An investigation into the potential neurobiological constructs and long-term biobehavioural effects of perinatal combined antiretroviral therapy (cART)-exposure in the deer mouse

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Dissertation submitted in fulfilment of the requirements for the degree *Magister of Scientiae in Pharmacology*

at the North-West University

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Examination: November 2021

Student number: 27358860

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in

Pharmacology

at the

North-West University (Potchefstroom Campus)

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POTCHEFSTROOM, SOUTH AFRICA

2021

* * *

In honor of my Oumi Sunshine, Estelle Stroebel.

Without your passion for life, unique perspective of the world and unfailing love, I would not be where I am today. You cultivated a space for free thought and the pursuit of passions in my lifetime that has instilled both a desire for greatness and an appreciation for simplicity. Thank you for every life lesson, daring adventure and unabashed dedication to your family. Thank you for your courage, for bravely loving and living and teaching us all how.

* * *

* * *

But he said to me, “My grace is sufficient for you, for my power is made perfect in weakness.” Therefore, I will boast all the more gladly of my weaknesses, so that the power of Christ may rest upon me. For the sake of Christ, then, I am content with weakness, insults, hardships, persecutions and calamities. For when I am weak, then I am strong.

2 Corinthians 12:9-10

* * *

Not only that, but we rejoice in our sufferings, knowing that suffering produces endurance, and endurance produces character, and character produces hope, and hope does not put us to shame, because God’s love has been poured into our hearts through the Holy Spirit who has been given to us.

Romans 5:3-5

* * *

Acknowledgments

This work is by no means a triumph I claim as my own. Rather this is a collection of lessons and failures and mistakes and golden nuggets of shared wisdom. I feel so humbled to be able to present to you all the polished version of my journey as I know you were all privy to the raw and rough beginning. Although this dissertation is limited to show only the evidence of my research findings, I wish I could present a separate version for all the incredible things I have learnt along the way. I know however that the evidence of battles won, and fears overcome are only evident once we face new challenges and meet new obstacles with new perspectives as this allows us to finally present our findings in the form of the kindness shown in our choices, renewed passion in our actions, and hope for our perspectives.

MY LORD AND SAVIOR: Thank you for your grace that carries me, your love that sustains me and your forgiveness that covers me. I owe all of this and more to you. I stand in awe at how you have orchestrated opportunities and been patient in your teachings. The depth of your love knows no end, for I, myself have pushed and failed and tested its limits, only to find that there are none. I pray that I keep your praise on my lips, my feet on your path and my eyes on you always.

For from him and through him and to him are all things. To him be glory forever. Amen. – *Romans11:36*

MY FAMILY: Firstly, thank you to my parents, JOSEPH and KIM STROEBEL, words cannot express how infinitely grateful I am to you both. Thank you for supporting me in everything. You both provide the roots I need to stay grounded and the freedom to spread my wings. Thank you my Dad, for your resolute strength and compassion, thank you for molding me and believing in me and for your wisdom in knowing just what to say and when to say it. You always make me feel a little taller and help me stand a little stronger and somehow you always seem to offer a new perspective because I know it is because you let me stand upon on your shoulders. My Mom, thank you for your incredible love, thank you for its all-encompassing width. I am forever grateful for the example you have set and the sacrifices you have made for me to be where I am and who I am today. Thank you for equipping me with a full toolbox to tackle life head on and for comforting me in the times I forget how to use them. Mom and Dad thank you for always having my back through these wonderfully full 30 years, I am honored to be your daughter.

Secondly, NATALIE, HELGAARD, HAYLIE and LLEWELLYN SMITH: My Nats, Thank you for your unselfish love and care. Thank you for always understanding me and bringing so much joy into my life. You are such an inspiration to me as a person, I have always looked up to you and I know I am a far better person for being your little sister. Thank you for always paving the way and instilling a confidence in

