

Chronic effects of pre-adolescent pharmacological and non-pharmacological interventions on depressive-like behaviour in rats

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Opgedra aan Alexa, Zilla en Ferdie Steyn.

Mag ek ook die voorbeeld vir Alexa

en die man vir Zilla wees

wie Pappa vir my en Mamma was.

A few years ago, the city council of Monza, Italy, barred pet owners from keeping goldfish in curved goldfish bowls. The measure's sponsor explained the measure in part by saying that it is cruel to keep a fish in a bowl with curved sides because, gazing out, the fish would have a distorted view of reality. But how do we know we have the true, undistorted picture of reality? Might not we ourselves also be inside some big goldfish bowl and have our vision distorted by an enormous lens? The goldfish's picture of reality is different from ours, but can we be sure it is less real?

Steven Hawking and Leonard Mlodinow, The Grand Design

Sitting high above the pentomic swamp, in the long shadow of the spilled blood and dreams of the founding fathers and founding mothers, I can see more than the average man sees. I imagine the cells, the nucleus of things. I see colours that evolved to speak to the smallest of eyes. Sacrifices that meet in this cataclysm of longing for what can and what can't, overcoming what cannot. In the majesty of the quantum world, in the beauty of building blocks, in the tiniest of elements, I glimpse the privilege of being. This alertness does not subtract, it adds. It is not our inheritance merely to abide in this beautiful world. It is our inheritance to understand it.

Unknown

Dad, you always told me: "*Don't you cry when you're down*",
But, Dad, there's a tear every time that I blink.
Oh, I'm in pieces - it's tearing me up, but I know a heart that's broke is a heart that's been loved;
So, I'll sing *Hallelujah*, you were an angel in the shape of my dad.
You got to see the person I have become,
Spread your wings and I know that when God took you back, he said: "*Hallelujah, you're home.*"

Adapted from Ed Sheeran's 'Supermarket Flowers'

ABSTRACT

Similar to adults, major depressive disorder (MDD) also affects children and adolescents, with comparable treatment outcomes. However, only two antidepressants, namely the serotonin reuptake inhibitors fluoxetine and escitalopram, are currently approved for the treatment of juvenile MDD. Additionally, the serotonin-noradrenaline reuptake inhibitor venlafaxine, is a popular ‘off-label’ treatment option for depressed juveniles. The efficacy of pro-serotonergic, and not pro-noradrenergic antidepressants in juvenile patients have been ascribed to the earlier maturation of the serotonergic, relative to the noradrenergic system. Nevertheless, the pathophysiology of MDD in juvenile patients generally appears to be comparable to that in adults. In this regard, increased central inflammation and oxidative stress damage are also observed in depressed juveniles, resulting in compromised neuroplasticity that ultimately contribute to decreased monoaminergic neurotransmission, the main target of the approved antidepressants. During childhood and adolescence dynamic and adaptable neurodevelopment renders the young brain vulnerable to possible long-term detrimental or beneficial effects of antidepressants. Our current knowledge of such long-term effects are limited, warranting further investigation.

Also, non-pharmacological interventions have attracted attention as possible augmentative strategies to currently approved therapies. Based on preliminary evidence, and due to their purported improved safety profiles, omega-3 essential fatty acid (ω -3 EFA) supplementation and exercise have been investigated for their possible antidepressant properties. Both these interventions beneficially affect numerous MDD-associated neurobiological processes, yet the exact mechanisms of action remain unknown. Nevertheless, similar to antidepressant treatment, how and to which extent such early-life interventions, either as mono-treatment or in combination with antidepressants, could affect the developing brain and behaviour later in life, also warrants further investigation.

The current project therefore investigated the early-life and lasting bio-behavioural effects of pre-pubertal pharmacological and non-pharmacological treatments in a stress-sensitive animal model of depression (i.e. Flinders Sensitive Line rat; FSL). Pre-pubertal male FSL rats were treated with saline (control; SAL), fluoxetine (FLX; 5 mg/kg/day), escitalopram (ESC; 10 mg/kg/day) or venlafaxine (VEN; 10 mg/kg/day), with or without dietary intervention (standard rat chow [STD] *or* ω -3 EFA coated rat chow [OM3]) or exercise (sedentary behaviour [SED] *or* low intensity exercise [EXE]) for fourteen days, starting from postnatal day 21 (PND21) to PND34 (i.e. pre-puberty). Drugs were administered subcutaneously, whereas ω -3 EFAs were administered via diet and low intensity forced exercise was introduced by a custom-built treadmill. Following chronic, pre-pubertal treatment, a sub-set of animals underwent behavioural analyses in the open field test (OFT) and forced swim test (FST) immediately following treatments on PND35 (i.e. puberty) and were euthanized on PND36 for neurochemical analyses. Another sub-set of animals received

the same pre-pubertal treatments, followed by no treatment for 26 days until PND60 (i.e. early adulthood) to determine lasting bio-behavioural effects and were euthanized on PND61 for neurochemical analyses. In addition, the main study was foregone by pilot studies to develop a juvenile forced exercise regimen and ω -3 EFA coated rat chow.

Only FLX treatment alone reduced early-life depressive-like behaviour and induced lasting antidepressant-like effects in FSL rats on PND60. Pre-pubertal EXE alone, decreased depressive-like behaviour and promoted behaviour associated with enhanced serotonergic neurotransmission, supported by increased cortical serotonin and hippocampal brain-derived neurotrophic factor (BDNF) concentrations in rats on PND35. This antidepressive-like behaviour became more pronounced later in life, with significant increased cortical monoamine concentrations observed on PND60. Contrary, OM3 treatment alone failed to improve depressive-like behaviour, yet putatively induced enhanced coping mechanisms, possibly masking any antidepressive-like behaviour on PND35 and PND60. Nevertheless, both non-pharmacological interventions displayed strong augmentation properties that lasted into early-adulthood. FLX+EXE combination treatment significantly decreased early-life depressive-like behaviour, but did not last into early-adulthood. When combined with EXE, VEN decreased depressive-like behaviour later in life via enhanced noradrenergic and serotonergic neurotransmission. Similarly, pre-pubertal ESC+OM3 treatment appeared ineffective in early-life, yet exerted long-lasting antidepressant-like properties into early-adulthood, associated with increasing serotonin turnover later in life. Finally, the triple combination of ESC+OM3+EXE showed great potential in reducing early-life depressive-like behaviour, with more pronounced antidepressant-like effects later in life.

In conclusion, the current project confirmed the use of FLX in pre-pubertal individuals with beneficial lasting effects. Furthermore, that the long-term effects of different classes of antidepressants were beneficially augmented by ω -3 EFA supplementation and low intensity exercise, suggests neurodevelopmental processes to be positively affected, resulting in improved behaviour later in life. Furthermore, the newly developed age-related, intensity-specific exercise regimen for pre-pubertal rodents is a novel improvement to existing regimens, as is the successful formulation of the ω -3 EFA supplementation coating on rat chow. Overall, the current project underlines the value of improved lifestyle and nutritional modifications to augment the pharmacological treatment of juvenile MDD, and in particular its potential value for long-lasting effects into adulthood.

Key terms

Juvenile depression, Neurodevelopment, Lasting-effects, Fluoxetine, Escitalopram, Venlafaxine, Low intensity exercise, Omega-3 supplementation, Flinders sensitive line rat, Depressive-like behaviour.

OPSOMMING

Soos by volwassenes, affekteer major depressiewe versteuring (MDV) ook kinders en tieners, met vergelykbare uitkomste. Slegs twee antidepressante, naamlik die serotonien-heropnameremmers fluoksetien en esitalopram, is egter tans goedgekeur vir die behandeling van jeug-verwante MDV. Verder is die serotonien-noradrenalin-heropnameremmer, venlafaksien, 'n populêre 'nie-registreerde behandelingsopsie' vir depressiewe jeugdiges. Die effektiwiteit van pro-serotonergiese, en nie pro-noradrenergiese antidepressante nie, in jeugdige pasiënte is toegeskryf aan die vroeër volwassewording van die serotonergiese, relatief tot die noradrenergiese sisteem. Desnieteenstaande wil dit oor die algemeen voorkom of die patofisiologie van MDV in jeugdige pasiënte vergelykbaar is met dié in volwassenes. In hierdie verband is verhoogde sentrale inflammasie en oksidatiewe stresskade ook waargeneem in depressiewe jeugdiges, wat resulteer in ingekorte neuroplastisiteit, wat uiteindelik bydra tot verminderde monoaminergiese neurotransmissie, die hoofteiken van goedgekeurde antidepressante. Gedurende die kinder- en tienerjare laat die dinamiese en aanpasbare neuro-ontwikkeling die jong, ontwikkelende brein kwesbaar vir potensieel nadelige of voordelige effekte van antidepressante. Ons huidige kennis van sodanige langtermyn-effekte is beperk, wat verdere ondersoeke regverdig.

Verder het nie-farmakologiese intervensies aandag getrek as moontlike potensieringstrategieë tot bestaande goedgekeurde terapieë. Gebaseer op voorlopige bewyse, en a.g.v. beweerde verbeterde veiligheidsprofile, is omega-3-essensiële vetsuur- (ω -3 EVS) aanvulling en oefening ondersoek vir potensieële antidepressant-eienskappe. Beide hierdie intervensies het 'n voordelige invloed op verskeie MDV-geassosieerde neurobiologiese prosesse, maar steeds is die presiese meganismes van werking onbekend. Soortgelyk aan antidepressantbehandeling, is dit nodig om verder ondersoek in te stel na hoe, en tot watter mate, sodanige vroeë-lewe-intervensies, as beide enkel-behandeling of in kombinasie met antidepressante, die ontwikkelende brein en gedrag later in die lewe kan beïnvloed.

Die huidige projek het daarom ondersoek ingestel na die vroeë-lewe en blywende bio-gedragseffekte van pre-pubertale farmakologiese en nie-farmakologiese behandelings in 'n stres-sensitiewe dieremodel van depressie (d.i. Flinders sensitiewe lyn-rotte; FSL). Pre-pubertale manlike rotte was met isotoniese soutoplossing (kontrole; SAL), fluoksetien (FLX; 5 mg/kg/dag), esitalopram (ESC; 10 mg/kg/dag) of venlafaksien (VEN; 10 mg/kg/dag) behandel, met of sonder dieëntervensie (standaard rotkos [STD] of ω -3 EVS-bedekte rotkos [OM3]) of oefening (sittende gedrag [SED] of lae-intensiteit oefening [EXE]) vir veertien dae, beginnende op nageboortedag 21 (NGD21) tot NGD34 (d.i. pre-puberteit). Geneesmiddels is subkutaneus toegedien en lae-intensiteit oefening was ingestel deur 'n pasgemaakte trapmeule. Na kroniese, pre-pubertale behandeling het 'n sub-indeling van die diere gedragsanalises in die oopveldtoets (OVT) en geforseeerde swemtoets (GST) ondergaan direk na afloop van die behandelings, op NGD35 (d.i. puberteit)

en het dan genadedood op NGD36 ontvang ter voorbereiding vir neurochemiese analises. 'n Volgende sub-indeling van die diere het dieselfde pre-pubertale behandelingstrategieë ontvang, maar alle behandelings is vanaf NGD35 onttrek, waarna bio-gedragsanalises eers op NGD60 (d.i. vroeë volwassenheid) uitgevoer is om blywende bio-gedragseffekte vas te stel. Hierdie groep diere het dan genadedood op NGD61 ontvang ter voorbereiding vir neurochemiese analises. Addisioneel hiertoe was die hoofstudie voorafgegaan deur lootsstudies om die jeugdige geforseerde oefenprogram en ω -3 EVS-bedekte rotkos te ontwikkel.

Slegs FLX-behandeling alleen het depressie-agtige gedrag in die vroeë lewe verminder en het blywende antidepressant-agtige effekte op NGD60 in FSL rotte geïnduseer. Pre-pubertale EXE alleen het depressie-agtige gedrag verminder en het gedrag wat geassosieer word met verhoogde serotonergiese neurotransmissie bevorder, ondersteun deur verhoogde konsentrasies van kortikale serotonien en hippokampus brein-verkreë neurotrofiese faktor (BVNF) in rotte op NGD35. Hierdie antidepressief-agtige gedrag was meer uitgespoken later in die lewe, met beduidende verhoging in kortikale monoamienkonsentrasies soos waargeneem op NGD60. Hierteenoor het OM3-behandeling gefaal om depressie-agtige gedrag te verbeter, maar vermeende geïnduseerde hanteringsmeganismes is bevorder, wat moontlik enige antidepressie-agtige gedrag kon maskeer op NGD35 en NGD60. Desnieteenstaande, het beide nie-farmakologiese intervensies sterk, potensierende eienskappe getoon wat tot in vroeë volwassenheid volgehou is. FLX+EXE-behandeling het vroeë-lewe depressie-agtige gedrag beduidend verminder, maar het nie tot volwassenheid volgehou nie. Wanneer met EXE gekombineer, het VEN depressie-agtige gedrag later in die lewe verlaag via verhoogde noradrenergiese en serotonergiese neurotransmissie. Net so het pre-pubertale ESC+OM3-behandeling geblyk om oneffektief te wees vroeg in die lewe, maar het dit langsdurende antidepressant-agtige eienskappe in vroeë volwassenheid getoon, geassosieer met verhoogde serotonergiese omset later in die lewe. Ten laaste het die drievoudige kombinasie van ESC+OM3+EXE groot potensiaal getoon om vroeë-lewe depressie-agtige gedrag te verminder, met meer uitgesproke antidepressant-agtige effekte later in die lewe.

Om op te som het die huidige projek bevestig dat die gebruik van FLX in pre-pubertale individue blywende voordelige effekte het. Verder, dat die langtermyn-effekte van verskillende klasse antidepressante potensierend bevoordeel was deur ω -3 EVS-aanvulling en lae-intensiteit oefening, suggereer dat neuro-ontwikkelingsprosesse positief beïnvloed word, wat lei tot verbeterde gedrag later in die lewe. Verder is die nuut-ontwikkelde ouderdomsverwante, intensiteitspesifieke oefeningsreeks vir pre-pubertale knaagdiere 'n nuwe verbetering tot bestaande reekse, so ook die suksesvolle formulering van die ω -3 EVS-bedekte rotkos. Oor die algemeen lig die projek die waarde van verbeterde leefstyl en aanpassings in voeding uit om die farmakologiese behandeling van jeugdige MDV te potensieer, en in besonder die potensiele waarde vir langdurende effekte tot vroeë volwassenheid.

Sleutelterme

Jeug-verwante depressie, Neuro-ontwikkeling, Lang-termyneffekte, Fluoksetien, Esitalopram, Venlafaksien, Lae-intensiteit oefening, Omega-3 aanvulling, Flinders sensitiwe lyn rot, Depressie-agtige gedrag.

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LIST OF ABBREVIATIONS

5HIAA	5-hydroxyundoleactic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HTTLPR	Serotonin-transporter-linked polymorphic region (serotonin transporter gene)
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)-tetralin
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACTH	Adrenocorticotrophic hormone
ADHD	Attention-deficit/hyperactivity disorder
ADS	Antidepressant discontinuation syndrome
AMP	Adenosine monophosphate
ANOVA	Analysis of variance(s)
BDNF	Brain-derived neurotrophic factor
CMI	Cell-mediated immunity
CREB	cyclic AMP response binding protein
CRF	Adrenocorticotrophic hormone-releasing factor
CRHR	Corticotrophin releasing hormone receptor
CRL	Control
CRP	C-reactive protein
DA	Dopamine
DAT	Dopamine transporter
DFP	Diisopropylfluorophosphonate
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DSM-V	Diagnostic and Statistical Manual of Mental Disorders, fifth edition

EPA	Eicosapentaenoic acid
ESC	Escitalopram
EXE	Exercise
FDA	Food and drug administration
FLX	Fluoxetine
FRL	Flinders resistant line
FSL	Flinders sensitive line
FST	Forced swim test
G x E	Gene-environment (hypothesis)
GABA	γ -aminobutyric acid
GR	Glucocorticoid receptor(s)
HDRS	Hamilton Depression Rating Scale
HPA	Hypothalamic-pituitary-adrenal
IDO	Indoleamine-2,3-dioxygenase
IFNγ	Interferon gamma
IGF-1	Insulin-like growth factor-1
IL	Interleukin
LPS	Lipopolysaccharides
mAChR	Muscarinic acetylcholine receptor
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor(s)
MDA	Malondialdehyde
MDD	Major depressive disorder
mRNA	Messenger ribonucleic acid
MT	Melatonergic receptor
NA	Noradrenaline (norepinephrine)

NAC	N-acetylcysteine
nAChR	Nicotinic acetylcholine receptor
NAT	Noradrenaline transporter
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
NRI	Noradrenaline reuptake inhibitor(s)
NT	Neurotrophin
NWU	North-West University
OFT	Open field test
OM3	Omega-3
p75^{NTR}	p75 neurotrophin receptor
PND	Postnatal day
PUFA	Polyunsaturated fatty acid(s)
REM	Rapid eye movement
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAL	Saline
SD	Sprague-Dawley
SED	Sedentary
SNP	Single nucleotide polymorphism
SNRI	Serotonin-noradrenaline reuptake inhibitor(s)

SSRI	Selective serotonin reuptake inhibitor(s)
TCA	Tricyclic antidepressant(s)
TH	Tyrosine hydroxylase
TNF	Tumour necrosis factor
TPH	Tryptophan hydroxylase
TRD	Treatment resistant depression
Trk	Tyrosine kinase receptor
VEGF	Vascular endothelial growth factor
VEN	Venlafaxine
VO_{2max}	Maximal oxygen uptake
vVO_{2max}	Velocity to reach VO _{2max}
WKY	Wistar Kyoto
ω-3 EFA	Omega-3 essential fatty acid(s)

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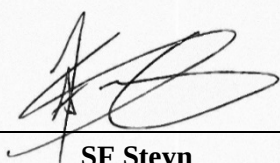
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DECLARATION

I, Stephanus Frederik Steyn hereby declare that all experimental work, planning, literature research, data capturing and interpretation, as well as writing the initial version of this thesis was conducted by myself. My supervisor (Professor Christiaan B Brink) funded the project while both he and the co-supervisor (Professor Brian H Harvey) assisted in the interpretation of results of the experimental work and proof read the thesis in preparation for its final version. All neurochemical analyses were conducted by myself with the assistance of the lab technicians, Mr Walter Dreyer and Mr Francois Viljoen. The method used to coat the vivarium rat chow with ω -3 oil was developed by Professor Jan Steenekamp (Pharmaceutics Department, NWU, Potchefstroom campus), whereas nutritional analysis was performed at the Cape Peninsula University of Technology's Functional Food Research Unit under the supervision of Professor Maretha Opperman. All statistical analyses were conducted by myself with the guidance of Laerd Statistics® (<https://statistics.laerd.com>) and the statistical consultation service of the North-West University, Potchefstroom.

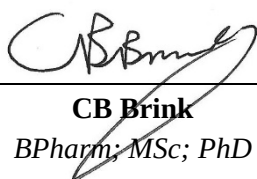


SF Steyn
BPharm; MSc

2017/11/17

Date

As supervisors, Professors CB Brink and BH Harvey, confirm that the above statement by Mr SF Steyn is true and correct.



CB Brink
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2017/11/20

Date



BH Harvey
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2017/11/23

Date

CHAPTER 1: INTRODUCTION

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1.1 Thesis layout

This thesis is compiled in article format, as prescribed and approved by North-West University (NWU). As such, the main body of the thesis is presented as three manuscripts (Chapters 3, 4 and 5) that have either been accepted or are in submission to international, peer reviewed neuroscience journals.

Chapter 1 provides a concise description of the project problem statement, study questions, study layout and expected outcomes and foreseen impact of the study. Chapter 2 contains the broad, overall literature background supporting the current PhD project as a whole. Next, chapter 3, 4 and 5 contain the key findings of the current project presented in three separate manuscripts. These manuscripts have been prepared according to the 'Instructions to Authors' provided by the respective journals for which it was prepared, and will be presented in the prescribed format. Importantly, the figures of these Chapters have been incorporated into the text to ease reading. Chapter 6 summarizes the key findings of the PhD project and concludes the thesis. The addendums contain a more detailed description of the materials and methods used throughout the current project, additional data and results not presented in the manuscripts, letters of permission of co-authors for subjecting manuscripts A, B and C for assessment purposes, and confirmations of article submissions or acceptance to peer reviewed neuroscience journals. Finally, all congress proceedings during the PhD period are presented at the end of the thesis.

The reference lists for each manuscript are presented at the end of the specific manuscript in accordance to the specific reference style indicated by the journal. All other referencing throughout the thesis was done with EndNote software and cited according to the Harvard style, as preferred by the NWU, and are presented at the end of Chapter 6.

This thesis is presented in United Kingdom (UK) English.

1.2 Problem statement

Major depressive disorder (MDD) is one of the most common and challenging mental conditions of our time; continually rising in prevalence and burden. In fact, depression is now estimated to become the leading cause of disability by the year 2030 (World Health Organization, 2012). This is of great concern since the economic burden caused by MDD (and other mental disorders) is already worrisome and could, according to the mentioned projection, have an even more significant impact on the economic and medical sectors in the near future. Of further concern is the low response rate, and subsequent adherence, of

depressed individuals to available pharmacological antidepressants and other treatment options. Importantly, MDD is not limited to adults, but also affects a significant number children and adolescents (Green *et al.*, 2005) with a similarly unsatisfactory therapeutic success rate as in the adult population. To this extent, roughly half of mental conditions are already established by the onset of puberty, yet more than half of juvenile patients do not receive appropriate interventions at a sufficiently early stage of development (Mental Health Foundation, 2017a). Therefore, juvenile depression contributes to the annual estimated € 21 billion global economic burden of juvenile mental diseases (Olesen *et al.*, 2012), as calculated from missed school days and overall academic decline (Owens *et al.*, 2012), altogether contributing to the disturbingly high estimated prevalence of disability within fifteen years.

What further complicates the mentioned scenario is that juvenile patients respond differently to antidepressants than adult patients. In fact, only two serotonergic antidepressants have been approved by the Food and Drug Administration (FDA) for the treatment of childhood and adolescent depression, yet along with a black-box warning of increased symptoms of suicidal ideation in younger patients (U.S. Food & Drug Administration, 2004). Interestingly, the majority of antidepressants primarily target a dysregulated monoaminergic system, yet only those increasing serotonergic, and not noradrenergic neurotransmission, are effective in juvenile patients. It is therefore argued that specifically the neurobiological basis of MDD may be different in juveniles (Hazell & Mirzaie, 2013; Hazell *et al.*, 1995; Keller *et al.*, 2001). In fact, these observations suggest a unique pathophysiology of monoaminergic circuits in post-pubertal depression (Axelson & Birmaher, 2001). Indeed, the maturation rate of the different monoamine neuronal pathways has been associated with the observed differences in response to antidepressants, and in particular the central serotonergic system is known to mature before the noradrenergic and dopaminergic systems, and thereby partly explaining the effectiveness of serotonin-selective antidepressants. In addition, genetic and environmental factors significantly contribute to the development of juvenile MDD (Hankin *et al.*, 2015; Silberg *et al.*, 2010), further complicating treatment strategies and clinical outcome in an already difficult-to-treat population. Regardless, as the developing brain is susceptible to external insults, early-life treatment could indeed present a window of opportunity where appropriate intervention could induce lasting effects later in life. However, whether and to which extent these effects are positive, neutral or negative remain unclear.

That pharmacological treatment options for the depressed juvenile patient are very limited, accentuates the need for novel, alternative and/or augmentative treatment strategies. In this regard, non-pharmacological strategies are currently receiving increased interest as alternative or adjunctive to pharmacological therapy to improve treatment outcome (Cala *et al.*, 2003; Hoffman *et al.*, 2011; Perraton *et al.*, 2010). This is of particular interest since untreated juvenile MDD is not only associated with other mental disorders, but is in itself a significant risk factor for juvenile suicide (Friedman & Leon, 2007); currently a leading cause of juvenile deaths worldwide (Hulvershorn *et al.*, 2011; South African Depression and Anxiety Group, 2017).

Exercise and omega-3 essential fatty acid (ω -3 EFA) supplementation are examples of such non-pharmacological strategies that have received growing interest and are suggested to hold small-to-modest benefits over that of placebo, and possibly even comparable effects to that of pharmacological antidepressants (Appleton *et al.*, 2015; Cooney *et al.*, 2013). In fact, according to the World Health Organization (WHO) non-pharmacological interventions are recommended for mild depression, whereas pharmacological antidepressants should be initiated in moderate to severe cases (World Health Organization, 2017a). Furthermore, the affordability, especially that of physical exercise, and perceived improved safety profile of these treatment strategies further contribute to their growing popularity. To this extent, ω -3 EFA supplementation and exercise contribute to, or are in line with the WHO's suggested 'protective factors' to be implemented in school-based programs to prevent juvenile MDD (World Health Organization, 2012). These 'protective factors' are implemented to enhance cognition and problem-solving abilities in children and adolescents, which are known to be impaired by MDD, and are enhanced by exercise (Hillman *et al.*, 2005; Hillman *et al.*, 2008) and ω -3 EFA supplementation (Wu *et al.*, 2004, 2008). Moreover, these intervention strategies have been reported to affect several of the neurological deficits underlying MDD and could therefore not only be effective in augmenting the antidepressant effects of pharmacological drugs, but may also prove effective in protecting a stress-sensitive individual from developing MDD later in life.

The current project therefore investigated the early-life and lasting or long-term (observed in early adulthood) effects of two different classes of antidepressants, i.e. selective serotonin reuptake inhibitors (i.e. fluoxetine, escitalopram) and serotonin- noradrenaline reuptake inhibitor (venlafaxine) with/without non-pharmacological augmentation therapies (i.e. low intensity exercise and/or ω -3 EFA supplementation) in a stress-sensitive animal model of depression. Different treatment strategies were implemented during pre-pubertal development, when the brain is suggested to be at its most susceptible to external influences, thereby possibly susceptible to long-term or lasting bio-behavioural alterations. To evaluate long-term or lasting bio-behavioural effects, analyses after juvenile treatment were performed after also a long treatment-free period (i.e. washout or withdrawal) and compared to that observed immediately following a chronic pre-pubertal exposure. The antidepressants investigated in the current project represented those approved for juvenile treatment (i.e. fluoxetine and escitalopram) as well as an antidepressant used 'off-label' in juveniles improved efficacy in resistant depression (i.e. venlafaxine). Additionally, fluoxetine and escitalopram, selectively target the serotonergic system which matures earlier in development, compared to the noradrenergic system. These different maturation rates of neurotransmitter pathways explain the effectiveness of these, and not noradrenergic antidepressants. That venlafaxine has a dual mechanism of action, targeting both serotonergic and noradrenergic neurotransmission, explains its effectiveness in depressed juveniles. Furthermore, a translational animal model of depression, the Flinders sensitive line (FSL) rat was implemented, which has well-demonstrated predictive and construct validity for adult MDD

as well as supportive, although limited, data supporting its value in accurately modelling the juvenile condition as well. Moreover, an age-related low intensity forced exercise regimen was developed to be responsive to increased exercise capacity of pre-pubertal ageing, in contrast to fixed exercise programmes commonly implemented in rodent studies (Cechetti *et al.*, 2008; Cechetti *et al.*, 2007; Kim *et al.*, 2015; Kim *et al.*, 2014; Lou *et al.*, 2008; Lou *et al.*, 2006; Lovatel *et al.*, 2013). Secondly, ω -3 EFA supplementation was introduced via coating of normal rat chow, thereby eliminating the need for additional administration stress. Finally, the use of the pre-pubertal FSL rat as a juvenile model for childhood depression was investigated and evaluated as very limited data regarding this topic is available. Importantly, the focus of the current PhD project excluded investigation into the role of genetic susceptibility, but rather the role of various intervention strategies in an approved genetic animal model of depression. Therefore, no Flinders Resistant Line (FRL) rat control line was included. Overall, the current project, according to our knowledge, is the first to investigate and develop an age-related, intensity specific exercise regimen for pre-pubertal FSL rats as well as investigate the immediate and long-term or lasting effects of different pharmacological and non-pharmacological interventions strategies (mono-, double- and triple-therapy) for juvenile MDD and its neurodevelopmental effects on bio-behaviour. In this regard, positive reports on the augmentation properties of antidepressant and exercise (or ω -3 supplementation) combination, and ω -3 supplementation and exercise combinations are available, yet the lasting effects of such treatment strategies are unknown, specifically when introduced during pre-pubertal development. Furthermore, whether a triple combination of antidepressant, exercise and ω -3 supplementation could be even more effective in reducing depressive-like behaviour, either during early-life or early adulthood, has not been investigated.

1.3 Study questions

The current project was therefore designed to address the following study questions as presented in Table 1-1, below.

Table 1-1: Study questions.

Study question		Applicable literature
1)	Are depressive- and anxiety-like behaviours and specific biomarkers (monoaminergic and neuroplasticity markers) thereof differently affected by two different classes of antidepressants (i.e. SSRI and SNRI), either during early-life following pre-pubertal treatment or later in life?	(Andersen & Navalta, 2004, 2011; Murrin <i>et al.</i> , 2007)
2)	Do non-pharmacological interventions (low intensity exercise and ω -3 supplementation) during pre-pubertal development have any early-life or lasting antidepressant-like effects and how do these effects compare to those observed in the antidepressant-treated groups?	(Coluccia <i>et al.</i> , 2009; Lopresti <i>et al.</i> , 2013; Lovatell <i>et al.</i> , 2013; Schoeman <i>et al.</i> , 2017)
Sub-question 2.1)	Does the vVO_{2max} capacity of the pre-pubertal male FSL rat increase with age?	
Sub-question 2.2)	Can ω -3 EFA supplementation be administered via normal rat diet to minimize administration stress and allow natural supplementation according to each subject's developmental needs?	
3)	Can the observed bio-behavioural effects of pre-pubertal antidepressant treatment be augmented by the non-pharmacological interventions (low intensity exercise and/or ω -3 EFA supplementation), both in early-life and during early adulthood?	(Gertsik <i>et al.</i> , 2012; Nemets <i>et al.</i> , 2002; Schoeman, 2015; Schoeman <i>et al.</i> , 2017; Su <i>et al.</i> , 2003; Trivedi <i>et al.</i> , 2011; Trivedi <i>et al.</i> , 2006a)

1.4 Project objectives

The specific objectives of the current study are discussed in response to each of the study questions as presented in above (Section 1.3).

Study question one

Chronically administer either fluoxetine or escitalopram (a selective serotonin reuptake inhibitor; SSRI) or venlafaxine (a serotonin-noradrenaline reuptake inhibitor; SNRI) during pre-pubertal development (i.e. postnatal day 21 (PND21) until PND34) and analyse bio-behavioural effects on PND35 (early-life) and PND60 (early-adulthood) in different subsets of animals.

Study question two

Sub-question 2.1

An approved method to indirectly determine the maximal oxygen uptake (VO_{2max}), expressed as vVO_{2max} (velocity to reach VO_{2max}), will be implemented to determine vVO_{2max} at different pre-pubertal ages (PND21, 23, 26, 28, 32 and 34) in male FSL rats. Consequently, low (55 %), moderate (70 %) and high (85 %) intensities will be calculated as percentages of the generated vVO_{2max} data. Finally, a comparison of early-life depressive-like effects behaviour will be analysed to identify the exercise intensity with the most robust antidepressant-like effects to be used for the remainder of the project (*Chapter 3*).

Sub-question 2.2

ω -3 EFA oil will be coated onto standard vivarium rat chow via a pan coating method. Furthermore, FSL rats will be given access to a measured amount of coated rat chow during pre-pubertal development (PND21 until PND35) to determine whether food intake or weight gain is adversely affected. Nutritional analyses on the different diets will also be performed to confirm successful coating.

**

After identifying the exercise intensity to be used for the remainder of the project and confirming the effective coating of vivarium rat chow, these treatment strategies will be compared to the effectiveness of the two mentioned antidepressants (i.e. escitalopram and venlafaxine) in terms of early-life efficacy and lasting effects. In this regard, both non-pharmacological intervention strategies will be administered chronically during pre-pubertal development (PND21 until PND35) whereafter bio-behaviour will be analysed and compared (*Chapters 4 and 5*).

Study question three

Non-pharmacological interventions (i.e. ω -3 supplementation and treadmill exercise) will be combined with specified antidepressant treatment, and administered chronically during pre-pubertal development (PND21 until PND35) whereafter early-life and lasting bio-behavioural effects will be analysed and compared. To this extent, both double and triple combination strategies will be investigated to determine and identify any augmentation potential, whether in early-life or in early-adulthood.

1.5 Project layout

Before studying the effects of the different interventions/treatment options, an age-related, intensity-specific exercise regimen was required. To determine the vVO_{2max} (velocity to reach VO_{2max}) for pre-pubertal male FSL rats, animals were randomly divided into two groups for Phase 1A (*Figure 1-1*). Both

test groups were familiarized to the treadmill from PND16 to PND20 and then subjected to the exhaustion test on the specified days (Group 1: PND21, 23 and 32; Group 2: PND23, 28 and 34) whilst receiving no drug treatment or nutritional supplementation. Animals were allowed to rest on the day following the exhaustion test, yet were subjected to a comfortable walking speed on the remaining days leading up to the next exhaustion test. Briefly, treadmill speed was constantly increased until the point of exhaustion, when the maximum speed reached and total time spent on the treadmill was used to calculate vVO_{2max} . This procedure established whether vVO_{2max} and pre-pubertal age are positively correlated, and in particular, determine the eventual treadmill speed required at a given age to reach the indicated percentage of vVO_{2max} in all subsequent experiments (Schoeman *et al.*, 2017). From the calculated maximum intensity, high intensity exercise was defined as 85 % of vVO_{2max} , moderate intensity exercise as 70 % vVO_{2max} (Kemi *et al.*, 2005) and low intensity exercise as 55 % vVO_{2max} (Belman & Gaesser, 1991). These intensities correlate with human athlete percentages of vVO_{2max} for low and moderate intensity exercise (Romijn *et al.*, 1995; Tabata *et al.*, 1996).

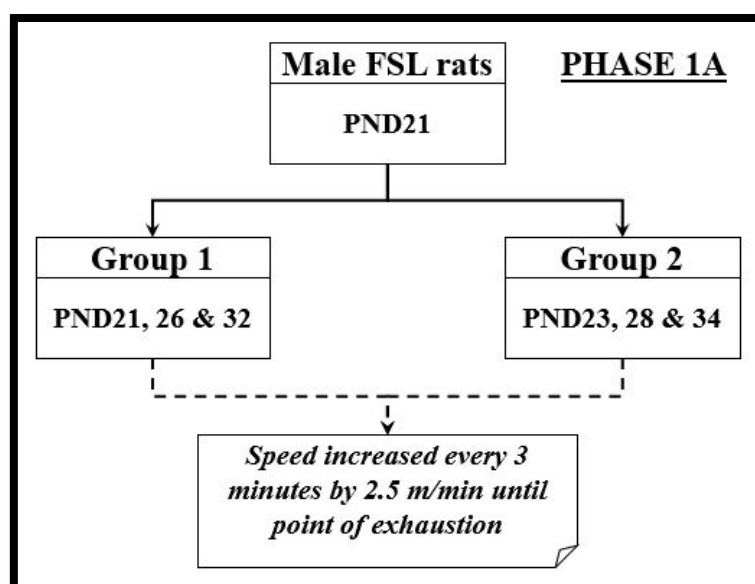


Figure 1-1: Graphical representation of Phase 1A (vVO_{2max} determination (exhaustion test)).

Figure 1-1 summarises the layout of Phase 1A of the current project where the vVO_{2max} , and consequently age-related, intensity specific exercise regimens for pre-pubertal male FSL rats was determined. Pre-pubertal male FSL rats were divided into two groups and subjected to the exhaustion test on the specified days. For the exhaustion test the speed of the treadmill was increased from an initial 2.5 m/min by 2.5 m/min every three minutes until the point of exhaustion. FSL: Flinders sensitive line. PND: Postnatal day.

**

Following Phase 1A, Phase 1B investigated the immediate effects of the different calculated age-related exercise intensity regimens to determine the exercise regimen with the most robust anti-depressant-like effects that would also be implemented in the subsequent Phases of the current study. Phase 1B therefore

assigned animals to three different exercise regimen groups where animals were subjected to the specific intensity from PND21 to PND34. Animals were then subjected to behavioural tests on PND35 to determine early-life depressive-like behaviour (*Figure 1-2*).

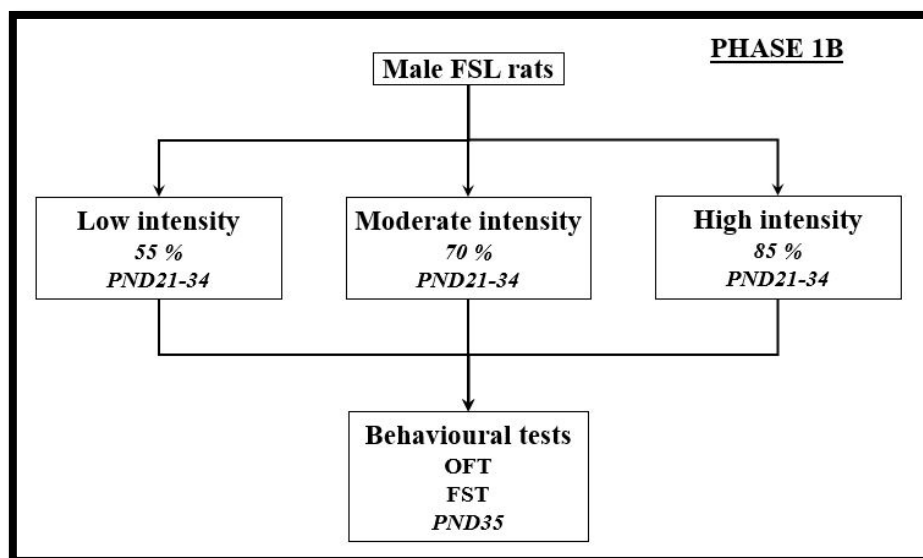


Figure 1-2: Graphical representation of Phase 1B (*Determining the exercise intensity regimen with the most robust antidepressant-like effects*).

Figure 1-2 summarises the layout of Phase 1B of the current project where the exercise intensity with the most robust antidepressive-like effects for pre-pubertal male FSL rats was determined. Pre-pubertal male FSL rats were divided into three groups and subjected to the specified intensity exercise from PND21 to PND34. Behavioural tests were performed on PND35. FSL: Flinders sensitive line. FST: Forced swim test. OFT: Open field test. PND: Postnatal day.

**

To determine whether coating of vivarium rat chow with ω -3 oil was indeed successful, an observation pilot study was implemented (*Phase 1C; Figure 1-3*). To this extent, animals were housed in pairs and given free access to 200 g coated rat chow from PND21 until PND35. The remaining rat chow was measured on a daily basis to determine the average food intake per cage. From the average cage intake, individual intake could be calculated to determine approximate ω -3 EFA content. No bio-behavioural analyses were performed at the end of Phase 1C.

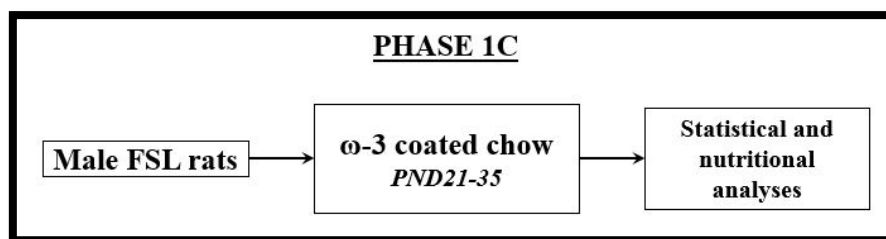


Figure 1-3: Graphical representation of Phase 1C (*Confirming the successful coating of vivarium rat chow*).

Figure 1-3 summarises the layout of Phase 1c of the current project where an observational study is performed to confirm the successful coating of standard vivarium rat chow with ω-3 oil. FSL: Flinders sensitive line. PND: Postnatal day.

**

For the subsequent Phases of the current study, the early-life and lasting effects of different intervention/treatment options were investigated, implementing the exercise regimen identified in Phases 1A and B. Specifically, pharmacological and non-pharmacological interventions were administered in various combinations during pre-pubertal development whereafter bio-behaviour was evaluated. The early-life effects of the mentioned treatments/interventions were studied in Phase 2 (*Figure 1-4*) whereas the lasting or long-term effects were investigated in Phase 3. Following pre-pubertal treatment/intervention, behavioural testing was performed on PND35, followed by euthanasia and neurochemical testing on PND36 to assess early-life effects. Behavioural tests included the Open field test (OFT) and the Forced swim test (FST), whereas the biological markers of depression analysed included frontal cortex monoaminergic and hippocampal brain-derived neurotrophic factor (BDNF) concentrations.

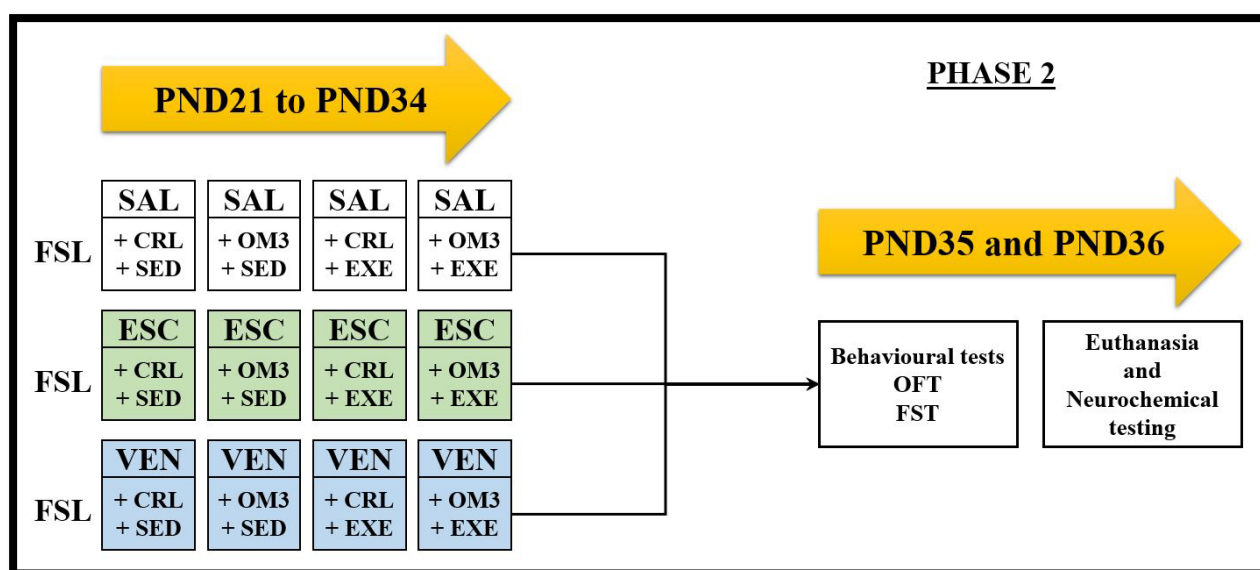


Figure 1-4: Graphical representation of Phase 2 (Early-life effects of treatment combinations).

Figure 1-4 summarises the layout of the first part of the current project where the early-life effects of the treatment combinations are analysed. Pre-pubertal male FSL rats will be treated with all possible combinations of drug, ω -3 EFA and low intensity exercise from PND21 to PND34. Treatment will be followed by behavioural testing on PND35 and euthanasia and neurochemical testing on PND36. ESC: Escitalopram (10 mg/kg/day sc). EXE: Low intensity exercise. FSL: Flinders sensitive line. FST: Forced swim test. OFT: Open field test. OM3: Omega-3 supplemented rat chow. PND: Postnatal day. SAL: Saline. SED: Sedentary. STD: Standard rat chow. VEN: Venlafaxine (10 mg/kg/day sc).

**

Finally, in Phase 3 the procedures of Phase 2 were repeated to also assess any long-term or lasting effects of different pre-pubertal treatment strategies in young adult FSL rats. Hence, animals received above mentioned pre-pubertal treatment strategies, however, hereafter rats underwent a 26-day wash-out period where they received no treatment/intervention. Importantly, subjects receiving ω -3 EFA coated rat chow returned to the standard diet, provided *ad libitum* during wash-out period. Subjects then underwent behavioural testing during their wake cycle on PND60 to assess and identify any lasting effects on locomotor activity and depressive-like behaviour (Figure 1-5). Again, FRL rats were not included in the current project as the focus was to investigate the role of various intervention strategies in an approved genetic animal model of depression and not to investigate the role of genetic susceptibility.

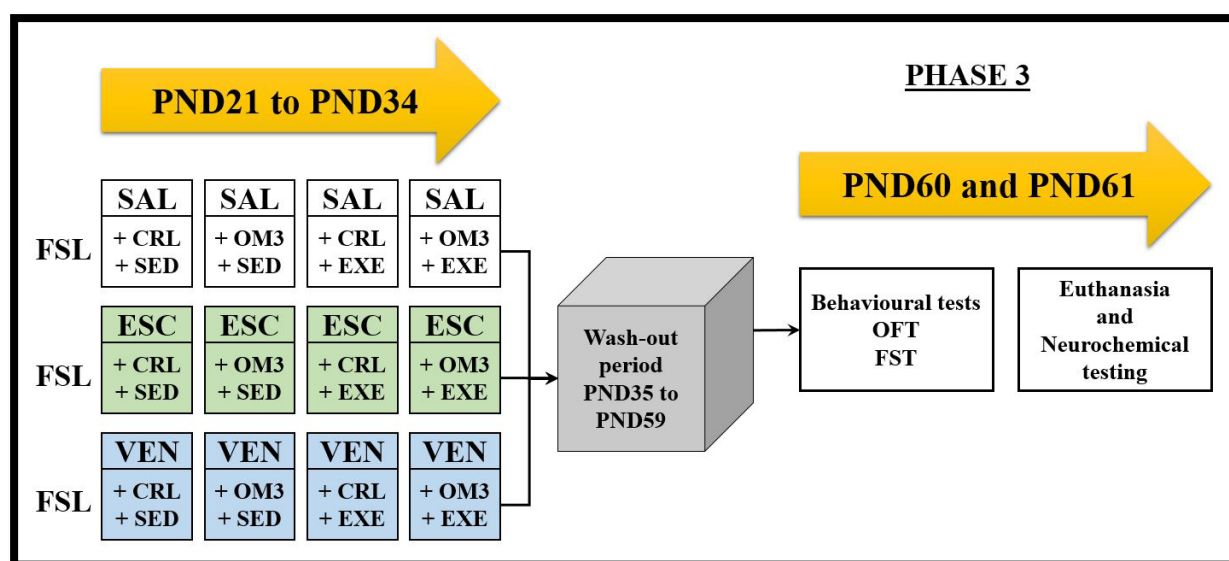


Figure 1-5: Graphical representation of Phase 3 (*Lasting effects of treatment combinations*).

Figure 1-5 summarises the layout of the second part of the current project where the lasting effects of the treatment combinations are analysed. Pre-pubertal male FSL rats will be treated with all possible combinations of drug, ω -3 EFA and low intensity exercise from PND21 to PND34. Treatment will be followed by a 26-day wash-out period where animals will be normal housed while receiving no further treatment and/or intervention. Groups which received ω -3 EFA supplementation will, during the wash-out period, receive normal rat chow. Behavioural testing will be performed on PND60 and euthanasia and neurochemical testing on PND61. ESC: Escitalopram (10 mg/kg/day sc). EXE: Low intensity exercise. FSL: Flinders sensitive line. FST: Forced swim test. OFT: Open field test. OM3: Omega-3 coated rat chow. PND: Postnatal day. SAL: Saline. SED: Sedentary. STD: Standard rat chow. VEN: Venlafaxine (10 mg/kg/day sc).

The main results of the various Phases are presented in Chapters 3 (development of age-related, forced exercise regimen, early-life and lasting effects of fluoxetine with/without exercise), 4 (early-life and lasting effects of escitalopram with/without ω -3 supplementation) and 5 (early-life and lasting effects of venlafaxine with/without exercise). Additionally, the early-life and lasting bio-behavioural effects of the triple combinations are presented in Addendum B.

1.6 Statistical analyses

The main results of the current project are presented in separate manuscripts. Therefore, a description of the specific statistical analyses is also presented in the relevant manuscript chapters, however, this section gives a broad overview of the statistical analyses performed throughout the project as whole. All statistical analyses were performed in IBM® SPSS® Statistics (version 24.0. Armonk, NY: IBM Corp) and GraphPad Prism® (version 6.0, San Diego California USA), assisted by Laerd Statistics® (<https://statistics.laerd.com>) and the statistical consultation service of the NWU.

Normality of the data was determined for all analyses with the Shapiro-Wilk test, where $p < 0.05$ indicated a violation of the assumption of normal data distribution. The assumption of homogeneity of variances was determined using the Levene's test for equality of variances where $p < 0.05$ indicated that the assumption

had been violated. Grubbs' test was used to determine the outlier in each data set with $\alpha = 0.05$ accepted as significant. In this regard, experimental group sizes are presented for each specific data set in Addendum B.

Three-way ANOVAs (analysis of variance) were performed on all data sets, regardless of normal distribution or homogeneity of variances because the three-way ANOVA is deemed robust enough and group sizes were approximately equal to compensate for these violations (Laerd Statistics, 2016c, 2016d). The three-way ANOVA determined whether a statistical significant three-way interaction between drug (SAL, ESC or VEN), diet (STD or OM3) and activity (SED or EXE) existed. Where a significant three-way interaction was identified, simple two-way interactions were analysed, followed by analysis of significant simple simple main effects and significant simple simple comparisons. However, where no significant three-way interaction was present, analyses for significant two-way interactions were performed, followed by analyses for significant simple main effects and significant pairwise comparisons (Laerd Statistics, 2016c).

In instances investigating two variables, normal two-way ANOVAs were performed, regardless of normal distribution or homogeneity of variances (Laerd Statistics, 2017b, 2017c). Furthermore, main effects were analysed followed by pairwise comparisons, regardless of whether a statistically significant interaction existed (Howell, 2009; Laerd Statistics, 2017d). In all instances, the Bonferroni post-hoc test was used for multiple comparison, unless stated otherwise.

When required, a one-way ANOVA was performed and followed by the Tukey post-hoc test for multiple comparisons when the assumption for homogeneity of variances were true. In instances where the assumption for homogeneity of variances were violated, the Welch ANOVA was performed, followed by the Games-Howell post-hoc test for multiple comparisons (Laerd Statistics, 2017a).

When comparing only two data points, the Independent-samples *t*-test with Welch's correction was used, regardless of normality of data distribution (Laerd Statistics, 2016a).

The Spearman's rank-order correlation test (r_s) was performed to analyse the correlation coefficient when the assumption for normality had been violated, while the Pearson's rank-order correlation test (r) was performed when the assumption was true. The strength of association was considered strong when $r > 0.5$ (Cohen, 1988; Laerd Statistics, 2016b). In all the above instances, a 5 % confidence limit for error was taken as statistically significant ($p \leq 0.05$) and data is reported with a 95 % confidence interval (95 % CI) of the mean difference.

Finally, effect magnitude indicators were calculated (Lakens, 2013) along with all statistical analyses, in line with statistical reporting guidelines (American Psychological Association, 2009; Cumming *et al.*, 2007;

Wilkinson, 1999) to indicate strong trends and rule out Type I (false positive) or Type II (false negative) errors (Cohen, 1988; Ellis, 2010). Effect magnitude for interactions were calculated with partial eta squared (η^2), where effect sizes were considered large when $\eta^2 \geq 0.14$, medium when $\eta^2 \geq 0.06$ and small when $\eta^2 \geq 0.01$ (Ellis, 2010). Furthermore, effect magnitude differences between specific groups were calculated by Cohen's d value (with a 95 % CI of the effect magnitude). Cohen's d value is an effect size indicator used to specify the standardized difference between two means, with effect sizes considered large when $d \geq 0.8$, medium when $d \geq 0.5$ and small when $d \geq 0.2$ (Cohen, 1988; Sullivan & Feinn, 2012). In all instances, only large effect magnitude indicators were considered significant.

1.7 Behavioural, neurochemical and nutritional analyses

Behavioural data of the OFT was carried out by an automated software system (Ethovision XT12; Noldus Information Technology BV, Wageningen, NLD), whereas the FST data was analysed with a continuous timer program (FST Scoreboard 2.0 software; Academic Support Services: Information Technology in Education, NWU, Potchefstroom campus, RSA) by the main researcher. Importantly, all recorded videos of manually scored behavioural data were first randomized and key-coded to blind the researcher to the behavioural groups and eliminate any researcher bias.

Neurochemical analyses were performed according to approved methods. To this extent, cortical monoaminergic concentrations were analysed according to published and validated method in our laboratories (Brand & Harvey, 2017a; Brand & Harvey, 2017b; Harvey *et al.*, 2006), whereas hippocampal BDNF concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) BDNF kit (Elabscience) according to the manufacturers' protocol.

Nutritional analyses of the rat chow were carried out in duplicate at the Functional Foods Research Unit, Cape Peninsula University of Technology, RSA.

1.8 Expected results and impact

The expected results of the current study are discussed in response to each of the study questions as presented above (*section 1.3*).

Study question one

We expect chronic, pre-pubertal fluoxetine and escitalopram, but not venlafaxine treatment, to induce early-life antidepressive-like effects in FSL rats. This is based on fluoxetine and escitalopram being the only FDA-approved antidepressants for juvenile MDD. Furthermore, we expect lasting bio-behavioural effects to be induced by all three antidepressants, since previous studies in our own laboratories have reported early-life treatment with antidepressants or central active stimulants to significantly affect behaviour later in life (Kruger, 2014; Kruger *et al.*, 2013; Mouton *et al.*, 2016; Steyn, 2011; Steyn *et al.*, 2011). However, since fluoxetine and escitalopram are FDA-approved, we expect the lasting antidepressive-like effects induced by these drugs to be at least neutral or greater and more beneficial than those observed by venlafaxine.

Study question two

Sub-question 2.1

We expect the vVO_{2max} -value of the pre-pubertal rat will steadily and consistently increase along with age. This increase is further expected to increase in such a manner that a significant difference in vVO_{2max} at the start and end of the intervention will be observed, necessitating an age-related and intensity-specific exercise regimen to optimally and accurately expose the individual to the required exercise intensity regimen. Finally, we expect low, and not high, intensity exercise to produce the most robust antidepressant-like bio-behavioural effects. This is in line with previous reports suggesting chronic, high intensity exercise to induce harmful effects compared to low or moderate intensity (Aguiar *et al.*, 2010; Kim *et al.*, 2003; Sun *et al.*, 2017).

Sub-question 2.2

Secondly, we expect that ω -3 EFA oil coating of the vivarium-provided rat chow will result in comparable amounts of daily chow eaten to those fed the standard (adequate) diet. Yet, the coated diet will provide significantly higher mean daily doses of ω -3 EFAs.

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Overall, based on literature, we expect pre-pubertal ω -3 EFA supplementation, but not low intensity exercise, to have both early-life and lasting antidepressant-like effects. Chronic supplementation with ω -3 EFA has been reported to yield long-term beneficial effects (Coluccia *et al.*, 2009), whereas the beneficial effects of chronic exercise appear to be transient (Berchtold *et al.*, 2005; Berchtold *et al.*, 2010; Greenwood *et al.*, 2012). Therefore, we expect chronic pre-pubertal exercise to induce early-life, but not lasting, antidepressant-like bio-behavioural alterations. Taken together, we expect both the non-pharmacological

interventions to induce early-life antidepressant-like effects, comparable to that induced by fluoxetine and escitalopram. However, we expect pre-pubertal antidepressant treatment to induce significantly greater antidepressant-like bio-behavioural effects later in life, compared to either non-pharmacological intervention.

Study question three

We expect chronic pre-pubertal low intensity exercise and ω -3 EFA supplementation to augment the early-life antidepressant-like effects of pre-pubertal antidepressant treatment, while simultaneously inducing significant lasting beneficial bio-behavioural alterations. Previous reports suggest ω -3 EFA supplementation to not only augment the effects of antidepressants at a therapeutic concentration (Gertsik *et al.*, 2012; Jazayeri *et al.*, 2010; Jazayeri *et al.*, 2008), but also enhance the effects of sub-therapeutic antidepressant concentrations (Laino *et al.*, 2010) and augment beneficial exercise-induced effects (Wu *et al.*, 2008). Similarly, exercise also augments pharmacological treatment (Hoffman *et al.*, 2011; Trivedi *et al.*, 2011; Trivedi *et al.*, 2006a) and contribute to the beneficial effects induced by ω -3 EFAs (Chytrova *et al.*, 2010; Joseph *et al.*, 2012). However, we expect the antidepressant-like effects induced by the fluoxetine and escitalopram combination strategies to be greater and more beneficial than those observed by any of the combinations with venlafaxine. Finally, we expect the triple combination of escitalopram, low intensity exercise and ω -3 EFA supplementation will induce the greatest immediate and lasting anti-depressive-like bio-behavioural alterations.

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The results of the current project will have significant impact on the already available literature and our understanding of juvenile depression and different treatment strategies. Firstly, the current project will contribute to the effectiveness of two different classes of antidepressants in juvenile patients, while simultaneously highlighting any possible lasting effects (whether positive, neutral or negative) that these drugs may have later in life.

Secondly, although conflicting evidence for the use of exercise and ω -3 supplementation as antidepressants are available, the WHO highlights the usefulness of non-pharmacological interventions in the treatment of MDD (World Health Organization, 2017a). That the dietary and lifestyle changes investigated in the current project might be more affordable than pharmacological antidepressants and are perceived to have a better safety profile, contributes to the growing popularity of these interventions. Therefore, although in an animal model of depression, the results of the current project will improve our understanding into the possible antidepressant properties of the different strategies as well as their potential to induce beneficial lasting effects. Furthermore, by establishing the $\dot{V}O_{2\max}$ capacity of the developing, pre-pubertal FSL rat, more accurate guidelines could be formulated regarding training regimens for juvenile patients; a novel

contribution to the available literature. Compared to a treadmill exercise program that remains constant for the intervention period, an age-related and intensity specific regimen might in fact be more effective in motivating the juvenile patient for a longer period; increasing patient adherence and possibly clinical outcome. Also, the majority of available literature investigates the effects of ω -3 EFA supplementation in relation to a deficient diet. Therefore, by comparing the effects of an ω -3 EFA supplemented diet to that of an adequate diet, the current results will contribute to our understanding of the value of complimentary supplements.

Thirdly, the possible augmenting effects of either of the non-pharmacological interventions on the pharmacological drugs could be significant in future treatment strategies for depressed juvenile patients. In this regard, insight into the possible augmenting mechanisms of action of the various treatment/intervention combinations may in fact open doors to other juvenile conditions treatment options, specifically the triple combinations initiated in the current project; a novel investigation at the time of the study layout.

Although the generated preclinical data cannot be directly extrapolated to humans, the current project will address, at least in part, the concerns regarding lasting effects of early-life interventions, both pharmacological and non-pharmacological. Furthermore, that we expect the non-pharmacological interventions to significantly augment the antidepressant-like effects of the pharmacological antidepressants could possibly lead to dosage adjustments in the depressed juvenile population. In addition, the data generated from the current project will also contribute to the use of the pre-pubertal FSL rat as an animal model for childhood depression, opening novel research avenues in our own laboratories.

1.9 Ethical considerations and approval

All experimental data are reported according to the National Centre for the Replacement Refinement and Reduction of Animals in Research's (NC3R's) ARRIVE (Animal Research: Reporting of *In Vivo Experiments*) guidelines (Kilkenny *et al.*, 2010) in order to promote reproducible, transparent, accurate, comprehensive, concise, logically ordered and well written manuscripts. In this regard, group sizes were comparable to that used in similar behavioural studies in our own laboratory which yielded statistical and/or practical significance (*Reduce*). Furthermore, due to the additional analyses of effect magnitude, a more accurate interpretation of the data would be generated even in instances where statistical significance might be missed due to sample size. Secondly, the researcher administering the injections and working with the animals has completed the Animal Handling course and has five years' experience of vivarium work. Also, all behavioural tests were performed according to approved and published protocols as well as previously accepted and approved Ethics applications at the NWU (*Refine*). Finally, as bio-behavioural alterations are required to be investigated and interpreted in regards to a very complex system (i.e. developing central nervous system), *in vivo* experimental work was required and therefore the current project could not be

performed in alternatives such as computer models or lower order vertebras (*Replace*). Finally, an animal monitoring sheet was used to monitor every test subject daily in order to determine any signs or symptoms of distress. The study was expected to provide relevant information to better understand the treatment of juvenile depression, thereby contributing to the relief of human suffering (*Justification & favourable risk-benefit ratio*).

All experiments conformed to the guidelines of the South African National Standards: The care and use of animals for scientific purposes (SANS 10386:2008) and were approved in accordance with the regulations set by the AnimCare animal research ethics committee (DoH reg. no. AREC-130913-015) of the NWU. Project ethics approval numbers **NWU-00148-14-A5** and **NWU-00373-16-A5** (*Appendix F*).

1.10 Conflict of interest

Jade Pharma Corporate (Pty) Ltd. (RSA) kindly sponsored the drugs used in the current study, while Nordic Naturals (USA) sponsored the ω -3 oil used for coating the vivarium rat chow. Finally, except for income from the primary employer and research funding to both Professors Christiaan B Brink and Brian H Harvey from the MRC, NRF, no financial support or compensation has been received from any individual or corporate entity over the past four years for research or professional services, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

CHAPTER 2: LITERATURE REVIEW

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Chapter 2 reviews current literature on major depressive disorder (MDD), with specific focus on juvenile MDD and treatment options thereof; the problem statement for the current study. In this regard, the epidemiology, diagnostic criteria and clinical presentation of MDD will be discussed, followed by a discussion of the current views on the aetiology of MDD, including various biological based hypotheses, current treatment options, role of neurodevelopment and the possible impact of early-life treatment later in life. Finally, a review of an animal model of depression will be discussed, concentrating on its usefulness modelling juvenile depression. Importantly, the majority of literature considers MDD in the adult population, in effect neglecting juvenile MDD. Reasons for the latter may include obvious ethical concerns with, and risks involved in clinical investigations in children as a vulnerable population. The literature review will therefore consider current theoretical background and clinical data on adult MDD, review what is known about juvenile MDD patients and compare the similarities and differences with the adult condition.

Furthermore, different pharmacological treatment options for MDD are discussed, again firstly reviewing the treatment options used in adult patients whereafter focus is shifted towards the limited treatment options for juvenile depression and the possible effects thereof on the developing brain and individual. In this regard, brain development is discussed in terms of the rat brain, however, it should be noted that similar development has been demonstrated (or delineated from supporting data) for humans, yet due to obvious reasons, very limited data regarding this topic are available. Also, two specific non-pharmacological interventions (*viz.* exercise and ω -3 EFA supplementation) will be reviewed, with a focus on possible antidepressant mechanisms of action and evidence of how they impact on behaviour and neurobiology. Important to note is that the term 'juvenile' is used throughout the thesis with reference to both children and adolescents, unless stated otherwise.

Finally, an overview of the animal model used in the current project (*viz.* FSL rat) is presented at the end of Chapter 2, with a specific focus on its use as an animal model for childhood depression. In this regard, a brief overview of the available literature regarding each of the different hypotheses of MDD's involvement in the specific adult animal model is presented. Also, although very limited, the data regarding the pre-pubertal FSL rat is compared to that of its adult counterpart to better understand the use of it as a pre-pubertal animal model for childhood depression in the current project, as well as to highlight the need for further research into the validation of animal models for childhood depression.

2.1 Epidemiology of major depressive disorder

According to the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-V), MDD is the alteration of a patient's cognition, affect, neurovegetative functions (those neuronal functions necessary to maintain life) and inter-episode remissions over a period of at least two weeks (although episodes last much longer in general) (American Psychiatric Association, 2013). Depression is further categorized to be either a bipolar affective disorder (presenting with both depressive and manic episodes) or a recurrent unipolar depressive disorder where the patient experiences repeated depressive episodes without manic episodes (World Health Organization, 2012, 2017a). MDD remains one of the most challenging mental health problems of our time and rising on a global scale. In fact, depression was reported to be the fourth leading cause of disease burden in the early 2000s, but was then estimated to be the second leading cause of disability by the year 2020 (Lopez & Murray, 1998) and the leading cause by 2030 (World Health Organization, 2012). Still, other reports suggest MDD to already be the main cause of overall disease burden (Mental Health Foundation, 2017c; Vos *et al.*, 2013). It is thus understandable that MDD (with co-morbid anxiety) contributes to one fifth of lost working days (Mental Health Foundation, 2017b), almost \$ 40 billion annual loss (Lépine & Briley, 2011) and a 29 % increase in global medical expenditures (Olchanski *et al.*, 2013).

Depression is estimated to affect more than 300 million people, worldwide (World Health Organization, 2017a), with a survey from seventeen countries reporting that 5 % of people to have suffered a depressive episode during the previous year (World Health Organization, 2012). Interestingly, although MDD affects adult females twice as much as adult males, reports regarding gender differences in juvenile patients have been mixed. Studies have suggested no gender differences in children (Hankin *et al.*, 1998), similar gender presentations to that of adults (Essau *et al.*, 2010) and even reversed adult gender presentations in pre-pubertal children (Cyranowski *et al.*, 2000). Still, important to note is that the term 'childhood' is used broadly in the literature and may in some instances even refer to adolescents. Nevertheless, literature appears to agree that females are generally at an increased risk for developing MDD following the onset of puberty (American Psychiatric Association, 2013; Cyranowski *et al.*, 2000; Hankin *et al.*, 1998; World Health Organization, 2017a). Yet a recent United Kingdom report indicated that 75 % of suicide victims were males (Office for National Statistics, 2016), suggesting that although females are more prone to develop MDD, male patients are at a significantly increased risk for taking their own lives. Importantly, although this report describes suicide in the general population, it is of note that suicide is defined by the National Statistic office as all deaths from intentional harm for persons aged ten and over, and deaths where the intent was undetermined for those aged fifteen and over (Office for National Statistics, 2016). On a global scale, suicide contributes to 1.4 % of all deaths, making it the seventeenth leading cause of death in 2015 (World Health Organization, 2016). However, in the South Africa, suicide contributes to 8 % of all

deaths, however, this figure only represents the reported data of academic hospitals and could therefore only represent a fraction of the real problem (South African Depression and Anxiety Group, 2017).

It was not until the 1970s that juvenile depression was recognised (Baker, 2006) and it has since been reported that as many as 10 % of adolescents, 2.8 % of children and 0.3 % of pre-schoolers do indeed suffer from MDD (Bhatia & Bhatia, 2007; Birmaher & Brent, 1998; Costello *et al.*, 2006; Costello *et al.*, 2003; Kozisek *et al.*, 2008). Furthermore, the lifetime prevalence of MDD in these individuals are estimated between 15 % and 20 % (Birmaher *et al.*, 1996), with as many as 70 % of depressed adolescents estimated to have a relapse within five years, as well as having an increased risk for developing MDD during adulthood (Richmond & Rosen, 2005). Similarly, school-aged children demonstrate a 40 % recurrence rate after two years and 70 % after five years (Kovacs *et al.*, 1984a; Kovacs *et al.*, 1984b; Luby *et al.*, 2009). Irrespective of the alarming lifetime prevalence rates, the immediate impact of juvenile depression is also of great concern. Developing years, especially adolescence, is known to be one of the most stressful periods in life; when peer pressure (including bullying and academic expectations) is at its highest and the conceivable life altering effects thereof on the individual are of great concern. This is of note, since an early report suggested that depressed juvenile patients are up to seven times more likely to commit suicide than those without (Gould *et al.*, 1998), partly explaining the high suicide rates currently observed in juvenile patients (Hulvershorn *et al.*, 2011; World Health Organization, 2017a). In fact, 35 % of youth suicides have been positively associated with MDD, with a similar gender presentation as observed in adults (Bridge *et al.*, 2006). Furthermore, in South Africa the teenage suicide rate has doubled over the past fifteen years (South African Depression and Anxiety Group, 2017), whereas suicide is considered the second leading global cause of deaths in individuals aged between 15 and 29 years (World Health Organization, 2017a). Nevertheless, juvenile MDD may also have long-term effects, such as increased risk for other mental disorders, including MDD and even treatment resistant depression (TRD) (Frodl *et al.*, 2010; Hatcher-Kay & King, 2003; Luby *et al.*, 2014; Luby *et al.*, 2009; McLaughlin *et al.*, 2010; Nanni *et al.*, 2012). Interestingly, attention-deficit/hyperactivity disorder (ADHD) has been strongly associated with MDD (Hinshaw *et al.*, 2006; Lahey *et al.*, 2007), particularly in girls, as well as being a very robust predictor of adolescent depression and even suicidal attempts between five and thirteen years later (Chronis-Tuscano *et al.*, 2010).

Similar to adult patients, not all juvenile patients respond to antidepressant treatment, yet unlike adults, treatment options for children and adolescents are very limited whilst also being associated with harmful and unwanted effects. Yet, as mentioned, untreated juvenile MDD may in itself lead to harmful immediate and long-term consequences, placing the prescribing medical practitioner in a particularly difficult situation having to weigh the possible harmful effects of early-life treatment against concerns of immediate and long-term consequences of untreated juvenile MDD itself (Friedman & Leon, 2007). Regardless, half of mental health conditions are already established by the age of fourteen (Kessler *et al.*, 2005; Mental Health

Foundation, 2017a), yet, more than 70 % of depressed juvenile patients do not receive appropriate treatment or diagnosis (Bhatia & Bhatia, 2007) at an appropriately early age. Together, this data necessitates early detection, diagnoses and treatment in the specific patient population to prevent any harmful or unwanted developing effects.

2.2 Signs, symptoms and diagnosis for juvenile depression

The presentation of juvenile depression, similar to the adult condition, includes both behavioural and emotional impairments, such as, but not limited to feelings of sadness, hopelessness, worthlessness, crying for no apparent reason, loss of interest/pleasure in normal activities and/or friends and family, trouble thinking, concentrating or making decisions, loss of energy, in- or hypersomnia, poor school performance, neglected appearance, self-harm and suicidal thoughts (Mayo Clinic, 2016b). When diagnosing MDD in juveniles, the general requirements for both adult and juvenile MDD diagnosis overlap, however, two additional criteria exist for the juvenile condition, i.e. irritable mood and insufficient weight gain during developmental years.

Table 2-1, below, summarizes the diagnostic criteria for both adult and juvenile MDD according to the DSM-V.

Table 2-1: Summary of the diagnostic criteria for MDD in adult and juvenile patients according to DSM-V (American Psychiatric Association, 2013).

Overlapping diagnostic criteria for adult and childhood MDD (present most of the day, nearly every day, as either indicated by either subject or observed by others)	Additional juvenile MDD criteria
Depressed mood.	Can be irritable mood.
Significant weight loss when not dieting or weight gain, or decrease/increase in appetite nearly every day.	Failure to make expected weight gain.
Markedly diminished interest or pleasure in all, or almost all, activities.	-
Insomnia or hypersomnia.	-
Psychomotor agitation or retardation.	-
Fatigue or loss of energy.	-
Feelings of worthlessness or excessive inappropriate guilt.	-
Diminished ability to think or concentrate, or indecisiveness.	-
Recurrent thoughts of death (not just the fear of dying), recurrent suicidal ideation.	-
The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.	
The episode is not attributable to the physiological effects of a substance or to another medical condition.	
The occurrence of the major depressive episode is not better explained by schizoaffective disorder, schizophrenia, schizophreniform disorder, delusional disorder, or other specified and unspecified schizophrenia spectrum and other psychotic disorders.	
There has never been a manic episode or a hypomanic episode.	

To accurately diagnose MDD, these symptoms must be present nearly every day, except for weight change and suicidal ideation. Interestingly, insomnia or fatigue are usually the most general presenting complaint, whereas sadness may initially be denied (or not self-recognised by the individual), but could surface through interviews with an appropriate healthcare professional (American Psychiatric Association, 2013). In addition, children younger than seven years may not be able to describe their state of mood and may therefore express their distress through vague somatic symptoms or even physical pain (Bhatia & Bhatia, 2007). Nevertheless, positive diagnosis of MDD, should be followed by appropriate categorisation of the condition. In this regard, an individual having difficulty performing his/her ordinary work and/or social activities, but does not cease function completely, is defined to suffer from mild MDD. Contrary, a person diagnosed with severe depression will most likely not be able to continue his/her social, professional or even domestic activities in addition to the mentioned impaired mood (World Health Organization, 2017a). It is clear that both emotional and behavioural changes are present in depressed children and adolescents, however, distinguishing these changes from ‘normal’ teenage behaviour can be difficult and it is therefore important for parents to distinguish normal social behaviour from adolescent personality changes and rather

seek professional help when they are concerned or uncertain. In this regard, irritability in children and adolescents is a key diagnostic symptom of juvenile MDD, yet is a very common emotion experienced by juveniles. In fact, irritability has been reported to be present in as many as 20 % of adolescents (Pickles *et al.*, 2010), besides its commonly described association with a variety of anxiety disorders, including generalized anxiety disorder (Stringaris, 2011).

According to data of the late 1990s, up to 50 % of individuals suffering from MDD also suffered from anxiety disorders (Kessler *et al.*, 1994; Kessler *et al.*, 1999; Kessler *et al.*, 1998). This comorbidity is in fact reported to be as high as 70 % in juveniles (Birmaher *et al.*, 1996) and could contribute to the presentation of other key diagnostic symptoms, such as decreased concentration, sleep disturbances (Axelson & Birmaher, 2001) and irritability. Of note is the findings of Wilkinson and colleagues (Wilkinson *et al.*, 2011) that indicated that non-suicidal behaviour, such as self-harm, feeling of hopelessness and anxiety disorder to be significant indicators of suicidal intent and should be attended to with the necessary concern. Therefore, a robust suicidal risk assessment must be completed on the first visit to a healthcare professional, categorizing the juvenile individual as either a high risk or protective (low risk) case profile, of which the former should be referred to a child and adolescent psychiatrist (Bhatia & Bhatia, 2007). Additional risk factors for a negative outcome of juvenile depression (to be managed with appropriate concern) include an environment that negatively impacts the individual's self-esteem over a considerable period of time (such as bullying and academic and/or peer problems), being a victim or witness of traumatic events (such as violence, sexual abuse parental divorce and/or death in the family), having other pre-existing mental conditions or a physical disability, familial history of MDD, being female, specific gene-variants, cigarette smoking and/or loss of a loved one, parent or romantic relationship (Bhatia & Bhatia, 2007; Mayo Clinic, 2016b).

