, Beiser et al. 2010), current work shows this may not be the case (Singh-Manoux, Dugravot et al. 2017), although depression in older individuals (aged over 65) may contribute to cognitive impairment (Richard, Reitz et al. 2013). Considered together, these findings are suggestive of common pathways for these conditions, and this suggests that therapies that can treat all these conditions are preferable to those that address only one condition or another.

One therapy with potential for the prevention of depression, anxiety and cognitive impairment with aging is physical exercise. Rodent studies have shown chronic voluntary wheel running can be effective for improving depression-like, anxiety-like, and cognition-like behaviours (van Praag, Christie et al. 1999, Duman, Schlesinger et al. 2008, Castilla-Ortega, Rosell-Valle et al. 2013). For instance, adult mice that had *ad libitum* access to running wheels for three or four weeks have showed reductions in immobility time in the forced swim test (FST) (Duman, Schlesinger et al. 2008, Cunha, Oliveira et al. 2013). Similarly, studies examining 2 to 4 weeks of running in adult male C57BL/6 (wild type, WT) mice found exercise increased centre crossings and time spent in the centre of the open field (Duman, Schlesinger et al. 2008, Salam, Fox et al. 2009) suggesting chronic running reduces anxiety-like behaviours in adult male mice. Exercise induced changes in cognition-like functioning are consistent with the benefits of exercise for depression-like and anxiety-like behaviours. Improvements in latencies to locate the platform in the Morris water maze have been found in adult mice and rats following 4 weeks of running (Liu, Chen et al. 2009, Yau, Lau et al. 2012). Moreover, the beneficial effects of exercise for anxiety-like and cognition-like behaviours are also evident in aging animals (Pietrelli, Lopez-Costa et al. 2012). Evidence of anxiolytic effects of exercise have been found in 18 month old animals, with increases in the time spent in the central zone, and reductions in time spent in the closed arms of the elevated plus maze (EPM) (Pietrelli, Lopez-Costa et al. 2012). In addition, chronic running improved cognition-like behaviours as was evident in a greater number of correct entries in the radial maze in aging animals (Pietrelli, Lopez-Costa et al. 2012).

Chronic wheel running therefore appears to reduce anxiety-like and depression-like behaviours, and improve aspects of cognition-like behaviours in adult mice and rats, and these changes are also apparent in older animals. Notwithstanding this, there is limited work investigating the effects of chronic exercise on depression-like behaviours during normal healthy aging and in older robust and healthy animals. Nevertheless, considered together, these findings suggest that longer term exercise over the lifespan or active aging may have greater anxiolytic and pro-cognitive effects, and may reduce depression-like behaviours occurring with aging, and may have potential for more extensive changes in these behaviours.

However, the majority of research investigating the effects of chronic voluntary running on these behaviours of interest have been conducted with adult animals (Duman, Schlesinger et al. 2008, Salam, Fox et al. 2009, Yau, Lau et al. 2012, Cunha, Oliveira et al. 2013, Ebada, Kendall et al. 2016, Jones, Gupton et al. 2016) and this raises questions about the effects of lifelong exercise on behaviours with healthy aging animals. We hypothesise that long term exercise is associated with favourable behaviours compared to healthy age matched control non-exercise mice. This study therefore aims to investigate the effects of lifelong voluntary wheel running on ageing-associated alterations in anxiety-like, depression-like, and cognition-like behaviours in normal healthy aging male and female C57BL/6 mice over the adult lifespan from young adulthood, middle age, to older animals.

Methods

Animals

Eight week old C57BL/6 (WT) mice (n = 80, 38 males and 42 females) were purchased from the University of Adelaide. We chose C57BL/6 mice because they have shown the behavioural responses to exercise of interest (van Praag, Shubert et al. 2005, Duman, Schlesinger et al. 2008, Clark, Brzezinska et al. 2009). Animals were first housed in IVC cages approximately 3-6 per cage and given at least 1 week to acclimatise to the facility prior to the commencement of the study. Mice were given *ad libitum* access to standard laboratory chow and water. Environmental conditions were maintained at 21±1 °C with a 12 hour light/dark cycle (lights on 7:00-21:00). This experiment was conducted and reported in accordance with the ARRIVE guidelines (Kilkenny, Browne et al. 2014) for reporting in vivo experiments, and the University of Adelaide Animal Ethics Committee approved this study (M216-12). Animal husbandry and welfare was conducted according to ethical and animal facility guidelines, and the numbers of animals used and their suffering were minimised wherever possible.

Experimental design

At 12 weeks of age approximately equal numbers of healthy male and female mice (total n = 80) were block randomised in pairs for treatment allocation according to three time-points of behavioural evaluation (a) young adulthood; b) middle age; c) older middle age). These three cohorts remained independent throughout the study. All mice were transferred to open top caging and housed in pairs the duration of the study (unless fighting in males necessitated separation). Control mice (n = 38) were housed in standard open top housing (48.5cm x 15.5cm x 12cm) and received no exercise running wheel intervention. Exercise mice (n = 42) were housed in open top plexiglass cages (37cm x 20.5cm x 13.5cm) to accommodate the running wheel (12cm x 5.5cm). Wheel revolutions were manually recorded weekly from a digital counter. Exercise mice had continuous access to running wheels until different stages of adulthood: young adulthood (Goh and Ladiges 2013), middle age (D'Antona, Ragni et al. 2010), or older middle age (Packer and Hoffman-Goetz 2015) (Table 2.1). Behavioural testing was then conducted for four weeks (Table 2.1).

Table 2. 1 Durations of exercise in ageing C57BL/6 mice.

Group	Start of exercise	Behavioural testing	No. of mice	Control (male: female)	Exercise (male: female)
Y	3 months	4 months	22	11 (6: 5)	11 (5: 6)
M	3 months	9 months	27	13 (6: 7)	14 (9: 7)
O	3 months	14 months	31	14 (7: 6)	17 (9: 12)

Behavioural testing

Behavioural testing was conducted over 4 weeks. Tests were conducted in the light phase of the cycle and completed in order of the least to most stressful. To minimise the possible stressful effects of prior testing there was a minimum of one day between tests (Camara, Corrigan et al. 2013). All testing was conducted utilising ANYmaze video tracking software (Stoelting Company (USA)). Tests were conducted in the following order 1) Home cage; 2) Open field; 3) Elevated zero maze; 4) Y maze; 5) Barnes maze; 6) Forced swim test. Testing areas were thoroughly cleaned between tests with F10SC Veterinary Disinfectant to remove any olfactory traces.

Locomotion

Baseline locomotion under non-stressful conditions was measured in the home cage with 2 day old bedding. The distance travelled was quantified for 5 minutes according to established protocols (Baune and Wiede 2008).

Open field test

We also quantified baseline locomotion and anxiety-like behaviours in more stressful conditions of the open field. Mice were placed in the North-West corner of a well-lit 40 x 40cm plexiglass box. The measures recorded included the distance travelled and time spent in the inner and outer zones of the field, and these were quantified for 5 minutes in line with published protocols (Baune, Wiede et al. 2008)

Elevated zero maze

Anxiety-like and exploratory behaviours were also investigated using the elevated zero maze (EZM). The EZM is a circular elevated platform (40cm high, a diameter of 50cm, and 5cm wide) with four quadrants. Two closed quadrants have inner and outer walls 27cm high while the open quadrants have none. Mice were placed on the centre of the southern open quadrant to enable exploration whilst we recorded times spent in the open quadrants and head dipping for 5 minutes.

Y maze

The Y maze was utilised to measure hippocampal dependent spatial recognition memory. The Y maze has 3 arms shaped as a 'Y' (35cm long, 5cm wide, and 10cm high) that are at 120° angle to one another (Choy, de Visser et al. 2008) and is undertaken in 2 phases.

Phase 1

Mice were placed in the lower (southern arm) and allowed 10 minutes of exploration with one of the lateral arms closed.

Phase 2

Mice were again placed into the southern arm of the maze thirty minutes following phase 1. In phase two, the mazes' three arms are open for exploration for 5 minutes. Healthy mice with intact hippocampal learning and memory have a preference for the exploration of a novel environment (Dulawa, Grandy et al. 1999). However if mice have memory impairments they will not recognise the novel arm resulting in a greater amount of time being spent in the familiar arm of the maze.

Barnes maze

We utilised the Barnes maze to examine spatial learning and memory, and cognitive flexibility. The Barnes maze is a well-lit round table 91cm in diameter with 19 false escape boxes and 1 genuine escape box.

Training days, days 1 -4

The latency to locate the escape box over four days of training was used as a measure of spatial learning. On each of the four training days mice were placed in the centre of the table under a removable chamber. Mice were given 3 minutes to learn the location of the escape box. Mice that failed to learn the box's location were guided to its location where they resided for 1 minute. Animals were given 3 trials per day on each of the 4 days of training.

Probe trials

On day 5, the table was rotated clockwise 90 degrees. Mice were again placed on the centre of the table, and given 3 minutes to locate the new location of the escape box. Latencies to locate the escape box on day 5 were measures of spatial memory, and the latency to identify the new escape box location were taken as a measures of cognitive flexibility because it constituted the time taken to change focus from the old location of the box, and identify its new location.

Forced swim test

Depression-like behaviour was quantified with the forced swim test. We quantified anhedonia type behaviour using immobility time as a measure of despair-like behaviour (Petit-Demouliere, Chenu et al. 2005). Immobility was defined as floating with or without the small movements that contribute to maintaining equilibrium, but that do not contribute to forward movement as in swimming or climbing.

Statistical methods

Primary outcomes were behaviours involved in the depression phenotype and included

anxiety-like, depression-like, and cognition-like behaviours. The Shapiro-Wilk test for normality was used. To accommodate and analyse exercise distances travelled from groups of difference sizes (Table 2.1), analyses of mean daily distances travelled was conducted using linear mixed-effects modelling clustering on mouse for repeated measures, and utilising an AR(1) convergence structure for best model fit. The assumptions of normal distribution of residuals and homoscedasticity were confirmed by visual inspection of scatterplots and histograms of the residuals and predicted values. One model was fit for the mean daily distances travelled over the first eight weeks, and another for distances travelled from one to six months. Two-way ANOVA with Sidak's post hoc test for multiple comparisons were conducted for analyses of i) commencement and completion animal weights (control vs. exercise; male vs. female); ii) Barnes maze data between control and exercise groups and within groups over the 4 training days, and iii) to investigate differences between male and female animals in control and exercise groups in all behavioural tests. All other tests comparing control and exercise groups within each age cohort including differences between male and female mice were analysed by unpaired student's t tests for normally distributed data, and Mann-Whitney tests for data that was not normally distributed. Linear mixed-effects models were performed in SPSS (version 24), and the analyses of all other data were performed in GraphPad Prism (6 version 008, and version 7) and the data presented are mean ± SE.

Results

Animals

T-test analyses of animal body weights in Y, M, and O mice at the start and end of the experiment showed all groups maintained healthy body weights over the experiment. Female M and O control mice weighed significantly less than male M and O control mice at the start of the experiment ($p = 0.006$ and $p = 0.01$), whilst Y and M female exercise mice weighed less than Y and M exercise male mice at the experiment start ($p = 0.001$ and $p = 0.005$). At the end of the experiment, only M control and exercise female mice weighed less than their male counterparts ($p = 0.03$ and $p = 0.01$) (Table 2.2).

Table 2. 2. Mouse body weights at the start and end of the experimental period.

Group	Start of experiment				End of experiment			
	Control		Exercise		Control		Exercise	
	m	f	m	f	m	f	m	f
Y	22.80 (±0.91)	20.40 (±0.24)	24.50 (±1.23)	19.6** (±0.61)	23.40 (± 1.32)	22.0 (±0.31)	24.2 (±0.02)	21.0 (±2.33)
M	25.83 (±0.87)	21.43** (±0.29)	26.14 (±1.14)	20.71** (±0.25)	23.0* (±1.59)	27.0* (±0.75)	32.29* (±1.24)	24.86* (±0.88)
O	22.22 (±0.40)	20.50* (±0.50)	21.42 (±0.45)	21.50 (±0.83)	26.6 (±1.03)	28.5 (±0.61)	27.30 (±0.47)	26.0 (±1.04)

Legend: Data presented are the means (± SEM) in grams *$p < 0.05$, and **$p < 0.01$, n = 11-17/group).

Distances travelled

To investigate the distances travelled by Y, M, and O mice over the experimental period we

performed linear mixed-effects modelling of the mean daily distances travelled by Y, M, and O mice over the first four weeks (Figure 2.1A), and the mean daily distances travelled by M and O mice from month one to six (Figure 2.1B). Analyses of mean daily distances travelled over the first four weeks by Y, M, and O mice found a non-significant interaction between group and time ($p = 0.213$), suggesting there were no significant differences over the four weeks in the associations between distances travelled and group, and this was confirmed by post hoc analyses with Bonferroni correction for multiple comparisons. Similarly, linear mixed-effects model analyses of mean daily distances travelled in M and O mice over months one to six found no significant group by time interaction ($p = 0.867$). However, following adjustment for multiple post-hoc comparisons with Bonferroni correction, a significant comparison was found showing M mice demonstrated daily mean distances travelled of 1.189km less than O mice in month four ($p = 0.042$) (Figure 2.1B). Interestingly however, there was an overall non-significant pattern of M female mice running longer distances in the M and O groups. Female M mice travelled greater distances than male M mice in months one, two, three, and five, and female O mice ran greater distances than male O mice in all months except month three, with a pattern of a trend towards significant differences in months seven, eight, nine, and 10 ($p = 0.085$, $p = 0.085$, $p = 0.067$, and $p = 0.062$) (Table 2.3). In addition, there were modest reductions in the distances travelled was evident over time in O mice from month four that continued through months seven, eight, nine, and ten, however these were not significant (compared to month four showing the greatest exercise: month 5 $p = 0.810$; month 6 $p > 0.786$; month 7 $p = 0.401$; month 8 $p = 0.302$; month 9 $p = 0.244$, month 10 $p = 0.096$, and month 11 $p = 0.737$), and are therefore unlikely to have effected behaviours.

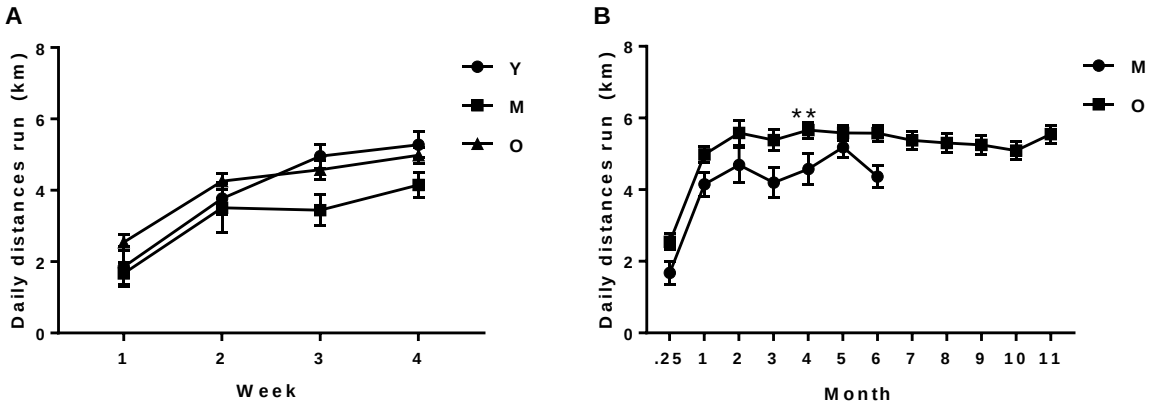

Figure 2.1. Distances travelled over the experimental period for C57BL/6 mice.
A) Mean daily distances travelled over months by M and O mice (Bonferroni post hoc correction for multiple comparisons significance level of 0.007; **p=0.006 for M vs. O mice, N = 11 mice/group).

Effects of exercise on behaviours

Baseline locomotion

Baseline locomotion was quantified in the non-stressful environment of the animals' home cages with running wheels removed. Unpaired t-tests found that compared to control mice, exercised mice travelled less distance in the home cage in Y and O mice but not at in M mice ($p = 0.001$, $p = 0.114$, and $p = 0.033$ respectively) (Figure 2.2A-C). Differences between males and females in the distances travelled were evident in all age groups. Exercise had a gender

effect in M and O mice (p = 0.007, p = 0.014), but not in Y mice (0.180). Post hoc analyses showed M female control mice and O male control mice travelled significantly greater distances compared to their male and female counterparts respectively (p = 0.001 and p = 0.004).

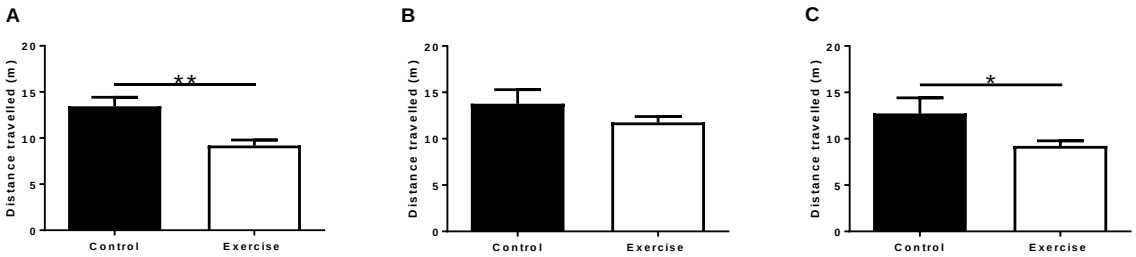

Figure 2.1. Baseline locomotion for C57BL/6 mice over the lifespan.
Distances travelled in the home cage for mice in A) Y mice, B) M mice, and C) O mice (**p < 0.01, *p < 0.05, N = 11-17mice/group).

Open field

Baseline locomotion was also quantified in the more stressful environment of the Open field. There was no difference in the distances travelled in this environment between control and exercise mice at 4 months (p =0.584) as was found by unpaired t-test, but exercised mice travelled significantly less than control mice in M mice and O mice (p = 0.0005, and p = 0.053) (Figure 2.3A-C). There was no effect of sex in distances travelled at any age.

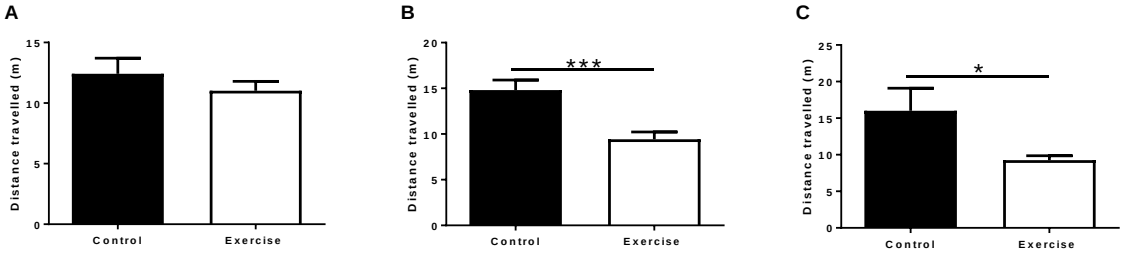

Figure 2.2. Distances travelled in the Open Field by C57BL/6 mice over the lifespan.
A) in Y mice, B) in M mice, and C) in O mice (***p < 0.001, *p = 0.05, N = 11-17mice/group).

Table 2. 3. Mean daily distances travelled by Y, M, and O C57BL/6 mice over the experimental period.

Age group	Month										
	1	2	3	4	5	6	7	8	9	10	11
Y	4.421 ± 0.680										
Male	5.927 ± 1.087										
Female	4.953 ± 0.144										
P value	0.231										
M	4.152 ± 0.307	4.689 ± 0.337	4.193 ± 0.416	4.576 ± 0.417	5.181 ± 0.287	4.362 ± 0.307					
Male	4.051 ± 0.168	4.480 ± 0.682	3.948 ± 0.424	4.663 ± 0.395	4.966 ± 0.465	3.597 ± 1.200					
Female	4.253 ± 0.676	4.897 ± 0.705	4.439 ± 0.738	4.489 ± 0.767	5.468 ± 0.259	5.0 ± 0.408					
P value	0.776	0.677	0.573	0.843	0.409	0.0125*					
O	4.986 ± 0.224	5.582 ± 0.360	5.383 ± 0.299	5.661 ± 0.224	5.585 ± 0.218	5.576 ± 0.216	5.378 ± 0.246	5.300 ± 0.262	5.252 ± 0.263	5.091 ± 0.249	5.548 ± 0.248
Male	4.950 ± 0.295	5.316 ± 0.625	5.396 ± 0.396	5.361 ± 0.297	5.284 ± 0.302	5.251 ± 0.303	4.922 ± 0.343	4.888 ± 0.364	4.815 ± 0.370	4.670 ± 0.355	5.275 ± 0.417
Female	5.028 ± 0.359	5.90 ± 0.265	5.386 ± 0.479	6.021 ± 0.320	5.946 ± 0.289	5.966 ± 0.271	5.842. ± 0.343	5.795 ± 0.330	5.777 ± 0.314	5.596 ± 0.285	5.767 ± 0.299
P value	0.867	0.432	0.992	0.146	0.134	0.100	0.085	0.085	0.067	0.062	0.340

Legend: Data presented are the mean +/- SEM (*male versus female, *$p < 0.05$, N = 11-17/group).

Anxiety-like behaviours

Open field

The centre regions of the open field are considered anxiogenic, so anxiety-like behaviour was measured by quantifying time spent in the centre of the Open Field. The unpaired t-test found no differences between control and exercise mice in centre time in Y mice ($p = 0.205$) (Figure 2.4). Compared to control mice, exercised mice spent significantly more time in the centre of the open field in M mice and O mice ($p = 0.021$ and $p = 0.026$). There was no main effect of the animals' sex on time spent in the centre of the open field in Y mice ($p = 0.140$), whereas a significant sex by treatment interaction effect was evident in M mice, and a significant main effect of sex was evident in O mice ($p = 0.025$ and $p = 0.039$). This remained significant in post hoc testing only in M mice, where male exercise mice displayed greater centre time than female exercise mice ($p = 0.027$).

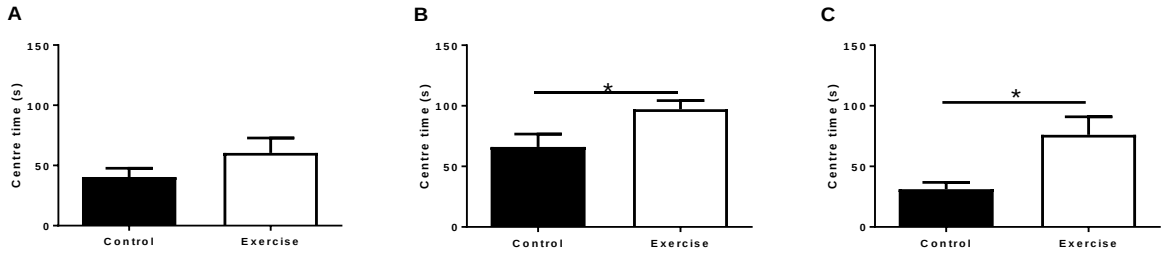

Figure 2 3. Time spent in the centre of the Open Field for C57BL/6 mice over the lifespan. Open Field centre time in A) Y mice, B) M mice, and C) O mice (*$p < 0.05$, N = 11-17mice/group).

Elevated zero maze

Anxiety-like behaviours were also assessed by measuring the time spent in the open quadrants of the Elevated Zero Maze. Interestingly, unpaired t-tests showed there were no significant differences between exercised mice and control mice in open quadrant time in Y or O mice ($p = 0.322$ and $p = 0.801$), whereas exercised mice demonstrated significantly greater time in the open quadrants of the EZM in M mice ($p = 0.036$) (Figure 2.5A-C).

Anxiety-like behaviours were further examined by quantifying head dipping behaviours over the edge of the maze. There were no differences between control and exercise mice in Y or O mice, but exercised mice demonstrated greater head dipping behaviour than control mice in M mice ($p < 0.0001$) (Figure 2.5D-F). There were no differences between male and female mice for time spent in the open quadrant at any age, or in the number of head dips for Y or O mice. However a significant effect of sex was evident in M exercise mice ($p = 0.005$), and Sidak's post hoc testing found significantly greater head dipping in female exercise mice compared to male exercise mice ($p = 0.021$).

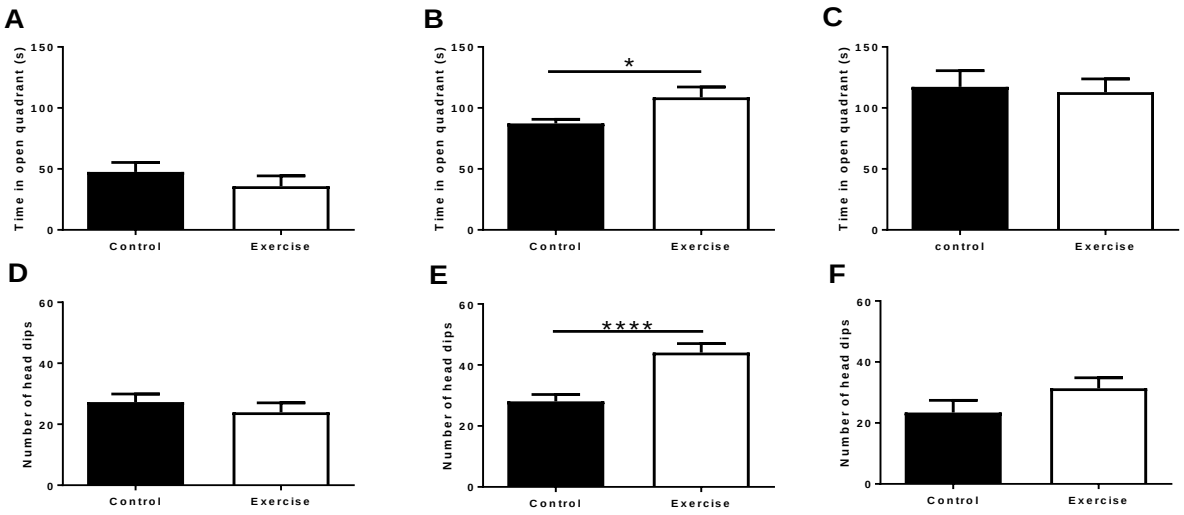

Figure 2.4. Anxiety-like behaviours in the EZM in middle aged C57Bl/6 mice.
Time in the open quadrants of the Elevated Zero Maze in A) Y mice, B) M mice, and C) O mice. Head dipping over the edge of the Elevated Zero Maze in D), Y mice E), M mice, and F) O mice (* = < 0.05, and ****p < 0.0001, N = 11-17mice/group).

Depression-like behaviour

Forced swim test

Immobility time in the Forced Swim Test is regarded as a measure of anhedonia or despair, and is thought to be a measure of depression-like behaviour. Contrary to expectations, compared to control mice, exercised mice demonstrated no differences in immobility time at any age (Figure 2.6). There were also no differences in immobility time found between male and female control or exercise mice at any age.

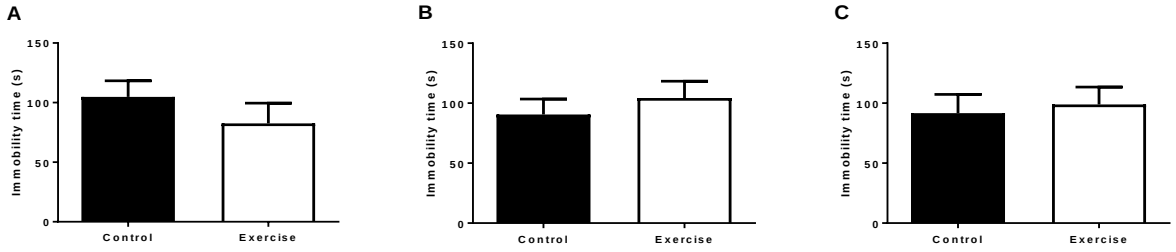

Figure 2.5. Depression-like behaviour in the Forced Swim Test in C57BL/6 mice.
A) In Y mice, B) M mice, and C) O mice (N = 11-17mice/group).

Cognition-like behaviour

Y maze

We investigated the effects of exercise on spatial recognition memory over the lifespan utilising the Y maze. Unpaired t-tests found no differences between control and exercised mice in spatial recognition memory in Y mice (p = 0.230), however exercise mice displayed an increased preference index in M mice (p = 0.015), that reduced with aging in O mice (p = 0.007) (Figure 2.7A-C). There was no main effect of sex noted at any age.

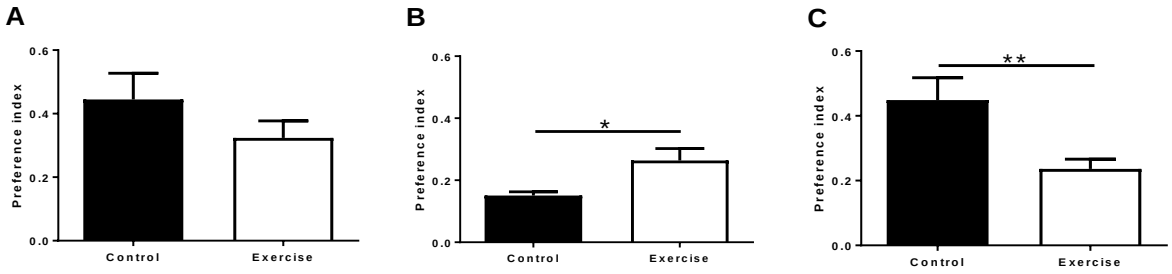

Figure 2.6. Preference index of recognition memory over the lifespan.
Recognition memory in A), Y mice B), M mice, and C) O mice (*p < 0.05, **p < 0.01, N = 11-17mice/group).