me that that gets less shaky with every year. HAYLIE your bright eyes and shining smile are but a glimpse of what is shining inside you. You are so incredibly kind and strong and already love so deeply, you truly brighten up the lives around you. LLEWELLYN your generosity and sweet nature mirrors your silent strength and the peace you carry. Your infectious smile and little giggles always bring so much joy. I look forward to seeing all that God has instore for the two of you. HELGAARD, thank you for seeing through all the chaos and talking straight in a world that loves confusion. Thank you for pushing me to be better and lovingly supporting me in the process. My Granny, LYNN DONKIN thank you for your tenderness and care even through all your suffering. Your quiet strength is inspirational. Thank you for your love and kindness and for always believing in me. My late Grandfather, Pop, KEN DONKIN, oh, how I would have loved for you to edit my dissertation and sit and debate the use of longwinded words. Thank you for your love and for giving me lifetime of memories. My Uncle, CHARLES DONKIN. Thank you for being the best uncle ever, thank you for being excited with and for me. Your unabashed authenticity is something that I admire and respect, you are a trailblazer, and you inspire me so. Your support from far is always so appreciated. I love you all so very much – SHMILY.

PROF DE WET WOLMARANS: In both versions of my dissertation, seen and unseen I have the privilege of acknowledging the incredible part you have played. You have impacted me as a person in so many ways. I am so grateful for the time you took to teach, mentor, and mold me on this journey. Thank you for pushing and pruning and stepping back to watch me grow. In all those moments I have felt your support and carefully looked for the lessons you had waiting at each step. Thank you for being intentional in your effort, meaningful in your care, and authentic in your ways. Your passion for life is contagious and your wisdom shines through your teaching skills and approach to life, all while remaining humble and true. Meeting your enthusiasm is and will always be a challenge I gladly accept. Thank you for believing in me, it is both and honor and privilege to be a part of your team. I look forward to working with you as we attempt to unravel the mysteries of creation.

DR HEATHER JASPAN: I would like to express my sincere gratitude for your guidance and insight offered throughout this project. It has been an incredible learning experience and I look forward to learning even more from you in the near future. Thank you for affording me the opportunity of visiting Cape Town and for giving me the opportunity to learn and grow as part of the team.

DR ANNA HAPPEL: Thank you so much for the amazing two years spent on this project. I am so grateful I had the opportunity to work with and learn from you in the lab. Your patience and mentoring meant the world. You opened my eyes and helped harness my skills. I eagerly look forward to working on future projects with you.

DR GEOFFREY DE BROUWER: Geoffrey, I really want to thank you for the exceptional person you are. And I mean that quite literally as I have never met anyone as unassumingly clever as you. The way you come alongside us and patiently teach and explain without any judgement is commendable. In amongst the calamities and pressure we felt during our MSc's you have led us on and set the pace and encouraged from behind, supported from the side and managed to train us in-between. Thank you for always being there, for sticking up for us, for your invaluable advice and kind listening ear. Your life-lessons and advice on how to survive an MSc were something I really relied upon, so thank you. I am so grateful to have been able to work alongside you and to grow and learn from you too. Thank you for being an exception and for silently showing us how.

PERSONNEL OF THE PHARMACOLOGY DEPARTMENT: I am so grateful for the opportunity to learn and work alongside such highly esteemed researchers. PROF TIAAN BRINK and PROF BRIAN HARVEY, thank you for all the insight and interest shown throughout my study. PROF LINDA BRAND, thank you for always checking in to make sure we are doing well. I really appreciate all the kindness you have shown me and everything that I have learnt from you throughout the years. DR STEPHAN STEYN, I really appreciate the initiative you took with Journal Club this year and I really feel that I benefitted both as a researcher and as a person. Thank you for being so open to questions and for always encouraging us to be the best version of ourselves. DR MADELEINE UYS, thank you so for your help in my time of need. I am beyond grateful for your ability to create calm in chaos and for solving what felt like an unsolvable equation. DR FRANCOIS VILJOEN, I would like to thank you for your help with the GSH and GSSG analysis on the LCMS. Thank you for always taking the time to teach and mentor and ensure I was prepared for every step. Your knowledge in this field was invaluable and I appreciate the time you took in sharing this with me. CYNTHIA DABHELIA: Thank you always being eager to help and assist, I appreciate it so.

NORTH-WEST UNIVERSITY: Thank you for the incredible opportunity to complete my studies on this wonderful campus. I have enjoyed every moment and feel privileged to be afforded this opportunity.