Taken together, MDD, in both juveniles and adults, have similar presentations with the described specific deviations. Importantly, the underlying neurobiology may be diverse. Yet due to obvious reasons, clinical research into the underlying pathophysiology of juvenile MDD remains very limited. The following sections will discuss these various alterations associated with juvenile MDD, as well as briefly review its clinical relevance.

2.3 Aetiology of major depressive disorder

The aetiology of MDD has been accurately described as heterogeneous, yet presenting clinically homogeneous (Judd *et al.*, 1998; Winokur, 1997). This implies that, although the symptoms, as reflected in the diagnostic criteria of MDD, may show close resemblance between individuals, the underlying neurobiology and environmental triggers may be vastly different between them. In fact, a range of diverse hypotheses of the neurobiological basis of MDD has been described (*see section 2.3.7 below*), all supported

by evidence from both pre-clinical and clinical studies, further supporting the idea of heterogeneity and inter-individual differences in the underlying neurobiology of MDD. It has been widely advocated that MDD has a genetic and environmental basis, denoted the gene X environment hypothesis of depression, (Caspi *et al.*, 2003) and whereby both hereditary factors, as well as physical and nurturing challenges and triggers may play a role in the development or manifestation of MDD. Therefore, the role of such environmental factors appear to be receiving growing attention. In this regard, there has been a growing interest in proposed complimentary or augmentative strategies in the treatment or prevention of psychological conditions, such as MDD to better treatment outcome. Exercise and ω -3 supplementation represent such non-pharmacological intervention strategies, both reported to beneficially affect some or all of the neurobiological hypotheses of MDD. Nevertheless, despite our better understanding of the pathophysiology of MDD, therapeutic outcome remain limited, and our complete understanding of the aetiology of MDD remains largely unknown.

This section will thus focus on and discuss various hypotheses of the biological basis of MDD in chronological order of origin. These will be discussed together with the lines of supporting evidence, and will reflect on their value and limitations. Importantly, whereas the respective hypotheses focus on different aspects and factors associated with MDD, no single hypothesis explains all phenomena associated with the condition, neither is any one sufficient as a solitary causal explanation. Rather, they usually complement one another (or sometimes even overlap) and add a piece of the puzzle that the other neglects. Hence, previous attempts to unify some of these hypotheses have been made (Andrews *et al.*, 2015; Daws *et al.*, 2007; Homberg *et al.*, 2014; Maes *et al.*, 2012a; Maes *et al.*, 2008; Maes *et al.*, 2011b; Maes *et al.*, 2011c; Maes *et al.*, 2007b; Maes *et al.*, 2009; Martinowich & Lu, 2008), yet focus is generally limited to two or three of the major hypotheses at a time. This section will thus attempt to discuss each hypothesis on its own merit and limit the discussion to the specific elements involved in the specific hypothesis and initially not elaborate on the overlapping effects with other hypotheses. Also, the majority of hypotheses have been constructed to reflect MDD as represented in the adult brain, whereas the focus of the current study is on juvenile depression. Therefore, following the discussion of the various neurobiological hypotheses of MDD, a unified hypothesis of MDD is discussed in section 2.4, connecting all the various hypotheses and linking it to juvenile depression, discussing how this is expected to manifest in the developing brain.

2.3.1 The monoamine hypothesis

The monoamine hypothesis was first put forward during the 1950's (Baumeister *et al.*, 2003; Bunney & Davis, 1965; Schildkraut, 1965) based on the observations that reserpine was depressogenic due to its monoaminergic depleting properties (Baumeister *et al.*, 2003; Nemeroff, 1998), and iproniazid improved depressive symptoms via its monoamine oxidase (MAO) inhibiting properties (Andrews *et al.*, 2015). Therefore, in its most basic form, the monoamine hypothesis explains an underactivity of monoamine

synthesis in the brain of the depressed individual as cause of the MDD symptomology. In fact, one of the earliest descriptions of this hypothesis states:

“One may speculate on the possible role of centrally active amines present in the brain in the normal activity and general responsiveness of an individual. An excess of these might result in irritability, restlessness and aggressiveness. In the opposite direction, a deficiency of these substances would result in depressions and general lassitude.” (Everett & Toman, 1959).

However, it is now understood that altered monoaminergic neurotransmission, and not a mere decrease in neurotransmitter concentration, partly underlies the aetiology of MDD. In fact, extracellular monoamine concentrations are significantly increased within minutes of drug administration (Andrews *et al.*, 2015; Bymaster *et al.*, 2002; Rutter & Auerbach, 1993), yet therapeutic improvement is only observed two to three weeks after treatment initiation (Hindmarch, 2001). Furthermore, the antidepressant tianeptine enhances, rather than inhibits, 5-hydroxytryptamine (5-HT; serotonin) reuptake, yet still produce therapeutic antidepressant effects (Brink *et al.*, 2006; Burghardt *et al.*, 2004; Hindmarch, 2001; Mennini *et al.*, 1987). Also, both hypo- (Tremblay *et al.*, 2005) and hyperdopaminergia have been associated with MDD (Conner *et al.*, 2008; Volkow *et al.*, 2007). Finally, the depletion of monoamine neurotransmitters have failed to induce depression in healthy subjects, but is reported to cause a relapse of depression in successfully treated patients and those with a familial history of MDD (Ruhé *et al.*, 2007). Altogether suggesting any mood elevation properties induced by monoaminergic antidepressants may in fact be due to gradually developing adaptations to the enhanced neurotransmission (Andrews *et al.*, 2015; Nestler *et al.*, 2002).

Regardless, altered density and sensitivity of cortical α_{2A} -receptors (Ordway *et al.*, 2003; Valdizán *et al.*, 2010), together with reduced NA concentrations (Ruhé *et al.*, 2007) are observed in depressed patients. Furthermore, that hypodopaminergia (Dunlop & Nemeroff, 2007; Krishnan & Nestler, 2008), decreased D₂ receptor density (Klimke *et al.*, 1999) and decreased dopamine (DA) signalling (Tremblay *et al.*, 2002) are associated with anhedonia (the inability to experience pleasure), confirm the involvement of the dopaminergic system in MDD. The involvement of 5-HT is suggested in post-mortem studies, indicating decreased 5-hydroxyindoleacetic acid (5-HIAA) and 5-HT concentrations in the limbic brain regions and decreased 5-HT_{2A} receptor density in depressed suicide victims (Leonard, 2003). Still, that almost all of the currently approved antidepressants produce mood elevating effects via monoaminergic related processes, confirms the role of an abnormal and sub-optimal functioning monoaminergic system in MDD patients. Clearly, altered monoaminergic neurotransmission does not fully explain the therapeutic effectiveness of monoamine-increasing antidepressants and highlight the complicated role that monoaminergic neurotransmitters play in the aetiology of MDD (Nutt *et al.*, 2007; Shelton & Tomarken, 2001).

2.3.2 The hypothalamic-pituitary-adrenal axis hyperactivity hypothesis

The hypothalamic-pituitary-adrenal (HPA) axis regulates body functions such as immune response and metabolism, but also has powerful effects on the brain (Pariante & Lightman, 2008). The HPA axis is responsible for the secretion glucocorticoids (cortisol in humans and corticosterone in rats) from the adrenal cortex (Long & Cakman, 2015; Pariante & Lightman, 2008). In the brain, glucocorticoids are responsible for the regulation of neuronal survival, neurogenesis, acquisition of new memories, emotional evaluation of events as well as structural configuration of anatomical brain structures such as the hypothalamus (Herbert *et al.*, 2006). Secretion of glucocorticoid is dependent on a diurnal (24 hour) circadian rhythm, with maximal levels typically produced at the onset of the active part of the day. Nevertheless, secretion of glucocorticoids is also affected by stress levels experienced by the individual during a physically or emotionally stressful event, defined as any challenge or threat, either real or implied, to homeostasis (McEwen, 2000). Stressful events that pose a threat to an individual, whether internal (e.g. prolonged food or water deprivation) or external (e.g. dangerous situations) (Selye, 1950), acute or novel, perceived or real results in an exaggerated secretion of glucocorticoids (Dallman *et al.*, 2004; Shepard *et al.*, 2000, 2003). During these acute stressful situations, the function of the increased glucocorticoids includes the facilitation of appropriate behaviour in response to the posed threat (e.g. fight or flight), thereby attempting to restore homeostasis. Secondly, glucocorticoid secretion also assist in the formation of memories regarding the nature and context of the threat in order to minimize the risk of recurrence (via avoidance strategies) or to better cope with a similar situation in the future (Herbert *et al.*, 2006). Finally, in the presence of inflammation, glucocorticoids are secreted as an anti-inflammatory or immune suppressing agent, preventing possible maladaptive effects to the body (Herbert *et al.*, 2006), thereby, again, restoring homeostasis.

However, chronic activation of the HPA axis does not result in these beneficial or homeostasis effects, in fact, chronic increased levels of glucocorticoids form the basis of the HPA axis hyperactivity hypothesis which was first proposed during the 1960's (Nemeroff, 1996) and postulates that a chronic overactive HPA axis along with a disturbed circadian rhythm contributes to the aetiology of MDD (Germain & Kupfer, 2008; Li *et al.*, 2013; Pariante & Lightman, 2008). Such a dysfunctional HPA axis has been associated with delayed onset of sleep, early-morning wakening, blunting of the normal morning cortisol peaks and daytime fatigue (Germain & Kupfer, 2008). However, chronic increased HPA activity is only partially responsible for observed depressed symptoms. In fact, the glucocorticoid receptors (GR) appear to be equally involved (Pariante, 2017), as chronically increased glucocorticoid concentrations desensitize these receptors, effectively resulting in GR resistance and an impaired negative feedback system (Charmandari *et al.*, 2005; Pariante & Lightman, 2008). This is supported by studies demonstrating that the HPA axis of depressed patients is not suppressed by the administration of an oral dose of dexamethasone, whereas a small oral dose of dexamethasone inhibits glucocorticoid secretion for up to 24 hours in healthy individuals

(Pariante & Lightman, 2008). Furthermore, a recent study reported increased inflammatory markers along with reduced anti-inflammatory cytokines and GR mRNA expression in depressed patients (Cattaneo *et al.*, 2013). Hyperactivity of the HPA axis, GR resistance and the resulting chronic exposure to glucocorticoids can lead to modifications of certain brain areas, such as the hippocampus (Holmes *et al.*, 2006; McEwen, 2000), which plays a critical role in memory and learning capabilities (Squire *et al.*, 1990). Hippocampal atrophy, and decreased cognitive function (Lupien *et al.*, 1999; Roozendaal *et al.*, 2004) have been widely linked to MDD and confirmed by post-mortem studies of depressed patients, showing up to a 10 % reduction in hippocampal volume (Koolschijn *et al.*, 2009; Lorenzetti *et al.*, 2009; Sheline *et al.*, 2003; Videbech & Ravnkilde, 2015). Other unwanted effects of HPA axis hyperactivity include reduced survival rates of immature neurons (Wong & Herbert, 2004) and altered neuroplasticity (McEwen, 2005), which have been positively associated with MDD.

Research data further suggest that patients chronically treated with glucocorticoids tend to develop depressive-like symptoms (Brown & Suppes, 1998). This observation is further mirrored in Cushing's disease patients, yet in both patient types (those chronically treated with glucocorticoids and those diagnosed with Cushing's disease), depressive symptoms are resolved once the excess cortisol have been resolved (Bertagna *et al.*, 2009; Herbert *et al.*, 2006; McEwen, 2005). Interestingly, it has been reported that between 40 % and 60 % of patients suffering from MDD have a dysfunctional diurnal cortisol rhythm, accompanied with elevated levels of cortisol (Block & Nemeroff, 2014; Hallonquist *et al.*, 1986; Sachar, 1974), suggesting that altered circadian rhythms play an important role in the extent to which the brain handles abnormal stress (Brand *et al.*, 2015). In fact, depressed patients display an increased and lasting peak in salivary cortisol after waking, whereas healthy patients display a return to normal decrease approximately sixty minutes after waking (Bhagwagar *et al.*, 2003, 2005). Finally, CRF antagonists have been proposed as promising candidates for the treatment for MDD (Beers, 2006), yet clinical application remain limited. Moreover, antidepressants but not antipsychotics, modulate glucocorticoid receptor sensitivity (Carvalho *et al.*, 2010), confirming the involvement of the HPA axis in MDD. In fact, antidepressants successfully reverse glucocorticoid resistance and normalize the impaired negative feedback system (Pariante *et al.*, 2012; Pariante *et al.*, 2004).

Overall, the HPA axis hyperactivity hypothesis proposes that an excess corticosteroid concentration can damage the brain and/or inhibit its normal function, either directly or by increasing its susceptibility to other harmful agents. The prolonged exposure of increased concentrations of corticosteroids may also blight both cognitive and affective functions (Herbert *et al.*, 2006) often observed in MDD patients.

2.3.3 The cholinergic super sensitivity hypothesis

A third hypothesis of the neurobiological basis of MDD is the cholinergic super sensitivity hypothesis, first suggested in the early 1970s by Janowsky and colleagues (Janowsky *et al.*, 1972; Janowsky *et al.*, 1974). This hypothesis suggests that an oversensitive cholinergic nervous system, in concert with decreased noradrenergic activity, underlies MDD (Janowsky *et al.*, 1972). In other words, stimulation of the cholinergic system may be depressogenic, whereas inhibition of the cholinergic system induces mood elevating behaviour (Carlton, 1963; Domino & Olds, 1968; Vaillant, 1967). In this regard, Janowsky and colleagues observed that organophosphate poisoning, which inhibits acetylcholinesterase (AChE) and consequently decreases the synaptic catabolism of acetylcholine (ACh, leading to elevated synaptic concentrations of ACh, induce depressive-like symptoms (Janowsky *et al.*, 1972; Janowsky *et al.*, 1974). Individuals exposed to such compounds (i.e. diisopropylfluorophosphonate (DFP)) appeared to have a higher prevalence for MDD (Gershon & Shaw, 1961; Rowntree *et al.*, 1950). Furthermore, that centrally acting AChE inhibitors such as physostigmine, but not the peripheral active neostigmine, produce similar effects to that observed by the organophosphate poisons (Janowsky *et al.*, 1974), support the central involvement of central ACh in MDD.

It is well known that ACh plays a key role in cognitive processes, such as attention, learning and memory (Blokland, 1995; Everitt & Robbins, 1997), processes known to be decreased in depressed patients (American Psychiatric Association, 2013; Jaeger *et al.*, 2006; McIntyre *et al.*, 2013). In fact, antidepressant treatment normalizes the super sensitivity of the cholinergic system receptors, yet this effect appears to be independent of the intrinsic anticholinergic properties of the antidepressant (Leonard, 2003). In the preclinical arena, the FSL rat was initially bred to be resistant to the effects of DFP, yet turned out to be more sensitive. It was consequently demonstrated that this rat line displays robust face, construct and predictive validity as a genetic translational model of human MDD (Overstreet *et al.*, 2005; Overstreet & Wegener, 2013). In fact, the FSL rat displays several behavioural characteristics correlating to that required to diagnose MDD in humans and including psychomotor retardation, decreased weight gain and appetite (Overstreet *et al.*, 2005) and elevated rapid eye movement (REM) sleep (Jindal *et al.*, 2002; Kupfer & Reynolds, 1992). Overall, that altered basal ACh levels were reported in several brain areas of the FSL, in relation to controls (Brand *et al.*, 2012), support the involvement of the cholinergic system in MDD.

Interestingly, the cholinergic super sensitivity hypothesis is not solely based on the effects of ACh on the muscarinic receptors. ACh binds to two broad types of receptors, namely muscarinic and nicotinic acetylcholine receptors (mAChR and nAChR). The role of mAChRs is implicated by studies mentioned above, as well as the rapid onset of antidepressant effects caused by the muscarinic antagonist, scopolamine (Drevets & Furey, 2010; Furey & Drevets, 2006), whereas the role of nAChRs is supported by the antidepressant potentiating effect of nicotinic receptor stimulation (Popik *et al.*, 2003). These observations

are mirrored in the clinical setting with a significantly higher prevalence of MDD in smokers compared to healthy controls (Glassman *et al.*, 1990). Moreover, smokers with a history of MDD are two to three times more likely to fail in attempts to stop smoking (Covey *et al.*, 1998), however, if these individuals do succeed, they generally tend to complain of depressive-like symptoms (Glassman *et al.*, 1990). This said, clinical evidence suggests that application of nicotine patches can reduce depressive symptoms (Salin-Pascual *et al.*, 1995; Salín-Pascual *et al.*, 1996). However, this improvement is attributed to the desensitization (rather than activation) of the nAChR by the chronic administration of low nicotine concentrations of the nicotine patch (Pidoplichko *et al.*, 1997; Reitstetter *et al.*, 1999; Tizabi *et al.*, 2000).

Finally, controversy regarding the cholinergic super sensitivity hypothesis also exist with neuroimaging studies reporting increased choline concentrations, the precursor of ACh, in the brains of depressed patients and its reversal after recovery from MDD (Charles *et al.*, 1994; Steingard *et al.*, 2000), as well as an increased number of mAChR binding sites in the frontal cortex brain area of suicide victims (Dagytė *et al.*, 2011). Yet, other studies were unable to confirm these observations (Gibbons *et al.*, 2009; Katerina *et al.*, 2004; Stanley, 1984). Furthermore, results on the efficacy of anticholinergic drugs in the treatment of MDD are inconsistent (Drevets & Furey, 2010; Furey & Drevets, 2006). Regardless, although the importance of the involvement of the cholinergic system in MDD remains uncertain, it is evident from the available literature that there seems to be a fine balance between the stimulation and the desensitization of the cholinergic receptors for the mood depressing or elevating effects to manifest. Therefore, although ACh seems to play a role in the aetiology of MDD, it is considered be a contributor rather than an independent causative factor (Picciotto *et al.*, 2008), and may even be linked to a personality dimension than MDD itself (Fritze *et al.*, 1995).

2.3.4 The immunological hypothesis

The first published article to highlight the connection between MDD and T-cell activation was published in 1990 (Maes *et al.*, 1990) and consequently led to the Monocyte-T-lymphocyte or Cytokine hypothesis of depression (Maes, 1993, 1995, 1999). However, because inflammation is generally accompanied by oxidative and nitrosative stress (O&NS), this hypothesis extends beyond inflammation, and includes other accompanying factors as well, such as the mentioned O&NS damage and the kynurenic pathway, which is involved in the metabolism of tryptophan, the precursor of 5-HT. Therefore, the influence of inflammation, oxidative stress and kynurenic pathway alterations on MDD is described under the umbrella term of the immunological hypothesis and will be referred to as such throughout this thesis.

Firstly, increased inflammation is mirrored by elevated levels of pro-inflammatory cytokines, such as interleukins 1 and 6 (IL-1 and IL-6), tumour necrosis factor alpha (TNF α) as well as increased activation of cell-mediated immunity (CMI), as measured by interferon gamma (IFN γ), C-reactive protein (CRP) and T-

lymphocyte numbers (Leonard & Maes, 2012; Maes, 2011; Maes *et al.*, 1995; Maes *et al.*, 2012c). These pro-inflammatory cytokines have been shown to induce symptoms strongly associated with MDD in the clinical and preclinical arenas, such as weight loss, reduced locomotor activity and exploratory behaviour, anhedonia and cognitive dysfunction (Maes, 1993; Maes *et al.*, 2009). Specifically, administration of IL-6 not only induces increased behavioural despair (depressive-like behaviour) in Sprague-Dawley rats in the FST (Wu & Lin, 2008), but also increase anxiety-like behaviour, psychomotor retardation, impaired cognition, decreased body weight, altered sleep patterns, anhedonia and augmented learned helplessness (Maes *et al.*, 2012c; Salome *et al.*, 2008). Furthermore, a recent study suggests depressed patients with the highest IL-6 levels, are also the least likely to respond to antidepressant treatment (Carvalho *et al.*, 2013), thereby supporting the role of central inflammation in MDD. Nevertheless, inflammation can be caused by various factors, such as increased translocation of Gram-negative bacteria (Maes *et al.*, 2008), decreased antioxidant levels and elevated oxidative and/or nitrosative stress (Bilici *et al.*, 2001; Maes *et al.*, 2000; Maes *et al.*, 2011a), mitochondrial damage (Gardner & Boles, 2011) and decreased concentrations of antioxidant nutrients such as zinc (Maes *et al.*, 1997) and ω -3 EFAs (Maes *et al.*, 1999a). Indeed, recent meta-analyses and research studies have confirmed the positive association of increased inflammation and MDD (Dowlati *et al.*, 2010; Howren *et al.*, 2009; Hughes *et al.*, 2012; Liu *et al.*, 2012; Wu & Lin, 2008). Interestingly, it appears that only a subset of the population (approximately 30 %) display depressive symptoms when administered exogenous inflammatory cytokines (Capuron *et al.*, 2002; Capuron & Miller, 2004; Lotrich *et al.*, 2007; Musselman *et al.*, 2001), suggesting that impaired resilience towards the central effects of inflammation may play a role.

Secondly, inflammation is generally accompanied by increased levels of O&NS (Maes *et al.*, 2012b; Maes *et al.*, 2012c) caused by either a decrease in central antioxidant defences or an increase in reactive oxygen and nitrogen species (ROS and RNS) concentrations. In this regard, ROS and RNS are by-products of normal oxygen and nitrogen cellular metabolism, however, when these concentrations are not adequately counterbalanced by antioxidant defences, an overproduction of free radicals (e.g. O_2^- and H_2O_2) result, leading to cellular damage (Brand *et al.*, 2015). Moreover, the brain consists of very high phospholipid and polyunsaturated fatty acid (PUFA) concentrations, which have high levels of oxygen consumption and low baseline levels of antioxidant enzymes (Camiletti-Moirón *et al.*, 2013), thereby rendering them (and ultimately the brain) highly vulnerable to O&NS damage. In fact, several studies have reported O&NS-induced damage to DNA, proteins, fatty acids and mitochondria (Gardner & Boles, 2011; Maes *et al.*, 2012a; Maes *et al.*, 2007a; Peet *et al.*, 1998), all of which have, at least in part, been associated with MDD. These observations are further supported by reports of increased concentrations of plasma malondialdehyde (MDA) (a marker of lipid peroxidation) (Brand *et al.*, 2015) and superoxide dismutase (SOD) activity (endogenous antioxidant) in depressed patients (Khanzode *et al.*, 2003; Sarandol *et al.*, 2007), with the latter suggesting increased ROS levels present in these patients. Indeed, elevated ROS levels have been

observed in post-mortem brain tissue studies of depressed patients (Michel *et al.*, 2010). Furthermore, that antioxidants such as N-acetylcysteine (NAC) and zinc are effective in ameliorating depressive-like behaviour (Berk *et al.*, 2014; Fernandes *et al.*, 2016; Swardfager *et al.*, 2013; Vashum *et al.*, 2014) and that antidepressant treatment attenuate activated O&NS pathways (Gałecki *et al.*, 2009; Khanzode *et al.*, 2003; Zafir *et al.*, 2009), confirm the key role of central oxidative stress in the aetiology of MDD. Overall, O&NS damage cause functional and/or chemical changes to mentioned molecules and structures, which in turn activate a defensive autoimmune response (Dantzer, 2009; Herbert *et al.*, 2006), altogether causing fatty acid and/or protein structural damage (Maes *et al.*, 2011c), contributing to the MDD symptomology.

Thirdly, as depicted in Figure 2-1, pro-inflammatory cytokine concentrations and glucocorticoids stimulate indoleamine-2,3-dioxygenase (IDO) and tryptophan dioxygenase (TDO) activity. These enzymes regulate the conversion of tryptophan to kynurenine (Maes *et al.*, 2011b; Sakash *et al.*, 2002), a pathway with both neurotoxic and neuroprotective properties. Increased central inflammation, as hypothesized to exist in MDD, alters this specific pathway, resulting in an imbalance of neuroprotective and neurotoxic metabolites. To this extent, an increase in IDO and TDO will result in a shift towards increased production of kynurenine, compared to 5-HT. Nevertheless, the synthesized kynurenine is then further metabolised into quinolinic and kynurenic acid, an excitotoxic N-methyl-D-aspartate (NMDA) receptor agonist (Chiarugi *et al.*, 2001; Schwarcz *et al.*, 1983; Tokita *et al.*, 2012) and antagonist (Perkins & Stone, 1982), respectively. Firstly, kynurenic acid exhibits neuroprotective properties, whereas the quinolinic acid pathway is associated with neurotoxic effects (Stone & Darlington, 2002). To this extent, 3-hydroxy kynurenine (3-OH-kynurenine) generates oxidative radicals (Dantzer *et al.*, 2011; Maes *et al.*, 2011b), inducing neuronal damage. Furthermore, an imbalance in the neuroprotective and neurotoxic kynurenine metabolite concentrations are associated with increased depressive-like behaviour in animals (O'Connor *et al.*, 2009) and humans (Cho *et al.*, 2017; Young *et al.*, 2016). In summary, it is proposed that such a chronic imbalance in the kynurenine pathway forms part of the neurobiological build of MDD (Busse *et al.*, 2015; Savitz *et al.*, 2015).

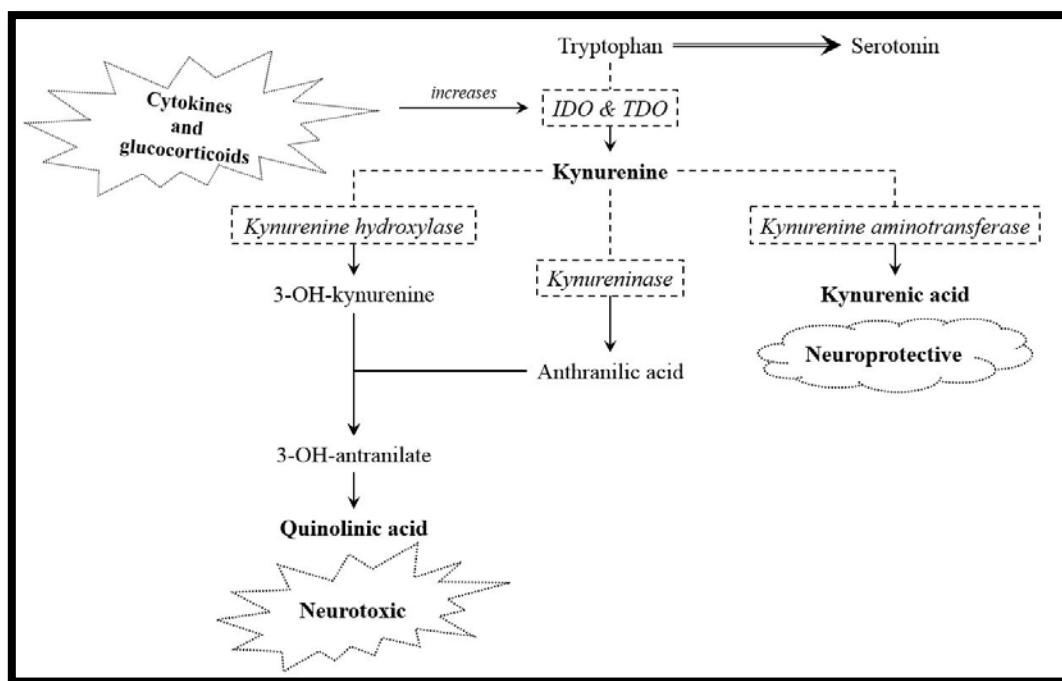


Figure 2-1: Simplified illustration of the kynurenine pathway (adapted from (Maes, 2011; Myint *et al.*, 2007)).

Figure 2-1 summarises the tryptophan catabolic pathway. Tryptophan, when not converted to serotonin, is catabolised into kynurenine, which is further metabolised to either the neuroprotective kynurenic acid or the neurotoxic quinolinic acid. IDO: indoleamine-2,3-dioxygenase. TDO: Tryptophan dioxygenase.

Finally, preclinical studies have supported the immunological hypothesis of MDD with administration of bacterial lipopolysaccharides (LPS) successfully inducing depressive-like behaviour in rodents, possibly due to activated inflammatory and O&NS pathways (Kubera *et al.*, 2013; Yirmiya, 1996). These depressive-like behaviours have subsequently also been reversed with chronic administration of fluoxetine (Kubera *et al.*, 2013) and support the idea that antidepressant efficacy may, in part, be due to anti-inflammatory mechanisms (Kubera *et al.*, 2001a; Kubera *et al.*, 2001b; Maes, 1999; Maes *et al.*, 1999b). Moreover, the antidepressant effects of fluoxetine are augmented by the concurrent use of celecoxib, a lipophilic selective cyclooxygenase-2 (COX-2) inhibitor (Akhondzadeh *et al.*, 2009), further highlighting the involvement of central inflammation in MDD. Likewise, fluoxetine treatment in LPS-administered rats also decreased superoxide concentrations (Kubera *et al.*, 2013), whereas treatment with the antioxidant NAC decreased depressive-like behaviour (Berk *et al.*, 2008), suggesting an antioxidant-like mechanism, in line with the current hypothesis of MDD. Lastly, ketamine, a non-competitive NMDA-receptor antagonist, has also been reported to exert antidepressant properties, even in TRD (Mathew *et al.*, 2010; Zarate *et al.*, 2006), whereas a variety of antidepressants have been reported to reduce inflammation via various mechanisms (Kenis & Maes, 2002; Kubera *et al.*, 2001a; Maes *et al.*, 1999b; Myint *et al.*, 2005), possibly adding to their therapeutic effects.

2.3.5 The glutamate/GABA hypothesis

Glutamate is the brain's endogenous excitatory neurotransmitter (Ramos-Chávez *et al.*, 2015), whereas γ -aminobutyric acid (GABA) is accepted as the major inhibitory neurotransmitter, mediating fast excitatory and inhibitory transmission, respectively (Brand *et al.*, 2015; Sanacora *et al.*, 2012). A dysregulated balance between these two opposing neurotransmitters could therefore lead to altered brain function and it is this imbalance of increased glutamate and decreased GABA concentrations that form the basis of the glutamate/GABA hypothesis (Mauri *et al.*, 1998; Mitani *et al.*, 2006; Petty, 1995; Sanacora *et al.*, 2002). The involvement of GABA in central disorders precedes the association of MDD and increased glutamate. Indeed, as far back as the 1980s, the GABA agonist, valproic acid, proved effective in the treatment of bipolar disorder (Emrich *et al.*, 1985), whereas the first mention of the involvement of glutamate in MDD, appeared during the 1990s with findings that suggested antagonists of the N-methyl-D-aspartate receptor (NMDAR) to have antidepressant-like effects (Trullas & Skolnick, 1990). Notably, the majority (80%) of brain synapses are excitatory (Douglas & Martin, 2007), suggesting that the brain is in general regulated by excitatory neurotransmission, and to a lesser extent by inhibitory transmission (including monoamines) (Sanacora *et al.*, 2012). Therefore, glutamate dysregulation could be a more significant treatment target, partly explaining its significant role in treating resistant depression. Regardless, glutamate transmission has been strongly associated with cognition where a disruption in the transmission could result in impaired fixation of memories (Citri & Malenka, 2008; Diamond *et al.*, 2004) and ultimately present as cognitive decline; a symptom strongly correlated with increased neural and dendrite death, especially in the hippocampus (reviewed by (Sanacora *et al.*, 2012)). In fact, excess levels of glutamate have been documented to cause neural degeneration and even death (Hardingham & Bading, 2010), possibly responsible for the structural changes in key brain areas associated with MDD. Furthermore, NMDAR activation, stimulates nitric oxide synthase (NOS) production (Dhir & Kulkarni, 2011) which is implicated in MDD via increased levels of its neuronal NOS (nNOS) form and antidepressant-like effects induced by NOS inhibitors (Dhir & Kulkarni, 2011).

Clinical findings further support the involvement of increased glutamate concentrations in MDD as increased plasma glutamate:glutamine ratios have been reported in depressed patients, in relation to healthy controls (Küçükibrahimoğlu *et al.*, 2009; Mauri *et al.*, 1998; Mitani *et al.*, 2006; Sanacora *et al.*, 2004). Additionally, increased plasma levels of GABA were observed in healthy patients, compared to the depressed ones (Küçükibrahimoğlu *et al.*, 2009). Furthermore, antidepressants, such as the SSRIs, not only decrease plasma glutamate levels, but also increase glutamine and GABA concentrations, ultimately leading to decreased depressive symptoms (Gambarana *et al.*, 1995; Kaufman *et al.*, 2001; Küçükibrahimoğlu *et al.*, 2009; Leonard, 2003; Maes *et al.*, 1998). Excitatory amino acid transporters, responsible for glutamate uptake, and glutamine synthetase (the enzyme responsible for the metabolism of glutamate to glutamine), have also been reported to be decreased in postpartum studies in depressed patients

(Bernard *et al.*, 2011; Miguel-Hidalgo *et al.*, 2010) providing additional support for the involvement of increased glutamate concentrations in MDD. Finally, the NMDAR antagonist, ketamine, reverse depressive-like behaviour in rodents, comparable to that seen with imipramine (Papp & Moryl, 1994). Moreover, lasting antidepressant responses were observed in unresponsive depressed patients, compared to those receiving saline (Berman *et al.*, 2000), whereas ketamine treatment proved effective as augmentation therapy for treatment resistant depression animals (Brand & Harvey, 2017b). Other NMDAR antagonists that have been reported to exert antidepressant effects, include memantine (Ossowska *et al.*, 1997) and lamotrigine (Kugaya & Sanacora, 2005). Various mechanisms of actions have been proposed for these observed antidepressant effects via the glutamatergic system, including reduced depolarization-stimulated glutamate release and expression of NMDARs (Bonanno *et al.*, 2005; Pittaluga *et al.*, 2007) and increased glutamate transporter expression (Moutsimilli *et al.*, 2005).

Continuing with this line of thought, the involvement of GABA in MDD has received less attention in relation to glutamate, yet limited literature suggests a significant role. To this extent, decreased cortical GABA concentrations in depressed patients have been noticed following SSRI or ECT treatment (Sanacora *et al.*, 2004; Sanacora *et al.*, 2002) via GABA_B receptor modulation. GABA_B receptors occur both pre- and post-synaptically in the hippocampus, where the former inhibits GABA (and other neurotransmitters, such as glutamate) release and the latter induces slow inhibitory potentials (Cryan & Kaupmann, 2005; Kulik *et al.*, 2003). Several studies have investigated GABA_B receptor antagonism as a possible treatment strategy for MDD with promising results. In this regard, decreased depressive-like behaviour in both the FST (Mombereau *et al.*, 2004) and learned helplessness (Nakagawa *et al.*, 1999) models were observed in both GABA_B knockout mice (genetically modified mice where the GABA_B receptors have been inactivated) and those treated with a GABA_B receptor antagonist (Mombereau *et al.*, 2004). Furthermore, these antidepressant-like behaviours, have been reversed by baclofen, a GABA_B receptor agonist (Nakagawa *et al.*, 1996), confirming the involvement of the inhibitory neurotransmission system in MDD.

The glutamate/GABA hypothesis has proven a valuable novel treatment target, having gained increased interest in recent years (Sanacora *et al.*, 2008). Particularly the use of ketamine as an alternative or adjunctive to antidepressants has received growing attention (Brand & Harvey, 2017b), largely due to its rapid and continued therapeutic effects (Maeng *et al.*, 2008). And although the mechanisms by which GABA_B receptor antagonism modulates behaviour are not yet fully understood, the potential as treatment alternative remain significant (Cryan & Kaupmann, 2005), especially since GABA_B is also implicated in anxiety (Pizzo *et al.*, 2017), a co-morbid condition/symptom of MDD.

2.3.6 The neuroplasticity hypothesis

By the end of the twentieth century, the involvement of neuroplasticity in the development and progress of MDD was suggested (D'Sa & Duman, 2002; Duman *et al.*, 2000; Jacobs *et al.*, 2000). The neuroplasticity hypothesis postulates that MDD symptoms occur due to a dysfunction in neuroplasticity mechanisms (Duman *et al.*, 1999), mediated by neurotrophins of which brain-derived neurotrophic factor (BDNF) is a key role player and has received the most attention (Groves, 2007). More specifically, a decrease in hippocampal BDNF concentrations occurs due to prolonged stress and lead to cognitive dysfunction and depressive symptoms, reversible with antidepressant therapy (Duman *et al.*, 1997; Duman & Monteggia, 2006). Overall, neuroplasticity describes the lifelong modification and reorganization of neural systems, single neurons, synapses, and receptors of the brain in order to adapt to aversive or stressful external and/or internal stimuli (Fuchs *et al.*, 2004; Zilles, 1992); absolutely vital processes for adequate functioning in a dynamic environment. These modifications are not only restricted to developing immature brain circuits, when neuroplasticity is more dynamic in relation to later in life. Data suggest that degeneration or cell death of mature neurons, particularly injured or degenerative cells, can be delayed by increased neuroplasticity-promoting proteins (Kerschensteiner *et al.*, 2003), possibly via increased neurogenesis and synaptogenesis.

Neuroplasticity is mediated by a family of protein growth (neurotrophic) factors, coined the neurotrophins (Kerschensteiner *et al.*, 2003), of which nerve growth factor (NGF) was the first to be identified (Cowan, 2001). However, the family of neurotrophins has since been expanded to include BDNF, neurotrophin 3 (NT-3), and NT-4/5 (Lewin & Barde, 1996) as well as insulin-like growth factor-1 (IGF-1) (Heck *et al.*, 1999; Kerner *et al.*, 2000). As mentioned, the neurotrophins are generally known for their cell survival-promoting effects, which are mediated by binding to several tyrosine kinases receptors (Trks) and activation of the antiapoptotic protein, bcl-2, expression (Yuan & Yankner, 2000). Yet, as depicted in Figure 2-2 below, stimulation of the TNF receptor-like molecule, p75 neurotrophin receptor (p75^{NTR}), can also trigger apoptosis (Bothwell, 1995; Fossati *et al.*, 2004; Groves, 2007; Segal & Greenberg, 1996), suggesting a fine balance between neurogenesis and neurodegeneration. Aversive stimuli, such as stress, have also been shown to decrease neurotrophin concentrations, especially BDNF, resulting in increased neuronal death and ultimately depressive mood (Almeida *et al.*, 2005). Altogether these form the basis of the neuroplasticity hypothesis. Importantly, although IGF-1 is involved in regulating cell growth and neuroplasticity (Anlar *et al.*, 1999; Carro *et al.*, 2003), and has been associated with antidepressant-like effects (Hoshaw *et al.*, 2005), its specific role in MDD remains unclear (Brand *et al.*, 2015). Another neurotrophin with limited data regarding its involvement in MDD, is the vascular endothelial growth factor (VEGF) which regulates angiogenesis (Ferrara, 2009) and may contribute to neuronal proliferation (Sun *et al.*, 2003). VEGF has been suggested to possibly counterbalance the neurodegenerative effects associated with MDD (Brand *et al.*, 2015) via increased angiogenesis and consequently neuronal growth. Of similar

interest is neuropeptide Y (NPY), a protein with widespread central and peripheral physiological and behavioural functions and of which expression is dependent on BDNF concentrations (Sibille, 2013). Nonetheless, NPY appears to be strongly associated with anxiety (Kautz *et al.*, 2016; Roseboom *et al.*, 2014; Schmeltzer *et al.*, 2016) and might therefore be a more accurate marker of anxiety than of depression, yet since these often present as co-morbid conditions, the involvement of NPY in MDD cannot be overlooked and could prove useful in MDD as a treatment target. In fact, voluntary wheel running increases hippocampal NPY concentrations in stress-sensitive, but not stress-resistant rats, inducing enhanced cell proliferation and antidepressant-like effects (Bjørnebekk *et al.*, 2006).

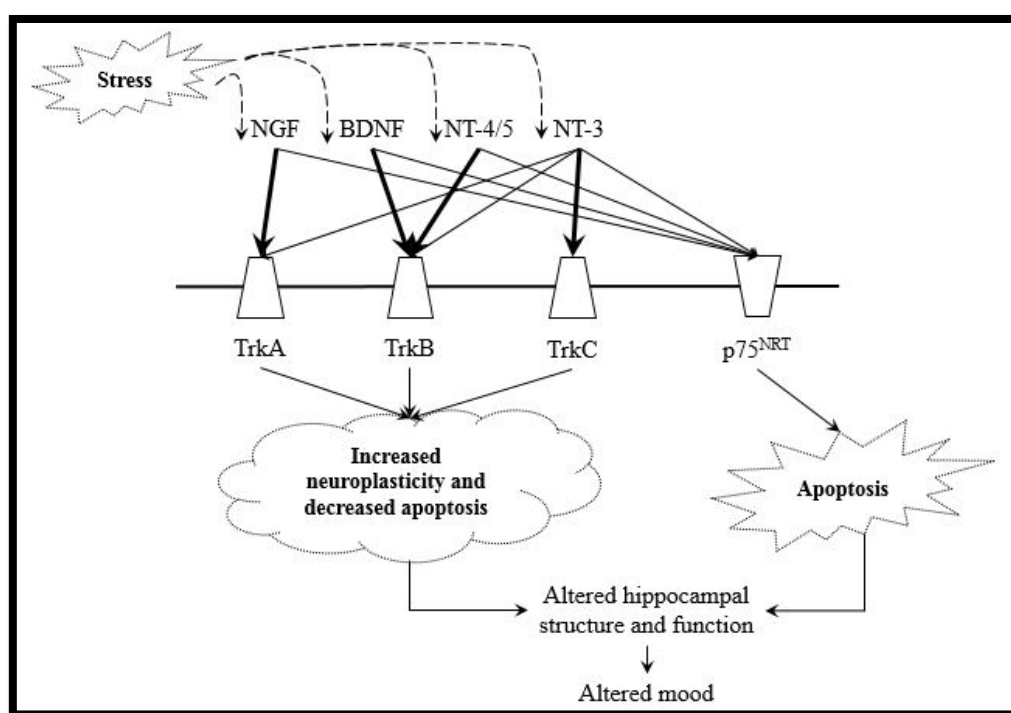


Figure 2-2: Simplified illustration of the binding patterns of neurotrophins to their receptors and their impact on the hippocampus and mood (adapted from (Fossati *et al.*, 2004; Groves, 2007; Kerschensteiner *et al.*, 2003)).

Figure 2-2 summarises the binding patterns of the neurotrophins to their different receptors. Binding to a Trk receptor with high affinity is represented by a thick arrow, while low affinity binding is represented by a thin arrow.

The effect of stress on the neurotrophins is represented by a broken line arrow. Stimulation of TrkA, B and C receptors by neurotrophins induce neuronal proliferation, survival and plasticity and decreased apoptosis leading to altered hippocampal structure and function and ultimately increased mood. Stimulation of p75^{NTR} can trigger apoptosis causing decreased hippocampal function and ultimately decreased mood. Neurotrophin concentrations are negatively affected by stress and can, therefore, also lead to decreased neuroplasticity and ultimately decreased mood. BDNF: Brain-derived neurotrophic factor. NGF: Nerve growth factor. NT-3: neurotrophin 3. NT-4/5: neurotrophin 4/5. p75^{NTR}: p75 neurotrophin receptor. TrkA: Tyrosine kinase receptor A. TrkB: Tyrosine kinase receptor B. TrkC: Tyrosine kinase receptor C.

However, not all neuroplasticity processes are beneficial (Fuchs *et al.*, 2004). In healthy patients, the beneficial effects of neuroplasticity (e.g. neurogenesis) outweigh the detrimental effects (e.g. neurodegeneration), yet, when this balance is reversed, the function of key brain structures, such as the

hippocampus and frontal cortex, might be adversely affected, as suggested in depressed patients (Fuchs *et al.*, 2004; Manji *et al.*, 2000; Manji *et al.*, 2003). In fact, hippocampal (and other brain regions) atrophy has been reported in depressed patients (Koolschijn *et al.*, 2009; Lorenzetti *et al.*, 2009; Sheline, 2003; Videbech & Ravnkilde, 2015) and rodents (Chen *et al.*, 2010), and successfully reversed with chronic antidepressant therapy (Aydemir *et al.*, 2005; Frodl *et al.*, 2008; Huang *et al.*, 2008). Other animal models have shown that stressful stimuli, such as social isolation (Barrientos *et al.*, 2003), chronic swim stress (Roceri *et al.*, 2004) and maternal separation (Roceri *et al.*, 2002) induce significant decreases in hippocampal BDNF mRNA and increase depressive-like behaviour. In contrast, this induced depressive-like behaviour has been reported to be reversed by various antidepressants (Coppell *et al.*, 2003; Dias *et al.*, 2003; Sen *et al.*, 2008), electroconvulsive therapy (Nibuya *et al.*, 1995), physical activity (Cotman *et al.*, 2007) and ω -3 EFAs (Wu *et al.*, 2004), possibly via increased BDNF protein expression and/or cyclic AMP (adenosine monophosphate) response binding protein (CREB), a transcription factor regulating BDNF expression (Nibuya *et al.*, 1996). Overall, that BDNF knockout mice only live a couple of days after birth, corroborates with the proposed vital role of neuroplasticity (Homborg *et al.*, 2014).

In accordance with the above mentioned preclinical reports, neuroimaging and post-mortem studies of depressed patients report decreased cortical thickness, neuronal size and numbers, reduced hippocampal volume (Manji *et al.*, 2001; Rajkowska, 2000; Sheline *et al.*, 2003) and decreased hippocampal BDNF, NT-3 and TrkB expression. Contrary, elevated neurotrophin levels were reported in patients receiving antidepressant treatment at the time of death (Dwivedi *et al.*, 2003; Karege *et al.*, 2005). It is therefore conceivable that a negative correlation between neurotrophic factor concentrations, such as BDNF, and MDD exist and could play an integral role in the aetiology of the disorder (Yulug *et al.*, 2009). To this extent, increased neurotrophic factor concentrations (Nibuya *et al.*, 1995; Nibuya *et al.*, 1996) and neurogenesis (Malberg *et al.*, 2000) have been suggested as possible mechanisms of action for antidepressant drugs as well as explain the delayed therapeutic effect of these drugs (Fossati *et al.*, 2004).

Taken together, the results suggest that neuroplasticity and neurotrophic factors play an integral role in the aetiology of MDD, and in particular that neurogenesis and neurodegeneration should be finely balanced and controlled in order to maintain normal and healthy brain function. Although arguments for and against this hypothesis exist, a host of strong evidence suggest a key role of neurotrophins in antidepressant treatment. Indeed, BDNF (and other neurotrophic factors) play an important role in modulating neuronal circuits and function, which may be compromised in MDD. Although reduced BDNF may not necessarily induce depressive-like behaviour or traits, it may represent a predisposing factor that, together with chronic stress, may manifest as MDD (Castrén *et al.*, 2007; Groves, 2007).

2.3.7 The gene-environment hypothesis

The gene-environment (G x E) hypothesis is based on the knowledge gained from the Dunedin birth cohort study (Caspi *et al.*, 2003). In this specific study, the researchers attempted to identify why stressful life experiences lead to depression in certain individuals, but not in others. The researchers concluded that an individual's response to environmental factors are determined by his/her genetic makeup. More specifically, the increased risk for developing MDD after a stressful or adverse life event is thought to be higher in individuals with a susceptible genetic build, whereas individuals with a more resilient build are less likely to have lasting mental consequences following a similar event (Kendler *et al.*, 2010). Therefore, the role of genetic susceptibility has been investigated with regards to a two-way interaction between a common functional or single nucleotide polymorphism (SNP) (genetic factor) and a stressful life event (environmental factor); together culminating to an increased risk for developing MDD. It has, however, been suggested that a single change in a genetic or environmental factor will have little or no significant effect on an individual's behaviour (Uher, 2008) due to degeneracy. In this regard, degeneracy describes the adaptive ability of a system to counterbalance any single change in order to minimize the impact thereof (van Swinderen & Greenspan, 2005). A basic example of this ability is seen in humans and laboratory animals with little or no adverse consequences, despite the absence of albumin (Buehler, 1978; Edelman & Gally, 2001). The G x E hypothesis therefore suggests that several factors have to co-occur to induce MDD (Uher, 2008) and that although stressful life events are regarded as one of the strongest predictors of depression onset, the occurrence of such a traumatic event has little or no impact in the absence of a pre-existing, genetic vulnerability (Brown *et al.*, 2008). To this extent, several significant SNPs have been associated with either increasing or decreasing an individual's risk for developing MDD following a stressful or adverse life event.

The first of these SNPs associated with an increased risk for developing MDD is found on the serotonin transporter gene (5-HTTLPR). Individuals with 5-HTTLPR 'short' (s) alleles (associated with decreased SERT without affecting its availability (Heils *et al.*, 1996; Karg *et al.*, 2011; Martin *et al.*, 2007)) are associated with an increased risk for developing MDD following stressful life events, whereas 'long' (l) allele homozygote individuals appear not to be affected to the same extent by similar traumatic events (Caspi *et al.*, 2003; Uher & McGuffin, 2008; Uher & McGuffin, 2010). Important to note is that psychiatric diseases (including MDD) do not appear to be more prevalent amongst 5-HTTLPR s allele carriers (Lasky-Su *et al.*, 2005). In fact, MDD appears to be just as prevalent in individuals with the 5-HTTLPR l allele carriers, but possibly due to reasons independent of environmental factors (Uher, 2008). To this extent, 5-HTTLPR s allele individuals (approximately 17 % of the population (Brown *et al.*, 2008; Caspi *et al.*, 2003)) appear to benefit more from positive environmental factors, such as social support (Kaufman *et al.*, 2006; Wilhelm *et al.*, 2006) and could therefore be more sensitive to environmental insults as well (Uher, 2008). Furthermore, sub-optimal functioning of SERT during early-life development may result in

increased extracellular 5-HT and subsequent desensitization and downregulation of 5-HT_{1A} receptors (Holmes *et al.*, 2003), presenting as increased anxiety- and depressive-like behaviour later in life (Ansorge *et al.*, 2004; Holmes, 2008; Holmes *et al.*, 2003). A possible explanation is based on the degeneracy principle which proposes that adaptive changes occur in the serotonergic system during early-life development in order to compensate for the genetic lowered expression of SERT (Uher, 2008). This genetic lowered expression of SERT is, however, exposed during periods of, or after, heightened stress (Sibille & Lewis, 2006; Uher & McGuffin, 2008; Uher & McGuffin, 2010), such as adolescence, rendering the 5-HTTLPR *s* individual more exposed to the effects of increased 5-HT concentrations (David *et al.*, 2005; Fabre *et al.*, 2000; Uher, 2008). In contrast, the 5-HTTLPR *l* allele carriers (approximately 31 % of the population (Brown *et al.*, 2008; Caspi *et al.*, 2003)), might be able to counterbalance the increased 5-HT via upregulating SERT function. Therefore, the combination of the less efficient 5-HTTLPR *s* allele, the downregulated and desensitized 5-HT_{1A} receptors, and a consequent traumatic event could therefore contribute to the onset of MDD (Goodyer *et al.*, 2009). Interestingly, a previous study found only the main effect of the 5-HTTLPR and 5-HT_{2A} alleles significant in interacting with environmental events to predict depression in females and not in males (Eley *et al.*, 2004), partly explaining the increased incidence of MDD in the female population, compared to males.

Relevant to the current study, the influence of early life experiences appears to play a vital role in the G x E hypothesis of MDD. The early developing years of an individual appear to be critical and may present a window of genomic plasticity, during which the expression of specific genes may adapt its function in such a manner to prepare the individual for a life under specific environmental conditions, as predicted on the basis of early life experiences (Champagne *et al.*, 2006; Lillycrop *et al.*, 2005; Seckl & Holmes, 2007; Weaver *et al.*, 2004). In effect, adverse events, such as parenting, neglect and abuse during early life development (Brown *et al.*, 2008) have been reported to alter gene expression towards a high-risk, high-resilience state which is maintained throughout the individual's life by epigenetic modifications of the DNA (Szyf *et al.*, 2008). These DNA modifications have been reported to alter the expression and function of the HPA axis (Heim *et al.*, 2000; Weaver *et al.*, 2004), immune system (Danese *et al.*, 2007) and serotonergic neurotransmission (Ansorge *et al.*, 2004; Kinnally *et al.*, 2008); all of which are associated with MDD and discussed elsewhere earlier.

A second genomic alteration associated with increased risk for developing MDD, is the BDNF gene variant, the *val*⁶⁶*met* allele (where valine (*val*) is substituted with methionine (*met*) in codon 66), present in 27 % of adolescents (Goodyer *et al.*, 2009). The *val*⁶⁶*met* allele is associated with decreased BDNF secretion and conversion of pro-BDNF to mature BDNF (Egan *et al.*, 2003). Moreover, the *val*⁶⁶*met* allele interacts with the 5-HTTLPR *s* allele to further sensitize the individual to positive and negative life events (Uher, 2008), and has been associated with decreased cognitive function, and BDNF and hippocampal N-acetylaspartate levels (Egan *et al.*, 2003; Hariri *et al.*, 2003). N-acetylaspartate is accepted as a marker of neuronal density

and/or integrity (Schuff *et al.*, 2001; Urenjak *et al.*, 1993), indicating towards reduced neuronal concentrations in carriers of this specific gene variant. In fact, the *val⁶⁶met* allele could also be partly responsible for hippocampal volume reduction, commonly seen in post-mortem studies of depressed patients (Bueller *et al.*, 2006; Gatt *et al.*, 2009; Pezawas *et al.*, 2004; Szeszko *et al.*, 2005). Finally, the *val⁶⁶met* gene variant has been positively associated with SSRI response (Bath *et al.*, 2012; Chen *et al.*, 2006; Yu *et al.*, 2012). Other gene variants associated with increased risk for developing MDD, include the corticotrophin releasing hormone receptor gene (CRHR1) (Bradley *et al.*, 2008) (associated with hyperactive cortisol production in response to traumatic events (Heim & Binder, 2012)), the T or C SNP on the 5-HT_{2A} receptor (Hrdina *et al.*, 1993), the allele of the 5-HT_{2C} receptor (associated with increased transcriptional activity (Eley *et al.*, 2004)), the high (*H*) and low (*L*) functional alleles of MAO_A (Deckert *et al.*, 1999), the protective 198bb allele of TPH (Eley *et al.*, 2004) and the -511C/T polymorphism on the IL-1 β (Younger *et al.*, 2003).

In conclusion, that MDD treatment options, whether pharmacological or psychological, are effective, indirectly supports the G x E hypothesis. In this regard, individuals with a sensitive genotype (i.e. 5-HTTLPR *s* allele and/or *val⁶⁶met* BDNF allele), and a consequent increased risk for developing MDD following a stressful life event, appear to benefit more from psychotherapeutic strategies. In contrast, depressed individuals with an environmentally insensitive genotype (i.e. 5-HTTLPR *l* allele and/or *val⁶⁶val* BDNF allele) may benefit more from pharmacological treatment strategies, i.e. antidepressants (Jennings *et al.*, 2008; Landén & Thase, 2006; Uher, 2008). In fact, depressed patients with the 5-HTTLPR *s* allele are reported to have a weaker response to pharmacological antidepressants, compared to the environmentally insensitive genotypes (Schatzberg *et al.*, 2005). These diverse sensitivities towards MDD treatment options might also explain why not all patients respond equally to the commonly used SSRI antidepressants and at the same time give insight into the understanding of the antidepressant effects of drugs such as tianeptine with its alternative mechanisms of action. Overall, the G x E hypothesis, does not completely explain the aetiology of MDD and in this regard, the evidence presented above have not been replicated in all studies (Brown *et al.*, 2008; Serretti *et al.*, 2007; Uher & McGuffin, 2008). Regardless, the G x E hypothesis of MDD remains valid (Cervilla *et al.*, 2007) and contributes to the unknown or complex cause and aetiology of MDD, especially considering the fragile and sensitive state of the developing individual during early-life that is sensitive towards both positive and negative influences (in the presence of genetic susceptibility) with possible long-term consequences (Andersen & Nalvalta, 2004, 2011).

2.4 A unifying hypothesis for major depression and its involvement in juvenile depression

Although the clinical symptoms and general presentation of MDD in children, adolescents and adults may largely overlap, the aetiologies may differ and be dependent on the underlying level of emotional and cognitive development as well as the brain structures involved in these processes (Goodyer, 2008; Winokur,

1997). Therefore, the question arises whether the neurobiological basis of MDD, as portrayed by the hypotheses discussed above, are also applicable to MDD in juvenile patients? Moreover, are there any distinct differences in the juvenile pathophysiology, compared to that in adult patients? Interestingly, one early review concluded that caution should be used when adult MDD is extrapolated to the juvenile condition, because data in the younger patient population are very limited. Of note, differences in volumetric dimensions, functional modalities and neural networks in the brain may, according to Hulvershorn and colleagues (Hulvershorn *et al.*, 2011), precede psychopathology and have causal implications for the onset of MDD, especially in the juvenile population, where such abnormalities can be augmented by internal (genotype) and/or external (environmental) influences. Whereas some observations suggest dissimilarities and alternative mechanisms involved in the underlying pathophysiology of juvenile MDD (Kaufman *et al.*, 2001), more recent data suggest striking similarities in mechanisms underlying adult and juvenile MDD. Below follows a discussion of evidence for both similarities and dissimilarities between adult and juvenile MDD:

Indeed, one of the most significant differences in the pathophysiology of juvenile MDD, in relation to the adult condition, concerns the HPA-axis hyperactivity hypothesis. Differently to adult patients, depressed children are not, or at least not as frequently as adults, reported to present with hypercortisolemia (Kaufman & Ryan, 1999; Ryan & Dahl, 1993), but rather with hypocortisolism. Still, preclinical research suggest CRF antagonists to ameliorate depressive-like behaviour in adolescent animals, comparable to that seen with fluoxetine (Bourke *et al.*, 2014), confirming the involvement of the HPA system. Nevertheless, evidence suggests that hypocortisolism, and not hypercortisolism, may be the result of chronic stress and consequent receptor desensitization, together explain the somewhat paradoxical observation of hypocortisolism in children growing up in less than optimal care conditions (Gunnar & Vazquez, 2001; Hart *et al.*, 1996). Interestingly, hypocortisolism has been identified in boys with disruptive and aggressive behaviour (Malkesman *et al.*, 2006a), which could relate to irritability, a key diagnostic criterion for childhood MDD (American Psychiatric Association, 2013). Furthermore, aggressive children have often been exposed to higher than normal levels of violent and disruptive circumstances, adding to an already stressful environment and thereby causing the reported hypocortisolism (Malkesman *et al.*, 2006a). However, reports of the contrary to this have also been published (Dockray *et al.*, 2009; Goodyer *et al.*, 1996), where, similar to adult patients, hypercortisolism was indeed detected in depressed juveniles. In fact, increased pituitary size has been noted in depressed juveniles (reviewed by (Hulvershorn *et al.*, 2011)), at least in part confirming the involvement of the glucocorticoid system in juvenile MDD. Finally, one recent report suggests a hypercortisol stress-response to be present only in adolescents with mild to moderate depression, whereas those with moderate to severe depression exhibit a blunted cortisol response (Harkness *et al.*, 2011). Another report suggests up to 70 % of depressed children to not respond to a dexamethasone suppression test, correlating with results found in juvenile animals (Kaufman *et al.*, 2001).

Also, an earlier report suggests that either hypo- or hypercortisolism can be present in MDD patients (melancholic or atypical depression), yet induce different behaviours, i.e. increased sleep and appetite and reduced concentration and energy levels, or decreased sleep and appetite and relative immunosuppression (Gold & Chrousos, 2002). Hence, it appears that the exact state of the juvenile HPA axis activity is uncertain and that both hyper- and hypocortisolemia have been observed in different validated animal models of depression (Brand *et al.*, 2015). This inconsistency may therefore not be vital to the overall presentation of the disorder, but rather represent a contributing factor. In fact, the early-life state of the HPA may continue to play a longer term role in the susceptibility of an individual to develop other psychological conditions later in life (Maniam *et al.*, 2014).

The other hypotheses of MDD appear to be similar to that described earlier in adult patients. In this regard, 5-HTTLPR *s* and/or MAOA *L* and/or BDNF (*val⁶⁶met*) genetic alterations are associated with greater fearfulness in children (Hayden *et al.*, 2007), biased attention to emotional pictures in adolescents (Perez-Edgar *et al.*, 2010) and significantly high depression ratings (Cicchetti *et al.*, 2007; Kaufman *et al.*, 2004; Lau *et al.*, 2010). Increased inflammation has also been observed in depressed children (Danese *et al.*, 2011) as well as in adults who experienced a stressful childhood (Danese *et al.*, 2009). That 5-HT plays a significant role in juvenile MDD is supported by altered neurosteroid 3- α , 5- α , tetrahydroprogesterone concentrations (involved in the regulation of GABA release), which are only increased by SSRIs (Gambarana *et al.*, 1995; Kaufman *et al.*, 2001). Therefore, it could be argued that a deficit in GABA concentration might also contribute to juvenile MDD. In fact, decreased plasma GABA concentrations have been documented in a subset of depressed juveniles (Prosser *et al.*, 1997). Altered monoaminergic neurotransmission has also been observed in juvenile patients with a study reporting increased midbrain SERT availability, compared to healthy controls (Dahlström *et al.*, 2000). To this extent, 5-HT may affect development and function of other monoaminergic systems as it has been reported to decrease DA release and is, at least in adults, partly dependent on the functionality of the noradrenergic system (Leonard, 2003). Finally, in line with adult patients, decreased hippocampal volume and thickness have also been reported in depressed juveniles (Peterson *et al.*, 2009; Rao *et al.*, 2010), suggesting impaired neuroplasticity and/or increased neurotoxicity resulting from an array of possible mechanisms (discussed further below). In fact, reduced activation of key brain areas during task-based functional neuroimaging, accompanied by decreased cognitive function during cognitive control tasks, are evident in depressed juveniles (Hulvershorn *et al.*, 2011), supporting the involvement of reduced neuroplasticity and altered glutamatergic neurotransmission.

Taken together, the neurobiological basis for juvenile MDD appears to be comparable to that of the adult patient, with subtle differences due to neurodevelopmental status. Importantly, the different neurobiological hypotheses of MDD should not be viewed as independent from one another, but are rather as interlinked, or as different pieces of the larger puzzle to be connected to give the full picture. Below is

therefore an attempt to unify the neurobiological hypotheses of MDD within the framework of early neurodevelopment, into a single overlapping hypothesis applicable to juvenile MDD.

As mentioned before, increasing monoamine concentrations with pharmacological drugs do not result in immediate clinical improvement of MDD. In fact, antidepressants have been shown to increase neuroplasticity (Aydemir *et al.*, 2005; Chen *et al.*, 2001; Matrisciano *et al.*, 2009), decrease central inflammation (Bah *et al.*, 2011; Piletz *et al.*, 2009) and oxidative stress (Eren *et al.*, 2007; Lee *et al.*, 2011; Stefanescu & Ciobica, 2012), inhibit HPA-axis hyperactivity (Holsboer, 2000) and decrease synaptic glutamate release (Musazzi *et al.*, 2011) in addition to its intended monoaminergic effects. Nevertheless, chronic altered monoaminergic neurotransmission remains the key target of MDD treatment strategies, yet the subsequent effects of sub-optimum monoaminergic neurotransmission and function are far reaching.

As summarized in Figure 2-3, monoaminergic neurotransmission is influenced by the genetic build of an individual. To this extent, individuals with the $MOA_A L$ allele (up to a 10-time lower activity than the $MOA_A H$ variant in MAO activity (Sabol *et al.*, 1998)), 5-HTTLPR s allele and/or a COMT gene variant have significantly different monoaminergic concentrations compared to their more resilient counterparts. Nevertheless, a strong interaction between monoaminergic function and neuroplasticity also exist. In fact, monoaminergic antidepressants, such as fluoxetine, activate BDNF TrkB receptors (Burke *et al.*, 2013; Saarelainen *et al.*, 2003) as part of their mechanism of action. However, blockade of these receptors, prevent antidepressant-induced neuroplasticity enhancing effects (Vetencourt *et al.*, 2008), whereas chronic fluoxetine treatment prevents stress-induced downregulation of BDNF in rats (Burke *et al.*, 2013). Furthermore, both 5-HT_{1A} and 5-HT_{1B} knockout mice display decreased hippocampal BDNF protein concentration (Sibille *et al.*, 2007; Wu *et al.*, 2012), supporting the survival and differentiation regulating effects of BDNF concentration on monoaminergic neurons (Angelucci *et al.*, 2005; Goodyer *et al.*, 2010; Martinowich & Lu, 2008) and that BDNF concentrations are positively associated with 5-HT-associated behaviour (Homberg *et al.*, 2014). Alternatively, normal monoaminergic neurotransmission is dependent on normal neuron function, which is regulated by neuroplasticity. To this extent, central inflammation may be elevated in response to a stress-induced increase in kynurenine metabolism, causing a shift towards the production of neurotoxic metabolites. In this regard, quinolinic acid and other neurotoxic metabolites of the kynurenine pathway, namely 3-hydroxykynurenine, 3-hydroxyanthranilic, stimulate NMDA receptors (Guillemin *et al.*, 2001; Takikawa, 2005), generate ROS and RNS, and increase O&NS damage (Maes *et al.*, 2011b), thereby inducing neuronal damage and death, ultimately resulting in decreased monoaminergic neurotransmission. Still, such a kynurenine pathway increase in O&NS, is also accompanied by increased central inflammation, which as depicted in Figure 2-3, has a direct effect on monoaminergic neurotransmission. In this regard, prolonged exposure to pro-inflammatory cytokines, such as IL-1 and TNF α induces a significant decrease in extracellular 5-HT, via increasing SERT (Katafuchi *et al.*, 2006) and 5-HT_{2A} receptor expression, and decreasing 5-HT_{1A} receptor expression (Kulikov *et al.*, 2010) and

serum tryptophan (Logan, 2003). Moreover, centrally IL-1 administration decrease NA concentrations in several limbic regions and increase 5-HT and DA metabolism (Song *et al.*, 2006), a stimulate free radical formation and increase O&NS (Simopoulos, 2011); altogether contributing to the initial decreased monoaminergic neurotransmission.

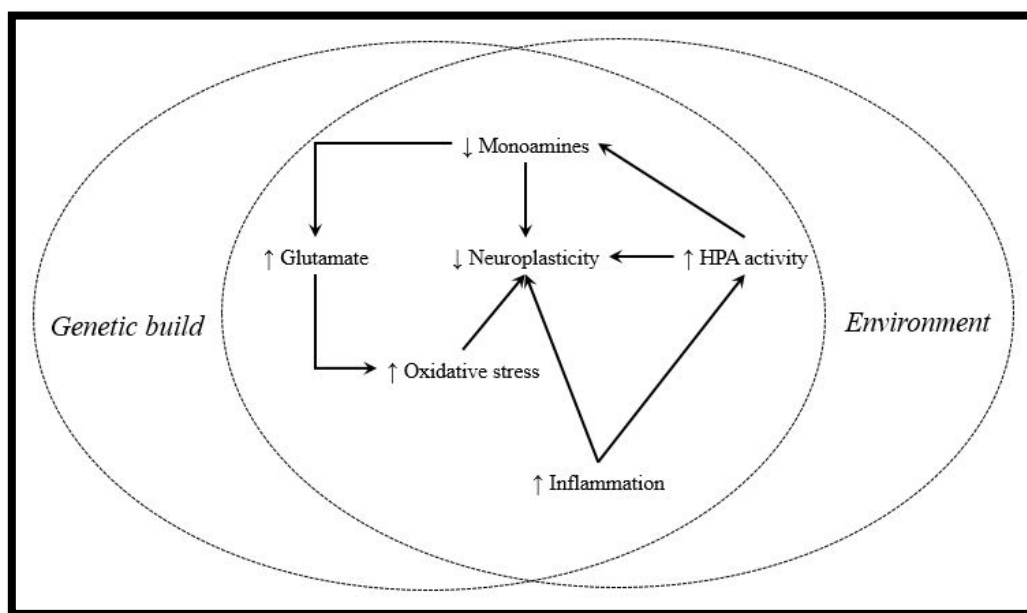


Figure 2-3: Graphical representation of a unified hypothesis of MDD.

Figure 2-3 illustrates the interaction of the different hypotheses of the neurobiological basis of MDD with each other. However, all of the proposed interactions are overall affected by the genetic build of an individual and environmental factors (insults) experienced. All interactions are indicated with a thick arrow, while an upregulation or increase of a specific parameter is indicated with an arrow pointing up in front of the specific parameter. A downregulation or decrease of a specific parameter is indicated with an arrow pointing down in front of the specific parameter. G x E: Gene-environment hypothesis. GABA: γ -aminobutyric acid. HPA: hypothalamic-pituitary-adrenal.

Nevertheless, that a strong interaction between central inflammation and HPA activity exist, further impacts the pathophysiology of MDD. In fact, increased central inflammation causes a surge in glucocorticoid production and due to the mentioned negative feedback system of the HPA axis in depressed patients, prolonged exposure to increased glucocorticoids may induce structural and functional alterations to key brain areas. In this regard, GRs play a central role in neuroplasticity processes, as evidenced by its involvement in the neuroprotective properties of antidepressants, and neurodegenerative properties of cortisol. In this regard, antidepressants and cortisol stimulate different patterns of GR phosphorylation and activation, leading to different GR-stimulated genes. In fact, antidepressants induce neurogenesis via upregulation of specific genes, such as p^{11} , p^{27} and p^{57} (Anacker *et al.*, 2011) which contribute to differentiation and development of new-born neurons (Gil-Perotin *et al.*, 2011) via GABAergic-mediated processes (Ge *et al.*, 2006; Tozuka *et al.*, 2005) and neural stem cell regulation (Furutachi *et al.*, 2013). Whereas, cortisol decreases neurogenesis via activation and stimulation of other GR-mediated genes, such as serum- and glucocorticoid-inducible kinase 1 (Anacker *et al.*, 2013a; Anacker *et al.*, 2013b). Regardless,

5-HTTLPR s carriers with chronic increased HPA activity are associated with decreased memory and hippocampal volume (O'Hara *et al.*, 2007), which are indicative or even the result of, at least, decreased neuroplasticity (Erickson *et al.*, 2011; Lucassen *et al.*, 2010; Mamounas *et al.*, 2000).

Of note, certain memory formation functions are most effective in the presence of high negative emotion, such as fear or sadness, and these emotions are often accompanied by increased glucocorticoid release. Therefore, a hyperactive HPA-axis, is expected to increase, rather than decrease, cognitive function. However, different responses to chronic and acute glucocorticoids exposure, are responsible for the altered cognitive function. Nevertheless, studies suggest a key interaction between glucocorticoids and monoaminergic neurotransmission, specifically NA, in cognitive function. In this regard, an intact noradrenergic system appears to be required for optimal memory formation (O'Hara *et al.*, 2007; Roozendaal *et al.*, 2006a; Roozendaal *et al.*, 2006b). Therefore, the chronic decreased monoaminergic neurotransmission and consequent altered receptor densities of a depressed individual, could indeed inhibit optimal functioning of such cognitive tasks, contributing to the symptomology of MDD and support the involvement of an interaction between these two systems.

Additionally, prolonged exposure to glucocorticoids further inhibits monoaminergic function and neuroplasticity via a stress-induced increase in IDO activity, resulting in increased breakdown of tryptophan and a subsequent increase in neurotoxic metabolites (Maes, 2011; Myint *et al.*, 2007). As mentioned earlier, these neurotoxic effects are in part caused by increased O&NS and NMDAR stimulation. However, it is of note that nitric oxide (NO) synthesis increase in response to glutamate release (Beers, 2006). This interaction may then theoretically increase the risk for O&NS damage and glutamate-induced neurotoxicity (Dawson *et al.*, 1991). Furthermore, NO are also inversely associated with neuroplasticity (Canossa *et al.*, 2002) and hippocampal neurogenesis (Zhu *et al.*, 2006). In fact, O&NS damages ω -3 PUFA membrane content, which in turn can cause alterations in cell membrane structure as well as receptor expression, including that of the monoamine system (Maes *et al.*, 1999a). Subsequently, binding of monoamines to membrane receptors and overall neurotransmission are thus inhibited by increased lipid peroxidation damage (Leonard & Maes, 2012; Maes *et al.*, 2007a; Muakkassah-Kelly *et al.*, 1982), due to neuron damage.

Taken together, although altered monoaminergic neurotransmission forms the cornerstone of current MDD treatment strategies, the effect of neuroplasticity appears to be more centrally involved in the pathophysiology of MDD. Therefore, the suggestion that early-life development may present a 'window of opportunity' when lasting or long-term effects can be induced, become even more relevant. In fact, although neuroplasticity is an ongoing process throughout an individual's lifetime, it is a more dynamic and adaptable process during early-life development. Therefore, the question arises of whether central acting interventions during early-life could indeed induce neurodevelopmental alterations to specifically

monoaminergic and neuroplasticity processes that the effects (whether positive or negative) can present as behavioural alterations later in life. Thus, the following sections will briefly review the different pharmacological treatment options available, including those approved for juvenile MDD and the specific non-pharmacological intervention relevant to the current project, the neurodevelopmental process of the developing brain, as well as the use of antidepressants in juveniles.