Barnes maze

The Barnes maze investigated spatial learning, memory, and cognitive flexibility over the lifespan. Mice were assessed on their capacity to learn the location of an escape box over four days of training and the latency to locate the box was taken as a measure of spatial learning. Curiously, Y exercise mice showed longer latencies to the escape box compared to control mice on training days 1, 3 and 4 ($p = 0.017$, $p = 0.011$ and $p < 0.001$) (Figure 2.8A) however this was largely explained by increased latencies for female mice to locate the escape box on days 2, 3, and 4 ($p = 0.026$, $p = 0.006$, and $p = 0.0003$). There were no differences in training day latencies evident in M mice, but exercise mice displayed increased latencies on day 2 in O mice ($p < 0.001$)(Figure 2.8B-C).

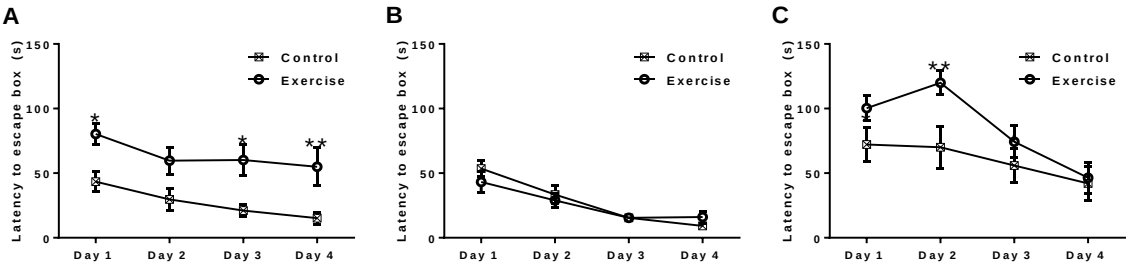

Figure 2.8. The Barnes maze over four days of training in C57BL/6 mice.
Latencies to the Barnes maze escape box in A) Y mice, B) M mice, and C) O mice (* = p < 0.05, ** p < 0.01, N = 11-17mice/group).

Following four days of training, a probe trial was conducted on day five where the position of the training day escape box was rotated clockwise 90 degrees. Latencies to locate the training day escape box location and the new escape box locations were recorded to quantify spatial retention memory and cognitive flexibility respectively. Compared to control mice, exercise mice Y and O mice displayed increased latencies to locate both the original box location and the new box location (Table 2.4). There were no differences between the sexes at any age to identify the old or the new locations of the escape box in the probe trial.

Table 2. 4. Barnes maze probe trial latencies to escape box locations during the Probe trial.

	Time to find training escape box location (s)		Time to find new escape box location (s)	
	Control	Exercise	Control	Exercise
Y mice	27.627 ± 17.021	68.864 ± 24.255†	69.155 ± 15.837	80.918 ± 20.950
M mice	7.736 ± 1.778	10.454 ± 2.740	62.369 ± 15.987	54.621 ± 8.929
O mice	62.557 ± 21.034	90.441 ± 19.390‡	87.964 ± 18.383	166.610 ± 6.396‡‡

Table 4. Barnes maze probe trial latencies to escape box locations during the Probe trial. ($^{\dagger}p < 0.05$, $^{\ddagger}p < 0.01$, $^{\ddagger\ddagger}p < 0.001$ compared to control mice, N = 11 -17 mice/group).

Research report summary

Previous research investigating the effects of exercise on anxiety-like, depression-like, and cognition-like behaviours has shown that chronic exercise can reduce anxiety-like, depression-like, and cognition-like impairment behaviours. However, these studies are commonly limited to exercise protocols of up to one month in male adult animals, resulting in a lack of understanding about the effects of longer-term or lifetime exercise on these behaviours. This research therefore addressed the question *"What are the effects of lifetime exercise on anxiety-like, depression-like, and cognition-like behaviours over the lifespan in young adult (Y), early middle age (M), and old (O) mice?"*

We compared the effects of exercise over the lifespan from 12 weeks of age to non-exercise control mice in Y, M, and O mice utilising the open field and EZM to investigate anxiety-like behaviours, the Y maze and Barnes maze to examine cognition-like behaviours, and the forced swim test to quantify depression-like behaviours.

Our results suggested the effects of lifetime exercise were not largely similar to the effects of chronic exercise on anxiety-like, depression-like, and cognition-like behaviours. As was anticipated, we found lifetime exercise reduced overt anxiety-like behaviours with ageing, and enhanced recognition memory in middle aged mice. However, contrary to our expectations, we found increased anxiety-like behaviours of freezing that appeared to impair spatial learning in Y female mice, and longer spatial memory and cognitive flexibility latencies arising from reduced distances travelled in Y and O mice. These results are discussed in detail in the Discussion in chapter six.

Chapter 3. TNF signalling via the TNF receptors mediates the effects of exercise on cognition-like behaviours.

Statement of Authorship

Title of Paper	TNF signalling via the TNF receptors mediates the effects of exercise on cognition-like behaviours.		
Publication Details	Morgan, J. A., G. Singhal, F. Corrigan, E. J. Jaehne, M. C. Jawahar and B. T. Baune (2018). "TNF signalling via the TNF receptors mediates the effects of exercise on cognition-like behaviours." Behavioural Brain Research 353: 74-82.		
Name of Principal Author (Candidate)	Julie A. Morgan		
Contribution to the Paper	Data collection utilising mouse models and wet lab techniques, data analyses, interpretation, writing and redrafting manuscripts. Overall percentage: 80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	28th November, 2018
Name of Co-Author	Dr Frances Corrigan		
Contribution to the Paper	Conception of the project, design of methodology or experimental protocol, teaching experimental techniques, editing and revision of manuscript drafts.		
Signature		Signature	28th November, 2018
Name of Co-Author	Dr Magdalene C. Jawahar		
Contribution to the Paper	Conception of the project, design of methodology or experimental protocol, teaching experimental techniques, editing and revision of manuscript drafts.		
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Name of Co-Author	Dr Emily J. Jaehne		
Contribution to the Paper	Conception of the project, design of methodology or experimental protocol, teaching experimental techniques, editing and revision of manuscript drafts.		
Signature		Signature	28th November, 2018
Name of Co-Author	Dr Gaurav Singhal		
Contribution to the Paper	Assistance with planning and conducting mouse husbandry and laboratory techniques.		
Signature		Signature	28th November, 2018
Name of Co-Author	Professor Bernhard T. Baune		
Contribution to the Paper	Conception of the project, design of methodology or experimental protocol, guidance about manuscript drafts.		
Signature		Signature	28th November, 2018

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

"TNF signalling via the TNF receptors mediates the effects of exercise on cognition-like behaviours."[2]

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Research report

TNF signalling via the TNF receptors mediates the effects of exercise on cognition-like behaviours.

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ABSTRACT

Background: Altered TNF levels are associated with cognitive impairment in depression, schizophrenia, bipolar disorder, and Alzheimer's disease (AD). Exercise improves cognition-like behaviours, reduces the expression of tumour necrosis factor alpha (TNF), and increases expression of the soluble TNF receptors soluble TNFR1 (sTNFR1) and sTNFR2. We suggest TNF and its receptors are involved in cognitive function and dysfunction, and investigate whether exercise mediates its effects on cognitive function via TNF and its receptors.
Methods: We utilised C57BL/6, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice to compare exercise to non-exercise control groups to investigate whether exercise exerts its effects on various types of cognition-like behaviours via TNF and its receptors.
Results: Recognition memory improved with exercise in WT mice, was impaired in $TNFR1^{-/-}$ exercise mice, showed non-significant impairment with exercise in $TNF^{-/-}$ mice, and no changes in $TNFR2^{-/-}$ mice. In spatial learning there were exercise related improvements in WT mice, non-significant but meaningful impairments evident in $TNFR1^{-/-}$ exercise mice, modest improvement in $TNF^{-/-}$ exercise mice, and potentially meaningful non-significant improvements in $TNFR2^{-/-}$ exercise mice. Moreover, WT and $TNFR2^{-/-}$ mice displayed noteworthy non-significant improvements in spatial memory, whereas $TNFR1^{-/-}$ exercise mice demonstrated non-significant spatial memory impairment. Exercise did not alter cognitive flexibility in any strain.
Discussion: TNF receptor signalling via the TNFR1 and TNFR2 appears to mediate the effects of exercise on cognitive-like behaviours. The potential for exercise to regulate human TNF and TNF signalling and cognitive dysfunction needs investigation under inflammatory conditions including depression and neuropsychiatric disorders.

[2]This chapter has been published and the text has been included here with minimal changes to the published article.

Research report preamble

The previous study investigated the effects of lifetime exercise on anxiety-like, depression-like and cognition-like behaviours over the lifespan and found the effects of lifetime exercise were not as similar as chronic exercise as we had anticipated. However, previous clinical research suggests that exercise might confer its effects on depression- and related behaviours through changes in pro-inflammatory cytokines, however the question of TNF signalling contributes to the effects of long-term exercise on cognition-like behaviours remains unknown. Thus, in this chapter, we investigate whether TNF signalling via the TNF receptors confers the effects of exercise on cognition-like behaviours.

There is growing evidence that altered TNF levels are associated with cognitive impairment in neuropsychiatric conditions such as depression, schizophrenia, bipolar disorder, and Alzheimer's disease (AD), making the modulation of TNF signalling without adverse side effects current therapeutic aim. Research investigating chronic exercise for cognitive function have demonstrated that exercise can improve cognition-like behaviours in rodents, reduce the expression of TNF, and increase expression of the sTNFR1 and sTNFR2. However there remains limited understanding about whether TNF signalling via the TNFR1 (predominantly neurodegenerative signalling) and TNFR2 (predominantly neuroprotective signalling) mediate the effects of exercise on cognitive-like function.

RESEARCH QUESTION: This research addresses the question *"Does TNF signalling via the TNFR1 and TNFR2 mediate the effects of exercise on cognition-like behaviours in C57BL/6, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice?"*

STUDY AIMS: The aim of this research is to investigate the TNF signalling mediated effects of long-term exercise on cognition-like behaviours by utilising voluntary wheel running in a transgenic mouse model of TNF, TNFR1, and TNFR2 knockout. We considered that exercise would improve cognitive function in WT and $TNFR1^{-/-}$ mice, but not in $TNFR2^{-/-}$ or $TNF^{-/-}$ mice.

This study utilised C57BL/6, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice to compare exercise to non-exercise control groups to investigate whether exercise effects various cognition-like behaviours via TNF and its receptors.

In this paper we report that recognition memory improved with exercise in WT mice. However we found impairments in recognition memory in $TNFR1^{-/-}$ exercise mice, non-significant impairment with exercise in $TNF^{-/-}$ mice, and no changes in $TNFR2^{-/-}$ mice. Exercise improved spatial learning in WT mice, and resulted in non-significant but meaningful impairments evident in $TNFR1^{-/-}$ exercise mice, modest improvement in $TNF^{-/-}$ exercise mice, and potentially meaningful non-significant improvements in $TNFR2^{-/-}$ exercise mice. Moreover, WT and $TNFR2^{-/-}$ mice displayed noteworthy non-significant improvements in spatial memory, whereas $TNFR1^{-/-}$ exercise mice demonstrated non-significant spatial memory impairment. Exercise did not alter cognitive flexibility in any strain.

Introduction

Cognitive impairment is a defining characteristic of neuropsychiatric conditions including depression, schizophrenia, bipolar disorder, mild cognitive impairment, and Alzheimer's disease (AD). Whilst TNF at physiological levels is required for normal cognitive functioning (Baune and Wiede 2008, Camara, Corrigan et al. 2013), altered TNF is significantly associated with cognitive impairment in depression, schizophrenia, bipolar disorder, and AD (Doganavsargil-Baysal, Cinemre et al. 2013, Lv, Tan et al. 2015, Bobinska, Galecka et al. 2017, Hennessy, Gormley et al. 2017, Kim, Lee et al. 2017). TNF signals via the membrane bound receptors TNFR1 and TNFR2, and the soluble TNF receptors sTNFR1 and sTNFR2. TNFR1 is found on most cells, whilst TNFR2 is found only on haematopoietic and endothelial cells (Grell, Douni et al. 1995, Morgan, Singhal et al. 2018). The soluble TNF receptors are found in circulating bloods and are formed by the cleavage of the extracellular component of membrane bound TNFR1 and TNFR2 into the soluble TNF receptor 1 (sTNFR1) and soluble TNF receptor 2 (sTNFR2) (Waetzig, Rosenstiel et al. 2005).

TNFR1 and TNFR2 are thought to have physiologically distinct functions. TNFR1 negatively regulates cellular proliferation and is distinct from the TNFR2, because it contains a death domain involved in triggering apoptosis cascades (Sedger and McDermott 2014), whereas TNFR2 is considered to have a neuroprotective function. This arises through the inhibition of caspase cascades, and the protection of neurons from glutamate induced excitotoxicity (Wajant, Pfizenmaier et al. 2003, Marchetti, Klein et al. 2004, Baune, Camara et al. 2012, Morgan, Singhal et al. 2018). Interestingly, the sTNFR1 plays noteworthy roles in the regulation of TNF in physiological conditions, and this occurs via the inhibition of the pro-inflammatory bioactivity of TNF signalling via the TNF receptors TNFR1 (Aderka, Engelmann et al. 1992, Pinckard, Sheehan et al. 1997). sTNFR1 is considered an antagonist for TNFR1, and together with its role in buffering circulating TNF may have beneficial effects in the context of TNF induced cognitive impairment. In physiological conditions, constitutive levels of TNF are critical for normal hippocampal neural plasticity, with TNF signalling via the TNFR1 and TNFR2 in concert considered to maintain normal cellular functioning (Albensi and Mattson 2000, Morgan, Singhal et al. 2018). However, a shift towards TNF signalling via the TNFR1 is thought to induce increases in cellular apoptosis contributing to neurodegeneration and cognitive impairment (Baune, Camara et al. 2012, Morgan, Singhal et al. 2018).

There may be potential for exercise to reduce pathologically elevated levels of TNF by altering TNF signalling and the TNF receptors. Acute exercise in healthy humans is characterised by marked changes in cytokine and cytokine receptor expression (Petersen and Pedersen 2005). Exercising muscle generated interleukin 6 (IL6) blunts the expression of TNF (Petersen and Pedersen 2005), but increases levels of the soluble TNF receptors sTNFR1 and sTNFR2, the interleukin 1 receptor antagonist (IL1ra), and the potent anti-inflammatory cytokine interleukin 10 (IL10) (Petersen and Pedersen 2005, Morgan, Singhal et al. 2018). Exercise generated IL6 therefore results in an overall anti-inflammatory environment that is considered to contribute to the anti-inflammatory effects of chronic exercise that may ameliorate cognitive impairment. Indeed, chronic exercise has demonstrated benefits for cognitive impairment and TNF levels in preclinical research. These include reductions in latencies to locate the hidden platform in the Morris water maze (Gibbons, Pence et al. 2014), reduced errors and improvements in spatial learning and spatial memory deficits in the radial arm water maze following four weeks of wheel running (Nichol, Parachikova et al. 2007,

Parachikova, Nichol et al. 2008, Nichol, Deeny et al. 2009), and improvements in short-term and long-term memory deficits (Nichol, Parachikova et al. 2007). In health, chronic exercise reduces hippocampal and peripheral TNF in mice (Keller, Keller et al. 2004, Nichol, Poon et al. 2008, Pervaiz and Hoffman-Goetz 2011) suggesting chronic exercise might improve cognitive-like impairment behaviours and slow the progression of neurodegeneration associated cognitive-impairment. Chronic exercise therefore has beneficial effects on aspects of cognitive impairment that may be associated with changes in TNF signalling via the TNF receptors however, its modulatory effect on cognitive function via TNF signalling via the TNFR1 and TNFR2 pathways remains unknown. This study therefore aims to investigate TNF signalling mediated effects of long-term exercise on cognition-like behaviours by applying voluntary wheel running in a transgenic mouse model of TNF, TNFR1, and TNFR2 knockout. Consistent with the requirement for TNF for normal cognitive performance, and the neuroprotective effects of TNFR2 and neurodegenerative effects of TNFR1, we hypothesised exercise would improve cognitive performance in WT and $TNFR1^{-/-}$ mice, but not in $TNFR2^{-/-}$ or $TNF^{-/-}$ mice.

Materials and methods

Animals

Eight week old C57BL/6 (WT) mice were purchased from the University of Adelaide or bred at University laboratory animal facility ($TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice). The C57BL/6 mouse strain deficient in TNF ($TNF^{-/-}$), TNFR1 ($TNFR1^{-/-}$) and $TNFR2^{-/-}$ were generated on a pure C57BL/6 genetic background (Körner, Cook et al. 1997, Camara, Corrigan et al. 2015). Due to breeding challenges with $TNFR2^{-/-}$ mice, these groups were smaller and the exercise group contained only one female (Table 4.1). Although the analyses of sex differences was therefore not possible, Chi-square tests found no significant differences in the proportions of male and female animals in control or exercise groups in any strain (Table 4.1). Animals were housed six per cage in IVC cages for one week to acclimatise to the facility until study commencement. Mice had continuous access to water and standard mouse chow, and environmental conditions were maintained at 21 ± 1°C with a 12 hour light/dark cycle (lights on 7:00-21:00). Ethics approval was granted by the University of Adelaide Animal Ethics Committee, and the number of animals and their suffering were minimised wherever possible.

Experimental design

Twelve week old $TNF^{-/-}$, $TNFR2^{-/-}$, or $TNFR1^{-/-}$ male and female mice were block randomised in pairs in approximately equal numbers to an exercise or control group for each strain. Animals were transferred in same sex pairs to open topped cages for control (48.5cm x 15.5cm x 12cm) or exercise mice (37cm x 20.5cm x 13.5cm, fitted with running wheels (12cm x 5.5cm) (Mini Mitter USA). Animal welfare was monitored daily. Exercise distances travelled in both the light and dark phases were manually recorded from running wheel digital counters weekly. Exercising mice had continuous access to running wheels for six months prior to behavioural testing. The mean monthly distances travelled were calculated from the weekly distances travelled. There were no adverse events during the experiment.

Table 3. 1 Treatment group numbers for mouse strains.

Strain	Control n (males: females)	Exercise n (males: females)	Male / female proportions
WT	19 (9: 10)	17 (7: 10)	0.721
$TNF^{-/-}$	19 (9: 10)	15 (8: 7)	0.732
$TNFR1^{-/-}$	17 (10: 7)	16 (9: 7)	0.863
$TNFR2^{-/-}$	6 (6:0)	6 (5: 1)	0.312

Legend: ^Chi-square test for significant differences in proportions of male and female control and exercise mice of all strains showed no significant differences between strains (adapted from (Morgan, Singhal et al. 2018)).

Behavioural assessments

Behavioural testing order was block randomised in pairs and conducted in the light phase of the cycle. Tests were conducted over three weeks from the least to most stressful to minimise the effects of stress impacting on test performance and the animals' welfare, and there was a minimum of 24 hours rest for animals between tests (Camara, Corrigan et al. 2013). Tests were conducted as follows: i) home cage locomotion, ii) open field locomotion iii) Y maze, and iv) Barnes maze (mice had previously undertaken testing for anxiety-like and depression-like behaviours as is reported in previous work from this group (Morgan, Singhal et al. 2018)). All tests were recorded utilising ANYmaze Video tracking software (Stoelting Company, USA), and testing areas were thoroughly cleaned with F10SC Veterinary Disinfectant between tests to remove olfactory cues.

Locomotor activity

Home cage locomotion

To investigate locomotion under non-stressful conditions, we quantified the locomotion of mice in their home cages on two day old bedding for 5 minutes according to published protocols (Baune and Wiede 2008).

Locomotion in the open field

We also examined whether locomotion in more stressful conditions of the open field was altered by exercise across strains. Mice were positioned in the North-West corner of the open field (a 40cm x 40cm plexiglass box) and the total distance travelled was recorded for 5 minutes according to published protocols (Baune and Wiede 2008).

Cognition-like behaviours

Recognition memory in the Y maze

Recognition memory was investigated with the Y maze preference index (time spent in the novel arm/total arm time). A lower Y maze preference index (YMPI) indicates poorer recognition memory, whereas a higher YMPI is suggestive of better recognition memory (Dulawa, Grandy et al. 1999, Camara, Corrigan et al. 2013). The Y maze is a "Y" shaped maze with arms 35cm long, 5cm wide, and 10cm high, and is conducted in two phases. Mice were

placed in the southern-most arm and allowed 10 minutes for exploration with one lateral arm closed. Thirty minutes later, mice were again placed into the southern arm with all arms open for five minutes exploration.

Barnes maze

Spatial learning, spatial memory, and cognitive-flexibility in the Barnes maze

Spatial learning, spatial memory, and cognitive flexibility were investigated utilising the Barnes maze. We utilised the latencies to identify the location of escape box over 4 days of training were utilised as a measure of spatial learning (Baune and Wiede 2008). On day 5, a probe trial was conducted, and the maze was rotated clockwise 90°. The latencies to a) locate the original box location was taken as a measure of spatial memory, and b) the latency to identify the new escape box location was utilised as a measure of cognitive flexibility.

The Barnes maze is a well-lit round table 91cm in diameter, with 19 false escape boxes and one true escape box. On the four training days, mice were placed in the centre of the table under a removable chamber. Mice were given three minutes to learn the location of the escape box. Mice failing to learn the box's location were guided to its location where they resided for one minute. Mice were given three trials daily, each 15 minutes apart. On day 5, mice were again placed in a removable chamber then given three minutes to identify the new location of the escape box.

Statistical methods

The Shapiro-Wilk test was performed to determine whether data was normally distributed for each outcome measure. Data conforming to a normal distribution were analysed with the appropriate parametric test (t-test or ANOVA) whereas data not passing the Shapiro-Wilk test ($p<0.05$) were analysed with non-parametric analyses (Mann-Whitney or Kruskal–Wallis tests). Sidak's correction for multiple comparisons was utilised for ANOVA post hoc comparisons. Differences between control and exercise mice in body weights within strains were analysed by two-way ANOVAs. Differences between control and exercise WT, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice in recognition memory, spatial memory and cognitive flexibility were conducted with individual students t-tests for normally distributed data, or the Mann-Whitney for non-normally distributed data. Barnes maze training day data and monthly distances travelled across strains over the experimental period were analysed by individual two-way repeated measures ANOVAs, whilst Barnes maze probe trial data including the spatial memory and cognitive flexibility measures were analysed with Mann-Whitney tests.

We also utilised individual two-way ANOVAs with Sidak's post hoc correction for multiple comparisons to investigate between strains differences in locomotor activity in the home cage and open field; Y maze recognition memory; spatial learning; spatial memory; and cognitive flexibility. All data are presented as the mean ± SEM, and all analyses were conducted in GraphPad Prism (Version 7) with the threshold for statistical significance set at $p<0.05$.

Results

Mouse body weights

Mice of all strains maintained body weights during the experimental period (Table 4.2).

Exercise distances travelled

All mouse strains undertook daily voluntary exercise in amounts that are consistent with distances travelled in the literature that result in physiological adaptations to exercise (van Praag, Christie et al. 1999, Marlatt, Potter et al. 2012, Morgan, Singhal et al. 2018). Two-way ANOVA analyses of the mean exercise distances travelled by all strains of exercise mice found no effect of time ($F(5, 270) = 0.9555$, $p=0.4456$) a significant effect of strain ($F(3, 270) = 6.538$, $p=0.0003$) with no time by strain interaction ($F(15, 270) = 0.2802$, $p=0.9967$) (Morgan, Singhal et al. 2018). Post hoc analyses with Sidak's correction for multiple comparisons found no significant differences between strains in the mean monthly distances travelled, and no significant changes in the mean monthly distances travelled over time within strains. This suggests any differences in behavioural tests did not arise because of differences in the mean distances travelled between strains (Figure 4.1).

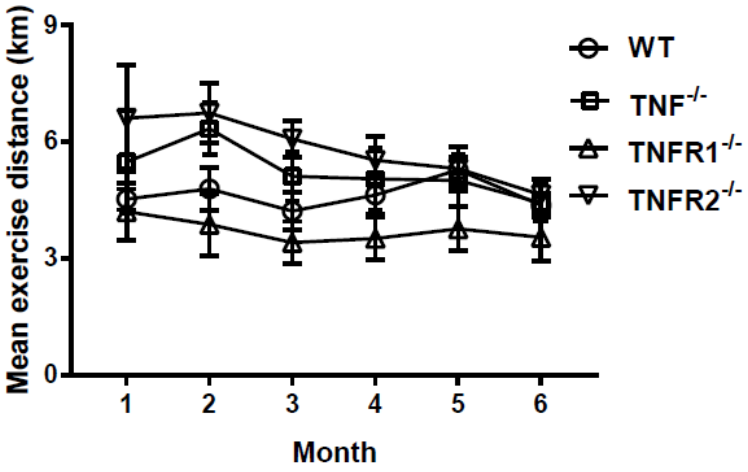


Figure 3.1 Mean exercise distances.
Mean monthly distances travelled by WT, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice over six months until nine months of age. Data presented are the mean ± SEM (n = 6 – 19/group, adapted from (Morgan, Singhal et al. 2018)).

Table 3. 2. Mean weights of control and exercise WT, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice.

	WT		$TNF^{-/-}$		$TNFR1^{-/-}$		$TNFR2^{-/-}$	
	Control (g)	Exercise (g)	Control (g)	Exercise (g)	Control (g)	Exercise (g)	Control (g)	Exercise (g)
Experiment start								
Combined	22.78 ± 0.5	23.17 ± 0.9	21.38± 0.8	22.08 ± 1.0	19.62 ± 0.9	20.7 ± 0.78	20 ± 1.04	20 ± 3.0
Male	24.5 ± 0.2	25.5 ± 1.11	23.57 ± 0.89	24.83 ± 1.42	22.67 ± 0.95	22.8 ± 0.66	25.5 ± 0.86	24.33 ± 2.3
Female	21.4 ± 0.4	20.83 ± 0.4	18.6 ± 0.5	19.71 ± 0.74	17 ± 0.3	18.6 ± 0.4	-	-
Experiment end								
Combined	28.78 ± 0.7***	27.75 ± 0.8**	26.54 ± 0.8***	25.08 ± 0.74	24.23 ± 1.2**	25 ± 0.8*	24.4 ± 1.28	-
Male	30.25 ± 0.75	29.67 ± 1.2	28.57 ± 0.99	27.5 ± 0.56	28.67 ± 0.95	27.4 ± 0.67	25.5 ± 0.86	24.33 ± 2.33
Female	27.6 ± 0.92	25.83 ± 0.3	24.17 ± 0.3	23 ± 0.53	20.43 ± 0.2	22.6 ± 0.4	-	-

Legend: Data presented are mean ± SEM found by 2-way repeated measures ANOVA of animal body weights of control vs. exercise mice within strains at experiment commencement compared to experiment end with Sidak's correction for multiple comparisons. All weights are in grams (g). - = unable to provide data due to only one female $TNFR2^{-/-}$ mouse (*p = < 0.05; **p < 0.01; ***p < 0.0001 compared to experiment start, n = 6-19/group, adapted from (Morgan, Singhal et al. 2018)).

Baseline locomotion

Locomotion in the home cage

Examination of the distances travelled in the home cage between control and exercise mice with unpaired student's t-tests found $TNFR1^{-/-}$ exercise mice demonstrated reduced locomotor activity ($p = 0.007$), with no changes evident between control and exercise mice in WT, $TNF^{-/-}$, or $TNFR2^{-/-}$ mice ($p = 0.083$, $p = 0.250$, and $p = 0.757$) (Figure 4.2A-D). As was previously outlined by this group (Morgan, Singhal et al. 2018), one-way ANOVA of differences in home cage locomotor activity between strains found significant effects of strain ($F (3, 96) = 5.277$, $p=0.002$) and treatment ($F (1, 96) = 6.385$, $p=0.013$). Post hoc analyses showed WT control mice travelled greater distance than $TNF^{-/-}$ control mice ($p = 0.03$), and WT exercise mice travelled greater distance than $TNFR1^{-/-}$ exercise mice ($p = 0.02$).

Locomotion in the open field

We further investigated locomotor activity across strains in the more stressful environment of the open field utilising t-test analysis within strains. T-test analyses found $TNFR2^{-/-}$ exercise mice displayed non-significant reductions in locomotor activity ($TNFR2^{-/-}$ $p = 0.374$) however exercise mice of all other strains displayed reduced locomotor activity compared to their respective control mice (WT $p = 0.0003$; $TNF^{-/-}$ $p < 0.0001$; and $TNFR1^{-/-}$ $p = 0.007$) (Figure 4.2E-H). Analyses of between strains differences found significant treatment ($F (1, 96) = 31$, $p<0.0001$) and strain effects ($F (3, 96) = 7.002$, $p=0.0003$), with WT control mice travelling greater distance in the open field than $TNFR1^{-/-}$ control mice ($p <0.0001$) (Morgan, Singhal et al. 2018).