SHARLENE LOWE: Thank you Sharlene, for the enthusiasm and perseverance you showed throughout the work we did together. I am grateful to have learnt from you and thank you for your support in those tough times.

DR HELEEN JANSE VAN RENSBURG: Thank you for your keen eye and insightful explanations. I really appreciate your willingness to help and kindness in doing so.

VIVARIUM PERSONNEL: To Dr Nico Minnaar, Mr. Cor Bester, and Mr. Kobus Venter and Mrs. Antoinette Fick. Thank you for your training and assistance throughout the course of this investigation, I learnt so much from you all and am so grateful for the opportunity to work with you.

MY DEAREST FRIENDS: SUNNY SEO: Sunny, I just want to say a massive thank you for your incredible support throughout our friendship. You were one of the precious few that got to see behind the scenes. We got to witness the battles fought, the lessons received and then rejoice in the victories. Thank you for fighting and celebrating alongside me. I am so grateful God brought us together. Our uniquely parallel paths have been both a comfort and inspiration to me. Thank you for being you and being true. You are true to your name and bring so much joy to the lives around you. Thank you for our incredible friendship that has grown and sustained us through all of this. 사랑과 우정에 감사드립니다. CLAUDIA VAN WYK: Cloudy, I want to express my gratitude for being both a comfort and support throughout this journey. We got to tackle so many things together and could share in both your and my journey at every step. Thank you for your continued honesty and authenticity in a world full of people pretending. Your confidence and ability to live so fiercely is contagious, and I am grateful for all the moments we could live fiercely together. CHRISNA MATTHEE: Kriek, it was incredible to be able to share and witness as we both grew and learnt and reached our goals. Thank you for always cheering me on and lending unique perspectives in how to tackle things we struggled with. Thank you for being part of this journey from the very beginning. LIZE KOK: I was so blessed to have met you. Thank you for your inspiring friendship. Thank you for your kind words of support and gentle nudging to understanding. Your words of comfort and advice have carried me through tough times. Thank you for that and for so much more. MONIQUE DELPORT: Thank you for being there before any of this was a reality, when I was choosing pharmacy. Thank you for your continued friendship and support throughout this journey. Thank you for your optimism and enthusiasm and for our incredible Kruger Park holiday in between all of this. I am so grateful to my amazing friends for the impact you all have had in my life.

***MY FELLOW SENIOR MASTER STUDENTS: It was truly an honor and privilege to rub shoulders with you all, both literally and figuratively. This journey would not have been the same without any of you. Thank you that we could learn from and with each other. VASTI THERON: Vasti, thank you for bringing life to the office with your vibrant personality and kind heart. Thank you for your unfiltered honesty and incredible loyalty, two qualities that I have grown to not only respect but love. It has been so amazing getting to know you. Thank you for sharing in the ups and downs that have been thrown our way and for even screaming with me in the hallway. ALEXIO DOS SANTOS: Alex, thank you for being there through both the fun times and the tough times. Thank you for your generous and brave heart and for always being so willing to help. I am so proud of you; despite doing such a different study that was met with its own challenges, you stayed true and strong and hopeful. Thanks for all the laughs and memories and most importantly the coffee runs to Toro. MARET DE RIDDER: Maret, thank you for all the late nights on the vivarium floor and the heart pouring-out sessions in-between. Your bravery and tireless efforts are something I admire so in you. It was so amazing to work with you both in the vivarium and in the office as we chatted and learnt from each other. STOFFEL VAN DER

WESTHUIZEN: Thank you for your warm and open heart, your infectious optimism and for all the honest moments shared. I really enjoyed every joke we shared and songs we hummed. Thanks for offering your silent strength and comfort. LARISSA KARSTEN, thank you that we could learn and work together in both the lab and the vivarium. I was so grateful to have shared in so many unique experiences and lessons with you. Thank you for all your support and friendship throughout the past two years; it has been so special. MARIE-LOUISE MULLER Thank you for being your sweet and true self. I thoroughly enjoyed our conversations and teamwork. Thank you for your kindness and fantastic sense of humor throughout the two years. RASEMOKO POLILE: Thank you for your friendliness and optimism. I really admired your constant hard work throughout the years.