2.5 Treatment options in major depressive disorder

As discussed earlier, several hypotheses regarding the aetiology of MDD have been developed during the last century, yet treatment options are still largely based upon altered monoaminergic neurotransmission as suggested by the monoamine hypothesis. However, antidepressants also address some of the issues raised by other hypotheses, to a greater or lesser extent. In fact, considering the uniting hypothesis of MDD discussed earlier, the intended drug targets may only partly contribute to the mood elevating properties, with other downstream effects contributing to the observed treatment outcome. Nevertheless, pharmacotherapy remains the first-line treatment of moderate to severe depression, whereas non-pharmacological treatment options are recommended as initiation therapy for mild depression (World Health Organization, 2017a). These non-pharmacological interventions include psychotherapy, electroconvulsive therapy, sleep deprivation and life-style modifications and are also used as augmentation to pharmacotherapy options. However, as the focus of this thesis is on only two specific non-pharmacological interventions, the following section will include an overview of only these specific non-pharmacological strategies.

Although numerous treatment options, both pharmacological and non-pharmacological, are available, only a third of patients achieve complete remission after a single antidepressant treatment trial (Trivedi *et al.*, 2006b). The success rate, however, increases to 60 % following numerous treatment trials, though the likelihood of remission decreases significantly after two consecutive failed interventions (Rush *et al.*, 2006). Apart from the low response rate of the available treatment options, as well as initial unpleasant side effects, the known delayed onset of action of the pharmacological treatment options have resulted in premature treatment withdrawals. Evidence indicates that monoamine concentrations are increased by as much as 300 % within hours of antidepressant treatment, yet significant therapeutic improvement is only observed weeks later (Delgado, 2004). Therefore, MDD treatment is suggested for at least six weeks before changing treatment, either within the same class of antidepressants or to another class (different mechanism of action), or employing augmentative drug therapies (Culpepper *et al.*, 2015; Philip *et al.*, 2010). Still, abrupt and premature withdrawal of antidepressant treatment is associated with the antidepressant discontinuation syndrome (ADS) which occurs in 20 % of patients who abruptly discontinue their antidepressant treatment after six weeks. ADS is characterized by mild flu-like symptoms, insomnia, nausea and sensory disturbances which cease once treatment is reinstated (Warner *et al.*, 2006). It is

therefore necessary to educate patients on what to expect with different treatment options and that immediate remission should not be expected. However, the side effect profile of the selected treatment option is not solely responsible for treatment cessation, poor response to antidepressants may also lead to weak patient adherence. In this regard, several predictors of poor response have been identified and include genetic alterations (SNPs) of the neurotransmitter systems, hepatic and/or neurotransmitter enzyme alterations, neuroplasticity status, HPA axis regulation and thyroid function (El-Hage *et al.*, 2013).

In conclusion, a vast number of factors should be considered when selecting and prescribing a suitable treatment option, whether pharmacological or non-pharmacological and in this regard, the following sections will briefly overview the pharmacological treatment options. Following this overview, a more detailed discussion regarding treatment options and applications thereof in juvenile patients (with special focus on escitalopram and venlafaxine) are discussed (*section 2.5.2*) as this time period and drugs are the main focus of the current project.

2.5.1 Pharmacotherapy

The main classes of antidepressants generally target the same monoamine deficiencies, whereas the second generation antidepressants (i.e. SSRIs, NRIs, SNRIs) do this with more specificity and improved side effect profile. The latest classes of antidepressants, such as vortioxetine, appear to be less target specific, yet induce significant reduction in depressive symptoms with mixed reports regarding side effect profile (Berhan & Barker, 2014; Fu & Chen, 2015; Mahableshwarkar *et al.*, 2015; Thase *et al.*, 2016), suggesting a movement towards a more holistic approach to MDD treatment as highlighted earlier (*Section 2.4*). Of note, although newer antidepressants are better tolerated, evidence supporting better efficacy of the newer drugs remains limited (Willner *et al.*, 2013). In fact, venlafaxine, sertraline, escitalopram, and mirtazapine remain of the most effective antidepressants (Cipriani *et al.*, 2009a; Montgomery *et al.*, 2007), however these studies were performed prior to the availability of vortioxetine. The pharmacological treatment options are numerous and are summarized in Table 2-2 below. The antidepressants are presented in three main classes, based on the selectivity of their mechanisms of action, i.e. non-selective, selective and atypical.

Table 2-2: Summary of antidepressants according to pharmacological classification.

Table 2-2 summarizes the different classes of antidepressants with key drug examples and a brief description of the mechanism of action and side effect profile of the specific class (Beers, 2006; Blier, 2010; Cheng *et al.*, 2007; DeBattista, 2015; Dwoskin *et al.*, 2006; Ferreri *et al.*, 2001; Gelenberg *et al.*, 2000; Horst & Preskorn, 1998; Jain *et al.*, 2013; Kasper *et al.*, 2000; Sanchez *et al.*, 2015; University of Cape Town, 2016; Wagstaff *et al.*, 2001; Willner *et al.*, 2013).

Classification	Drug examples	Mechanism of action	Side effect profile
Non-selective targets			
Monoamine oxidase inhibitors	Moclobomide, Selegiline, Tranylcypromine	Inhibits catabolic enzyme (MAO), resulting in increased levels of 5-HT, NA and DA	Dietary side effects
Tricyclic antidepressants	Amitriptyline, Desipramine, Imipramine, Nortriptyline,	Amplifies 5-HT and NA by inhibiting reuptake mechanisms	Anticholinergic, cardio toxic, drowsiness
Serotonin-norepinephrine reuptake inhibitors	Duloxetine, Milnacipran, Venlafaxine	Amplifies 5-HT and NA by inhibiting reuptake mechanisms	Visual disturbances, sweating, fatigue, sexual dysfunction
Selective targets			
Selective serotonin reuptake inhibitors	Citalopram, Escitalopram, Fluoxetine, Paroxetine, Sertraline	Amplifies 5-HT by selectively inhibiting 5-HT reuptake	Agitation, emotional blunting, sexual dysfunction
Norepinephrine reuptake inhibitors	Atomoxetine, Reboxetine	Amplifies NA by selectively inhibiting NA reuptake	Increased sweating, dry mouth
Atypical			
5-HT ₂ antagonists	Nefazodone, Trazodone	Antagonizes 5-HT _{2A} and weak inhibitor of SERT (and NAT)	Weight loss, dry mouth
α_2 antagonists	Mianserin	Antagonizes α_2	Drowsiness, bone marrow suppression
α_2 and 5-HT ₂ antagonists	Mirtazapine	Antagonizes α_2 and 5-HT _{2A} , 2C & 3	Sedation, irritability, muscle soreness
5-HT reuptake enhancers	Tianeptine	Increases 5-HT uptake (and increases DA and NA)	GI and CNS disturbances
Transporter inhibitor	Bupropion	Amplifies DA (and NA) by inhibiting transporters	Hallucinations, tachycardia, visual disturbances
Melatonergic agonists	Agomelatine	Stimulates MT ₁ & 2 and antagonizes 5-HT _{2C}	Liver toxicity
Diverse	Vortioxetine	Amplifies 5-HT by inhibiting SERT, antagonizing 5-HT _{1D} , 3, & 7, and stimulating 5-HT _{1A} & B	Headache, nausea, diarrhoea

The choice of antidepressant is largely based on the clinical considerations of the patient and should be evaluated on an individual basis. These considerations include cost and availability of drug, side effect profile and potential drug interactions as well as patient history regarding drug response and patient preference (DeBattista, 2015; World Health Organization, 2017a). Other factors such as gender, medical status and age should also be considered when prescribing antidepressants. In this regard, only fluoxetine and escitalopram are approved by the USA's FDA for the treatment of juvenile depression (Mayo Clinic, 2016a; U.S. Food & Drug Administration, 2009). Furthermore, the SSRIs remain the most commonly prescribed first-line antidepressants, possibly due to their acceptable tolerability profile and cost effectiveness (DeBattista, 2015). As mentioned, the side effect profiles of the different drugs also affect the choice of treatment and although summarized in Table 2-2 along with the different mechanisms of action, it is worth reviewing the key points and highlights of the different pharmacological treatment options as well as elaborate on how their mechanism(s) of action link with the postulated neurobiological bases of depression.

2.5.1.1 Non-selective antidepressants

Firstly, the MOAIs act by inhibiting MAO, the catabolic enzyme responsible for monoamine breakdown, and thereby increasing the monoamine concentration in the synaptic cleft (Katzung, 2015) which is, according to the monoamine hypothesis, the reason for its antidepressant effects. The most significant side effect associated with the MAOIs is the so called 'cheese reaction'. This reaction is the consequence of increased concentrations of tyramine, found in many fermented food including cheese, which cannot be catabolized by MAO due to the inhibiting effects of the MAOIs and consequently lead to significantly increased NA release and a severe hypertensive reaction (Youdim & Bakhle, 2006; Youdim & Weinstock, 2004). Two types of MAO exist, namely A (MAO_A) and B (MAO_B), with the former found primarily in dopaminergic and noradrenergic neurons, and the latter in serotonergic and histaminergic neurons (DeBattista, 2015). Tranylcypromine non-selectively inhibits both MAO_{A&B} and although effective in treating depressive symptoms, could induce the 'cheese reaction' when combined with certain dietary intake, contributing to treatment cessation. In an attempt to successfully treat MDD without the dietary side effects associated with these drugs, the selective MAOIs were developed. In this regard, selegiline was the first MAO_B specific drug which proved to be devoid of the 'cheese reaction' side effect, yet had no antidepressant actions (Youdim & Weinstock, 2004). However, at higher dosages, selegiline loses its specificity and inhibits both MAO_{A&B} and is therefore currently used as an antiparkinsonian drug (DeBattista, 2015; University of Cape Town, 2016). Moclobomide selectively inhibits MAO_A and successfully improves depressive symptoms, but as MAO_A is more abundant than MAO_B, drugs selectively inhibiting MAO_A is consistently associated with the reported 'cheese reaction' (Youdim & Bakhle, 2006) and might explain why MAOIs are not as popular as initial development might have thought.

Secondly, as mentioned earlier, the TCAs were one of the first classes of antidepressants to be developed. These drugs primarily inhibit the reuptake of 5-HT and NA from the synaptic cleft, thereby increasing the concentration of these neurotransmitters. However, TCAs differ from one another with regard to their selectivity for NAT or SERT, with clomipramine and imipramine having a higher affinity for SERT, whereas desipramine, the metabolite of imipramine, and nortriptyline having a higher affinity for NAT (DeBattista, 2015). The TCAs, however, are also considered multipotent drugs and therefore do not only affect these specific neurotransmitter systems, but bind to other receptors, such as the histaminergic cholinergic and adrenergic receptors as well. Due to their multipotent characteristics, the TCAs are associated with anticholinergic side effects, such as dry mouth and constipation, sedation due to antagonism of the histamine H₁ receptors, severe orthostatic hypotension because of α -receptor blockade and cardiac arrhythmias. Yet, despite the supposedly unfavoured side effect profile of the TCAs, it remains a successful treatment option (Arroll *et al.*, 2005).

Finally, the SNRIs are similar in their mechanism of action to the TCAs, however, these drugs do not display the same potent side effect profile, due to increased target specificity, and are therefore often favoured over the TCAs. Although the SNRIs were developed to inhibit both the 5-HT and NA reuptake mechanisms, this class of antidepressants show greater affinity towards the SERT, especially venlafaxine, and has limited effects on DA (DeBattista, 2015; Gutierrez *et al.*, 2003). However, when venlafaxine is administered at higher doses (> 150 mg), a shift in its affinity for SERT is observed, to a more balanced inhibition of both SERT and NAT (Gutierrez *et al.*, 2003). Regardless, increased serotonergic and noradrenergic concentrations of the SNRIs result in adverse effects similar to those observed by the SSRIs (*discussed in section 2.5.1.2*) and noradrenergic effects, such as hypertension, insomnia and/or agitation, all associated with increased NA concentrations. The SNRIs remain in general the first choice in the treatment of depression, especially in treatment resistant depression, and venlafaxine also showing better clinical outcome when compared to the more popular SSRIs (Nemeroff *et al.*, 2008; Thase *et al.*, 2001).

2.5.1.2 Selective antidepressants

The two classes of antidepressants belonging to the selective target group include the SSRIs and the NRIs. These classes were developed to more specifically target the monoamine deficiencies hypothesized to underlie MDD as well as reduce side effects associated with the initial antidepressant classes. In this regard the SSRIs selectively inhibit SERT in order to increase 5-HT synaptic concentrations, whereas the NRIs selectively inhibit NAT; ultimately increasing NA concentrations (DeBattista, 2015). Both these classes of antidepressants increase their respective neurotransmitters without affecting cholinergic and histaminergic receptors and are therefore considered superior with regards to side effect profiles. As mentioned, the SSRIs remain the most popular antidepressant and first-line treatment choice, possibly due to their acceptable tolerability profile and absence of severe side effects compared to the TCAs (Celada *et*

al., 2004; DeBattista, 2015). More specifically, escitalopram and sertraline have been suggested to be the most superior antidepressants when considering efficacy and tolerability in a recent meta-analysis study (Cipriani *et al.*, 2009a). However, the SSRIs are not void of side effects and it is reported that as much as 40 % of patients treated with SSRIs experience sexual dysfunction (DeBattista, 2015), one of the main contributors to failed treatment adherence. Furthermore, as 5-HT is not just increased in the brain, but throughout the body, increased serotonergic activity in the gut may cause nausea, diarrhoea and other gastrointestinal symptoms (DeBattista, 2015). Finally, SSRIs are associated with significant weight gain which may also affect treatment compliance and lead to treatment discontinuation.

The efficacy of the NRIs, specifically reboxetine has been reported to be as effective as (Holm & Spencer, 1999; Papakostas *et al.*, 2008) or even superior to SSRIs (Andreoli *et al.*, 2002). In addition, drugs belonging to this class have been reported be free of sexual dysfunction, commonly associated with the SSRIs (Clayton *et al.*, 2003). Nevertheless, systemic reviews and meta-analysis suggest reboxetine to be the drug least tolerated and should therefore be avoided as first-line therapy (Cipriani *et al.*, 2009a; Eyding *et al.*, 2010), due to inferior efficacy and safety compared to placebo or SSRIs (Eyding *et al.*, 2010). Regardless, such target-selective antidepressants remain clinically relevant and a popular treatment option.

2.5.1.3 Atypical antidepressants

The atypical antidepressants refer to drugs with unrelated chemical structures and mechanisms of action (drug targets) that differ from those discussed above. The development of the drugs in this class resulted from the need for drugs with a different spectrum of superior efficacy, with enhanced tolerability as well as an earlier onset of action (Kent, 2000). Although these drugs may in some instances be useful in patients that do not respond to other drugs, mostly due to inter-individual differences, they all affect monoaminergic neurotransmission in some way or another and have not been demonstrated to be superior in general.

Firstly, the receptor antagonists include, amongst others, nefazodone and trazodone. These antidepressants are primarily 5-HT_{2A} antagonists with varying transporter inhibiting effects on both the 5-HT and NA systems (DeBattista, 2015; Fontaine *et al.*, 1994), resulting in increased release of the specific neurotransmitters. While antagonism of the 5-HT_{2A} receptors lead to antidepressant, anti-anxiety and antipsychotic effects, stimulation thereof is thought to be responsible for the hallucinogenic and anxiogenic effects associated with the recreational drug, lysergic acid diethylamide (LSD) (DeBattista, 2015). Interestingly, antagonism of the 5-HT_{2A} receptor is also associated with increased cognitive function (Meltzer, 1999), which may add value to the clinical outcome in treating depressed patients. A second group of receptor antagonists is the α_2 receptor antagonists, such as mianserin. A tetracyclic antidepressant with noradrenergic enhancing properties. Mianserin increases noradrenergic neurotransmission by antagonizing the pre-synaptic α_2 receptors, responsible for the inhibition of NA release (Ferreri *et al.*, 2001;

Katzung, 2015). Finally, mirtazapine is an example of an antidepressant with both noradrenergic and serotonergic receptor antagonizing effects. However, unlike nefazodone and trazodone, mirtazapine primarily antagonizes the 5-HT_{2C} receptor, while also causing downregulation of the 5-HT_{2A} receptors (University of Cape Town, 2016). Stimulation of the 5-HT_{2C} receptor indirectly inhibits frontal cortex DA and NA release via activation of the inhibitory GABAergic neurons (Gobert *et al.*, 2000; Millan *et al.*, 1998; Pessia *et al.*, 1994), therefore, by antagonizing the 5-HT_{2C} receptor would lead to an increase in these two neurotransmitters without significantly affecting 5-HT concentrations (Millan *et al.*, 2003). Furthermore, as discussed earlier, antagonism (or the downregulation) of the 5-HT_{2A}, will lead to increased monoamine neurotransmitter concentrations and when combined with the α_2 receptor antagonism-induced increase of noradrenergic concentrations, the antidepressant effects of mirtazapine may be augmented.

Secondly, and probably most interesting, is the 5-HT reuptake enhancer, tianeptine. In contrast to the popular reuptake inhibitors discussed earlier, as well as the monoamine deficiency highlighted by the monoamine hypothesis, tianeptine decreases, rather than increases, neurotransmitter concentrations in the synaptic cleft (Brink *et al.*, 2006; Burghardt *et al.*, 2004; Wagstaff *et al.*, 2001). Yet, the clinical outcome with tianeptine remains successful in comparison with both the TCAs and SSRIs as well as with regards to side effect profile (Ridout & Hindmarch, 1999; Von Frenckell *et al.*, 1990; Wagstaff *et al.*, 2001; Waintraub *et al.*, 2002). The antidepressant effects of tianeptine has been attributed, in part, to the reduction in HPA response, increased NA and DA levels in several brain regions as well as hippocampal neuroprotective properties (Brink *et al.*, 2006; Koe *et al.*, 2002; McEwen & Olie, 2005; Plaisant *et al.*, 2003; Wagstaff *et al.*, 2001).

The third type of diverse antidepressant is bupropion, an antidepressant with a complicated and not fully understood mechanism of action (Horst & Preskorn, 1998). However, the reuptake inhibiting properties of primarily DA, but also NA, but not 5-HT (DeBattista, 2015; Horst & Preskorn, 1998) remain significant and partially explains its effectiveness as antidepressant. Furthermore, bupropion has been shown to also increase the vesicular monoamine transporter-2 action, the transporter responsible for increasing DA, NA and 5-HT vesicle concentrations (Foley *et al.*, 2006) and ultimately increase these neurotransmitter concentrations. Although effective as antidepressant, bupropion is also used in the treatment of nicotine addiction (University of Cape Town, 2016).

Fourthly, the circadian rhythm affecting antidepressant, agomelatine acts as an agonist on the melatonin 1 and 2 (MT_{1&2}) receptors, whilst antagonizing 5-HT_{2C} receptors (Guardiola-Lemaitre *et al.*, 2014; Millan *et al.*, 2003). The stimulation of the MT_{1&2} receptors normalize the dysfunctional circadian rhythm, while antagonizing the 5-HT_{2C} receptors lead to an increase in monoamine neurotransmitter concentrations (Guardiola-Lemaitre *et al.*, 2014). Apart from the mentioned novel mechanism of action, a recent review

paper also associates agomelatine with increased neurogenesis, decreases glutamate release and downregulates pro-inflammatory cytokines (Guardiola-Lemaitre *et al.*, 2014).

The fifth drug classified as a diverse antidepressant is vortioxetine which has a multimodal mechanism of action on the serotonergic system with both 5-HT receptor modulation and SERT inhibiting properties. Specifically, vortioxetine has antagonistic properties on the 5-HT_{1D}, 5-HT₃ and 5-HT₇ receptors, while simultaneously acting as a full and partial agonist on the 5-HT_{1A} and 5-HT_{1B} receptors, respectively and inhibiting 5-HT reuptake (Bang-Andersen *et al.*, 2011; DeBattista, 2015). Vortioxetine was initially developed to combine the SERT-inhibiting as well as the 5-HT_{1A}-stimulating properties of approved antidepressants, resulting in enhanced antidepressant effects (Blier *et al.*, 1997; Blier & Ward, 2003). Interestingly, presynaptic 5-HT_{1D} receptors are responsible for regulating 5-HT release in humans, however this function is regulated by 5-HT_{1B} in rodents (Homberg *et al.*, 2014). Furthermore, antagonizing the 5-HT₃ receptor might further prevent the nausea observed during SSRI and SNRI treatments (Bang-Andersen *et al.*, 2011), while 5-HT₇ receptor antagonism results in a further increase of extracellular 5-HT levels (Bonaventure *et al.*, 2012). Overall, vortioxetine has proved to be successful in treating patients not responding to the more popular SSRIs or SNRIs, while also inducing significant cognitive enhancement without the sexual adverse effects, associated with other antidepressant classes (Sanchez *et al.*, 2015).

2.5.2 The use of antidepressants in juvenile patients

It was not until five decades ago that MDD in paediatrics was accepted and realized to be a significant problem (Baker, 2006), however, no approved pharmacological treatment options were available until the early 2000s (Delate *et al.*, 2004). Since the acceptance of juvenile MDD, pharmacological treatment options for adults have expanded along with our understanding of the possible causes and underlying discrepancies of MDD, yet current FDA-approved treatment for juvenile depression is limited to only two SSRIs *viz.* fluoxetine (patients eight years and older) and escitalopram (patients twelve years and older) (Mayo Clinic, 2016a; U.S. Food & Drug Administration, 2009). The older TCAs are ineffective in treating children and only marginally effective in adolescents (Hazell & Mirzaie, 2013), while others report TCA therapy to cause sudden death in paediatric patients (Varley, 2001). The relationship between the mentioned sudden death and TCA usage in paediatric patients remain unclear, but cardiotoxicity is considered a significant contributor (Bell & Efron, 2015). To this extent, baseline and routine ECG monitoring is recommended in juveniles if indeed treated with TCAs (Varley, 2001).

Remarkably, other classes of antidepressants are prescribed to juvenile patients according to 'off-label' uses (Mayo Clinic, 2016a; Zito *et al.*, 1998) and may partly explain the significant increase in antidepressant prescription and use in juvenile patients during the late twentieth century (Delate *et al.*, 2004; Olfson *et al.*, 2002; Zito *et al.*, 2003; Zito *et al.*, 2002). More recently, antidepressant-use in the USA have been reported

to be three-fold higher than that of Western European countries (Zito *et al.*, 2006). Nonetheless, antidepressant-use during early-life development is associated with increased suicidal thinking and behaviours, especially during the first few months of treatment and shortly after dosage alterations (Mayo Clinic, 2016a) and should thus be continuously monitored for any significant behaviour alterations. Importantly, sudden discontinuation of antidepressant treatment can also lead to ADS in juvenile patients, which further complicates the treatment approach for these specific patients. Therefore, a black box warning regarding increased suicidality risk associated with these drugs are now required by the FDA (Libby *et al.*, 2007; U.S. Food & Drug Administration, 2004). Oppositely, untreated juvenile depression has an inherent and possibly greater risk by itself to induce suicidal behaviour (Friedman & Leon, 2007; Richmond & Rosen, 2005). Incidentally, a notable argument is made by Friedman and Leon (Friedman & Leon, 2007) that the current black box warning may in fact confuse the medical practitioner as to whether antidepressants should be prescribed, while simultaneously discourage juvenile patients and/or families from seeking pharmacological treatment. Furthermore, and possibly most intriguing, is the association of increased suicidal behaviour and antidepressant use, compared to the effects of untreated depression. In this regard, Friedman and Leon propose that suicidal ideation is the core symptom of MDD and it is therefore almost impossible to determine whether suicidal symptoms are due to medication or the underlying illness. To this extent, it is known that antidepressant initiation in certain patients induces an energy surge or even agitation before elevating the mood and could therefore prompt the individual to act on his/her pre-existing suicidal impulses. The authors thus ask whether a warning such as “*untreated depression and psychiatric illness carry a significant risk for suicidal behaviour*” would not be more appropriate, compared to the current one. Nonetheless, the current black box warning could indeed affect the treatment strategy of the individual and/or medical practitioner and it is therefore of utmost importance to accurately investigate the long-term outcome of juvenile patients treated with antidepressants as well as explore novel treatment and/or augmentation strategies.

As mentioned, pharmacological treatment options for juvenile patients are very limited with the TCAs not being effective in the treatment of juvenile, specifically pre-adolescent depression and have therefore led to researchers investigating why juveniles respond differently to antidepressants than their adult counterparts. Firstly, it is important to note that all antidepressants were developed for adult patients and as children are not little adults, pharmacokinetics and -dynamics cannot be expected to be similar in the two patient groups. One of the first noticeable differences between the two patient groups are the maturity level brain development. For example, the earlier maturation of the serotonergic system, compared to the adrenergic and dopaminergic systems (see *section 2.6*). Secondly, the pharmacokinetic profiles of juveniles differ to that of adults (McLeod & Evans, 1992) with hepatic enzymes, being low at birth after which it increases before declining again later in life. In fact, hepatic enzymes have been shown to increase exponentially over the first few days in an animal study (Atterberry *et al.*, 1997), supporting increased drug

metabolism during developing years. Therefore, paediatric dosages should be adapted in order to achieve similar drug levels as in adults (Kozisek *et al.*, 2007); with juveniles requiring lower dosages in order to achieve similar adult dosages required for behavioural or receptor density alterations (Bylund & Reed, 2007).

Currently, only fluoxetine and escitalopram are approved for MDD treatment in children and adolescents with other antidepressants, including other SSRIs, shown to be ineffective or inconclusive with regards to efficacy (Hetrick *et al.*, 2012). In this regard, escitalopram is considered the SSRI with the greatest selectivity for SERT (Chen *et al.*, 2005; Owens *et al.*, 2001) with no or very low affinity for other enzymes or receptors (Cipriani *et al.*, 2009b), which had originally been expected to yield greater clinical efficacy and fewer side effects (Owens *et al.*, 2001). Escitalopram is the pure *S*-enantiomer of the racemic citalopram, which has previously been shown to be responsible for the SERT-inhibiting effects associated with citalopram, whereas the *R*-enantiomer has no significant inhibiting effects (Hyttel *et al.*, 1992). In comparison with fluoxetine, the only other antidepressant approved for juvenile use, escitalopram is reported to have similar response, success and tolerability rates, however, escitalopram seems to induce the greater reduction in depressive symptoms (Cipriani *et al.*, 2009b). Because escitalopram, like other antidepressants, require chronic treatment before clinical improvement is seen or experienced, it has been suggested that monoamine concentration increase might only partly explain the improvement in symptoms and that other downstream effects may be responsible for therapeutic outcome. In this regard, escitalopram has been reported to increase neurogenesis in pubertal animals (Jayatissa *et al.*, 2006; Jayatissa *et al.*, 2008; Kozisek *et al.*, 2008); possibly leading to cognitive improvements (Jorge *et al.*, 2010). Furthermore, escitalopram is reported to decrease both pro-inflammatory (Bah *et al.*, 2011) and oxidative stress (Lee *et al.*, 2011; Stefanescu & Ciobica, 2012) markers, and decrease synaptic glutamate release (Musazzi *et al.*, 2011) as well decrease social anxiety disorder symptoms in children and adolescents (Isolan *et al.*, 2007). However, results suggesting escitalopram treatment in children to be unsuccessful are also available (Wagner, 2005; Wagner *et al.*, 2006), yet preclinical data suggests escitalopram to be effective in the treatment of pre-pubertal animals (Kozisek *et al.*, 2008; Reed *et al.*, 2009; Reed *et al.*, 2008). Taken together, escitalopram remains one of only two FDA-approved drugs for the treatment of juvenile depression and because of the greater specificity for SERT, compared to other SSRIs, the improved clinical outcome is expected.

Interestingly, although not FDA-approved, venlafaxine remains a popular ‘off-label’ antidepressant in treating juvenile depression (Lee *et al.*, 2012; Volkers *et al.*, 2007; Zito *et al.*, 2008) with strong anxiolytic properties in juvenile patients (March *et al.*, 2007; Rynn *et al.*, 2007). Venlafaxine is a potent inhibitor of both SERT and NAT and a weak inhibitor of DAT (Cipriani *et al.*, 2007; Horst & Preskorn, 1998), without significantly affecting adrenergic, cholinergic, histaminergic and even 5-HT₂ receptors (Bolden-Watson & Richelson, 1993; Cusack *et al.*, 1994; DeBattista, 2015). However, venlafaxine is also able to increase

neuroplasticity (Aydemir *et al.*, 2005; Matrisciano *et al.*, 2009), decrease oxidative stress (Eren *et al.*, 2007) and pro-inflammatory cytokines (Piletz *et al.*, 2009) and inhibit depolarization-dependent glutamate release (Musazzi *et al.*, 2011) in addition to its monoamine reuptake inhibiting properties. The advantage of venlafaxine treatment is that at lower doses, venlafaxine mimics the pharmacodynamic profile of the commonly used SSRIs (DeBattista, 2015), however, by increasing the dose, a shift towards a more balanced inhibiting effect of both SERT and NAT are achieved (Gutierrez *et al.*, 2003) and possibly increasing the likelihood of response. This dual mechanism of action would lead to individuals being treated with an SSRI-mimicking drug, yet when not responding, a dose increase option that could lead to improved outcomes. Therefore, a single mechanism of action antidepressant, such as the SSRIs, may prove problematic in non-responders (Horst & Preskorn, 1998). Regardless, venlafaxine has been reported to have a more rapid onset of action (Horst & Preskorn, 1998) as well as being more effective in treating severe depression (Bauer *et al.*, 2013) and TRD (Corya *et al.*, 2006; Thase *et al.*, 2000), compared to other antidepressants which may further explain its popularity in treating MDD. In fact, in 2005, venlafaxine was one of the best sold antidepressants (Cipriani *et al.*, 2007). Also, according to a Cochrane review, venlafaxine is suggested to be comparable to escitalopram with regards to efficacy and tolerability (Cipriani *et al.*, 2009b).

Overall, antidepressant-use under juvenile patients is significant, yet although improvement in depressive symptoms are observed, the lasting effects that these drugs may have later in life remain unknown. To this extent, brain development has been described as a ‘use it or lose it’ system, where the brain becomes wired to match the requirements of or compensate for its environment (Andersen, 2003). Therefore, chronic drug exposure may represent an important environmental factor, which according to the G x E hypothesis, may have an important influence in the development of certain brain structures and/or functions that could yield changes in the mature individual exposing either subtle or obvious differences in the response to environmental factors later in life (Murrin *et al.*, 2007). Actually, early-life behavioural interventions can have positive effects in establishing and improving psychological and behavioural functioning (Dawson, 2008; Sullivan, 2003), whereas childhood adversities, such as maltreatment, can have a negative impact on mental health (Andersen & Teicher, 2008, 2009) later in life. However, failure to appropriately treat such childhood disorders may increase the probability of psychological problems later in life (Beesdo *et al.*, 2007; Edwards *et al.*, 2003; Felitti *et al.*, 1998). In contrast, the long-term effects that early-life experiences can induce, together with the use of antidepressants during these developing years, could lead to significant bio-behavioural alterations, either positive or negative later on. Therefore, early-life development could therefore represent a very specific window of opportunity where appropriate intervention strategies can induce significant long-term bio-behavioural effects.

Indeed, according to the ‘equal but opposite’ hypothesis, long-term effects of centrally acting drugs, such as antidepressants in juvenile patients differ remarkably to that of adults (Andersen, 2005; Andersen &

Navalta, 2004, 2011) (*Figure 2-4*). In this regard, chronic administration of centrally acting drugs, such as antidepressants, induce specific structural and/or functional changes in certain brain areas. However, the mature brain (i.e. adults) only temporarily adapts to these induced changes and return to its original state within a few weeks following drug withdrawal (Andersen & Navalta, 2011). In contrast, the developing brain (i.e. juvenile) does not temporarily accommodate these drug-induced changes. In fact, the drug is reported to integrate into the brain and alter developmental trajectories following withdrawal (Andersen & Navalta, 2011). More specific, if the drug was present in the brain during programming of a specific phenotype (the set of observable characteristics of an individual resulting from the interaction of its genotype with the environment), the drug (or the effect thereof, such as increased monoamine neurotransmitter concentrations) could be considered as part of the natural environment and might later develop into a deficit state. This is especially relevant, considering the natural overproduction and pruning processes of brain development, discussed later. A report that support this hypothesis include antipsychotics that reduce DA activity acutely in juveniles, but increases DA concentrations later in life (Moran-Gates *et al.*, 2006). Similarly, early-life fluoxetine seems to decrease depressive-like symptoms in pre-pubertal rats (Bylund & Reed, 2007; Schoeman *et al.*, 2017), whereas both depressive- and anxiety-like behaviour is increased later in life (Oh *et al.*, 2009). In both instances, the immediate effects of the central acting drugs appear to be positive and comparable to that observed in adult patients, yet the long-term effects of these drugs differ remarkably from that expected in adult patients (*Figure 2-4*). Finally, the ‘equal but opposite’ hypothesis appears to expand further than monoamine or neurotransmitter effects. In fact, different responses to SERT inhibition during early-life and adulthood has also been reported; stating that inhibition of SERT during early-life development results in increased extracellular 5-HT concentrations, accompanied by downregulated BDNF gene expression. However, SERT inhibition during adulthood also results in increased extracellular 5-HT concentrations, yet along with increased, rather than decreased, BDNF gene expression (Homberg *et al.*, 2014).

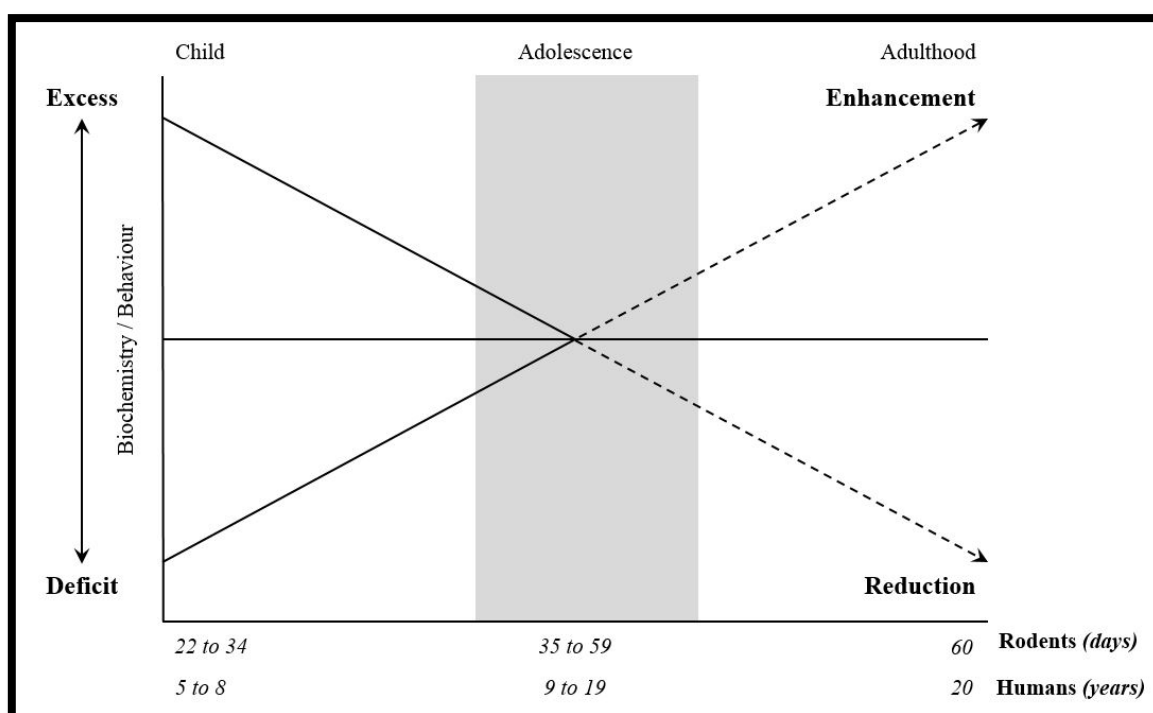


Figure 2-4: Illustrative representation of the 'equal but opposite' hypothesis (adapted from (Andersen, 2005; Andersen & Navalta, 2004, 2011)).

Figure 2-4 illustrates the 'equal but opposite' hypothesis which suggests different trajectories of response can be observed following central acting drugs, such as antidepressants, depending on the baseline level of a behaviour and/or biochemical marker. If such a specific bio-behavioural marker is pathologically elevated during childhood development, additional stimulating exposure could reduce its expression by adulthood. On the contrary, baseline deficit bio-behavioural markers are predicted to benefit from early levels of blockade to challenge the 'natural' baseline values and overcome the deficit later on. The arrows demonstrate the need for continued maturation before the full benefit of early pharmacotherapy is realized.

Taken together, although treatment of MDD in general is not straight forward, a significant percentile of the adult population appears to improve with antidepressant-use. However, treatment of childhood MDD may not be as easy as is in their adult counterparts, especially considering the possible opposing long-term effects. Excitingly, the developing brain may not only be susceptible to the negative effects caused by early-life treatment, but may represent certain 'windows of opportunities' where alterations to the developing central nervous system may induce positive effects later in life. In this regard, pre-adolescence (or pre-puberty in rodents) may represent such a window where alterations via pharmacological and/or non-pharmacological interventions may produce long-term improvements in individuals genetically susceptible to developing MDD.

2.5.3 Non-pharmacological interventions

2.5.3.1 Omega-3 essential fatty acids

Omega-3 essential fatty acids (ω -3 EFAs) are long-chain polyunsaturated fatty acids (PUFAs) found in various plant sources, such as flaxseed, walnut and canola oils, as well as marine life, such as fish and algae

(World Health Organization, 2011). ω -3 Oils are essential to the human diet (i.e. humans depend on dietary supplementation, therefore referred to as EFAs), because of the inability of the human body to synthesize it endogenously (World Health Organization, 2011; Youdim *et al.*, 2000). Eicosapentaenoic acid (EPA) (20:5n-3) and docosahexaenoic acid (DHA) (22:6n-3) are the highly biologically active building blocks of PUFAs, especially to support the structure and function of membrane proteins, in which receptors, enzymes and cellular transport molecules are embedded (Youdim *et al.*, 2000). The ω -3 EFAs obtained from plant sources are usually in the form of the parent ω -3 EFA, α -linolenic acid (ALA, 18:3n-3), which can be endogenously converted by humans to the active forms of EPA and DHA, however, at a relatively ineffective rate of between only 10 % and 15 % (Eaton & Konner, 1985). Interestingly, this conversion rate is reported to be higher in women, compared to men, as well as during pregnancy, compared to non-pregnant women (Williams & Burdge, 2006), yet dietary ALA intake does not appear to increase DHA blood lipids in either pregnant women or their new-born infants (De Groot *et al.*, 2004).

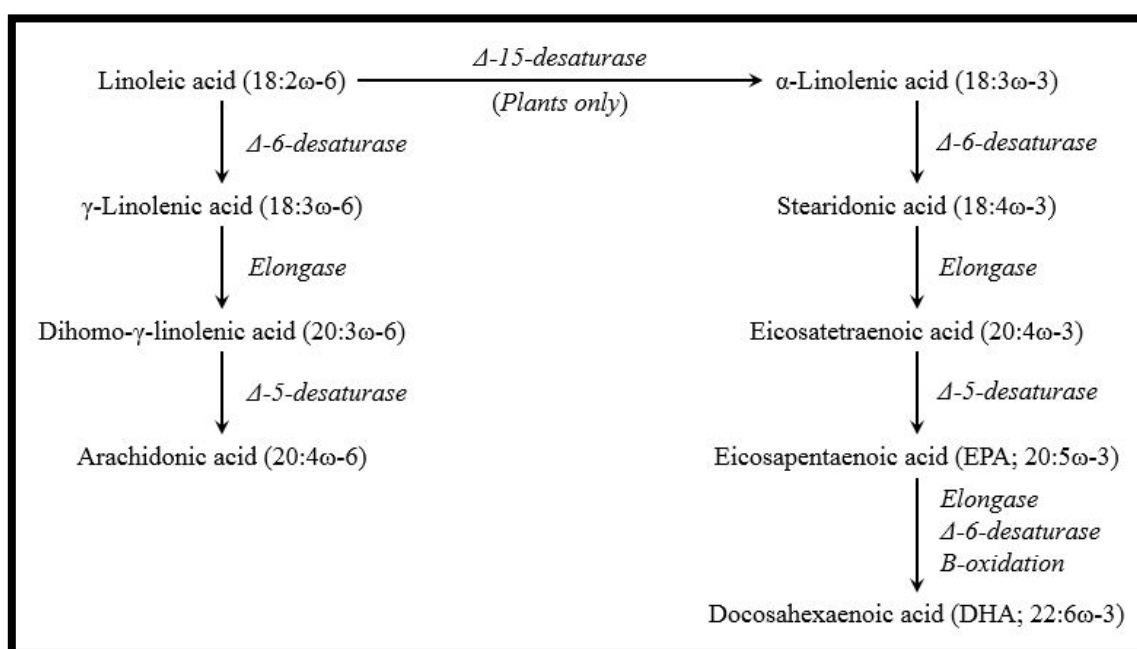


Figure 2-5: Omega-3 fatty acid metabolism

As depicted in Figure 2-5, ALA is synthesized from the dietary gained ω -6 FA, linoleic acid (LA, 18:2), by Δ -15-desaturase. Mammals do not possess the Δ -15-desaturase enzyme and can therefore not synthesize their own ω -3 EFAs. Regardless, mammals can metabolize dietary supplemented ALA to more unsaturated derivatives such as EPA and DHA via desaturation and elongation. Important to note is that Δ -5-desaturase is involved in the conversion of ALA to EPA as well as for LA to arachidonic acid (AA), and is therefore in constant competition with the respective pathways (Calder, 2012). The rate limiting step in this metabolic process is the desaturation of ALA and LA by Δ -6-desaturase of which the former pathway is the preferred substrate of the enzyme. Yet, LA is much more present in most mammalian diets and therefore metabolism generally shifts towards the production of AA, rather than EPA and DHA. In fact, it is

suggested that LA is consumed in amounts up to 20-times that of ALA in common a Western diet (Calder, 2012). Moreover, it is estimated that the average Western and urban sub-continental diet consists of at least a 15:1 ω -6: ω -3 ratio makeup, compared to the recommended ratio of 1-4:1 (Mayo Clinic, 2013; Simopoulos, 2002, 2011). Contrary, rural Indian and Japanese diets are estimated to have a 4:1 ω -6: ω -3 ratio, whereas the Palaeolithic (and probably the Mediterranean) diet contains more ω -3 than ω -6 EFAs (Simopoulos, 2002).

Overall, countries where marine dominated diets are common, have significantly lower reports of depressed patients, compared to countries with alternative diets (Cott & Hibbeln, 2001; Magnusson *et al.*, 2000). In this regard, these lower ratio intake diets are considered a pivotal contributing factor to the antidepressant effects of ω -3 oils. In fact, the role of ω -3 EFAs in the treatment or prevention of MDD is based on studies and meta-analyses where low fish intake was associated with a greater likelihood of developing depressive symptoms, compared to individuals who followed a high fish intake diet (Grosso *et al.*, 2016; Lai *et al.*, 2014; Li *et al.*, 2016; Smith *et al.*, 2014). Furthermore, depressed patients exhibit significantly lowered ω -3 EFA concentrations (Assies *et al.*, 2010; Maes *et al.*, 1999a; McNamara *et al.*, 2007), compared to healthy controls. More recent studies confirmed the antidepressant effects of ω -3 EFAs (Lespérance *et al.*, 2010; Lin & Su, 2007; Rondanelli *et al.*, 2010) with varying support for EPA and DHA as responsible agent (Dyall, 2015). Nevertheless, DHA is the most abundant ω -3 FA in the mammalian brain, strongly regulated by age and diet (Innis, 2005; Innis, 2007), and associated with a significant role in the development of MDD. However, the exact mechanism of action of ω -3 EFAs responsible for the antidepressant-like effects remain unknown but increased neuroplasticity, normalization of monoaminergic and glutamatergic concentrations, and decreased oxidative stress and inflammation have previously been described as possible mechanisms, which are discussed in the sections below. Moreover, that the exact contribution of EPA and DHA to the antidepressant properties also remain uncertain. Regardless, EPA appears to have the greater effect on depressive behaviour as evidenced by various studies (Martins, 2009; Mischoulon *et al.*, 2009; Peet & Horrobin, 2002; Su *et al.*, 2014). Yet, as EPA is metabolized to DHA, it may not fully explain the observed antidepressant effect and mechanism. In this regard, studies supporting the synergistic effect of EPA and DHA on MDD are also available. In fact, studies suggest a high EPA dose combined with a lower DHA dose to relieve depressive symptoms (Nemets *et al.*, 2006; Su *et al.*, 2003), yet a higher DHA:EPA treatment strategy to not induce comparable effects (Meyer *et al.*, 2013; Rogers *et al.*, 2008).

2.5.3.1.1 Effect on monoaminergic neurotransmission

Various studies have reported antidepressant effects following chronic ω -3 supplementation. In fact, a single study reported daily 1000 mg EPA supplementation for eight weeks, to be equally effective in reducing Hamilton Depression Rating Scale (HDRS) scores in adults, compared to daily 20 mg fluoxetine administration (Jazayeri *et al.*, 2008). The combination of EPA and monoaminergic antidepressants,

however, appear to be significantly more effective in decreasing HDRS scores, compared to mono-treatments (Gertsik *et al.*, 2012; Jazayeri *et al.*, 2008). Moreover, ω -3 supplementation potentiated the antidepressant-like effects of sub-therapeutic doses of fluoxetine and mirtazapine in adult Wistar rats (Laino *et al.*, 2010), further supporting its value as MDD treatment option. To this extent, ω -3 deficiency has been associated with lowered 5-HT, NA and DA neurotransmission (Chalon, 2006; Kudas *et al.*, 2004), whereas ω -3 supplementation normalizes brain monoaminergic levels (Vancassel *et al.*, 2008) through enhancement of proteins responsible for synaptic vesicle release (Pongrac *et al.*, 2007). Furthermore, early-life ω -3 EFA deficiency results in decreased release and vesicle concentrations of 5-HT and DA neurotransmitters later in life, whereas ω -3 EFA supplementation within the first two weeks of life restored concentrations comparable to subjects brought up on an adequate diet throughout their life (Chalon, 2006; Kudas *et al.*, 2004). Also, animals fed an ω -3 deficient diet display a significantly decrease in vesicular DA release, following tyramine stimulation, suggesting decreased DA concentrations (Zimmer *et al.*, 2000). This ω -3 deficiency induced hypodopaminergia, specifically in the cortical regions of the brain, can cause striatal hyperdopaminergia, resulting in learning deficits and hyperactive locomotion, often associated with ω -3 deficiency (Lavialle *et al.*, 2010; Mathieu *et al.*, 2008; Vancassel *et al.*, 2007). Interestingly, ω -3 EFA deficiency during various developmental stages (i.e. perinatal, pre- and post-pubertal) induce significant decreases in cortical DHA composition during adulthood. However, in ovariectomized female rats, only ω -3 EFA deficiency during perinatal development led to reduced cortical serotonergic concentration, whereas cortical serotonergic concentration was increased, without affecting metabolism, in pre-pubertal deficient rats and unchanged in post-pubertal deficient rats (McNamara *et al.*, 2009). These results suggest that the monoaminergic effects of ω -3 deficiency are independent of ovarian hormones, yet sensitive at different maturation stages with different long-term effects.

2.5.3.1.2 *Effect on cholinergic neurotransmission*

Dietary ω -3 EFA concentrations furthermore affects cholinergic neurotransmission. In fact, a diet deficient of ω -3 EFAs significantly increases hippocampal ACh release, compared to controls (Äid *et al.*, 2003). That KCl administration produced a significant reduction in hippocampal ACh release in ω -3 deficient rats, suggests that the reported ACh increase could be a spontaneous phenomenon, resulting in depleted vesicular stores and ACh outflow (Äid *et al.*, 2003).

2.5.3.1.3 *Effect on neuroplasticity*

Furthermore, that chronic decreased monoaminergic neurotransmission is associated with decreased neuroplasticity (Sibille *et al.*, 2007; Wu *et al.*, 2012) is of note since ω -3 supplementation has been reported to increase neuroplasticity (Ikemoto *et al.*, 2000; Sánchez-Villegas *et al.*, 2011; Wu *et al.*, 2004), synaptic signal transduction (Chalon, 2006; McNamara & Carlson, 2006) and differentiation of neural stem cells

(Katakura *et al.*, 2013). To this extent the mammalian brain is predominantly comprised of different mono- and polyunsaturated FAs, such as DHA (McNamara & Carlson, 2006) with the majority (up to 22 % of total FAs) found in the hippocampus and frontal cortex (McNamara & Carlson, 2006). Therefore, alterations in these FA concentrations could have significant effects on brain development and function, especially during early-life development. In fact, the majority of total brain FA accumulation takes place shortly before birth (third trimester) (Clandinin *et al.*, 1980; Martínez & Mougan, 1998) and plateaus around the time of birth. However, cortical DHA composition is approximately 9 % of total FAs at the time of birth, whereafter it continues to increase throughout early development (Green *et al.*, 1999; Green & Yavin, 1996). Furthermore, optimal neuro- and synaptogenesis are dependent on lipid composition (Martin & Bazan, 1992), however only DHA, and not EPA, is reported to improve neurite growth in rats across all developmental stages (i.e. pre-pubertal, young adulthood and aged) (Robson *et al.*, 2010). In addition, gestational and early-life ω -3 deficiency in rats, induced significant reductions in frontal cortex DHA composition across two generations (McNamara & Carlson, 2006), increased anxiety-like behaviour and decreased neuroplasticity markers (Bhatia *et al.*, 2011) in adult animals. Also, dietary ω -3 PUFA intake is strongly correlated with peripheral BDNF concentrations in adolescents (Ferreira *et al.*, 2014) and cognitive function (Drapeau *et al.*, 2003), further confirming the involvement of ω -3 EFAs in neuroplasticity throughout life. Children and adolescents born preterm, are reported to display a higher incidence of cognitive and motor impairments, compared to age-related full-term controls (Bhutta *et al.*, 2002; Cooke & Foulder-Hughes, 2003; Foulder-Hughes & Cooke, 2003). Finally, ω -3 deficiency during embryonic development inhibits normal neuronal development (Bertrand *et al.*, 2006), whereas ω -3 EFA supplementation, specifically DHA, promotes neurogenesis (Beltz & Sandeman, 2003) and inhibits cell death (Kawakita *et al.*, 2006). Postnatal ω -3 deficiency also has significant effects on neurocognitive functions. To this extent, infants fed DHA-deficient formula milk exhibit several signs of neurological and –cognitive dysfunctions, compared to infants fed DHA supplemented formula or breast milk (Helland *et al.*, 2003; Khedr *et al.*, 2004). Importantly, ω -3 breastmilk content is influenced by factors such as maternal dietary patterns. Overall, deficient dietary ω -3 intake in juveniles is considered a modifiable risk factors for mental illness (Sinn *et al.*, 2010), particularly due to its neuroplasticity enhancing effects.

2.5.3.1.4 Effect on glutamatergic neurotransmission

With regards to the cognitive enhancing effects of ω -3 EFAs, ω -3 PUFAs are documented to influence glutamatergic neurotransmission and plasticity (Esposito *et al.*, 2013); key factors in the memory and learning process. Firstly, glutamate is associated with learning and memory processes due to its significant involvement in Alzheimer's disease. In this regard, hippocampal glutamate reuptake is impaired in demented patients, whereas glutamatergic release is augmented by beta-amyloids (Arias *et al.*, 1995; Parpura-Gill *et al.*, 1997) and central oxidative stress damage (Lauderback *et al.*, 2001). Increased beta-

amyloid production further increases oxidative stress and neuron damage (Sultana *et al.*, 2009), contributing to impaired cognitive functions.

Secondly, the plasticity of the glutamatergic synapse is characterized by two key processes, long-term potentiation (LTP) and long-term depression (LTD) (Denis *et al.*, 2013). LTP is the glutamatergic synaptic response to a specific input to enhance memory formation with increased glutamate neurotransmission, whereas LTD is the persistent decrease in glutamatergic neurotransmission, inducing memory clearance (Denis *et al.*, 2013; Yau *et al.*, 2016). Chronic ω -3 deficiency leads to impaired LTP (Cao *et al.*, 2009), which can be reversed with sufficient ω -3 PUFA supplementation (Kawashima *et al.*, 2010). To this extent, ω -3 PUFAs are thought to be responsible for improved cognitive function, partly via its neuroplasticity enhancing (Kelly *et al.*, 2011) and glutamatergic stabilizing (Dyall *et al.*, 2007) properties in the hippocampus. Glutamate homeostasis is regulated by astrocytes (Danbolt, 2001; Maragakis & Rothstein, 2004), which contain a high membrane concentration of DHA (Champeil-Potokar *et al.*, 2004). In the presence of increased synaptic glutamate, microglial cells are activated to release pro-inflammatory cytokines, stimulating astrocytes to increase glutamate uptake and release neurotrophic factors (Denis *et al.*, 2013; Grntal *et al.*, 2009). Therefore, that chronic ω -3 deficiency leads to decreased LTP could be explained by sub-optimum functioning of the astrocytes, resulting in a disruption in glutamatergic homeostasis, increased extracellular glutamate concentrations, stimulation of the arachidonic cascade, increased excitotoxicity and ultimately neuron damage. This is supported by reports indicating that chronic ω -3 PUFA deficiency reduces astrocyte function, compared to animals fed a sufficient diet (Latour *et al.*, 2013), as well as by clinical trials reporting cognitive improvement following chronic, high doses of ω -3 PUFA supplementation (Lee *et al.*, 2013; Sinn *et al.*, 2012).

2.5.3.1.5 Effect on HPA activity

Furthermore, that genetic modifications influence monoaminergic concentrations and function has been reported by various studies. In this regard, certain genetic variants, such as 5-HTTLPR s and the BDNF *val*⁶⁶*met* alleles, significantly increases the risk for developing MDD (Caspi *et al.*, 2003; Uher, 2008; Uher & McGuffin, 2008; Uher & McGuffin, 2010), particularly following a traumatic or stressful event. However, individuals with such susceptible genetic build has also been reported to present with increased HPA axis activity, possibly due to an ineffective negative feedback system (Jabbi *et al.*, 2007), as observed in depressed patients (McEwen, 2005). Increased HPA activity, and the consequent continuous exposure to increased glucocorticoids, may inhibit monoamine transporter binding (Figlewicz, 1999; Willner *et al.*, 2013) and extracellular concentrations, inhibit neuroplasticity (McEwen, 2005; Wong & Herbert, 2004) and impair working memory (Lupien *et al.*, 1999; Roozendaal *et al.*, 2004). Therefore, it is of note that high ω -3 EFA intake is associated with low HPA axis disturbances (García-Prieto *et al.*, 2007) as well as preventing stress-induced glucocorticoid spikes (Delarue *et al.*, 2003). Contrary, ω -3 deficiency increases

glucocorticoid receptor concentrations in various brain regions, compared to an adequate diet (Bhatia *et al.*, 2011), thereby enhancing the mentioned glucocorticoid-associated effects. Furthermore, chronic EPA supplementation significantly decreases plasma cortisol (and corticosterone) levels (Jazayeri *et al.*, 2010; Song *et al.*, 2007), whilst also showing antidepressant activity comparable to that of fluoxetine (Jazayeri *et al.*, 2008). Importantly, DHA is a metabolite of EPA (*Figure 2-5*) and could therefore be responsible for the observed effects. In fact, most studies do not differentiate between the effects of EPA and DHA (Gorjão *et al.*, 2009) and therefore a synergistic, rather than two separate effects have recently been proposed with regards to antidepressant effects (Song *et al.*, 2016).

2.5.3.1.6 Effect on central inflammation

Further contributing to increased HPA activity is increased central inflammation (Biesmans *et al.*, 2013; Henry *et al.*, 2008). To this extent, increased central inflammation induce glucocorticoid secretion and increased oxidative (and nitrosative) stress damage (Dantzer *et al.*, 2011; Herbert *et al.*, 2006). This increase in oxidative (and nitrosative) stress is of great importance, especially considering the high lipid composition (specifically DHA) of the brain. The chemical structure of DHA makes it highly sensitive to oxidation and the harmful effects thereof, such as neuronal damage. For instance, chronic ω -3 deficiency during early-life development causes an increase in pro-inflammatory cytokine levels later in life. Furthermore, the increase in inflammatory markers further increase serotonergic metabolism in the hippocampus and frontal cortex, suggesting an inverse relationship between inflammatory markers and serotonergic concentrations (McNamara *et al.*, 2010a). In support, others have found significantly decreased EPA and/or DHA blood levels (Assies *et al.*, 2010; Maes *et al.*, 1999a; McNamara *et al.*, 2010b), increased pro-inflammatory cytokine production (Dowlati *et al.*, 2010; Kling *et al.*, 2007; Miller *et al.*, 2009) and increased central 5-HT metabolism (Barton *et al.*, 2008) in depressed patients, compared to controls. Yet, these inflammatory and monoaminergic changes could be normalized by sufficient ω -3 supplementation (McNamara *et al.*, 2010a). In this regard, both EPA and DHA display central anti-inflammatory properties. Firstly, cortical cyclooxygenase-2 (COX-2) is increased in rat brain following chronic ω -3 deprivation (Rao *et al.*, 2007), yet inhibited by EPA supplementation (Nieves & Moreno, 2006). EPA also serves as a precursor for eicosanoids which antagonize the effects of the AA-derived eicosanoids (Brock & Peters-Golden, 2007; Harizi *et al.*, 2008). Furthermore, EPA competes with AA for phospholipase A₂ (PLA₂), reducing pro-inflammatory AA-derived eicosanoids production and produces E-series resolvins, via COX-1 and 5-lipoxygenase (5-LOX). Resolvin E₂ modulates inflammation by regulating neutrophil migration and increasing production of anti-inflammatory cytokine, IL-10 (Song *et al.*, 2016). Furthermore, ω -3 EFA deficiency not only reduces ω -3 EFA brain content, but also increases ω -6 and AA brain concentrations (Bhatia *et al.*, 2011) and upregulates the enzymatic conversion of AA to prostaglandin E₂ (PGE₂) (Rao *et al.*, 2007), resulting in increased pro-inflammatory cytokine synthesis (Wang *et al.*, 2010). Secondly, DHA induces anti-inflammatory properties via competing with AA for the

production of cytokines. To this extent a balanced ratio (1-2:1) of AA:DHA reduces the release of pro-inflammatory cytokines, whereas it is increased in unbalanced ratios (Cotogni *et al.*, 2011). In fact, a positive correlation between erythrocyte as well as plasma AA: EPA + DHA ratio and vulnerability to develop depression has been identified (Adams *et al.*, 1996; Lotrich *et al.*, 2013). Finally, DHA also produces anti-inflammatory resolvins (D-series; resolvins produced from DHA) (Song *et al.*, 2016), further contributing to the anti-inflammatory mechanism of action. Other possible mechanisms include reduced expression of adhesion molecules on the surfaces of leucocytes (Miles *et al.*, 2000; Yamada *et al.*, 2008), and inhibited migration of neutrophils to inflammatory sites and pro-inflammatory cytokine production (Serhan *et al.*, 2008; Serhan & Savill, 2005). Interestingly, genetic variants may also contribute to the incidence of inflammation-induced depression. In this regard, Su and colleagues reported individuals with a sensitive COX-2 variant to present with decreased erythrocyte DHA concentrations and consequently an increased risk for IFN α -induced depression (Su *et al.*, 2010). Nevertheless, central inflammation causes oxidative (and nitrosative) stress damage, which has also been associated with ω -3 EFA intake. A diet high in ω -3 EFAs suppresses the production of ROS, induced by bacterial endotoxin lipopolysaccharides (LPSs) in brain microglia (Corsi *et al.*, 2015), preventing neural damage (Liu *et al.*, 2014). In addition EPA and DHA display neuroprotective effects via the inhibition NO production (Corsi *et al.*, 2015; Lluís *et al.*, 2013) and activation of Nrf2 (nuclear factor-erythroid 2-related factor 2), which stimulates various antioxidant and detoxification enzymes (Li *et al.*, 2008; Nguyen *et al.*, 2009).

Taken together, available data suggest a definite role for ω -3 EFAs treatment strategy in MDD. Several aspects of neurobiology associated with MDD are in fact beneficially affected, including neurodevelopment. However, the exact mechanism of action of ω -3 EFAs remain unknown.

2.5.3.2 Exercise

Sedentary lifestyles are associated with increased risks of several life crippling conditions, such as obesity, cardiovascular diseases, type 2 diabetes, osteoporosis, cancer and MDD (Johnson *et al.*, 2002; Kremer *et al.*, 2014; Page *et al.*, 2010). Yet, data suggest that the risk for these conditions are significantly lowered by increased physical activity (Da Silva *et al.*, 2012; Dishman *et al.*, 2006; Song *et al.*, 2012). The beneficial effects of exercise have been accredited to increased hippocampal vascularization (Heo *et al.*, 2014) and neuroplasticity (van Praag, 2009; van Praag *et al.*, 1999a; van Praag *et al.*, 1999b; Vivar *et al.*, 2012), improved cognitive function (Dishman *et al.*, 2006) and even decreased β -amyloid plaques, a marker of Alzheimer's disease (Adlard *et al.*, 2005). Exercise has also been reported to induce comparable antidepressant effects to that of placebo and pharmacotherapy drugs (Cooney *et al.*, 2014; Cooney *et al.*, 2013) and is probably the most affordable treatment strategy that can be incorporated into a MDD treatment regimen. In fact, the WHO prescribes non-pharmacological interventions as initiation therapy in mildly depressed patients, whereas pharmacotherapy should only be initiated in moderate to severe cases (World

Health Organization, 2017a). Furthermore, exercise has numerous, well-studied beneficial effects in the cardiovascular system, yet its exact mechanism of action in central conditions, such as MDD are less known. Enhanced monoaminergic neurotransmission and neuroplasticity, and decreased oxidative stress damage and glucocorticoid stress response have all been associated with increased physical activity and could therefore have a significant and possible lasting impact on the developing individual.

Babyak and colleagues (Babyak *et al.*, 2000) performed a ten month follow-up study where depressed patients were treated with either aerobic exercise, sertraline (an SSRI) or the combination thereof for four months. Treatment outcome across all three groups were significant and comparable following treatment, yet patients in the exercise alone group reported significantly lower relapse rates, compared to the other two treatment groups six months after the treatment period. Furthermore, continuous exercising after the intervention period was further associated with a reduced probability to diagnose MDD. Similarly, Blumenthal and colleagues (Blumenthal *et al.*, 2007) reported improved treatment outcome in MDD patients including exercise in the treatment regimen, yet reported no significant difference when exercise was performed in a group, compared to solitary exercise, in line with a previous report (Glenister, 1996). Results further suggested that treatment outcome (exercise or antidepressant) is similar in different depression severities. This is of particular note, since social interaction has been accredited to at least in part be responsible for the antidepressant effects of exercise (Ransford, 1981) and that non-pharmacological interventions should only be initiated in less severe cases. Other studies that also suggest exercise to augment antidepressant treatment are those conducted by Russo-Neustadt and colleagues (Russo-Neustadt *et al.*, 1999; Russo-Neustadt *et al.*, 2001; Russo-Neustadt *et al.*, 2004).

2.5.3.2.1 *Effect on monoaminergic neurotransmission*

With regards to the mechanism of these antidepressant effects of exercise, clinical studies have shown that the NA, 5-HT and DA systems are all positively affected by exercise (Lin & Kuo, 2013). Specifically, tryptophan and 5-HT concentrations are both increased by exercise and remain increased one hour after moderate treadmill exercise in aged individuals (Melancon *et al.*, 2012). Wheel running also decrease depressive-like behaviour via downregulating hippocampal 5-HT_{1A} receptors, in line with chronic SSRI treatment (Blier & Ward, 2003; Raap *et al.*, 1999), as well as upregulates 5-HT_{2A} receptors; both receptors associated with cognitive function (Chen *et al.*, 2008; Chennaoui *et al.*, 2000; Renoir *et al.*, 2012). Similarly, exercise also increases tyrosine hydroxylase, the enzyme responsible for metabolizing tyrosine to dihydroxyphenylalanine (DOPA), while reducing D₂ autoreceptor mRNA (Foley & Fleshner, 2008; Kim *et al.*, 2011). These observations suggest that exercise can increase DA synthesis and decrease D₂ autoreceptor-mediated inhibition of DA (Foley & Fleshner, 2008). In fact, the commonly known phenomenon, “runner’s high” has been explained by the exercise-induced dopaminergic enhancement via increased endocannabinoid-1 (eCB₁) release into the mesolimbic system, relieving the inhibitory effect of

CB₁-expressing GABAergic terminals on the dopaminergic neurons (Lupica & Riegel, 2005; Piomelli, 2003). However, as discussed earlier, mood enhancement is only observed following chronic and not acute antidepressant treatment, even though monoaminergic concentrations are significantly increased shortly after drug administration. Similarly, additional mechanisms have been proposed to be involved in the mood elevating properties of exercise, such as increased endorphin production (Ernst *et al.*, 2006) and increased mitochondrial energy generation (Boushel *et al.*, 2014; de Sousa *et al.*, 2014). Regardless, the antidepressant effects of exercise appear to be intensity-dependent, as three weeks of moderate exercise increased 5-HT_{2A} receptor number as well as 5-HT-transporter (5-HTT) levels, whereas four weeks of strenuous exercise do not affect 5-HTT and reduce, rather than increase, 5-HT_{2A} receptor number (Weicker & Strüder, 2001). Similarly, vigorous treadmill exercise for thirty minutes had no effect on D₂ receptor concentrations (Wang *et al.*, 2000). Interestingly, the serotonergic increase, induced by strenuous exercise can contribute to the central fatigue following such exercise (Lin & Kuo, 2013) and therefore contribute to the general observation that strenuous exercise may not be as beneficial as moderate and low, chronic exercise exposure (Schoeman *et al.*, 2017). Remarkably, administration of a 5-HT agonist and antagonist prior to an exhaustion test, increased and decreased animal's time to reach exhaustion, respectively (Bailey *et al.*, 1993), proposing a putative link between basal 5-HT levels and the subject's ability, and ultimately response, to an exercise intervention. Finally, chronic treadmill and wheel running also increased NA hippocampal concentrations (Dunn *et al.*, 1996; Sarbadhikari & Saha, 2006). This is of note since cognitive function is decreased in depressed patients and that by antagonizing β -adrenoreceptors with specific antagonists prior to an exercise intervention, the associated exercise-induced enhancement of memory can be prevented (Ebrahimi *et al.*, 2010; Van Hooymissen *et al.*, 2004). Overall, exercise alters neurotransmitter concentrations and transmission to such an extent that it is significantly increased, compared to concentrations found in sedentary controls (Dishman *et al.*, 1997).

2.5.3.2.2 Effect on neuroplasticity

Various groups have investigated the neuroplasticity enhancing effects of exercise (Bjørnebekk *et al.*, 2005; Ji *et al.*, 2014; Kitamura *et al.*, 2003; Marlatt *et al.*, 2013; Rhodes *et al.*, 2003; Stranahan *et al.*, 2006; van Praag *et al.*, 1999a; van Praag *et al.*, 1999b; Vivar *et al.*, 2012; Widenfalk *et al.*, 1999). Neeper and colleagues, however, were the first group to report on the neurotrophic enhancing effects of exercise (Neeper *et al.*, 1995; Vivar *et al.*, 2012) and it is now generally accepted that exercise induces antidepressant effects via enhanced neurogenesis, neuroplasticity and dendritic remodelling. Indeed, in rodents hippocampal BDNF gene and protein expression is elevated by free access to running wheels and remain elevated up to two weeks after removal of running wheels (Berchtold *et al.*, 2005). These effects appear to be age dependant (Kim *et al.*, 2004). Titterness and colleagues found voluntary exercise to enhance LTP in pre-pubertal mice (PND22), without altering BDNF concentrations (Titterness *et al.*, 2011). However, in our laboratory, we observed only a strong trend for low intensity exercise to decrease depressive-like

behaviour in pre-pubertal FSL male rats (Schoeman *et al.*, 2017). Other studies have also demonstrated significant neurotrophic enhancing effects in the hippocampus following exercise intervention (Cooper *et al.*, 2017; Vivar *et al.*, 2016), supporting the cognitive enhancement effects induced by running. In fact, one study reported the neurogenic enhancing effects of voluntary exercise to be significantly greater than that of fluoxetine or duloxetine (Marlatt *et al.*, 2010), whereas another reported exercise to augment the neuroplasticity enhancing effects of DHA (Chytrova *et al.*, 2010). Moreover, Erickson and colleagues found that physical activity is positively associated with gray matter volume and improved cognitive function later in life. Yet the beneficial effects appear to be dependent on quantity (intensity) of exercise, as exceeding a certain point of exercise quantity (intensity) had no additional benefit (Erickson *et al.*, 2010; Kim *et al.*, 2003). Moreover, high intensity exercise does not induce the same beneficial effects as observed with low or moderate intensity exercise. To this extent, lower exercise intensities induce beneficial neuroplasticity enhancing effects, whereas strenuous exercise either had no effect or negatively affected neuroplasticity (Inoue *et al.*, 2015; Lou *et al.*, 2008; Shih *et al.*, 2013; Sun *et al.*, 2017). Other factors that influence the neurogenesis enhancing effects of exercise include type of exercise, i.e. forced or voluntary (Leasure & Jones, 2008), and genetic background (Clark *et al.*, 2011; Merritt & Rhodes, 2015). Nevertheless, that exercise also enhance angiogenesis could further explain the mechanism of increased neurogenesis. To this extent, a recent study reported VEGF to be increased by exercise and stimulate hippocampal neurogenesis via increased blood flow (Rich *et al.*, 2017). Moreover, increased running-induced cerebral angiogenesis, appear to be dependent on a specific lactate receptor (i.e. hydroxycarboxylic acid receptor 1 (HCAR₁)) (Morland *et al.*, 2017). Overall, exercise decreased depressive symptoms partly via enhancing neuro- and synaptogenesis and improving neuroplasticity, however, these beneficial effects appears to be dependent on a normal functioning monoaminergic system (Ivy *et al.*, 2003).

2.5.3.2.3 Effect on inflammation and oxidative stress

The involvement of exercise on inflammation and oxidative stress damage has been extensively investigated. That exercise both induce and inhibit inflammation is also well documented and appear to be dependent on the duration and intensity of the exercise regimen (Teixeira de Lemos *et al.*, 2011). The anti-inflammatory and antioxidant effects of exercise are of specific interest for possible treatment or augmentation strategies in neurodegenerative diseases, such as MDD. To this extent, low and moderate, but not high, exercise intensities prove especially effective in ameliorating the inflammatory and oxidative stress deficiencies associated with MDD (Moylan *et al.*, 2013). Initially, acute or high intensity bouts of exercise produce a surge in pro-inflammatory cytokines, such as IL-6, and ROS and RNS which could overwhelm the endogenous defence systems, leading to neuronal damage (Moylan *et al.*, 2013). However, chronic low to moderate intensity exercise regimens also enhance IL-6 production, yet to a lesser extent compared to acute bouts of exercise (Ploeger *et al.*, 2009). To this extent, low-grade inflammation (marked by IL-6 concentrations) stimulates anti-inflammatory cytokine and reduces pro-inflammatory cytokine

production (Moynlan *et al.*, 2013; Starkie *et al.*, 2003), and increases oxidative and nitrosative stress defences (Moynlan *et al.*, 2013). Of note, IL-6 is the first classified myokine (a cytokine produced and released from contracting skeletal muscle fibres) (Pedersen *et al.*, 2003; Pedersen & Fischer, 2007). Interestingly, the initial increase of inflammation observed following an acute bout of exercise lasts longer in patients with inflammatory diseases, compared to healthy controls (Ploeger *et al.*, 2009). This is of note, since MDD is associated with increased inflammation and may therefore alter the expected response expected from exercise.

Taken together, exercise beneficially affects various biological markers associated with MDD. Therefore, the implementation of exercise as augmentation strategy is relevant and worth investigating. However, whether and to which extent (beneficial or detrimental) early-life exercise may impact the developing brain later in life remains unknown. And although the neuroplasticity enhancing effects of exercise appear to be transient in adults, the long-term effects on depressive and anxiety behaviour, particularly in juveniles, may be different and warrant further investigation. Therefore, the following section will review juvenile brain development as introduction for the potential long-term effects possibly induced by pharmacotherapy, non-pharmacological interventions or the combination thereof.

2.6 Juvenile brain development

As pointed out earlier, juvenile and adult patients respond differently to antidepressant therapy, possibly due to different maturation rates of brain development. One of the most significant observations suggesting different brain developmental rates is the ineffectiveness of the noradrenergic TCAs in children and adolescents. Yet, drugs that target the serotonergic system, such as the SSRIs, have been shown to be effective and are, in fact, the only approved treatment options for this specific population group. As the majority of brain developmental information has been generated from animal studies, specifically rats, it is important to keep in mind that brain development, relative to time of birth, differs greatly between species (Dobbing & Sands, 1979; Murrin *et al.*, 2007). For example, brain weight of rats at birth is similar to that of humans at the end of the second trimester (Murrin *et al.*, 2007), while brain development of Rhesus monkeys at birth is similar to that of human teenagers (Dobbing & Sands, 1979). Regardless, the basic developmental process as well as the impact of alterations in normal development, remain similar across mammalian species (Murrin *et al.*, 2007). It is further important to understand that puberty and adolescence are not synonymous, yet both these terms are used throughout the thesis with the former used in reference to animals and the latter referring to humans, unless stated otherwise. In the true definitions of these terms, puberty refers to the sexual maturation of the individual and can somewhat be pinpointed to a specific event, such as gonadarche (Graber & Brooks-Gunn, 1998). On the other hand, adolescence refers to the developmental period of an individual transiting from childhood into adulthood (Pickles *et al.*, 1998) and therefore extends over numerous years, including the onset of puberty.