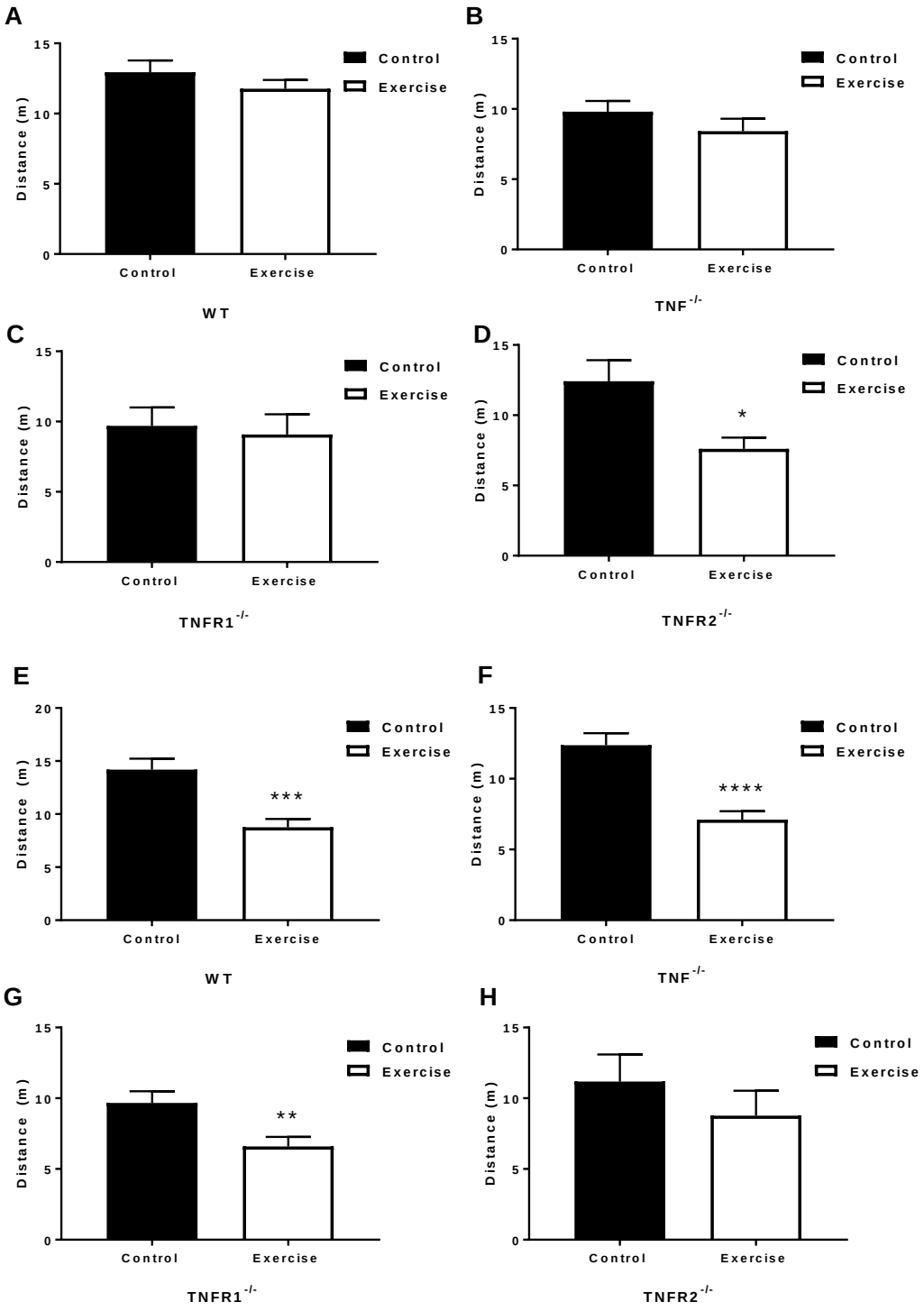


Figure 3.2. Distances travelled in the home cage and open field.
Distances travelled in the home cage (A-D), and the open field (E-H) by WT, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ control and exercise mice (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n = 6 – 19/group).

Cognition-like behaviours

Recognition memory in the Y maze

To investigate the roles of TNF receptor signalling in conferring the effects of exercise on recognition memory, we calculated the Y maze preference index (YMPI) (novel arm time/total

arm time) as a measure of recognition memory. Exercise significantly improved recognition memory for WT exercise mice compared to WT control mice, and impaired recognition memory for TNFR1$^{-/-}$ exercise mice compared to TNFR1$^{-/-}$ control mice (p = 0.017 and p = 0.007) and there were no changes in recognition memory in TNF$^{-/-}$ (p = 0.058) or TNFR2$^{-/-}$ mice (p = 0.926) (Figure 4.3A-D). Analyses of differences between strains in recognition memory found no effects of strain or treatment (F (3, 96) = 1.671, p=0.178 and F (1, 96) = 2.272, p=0.135).

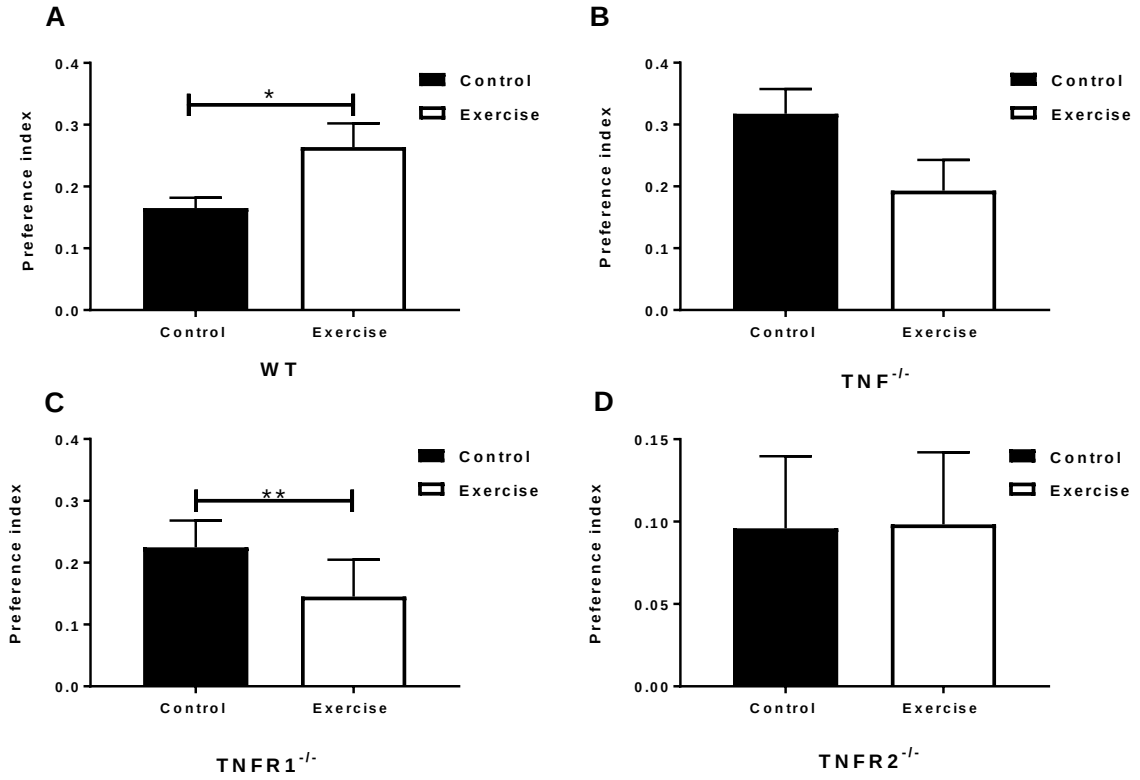


Figure 3.3. Recognition memory in the Y maze.
Recognition memory in nine month old control and exercise WT, TNF$^{-/-}$, TNFR1$^{-/-}$, and TNFR2$^{-/-}$ mice. Data presented are the mean ± SEM for the recognition memory preference index in the Y maze in A) WT, B) TNF$^{-/-}$, C) TNFR1$^{-/-}$, and D) TNFR2$^{-/-}$ mice (*p < 0.05, **p < 0.01, n = 6 – 19/group).

Barnes maze

Spatial learning in the Barnes maze

We investigated the roles of TNF signalling in conferring the effects of exercise on spatial learning, spatial memory, and cognitive-like flexibility in the Barnes maze. A significant main effect of exercise was evident in spatial learning in knockout strains but not in WT mice (p = 0.125) as was found by individual two-way ANOVAs of training day escape box latencies over the four training days. These revealed overall detrimental effects of exercise for spatial learning for TNF$^{-/-}$ mice and TNFR1$^{-/-}$ mice (p = 0.011; p = 0.006), and beneficial effects for TNFR2$^{-/-}$ mice (p = 0.044) (Figure 4.4A-D). However, post hoc analyses found no significant differences between control and exercise mice in any strain (Figure 4.4A-D). Individual one-way ANOVAs of control and exercise group latencies to the escape box for each of the Barnes maze training days found no effects of strain or treatment on days one, two and four, but a significant effect of treatment on day three (F (1, 96) = 4.768, p=0.031) with no effect of strain (F (3, 96) = 1.185, p=0.319).

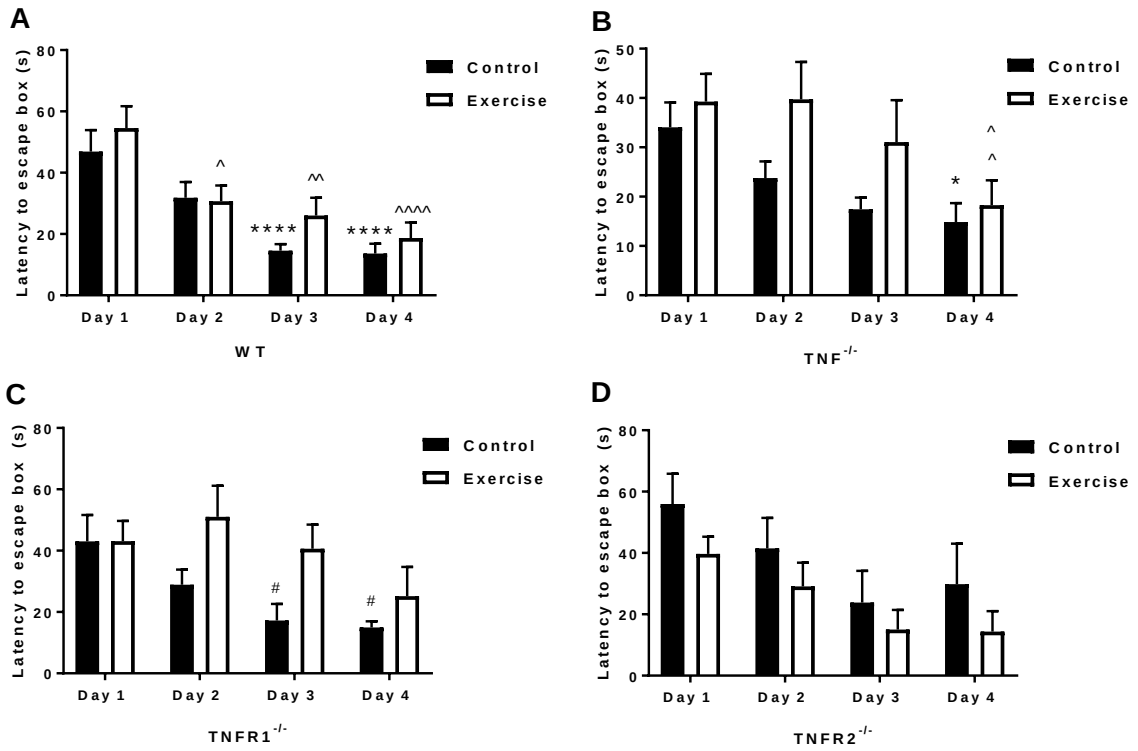
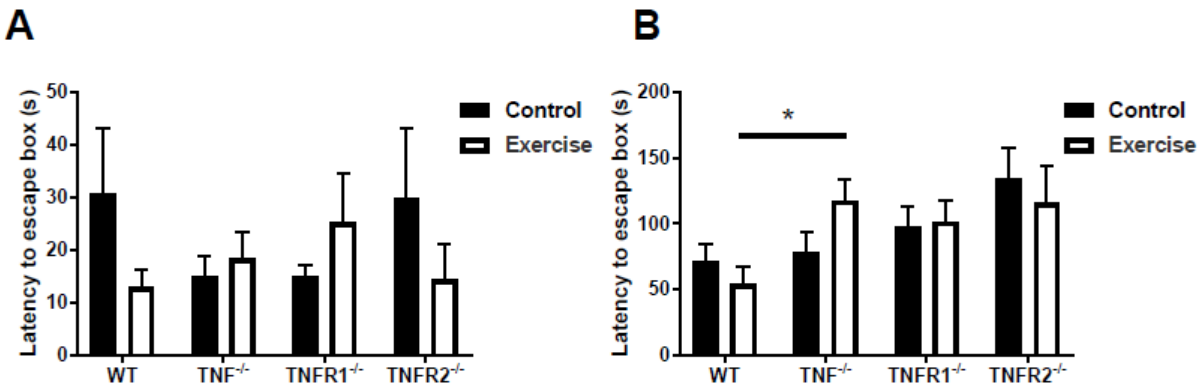


Figure 3.4. Barnes maze training day latencies to the escape box over four days of training.
Training day spatial learning latencies in A) WT, B) $TNF^{-/-}$, C) $TNFR1^{-/-}$, and D) $TNFR2^{-/-}$ mice (WT****p<0.0001 control day 1 vs. day 3, and ****p control day 1 vs. day 4; ^p<0.02 WT exercise day 1 vs. day 2; ^^p=0.003 WT exercise day 1 vs. day 3; ^^^p=0.0001 WT exercise day 1 vs. day 4; $TNF^{-/-}$ *p=0.03 control day 1 vs. day 4, $TNF^{-/-}$ ^p=0.04 exercise day 1 vs. day 4, and day 2 vs. day 4; $TNFR1^{-/-}$ #p=0.03 day 1 vs. day 4; $TNFR1^{-/-}$ #p=0.02 day 1 vs. day 4, n = 6-19/group).

Spatial memory in the Barnes maze

On day five, a probe trial was conducted where the escape box was moved to a new location 90° to the right. The time taken to identify the original escape box location was taken as a measure of spatial memory. T-test analyses found no differences between control and exercise mice of any strain in spatial memory (Figure 4.5A). In addition, one-way ANOVA of the differences between control and exercise mice in spatial memory across strains found no effects of strain or treatment ($F(3, 102) = 0.2068$, $p=0.891$ and $F(1, 102) = 0.6342$, $p=0.427$) (Figure 4.5A).



. Barnes maze spatial memory and cognitive flexibility.

Spatial memory in WT, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice exposed to either control or exercise conditions showing significant differences in control vs exercise mice within strains and between strain differences (A). Cognitive flexibility in WT, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice exposed to either control or exercise conditions showing significant differences in control vs exercise mice within strains and between strain differences (B). Data presented are the mean ± SEM found by t-test (within strains) and two-way ANOVAs (between strains) in seconds (*denotes significant difference between WT exercise and $TNF^{-/-}$ exercise mice, n = 6 – 19/group, threshold for significance set at $p < 0.05$).

Cognitive flexibility in the Barnes maze

We utilised the latency to identify the new escape box location as a measure of cognitive flexibility in the Probe trial because it involves the time taken to shift focus from the old escape box to identifying the new escape box location. There were no differences in cognitive flexibility between control and exercise mice in any strain found by individual t-tests (Figure 4.5B). However two-way ANOVA analyses of cognitive flexibility between strains found a significant strain effect ($F (3, 103) = 3.817$, $p=0.012$) with no effect of exercise ($F (3, 103) = 1.308$, $p=0.276$). Post hoc analyses found WT exercise mice had significantly shorter latencies to the escape box than $TNF^{-/-}$ exercise mice (Figure 4.5B).

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Research summary

Cognitive impairment in neuropsychiatric conditions commonly involves altered TNF expression, and changes in the levels of TNFR1, TNFR2, and the sTNFR1 and sTNFR2, necessitating safe interventions to modulate TNF signalling. Whilst there are exceptions, exercise has demonstrated efficacy for both improving cognitive functioning, and altering TNF and TNF signalling via the TNF receptors, thus suggesting potential for exercise to benefit TNF signalling in cognitive dysfunction.

This study investigated the effects of six months of voluntary wheel running exercise on cognitive functioning mediated by TNF signalling via the TNF receptors TNFR1 and TNFR2 in a mouse model of TNF, TNFR1, and TNFR2 deficit.

The research found exercise significantly improved recognition memory and spatial learning in WT mice. However the majority of findings in knockout strains were suggestive of potentially meaningful differences with exercise in the absence of significant behavioural changes. These included non-significant but meaningful impairments in spatial learning in $TNFR1^{-/-}$ exercise mice, and non-significant improvements in exercise $TNFR2^{-/-}$ mice. Exercise related spatial memory results showed non-significant improvements in WT and $TNFR2^{-/-}$ mice, and non-significant impairments in $TNFR1^{-/-}$ mice. There were no notable changes in cognitive flexibility in any strain. These results are discussed in further detail in the Discussion in chapter six.

Chapter 4. Exercise related anxiety-like behaviours are mediated by TNF receptor signalling, but not depression-like behaviours.

Statement of Authorship

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Name of lead author	Julie A. Morgan		
Contribution to the Paper	Data collection utilising mouse models and wet lab techniques, data analyses, interpretation, writing and redrafting manuscripts. Overall percentage: 80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	28th November, 2018
Name of Co-Author	Dr Frances Corrigan		
Contribution to the Paper	Conception of the project, design of methodology or experimental protocol, teaching experimental techniques, editing and revision of manuscript drafts.		
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Signature		Signature	28th November, 2018
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Contribution to the Paper	Assistance with planning and conducting mouse husbandry and laboratory techniques.		
Signature		Signature	28th November, 2018
Name of Co-Author	Professor Bernhard T. Baune		
Contribution to the Paper	Conception of the project, design of methodology or experimental protocol, guidance about manuscript drafts.		
Signature		Signature	28th November, 2018

By Signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

"Exercise related anxiety-like behaviours are mediated by TNF receptor signalling, but not depression-like behaviours."[3]

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Research report

Exercise related anxiety-like behaviours are mediated by TNF receptor signaling, but not depression-like behaviours

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ABSTRACT

Depression can involve disrupted pro-inflammatory TNF signaling via the TNF receptors TNFR1 and TNFR2, or the soluble TNF receptors sTNFR1 and sTNFR2. However, exercise might attenuate pro-inflammatory signaling in depression and related anxiety. We hypothesized that six months voluntary wheel running exercise would improve depression-like and anxiety-like behaviours in WT and TNFR1$^{-/-}$ mice, but not in TNF$^{-/-}$ and TNFR2$^{-/-}$ mice compared to their respective control mice.

Methods: We investigated the effects of six months voluntary wheel running exercise on open field (OF) and elevated zero maze (EZM) anxiety-like behaviours, and forced swim test (FST) depression-like behaviours in control and exercise WT, TNF$^{-/-}$, TNFR1$^{-/-}$, and TNFR2$^{-/-}$ mice with two-way ANOVAs.

Results: Exercise reduced of anxiety-like behaviours in TNFR2$^{-/-}$ exercise mice compared to their respective controls. Compared to WT control mice, WT exercise mice displayed significantly reduced EZM anxiety-like behaviours. There were no exercise related changes in FST immobility time. Between-strains analyses found WT control and exercise mice displayed reduced EZM anxiety-like behaviours compared to TNF$^{-/-}$ and TNFR1$^{-/-}$ control and exercise mice, and WT exercise mice displayed reduced anxiety-like behavior compared to TNFR2$^{-/-}$ exercise mice.

Discussion: Exercise associated TNFR1 and TNFR2 signaling in concert in WT exercise mice mediated reductions in aspects of anxiety-like behaviours. These findings are consistent with the current view that imbalances in TNF signaling are involved in disrupted affect. Additional studies are needed to further explore the roles of exercise related TNFR1 and TNFR2 signaling in anxiety-like and depression-like behaviours.

[3] This chapter has been published and the text has been included here with minimal changes to the published article.

Research report preamble

In the previous chapter, we investigated whether TNF signalling via the TNF receptors confers the effects of exercise on cognition-like behaviours. However, the roles of TNF signalling in mediating the effects of long-term exercise on depression-like and related anxiety-like behaviours during ageing remains unknown. Prior research has shown that depression can involve disrupted pro-inflammatory tumour necrosis factor alpha (TNF) signalling via the TNF receptors TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2), or the soluble TNF receptors sTNFR1 and sTNFR2. There is also evidence exercise could have anti-inflammatory effects, including the possible modulation of TNF and its receptors through changes in TNF signalling via the neurodegenerative TNFR1 and neuroprotective TNFR2. Exercise may therefore have potential to attenuate pro-inflammatory signalling in depression and related anxiety, however this possibility has not been investigated to date.

RESEARCH QUESTION: This research sought to address the question *"Does TNF signalling via the TNF receptors TNFR1 and TNFR2 mediate the effects of exercise on depression- and related anxiety-like behaviours?"*

STUDY AIM: The aim of this study was to investigate the roles of TNF signalling via the TNFR1 and TNFR2 in mediating the effects of exercise on depression-like and related anxiety-like behaviours in a mouse model of TNF, TNFR1, and TNFR2 deficit ($TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice) compared to no-exercise control mice.

RESEARCH HYPOTHESIS: We hypothesized that six months of voluntary wheel running exercise would improve anxiety-like and depression-like behaviours in WT and $TNFR1^{-/-}$ mice, but not in $TNF^{-/-}$ and $TNFR2^{-/-}$ mice compared to their respective control mice.

This research investigated the effects of six months voluntary wheel running exercise on open field (OF) and elevated zero maze (EZM) anxiety-like behaviours, and forced swim test (FST) depression-like behaviours in control and exercise WT, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice. We investigated behavioural differences between exercise and their respective control mice within each strain, and differences between control and exercise mice between strains.

In this article we report that compared to their respective control mice, exercise reduced OF anxiety-like behaviours in $TNFR2^{-/-}$ mice, and WT exercise mice displayed significantly reduced EZM anxiety-like behaviours. However interestingly, there were no exercise related changes in FST immobility time compared to controls in any strain. Analyses of behavioural differences between strains found WT control and exercise mice displayed reduced EZM anxiety-like behaviours compared to $TNF^{-/-}$ and $TNFR1^{-/-}$ control and exercise mice, and WT exercise mice displayed reduced anxiety-like behaviour compared to $TNFR2^{-/-}$ exercise mice.

Introduction

Tumour necrosis factor alpha (TNF) signalling via the membrane bound TNF receptors tumour necrosis factor receptor 1 (TNFR1) and tumour necrosis factor receptor 2 (TNFR2) TNFR2, and the soluble TNF receptors soluble TNFR1 (sTNFR1) and soluble TNFR2 (sTNFR2) are implicated in the pathophysiology of depression and related anxiety (Kohler et al., 2017; Rizavi et al., 2016; Smith, 1991). Patients with depression and/or anxiety have demonstrated significant changes in the levels of TNF and the TNF receptors in cells, plasma, and serum (Dowlati et al., 2010; Dunjic-Kostic et al., 2013; Himmerich et al., 2008; Kohler et al., 2017; Liu et al., 2012; Maes et al., 1998; Muthuramalingam et al., 2016; Vieira et al., 2010), and whilst no differences have also been found (Euteneuer et al., 2012), these findings are in the minority. Recent meta-analyses have shown chronically elevated TNF was associated with treatment non-response (Strawbridge et al., 2015) and TNF has consequently become a potential biomarker for depression (Martinez-Cengotitabengoa et al., 2016).

Preclinical research has investigated the roles of TNF and the TNF receptors in depression-like and anxiety-like behaviours. The administration of exogenous TNF increased immobility time in the Porsolt forced swim test (Kaster et al., 2012; Palin et al., 2009), whilst mouse models of TNF receptor deficit utilising $TNFR1^{-/-}$ or $TNFR2^{-/-}$ mice have shown reductions in immobility time in the forced swim test (FST) in both $TNFR1^{-/-}$ (Kaster et al., 2012; Simen et al., 2006) and $TNFR2^{-/-}$ mice (Simen et al., 2006), suggesting that TNF signalling via both receptors could be involved in the aetiology of depression-like behaviours. Mice deficient in TNF signalling via TNFR1 and TNFR2 demonstrated increased time spent in the centre of the open field (OF), suggestive of reductions in anxiety-like behaviours in the context of TNF receptor deficits (Patel et al., 2010). Collectively the disruption of TNF and the TNF receptors in patients with depression and the involvement of TNF signalling via the TNFR1 and TNFR2 in anxiety-like and depression-like behaviours suggests significant involvement of TNF signalling via both the TNFR1 and TNFR2 in these behaviours.

TNFR1 and TNFR2 have distinctive expression and roles. TNFR1 is expressed on most cells, and is involved in the negative regulation of cellular proliferation, and the activation of cellular apoptosis (Baune et al., 2012; Cui et al., 2011; Grell et al., 1995; Iosif et al., 2006; Wajant et al., 2003). In contrast, TNFR2 is only expressed on endothelial cells and cells of haematopoietic lineage and microglia, oligodendrocytes, and neuronal subtypes in the brain (Arnett et al., 2001; Dopp et al., 2002; Grell et al., 1995; McCoy and Tansey, 2008; Yang et al., 2002). TNFR2 is considered to have a neuroprotective role through inhibiting caspase initiated apoptosis and by the protection of neurons from glutamate related excitotoxicity (Baune et al., 2012; Heir and Stellwagen, 2015; Wajant et al., 2003). In constitutional conditions, sTNFR1 has an antagonist function to pro-inflammatory TNF signalling via the TNFR1 (Pinckard et al., 1997). In health, TNF signalling that is balanced between the TNFR1 and TNFR2 is thought to contribute to the maintenance of normal cellular functioning, however a shift towards greater TNF signalling via the TNFR1 is considered to contribute to TNF associated neuropathology and neurodegeneration (Baune et al., 2012). This suggests the modulation of TNF signalling in depression is an important therapeutic aim.

Exercise is a therapy with few risks and side effects. Previous research has found exercise can have significant effects on TNF and the TNF receptors that could contribute to the modulation of altered TNF signalling in depression. During acute aerobic exercise, major working muscles exponentially increase the expression of interleukin 6 (Petersen and Pedersen, 2005; Petersen

and Pedersen, 2006) that blunts TNF expression, with increases the soluble TNF receptors sTNFR1 and sTNFR2 (Petersen and Pedersen, 2005; Petersen and Pedersen, 2006). This results in a net increase in anti-inflammatory factors including IL1ra, IL10, and the sTNFR1, suggesting that chronic exercise could contribute to chronically reduced TNF levels. Preclinical studies of chronic exercise have shown evidence of this, with reductions in TNF levels following three or 16 weeks of voluntary wheel running (Liu et al., 2013; Pervaiz and Hoffman-Goetz, 2011) suggesting that chronic exercise could contribute to reducing TNF, and may alter TNF signalling via the TNF receptors with associated benefits for anxiety-like and depression-like behaviours. Indeed four weeks of wheel running increased the time spent in the central regions of the OF and open arms of elevated plus maze (EPM), suggesting a reduction in anxiety-like behaviours (Binder et al., 2004a; Duman et al., 2008b), whilst three to four weeks of running reduced immobility time in the FST (Cunha et al., 2013; Duman et al., 2008a) and tail suspension test (Cunha et al., 2013). Considered together, the effects of exercise on TNF, the TNF receptors, and anxiety-like and depression-like behaviours suggest that exercise might confer benefits for anxiety-like and depression-like behaviours arising from altered TNF signalling via the TNF receptors. However, the question of whether TNF signalling via the TNF receptors mediates the effects of exercise on anxiety-like and depression-like behaviours remains unknown. This study therefore sought to investigate the roles of TNF signalling via the TNF receptors TNFR1 and TNFR2 in mediating the effects of exercise on depression-like and related anxiety-like behaviours. We utilised genetically modified mouse models with TNF and TNF receptor knockout; including wild type (WT), tumour necrosis factor alpha knockout ($TNF^{-/-}$), tumour necrosis factor alpha receptor 1 knockout ($TNFR1^{-/-}$), and tumour necrosis factor alpha receptor 2 knockout ($TNFR2^{-/-}$) mice compared to the WT control strain. We provided mice with ad libitum voluntary wheel running exercise from three months to nine months of age (for six months). We hypothesized exercise would improve depression-like and anxiety-like behaviours in WT and $TNFR1^{-/-}$ mice arising from advantageous exercise associated TNF signalling via all TNF receptors in WT mice, and from the neuroprotective role of TNFR2 signalling in $TNFR1^{-/-}$ mice (Baune et al., 2012). However, we anticipated no exercise related changes in $TNF^{-/-}$ and $TNFR2^{-/-}$ exercise mice because exercise has no effect on TNF in the absence of TNF signalling in $TNF^{-/-}$ mice, and because TNF signalling via the TNFFR1 involves the negative regulation of cellular proliferation and the activation of cellular apoptosis (Wajant et al., 2003).

Methods

Animals

We purchased approximately equal numbers of eight week old male and female mice from The University of Adelaide (C57BL/6 or WT) or bred mice at the University Laboratory animal facility ($TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$). Due to breeding challenges with $TNFR2^{-/-}$ mice, control and exercise $TNFR2^{-/-}$ groups were smaller than other groups (Table 3.1). Mice were housed six per IVC cage for one week to acclimatize the animal facility prior to starting the experiment. Mice had *ad libitum* access to standard mouse chow and water and environmental conditions were maintained at 21 ± 1 °C with a 12-hour light/dark cycle (lights on 7:00-21:00). The number of mice and their suffering were minimized wherever possible, and this study was granted ethics approval by the University of Adelaide Animal Ethics Committee.