TO THE DEER MICE TEAM: Thank you for your unbelievable hard work with the deer mice, we were a mean team - ANÉ LOMABAARD Thank you for your kindness and awesome conversations. You have an incredible heart and even more amazing mind. BIANCA HURTER, my mentee and resident beamer, you are so awesome, and it was such a pleasure to work with you. Thank you for the awesome teamwork and all the fun in the office and the vivarium DEVAN SAAIMAN: Thank you for your kindness and friendliness for being so genuine and for checking up on me during the entire ride of the Anaconda. JOHANN BURKE: Jo, Jo and nothing but the Jo, thank you for our meaningful conversations and awesome ministry moments. Thank you for speaking into my life.

TO MY FELLOW JUNIOR MASTER STUDENTS: DANIEL VAN RENSBURG: My fellow Klofie, and favorite FNPG-groupie, thank you for the incredible discussions and conversations we have had. You have an incredible way of thinking and I've enjoyed our intellectual sparing. Thank you for dragging me along to Biochem and letting me share in your excitement. Your PCR advice was indispensable. ESTMIA VAN DER WALT, thank you for being sweet and kind in all you do. ASHLEIGH WHITNEY, the one that **doesn't** annoy me. You're a lovely and generous person. HENLO OOSTHUIZEN, thank you for always checking in and showing genuine interest in my study. MARELI ROETS, thank you for your friendliness and enthusiasm.

TO MY FELLOW DOCTORAL STUDENTS: JOHANE GERICKE: Hello Felicia, thank you so much for all your awesome tips and tricks before and now in my M for which I am so grateful for. Thanks for giving me the official introductory tour and for your warm welcome into research. CAILIN VAN STADEN: Thank you for all your advice before and during my M, I really appreciate it. JUANDRÉ SAAYMAN: thank you for your advice about balance in a time I really needed to hear it, and CRYSTAL LUBBE, thanks for the quick catch up and for always being eager to help.

"I know that I am a part of something so tremendous, so great that, if for this reason only, it is holy." – *Lawrence LeShan*

Congress proceedings

- STROEBEL, J., HAPPEL, A.U., JASPAN, H.B., and WOLMARANS, D. (2021). Oxidative stress and the long-term behavioral effect of perinatal combined antiretroviral treatment (cART)-exposure in a pre-clinical model of compulsivity. Presented at the 54th Annual South African Society for Basic and Clinical Pharmacology (SASBCP) Conference (online), 22nd October 2021.

Abstract

South Africa has one of the largest combination antiretroviral treatment (cART[1]) programs in the world with an estimated 7.52 million individuals that were living with human immunodeficiency virus (HIV[2]) by the end of 2018. One meaningful development of this program, i.e. a treat-all approach, ensures that even pregnant women, regardless of CD4[3] count, have access to cART. This led to a reduction in the mother-to-child—or vertical—HIV transmission rate to below 2 %. Despite the fact that cART successfully reduces the HIV viral load, it is known to have HIV-unrelated adverse physiological effects, i.e. oxidative stress and proinflammatory responses. In turn, oxidative stress and central nervous system (CNS[4]) pro-inflammatory responses, either alone or presenting concurrently, have been associated with the manifestation, altered prognosis and poor treatment response of psychiatric conditions, e.g. major depressive disorder (MDD[5]) and bipolar disorder (BD[6]).

Against this background, infants born HIV-uninfected but exposed to both HIV and cART in-utero (iHEU)[7] and perinatally, i.e. the weeks immediately before and after birth, may be especially at risk. Observational studies indicate that some of these children progress to present with increased morbidities, including neuropsychological difficulties. Indeed, considering the neurodevelopmental window, during which neurulation, cell proliferation, axonal outgrowth and synaptogenesis occur, it is likely that infants of mothers that were treated with cART throughout pregnancy and the breast-feeding period, may be at risk for developing biobehavioral complications during the later stages of life. However, the extent to which perinatal cART exposure may result in long-term biobehavioral aberrations remains to be established, since the long-term study of children born from HIV-positive mothers, remains problematic.