This section will review the available literature on the developmental process of brain circuitry with special focus on the maturation rates of the serotonergic and noradrenergic systems. The different developmental processes and rates of these specific neurotransmitter systems have been put forward as partial explanation why the more noradrenergic-affecting antidepressants, such as the TCAs are ineffective in the treatment of juvenile depression, whilst antidepressants targeting the serotonergic system, such as the SSRIs, are effective and approved in these patients (Murrin *et al.*, 2007). Furthermore, the mammalian brain is predominantly composed of different mono- and polyunsaturated fatty acids and, therefore, the development of fatty acids will also be touched upon. Importantly, DHA comprise as much as 20 % of total fatty acid brain composition (McNamara & Carlson, 2006) and has the most rapid growth rate of all the fatty acids (Green & Yavin, 1996). Therefore, DHA maturation will form the main focus of the fatty acid development review. However, EPA also plays an important role as messenger in CNS cells due to its faster metabolism (Mercier *et al.*, 2012) and is therefore, similar to DHA, critical for neural development (Song *et al.*, 2016).

Again, as the majority of information of brain development has been generated from animal studies, specifically rats, this section will focus on the developing process in these animals and only refers to clinical data where deemed necessary. Importantly, to place the developmental process of the rat brain into context with that of the human brain, it should be noted that prenatal rodent development occurs over a 21-day gestational period, whereas human development occurs over a period of forty weeks, divided into three trimesters. Incidentally, the prenatal developmental period of the rat resembles the first two trimesters of the human developing process, with the end of the third trimester's brain development being compared to that of the first three weeks of a rat pup's life (Kepser & Homberg, 2015). The onset of puberty and start of adolescence in humans is generally accepted to be between twelve and eighteen years of age (Spear, 2007), however, genetics, environment and nutrition may have significant effects on the onset thereof. In fact, emerging signs of adolescence may present as early as eight years in females (Kenney *et al.*, 2011; Spear, 2000; Spear, 2007) and last until 25 years of age (Parker, 1991). This developmental phase is similar to the PND35 old rat, whereas adulthood is considered from PND60 onwards (Malkesman & Weller, 2009; Panksepp, 1998) (Figure 2-6).

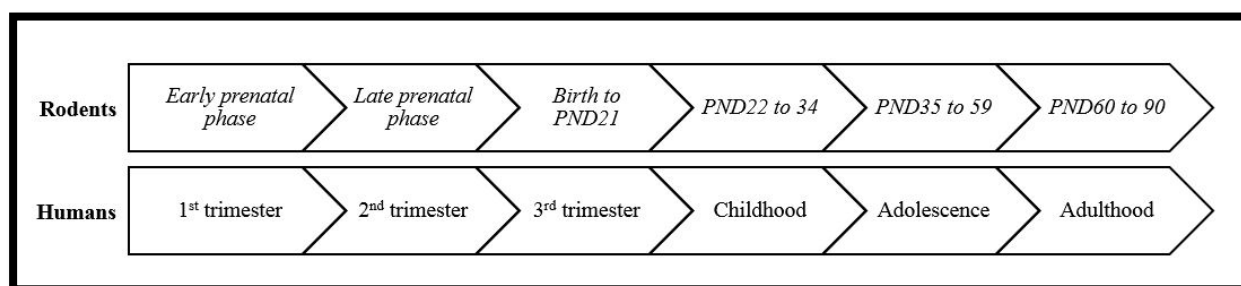


Figure 2-6: Summarized comparison of rodent and human developmental phases (adapted from (Brenhouse & Andersen, 2011; Kepser & Homberg, 2015; Malkesman & Weller, 2009; Panksepp, 1998)).

In rats, neurotransmitter-containing neurons are already detectable in the embryo. In fact, serotonergic neurons are already detected in the 8 mm embryo, with dopaminergic and noradrenergic neurons detected later in the 9 mm and 11 mm embryo, respectively (Golden, 1982). In similar fashion to the overall maturation process of these neurotransmitters, the serotonergic system is detectable before that of the noradrenergic system. Specifically, by gestational day 10 (G10), the noradrenergic neurons start to differentiate (Lauder & Bloom, 1974; Thomas *et al.*, 1995) by which time SERT mRNA is already detectable (Hansson *et al.*, 1998). However, even before G10, peak levels of the 5-HT synthesis enzyme, TPH, is already detected (Rho & Storey, 2001), whilst TH, the rate-limiting enzyme responsible for NA synthesis, is only detectable at G15 (Chugani *et al.*, 1999; Kato *et al.*, 1982; Murrin *et al.*, 2007). The majority of brain neuro- and synaptogenesis for most brain regions take place between G14 and G17 (Green *et al.*, 1999), however, these processes may also take place at an earlier (Brady *et al.*, 1989; Marchand & Lajoie, 1986) or later stage (Brückner *et al.*, 1976) in other regions. Therefore, that total fatty acid concentrations peak around this time (Green *et al.*, 1999) supports the importance of structural lipids for normal neurogenesis and brain development. On a side note, cholinergic neurogenesis is also detected between G12 and G17 (Brady *et al.*, 1989). After G17, the accumulation rate of all fatty acids plateaus up to birth, except that of DHA (Green *et al.*, 1999; Green & Yavin, 1996). To this extent, synapses are particularly DHA enriched (Wurtman *et al.*, 2009) and the surge in DHA accumulation before birth could therefore indicate preparation for ongoing synaptogenesis of a mature system (Martin & Bazan, 1992). As differentiation of the neurons are now already underway, projections of the serotonergic and noradrenergic neurons reach the cortex by G17 and G18, respectively (Lauder & Bloom, 1974; Markus & Petit, 1987; Wallace & Lauder, 1983) with the serotonergic neurons already representing adult distributions two days before birth i.e. G19 (Aitken & Törk, 1988; Wallace & Lauder, 1983).

At the time of birth, serotonergic receptor density in the rat brain is at its highest, surpassing that observed during adulthood (Daval *et al.*, 1987; Murrin *et al.*, 2007) and might even already be fully functional (Whitaker-Azmitia *et al.*, 1987), accompanied by a functional serotonergic reuptake system (Enjalbert *et al.*, 1978; Nelson *et al.*, 1980; Tissari, 1975). In contrast, noradrenergic receptors are only expressed from postnatal day 1 (PND1) onwards (Winzer-Serhan & Leslie, 1997; Winzer-Serhan *et al.*, 1996b) and generally not during prenatal development (Murrin *et al.*, 2007). However, α_{2A} mRNA and low levels of α_1 adrenergic receptors have been reported to be detectable just before or at the time of birth (Morris *et al.*, 1980; Winzer-Serhan *et al.*, 1996a). Interestingly, NMDA receptors are also already functional at the time of birth (Insel *et al.*, 1990). Furthermore, DHA represents approximately 9 % of human total fatty acid cortical composition at the time of birth (McNamara & Carlson, 2006).

Several developing processes take place after birth, including a DHA concentration increase. In fact, DHA accumulates at a rapid rate of approximately 14.5 mg/week during the third trimester in humans (Clandinin *et al.*, 1980; Martinez, 1992) (i.e. between birth and PND21 in rats; see *Figure 2-6*) and continues to

accumulate to such an extent that approximately 15 % of human total cortical fatty acids comprise of DHA by the age of twenty years (McNamara & Carlson, 2006). However, regarding monoaminergic maturation, a similar general pattern to prenatal development is seen. In this regard, the serotonergic reuptake system is already functioning, whereas low levels of noradrenergic reuptake transporters are only detected at PND5 and peak by PND20 (Murrin *et al.*, 2007) when adult levels of α_1 receptors have also been detected (Morris *et al.*, 1980). Seven days after birth there is a significant growth of serotonergic dendrites (Lidov & Molliver, 1982; Loizou & Salt, 1970), to such an extent that administration of 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino)-tetralin), a 5-HT_{1A} agonist, already induces serotonin syndrome in 7-day old Sprague-Dawley pups (Darmani & Ahmad, 1999). By PND14 a significant increase in striatal TH concentrations are observed, indicating towards further development in both the noradrenergic and dopaminergic systems (Murrin *et al.*, 2007) and is supported by a significant increase in noradrenergic neuron firing rates (Nakamura *et al.*, 1987). Furthermore, 5-HT₂ receptor densities already exceed that of the adult rat two weeks after birth and start to decline towards adult levels (Roth *et al.*, 1991). In fact, these receptor densities are already so significant that administration of a 5-HT_{2A/C} agonist at such a young age induces ‘wet dog shakes’ in pre-pubertal rats (Darmani & Ahmad, 1999), confirming the functionality of these receptors. Noradrenergic receptor densities significantly increase and also reach adult levels by PND15 (Happe *et al.*, 2004; Harden *et al.*, 1977; Morris *et al.*, 1980; Pittman *et al.*, 1980; Slotkin *et al.*, 1990) at which time the overall synaptogenesis of the noradrenergic and serotonergic systems are 55 % and 75 %, respectively, completed (Lauder & Bloom, 1975). Interestingly, by PND18 the noradrenergic system in the cerebellum and brainstem reaches maturity (Konkol *et al.*, 1978), however, this is an exception to the general rule that the noradrenergic system matures after the serotonergic one. At three weeks of age, the serotonergic dendrite network is already similar to that of the adult rat brain (Dori *et al.*, 1996; Lidov & Molliver, 1982; Loizou & Salt, 1970). From PND21 onwards, 5-HT concentrations continue to increase and slowly decreases towards adult concentrations (Loizou, 1972; Whitaker-Azmitia, 2005). At the same time (PND21) a significant increase in TH concentrations in the cortex is observed, again indicating further maturation of the noradrenergic system. Interestingly, cortex TH concentrations indicate NA activity, whereas striatal concentrations are associated with DA activity (Murrin *et al.*, 2007). In support, noradrenergic α_2 receptor stimulation is at its highest during the fourth postnatal week (Happe *et al.*, 1999) whereas NMDA development peaks around the same time (Insel *et al.*, 1990) and follows a similar decrease towards adulthood. An interesting observation to note here, with regards to the glutamate system, is that the NMDA antagonist, ketamine, does not produce hallucinations in children as it does in adults (Hirsch *et al.*, 1997), suggesting different or additional (mature) pathways involved in the adult response. As mentioned earlier, DHA concentrations continue to increase after birth, plateauing only on PND21. In fact, at this time, DHA makes up as much as 20 % of brain total fatty acids (Green *et al.*, 1999; Green & Yavin, 1996). By PND35 the noradrenergic system has reached maturity (Konkol *et al.*, 1978; Loizou & Salt, 1970; Morris *et al.*, 1980; Murrin *et al.*, 2007), although one report suggests noradrenergic synapses to only

reach adulthood by PND60 (Markus & Petit, 1987). Regardless, the general consensus is that the serotonergic system matures before the onset of puberty, whilst the adult similarities of the noradrenergic system is only reached with or after pubertal onset. In accordance with the mentioned continuous DHA increase during the first twenty years, it is important to note that similar linear increases are observed in frontal gray and white matter during the same period (Giedd *et al.*, 1999; Tisserand *et al.*, 2002), again suggesting a key role of brain fatty acid composition in normal brain development.

The available literature on human brain development is understandably limited as ethical and practical factors prevent researchers to fully investigate this process. In fact, four decades ago, Dobbing and Sands pointed out that apart from these ‘problematic’ factors, the investigation of human brain development remains complicated, nonetheless, when they commented:

“Perhaps it is worth reflecting that (the) difficulties of practicality would be formidable even if there were no ethical problems, due partly to the very long time span of human growth and development as well as to its complexity. Some would also say the human environment was much too complicated to bear analysis compared with that of other ‘lower’ species, but that is an assumption which may be false, at least for the behavioural scientist.” (Dobbing & Sands, 1979).

Increasingly, research data confirm complex processes for the human brain development (Murrin *et al.*, 2007). Therefore, the earlier maturation of the serotonergic system in the pre-adolescent individual has been accredited for the effectiveness of the SSRIs in this patient population, whilst the ineffectiveness of noradrenergic targeting antidepressants, such as the TCAs, might be explained by an immature noradrenergic neurotransmitter system. Nevertheless, more research into the development of the rodent brain is needed with comparisons, where possible, to that of the human brain to better understand this process as well as accurately determine the possible effects of central acting drugs in juvenile patients. In this regard, and specific to the current topic, robust animal models of juvenile depression is required. The following section will therefore discuss the specific animal model used in the current project, i.e. male FSL rats as well as review available literature on other animal models for juvenile depression.

2.7 Flinders sensitive line rat as a juvenile animal model of childhood depression

There are at least eighteen (adult) animal models of depression, which can be divided into different model types, such as genetic, stress, pharmacological and diverse (Overstreet, 1993, 2002). General guidelines were set out by McKinney and Bunney (McKinney & Bunney, 1969) in order to evaluate the validity of an animal model and its ability to effectively describe the human occurrence and condition. According to these guidelines, an effective animal model should adhere to or display all three of the following criteria (Willner, 1986; Willner, 1991b):

- 1) **Face validity:** Be phenomenologically similar to the syndrome it is imitating.
- 2) **Predictive validity:** Predicts the effects of certain drugs in a similar manner as to those observed clinically.
- 3) **Construct validity:** Has a theoretical rationale allowing the application of the specific phenomenon to non-human species.

However, although the mentioned guidelines are used to estimate the value of an animal model, it should be noted that an animal model can still be accurate in providing vital information on a human phenomenon, even if not all of the criteria above are met (Willner, 1984, 1991a). This consideration is echoed by McKinney when the author states the following:

“There will likely never be an animal model in any field of medicine that is a perfect fit with the human condition, rather the emphasis in the development and study of disease models in animals needs to be on specific components of the human illness.” (McKinney, 2001)

The Flinders sensitive line (FSL) rat is such a widely accepted animal model of depression with more than 100 international research articles featuring this specific animal model (Overstreet & Wegener, 2013). The FSL rat resulted from inbreeding of the Sprague-Dawley line (El Yacoubi & Vaugois, 2007; Hascup *et al.*, 2011) to create a rat line genetically resistant to the organophosphate, DFP. However, this was unsuccessful and produced one rat line proved to be more sensitive, rather than resistant, to the irreversible anticholinesterase agent (Russell & Overstreet, 1987). Important to note is that the Flinders Resistant Line (FRL) rat, commonly used as a control to for the FSL, is more resistant to DFP, only compared to the FSL rats, and not to outbred control rats (Overstreet *et al.*, 1979; Russell & Overstreet, 1987).

Although used as an animal model for childhood (and adolescent) depression in the current project, the FSL rat is primarily and generally used as a model for adult depression. Nevertheless, research into the identification of a suitable animal model for childhood depression is underway and gaining more interest (Malkesman *et al.*, 2009; Malkesman *et al.*, 2006a; Malkesman *et al.*, 2005; Malkesman *et al.*, 2006b; Malkesman *et al.*, 2007; Malkesman & Weller, 2009). In fact, two of the most frequently studied models, the Wistar Kyoto (WKY) and the FSL both show promise with distinct differences in ‘depression’ profiles. The juvenile FSL rat appears to more accurately mimic atypical depression behaviour (showing no co-morbid symptoms of anxiety), whereas the pre-pubertal WKY rat may represent an animal model for melancholic depression (showing severe co-morbid symptoms of anxiety) (Malkesman & Weller, 2009).

The pre-pubertal FSL rat was used as an animal model for childhood depression in the current study not only to investigate the lasting effects of different antidepressant treatments and/or interventions, but to also contribute to the limited data regarding the predictive validity criteria of this specific rat line. How these treatment and/or intervention options will affect the depressive-like behaviour of the pre-pubertal FSL rat

will be better understood after reviewing the available literature regarding the specific rat line, the different hypotheses of MDD as well as its specific role in the juvenile animal model. Therefore, the following section will briefly present the available literature in this regard in order to better interpret the bio-behavioural data of the current study.

2.7.1 Relevance of different major depression hypotheses in the Flinders sensitive line rat

With regards to the different hypotheses of MDD, the FSL rat displays similarities, to a greater or lesser extent, to each of these aetiology theories. These similarities are discussed below and summarized in Table 2-3.

Table 2-3: Comparison of key findings in FSL rats and observations in depressed patients (adapted from (Hascup *et al.*, 2011; Liu *et al.*, 2017; Malkesman *et al.*, 2009; Malkesman & Weller, 2009; Melas *et al.*, 2012; Overstreet *et al.*, 2005; Wegener *et al.*, 2010)).

Theoretical model / Hypothesis	Significant observation in depressed patients	Significant finding in adult FSL rat	Significant finding in pre-pubertal FSL rat
Monoamine	Increased 5-HT _{1A} sensitivity	Increased and decreased 5-HT _{1A} sensitivity	Abnormal levels of monoamines
	No change in NA receptor sensitivity	Altered NA receptor sensitivity	
	Decreased DA transporter	Decreased DA transporter and extracellular concentrations	
HPA	Elevated cortisol	Altered corticosterone levels	Decreased corticosterone levels
Cholinergic	Increased cholinergic sensitivity	Increased cholinergic sensitivity	No available information
	No antidepressant effect of cholinergic antagonists	No change in time spent immobile in FST with cholinergic antagonists	
	Increased REM sleep duration (a cholinergic mediated process)	Increased REM sleep (a cholinergic mediated process)	
Immunological	Increased oxidative stress markers	Increased NOS and levels following stress	No available information
	Increased kynurenine pathway metabolites	Decreased immune defences	
Neuroplasticity	Decreased NPY levels	Decreased NPY levels	Decreased BDNF levels
	Normal neurotrophin levels	Decreased neurotrophin levels	
Glutamate/GABA	Decreased GABA levels	Decreased GABA levels	Unaltered glutamate concentrations
	Increased glutamate levels	Increased glutamate levels	
G x E	Increased risk for developing MDD when carrier of the 5-HTTLPR s allele	Decreased gene expression associated with increased depressive-like behaviour	No available information

Firstly, pre-pubertal FSL rats, similar to adult rats, display altered monoamine concentrations in some, but not all, of the measured brain regions when compared to SD controls (Malkesman & Weller, 2009). Moreover, it appears that the developmental profiles of these monoamine neurotransmitters differ between juvenile and adult FSL rats, yet depressive-like symptoms are visible in both stages of life (Malkesman *et al.*, 2006a; Shayit *et al.*, 2003; Zangen *et al.*, 1997, 1999). In this regard, 5-HT concentrations during puberty differed from both new-born and adult FSL rats, with lower levels of 5HIAA observed during puberty (i.e. PND35), yet similar levels between new-borns and adults were reported. Differences in DA concentrations were also observed in juvenile FSL rats, accompanied with altered metabolism rates of both 5-HT and DA at PND35, compared to age-related SD controls (Malkesman *et al.*, 2007). Furthermore, brain monoamine concentrations of new-born FSL rats appear to be similar to that of new-born SD controls, nevertheless, a decrease in 5-HT and DA (and their respective metabolites) is observed around puberty for both strains with significant differences. These differences are even more pronounced in adult FSL and SD rats where monoamine levels continue to decrease in SD rats, but return to similar (or even higher) levels in the FSL adult, compared to new-borns (Malkesman *et al.*, 2007). Interestingly, although altered NA concentrations has been reported, it has been suggested that the FSL rat is not a suitable model for the NA hypothesis of MD (Hascup *et al.*, 2011) and might more accurately model depression in a serotonergic manner as lower 5-HT levels, compared to both adult FRL and SD controls have been reported (Hasegawa *et al.*, 2006).

Secondly, pre-pubertal FSL rats display significantly lower concentrations of plasma corticosterone and ACTH under baseline conditions (Malkesman *et al.*, 2006a; Malkesman & Weller, 2009). This is of note since earlier reports theorize that the increased HPA hormone (cortisol in humans and corticosterone in rats) concentrations, may be the result of hypersecretion of CRF and ACTH (Kalin *et al.*, 1982; Nemeroff *et al.*, 1984). However, adult FSL rats have also been reported to have decreased ACTH (but not corticosterone) levels, compared to adult FRL rats (Owens *et al.*, 1991), suggesting that the hypocortisolism observed in pre-pubertal FSL rats, continue into adulthood. To this extent, Malkesman and colleagues (Malkesman *et al.*, 2006a) theorize that, as with children growing up in stressful environments, FSL pups grow up with ‘depressed’ mothers which creates a stressful housing environment leading to less than optimal care (Lavi-Avnon *et al.*, 2005a; Lavi-Avnon *et al.*, 2005b) and ultimately resulting in hypocortisolism. This observation of hypocortisolism in the pre-pubertal FSL rat is in line with clinical reports of depressed children and adolescents that do not display the expected hypercortisolism as depressed adults, but rather hypocortisolism (Kaufman & Ryan, 1999).

Thirdly, in line with the hypothesized super sensitivity of the cholinergic system in depressed patients, the FSL rat also displays increased sensitivity to cholinergic agonists (Overstreet, 1993, 2002). However, although bred for altered cholinergic sensitivity, the cholinergic characteristics of the FSL rat may not be its most prominent feature in modelling MDD (Overstreet *et al.*, 2005). In fact, a novel hypothesis for

depressive-like behaviour of FSL rats has recently been suggested to better explain greater sensitivity towards other non-cholinergic affecting agents than to those affecting the cholinergic system. According to the proposed hypothesis an overexpressed potassium channel in key brain areas could, at least in part, be responsible for the observed increased sensitivity towards multiple receptors in the FSL rat (Overstreet & Wegener, 2013). This hypothesis, however, remains to be confirmed, yet does open novel and exciting avenues to explore regarding the FSL rat as animal model of depression as well as innovative antidepressant treatment options. Regardless, the cholinergic characteristics of the FSL rat remains valuable as a recent study in our laboratories found increased ACh concentrations in the frontal cortex of the adult FSL rat, but decreased hippocampal concentrations compared to FRL controls (Brand *et al.*, 2012). Also, a significant increase in nAChRs density, but not affinity, has been reported between the FSL and FRL rat lines (Auta *et al.*, 2000), supporting the clinical reported antidepressant effects of nicotine receptor stimulation. The involvement of the cholinergic system has also been identified to as a potential avenue in MDD treatment options (Brink *et al.*, 2008; Liebenberg *et al.*, 2010; Tizabi *et al.*, 2000).

Fourthly, although no significant information specifically regarding the pre-pubertal FSL rat and the immunological hypothesis of MDD could be found, available data concerning the adult FSL rat will be discussed. According to a previous study in our laboratories, the researchers reported no differences in brain oxidative stress markers between adult FSL and FRL subjects (Mokoena *et al.*, 2015). Regarding inflammation, natural killer cells (an essential component of the innate immune system and crucial in the host's defence against bacterial and viral infection) are significantly lower in the FSL rat, compared to the FRL control (Friedman *et al.*, 1996). Additionally, FSL rats are significantly more susceptible to the induction of anaphylactic shock (Djuric *et al.*, 1995), oxidative stress response (Wegener *et al.*, 2010) as well as present with deficiencies in their T-helper cell mediated immunity function (Friedman *et al.*, 2002; Strenn *et al.*, 2015). Frontal cortex kynurenic acid concentrations are also decreased in FSL, compared to FRL rats, suggesting altered metabolism of the kynurenine pathway (Liu *et al.*, 2017). Altogether, these findings suggests that the FSL rat line may be more susceptible to the consequences of inflammation and oxidative stress, ultimately adding to the phenotypic depressive-like behaviour supporting its use as an animal model for depression.

Fifthly, and in line with the neuroplasticity hypothesis of MDD, hippocampal BDNF concentrations are significantly lower in PND35 FSL rats, compared to SD controls of a similar age (Malkesman *et al.*, 2009). Remarkably, although decreased BDNF has been associated with hypercortisolism (McEwen, 1999), the fact that the pre-pubertal FSL rat displays hypocortisolism, may suggest a different mechanism involved as it has also previously been proposed that increased HPA hormones may not fully explain decreased cell proliferation (Malberg & Duman, 2003). Nonetheless, a previous report supporting the notion of decreased neuroplasticity in the FSL rat line explained decreased hippocampal volume, compared to FRL controls (Chen *et al.*, 2010) also exist.

With regards to the glutamate/GABA hypothesis of MDD, very little is known about the GABAergic/glutamatergic status of the adult FSL (and FRL) rat, and therefore information concerning the pre-pubertal model is even scarcer. Regardless, a recent study reported similar resting glutamate concentrations in the brain between young (three to six months of age) FSL rats and young and old (twelve to fifteen months of age) FRL rats. The older FSL rat line showed significantly increased resting glutamate concentrations, compared to FRL rats of similar age, as well as to younger FSL and FRL rats (Hascup *et al.*, 2011). This observation, along with the lack of anxiety-like behaviour in pre-pubertal FSL rats (Braw *et al.*, 2006; Malkesman *et al.*, 2005), yet increased anxiogenic behaviour in older versions (Overstreet *et al.*, 2004; Overstreet *et al.*, 1990) led the authors to conclude that the FSL rat may display increased depressive-like behaviour with aging together with a shift in the model's anxiety/depression profile. An important observation in the FSL rat model is the glutamate overflow reported in the older FSL rat. In this regard, increased extrasynaptic glutamate concentrations were also reported in the older, but not younger FSL rat line, possibly caused by a down regulation in glutamate uptake system due to excessive synaptic stimulation or decreased glutamate transporters (Hascup *et al.*, 2011; Pittenger *et al.*, 2007). Inhibitory glutamatergic receptors have also been reported to be decreased in the FSL rat line (Kovačević *et al.*, 2012; Matrisciano *et al.*, 2008)

Finally, although the involvement of genetic susceptibility in the depressive symptoms are receiving more and more attention, the available literature on the genetic construct of the FSL (and FRL) rat appears to be very limited and therefore warrants further investigation. In this regard, decreased hippocampal *P11* (*S100A10*) gene expression (associated with increased DNA methylation) has been reported in the FSL rat, compared to the FRL control and subsequently increased by antidepressant therapy (Melas *et al.*, 2012). Importantly, this observation was observed in three month old FSL rats which was classified as 'young' by the authors as 'older' animals were twelve to fifteen months of age. However, since this age does not fit into our definition of 'pre-pubertal', this observation may not be a true representation of juvenile FSL rats and should be interpreted with caution. Regardless, this reported decreased gene expression is in line with post-mortem brain tissue studies of depressed individuals (Anisman *et al.*, 2008), suggesting an underlying putative genetic deficit in the FSL rat responsible for the observed depressive-like behaviour.

2.7.2 Relevance of behavioural alterations

With regards to the behaviour of these animals, several reports have supported the use of the pre-pubertal FSL rat as an animal model for childhood depression. The following sections will thus briefly review the relevance of certain behavioural parameters supporting the use of the juvenile FSL rat in the current project, yet indicating that special attention should be given to interpretation of the data as behaviour of juvenile animals may in certain aspects differ to that generally observed (and expected) in adult counterparts.

2.7.2.1 Locomotor activity

Confounding results regarding the locomotor activity of the FSL, compared to FRL controls, are available with higher (Strenn *et al.*, 2015) and similar (Fischer *et al.*, 2012) locomotor activity being reported. Regardless, as implemented in the current study the locomotor activity measurement of the animal model is used in combination with the data of the FST. In this regard, the locomotor activity of the animals is used to determine whether the possible antidepressant-like effect of the treatment/intervention options are indeed altered psychomotor activity. Therefore, regardless of whether the locomotor activity of the FSL rat (whether pre-pubertal or adult) is higher or similar to the FRL control, this parameter acts as a support to that of the FST in the same animal and should also be interpreted as such. Moreover, altered general locomotor activity is not listed as one of the diagnostic criteria for juvenile MDD by the DSM-V (Table 2-1), again supporting the idea that locomotor activity should not be interpreted as a stand alone parameter, but rather in a supporting role.

2.7.2.2 Anxiety-like behaviour

Although anxiety is comorbid with MDD, pre-pubertal FSL animals are not known to display such behaviour, compared to pre-pubertal SD or FRL counterparts in the OFT or EPM (Braw *et al.*, 2006; Malkesman *et al.*, 2005). However, older FSL rats (three months) display similar anxiety-like behaviour in the EPM, compared to FRL controls, yet display significantly increased anxiogenic behaviour, compared to age-matched FRL rats in other, but not all, behavioural tests such as the social interaction test (SIT) and active avoidance tasks (Overstreet *et al.*, 1995; Overstreet *et al.*, 2004; Overstreet *et al.*, 1990; Wegener *et al.*, 2012). Although anxiety does not form part of the diagnostic criteria for juvenile MDD, psychomotor agitation does (Table 2-1) and could mistakenly be measured as decreased anxiety in a test such as the OFT where the animal could spend more time mobile and thus possibly spending more time in the centre zone of the test. Hence, caution should therefore be used when interpreting behaviour, especially since the FSL animal is, in general, not known to exhibit anxiety-like behaviour.

2.7.2.3 Depressive-like behaviour

Depressive-like behaviour or behavioural despair has been observed in PND21 rat pups, suggesting that animals can indeed model behaviour comparable to that of depressed children (Abel, 1993; Reed, 2008; Reed *et al.*, 2008). This observation is supported in the pre-pubertal male FSL rat model since baseline conditions showed the pre-pubertal FSL rat displayed increased immobility time in the FST, compared to controls (Malkesman *et al.*, 2006a); of note, pre-pubertal Sprague-Dawley (SD) rats were used as controls and not FRL rats. Remarkably, although the pre-pubertal FSL rat weighed significantly less than the control SD rats, these mass differences were concluded not to affect time spent immobile in the FST. This is of importance since a more recent review highlights body mass as a significant factor in FST result outcome

(Bogdanova *et al.*, 2013). However, the literature referenced in the specific review investigated adult rats and could possibly not be applicable to pre-pubertal animals, yet the mass difference between the pre-pubertal FSL and SD rats are in line with weight alterations observed in children diagnosed with MD as is depressed mood, feelings of worthlessness and recurrent thoughts of death (American Psychiatric Association, 2013) that could be highlighted by the FST.

2.8 Synopsis

MDD is not limited to the adult population as it affects a significant number of the paediatric individuals as well. In fact, the number of children and adolescents diagnosed with MDD are increasing, yet appropriate treatment is not initiated at an appropriate young age. Possible contributing factors to the late treatment initiation in depressed juveniles are firstly, the limited number of antidepressants available, and secondly, the increased risk for developing suicidal behaviour during the initial treatment period. In addition, the long-term or lasting effects of early-life treatment remain unknown and therefore places the medical practitioner in a situation weighing the unknown long-term effects against the short-term consequences of untreated juvenile MDD.

The neurobiological pathophysiology of juvenile MDD is comparable to that described in the adult patient. However, although antidepressants mainly target a deficient monoaminergic system, suggesting decreased monoaminergic neurotransmission to be the cornerstone of MDD pathophysiology. Furthermore, that neuroplasticity is more dynamic and adaptable process during early-life development, could suggest that significant early-life influences could have lasting effects. In fact, early-life stress leads to increased risk for developing central conditions, including MDD. Therefore, could early-life treatment positively affect neuroplasticity in such a way that beneficial effects are induced and observed later in life?

In addition to the limited pharmacological treatment options, exercise and ω -3 EFA supplementation are receiving growing attention as augmentative treatment strategies, partly due to their perceived improved safety profile and relative ease of accessibility. Nevertheless, both these non-pharmacological interventions beneficially influence several of the neurobiological hypotheses of MDD, including neuroplasticity, suggesting augmentation potential to not only the therapeutic effects of antidepressant treatment, but also possible lasting effects. Therefore, we investigated whether pre-pubertal treatment with antidepressants with or without non-pharmacological intervention strategies could indeed induce lasting effects in an approved animal model of depression (i.e. FSL rat). To this extent, although literature regarding the neurobiological characteristics of the pre-pubertal FSL rat is limited, the available information suggests these characteristics to be in line with that described in the adult FSL rat and be an accurate model of juvenile MDD.

CHAPTER 3: MANUSCRIPT A

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AUTHOR CONTRIBUTIONS

Jacobus C. Schoeman conducted the behavioural and neurochemical experiments, did the initial data work-up and statistical analyses, and wrote the first draft of the manuscript.

Stephanus F. Steyn assisted in designing and conducting the behavioural experiments of the VO_{2max} determination test, the initial data work-up and statistical analyses. Furthermore, SF Steyn assisted in the interpretation of the study data and finalized the manuscript for submission. As indicated in the published manuscript, both SF Steyn and JC Schoeman contributed equally to the published manuscript.

Brian H. Harvey advised on the study design and proofread the final manuscript.

Christiaan B. Brink designed and supervised the study and assisted in the interpretation of the study data, finalized the manuscript for publication and was corresponding author in the submission of the final manuscript to *Behavioural Brain Research*.

IMPORTANT INFORMATION

- Instructions to the author can be viewed online at <https://www.elsevier.com/journals/behavioural-brain-research/0166-4328/guide-for-authors>.
- As per the instructions to the author, figures, tables and legends are provided at the end of the manuscript.
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- Confirmation of acceptance of this manuscript by *Behavioural Brain Research* is presented in Addendum D.



Long-lasting effects of fluoxetine and/or exercise augmentation on bio-behavioural markers of depression in pre-pubertal stress sensitive rats



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HIGHLIGHTS

- Maximal exercise intensity (as estimated by VO₂max) in rats increases with age and should be adapted accordingly.
- Pre-pubertal fluoxetine and fluoxetine plus low intensity exercise exerts early antidepressant-like effects.
- Pre-pubertal low intensity exercise or low dose fluoxetine exerts lasting antidepressant-like effects into early adulthood.
- The combination of fluoxetine with low intensity exercise does not exert any lasting effects on depressive-like behaviour.
- Exercise, fluoxetine and the combination thereof increased hippocampal superoxide dismutase in early-adulthood.

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ABSTRACT

Juvenile depression is of great concern with only limited treatment currently approved. Delayed onset of action, low remission and high relapse rates, and potential long-lasting consequences further complicates treatment and highlights the need for new treatment options. Studies reporting on long-lasting effects of early-life treatment have reported conflicting results, with the pre-adolescent period mostly overlooked. The anti-depressive effect of exercise, as a possible treatment option or augmentation strategy, is dependent on age and exercise intensity. We investigated the immediate (i.e. postnatal day 35 (PND35)) and lasting (PND60 to PND61) effects of pre-pubertal (PND21 to PND34) fluoxetine and/or exercise on bio-behavioural markers of depression and oxidative stress in stress sensitive Flinders Sensitive Line rats. Low, but not moderate, intensity exercise or 5, but not 10, mg/kg/day fluoxetine displayed anti-depressant-like properties at PND35. Pre-pubertal treatment with 5 mg/kg/day fluoxetine or low intensity exercise exerted lasting anti-depressive-like effects into adulthood, whereas the combination of these two treatments did not. Furthermore, the combination of fluoxetine plus exercise reduced hippocampal BDNF levels as compared to exercise alone, which may explain the latter findings. In all treatment groups hippocampal SOD activity was significantly increased at PND61, suggesting an increased anti-oxidant capacity in adulthood. In conclusion, the data confirm the anti-depressant-like properties of both early-life fluoxetine and exercise in a genetic animal model of depression. However, optimal lasting effects of early-life interventions may require adjustment of antidepressant dose and/or exercise intensity to developmental age, and that a combination of antidepressant and exercise may not necessarily be augmentative.

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1. Introduction

Major depressive disorder (MDD) is one of the most challenging mental health problems of our time, affecting an estimated 350

million people worldwide, at any given time point [1]. Children are also affected and due to increased awareness and a rise in the number of juveniles diagnosed with MDD, it has become the most common mental health disorder in this age group [1]. In fact, MDD has an estimated prevalence of 2–5% in children, associated with a fourfold enhanced risk of enduring or reoccurring in adulthood [2]. Also, severe depression often leads to suicide [3], making it the fourth leading cause of death in pre-adolescent children [4]. An increase in the prescription rate of antidepressants in this age group has also been documented, further highlighting the need for safe and effective treatment options in this age group.

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During pre-adolescence the serotonin pathway is already matured, whereas the noradrenergic and dopaminergic pathways are still developing, so that drugs that modulate serotonergic neurotransmission are more likely to be effective than those modulating other systems [5]. That said, current treatment options for childhood or adolescent depression are limited to fluoxetine and escitalopram, both selective serotonin reuptake inhibitors (SSRIs). These two agents are approved by the United States' Food and Drug Administration (FDA) for use in children older than 8 and 12 years, respectively [6–8]. Other antidepressants, such as the tricyclic antidepressants and other SSRIs, have been shown to be ineffective (or inconclusive) in children [5,9]. In addition, a black box warning was issued by the FDA in September 2004 highlighting an increased risk of suicidal ideation in juveniles treated with SSRIs [10]. Lastly, the potential long-term consequences of early-life treatment in the developing brain have become a great concern in recent years. This has rendered paediatric prescribing a daunting task, where prescribers have to weigh the risks and benefits of early-life treatment against the unknown risks of neurodevelopmental impact and consequent outcome later in life.

A few studies focused on the potential long-term consequences of early-life exposure to centrally acting stimulants and antidepressants, in an attempt to shed some light on how such stimuli during the complex process of brain development could alter the brain's functional integrity in adulthood [11]. Interventions during neurodevelopment, such as drug use, presents a window to induce permanent neurodevelopmental alterations [12]. Some pre-clinical data suggest negative outcomes of early-life treatment with SSRIs, such as arrested development of spine density [13], decreased density of the serotonin transporters [14], reduced body-weight, reduced sexual functioning and/or increased anxiety [15], increased depressive-like behaviour [16] and decreased locomotor activity [17]. However, it has been suggested that pharmacotherapy during an appropriate developmental period could alter the course of development to exert lasting beneficial effects [12]. This idea is not farfetched and it has been demonstrated that fluoxetine treatment of rodents during puberty may produce significant antidepressant-like effects and positively influence responsiveness to rewarding and aversive stimuli in adulthood [18].

Importantly, several methodological and other differences exist between studies, which may explain contradictory research findings. For example, most animal studies employ healthy rodents, without any genetic predisposition to display depressive-like behaviour, consequently limiting the translational value of the findings. Also, although striking similarities between the age-related neurodevelopment in human and other mammals have been well-documented, these are not absolute. As such, key aspects of pre-pubertal rodent neurodevelopment can be translated to that of a human child (4–12 years), the last half of which antidepressant treatment is often indicated [1]. Importantly, this would represent a vulnerable time in neurodevelopment to investigate the effects of potential interventions in early life.

Even when antidepressants may exert beneficial therapeutic effects, antidepressant treatment is associated with bothersome side-effects, such as a delayed onset of action [18], low remission rates and a high rate of relapse following discontinuation [19], highlighting the need for new treatment modalities. Such interventions typically include psychotherapy, support groups and life-style adjustments. Exercise is one such treatment option and although the efficacy of exercise has been demonstrated in adults [20–22], children [23] and rodents [24], the data in children remain limited.

Exercise has also been proposed as an augmentative strategy to antidepressant treatment due to the putative synergistic effects with antidepressants as well as the advantage of being a relatively safe and low cost intervention. The antidepressant effects of exercise have been suggested to result from increased levels of

monoamines, neurotrophins, anti-inflammatory markers and anti-oxidants [22,25,26], whereas depression is widely regarded as an inflammatory and pro-oxidative state [27]. Immediate effects of exercise seem to be dependent upon age as well as exercise intensity [11,28]. One study reported low and high intensity exercise to induce different neurochemical effects during different developmental stages. In particular, high, but not low, intensity exercise during pre-pubertal development of Wistar rats was associated with increased pro-inflammatory cytokine levels, whereas low, but not high, intensity exercise in the same age-period increased cell proliferation rate in the dentate gyrus [28]. However high, but not low, intensity exercise in pubertal rats significantly increased proliferative cell density and anti-inflammatory cytokine concentrations [28]. This report lends further support to the idea that treatment could have differential effects depending on the time of exposure during neurodevelopment. Nevertheless, few studies have explored the potential lasting effects of exercise as a treatment option for depression.

The current study initially investigated whether the maximum oxygen consumption capacity (VO_{2max}) of the pre-pubertal Flinders Sensitive Line (FSL) rat increases with age, using an approved indirect approach. Thereafter it was determined whether pre-pubertal low or moderate exercise intensity, and low or high dose of fluoxetine treatment yields antidepressant-like effects immediately following intervention or treatment. Finally, immediate antidepressant-like behavioural effects of the combination of low intensity exercise and low dose fluoxetine was investigated. In the subsequent long-term study, we investigated the lasting effects of pre-pubertal low dose fluoxetine, low intensity exercise, or the combination of fluoxetine and exercise into early-adulthood, i.e. following a washout period of 26 days. The studies aimed to determine whether pre-puberty presents a window of opportunity, specifically in genetically susceptible rats, to exert lasting beneficial effects on behavioural and tissue biomarkers of depression (BDNF) and oxidative stress (lipid peroxidation, superoxide dismutase activity). In this regard, no Flinders Resistant Line (FRL) control animals were included in the current study, since the study did not aim to investigate the role of genetic susceptibility, but rather to investigate the role of the various interventions in an approved genetic animal model of depression. We also explored whether the combination of fluoxetine and exercise will yield an augmentative long-lasting anti-depressant-like effect in adulthood.

2. Materials and methods

2.1. Animals

Male Flinders Sensitive Line (FSL; $n=179$) and Flinders Resistant Line (FRL; $n=12$) rats were bred, supplied and housed at the Vivarium (SAVC reg. no. FR15/13458; SANAS GLP compliance no. G0019) of the Pre-Clinical Drug Development Platform (PCDDP), North-West University. The original rat colonies were obtained from Dr David H Overstreet, University of North Carolina, Chapel Hill, North Carolina, USA. The FSL rat is a validated genetic animal model of depression, displaying face, construct and predictive validity, whereas the FRL rat serves as a model control [29,30]. All experiments were approved by the AnimCare animal research ethics committee (NHREC reg. no. AREC-130913-015) of the North-West University (ethics approval no. NWU-00148-14-A5), and all animals were maintained and all procedures performed in studies involving animals were in accordance to the code of ethics in research, training and testing of drugs in South Africa and complied with national legislation.

The study aimed to employ 16 rats per test group. However, smaller numbers were sometimes employed due to a few non-

running animals that were excluded from the study [31], two animals that were immediately removed from the study due to minor foot injuries on the treadmill (followed by measures taken to avoid recurrence), as well as lower birth rates at the requested dates.

The rats were housed in groups of three with corncob bedding changed weekly and the environmental temperature maintained at $22 \pm 1^\circ\text{C}$ and a relative humidity of $55 \pm 10\%$. A 12 h light/dark cycle (lights on at 06:00 and off at 18:00) was followed and food and water were provided *ad libitum*. Pups were weaned on postnatal day 21 (PND21) and body weight was measured daily during treatment from PND21 until PND34. From PND35 to PND60 rats were housed under normal conditions, without any treatment. Animal wellbeing was routinely monitored on specially developed monitoring sheets throughout the study, and were monitored during and after each injection and exercise session. In addition, animals were handled daily by the researcher from PND16 onwards to familiarise the animals to human handling.

2.2. Drug treatment

Animals received either saline (vehicle) or fluoxetine HCl (5 or 10 mg/kg) (a kind gift from Jade Pharmaceuticals, South Africa; fluoxetine HCl USP, batch no.: FX/1505001) via subcutaneous (sc) administration once daily between 07:00 and 10:00 from PND21 to PND34. A 14 day treatment period is generally considered sub-chronic and longer than the minimum required to produce antidepressant-like effects in FSL rats [32]. The subcutaneous route of administration has a predictable bioavailability comparable to that of an intraperitoneal injection, although injection stress is less particularly in young rats. Fluoxetine too is successfully delivered via sc administration [33]. The period between PND21 to PND34 represents pre-puberty in rodents when the serotonergic pathway is already matured, while the noradrenergic and dopaminergic pathways are still developing [5,34]. After treatment and as indicated, rats were either submitted to behavioural analyses on the following day (PND35) or left in normal housing conditions during a period of 26 days of drug washout, until behavioural analyses on PND60 (early adulthood). Brain dissection for neurochemical testing occurred on PND61.

2.3. Exercise

Forced exercise was introduced using a custom-built treadmill, comprising a single treadmill belt (51 mm (w) $\times$ 96 mm (d)) and six shocking grids (14 mm (w) $\times$ 21 mm (d)) installed at the back of the treadmill. Six removable running lanes (14 mm (w) $\times$ 66 mm (d) $\times$ 14 mm (h)) with black opaque vertical walls and a clear top cover was placed over the belt in order to accommodate six subjects at a time. The speed range of the treadmill was between 2.0 m/min and 70 m/min with programmable increments of 0.01 m/min.

2.3.1. Familiarization

Prior to the commencement of exercise, all animals were familiarised to the treadmill from PND16 to PND20. During this period, the animals experienced 5 consecutive days [35–37] of comfortable walking for 10 min/day [38].

2.3.2. Reinforcement

Rats were motivated to run on the treadmill by means of negative reinforcement [31]. To this end a shocking grid, yielding sufficient electrical shock (1 mA, 3 Hz) to be uncomfortable but not painful or harmful, was installed at the rear-end of the treadmill. Importantly, animals that stopped running during familiarisation and the chronic exercise regimen, even after reinforcement, were removed from the treadmill after five seconds and subsequently

used as control rats [31]. We observed a small number of rats (<10%) who displayed such “non-runner” behaviour, in line with previous reports [31]. During the exhaustion test, exhaustion was considered whenever a rat touched the grid four times within a period of 1 min, where after the subject was immediately removed from the treadmill.

2.3.3. Exhaustion test

Animals were familiarized to the treadmill as described above. Thereafter, animals ($n = 32$) were randomly divided into two groups ($n = 16$ each) and submitted to alternate day exhaustion tests performed one hour after commencement of the rodent wake cycle (19:00–22:00) to allow initial foraging, with one group on PND21, 26 and 32 and the other on PND23, 28 and 34. More specific, animals were allowed to rest on the day following the exhaustion test, yet were subjected to a comfortable walking speed on the remaining days leading up to the next exhaustion test.

For the exhaustion testing, rats were subjected to treadmill running to determine the maximum speed (exercise intensity) at which the FSL rats can run and as an indirect estimate of $\text{VO}_{2\text{max}}$, similarly to methods described before [39–43]. Each rat started at a low initial speed (2.5 m/min) which was increased with 2.5 m/min every 3 min until the point of exhaustion (rat touching the shock grid four times within 1 min). The time to fatigue (in min) and workload (in m/min) were expressed as indexes of exercise capacity and as indirect estimates of $\text{VO}_{2\text{max}}$ [39–44]. This procedure was used to establish whether $\text{VO}_{2\text{max}}$ increases as rats grow in size and weight from PND21 to PND34, and in particular, to determine the eventual treadmill speed required at a given age to reach the indicated percentage of $\text{VO}_{2\text{max}}$ in all subsequent experiments.

Maximum exercise intensity (i.e. where $\text{VO}_{2\text{max}} = 100\%$) at which FSL rats can run over a period of 20 min was determined as follows:

Max intensity speed

$$= \frac{\text{time to exhaustion (min)} \times \text{speed at point of exhaustion}}{\text{exercise period (20 min)}}$$

From the maximum intensity, high intensity exercise was defined as $\text{VO}_{2\text{max}} = 85\%$, moderate intensity exercise as $\text{VO}_{2\text{max}} = 70\%$ [45] and low intensity exercise as $\text{VO}_{2\text{max}} = 55\%$ [46]. These intensities correlate with human athlete percentages of $\text{VO}_{2\text{max}}$ for low and moderate intensity exercise [47,48].

2.3.4. Chronic exercise regimen

Before the start of the exhaustion test, animals were familiarized to the treadmill as described above. Animals were subjected to sedentary (0.0 m/min), low, moderate and high intensity treadmill exercise once daily (between 18:00 and 22:00) for 30 min from PND21 to PND34. Low intensity, moderate as well as high intensity exercise were used as determined in the previous section. However, high intensity exercise was immediately suspended upon observation of minor foot injuries and the reporting of an adverse event (procedure according to NWU ethics guidelines).

Sedentary animals were removed from their cages and placed on a still-standing (mock) treadmill for 30 min during the time other rats underwent treadmill running. During the 30 min of daily training, the first 5 min were at a comfortable walking speed (33% of the intended exercise intensity), where after the intensity was increased to 67% of the intended intensity for 5 min (end of warm-up session) and then to 100% of the intended speed for the final 20 min of the 30 min session [38,49–52]. After treatment rats were either submitted to behavioural analyses on the following day (PND35) or left in normal housing conditions during a period of 26 days of no intervention, until behavioural analyses on PND60 (early adulthood) and brain dissection for neurochemical testing on

PND61. In line with a previous study, neurochemical tests were performed on the same animals that underwent behavioural testing [53].

2.4. Behavioural analyses

After the 14-day period of treatment (vehicle/fluoxetine, sedentary/exercise or drug plus exercise), animals were subjected to the least stressful open field test (locomotor activity) followed by the more stressful forced swim test (FST). We demonstrated before that under our experimental conditions, foregoing tests do not affect the outcome of the subsequent consecutive tests if they are ordered from least to most stressful [54]. The behavioural tests were performed for all treatment groups either early after the pre-puberty intervention period (i.e. injection and/or exercise) on PND35, or later in life after drug washout (withdrawal) and normal housing on PND60, representing early adulthood [34,55,56]. Testing commenced one hour after the start of the dark cycle (i.e. 19:00) in order to ensure normal initial foraging and activity of nocturnal animals. Tests were carefully spaced to allow 30 min between each test in order for animals to habituate to the environment.

2.4.1. Open field test

The open field test (OFT) is commonly used to measure locomotor activity, a parameter of the general ability of the animal to move and negotiate its surroundings.

The apparatus consisted of a 1 m² test arena, digitally marked with sixteen 25 × 25 cm smaller squares, and surrounded by opaque black, vertical walls [57]. On the day of testing, following initial foraging and habituation (see above), each rat was placed in the centre of the arena and allowed to explore the environment for 5 min under red light (80 lx) [57]. During this time, rats were videotaped by a camera mounted above the test arena and subsequently scored using Ethovision XT 11 software (Noldus Information Technology BV, Wageningen, Netherlands). The total number of lines crossed during the session was used as a measure of general activity.

2.4.2. Forced swim test

The forced swim test (FST) is widely used to assess depressive-like behaviour in rodents [58]. Lucki [59] adjusted the FST to distinguish between serotonergic and noradrenergic-directed behaviours [60]. The FSL rat represents a well described validated animal model of depression that displays depressive-like behaviour (enhanced immobility) without the pre-conditioning swim trial 24 h prior to the testing swim trial [29].

The FST was performed as described previously [61]. The apparatus consists of four cylindrical tanks ((40 cm (h) × 20 cm (d)), each filled to a depth of 30 cm with water, maintained at 25 ± 1 °C. The test was performed during the dark cycle under red light (80 lx). On the day of testing, following initial foraging and habituation, each rat was placed in the cylinder and allowed to swim for 7 min. A digital camera mounted in front of the cylinders allowed recording of swim behaviour. The first and last minute of the 7-min scoring period was discarded, with the remaining 5 min used for scoring by an experimenter blind to the test group.

Behaviour scored included immobility, climbing and swimming. Immobility was defined as floating with no active movements made, except those necessary to keep the rat's head above water. Escape behaviours included upward-directed movements of the forepaws along the inside of the swim cylinder (climbing), and horizontal movements throughout the cylinder that included crossing into another quadrant (swimming) [57,60,62–64]. Enhanced adrenergic neurotransmission has been associated with increased climbing, whereas enhanced serotonergic neurotransmission has been associated with increased swimming behaviour [62,65].

Importantly, the abovementioned association of behavioural markers with said monoaminergic neurotransmitter systems provides cues for further investigation (i.e. not conclusive), to be confirmed by further neurobiological investigations, and should hence be interpreted as such.

2.5. Molecular studies

Animals were euthanized within 16 h following the last behavioural test (i.e. PND61). Molecular studies on hippocampal tissue were performed according to methods described below.

2.5.1. Hippocampal tissue preparation

On PND61, i.e. 27 days after the last treatment and/or exercise session and within 16 h after behavioural assessments, rats were euthanized by means of decapitation, the whole brain removed and placed in ice-cold double distilled water (ddH₂O), where after the total hippocampi and frontal cortices were dissected out on an ice-cooled dissection slab, snap frozen in liquid nitrogen and stored at –80 °C until the day of analysis, as described previously [66].

2.5.1.1. Lipid peroxidation. Brain tissue samples were collected and prepared as described above (see *Brain tissue Preparation*). Lipid peroxidation was determined using a lipid peroxidation colorimetric assay kit from Biovision™, and performed according to the manufacturer's protocol. Briefly, 10 mg wet weight of hippocampal tissue was used to prepare a suitable homogenate for analysis. 200 µl of sample (homogenate) was pipetted into a centrifugable tube. 600 µl of thiobarbituric acid (TBA) was added and incubated in glass vials at 95 °C for 60 min. The vials were cooled on ice to room temperature. 200 µl of the reaction mixture was pipetted into a 96-well microplate for spectrophotometric analysis at 532 nm. Data was expressed as nmol malondialdehyde (MDA), an indication of lipid peroxidation, formed/mg protein.

2.5.1.2. Superoxide dismutase. Brain tissue samples were collected and prepared as described above (see *Brain tissue Preparation*). Percentage superoxide dismutase (SOD) inhibition was determined using a SOD Activity Assay Kit from Biovision™ and performed according to the manufacturer's protocol. Briefly, 10 mg wet weight of hippocampal tissue was used to prepare a homogenate for analysis. 20 µl of supernatant was pipetted into a 96-well plate containing 200 µl of water soluble tetrazolium (WST) working solution and 20 µl of enzyme working solution. Spectrophotometric analyses were performed at 450 nm in a microplate reader were done after incubating the plate at 37 °C for 20 min. Data was expressed as SOD activity (inhibition rate%).

2.5.1.3. BDNF. Brain tissue samples were collected and prepared as described above (see *Brain tissue Preparation*). Hippocampal brain-derived neurotrophic factor (BDNF) levels were determined using an enzyme-linked immunosorbent assay (ELISA) BDNF kit (Thermo Scientific) according to the manufacturers' protocol. Briefly, 10 mg of hippocampal tissue was used to prepare a homogenate as previously described by Kolbeck and colleagues [67]. The supernatant (centrifuged homogenate) was transferred to a 96-well plate coated with an anti-BDNF antibody (1:1000). Plates were incubated for 2.5 h at room temperature, after which the solution was discarded and the plate washed 4 times with 1 × wash buffer. Thereafter 100 µl of 1 × prepared biotinylated antibody were added to each well and incubated for 1 h at room temperature with gentle shaking. The wash step was repeated after incubation. 100 µl of Streptavidin-HRP solution were then pipetted to each well and incubated for 45 min with gentle shaking and the wash step repeated. Finally, 100 µl of tetramethyl benzidine (TMB) substrate

was pipetted into each well and incubated for 30 min at room temperature in a dark room with gentle shaking. Spectrophotometric analysis in a plate reader was carried out after a colour reaction with TMB and quantified at 450 nm. The results were obtained from a standard curve plotted according to the manufacturers' instructions, and expressed as pg/ml.

2.6. Statistical analyses

Normality of the data was determined using the Shapiro-Wilk test with $p > 0.05$ indicating that the assumption for normality had been violated. When comparing only two data points, the Student's *t*-test was used, regardless of normality distribution [68]. For multiple comparisons of data a non-parametric two-way ANOVA based on ranked data was performed if the assumption of normality was violated. In other instances, an ordinary two-way ANOVA was used, and when this analysis indicated interaction between the main factors (i.e. drug and exercise treatment, respectively), it was followed by the Dunnett or Tukey post-hoc test depending on the purpose of analysis. The Dunnett post-hoc test was used when the mean of all treatment groups were compared to that of the control group, whereas the Tukey post-hoc test was used for multiple comparison of all groups. Three-way analyses were not necessary, since the third main factor, namely age at PND35 vs. PND60 was not directly compared. When analysing the correlation coefficient, the Spearman's rank-order correlation test was performed when the assumption of normality had been violated. A 5% confidence limit for error was taken as statistically significant ($p \leq 0.05$). Finally, statistical analysis was followed by calculation of the Cohen's *d* value, in order to establish the practical significance of effect magnitude where no statistical significance was present, but a trend was apparent. Cohen's *d* value is an effect size used to indicate the standardized difference between two means, where effect sizes were considered large when $d \geq 0.8$, as reported by Cohen [69].

GraphPad Prism[®] version 6 (GraphPad Software, San Diego California USA, www.graphpad.com) was used for statistical analysis and graphical presentations, except for non-parametric statistical analyses when indicated, where IBM SPSS Statistics version 22 was used (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). All analyses were performed under the guidance of the statistical consultation service of the North-West University, Potchefstroom.

3. Results

3.1. Immediate effects

Fig. 1 represents the relationship between maximal treadmill speed and postnatal age in FSL rats.

The Shapiro-Wilk test indicates that the assumption of normality has been violated for all data sets ($p < 0.05$), except PND34, so that the non-parametric correlation test i.e. Spearman's rank-order correlation test was performed. The linear relationship between age and maximal speed within the measured age range is described by $y = 1.856x - 30.68$ (where *x* is the age in postnatal days and *y* is the maximal treadmill speed in m/min for 20 min to achieve VO_{2max}) with a strong positive correlation between maximal treadmill speed and age, $r_s(4) = 0.9429$. The slope of the regression line also significantly deviates from zero ($F(1,88) = 24.55$, $p \leq 0.0001$) and the unpaired Student's *t*-test indicates that the maximal treadmill speed increased significantly from PND21 (7.44 ± 1.02 m/min) to PND34 (36.20 ± 4.682 m/min) ($t(29) = 6.187$, $p \leq 0.0001$).

Fig. 2 represents the behaviour of FSL rats in open field test (OFT) and the forced swim test (FST) on PND35, following different intensities of pre-pubertal forced exercise.

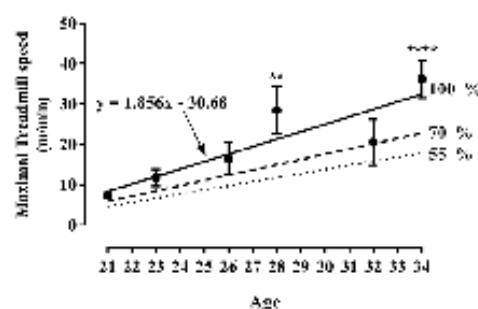


Fig. 1. Pilot study on indirect VO_{2max} across pre-pubertal development in FSL rats. Increase in maximal exercise intensity over time, represented by maximal treadmill speed in m/min from PND21 to PND34 in FSL rats, $n = 16$ rats per group, as non-runners were excluded. Data points are mean $\pm$ SEM and the line represent the correlation between speed and age, defined by the equation $y = 1.855x - 30.68$, with $r_s(4) = 0.9429$ (Spearman's rank-order correlation). ** $p \leq 0.01$ vs. PND21; **** $p \leq 0.0001$ vs. PND21. PND = postnatal day.

We confirmed that FSL rats display enhanced immobility in the forced swim test (FST), as compared to the FRL rat (192.8 ± 4.01 vs. 151.9 ± 5.3 s per 5 min; $p \leq 0.0001$); validating the FSL translational animal model under our experimental conditions (data not shown).

The Shapiro-Wilk's test indicates that the assumption of normality was true for all data sets in Fig. 2 ($p > 0.05$), so that the ordinary one-way ANOVA could be applied. In Fig. 2A no statistically significant differences or large effect sizes were observed in the number of line crossings in the OFT ($F(2,44) = 0.1547$, $p = 0.8572$, $d < 0.8$). In Fig. 2B an ordinary one-way ANOVA of the data indicates no statistically significant differences in immobility between treatment groups in the FST ($F(2,45) = 2.698$, $p = 0.0783$). However, Cohen's *d* value indicates a large effect size of low intensity exercise on immobility relative to that of sedentary controls (162.3 ± 6.7 vs. 138.9 ± 7.1 s; $d = 0.85$). In Fig. 2C no statistically significant differences or large effect sizes were observed in the climbing behaviour in the FST between any of the groups ($F(2,39) = 0.3149$, $p = 0.7317$, $d < 0.8$). In Fig. 2D an ordinary one-way ANOVA of the data indicates statistically significant differences between treatment groups ($F(2,39) = 6.957$, $p = 0.0026$). The Tukey's post-hoc test further indicates that only low intensity exercise altered swimming relative to the sedentary controls in the FST (48.4 ± 3.0 vs. 32.3 ± 2.6 s; $p \leq 0.01$).

Fig. 3 represents the behaviour of FSL rats in open field test (OFT) and the forced swim test (FST) on PND35, following different sub-chronic doses of fluoxetine.

The Shapiro-Wilk's test indicates that the assumption of normality was true for all data sets in Fig. 3 ($p > 0.05$), so that the ordinary one-way ANOVA could be applied.

In Fig. 3A an ordinary one-way ANOVA of the data ($F(2,19) = 3.931$, $p = 0.0373$) indicates statistically significant differences between treatment groups, with Tukey's post-hoc analyses indicating that 10 mg/kg/day fluoxetine significantly decreased the number of line crossings when compared to the vehicle control (123.7 ± 11.9 vs. 159.5 ± 10.0 ; $p \leq 0.05$). Furthermore, Cohen's *d* value indicates a large effect size of 5 mg/kg/day fluoxetine on the number of line crossings as compared to vehicle control (130.0 ± 6.7 vs. 159.5 ± 10.0 ; $d = 1.041$).

In Fig. 3B an ordinary one-way ANOVA of the data ($F(2,19) = 6.911$, $p = 0.0056$) indicates statistically significant differences regarding immobility between the various treatment groups. The Tukey's post-hoc analysis indicates that 5 mg/kg/day fluoxetine significantly decreased immobility in the FST at PND35 when compared to the vehicle control (148.4 ± 26.0 vs. 190.7 ± 9.7 s; $p \leq 0.05$).

Determining exercise intensity FSL rats (PND35)

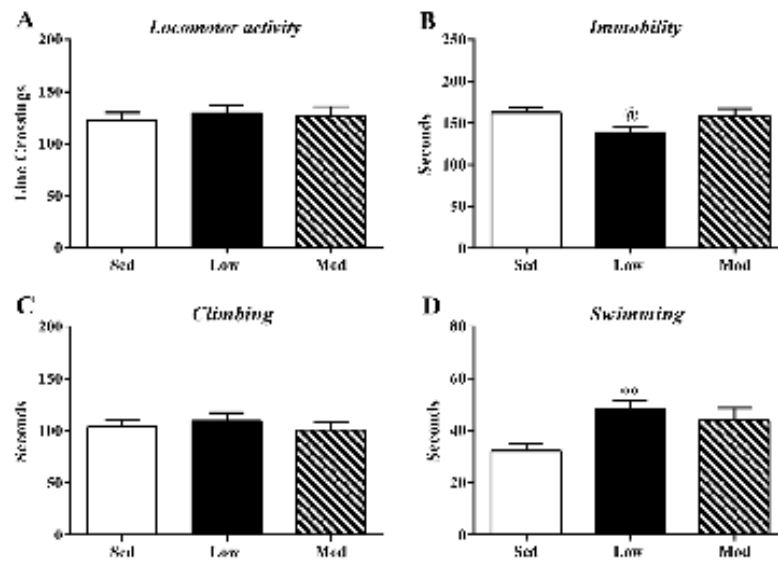


Fig. 2. Immediate effects of different exercise intensities on depressive-like behaviour and locomotor activity of FSL rats on PND35.

(A) Number of line crossings in the OFT after treatment with low ($n = 15$) and moderate intensity exercise ($n = 16$) when compared to a sedentary control ($n = 16$). (B) Immobility in the FST on PND35. (C) Climbing in the FST on PND35. (D) Swimming in the FST on PND35. Data points represent the mean ± SEM. Statistical analyses are reported in the text, with * $p \leq 0.05$ vs. control; # $p \leq 0.05$ vs. control. Sed: Sedentary. Low: Low intensity exercise (55% VO_{2max}). Med: Medium intensity exercise (70% VO_{2max}).

Determining fluoxetine dose FSL rats (PND35)

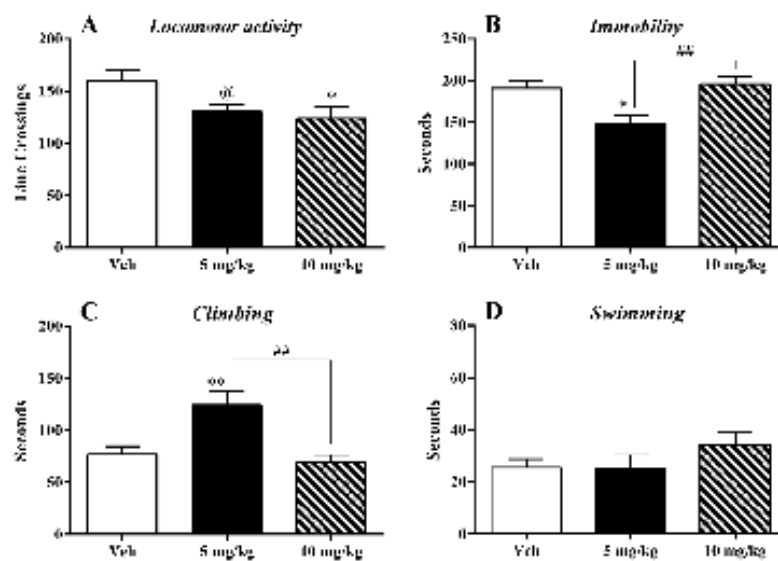


Fig. 3. Immediate effects of different fluoxetine doses on depressive-like behaviour and locomotor activity of FSL rats on PND35.

(A) Number of line crossings on PND35 in the OFT after 5 mg/kg/day (n = 7) and 10 mg/kg/day fluoxetine (n = 7) and saline (vehicle) treatment (n = 8). (B) Immobility on PND35. (C) Climbing in the FST on PND35. (D) Swimming in the FST on PND35. Data points represent the mean ± SEM. Statistical analyses are reported in the text, with * $p \leq 0.05$ vs. control; ## $p \leq 0.01$ vs indicated test group; # $p \leq 0.05$ vs control. 5 mg/kg: Low fluoxetine dose. 10 mg/kg/day: High fluoxetine dose. Veh: Vehicle saline control.

Also, immobility following 10 mg/kg fluoxetine was significantly enhanced than that observed with 5 mg/kg/day (195.4 ± 9.5 vs. 148.4 ± 26.0 s; $p \leq 0.01$), but not relative to vehicle control.

In Fig. 3C the ordinary one-way ANOVA of the data ($F[2,19] = 9.297$, $p = 0.0015$) indicates statistically significant differences regarding time spent climbing between the various treatment groups. The Tukey's post-hoc analysis indicates that 5 mg/kg/day fluoxetine significantly increased the time spent climbing in the FST at PND35 when compared to the vehicle control (124.4 ± 13.5 vs. 77.0 ± 7.4 s; $p \leq 0.01$). Furthermore, the time spent climbing was significantly less following treatment with 10 mg/kg/day, compared to 5 mg/kg/day fluoxetine (68.8 ± 7.5 vs. 124.4 ± 13.5 s; $p \leq 0.01$).

In Fig. 3D an ordinary one-way ANOVA of the data and calculation of Cohen's d -value ($F[2,19] = 1.431$, $p = 0.2637$, $d < 0.8$) indicated no significant differences in the swimming behaviour in the FST between any of the groups.

Fig. 4 represents the behaviour of FSL rats in open field test (OFT) and the forced swim test (FST) on PND35, following no pre-pubertal treatment or a combination of low intensity pre-pubertal forced exercise plus sub-chronic administration of 5 mg/kg/day fluoxetine.

The Shapiro-Wilk's test indicates that the assumption of normality was true for all data sets in Fig. 4 ($p > 0.05$), so that the unpaired Student's t -test could be applied.

In Fig. 4A, the unpaired Student's t -test indicates that the number of line crossing was unaffected by exercise plus fluoxetine, as compared to the sedentary plus vehicle control group (124.8 ± 7.5 vs. 113.6 ± 7.7 ; $t(22) = 1.038$, $p = 0.3105$, $d < 0.8$). In Fig. 4B, the combination of low intensity exercise plus 5 mg/kg/day fluoxetine significantly decreased immobility in the FST when compared to the sedentary plus vehicle control (142.3 ± 10.1 vs. 179.0 ± 8.3 s; $t(22) = 2.803$, $p \leq 0.0104$). In Fig. 4C the time spent climbing in the FST was unaffected by exercise plus fluoxetine, as compared to the sedentary plus vehicle control group (87.7 ± 5.7 vs. 77.8 ± 7.6 s; $t(22) = 1.040$, $p = 0.3095$, $d < 0.8$). In Fig. 4D the combination of low intensity exercise plus 5 mg/kg/day fluoxetine significantly increased the time spent swimming in the FST, compared to the sedentary plus vehicle control (67.7 ± 5.6 vs. 40.9 ± 5.6 s; $t(22) = 3.411$, $p = 0.0025$).

3.2. Lasting effects

Fig. 5 represents lasting behavioural effects in FSL rats in open field test (OFT) and the forced swim test (FST) on PND60, following either no pre-pubertal treatment, treatment with low intensity forced exercise, treatment with sub-chronic administration of 5 mg/kg/day fluoxetine, or treatment with the combination of low intensity forced exercise plus 5 mg/kg/day fluoxetine.

The Shapiro-Wilk's test indicates that the assumption of normality was true for all data sets in Fig. 5 ($p > 0.05$), so that the ordinary two-way ANOVA could be applied.

In Fig. 5A the two-way ANOVA ($F[1,44] = 0.1604$, $p = 0.6907$) of the data indicates no statistically significant interaction between drug treatment and exercise regarding the number of lines crossed in the open field test (OFT). Therefore, no post-hoc analyses could be performed. The main factor effect of fluoxetine treatment was significant ($F[1,44] = 7.346$, $p = 0.0095$), whereas the main factor effect of exercise was not ($F[1,44] = 2.622$, $p = 0.1132$). Cohen's d value indicates a large effect size difference of fluoxetine plus exercise on number of line crossings, compared to the sedentary plus vehicle control group (101.3 ± 8.2 vs. 132.3 ± 9.3 ; $d = 0.962$).

In Fig. 5B the two-way ANOVA of the data ($F[1,44] = 29.32$; $p \leq 0.0001$) indicates a statistically significant interaction between exercise and fluoxetine treatment interventions regarding immobility on PND60. The Tukey's post-hoc analyses for multiple

comparison indicates that low intensity exercise alone significantly decreases immobility in the FST as compared to the sedentary control plus vehicle control group (150.5 ± 5.9 vs. 193.8 ± 4.1 s; $p \leq 0.0001$). Administration of 5 mg/kg/day fluoxetine alone also reduces immobility, compared to the sedentary control plus vehicle control group (193.8 ± 4.1 vs. 162.1 ± 4.0 s; $p \leq 0.01$). However, the combination of low intensity exercise plus fluoxetine failed to significantly reduce immobility compared to the sedentary plus vehicle control group (183.5 ± 8.7 vs. 193.8 ± 4.1 s; $p > 0.05$). Also, immobility in the combination treatment was significantly more compared to exercise alone (183.5 ± 8.7 vs. 150.5 ± 5.9 s; $p \leq 0.01$). Furthermore, a large effect size (Cohen's d value = 1.026) is seen for the difference between the vehicle plus exercise group and the fluoxetine plus exercise group, whereas the effect sizes were small between other groups, thereby ruling out Type I (false positive) or II (false negative) errors [69,70] and supporting the calculated statistically significant differences.

In Fig. 5C, the two-way ANOVA of the data ($F[1,44] = 18.68$, $p \leq 0.0001$) indicates a significant interaction between exercise and the fluoxetine treatment interventions regarding climbing behaviour in the FST. The Tukey's post-hoc analysis for multiple comparison indicates that low intensity exercise alone significantly increases climbing behaviour in the FST, compared to the sedentary plus vehicle control group (88.3 ± 4.7 vs. 56.3 ± 3.2 s; $p \leq 0.001$). Administration of 5 mg/kg/day fluoxetine alone also increases climbing behaviour, compared to the sedentary plus vehicle control group (76.0 ± 5.0 vs. 56.3 ± 3.2 s; $p \leq 0.05$). However, the combination of exercise plus fluoxetine did not significantly alter the climbing behaviour, compared to the sedentary plus vehicle control group (67.4 ± 5.6 vs. 56.3 ± 3.2 s; $p > 0.05$). Also, the climbing behaviour of the combination of exercise plus fluoxetine was significantly reduced as compared to the sedentary plus vehicle control group (67.4 ± 5.6 vs. 88.3 ± 4.7 s; $p \leq 0.05$).

In Fig. 5D the two-way ANOVA ($F[1,44] = 4.316$, $p = 0.0436$) indicated a significant interaction between drug treatment and lifestyle intervention regarding swimming behaviour in the FST. However, the Tukey's post-hoc analyses indicated no significant differences in swimming behaviour between any of the treatment groups. Cohen's d value indicates a large effect size difference between the sedentary plus vehicle control group and the exercise alone group (46.6 ± 2.7 vs. 56.2 ± 3.0 s; $d = 0.913$).

Table 1 represents hippocampal levels of the respective biomarkers on PND61 in FSL rats, before and after the indicated pre-pubertal treatment interventions.

According to the Shapiro-Wilk's test as an analysis of variance for normality, the assumption of normality was met for BDNF and MDA, but not for SOD data set. Non-parametric analysis was therefore applied to the SOD data. Regarding BDNF levels the two-way ANOVA of the data ($F[1,35] = 6.030$, $p = 0.0192$) indicates a significant interaction between exercise and fluoxetine treatment interventions on PND61. The Tukey's post-hoc analyses for multiple comparison indicates that there were no significant differences between the different treatment regimens and the sedentary plus vehicle control group. However, exercise alone significantly increased hippocampal BDNF levels, compared to exercise plus fluoxetine ($27,440 \pm 823.9$ vs. $24,767 \pm 354.1$ pg/ml; $p \leq 0.05$). No differences in hippocampal MDA levels were determined between any of the treatment groups. Hippocampal SOD concentrations were significantly increased later in life, compared to the vehicle plus sedentary controls for, all treatment groups.