Experimental design

At 12 weeks of age, WT, $TNF^{-/-}$, $TNFR1^{-/-}$, or $TNFR2^{-/-}$ male and female mice were block randomized in pairs for allocation to no exercise control or running wheel exercise groups. Breeding challenges resulted in the $TNFR2^{-/-}$ strain groups being smaller than other groups, and there was only one female $TNFR2^{-/-}$ mouse in the $TNFR2^{-/-}$ exercise group. As such, this group was not powered for the analyses of sex differences in behaviours so results about sex differences are not reported (Table 3.1). All mice were transferred to open topped PVC cages at treatment allocation for the control condition (48.5cm x 15.5cm x 12cm) or exercise condition (37cm x 20.5cm x 13.5cm) that were fitted with running wheels (12cm x 5.5cm). Animal welfare was monitored daily, and mice were weighed weekly. Exercising mice had *ad libitum* access to running wheels until the completion of behavioural testing. Running wheel distances travelled were manually recorded weekly from electronic counters attached to each wheel. We calculated the mean monthly exercise distances from weekly exercise distances, and the distances travelled per mouse were calculated by dividing the monthly distances by two (the number of animals per cage). There were no adverse events during the experiment.

Table 4.1. Control and exercise WT, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mouse group sizes.

Strain	Control n (males: females)	Exercise n (males: females)
WT	19 (9: 10)	17 (7: 10)
$TNF^{-/-}$	19 (9: 10)	15 (8: 7)
$TNFR1^{-/-}$	17 (10: 7)	16 (9: 7)
$TNFR2^{-/-}$	6 (5:1)	6 (6: 0)

Legend: WT = wild type; $TNF^{-/-}$ = tumour necrosis factor alpha knockout; $TNFR1^{-/-}$ = tumour necrosis factor alpha receptor 1 knockout; $TNFR2^{-/-}$ = tumour necrosis factor alpha receptor 2 knockout.

Behavioural assessments

To investigate the effects of TNF signalling via the TNFR1 and TNFR2 on anxiety-like and depression-like behaviours, behavioural testing was conducted with all mice over four weeks. Testing was conducted in the light phase of the cycle between 8.30am – 11.30am and was performed in the order of least to most stressful with a minimum of 24 hours between each test to minimize the effects of stress arising from testing on the animals' welfare and possible effects on test performances. Tests were conducted in the following order 1) Home cage locomotor activity; 2) OF locomotor activity; 3) OF anxiety-like behaviour;4) Elevated zero maze (EZM) anxiety-like behaviour; 5) FST. Behavioural tests were recorded utilising ANYmaze Video tracking software (Stoelting Company (USA)), and olfactory cues were removed between tests by cleaning testing areas with Veterinary Disinfectant (F10SC).

Locomotion

Home cage locomotion

To investigate the effects of TNF signalling via the TNFR1 and TNFR2 on home cage locomotor

activity, we examined the distances travelled in the animals' home cages on two day old bedding for five minutes in line with published protocols (Baune and Wiede 2008).

Locomotion in the open field

To investigate the effects of TNF signalling via the TNFR1 and TNFR2 on locomotor activity in a more stressful novel environment, we quantified the distances travelled in the OF (a 40cm x 40cm plexiglass box). Mice were placed in the North-West corner of the field and the total distance and time spent in the inner and outer regions of the field were recorded for five minutes in accordance with published protocols (Baune and Wiede 2008).

Anxiety-like behaviours

Anxiety-like behaviours in the open field

TNF signalling mediated effects of exercise on anxiety-like behaviours were investigated by quantifying the time spent in the inner zone over the time spent in the outer zone of the OF (inner time/outer time, the inner: outer ratio). Mice were placed in the North-West corner of the box for five minutes exploration, and the distance travelled and time spent in the inner and outer zones of the field were recorded (Baune and Wiede 2008).

Anxiety-like behaviours in the elevated zero maze

Anxiety-like behaviours were further investigated in the EZM. The EZM is an elevated circular platform with four quadrants (50cm in diameter, 40cm high and 5cm wide). The maze has two closed quadrants with inner and outer walls 27cm high, while the open quadrants have none. Mice were placed on the centre of the open southern quadrant to enable five minutes of exploration whilst the time spent in the open and closed quadrants was recorded.

Depression-like behaviours

Depression-like behaviours in the Porsolt forced swim test

TNF signalling mediated effects of exercise on depression -like behaviours were investigated utilising immobility time in the PRSoft forced swim test (FST) (Porsolt, Anton et al. 1978). Immobility was defined as floating with or without the small movements that contribute to maintaining equilibrium, but that do not contribute to other movements such as swimming or climbing.

Statistical methods

To test the hypothesis that the effects of exercise on anxiety-like and depression-like behaviours are mediated by TNF signalling via the TNFR1 and TNFR2, we performed the Shapiro-Wilk test to ascertain whether data was normally distributed for each outcome measure. We then performed two factor ANOVAs (of genotype by intervention, and the genotype by intervention interaction) to quantify the effects of these factors on home cage locomotor activity; open field locomotor activity; OF inner: outer ratio; EZM open: closed quadrant ratio; and FST immobility time. Post hoc analyses were conducted to investigate differences between control and exercise mice within each strain, and the differences

between strains of control and exercise mice. Repeated measures ANOVAs were performed to investigate differences in body weights between control and exercise mice male and female mice at the start and end of the experiment, and to compare the exercise distances travelled over the six month experimental period between exercise WT, $TNF^{-/-}$, $TNFR1^{-/-}$ or $TNFR2^{-/-}$ exercise mice (strain by time) with Sidak's correction for multiple comparisons. We analysed data from 115 mice with the threshold for statistical significance level set at <0.05 for all tests. The data presented are the mean ± SEM. All statistical analyses were conducted with GraphPad Prism (Version 7).

Results

Mouse body weights

Examination of mouse body weights showed that all strains maintained healthy body weights over the experimental period, with WT and $TNFR1^{-/-}$ control and exercise mice, and TNF control mice displaying significant weight gain over the experimental period consistent with development from young adulthood to middle age (Table 3.2).

Exercise distances travelled

Two way repeated measures ANOVA analyses of the distances travelled over the experimental period showed there were no significant differences in the mean monthly distances travelled between strains (Figure 3.1). All mice therefore ran comparable distances over the experiment, and these were consistent with the distances run by mice in previous studies (Marlatt, Potter et al. 2012, Morgan, Singhal et al. 2018), with no differences between groups that might contribute to behavioural changes.

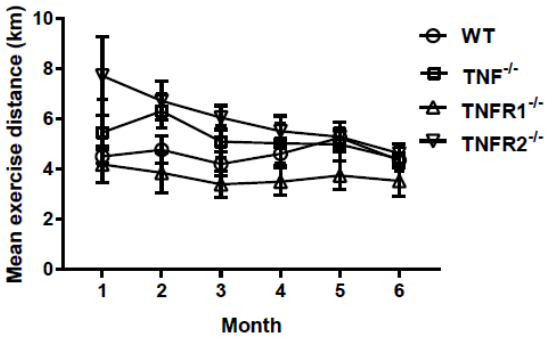

Figure 4.1. Mean monthly distances travelled until nine months of age.
Exercise distances travelled in WT, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice until aged nine months. Data presented are the mean ± SEM (n = 6 – 19/group, threshold for significance set at $p < 0.05$).

Table 4.2. Mean weights of WT, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice over the experimental period.

	WT		$TNF^{-/-}$		$TNFR1^{-/-}$		$TNFR2^{-/-}$	
	Control (g)	Exercise (g)	Control (g)	Exercise (g)	Control (g)	Exercise (g)	Control (g)	Exercise (g)
Experiment start								
Combined	22.78 ± 0.5	23.17 ± 0.9	21.38± 0.8	22.08 ± 1.0	19.62 ± 0.9	20.7 ± 0.78	20 ± 1.04	20 ± 3.0
Male	24.5 ± 0.2	25.5 ± 1.11	23.57 ± 0.89	24.83 ± 1.42	22.67 ± 0.95	22.8 ± 0.66	25.5 ± 0.86	24.33 ± 2.3
Female	21.4 ± 0.4	20.83 ± 0.4	18.6 ± 0.5	19.71 ± 0.74	17 ± 0.3	18.6 ± 0.4	-	-
Experiment end								
Combined	28.78 ± 0.7***	27.75 ± 0.8**	26.54 ± 0.8***	25.08 ± 0.74	24.23 ± 1.2**	25 ± 0.8*	24.4 ± 1.28	24.33 ± 2.33^
Male	30.25 ± 0.75	29.67 ± 1.2	28.57 ± 0.99	27.5 ± 0.56	28.67 ± 0.95	27.4 ± 0.67	25.5 ± 0.86	24.33 ± 2.33^
Female	27.6 ± 0.92	25.83 ± 0.3	24.17 ± 0.3	23 ± 0.53	20.43 ± 0.2	22.6 ± 0.4	-	-

Legend: WT = wild type; $TNF^{-/-}$ = tumour necrosis factor alpha knockout; $TNFR1^{-/-}$ = tumour necrosis factor alpha receptor 1 knockout; $TNFR2^{-/-}$ = tumour necrosis factor alpha receptor 2 knockout; - = only one female $TNFR2^{-/-}$ mouse; ^all male $TNFR2^{-/-}$ exercise group. Data presented are mean ± SEM of commencement body weight versus completion body weight as was found by two way repeated measures ANOVA with Sidak's correction for multiple comparisons. All body weights are in grams (g), and mice were aged 12 weeks at experiment start, and 9 months at the end of the experiment (*p = < 0.05; **p < 0.01; ***p < 0.0001, threshold for significance set at p < 0.05).

Behavioural assessments

Locomotor activity in the home cage

To determine whether TNF signalling via the TNF receptors affected baseline locomotor activity we quantified locomotor activity in the animals' home cages. Investigation into home cage locomotor activity utilising two way ANOVA found significant effects of strain ($F(3, 96) = 5.277$, $P=0.002$) and treatment ($F(1, 96) = 6.385$, $P=0.013$), with no strain by treatment interaction ($F(3, 96) = 0.9103$, $P=0.439$). Post hoc analyses with Sidak's' correction for multiple comparisons found $TNFR1^{-/-}$ exercise mice travelled significantly less distance in the home cage compared to $TNFR1^{-/-}$ control mice ($p=0.014$), but there were no differences in the distances travelled between control and exercise mice in WT, $TNF^{-/-}$, or $TNFR2^{-/-}$ mice (Figure 3.2A, Table 3.3). However, WT control mice travelled significantly further than $TNF^{-/-}$ control mice ($p = 0.03$), and WT exercise travelled greater distances than $TNFR1^{-/-}$ exercise mice ($p = 0.02$) (Figure 3.2A).

Locomotor activity in the open field

We also investigated if TNF signalling via the TNF receptors affected locomotor activity in the more stressful novel environment of the OF. Two way ANOVA of locomotor activity in the open field found significant effects of treatment and strain ($F(1, 96) = 31$, $P<0.0001$ and $F(3, 96) = 7.002$, $P=0.0003$), and no effect of a treatment by strain interaction ($F(3, 96) = 1.532$, $P=0.211$). Post hoc analyses showed that WT and $TNF^{-/-}$ exercise mice travelled significantly greater distance in the open field than their respective control mice ($p < 0.0001$, and $p = 0.0002$) (Figure 3.2B, Table 3.3), and that WT control mice travelled significantly greater distance than $TNFR1^{-/-}$ control mice ($p < 0.0001$) (Figure 3.2B).

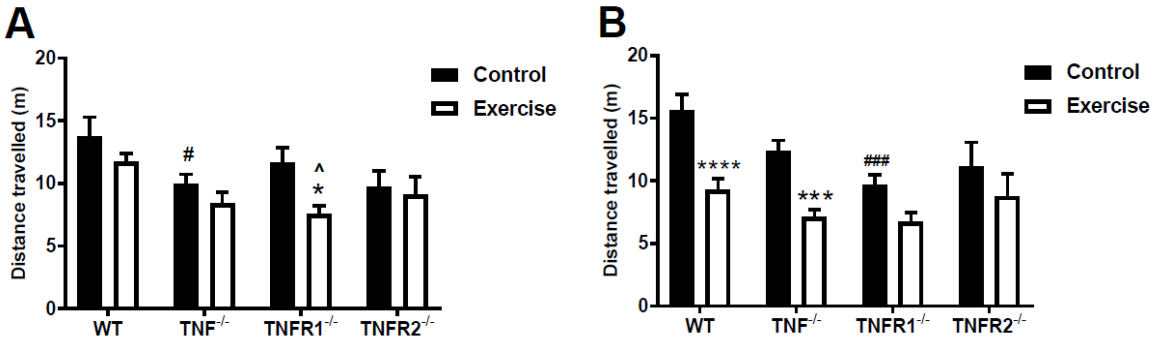

Figure 4.2. Locomotor activity in control and exercise WT, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice.
Data presented show significant differences in control vs exercise mice within strains and between strain differences. Data presented are the mean ± SEM found by two-way ANOVAs (*denotes significant difference between control and exercise mice within strains, # denotes significant difference compared to WT control mice, and ^denotes significant difference compared to WT exercise mice; #$p < 0.05$, *$p < 0.05$, ^$p < 0.05$; ****$p < 0.0001$, ***$p < 0.001$, ###$p < 0.001$, $n = 6 – 19$/group, threshold for significance set at $p < 0.05$).

Anxiety-like behaviours

Anxiety-like behaviours in the open field

To investigate whether TNF signalling via the TNF receptors mediated the effects of exercise on anxiety-like behaviours in the OF, we examined the OF inner: outer ratio (time spent in

inner regions over the time spent in outer regions of the OF). A high ratio is suggestive of reduced anxiety-like behaviours. Two-way ANOVA analyses of the open field inner: outer ratio found a significant treatment effect (F (1, 96) = 11.55, P=0.001) with no strain effect (F (3, 96) = 1.126, P=0.342), and a significant treatment by strain interaction (F (3, 96) = 3.03, P=0.033). Post hoc analyses of differences between control and exercise mice within strains found TNFR2$^{-/-}$ exercise mice displayed a significantly higher inner: outer ratio than TNFR2$^{-/-}$ control mice (p = 0.003) (Figure 3.3A, Table 3.3). There were no differences in open field anxiety-like behaviour were evident between strains in post hoc analyses (Figure 3.3A).

Anxiety-like behaviours in the elevated zero maze

We further investigated whether TNF signalling via the TNF receptors mediated the effects of exercise on anxiety-like behaviours by quantifying the open: closed quadrant ratio (open quadrant time over closed quadrant time) in the EZM. A ratio closer to one is suggestive of reduced anxiety-like behaviour. Two-way ANOVA analyses of the EZM open: closed quadrant ratio found a significant effect of strain (F (3, 96) = 20.76, P<0.0001), no effect of treatment (F (1, 96) = 3.096, P=0.081), and a treatment by strain interaction (F (3, 96) = 3.906, P=0.011). Sidak's post hoc analyses showed that WT exercise mice had a significantly higher open: closed quadrant ratio compared to WT control mice (p= 0.004) (Figure 3.3B, Table 3.3). Post hoc analyses of differences between strains found WT control mice displayed higher open: closed quadrant ratios than TNF$^{-/-}$ and TNFR1$^{-/-}$ control mice (p= 0.027 and p= 0.025). In addition, WT exercise mice significantly higher ratios than all exercise knock out mice (TNF$^{-/-}$ p <0.0001, TNFR1$^{-/-}$ p <0.0001, and TNFR2$^{-/-}$ p = 0.010), indicating significantly reduced EZM anxiety-like behaviours in WT exercise mice compared to all strains of knockout exercise mice (Figure 3.3B).

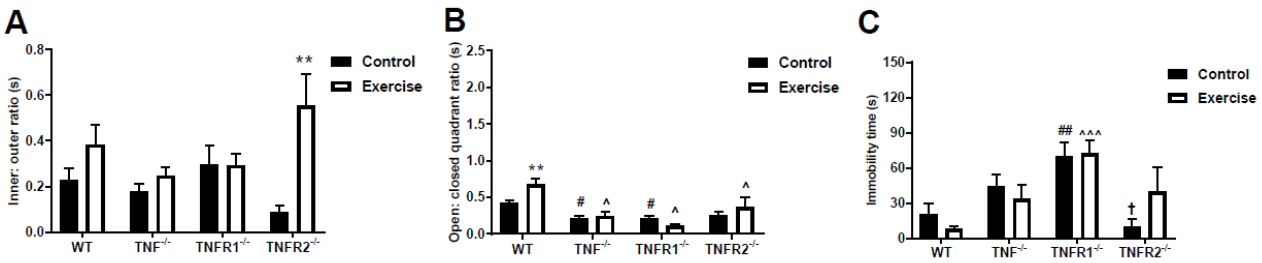

Figure 4.3. Anxiety-like and depression-like behaviours in WT, TNF$^{-/-}$, TNFR1$^{-/-}$, and TNFR2$^{-/-}$ mice.
(A) The open field inner: outer ratio in WT, TNF$^{-/-}$, TNFR1$^{-/-}$, and TNFR2$^{-/-}$ mice exposed to either control or exercise conditions showing significant differences in control vs exercise mice within strains and between strain differences. (B) The open: closed quadrant ratio in WT, TNF$^{-/-}$, TNFR1$^{-/-}$, and TNFR2$^{-/-}$ mice exposed to either control or exercise conditions showing significant differences in control vs exercise mice within strains and between strain differences. (C) Forced swim test immobility time in WT, TNF$^{-/-}$, TNFR1$^{-/-}$, and TNFR2$^{-/-}$ mice exposed to either control or exercise conditions showing significant differences in control vs exercise mice within strains and between strain differences. (*denotes significant difference between control and exercise mice within strains, # denotes significant difference compared to WT control mice, ^denotes significant difference compared to WT exercise mice, †denotes significant difference to TNFR1$^{-/-}$ control mice; **p < 0.01, #p < 0.05, ^p < 0.05, ##p < 0.05, ^^^p < 0.001, †p < 0.05, n = 6 – 19/group, threshold for significance set at p < 0.05).

Depression-like behaviours

Depression-like behaviours in the forced swim test

To investigate whether TNF signalling mediated the effects of exercise on depression-like behaviours, we quantified immobility time as a measure of despair-like behaviour in the FST. Examination of FST immobility time utilising two-way ANOVA found no effect of treatment ($F(1, 95) = 0.05917$, $P=0.8083$), but a significant effect of strain ($F(3, 95) = 9.946$, $P<0.0001$) with no treatment by strain interaction ($F(3, 95) = 0.8764$, $P=0.4562$). Sidak's post hoc analyses found no significant differences between control and exercise mice within any strain (Figure 3C, Table 3.3). However two-way ANOVA analyses of between strains differences in FST immobility time showed that WT control mice demonstrated significantly less immobility time than $TNFR1^{-/-}$ control mice ($p=0.009$), and $TNFR1^{-/-}$ control mice displayed significantly longer immobility time than $TNFR2^{-/-}$ control mice ($p=0.017$). In addition, WT exercise mice displayed significantly less immobility time than $TNFR1^{-/-}$ exercise mice ($p=0.0004$) (Figure 3.3C).

Table 4.3. The effects of exercise on behaviours within strains of WT, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice.

TEST	WT	$TNF^{-/-}$	$TNFR1^{-/-}$	$TNFR2^{-/-}$
Home cage locomotion	No change	No change	Exercise reduced home cage locomotor activity*	No change
OF locomotion	Exercise reduced locomotor activity in the novel open field****	Exercise reduced locomotor activity in the novel open field***	No change	No change
OF ratio	No change	No change	No change	Exercise reduced anxiety**
EZM ratio	Exercise reduced anxiety-like behaviour**	No change	No change	No change
FST immobility time	No change	No change	No change	No change

Legend: WT = wild type; $TNF^{-/-}$ = tumour necrosis factor alpha knockout; $TNFR1^{-/-}$ = tumour necrosis factor alpha receptor 1 knockout; $TNFR2^{-/-}$ = tumour necrosis factor alpha receptor 2 knockout; OF = open field; EZM = elevated zero maze; FST = forced swim test. Data presented were found by two-way ANOVAs utilising Sidak's post hoc correction for multiple comparisons, and all data are compared within strains to same strain control mice (WT n=36; $TNF^{-/-}$ n=34; $TNFR1^{-/-}$ n=33; $TNFR2^{-/-}$ n=12; *p = < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, threshold for significance set at $p < 0.05$).

Research summary

Prior clinical and preclinical research has found TNF signalling via the TNFR1 and the TNFR2 may be involved in the pathophysiology of depression and related anxiety. Clinical research investigating the effects of exercise on TNF signalling have shown acute exercise blunts TNF expression, and chronic exercise in mice reduces TNF, suggesting chronic exercise could benefit pathological TNF signalling in depression and anxiety. However the question of whether TNF signalling via the TNFR1 and TNFR2 mediates the effects of exercise on depression and anxiety-like behaviours remains unexamined. This report has described our investigation of the question *"Does TNF signalling via the TNFR1 and TNFR2 mediate the effects of exercise on depression-like and anxiety-like behaviours?"*

We examined the effects of six months of wheel running exercise on anxiety- and depression-like behaviours in a mouse model of WT, $TNF^{-/-}$, $TNFR1^{-/-}$, and $TNFR2^{-/-}$ mice compared within strains to their respective no-exercise control mice. We also examined differences between strains of control and exercise mice.

This research found significant reductions in OF anxiety-like behaviours in $TNFR2^{-/-}$ exercise mice, and reduced EZM anxiety-like behaviours in WT exercise mice compared to their respective control mice, but no exercise related changes in FST depression-like behaviours in any strain. Further analyses of within strains differences showed reduced EZM anxiety-like behaviours in WT control mice compared to $TNF^{-/-}$ and $TNFR1^{-/-}$ control mice, and reduced EZM anxiety-like behaviours in WT exercise mice compared to exercise mice of all knockout strains. These results are discussed in detail in chapter six, the Discussion.

Chapter 5. Exercise cessation induces depression-like, anxiety-like, and impaired cognition-like behaviours and altered hippocampal gene expression.

Statement of Authorship

Title of Paper	Ceasing exercise induces depression-like, anxiety-like, and impaired cognition-like behaviours and hippocampal dysfunction.		
Publication Details	In press for publication with *Brain Research Bulletin*.		
Name of Principal Author (Candidate)	Julie A. Morgan		
Contribution to the Paper	Conception of the project, design of methodology or experimental protocol, data collection utilising mouse models and wet lab techniques, data analyses, interpretation, writing and redrafting manuscripts (90%).		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	10th April, 2019
Name of Co-Author	Dr Frances Corrigan		
Contribution to the Paper	Conception of the project, design of methodology or experimental protocol, teaching experimental techniques, editing and revision of manuscript drafts.		
Signature		Signature	28th November, 2018
Name of Co-Author	Dr Magdalene C. Jawahar		
Contribution to the Paper	Conception of the project, design of methodology or experimental protocol, teaching experimental techniques, editing and revision of manuscript drafts.		
Signature		Signature	28th November, 2018
Name of Co-Author	Dr Emily J. Jaehne		
Contribution to the Paper	Conception of the project, design of methodology or experimental protocol, teaching experimental techniques, editing and revision of manuscript drafts.		
Signature		Signature	28th November, 2018
Name of Co-Author	Dr Gaurav Singhal		
Contribution to the Paper	Assistance with planning and conducting mouse husbandry and laboratory techniques.		
Signature		Signature	28th November, 2018
Name of Co-Author	Professor Bernhard T. Baune		
Contribution to the Paper	Conception of the project, design of methodology or experimental protocol, guidance about manuscript drafts.		
Signature		Signature	28th November, 2018

By Signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

"Ceasing exercise induces depression-like, anxiety-like, and impaired cognitive-like behaviours and altered hippocampal gene expression."[4]

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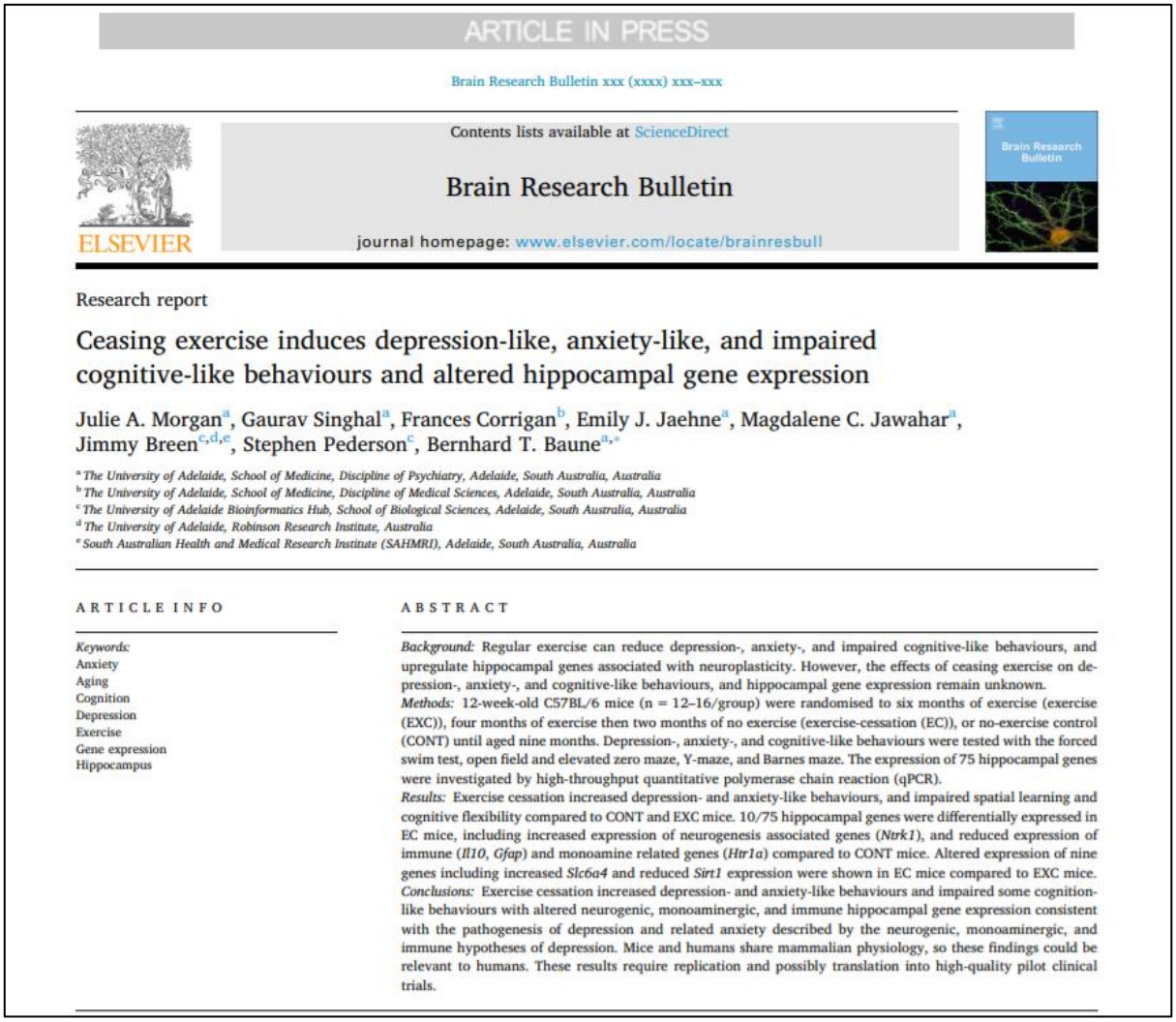

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Research report

Ceasing exercise induces depression-like, anxiety-like, and impaired cognitive-like behaviours and altered hippocampal gene expression

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ABSTRACT

Background: Regular exercise can reduce depression-, anxiety-, and impaired cognitive-like behaviours, and upregulate hippocampal genes associated with neuroplasticity. However, the effects of ceasing exercise on depression-, anxiety-, and cognitive-like behaviours, and hippocampal gene expression remain unknown.
Methods: 12-week-old C57BL/6 mice (n = 12–16/group) were randomised to six months of exercise (exercise (EXC)), four months of exercise then two months of no exercise (exercise-cessation (EC)), or no-exercise control (CONT) until aged nine months. Depression-, anxiety-, and cognitive-like behaviours were tested with the forced swim test, open field and elevated zero maze, Y-maze, and Barnes maze. The expression of 75 hippocampal genes were investigated by high-throughput quantitative polymerase chain reaction (qPCR).
Results: Exercise cessation increased depression- and anxiety-like behaviours, and impaired spatial learning and cognitive flexibility compared to CONT and EXC mice. 10/75 hippocampal genes were differentially expressed in EC mice, including increased expression of neurogenesis associated genes (*Ntrk1*), and reduced expression of immune (*Il10*, *Gfap*) and monoamine related genes (*Htr1a*) compared to CONT mice. Altered expression of nine genes including increased *Slc6a4* and reduced *Sirt1* expression were shown in EC mice compared to EXC mice.
Conclusions: Exercise cessation increased depression- and anxiety-like behaviours and impaired some cognition-like behaviours with altered neurogenic, monoaminergic, and immune hippocampal gene expression consistent with the pathogenesis of depression and related anxiety described by the neurogenic, monoaminergic, and immune hypotheses of depression. Mice and humans share mammalian physiology, so these findings could be relevant to humans. These results require replication and possibly translation into high-quality pilot clinical trials.