Therefore, the broad aim of this work was to investigate potential cART (i.e. a combination of tenofovir and emtricitabine)-induced biobehavioral alterations, as it pertains to oxidative stress (as measured by reduced to oxidized glutathione ratio; GSH/GSSG[8]), immune signaling (as reflected by both pro-inflammatory and anti-inflammatory cytokines), and behavioral expression over the different developmental phases of life in an uninfected and validated naturalistic animal model of a psychiatric phenotype, i.e. compulsive-like behavior as expressed by deer mice *(Peromyscus maniculatus bairdii)*. This model system was chosen for this purpose, since a subpopulation, i.e. 30 – 35 %, of deer mice,

[1] combination antiretroviral treatment
[2] human immunodeficiency virus
[3] cluster of differentiation antigen 4
[4] central nervous system
[5] major depressive disorder
[6] bipolar disorder
[7] HIV-uninfected, but HIV/cART exposed infants
[8] reduced to oxidized glutathione ratio

irrespective of sex, naturalistically express persistent and repetitive large nest building (LNB[1]) behavior. Thus, by comparing the biobehavioral profile of normal nesting (NNB[2]) and LNB expressing animals that were perinatally exposed to either normal water or cART[3], the immediate and long-term effects of said exposure in a standardized normal and compulsive-like cohort, can be explored. The GSH/GSSG[4] ratio was chosen as a marker of oxidative stress, since perturbations in this system have been shown in clinical obsessive-compulsive disorder (OCD[5]) and in deer mice presenting with another compulsive-like phenotype, i.e. high motor stereotypy, before. It was thus of value to expand on these findings in the present investigation. In terms of inflammatory profile, we highlighted interleukin (IL[6])-1β, IL-6, IL-10, IL-17, tumor necrosis factor-alpha (TNF-α[7]), and interferon-gamma (IFN-γ[8]) for investigation, as these have variably been implicated both in clinical OCD as well as in preclinical models of the disorder. Briefly, we hypothesized that perinatal cART exposure will induce oxidative stress and pro-inflammatory signaling in female and pregnant deer mice, as well as in their respective 3- and 14-week-old offspring. Given the reported associations between such aberrations and neuropsychiatric symptomology as alluded to above, we further expected that perinatal cART exposure would associate with an inflated and exacerbated degree of LNB expression later in life as compared to the behavior of the control-exposed cohort.

* * *

Three groups of 20 deer mouse dams were exposed to either normal water (control) or cART (tenofovir 60 mg/kg/day and emtricitabine 40 mg/kg/day, *n* = 10 per exposure; ethical approval nr.: **NWU[9]-00524-20-A5**) as follows: Group 1, all non-pregnant, was exposed to either intervention for 21 days and euthanized prior to pairing. Groups 2 and 3, all pregnant, were exposed from 21 days prior to mating until the end of nursing. Pups born from Group 2 were euthanized at 21 days (3-week-old group), while Group 3 pups were raised until adulthood when compulsivity testing, i.e. nesting assessment, was performed (14-week-old group). After collecting the whole-brain tissues of all animals, samples were analyzed for the GSH/GSSG ratio and cytokine profile (IL-1β, IL-6, IL-10, IL-17, IFN-γ, and TNF-α).

[1] large nest building
[2] normal nest building
[3] combination antiretroviral treatment
[4] reduced to oxidized glutathione
[5] obsessive-compulsive disorder
[6] interleukin
[7] tumor necrosis factor-alpha
[8] interferon-gamma
[9] North-West University

Our main findings were that 1) both pregnancy and cART[1] significantly decreased the whole-brain GSH/GSSG[2] ratios in dams, 2) cART-, compared to control-exposure, varyingly decreased the concentrations of most cytokines measured in non-pregnant and pregnant dams, 3) differences in cytokine expression between control- and cART-exposed offspring only became apparent by the age of 14 weeks, in that cART-exposed offspring presented with significantly lower IL[3]-10, but higher IL-17 and IFN-γ[4] concentrations than their age-matched control-exposed counterparts, 4) that cART-, compared to control-exposed LNB-expressing animals, generated lower total nesting scores, and 5) that the whole-brain concentrations of most cytokines were higher in cART-exposed 14-week-old NNB[5]-, compared to LNB[6]-expressing offspring.