4. Discussion

In the current study maximal exercise intensity (or $\dot{V}O_{2max}$) increased directly proportional to age across pre-pubertal devel-

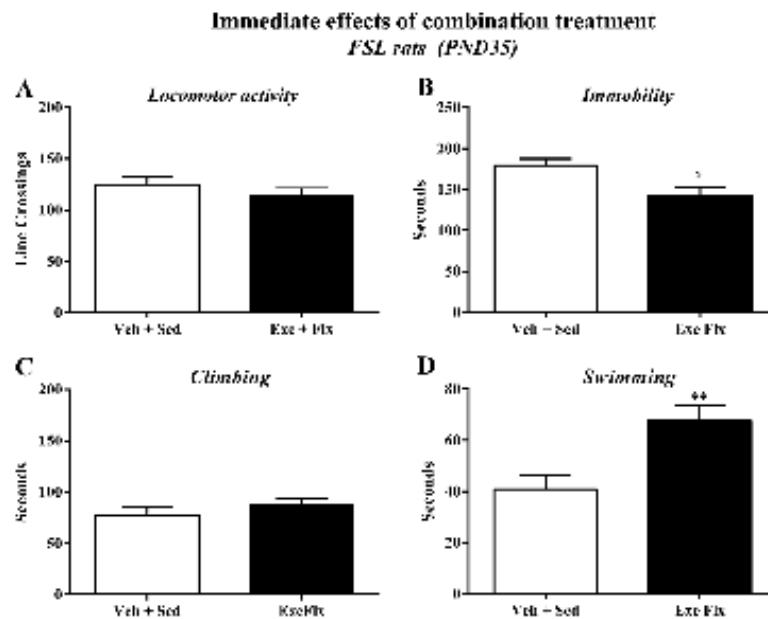


Fig. 4. Immediate effects of combination therapy on depressive-like behaviour and locomotor activity of FSL rats on PND35.

(A) Number of line crossings in the OET on PND35 after treatment with low intensity exercise ($n=12$), fluoxetine 5 mg/kg/day ($n=12$) and the augmentation of fluoxetine 5 mg/kg/day with low intensity exercise ($n=12$) compared to the vehicle control ($n=12$). (B) Immobility in the FST on PND35. (C) Climbing in the FST on PND35. (D) Swimming on PND35 in the FST. Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text, with * $p \leq 0.05$ vs. control, ** $p \leq 0.0001$ vs. control. Exe: Exercise (low intensity), Ftx: Fluoxetine (5 mg/kg/day), Sed: Sedentary, Veh: Vehicle saline control.

Table 1

Biomarkers on PND61 in pre-pubertal FSL rats treated with low intensity exercise, fluoxetine 5 mg/kg/day and the combination of fluoxetine and exercise.

	Vehicle			Fluoxetine		
	Sedentary	Exercise		Sedentary	Exercise	
BDNF	25403 $\pm$ 647.9	25917 $\pm$ 672.6	ns	27440 $\pm$ 823.9	24767 $\pm$ 354.1	#
MDA	12.6 $\pm$ 1.3	15.1 $\pm$ 0.8	ns	13.3 $\pm$ 0.9	12.6 $\pm$ 0.9	ns
SOD	73.8 $\pm$ 3.4	90.2 $\pm$ 1.1	****	90.2 $\pm$ 1.73	87.3 $\pm$ 2.4	**

(BDNF): Brain-derived neurotrophic factor. Hippocampal levels of BDNF (pg/ml) on PND61, after 27 days washout following treatment with low intensity exercise plus vehicle ($n=10$) fluoxetine 5 mg/kg/day plus sedentary ($n=10$) and the combination of exercise and fluoxetine ($n=10$) when compared to a vehicle plus sedentary control ($n=10$) as measured with a Rat BDNF ELISA kit (Thermo Scientific). (MDA): Malondialdehyde nmol/mg tissue on PND61, indicating levels of hippocampal lipid peroxidation. (SOD): Hippocampal SOD activity (SODX inhibition) on PND61. Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text, with ns= non-significant ** $p \leq 0.01$ vs. control (i.e. vehicle sedentary), *** $p \leq 0.001$ vs. control, **** $p \leq 0.0001$ vs. control, # $p \leq 0.05$ vs. test group (i.e. vehicle exercise).

opment of the FSL rat (Fig. 1). This is supported by the strong linear correlation between maximal treadmill speed (m/min) and age (PND) as described by the equation provided, a statistically significant increase in maximal intensity from PND21 to PND34, and the slope of the linear relationship that significantly deviates from zero. This finding highlights the need to adapt the running speed for the targeted exercise intensity according to age, rather than keeping it constant throughout treatment, as done in previously reported studies [28,51,71,72]. VO_{2max} , an accurate predictor of athletic/exercise ability limit [73–75], is also different in humans for children and adults [76], so that this result is not entirely unexpected. To our knowledge, this is the first time that an age-related exercise regimen has been developed in pre-pubertal rodents.

The data obtained from the FST suggest a low for antidepressant-like behaviour following low (55% VO_{2max}) pre-pubertal exercise for 14 days (Fig. 2B), without corresponding change in locomotor activity (Fig. 2A), suggesting altered psychomotor activity. The latter seems related to enhanced serotonergic activity (swimming behaviour – Fig. 2D) and not to altered

noradrenergic activity (climbing behaviour – Fig. 2C), although this needs confirmation by follow-up neurochemical studies. No antidepressant-like behaviour was apparent following moderate (70% VO_{2max}) intensity pre-pubertal exercise (Fig. 2B). Our findings are in line with a previous report of antidepressant-like effects of low intensity treadmill exercise from PND21–30 in Sprague Dawley rats [72], as well as several other reports suggesting beneficial effects of low intensity exercise on physiological and psychological resilience. Hence, so that low intensity exercise, more than moderate and high intensity exercise, has been recommended as the most desirable approach to study antidepressant-like effects in rodents [28,51,71,72] and thus represents a possible augmentative strategy in the treatment of depression. Current studies in our laboratories are underway to investigate the role of genetic susceptibility in the differential effects of non-pharmacological interventions in stress-sensitive FSL versus control FRL rats, particularly since few studies suggest a role for genetic susceptibility [25,77,78].

Interestingly, we found that only low-dose (5 mg/kg/day), and not the higher dose of 10 mg/kg/day fluoxetine, significantly

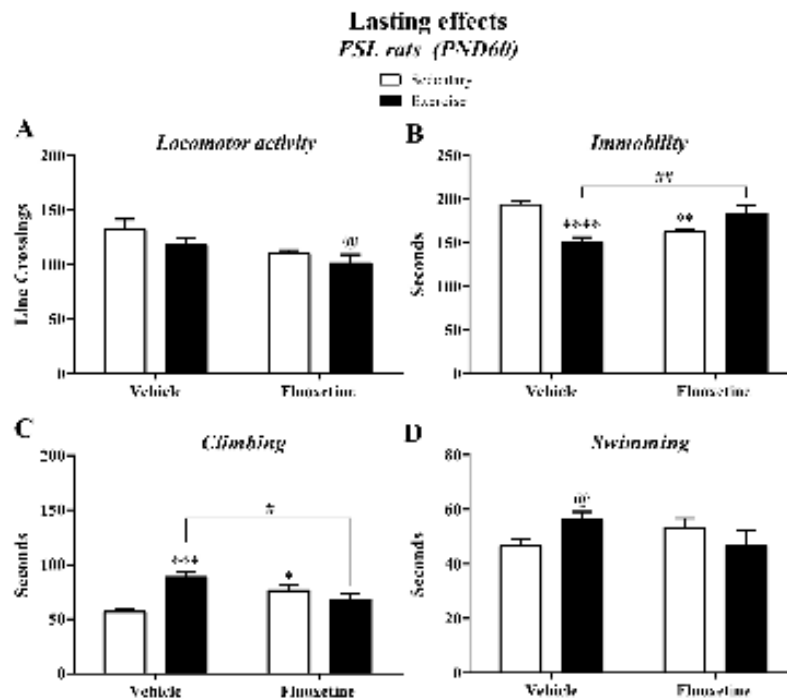


Fig. 5. Lasting effects on behaviour of FSL rats on PND60 after pre-pubertal treatment with low intensity exercise, fluoxetine 5 mg/kg/day and the combination of fluoxetine with exercise.

(B) Number of line crossings in the OFT on PND60, after 26 days washout following pre-pubertal treatment with low intensity exercise ($n = 12$), fluoxetine 5 mg/kg/day ($n = 12$) and the augmentation of fluoxetine 5 mg/kg/day with low intensity exercise ($n = 12$) compared to the vehicle control ($n = 12$). (B) Immobility in the FST on PND60. (C) Climbing in the FST on PND60. (D) Swimming on PND60 in the FST. Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text, with * $p \leq 0.05$ vs. control, **** $p \leq 0.0001$ vs. control, # $p \leq 0.05$ vs. indicated test group, ## $p \leq 0.01$ vs. indicated test group; @ $d \geq 0.8$ vs. control.

decreased immobility in the FST in pre-pubertal rats on PND35 (Fig. 3B). In this regard, noradrenergic neurotransmission (climbing behaviour – Fig. 3C), and not serotonergic neurotransmission (swimming behaviour – Fig. 3D), was significantly increased by the low, but not the high dose of fluoxetine. The decreased depressive-like behaviour induced by the low dose fluoxetine is supported by previous findings that 5 mg/kg/day fluoxetine is sufficient to inhibit the serotonin transporter in juvenile rats [79,80]. However, the increase in noradrenergic-associated climbing behaviour and not swimming behaviour (serotonergic), as observed in the current study, was unexpected and warrants future investigation, e.g. to assess central monoamine levels and receptor density. Nevertheless, increased noradrenaline-mediated suppression of 5-HT release via α_2 -heteroreceptors may explain this phenomenon [81,82]. A previous study [83] showed that PND35-old FSL rats have significantly lower concentrations of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5HIAA) compared to either adult or new-born FSL rats, yet still displayed depressive-like symptoms at any of the three ages. Moreover, Sprague-Dawley and FSL rats at PND35-old display different concentrations of 5HT and 5HIAA and in the nucleus accumbens [83], all demonstrating that the status and profiles of monoaminergic systems may be significantly different in pre-pubertal versus adult animals. Also of note, locomotor activity (Fig. 3A) was significantly reduced following the higher dose of fluoxetine, with only a trend following the lower dose, which may have blunted any antidepressant-like activity (Fig. 3B) by the higher dose fluoxetine. Rats typically receive a 10-times higher dose of fluoxetine than humans, due to increased

liver metabolism, so that a dosage dose of 20 mg/day in humans (i.e. 0.3–0.9 mg/kg/day) [10,72], corresponds to 3–9 mg/kg/day in rodents (i.e. the lower dose of 5 mg/kg/day in the current study). The higher dose of 10 mg/kg/day fluoxetine in rodents would therefore correspond to a dose of about 40 mg/day in humans, which may be associated with more locomotor effects [84]. Hence, this may also explain the significantly decreased number of line crossings in the OFT (Fig. 3A) as well as unaltered depressive-like behaviour (Fig. 3B) [60] in the current study. In light of these findings, we used the lower dose of fluoxetine in the main study (see discussion of Fig. 5 below).

Low-dose fluoxetine plus low intensity exercise promoted significant antidepressant-like behaviour (immobility – Fig. 4B) immediately following the sub-chronic intervention (i.e. at PND35). Whereas the monotherapy of low intensity exercise and low dose fluoxetine seemingly enhanced serotonergic and noradrenergic neurotransmission, respectively (Figs. 2 D and 3 C), the combination therapy only increased serotonergic neurotransmission (swimming behaviour – Fig. 4D) and not noradrenergic neurotransmission (climbing behaviour – Fig. 4C). These observations require confirmation via assessment of central monoamine levels and receptor density. Indeed, the abrogating effect of serotonergic agents, especially SSRIs, on the release of frontal cortical noradrenaline is a well-described phenomenon [81]. That fluoxetine plus exercise did not change (in particular did not enhance) locomotor activity (Fig. 4A), confirms that the observed effect in the FST is related to a psychomotor and not a locomotor effect. This finding is in line with several previous reports of the beneficial

effects of exercise and antidepressant combination treatments in adult rodents [85,86], human adolescents [87] and human adults [24,25].

Based on the results from the immediate effects of pre-pubertal exercise and fluoxetine, we selected low intensity exercise, 5 mg/kg/day fluoxetine and the combination thereof for studying long-lasting effects into adulthood. Immobility data on PND60 (Fig. 5B) demonstrates that low intensity pre-pubertal exercise alone exerts long-lasting anti-depressive-like effects into early adulthood and remarkably so following 26 days of normal housing without intervention. The decreased immobility was not accompanied by corresponding change in locomotor activity (Fig. 5A), suggesting that the effect reflects psychomotor (i.e. antidepressant) and not locomotor effects. Furthermore, this effect seems to be attributed to long-term enhanced noradrenergic neurotransmission (evinced by increased climbing behaviour, Fig. 5C) [88], as well as a trend for increased serotonergic neurotransmission (evinced by a trend for increased swimming behaviour, Fig. 5D) [89]. These results warrant further investigation into how this may relate to corresponding changes in monoamine neurotransmission and levels. Earlier preclinical work in adult Wistar rats, has shown that increased chronic physical activity may lead to enhanced serotonin neurotransmission, specifically by increasing tryptophan hydroxylase-2, the rate-limiting enzyme of serotonin synthesis [90], yet increased noradrenaline and dopamine has also been suggested [24,91]. Nevertheless, the latter were observed immediately after completion of the exercise regimen, and not following a chronic sedentary period as in the current study. Pre-pubertal exercise may thus modulate neurodevelopment in such a manner that it has long-lasting effects on other developing neurotransmitter systems [92,93]. To our knowledge, this is the first report of long-lasting beneficial effects of pre-pubertal exercise into early adulthood, and with translational relevance in a genetic rodent model of depression. Whereas current systematic reviews show a small benefit in favour of physical activity, compared to sedentary behaviour, and no benefit over pharmacological therapies [94,95], the current study may suggest as a working hypothesis, for further investigation, that pre-adolescent, low intensity exercise in humans may offer significant lasting antidepressant-like effects, manifesting later in life.

Similarly, pre-pubertal exposure to sub-chronic low-dose fluoxetine in the current study significantly decreased depressive-like behaviour in early adulthood, following a 26-day washout period (Fig. 5B), that could also be attributed to enhanced noradrenergic, but not serotonergic activity (Fig. 5C and D), similar to what was observed immediately after intervention on PND35 (Fig. 3C). This was again unexpected, since fluoxetine as an SSRI, is expected to enhance serotonergic neurotransmission [96], and thus swimming behaviour. However, an earlier report demonstrated unaffected swimming behaviour in adult Sprague-Dawley rats directly following chronic fluoxetine treatment [89], however require further investigation and confirmation by neurobiological analyses. Early-life serotonergic stimulation (PND25–39) has been shown to increase concentrations of the serotonin transporter in the frontal cortex later in life (PND90), but not of the noradrenaline transporter [80]. In fact, increased serotonergic transporter-mediated lowering of synaptic levels of serotonin, or increased α_2 -heteroreceptors mediated suppression of 5-HT release [81] may explain why only noradrenergic neurotransmission is up-regulated by pre-pubertal fluoxetine. Recent data from our laboratory suggest that in FSL rats pre-pubertal fluoxetine exposure, followed by a washout period, suppresses stressor-induced release of serotonin and norepinephrine in the prefrontal cortex, yet increase basal dopamine concentrations [97]. Taken together, these results suggest that early-life stimulation of a specific neurotransmission system may indeed affect neurotransmitter

function and responses later in life, where such lasting effects may be attributed to fluoxetine-induced dysregulation by serotonergic neuron projections during pre-pubertal development [80]. Indeed, others have reported serotonergic outgrowth to be inhibited by elevated serotonin concentrations, especially when increased during early-life development [92]. Moreover, chronic SSRI administration has been reported to desensitize the 5-HT_{1A} auto receptors [98,99], responsible for decreasing net 5-HT release via a negative feedback mechanism [98], in adult rodents but not juveniles [100], suggesting that underlying mechanisms differ in the juvenile brain, compared to the adult brain, and warrants further investigation.

The combination of fluoxetine plus exercise, however, did not affect immobility or coping behaviours in the FST on PND60, compared to the control groups (Fig. 5B–D). Yet the main factor effect of fluoxetine (from the two-way ANOVA) and large effect size in Fig. 5A indicates that fluoxetine overall reduces locomotor activity, which could potentially have blunted any antidepressant-like behaviour (reduced immobility) in the fluoxetine plus exercise group (Fig. 5B). Alternatively, the “equal but opposite” hypothesis proposes that an over sensitized response (resulting from exercise plus fluoxetine) in early-life could lead to an opposite outcome in adulthood [13,101]. Furthermore, that both low intensity exercise alone and fluoxetine alone significantly increased the climbing behaviour of the animals, yet combination therapy did not significantly affect this behaviour is of particular interest. In fact, the climbing behaviour of the fluoxetine plus exercise group was significantly decreased compared to the exercise alone group (Fig. 5C), suggesting that fluoxetine reversed the exercise-induced effect on climbing behaviour. The “neural Darwinism” theory, postulates that the brain “selects” to retain those synapses into adulthood that allow adaptation to the required environmental needs, by controlling the natural synapse pruning process [102]. In this regard, the pre-pubertal central nervous system is now subjected to two interventions with possible lasting effects on the noradrenergic system, and therefore any further development of this system is arrested [13]. Therefore, would this theory be applicable to the data in the current study, it may explain the decreased noradrenergic-related behaviour (climbing) of the combination therapy group, compared to the exercise alone group. The status of monoaminergic neurotransmission in this treatment group needs further investigation.

Increased immobility in the FST and reduced exploratory behaviour have also been reported with early-life (PND0–21) increase in central serotonin [16,17], as would be expected from fluoxetine and exercise treatment [24,25,60,62]. This has also been associated with increased anxiety, reduced aggression, increased REM sleep and anhedonia, as well as decreased impulsivity and improved learning and memory [17]. That serotonergic neurodevelopment achieves functional maturity earlier than noradrenergic neurodevelopment [5] has been proposed to explain why SSRIs, but not tricyclic antidepressants, are effective in treating depression in children and adolescents. Data of the current study furthermore support the idea that noradrenergic neurodevelopment may be modulated by serotonin, explaining the observed long-term effects of SSRIs in early-life. Moreover, the integral role of serotonin in neurodevelopment, reaching peak levels in early-life under normal conditions (PND21) [92], may prove vital in understanding the observed lasting effects of pharmacologically elevated serotonin levels. In particular, pre-pubertal modulation of serotonin levels may also affect the development and maturation of other monoamine systems [5,103], whereas the degree of modulation of serotonin may also affect outcome, as demonstrated by the differential outcomes in the current study between exercise alone, fluoxetine alone and the combination therapy.

That MDD is associated with impaired hippocampal function, is well described [104,105]. The impact of MDD on the hippocampus is most prominent during early-life when the developing

brain is particularly vulnerable [104] to insults such as oxidative stress [106], and susceptible to alterations in neurotrophins such as BDNF [107]. Yet these effects have been reportedly reversed by fluoxetine or exercise during adulthood [22,25,26]. In the current study exercise or fluoxetine alone did not alter BDNF levels (Table 1), suggesting that neurotrophic factors do not explain the lasting antidepressant-like effects observed in the current study. However, the combination of pre-pubertal exercise plus fluoxetine induced a significant long-lasting decrease in hippocampal BDNF levels (Table 1), which may suggest a neurobiological basis for a putative blunting effect on any antidepressant-like effects of the combination therapy. Previous reports have shown BDNF levels to be increased by both exercise [28,51,85,108,109] and fluoxetine [110,111] in both juvenile and adult animals, although measured as immediate effects after the intervention. Another study showed that the combination of exercise plus fluoxetine increases hippocampal BDNF mRNA levels to a greater extent than either intervention alone [85,86], but again these data do not reflect lasting effects into adulthood. In fact, the increase in BDNF following chronic exercise has been shown to last a maximum of 14 days, before returning to baseline [112], which could also explain why our data did not demonstrate any long-lasting changes in BDNF levels following pre-pubertal treatments.

Table 1 also suggests that neither pre-pubertal exercise, fluoxetine nor the combination of exercise plus fluoxetine significantly altered hippocampal peroxidation levels (MDA in nmol/mg) later in life, suggesting that none of the interventions induced long-lasting hippocampal oxidative damage. This is of note since exercise, specifically short bouts of high intensity, has previously been reported to increase free radical production to such an extent that antioxidant defences are overwhelmed, resulting in lipid peroxidation damage [113]. Yet, short periods of low intensity exercise seems to reduce plasma levels of MDA [114], while chronic periods of exercise appears to inhibit free radical concentration and protein or lipid damage increases [19,41]. Furthermore we did not observe any differences in hippocampal MDA in the fluoxetine treated group (Table 1), although studies in humans have found fluoxetine to decrease levels of MDA immediately following sub-chronic treatment [115], while others have reported antidepressant withdrawal to induce oxidative stress damage [116]. Finally, disturbances in SOD activity are generally found in depressed patients, suggesting that increased SOD concentrations play an important role in preventing oxidative damage [117], as well as exert antidepressant-like properties in rats [118]. In this regard, SOD was found to be increased in all of the treatment groups later in life in the current study (Table 1), compared to the sedentary controls and may therefore indicate towards a long-lasting increased anti-oxidant capacity following either SSRI treatment [115,119] or exercise [120]. The increased hippocampal SOD concentrations in both the exercise alone and fluoxetine sedentary groups support the lasting decreased depressive-like behaviour observed in the FST (Fig. 5B).

5. Conclusion

Long-lasting effects of early-life treatment have become a great concern in recent years as several studies have found paradoxical outcomes in both neurology and behaviour in adulthood after early-life interventions. The current study suggests that the timing, as well as the intensity or dose of the intervention may be a critical factor to determine long-lasting outcome, including whether the early-life intervention has beneficial or detrimental effects into adulthood. As such, the pre-pubertal exercise intensity and fluoxetine dose determined behavioural outcomes in early adulthood, and furthermore low intensity exercise or fluoxetine alone, but not the combination thereof, yielded long-lasting antidepressant-like

effects under our experimental conditions. That the combination thereof under our experimental conditions was pro-depressant is a startling revelation that warrants further investigation. The study also supports the idea that pre-pubertal modulation of serotonin may affect the neurodevelopment of other monoaminergic systems, in particular that of the noradrenergic system, to explain long-lasting effects.

Lastly, the findings of the current study highlight the need for pre-clinical studies on the effect of exercise in rodents, to adapt exercise intensity according to age, rather than keeping it constant throughout development.

Prospective studies should investigate long-lasting neurobiological effects of pre-pubertal fluoxetine and exercise, in particular its modulating effects on dopaminergic, serotonergic and noradrenergic neurotransmission, in order to elucidate the observed differential effects and associated mechanisms. The role of genetic susceptibility to depression should also be investigated.

Declaration of interest

There is no actual or potential conflict of interest in relation to this article to declare.

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CHAPTER 4: MANUSCRIPT B

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Article to be submitted to *Behavioural Brain Research* titled:

The lasting augmentation of antidepressive-like bio-behavioural effects following pre-pubertal escitalopram and omega-3 supplementation in stress-sensitive rats

AUTHOR CONTRIBUTIONS

Stephanus F. Steyn assisted in designing the current project. SF Steyn also conducted all bio-behavioural experiments, performed the data work-up and statistical analyses, assisted in the interpretation of the study data, wrote the first draft of the manuscript and finalized it for submission.

Brian H. Harvey advised on the study design and proofread the final manuscript.

Christiaan B. Brink designed and supervised the study and assisted in the interpretation of the study data, finalized the manuscript for publication and was corresponding author in the submission of the final manuscript to *Behavioural Brain Research*.

IMPORTANT INFORMATION

- Instructions to the author can be viewed online at <https://www.elsevier.com/journals/behavioural-brain-research/0166-4328/guide-for-authors>.
- As per the instructions to the author, figures and legends are provided at the end of the manuscript.
- Consent for the manuscript to be assessed as part of the PhD thesis of SF Steyn is presented in Addendum C.

Sustained augmentation of antidepressive-like bio-behavioural effects following pre-pubertal escitalopram and omega-3 supplementation in stress-sensitive rats

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Highlights

- ω -3 EFA supplementation augments the antidepressive-like effects of sub-therapeutic escitalopram treatment.
- The combination of escitalopram and ω -3 EFA supplementation exerts long-term neurodevelopmental antidepressant-like behaviour into early-adulthood, following a chronic wash-out period.
- Improved depressive-like behaviour of the combination treatment strategy was observed despite reduced early-adulthood locomotor activity.
- Pre-pubertal ω -3 EFA supplementation might induce neurodevelopmental coping mechanisms, lasting into early-adulthood.

Keywords

Escitalopram; Omega-3; Pre-pubertal treatment, Lasting effects, Augmentation, Flinders Sensitive Line rats

Abstract

Major depressive disorder (MDD) affects a significant number of children and adolescents, yet treatment options for this population remain very limited. Escitalopram (ESC) is one of only two antidepressants approved as treatment for juvenile depression. Still, delayed onset of action, and immediate and possible lasting side effects contribute to low patient adherence, and places the medical prescriber in a difficult situation weighing the potential long-term effects of juvenile treatment against the known consequences of untreated MDD. Research into alternative or augmentation strategies and their long-term effects are needed to improve clinical outcome and better our understanding of the long-term consequences of early-life treatment. We investigated the early-life (postnatal day 35 (PND35))

and lasting (PND60) bio-behavioural effects of pre-pubertal (PND21 to PND34) escitalopram (ESC) and/or ω -3 supplementation (OM3) in stress sensitive Flinders Sensitive Line rats. Only ESC treatment showed a strong trend to decrease depressive-like behaviour via significantly increased climbing behaviour. However, OM3 treatment reduced PND35 locomotor activity and increased hippocampal neuroplasticity, suggesting improved coping behaviour and masking of possible antidepressant-like effects. Reduced locomotor activity lasted into early-adulthood, despite a chronic wash-out period. Regardless, early-adulthood antidepressant-like behaviour was only observed in the combination treatment (ESC+OM3) group, despite a significant increase in serotonin turnover, suggesting strong neurodevelopmental process to be involved. Taken together, the combination of ESC and OM3 might induce beneficial neurodevelopmental effects in a stress-sensitive population, suggesting a possible role in current treatment strategies.

1 Introduction

Major depressive disorder (MDD) is estimated to become the second leading cause of disability by the year 2020 [1] and the leading cause by 2030 [2]. The epidemiological backdrop is further fraught by increased awareness and diagnosis of juvenile depression, as well as limited treatment options and concerns about acute and long-term untoward effects. An estimated 10 % of adolescents, 2.8 % of children and 0.3 % of pre-schoolers suffer from MDD [3-7], resulting in a significant increase in missed school days and overall academic decline [8], altogether contributing to the alarming economic burden of juvenile MDD [9]. Furthermore, depressed children demonstrate a 40 % recurrence rate after two years and 70 % after five years [10-12] as well as an overall increased risk to develop treatment resistant depression in later in life [12-17]. MDD is strongly associated with the risk of suicide, and this is of particular concern in juvenile patients. In fact, suicide is the fourth and second leading causes of death in children [18] and adolescents, respectively [19], with 35% of juvenile suicides positively associated with MDD [20]. Therefore, the short- and long-term consequences of untreated juvenile MDD are of equal concern, necessitating novel treatment strategies.

Research into the pathophysiology of juvenile MDD is limited due to complications in undertaking extensive bio-behavioural investigations in this population. Nevertheless, evidence from pre-clinical and limited clinical investigations suggest several similarities between juvenile and adult depression. However, only fluoxetine and escitalopram, both selective serotonin reuptake inhibitors (SSRIs), have been shown to be effective and have been approved for the treatment of juvenile MDD [21]. On the other hand, noradrenaline selective antidepressants are ineffective in this group of patients [22], suggesting that impaired serotonergic neurotransmission contributes significantly to the pathophysiology of juvenile MDD. This selective response to serotonergic antidepressants has been partly explained by the earlier maturation of the serotonergic neurotransmitter system, compared to that of the noradrenergic system [22]. Yet, even the SSRIs have a black-box warning indicating an increased

risk of suicidal behaviour in children and adolescents with onset of treatment. This may place the prescribing medical practitioner in a particular difficult situation weighing the possible harmful effects of early-life antidepressant treatment against concerns of immediate and long-term consequences of untreated juvenile MDD [23]. Furthermore, whether and to what extent early-life antidepressant treatment may impact susceptibility to depressive symptoms in adulthood, remains unclear.

Alternative or augmentation treatment strategies to conventional antidepressants in the juvenile population are therefore of interest. In this regard, the World Health Organization (WHO) recommends that non-pharmacological interventions be implemented as first line therapy for mild depression in juveniles, whereas pharmacological antidepressants be initiated in moderate to severe cases [19]. Dietary adjustments, such as omega-3 essential fatty acid (ω -3 EFA) supplementation represents such a non-pharmacological treatment strategy, with suggested potential benefits in depression. To this extent, a recent Cochrane review suggest ω -3 EFA supplementation to have small-to-modest benefits over that of placebo, and comparable effects to that of pharmacological antidepressants [24]. Furthermore, ω -3 EFA supplementation also shows promise in treating depressed adolescents, unresponsive to SSRI treatment [25]. However, the exact mechanism of action of this dietary intervention strategy remains unclear, although, increased monoamine concentrations [26, 27], enhanced neuroplasticity [28, 29] and decreased central inflammation [30, 31] and oxidative stress damage [32, 33], have been suggested to underlie the effectiveness of ω -3 EFA supplementation. That ω -3 polyunsaturated fatty acids (PUFAs), specifically docosahexaenoic acid (DHA), constitute a significant amount of total brain fatty acids [34, 35] and play a key role in neuronal growth [36], could present a relatively unexplored treatment strategy with potentially long-term benefit [37]. However, its effectiveness in juveniles is not fully explored or understood while there is uncertainty regarding its long-term impact on the developing brain.

Recent studies in our laboratory have demonstrated that prenatal and shortly after birth antidepressant treatment induces lasting effects later in life [38-40]. Thus pre-puberty represents a developmental period that is both susceptible to external insults and amenable to pharmacological intervention with long-lasting effects. The current study therefore investigated the immediate and lasting effects of sub-chronic pre-pubertal (PND21 to PND34) escitalopram, ω -3 EFA supplementation, and the combinations thereof on depressive-like behaviour in a genetic animal model of depression, i.e. the Flinders Sensitive Line (FSL) rat. Immediate effects were measured directly following the pre-pubertal treatment, whereas the lasting effects were investigated in early adulthood (PN day?), following a 26-day washout/cessation period. Importantly, the effects of ω -3 EFA supplementation were compared to that observed with a standard ω -3 EFA sufficient diet, and not to a deficient diet. Furthermore, the treatment strategies were investigated only in FSL rats, so that we did not investigate the role of genetic

susceptibility by comparing the responses to those in healthy control animals (i.e. Flinders resistant line, FRL rats).

2 Materials and methods

2.1 Test subjects and treatment strategies

2.1.1 Animals

Male Flinders Sensitive Line (FSL $n = 91$) rats were bred, supplied and housed at the vivarium (SAVC reg. no. FR15/13458; SANAS GLP compliance no. G0019; AAALAC accreditation international file #1717) of the Pre-Clinical Drug Development Platform (PCDDP), NWU, RSA. The original rat colonies were obtained from Dr David H Overstreet, University of North Carolina, Chapel Hill, North Carolina, USA. The FSL rat is a validated genetic animal model of depression, displaying face, construct and predictive validity [41, 42]. Importantly, the exaggerated 8-hydroxy-2-(di-N-propylamino)tetralin (8-OH-DPAT) induced hypothermic response in FSL rats [43], as well as enhanced immobility in the forced swim test, relative to Flinders Resistant Line (FRL) rats were confirmed at the start of the study.

All animals were maintained and all procedures performed in studies involving animals were in accordance to the code of ethics in research, training and testing of drugs in South Africa and complied with national legislation. Animals were randomly divided into four test groups: **1)** vehicle control plus standard diet (**SAL+STD**), **2)** escitalopram plus standard diet (**ESC+STD**), **3)** vehicle control plus ω -3 EFA supplemented diet (**SAL+OM3**), **4)** escitalopram plus ω -3 EFA supplemented diet (**ESC+OM3**) with approximately 12 rats per group. Rats were group-housed three per cage, with corncob bedding changed weekly and the environmental temperature maintained at 22 ± 1 °C and a relative humidity of 55 ± 10 %. A 12-hour light/dark cycle was followed with water provided *ad libitum* and 200 g rat chow (either normal rat chow or ω -3 EFA supplemented) provided daily. Pups were weaned on postnatal day 21 (PND21) and body weight was measured daily during treatment from PND21 until PND34. Furthermore, animals were handled daily to habituate them to human contact from PND16. From PND35 until PND60 rats were housed under normal conditions, without any treatment. Animal wellbeing was routinely monitored on specially developed monitoring sheets throughout the study, and were monitored during and after each injection.

The period between PND21 to PND34 represents pre-puberty in rodents when the serotonergic pathway is already matured, while the noradrenergic and dopaminergic pathways are still developing [22, 44]. After treatment, rats were either submitted to behavioural analyses on the following day (PND35; early-life) or left in normal housing conditions with normal rat chow during a period of 26 days of neurodevelopment and maturing, serving also as a treatment washout/cessation, until behavioural

analyses on PND60 (early adulthood). Animals were sacrificed by decapitation within 24 hours following completion of behavioural analyses, where frontal cortices and hippocampi were dissected and stored for neurobiological analyses.

2.1.2 Drug treatment

Animals received either saline (vehicle) or escitalopram oxalate (10 mg/kg) (a gift from Jade Pharmaceuticals, RSA) via subcutaneous (sc) administration once daily from PND21 to PND34. Dosage was selected in line with a previous report indicating higher (i.e. 10 mg/kg/day), and not lower acute doses of escitalopram to be effective in inducing antidepressant-like behaviour in pre-adolescent rats [45]. Furthermore, daily 10/mg/kg/day may represent a sub-therapeutic dose [46], thereby allowing us to investigate true augmentation properties of escitalopram and ω -3 EFA supplementation. Nevertheless, the subcutaneous (sc) route of administration has a predictable bioavailability comparable to that of an intraperitoneal injection, although injection stress is less, particularly in young rats, and was therefore used in the current study, in line with previous work [38, 40].

2.1.3 Omega-3 supplementation, daily food intake and content analysis

Normal vivarium rat chow was coated with omega-3 essential fatty acid oil (a gift from Nordic Naturals, USA; ProOmega[®], batch: 153003). Briefly, a 10 % (v/v) ω -3 EFA oil and chloroform solution was made and then sprayed onto the rat chow by an automatic spray gun used for tablet coating. Following formulation, the coated rat chow was stored in an air tight container in a refrigerator (8 °C) until use. Daily food intake per animal was calculated by weighing the remaining food per cage every day. The daily ω -3 EFA content for each animal were then calculated by dividing the food eaten per cage by the number of animals per cage, and then further worked up using each subject's body weight and the results from the ω -3 EFA content analyses to calculate for each animal the daily ω -3 EFA intake in mg, per g body weight.

Samples of both the standard and ω -3 EFA coated chow were analysed for ω -3 EFA content at The Functional Foods Research Unit, Cape Peninsula University of Technology, RSA (see Results). Analyses were carried out in duplicate, with samples from two separate batches weighed and the average fatty acid content calculated. A single run was performed on a silica plate, using a non-polar solvent mixture for thin layer chromatography determination.

2.2 Behavioural analyses

After the 14-day treatment period, animals were subjected to the open field test (OFT) followed by the more stressful forced swim test (FST). It has previously been demonstrated before that under our experimental conditions, foregoing tests do not affect the outcome of the subsequent consecutive tests

if they are ordered from least to most stressful [47]. As before [38, 40], the behavioural tests were performed for all treatment groups either early following the pre-puberty intervention period on PND35, or later in life after treatment washout and normal housing on PND60, representing early adulthood [44, 48, 49]. Testing commenced one hour after the start of the dark cycle to accommodate initial foraging and activity of the nocturnal animals. Tests were carefully spaced to allow 30 min between each test for animals to habituate to the environment.

2.2.1 Open field test

The open field test (OFT) is commonly used to measure locomotor activity, a parameter of the general ability of the animal to move and negotiate its surroundings, as well as anxiety-like behaviour [50-52], of which the latter correlates with results of other robust behavioural tests, such as the elevated plus maze [53]. The OFT apparatus in the current study consisted of a 1 m² test arena, surrounded by opaque black, vertical walls. As before [40], on the day of testing, each rat was placed in the centre of the arena and allowed to explore the environment for 5 min under red light (80 lx) [40]. During this time, rats were videotaped by a camera mounted above the test arena and subsequently scored using Ethovision XT12 software (Noldus Information Technology BV, Wageningen, NLD). Total distance moved during the session was used as a measure of general activity. The total time spent in the centre zone (50 cm²) was used as a measure of anxiety-like behaviour, with a reduction indicating increased anxiety-like behaviour.

2.2.2 Forced swim test

The forced swim test (FST) is widely used to screen for antidepressant-like in rodents [54], and by implication also discerns depressive-like behaviour. An adjusted version of the FST distinguishes between serotonergic and noradrenergic-directed behaviours [55, 56]. The FSL rat is a validated genetic animal model of depression that displays increased depressive-like behaviour (enhanced immobility), without requiring the pre-conditioning swim trial 24 hours prior to the testing swim trial [41].

The FST was performed as previously described by our laboratory group [40]. Briefly, the apparatus consisted of four cylindrical tanks ((40 cm (*h*) x 20 cm (*d*)), each filled to a depth of 30 cm with water, maintained at 25 ± 1°C. The test was performed during the dark cycle under red light (80 lx). On the day of testing, each rat was placed in the cylinder where behaviour was recorded and analysed for 5 min by an experimenter blind to the test group [57].

Behaviour was scored with a manual continuous timer software (FST Scoreboard 2.0 software; Academic Support Services: Information Technology in Education, NWU, RSA), previously validated against the 5 s time-sampling technique [38]. Scored behaviour included immobility (floating with no active movements made, except those necessary to keep the rat's head above water), swimming (horizontal movements throughout the cylinder that included crossing into another quadrant) and

struggling (upward-directed movements of the forepaws along the inside of the swim cylinder) [55, 58]. Of note, although total time spent diving was recorded, this specific behaviour was not included in the final depressive-like behaviour analysis of the animals due to being episodic and seemingly not corresponding with specific treatment effects [55]. Enhanced adrenergic neurotransmission is associated with increased struggling, whereas enhanced serotonergic neurotransmission is associated with increased swimming behaviour [58, 59].

2.3 Neurobiological analyses

2.3.1 Quantitative analysis of frontal cortical monoamines and metabolites

Quantitative monoaminergic concentrations were analysed as previously described by our laboratory group [60-62]. In this regard, whole- or regional brain monoamine analysis represent total levels of serotonin (5-hydroxytryptamine; 5-HT); extracellular and unreleased from nerve terminals [63]. Therefore, several indices of central serotonergic activity may be applied, including 5-HT and 5-HIAA (5-hydroxyindoleacetic acid) levels, as well as the 5-HIAA/5-HT ratio [61, 64]. Following sacrifice of rats by decapitation, total frontal cortices and hippocampi were dissected on an ice-cooled dissection slab and snap frozen in liquid nitrogen and stored at -80 °C until day of analysis. Neurobiological samples were pooled in pairs and analysed accordingly. Pooling of biological samples have been shown to be a valid method to, amongst others, improve cost-effectiveness without affecting biological variation [65]. In cases where an odd number of biological samples were available, the remaining sample would be analysed individually. Quantification of noradrenaline (NA) and 5-HT and the mentioned metabolites were performed by high performance liquid chromatography (HPLC) coupled with electrochemical detection (ECD). An Agilent 1200 series HPLC (Agilent Technologies, California, USA), equipped with an isocratic pump, auto sampler and coupled to an ESA Coulochem III Electrochemical detector (Dionex, California, USA), and Chromeleon® Chromatography Management System software (version 6.8), was used. NA, 5-HT and 5-HIAA concentrations in the tissue samples were determined by comparing the peak area of each monoamine to that of the monoamine standards (range 5-50 ng/ml). Linear standard curves (regression coefficient > 0.95) were found in this range. Monoamine concentrations were expressed as ng/g wet weight of tissue.

2.3.2 Quantitative analysis of hippocampal brain-derived neurotrophic factor

Hippocampal tissue samples were collected and prepared as described above. Hippocampal brain-derived neurotrophic factor (BDNF) concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) BDNF kit (Elabscience) according to the manufacturers' protocol. Briefly, the pooled hippocampal samples were weighed and homogenized in phosphate buffered saline (PBS) and centrifuged for 5 min at 5 000 xg. The supernatant was transferred to the 96-well plate and

incubated for 90 min at 37 °C, whereafter the liquid was removed, biotinylated detection antibody added and incubated for another 60 min at 37 °C. The samples were aspirated and washed three times, whereafter 100 µl horseradish peroxidase (HRP) conjugate was added and incubated for 30 min at 37 °C. Samples were again aspirated and washed five times before 90 µl substrate reagent were added to samples and incubated for 15 min at 37 °C. Finally, 50 µl of stop solution was added and samples read at 450 nm. Results were obtained from a standard curve plotted and expressed as pg/ml.

2.4 Statistical analyses

Normality of the data and homogeneity of variances were determined using the Shapiro-Wilk test and Levene's test for equality of variances, respectively. In both instances, $p < 0.05$ indicated that the respective assumption had been violated. The Grubbs' test was used to determine any outlier in each data set with $\alpha = 0.05$ accepted as significant. In this regard, experimental group sizes are presented for each specific data set [66], however, it was rarely necessary to exclude any data points and clearly indicated in the figure and table legends.

Normal two-way analysis of variances (ANOVAs) were performed on all data sets, regardless of normal distribution or homogeneity of variances [67, 68]. The two-way ANOVA determined whether a statistically significant two-way interaction between drug (SAL or ESC) and diet (STD or OM3) existed. Regardless of whether a statistically significant interaction existed or not, main effects were analysed followed by pairwise comparisons [69, 70] with the Bonferroni post-hoc test. When comparing only two data points, the Independent-samples *t*-test with Welch's correction was used, regardless of normality distribution [71]. The Spearman's rank-order correlation test (r_s) was performed to analyse the correlation coefficient when the assumption for normality had been violated, whereas the Pearson's rank-order correlation test (r) was performed when the assumption was true. The strength of association was considered strong when $r > 0.5$ [72, 73]. In all instances, a 5 % confidence limit for error was taken as statistically significant ($p \leq 0.05$) for all analyses and data is reported with a 95 % confidence interval (CI) of the mean difference.

Finally, effect size indicators were calculated [74] along with all statistical analyses, in line with statistical reporting guidelines [75-77] to indicate strong trends and rule out Type I (false positive) or Type II (false negative) errors [72, 78]. Effect size for interactions were calculated with partial eta squared (η^2), where effect sizes were considered large when $\eta^2 \geq 0.14$ [78]. Furthermore, effect size differences between specific groups were calculated by Cohen's *d* value (with a 95 % CI of the effect size). Cohen's *d* value is an effect size indicator used to specify the standardized difference between two means, with effect sizes considered large when $d \geq 0.8$ [72, 79]. In all instances, only large effect size indicators were considered significant.

ANOVA statistical analyses were performed in IBM® SPSS® Statistics (version 24.0, Armonk, NY: IBM Corp) and GraphPad Prism® (version 6.0, San Diego California USA), assisted by Laerd Statistics® (<https://statistics.laerd.com>) and the statistical consultation service of the North-West University, Potchefstroom.

3 Results

3.1 Omega-3 fatty acid intake

Content analyses indicated that the control diet contained 150 ± 0.05 mg/100 g eicosapentaenoic acid (EPA) and 130 ± 0.05 mg/100 g docosahexaenoic acid (DHA). Following coating with ω -3 EFA oil, the rat chow contained 310 ± 0.05 mg/100 g and 260 ± 0.05 mg/100 g EPA and DHA, respectively. Coincidentally, this represents an approximate 100 % increase (doubling) of the ω -3 EFA content in the enriched diet (OM3) relative to the standard control diet (STD).

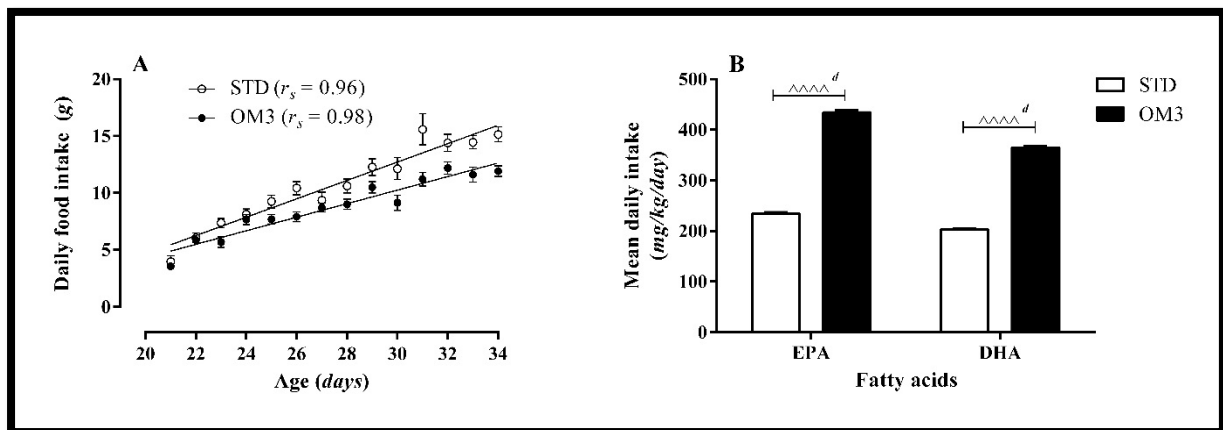


Figure 1: Daily food and ω -3 EFA intake from PND21 until PND34.

(A) Daily food intake of control and ω -3 EFA supplemented diets during pre-pubertal intervention period (PND21-34). (B) Mean daily EPA and DHA intake during pre-pubertal intervention period. Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text, with **** $p \leq 0.0001$ vs. STD; d large effect size ≥ 0.8 . OM3: Omega-3 supplemented diet. STD: Standard (adequate) diet.

Fig. 1 represents the nutritional intake of animals fed the control or ω -3 EFA supplemented diets.

Daily food intake data sets were not normally distributed for all treatment groups ($p < 0.05$) and no outliers were removed from the data. There were strong correlations between age and daily food intake for all test groups ($r_s > 0.9$). However, no significant differences between the slopes of food intake of SAL+STD and ESC+STD ($F[1, 183] = 0.165$; $p = 0.685$), or between SAL+OM3 and ESC+OM3 treatment groups ($F[1, 234] = 0.026$; $p = 0.873$), respectively, were observed (*data not shown*). Therefore, the data of subjects fed the STD diet and those fed the OM3 diet, could be pooled to determine whether any difference in daily food intake existed between these groups (irrespective of drug). In Fig. 1A, the slopes of the daily intake over time of the combined STD treatment groups ($y =$

0.8073x - 11.50) differed statistically significantly from that of the combined OM3 treatment groups ($y = 0.5949x - 7.595$) ($F[1, 421] = 14.058$; $p \leq 0.0005$). Furthermore, specific ω -3 EFA content could be compared between the two diets (irrespective of drug). In this regard, there was a weak positive correlation between daily EPA and DHA intake (measured as mg/kg) and age for animals fed the STD diet ($r_s(470) = 0.275$; $p = 0.050$) and those fed the OM3 diet ($r_s(614) = 0.147$; $p \leq 0.0005$) (*data not shown*). An independent-samples-*t*-test was consequently run to determine whether differences in mean overall ω -3 EFA (EPA and DHA) intake existed between STD and OM3 diets (measured as mg/kg/day). In Fig. 1B, the mean EPA intake of the OM3 diet was 199.7 mg/kg/day (95 % CI, 189.3 to 210.2 mg/kg/day) higher compared to those fed the STD diet (434.3 ± 4.749 vs. 234.6 ± 2.384 mg/kg/day; $t(890.1) = 37.59$; $p \leq 0.0005$; $d = 2.104$, 95 % CI, 1.955 to 2.253). Similarly, the mean DHA intake of the OM3 diet group was 161.0 mg/kg/day (95 % CI, 152.2 to 169.8 mg/kg/day) higher compared to those fed the STD diet (364.3 ± 3.983 vs. 203.3 ± 2.066 mg/kg/day; $t(904.9) = 35.87$; $p \leq 0.0005$; $d = 2.013$, 95 % CI, 1.866 to 2.159). Overall an average increase of 58 % of daily ω -3 EFA intake was observed in the OM3 group in relation to the STD group.

3.2 Body weight gain

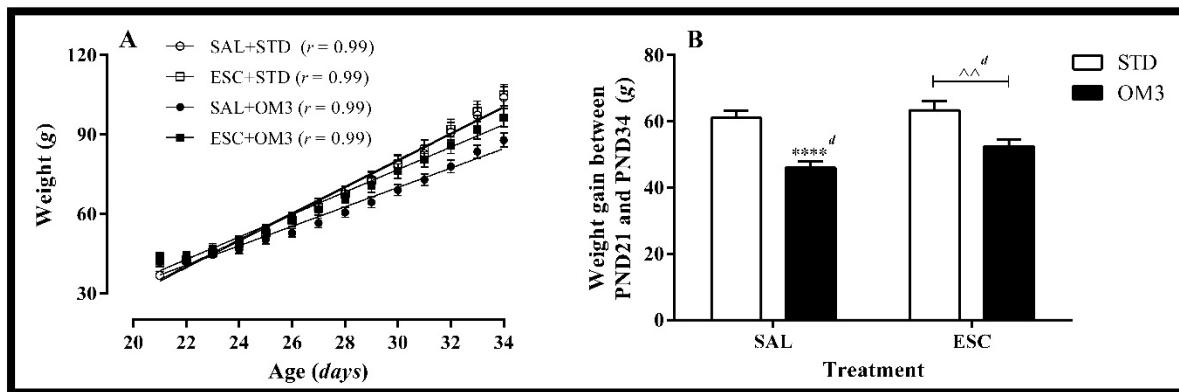


Figure 2: Weight gain from PND21 until PND34.

(A) Daily body weight during pre-pubertal intervention period (PND21-34) of all treatment groups. (B) Mean weight gained during pre-pubertal intervention period for SAL+STD ($n = 23^a$), SAL+OM3 ($n = 24$), ESC+STD ($n = 24$) and ESC+OM3 ($n = 20^a$). Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text, with d large effect size ≥ 0.8 (vs. SAL+STD when alone). ESC: Escitalopram (10 mg/kg/day). OM3: Omega-3 coated diet. SAL: Saline (control). STD: Standard (adequate) diet. ^a Less than 12 animals due to lower than expected birth rates at the requested date. ^b Outlier identified and removed from group due to not representing the target population.

Fig. 2 represents the relationship between body weight and postnatal age in male FSL rats.

In Fig. 2A the data was normally distributed for daily body weight ($p > 0.05$) and no outliers were removed from the data. There were no statistical significant differences of initial body weight (PND21) between the different test groups ($F[1, 87] = 0.223$, $p = 0.638$; $\eta^2 = 0.003$) (*data not shown*), however, strong positive correlations between age and weight gain for all test groups ($r > 0.9$) as well as significant differences between the slopes of pre-pubertal body weight of the different intervention

groups existed ($F[3, 1258] = 14.722$; $p < 0.0001$) (SAL+STD: $y = 5.036x - 71.10$; ESC+STD: $y = 5.020x - 70.16$; SAL+OM3: $y = 3.666x - 39.99$; ESC+OM3: $y = 4.241x - 50.49$). In Fig. 2B mean pre-pubertal weight gain data between PND21 and PND34 were not normally distributed for all test groups ($p < 0.05$), yet had homogeneity of variances ($p = 0.131$). No outliers were removed from the data set. Furthermore, there was no statistically significant two-way interaction between drug and diet for pre-pubertal weight gain ($F[1, 87] = 0.779$, $p = 0.380$; $\eta^2 = 0.009$). However, there was a statistically significant main effect of diet ($F[1, 87] = 32.101$, $p \leq 0.0005$; $\eta^2 = 0.270$), that OM3 decreased overall pre-pubertal weight gain by 12.9 g (95 % CI, 8.4 to 17.5 g). Specifically, SAL+OM3 decreased pre-pubertal weight gain by 14.9 g (95 % CI, 6.4 to 23.5 g) compared to SAL+STD (46.1 ± 1.9 vs. 60.0 ± 2.1 g; $p \leq 0.0005$; $d = 1.566$, 95 % CI, 0.9 to 2.2). Similarly, ESC+OM3 decreased pre-pubertal weight gain by 10.9 g (95 % CI, 2.0 to 19.8 g) compared to ESC+STD (52.5 ± 2.2 vs. 63.4 ± 2.8 g; $p = 0.008$; $d = 0.937$, 95 % CI, 0.3 to 1.6)

3.3 Open field test

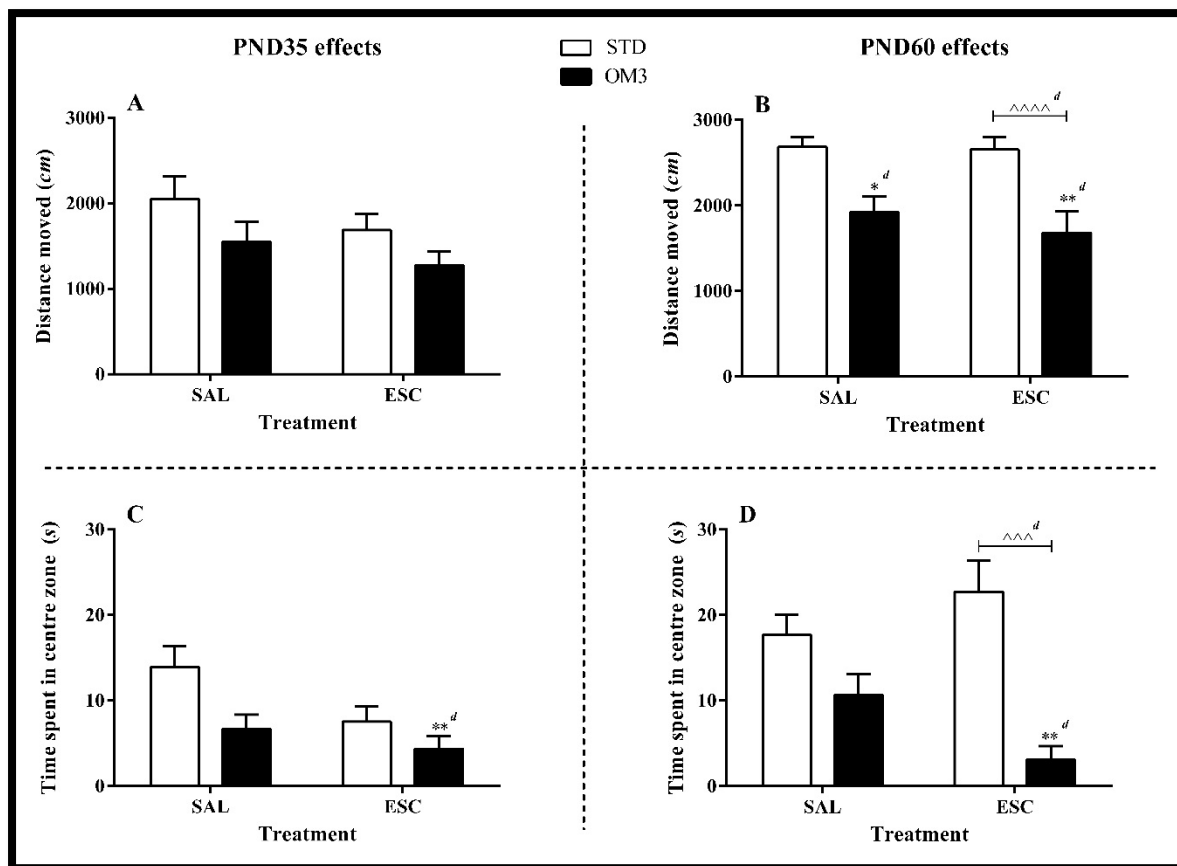


Figure 3: Early and lasting effects of pharmacological and non-pharmacological interventions on locomotor activity and anxiety-like behaviour of FSL rats on PND35 and PND60.

(A) Distance moved in the OFT following treatment of SAL+STD ($n = 12$), SAL+OM3 ($n = 12$), ESC+STD ($n = 12$) or ESC+OM3 ($n = 10^a$) on PD35. (B) Distance moved in the OFT after washout period on PND60 (SAL+STD $n = 10^{a,b}$; SAL+OM3 $n = 12$; ESC+STD $n = 12$; ESC+OM3 $n = 10^a$). (C) Time spent in the centre zone of the OFT on PND35 (SAL+STD $n = 12$; SAL+OM3 $n = 12$; ESC+STD $n = 11^b$; ESC+OM3 $n = 10^a$). (D) Time spent in the centre zone of the OFT PND60 (SAL+STD $n = 10^{a,b}$; SAL+OM3 $n = 12$; ESC+STD $n =$

12; ESC+OM3 $n = 9^{a,b}$). Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text, with * $p \leq 0.05$, ** $p \leq 0.01$ vs. SAL+STD; ^^^ $p \leq 0.001$, ^^^^ $p \leq 0.0005$ vs. indicated test group; d large effect size ≥ 0.8 (vs. SAL+STD when alone). ESC: Escitalopram (10 mg/kg/day). OM3: Omega-3 coated diet. SAL: Saline (control). STD: Standard (adequate) diet. ^{a)} Less than 12 animals due to lower than expected birth rates at the requested date. ^{b)} Outlier identified and removed from group due to not representing the target population.

Fig. 3 represents the early and lasting behavioural effects of the FSL rats in the OFT on PND35 and PND60.

In Fig. 3A, distance moved data of PND35 rats were normally distributed ($p > 0.05$) and had homogeneity of variances ($p = 0.181$). There was no statistically significant two-way interaction between drug and diet ($F[1, 42] = 0.042$, $p = 0.839$; $\eta^2 = 0.001$). However, there was a statistically significant main effect of diet ($F[1, 42] = 4.298$, $p = 0.044$; $\eta^2 = 0.093$), but not of drug ($F[1, 42] = 2.085$, $p = 0.156$; $\eta^2 = 0.047$). Total distance moved was 456.1 cm (95 % CI, 12.1 to 900.1 cm) lower in OM3 treatment groups compared to STD treatment groups, yet no significant differences could be identified within the main effect.

In Fig. 3B, distance moved data of PND60 rats were normally distributed ($p > 0.05$), yet had no homogeneity of variances ($p = 0.043$). There was no statistically significant two-way interaction between drug and diet ($F[1, 40] = 0.336$, $p = 0.565$; $\eta^2 = 0.008$). However, there was a statistically significant main effect of diet ($F[1, 40] = 23.519$, $p \leq 0.0005$; $\eta^2 = 0.370$), but not of drug ($F[1, 40] = 0.561$, $p = 0.458$; $\eta^2 = 0.014$). In line with the main effect of diet, SAL+OM3 decreased distance moved by 767.0 cm (95 % CI, 61.8 to 1472.2 cm) compared to SAL+STD (1918.1 ± 184.3 vs. 2685.1 ± 115.1 cm; $p = 0.026$; $d = 1.510$, 95 % CI, 0.6 to 2.5). Similarly, ESC+OM3 decreased distance moved by 975.4 cm (95 % CI, 461.9 to 1488.8 cm) compared to ESC+STD and (1679.4 ± 251.2 vs. 2654.7 ± 143.7 cm; $p \leq 0.0005$; $d = 1.578$, 95 % CI, 0.6 to 2.5). However, ESC+OM3 also decreased distance moved by 1005.8 cm (95 % CI, 269.2 to 1742.3 cm) compared to SAL+STD (1679.4 ± 251.2 vs. 2685.1 ± 115.1 cm; $p = 0.003$; $d = 1.716$, 95 % CI, 0.7 to 2.7).

In Fig. 3C, time spent in centre zone data of PND35 rats were not normally distributed for all test groups ($p < 0.05$), yet had homogeneity of variances ($p = 0.157$). There was no statistically significant two-way interaction between drug and diet ($F[1, 41] = 1.095$, $p = 0.302$; $\eta^2 = 0.026$). However, there were statistically significant main effects of diet ($F[1, 41] = 7.116$, $p = 0.011$; $\eta^2 = 0.148$) and drug ($F[1, 41] = 4.992$, $p = 0.031$; $\eta^2 = 0.109$). ESC (irrespective to diet) and OM3 (irrespective to drug) decreased overall centre zone time by 4.4 s (95 % CI, 0.4 to 8.3 s) and 5.2 s (95 % CI, 1.3 to 9.1 s). Furthermore, only ESC+OM3 decreased centre zone time by 9.6 s (95 % CI, 1.8 to 17.3 s) compared to SAL+STD (4.3 ± 1.5 vs. 13.9 ± 2.5 s; $p = 0.009$; $d = 1.400$, 95 % CI, 0.5 to 2.3).

In Fig. 3D, time spent in centre zone data of PND60 rats were not normally distributed for all test groups ($p < 0.05$), yet there was homogeneity of variances ($p = 0.083$). There was a statistically significant

two-way interaction between drug and diet on PND60 ($F[1, 39] = 4.922, p = 0.032; \eta^2 = 0.112$), supported by a statistically significant main effect of diet ($F[1, 39] = 22.404, p \leq 0.0005; \eta^2 = 0.365$). Hence, OM3 decreased overall centre zone time by 13.4 s (95 % CI, 7.7 to 19.1 s) compared to STD (irrespective of drug). Furthermore, ESC+OM3 decreased centre zone time by 14.6 s (95 % CI, 2.9 to 26.3 s) compared to SAL+STD (3.1 ± 1.6 vs. 17.7 ± 2.3 s; $p = 0.008; d = 2.469$, 95 % CI, 1.3 to 3.7) and by 19.6 s (95 % CI, 8.4 to 30.9 s) compared to ESC+STD (22.7 ± 3.7 s; $p = 0.0001; d = 0.914$, 95 % CI, 0.0 to 1.8).

3.3 Forced swim test

Fig. 4 represents the early and lasting behavioural effects of the FSL rats in the FST on PND35 and PND60.

In Fig. 4A, time spent immobile data of PND35 rats were normally distributed ($p > 0.05$) and had homogeneity of variances ($p = 0.482$). There was no statistically significant two-way interaction between drug and diet ($F[1, 42] = 2.868, p = 0.098; \eta^2 = 0.064$). However, there was a statistically significant main effect of diet ($F[1, 42] = 9.381, p = 0.004; \eta^2 = 0.183$), but not of drug ($F[1, 42] = 1.723, p = 0.196; \eta^2 = 0.039$). The main effect of diet was only significant when combined with ESC ($F[1, 42] = 10.798, p = 0.002; \eta^2 = 0.205$). SAL+OM3 decreased time spent immobile by 33.9 s (95 % CI, 4.3 to 63.6 s) compared to ESC+STD (163.7 ± 6.4 vs. 197.6 ± 8.6 s; $p = 0.017; d = 1.330$, 95 % CI, 0.4 to 2.2). Similarly, ESC+OM3 increased time spent immobile by 36.9 s (95 % CI, 5.8 to 68.0 s) compared to ESC+STD (200.5 ± 6.2 vs. 163.7 ± 6.6 s; $p = 0.012; d = 1.799$, 95 % CI, 0.8 to 2.8).

In Fig. 4B, time spent immobile data of PND60 rats were normally distributed ($p > 0.05$), yet had no homogeneity of variances ($p = 0.016$). There was no statistically significant two-way interaction between drug and diet ($F[1, 41] = 1.650, p = 0.206; \eta^2 = 0.039$). However, there was a statistically significant main effect of diet ($F[1, 41] = 4.289, p = 0.045; \eta^2 = 0.095$), but not of drug ($F[1, 41] = 1.327, p = 0.256; \eta^2 = 0.031$). The main effect was only significant when combined with ESC ($F[1, 41] = 5.491, p = 0.024; \eta^2 = 0.118$), however, the difference between ESC+OM3 and ESC+STD did not reach statistical significance, yet a large effect size difference existed (197.6 ± 10.9 vs. 224.9 ± 5.0 s; $p = 0.144; d = 1.082$, 95 % CI, 0.2 to 2.0).

In Fig. 4C, time spent swimming data of PND35 rats were not normally distributed for all test groups ($p < 0.05$), yet had no homogeneity of variances ($p = 0.037$). There was no statistically significant two-way interaction between drug and diet ($F[1, 40] = 0.011, p = 0.917; \eta^2 < 0.0005$), nor any statistically significant main effects.

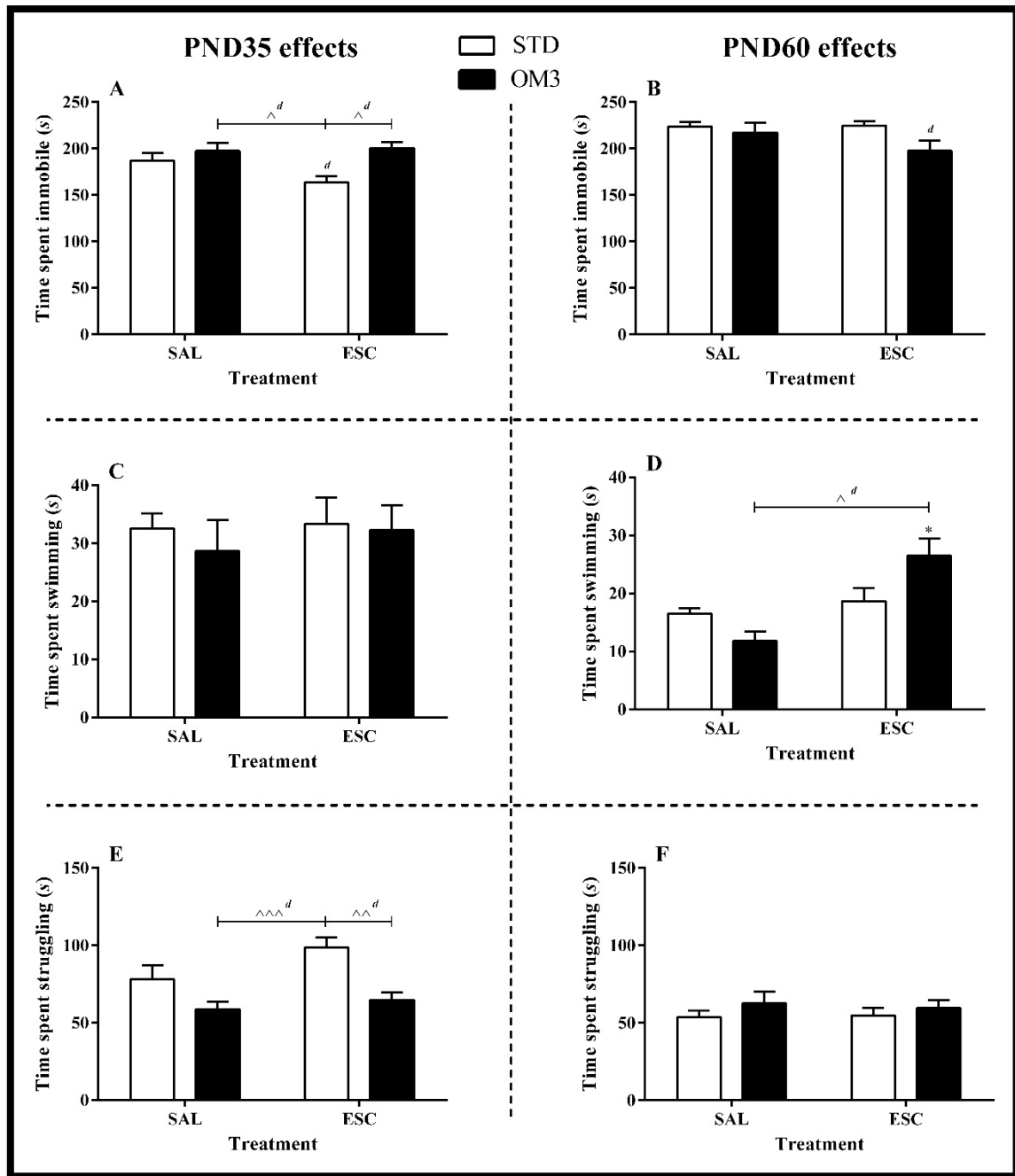


Figure 4: Early and lasting effects of pharmacological and non-pharmacological interventions on depressive-like behaviour of FSL rats on PND35 and PND60.

(A) Time spent immobile in the FST following treatment of SAL+STD ($n = 12$), SAL+OM3 ($n = 12$), ESC+STD ($n = 12$) or ESC+OM3 ($n = 10^a$) on PD35. **(B)** Time spent immobile in the FST on PND60 (SAL+STD $n = 11^a$; SAL+OM3 $n = 12$; ESC+STD $n = 12$; ESC+OM3 $n = 10^a$). **(C)** Time spent swimming in the FST on PND35 (SAL+STD $n = 12$; SAL+OM3 $n = 11^b$; ESC+STD $n = 12$; ESC+OM3 $n = 9^{a,b}$). **(D)** Time spent swimming in the FST on PND60 (SAL+STD $n = 10^{a,b}$; SAL+OM3 $n = 12$; ESC+STD $n = 12$; ESC+OM3 $n = 10^a$). **(E)** Time spent struggling in the FST on PND35 (SAL+STD $n = 12$; SAL+OM3 $n = 11^b$; ESC+STD $n = 12$; ESC+OM3 $n = 10^a$). **(F)** Time spent struggling in the FST (SAL+STD $n = 11^a$; SAL+OM3 $n = 12$; ESC+STD $n = 12$; ESC+OM3 $n = 9^{a,b}$). Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text with * $p \leq 0.05$ vs. SAL+STD; ^ $p \leq 0.05$, ^^ $p \leq 0.01$, ^^^ $p \leq 0.001$ vs. indicated test group; d large effect size ≥ 0.8 (vs. SAL+STD when alone). ESC: Escitalopram (10 mg/kg/day). OM3: Omega-3 coated diet. SAL: Saline (control). STD: Standard (adequate) diet. ^{a)} Less than 12 animals due to lower than expected birth rates at the requested date. ^{b)} Outlier identified and removed from group due to not representing the target population.

In Fig. 4D, time spent swimming data of PND60 rats were normally distributed ($p > 0.05$), yet had no homogeneity of variances ($p = 0.007$). There was a statistically significant two-way interaction between drug and diet ($F[1, 40] = 8.790$, $p = 0.005$; $\eta^2 = 0.180$). ESC+OM3 (26.5 ± 3.0 s) increased swimming by 14.7 s (95 % CI, 6.4 to 23.0 s) compared to SAL+OM3 (11.8 ± 1.6 s; $p \leq 0.0005$; $d = 2.009$, 95 % CI, 1.0 to 3.0) and by 10.0 s (95 % CI, 1.3 to 18.6 s) compared to the SAL+STD group (16.5 ± 0.1 s; $p = 0.016$; $d = 1.481$, 95 % CI, 0.5 to 2.5).

In Fig. 4E, time spent struggling data of PND35 rats were all normally distributed ($p > 0.05$) and had homogeneity of variances ($p = 0.111$). There was no statistically significant two-way interaction between drug and diet ($F[1, 41] = 1.168$, $p = 0.286$; $\eta^2 = 0.028$). However, there was a statistically significant main effect of diet ($F[1, 41] = 15.337$, $p \leq 0.0005$; $\eta^2 = 0.272$), but not of drug ($F[1, 41] = 3.869$, $p = 0.056$; $\eta^2 = 0.086$). Compared to ESC+STD (98.7 ± 6.4 s), time spent struggling was decreased by 33.9 s (95 % CI, 7.0 to 60.8 s) and by 39.9 s (95 % CI, 13.6 to 66.1 s), respectively by ESC+OM3 (64.9 ± 4.9 s; $p = 0.001$; $d = 1.824$, 95 % CI, 0.8 to 2.8) and SAL+OM3 (58.9 ± 5.0 s; $p = 0.001$; $d = 2.113$, 95 % CI, 1.1 to 3.1).

In Fig. 4F, time spent struggling data of PND60 rats were all normally distributed ($p > 0.05$) and had homogeneity of variances ($p = 0.156$). There was no statistically significant two-way interaction between drug and diet ($F[1, 40] = 0.123$, $p = 0.727$; $\eta^2 = 0.003$), nor any statistically significant main effects.

3.5 Neurobiological tests

Fig. 5 represents the early and lasting frontal cortical serotonergic turnover and hippocampal neuroplasticity concentrations of the FSL rats on PND35 and PND60.

In Fig. 5A, frontal cortical 5-HIAA/5-HT data of PND35 rats were not normally distributed for all test groups ($p < 0.05$), yet had homogeneity of variances ($p = 0.222$), but there was no statistically significant two-way interaction between drug and diet on PND35 ($F[1, 14] = 1.159$, $p = 0.069$; $\eta^2 = 0.217$), nor any statistically significant main effects.

In Fig. 5B, frontal cortical 5-HIAA/5-HT data of PND60 rats were normally distributed for all test groups ($p > 0.05$), yet did not have homogeneity of variances ($p = 0.002$). There was a statistically significant two-way interaction between drug and diet on PND60 ($F[1, 14] = 15.105$, $p = 0.002$; $\eta^2 = 0.519$). ESC+OM3 increased 5-HIAA/5-HT ratio by 6.61 (95 % CI, 2.00 to 11.24) compared to SAL+STD (9.90 ± 2.14 vs. 3.30 ± 0.81 ; $p = 0.004$; $d = 2.407$, 95 % CI, 0.69 to 4.13), by 7.05 (95 % CI, 2.42 to 11.68) compared to SAL+OM3 (9.90 ± 2.14 vs. 2.85 ± 0.34 ; $p = 0.002$; $d = 1.010$, 95 % CI, -0.39 to 2.41) and by 7.86 (95 % CI, 2.97 to 12.74) compared to ESC+STD (9.90 ± 2.14 vs. 2.04 ± 0.23 ; $p = 0.001$; $d = 2.407$, 95 % CI, 0.69 to 4.13).