[4] This chapter has been published and the text has been included here with minimal changes to the published article.

Research report preamble

In chapter two, we found reductions in OF anxiety with ageing in WT mice, no exercise related changes in depression-like behaviours over the lifespan, and exercise related anxiety-like behaviours limited cognition-like functioning in both Y and O mice. Interestingly, in chapter three we demonstrated impaired spatial learning in $TNF^{-/-}$ exercise mice and impaired recognition memory and spatial learning in $TNFR1^{-/-}$ exercise mice. However, we also found largely non-significant but meaningful exercise related TNF signalling in spatial memory and cognitive flexibility. In chapter four we found TNFR1 signalling related reductions in OF anxiety-like behaviour, and an unanticipated lack of TNF signalling mediated effects of exercise on depression-like behaviours.

Notwithstanding this however, as was outlined in chapters one and two, previous preclinical research has shown chronic exercise related reductions in depression- and anxiety-like behaviours, and improvements in cognitive-like behaviours and cognition-like impairments. Moreover, research into the mechanisms of efficacy of exercise for these behaviours have shown regular exercise alters factors that contribute to hippocampal mediated affect, learning, and memory. For instance, exercise increases levels of serum BDNF with resultant increases in hippocampal neurogenesis. Exercise also reduces pro-inflammatory TNF both in the periphery and brain. Given these benefits of exercise for hippocampal mediated anxiety-, depression-, and cognition-like behaviours and physiology, the cessation of exercise could have adverse effects on behaviours and hippocampal physiology. However, the effects of ceasing exercise on depression-, anxiety-, and cognitive-like behaviours, and hippocampal gene expression remain unknown.

RESEARCH QUESTION: This research report responds to the question *"What are the effects of exercise cessation on anxiety-, depression-, and cognition-like behaviours, and hippocampal physiology?"*

STUDY AIMS: The aim of this study is to investigate the effects of exercise cessation on depression-, anxiety-, and cognition-like impairment behaviours and hippocampal function.

This research utilised 12-week-old C57BL/6 mice (n=12-16/group). Mice were randomised to no-exercise control (CONT), six months of exercise (exercise mice (EXC)), or four months of exercise followed by two months of no exercise (exercise cessation mice (EC)) until nine months of age. At aged nine months, depression-, anxiety-, and cognitive-like behaviours were tested with the forced swim test, open field and elevated zero maze, and Y-maze and Barnes maze respectively. The expression of 75 hippocampal genes of interest were also investigated by high-throughput quantitative polymerase chain reaction (qPCR).

This chapter reports that the cessation of exercise significantly increased anxiety-, depression-, and cognition-like impairment behaviours, and significantly altered 10 of 75 hippocampal genes of interest compared to no-exercise control mice and that continued exercise. Interestingly, gene expression changes were suggestive of alterations to immune and neurotrophic functions (*IL10*, *Gfap*, and *Ntrk1*), and altered serotonergic, dopaminergic, and glutamatergic neurotransmission (*Htr1a*, *Slc6a4*). Changes in hippocampal gene expression seem in broad agreement with the immune, monoaminergic, and neurotrophic hypotheses of major depression.

Introduction

Preclinical and clinical research has largely reported exercise can improve prevalent conditions such as depression, related anxiety, and cognitive impairment. Mouse models of chronic exercise have shown exercise can reduce depression-like behaviours, attenuate anxiety-like behaviours, and reduce impaired spatial learning and memory in adult mice (Binder et al., 2004, Duman et al., 2008, Salam et al., 2009) although inconsistencies are also evident (Pietropaolo et al., 2006, Fuss et al., 2010). Nevertheless, preclinical research utilizing as little as three weeks of wheel running have shown exercised mice display reduced depression-like behaviours of immobility time in the forced swim test (Duman et al., 2016) and spend greater time in the anxiety provoking regions of the open field and elevated plus maze (Binder et al., 2004, Duman et al., 2008). Wheel running or treadmill exercise for three to six weeks significantly reduced the latencies and path length to identify the hidden location of the platform in the Morris water maze (Parachikova et al., 2008, Kim et al., 2013), and improvements in cognitive-like behaviours are also found in older animals (van Praag et al., 2005, Marlatt et al., 2012). Regular exercise can also contribute to beneficial changes in the hippocampus involved in maintaining healthy mood and cognitive functioning.

Clinical trials have shown exercise can involve changes in anatomical, neurotrophic, and immune factors in humans. Studies in adults and older adults have shown changes in fitness are associated with increased regional brain volumes of the total, left, and right hippocampus, that can arise from increases in myelination (Thomas et al., 2016, Li et al., 2017) and increased brain connectivity that improves signal activation in the cingulate gyrus and anterior cingulate cortex (Li et al., 2017). Greater changes in fitness can involve greater changes in tissue density (Kleemeyer et al., 2016, Li et al., 2017), and changes in anterior hippocampal volume have been associated with upregulated serum BDNF, neural plasticity, and memory improvements (Erickson et al., 2011, Schulkin 2016, Vedovelli et al., 2017, Gmiąt et al., 2018). Interestingly, higher exercise associated BDNF has been shown to predict remission from major depressive disorder (Rethorst et al., 2017), and BDNF has been suggested as a candidate biomarker for exercise treatment of depression (Hearing et al., 2016). There are additional potential benefits from exercise for innate and adaptive immunity. Clinical research has demonstrated that acute exercise involves the interleukin 6 (IL6) mediated blunting of tumour necrosis factor (TNF), with increased expression of the anti-inflammatory cytokine interleukin 10 (IL10) (Petersen et al., 2005), suggesting possible modulatory effects of exercise on these cytokines with regular exercise. Moreover, exercising ageing individuals show less age-related declines in aspects of T-cell functioning compared to age-matched non-exercising control individuals (Yan et al., 2001).

The mechanisms involved in exercise associated changes in anatomical, neurotrophic, and immune functioning have been examined in preclinical mechanistic studies. Chronic running enhances hippocampal neurogenesis related synaptic plasticity in adult and older running mice compared to control mice (Van Praag et al., 1999, van Praag et al., 1999). This is thought to arise from exercise related increases in hippocampal BDNF (Rasmussen et al., 2009, Yu et al., 2014) and favourable changes in serotonin transporter and receptor mediated monoamine transmission (Greenwood et al., 2005, Nishii et al., 2017). Furthermore, chronic exercise also reduces hippocampal tumour necrosis factor (TNF) and the TNF: interleukin 10 (IL10) ratio (TNF: IL10 ratio), suggestive of improvements in the balance between pro- and anti-inflammatory hippocampal cytokines suggested by clinical research (Nichol et al., 2008, Pervaiz et al., 2011).

Regular running also reverses senescent hippocampal gene expression and increases the expression of hippocampal genes associated with neuroplasticity such as *Bdnf* and *Sirt1*

(Berchtold et al., 2005, Stranahan et al., 2010, Kohman et al., 2011, Steiner et al., 2011, Abe-Higuchi et al., 2016, Kim et al., 2016). Given that *Bdnf* and *Sirt1* are thought to have a role in the pathogenesis and attenuation of major depression in humans (Brunoni et al., 2008, Teche et al., 2013, Molendijk et al., 2014, Converge Consortium 2015), exercise may have potential for preventing and ameliorating the pathogenesis of senescence contributing to these conditions. Moreover, exercise has also increased hippocampal astrocytic GFAP and markers of astrocytic ageing in mice and rats, suggesting that exercise may contribute to maintaining healthy astrocytic functioning (Latimer et al., 2011, Saur et al., 2014).

Exercise attenuated anxiety-like and depression-like behaviours, improvements in cognitive-like behaviours, and enhanced neuroplasticity, innate immunity and monoaminergic metabolism, and reversed hippocampal gene expression senescence suggests that ceasing exercise might have detrimental effects. Indeed the cessation of exercise in humans may contribute to increases in depressive symptoms (Morgan et al., 2018), and exercise cessation in adult mice has increased anxiety-like behaviours, impaired spatial learning and memory, and significantly reduced neurogenesis and numbers of immature neurons (Kim et al., 2013, Nishijima et al., 2013). However, the effects of ceasing exercise in older adult mice remains unknown, and investigation into the specific mechanisms involved in the adverse effects of exercise cessation are needed. This study investigates a mouse model of exercise cessation (EC) compared to no-exercise control (CONT) and exercise control (EXC) groups to examine the effects of exercise cessation in mammalian physiology that may translate to humans. The primary aim of this study is to investigate the effects of ceasing long-term exercise on depression-, and related anxiety- and cognitive-like behaviours in nine month-old C57BL/6 mice compared to mice that do not exercise and continue exercise. Our secondary aim is to investigate exercise cessation related changes in hippocampal gene expression. We hypothesise that exercise cessation will increase depression-, anxiety-, and cognitive-like impairment behaviours and contribute to significant changes in hippocampal gene expression.

Materials and Methods

Animals

Eight week old male and female C57BL/6 mice were purchased from The University of Adelaide and socially housed six per cage for four weeks until the commencement of the experiment. Mice had *ad libitum* access to standard mouse chow and water and environmental conditions were maintained at 21 ± 1°C with a 12-hour light/dark cycle (lights on 7:00-21:00). This study was approved by The University of Adelaide ethics committee and was conducted to minimise animal suffering wherever possible.

Experimental design

This study utilised a randomised study design with positive control mice undertaking six months of exercise (exercise control, n=16), and experimental exercise cessation mice undertaking four months of exercise (EC, n=12). Twelve week old C57BL/6 mice (n = 28) were block randomised in pairs (males and females separately) to exercise control or exercise cessation groups unless fighting necessitated separation of male mice into single housing. Mice were transferred to open topped cages. Cage dimensions were 37cm x 20.5cm x 13.5cm for the accommodation of running wheels. All mice were handled weekly between 9-10am for health checks, weighing, and the recording of distances run by via a digital

counting mechanism (MiniMitter Incorporated USA). All mice commenced behavioural testing at nine months of age. This study is reported in accordance with guidelines for the reporting of animal studies, the ARRIVE guidelines (Kilkenny et al., 2014). The University of Adelaide Animal Ethics Committee approved this study (approval number M216-12).

Behavioural testing

To investigate the effects of exercise cessation on depression-like, anxiety-like, and cognitive-like behaviours, behavioural testing was conducted in the light phase between 8.30am and 3.30pm over four weeks. Testing was conducted during the light phase to enable *ad libitum* wheel running during the dark phase. Tests were performed with individual mice in random order, and tests were conducted from least to most stressful to minimise the possible stressful effects of testing (Lad et al., 2010). Tests were performed in the following order; i) Open field; ii) Elevated zero maze; iii) Y maze; iv) Barnes maze; v) Forced swim test. One un-blinded assessor collected data for all tests. All testing was recorded with ANYmaze video tracking software (Stoelting Co. USA) and testing areas were thoroughly cleaned with F10 veterinary disinfectant between tests to remove olfactory traces.

Distances travelled

Distances travelled in the home cage

The distances travelled in non-stressful conditions in the home cage with two day old bedding and running wheels removed were recorded for five minutes in line with previously published protocols (Baune et al., 2008).

Distances travelled in the open field

We also examined the distances travelled in the more stressful environment of the open field. The open field is a well-lit 40cm x 40cm plexiglass box. Mice were placed in the North-western corner of the field and allowed 5 minutes to explore the field according to previously published protocols (Morgan et al., 2018).

Depression-like behaviours

Depression-like behaviours in the forced swim test

The forced swim test was conducted utilising immobility time as a measure of depression-like behaviour as in previously published protocols [50]. Mice were placed in a 4L tank of water (diameter of 20cm) filled to a depth of 40cm (at 23-24 degrees) and behaviours were recorded for six minutes. One trial was conducted per animal to minimise distress associated with testing, and immobility was defined as the absence of swimming or struggling movements resulting in forwards or climbing movements.

Anxiety-like behaviours

Anxiety-like behaviours in the open field

Anxiety-like behaviours in the open field were investigated by quantifying the time spent in the centre regions of the open field. Mice were placed in the corner of the field and allowed five minutes exploration whilst time spent in the inner and outer regions of the field were recorded according to previously published protocols (Camara et al., 2015).

Anxiety-like behaviours in the elevated zero maze

Anxiety-like behaviours were further investigated in the elevated zero maze (EZM) utilising the time spent in the open quadrants of the maze as a marker of anxiety-like behaviours. The elevated zero maze is an elevated circular platform (a diameter of 50cm, 40cm high and 5cm wide) with two open quadrants without walls, and two 27cm high walled closed quadrants. Mice were placed on the southern open quadrant of the apparatus and allowed 5 minutes for exploration of the maze whilst the time spent in the open and closed quadrants was recorded in accordance with previously published protocols (Camara et al., 2015).

Cognitive-like behaviours

Recognition memory in the Y maze

Recognition memory was quantified utilising time spent in the novel arm of the Y maze as was conducted in previously published protocols [49]. The Y maze has three arms with walls 10cm high and 5cm wide shaped as a 'Y' and angled 120° to one another. This test is conducted in two phases. Firstly, mice were placed in the Southern-most arm of the maze with one lateral arm closed for 10 minutes exploration of the maze. Then in phase two, after 30 minutes in the home cage, mice were again placed in the Southern-most arm for five minutes exploration with all arms open, and the time spent in each arm was recorded. Mice with intact hippocampal learning and memory prefer to explore the novel arm of the maze, whereas mice with memory impairments will not recognise the familiar arms and will therefore spend less time exploring the novel arm (Dulawa et al., 1999).

Cognitive-like behaviours in the Barnes maze

Spatial learning, spatial memory and cognitive flexibility were investigated utilising the Barnes maze in line with previously published protocols (Morgan et al., 2018). The Barnes maze is an exposed well-lit table with 19 false escape boxes and one true escape box around its periphery.

Phase 1

Mice were placed in the centre of the table under a removable chamber, then allowed three minutes to identify the location of the escape box. Mice failing to identify the boxes' location were gently guided to the true escape box where they remained for one minute. Mice undertook three trials daily for four days, each separated by 15 minutes.

Phase 2

On day five, the location of the escape box was rotated 90 degrees clockwise, and mice were again placed in a chamber in the table centre. Mice were given three minutes to identify the

new location of the escape box, and the latency to locate the original escape box location was recorded as a measure of spatial memory. The latency to identify the new box location was utilised as a measure of cognitive flexibility because it constitutes the time taken to change focus from the old escape box location to identify the new location of the box.

Cellular and molecular investigations

To examine the effects of ceasing exercise on salient cellular and molecular factors, we utilised hippocampal differential gene expression and examined hippocampal microglia and astrocytes utilising immunohistochemistry (IHC).

Gene expression analyses

To investigate the effects of exercise cessation on hippocampal gene expression, we investigated 75 genes of interest thought to be involved in the pathophysiology of depression, anxiety, and cognitive impairment in the literature, including those altered by exercise (Appendix Table A1, n=6/group). These included the following factors:

1. Monoamines (Ressler et al., 2000, Greenwood et al., 2005, Dantzer et al., 2008, Liu et al., 2013, Harkin 2014, Real et al., 2015)
2. Growth factors (Martinowich et al., 2007, Chen et al., 2009, Gomes da Silva et al., 2012, Maes et al., 2012, Agudelo et al., 2014, Egeland et al., 2015)
3. Cytokines and cytokine receptors (Petersen et al., 2005, Chennaoui et al., 2008, Maes 2011, Baune et al., 2012, Speisman et al., 2013)
4. T cells and T cell polarisation (Renault et al., 2009, Kubera et al., 2011, Maes et al., 2012, Lovatel et al., 2013, Souza et al., 2013, Speisman et al., 2013, Walsh et al., 2013, Maes et al., 2014, Cai et al., 2015, Song et al., 2017)
5. Cellular apoptosis (Brentnall et al., 2013) (Packer et al., 2012) 81].
6. Glial and astrocytic factors (Kohman et al., 2012, Kohman et al., 2012, Bernardi et al., 2015)
7. Reactive oxygen species and oxidative factors (Maes et al., 2011, Marosi et al., 2012)
8. The hypothalamic-pituitary-adrenal axis (Sapolsky 2000, Egeland et al., 2015), and
9. Mitochondrial markers (Steiner et al., 2011, Karabatsiakis et al., 2014, Rossignol et al., 2015).

Hippocampal genes of interest were examined by high-throughput quantitative polymerase chain reaction (BioMark HD^{TM}, Fluidigm Inc., USA). Quantification of gene mRNA levels were performed using TaqMan assays (Life Technologies, ThermoFisher, Australia) in the high-throughput qPCR system from BioMarkHD^{TM} (Fluidigm Inc., USA). Brains were dissected on a petri-dish on ice. The cerebellum and prefrontal cortex were dissected, then the hippocampus was dissected from the cortex and subcortex and stored in containing RNALater (Ambion, Life technologies) and stored at -80° C until processing. RNA for exercise (n=7) and exercise cessation mice (n=5) as later extracted utilising the PureLink RNA mini extraction kit (Ambion) following the manufacturer's instructions. Total RNA was then subjected to reverse transcription using the SuperScript III first-strand cDNA synthesis system (Invitrogen, Australia) according to manufacturer's instructions. Samples were then prepared for high-throughput qPCR in BioMark HD^{TM} (Fluidigm Inc., USA) using a single 14-cycle Pre-amplification consisting of 20ng of each cDNA sample mixed with pooled TaqMan assays (79 assays listed in Table 3) and PreAmp Master Mix (Fluidigm Inc., USA). Quantitative

PCR was performed for each TaqMan assay for each sample in a 96.96 dynamic array nanofluidic chip (Fluidigm Inc., USA). A total of 79 X 96 (Assays x Samples in duplicates) PCR reactions were performed. Cycle threshold (Ct) values were generated by Fluidigm Real-time PCR analysis software (Fluidigm Inc., USA). Analyses of PCR data were conducted utilising the R package as is outlined in section 2.8.

Immunohistochemistry

A subset of 19 mice (n = 6-7 / group) were sacrificed for immunohistochemistry immediately following behavioural testing with a fatal dose of pentobarbitol (60mg/kg). Mice were then perfused with 10% neutral buffered formalin via left atrial cardiac injection. Brains were stored in 10% neutral buffered formalin until fixation in paraffin wax. Hippocampal tissues were sectioned 5µm thick, 150µm apart commencing from the anterior hippocampus from approximately coronal level 67 relative to Bregma at -1.255mm until approximately coronal level 78 at Bregma -2.355mm. The six sections were selected based on whether they were the clearest representation of the anatomy of the Bregma level from which they had come. Slides were dewaxed and dehydrated, and endogenous peroxidases blocked prior to antigen retrieval in either citrate or TRS (Dako). Normal horse serum to block non-specific binding was applied for overnight incubation followed by the primary antibody on the second day (GFAP, Dako 1:40000; IBA1, Abcam 1:1000). The relevant species of secondary antibody (Abacus, 1:250) was applied prior to streptavidin peroxidase. Detection of the antigen-antibody complex was achieved using diaminobenzadine (Sigma) before counterstaining with haematoxylin, slide dehydration and slide mounting using DePex. Slides were scanned using the Nanozoomer scanner (Hamamatsu) and counting utilising NDP.view2. Positively stained cells were manually counted in six hippocampal sections per animal. Sections were magnified 20X and counting was conducted blinded to treatment group utilising *a priori* determined criteria for the classification of positively stained cells. These criteria included 1) a discernible cellular membrane, 2) greater than half the cell positively stained consistent with the staining density and intensity of an *a priori* selected stained reference cell.

Statistical methods

To investigate the effects of ceasing exercise on depression-like, anxiety-like, and cognitive-like behaviours we investigated two groups of more than 11 mice (no-exercise control n = 17, exercise control n = 16, and exercise cessation n = 12). Power calculations assume the need to detect effects at the 5% alpha level with 80% power. Based on available data, we anticipated a minimum difference of 45 seconds in forced swim test immobility time between exercise cessation mice compared exercise control mice. Assuming constant variance and equal sized groups, a sample of 11 mice per group would be required to detect this effect.

Analyses of differences between mice undertaking no exercise (CONT), performing six months exercise (exercise control, EXC), mice ceasing four months of exercise for eight weeks exercise cessation (exercise cessation mice, EC) were largely conducted utilising one-way NAOVAs. Individual one-way ANOVAs were conducted for baseline locomotion in the home cage and open field, open field and EZM anxiety-like behaviours, FST immobility time, the Y maze, and Barnes maze spatial memory and cognitive flexibility. Individual two-way repeated measures ANOVAs were performed to determine differences between exercise

control and exercise cessation mice in animal body weights (at experiment commencement and completion), and for each of the Barnes maze training days (days one, two, three, and four). There were no outliers excluded from any statistical analyses for behavioural testing.

Analyses of qPCR sample measurements taken from the 96.96 well Biomark Gene Expression Dynamic Array was performed in R. Input expression values of house-keeping genes (*B2m*, *Gapdh*, *Gusb* and *Hprt*) were compared across all samples to identify outlier samples. Delta Ct values of all genes were then normalised against the geometric mean of house-keeping gene expression values. We removed outliers and pooled duplicate samples for each mouse for analyses. Mixed-effects models were fit to estimate the changes in Delta Ct across treatments within each sample, and adjustments for multiple comparisons was conducted using the R package *multcomp* (Hothorn et al., 2008).

Results

Animal body weights

We utilised roughly equal numbers of male and female mice in each group (exercise control males: females: 6: 8; exercise cessation males: females 6:6). Mice in all groups maintained healthy body weights over the experiment (Table 1). There were no differences between EXC and EC mice in bodyweight prior to the cessation of exercise by EC mice ($F(1, 66) = 2.201$, $p=0.1427$) (Table 1) as was found by two-way ANOVAs. There were no adverse events over the experimental period.

Table 5.1. Mean body weights of CONT, EXC, and EC mice during the experimental period.

	CONT	EXC	EC
Commencement	23.4g ± 0.7g	23.4g ± 0.9g	20.9g ± 0.7g
Completion			
Prior to exercise cessation	29.0g ± 1.1g	26.8g ± 0.8g	26g ± 0.5g
Post exercise cessation mean	29.54g ± 1.1g	28.07g ± 0.7g	27.17g ± 0.8g

Legend: Data presented are the mean ± SEM; all weights are in grams (g) (CONT = no-exercise control; EXC = exercise control; EC = exercise then exercise cessation, n = 12-17/group).

Exercise distances travelled

Over the six-month experiment EXC mice had continuous wheel access, and EC mice had four months wheel access then eight weeks of no wheel access until nine months of age (Fig. 1). With the exception of month one ($p=0.031$) there were no significant differences in the mean monthly distances travelled between exercise control and exercise cessation mice until the cessation of exercise for exercise cessation mice at the end of month four. The distances run by all exercising mice were comparable to the distances travelled by mice in previous research (Figure 1) (van Praag et al., 2005, Marlatt et al., 2012).

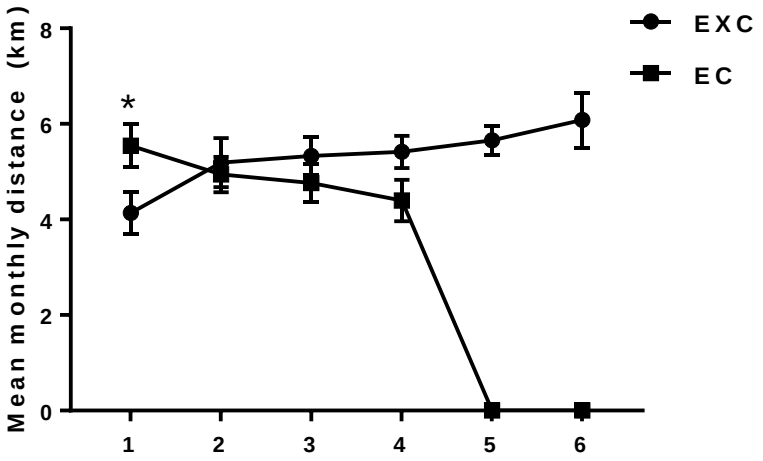

Figure 5.1. Wheel running behaviours in nine month old C57BL/6 mice.
Mean monthly distances run over the experimental period in exercise and exercise cessation mice (a), and immobility time in the forced swim test in exercise and exercise cessation mice (b) (*p<0.05, EXC = exercise control; EC = exercise cessation, n=12-16/group).

Baseline locomotion

Distances travelled in the home cage

Examination of the distances travelled in the home cages showed there were no differences between CONT, EX, and EC mice in the distances travelled in the home cage (ANOVA p=0.167) (Figure 2A).

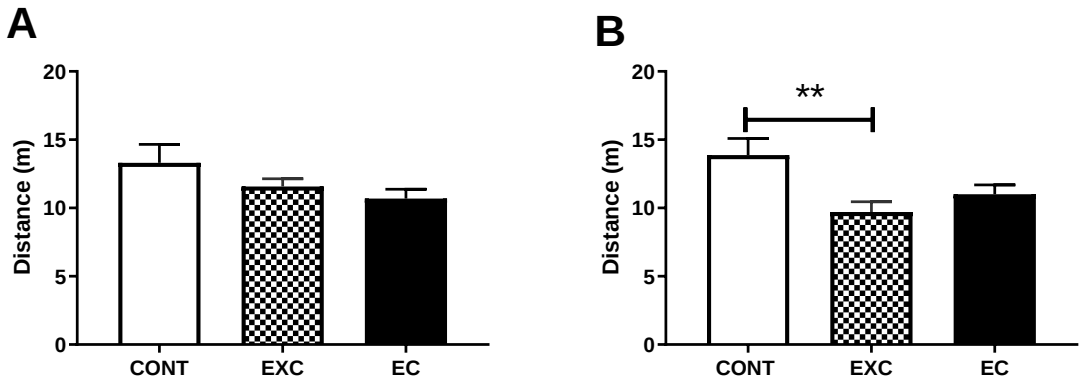

Figure 5.2. Baseline locomotor activity in nine-month old C57BL/6 CONT, EXC, and EC mice. A) Distances travelled in the home cage, and B) Distances travelled in the open field (CONT= no-exercise control; EXC = exercise control; EC = exercise cessation; *p<0.05, **p<0.01, n=12-17/group).

Distances travelled in the open field

Investigation into the distances travelled in the open field showed that compared to CONT mice, EXC mice showed a significant reduction in the distance travelled in the open field

(p=0.008). There were no differences in the distances travelled between CONT and EXC mice or between EXC and EC mice (p=122 and p=651) (Figure 2B).

Depression-like behaviours

Depression-like behaviours in the forced swim test

We quantified immobility time in the forced swim test as a measure of depression-like behaviours arising from exercise cessation. One way ANOVA of immobility time in the forced swim test found no differences in immobility time between CONT and EXC mice (p=767), however EC mice demonstrated significantly more immobility time than both CONT and EXC mice (both p<0.0001) (Figure 3).

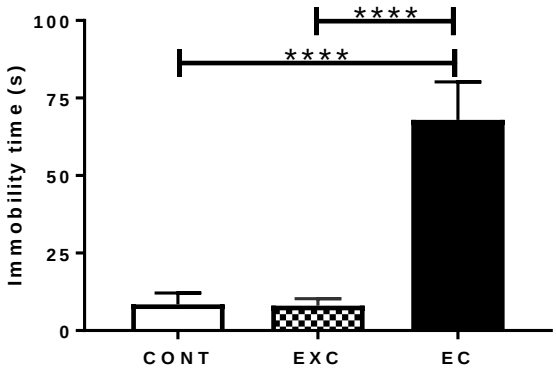

Figure 5.3. Immobility time in the forced swim test in CONT, EXC, and EC mice (CONT= no-exercise control; EXC = exercise control; EC = exercise cessation; ****p<0.0001, n=12-17/group).

Anxiety-like behaviours

Anxiety-like behaviours in the open field

Investigation into differences in open field anxiety-like behaviours found compared to CONT mice, EXC mice spent significantly longer in the centre regions of the open field (p=0.002), whereas EC mice spent significantly less time in the central regions of the open field (p=0.043). EC mice also spent less time in the centre of the field compared to EXC mice (p<0.0001) (Figure 4A).

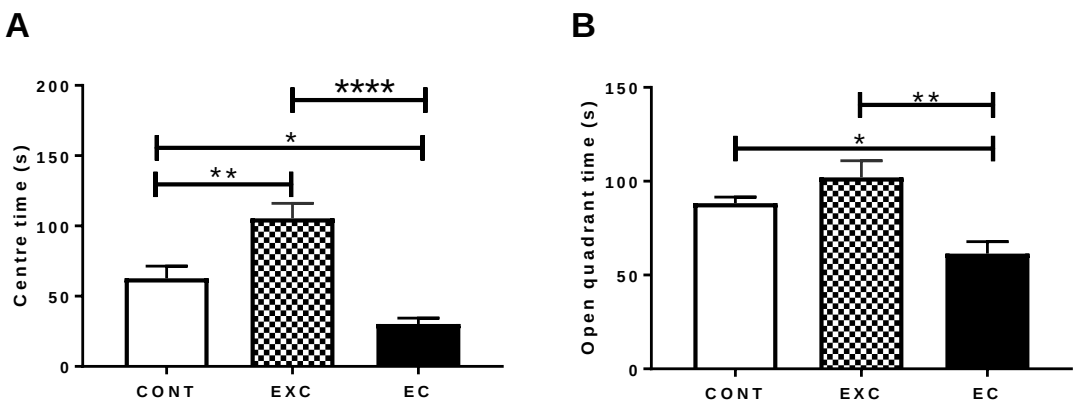

Figure 5.4. Anxiety-like behaviours in nine month old CONT, EXC, and EC mice. A) Time spent in the centre regions of the open field, and B) Time spent in the open quadrants of the elevated zero maze (CONT= no-exercise control; EXC = exercise control; EC = exercise cessation; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n=12-17/group).