* * *

As it is difficult to distinguish between the effects of maternal cART administration and the effects of HIV itself on the long-term development of affected children in a human cohort, this investigation was designed as a longitudinal exploration of the effects of cART on oxidative stress, immune signaling and behavioral expression in a naturalistic model of compulsive-like behavioral expression. Given the main findings of this work alluded to above, we successfully showed that such interactions between indirect exposure to cART during the perinatal period and central nervous system oxidative and inflammatory processes, do indeed exist and that the deer mouse model is a useful framework in which to study said interactions. Specifically, we showed that perinatal cART exposure exacerbates oxidative stress and suppresses immune signaling in pregnant vs non-pregnant mice. We further showed that cART-exposed NNB- and LNB-expressing offspring differ in terms of their immunological response to said exposure, with the long-term pro-inflammatory effects of cART exposure being masked in LNB-expressing offspring. Collectively, this data highlights a dichotomous influence of cART on the immediate and long-term functioning of the immune system. We also showed that LNB uniquely associates with a perturbed brain-immune crosstalk fingerprint and that future investigations using the deer mouse model of compulsive-like behavior will be vital for an expanded understanding of related concepts.

Keywords

combination antiretroviral treatment, deer mouse, perinatal exposure, oxidative stress, cytokine, inflammation, obsessive-compulsive disorder

[1] combination antiretroviral treatment
[2] reduced to oxidized glutathione ratio
[3] interleukin
[4] interferon-gamma
[5] normal nest building
[6] large nest building

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1 Introduction

1.1 Dissertation approach and layout

This dissertation has been prepared in article format, as specified by the North-West University (NWU[1]), South Africa. The main body will be presented in the form of a journal article, namely Chapter 3, which will report particulars of the experimental work, the results, and the main findings of the present investigation. This chapter will be submitted for publication in an internationally accredited, peer-reviewed journal, i.e., '*Pharmacology, Biochemistry and Behavior*'.

The complete dissertation will comprise four chapters. Chapter 1 offers a concise summary of the investigation, including the problem statement, working hypothesis, study aims and objectives, experimental layout, and the ethical considerations. Chapter 2 provides a detailed literature review which will lay the foundation of the investigation presented in Chapter 3. Last, Chapter 4 contains a broad summary of the complete dissertation as well as future considerations.

One addendum is included. Addendum A contains supplementary results that are not provided in Chapter 3 as well as extra detail concerning the methodology employed during the course of this work.

Chapter 3 was prepared according to the "Instructions to Authors" as specified by *'Pharmacology, Biochemistry and Behavior'*. As such, the remainder of this dissertation is written in US English and referenced in the style of said journal.

* * *

[1] North-West University

1.2 Problem statement

South Africa operates one of the largest antiretroviral treatment (ART[1]) programs in the world, with an estimated 7.52 million individuals living with human immunodeficiency virus (HIV[2]) by the end of 2018 (Vagiri et al., 2018). The country's national ART program initiated a new treat-all approach in 2016, providing ART to all HIV-positive individuals regardless of CD4[3] count to increase both the successful prevention of the condition and harness the therapeutic advantages of early treatment (Maughan-Brown et al., 2018). Women are disproportionately affected by HIV, with factors such as poverty, gender-based violence, and their generally poor socio-economic status contributing to this alarming statistic. By 2017, 26 % of women in South Africa were living with HIV, compared to 15 % of men in the same year (Simbayi et al., 2019). With this statistic in mind, it is important to note that with the implementation of the combination antiretroviral treatment (cART[4]) program, which specifically also includes strategies for the prevention of mother-to-child transmission (PMTCT[5]), South Africa has made significant progress in reducing the mother-to-child—or vertical—transmission of HIV.