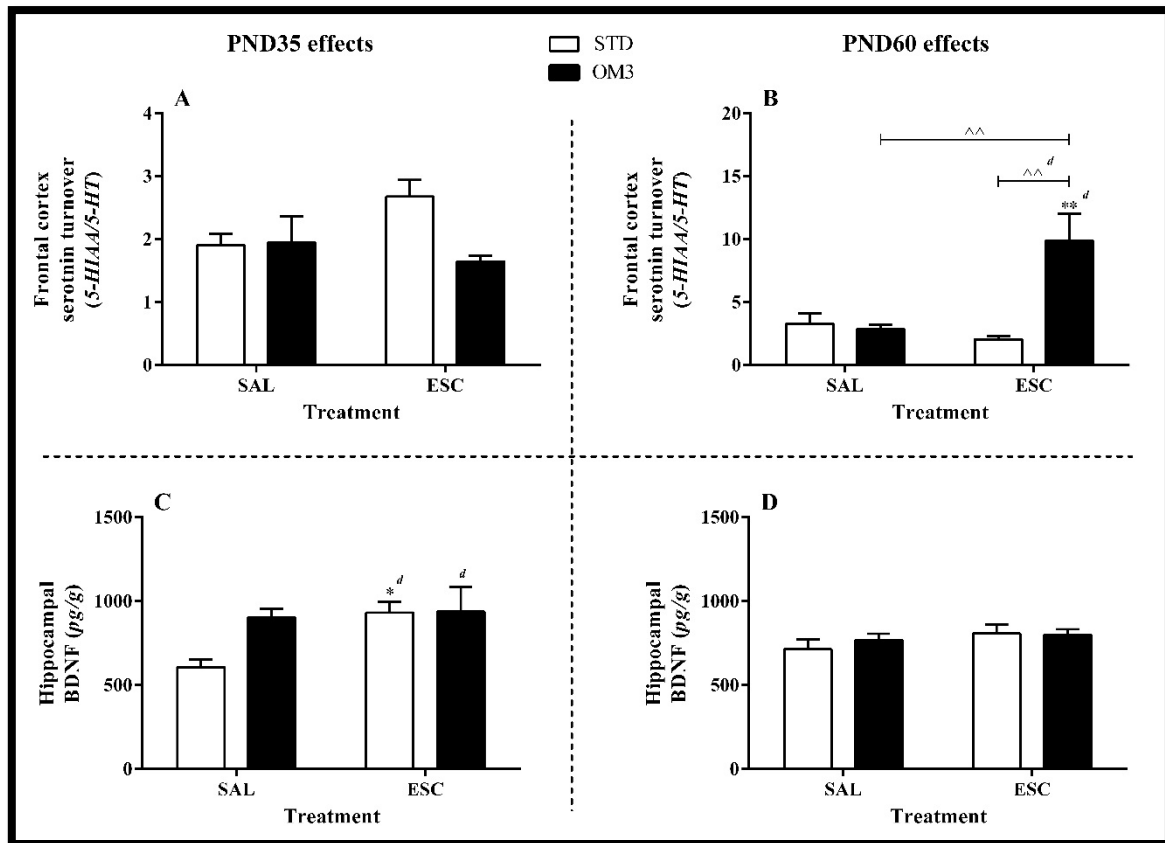


Figure 5: Early and lasting effects of pharmacological and non-pharmacological interventions on frontal cortical serotonergic turnover and hippocampal neuroplasticity markers of FSL rats on PND35 and PND60.

(A) Frontal cortical serotonin turnover following treatment of SAL+STD ($n = 6$), SAL+OM3 ($n = 3$), ESC+STD ($n = 6$) or ESC+OM3 ($n = 3$) on PD35. (B) Frontal cortical serotonin turnover following treatment on PND60 (SAL+STD $n = 5$; SAL+OM3 $n = 5$; ESC+STD $n = 4$; ESC+OM3 $n = 4$). (C) Hippocampal BDNF concentrations on PND35 (SAL+STD $n = 6$; SAL+OM3 $n = 4$; ESC+STD $n = 6$; ESC+OM3 $n = 4$). (D) Hippocampal BDNF concentrations on PND60 (SAL+STD $n = 4$; SAL+OM3 $n = 5$; ESC+STD $n = 5$; ESC+OM3 $n = 3$). Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text, with d large effect size ≥ 0.8 (vs. SAL+STD when alone). ESC: Escitalopram (10 mg/kg/day). OM3: Omega-3 coated diet. SAL: Saline (control). STD: Standard (adequate) diet.

In Fig. 5C, hippocampal BDNF concentration data of PND35 rats were not normally distributed for all test groups ($p < 0.05$), yet had homogeneity of variances ($p = 0.268$). There was no statistically significant two-way interaction between drug and diet on PND35 ($F[1, 16] = 3.286$, $p = 0.089$; $\eta^2 = 0.170$). However, there was a statistically significant main effect of drug ($F[1, 16] = 5.108$, $p = 0.038$; $\eta^2 = 0.242$), but not of diet ($F[1, 16] = 3.602$, $p = 0.076$; $\eta^2 = 0.184$), so that ESC increased overall BDNF concentrations by 180.5 pg/g (95 % CI, 11.2 to 349.7 pg/g) compared to SAL (irrespective of diet). In line with the main effect of drug, ESC+STD increased BDNF concentrations by 325.2 pg/g (95 % CI, 21.4 to 629.0 pg/g) compared to SAL+STD (605.3 ± 47.2 vs. 930.5 ± 65.0 pg/g; $p = 0.032$; $d = 2.562$, 95 % CI, 1.0 to 4.1). Although the mean difference between ESC+OM3 and SAL+STD did not reach statistical significance, a large effect size was identified ($p = 0.058$; $d = 1.828$, 95 % CI, 0.3 to 3.3).

In Fig. 5D, hippocampal BDNF concentration data of PND60 rats were normally distributed for all test groups ($p > 0.05$), and had homogeneity of variances ($p = 0.558$). There was no statistically significant two-way interaction between drug and diet on PND60 ($F[1, 13] = 0.393$, $p = 0.542$; $\eta^2 = 0.029$), nor were there any statistical significant main effects.

Table 1 represents the early and lasting frontal cortical noradrenergic and serotonergic concentrations of the FSL rats on PND35 and PND60.

On PND35, frontal cortical NA data were normally distributed for all test groups ($p > 0.05$), and had homogeneity of variances ($p = 0.528$), but there was no statistically significant two-way interaction between drug and diet ($F[1, 14] = 1.130$, $p = 0.306$; $\eta^2 = 0.075$). However, there was a statistically significant main effect of drug ($F[1, 14] = 4.978$, $p = 0.043$; $\eta^2 = 0.262$), but not of diet ($F[1, 14] = 3.431$, $p = 0.085$; $\eta^2 = 0.197$). ESC increased overall NA concentrations by 14.4 ng/g (95 % CI, 0.6 to 28.3 ng/g) compared to SAL (irrespective of diet), yet no significant differences could be identified within the main effect.

On PND60, frontal cortical NA data were normally distributed for all test groups ($p > 0.05$), and had homogeneity of variances ($p = 0.150$), but there was no statistically significant two-way interaction between drug and diet ($F[1, 14] = 4.018$, $p = 0.065$; $\eta^2 = 0.223$). However, there was a statistically significant main effect of diet ($F[1, 14] = 6.542$, $p = 0.023$; $\eta^2 = 0.318$), but not of drug ($F[1, 14] = 1.045$, $p = 0.324$; $\eta^2 = 0.069$). The main effect of diet was only significant when combined with SAL ($F[1, 14] = 11.708$, $p = 0.004$; $\eta^2 = 0.455$). SAL+OM3 increased NA concentrations by 27.0 ng/g (95 % CI, 2.8 to 51.2 ng/g) compared to SAL+STD (92.9 ± 3.8 vs. 65.9 ± 3.0 ng/g; $p = 0.025$; $d = 4.117$, 95 % CI, 1.9 to 6.3).

On PND35, frontal cortical 5-HT data were normally distributed for all test groups ($p > 0.05$), and had homogeneity of variances ($p = 0.889$), but there was no statistically significant two-way interaction between drug and diet on PND35 ($F[1, 14] = 0.676$, $p = 0.425$; $\eta^2 = 0.046$). However, there was a statistically significant main effect of diet ($F[1, 14] = 5.724$, $p = 0.031$; $\eta^2 = 0.290$), but not of drug ($F[1, 14] = 2.735$, $p = 0.120$; $\eta^2 = 0.163$), yet no significant differences could be identified within the main effect.

On PND60, frontal cortical 5-HT data were normally distributed for all test groups ($p > 0.05$), and had homogeneity of variances ($p = 0.443$). There was a statistically significant two-way interaction between drug and diet on PND60 ($F[1, 14] = 36.350$, $p < 0.0005$; $\eta^2 = 0.722$) as well as a statistically significant main effect of drug ($F[1, 14] = 12.220$, $p = 0.004$; $\eta^2 = 0.466$). SAL+OM3 increased 5-HT by 51.2 ng/g (95 % CI, 26.3 to 76.0 ng/g), compared to SAL+STD (119.2 ± 10.0 vs. 68.1 ± 9.4 ng/g; $p = 0.004$; $d = 2.641$, 95 % CI, 0.9 to 4.3). Contrary, ESC+OM3 decreased 5-HT by 82.6 ng/g (95 % CI, 56.3 to 109.0

ng/g), compared to SAL+OM3 (36.6 ± 7.3 vs. 119.2 ± 10.0 ng/g; $p < 0.0005$; $d = 4.836$, 95 % CI, 2.2 to 7.4) and by 53.5 ng/g (95 % CI, 25.7 to 81.2 ng/g), compared to ESC+STD (36.6 ± 7.3 vs. 90.1 ± 5.0 ng/g; $p = 0.006$; $d = 4.956$, 95 % CI, 2.2 to 7.8).

On PND35, frontal cortical 5-HIAA data were not normally distributed for all test groups ($p < 0.05$), yet had homogeneity of variances ($p = 0.530$). There was no statistically significant two-way interaction between drug and diet on PND35 ($F[1, 14] = 1.273$, $p = 0.278$; $\eta^2 = 0.083$), nor any statistically significant main effects.

On PND60, frontal cortical 5-HIAA data were normally distributed for all test groups ($p > 0.05$), and had homogeneity of variances ($p = 0.441$). There was no statistically significant two-way interaction between drug and diet ($F[1, 14] = 0.016$, $p = 0.900$; $\eta^2 = 0.001$). However, there was a statistically significant main effect of diet ($F[1, 14] = 42.074$, $p < 0.0005$; $\eta^2 = 0.750$), but not of drug ($F[1, 14] = 0.381$, $p = 0.547$; $\eta^2 = 0.026$). In line with the main effect of diet, SAL+OM3 increased 5-HIAA by 131.5 ng/g (95 % CI, 72.1 to 190.0 ng/g) compared to SAL+STD (327.5 ± 23.3 vs. 196.4 ± 24.4 ng/g; $p = 0.002$; $d = 2.755$, 95 % CI, 1.0 to 4.5). Similarly, ESC+OM3 increased 5-HIAA by 136.3 ng/g (95 % CI, 70.4 to 202.2 ng/g) compared to ESC+STD (317.4 ± 9.0 vs. 181.1 ± 15.4 ng/g; $p = 0.003$; $d = 6.247$, 95 % CI, 2.9 to 9.6). Furthermore, ESC+OM3 also increased 5-HIAA by 121.0 ng/g (95 % CI, 31.5 to 210.4 ng/g) compared to SAL+STD ($p = 0.006$; $d = 3.220$, 95 % CI, 1.2 to 5.2).

Table 1: Early and lasting effects of pharmacological and non-pharmacological interventions on frontal cortical monoaminergic and hippocampal neuroplasticity markers of FSL rats on PND35 and PND60.

Frontal cortical NA, 5-HT and 5-HIAA concentrations following treatment of SAL+STD ($n = 6$), SAL+OM3 ($n = 3$), ESC+STD ($n = 6$) or ESC+OM3 ($n = 3$) on PD35. Frontal cortical NA, 5-HT and 5-HIAA concentrations on PND60 (SAL+STD $n = 5$; SAL+OM3 $n = 5$; ESC+STD $n = 4$; ESC+OM3 $n = 4$). Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text with ** $p \leq 0.01$ vs. SAL+STD; ^ $p \leq 0.01$, ^^^ $p \leq 0.0005$ vs. indicated test group; d large effect size ≥ 0.8 . 5-HIAA: 5-hydroxyundoleactic acid. 5-HT: 5-hydroxytryptamine (serotonin). BDNF: Brain-derived neurotrophic factor. ESC: Escitalopram. NA: Noradrenaline. ns. Non-significant. OM3: Omega-3 coated diet. PND: Postnatal day. SAL: Saline (control). STD: Standard (adequate) diet.

Marker	EARLY-LIFE (PND35)					EARLY ADULTHOOD (PND60)				
	SAL		ESC		Difference between groups	SAL		ESC		Difference between groups
	STD (Group 1)	OM3 (Group 2)	STD (Group 3)	OM3 (Group 4)		STD (Group 1)	OM3 (Group 2)	STD (Group 3)	OM3 (Group 4)	
NA (ng/g)	64.0 $\pm$ 7.6	82.8 $\pm$ 3.4	85.3 $\pm$ 3.7	90.4 $\pm$ 4.8	ns	65.9 $\pm$ 2.7	92.9 $\pm$ 3.8	71.7 $\pm$ 2.0	75.0 $\pm$ 11.9	1 vs. 2*** ^d
5-HT (ng/g)	98.6 $\pm$ 9.6	118.6 $\pm$ 18.4	109.2 $\pm$ 10.1	150.2 $\pm$ 14.1	ns	68.1 $\pm$ 9.4	119.2 $\pm$ 10.0	90.1 $\pm$ 5.0	36.6 $\pm$ 7.3	1 vs. 2*** ^d 2 vs. 4^^^ ^d 3 vs. 4^^ ^d
5-HIAA (ng/g)	190.6 $\pm$ 25.2	216.6 $\pm$ 19.2	287.3 $\pm$ 31.9	244.5 $\pm$ 8.7	ns	196.4 $\pm$ 24.2	327.5 $\pm$ 23.3	181.1 $\pm$ 15.36	317.4 $\pm$ 9.0	1 vs. 2*** ^d 1 vs. 4*** ^d 3 vs. 4^^ ^d

4 Discussion

Daily food intake and pre-pubertal weight gain

Normal rat chow was successfully coated with ω -3 EFAs to contain double EPA and DHA content, compared to the STD diet. Daily food intake was unaffected by ESC treatment, and although ω -3 EFA coating significantly reduced overall intake by roughly 17 % over two weeks from PND21 to PND34 (irrespective of drug) (Fig. 1A), the overall ω -3 EFA intake was approximately 58 % higher in the OM3 treatment groups (Fig. 1B) in relation to the STD treatment groups. Pre-pubertal weight gain of FSL male rats increased directly proportional to age for all treatment strategies, yet the rate of weight gain (i.e. regression slopes of lines in Fig. 2A) differed significantly between the different test groups. To this extent, ω -3 EFA coating significantly reduced mean pre-pubertal weight gain, compared to animals fed the standard diet between PND21 and PND34 (Fig. 2B), despite initial body weight being comparable. This is in line with previous clinical reports suggesting ω -3 EFA supplementation to cause weight loss in obese juveniles [80-82] and is of note, since body weight has previously been positively associated with depressive-like behaviour in the FST [83]. However, according to the current data, OM3 treated animals weighed significantly less on PND34, yet spent more time immobile in the FST on PND35 in relation to control-fed animals, suggesting body weight to not affect depressive-like behaviour of the pre-pubertal FSL rat in the FST.

Locomotor activity

Pre-pubertal escitalopram treatment did not significantly affect locomotor activity (i.e. comparing ESC+STD to SAL+STD treatment), neither immediately following early-life treatment (PND35) nor following normal housing until early adulthood (PND60) (Fig. 3A and B). However, pre-pubertal ω -3 EFA supplementation significantly reduced locomotor activity (i.e. comparing OM3 treatment groups to STD treatment groups in Fig. 3A and B), both immediately following early-life treatment (PND35) and following normal housing until early adulthood (PND60). In fact, Fig. 3A indicates a general reduction in early-life locomotor activity induced by OM3 treatment groups relative to STD treatment groups (irrespective of drug and supported by a significant main effect of diet). This is in line with literature demonstrating decreased ambulatory activity in animals fed an ω -3 EFA supplemented diet [84, 85], whereas increased locomotor activity or hyperactivity in animals fed an ω -3 EFA deficient diet have been observed [86, 87]. Of note, our data indicate that the reduction in locomotor activity by pre-pubertal ω -3 EFA is long-lasting, still observed in early-adulthood, following more than 3 weeks of standard diet (Fig. 3B). In fact, this lasting effect appears to be more profound later in life, compared to early-life behaviour. In this regard, both OM3 treatment groups displayed significant reductions in locomotor activity relative to their respective STD treatment controls, yet the locomotor activity of the

combination treatment (ESC+OM3) was now also significantly reduced compared to SAL+STD, suggesting augmentation of effect by the escitalopram- ω -3 EFA combination. These findings are supported by previous reports that showed ω -3 EFA supplementation initiated from weaning (PND21), adequately normalized brain ω -3 PUFA concentrations later in life (PND60) in animals born to ω -3 EFA deficient dams [88]. Importantly, none of the animals in the current study were either bred into or nurtured on an ω -3 EFA deficient diet prior to weaning, so that our study uniquely investigated the effect of ω -3 EFA supplementation and not of the reversal of deficiency. It can therefore be concluded that brain ω -3 EFA levels were comparable across all treatment groups at the beginning of the intervention period (i.e. PND21), and although not analysed in the current study, ω -3 EFA supplementation is expected to have increased these brain ω -3 EFA concentrations [89] in OM3 treatment groups. Furthermore, following a chronic wash-out period where a supplemented (and not a deficient) diet was fed could have maintained these increased levels into early-adulthood and induced the observed effects. However, this explanation warrants the support of ω -3 EFA brain content analyses in prospective studies.

Anxiety-like behaviour

Early-life (PND35) anxiety-like behaviour, indicated by a decrease in the time spent in the centre zone, was increased only by the combination treatment (ESC+OM3; Figure 3C), in relation to controls (SAL+STD). Of note, SSRIs are known to have significant effects on anxiety-like behaviour, yet in the current study the mean difference between escitalopram mono-treatment and age-matched controls did not reach statistical significance (ESC+STD vs. SAL+STD; Fig. 3C). Nevertheless, the significant main effect of diet suggests general anxiety-like behaviour in PND35 animals to be increased by OM3 treatment, compared to STD treatment groups (irrespective of drug). This is of note since previous studies suggest an ω -3 EFA deficient, and not supplemented diet, to induce anxiogenic-like behaviour, yet be reversed by a ω -3 EFA supplementation [90, 91]. However, others have observed similar anxiogenic-like behaviour in ω -3 EFA supplemented mice, compared to adequate fed controls [92, 93], with unaltered anxiety-like behaviour also being reported [94]. Interestingly, compared to an adequate diet, ω -3 EFA deficiency is suggested to induce slower habituation to novel environments [95, 96] and increased exploratory behaviour [97]. Therefore, ω -3 EFA supplementation could have decreased habituation time and consequent exploratory behaviour in the OFT of OM3 treatment groups, thereby presenting as decreased locomotor activity (Fig. 3A) and being falsely interpreted as increased anxiety-like behaviour (Fig. 3C). In fact, increased adaptive coping behaviour has been observed in ω -3 EFA supplemented rodents [98], possibly due to enhanced neurogenesis and neuroplasticity [99]. Alternatively, increased 5-HT concentrations via inhibition of the serotonin transporter (5-HTT) during early-life development aversively affects social behaviour in juvenile rats, and may also be responsible for anxiety, and corroborated by the elevated albeit non-significant anxiogenic effect of ESC. To this

extent, social playful behaviour, such as chasing, was reduced in juvenile 5-HTT knockout rats [100]. That ω -3 EFA supplementation is associated with increased serotonergic transmission [27] could therefore have reduced such playful behaviour, explaining the observed overall reduction in locomotor activity and apparent increase in anxiety-like behaviour. That ω -3 EFA brain content, and not dietary content, is considered a strong regulator of behaviour [97] supports our hypothesis that ω -3 EFA brain levels of OM3 treatment groups remained elevated throughout the wash-out period, inducing behavioural alterations later in life. In fact, anxiety-like behaviour of PND60 rats (Fig. 3D) were increased in OM3 treatment groups, compared to STD treatment groups (irrespective of drug and supported by a significant main effect of diet). Moreover, only rats treated with the combination strategy (ESC+OM3) spent significantly less time inside the centre zone of the OFT compared to age-matched controls (SAL+STD), indicative of increased anxiety-like behaviour later in life. However, these results could again be the result of decreased locomotor activity and a possible improved coping mechanism to be confirmed in prospective studies. To this extent, the anxiogenic effect of ω -3 EFA supplementation has been shown to be more significant under high stress, compared to low stress situations [96], suggesting additional anxiety models to further investigate the current behaviour.

Depressive-like behaviour

Daily high doses of ω -3 EFA have been reported to induce antidepressant-like effects [101-104] in adults [105, 106] and children [104], however, others have not been able to reproduce similar findings [107-109]. As such, data from the current study indicated no early antidepressant-like effect of pre-pubertal OM3 treatment in PND35 FSL rats (Fig. 4A), in line with a previous report in pubertal [110] and older animals [98]. However, the overall reduction in early-life locomotor activity in OM3 treatment groups (compare Fig. 3A), could have masked potential antidepressant-like effects of OM3 treatment. Furthermore, the possible improved coping behaviour of ω -3 EFA supplementation could also explain the current results. Regardless, pre-pubertal OM3 treatment also had no significant effect on depressive-like behaviour in early-adulthood in relation to controls (Fig. 4B). Again, it is important to consider that the decreased locomotor activity of OM3 treatment groups (compare Figure 3B) could again have masked any antidepressant-like effects.

Of note, pre-pubertal escitalopram treatment alone did not significantly affect depressive-like behaviour relative to age-matched controls (PND35; Fig. 4A). Importantly, a previous study found a seven day, 10 mg/kg twice daily escitalopram treatment to be effective in reducing depressive-like behaviour in PND28 Sprague-Dawley rats [46]. Therefore, the current treatment regimen represented sub-therapeutic dosing of escitalopram in PND21 FSL rats. Moreover, a double-blind, randomized, trial found 10-20 mg/kg/day escitalopram to be ineffective in improving depression scores in juveniles aged between 6 and 17 years [111]. However, when differentiating between children (6-11 years) and adolescents (12-17 years), escitalopram was found to be significantly more effective than placebo

treatment [111-113]. To this extent, PND21 to 34 represents pre-pubertal development in rats and correlates with childhood in humans [114], explaining unaltered antidepressant-like behaviour. Nevertheless, escitalopram mono-treatment appeared to be effective in reducing early-life depressive-like behaviour when compared to treatment groups that included ω -3 EFA supplementation (i.e. comparing ESC+STD to SAL+OM3 or ESC+OM3 treatment) immediately following early-life treatment (PND35; Fig. 4A). Escitalopram is known to increase 5-HT levels via inhibition of reuptake mechanisms, yet in the current study there was no significant increase in serotonergic-associated behaviour (i.e. swimming behaviour [58, 59, 115]) immediately following early-life treatment (PND35; Fig. 4C). Rather, increased struggling behaviour was observed (Figure 4E), where this behaviour has been positively associated with enhanced noradrenergic neurotransmission [58, 59, 115]. This somewhat paradoxical observation is in line with similar observations with fluoxetine in a previous study [40]. That PND35 FSL rats have been shown to display significantly lower serotonergic concentrations, compared to both new-born and adult FSL rats, yet still display depressive-like behaviour at all three stages [116], suggest a fine interplay between the developing monoaminergic systems in pre-pubertal brain. In particular, the already mature serotonergic pathway could have affected the neurodevelopment and signalling of other monoaminergic systems, such as the noradrenergic system, to now play a role in the observed antidepressant-like response. This, however, warrants investigation in prospective studies.

Furthermore, Fig. 4B indicates that the strong trend of pre-pubertal escitalopram mono-treatment (ESC+STD) to induce early-life antidepressant-like effects did not last into early-adulthood (PND60). However, a strong trend for the pre-pubertal combination treatment (ESC+OM3) to induce antidepressant-like behaviour later in life, compared to age-matched controls (SAL+STD) developed. This is of particular interest, since ESC+OM3 had no beneficial effect on depressive-like behaviour early in life (PND35), nor did the two respective mono-treatment strategies induce similar long-term effects, despite ESC+STD treatment improved early-life depressive-like behaviour. Therefore, the current data suggests the combination treatment to induce beneficial, neurodevelopmental behaviour alterations only observed later in life (PND60). Of note, this apparent decrease in depressive-like behaviour was observed despite a significant reduction in locomotor activity (Fig. 3B), suggesting a true reflection of psychomotor alteration. In support of the apparent neurodevelopmental antidepressant-like effect of ESC+OM3, is the significant increase in swimming behaviour of the ESC+OM3 treatment group later in life (Fig. 4D), indicative of enhanced serotonergic neurotransmission and supporting the abovementioned trend of decreased depressive-like behaviour. To this extent, when increased swimming behaviour is observed in the presence of a non-significant (or strong trend) decrease in time spent immobile, the data is still indicative of antidepressant-like effects [46]. That neither mono-treatment strategy induced similar antidepressant-like effects, yet the combination treatment did, suggests strong augmentative, neurodevelopmental effects of OM3 and

ESC. In fact, although data on the long-term or lasting effects of early-life antidepressant and even more so, dietary supplementation are limited, the proposed beneficial augmentation properties of ω -3 EFA supplementation on antidepressants are well reported [106, 117-120] and therefore support the current findings.

Monoaminergic and neuroplasticity bio-markers

None of the individual pre-pubertal treatment strategies significantly modulated monoamine concentrations (Table 1) or serotonin turnover (Fig. 5A) in the frontal cortex immediately following early-life treatment (PND35). However, for the NA concentration (Table 1), the main effect of drug (irrespective of diet), suggested that escitalopram enhanced NA concentration in the prefrontal cortex immediately following early-life treatment (PND35). The latter effect supports the observed corresponding increase in noradrenergic-associated behaviour (i.e. struggling; Fig. 4E) and is also supported by similar previous findings with fluoxetine [40]. That escitalopram increased NA, and not 5-HT concentrations (Table 1), can also be explained by the NA-mediated suppression of 5-HT release via α_2 -heteroreceptor stimulation [121, 122].

However, this effect of escitalopram on NA concentration in the prefrontal cortex did not last into early-adulthood. To the contrary, the concentrations of 5-HT and that of its metabolite 5-HIAA in the prefrontal cortex were significantly affected by escitalopram, ω -3 EFA supplementation and the combination thereof (Table 1), and of particular importance, serotonin turnover was significantly enhanced by the combination treatment group (ESC+OM3) relative to other treatment groups. This provides striking support for the findings from our behavioural data (Figure 4D), where the corresponding treatment group showed long lasting enhanced swimming behaviour, indicative of enhanced serotonergic neurotransmission.

Increased metabolism of 5-HT may also be associated with a surge in the activity of the kynurenine metabolic pathway, potentially increasing concentrations of neurotoxic metabolites [123]. To this extent, hippocampal BDNF concentrations were comparable across all treatment groups later in life (Fig. 5D). Therefore, any potential neurotoxic effects of enhanced serotonergic turnover are not reflected in the BDNF data. In this regard, future studies should investigate the effects of similar treatment strategies on serotonergic neurotransmission in terms of receptor density and binding analyses. Nevertheless, that early-life serotonergic neurotransmission was unaffected by combination treatment, yet significantly affected later in life, confirms neurodevelopmental processes to be involved.

Both ω -3 EFA supplementation [28, 124, 125] and escitalopram [7, 126, 127] have been associated with enhanced neuroplasticity. Yet in the current study, only escitalopram (ESC+STD), and not ω -3 EFA supplementation mono-treatment (SAL+OM3) significantly increased hippocampal BDNF

immediately following early-life treatment (PND35; Fig. 5C). However, when used in combination (ESC+OM3), the treatment strategy showed a strong trend to increase juvenile hippocampal BDNF as marker of neuroplasticity, relative to age-matched controls (SAL+STD). Taken together with the discussed anxiety-like behaviour above, the increase in hippocampal BDNF concentrations by ESC treatment with and without ω -3 EFA supplementation, supports the hypothesized improved coping mechanism responsible for the apparent increase in early-life anxiety-like behaviour, despite the difference between ESC+STD and SAL+STD not reaching statistical significance. Exercise is also known to have strong neuroplasticity enhancing effects, yet in other studies increased concentrations of BDNF return to baseline within two weeks following exercise cessation [128, 129], while pre-pubertal fluoxetine treatment also had no effect on hippocampal BDNF concentrations following a chronic wash-out period [40]. Together this suggests the current treatment strategies to only induce transient neuroplasticity enhancing benefits, yet strong, long-term augmentation properties to improve depressive-like behaviour in early-adulthood.

5 Conclusion

Non-pharmacological intervention strategies are particularly attractive considering their perceived improved safety profile and general ease of accessibility, although invariably not much is known of their overall efficacy or mechanism of action. In the current study, ω -3 EFA supplementation was investigated for its potential as augmentative strategy to an already approved pharmacological treatment option in juvenile MDD, escitalopram at a possible sub-therapeutic dose. Although both interventions have been shown to induce antidepressant-like behaviour, escitalopram mono-treatment showed a strong trend to improve depressive-like behaviour in relation to age-matched controls, whereas any beneficial effect of OM3 treatment may have been masked by reduced locomotor activity. Regardless, the apparent antidepressant-like effect of sub-therapeutic ESC treatment did not last into early-adulthood. In fact, later in life, only animals treated with the combination of escitalopram and ω -3 EFAs displayed decreased depressive-like behaviour, despite general locomotor activity being reduced and serotonergic turnover increased, while this was also associated with elevated hippocampal BDNF levels. In fact, BDNF is widely regarded as an important contributor to cellular resilience and to prevent the untoward effects of psychosocial stressors on brain structure, function and integrity [130]. BDNF is a distinct marker of stress adaptation, so that along with glucocorticoids and catecholamines, it plays an important link in understanding individual differences in vulnerability and resilience to anxiety, mood and stress disorders [131]. Anxiety-like behaviour was increased by the combination treatment in both stages of life, although improved coping mechanisms could be responsible for the observed behaviour. However, the aforementioned elevation in BDNF following combined treatment may underpin this rather paradoxical finding. A recent study explains how BDNF may mediate undesirable redox and metabolic changes that are associated with the development of a mood disorder [132], thus counter-regulatory. Nevertheless, further investigation into the apparent anxiogenic-like properties of ω -3 EFA supplementation is warranted. Overall, the results of the current study highlight the importance and value of ω -3 EFA supplementation in the prevention of behavioural deficiencies in later life. The impact of this data is seen in light of the significant ω -3 EFA content decline observed in the general Western diet [30]. Importantly, none of the investigated treatment strategies induced any unwanted or negative depressive-like alterations later in life, suggesting that escitalopram treatment, but not ω -3 EFAs, is effective in reducing early-life depressive-like symptoms. Neither mono-treatment strategy had a negative influence on depressive-like behaviour in a stress-sensitive subject. Contrary, ω -3 EFA supplementation shows potential as an augmentative strategy to escitalopram treated juveniles by possibly improving coping mechanisms induced in early-life and decreasing depressive-like behaviour later in life, following cessation of chronic treatment.

6 Compliance with Ethical Standards

All experiments conformed to the guidelines of the South African National Standards. The care and use of animals for scientific purposes (SANS 10386:2008) and were approved in accordance with the regulations set by the AnimCare animal research ethics committee (NHREC reg. no. AREC-130913-015) of the NWU, project ethics approval numbers NWU-00148-14-A5 and NWU-00373-16-A5.

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8 Conflict of interest

Jade Pharma Corporate (Pty) Ltd. (RSA) kindly sponsored the drugs used in the current study and Nordic Naturals (USA) sponsored the omega-3 oil that was used in coating of the vivarium rat chow. Except for income from the primary employer and research funding to both Professors Christiaan B Brink and Brian H Harvey from the MRC, NRF (and the above-mentioned exceptions), no financial support or compensation has been received from any individual or corporate entity over the past four years for research or professional services, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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CHAPTER 5: MANUSCRIPT C

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Article to be submitted to *Developmental Neuroscience* titled:

Pre-pubertal low intensity exercise augments venlafaxine treatment outcome in Flinders Sensitive Line rats by inducing lasting bio-behavioural effects into early adulthood

AUTHOR CONTRIBUTIONS

Stephanus F. Steyn assisted in designing the current project. SF Steyn also conducted all bio-behavioural experiments, performed the data work-up and statistical analyses, assisted in the interpretation of the study data, wrote the first draft of the manuscript and finalized it for submission.

Brian H. Harvey advised on the study design and proofread the final manuscript.

Christiaan B. Brink designed and supervised the study and assisted in the interpretation of the study data, finalized the manuscript for publication and was corresponding author in the submission of the final manuscript to *Developmental Neuroscience*.

IMPORTANT INFORMATION

- Instructions to the author can be viewed online at <https://www.karger.com/Journal/Home/224107>.
- As per the instructions to the author, figures and legends are provided at the end of the manuscript.
- Consent for the manuscript to be assessed as part of the PhD thesis of SF Steyn is presented in Addendum C.

Pre-pubertal low intensity exercise augments venlafaxine treatment outcome in Flinders Sensitive Line rats by inducing lasting bio-behavioural effects into early adulthood

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Abstract

A significant number of the world's adolescent population is considered to be insufficiently active. This is of concern, considering decreased physical activity to be positively associated with depressive symptoms and major depressive disorder (MDD) incidence. In fact, the use of antidepressants in children and adolescents are increasing, suggesting a rise in juvenile MDD prevalence. However, approved pharmacological treatment options remain limited, partly due to the possible unknown lasting effects of early-life treatment. Therefore, interest in non-pharmacological strategies is gaining popularity, largely due to the perceived improved safety profile. In this regard, low to moderate intensity exercise is especially attractive, due to its antidepressant-like effects and strong augmentation properties in combination with pharmacological antidepressants. Nevertheless, early-life development might present a unique 'window of opportunity' to induce long-term beneficial effects in individuals treated with central acting drugs, such as antidepressants. Therefore, we investigated the bio-behavioural effects of pre-pubertal (PND21 to PND34) venlafaxine and/or low intensity exercise on anxiety- and depressive-like behaviour during early-life (postnatal day 35 (PND35)) and early-adulthood in stress sensitive Flinders Sensitive Line rats, looking at behavioural outcomes and corresponding cortical and hippocampal monoamines and markers of neuroplasticity, i.e. BDNF. To

this extent, pre-pubertal treatment was followed by a 26-day wash-put/sedentary period until PND60 (early-adulthood). Pre-pubertal, low intensity exercise increased overall early-life coping strategies (viz. swimming behaviour), without affecting immobility, indicating towards an overall early-life antidepressant-like effect. The antidepressant-like effect of pre-pubertal exercise became more pronounced later in life (PND60) when it reduced immobility (depressive-like behaviour) and increased swimming behaviour, despite a significant reduction in locomotor activity. Importantly, this was observed with *and* without venlafaxine treatment during early-adulthood. Furthermore, that cortical noradrenaline and serotonin remained increased later in life (irrespective of drug), supported the observed increase in escape-directed behaviour, underscoring a monoaminergic basis to the observed antidepressant and enhanced coping effects of low intensity exercise, but also of venlafaxine and the combination? Importantly, that venlafaxine treatment alone, did not induce any antidepressant like-behaviour at either stage of life, yet when combined with low-intensity exercise, long-term antidepressant-like effects were observed, suggests true augmentation potential for such a treatment strategy. Nevertheless, the early-life exercise-induced increase in hippocampal BDNF was only temporary, returning to levels, comparable to controls later in life. We conclude that, chronic pre-pubertal, low intensity exercise may induce pronounced early-life monoaminergic and neuroplasticity enhancing effects that beneficially influence neurodevelopment and present as decreased antidepressant-like behaviour later in life. Furthermore, pre-pubertal low intensity exercise could augment the long-term effects of early-life venlafaxine treatment, suggesting a potential beneficial treatment strategy.

Keywords

Venlafaxine; Exercise; Neurodevelopment; Lasting effects; Depressive-like behaviour; Pre-puberty, FSL, Augmentation

1 Introduction

The role of exercise in major depressive disorder (MDD) has been extensively researched, yet its involvement and/or value in a depressed juvenile population are less known. Currently, more than 80 % of the world's adolescent population is insufficiently physically active [1]. Furthermore, only 30 % of the Western population take part in sufficient amounts of weekly exercise, of which up to 50 % discontinue their exercise activities within 3 to 6 months [2]. This is of concern since a significant number of children and adolescents suffer from MDD [3-7] and is further fraught by the inverse correlation of physical activity and MDD incidence [8,9]. Therefore, exercise may serve as a valuable adjunctive strategy in the treatment of juvenile MDD. In fact, exercise is associated with various neurobiological improvements that contribute to antidepressant-like effects. In this regard, clinical and preclinical studies have demonstrated that chronic low to moderate, but not high intensity exercise,

enhances monoaminergic neurotransmission [10], neuroplasticity [11] and anti-oxidant defences, and ameliorates central inflammation [12]. Additionally, human and animals studies have reported on the positive augmentative properties of exercise on antidepressant treatment [13-15]. Nevertheless, appropriately approved antidepressant treatment options for juvenile MDD patients are limited to only fluoxetine and escitalopram [16], although venlafaxine, a serotonergic-noradrenergic reuptake inhibitor (SNRI) is a popular 'off-label' antidepressant to be considered in treatment resistant juvenile depression [17-19]. The effectiveness and rationale for this indication is unclear and controversial.

However, concern regarding juvenile treatment with pharmacological strategies, such as antidepressants, are associated with adverse effects and generally unknown lasting or long-term effects. To this extent, all antidepressants are now required to be marketed with a black box warning [20], highlighting the increased risk of suicidality in paediatric patients. Contrary, untreated juvenile MDD in itself is an obvious risk for suicidal behaviour, thereby placing prescribing medical practitioners in a peculiar situation where the value of an antidepressant is questioned with regards to immediate and possible long-term effects [21]. Therefore, research into the long-term effects of early-life antidepressant and/or augmentation strategies are required to assist medical practitioners in prescribing safe treatment strategies with lasting beneficial effects. In this regard, early-life development has been described as a possible 'window of opportunity' [22] where interventions could induce lasting neurodevelopmental alterations, benefitting the individual later in life. To this extent, exercise during early-life, with or without drug treatment, could potentially benefit the individual later in life

According to earlier data from our laboratories, venlafaxine treatment during pre- and early postnatal development induces late-life beneficial antidepressive-like behaviour in an animal model of depression [23,24]. Therefore, that both venlafaxine [25] and exercise [10] induce antidepressant-like effects, via different pathways, could suggest augmentative potential. In this regard, venlafaxine is a known dual inhibitor of noradrenergic and serotonergic reuptake mechanisms [26], whereas exercise affects various other neurobiological pathways associated with MDD pathophysiology. In fact, as mentioned, low intensity exercise enhances neuroplasticity [11] and anti-oxidant defences, and ameliorates central inflammation [12]; altogether alleviating depressive-like symptoms. Therefore, the current study investigated the lasting or long-term effects of pre-pubertal (postnatal day 21 (PND21) until PND34) venlafaxine treatment, at a possible sub-therapeutic dose, low intensity exercise and the combination thereof, on anxiety- and depressive-like behaviour in a stress-sensitive, genetic animal model of depression, the Flinders Sensitive Line (FSL) rat. In addition, key neurochemical and neuroplasticity markers were assessed in selected limbic brain regions, including total monoamine NA and 5HT levels, as well as brain derived neurotrophic factor (BDNF). To determine whether the observed bio-behaviour indeed involves neurodevelopmental mechanisms, a subset of animals were analysed immediately

following chronic pre-pubertal intervention on PND35 (i.e. onset of puberty) and another subset following a long-term treatment-free period at PND60 (i.e. early-adulthood) [27].

2 Materials and methods

2.1 Test subjects and treatment strategies

2.1.1 Animals

Male Flinders Sensitive Line (FSL; $n = 92$) rats were bred, supplied and housed at the vivarium (SAVC reg. no. FR15/13458; SANAS GLP compliance no. G0019; AAALAC accreditation international file #1717) of the Pre-Clinical Drug Development Platform (PCDDP), NWU, RSA. The FSL rat is a validated genetic animal model of depression, displaying face, construct and predictive validity [28,29]. The original rat colonies were obtained from Dr David H Overstreet, University of North Carolina, Chapel Hill, USA. All animals were maintained and all procedures involving animals were in accordance to the code of ethics in research, training and testing of drugs in South Africa and complied with national legislation. Importantly, the exaggerated 8-hydroxy-2-(di-N-propylamino)tetratin (8-OH-DPAT) induced hypothermic response in FSL rats [30], as well as enhanced immobility in the forced swim test, relative to Flinders Resistant Line (FRL) rats were confirmed at the start of the study.

Animals were randomly divided into four test groups: **1)** saline control + sedentary (**SAL+SED**), **2)** venlafaxine + sedentary (**VEN+SED**), **3)** saline control + low intensity exercise (**SAL+EXE**), **4)** venlafaxine + low intensity exercise (**VEN+EXE**) with approximately 12 rats per group. Rats were group-housed three per cage, with corncob bedding changed weekly and the environmental temperature maintained at 22 ± 1 °C and a relative humidity of 55 ± 10 %. A 12-hour light/dark cycle was followed with food and water provided *ad libitum*. Pups were weaned on postnatal day 21 (PND21) and body weight measured daily during pre-pubertal treatment (PND21 until PND34). From PND35 to PND60 rats were housed under normal conditions, without any treatment/intervention. Animal wellbeing was routinely monitored during and after each injection and exercise session.

The period between PND21 to PND34 represents pre-puberty in rodents when the serotonergic pathway is already matured, while the noradrenergic and dopaminergic pathways are still developing [31,32]. After treatment, rats were either submitted to behavioural analyses on the following day (PND35; early-life) or left in normal housing conditions with normal rat chow during a period of 26 days of neurodevelopment and maturing, serving also as a treatment washout/cessation, until behavioural analyses on PND60 (early adulthood). Animals were sacrificed by decapitation within 24 hours following completion of behavioural analyses, where frontal cortices and hippocampi were dissected and stored for neurobiological analyses.

2.1.2 Drug treatment

Animals received either saline (vehicle) or venlafaxine HCl (10 mg/kg/day) (a gift from Jade Pharmaceuticals, RSA) via subcutaneous (sc) administration once daily from PND21 to PND34. Currently, because venlafaxine not being an approved treatment option for juvenile MDD, yet a popular ‘off-label’ strategy, no dosage guidelines for its use in children are available. However, one group suggests a dose between 37.5 mg/day and 75 mg/day might be successful [33]. In this regard, a dose of 10 mg/kg/day has been shown to be successful in inducing long-term behavioural alterations in FSL offspring when administered to pregnant dams [23]. Nevertheless, it is important to note that venlafaxine was not used a positive control in the current study, but merely as an alternative treatment option with augmentation potential and that the selected dose was expected to be sub-therapeutic. A 14-day treatment period is generally considered sub-chronic and longer than the minimum required to produce antidepressant-like effects in FSL rats [34]. The subcutaneous route of administration has a predictable bioavailability comparable to that of an intraperitoneal injection, although injection stress is less, particularly in young rats, and was therefore used in the current study, in line with previous work [35].

2.1.3 Exercise regimen

An age related, low intensity exercise regimen was implemented as previously described [35]. In short, a custom-built, programmable treadmill, comprising a single treadmill belt (51 mm (w) x 96 mm (d)) and six shocking grids (14 mm (w) x 21 mm (d)), yielding sufficient electrical shock (1 mA, 3 Hz) to be uncomfortable but not painful or harmful installed at the back of the treadmill was used. Six removable running lanes (14 mm (w) x 66 mm (d) x 14 mm (h)) with black opaque vertical walls and a clear top cover were placed over the belt to accommodate six subjects at a time.

All animal subjects were familiarized to the treadmill from PND16 until PND20 to reduce injury risk as well as identify any “non-runners”. In this regard, animals were subjected to a daily 10 min routine with gradual daily increase in treadmill speed where “non-runners” were removed from the study. Subjects of the exercise intervention group were subjected to a daily age-related, low intensity exercise regimen, according to data from our own laboratory. Sedentary animals were removed from their cages and placed on a still-standing (mock) treadmill for 30 min during the time other rats underwent treadmill running. During the 30 min of daily training, the first 5 min were at a comfortable walking speed (33% of the intended exercise intensity), where after the intensity was increased to 67 % of the intended intensity for 5 min (end of warm-up session) and then to 100 % of the intended speed for the final 20 of the 30-min session [35]. In this regard, treadmill speed for the 20-min training session increased

from 4.6 m/min on PND21 to 17.8 m/min on PND34. All exercise interventions (and sedentary controls) were performed during the dark cycle.

2.2 Behavioural analyses

After the 14-day treatment period, animals were subjected to the open field and forced swim tests. Data from our own laboratory indicated that foregoing tests do not affect the outcome of the subsequent consecutive tests if they are ordered from least to most stressful [36]. As before [35], the behavioural tests were performed for all treatment groups either early after the pre-puberty intervention period on PND35, or later in life after washout (withdrawal) and normal housing on PND60. Testing commenced one hour after the start of the dark cycle to ensure normal initial foraging and activity of nocturnal animals. Tests were carefully spaced to allow 30 min between each test for animals to habituate to the environment.

2.2.1 Open field test

The open field test (OFT) is commonly used to measure locomotor activity, a parameter of the general ability of the animal to move and negotiate its surroundings, as well as anxiety-like behaviour [37-39], of which the latter correlates with results of other robust behavioural tests, such as the elevated plus maze [40]. The OFT apparatus in the current study consisted of a 1 m² test arena, surrounded by opaque black, vertical walls. As before [35], on the day of testing, each rat was placed in the centre of the arena and allowed to explore the environment for 5 min under red light (80 lx) [35]. During this time, rats were videotaped by a camera mounted above the test arena and subsequently scored using Ethovision XT12 software (Noldus Information Technology BV, Wageningen, NLD). Total distance moved during the session was used as a measure of general activity. The total time spent in the centre zone (50 cm²) was used as a measure of anxiety-like behaviour, with a reduction indicating increased anxiety-like behaviour.

2.2.2 Forced swim test

The forced swim test (FST) is widely used to screen for antidepressant activity and to assess depressive-like behaviour in rodents [41]. An adjusted version of the FST distinguishes between serotonergic and noradrenergic-directed behaviours [42]. The FSL rat is a validated genetic animal model of depression that displays depressive-like behaviour (enhanced immobility) without the pre-conditioning swim trial 24 hours prior to the testing swim trial [28].

The FST was performed as described by our laboratory group [35]. Briefly, the apparatus consisted of four cylindrical tanks ((40 cm (*h*) x 20 cm (*d*)), each filled to a depth of 30 cm with water, maintained at 25 ± 1°C. The test was performed during the dark cycle under red light (80 lx). On the day of testing,

each rat was placed in the cylinder where behaviour was recorded and analysed for 5 min by an experimenter blind to the test group [43].

Behaviour was scored with a manual continuous timer software (FST Scoreboard 2.0 software; Academic Support Services: Information Technology in Education, NWU, RSA), previously validated against the 5 s time-sampling technique [44]. Scored behaviour included immobility (floating with no active movements made, except those necessary to keep the rat's head above water), swimming (horizontal movements throughout the cylinder that included crossing into another quadrant) and struggling (upward-directed movements of the forepaws along the inside of the swim cylinder) [42,45]. Of note, although total time spent diving was recorded, this specific behaviour was not included in the final depressive-like behaviour analysis of the animals due to being episodic and seemingly not corresponding with specific treatment effects [42]. Enhanced adrenergic neurotransmission is associated with increased struggling, whereas enhanced serotonergic neurotransmission is associated with increased swimming behaviour [45,46].

2.3 Neurobiological analyses

2.3.1 Quantitative analysis of frontal cortical monoamines

Quantitative monoaminergic concentrations were analysed as previously described by our laboratory group [47]. Following sacrifice of rats by decapitation, total hippocampi and frontal cortices were dissected on an ice-cooled dissection slab and snap frozen in liquid nitrogen and stored at -80 °C until day of analysis. Neurobiological samples were pooled in pairs and analysed accordingly. Pooling of biological samples have been shown to be a valid method to, amongst others, improve cost-effectiveness without affecting biological variation [48]. In cases where an odd number of biological samples were available, the remaining sample would be analysed individually. Quantification of noradrenaline (NA) and 5-HT was performed by high performance liquid chromatography (HPLC) coupled with electrochemical detection (ECD). An Agilent 1200 series HPLC (Agilent Technologies, California, USA), equipped with an isocratic pump, auto sampler and coupled to an ESA Coulochem III Electrochemical detector (Dionex, California, USA), and Chromeleon® Chromatography Management System software (version 6.8), was used. NA and 5-HT concentrations in the tissue samples were determined by comparing the peak area of each monoamine to that of the monoamine standards (range 5-50 ng/ml). Linear standard curves (regression coefficient > 0.95) were found in this range. Monoamine concentrations were expressed as ng/g wet weight of tissue.

2.3.2 Quantitative analysis of hippocampal brain-derived neurotrophic factor

Hippocampal tissue samples were collected and prepared as described above. Hippocampal BDNF concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) BDNF kit

(Elabscience) according to the manufacturers' protocol. Briefly, the pooled hippocampal samples were weighed and homogenized in phosphate buffered saline (PBS) and centrifuged for 5 min at 5 000 xg. The supernatant was transferred to the 96-well plate and incubated for 90 min at 37 °C, whereafter the liquid was removed, biotinylated detection antibody added and incubated for another 60 min at 37 °C. The samples were aspirated and washed three times, before 100 µl horseradish peroxidase (HRP) conjugate was added and incubated for 30 min at 37 °C. Samples were again aspirated and washed five times before 90 µl substrate reagent were added to samples and incubated for 15 min at 37 °C. Finally, 50 µl of stop solution was added and samples read at 450 nm. Results were obtained from a standard curve plotted and expressed as pg/ml.

2.4 Statistical analyses

Normality of the data and homogeneity of variances were determined using the Shapiro-Wilk test and Levene's test for equality of variances, respectively. In both instances, $p < 0.05$ indicated that the respective assumption had been violated. The Grubbs' test was used to determine any outlier in each data set with $\alpha = 0.05$ accepted as significant, however, less than 10 % of the total number of subjects were removed due to either not representing the target population or displaying "non-running" behaviour. In this regard, experimental group sizes are presented for each specific data set in the figure legend.

Normal two-way analysis of variances (ANOVAs) were performed on all data sets, regardless of normal distribution or homogeneity of variances [49,50]. The two-way ANOVA determined whether a statistically significant two-way interaction between drug (SAL or VEN) and activity (SED or EXE) existed. Irrespective of the significance of the ANOVA-identified interaction, main effects were analysed followed by pairwise comparisons [51] with the Bonferroni post-hoc test. In all instances, a 5 % confidence limit for error was taken as statistically significant ($p \leq 0.05$) for all analyses and data is reported with a 95 % confidence interval (CI) of the mean difference.

Finally, effect magnitude indicators were calculated [52] along with all statistical analyses, in line with statistical reporting guidelines [53,54] to indicate strong trends and rule out Type I (false positive) or Type II (false negative) errors [55,56]. Effect magnitude for interactions were calculated with partial eta squared (η^2), where effect sizes were considered large when $\eta^2 \geq 0.14$ [56]. Furthermore, effect magnitude differences between specific groups were calculated by Cohen's d value (with a 95 % CI of the effect magnitude). Cohen's d value is an effect size indicator used to specify the standardized difference between two means, with effect sizes considered large when $d \geq 0.8$ [57]. In all instances, only large effect magnitude indicators were considered significant.

ANOVA statistical analyses were performed in IBM® SPSS® Statistics (version 24.0, Armonk, NY: IBM Corp) and GraphPad Prism® (version 6.0, San Diego California USA), assisted by Laerd Statistics® (<https://statistics.laerd.com>) and the statistical consultation service of the North-West University, Potchefstroom.

3 Results

Figure 1 represents early and lasting behavioural effects of the FSL rats in the OFT on PND35 and PND60.

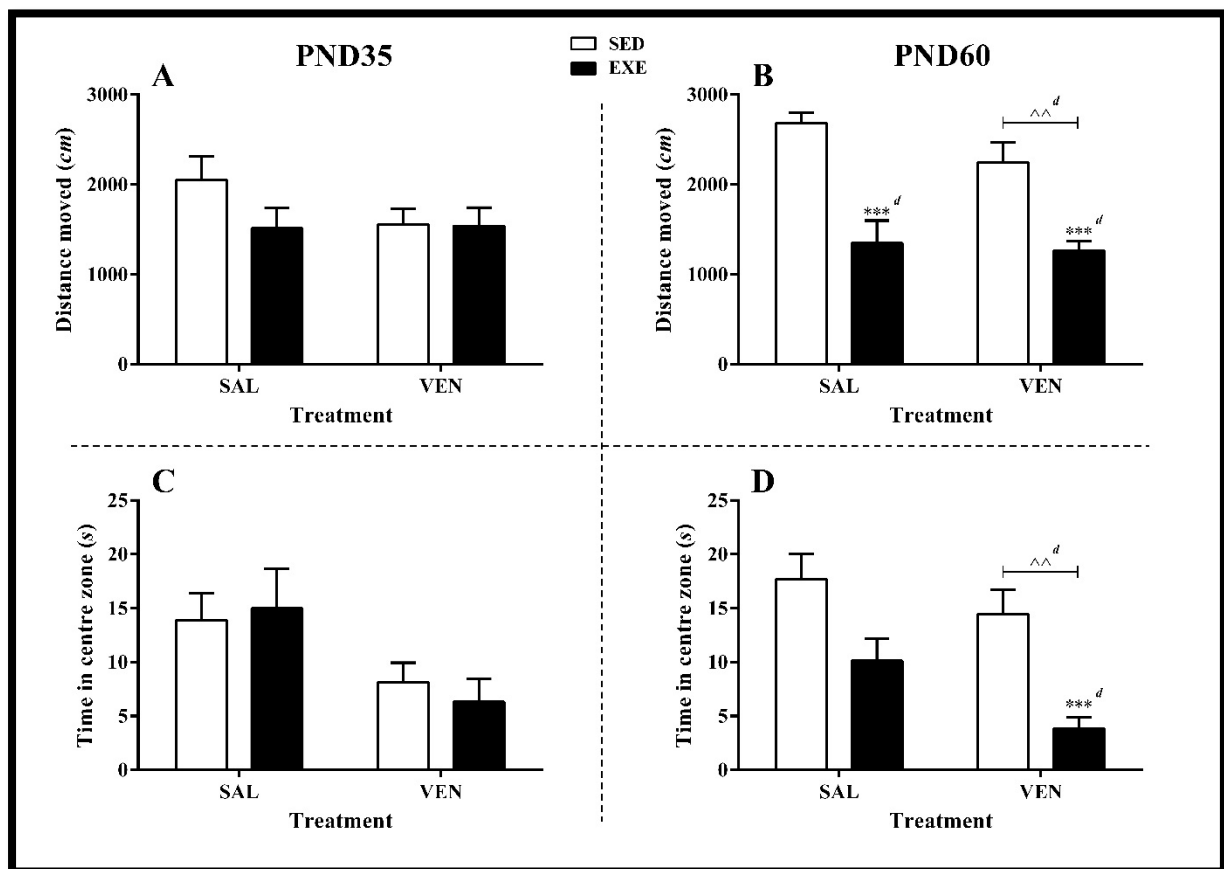


Figure 1: Early and lasting effects of pharmacological and non-pharmacological interventions on locomotor activity and anxiety-like behaviour of FSL rats on PND35 and PND60.

(A) Distance moved in the OFT on PND35 after treatment with SAL+SED ($n = 12$), SAL+EXE ($n = 11^a$), VEN+SED ($n = 12$) or VEN+EXE ($n = 12$). **(B)** Distance moved in the OFT on PND60 (SAL+SED $n = 10^{a,b}$; SAL+EXE $n = 12$; VEN+SED $n = 12$; VEN+EXE $n = 10^a$). **(C)** Time in centre zone in the OFT on PND35 (SAL+SED $n = 12$; SAL+EXE $n = 10^{a,b}$; VEN+SED $n = 12$; VEN+EXE $n = 12$). **(D)** Time in centre zone in the OFT on PND60 (SAL+SED $n = 10^{a,b}$; SAL+EXE $n = 12$; VEN+SED $n = 12$; VEN+EXE $n = 10^a$). Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text with *** $p \leq 0.001$ vs. SAL+STD; ^ $p \leq 0.01$ vs. indicated test group; d large effect magnitude ≥ 0.8 . EXE: Exercise (low intensity). OFT: Open field test. PND: Postnatal day. SAL: Saline (control). SED: Sedentary. VEN: Venlafaxine. ^a) Less than 12 animals due to lower than expected birth rates at the requested date. ^b) Outlier identified and removed from group due to not representing the target population.

In Figure 1A, distance moved data of PND35 rats were normally distributed ($p > 0.05$) and had homogeneity of variances ($p = 0.689$). There was no statistically significant two-way interaction

between drug and activity ($F[1, 43] = 1.426, p = 0.239; \eta^2 = 0.032$), nor were there any statistically significant main effects.

In Figure 1B, distance moved data of PND60 rats were normally distributed ($p > 0.05$) and had homogeneity of variances ($p = 0.128$). There was no statistically significant two-way interaction between drug and activity ($F[1, 40] = 0.794, p = 0.378; \eta^2 = 0.019$). However, there was a statistically significant main effect of activity ($F[1, 40] = 34.627, p < 0.0005; \eta^2 = 0.464$), but not of drug ($F[1, 40] = 1.775, p = 0.190; \eta^2 = 0.042$), so that EXE decreased overall locomotor activity by 1161.0 cm (95 % CI, 762.3 to 1559.8 cm) compared to SED (irrespective of drug). Furthermore, compared to SAL+SED (2685.1 ± 115.1 cm), distance moved was 1336.9 cm (95 % CI, 562.3 to 2111.4 cm) lower in SAL+EXE (1348.3 ± 249.9 cm; $p = 0.0001; d = 2.041$, 95 % CI, 1.0 to 3.1), and 1423.9 cm (95 % CI, 614.9 to 2232.8 cm) lower in VEN+EXE (1261.3 ± 110.1 cm; $p = 0.0001; d = 4.215$, 95 % CI, 2.6 to 5.8). Similarly, VEN+EXE decreased distance moved by 985.2 cm (95 % CI, 210.6 to 1759.7 cm) compared to VEN+SED (2246.5 ± 221.2 cm; $p = 0.006; d = 1.681$, 95 % CI, 0.7 to 2.7).

In Figure 1C, time spent in centre zone data of PND35 rats were not normally distributed for all test groups ($p < 0.05$), however, there was homogeneity of variances ($p = 0.562$). There was no statistically significant two-way interaction between drug and activity ($F[1, 42] = 0.336, p = 0.565; \eta^2 = 0.008$). However, there was a statistically significant main effect of drug ($F[1, 42] = 8.185, p = 0.007; \eta^2 = 0.163$), but not of activity ($F[1, 42] = 0.022, p = 0.883; \eta^2 = 0.001$), yet no treatment specific differences were identified by the multiple comparison test.

In Figure 1D, time spent in centre zone data of PND60 rats were normally distributed for all test groups ($p > 0.05$), and there was homogeneity of variances ($p = 0.131$). There was no statistically significant two-way interaction between drug and activity ($F[1, 40] = 0.534, p = 0.469; \eta^2 = 0.013$). However, there were statistically significant main effects of activity ($F[1, 40] = 19.422, p < 0.0005; \eta^2 = 0.327$) and of drug ($F[1, 40] = 5.393, p = 0.025; \eta^2 = 0.119$), that VEN (irrespective of activity) and EXE (irrespective of drug) decreased overall time spent in the centre zone by 4.8 s (95 % CI, 0.6 to 9.0 s) and 9.0 s (95 % CI, 4.9 to 13.3 s) compared to SAL and SED, respectively. Furthermore, VEN+EXE (3.8 ± 1.1 s) decreased time spent in centre zone by 13.9 s (95 % CI, 5.4 to 22.3 s) and by 10.6 s (95 % CI, 2.5 to 18.7 s) compared to SAL+SED (17.7 ± 2.3 s; $p = 0.0003; d = 2.557$, 95 % CI, 1.4 to 3.7) and VEN+SED (14.4 ± 2.3 s; $p = 0.005; d = 1.762$, 95 % CI, 0.8 to 2.8), respectively.

Figure 2 represents early and lasting behavioural effects of the FSL rats in the FST on PND35 and PND60.

In Figure 2A, time spent immobile data of PND35 rats were not normally distributed for all test groups ($p < 0.05$), yet there was homogeneity of variances ($p = 0.567$). There was no statistically significant two-way interaction between drug and activity ($F[1, 43] = 0.010$, $p = 0.921$; $\eta^2 < 0.0005$), nor were there were any statistically significant main effects.

In Figure 2B, time spent immobile data of PND60 rats were normally distributed for ($p > 0.05$), yet had no homogeneity of variances ($p = 0.024$). There was no statistically significant two-way interaction between drug and activity ($F[1, 41] = 0.660$, $p = 0.421$; $\eta^2 = 0.016$). However, there was a statistically significant main effect of activity ($F[1, 41] = 27.466$, $p < 0.0005$; $\eta^2 = 0.401$), but not of drug ($F[1, 41] = 1.334$, $p = 0.255$; $\eta^2 = 0.032$). Compared to SAL+SED (223.8 ± 5.2 s), time spent immobile was 27.2 s (95 % CI, 3.4 to 51.0 s) lower in SAL+EXE (196.7 ± 8.1 s; $p = 0.017$; $d = 1.216$, 95 % CI, 0.3 to 2.1), and 39.3 s (95 % CI, 14.4 to 64.2 s) lower in VEN+EXE (184.6 ± 5.9 s; $p = 0.0005$; $d = 2.319$, 95 % CI, 1.2 to 3.4). Similarly, VEN+EXE decreased time spent immobile by 37.2 s (95 % CI, 12.8 to 51.6 s) compared to VEN+SED (221.7 ± 4.5 s; $p = 0.0008$; $d = 2.290$, 95 % CI, 1.2 to 3.4).

In Figure 2C, time spent swimming data of PND35 rats were not normally distributed for all test groups ($p < 0.05$), nor was there homogeneity of variances ($p = 0.008$). There was no statistically significant two-way interaction between drug and activity ($F[1, 43] = 0.161$, $p = 0.690$; $\eta^2 = 0.004$). However, there was a statistically significant main effect of activity ($F[1, 43] = 5.418$, $p = 0.025$; $\eta^2 = 0.112$), but not of drug ($F[1, 43] = 0.008$, $p = 0.928$; $\eta^2 < 0.0005$). EXE increased overall swimming time by 9.1 s (95 % CI, 1.2 to 17.1 s) compared to SED treatment groups (irrespective of drug), yet no treatment specific differences were identified by the multiple comparison test.

In Figure 2D, time spent swimming data of PND60 rats were not normally distributed for all test groups ($p < 0.05$), nor was there homogeneity of variances ($p = 0.003$). There was no statistically significant two-way interaction between drug and activity ($F[1, 39] = 0.161$, $p = 0.690$; $\eta^2 = 0.004$). However, there was a statistically significant main effect of activity ($F[1, 39] = 25.928$, $p < 0.0005$; $\eta^2 = 0.399$), but not of drug ($F[1, 39] = 1.560$, $p = 0.219$; $\eta^2 = 0.038$). In line with the main effect of activity, SAL+EXE increased time spent swimming by 11.9 s (95 % CI, 3.5 to 20.3 s) compared to SAL+SED (28.4 ± 3.0 vs. 16.5 ± 1.0 s; $p = 0.002$; $d = 1.561$, 95 % CI, 0.6 to 2.5). Similarly, VEN+EXE increased time spent swimming by 10.2 s (95 % CI, 1.6 to 18.8 s) compared to VEN+SED (24.8 ± 2.5 vs. 14.7 ± 1.1 s; $p = 0.002$; $d = 1.788$, 95 % CI, 0.8 to 2.8).

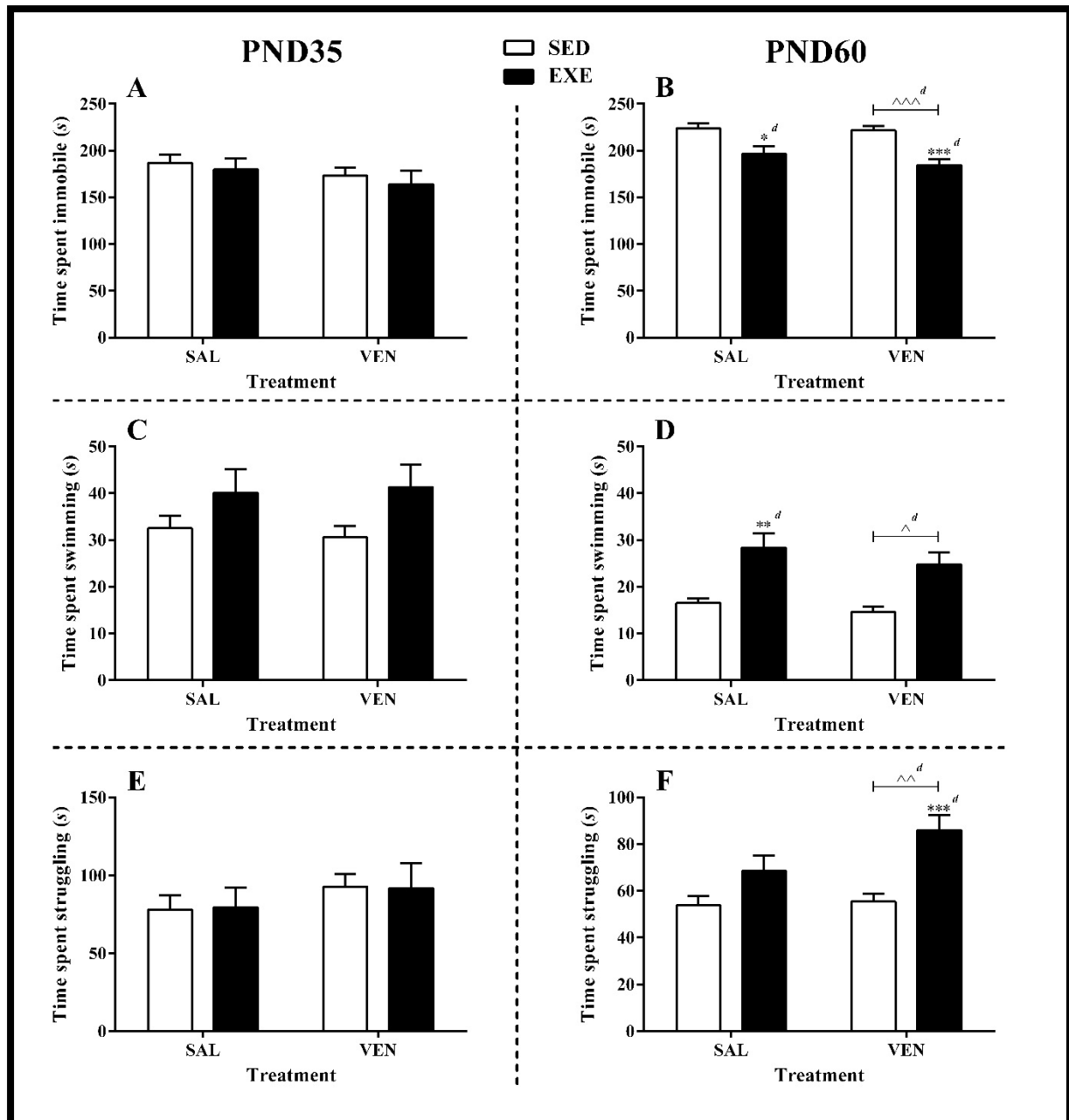


Figure 2: Early and lasting effects of pharmacological and non-pharmacological interventions on depressive-like behaviour of FSL rats on PND35 and PND60.

(A) Time spent immobile in the FST on PND35 after treatment with SAL+SED ($n = 12$), SAL+EXE ($n = 11^a$), VEN+SED ($n = 12$) or VEN+EXE ($n = 12$). (B) Time spent immobile in the FST on PND60 (SAL+SED $n = 11^a$; SAL+EXE $n = 12$; VEN+SED $n = 12$; VEN+EXE $n = 10^a$). (C) Time spent swimming in the FST on PND35 (SAL+SED $n = 12$; SAL+EXE $n = 11^a$; VEN+SED $n = 12$; VEN+EXE $n = 12$). (D) Time spent swimming in the FST on PND60 (SAL+SED $n = 10^{a,b}$; SAL+EXE $n = 12$; VEN+SED $n = 11^b$; VEN+EXE $n = 10^a$). (E) Time spent struggling in the FST on PND35 (SAL+SED $n = 12$; SAL+EXE $n = 11^a$; VEN+SED $n = 12$; VEN+EXE $n = 12$). (F) Time spent struggling in the FST on PND60 (SAL+SED $n = 11^a$; SAL+EXE $n = 12$; VEN+SED $n = 12$; VEN+EXE $n = 10^a$). Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text with * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs. SAL+STD; ^ $p \leq 0.05$, ^^ $p \leq 0.01$, ^^ $p \leq 0.001$ vs. indicated test group; d large effect magnitude ≥ 0.8 . EXE: Exercise (low intensity). FST: Forced swim test. PND: Postnatal day. SAL: Saline (control). SED: Sedentary. VEN: Venlafaxine. ^a Less than 12 animals due to lower than expected birth rates at the requested date. ^b Outlier identified and removed from group due to not representing the target population.

In Figure 2E, time spent struggling data of PND35 rats were not normally distributed for all test groups ($p < 0.05$), yet there was homogeneity of variances ($p = 0.370$). There was no statistically significant two-way interaction between drug and activity ($F[1, 43] = 0.013$, $p = 0.911$; $\eta^2 < 0.0005$), nor were there were any statistically significant main effects.

In Figure 2F, time spent struggling data of PND60 rats were not normally distributed for all test groups ($p < 0.05$), nor was there homogeneity of variances ($p < 0.0005$). There was no statistically significant two-way interaction between drug and activity ($F[1, 41] = 2.277$, $p = 0.139$; $\eta^2 = 0.053$). However, there was a statistically significant main effect of activity ($F[1, 41] = 18.266$, $p < 0.0005$; $\eta^2 = 0.308$), but not of drug ($F[1, 41] = 3.263$, $p = 0.078$; $\eta^2 = 0.074$), that EXE increased overall struggling time by 22.6 s (95 % CI, 11.9 to 33.2 s) compared to SED (irrespective of drug). Furthermore, VEN+EXE (86.0 ± 6.4 s) increased time spent struggling by 32.1 s (95 % CI, 10.7 to 53.5 s) and by 30.5 s (95 % CI, 9.6 to 51.5 s) compared to SAL+SED (53.9 ± 4.1 s; $p = 0.0009$; $d = 1.984$, 95 % CI, 0.9 to 3.0) and VEN+SED (55.4 ± 3.3 s; $p = 0.001$; $d = 2.005$, 95 % CI, 1.0 to 3.0), respectively.

Figure 3 represents the early and lasting monoaminergic effects as measured in the frontal cortex of the FSL rats on PND35 and PND60.

In Figure 3A, PND35 frontal cortical NA data were normally distributed for all test groups ($p > 0.05$), and had homogeneity of variances ($p = 0.169$). There was no statistically significant two-way interaction between drug and activity ($F[1, 18] = 0.053$, $p = 0.821$; $\eta^2 = 0.003$). However, there was a statistically significant main effect of activity ($F[1, 18] = 10.601$, $p = 0.004$; $\eta^2 = 0.371$), but not of drug ($F[1, 18] = 0.583$, $p = 0.455$; $\eta^2 = 0.031$). EXE increased overall NA concentrations by 15.1 ng/g (95 % CI, 5.3 to 24.8 ng/g) compared to SED (irrespective of drug), yet no treatment specific differences were identified by the multiple comparison test.

In Figure 3B, PND60 frontal cortical NA data were not normally distributed for all test groups ($p < 0.05$), yet had homogeneity of variances ($p = 0.694$). There was no statistically significant two-way interaction between drug and activity ($F[1, 17] = 2.588$, $p = 0.126$; $\eta^2 = 0.132$). However, there was a statistically significant main effect of activity ($F[1, 17] = 83.705$, $p < 0.0005$; $\eta^2 = 0.831$), but not of drug ($F[1, 17] = 1.867$, $p = 0.190$; $\eta^2 = 0.099$). Compared to SAL+SED (65.9 ± 2.7 ng/g), NA concentration was 24.4 ng/g (95 % CI, 11.2 to 37.5 ng/g) higher in SAL+EXE (90.3 ± 3.0 ng/g; $p = 0.0002$; $d = 3.972$, 95 % CI, 1.9 to 6.0) and 34.0 ng/g (95 % CI, 20.8 to 47.1 ng/g) higher in VEN+EXE (99.9 ± 3.5 ng/g; $p < 0.0001$; $d = 5.019$, 95 % CI, 2.6 to 7.4), respectively. Similarly, VEN+EXE increased NA concentration by 34.8 ng/g (95 % CI, 20.7 to 48.8 ng/g) compared to VEN+SED (65.1 ± 3.3 ng/g; $p < 0.0001$; $d = 4.953$, 95 % CI, 2.4 to 7.5).