Anxiety-like behaviours in the elevated zero maze

The effects of ceasing exercise on anxiety-like behaviours were further characterized in the EZM by quantifying the time spent in the open quadrants of the maze. There was no change in the time spent in the open quadrants in EXC mice compared to CONT mice (p=0.346). However, EC mice displayed significantly less time spent in the open quadrants of the maze compared to both CONT and EXC mice (p=0.047 and p=0.001), suggestive of increased anxiety-like behaviour in EC mice (Figure 4B).

Cognitive-like behaviours

Cognitive-like behaviours in the Y-maze

The effects of ceasing exercise on cognitive-like behaviours were investigated with the Y-maze and the Barnes maze. There were no differences between CONT and EXC mice in the time spent in the novel arm of the Y maze (p=979). There was a trend towards a significant reduction in novel arm time between EXC mice and EC mice (p=0.055), and EC displayed less time in the novel arm time than CONT mice (p=0.028) (Figure 5A).

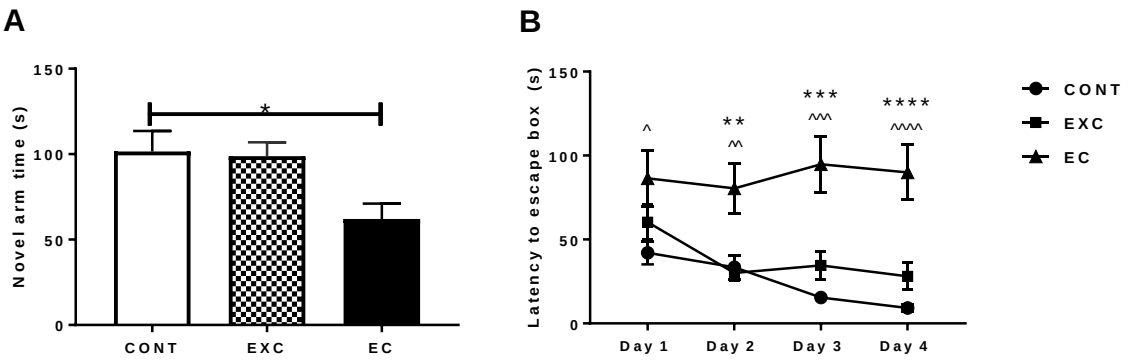

Figure 5.5. Cognitive-like behaviours in nine month old exercise control and exercise cessation C57BL/6 mice. A) Time spent in the novel arm of the Y-maze (*p<0.05), and B) Barnes maze latencies to identify the escape box location over four training days (CONT= no-exercise control; EXC = exercise control; EC = exercise cessation; (5B) CONT vs. EC: ^p<0.05, ^^p<0.01, ^^^p<0.001, ^^^^p<0.0001; EXC vs.EC **p<0.01, ***p<0.001, ****p<0.0001, n=12-17/group).

Cognitive-like behaviours in the Barnes maze

We investigated the effects of ceasing exercise on spatial learning, spatial memory, and cognitive flexibility with the Barnes maze training days and probe trial. Analyses of latencies to the escape box with two-way repeated measures ANOVA found no effect of time ($F(3, 126) = 1.047$, $p=0.374$) and a significant effect of treatment for spatial learning ($F(2, 42) = 11.13$, $p=0.0001$).

Post hoc analyses with Tukey's correction for multiple comparisons found no differences between CONT and EXC mice on any training day (day one p=0.415, day two p=652, day three p=0.880, and day four p=0.932). Compared to CONT mice however, EC mice had longer latencies on training days one, three, and four (p=0.018, p=0.001, and p=0.002). Similarly, compared to EXC mice, EC displayed significantly longer latencies to the escape box on days two, three, and four (p=0.00, p<0.001, and p<0.0009) (Figure 5B).

There were no differences between CONT, EXC, and EC mice in spatial memory during the probe trial (ANOVA p=0.563) (Figure 6A), however EC mice demonstrated significantly longer latencies to the escape box as a measure of cognitive flexibility compared to both CONT and EXC mice (p=0.03 and p=0.01 respectively).

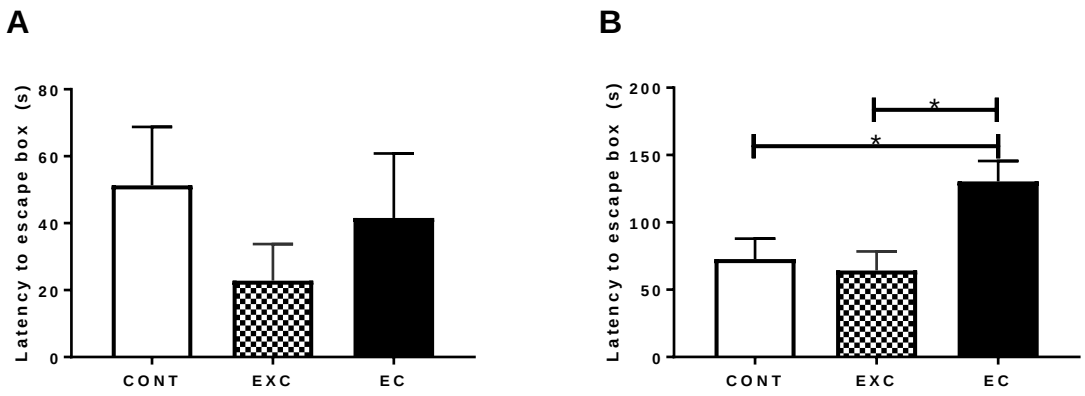

Figure 5.6. Cognitive-like behaviours in nine month old exercise control and exercise cessation C57BL/6 mice. a) Latencies to identify the escape box as a measure of spatial memory in the Barnes maze, b) Latencies to the new location of the escape box in the Barnes maze probe trial (CONT= no-exercise control; EXC = exercise control; EC = exercise cessation; ****p<0.0001, n=12-17/group).

Molecular results

Hippocampal gene expression

The effects of ceasing exercise on hippocampal gene expression were investigated with high-throughput qPCR (Table 2). Compared to CONT mice, EXC mice differentially expressed two monoaminergic related genes, and EC mice differentially expressed one monoaminergic and three inflammation related genes. Nine genes associated with neurogenesis, monoamines, and inflammation were differentially expressed between EXC and EC mice. These results suggest that both exercise and exercise cessation alters hippocampal gene expression.

Table 5.2. Significantly differentially expressed genes between CONT, EXC, and EC mice.

Comparison/ Gene	Estimate (coefficient of model fit)	Std. Error	Z score	p value	False Discovery Rate
EXC vs. CONT					
Slc6a4	-5.110303	1.357896	-3.763397	0.000167	0.012
Tph2	-4.103958	1.209907	-3.391969	0.000693	0.025
EC vs. CONT					
IL10	-5.1205775	1.1808710	-4.336270	1.449e-05	0.003
Gfap	-1.1374163	0.3454366	-3.292692	9.923e-04	0.022
Ntrk1	3.9875238	1.266777	3.063501	1.787e-03	0.036
Htr1a	-1.650551	0.5404401	-3.054087	2.257e-03	0.038
EC vs. EXC					
Slc6a4	5.0815991	1.284913	3.956426	7.607e-05	0.005
Th	4.2552015	1.1404307	3.731223	1.905e-04	0.007
Tgfbr2	-0.6136659	0.1778976	-3.449546	5.615e-04	0.010
Slc6a3	7.5913960	2.2550767	3.366358	7.616e-04	0.010
Sirt1	-0.4727714	0.1389780	-3.401773	6.695e-04	0.010
Tph2	3.8056496	1.1370566	3.346931	8.171e-04	0.010
Ntrk1	3.9111037	1.2766777	3.063501	2.187e-03	0.023
Gfap	-0.9339696	0.3117536	-2.995858	2.736e-03	0.025
Nlrp3	-0.9315195	0.3346773	-2.783336	5.380e-03	0.044

Legend: CONT = no-exercise control; EXC = exercise control; EC = exercise cessation; data presented were found with linear mixed-effects model of the treatment groups. Coefficients of the model fit and standard errors are detailed, with test statistics (Z-score) and false discovery rate calculated after correcting for multiple tests.

Immunohistochemistry

Investigation into exercise cessation related hippocampal expression of GFAP and IBA1 revealed no effect of exercise or exercise cessation for IBA1 stained cells (ANOVA $p = 0.266$). There was no difference between CONT and EXC mice in GFAP positively stained astrocytes, however EC displayed a significant reduction in GFAP positive astrocytes compared to CONT mice ($p=0.041$) (Figure 7).

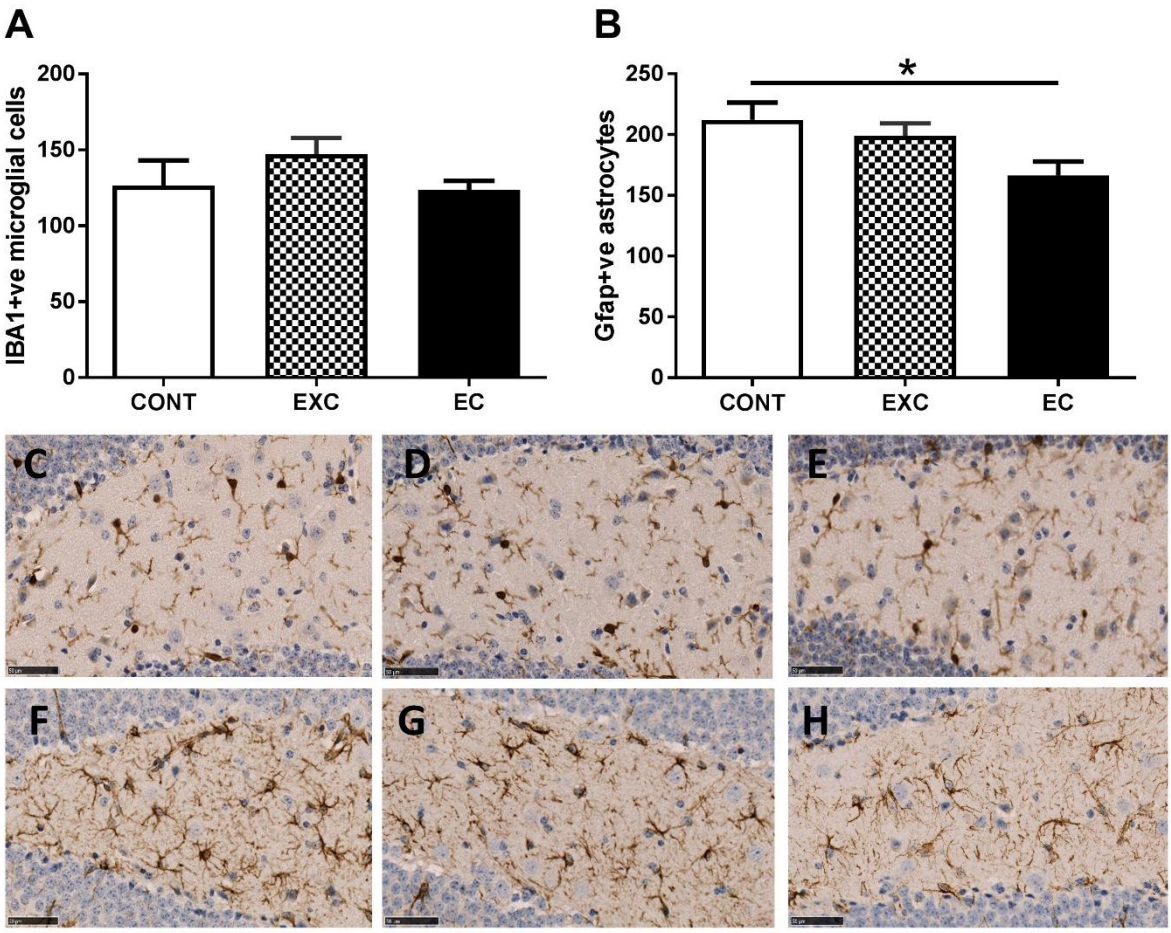

Figure 5.7. Exercise cessation related hippocampal expression of IBA1 and GFAP.
A) IBA1 and B) GFAP cells in nine month old CONT, EXC, and EC mice. Compared to CONT (C) and EXC (D) mice, there were no differences in the numbers of IBA1 stained microglia in EC mice (E) ($p = 0.2$). Compared to CONT (F) and EXC (G) mice, EC mice (H) demonstrated a significant reduction in the expression of positively stained GFAP stained GFAP astrocytes (CONT = no-exercise control; EXC = exercise control; EC = exercise cessation), scale bar = 50µm, *$p = 0.03$, n = 12-17/group).

Research report summary

Extensive research suggests that exercise can reduce anxiety-, depression-, and cognition-like impairment behaviours in mice and rats, and preclinical research has demonstrated some of the mechanisms in the hippocampus involved in conferring these effects. These include increases in neurotrophins such as BDNF that are associated increases in hippocampal neurogenesis, reductions in the inflammatory cytokine TNF, and advantageous changes in hippocampal gene expression including increases in *Sirt 1* mediated mitochondrial biogenesis. These exercise related changes suggest the cessation of exercise may have detrimental effects on anxiety-, depression-, and cognition-like impairment behaviours and hippocampal functioning. This research therefore addressed the question *"What are the effects of ceasing exercise on anxiety-, depression-, and cognition-like behaviours, and hippocampal physiology?"*

We randomised 28 C57BL/6 mice to exercise (for six months) or exercise followed by exercise cessation (four months exercise then two months no exercise) until nine months of age. Anxiety-like behaviours were then quantified with the OF and EZM, depression-like behaviours were investigated with the FST, and cognition-like behaviours were tested with the Y maze and Barnes maze. Hippocampal gene expression of 75 genes of interest was investigated with qPCR.

This article has reported that compared to exercise mice, mice that ceased exercise displayed significant increases in depression-, anxiety-, and impaired cognitive-like behaviours. Moreover, sixteen of the 75 hippocampal genes of interest were differentially expressed between exercise cessation mice and exercise control mice. These included reduced *Sirt1* related cell cycle signalling in the glucagon, AMPK, FOXO, and cellular senescence signalling pathways, altered neurotrophic signalling (via reduced *Tgfbr2* and *Smad3*), and altered serotonergic (increased *Slc6a4, Tph2 Ido1*), dopaminergic (increased *Slc6a3, Th*), and glutamatergic (reduced *Grin2b, Gria1, Gria2*) synaptic transmission. These results are detailed further in chapter six, the Discussion.

Chapter 6. Discussion.

Discussion preamble

In the discussion chapter that follows, the findings of the preceding results chapters are discussed in turn. The chapter commences with the first hypothesis:

Hypothesis one from chapter two: "*Long term exercise is associated with favourable behaviours compared to healthy age matched control non-exercise mice*".

This is followed by an outline of the chapter two discussion, the limitations of the study, and the chapter two conclusions. The hypothesis, discussion outline, discussion, limitations, and conclusions are then detailed for each of the remaining results chapters in turn:

Hypothesis two from chapter three: "*Exercise will improve depression-like and anxiety-like behaviours in WT and TNFR1$^{-/-}$ mice, without exercise related changes in TNF$^{-/-}$ and TNFR2$^{-/-}$ exercise mice*"

Hypothesis three from chapter four: "E*xercise will improve cognitive performance in WT and TNFR1$^{-/-}$ mice, but not in TNFR2$^{-/-}$ or TNF$^{-/-}$ mice*"

Hypothesis four from chapter five: "*Exercise cessation will increase depression-, anxiety-, and cognitive-like impairment behaviours and change hippocampal gene expression*"

Hypothesis 1

Long-term exercise will be associated with favourable behaviours compared to healthy age matched control non-exercise mice.

Hypothesis 1 discussion outline

Lifetime exercise may increase neurogenesis-associated anxiety. This could be an evolutionary conserved adaptation with adverse impacts on cognition-like function, with particularly pronounced effects in Y female mice with intact sex hormones. These issues require careful investigation in future preclinical research.

Discussion

This study has investigated the effects of lifetime exercise on anxiety-, depression-, and cognition-like behaviours in Y, M, and O mice. Interestingly, although our results revealed exercise reduced OF anxiety-like behaviours in line with our expectations, only M exercise mice displayed reduced anxiety-like behaviours in the EZM, and no exercise related changes to depression-like behaviours were evident. Similarly, whilst our results revealed exercise enhanced recognition memory in M mice, apparently reduced recognition memory was evident in Y and O exercise mice. Although an apparent reduction in recognition memory in O exercise mice and potentially impaired spatial memory in Y exercise mice seems counterintuitive, these behaviours are nevertheless a product of lifetime exercise. We consider that given exercise induced hippocampal neurogenesis is associated with increased anxiety-like behaviours in anxiety provoking tests (Fuss, Ben Abdallah et al. 2010, Onksen, Briand et al. 2012), it is possible that exercise related increases in anxiety were contributing factors to the apparently reduced recognition memory and spatial learning performance in our results.

In addition, our results also revealed control mice displayed elevated levels of baseline home cage locomotion across the lifespan, suggesting potentially pathologically elevated baseline activity levels. Lifetime exercise reduced home cage locomotion in Y and O mice, and reduced locomotion in the novel environment of the OF in M and O mice, with Y female mice travelling further in the home cage than male mice, and O male mice travelling further in the home cage than female mice. Other effects of sex on behaviours were evident predominantly in M mice, with male mice displaying greater centre time than female M mice, and female M mice showing greater head dipping exploration of the EZM than male mice.

Lifetime exercise alters baseline locomotor activity

Baseline locomotion may impact significantly on behavioural results. Our results revealed control mice show elevated levels of home-cage locomotor activity that remained stable across the lifespan. Consistent with previous work conducted with C57BL/6 mice, Y exercise mice showed reduced home cage locomotor activity (Duman, Schlesinger et al. 2008), although this did not reach significance in M mice. However, M exercise mice have exercised from 12 weeks until nine months of age – the six month duration of the adult lifespan, and therefore demonstrate all the health benefits of regular exercise at this age. In particular, this is likely to include exercise induced upregulation of the expression of Sirtuin 1 (Sirt 1) that activates PGC1a (peroxisome-proliferator-activated receptor-y or PPARGC1A) to orchestrate mitochondrial biogenesis and mitochondrial copy number (Dietrich, Andrews et al. 2008,

Bayod, Del Valle et al. 2011, Steiner, Murphy et al. 2011, Lezi, Swerdlow et al. 2014, Bayod, Guzman-Brambila et al. 2015). Upregulated mitochondrial biogenesis allows for greater mitochondrial respiratory chain function that enables ATP production for cellular functioning to meet the body's energy needs. It is possible this may have contributed to greater home cage locomotor activity in M mice that was non-significantly different from M control mice. Moreover, whilst ageing involves declines in mitochondrial biogenesis and exercise can ameliorate such declines (Koltai, Hart et al. 2012, Kang, Chung et al. 2013), it seems reasonable that increases in home cage locomotor activity to control levels would not be anticipated in O mice, in agreement with our results.

It is also noteworthy that Y female mice elected to travel significantly greater distances in the home cage than Y male mice. This could suggest that Y female mice require greater baseline levels of physical activity than Y male mice to maintain health during young adulthood, and raises some interesting issues in the context of anxiety-like, depression-like, and cognitiion-like behaviours in the context of ageing. The female sex hormone estradiol (E2) has significant effects on stress, with higher levels of E2 resulting in greater psychosocial stress than lower levels, and may be a mechanism involved in the prevalence of affective disorders in women (Newhouse 2014). Furthermore, E2 interacts with exericse. Exercise induced neurogengesis is transiently increased with estogen (Tanapat, Hastings et al. 1999), and wheel running for 1 or 6 months in middle aged female C57BL/6 mice induces similar levels of hippocampal neurogenesis and improves spatial memory (Marlatt, Potter et al. 2012). Exercise also has comparable effects to E2 on attention and memory (Marosi, Felszeghy et al. 2012). Furthermore, exercise may be more effective at attenuating stress hormones if estrogen is present (Jones, Gupton et al. 2016). Given these factors, it may be that female mice self-select levels of exericse that are optimal for balancing and maintaining healthy levels of female sex hormones, hippocampal neurogensis, and adaptive behaviours. Given that depression and cognitive impairment during ageing is more prevalent in human females than males, additional well powered studies are clearly needed to investigate and elucidate these issues.

Long-term exercise in Y mice had no effect on locomotor activity in the novel context of the open field, and this is consistent with previous findings of a lack of correlation between exercise and locomotor activity in the open field (during the light phase) in young adult mice at 8 weeks of age (r +0.09, ns) (Pietropaolo, Sun et al. 2008). However, reduced activity was evident with exercise in M and O mice, contrary to results from previous research showing exercise related increases in activity in this test (Dishman, Dunn et al. 1996, Marques-Aleixo, Santos-Alves et al. 2015), however species (mouse cf. rat) and running protocol (running wheel cf. treadmill running) are likely to account for these differences. Reduced locomotor activity in the OF is thought to be indicative of anxiety-like behaviour (Tang, Orchard et al. 2002), suggesting that the reduced locomotor activity in M and O mice may be related to exercise related increases in anxiety in this test.

Exercise associated neurogenesis may increase anxiety and impair cognition-like function

There is potentially further evidence of exercise related anxiety in our results. Work by Fuss et.al. (2010) has shown that chronic wheel running induced hippocampal neurogenesis in single housed male C57BL/6J mice had large correlations with markers of anxiety-like behaviours such as the total distance travelled in the light-dark box (r -0.78, p = 0.001) and rearings in the open field (r -0.67, p = 0.011). Onksen et.al (2012) further demonstrated that both male and female singly housed mice homozygous for the Cre/lox -conditional allele of ATR ($ATR^{f/f}$) (ataxia telangiectasia-mutated and rad-3-related protein; a cell cycle kinase necessary for normal levels of hippocampal neurogenesis) displayed increases in anxiety-like

behaviours of reduced open field centre time and distance travelled in the light compartment of the light-dark box following 4 weeks of wheel running. Although mice in the present study were socially housed, these findings are suggestive of a mechanism that plausibly explains increases in anxiety-like behaviours arising from exercise in the present study.

Interestingly, and contrary to our expectations, there was no exercise related improvement in recognition memory in Y mice – indeed a non-significant reduction was evident. Further investigations revealed no significant differences between Y control mice and Y exercise mice in the distances travelled or the time spent freezing in the maze, so the reasons for this reduction are unclear, and are contrary to previous work finding exercise related enhancement of recognition memory in adult mice aged 12 weeks (mediated by increases in neurogenesis) (Van der Borght, Havekes et al. 2007). Our investigation into the effects of lifetime exericse in M mice revealed M exercise mice displayed signifcantly better recognition memory than M control mice at this age, in agreement with our expectations. Whilst there are a paucity of studies examining the effects of exericse on recognition memory in M and O mice, it seems likely that increases in hippocampal neurogenesis would be involved in mediating exercise effects. It is possible that exercise associated in increases in hippocampal neurogenesis contributed to a significant reduction in recognition memory in O exercise mice compared to control O mice. However it is possible that exercise related fatigue also contributed to the reduction in exploration of the novel arm of the Y maze in this age group, or that the combination of these factors contributed to impaired performance. Examination of the distances travelled by O mice in the Y maze revealed O exercise mice travelled significantly less distance than control O mice ($p = 0.014$). Given that reductions of locomotor activity in novel contexts is considered to reflect increased anxiety-like behaivour (Tang, Orchard et al. 2002), significantly reduced distance in the Y maze by O exercise mice is suggestive of an exercise related increase in neurogenesis that may have contributed to greater anxiety, with reduced exploration of the maze that subsequently limited cognitive performance in this test. This may also have been effected by exercise related fatigue. Additional work is clearly required to elucidate whether exercise related neurogenesis is correlated with rodent performances in other tests where anxiety might confound results, such as the EZM and Barnes maze indices of spatial learning, memory, and cognitive flexibility.

Our investigations into the effects of lifetime exericse on spatial learning in the Barnes maze revealed behaviours that were contrary to our expectations. We revealed poorer performance in spatial learning and spatial memory in Y exercise mice compared to Y control mice on days one, three, and four, and no effect of exercise in M or O mice with the exception of longer latencies to locate the escape box for O exericse mice on day two of training. Exercise has been demonstrated to increase hippocampal neurogenesis in both adult and ageing mammals (van Praag, Christie et al. 1999, van Praag, Shubert et al. 2005), so it seems likley that our unanticipated results are related to the effects of lifetime exericse. Whilst there were no differences in the distances to the escape box in Y exericse mice compared to Y control mice, Y exercise mice displayed longer freezing times in all four training days (day 1 33.356 ± 7.581 vs 57.663 ± 10.071; day 2 18.571 ± 4.182 vs 34.003 ± 7.754 ; day 3 23.681 ± 4.768 vs 42.551 ± 10.253; and day 4 25.141 ± 5.457 vs 30.262 5.290, $p = 0.002$) suggesting exercise related neurogenesis contributed to greater anxiety-like behavours that increased the latencies to the escape box and may have impaired spatial learning in this test. Interestingly, female latencies to the escape box were significantly longer than males on days two, three, and four, suggesting that overall, exercise may have contributed to greater anxiety that may have extended the escape box latencies for Y female mice, thereby increasing the mean latencies for the Y exericse group. This hypothesis was confirmed by two way ANOVA of sex differences in freezing time that showed significant effect of sex ($p < 0.003$) with post hoc analyses revealing

significantly greater freezing by Y female mice compared to Y male mice on all four training days (all $p < 0.0001$). Whilst exercise related increased anxiety may have impaired spatial learning in Y exercise mice, exercise reduced anxiety in M mice, and this appears to have resulted in reductions in latencies to the escape box that were comparable to M control mice levels, although exercise did not enhance spatial learning beyond that shown by M control mice.

The probe trial investigations into spatial memory and cognitive flexibliity revealed that Y exercise mice displayed longer latencies to identify the locations of the old and new boxes as measures of spatial memory and cognitive flexibility respectively, suggesting that anxiety may have also contributed to impairment in these meaures. Interestingly, analysis of the distances travelled between control and exercise mice in the Probe trial revealed that Y exercise mice travelled significantly less than Y control mice consistent with reductions in home cage locomotor acitivty, and suggesting that neurogenesis associated anxiety is likely to have impacted on spatial memory performance in Y exercise mice. O exercise mice demonstrated singificantly longer latencies to identifiy the location of the new escape box as a measure of cognitive flexibility. Further t-test investigations into the distances travelled and freezing time in this test revealed no significant differences in the distances travelled ($p = 0.090$) but a significant increase in freezing time for O exericse mice compared to O control mice ($p = 0.001$), suggesting exercise related anxiety associated with hippocampal neurogenesis may have persisted into old age and affected cognitive flexibility in O mice.

Chronic exercise can reduce anxiety-like, depression-like, and cognition-like impairment behaviours

Our results of no lifetime exercise related changes in depression-like behaviours and potential impairments to cognition-like behaviours belies the notable range of advantageous behavioural, cellular, and molecular adaptations associated with exericse suggesting that lifetime exericse may be beneficical and attenuate ageing related anxiety, depression, and cognitive impairment. Chronic exercise has long been considered to reduce emotionality (Tharp and Carson 1975), however recent research has shown chronic exercise can reduce anxiety-like (Fulk, Stock et al. 2004) and depression-like behaviours (Duman, Schlesinger et al. 2008, Salam, Fox et al. 2009) with even low intensity exercise (Otsuka, Nishii et al. 2016). Exercise can also normalise and improve cogntion-like function and impairment (Marlatt, Potter et al. 2012), and has protective effects against cognitive decline in ageing mice (Kobilo, Guerrieri et al. 2014). However the increasing interest in the therapeutic effects of exericse has revealed inconsistencies, with occasional studies finding exercise induced increases in anxiety that seem to be associated with neurogenesis levels as was noted above (Grace, Hescham et al. 2009, Fuss, Ben Abdallah et al. 2010, Onksen, Briand et al. 2012). Nevertheless, chronic exericse also confers resilience to stress induced anxiety-like and depression-like behaviours arising from trauma and post traumatic stress (Patki, Li et al. 2014, Kochi, Liu et al. 2017), and cognitive-like changes arising from early life stress (James, Campbell et al. 2014). Lifestyle related stressors such as sleep deprivation related memory impairment (Salari, Sheibani et al. 2015), smoking cessation associated stress (Motaghinejad, Fatima et al. 2016), and the detrimental effects of a high fat diet on memory during ageing (Spencer, D'Angelo et al. 2017) are also reduced with chronic exericse.