Although cART successfully reduces the HIV viral load, it is known to have HIV-unrelated adverse effects, i.e. oxidative stress (M Gallego-Escuredo et al., 2010) and pro-inflammatory responses (Akay et al., 2014; Kaplan et al., 2015; Manda et al., 2011). In turn, oxidative stress and central nervous system (CNS[6]) pro-inflammatory responses, either alone or presenting concurrently, have been associated with the manifestation, altered prognosis and poor treatment response of psychiatric conditions (de Sousa et al., 2014; Lindqvist et al., 2017; Ng et al., 2008). Such potentially harmful effects are not only restricted to pregnant and breastfeeding mothers. Despite the fact that the provision of lifelong cART to HIV positive women, i.e. the so-called Option B+ era, resulted in vertical HIV transmission rates of lower than 2 % (Chi et al., 2013), observational studies have shown uninfected infants that have been exposed to both HIV and cART (iHEU[7]) perinatally, i.e. the weeks immediately before to after birth, progress to present with increased morbidities, including neuropsychological difficulties (Ajaykumar, 2019; Cespedes and Aberg, 2006). However, the extent to which perinatal cART exposure may result in long-term biobehavioral aberrations remains to be established since the long-term study of children born from HIV-positive mothers remains problematic. Considering the long-lasting fundamental processes that transpire during early life neurodevelopmental windows (Nelson, 2002), e.g. neurulation, cell proliferation, axonal outgrowth and synaptogenesis (Lawson et al., 2016; Nelson, 2002), it is

[1] antiretroviral treatment
[2] human immunodeficiency virus
[3] cluster of differentiation antigen 4
[4] combination antiretroviral treatment
[5] prevention of mother-to-child transmission
[6] central nervous system
[7] HIV-uninfected, but HIV/cART exposed infants

possible that infants of mothers that have been treated with cART[1] while pregnant and breast-feeding may be at risk to develop neuropsychiatric complications during the later stages of life as a result of said passive and involuntary drug exposure.

Since long-term studies in humans that aim to explore the lasting effects of such exposure in adulthood are problematic, the intended significance of the current project will lie in its approach to investigate and highlight cART-induced biobehavioral changes as they transpire over two different developmental phases of life, i.e. in juvenile and adult mice, in an uninfected and validated naturalistic animal model of psychiatric illness, *viz.* the deer mouse model of compulsivity. Specifically, we will apply the deer mouse model to shed more light on the question of whether perinatal cART exposure in uninfected offspring may contribute to biobehavioral anomalies as reflected on the levels of oxidative stress, immune signaling and compulsive-like behavioral expression. Indeed, such an approach might provide valuable insights into the long-term outcomes of perinatal cART exposure in offspring. By studying a spontaneous, neurodevelopmental model of a psychiatric phenotype, i.e. compulsive-like large nest building (LNB[2]), this study is especially suited to investigate the potential long-term sequelae of perinatal cART exposure, as a similar focus is difficult to implement in clinical studies. Also, studies that can accurately delineate the specific contributions of HIV[3] and cART respectively on the long-term health outcomes in humans are inherently unethical, as mothers diagnosed with HIV should receive cART treatment.

* * *

[1] combination antiretroviral treatment
[2] large nest building
[3] human immunodeficiency virus

1.3 Research hypothesis

Broadly, we hypothesize that combined exposure to two drugs that are commonly employed in the cART[1] regimen of pregnant and breastfeeding women, i.e. tenofovir disoproxil fumarate (TDF[2]) and emtricitabine (FTC[3]), but not to normal tap water (control) during the perinatal period, will induce specific biological alterations in exposed females and dams and their juvenile offspring. These will include increased central oxidative stress and a pro-inflammatory immune state. Further, we hypothesize that said cART-associated alterations will induce detectable changes in the later-life behavioral expression of offspring. More specifically, cART exposure is expected to induce a greater severity of a naturally occurring compulsive-like behavioral phenotype, i.e. large nest building (LNB[4]) as expressed by deer mice. We will not afford attention to the newest drug in the South African armament against HIV[5], i.e. dolutegravir, since this drug had not been used in pregnant women at the time of the conceptualization of this work (Hill et al., 2018). In more specific terms, the current hypothesis can be stratified into two broad hypotheses as follows:

Hypothesis 1: Deer mouse females or dams exposed to cART for 21 days, or alternatively, from 21 days prior to being mated, and their juvenile 3-week-old offspring (where applicable), will present with distinct CNS[6] oxidative stress and pro-inflammatory marker profiles, compared to females or dams and offspring (where applicable) exposed to normal tap water only over the same period. We further hypothesize that these differences will be observed at two separate time points, *viz.* (i) in non-mated, non-pregnant female mice after 21 days of treatment and (ii) after pregnancy and nursing, during which time animals would have continued to receive either normal tap water or oral cART solutions.