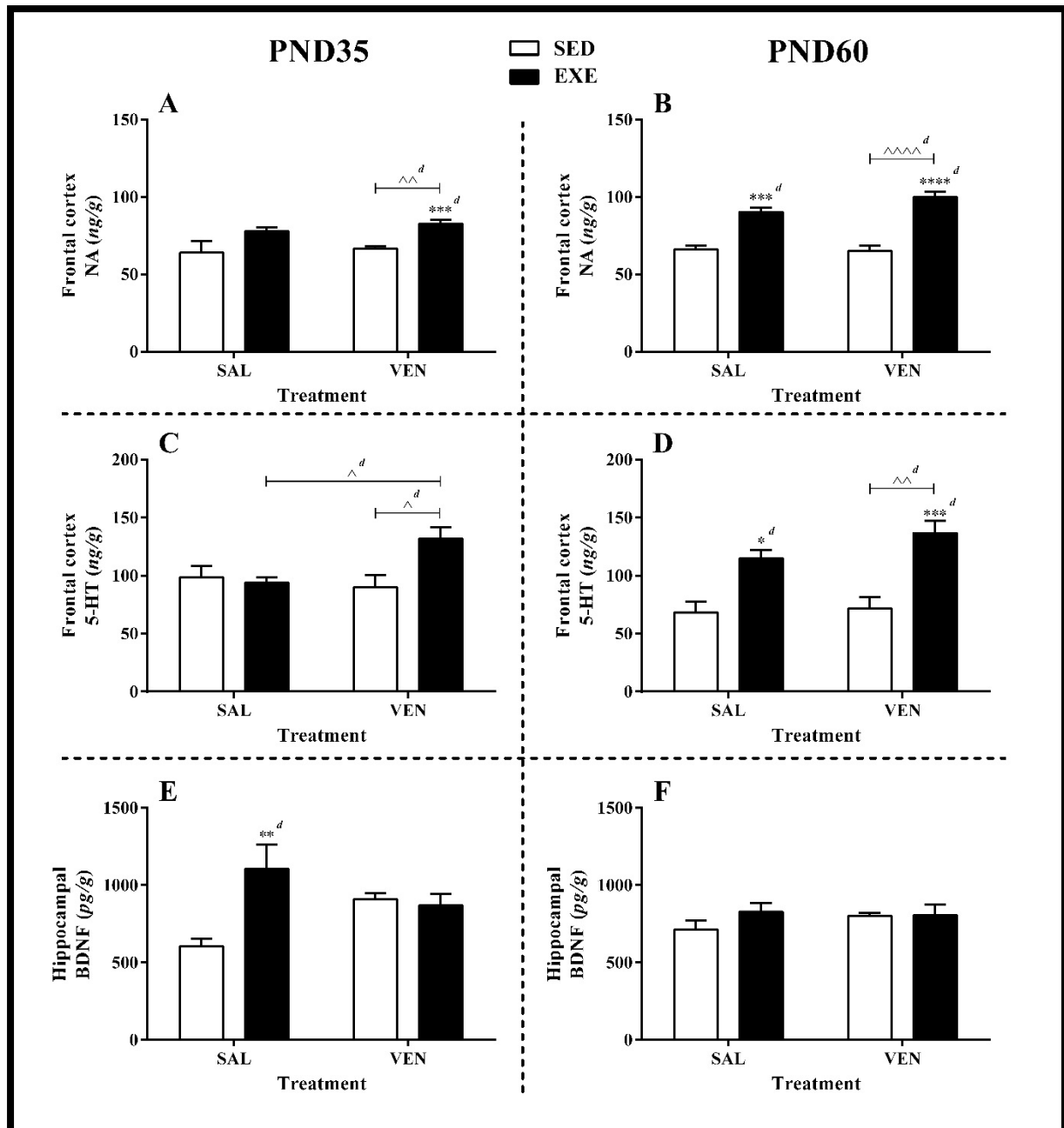


Figure 3: Early and lasting effects of pharmacological and non-pharmacological interventions on depressive-like behaviour of FSL rats on PND35 and PND60.

(A) Frontal cortical NA concentrations on PND35 after treatment with SAL+SED ($n = 6$), SAL+EXE ($n = 6$), VEN+SED ($n = 5$) or VEN+EXE ($n = 5$). (B) Frontal cortical NA concentrations on PND60 (SAL+SED $n = 5$; SAL+EXE $n = 6$; VEN+SED $n = 4$; VEN+EXE $n = 6$). (C) Frontal cortical 5-HT concentrations on PND35 (SAL+SED $n = 6$; SAL+EXE $n = 6$; VEN+SED $n = 5$; VEN+EXE $n = 5$). (D) Frontal cortical 5-HT concentrations on PND60 (SAL+SED $n = 5$; SAL+EXE $n = 6$; VEN+SED $n = 4$; VEN+EXE $n = 6$). (E) Hippocampal BDNF concentrations on PND35 (SAL+SED $n = 6$; SAL+EXE $n = 6$; VEN+SED $n = 6$; VEN+EXE $n = 6$). (F) Hippocampal BDNF concentrations on PND60 (SAL+SED $n = 4$; SAL+EXE $n = 6$; VEN+SED $n = 4$; VEN+EXE $n = 6$). Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text with * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ vs. SAL+STD; ^ $p \leq 0.05$, ^^ $p \leq 0.01$, ^^^ $p \leq 0.0001$ vs. indicated test group; d large effect magnitude ≥ 0.8 . EXE: Exercise (low intensity). 5-HT: 5-hydroxytryptamine (serotonin). BDNF: Brain-derived neurotrophic factor. NA: Noradrenaline. PND: Postnatal day. SAL: Saline (control). SED: Sedentary. VEN: Venlafaxine. ^{a}) Less than 12 animals due to lower than expected birth rates at the requested date. ^{b}) Outlier identified and removed from group due to not representing the target population.

In Figure 3C, PND35 frontal cortical 5-HT data were normally distributed for all test groups ($p > 0.05$), and had homogeneity of variances ($p = 0.320$). There was a statistically significant two-way interaction between drug and activity ($F[1, 18] = 7.099$, $p = 0.016$; $\eta^2 = 0.283$), supported by a statistically significant main effect of activity ($F[1, 18] = 4.513$, $p = 0.048$; $\eta^2 = 0.200$), so that EXE increased overall 5-HT by 18.6 ng/g (95 % CI, 0.2 to 37.0 ng/g) compared to SED (irrespective of drug). Furthermore, VEN+EXE (131.8 ± 9.7 ng/g) increased 5-HT by 37.9 ng/g (95 % CI, 1.3 to 74.6 ng/g) compared to SAL+EXE (93.9 ± 4.6 ng/g; $p = 0.040$; $d = 2.509$, 95 % CI, 0.9 to 4.1), and by 41.9 ng/g (95 % CI, 3.6 to 80.3 ng/g) compared to VEN+SED (89.9 ± 10.6 ng/g; $p = 0.027$; $d = 2.072$, 95 % CI, 0.5 to 3.6).

In Figure 3D, PND60 frontal cortical 5-HT data were normally distributed for all test groups ($p > 0.05$), and had homogeneity of variances ($p = 0.690$). There was no statistically significant two-way interaction between drug and activity ($F[1, 17] = 0.943$, $p = 0.345$; $\eta^2 = 0.053$). However, there was a statistically significant main effect of activity ($F[1, 17] = 35.338$, $p < 0.0005$; $\eta^2 = 0.675$), but not of drug ($F[1, 17] = 1.783$, $p = 0.199$; $\eta^2 = 0.095$), that EXE increased 5-HT concentration by 56.1 ng/g (95 % CI, 36.2 to 76.0 ng/g) compared to SED (Irrespective of drug). Furthermore, SAL+EXE increased 5-HT by 46.9 ng/g (95 % CI, 19.7 to 74.1 ng/g) compared to SAL+SED (115.0 ± 7.0 vs. 68.1 ± 9.4 ng/g; $p = 0.002$; $d = 2.734$, 95 % CI, 1.1 to 4.4). Similarly, VEN+EXE increased 5-HT by 65.2 ng/g (95 % CI, 36.2 to 94.3 ng/g) compared to VEN+SED (136.7 ± 10.5 vs. 71.5 ± 10.0 ng/g; $p < 0.0005$; $d = 3.081$, 95 % CI, 1.2 to 5.0).

In Figure 3E, PND35 hippocampal BDNF data were not normally distributed for all test groups ($p < 0.05$), nor was there homogeneity of variances ($p = 0.010$). There was a statistically significant two-way interaction between drug and activity ($F[1, 20] = 8.283$, $p = 0.009$; $\eta^2 = 0.293$), supported by a statistically significant main effect of activity ($F[1, 20] = 5.987$, $p = 0.024$; $\eta^2 = 0.230$), so that EXE increased overall BDNF by 229.4 pg/g (95 % CI, 33.8 to 424.9 pg/g) compared to SED (irrespective of drug). Furthermore, SAL+EXE increased BDNF concentration by 499.2 pg/g (95 % CI, 111.1 to 887.3 pg/g) compared to SAL+SED (1104.5 ± 160.1 vs. 605.3 ± 47.2 pg/g; $p = 0.007$; $d = 1.892$, 95 % CI, 0.5 to 3.3).

In Figure 3F, PND60 hippocampal BDNF data were normally distributed for all test groups ($p > 0.05$), and had homogeneity of variances ($p = 0.185$). There was no statistically significant two-way interaction between drug and activity ($F[1, 20] = 0.776$, $p = 0.391$; $\eta^2 = 0.046$, nor were there were any statistically significant main effects.

4 Discussion

Pre-pubertal low intensity exercise, but not venlafaxine, decreases locomotor activity later in life.

In line with a recent study in our laboratory [35], pre-pubertal low intensity exercise had no significant effect on early-life locomotor activity as measured in the OFT (Figure 1A) correlating with other reports in juvenile [58] and older rats [59]. Interestingly, voluntary wheel running has been shown in other studies to increase general locomotor activity, whereas forced treadmill exercise has been shown to decrease early-life locomotor activity [60], possibly via stress of being deprived of habitual running [61] or the stress induced by the treadmill itself [62]. However, in the current study a pre-pubertal, age-related low intensity exercise regimen was implemented as validated and used in our laboratory before [35]. Briefly, treadmill speed was increased daily according to the running capacity, as determined by 55% of the age-related velocity to reach VO_{2max} (vVO_{2max}), of the pre-pubertal FSL rat for the entire intervention period [35]. This age-appropriate exercise regimen improves on previously used constant treadmill speed throughout the intervention period [63-66] and is also expected to significantly reduce stress levels, compared to other forced exercise regimens, putatively resulting in the observed unaltered locomotor activity in the current study. Following a 26-day sedentary period, exercise in both EXE treatment groups significantly reduced locomotor activity relative to their respective SED controls (Figure 1B). Moreover, on PND60 the reduction in locomotor activity induced by the combination of venlafaxine and exercise (VEN+EXE) was also significant compared to drug-free, sedentary controls (SAL+SED). In this regard, Grace and colleagues [67] previously reported a significant reduction in young adult rats immediately following pre-pubertal wheel running. And although only observed in PND60 rats in the current study, such a reduction in locomotor activity was concluded to be indicative of increased anxiety-like behaviour. Therefore, although methodological differences exist between the current and referenced study, this hypothesis was investigated by other behavioural parameters as measured in the OFT (see below).

Pre-pubertal venlafaxine and low intensity exercise display anxiogenic-like properties later in life.

In accordance with the locomotor activity displayed on PND60, overall anxiety-like behaviour was worsened by low intensity exercise and venlafaxine treatment strategies (both supported by significant main effects). A particularly robust long lasting increase in anxiety-like behaviour was observed following the combination of pre-pubertal venlafaxine and exercise (VEN+EXE treatment groups) following a 26 day treatment-free period, compared to sedentary controls (SAL+SED) and the venlafaxine mono-treatment group (VEN+SED) (Figure 1D). In previous studies forced treadmill running, but not voluntary wheel running, has been shown to reduce the number of centre zone entries in the OFT, compared to sedentary controls [62], possibly due to putative treadmill-induced stress. However, in the current study, such anxiogenic-like effects were only observed later in life and not

immediately following a chronic exercise intervention period (Figure 1C). In fact, on PND35, anxiety-like behaviour was comparable across all treatment groups. Nevertheless, that anxiety-like behaviour increased later in life following pre-pubertal combination treatment and a 26-day sedentary/withdrawal period, could in fact be the result of decreased locomotor activity and not the cause thereof. Also, exercise can be addictive [68] and is known to induce negative symptoms such as increased anxiety and depression upon withdrawal. Even a translational animal model of depression, such as the FSL rat, significantly increase their daily distance of running when given free access to a running wheel over a period of time [69], supporting a putative addictive-like behaviour. However, a sedentary/withdrawal period of 26-days, as implemented in the current study, is expected to be significant enough to recover from such adverse withdrawal effects. At the same time, abrupt exercise cessation in spontaneously exercising rats, induces downregulation of hippocampal BDNF mRNA that last as long as ten days [70], which could have longer lasting detrimental effects. In fact, that the neuroplasticity enhancing effects of exercise are well documented [71], and demonstrated here (see below), and associated with antidepressant and anxiolytic-like effects. However, such neuroplasticity enhancing effects are only temporary, returning to baseline, but not to sub-baseline levels within two weeks following exercise cessation [72], thereby explaining the unaltered, and not detrimental behavioural observations. Nevertheless, that pre-pubertal venlafaxine and low intensity exercise combination treatment (VEN+EXE) significantly altered OFT behaviour later in life, suggests an augmentative, neurodevelopmental mechanism to be at work, posing the risk of other long-term behavioural effects. To this extent, the clinical relevance of exercise as treatment option for anxiety disorders remain uncertain, whereas its role in depression is generally more beneficial [73,74]. Therefore, to elaborate on the current behavioural data, depressive-like behaviour was also analysed in the FST.

Pre-pubertal low intensity exercise, but not venlafaxine, induces early and lasting antidepressant-like effects.

None of the current pre-pubertal treatment strategies induced any significant early-life behavioural changes in PND35 FSL rats (Figure 2A). That venlafaxine alone (VEN+SED) was also ineffective in reducing early-life depressive-like behaviour is in line with a previous report indicating that venlafaxine is not effective in reducing depression symptoms in children, despite being effective in adolescents [75]. Taken together with the popular ‘off-label’ use of venlafaxine, and consequent unavailable official dosage guidelines for childhood depression [33], it is not surprising that a dose the selected dose of 10 mg/kg/day to be ineffective in alleviating depressive-like symptoms in animals modelling the childhood period in humans [76]. However, despite unaltered early-life depressive-like behaviour, pre-pubertal exercise (irrespective of drug) had a significant effect on early-life serotonergic-associated behaviour (i.e. swimming; Figure 2C), as supported by a significant main effect of exercise. Of note, such increased escape-directed behaviour in the presence of unaltered depressive-like behaviour (i.e. time

spent immobile) in the FST is indicative of an antidepressant-like effect, specifically in juvenile animals [77]. The results are also supported by previous data from our laboratory [35].

Later in life (PND60), following a 26-day treatment-free period, low intensity exercise yielded a significant lasting reduction in depressive-like behaviour in both EXE treatment groups, compared to age-matched controls (SAL+SED; Figure 2B). Moreover, that time spent immobile was also significantly less in the venlafaxine plus exercise combination treatment group (VEN+EXE) relative to venlafaxine alone (VEN+SED), supports the hypothesis of an augmentative mechanism to be at play. Of note is the improved depressive-like behaviour observed despite a significant decrease in early-adult locomotor activity (Figure 1B). Therefore, the observed decrease in time spent in the centre zone of the OFT on PND60 (Figure 1D) might indeed be a consequence of reduced ambulatory activity induced in young adult FSL rats, and not due to enhanced anxiety. Furthermore, escape-directed behaviour (swimming) of PND60 rats was significantly increased in both EXE treatment groups in relation to their respective SED controls, suggestive of serotonergic neurotransmission to be enhanced by pre-pubertal low intensity exercise, followed by a 26-day sedentary period. Similarly, both EXE treatment groups also significantly increased early-adulthood, struggling behaviour compared to SED controls (irrespective of drug, and supported by a significant main effect) (Figure 2F), suggestive of enhanced noradrenergic neurotransmission. Yet this effect was most pronounced following combination treatment strategy compared to both SAL+SED and VEN+SED treatment groups. Therefore, in addition to the abovementioned enhanced serotonergic neurotransmission, adrenergic neurotransmission [45,46] was also enhanced later in life, suggesting a strong neurodevelopmental effect of low intensity exercise when introduced during early-life development. The long-term beneficial effects of low intensity exercise are supported by our previous work [35] and suggest an age-related, low intensity exercise regimen during developmental years to induce specific neurodevelopmental alterations that could benefit an individual, sensitive to external stressors, with regards to depressive symptoms. Furthermore, that both exercise [10] and venlafaxine [26] are known to have adrenergic and serotonergic neurotransmission enhancing effects, could augment the treatment outcome. To this extent, although the combination treatment intervention (VEN+EXE) did not induce beneficial early-life behavioural alterations, its long-term (neurodevelopmental) effects were significant; apparently via enhanced neurotransmission of both mentioned neurotransmitters. Taken together with the ineffectiveness of venlafaxine mono-treatment (VEN+SED) in improving overall depressive-like behaviour in both age groups, yet be effective when combined with low intensity exercise, could in fact support such an augmentation mechanism. In this regard, cortical monoaminergic concentrations as well as a marker of hippocampal neuroplasticity was analysed, as discussed below.

Pre-pubertal venlafaxine treatment enhances monoaminergic concentrations only when combined with low intensity exercise.

Overall, chronic, pre-pubertal low intensity exercise had a significant lasting effect on cortical noradrenergic and serotonergic concentrations, relative to age-matched sedentary controls. To this extent, the pronounced increase of cortical noradrenaline concentrations during early adulthood in both EXE treatment groups compared to their respective SED controls (Figure 3B), suggests a lasting enhancement of noradrenergic neurotransmission to be induced by pre-pubertal low intensity exercise, even after a 26-day sedentary period. In fact, such a lasting increase in noradrenergic concentration supports the overall increase in noradrenergic-associated behaviour (i.e. struggling; Figure 2F), observed in the FST on PND60. The increase in overall cortical noradrenaline concentrations (irrespective of drug and supported by a significant main effect) (Figure 3A) translated into noradrenergic-associated escape behaviour on PND60 (Figure 3F, but not on PND35 (Figure 2E)). That early-life neurobiological data do not appear to support corresponding behaviour, could be explained by the immature noradrenergic neurotransmitter system in pre-pubertal rats. To this extent, the noradrenergic neurotransmitter system only reaches maturity by PND35 [31], implying that pre-pubertal treatment enhanced concentrations of a developing system, arguably resulting in behaviour different to that expected in a mature system. Nevertheless, that pre-pubertal low intensity exercise, and not venlafaxine, induced lasting beneficial noradrenergic-associated effects (Figure 3B), suggests a noradrenergic mechanism of action to be responsible for the lasting antidepressant-like effects observed on PND60 (Figure 2B). Furthermore, that venlafaxine treatment alone (VEN+SED) did not induce any significant noradrenergic increase, supports the sub-therapeutic dose of venlafaxine.

In addition and supportive of increased serotonergic-associated behaviour (i.e. swimming; Figure 2D) later in life, cortical serotonin levels were also increased in both EXE, but not VEN, treatment groups relative to their respective SED controls (Figure 3D), suggesting serotonergic antidepressant-like effects to also be involved. Similarly, early-life serotonin concentrations were also increased in the combination treatment group (VEN+EXE) compared to either exercise (SAL+EXE) or venlafaxine (VEN+SED) mono-treatment groups, suggestive of augmentation and again suggesting a sub-therapeutic dose of venlafaxine. However, this overall exercise-induced increase in cortical serotonin (supported by a significant main effect of activity) (Figure 3C), translated into a significant increase in overall serotonergic-associated behaviour (i.e. swimming) both immediately following intervention on PND35 (Figure 2C) and following a 26-day sedentary period in PND60 (Figure 2D). That the serotonergic-associated behaviour correlates with the serotonergic increase, supports an earlier maturation of the serotonergic system, relative to that of the noradrenergic system. Overall, that pre-pubertal low intensity exercise, and not venlafaxine, had significant early-life noradrenergic and serotonergic enhancing effects, suggests a significant role for low intensity exercise in juvenile MDD

treatment strategies. In fact, that pre-pubertal low intensity exercise, with and without venlafaxine treatment, induced lasting enhancement of monoaminergic concentrations with corresponding increased escape-directed behaviour, supports the value of increased activity during early-life development.

Moreover, that hippocampal BDNF concentrations were increased by low intensity exercise alone immediately following the intervention period on PND35 (Figure 3E), may have resulted in long-term neuroplasticity and neurodevelopmental changes, translating into the overall increase in swimming behaviour in EXE treatment groups described above. This increase in BDNF levels was not seen 26 days after the exercise intervention on PND60 (Figure 3F) – see discussion below. To this extent swimming behaviour has been positively associated with hippocampal BDNF concentrations [13]. Furthermore, decreased hippocampal BDNF levels are observed in suicidal subjects [78], whereas antidepressant treatment normalizes these levels [79], together confirming enhanced neuroplasticity to alleviate depressive symptoms. Yet according to the current data, the increase in early-life hippocampal BDNF concentrations only reached significance in the SAL+EXE, and not in VEN+EXE treatment group, and not the venlafaxine alone treatment group (VEN+SED). This is of note, since the data therefore suggests the beneficial effects of low intensity exercise on neuroplasticity via enhanced BDNF expression to be inhibited by pre-pubertal venlafaxine treatment. In this regard, venlafaxine, although effective in treating adult depression and treatment resistant depression [80,81], may not display the same benefits in juvenile MDD. Nevertheless, hippocampal BDNF concentrations returned to baseline levels on PND60, across all treatment groups (Figure 3F). This is in line with reports suggesting the neuroplasticity enhancing effects of exercise to be transient [72,82] and correlates with our previous report [35]. Of note, in the mentioned study, animals treated with a combination of low intensity exercise and fluoxetine, displayed increased hippocampal BDNF levels later in life, compared to those only receiving pre-pubertal exercise, yet without improving depressive-like behaviour. Therefore, that the combination of low intensity exercise and sub-therapeutic venlafaxine, and not low intensity exercise plus fluoxetine [35], induced long-term beneficial effects could suggest a role for such (or similar) a treatment strategy in the current approach to juvenile MDD treatment.

5 Conclusion

Although not FDA-approved, venlafaxine remains a popular ‘off-label’ treatment option for juvenile MDD [17-19] with no official dosage guidelines. Furthermore, venlafaxine’s mechanism of action targets both the noradrenergic and serotonergic neurotransmitter pathways at high doses, making it a valuable drug in the treatment of resistant depression in adults and possibly explains why it may be considered in some juvenile patients. However, venlafaxine during pre-pubertal development may also induce long-term bio-behavioural alterations later in life, and therefore the possible acute benefits of treatment may be outweighed by unknown effects later in life. In the current study, we aimed to

investigate such long-term effects of juvenile venlafaxine treatment, compare it to chronic low intensity exercise and investigate the augmentative potential of these treatment strategies on early-life depressive- and anxiety-like bio-behaviour. None of the investigated treatment strategies induced any significant effect in early-life depressive- and anxiety-like behaviour immediately following the interventions on PND35, while only low intensity exercise alone worked in early-adulthood, following withdrawal. However, that swimming behaviour was significantly increased on PND35, indicates towards the potential benefit of an age-related, low intensity exercise regimen. Nevertheless, that pre-pubertal low intensity exercise (irrespective of drug) improved depressive-like behaviour, supports the implementation thereof in current juvenile MDD treatment regimens. Importantly, long-term enhancement of depressive-like behaviour was observed following 26 days intervention-free period on PND60 (early adulthood), suggesting lasting beneficial effects of pre-pubertal low intensity exercise. In this regard, both NA and 5-HT concentrations were also increased by the combination treatment strategy at both ages, suggesting improved monoaminergic transmission to be responsible for the antidepressant-like effects. The results of the current study therefore suggest an age-related, low intensity exercise program to be of particular value in treatment strategies of juvenile MDD with the potential to induce long-term beneficial effects with or without early-life venlafaxine treatment. Moreover, low intensity exercise may augment sub-therapeutic doses of venlafaxine, which is of importance considering the lack of dosage guidelines.

6 Compliance with Ethical Standards

All experiments conformed to the guidelines of the South African National Standards: The care and use of animals for scientific purposes (SANS 10386:2008) and were approved in accordance with the regulations set by the AnimCare animal research ethics committee (NHREC reg. no. AREC-130913-015) of the NWU, project ethics approval numbers NWU-00148-14-A5 and NWU-00373-16-A5.

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8 Conflict of interest

Jade Pharma Corporate (Pty) Ltd. (RSA) kindly sponsored the drugs used in the current study and Nordic Naturals (USA) sponsored the omega-3 oil that was used in coating of the vivarium rat chow. Professor Brian H Harvey has participated in advisory boards and received honoraria from Servier®, and has received research funding from Servier® and Lundbeck® over the past four years. Finally, except for income from the primary employer and research funding to both Professors Christiaan B

Brink and Brian H Harvey from the MRC, NRF (and the above-mentioned exceptions), no financial support or compensation has been received from any individual or corporate entity over the past four years for research or professional services, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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CHAPTER 6: SUMMARY, CONCLUSION, LIMITATIONS AND FUTURE DIRECTIONS

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The results of the current project were presented as three different manuscripts in this thesis, with additional data included in Addendum B. Therefore, it was considered useful to provide a concise summary of all the results of the project as a whole, together with a general discussion. Table 6-1 provides a summary of how the results (research answers) addressed the original objectives (research questions). This is followed by a final conclusion, elaborating on the impact of the entire project, and finally closing remarks on limitations of the current project and suggestions for prospective studies.

6.1 Overall summary and discussion

The key findings of the current project include:

6.1.1 Primary findings

- 1) Only pre-pubertal fluoxetine, but not escitalopram nor venlafaxine, treatment improves early-life depressive-like behaviour, and exerts lasting antidepressant-like effects into early-adulthood.
- 2) Pre-pubertal low intensity exercise alone increases early-life serotonergic-associated escape-directed behaviour, indicative of antidepressant-like properties and exerts lasting antidepressant-like properties into early-adulthood via enhanced noradrenergic and serotonergic neurotransmission.
- 3) The combination of pre-pubertal low intensity exercise and fluoxetine only improves early-life depressive-like behaviour, whereas low intensity exercise augments the bio-behavioural effects of venlafaxine during pre-pubertal development to exert long-term antidepressant-like behaviour in early-adulthood.
- 4) The combination of pre-pubertal escitalopram and ω -3 EFA supplementation exerts long-term antidepressive-like behaviour in early-adulthood, despite a reduction in locomotor activity.
- 5) Pre-pubertal ω -3 EFA supplementation may induce improved coping behaviour in early-life, incorrectly presenting as increased anxiety-like behaviour and unaltered depressive-like behaviour.

6.1.2 Secondary findings

- 1) Maximal running capacity in pre-pubertal FSL rats increases with age and should be adapted accordingly when implementing a forced exercise regimen.
- 2) Standard vivarium rat chow is successfully coated with ω -3 EFA oil to contain double the amount of EPA and DHA compared to standard rat chow.

Major depressive disorder (MDD) amongst children and adolescents is a real and growing concern. In fact, current prevalence in juvenile patients is expected to significantly contribute to the increasing economic burden of MDD, and overall disability within the next fifteen years. Of further concern is the increased risk for suicidal behaviour in juvenile patients with treatment onset (Friedman & Leon, 2007), yet a similar consequence of untreated MDD also exist. Regardless, antidepressant treatment strategies in the juvenile population remain restricted, putatively due to different maturation rates of monoaminergic pathways, but also due to unknown lack of knowledge about lasting effects, induced by early-life treatment. Altogether, this places the medical prescriber in a difficult situation, weighing the immediate effects against the possible, unknown long-term outcome. Currently, only two selective serotonergic reuptake inhibitors (SSRIs), fluoxetine and escitalopram, are approved for MDD treatment in juvenile patients, yet venlafaxine, a serotonergic-noradrenaline reuptake inhibitor (SNRI), is a popular ‘off-label’ choice for treatment-resistant MDD in children and adolescents. That serotonergic, and not noradrenergic antidepressants are effective in improving juvenile MDD is explained by the earlier maturation of the serotonergic pathway compared to the noradrenergic pathway. Still, as mentioned before, the possible lasting or long-term effects of chronic treatment during early-life development remain unknown. In addition, that neuroplasticity processes are very dynamic and more adaptable during early-life development, in relation to that of the mature brain, could present a ‘window of opportunity’ where appropriate early-life intervention could indeed induce beneficial neurodevelopmental effects, with positive consequences later in life. In fact, neuroplasticity plays a central role in the neurobiological pathophysiology that appropriate significant influences could indeed have lasting or long-term beneficial effects.

Study question one

Therefore, approved juvenile antidepressants (i.e. fluoxetine and escitalopram), and a popular ‘off-label’ antidepressant (i.e. venlafaxine) for juvenile MDD was compared in terms of their early-life and possible lasting effects on depressive- and anxiety-like bio-behaviour in an animal model of depression. First of all, fluoxetine (FLX), but not escitalopram (ESC) nor venlafaxine (VEN), improved early-life depressive-like behaviour, possibly via increased noradrenergic neurotransmission, evidenced by increased noradrenergic-associated behaviour (i.e. climbing/struggling behaviour) in PND35 FSL rats. In fact, as discussed in Chapter 4, despite overall cortical noradrenaline concentrations being increased by pre-pubertal

escitalopram treatment during early-life, no significant antidepressant-like effects were observed, confirming a sub-therapeutic dose. Of note, only pre-pubertal fluoxetine induced lasting behavioural effects into early-adulthood. Moreover, this improved depressive-like behaviour could be attributed to lasting enhanced noradrenergic neurotransmission, evidenced by increased climbing (struggling) behaviour of PND60 FSL rats. In addition, neither escitalopram, nor venlafaxine significantly affected early-life anxiety-like behaviour, when administered during pre-pubertal development. This is of note since early-life antidepressant-use is associated with an increased suicide risk (Friedman & Leon, 2007; U.S. Food & Drug Administration, 2004). Moreover, increased suicidal behaviour risk is further increased by comorbid anxiety among depressed adolescents (Wunderlich *et al.*, 1998). Therefore, that neither of these antidepressants negatively affected early-life and early-adulthood anxiety- and depressive-like behaviour, at least suggests no harmful effects induced by these specific early-life antidepressant treatment strategies. However, these observations require confirmation through further investigation into a broader spectrum of behavioural and neurobiological analyses.

Study question two

Sub-question 2.1

Nevertheless, that therapeutic success in depressed juveniles are comparable to that observed in adult patients, and that antidepressant-use is known to induce side-effects, contribute to reduced patient adherence and even treatment cessation. Therefore, non-pharmacological strategies, such as exercise and ω -3 EFA supplementation, are receiving growing interest as alternative or augmentation strategies to currently used antidepressants to improve clinical outcome. Such lifestyle and nutritional interventions are generally more accessible and perceived to have an improved safety profile compared to prescribed antidepressants. In fact, both exercise and ω -3 EFA supplementation is associated with antidepressant properties via various beneficial effects on the neurobiological pathophysiology of MDD. To this extent, we developed an age-related intensity specific treadmill exercise regimen where treadmill speed is increased daily in accordance with the increasing $\dot{V}O_{2\max}$ -capacity of the pre-pubertal male FSL rat. Such an exercise regimen would accurately expose pre-pubertal test subjects to a specific exercise intensity, in accordance with age. In fact, according to our data, an age-related low intensity exercise regimen, and not moderate or high intensities, improved early-life depressive-like behaviour.

Sub-question 2.2

Nevertheless, ω -3 EFA supplementation is also associated with antidepressant properties. However, administration stress of oral gavage may be significant in affecting behaviour in a stress-sensitive animal model, especially when combined with the drug administration stress implemented in the current project. Therefore, we developed a method where ω -3 EFA oil was successfully coated to the vivarium rat chow,

resulting in doubling of the ω -3 EPA and DHA content in relation to standard rat chow. Furthermore, by administering ω -3 EFAs via rat chow, administration stress is eliminated and each subject is able to supplement according to its natural daily food intake capacity.

Pre-pubertal low intensity exercise significantly increases early-life serotonergic-associated behaviour (i.e. swimming), indicative of antidepressant-like properties. In fact, depressive-like behaviour (i.e. time spent immobile in the FST) shows a strong trend to be decreased in early-life, supporting the antidepressant-like potential. Of note, swimming behaviour has also been positively associated with brain-derived neurotrophic factor (BDNF) concentrations (Russo-Neustadt *et al.*, 2001) which was significantly increased during early-life development by low-intensity exercise and not by venlafaxine treatment. Therefore, chronic pre-pubertal low intensity exercise could prove useful as treatment strategy for juvenile MDD with superior early-life bio-behavioural effects, at least compared to venlafaxine treatment.

Regarding nutritional supplementation, pre-pubertal ω -3 EFA supplementation induced significant early-life neuroplasticity enhancing effects in pre-pubertal FSL rats, whereas escitalopram treatment did not. Nevertheless, early-life depressive-like behaviour was unaffected by pre-pubertal ω -3 EFA supplementation, yet appeared to induce anxiogenic-like behaviour. In this regard, overall locomotor activity was also decreased in ω -3 supplemented animals during early-life, possibly suggestive of improved coping mechanisms due to the enhanced neuroplasticity properties of ω -3 EFA supplementation (Crupi *et al.*, 2013; Gonzales *et al.*, 2015). Therefore, the apparent increase in anxiety-like behaviour may indeed be a false-positive observation to be confirmed in prospective studies. Regardless, this suggested falsely interpreted anxiogenic-like behaviour lasted into adulthood, with ω -3 supplemented animals again displaying comparable depressive-like behaviour during early-adulthood. Importantly, that animals were fed an ω -3 adequate diet during the wash-out period, could have maintained elevated levels of ω -3 EFAs, inducing similar effects during early-adulthood to that observed during early-life. However, this hypothesis requires further investigation. Taken together, that pre-pubertal ω -3 EFA supplementation alone did not improve depressive-like behaviour either during early-life or early-adulthood, but escitalopram treatment did, suggests that escitalopram treatment during pre-pubertal development might be superior to ω -3 EFA supplementation alone in improving early-life depressive-like behaviour.

Study question three

That both exercise and ω -3 EFA supplementation displayed significant antidepressant-like potential with overall beneficial long-term effects, suggests a role as augmentation strategy to current juvenile MDD treatment options. Therefore, the augmentation potential of low intensity exercise on either fluoxetine (Chapter 3), escitalopram (Addendum B) or venlafaxine (Chapter 5) treatment was investigated, whereas

the augmentation potential of ω -3 EFA supplementation on escitalopram treatment, at a sub-therapeutic dose was investigated in terms of early-life and lasting bio-behaviour.

Pre-pubertal low intensity exercise in combination with fluoxetine, but not escitalopram or venlafaxine, improved early-life depressive-like behaviour, supporting the value of early-life exercise in the treatment of juvenile MDD. However, when combined with venlafaxine, and not fluoxetine, pre-pubertal low intensity exercise induced antidepressive-like behaviour during early-adulthood, apparently via enhanced serotonergic and noradrenergic neurotransmitter mechanisms. This is of note, since the combination of low intensity exercise and venlafaxine during early-life development did not appear to be effective shortly following chronic treatment, yet improved depressive-like behaviour following extended period with no treatment, suggesting neurodevelopmental effects to be induced, partly via the strong documented neuroplasticity enhancing effects of low intensity exercise (van Praag, 2009; van Praag *et al.*, 1999a; van Praag *et al.*, 2014; van Praag *et al.*, 1999b; Vivar *et al.*, 2016; Vivar *et al.*, 2012).

The combination of ω -3 EFA supplementation and escitalopram treatment during pre-pubertal development, did not improve early-life depressive-like behaviour, yet as with ω -3 EFA supplementation alone, locomotor activity and anxiety-like behaviour, where respectively reduced and increased, again suggesting possible improved coping mechanisms involved. However, pre-pubertal treatment with this combination showed strong potential (evidenced by a strong trend for decreased depressive-like behaviour and significant increased escape-directed behaviour in the FST) for long-term antidepressant-like effects. These antidepressant-like effects were not observed when ω -3 EFA supplementation was combined with venlafaxine (*Addendum B*). Interestingly, the combination of low intensity exercise and ω -3 EFA supplementation during pre-pubertal development exerted antidepressant-like effects in early-adulthood, but not during early-life (*Addendum B*), suggesting a valuable strategy to be incorporated into current juvenile treatment regimens.

Of note, no significant early-life antidepressant-like effects were observed when ω -3 EFA supplementation and low intensity exercise were both combined with either escitalopram or venlafaxine (*Addendum B*). However, the triple combination of escitalopram, ω -3 EFA supplementation and low intensity exercise during early-life development induced long-term antidepressant-like behaviour during early-adulthood in relation to age-matched controls, possibly via enhanced serotonergic neurotransmission, evidenced by increased swimming behaviour. Altogether suggesting beneficial neurodevelopmental effects of such (or similar) an early-life treatment strategy.

A summary of the main results discussed above, are summarized in Table 6-1, below in response to each of the study questions presented at the start of the current thesis.

Table 6-1: Study questions and final outcome.

Study question	Final study outcome
1) Are depressive- and anxiety-like behaviours and specific biomarkers thereof differently affected by two different classes of antidepressants, either during early-life following pre-pubertal treatment or later in life?	In our animal model, only FLX (<i>Chapter 3</i>) is effective in improving early-life (PND35) depressive-like behaviour, whereas ESC (<i>Chapter 4</i>) VEN mono-treatments are not (<i>Chapter 5</i>). Furthermore, only FLX induced improved depressive-like behaviour later in life (PND60). Importantly, pre-pubertal ESC and VEN mono-treatments did not induce any harmful bio-behavioural alterations during early-adulthood in our animal model.
2) Do non-pharmacological interventions (low intensity exercise and ω -3 EFA supplementation) during pre-pubertal development have any early-life or lasting antidepressant-like effects and how do these effects compare to those observed in the antidepressant-treated groups?	EXE (irrespective of drug) induces significant increased escape-directed behaviour during early-life (PND35), suggesting antidepressant-like potential via serotonergic and neuroplasticity enhancement. Yet, such behaviour becomes more pronounced later in life, suggesting pre-pubertal low intensity exercise to induce lasting beneficial effects, possibly via enhanced neurodevelopmental serotonergic and noradrenergic processes (<i>Chapters 3 and 5</i>). Contrary, OM3 is ineffective in improving early-life depressive-like behaviour with no apparent neurodevelopmental effects later in life. However, OM3 might induce lasting improved coping mechanisms via increased neuroplasticity, explaining the apparent unaltered depressive-like behaviour (<i>Chapter 4</i>).
<i>Sub-question 2.1)</i> Does the vVO_{2max} capacity of the pre-pubertal male FSL rat increase with age?	Yes. A strong positive correlation between pre-pubertal age and vVO_{2max} capacity exist (<i>Chapter 3</i>). In fact, age-related and intensity specific treadmill exercise regimens can be calculated according to vVO_{2max} data; a novel finding with unique possibilities in further exploring the antidepressant-like effect of forced exercise.
<i>Sub-question 2.2)</i> Can ω -3 EFA supplementation be administered via normal rat diet to minimize administration stress and allow natural supplementation according to each subject's developmental needs?	Yes. Normal vivarium rat chow was successfully coated with ω -3 oil to contain double the amount of DHA and EPA compared to standard rat chow (<i>Chapter 4 and Addendum B</i>). Oral administration stress is eliminated with the developed method, resulting in animals of different body weight to be supplemented with ω -3 EFAs according to each animal's natural food intake capacity.
3) Can the observed bio-behavioural effects of pre-pubertal antidepressant treatment be augmented by low intensity exercise and/or ω -3 EFA supplementation, both in early-life and during early adulthood?	Only pre-pubertal FLX+EXE treatment improved early-life depressive-like behaviour (<i>Chapter 3</i>). However, both pre-pubertal ESC+OM3 (<i>Chapter 4</i>) and VEN+EXE treatment (<i>Chapter 5</i>) induced neurodevelopmental beneficial alterations later in life. These behavioural improvements are supported by enhanced serotonergic and noradrenergic concentrations. Similarly, the triple combination of ESC+EXE+OM3 was not effective during early-life, yet was the only strategy to induce significant decreased depressive-like behaviour later in life, suggesting a novel treatment strategy with long-term benefits (<i>Addendum B</i>).

6.2 General conclusion

The current project investigated the antidepressant-like potential of two specific and popular non-pharmacological interventions, i.e. low intensity exercise and ω -3 EFA supplementation as augmentation strategies to current used antidepressants in juvenile MDD. The main focus of the study was the possible lasting effects that could theoretically be induced by early-life treatment. To this extent we showed that current approved juvenile antidepressant, fluoxetine, is effective in alleviating early-life depressive symptoms and suggest that escitalopram might be more effective older (i.e. adolescent) individuals. Furthermore, although venlafaxine is a popular ‘off-label’ treatment option, it appears to be inferior to the approved antidepressants in treating juvenile MDD, specifically due to no official dosing guidelines. Nevertheless, that only fluoxetine induced lasting antidepressant-like effects, yet no negative effects were observed with escitalopram and venlafaxine, could contribute to the understanding of long-term therapeutic outcome when treating depressed children and adolescents with pharmacological antidepressants, if the current pre-clinical data could be extrapolated to the clinical setting.

Furthermore, both low intensity exercise and ω -3 EFA supplementation during early-life development exert long-term antidepressant-like bio-behaviour when combined with escitalopram and venlafaxine, but not fluoxetine. However, early-life ω -3 EFA supplementation, with or without escitalopram (and possibly similar treatment) may enhance overall coping mechanisms to an extent where an overall improvement in depressive-like behaviour is indeed induced, yet falsely presents unaltered. Nevertheless, the importance of optimal nutritional supplementation and sufficient physical activity, is seen in the light of a modern Western diet with declining amounts of ω -3 EFAs (Calder, 2012) and general lifestyle with increasing sedentary behaviour (World Health Organization, 2017b), both of which are inversely associated with MDD. Therefore, that combination treatment strategies may induce long-term beneficial bio-behavioural effects, despite a chronic wash-out period, could be indicative of novel treatment strategies to be implemented in depressed children and adolescents.

Overall, despite the strong augmentation properties of the specific non-pharmacological intervention strategies, the importance of a healthy lifestyle, especially during early-life development, when neuroplasticity processes are more dynamic and adaptable, relative to a mature brain, is highlighted in the current project. That neuroplasticity may play a central role in the pathophysiology of MDD and beneficially affected by the various treatment strategies, could possibly support a holistic approach to juvenile MDD treatment, with lasting beneficial effects.

6.3 Limitations and future directions

Finally, a number of limitations have been identified in the current project to be addressed in future studies. These include:

- 1) The current project only investigated the lasting effects of pre-pubertal pharmacological and non-pharmacological interventions in a stress-sensitive animal model of depression (i.e. Flinders sensitive line rat). Although the focus of the current project was not to investigate the effect of genetic susceptibility on such intervention strategies, it would be valuable to investigate the interaction between different genetically susceptible species. In this regard, future studies should investigate whether the identified effective treatment strategies, would indeed produce similar bio-behavioural alterations in a stress-resistant animal model (i.e. Flinders resistant line rat) or whether these effects are limited to individuals with a natural increased risk for depressive-like behaviour.
- 2) As discussed in Chapter 4, pre-pubertal ω -3 EFA supplementation may enhance coping mechanisms that might have masked any antidepressant-like effects. Therefore, future studies should further investigate this proposed effect with a broader spectrum of bio-behavioural analyses, including other behavioural tests, such as the Elevated plus maze, the Morris water maze, Tail suspension test and Novel object recognition test. In addition, corticosterone should also be analysed to highlight any stress-related responses.
- 3) No ω -3 EFA brain content was analysed in the current project, yet nutritional analyses and bio-behavioural analyses suggested successful ω -3 EFA supplementation to all OM3 treatment groups. Therefore, future studies should also analyse brain and peripheral ω -3 concentrations to confirm the current results as well as validate the developed method of food coating as implemented in the current project.
- 4) ω -3 EFAs reduce central inflammation as part of their proposed mechanism of action. Therefore, it will be of value for future studies to further investigate the anti-inflammatory effects of ω -3 EFA supplementation on the proposed beneficial lasting effects. Analyses of redox markers, such as superoxide dismutase and malondialdehyde would further elaborate on the suggested improved coping mechanisms to be induced by ω -3 EFA supplementation.
- 5) The current project investigated the augmentation effects of low intensity exercise and ω -3 EFA supplementation on escitalopram and venlafaxine at two specific doses, suggestive of sub-therapeutic doses. To this extent, future dose range studies of these and other antidepressants, specifically in juvenile animal models would be of value to perhaps uncover additional lasting bio-behavioural effects.

BIBLIOGRAPHY

APPENDIX A: MATERIALS AND METHODS

This appendix will elaborate on materials and methods in more details than discussed in the manuscripts of Chapters 3, 4 and 5. In particular, it will provide additional background and descriptions of the formulation of the omega-3 essential fatty acid (ω -3 EFA) supplemented rat chow, the establishment of an age-related forced exercise regimen for juvenile Flinders Sensitive Line (FSL) rats, the open field test and forced swim test.

A.1 Omega-3 essential fatty acid supplementation

Omega-3 EFA was sponsored by Nordic Naturals (Watsonville, California, USA) in oil form (ProOmega, *batch: 153003, manufacture date: 09/2015*) and the certificate of analysis is presented in Addendum B.

The method used to coat the rat chow with the ω -3 EFA was created with the help of Professor Jan Steenekamp of Pharmaceutical services of the NWU, Potchefstroom campus. Briefly, a 10 % (v/v) ω -3 oil and chloroform solution was made. The solution was then sprayed onto the rat chow by an automatic spray gun used for pan coating (10 s duration, 20 s interval). Following formulation, the coated rat chow was stored in an air tight container in a refrigerator (8 °C) until use.

Although the data of the current project is presented as separate manuscripts, the overall ω -3 EFA intake for the entire current project resembled that of the individual nutritional data of the relevant manuscripts. The overall nutritional data results are therefore presented in Addendum B.

A.2 Age-related, intensity specific exercise regimen

A description of the treadmill's physical appearance can be found in Chapters 3 and 5. Below is a picture of the treadmill as described and implemented in the respective manuscripts and current project.

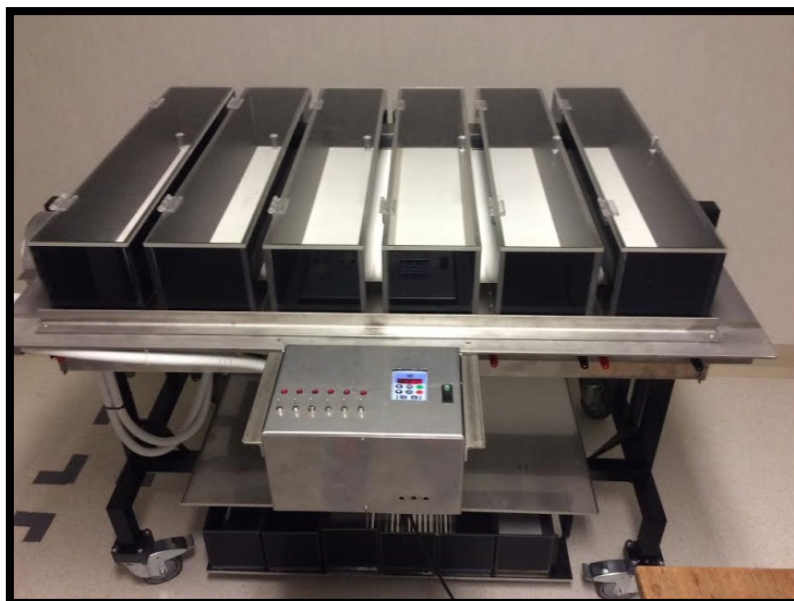


Figure 6-1: A picture of the custom-built treadmill used in the current project.

Figure 6-1 displays the custom-built, programmable treadmill used in the current project. The treadmill comprises a single treadmill belt (51 mm (w) x 96 mm (d)) and six shocking grids (14 mm (w) x 21 mm (d)) installed at the back of the treadmill. Six removable running lanes (14 mm (w) x 66 mm (d) x 14 mm (h)) with black opaque vertical walls and a clear top cover were placed over the belt to accommodate six subjects at a time. Treadmill speed for the 20-min training session ranged from 4.6 m/min on PND21 to 17.8 m/min on PND34.

A.2.1 Familiarization

Before the start of an exercise regimen, animals were introduced to the treadmill in order to familiarise the subjects to the treadmill and exercise, as well as give the researchers the opportunity to exclude any “non-runners” from the study. A non-runner was defined as a rat which refused to run even with negative reinforcement. The familiarisation period consisted of five consecutive days (Barnard *et al.*, 1974; Brooks *et al.*, 1984; Wisløff *et al.*, 2001) (PND16-20) of running for 10 minutes/day (Arida *et al.*, 1999) at a speed where the animal could walk comfortably at 0° incline. Familiarization also lowered the risk of foot injuries. If foot injuries were present, the situation was evaluated and treated accordingly, if it persisted for more than a few days the rat was excluded from the study.

The protocol used for the familiarisation period is presented in Table 6-2, below:

Table 6-2: Familiarisation protocol between PND16 and PND20.

Time point (minutes)	Speed at specific age (m/min)				
	PND16	PND17	PND18	PND19	PND20
0-1	0.0	2.0	2.0	2.0	2.5
1-2	0.0	2.0	2.0	2.0	2.5
2-3	0.0	2.0	2.0	2.0	2.5
3-4	2.0	2.0	2.5	3.0	3.5
4-5	2.0	2.0	2.5	3.0	3.5
5-6	2.0	2.1	REST	3.0	3.5
6-7	2.0	2.2	2.5	4.0	4.5
7-8	2.0	2.3	3.0	4.0	4.5
8-9	2.0	2.4	3.0	4.0	4.5
9-10	2.0	2.5	3.0	4.5	STOP

A.2.2 Exercise regimen

The age-related, intensity specific exercise regimen was calculated according the data obtained from the Exhaustion test, discussed in Chapter 3. In this regard, treadmill speed was calculated at 55 % of the values generated by the equation of $y = 1.856x - 30.68$ for low intensity and 70 % for moderate. Therefore, treadmill speed for the final 20-min session, ranged from 4.6 m/min on PND21 to 17.8 m/min on PND34.

A.3 Behavioural tests

The following section gives detail regarding each of the different behavioural tests used in the current study. As mentioned in Chapter 1 (*Ethical considerations and approval*), all tests were performed in accordance with the required guidelines and with prior ethical approval from the required body. To reduce the number of animals used in the current study, test subjects were subjected to a battery of tests, arranged from least stressful/invasive to most stressful/invasive i.e. open field test followed by the forced swim test. All behavioural tests were performed during the animal's dark cycle to yield the most accurate behavioural data from the animals. A brief description and history of the behavioural test used in the current study is discussed below.

A.3.1 Open field test

The open field test (OFT) was originally described by Hall in 1934 and was developed to measure the emotional state of the test subject (Prut & Belzung, 2003). Hall evaluated the behaviour of rodents in the

circular arena (1.2 m in diameter with 0.45 m high walls) and observed that emotional rats entered the central part of the arena fewer times than normal rats and they also had higher levels of defecation (Hall, 1934). The ability of the OFT to measure anxiety-like behaviour (along with locomotor activity) is what made this test one of the most popular procedures in animal psychology (Hall, 1934; Prut & Belzung, 2003). Since its introduction, many versions or variations of the test have been developed and include arenas of different shapes (such as circular, square or rectangular), lighting (lightning from above the arena or underneath the arena), the presence of objects within the arena, such as platforms or tunnels starting position of the test subject (centre of arena or close to the walls of the arena) and time spent in the arena (varies from 2 to 20 minutes, but generally 5 minutes). However, despite the difference in setup of the test, the basis of the test remains the same in that the animal is placed inside the arena and left to freely explore the arena for the predefined time. The time spent in the central part of the arena is indicative of decreased anxiety-like behaviour (the ratio of central/total locomotion, latency to enter the central part of the arena and ratio of central/corners time spent can also be used). Additionally, the general locomotor activity of the animal can also be measured by means of different parameters (number of line crossings marked on the floor or total distance travelled calculated by automated tracking systems) (Overstreet & Griebel, 2004; Prut & Belzung, 2003). The success and popularity of the OFT to identify anxiety-like behaviour is built on two factors that trigger anxiety-like behaviour in animals; individual testing (the animal is separated from its housed or social group) and agoraphobia (fear of feeling trapped, helpless or embarrassed). Important to note, when evaluating rodents in the OFT, behaviour of these species mainly depend on their tactile sensory factors. In this regard, it has been reported that animals without vibrissae (whiskers) show low levels of thigmotaxis (the behaviour of walking close to the walls of the arena), resulting in increased number of entries into the central zone which could ultimately be misinterpreted as anxiolytic-like behaviour. Other factors that may also influence (but not trigger) the anxiety-like behaviour of the animals in the OFT include food and water intake and lighting conditions (Prut & Belzung, 2003; Walsh & Cummins, 1976).

As mentioned, the OFT is also used to assess general locomotor activity. This parameter is analysed in combination with the FST to support the observed depressive-like behaviour of the animals and to rule out any false-negative results. Because the results of the FST and the interpretation thereof are dependent on the time spent immobile, it is important to determine whether the general locomotor activity of the animal was affected by the specific intervention or treatment. For instance, when an animal presents with decreased time spent immobile in the FST, but also shows significantly increased locomotor activity, compared to controls, the physiological effect on locomotor activity may be misinterpreted as a psychological effect (Slattery & Cryan, 2012). However, since the comorbidity of anxiety and MDD is very high, it is suggested that depression and anxiety not be investigated in isolation, but rather together and therefore the OFT acts as a valuable indicator of anxiety-like behaviour.

The apparatus used in our laboratory and in the current study consisted of four square arenas (1 m²), surrounded by opaque black walls (*Figure 6-2*). Animals were placed in the centre of the arena on the day of testing and allowed to freely explore the arena for 5 minutes under red light of 80 lux intensity (Schoeman *et al.*, 2017). Time spent in the arena was recorded via a camera mounted above the test arena. Behaviour during the test session was analysed using Ethovision XT12 software (Noldus Information Technology BV, Wageningen, NLD). Behavioural parameters measured in the OFT included total distance covered, average velocity travelled by, total time mobile and immobile; indicative of general locomotor activity. Parameters indicative of anxiety-like behaviour include total time spent in the centre zone of the arena and the corners, number of entries into the centre zone as well as ratio of time spent moving and time spent immobile. These anxiety-like parameters have been reported to positively correlate with those measured by other anxiety-like behavioural tests, such as the Elevated plus maze (Bergami *et al.*, 2009; Bhatia *et al.*, 2011).

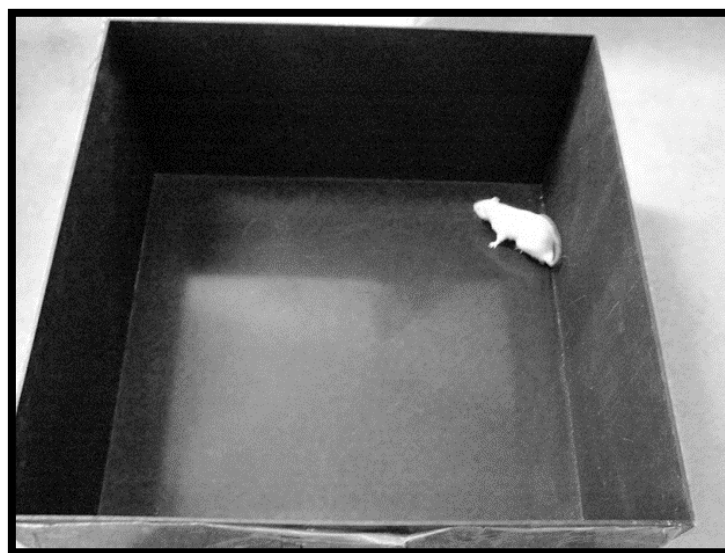


Figure 6-2: A picture of the OFT arena as used in the current project.

A.3.2 Forced swim test

The forced swim test (FST) is a behavioural test that assesses antidepressant activity across a broad spectrum of antidepressant agents (Borsini & Meli, 1988). The test is based on the observation that after rats have been placed in an inescapable environment i.e. a water filled cylinder, they will initially try and escape via escape-directed behaviour (such as swimming, struggling/climbing and diving) (Armario *et al.*, 1988). These escape-directed behaviours are then followed by the development of an immobile posture (described as floating) which has been linked with either a failure of perseverance in escape-directed actions (i.e. behavioural despair) or the development of passive behaviour that separates the animal from active forms of coping with stressful stimuli (Cryan & Mombereau, 2004; Lucki, 1997; Petit-Demouliere *et al.*, 2005).

The test was originally described by Porsolt and colleagues (Porsolt *et al.*, 1977) four decades ago, however, a modified version of the test has since been proposed and is currently (along with the original version) one of the most widely used behavioural test for depressive-like behaviour, mainly due to the ease of use, inter-laboratory reliability and specificity (Slattery & Cryan, 2012). The original test included two separate sessions where the animals were exposed to the water filled cylinder. Animals first underwent a Pre-swim session where they were left in the water filled cylinder for fifteen minutes and then returned to their home cages. After 24 hours, the subjects were again placed in the FST cylinders, only for five minutes, for the Test session. The Pre-swim session ensures that the animals quickly adopt an immobile posture during the Test session, which enables the effects of the test compounds to be easily observed (Borsini & Meli, 1988; Cryan *et al.*, 2002; Detke & Lucki, 1995; Lucki, 1997). The behaviours of the animals during the second swim session was scored and subsequently analysed. When antidepressants were administered between the two swim sessions, it was observed that subjects persisted in their escape-directed behaviour for longer periods of time, compared to controls (Cryan *et al.*, 2002).

The original version of the FST, however, is unreliable in detecting the effects of SSRIs, such as fluoxetine (Lucki, 1997). Therefore, a modified version of the test was proposed in order to increase the sensitivity of the test, especially with regards to the antidepressant effects of SSRIs. These modifications included the increase of the water depth from the traditional fifteen to thirty centimetres which led to a significant decrease in the total time spent immobile, because the animal was no longer in contact with the bottom of the cylinder (Lucki, 1997). Also, more emphasis was placed on the escape-directed behaviour which have since been associated with alterations of different neurotransmitters. To this extent, swimming is associated with an increase in serotonin, while an increase in noradrenaline is associated with increased time spent climbing or struggling (Cryan & Lucki, 2000; Detke *et al.*, 1995; Hemby *et al.*, 1997).

Interestingly, when using the FST to analyse the antidepressant activities of interventions in the Flinders Sensitive Line (FSL) rat, the original Porsolt FST protocol does not have to be followed. In fact, a modified version of the original test, where no Pre-swim session is required, is acceptable when screening these specific animal models. The reason being that the FSL rat is highly immobile without any pre-exposure to the FST (Overstreet *et al.*, 2005; Pucilowski & Overstreet, 1993; Schiller *et al.*, 1992). Another modification to the original Porsolt FST protocol is that the FSL rats can be tested 24 hours after the last antidepressant administration of a fourteen day treatment regimen (Overstreet & Wegener, 2013). The above mentioned protocol was also used in the current study.

The different behaviours observed in the FST are generally defined as follows:

- **Immobility:** Floating with no additional activity observed other than that required to keep the rat's head above the water line (Hédou *et al.*, 2001; Porsolt *et al.*, 1978).

- **Swimming:** Movements that are usually horizontal with the water level throughout the cylinder that also includes crossing into another quadrant of the cylinder (Porsolt *et al.*, 1978).
- **Struggling (also known as Climbing/Thrashing):** Any upward-directed movements of the forepaws along the side of the cylinder. These actions are significantly more intense than swimming and has thus also been described as thrashing (Cryan *et al.*, 2002; Porsolt *et al.*, 1978).

Although diving is also classified or interpreted as an escape-directed activity, and head shaking is also observed in animals in the FST, these behaviours are generally not included in the analysis of the animal because they are episodic (instead of continuous) and do not seem to correspond with specific treatment effects (Cryan *et al.*, 2005). In other studies, diving actions (but not head shaking) has been grouped together with swimming action of the animal (Piubelli *et al.*, 2011). However, in the current study, although the total time spent diving by the animal was recorded, this specific behaviour was not included in the final depressive-like behaviour analysis of the animals. Nevertheless, the FST apparatus used in our laboratory, and in the current project, consists of four cylindrical tanks (40 cm high and 20 cm in diameter) spaced next to each other. An example of one such cylindrical tank is presented in Figure 6-3. As per the modified version of the FST, the water level in each of the cylinders was 30 cm deep with water at a temperature of 25 ± 1 °C. As described earlier, all behavioural tests were performed during the animals' dark cycle. During the test, each subjected was placed in a water filled cylinder and left to swim for seven minutes, whilst the behaviour of the animal was recorded by a camera mounted in front of the apparatus. Behaviour was scored from the recorded video from the mid five minutes of the test by experimenters blind to the different treatment groups.



Figure 6-3: A picture of one of the four cylindrical tanks of the FST.

APPENDIX B: SUPPLEMENTARY DATA AND RESULTS

Appendix B contains all supplementary data, relevant to the main findings as presented in Chapters 3, 4 and 5. First of all, the nutritional analyses results of the ω -3 EFA oil used to coat the vivarium rat chow is presented in B.1, followed by the nutritional content analyses of both the standard and ω -3 EFA coated rat chow (B.2).

Secondly, the results of the pilot study (Phase 1C), investigating whether animals would indeed eat the coated rat chow are presented in Appendix B.3.

Finally, results pertaining to the triple combination effects of Phases 2 and 3 (Section 1.5), are presented in section B.4 of Appendix B. The results of the triple combination effects were not included in the manuscripts, as presented in Chapters 4 and 5, due to the magnitude of the analyses that needed to be performed (i.e. Three-way ANOVA) and consequently may have failed to highlight significant treatment differences due to group size. Regardless, the results gained from the behavioural analyses indicate potential novel treatment strategies for juvenile MDD to be confirmed and elaborated on in future studies. To this extent, only behavioural results of triple combination treatment strategies of escitalopram and venlafaxine are presented in Appendix B, without any neurochemical analyses. Neurochemical analyses, relevant to significant behavioural alterations are presented and discussed in Chapters 4 and 5.

B.1 ProOmega oil certificate of analysis received with product

The certificate of analysis for ProOmega oil, batch number: 53004, manufactured by Nordic Naturals USA is presented below.



Certificate of Analysis

Product: ProOmega, 8oz.	Bulk Liquid Lot Nr.: 153003	Bottle Lot Nr: 153004
Manufacture Date: September, 2015	Item Nr.: PUS-12126	Shelf Life: Three years from manufacture date

Oxidation	Test Method	Limits	Assay Result
ACID	AOCS Cd 3d-63	NMT 3.0 KOH/g	0.2 KOH/g
PEROXIDE	AOCS Cd 8b-90	NMT 5.0 meq/kg	1.6 meq/kg
ANISIDINE**	AOCS Cd 18-90	NMT 20.0	3.6
TOTOX*** (TOTAL OXIDATION)	Calculation	NMT 26.0 meq/kg	6.8 meq/kg

**Because of limitations of available testing methods, the anisidine value is determined using testing from pre-flavored oil.

***Totox values are computed from testing performed on pre-flavored oil.

Heavy Metals	Test Method	Limits	Assay Result
ARSENIC	USEPA 1638	NMT 0.1 mg/kg (ppm)	< 0.02 ppm
CADMIUM	USEPA 1638	NMT 0.1 mg/kg (ppm)	< 0.02 ppm
LEAD	USEPA 1638	NMT 0.1 mg/kg (ppm)	< 0.02 ppm
MERCURY	USEPA 1631	NMT 0.1 mg/kg (ppm)	< 0.0006 ppm

Environmental Toxins	Test Method	Limits	Assay Result
POLYCHLORINATED BIPHENYLS (PCBs)	USEPA 1668 A/C	NMT 0.09 mg/kg (ppm)	0.0001 ppm
DIOXIN-LIKE PCBs (non-ortho & mono-ortho)	USEPA 1668 A/C	NMT 1.0 pg/g (ppt)	0.0004 ppt
DIOXINS & FURANS (WHO TEQ)	USEPA 1613B	NMT 2.0 pg/g (ppt)	Not Detected

Microbial Analysis	Test Method	Limits	Assay Result
PLATE COUNT	USP 37 <2021>	<1000 cfu/g	Negative
STAPHYLOCOCCUS AUREUS	USP 37 <2022>	Absent	Absent
ESCHERICHIA COLI	USP 37 <2022>	Absent	Absent
SALMONELLA	USP 37 <2022>	Absent	Absent
YEAST & MOLD	USP 37 <2021>	<100 cfu/g	Negative

Fatty Acid Profile	Test Method	Label Claim (mg/serving)	Assay Result
EICOSAPENTAENOIC ACID (EPA ; 20:5 (n-3))	AOCS CE 1b-89	1450 mg	1460.3 mg
DOCOSAHEXAENOIC ACID (DHA ; 22:6 (n-3))	AOCS CE 1b-89	1060 mg	1071.5 mg
TOTAL OMEGA-3	AOCS CE 1b-89	2960 mg	2956.9 mg
OTHER OMEGAS	AOCS CE 1b-89	450 mg	425.1 mg

Radioactivity	Test Method	Assay Result
Radioactivity - Cesium 134	γ-spectrometry / Gamma-spectrometry	<3 Bq/Kg
Radioactivity - Cesium 137	γ-spectrometry / Gamma-spectrometry	<3 Bq/Kg
Radioactivity - Iodine 131	γ-spectrometry / Gamma-spectrometry	<3 Bq/Kg

Prepared By	Reviewed By
 Quality Assurance	 Quality Assurance

B.2 Nutritional analysis of rat chow's EFA content (Certificates of analysis)

The EFA content analysis of the ProOmega oil and rat chow was performed at the Cape Peninsula University of Technology. The results of the ProOmega oil, the ω -3 EFA coated and standard rat chow are presented below. The results include total fatty acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) content. In addition, the peroxide and conjugated diene levels of all three samples were analysed. These levels represent the breakdown process of the EFA oil.

All samples analysed were carried out in duplicate with two separate samples weighed and the average fatty acids calculated. Duplicate analyses were also done for peroxide, conjugated diene determinations. A single run was done for thin layer chromatography determination on the silica plate using a non-polar solvent mixture.

Interestingly, the peroxide and conjugated dienes levels of the oil used to coat the rat chow with are significantly higher than the acceptable limit of 5 meqO₂/kg oil and 18 μ mol/g oil, respectively. The influence of these elevated levels did, however, not seem to negatively affect the behaviour of the ω -3 EFA supplemented animals as observed in the monitoring of the test subjects.

B.2.1 ProOmega oil



Pro_Omega_Oil
B/N:53004

<i>Pro_Omega_Oil</i>			
<i>B/N:53004</i>			
Fatty Acids	Total Fatty Acids (mg)/1g of Oil		
	A	B	Average
12:0	1.0	0.5	0.7
14:0	2.7	2.9	2.8
14:1	0.1	0.2	0.1
16:0	23.7	24.3	24.0
16:1	9.1	9.4	9.2
C17:0	0.0	0.0	0.0
18:0	28.1	28.9	28.5
18:1 n-9	61.6	62.9	62.3
18:1 n-11	20.7	21.1	20.9
18:2 n-6	8.0	8.1	8.1
18:3 n-6	1.5	1.5	1.5
18:3 n-3	3.1	3.2	3.1
20:0	2.1	2.0	2.0
20:1 n-9	21.6	22.1	21.8
20:2 n-6	2.0	2.0	2.0
20:3 n-6	1.9	1.9	1.9
20:4 n-6	11.7	12.0	11.9
20:3 n-3	0.0	0.0	0.0
22:0	1.4	0.6	1.0
22:1 n-9	11.0	11.2	11.1
20:5 n-3	254.7	260.1	257.4
22:2 n-6	0.1	0.2	0.1
24:0	1.2	1.2	1.2
22:4 n-6	5.3	5.4	5.4
22:5 n-6	0.0	0.0	0.0
24:1	4.2	3.0	3.6
22:5 n-3	40.8	41.8	41.3
22:6 n-3	204.8	210.0	207.4
Total fatty acids			729.3/1000 mg oil

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B.2.2 Control rat chow (uncoated)

Rat_Food
Control

<i>Rat_Food</i>			
<i>Control (uncoated)</i>			
Fatty Acids	Total Fatty Acids (mg)/1g of Food Sample		
	A	B	Average
12:0	0.1	0.1	0.1
14:0	0.5	0.5	0.5
14:1	0.0	0.0	0.0
16:0	3.9	3.9	3.9
16:1	0.5	0.5	0.5
C17:0	0.0	0.0	0.0
18:0	0.8	0.9	0.8
18:1 n-9	5.7	5.8	5.8
18:1 n-11	0.4	0.4	0.4
18:2 n-6	10.8	10.8	10.8
18:3 n-6	0.0	0.0	0.0
18:3 n-3	0.9	0.9	0.9
20:0	0.0	0.0	0.0
20:1 n-9	0.3	0.3	0.3
20:2 n-6	0.0	0.0	0.0
20:3 n-6	0.0	0.0	0.0
20:4 n-6	0.1	0.1	0.1
20:3 n-3	0.0	0.0	0.0
22:0	0.0	0.0	0.0
22:1 n-9	0.1	0.1	0.1
20:5 n-3	1.5	1.5	1.5
22:2 n-6	0.0	0.0	0.0
24:0	0.1	0.1	0.1
22:4 n-6	0.0	0.0	0.0
22:5 n-6	0.0	0.0	0.0
24:1	0.1	0.1	0.1
22:5 n-3	0.2	0.2	0.2
22:6 n-3	1.3	1.3	1.3
Total fatty acids			27.3 mg /1g food

Please note that the EPA (20:5 n-3) and DHA (22:6 n-3) concentrations are 1.5 mg/g (150 mg/100 g) and 1.3 mg/g (130 mg/100 g), respectively.

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B.2.3 Omega-3 EFA supplemented rat chow (coated)

Rat_Food
Coated

<i>Rat_Food</i>			
<i>Coated</i>			
Fatty Acids	Total Fatty Acids (mg)/1g of Food Sample		
	A	B	Average
12:0	0.1	0.1	0.1
14:0	0.5	0.5	0.5
14:1	0.0	0.0	0.0
16:0	4.2	4.3	4.2
16:1	0.6	0.6	0.6
C17:0	0.0	0.0	0.0
18:0	1.0	1.1	1.1
18:1 n-9	6.5	6.7	6.6
18:1 n-11	0.5	0.5	0.5
18:2 n-6	11.5	11.8	11.7
18:3 n-6	0.0	0.0	0.0
18:3 n-3	1.0	1.1	1.0
20:0	0.0	0.0	0.0
20:1 n-9	0.4	0.4	0.4
20:2 n-6	0.0	0.0	0.0
20:3 n-6	0.0	0.0	0.0
20:4 n-6	0.1	0.1	0.1
20:3 n-3	0.0	0.0	0.0
22:0	0.1	0.1	0.1
22:1 n-9	0.1	0.1	0.1
20:5 n-3	3.1	3.2	3.1
22:2 n-6	0.1	0.1	0.1
24:0	0.1	0.1	0.1
22:4 n-6	0.1	0.1	0.1
22:5 n-6	0.0	0.0	0.0
24:1	0.1	0.1	0.1
22:5 n-3	0.5	0.5	0.5
22:6 n-3	2.5	2.6	2.6
Total fatty acids			33.6 mg/1g food
EPA			310 mg/100 g food
DHA			260 mg/100 g food



Cape Peninsula
University of Technology

Peroxide Value (meqO₂/kg of food sample) 4.24

Conjugated Dienes (μmol/g of food sample) 6.46

Thin Layer Chromatography Results *Only Glycerides Present*

Please note: The fatty acid (FA) percentages are not based on the area percentages but rather on the FA quantities in the oil/food samples based on the calculations with inclusion of the internal standard.

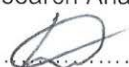
The oil and/or rat food samples analyses were carried out in duplicate with two separate samples weighed and the average FAs amount calculated. Duplicate analyses were also done for peroxide, conjugated diene determinations. A single run was done for thin layer chromatography determination on the silica plate using a non-polar solvent mixture.

Functional Foods Research Unit (FFRU)

Head: Prof. A.J.S. Benadè


.....
06/05/16

Research Analyst: Mr. Lloyd Matsheka


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2016/05/06

B.3 Additional results not presented in manuscripts

B.3.1 Nutritional results of coated rat chow (*Pilot study, Phase 1C*)

Nutritional content analyses indicated that the control diet contained 150 ± 0.05 mg/100 g eicosapentaenoic acid (EPA) and 130 ± 0.05 mg/100 g docosahexaenoic acid (DHA). Following coating with ω -3 EFA oil, the rat chow contained 310 ± 0.05 mg/100 g and 260 ± 0.05 mg/100 g EPA and DHA, respectively. Coincidentally, this represents an approximate 100 % increase (doubling) of the ω -3 EFA content in the enriched diet relative to the standard control diet. Put differently, the ω -3: ω -6 ratio decreased from 1:3 in the standard rat chow to 1:2 in the supplemented rat chow.

Additionally, a pilot study (Phase 1C) established that coating of standard vivarium rat chow did not adversely affect daily food intake and body weight of pre-pubertal FSL rats. Importantly, the effect of coated rat chow was not compared to that of standard rat chow. In fact, the objective of Phase 1C was only to establish that animals fed the coated diet from weaning, do indeed gain weight and increase their daily food intake. A comparison to the effects of standard vivarium rat chow was investigated following successful coating and later on below.

Strong positive correlations ($r > 0.9$) were observed between pre-pubertal age and body weight (Figure 6-4A), between pre-pubertal age and daily food intake (Figure 6-4B), as well as between body weight and daily food intake (Figure 6-4C) during pre-pubertal development, thereby confirming the use of the coated rat chow throughout the remainder of the project.