The peripheral benefits of chronic exercise include the maintenance of lean body mass during ageing (the prevention of sarcopenia) (McMullan, Kelly et al. 2016), and reductions in resting glucose, triglycerides, and cholesterol levels (Lalanza, Sanchez-Roige et al. 2012). There are also central nervous system (CNS) benefits such as increases in cortical capillary volume and

capillary length (Wang, Chen et al. 2015), greater blood flow in limbic brain regions following chronic stress pathophysiology (Huang, Dong et al. 2017), and the attenuation of HPA axis responses to psychological stressors (Droste, Chandramohan et al. 2007). Chronic exercise involves brain region specific changes in physiology that modulate cardiovascular, circadian, and metabolic status (Morgan, Corrigan et al. 2015), and may be protective of telomere length that is considered a marker of cellular ageing (Botha, Grace et al. 2012, Ludlow, Ludlow et al. 2013). There is also well known exercise related upregulation of neurotrophins and signalling proteins including NGF (nerve growth factor), BDNF (brain derived neurotrophic factor), and TrkB (*Tropomyosin receptor kinase B*) that are required for the survival of new neurons in the hippocampus (Gustafsson, Liang et al. 2011, Bechara, Lyne et al. 2014, Hong, Lee et al. 2015) and are associated with enhanced spatial learning and recognition memory (Vaynman, Ying et al. 2004, Hopkins and Bucci 2010). Interestingly, whilst a blocked wheel increased cell proliferation, only running increased the number of new neurons in a dose dependent manner (Dostes, Dubreucq et al. 2016), with increases in new neuron numbers of 200% in exercising mice (Marlatt, Lucassen et al. 2010) that may be a contributing factor to reducing CA1 and dentate gyrus atrophy following stress (Li, Zhu et al. 2017). Finally, exercise is increasingly demonstrating inter-generational benefits such as enhancing learning and memory in the offspring of both maternal and paternal exericsing parents (Yin, Wang et al. 2013, Gomes da Silva, de Almeida et al. 2016). In contrast to this range of benefits from exercise, the available evidence about the effects of cessation of exericse in the brain from preclinical research shows the cessation of chronic exercise increases anxiety-like behaviours and cognitive-like impairment with reductions in BDNF and hippocampal neurogenesis (Kim, Ji et al. 2013, Nishijima and Kita 2015), suggesting that exercise may need to be sustained to maintain its benefits for the CNS during ageing.

Limitations

A limitation of this research is the absence of biological investigations to examine the mechanisms associated with lifetime voluntary exericse. Mice were not singly housed because this is a stressor (Fitzgerald, Yen et al. 2019) that could confound our outcomes of interest, however our exercise distance data measured for mice pairs was less accurate than had mice been housed singly.

Conclusions

In conclusion, to the authors' knowledge, this study is the first to investigate the impacts of lifetime exericse in normal healthy aging mice over the lifespan. Our results support the hypothesis that lifetime exericse reduces (OF) anxiety-like behaviours in M and O mice. Interestingly, and contrary to our expectations, we reveal no exercise related changes in depression-like behaviours, and possible exercise related impairment to spatial learning in Y mice, that largely normalised in M and O mice, however no exercise induced improvements were evident compared to control M and O mice. However interestingly, our results also suggest exercise related increases in anxiety-like behaviours that are likely due to exercise associated increases in hippocampal neurogenesis, consistent with the findings of previous studies (Fuss, Ben Abdallah et al. 2010, Onksen, Briand et al. 2012). We reveal likely exercise related anxiety-like behaivors of reduced distances travelled in O mice and freezing in Y mice that appear to have contributed to the unanticipated reduced recognition memory and spatial learnning respectively. These findings highlight that the effects of lifetime exercise are not simply an extension of the effects of chronic exercise. Additional well powered studies are required to elucidate the impacts of lifetime exercise on anxiety-like, depression-like, and

cognition-like behaivours, the associated mechanisms involved, and the sex specific effects of exercise for female mice in particular given the prevalence of depression and dementia in ageing human females.

Declaration of interest

The authors have no actual or potential conflicts of interest to disclose that could inappropriately influence, or be perceived to influence this work.

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Hypothesis 2

We hypothesised exercise would improve depression-like and anxiety-like behaviours in WT and TNFR1$^{-/-}$ mice arising from advantageous exercise associated TNF signalling via all TNF receptors in WT mice, and from the neuroprotective role of TNFR2 signalling in TNFR1$^{-/-}$ mice (Baune et al., 2012). We anticipated no exercise related changes in TNF$^{-/-}$ and TNFR2$^{-/-}$ exercise mice because exercise has no effect on TNF in the absence of TNF signalling in TNF$^{-/-}$ mice, and because TNF signalling via the TNFFR1 involves the negative regulation of cellular proliferation and the activation of cellular apoptosis (Wajant et al., 2003).

Hypothesis 2 discussion outline

Exercise associated TNFR1 and TNFR2 signalling in concert in WT exercise mice mediated reductions in aspects of anxiety-like behaviours. These findings are consistent with the current view that imbalances in TNF signalling are involved in disrupted affect. Additional studies are needed to further explore the roles of exercise related TNFR1 and TNFR2 signalling in anxiety-like and depression-like behaviours.

Discussion

To the author's knowledge, this is the first study to investigate TNF signalling mediated effects of exercise on anxiety-like and depression-like behaviours. We utilized a mouse model of TNF receptor signalling including WT, TNF$^{-/-}$, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ control and exercise mice to test the hypothesis that six months voluntary wheel running exercise would improve anxiety-like and depression-like behaviours in WT and TNFR1$^{-/-}$, but not in TNF$^{-/-}$ or TNFR2$^{-/-}$ mice. Our results provided limited results supporting our hypothesis that exercise would improve anxiety-like and depression-like behaviours in WT and TNFR1$^{-/-}$, but not in TNF$^{-/-}$ or TNFR2$^{-/-}$ mice. Compared to their respective control mice, exercise reduced OF anxiety-like behaviours in TNFR2$^{-/-}$ mice and EZM anxiety-like behaviours in WT exercise, with no other changes in EZM anxiety-like behaviours or FST depression-like behaviours.

We first examined the effects of TNF signalling via the TNF receptors on locomotion in the home cage, and in the more stressful novel environment of the open field. Interestingly, only TNFR1$^{-/-}$ exercise mice displayed reduced home cage locomotion compared to TNFR1$^{-/-}$ control mice, and WT exercise mice demonstrated reduced locomotion compared to WT control mice in the more stressful environment of the open field, suggesting exercise related anxiety in the more stressful context of a novel environment. We hypothesised exercise related reductions in anxiety-like behaviours in WT and TNFR1$^{-/-}$ mice, and no changes in anxiety-like behaviours in TNF$^{-/-}$ and TNFR2$^{-/-}$ mice, and investigated whether TNF signalling was involved in the effects of exercise on anxiety-like behaviours in the OF and EZM. We found little support for our hypothesis because compared to their relative control mice, WT exercise mice showed reduced anxiety-like behaviour in the EZM, and contrary to our hypothesis, TNFR2$^{-/-}$ exercise mice demonstrated significantly less anxiety-like behaviour in the OF. This suggests that TNF signalling via the TNFR1 was anxiolytic in the OF.

Previous research has investigated the effects of TNF and TNF receptor deficits on anxiety-like behaviours and depression-like behaviours in the open field (Camara, Corrigan et al. 2013); Patel et al., 2010; Simen et al., 2006b). These studies demonstrated mixed findings in both TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice, including no changes in OF centre time (Simen et al., 2006b) and reductions in OF anxiety-like behaviours (Patel et al., 2010). Reductions in OF anxiety-like behaviours in TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice are consistent with our findings of reductions in OF

anxiety-like behaviours in TNFR2$^{-/-}$ exercise mice. TNFR2$^{-/-}$ mice signal TNF via the TNFR1, so reductions in OF anxiety in TNFR2$^{-/-}$ exercise mice compared to TNFR2$^{-/-}$ control mice are also suggestive that exercise related mechanisms associated with TNFR1 signalling contribute to attenuating anxiety-like behaviours in the OF. Acute exercise induces increases in the cleaving of TNFR1 into sTNFR1 with a net anti-inflammatory effect (Petersen and Pedersen, 2005; Petersen and Pedersen, 2006), so lifetime exercise in our TNFR2$^{-/-}$ mice may have reduced pro-inflammatory TNF signalling with associated reductions in OF anxiety-like behaviours. Additional research is needed to investigate exercise related TNF signalling, and the effects of changes in TNF receptor expression on anxiety. Our results about exercise related reductions in anxiety-like behaviours in the OF are also consistent with previous findings of reductions in OF anxiety-like behaviours with moderate treadmill running or wheel running exercise (Binder, Droste et al. 2004, Salim, Sarraj et al. 2010, Patki, Li et al. 2014). However, our results extend previous results by suggesting that TNF signalling via the TNFR1 mediates exercise related reductions in OF anxiety-like behaviours.

We further investigated whether TNF signalling mediates the effects of exercise on anxiety-like behaviours in the EZM. Interestingly, there were no differences in EZM anxiety-like behaviours between TNF$^{-/-}$, TNFR1$^{-/-}$, and TNFR2$^{-/-}$ exercise and control mice, suggesting that the absence of TNF signalling does not impact on the effects of exercise on EZM anxiety-like behaviours, and that TNF signalling via the TNFR1 and TNFR2 do not mediate the effects of exercise on anxiety-like behaviours in the EZM. However, compared to WT control mice, WT exercise mice displayed significantly reduced EZM anxiety-like behaviours. WT exercise mice had intact TNF signalling via the TNFR1 and TNFR2. Previous studies have shown exercise related increased cleaving of TNFR1 into the anti-inflammatory sTNFR1 (Petersen and Pedersen 2005, Petersen and Pedersen 2006). It is possible that exercise related sTNFR1 signalling in WT exercise mice might have played a role in reductions in EZM anxiety-like behaviours in this strain. However, additional research investigating the molecular effects of exercise on TNF signalling via the TNF receptors is needed to clarify this hypothesis.

Our between strains analyses showed that WT control mice demonstrated significantly reduced EZM anxiety-like behaviours compared to TNF$^{-/-}$ and TNFR1$^{-/-}$ control mice, and WT exercise mice displayed reduced EZM anxiety-like behaviours compared to all knockout strains exercise mice. Reduced EZM anxiety in WT control mice compared to TNF$^{-/-}$ control mice suggests that intact TNF signalling may be more beneficial for attenuating anxiety-like behaviours than no TNF signalling. Reduced EZM anxiety-like behaviour in WT exercise compared to TNF$^{-/-}$ exercise mice is further evidence that intact TNF signalling via both the TNFR1 and TNFR2 has greater benefits for attenuating EZM anxiety-like behaviour than the absence of TNF signalling in the context of exercise. Reduced EZM anxiety in WT exercise mice than in TNFR1$^{-/-}$ and TNFR2$^{-/-}$ exercise mice suggests exercise associated increases in the cleaving of the membrane bound receptors TNFR2 and TNFR1 in intact animals reduces anxiety-like behaviours. In contrast to this, exercise associated increases in the soluble TNF receptors in the absence of either of the receptors results in imbalances in TNF signalling and increases in EZM anxiety-like behaviours.

We hypothesised exercise would reduce depression-like behaviours in WT and TNFR1$^{-/-}$ mice, and not in TNF$^{-/-}$ mice and TNFR2$^{-/-}$ mice. Contrary to our hypothesis, we found no exercise related changes in depression-like behaviours in any knockout exercise mice, suggesting a lack of involvement of TNFR1 or TNFR2 signalling in mediating the effects of exercise on depression-like behaviours, and that the absence of TNF signalling also does not impact exercise mediated effects on depression-like behaviours in the FST. Interestingly, we also found no changes in depression-like behaviours in WT exercise mice compared to WT control mice, contrary to previous work (Duman, Schlesinger et al. 2008, Cunha, Oliveira et al. 2013),

and the reasons for this seem unclear.

It is interesting to note our investigations of between strains differences in immobility time found TNFR1$^{-/-}$ control mice displayed longer immobility time than TNFR2$^{-/-}$ control mice. TNFR1$^{-/-}$ control mice have intact TNF signalling via the TNFR2, and TNFR2 is thought to be neuroprotective (Baune, Camara et al. 2012). However, increased immobility time in TNFR1$^{-/-}$ control mice seems contrary to TNFR2 mediated neuroprotection. It is possible that the absence of TNF signalling via the TNFR1 for the negative regulation of exercise-associated hippocampal cellular proliferation resulted in an unregulated excess of proliferation that disrupted affect and increased depression-like behaviours in this strain. However, additional research is needed to clarify the effects of exercise on TNF signalling via the TNFR2, and TNFR2 mediated affective behaviours.

The regulation of TNF signalling in patients displaying disrupted TNF levels and signalling in depression is a worthy clinical aim that warrants careful investigation. Efforts to block TNF signalling have found varied results (Krügel, Fischer et al. 2013, Sedger and McDermott 2014), and treatment with exogenous TNF can involve unforeseen biological consequences arising from blocking the wide-ranging functions of the protein (Sedger and McDermott 2014). Exercise may have potential to regulate TNF signalling without the adverse side effects associated with TNF pharmacotherapies. Indeed, one clinical study found higher baseline levels of TNF were associated with better treatment response to a 12 week exercise program in patients with depression ($p<0.0001$) (Rethorst, Toups et al. 2013). Exercise related changes in membrane bound and soluble TNF receptors (Petersen and Pedersen 2005, Petersen and Pedersen 2006) may have contributed to these results, and this hypothesis requires careful investigation in future studies to ascertain its potential clinical utility in the treatment of depression.

Exercise involves few risks and adverse side effects. It would be informative to investigate the effects of exercise in mouse models of TNF deficit utilising a chromic mild stress paradigm to explore the effects of TNF signalling in anxiety-like and depression-like behaviours. Future research ought to investigate the effects of a range of exercise protocols for reducing TNF in anxiety-like and depression-like behaviours that may translate to human conditions. These could including different types (aerobic, weight training, and mixed programs); durations (weeks and months); intensities (light, moderate, and high intensity); and frequencies (continuous exercise access and a range of translatable intermittent exercise programs). Such studies could ascertain whether exercise has therapeutic efficacy for altering TNF signalling and TNF receptor expression in depression and anxiety.

Limitations

Due to challenges with breeding TNFR2$^{-/-}$ mice the control and exercise TNFR2$^{-/-}$ mouse groups were smaller than WT, TNF$^{-/-}$, or TNFR1$^{-/-}$ mice, which might have limited the statistical power to detect smaller differences between strains.

Conclusions

To the authors' knowledge, this is the first study investigating TNF signalling mediated effects of exercise on anxiety-like and depression-like behaviours. We hypothesised six months voluntary wheel running exercise would reduce anxiety-like and depression-like behaviours in WT and TNFR1$^{-/-}$ mice, but not in TNF$^{-/-}$ or TNFR2$^{-/-}$ mice and found limited evidence in support of our hypothesis. Exercise reduced OF anxiety-like behaviours in TNFR2$^{-/-}$ exercise mice, and

reduced EZM anxiety-like behaviours in WT exercise mice compared to their respective control mice. FST depression-like behaviours were unchanged in both WT and knockout strains. Exercise associated TNF signalling via the TNFR1 including possible signalling via the sTNFR1 appears to mediate the effects of exercise on OF anxiety-like behaviours, whereas TNF signalling via the both membrane bound receptors with possible signalling via the soluble receptors sTNFR1 and sTNFR2 seems to mediate the effects of exercise on EZM anxiety-like behaviours. In spite of providing limited support of our hypothesis, these results are consistent with the current understanding of exercise associated cleaving of the TNFR1 into the anti-inflammatory signalling sTNFR1, TNFR2 cleaving into sTNFR2, and of TNF signalling in concert contributing to the maintenance of brain health. Further investigation into the TNF mediated effects of exercise are needed to investigate its potential as a therapy for modifying disrupted TNF signalling in human depression. Future research ought to include specific investigations into the potential differences in exercise mediated TNF signalling via the TNF receptors between males and females, and the effects of a range of exercise program parameters and associated impacts on affect in populations with TNF receptor imbalances.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

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Hypothesis 3

Here we hypothesised exercise would improve cognitive performance in WT and TNFR1$^{-/-}$ mice, but not in TNFR2$^{-/-}$ or TNF$^{-/-}$ mice, consistent with the requirement for TNF for normal cognitive performance, and the neuroprotective effects of TNFR2 and neurodegenerative effects of TNFR1.

Hypothesis 3 discussion outline

TNF receptor signalling via the TNFR1 and TNFR2 appears to mediate the effects of exercise on cognitive-like behaviours. The potential for exercise to regulate human TNF and TNF signalling and cognitive dysfunction needs investigation under inflammatory conditions including depression and neuropsychiatric disorders.

Discussion

To the authors' knowledge, this is the first study to examine the salient question of whether TNF signalling via the TNFR1 and TNFR2 confers the effects of exercise on cognition-like behaviours. We hypothesised that exercise would improve cognitive performance in WT and TNFR1$^{-/-}$ mice, but not in TNFR2$^{-/-}$ or TNF$^{-/-}$ exercise mice compared to their respective control mice. We found modest support for our hypothesis. Recognition memory was improved with exercise in WT mice, but impaired in TNFR1$^{-/-}$ mice, and had no effects in TNFR2$^{-/-}$ mice. Interestingly however, with the exception of WT mice, two-way ANOVAs found a significant main effect of exercise for spatial learning across all knockout strains. These showed detrimental effects of exercise for spatial learning in TNF$^{-/-}$ and TNFR1$^{-/-}$ mice and beneficial effects of exercise for TNFR2$^{-/-}$ mice, however post hoc testing found no significant differences between control and exercise mice of any strain. There were also no significant differences between control and exercise mice in spatial memory or cognitive flexibility in any strain. Collectively our results suggest that TNF signalling via the TNF receptors are involved in conferring the effects of exercise on recognition memory and spatial learning, but not spatial memory or cognitive flexibility.

Interestingly, recognition memory was improved by exercise in WT mice, impaired by exercise in TNFR1$^{-/-}$ mice, showed a trend towards memory deficits in TNF$^{-/-}$ mice, and had no effects in TNFR2$^{-/-}$ mice. Improvements in recognition memory in WT exercise mice is suggestive that TNF signalling via the TNFR1 and TNFR2 in concert confer the effects of exercise that improved recognition memory in this strain. However, the trend towards a significant reduction in recognition memory in TNF$^{-/-}$ exercise mice compared to TNF$^{-/-}$ control mice belies that TNF$^{-/-}$ exercise mice displayed a Y maze preference index that was approximately half that of control mice. This is evidence of meaningful impairments in recognition memory with exercise in this strain that are consistent with previous literature showing that TNF contributes to molecular aspects of cognitive functioning including synaptic plasticity, neurogenesis, and neuromodulation (McAfoose and Baune 2009).

Impaired recognition memory in TNFR1$^{-/-}$ exercise mice suggests exercise is detrimental for TNF signalling via the TNFR2 in the absence of TNFR1 signalling. Given the potential for reduced locomotor activity in exercise TNFR1$^{-/-}$ mice, we examined whether TNFR1$^{-/-}$ exercise mice travelled reduced distance in the Y maze, and found significantly less distance was travelled by TNFR1$^{-/-}$ exercise mice compared to their control counterparts ($p=0.001$). This could suggest that the reductions in time spent in the novel arm of the maze may have arisen from reduced distances travelled in the maze rather than reduced time exploring the novel

arm of the maze *per se*. However, it is also possible TNFR1$^{-/-}$ exercise mice travelled less distance due to a reduced preference for the novel arm of the maze, and this seems more likely because healthy mice are known to demonstrate a preference for exploring novelty rather than familiarity (Dulawa, Grandy et al. 1999). Previous work has shown TNFR1$^{-/-}$ mice display elevated levels of hippocampal neurogenesis (Chen and Palmer 2013). This study involves the absence of TNFR1 related negative regulation of neurogenesis in TNFR1$^{-/-}$ exercise mice. Increased neurogenesis arising from exercise and the absence of negative modulation of neurogenesis in TNFR1$^{-/-}$ exercise mice is therefore likely. Previous work has shown that phospholipase C-β1 knockout (PLC-β1$^{-/-}$) mice have elevated levels of neurogenesis in the granule cell layer of the hippocampus compared to WT littermates (Manning, Ransome et al. 2012). Interestingly, mice with elevated neurogenesis also demonstrated aberrant migration of mature granule cell layer neurons and impaired hippocampal dependent location recognition, indicating that elevated neurogenesis can contribute to impaired cognition-like behaviours. An absence of TNFR1 mediated negative regulation of neurogenesis may therefore have disrupted normal recognition memory, however this hypothesis requires investigation in future research.

Investigation into spatial learning latencies found no effect of exercise in WT mice, but significant main effects of exercise in TNF$^{-/-}$, TNFR1$^{-/-}$, and TNFR2$^{-/-}$ mice. Notably, exercise was detrimental for spatial learning in TNF$^{-/-}$ and TNFR1$^{-/-}$ mice, but was beneficial for TNFR2$^{-/-}$ mice. These results suggest that TNF is required for normal spatial learning consistent with previous findings by this group (Baune and Wiede 2008, Camara, Corrigan et al. 2013), and that TNF signalling via the TNFR1 and TNFR2 has significant roles in conferring the effects of exercise on spatial learning. As was noted above, TNFR1 deficit in TNFR1$^{-/-}$ mice may have increased levels of neurogenesis (Chen and Palmer 2013) contributing to impaired cognition-like behaviours in this strain with exercise. In contrast, exercise improved spatial learning in TNFR2$^{-/-}$ mice. TNFR2$^{-/-}$ mice have previously demonstrated *reduced* hippocampal neurogenesis (Chen and Palmer 2013). Exercise increases hippocampal neurogenesis [31], which may have elevated their previous lowered levels in TNFR2$^{-/-}$ exercise mice, improving spatial learning in this strain. This finding also provides further evidence for the contention that balanced signalling between the TNF receptors is needed to prevent neuropathology. TNF$^{-/-}$ exercise mice displayed a non-significant trend towards impaired spatial learning. TNF$^{-/-}$ mice lack TNF, and therefore lack the capacity for TNF signalling induced cellular apoptosis. Ordinarily this occurs via the TNFR1 death domain, FADD, and caspase 8 to negatively regulate neurogenesis and maintain healthy hippocampal cell numbers [32]. In the exercise condition, working muscle generated IL6 blunts TNF expression thereby reducing the available TNF for participating in the TNFR1 FADD/caspase 8 pathway, suggesting potential reductions in cellular apoptosis during exercise. However, during exercise in our TNF$^{-/-}$ exercise mice, the absence of TNF seems likely to involve an absence of TNF mediated cellular apoptosis via the TNFR1 FADD/caspase 8 pathway that may have contributed to aberrant hippocampal cell populations, and may have adversely affected spatial learning in this strain. Together these results highlight the importance of TNF signalling in concert via the TNFR1 and TNFR2 to maintain brain health, and of physiological levels of TNF for cellular regulation during neurogenesis and during exercise in particular.

We found no exercise related changes in spatial memory between control and exercise mice within strains, contrary to our expectations. Further examination of the distances travelled between control and exercise mice within strains found no differences in the distances travelled that might elucidate these results. Nevertheless, WT and TNFR2$^{-/-}$ exercise mice displayed latencies to the escape box that were approximately half those of their respective control mice, suggesting that exercise may induce meaningful changes in spatial memory in

spite of the lack of statistical differences. These results therefore support the view that TNF signalling in concert via the TNFR1 and TNFR2 confer the beneficial effects of exercise on spatial memory, and that the negative regulation of cell populations is essential for the functioning of normal spatial memory. In contrast, TNFR1$^{-/-}$ exercise mice showed spatial memory latencies that were nearly double those of TNFR1$^{-/-}$ control mice, suggestive of further evidence supporting the contention that the absence of TNF signalling via the TNFR1 in the context of exercise could contribute to unregulated increases in cell populations that could be detrimental to spatial memory.

Interestingly, we also found no significant differences in cognitive flexibility between control and exercise mice within strains. Notwithstanding this, exercise WT and TNFR2$^{-/-}$ mice displayed latencies to new location of the escape box that were less than half those of their respective control mice. This suggests that in spite of no statistically significant reductions in latencies in these strains, latency reductions arising from exercise do appear meaningful. In addition, TNFR1$^{-/-}$ exercise mice display latencies that are close to double those of TNFR1$^{-/-}$ control mice. The non-significant improvements in cognitive flexibility in WT exercise mice and the deterioration in cognitive flexibility in TNF$^{-/-}$ exercise mice are consistent with our previous findings of improvements in recognition memory in WT exercise mice and impairments in recognition memory in TNF$^{-/-}$ exercise mice. These findings therefore support the view that TNF signalling on concert via the TNFR1 and TNFR2 contributes to enhancing cognitive flexibility, whereas TNF deficit contributes to the deterioration of cognitive flexibility in TNF$^{-/-}$ exercise mice. Considered together, these results suggest that TNF signalling via the TNFR1 and TNFR2 may contribute to spatial memory and cognitive flexibility, and are consistent with literature finding exercise associated improvements in spatial memory (Ang, Dawe et al. 2006, Cassilhas, Lee et al. 2012, Vilela, Muller et al. 2016). This further suggests the TNF receptors may be involved in this aspect of cognition-like functioning. We have investigated a voluntary wheel running mouse model of exercise that translates to voluntary human self-selected exercise. However, there are a range of exercise variables that could contribute to changes in TNF signalling via the TNF receptors, and these require investigation in future research. These include the effects of different types (treadmill vs. wheel running), different intensities (low, moderate, and high intensity), and types of exercise (resistance or strength exercise), for different durations (weeks, months, or long term/years). Additional research is required to replicate or otherwise elucidate the involvement of TNF signalling in cognition-like behaviours.

Limitations

Due to challenges with breeding the TNFR2$^{-/-}$ mice, the TNFR2$^{-/-}$ mouse groups had only one female TNFR2$^{-/-}$ mouse in the TNFR2 exercise group (Table 1) and group sizes were smaller than groups of other strains. In addition, this experiment was conducted with data collectors, assessors, and analysts unblinded to treatment groups.

Conclusions

This is the first study to investigate the salient question of whether TNF signalling via the TNF receptors TNFR1 in TNFR2 confer the effects of exercise on cognition-like behaviours. Impaired recognition memory in TNFR1$^{-/-}$ exercise mice occurred in the absence of TNFR1 mediated negative regulation of hippocampal cell populations. This suggests exercise related increased neurogenesis may have been abnormally high with resultant deficits in recognition memory in TNFR1$^{-/-}$ exercise mice. This result supports the current view that TNF signalling in concert via the TNFR1 and TNFR2 is necessary for neuroprotection and brain health. In

addition, deficits in spatial learning in $TNF^{-/-}$ exercise mice indicates that TNF signalling is required for normal cognitive function and is consistent with previous reports, whereas spatial learning deficits in $TNFR1^{-/-}$ exercise mice is further evidence of the role of TNF signalling via the TNFR1 to negatively regulate and maintain normal hippocampal cell populations. There were no significant effects of exercise on spatial memory or cognitive flexibility, however noteworthy non-significant improvements in spatial memory in WT and $TNFR2^{-/-}$ exercise mice, and non-significantly impaired spatial memory in $TNFR1^{-/-}$ exercise mice indicated meaningful changes in spatial memory behaviours with exercise, suggesting the possible involvement of TNF signalling via the TNFR1 and TNFR2 in spatial memory. Our results suggest exercise may have potential to alter aspects of cognitive function in ways that might be beneficial for humans, and additional exercise protocols therefore need investigation. The potential for exercise to regulate TNF and TNF signalling in contexts of disrupted TNF physiology and cognitive dysfunction is an important line of investigation for depression, schizophrenia, bipolar disorder, Alzheimer's disease, and other dementias. Additional research to investigate how exercise effects peripheral and central nervous system TNF and TNF signalling via the TNFR1, TNFR2, sTNFR1, and sTNFR2 in these conditions are required.

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Table 4.1, Table 4.2, and Figure 4.1 are reprinted from Brain Research, 1695, Morgan, Julie A., Singhal, Gaurav, Corrigan, Frances, Jaehne, Emily J., Jawahar, Magdalene C., Baune, Bernhard T., "Exercise related anxiety-like behaviours are mediated by TNF receptor signalling, but not depression-like behaviours", 10-17, Copyright (2018), with permission from Elsevier.

Hypothesis 4

We hypothesise that the cessation of exercise will increase depression-, anxiety-, and cognitive-like impairment behaviours with related alterations in hippocampal gene expression.

Hypothesis 4 discussion outline

In this discussion of the results of chapter 4, exercise cessation related increases in depression-, anxiety-, and cognitive-like impairment behaviours are discussed. The exercise cessation related hippocampal gene expression changes appeared suggestive of altered immune, neurogenic, and monoaminergic functioning. The findings of this study suggest that replication and translation into high-quality pilot clinical trials may be warranted.

Discussion

This study investigates the hypothesis that ceasing exercise for eight weeks after four months of exercise will increase depression-like and related anxiety-like and impaired cognition-like behaviours with changes in hippocampal gene expression in C57BL/6 mice at nine months of age. We had 80% power to detect the anticipated minimum difference in FST immobility time between EC mice compared to CONT and EXC mice with a 5% alpha level. There were no differences between groups in home cage locomotion. Whilst EXC mice demonstrated a reduction in locomotor activity in the more stressful environment of the open field, there were no changes in locomotor activity in EC mice. In support of our hypothesis, compared to CONT and EXC mice, mice that ceased exercise displayed significant increases in depression-like behaviours in the FST, increased OF anxiety- and EZM anxiety-like behaviours, and spatial learning and cognitive flexibility deficits in the BM. Exercise cessation mice also spent less time in the novel arm of the Y maze compared to CONT mice, with a trend toward a significant reduction in novel arm time between EC mice and EXC mice, however it is possible this is chance finding, and this will be discussed in detail below. The behavioural changes were accompanied by changes in hippocampal gene expression, with 10 of the 75 genes of interest significantly differentially expressed between EC mice and CONT and EXC mice. These included four differentially expressed genes in EC mice compared to constitutional CONT mice levels (IL10, Gfap, neurotrophic receptor tyrosine kinase 1 (Ntrk1), and the serotonin 1a receptor (Htr1a)). Compared to EXC mice, EC mice demonstrated six differentially expressed genes (Slc6a4, tyrosine hydroxylase (Th), transforming growth factor beta receptor 2 (Tgfbr2), the dopamine transporter (Slc6a3), Sirtuin 1 (Sirt1), Tph2, Ntrk1, Gfap, and the NLR family pyrin domain containing 3 (Nlrp3)), suggestive of possible disruption of hippocampal physiology following exercise cessation (Figure 7).