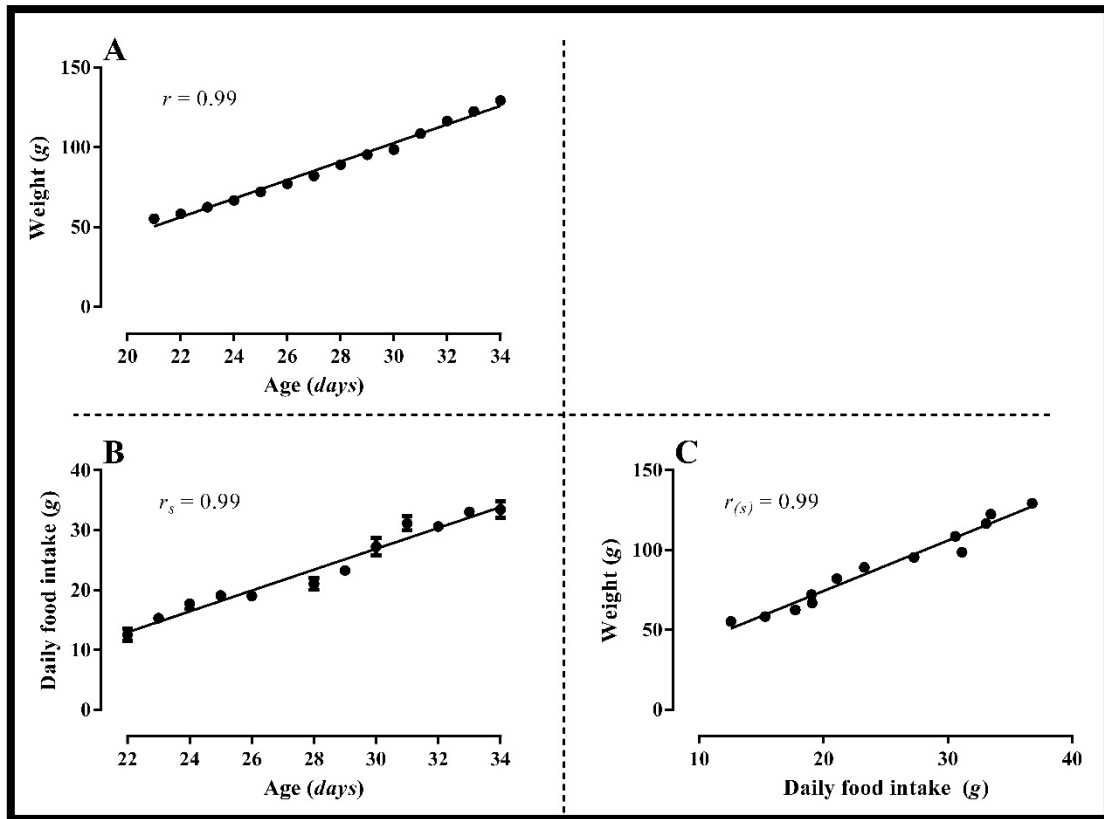


Figure 6-4: Results of Phase 1C.

(A) Correlation between pre-pubertal age and body weight following exposure to ω -3 coated rat chow. **(B)** Correlation between pre-pubertal age and daily food intake. **(C)** Correlation between pre-pubertal daily food intake and body weight.

Following successful completion of Phase 1C, the effects of ω -3 supplementation during pre-pubertal development could be investigated as part of the main objectives of the current project. To this extent, a linear regression analysis was run to determine whether ω -3 EFA intake (EPA and DHA) increased with age between PND21 and PND34. Data were not normally distributed for all test groups ($p > 0.05$) and no outliers were removed from the data set. There were strong correlations between age and daily food intake for all test groups ($r_s > 0.9$). No significant differences were identified between the slopes of daily food intake for sedentary animals fed the standard diet (irrespective of drug) (DRG+SED+STD) ($F[2, 237] = 0.768, p = 0.465$), sedentary animals fed supplemented diet (irrespective of drug) (DRG+SED+OM3) ($F[2, 358] = 0.839, p = 0.433$), exercised animals fed control diet (irrespective of drug) (DRG+EXE+STD) ($F[2, 344] = 0.557, p = 0.543$) or exercised animals fed supplemented diet (irrespective of drug) (DRG+EXE+OM3) ($F[2, 340] = 0.345, p = 0.708$). Therefore, the slopes could be pooled to determine whether exercise or diet had a significant effect on the amount of food consumed per animal per day.

The data of the combined slopes indicated strong correlations between age and daily food intake for all test groups ($r_s > 0.9$). However, the slopes of daily food intake data differed significantly between SED+STD

and SED+OM3 groups ($F[1, 603] = 10.782, p = 0.001$) and between EXE+STD and EXE+OM3 ($F[1, 692] = 5.929, p = 0.015$). In contrast, there were no significant difference in daily food intake data between SED+STD and EXE+STD treatment groups ($F[1, 589] = 0.017, p = 0.894$) or between SED+OM3 and EXE+OM3 treatment groups ($F[1, 706] = 0.779, p = 0.378$) (*data not shown*). Therefore, linear regression and the Spearman's rank-order correlation (r_s) were run to determine the different daily intake of ω -3 EFA (EPA and DHA) between the two types of diet.

Daily EPA and DHA intake was statistically significantly predicted by pre-pubertal age for both STD ($F[1, 1624] = 27.79, p < 0.0001; y = 1.670x + 191.0$) and OM3 treatment groups ($F[1, 1919] = 48.66, p < 0.0001; y = 4.472x + 319.2$). There was a weak positive correlation between daily EPA and DHA intake and age for animals fed the STD ($r_s(1624) = 0.090, p < 0.0005$) and OM3 treatment groups ($r_s(1919) = 0.150, p < 0.0005$). Finally, the mean daily intake of EPA and DHA for both diets were determined with the independent-samples *t*-test. To this extent, the assumption for homogeneity of variances were violated for all test groups ($p < 0.0005$). The mean EPA intake of the control fed diet was 205.16 mg/kg (95 % CI, 199.44 to 210.88 mg/kg) lower compared to those fed the supplemented diet (236.940 ± 1.287 vs. 442.100 ± 2.618 mg/kg; $t(2768.562) = 70.334; p < 0.0005; d = 2.244, 95\% \text{ CI}, 2.160 \text{ to } 2.328$). Similarly, the mean DHA intake of the control fed diet was 165.443 mg/kg (95 % CI, 160.618 to 170.275 mg/kg) lower compared to those fed the supplemented diet (205.348 ± 1.115 vs. $370.794 \pm 2.196; t(2816.597) = 67.185; p < 0.0001; d = 2.147, 95\% \text{ CI}, 2.064 \text{ to } 2.230$).

B.4 Early-life and lasting effects of triple combination treatment strategies

B.4.1 Body weight

In Figure 6-5A, pre-pubertal weight gain rate showed a strong positive correlation with age for all test groups ($r_s > 0.9$), yet the slopes (i.e. weight gain rate) of the different treatment groups differed significantly ($F[11, 3879] = 6.641, p < 0.0001$), suggesting significant differences in weight gain rate induced by the various treatment strategies. To this extent, in Figure 6-5B no statistically significant three-way interaction between drug, diet and activity existed ($F[2, 265] = 2.564, p = 0.079; \eta^2 = 0.019$) on total weight gain between PND21 to PND34. However, there was a statistically significant two-way interaction between drug and diet ($F[2, 265] = 3.234, p = 0.041; \eta^2 = 0.024$).

The simple main effect of drug was only statistically significant when combined with STD ($F[2, 265] = 4.033, p = 0.019; \eta^2 = 0.030$) and not with OM3 ($F[2, 265] = 0.626, p = 0.536; \eta^2 = 0.005$). Secondly, the simple main effect of diet was statistically significant when combined with SAL ($F[1, 265] = 24.414, p < 0.0005; \eta^2 = 0.084$) and ESC ($F[1, 265] = 20.758, p < 0.0005; \eta^2 = 0.073$), but not when combined with VEN ($F[1, 265] = 2.694, p = 0.102; \eta^2 = 0.010$). Pre-pubertal weight gain was 12.6 g (95 % CI, 7.6 to 17.6 g) lower in SAL+OM3, compared to SAL+STD animals ($p \leq 0.0005; d = 1.137, 95\% \text{ CI}, 1.0 \text{ to } 1.6$) as

was it in ESC+OM3 compared to ESC+STD animals ($p \leq 0.0005$; $d = 0.901$, 95 % CI, 0.5 to 1.3) (irrespective of activity). Furthermore VEN+STD animals weighed 6.8 g (95 % CI, 0.6 to 12.9 g) less than ESC+STD animals ($p = 0.025$; $d = 0.495$, 95 CI, 0.1 to 0.9) (irrespective of activity).

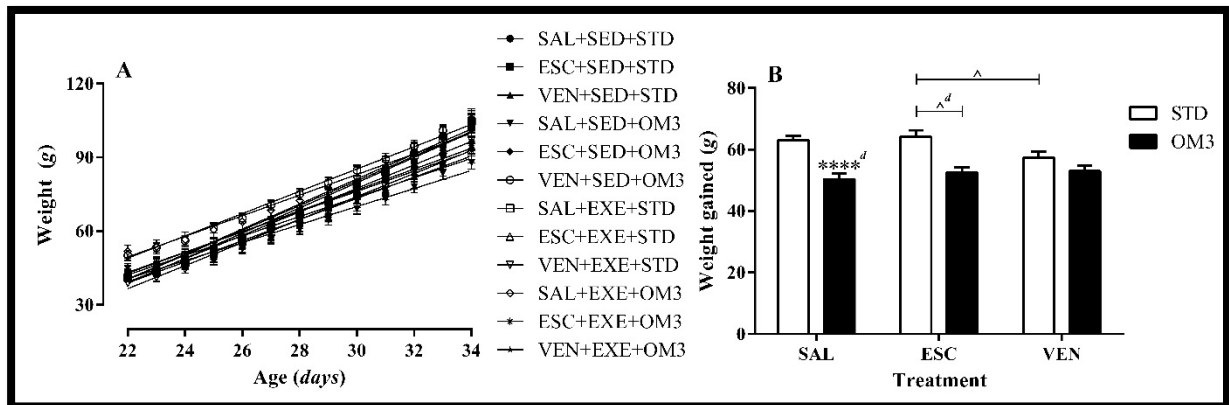


Figure 6-5: Overall effect of different interventions on body weight.

(A) Daily body weight during pre-pubertal intervention period (PND21-34) of all treatment groups. (B) Weight gained during pre-pubertal intervention period of drug treatment with the significant simple main effects of drug and diet (irrespective of exercise) as highlighted by the three-way ANOVA. Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text, with **** $p < 0.0005$ vs. SAL+STD; ^ $p \leq 0.05$ vs. indicated test group; d large effect size ≥ 0.8 .

Taken together, although weight gain rates differed between treatment groups, it appears that physical activity had no effect on overall pre-pubertal weight gain. In fact, ω -3 EFA supplementation appeared to reduce overall pre-pubertal weight gain, compared to animals fed the control diet. This difference was significant between SAL+STD and SAL+OM3, and between ESC+STD and ESC+OM3 (irrespective of exercise). That weight appeared to be unaffected in the venlafaxine-treated group is unexpected as recent data suggests venlafaxine to cause weight loss in adolescents (Reekie *et al.*, 2015) and with the overall weight reduction caused by ω -3 EFA supplementation, it would be expected to have a similar effect than in the other pharmacotherapy groups. Regardless, recent reports (Arnold *et al.*, 2017; Bigornia *et al.*, 2017; Simopoulos, 2016) on the effect of ω -3 EFA supplementation on weight indicate a negative correlation between consumption and weight gain and therefore support the current results. Although not measured in the current project, a recent report (Christian *et al.*, 2017) suggests a strong inverse correlation of juvenile weight and fatty acid accumulation. This is of note, since ω -3 EFA supplementation caused a significant reduction in pre-pubertal weight gain and could, according to this report, simultaneously induce increased fatty acid concentrations, even during the wash-out period (i.e. PND35 to PND60) when animals returned to an adequate diet.

B.4.2 Locomotor activity

In Figure 6-6A, distance moved data on PND35 were normally distributed for all test groups ($p > 0.05$) and had homogeneity of variances ($p = 0.205$). There was no statistically significant three-way interaction

between drug, diet and activity ($F[2, 129] = 0.197, p = 0.821; \eta^2 = 0.003$). However, there was a statistically significant two-way interaction between diet and activity ($F[1, 129] = 4.642, p = 0.003; \eta^2 = 0.035$), supported by a statistically significant simple main effect of diet when combined with SED ($F[1, 129] = 4.020, p = 0.047; \eta^2 = 0.030$). In this regard, overall distance moved was decreased by 356.0 cm (95 % CI, 4.7 to 707.3 cm) by SED+OM3, compared to SED+STD (irrespective of drug) ($p = 0.047$). Regardless, no treatment specific differences, compared to SAL+SED+STD controls were identified by the Dunnett post-hoc test.

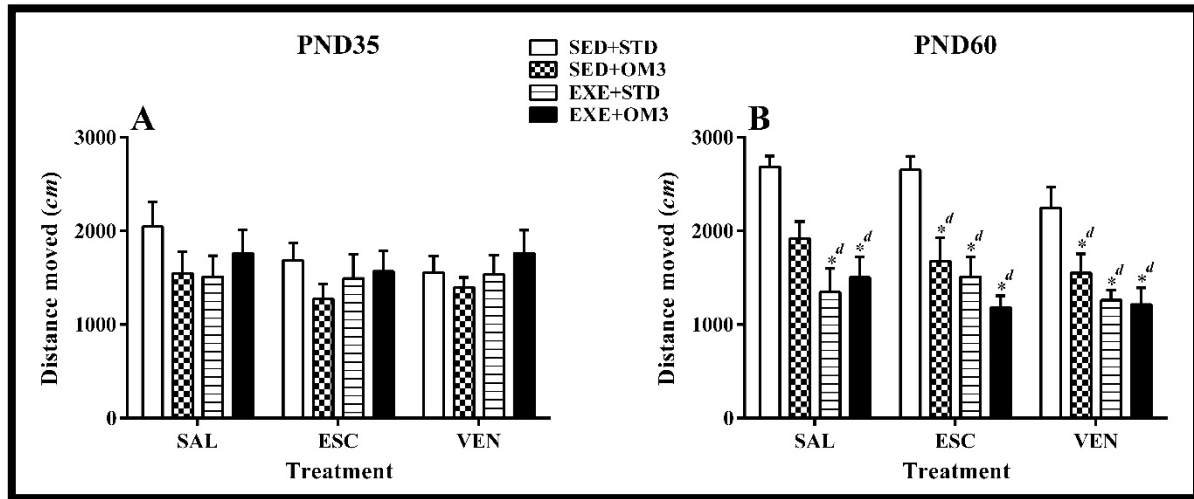


Figure 6-6: Three-way interaction data on locomotor activity on PND35 and PND60.

(A) Total distance moved in the OFT on PND35, following various treatment strategies during pre-pubertal development (i.e. PND21 until PND34). **(B)** Total distance moved in the OFT on PND60, following a 26-day wash-out/withdrawal period. Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text, with * indicating statistical significance and *d* a large effect magnitude difference compared to SAL+SED+STD.

In Figure 6-6B, distance moved data on PND60 were normally distributed for all test groups ($p > 0.05$) and had homogeneity of variances ($p = 0.279$). There was no statistically significant three-way interaction between drug, diet and activity ($F[2, 122] = 0.171, p = 0.843; \eta^2 = 0.003$). However, there was a statistically significant two-way interaction between diet and activity ($F[1, 122] = 10.912, p = 0.001; \eta^2 = 0.082$), supported by a statistically significant simple main effect of diet when combined with SED ($F[1, 122] = 26.063, p \leq 0.0005; \eta^2 = 0.176$) and of activity, regardless of diet ($F[1, 122] = 53.552, p \leq 0.0005; \eta^2 = 0.305$ and $F[1, 122] = 6.992, p = 0.009; \eta^2 = 0.054$). In this regard, compared to SED+STD, distance moved was 12.6 cm (95 % CI, 497.5 to 1127.7 cm) lower in SED+OM3 ($p \leq 0.0005$) (irrespective of drug) and 1154.8 cm (95 % CI, 842.3 to 1467.3 cm) lower in EXE+STD ($p \leq 0.0005$) (irrespective of drug). Furthermore, distance was decreased by 417.4 cm (95 % CI, 104.9 to 729.8 cm) by EXE+OM3 compared to SED+OM3 ($p = 0.009$) (irrespective of drug). Specifically, the treatment strategies that significantly reduced locomotor activity in relation to SAL+SED+STD are summarized below:

Table 6-3: Multiple comparison summary of PND60 locomotor activity according to Dunnet's post-hoc test.

Control	Treatment strategy	Mean difference (95 % CI)	<i>p</i> -value	<i>d</i> -value (95 % CI)
SAL+SED+STD	SAL+EXE+STD	-1336.9 cm (569.0 to 2104.8 cm)	≤ 0.0001	2.041 (1.0 to 3.1)
	SAL+EXE+OM3	-1182.4 cm (398.8 to 1966.0 cm)	0.0005	2.089 (1.0 to 3.2)
	ESC+SED+OM3	-1005.8 cm (203.7 to 1807.8 cm)	0.0063	1.716 (0.7 to 2.7)
	ESC+EXE+STD	-1172.7 cm (389.1 to 1956.3 cm)	0.0006	2.184 (1.1 to 3.3)
	ESC+EXE+OM3	-1505.9 cm (738.0 to 2273.8 cm)	≤ 0.0001	3.843 (2.4 to 5.3)
	VEN+SED+OM3	-1134.0 cm (332.0 to 1936.1 cm)	0.0014	2.263 (1.1 to 3.4)
	VEN+EXE+STD	-1423.9 cm (621.8 to 2225.9 cm)	≤ 0.0001	4.215 (2.6 to 5.8)
	VEN+EXE+OM3	-1470.6 cm (702.7 to 2238.6 cm)	≤ 0.0001	2.947 (1.7 to 4.2)

None of the treatment strategies induced any significant effects on early-life locomotor activity. Yet, following a chronic wash-out/withdrawal period, low intensity exercise alone and in combination with ω -3 EFA supplementation significantly decreased locomotor activity later in life. Interestingly, neither escitalopram nor venlafaxine affected locomotor activity later in life, yet when combined with either low intensity exercise and/or ω -3 EFA supplementation, early-adulthood locomotor activity was significantly reduced compared to age-matched controls (SAL+SED+STD).

B.4.3 Anxiety-like behaviour

In Figure 6-7A, time spent in centre zone data were not normally distributed for all test groups ($p < 0.05$), yet there was homogeneity of variances ($p = 0.180$). There was no statistically significant three-way interaction between drug, diet and activity ($F[2, 125] = 0.132, p = 0.877; \eta^2 = 0.002$). However, there was a statistically significant two-way interaction between drug and diet ($F[1, 125] = 4.102, p = 0.019; \eta^2 = 0.062$), supported by a statistical significant simple main effect of drug when combined with STD ($F[2, 125] = 4.447, p = 0.014; \eta^2 = 0.066$) and of diet when combined with SAL ($F[1, 125] = 6.429, p = 0.012; \eta^2 = 0.049$). In this regard, overall time spent in centre zone was decreased by 7.2 s (95 % CI, 1.2 to 13.2 s) by VEN+STD, compared to SAL+STD (irrespective of activity) ($p = 0.012$) and by 6.2 s (95 % CI, 1.4 to 11.1 s) by SAL+OM3, compared to SAL+STD (irrespective of activity) ($p = 0.012$). Regardless, no

treatment specific differences, compared to SAL+SED+STD controls were identified by the Dunnett post-hoc test.

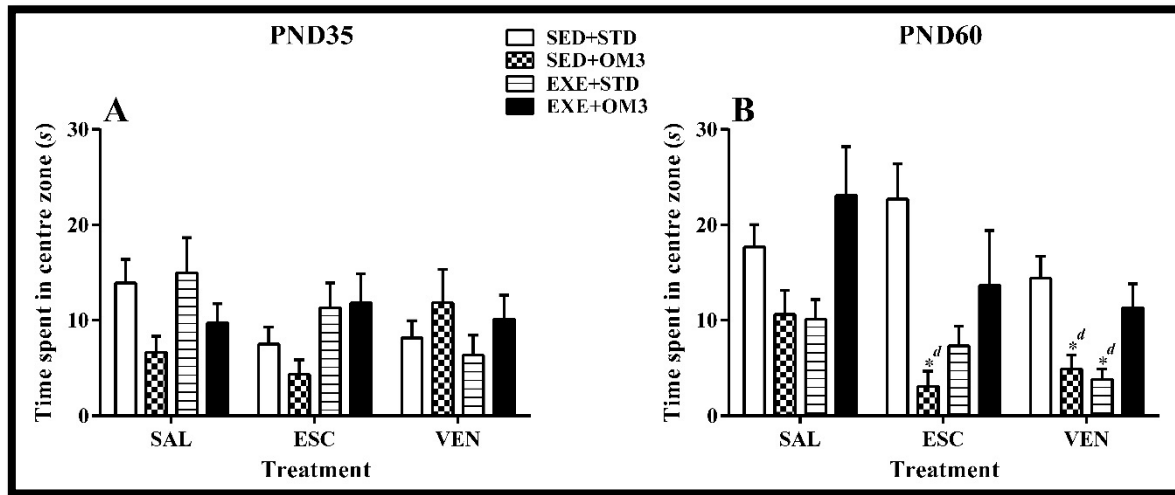


Figure 6-7: Three-way interaction data on time spent in centre zone on PND35 and PND60. (A) Time spent in the centre zone of the OFT on PND35, following various treatment strategies during pre-pubertal development (i.e. PND21 until PND34). (B) Time spent in the centre zone of the OFT on PND60, following a 26-day wash-out/withdrawal period. Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text, with * indicating statistical significance and *d* a large effect magnitude difference compared to SAL+SED+STD.

In Figure 6-7B, time spent in centre zone data were not normally distributed for all test groups ($p < 0.05$), nor was there homogeneity of variances ($p = 0.002$). There was no statistically significant three-way interaction between drug, diet and activity ($F[2, 120] = 0.202, p = 0.817, \eta^2 = 0.003$). However, there was a statistically significant two-way interaction between diet and activity ($F[1, 120] = 29.876, p \leq 0.0005, \eta^2 = 0.199$), supported by statistical significant simple main effects of diet, irrespective of activity ($F[1, 120] = 18.173, p \leq 0.0005, \eta^2 = 0.132$ and $F[1, 120] = 12.016, p = 0.001, \eta^2 = 0.091$) and of activity, irrespective of diet ($F[1, 120] = 19.106, p \leq 0.0005, \eta^2 = 0.137$ and $F[1, 120] = 11.335, p = 0.001, \eta^2 = 0.086$). In this regard, EXE+STD decreased centre zone time by 11.0 s (95 % CI, 5.9 to 16.1 s) compared to SED+STD ($p \leq 0.0005$) (irrespective of drug), whereas EXE+OM3 increased centre zone time by 8.9 s (95 % CI, 3.8 to 14.0 s) compared to EXE+STD ($p = 0.001$) (irrespective of drug). Specifically, the treatment strategies that significantly reduced time spent in centre zone, in relation to SAL+SED+STD are summarized below:

Table 6-4: Multiple comparison summary of PND60 centre zone time according to Dunnet's post-hoc test.

Control	Treatment strategy	Mean difference (95 % CI)	<i>p</i> -value	<i>d</i> -value (95 % CI)
SAL+SED+STD	ESC+SED+OM3	-14.6 s (1.7 to 27.5 s)	0.018	2.469 (1.3 to 3.6)
	VEN+SED+OM3	-12.8 s (0.2 to 25.4 s)	0.044	2.192 (1.1 to 3.3)
	VEN+EXE+STD	-13.9 s (1.3 to 26.5 s)	0.023	2.557 (1.4 to 3.7)

Overall, none of the treatment strategies induced any anxiolytic or anxiogenic effects during early-life development (i.e. PND35; Figure 6.7A), despite a significant main effect, suggested venlafaxine mono-treatment (irrespective of activity) and ω -3 EFA supplementation alone (irrespective of activity) to increase early-life anxiety-like behaviour. Regardless, the pre-pubertal combination of escitalopram and ω -3 EFA supplementation (ESC+OM3+SED), as well as venlafaxine with ω -3 EFA (VEN+OM3+SED) or low intensity exercise (VEN+STD+EXE) treatment strategies significantly increased anxiety-like behaviour later in life. However, as discussed in Chapter 4, ω -3 EFA supplementation could have induced lasting improved coping behaviour, explaining the observed behaviour. In addition, that anxiety-like behaviour was increased during early-adulthood by pre-pubertal venlafaxine-low intensity exercise combination, could in fact be a result of decreased locomotor activity as discussed in Chapter 5.

B.4.4 Depressive-like behaviour

B4.4.1 Immobility

In Figure 6-8A, time spent immobile data on PND35 were not normally distributed for all test groups ($p < 0.05$), yet there was homogeneity of variances ($p = 0.507$). There was no statistically significant three-way interaction between drug, diet and activity ($F[2, 129] = 0.2080$, $p = 0.129$; $\eta^2 = 0.031$) nor any statistically significant two-way interactions and consequently no treatment specific differences, relative to SAL+SED+STD controls were identified by the Dunnet post-hoc test.

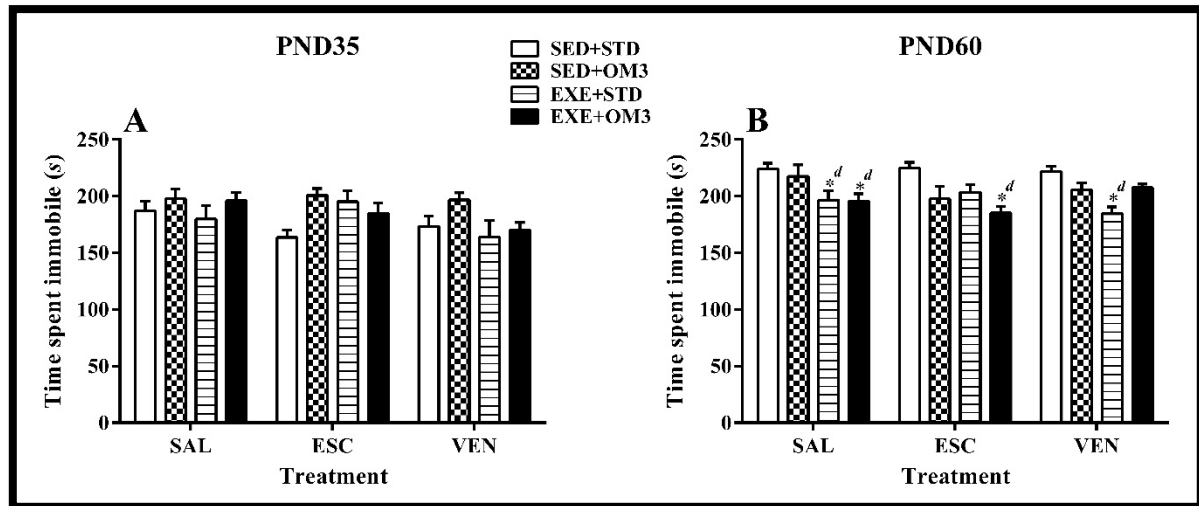


Figure 6-8: Three-way interaction on time spent immobile on PND35 and PND60.

(A) Time spent immobile in the FST on PND35, following various treatment strategies during pre-pubertal development (i.e. PND21 until PND34). (B) Time spent immobile in the FST on PND60, following a 26-day wash-out/withdrawal period. Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text, with * indicating statistical significance and *d* a large effect magnitude difference compared to SAL+SED+STD.

In Figure 6-8B, time spent immobile data on PND60 were normally distributed for all test groups ($p > 0.05$), yet there was no homogeneity of variances ($p = 0.002$). There was no statistically significant three-way interaction between drug, diet and activity ($F[2, 122] = 1.812, p = 0.168; \eta^2 = 0.029$). However, there were statistically significant two-way interactions between drug and diet ($F[2, 122] = 3.870, p = 0.023; \eta^2 = 0.060$) as well as between diet and activity ($F[1, 122] = 5.125, p = 0.025; \eta^2 = 0.040$). Firstly, the simple main effect of drug was statistically significant when combined with OM3 ($F[2, 122] = 3.153, p = 0.046; \eta^2 = 0.049$), whereas the simple main effect of diet was only statistically significant when combined with ESC ($F[1, 122] = 10.995, p = 0.001; \eta^2 = 0.083$). In this regard, overall time spent immobile was decreased by 22.8 s (95 % CI, 9.2 to 36.4 s) by ESC+OM3 compared to ESC+STD ($p = 0.001$) (irrespective of activity).

Secondly, the simple main effect of diet was statistically significant when combined with SED ($F[1, 122] = 8.891, p = 0.003; \eta^2 = 0.068$), whereas the simple main effect of activity was statistically significant when combined with STD ($F[1, 122] = 26.718, p < 0.0005; \eta^2 = 0.180$). In this regard, compared to SED+STD, overall time spent immobile in the FST was decreased by 16.6 s (95 % CI, 5.6 to 27.7 s) by SED+OM3 ($p = 0.003$) (irrespective of drug) and by 28.6 s (95 % CI, 17.6 to 39.6 s) by EXE+STD ($p < 0.0005$) (irrespective of drug).

Overall, the specific treatment strategies that significantly reduced time spent immobile in relation to SAL+SED+STD are summarized below:

Table 6-5: Multiple comparison summary of PND60 immobility time according to Dunnet's post-hoc test.

Control	Treatment strategy	Mean difference (95 % CI)	<i>p</i> -value	<i>d</i> -value (95 % CI)
SAL+SED+STD	SAL+EXE+STD	-27.2 s (0.7 to 53.7 s)	0.041	1.216 (0.3 to 2.1)
	SAL+EXE+OM3	-28.1 s (1.0 to 55.1 s)	0.038	1.522 (0.6 to 2.5)
	ESC+EXE+OM3	-38.7 s (11.6 to 65.7 s)	0.001	2.211 (1.2 to 3.3)
	VEN+EXE+STD	-39.3 s (11.6 to 67.0 s)	0.001	2.319 (1.2 to 3.4)

None of the treatment strategies induced any antidepressive-like behaviour during early-life development. This is of note, since the lack of antidepressant-like effects of escitalopram and venlafaxine treatments alone, support a sub-therapeutic dose to have been used with augmentation potential (*please refer to Chapters 4 and 5*). In this regard, pre-pubertal low intensity exercise alone (SAL+STD+EXE) and in combination with ω -3 EFA supplementation (SAL+OM3+EXE) induced long-term antidepressant-like behaviour in early-adulthood (Figure 6-8B). Furthermore, as discussed in Chapter 5, low intensity exercise (VEN+STD+EXE) augmented the antidepressant-like properties of venlafaxine (VEN+STD+EXE) treatment later in life. Of note, the antidepressant-like properties of pre-pubertal escitalopram treatment was augmented when combined with ω -3 EFA supplementation and low intensity exercise (ESC+OM3+EXE), suggestive of a novel treatment strategy for juvenile MDD during early-life development. Importantly, according to Chapter 4, the combination of escitalopram and ω -3 EFA supplementation showed a strong trend to also be effective in reducing depressive-like behaviour later in life, yet this is not indicated in Figure 6-8B.

B.4.4.2 Swimming

In Figure 6-9A, time spent swimming data on PND35 were not normally distributed for all test groups ($p < 0.05$), nor was there homogeneity of variances ($p < 0.0005$). There was no statistically significant three-way interaction between drug, diet and activity ($F[2, 127] = 2.967, p = 0.055; \eta^2 = 0.045$). However, there was a statistically significant two-way interaction between diet and activity ($F[1, 127] = 4.377, p = 0.038; \eta^2 = 0.033$), supported by statistical significant simple main effects of diet when combined with EXE ($F[1, 127] = 4.363, p = 0.039; \eta^2 = 0.033$) and of activity when combined with OM3 ($F[1, 127] = 16.968, p < 0.0005; \eta^2 = 0.118$). In this regard, EXE+OM3 increased overall swimming time by 8.0 s (95 % CI, 0.4 to 15.5 s) compared to EXE+STD (irrespective of drug) ($p = 0.039$) and by 16.2 s (95 % CI, 8.4 to 23.9 s)

compared to STD+OM3 (irrespective of drug). Specifically, the treatment strategies that significantly reduced time spent in centre zone, in relation to SAL+SED+STD are summarized below:

Table 6-6: Multiple comparison summary of PND35 swimming time according to Dunnet's post-hoc test.

Control	Treatment strategy	Mean difference (95 % CI)	<i>p</i> -value	<i>d</i> -value (95 % CI)
SAL+SED+STD	ESC+EXE+OM3	21.0 s (2.7 to 39.3 s)	0.015	0.920 (0.1 to 1.8)

In Figure 6-9B, time spent swimming data on PND60 were not normally distributed for all test groups ($p < 0.05$), nor was there homogeneity of variances ($p = 0.001$). There was no statistically significant three-way interaction between drug, diet and activity ($F[2, 120] = 0.251, p = 0.778; \eta^2 = 0.004$). However, there was a statistically significant two-way interaction between drug and diet ($F[2, 120] = 10.969, p < 0.0005; \eta^2 = 0.155$), supported by statistical significant simple effects of drug when combined with OM3 ($F[2, 120] = 15.863, p < 0.0005; \eta^2 = 0.209$) and of diet when combined with ESC ($F[1, 120] = 15.644, p < 0.0005; \eta^2 = 0.115$) and VEN ($F[1, 120] = 12.425, p = 0.001; \eta^2 = 0.094$). In this regard, compared to SAL+OM3, overall time spent swimming was increased by 13.0 s (95 % CI, 6.9 to 19.2 s) and 11.3 seconds (95 % CI, 5.2 to 17.5 s) by ESC+OM3 ($p < 0.0005$) and VEN+OM3 ($p < 0.0005$) (irrespective of activity), respectively. Furthermore, overall time spent swimming was increased by 10.1 s (95 % CI, 5.1 to 15.2 s) by ESC+OM3 compared to ESC+STD ($p < 0.0005$) (irrespective of activity), whereas VEN+OM3 increased overall time spent swimming was increased by 9.1 s (95 % CI, 4.0 to 14.2 s), compared to VEN+STD ($p = 0.001$) (irrespective of activity).

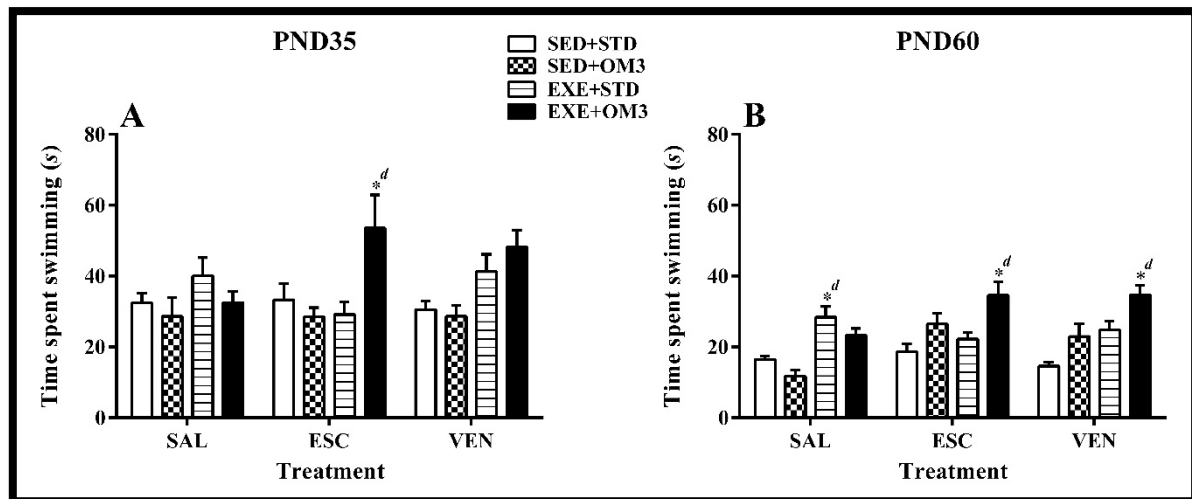


Figure 6-9: Three-way interaction on time spent swimming on PND35 and PND60.

(A) Time spent swimming in the FST on PND35, following various treatment strategies during pre-pubertal development (i.e. PND21 until PND34). (B) Time spent swimming in the FST on PND60, following a 26-day wash-out/withdrawal period. Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text, with * indicating statistical significance and *d* a large effect magnitude difference compared to SAL+SED+STD.

Overall, the specific treatment strategies that significantly reduced time spent swimming in relation to SAL+SED+STD are summarized below:

Table 6-7: Multiple comparison summary of PND60 swimming time according to Dunnet's post-hoc test.

Control	Treatment strategy	Mean difference (95 % CI)	<i>p</i> -value	<i>d</i> -value (95 % CI)
SAL+SED+STD	SAL+EXE+STD	11.9 s (1.8 to 22.0 s)	0.012	1.561 (0.6 to 2.5)
	ESC+EXE+OM3	18.1 s (8.0 to 28.2 s)	≤ 0.0001	1.924 (0.9 to 2.9)
	VEN+EXE+OM3	18.2 s (8.1 to 28.3 s)	0.001	2.702 (1.5 to 3.9)

In support of the potential role for the triple combination escitalopram treatment strategy (i.e. ESC+OM3+EXE) in juvenile MDD treatment, early-life swimming behaviour was significantly increased by this combination. And as discussed in Chapters 4 and 5, such an increase in escape-directed behaviour in the presence of unaltered depressive-like behaviour (i.e. immobility) is indicative of an antidepressant-like effect. Moreover, this apparent serotonergic-associated antidepressant-like effect lasted into early-adulthood, explaining the observed decrease in depressive-like behaviour, observed in Figure 6-8 above. In addition, the combination of low intensity exercise, ω -3 EFA supplementation and venlafaxine (VEN+OM3+EXE) also increased swimming behaviour later in life when compared to age-matched controls. Again suggestive of a serotonergic antidepressant-like mechanism to be involved.

B4.4.3 Struggling

In Figure 6-10A, time spent swimming data on PND35 were not normally distributed for all test groups ($p < 0.05$), nor was there homogeneity of variances ($p = 0.044$). There was no statistically significant three-way interaction between drug, diet and activity ($F[2, 127] = 0.030$, $p = 0.970$; $\eta^2 < 0.0005$) nor any statistically significant two-way interactions and consequently no treatment specific differences, compared to SAL+SED+STD controls were identified by the Dunnet post-hoc test..

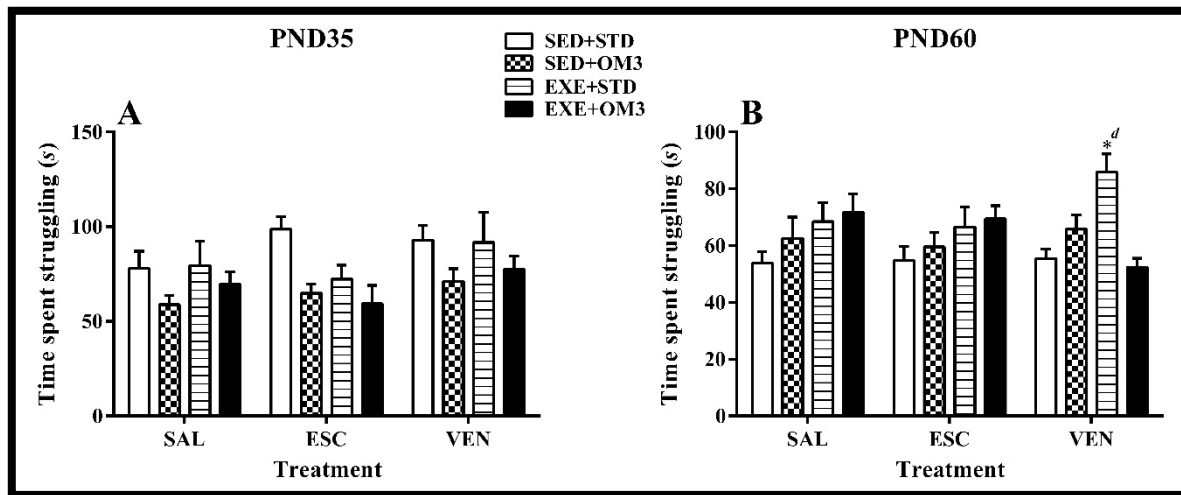


Figure 6-10: Three-way interaction on time spent struggling on PND35 and PND60.

(A) Time spent struggling in the FST on PND35, following various treatment strategies during pre-pubertal development (i.e. PND21 until PND34). (B) Time spent struggling in the FST on PND60, following a 26-day wash-out/withdrawal period. Data points represent the mean $\pm$ SEM. Statistical analyses are reported in the text, with * indicating statistical significance and d a large effect magnitude difference compared to SAL+SED+STD.

Figure 6-10B, time spent struggling data on PND35 were not normally distributed for all test groups ($p < 0.05$), nor was there homogeneity of variances ($p = 0.032$). There was a statistically significant three-way interaction between drug, diet and activity ($F[2, 121] = 4.340$, $p = 0.015$; $\eta^2 = 0.067$). In addition, there were statistically significant two-way interactions between drug and diet when combined with EXE ($F[2, 121] = 12.890$, $p < 0.0005$; $\eta^2 = 0.176$) and with SED ($F[2, 121] = 721.353$, $p < 0.0005$; $\eta^2 = 0.923$). The Statistical significant simple simple main effects were identified when activity was combined with SED ($F[2, 121] = 3.487$, $p = 0.034$; $\eta^2 = 0.054$) and OM3 ($F[2, 121] = 3.800$, $p = 0.025$; $\eta^2 = 0.059$). To this extent, the only statistically significant treatment strategy to increase struggling time in relation to SAL+SED+STD controls was VEN+EXE+STD (53.9 ± 4.1 s vs. 86.0 ± 6.4 s, $p = 0.001$; $d = 1.984$, 95 % CI, 0.9 to 3.0).

None of the treatment strategies significantly affected early-life struggling behaviour. Yet, compared to age-matched controls the combination of venlafaxine and low intensity exercise (VEN+STD+EXE) significantly increased struggling behaviour on PND60 (please see Chapter 5 for discussion).

B.5 Experimental group details

Group sizes for the different behavioural analyses of results presented in Appendix B are presented below.

Table 6-8: Experimental group sizes (Early-life effects; PND35).

Treatment	<i>n</i>	Treatment	<i>n</i>	Treatment	<i>n</i>
Weight gained from PND21-34					
SAL+SED+STD	23 ^a	ESC+SED+STD	24	VEN+SED+STD	24
SAL+SED+OM3	24	ESC+SED+OM3	20 ^a	VEN+SED+OM3	22 ^a
SAL+EXE+STD	23	ESC+EXE+STD	24	VEN+EXE+STD	22 ^a
SAL+EXE+OM3	23 ^a	ESC+EXE+OM3	24	VEN+EXE+OM3	24
Time spent immobile in FST					
SAL+SED+STD	12	ESC+SED+STD	12	VEN+SED+STD	12
SAL+SED+OM3	12	ESC+SED+OM3	10 ^a	VEN+SED+OM3	12
SAL+EXE+STD	11 ^a	ESC+EXE+STD	12	VEN+EXE+STD	12
SAL+EXE+OM3	12	ESC+EXE+OM3	12	VEN+EXE+OM3	12
Time spent swimming in FST					
SAL+SED+STD	12	ESC+SED+STD	12	VEN+SED+STD	12
SAL+SED+OM3	11 ^b	ESC+SED+OM3	9 ^{a,b}	VEN+SED+OM3	12
SAL+EXE+STD	10 ^a	ESC+EXE+STD	12	VEN+EXE+STD	12
SAL+EXE+OM3	12	ESC+EXE+OM3	12	VEN+EXE+OM3	12
Time spent struggling in FST					
SAL+SED+STD	12	ESC+SED+STD	12	VEN+SED+STD	12
SAL+SED+OM3	11 ^b	ESC+SED+OM3	10 ^a	VEN+SED+OM3	12
SAL+EXE+STD	11 ^a	ESC+EXE+STD	12	VEN+EXE+STD	12
SAL+EXE+OM3	12	ESC+EXE+OM3	11 ^b	VEN+EXE+OM3	12
Distance moved in OFT					
SAL+SED+STD	12	ESC+SED+STD	12	VEN+SED+STD	12
SAL+SED+OM3	12	ESC+SED+OM3	10 ^a	VEN+SED+OM3	12
SAL+EXE+STD	11 ^a	ESC+EXE+STD	12	VEN+EXE+STD	12
SAL+EXE+OM3	12	ESC+EXE+OM3	12	VEN+EXE+OM3	12
Time spent in centre zone of OFT					
SAL+SED+STD	12	ESC+SED+STD	11 ^b	VEN+SED+STD	12
SAL+SED+OM3	12	ESC+SED+OM3	10 ^a	VEN+SED+OM3	11 ^b
SAL+EXE+STD	10 ^{a,b}	ESC+EXE+STD	12	VEN+EXE+STD	12
SAL+EXE+OM3	12	ESC+EXE+OM3	12	VEN+EXE+OM3	11 ^b

Table 6-9: Experimental group sizes (Lasting effects; PND60).

Treatment	n	Treatment	n	Treatment	n
Time spent immobile in FST					
SAL+SED+STD	11 ^a	ESC+SED+STD	12	VEN+SED+STD	12
SAL+SED+OM3	12	ESC+SED+OM3	10 ^a	VEN+SED+OM3	10 ^a
SAL+EXE+STD	12	ESC+EXE+STD	11 ^a	VEN+EXE+STD	10 ^a
SAL+EXE+OM3	11 ^a	ESC+EXE+OM3	11 ^b	VEN+EXE+OM3	12
Time spent swimming in FST					
SAL+SED+STD	10 ^{a,b}	ESC+SED+STD	12	VEN+SED+STD	11 ^b
SAL+SED+OM3	12	ESC+SED+OM3	10 ^a	VEN+SED+OM3	10 ^a
SAL+EXE+STD	12	ESC+EXE+STD	10 ^{a,b}	VEN+EXE+STD	10 ^a
SAL+EXE+OM3	11 ^a	ESC+EXE+OM3	12	VEN+EXE+OM3	12
Time spent struggling in FST					
SAL+SED+STD	11 ^a	ESC+SED+STD	12	VEN+SED+STD	12
SAL+SED+OM3	12	ESC+SED+OM3	9 ^{a,b}	VEN+SED+OM3	10 ^a
SAL+EXE+STD	12	ESC+EXE+STD	11 ^a	VEN+EXE+STD	10 ^a
SAL+EXE+OM3	11 ^a	ESC+EXE+OM3	11 ^b	VEN+EXE+OM3	12
Distance moved in OFT					
SAL+SED+STD	10 ^{a,b}	ESC+SED+STD	12	VEN+SED+STD	12
SAL+SED+OM3	12	ESC+SED+OM3	10 ^a	VEN+SED+OM3	10 ^a
SAL+EXE+STD	12	ESC+EXE+STD	11 ^a	VEN+EXE+STD	10 ^a
SAL+EXE+OM3	11 ^a	ESC+EXE+OM3	12	VEN+EXE+OM3	12
Time spent in centre zone of OFT					
SAL+SED+STD	10 ^{a,b}	ESC+SED+STD	12	VEN+SED+STD	12
SAL+SED+OM3	12	ESC+SED+OM3	9 ^{a,b}	VEN+SED+OM3	10 ^a
SAL+EXE+STD	12	ESC+EXE+STD	11 ^a	VEN+EXE+STD	10 ^a
SAL+EXE+OM3	10 ^{a,b}	ESC+EXE+OM3	11 ^b	VEN+EXE+OM3	12

^{a)} Less than 12 animals due to “non-runner” behaviour and/or lower than expected birth rates at the requested date.

^{b)} Outlier identified and removed from group as assessed by Grubb’s test, due to not representing the target population. ESC: Escitalopram (10 mg/kg/day). EXE: Exercise (low intensity). FST: Forced swim test. OFT: Open field test. OM3: ω -3 coated diet. SED: Sedentary. STD: Standard diet. VEN: Venlafaxine (10 mg/kg/day).

APPENDIX C: CO-AUTHOR'S LETTER OF CONSENT



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13 November 2017

The Examiner
PhD Thesis of SF Steyn

Dear Examiner

Permission to Mr SF Steyn to Include Manuscripts in his Thesis for Examination Purposes for a PhD Degree

As study promotor and senior corresponding author on one published manuscript and two manuscripts to be submitted in the near future, understanding that these were co-authored by Mr Stephanus F Steyn, I hereby approve that the manuscripts be included in Chapters 3, 4 and 5 as part of the requirements for fulfilment of the PhD degree, and that these manuscripts be submitted for examination of Mr Steyn's thesis.

The three articles are as follows:

Manuscript A

Long-lasting effects of fluoxetine and/or exercise augmentation on bio-behavioural markers of depression in pre-pubertal stress sensitive rats

Published: Behavioural Brain Research (2017) 323:86–99. <http://dx.doi.org/10.1016/j.bbr.2017.01.043>

Manuscript B

The lasting augmentation of antidepressive-like bio-behavioural effects following pre-pubertal escitalopram and omega-3 supplementation in stress-sensitive rats

To be submitted to Behavioural brain research

Manuscript C

Chronic, pre-pubertal low intensity exercise induces beneficial bio-behavioural effects in older stress-sensitive rats following chronic exercise cessation

To be submitted to Developmental neuroscience

I trust that you will find this in order.

Yours sincerely

Prof Christiaan Beyers Brink
Professor of Pharmacology

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Original details: (10073892) C:\Users\NWUSER\Documents(2) Research(1) Students(2) Postgraduate Students & Postdocs(2015-2016 Steyn, Stephan(PhD(1) ProefskrifAppendix permission.docm
13 November 2017

APPENDIX D: CONFIRMATION OF MANUSCRIPT ACCEPTANCE

Manuscript A – Behavioural brain research

This document has been formatted without content change in the style of the current thesis. Original correspondence are available on request.

25 January 2017

Ref: BBR_2016_258_R1

Title: Long-lasting effects of fluoxetine and/or exercise augmentation on bio-behavioural markers of depression in pre-pubertal stress sensitive rats

Journal: Behavioural Brain Research

Dear Professor. Brink,

I have now received a re-review of the above referenced manuscript, and as you can see, the reviewer was favorably impressed with your revised manuscript. The revised version adequately addresses the issues raised by the reviewer, and therefore, I am pleased to inform you that it is now acceptable for publication in Behavioural Brain Research. The paper will be forwarded directly to the publisher, who will be in contact with you regarding the publication schedule in due course.

Thank you for submitting your work to Behavioural Brain Research. We hope you consider us again for future submissions.

Kind regards,

Stephen Maren

Editor-in-Chief

Behavioural Brain Research

Comments from the editors and reviewers:

All of the comments have been incorporated into the amended text.

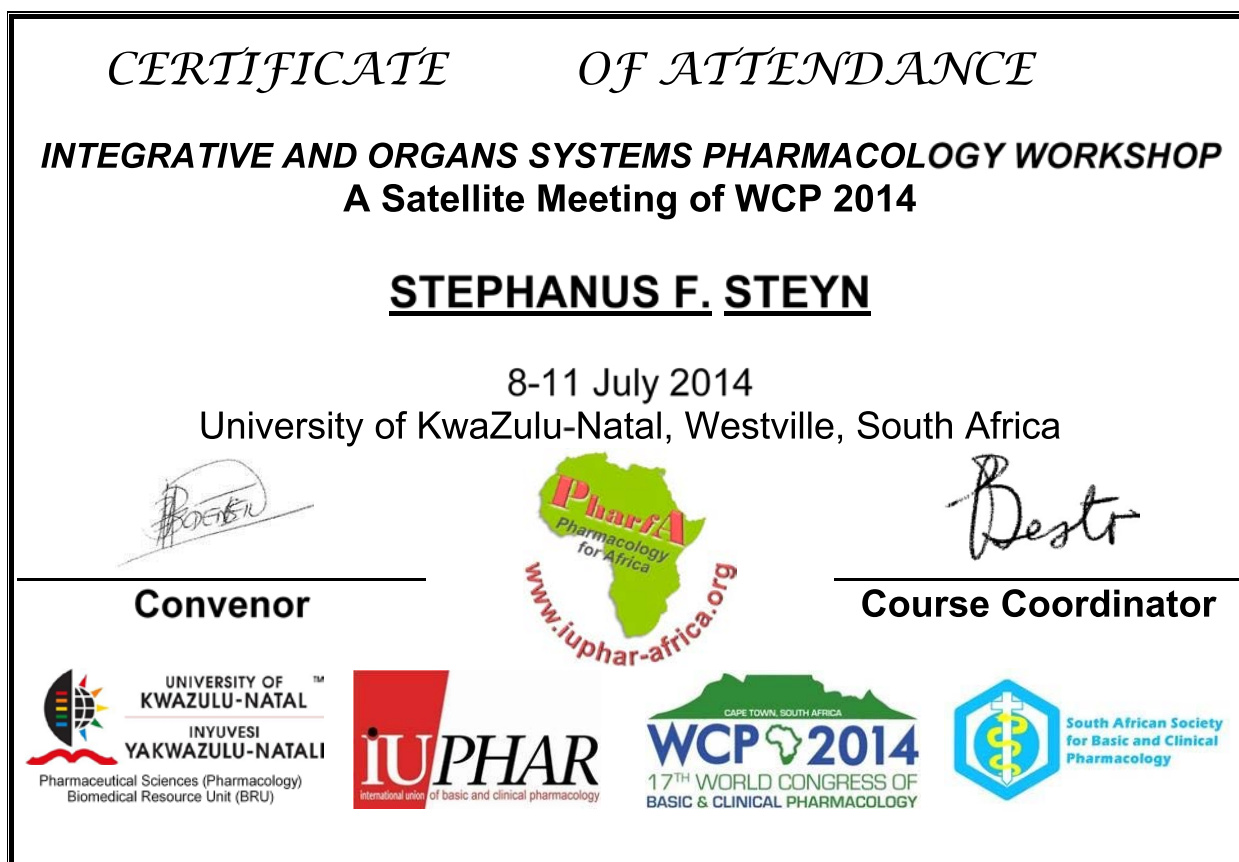
APPENDIX E: CONGRESS PROCEEDINGS

Presented below are the available attendance certificates of all the congresses/workshops attended during the course of the project as well as the abstracts of the posters/presentations delivered at the relevant congress.

The congresses/workshops attended include:

- 1) Integrative and Organs Systems Pharmacology Workshop (2014) (*certificate of attendance*)
- 2) 17th World Congress of Basic and Clinical Pharmacology (2014) (*certificate of attendance*)
- 3) SASBCP & TOXSA's Pharmacology and Toxicology Congress (2015) (*abstract of oral presentation*)
- 4) All Africa Congress on Pharmacology and Pharmacy (2016) (*abstract of oral presentation and certificate of attendance*)
- 5) The South African Annual Pharmacology Conference (2017) (*abstract of poster presentation*)

E.1 Integrative and Organs Systems Pharmacology Workshop (2014)



E.2 17th World Congress of Basic and Clinical Pharmacology (2014)



22 July 2014

CERTIFICATE OF ATTENDANCE

This is to certify that

Mr Stephan Steyn

Attended the 17th World Congress of Basic and Clinical Pharmacology
(WCP2014) held at the Cape Town International Convention Centre (CTICC) in Cape
Town, South Africa from 13-18 July 2014.

Prof Douglas W Oliver
President: WCP2014
Tel: +27 (0)11 463 5085
carina@soafrica.com
www.wcp2014.org

Page 1



South African Society
for Basic and Clinical
Pharmacology



E.3 SASBCP & TOXSA's Pharmacology and Toxicology Congress (2015)

The following abstract was presented as symposium speaker at the SASBCP & TOXSA's Pharmacology and Toxicology Congress, held at The Wits Club, Wits University, South Africa between 31 August and 2 September 2015.

AGE-RELATED, INTENSITY-SPECIFIC EXERCISE AS AN AUGMENTATION STRATEGY IN THE TREATMENT OF DEPRESSION

SF Steyn¹, JC Schoeman¹, BH Harvey², CB Brink¹

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²) Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North- West University, Potchefstroom, North West, South Africa

The management of major depressive disorder (MDD) is known to be challenging, and in particular drug treatment is associated with delayed onset of action, treatment resistance and several side effects that are often not well tolerated and result in non-compliance. Children and adolescents also suffer from MDD and of particular concern is the increased prescription rate of antidepressants in juvenile patients. This age group is associated with rapid growth, including neuronal development and maturation, leaving them vulnerable to long-term effects of antidepressant usage. Alternative or augmentation treatment strategies have hence been proposed, including life-style adjustments which include exercise and diet. The current presentation will review the potential role of chronic intensity-specific exercise as augmentation strategy in the treatment of depression in both the clinical and pre-clinical arenas, as well as briefly explain a pilot study investigating an age-related, intensity-specific exercise regimen in a translational animal model of depression. Clinical data have shown chronic low or moderate intensity exercise to decrease depression-rating scores and serve as a coping mechanism for stress. In addition, neurochemical results of animal studies have found that chronic low and moderate intensity exercise, increase neuroplasticity and cell survival, decrease oxidative stress, normalise stress-induced corticosterone concentrations and alter monoamine neurotransmission. However, these beneficial effects are not reported for chronic high intensity exercise, suggesting beneficial effects of exercise to be intensity-specific. Current data suggest an important role for life-style in the management of mood disorders and may prove to be a possible augmentation strategy to pharmacological treatment of depressed patients.

E.4 All Africa Congress on Pharmacology and Pharmacy (2016)

The following abstract was presented as an oral presentation at the All Africa Congress on Pharmacology and Pharmacy's Young Scientist Competition, held at Misty Hills Hotel and Conference Centre, Muldersdrift, South Africa between 5 and 7 October 2016.

**LASTING EFFECTS OF CHRONIC EARLY-LIFE EXPOSURE TO
ANTIDEPRESSANTS AND NON-PHARMACOLOGICAL
INTERVENTIONS ON DEPRESSIVE-LIKE BEHAVIOUR IN YOUNG
ADULT STRESS-SENSITIVE RATS**

Stephanus F Steyn¹, Brian H Harvey², Christiaan B Brink¹

¹⁾ Division of Pharmacology, School of Pharmacy, Faculty of Health Sciences, North-West University,
Potchefstroom, North West, South Africa

²⁾ Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North- West University,
Potchefstroom, North West, South Africa
E-mail-address: Tiaan.Brink@nwu.ac.za

Purpose: Major depression is a serious disorder, associated with suffering, severely impaired quality of life and even enhanced suicide rates. Juvenile depression is a major concern, with suicide being the second leading cause of adolescent mortalities worldwide. Pharmacotherapy for this age group is very limited, with only fluoxetine and escitalopram as approved treatment options. Due to side-effects and concerns about their effects on neurodevelopment and late-life outcome, complementary medicines and interventions have been advocated, notably omega-3 fatty acid supplementation and exercise. Both these non-pharmacological interventions have been shown to positively affect pathological parameters associated with depression, suggesting a role for their use as augmentation strategies to drug therapy in juvenile depression. The current study investigated the potential augmentative, long-lasting effects of pre-pubertal exercise and/or omega-3 fatty acids on juvenile antidepressant treatment, as observed in early adulthood in stress-sensitive rats.

Methods: Male Flinders Sensitive Line (FSL) rats (± 12 per group) received either indicated treatments from postnatal day 21 (PND21) to PND34 (pre-puberty), including either escitalopram (10 mg/kg/day sc), venlafaxine (10 mg/kg/day sc) or vehicle control with or without simultaneous exposure to normal or omega-3 (OM3) supplemented rat chow, or no or low intensity exercise (EXE) (ethics approval no. NWU-00148-14-A5). Thereafter rats were housed normally until PND60 and then subjected to the open field and the forced swim tests to assess lasting effects on locomotor activity and immobility (i.e. depressive-like behaviour).

Results: Both EXE and OM3 alone, and in combination with either antidepressant, reduced locomotor activity, compared to either saline control or antidepressant-treated groups on PND60. Venlafaxine plus

OM3 decreased immobility, compared to saline control and drug-treated groups. EXE alone, and in combination with either antidepressant, also decreased immobility, compared to saline control and drug-treated groups. Data on the effect of the triple combination of drug, OM3 and EXE are currently underway.

Conclusions: Chronic pre-pubertal administration of antidepressants does not appear to have any beneficial lasting effects on depressive-like behaviour during early adulthood. However, exercise alone as well as the combination with either escitalopram or venlafaxine and omega-3 in combination with either antidepressant exerts a positive effect on depressive-like behaviour later in life. It remains to be seen from current studies whether triple combination of drug, omega-3 and exercise may augment the observed effects.

E.5 The South African Annual Pharmacology Conference (2017)

The following abstract was presented as a poster presentation at the Annual South Africa Pharmacology Conference's Young Scientist Competition, held at The University of the Free State, Bloemfontein, South Africa between 1 and 4 October 2017.

IMMEDIATE AND LASTING EFFECTS OF EARLY-LIFE ESCITALOPRAM, EXERCISE AND OMEGA-3 SUPPLEMENTATION ON DEPRESSIVE- AND ANXIETY-LIKE BEHAVIOUR IN FSL RATS

Stephanus F. Steyn, Brian H. Harvey, Christiaan B. Brink

Division of Pharmacology, Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences,
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Correspondence: Stephan.steyn@nwu.ac.za

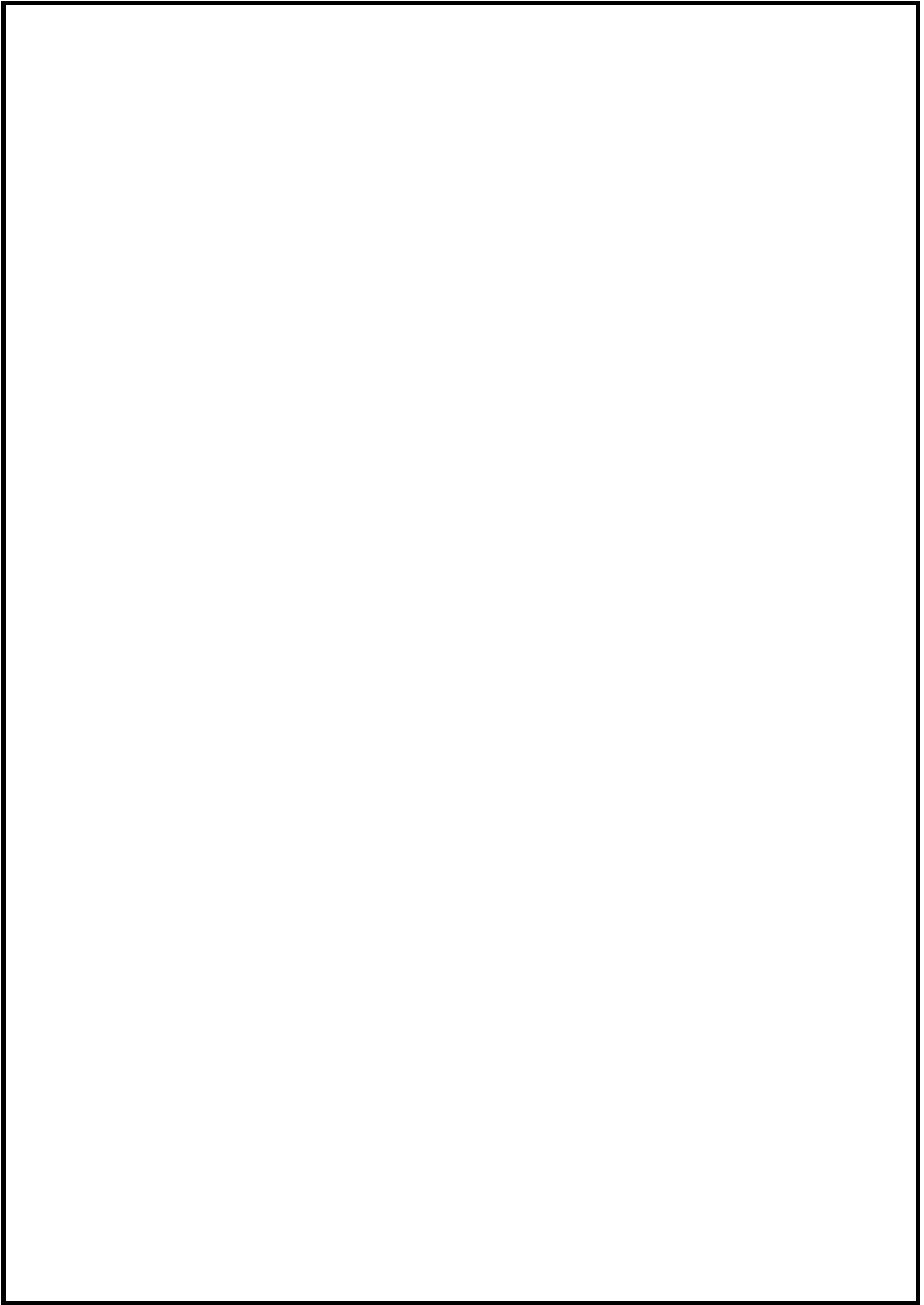
Introduction and Aim: Juvenile depression is a global concern with suicide being the second leading cause of adolescent mortalities worldwide. Pharmacotherapy for this age group is limited to only two serotonergic antidepressants, including escitalopram, partly due to the early maturation of this neurotransmitter system. Yet, juvenile antidepressant therapeutic outcome remains comparable to that of adult patients. Complementary treatment strategies, such as omega-3 fatty acid supplementation and exercise are gaining popularity as adjunctive treatment options, due to reported and postulated physiological, neurological and behavioural effects and perceived lower risk. These interventions hold to potential to augment pharmacotherapy and improve treatment outcome. The current study investigated the latter potential, and in particular whether early-life treatment had any lasting effects on the depressive- and anxiety-like behaviour in a stress-sensitive animal model of depression.

Methods: Male Flinders Sensitive Line (FSL) rats (± 12 per group) received saline control (SAL) or escitalopram (ESC) (10 mg/kg/day sc) with various combinations of simultaneous normal (CRL) or omega-3 (OM3) supplemented rat chow, and/or no (SED) or low intensity exercise (EXE) (ethics approval # **NWU-00148-14-A5** and **NWU-00373-16-A5**) from postnatal day 21 (PND21) to PND34 (pre-puberty). Thereafter rats were either subjected to behavioural testing on PND35 or normally housed until PND60 and then subjected to the open field (OFT) and the forced swim tests (FST), to assess immediate and lasting effects, respectively, on locomotor activity, anxiety- and depressive-like behaviour. The project was funded by the Centre of Excellence for Pharmaceutical Research of the NWU, the South African Medical Research Council and the National Research Foundation.

Results: Vivarium rat chow was successfully coated with OM3, containing double the amount of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) compared to uncoated chow. None of the

pre-pubertal treatment strategies had any immediate, early-life effects on body weight, locomotor activity or centre zone/corner time ratio. However, a large effect size difference in FST immobility time was observed between ESC/CRL/SED and SAL/CRL/SED on PND35. On PND60, there were no significant three-way interaction effects on behaviour, yet distance moved in the OFT was significantly decreased by diet and exercise (irrespective of drug). A significant diet*exercise interaction also highlighted decreased centre zone/corner time ratios in CRL/EXE and OM3/SED groups, compared to CRL/SED (irrespective of drug). Finally, no significant long-term effects on depressive-like behaviour were identified on PND60.

Conclusion: Only pre-pubertal ESC mono-therapy induced a strong trend for an immediate, yet transient decrease in early-life depressive-like behaviour. Neither pre-pubertal OM3 supplementation nor EXE (irrespective of drug) induced an immediate effect on anxiety-like behaviour, yet both strategies increased anxiety-like behaviour later in life. Literature suggests OM3 supplementation to improve stress-induced coping responses, evinced by shorter environmental habituation times. This may explain the observed reduced ambulatory activity and consequent false-positive interpretation of enhanced anxiety-like behaviour, as well as possible masking of altered immobility in the FST. Therefore, EXE may induce similar adaptive responses, yet these observations will be confirmed with other behavioural models and neurochemical analyses in prospective studies. In conclusion, early-life complimentary interventions may induce long-term effects in stress-sensitive individuals.



CERTIFICATE OF CPD ATTENDANCE

I, the undersigned, acting as representative of the aforementioned CPD Provider,
hereby certify that

Mr Stephan Steyn

P29959

Attended

**The South African Annual Pharmacology Conference of the
South African Society for Basic and Clinical Pharmacology
(SAPHARM-2017)**

Date: 01 – 04 October 2017

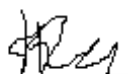
Venue: The Faculty of Health Sciences, University of the Free State, Bloemfontein

The Medical and Dental Professional Board's approved CPD reference number is as
follows:

MDB004/011/06/2017

I certify that the said practitioner qualifies for general CEU's obtained as follows:

Level 1	16



Signature on behalf of provider:
Prof A Walubo

Date: 13 October 2017

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CERTIFICATE OF CPD ATTENDANCE

I, the undersigned, acting as representative of the aforementioned CPD Provider,
hereby certify that

Mr Stephan Steyn

P29959

Attended

**The South African Annual Pharmacology Conference of the
South African Society for Basic and Clinical Pharmacology
(SAPHARM-2017)**

Date: 01 – 04 October 2017

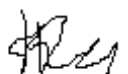
Venue: The Faculty of Health Sciences, University of the Free State, Bloemfontein

The Medical and Dental Professional Board's approved CPD reference number is as
follows:

MDB004/012/06/2017

I certify that the said practitioner qualifies for general CEU's obtained as follows:

Level 2	10



Signature on behalf of provider:
Prof A Walubo

Date: 13 October 2017

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APPENDIX F: ETHICAL APPROVAL

Presented below are the ethical approval certificates as issued by the NWU AnimCare animal research ethics committee.



NORTH-WEST UNIVERSITY
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Institutional Research Ethics Regulatory Committee
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ETHICS APPROVAL CERTIFICATE OF PROJECT AMMENDMENTS

Based on approval by AnimCare Animal Research Ethics Committee (AREC-130913-015), the North-West University Institutional Research Ethics Regulatory Committee (NWU-IRERC) hereby approves the amendments to your project as indicated below. This implies that the NWU-IRERC grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

Project title: *The effects of chronic exposure of pre-adolescent stress-sensitive rats to antidepressants and non-pharmacological interventions on stress-related behaviour and biomarkers in early adulthood.*

Project Leader: Prof CB Brink

Ethics number: N W U - 0 0 1 4 8 - 1 4 - A 5

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Approval date: 2015-09-14 **Expiry date:** 2016-09-14 **Category:** N/A

Special conditions of the approval (if any): None

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- The project leader (principle investigator) must report in the prescribed format to the NWU-IRERC:
 - annually (or as otherwise requested) on the progress of the project,
 - without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.
- The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-IRERC. Would there be deviation from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.
- The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-IRERC and new approval received before or on the expiry date.
- In the interest of ethical responsibility the NWU-IRERC retains the right to:
 - request access to any information or data at any time during the course or after completion of the project;
 - withdraw or postpone approval if:
 - any unethical principles or practices of the project are revealed or suspected,
 - it becomes apparent that any relevant information was withheld from the NWU-IRERC or that information has been false or misrepresented,
 - the required annual report and reporting of adverse events was not done timely and accurately,
 - new institutional rules, national legislation or international conventions deem it necessary.

The IRERC would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the IRERC for any further enquiries or requests for assistance.

Yours sincerely
Linda du Plessis
Digitally signed by Linda du Plessis
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ETHICS APPROVAL CERTIFICATE OF STUDY

Based on approval by AnimCare Animal Research Ethics Committee (AREC-130913-015) on 29/11/2016 after being reviewed at the meeting held on 23/11/2016, the North-West University Institutional Research Ethics Regulatory Committee (NWU-IRERC) hereby approves your study as indicated below. This implies that the NWU-IRERC grants its permission that provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: The behavioural and neurobiological effects of antidepressants and nonpharmacological interventions in pre-pubertal FSL and FRL rats.

Study Leader/Supervisor: Prof Tiaan Brink
Student: Mr Stefan Steyn

Ethics number: NWU - 00373 - 16 - A5
Institution Study Number Year Status
Status: S = Submission, R = Re-Submission, P = Provisional Authorisation, A = Authorisation

Application Type: New Application - Standard Project
Commencement date: 2016-11-29
Category: 4

Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation up to a maximum period of three years.

Special conditions of the approval (if applicable):

- Any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the AnimCare. Ethics approval is required BEFORE approval can be obtained from these authorities.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- The study leader (principle investigator) must report in the prescribed format to the NWU-IRERC via AnimCare:
 - annually (or as otherwise requested) on the monitoring of the study, and upon completion of the study
 - without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.
- Annually a number of studies may be randomly selected for an external audit.
- The approval applies strictly to the proposal as stipulated in the application form. Would any changes to the proposal be deemed necessary during the course of the study, the study leader must apply for approval of these amendments at the AnimCare, prior to implementation. Would there be deviation from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.
- The date of approval indicates the first date that the study may be started.
- In the interest of ethical responsibility the NWU-IRERC and AnimCare retains the right to:
 - request access to any information or data at any time during the course or after completion of the study;
 - to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.
 - withdraw or postpone approval if:
 - any unethical principles or practices of the study are revealed or suspected,
 - it becomes apparent that any relevant information was withheld from the AnimCare or that information has been false or misrepresented,
 - the required amendments, annual (or otherwise stipulated) report and reporting of adverse events or incidents was not done in a timely manner and accurately,
 - new institutional rules, national legislation or international conventions deem it necessary.
- AnimCare can be contacted for further information or any report templates via Ethics-AnimCare@nwu.ac.za or 018 299 2107.

The IRERC would like to remain at your service as scientist and researcher, and wishes you well with your study. Please do not hesitate to contact the IRERC or AnimCare for any further enquiries or requests for assistance.

Yours sincerely

Linda du Plessis

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Prof Linda du Plessis
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