Our findings of significant increases in anxiety-like and cognitive-like behaviours are consistent with previous research showing increased anxiety-like and cognition-like impairment behaviours after the cessation of exercise in mice (Kim et al., 2013, Nishijima et al., 2013) and in clinical studies (Morgan et al., 2018). However, we found the cessation of exercise increased depression-like behaviours, contrary to previous results (Nishijima et al., 2013). This might occur for several reasons, including the different ages when starting exercise, different durations of exercise, and different ages when ceasing exercise. Mice in the present study started exercise when they were considerably older than mice in previous research (12 weeks vs. four weeks), exercised for a greater proportion of the adult lifespan (four months vs. eight weeks or 69% vs. 38% of the adult lifespan), and were

considerably older than mice in other studies when behaviours were tested (nine months vs five months) (Nishijima et al., 2013). Given these differences, it seems possible that exercise for 69% of the adult lifespan became normal for mice in this study. It follows that ceasing exercise after exercising for nearly 70% of the adult lifespan could be a major change from the norm, with likely greater impacts on hippocampal gene expression and behaviours than the cessation of shorter durations of exercise of 38% of the lifespan for instance (Nishijima et al., 2013). Mice in the current study may also have had some ageing related vulnerability to depression-like behaviours that were not characteristic of the younger mice in previous research. Our findings of increased anxiety-like behaviours and impaired spatial learning are therefore consistent with previous results, although our results also suggest that older mice might develop depression-like behaviours from ceasing longer-term exercise.

Stopping exercise also changed cognition-like function. EC mice displayed significantly impaired spatial learning on days two, three, and four of training in the Barnes maze compared to CONT and EXC mice. Deficits in cognitive flexibility in the probe trial were also evident in EC mice compared to CONT and EXC mice. It is possible that the stressful open and well-lit environment of the Barnes maze contributed to a reduction in locomotor activity and longer latencies to locate the escape box over the training days for spatial learning in this test, although reduced locomotor activity was not evident in the open field test. However, spatial learning deficits in the Barnes maze are consistent with previous work showing deficits in spatial learning in the radial arm maze after ceasing four weeks swimming in four-month-old mice (Kim et al., 2013). EC mice spent around half the time CONT and EXC mice spent in the novel arm of the Y maze. This difference was significant compared to CONT mice and was a trend towards significance in EXC mice. However, CONT and EXC mice spent about 100s (one third of testing time) in the novel arm of the maze, and given there are three arms of the maze, it is possible these results could be a chance occurrence. Our Y maze results therefore seem inconclusive. Nevertheless, our exercise cessation related spatial learning and cognitive flexibility deficits seem consistent with previous research (Kim et al., 2013, Nishijima et al., 2013).

To the authors' knowledge, this is the first study to investigate the effects of ceasing exercise on hippocampal gene expression. EC mice demonstrated differentially expressed hippocampal genes suggestive of changes in neurotrophic (Ntrk1 and Tgfbr2), inflammatory (IL10, Gfap, Nlrp3 and Sirt1), and monoaminergic gene expression (Htr1a, Slc6a4, Tph2, Slc6a3, and Th). These results are in agreement with current literature suggesting the involvement of these factors in the pathogenesis of depression and associated anxiety and cognitive impairment (Jacobs et al., 2000, Dantzer et al., 2008) (Schiepers et al., 2005, Millan et al., 2012, Miller et al., 2015). Four genes were differentially expressed between EC and CONT mice, including Ntrk1 (that codes for TrkB a brain derived neurotrophic factor (BDNF) receptor), IL10, Gfap, and Htr1a, and interestingly this is suggestive of significant changes from constitutive levels. Some overlap was evident in the differentially expressed genes between EXC and EC mice (Gfap and Ntrk1), and between gene expression and immunohistochemistry results in EC mice (Gfap/GFAP). Compared to EXC mice, EC mice demonstrated changed expression of Slc6a4, Tph2, Slc6a3, Th, Ntrk1, Tgfbr2, Gfap, Nlrp3 and Sirt1. Sirt1 however has more broad ranging functions and is discussed further below.

Significant changes in the expression of hippocampal Ntrk1 (TrkB) and Tgfbr2 may have contributed to impaired cognition-like and depression-like behaviours. Ntrk1 codes for the BDNF receptor TrkB that is involved in regulating hippocampal neurogenesis and cellular proliferation (Li et al., 2008), whilst Tgfbr2 contributes to neuronal differentiation,

maturation, and the survival of newly born cells (Kandasamy et al., 2014). Significantly reduced expression of Tgfbr2 is suggestive of a limited capacity for Tgfb signalling related neuronal differentiation, maturation, and survival that together with increased Ntrk1 are suggestive of disruption to aspects of neurogenesis, cellular proliferation, and differentiation. These results are consistent with reduced neurogenesis and impaired cognition-like behaviours found with exercise cessation in previous research on this topic (Nishijima et al., 2013). These results are also consistent with the neurogenic theory of depression and anxiety postulated reductions in hippocampal neurogenesis in the pathogenesis of depression (Miller et al., 2015, Nishii et al., 2017).

The cessation of exercise significantly changed the expression of several monoaminergic metabolism related genes. Exercise cessation reduced hippocampal expression of Htr1a from constitutive CONT levels, and significantly increased expression of Slac6a4, Tph2, Slc6a3, and Th compared to EXC mice. Htr1a has implicated in anxiety and thus may have contributed to exercise cessation related anxiety-like behaviours. Slc6a4 and Slc6a3 regulate extracellular serotonin and dopamine, whilst Tph2 and Th are the rate-limiting enzymes for their synthesis. It follows that exercise cessation related increases in the expression of the Slc6a4 and Slc6a3 may have altered levels of serotonin and dopamine in the synaptic cleft. Increased expression of Tph2 and Th in EC mice is thus suggestive of altered serotonergic and dopaminergic metabolism. Collectively these results are suggestive of changes in hippocampal monoaminergic metabolism related to increased depression-like and anxiety-like behaviours. These results are also broadly consistent with the monoaminergic dysregulation characterized by the monoaminergic theory of depression (Schildkraut 1965, Baune et al., 2008).

Ceasing exercise significantly reduced the expression of hippocampal Gfap, IL10, and Nlrp3 that are involved in regulating pro and anti-inflammatory immune functioning. This is suggestive of possible altered immune regulation that may have contributed to impairments in cognition-like behaviours in EC mice. Gfap is a cytoskeletal filament protein that contributes to astrocytic morphology, astrocyte to neuron signalling, and mounting protective reactive gliosis (Pekny et al., 2005, Lobo-Silva et al., 2016), whereas IL10 is expressed by astrocytes and microglia to regulate neurogenesis and neuronal survival and prevent apoptosis and excessive inflammatory responses (Ramboz et al., 1998, Pekny et al., 2005). The Nlrp3 inflammasome detects pathogen specific proteins (PAMPS) and damage associated proteins (DAMPS) and activates immune system responses (Kanneganti et al., 2007, Franchi et al., 2009, Singhal et al., 2014). Significant reductions in Gfap, IL10, and Nlrp3 are therefore suggestive of possible detrimental changes to astrocytic morphology, astrocyte to neuron signalling, and protective gliosis. There could also be potentially limited dampening of excessive inflammatory responses, and blunted Nlrp3 mediated immune responses associated with increased depression-like and anxiety-like behaviours, and impaired cognition-like behaviours. This immune disruption is broadly in line with the immune disruption described by the inflammatory theory of depression (Smith 1991, Zorrilla et al., 2001, Dantzer et al., 2008).

Ceasing exercise significantly reduced Sirt1 in EC mice compared to EXC mice. Sirt1 mediated mitochondrial biogenesis and adenosine triphosphate production provides the energy needs for all brain and hippocampus functions. This includes the regulation of neurogenesis, the synthesis and metabolism of monoamines, cytokine expression and signalling, and the promotion of neuronal and astrocytic morphology that is involved with long-term potentiation, learning, and memory (Abe-Higuchi et al., 2016) (Michan et al., 2010, Codocedo et al., 2012, Liu et al., 2013, Satoh et al., 2013). Sirt1 associated mitochondrial biogenesis in conjunction with peroxisome proliferator-activated receptor-

gamma coactivator (PGC1α) in the hippocampus mediates depression-like behaviours in mice (Agudelo et al., 2014, Aguiar et al., 2014, Abe-Higuchi et al., 2016), and a recent GWAS identified a significant Sirt1 locus association with melancholic depression (Converge Consortium 2015). Reduced Sirt1 and otherwise altered hippocampal gene expression in EC mice therefore supports our hypothesis of altered hippocampal gene expression with the cessation of exercise, and is consistent with the postulated neurogenic, immune, and monoaminergic pathogenesis of depression and related anxiety and cognitive impairment (Schildkraut 1965, Smith 1991, Jacobs et al., 2000, Dantzer et al., 2008, Krishnan et al., 2008, Millan et al., 2012, Miller et al., 2015).

We found modest effects of exercise in EXC mice compared to CONT mice, with no changes in baseline locomotor activity in the home cage, but reduced locomotion in the more stressful context of the open field compared to CONT mice. Compared to CONT mice, EXC mice spent more time in the central regions of the open field but did not display any changes in time spent in the open quadrants of the EZM. Compared to CONT mice, EXC mice displayed similar time spent in the novel arm of the Y maze, although, as was noted above the time spent in the novel arm of the maze by CONT and EXC mice was one third of the total time in a test with three arms. It is therefore possible that the time spent in the novel arm by CONT and EXC mice was a chance occurrence. There were also no changes in latencies to the escape box as indices of spatial learning, spatial memory, or cognitive flexibility. It is possible that testing stress related reductions in locomotor activity contributed to these results, as could be suggested by reductions in locomotor activity in the open field. Notwithstanding this, compared to CONT mice, EXC mice displayed significant reductions in the expression of hippocampal tryptophan hydroxylase (Tph2) and the serotonin transporter (Slc6a4) that may have contributed to the reduced anxiety-like behaviours evident in the open field.

Slc6a4 and Tph2 are implicated in the pathogenesis of anxiety, and Tph2 has been suggested as potential therapeutic target for stress related anxiety (Lesch et al., 2003, Chen et al., 2013), making the reductions in the expression of these genes with exercise consistent with previous results. Interestingly however, our results of non-significant reductions in FST immobility time in EXC mice, and no exercise associated improvements in recognition memory, spatial learning, spatial memory, or cognitive flexibility, and this is inconsistent with much of the literature (van Praag et al., 1999, Li et al., 2013, Wood et al., 2015, Huang et al., 2017). However, exercise mice in previous research have undertaken exercise for between approximately 25% (Duman et al., 2008, Li et al., 2013, Huang et al., 2017) or 43% (van Praag et al., 1999) of the lifespan of the included animals, whereas in the current study EXC mice exercised for 69% of the lifespan. It is possible that shorter durations of exercise involve greater behavioural and physiological changes, and that longer-term exercise (for 69% of the lifespan) becomes the physiological norm with fewer behavioural or physiological changes over time. These results highlight additional research is needed to investigate the behavioural and hippocampal gene expression adaptations arising from long-term exercise.

Limitations

Although we block randomized treatment allocation for mice, we did not have sufficient numbers in the groups to conduct the analyses of sex differences in behaviours or molecular methods. In addition, the individuals conducting data collection and analyses were not blinded to treatment group. This paper directly examined molecular pathways associated with depression. Future studies could also incorporate those associated with

anxiety alone (rather than anxiety associated with depression), like the endocannabinoid system. Levels of endocannabinoids increase after acute exercise in animals and humans and are thought to relate to the euphoric sensations experienced after intense exercise (Basso et al., 2017). Of note modulation of the endocannabinoid system can modulate anxiety (Ruehle et al., 2012) and could be investigated in future research on exercise cessation.

Conclusions

We have investigated the effects of ceasing exercise on depression- and related anxiety- and cognition-like behaviours and hippocampal gene expression. Overall, the cessation of exercise increased depression- and anxiety-like behaviours, and impaired spatial learning and cognitive flexibility cognition-like behaviours in EC mice. Hippocampal gene expression was also altered in EC mice. Gene expression changes in EC mice were suggestive of changes in neurogenesis, monoaminergic metabolism, and immune function that are broadly consistent with pathophysiological aspects of depression and associated anxiety and cognitive impairment hypothesized by the neurogenic, inflammatory, and monoaminergic hypotheses of depression. As mice and humans share mammalian physiology, our results suggest ceasing exercise in early middle-aged humans could increase depression, anxiety, and cognitive deficits with related changes in hippocampal functioning. This concerning possibility requires careful investigation in high quality clinical trials. Future research ought to also investigate whether exercise cessation related detrimental behavioural and neurobiological changes are reversible with exercise recommencement.

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Ethical approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed, and all procedures performed in this study were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

Declarations of Interest: None.

Chapter 7. Significance and future directions

Preamble to the significance and future directions

In this final section of the thesis, is a brief summary of major findings that provides a foundation for the consideration of the significance and possible future directions for research in the field. Chapter two on the effects of exercise on anxiety-like, depression-like, and cognition-like behaviours over the adult lifespan of WT mice suggests that: i) exercise related anxiety could be a phenomenon worthy of future research in young women in particular; ii) there is also potential for exercise related anxiety in older populations that might warrant investigation, and; iii) given our null findings about exercise effects on depression-like behaviours over the lifespan and the increasing evidence of immune involvement in depression, the characterisation of immune profiles arising from exercise over the lifespan could be informative.

Chapters three and four examining whether TNF signalling mediates the effects of exercise on anxiety-like, depression-like, and cognition-like behaviours suggests: i) further work is required to replicate our findings about the TNF signalling mediated effects of exercise in anxiety-like, depression-like, and cognition-like behaviours; ii) The involvement of TNF and the sTNFR1 and sTNFR2 in both Major Depressive Disorder (MDD) and acute exercise physiology indicates that future trials ought to test whether exercise alters the proportions of membrane bound TNF receptors to soluble TNF receptors with subsequent changes in depressive symptoms.

Chapter five has examined the effects of exercise cessation on anxiety-like, depression-like, and cognition-like behaviours and hippocampal neurobiology. The findings of this study are suggestive that; i) exercise cessation at different life stages could involve detrimental changes in mood and cognition, and this possibility requires investigation in future trials; ii) if significant reductions in exercise have similar effects on affect and cognition, there may be cause for concern about international physical activity guidelines that involve a significant reduction in exercise from 17 years of age to the age of 18; iii) longitudinal survey analyses that include data linkage access could provide valuable data about the associations between exercise cessation and increases in depressive symptoms or poorer cognitive performance.

Significance and future directions

This work aimed to investigate the effects of long-term exercise and ceasing long-term exercise on anxiety-like, depression-like, and cognition-like behaviours, and neurobiology. To this end, chapter two investigated the effects of lifelong exercise on anxiety-like, depression-like, and cognition-like behaviours over the lifespan. The potential mediating influence of TNF signalling via the TNF receptors TNFR1 and TNFR2 in anxiety-like and depression-like behaviours, and cognition-like behaviours were then investigated in chapters three and four respectively. Chapter five investigated the effects of exercise cessation on anxiety-like, depression-like, and cognition-like behaviours and hippocampal neurobiology. The rodent model research included in this work were conducted because they have potential to inform about mammalian and therefore human behaviours and physiology. The significance of these studies, and possible directions for future research are thus considered in this context.

Results from chapter two offered limited support for our hypothesis of lifetime exercise related improvements in anxiety-like, depression-like, and cognition-like behaviours over the adult lifespan of WT mice. Major findings from this chapter included:

- reductions in OF anxiety-like behaviours in M and O mice, but not in Y mice
- no changes in depression-like behaviours
- increases in anxiety related freezing in Y (female) mice limiting Barnes maze spatial learning, and
- reduced distances travelled in O mice Barnes maze spatial memory with longer latencies to locate the escape box

These results were largely unanticipated, and interestingly, could suggest that lifetime exercise involves thus far un-investigated mechanisms affecting changes in anxiety and depression-like behaviours that remain to be elucidated. It is possible these could be different in O mice in some respects from the mechanisms involved in chronic exercise in Y mice such as increases in BDNF and hippocampal neurogenesis (Fuss, Ben Abdallah et al. 2010). Our findings of exercise related increases in anxiety-like behaviours are consistent with previous findings of exercise related increases in anxiety-like behaviours (Fuss, Ben Abdallah et al. 2010, Onksen, Briand et al. 2012). Moreover, exercise related cognitive impairment in older mice is of concern in the context of the high prevalence of dementias (Nichols, Szoeke et al. 2018). These findings add further evidence that exercise associated anxiety is a phenomenon requiring future research in humans. It could be that there are subgroups of individuals in younger and older cohorts who experience exercise related increases in anxiety in humans. Possible directions for future research in this area could include examination of the effects of different types, intensities, or frequencies of exercise on exercise related anxiety, and associated cognitive impairments.

Our results also indicated no changes in depression-like behaviours across the lifespan, contrary to our expectations. Whilst these findings are counterintuitive and the reasons for null findings remain to be clarified, they are a result of lifetime exercise. One possibility is that lifetime exercise – like a lifetime involving more limited physical activity – is the physiological norm. It follows that only perturbations from the norm might have disruptive effects on physiology that contribute to behavioural changes, as was demonstrated in chapter five. The aetiology and pathophysiology of depression is now considered to involve significant innate and adaptive immune changes in humans (Howren, Lamkin et al. 2009, Dowlati, Herrmann et al. 2010, Maes 2011, Strawbridge, Arnone et al. 2015, Kohler, Freitas et al. 2017, Kappelmann, Lewis et al. 2018) suggesting that the characterisation of immune profiles arising from lifetime exercise could be an informative direction for future investigation.

Chapters three and four extended our findings from chapter one, and investigated whether TNF signalling via the TNFR1 and TNFR2 mediated the effects of long-term exercise on anxiety-like, depression-like, and cognition-like behaviours. These chapters included major results from within strains comparisons of:

- reduced OF anxiety-like behaviours in TNFR2$^{-/-}$ exercise mice
- reduced EZM anxiety-like behaviours in WT exercise mice
- no changes in depression-like behaviours in any strain
- impaired recognition memory and spatial learning in TNFR1$^{-/-}$ exercise mice
- deficits in TNF$^{-/-}$ exercise mice in spatial learning
- no changes in spatial memory or cognitive flexibility in any strain

Deficits in spatial learning in TNF$^{-/-}$ exercise mice were in agreement with our hypothesis of no TNF signalling benefits in TNF$^{-/-}$ exercise mice due to the absence of TNF signalling in this strain. However, no changes in depression-like behaviours, spatial memory, or cognitive flexibility in any strain were contrary to our expectations. These initial findings require replication and further investigation in future mouse model research.

However, TNF signalling in human Major Depressive Disorder (MDD) is an area that requires careful and thorough investigation in particular. MDD can involve increases in TNF and changes in the levels of sTNFR1 and sTNFR2, and TNF is increasingly associated with treatment resistant depression (Dowlati, Herrmann et al. 2010, Strawbridge, Arnone et al. 2015, Sowa-Kucma, Styczen et al. 2018, Strawbridge, Young et al. 2018). Interestingly, a clinical trial examining the effects of exercise for MDD by Rethorst et al. (2013) has demonstrated that higher baseline TNF was associated with greater treatment response to exercise, in-spite of no significant changes in TNF. Furthermore, higher TNF at baseline predicted better outcomes with exercise treatment (Rethorst, Toups et al. 2013). Interestingly however, the effects of exercise on TNF receptors, including the exercise associated cleaving of the membrane bound TNF receptors in MDD remains unknown. Future research examining the effects of exercise on TNF in MDD should therefore investigate the effects of exercise on the cleaving of membrane bound TNF receptors into the soluble TNF receptors sTNFR1 and sTNFR2. Speculatively speaking, it could be that the cleaving of membrane bound TNF receptors into the soluble TNF receptors is a mechanism contributing to the efficacy of exercise for reducing MDD. It could also be that TNF receptor signalling changes in the proportions of membrane bound to soluble TNF receptors are involved in cognitive impairments found in MDD (Bobinska, Galecka et al. 2017). These timely hypotheses clearly require careful testing in future clinical trials.

The fourth chapter of this thesis investigated the effects of exercise cessation on anxiety-like, depression-like, and cognition-like behaviours and hippocampal neurobiology. This chapter demonstrated exercise cessation had detrimental effects on behaviours compared to mice that continued exercising, in agreement with our hypothesis of exercise cessation related adverse effects on behaviours and hippocampal neurobiology. Our results showed that exercise cessation in WT mice:

- increases in OF and EZM anxiety-like behaviours
- increases in depression-like behaviours in the FST
- evidence of impaired Y maze recognition memory
- impaired Barnes maze spatial learning and spatial memory
- altered the expression of 16 of 75 hippocampal genes of interest, with associated changes to the majority of cell signalling pathways (AMPK, FOXO, glucagon, cellular

senescence, JAK-STAT) and synaptic transmission pathways (serotonergic, dopaminergic, and glutamatergic transmission).

- There were no significant effects of exercise cessation on TNF signalling

These adverse effects of exercise cessation on affect, cognitive function, and neurobiology are concerning when considered in the context of periods of significant reductions in physical activity over the lifespan, such as in adolescence, young adulthood, and older adulthood (Anderssen, Jacobs Jr et al. 1996, Caspersen, Pereira et al. 2000). Future clinical research about the effects of exercise cessation should include examinations of whether reductions in physical activity and exercise at these life stages contributes to acute or subacute increases in anxiety, depression, and cognitive function, and could include the quantification of relevant biomarkers such as BDNF, cortisol, and inflammatory bloods based markers including TNF. This would add to initial (limited) evidence found by our group showing that the cessation of exercise can increase depressive symptoms (Morgan, Olagunju et al. 2018).

It is also possible that significant reductions in exercise might also detrimentally affect mood and cognition, however this possibility remains unknown. As such, future research could investigate whether the international guidelines for physical activity for adolescents and adults constitute a reduction in exercise that contribute to changes in mood or cognition. World Health Organisation (WHO) recommendations indicate children and adolescents up to 17 years of age should undertake at least 60 minutes of moderate to vigorous intensity physical activity daily to improve control over symptoms of anxiety and depression (World Health Organization 2011). However, WHO and Australian guidelines encourage adults to undertake at least 2.5 hours of moderate activity weekly to prevent depression, or 75 minutes of vigorous activity for added health benefits (Department of Health 2017, World Health Organisation 2017). This means that 17 year olds turning 18 who follow the WHO or Australian guidelines significantly reduce the exercise undertaken weekly from 420 minutes to 150 minutes. It remains unknown whether such a considerable reduction in exercise at this age has any meaningful effects on mood or cognition.

A possible way of addressing this issue could be to perform analyses of longitudinal surveys of young adult populations that include regular detailed questions about the amounts of exercise undertaken. Groups that undertake the public health dose of exercise (as is recommended by international guidelines for physical activity) and who then stop exercise could be analysed to ascertain whether changes in mood or cognition are evident following the cessation or reduction of exercise. Furthermore, surveys with data linkage to hospital admissions could provide data about potential changes in mental health status following significant reductions in exercise. Such research could provide valuable information about the chronic effects of exercise cessation on mood and cognition, including possible survival analyses and health related costs based on hospital admissions.

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Appendix Table A1. Hippocampal genes of interest investigated via quantitative polymerase chain reaction (qPCR).

Gene Symbol	Gene name	TaqMan assay ID
Slc6a4	solute carrier family 6 (neurotransmitter transporter; serotonin)	Mm00439391_m1
Htr1a	5-hydroxytryptamine (serotonin) receptor 1A	Mm00434106_s1
Htr1b	5-hydroxytryptamine (serotonin) receptor 1B	Mm00439377_s1
Htr2a	5-hydroxytryptamine (serotonin) receptor 2A	Mm00555764_m1
Grin2a	glutamate receptor; ionotropic; NMDA2A (epsilon 1)	Mm00433802_m1
Grin2b	glutamate receptor; ionotropic; NMDA2B (epsilon 2)	Mm00433820_m1
Gria1	glutamate receptor; ionotropic; AMPA1 (alpha 1)	Mm00433753_m1
Gria2	glutamate receptor; ionotropic; AMPA2 (alpha 2)	Mm00442822_m1
Ido1	indoleamine 2;3-dioxygenase 1	Mm00492590_m1
Tph2	tryptophan hydroxylase 2	Mm00557715_m1
Kynu	kynureninase (L-kynurenine hydrolase)	Mm00551012_m1
Ppard	peroxisome proliferator activator receptor delta	Mm00803184_m1
Bdnf	brain derived neurotrophic factor	Mm04230607_s1
Ntrk1	neurotrophic tyrosine kinase; receptor; type 1	Mm01219406_m1
Ntrk2	neurotrophic tyrosine kinase; receptor; type 2	Mm00435422_m1
Creb1	cAMP responsive element binding protein 1	Mm00501607_m1
Ppargc1a	peroxisome proliferative activated receptor; gamma; coactivator 1 alpha	Mm01208835_m1
Il1a	interleukin 1 alpha	Mm00439620_m1
Il1b	interleukin 1 beta	Mm00434228_m1
Il1r1	interleukin 1 receptor; type I	Mm00434237_m1
Ngf	nerve growth factor	Mm00443039_m1
Il6	interleukin 6	Mm00446190_m1
Il2	interleukin 2	Mm00434256_m1
Tnfrsf1a	tumor necrosis factor receptor superfamily; member 1a	Mm00441883_g1
Tnfrsf1b	tumor necrosis factor receptor superfamily; member 1b	Mm00441889_m1
Il10	interleukin 10	Mm01288386_m1
Tgfb1	transforming growth factor; beta 1	Mm01178820_m1
Ifng	interferon gamma	Mm01168134_m1
Il4	interleukin 4	Mm00445259_m1
Il6ra	interleukin 6 receptor; alpha	Mm00439653_m1
Il6st	interleukin 6 signal transducer	Mm00439665_m1
Tnf	tumor necrosis factor	Mm00443258_m1
Il12a	interleukin 12a	Mm00434169_m1
Il12b	interleukin 12b	Mm01288989_m1
Tbx21	T-box 21	Mm00450960_m1
Gata3	GATA binding protein 3	Mm00484683_m1
Foxp3	forkhead box P3	Mm00475162_m1
Smad2	SMAD family member 2	Mm00487530_m1
Smad3	SMAD family member 3	Mm01170760_m1
Foxo3	forkhead box O3	Mm01185722_m1
Nlrp3	NLR family; pyrin domain containing 3	Mm00840904_m1
Casp3	caspase 3	Mm01195085_m1
Casp7	caspase 7	Mm00432322_m1

Bcl2	B cell leukemia/lymphoma 2	Mm00477631_m1
Bag1	BCL2-associated athanogene 1	Mm01208593_m1
Igf1	insulin-like growth factor 1	Mm00439560_m1
Aif1	allograft inflammatory factor 1	Mm00479862_g1
Gfap	glial fibrillary acidic protein	Mm01253033_m1
Glul	glutamate-ammonia ligase (glutamine synthetase)	Mm00725701_s1
S100b	S100 protein; beta polypeptide; neural	Mm00485897_m1
Sod1	superoxide dismutase 1; soluble	Mm01344233_g1
Sod2	superoxide dismutase 2; mitochondrial	Mm01313000_m1
Gpx1	glutathione peroxidase 1	Mm00656767_g1
Cat	catalase	Mm00437992_m1
Prkaa1	protein kinase; AMP-activated; alpha 1 catalytic subunit	Mm01296700_m1
Prkaa2	protein kinase; AMP-activated; alpha 2 catalytic subunit	Mm01264789_m1
Nr3c1	nuclear receptor subfamily 3; group C; member 1	Mm00433832_m1
Tgfbr1	transforming growth factor; beta receptor I	Mm00436964_m1
Tgfbr2	transforming growth factor; beta receptor II	Mm03024091_m1
Crhr1	corticotropin releasing hormone receptor 1	Mm00432670_m1
Crh	corticotropin releasing hormone	Mm01293920_s1
Sirt1	sirtuin 1	Mm01168521_m1
Cs	citrate synthase	Mm00466043_m1
Uqcrc1	ubiquinol-cytochrome c reductase core protein 1	Mm00445911_m1
Actb	actin; beta	Mm02619580_g1
Hprt	hypoxanthine guanine phosphoribosyl transferase	Mm03024075_m1
Gusb	glucuronidase; beta	Mm01197698_m1
B2m	beta-2 microglobulin	Mm00437762_m1
Slc6a3	solute carrier family 6 (neurotransmitter transporter; dopamine); member 3	Mm00438388_m1
Th	tyrosine hydroxylase	Mm00447557_m1
Mecp2	methyl CpG binding protein 2	Mm01193537_g1
Dnmt1	DNA methyltransferase (cytosine-5) 1	Mm01151063_m1
Sgk1	serum/glucocorticoid regulated kinase 1	Mm00441380_m1
Egr1	early growth response 1	Mm00656724_m1
Dad1	defender against cell death 1	Mm01319221_m1
Dlg4	discs; large homolog 4 (Drosophila)	Mm00492193_m1
Casp4	caspase 4; apoptosis-related cysteine peptidase	Mm00432304_m1
Tac1	tachykinin 1	Mm01166996_m1