Hypothesis 2: Over the long term, perinatal cART exposure will cause similar biological changes as alluded to above, i.e. a central pro-oxidative and -inflammatory state, in the adult, 14-week-old offspring of cART-, compared to water-exposed dams. We further hypothesize that such physiological alterations will associate with exacerbated nesting expression in cART-exposed offspring, when compared to the offspring from water-exposed dams as assessed at 14 weeks of age.

* * *

[1] combination antiretroviral treatment
[2] tenofovir disoproxil fumarate
[3] emtricitabine
[4] large nest building
[5] human immunodeficiency virus
[6] central nervous system

1.4 Study aims and objectives

1.4.1 Aims

The present work had two broad aims:

1) Investigating the influence of cART[1] exposure on brain markers of oxidative stress, i.e. the reduced to oxidized glutathione redox (GSH/GSSG[2]) index, and the inflammatory profile, i.e. interleukin (IL[3])-1β, IL-6, IL-10, IL-17, interferon-gamma (IFN-γ[4]), and tumor necrosis factor-alpha (TNF-α[5]) in both non-pregnant and pregnant adult (13 weeks) female deer mice, as well as in their respective 3- and 14-week-old offspring, and
2) exploring the influence of perinatal cART exposure on the long-term behavioral nesting expression of the adult offspring of both water- and cART-exposed dams.

1.4.2 Objectives

To realize the aforementioned aims, the following objectives were met:

i) 20 adult female deer mice were exposed to either normal tap water (n = 10) or cART solutions (n = 10; TDF[6] 60 mg/kg/day and FTC[7] 40 mg/kg/day; Nair and Jacob (2016)) for 21 days after which they were euthanized;
ii) 40 deer mouse dams were paired with a single male animal each. 20 pairs were exposed to normal tap water (control) and 20 pairs to cART from 21 days prior to being mated, until the last day of nursing, i.e. when pups were at the age of 3 weeks. Dams were exposed for 63 days in total, *viz.* for 21 days prior to mating, 21 days during pregnancy, and 21 days during the nursing period. Dams in this group were then euthanized;
iii) The pups born in (ii) were divided into two groups. The first group of pups (n = 42 per exposure cohort irrespective of sex) were euthanized at the age of 21 days. The second group (water-exposed: n = 36, 22 males, 14 females; cART-exposed: n = 38, 16 males, 22 females), were reared until adulthood under normal laboratory conditions in the absence of cART exposure (all pups were included, irrespective of sex);

[1] combination antiretroviral treatment
[2] reduced to oxidized glutathione ratio
[3] interleukin
[4] interferon-gamma
[5] tumor necrosis factor-alpha
[6] tenofovir disoproxil fumarate
[7] emtricitabine

iv) The nest building expression of all the adult offspring as referred to in (iii) was conducted at the age of 13 weeks. These animals were then euthanized at age 14 weeks, and

v) For all of the animals referred to in (i) – (iv) above, the same biological analyses were performed (see **paragraph 1.4.1** above).

1.5 Project layout

To address the aims of the proposed work, this investigation was divided into 2 phases:

1.5.1 Phase 1 – Investigation in female mice, dams, and juvenile offspring

To investigate and elucidate the influence of cART[1]-exposure on markers of oxidative stress and immune signaling in both non-pregnant and pregnant adult female deer mice, as well as the juvenile offspring of the latter, 60 female deer mice were separated into 6 groups (**Figure 1-1**):

- Groups (**i**) and (**ii**) (*n* = 10 per group; age 13 weeks) received either normal water (**i**) or cART (**ii**) for 21 days. These animals were euthanized without pairing them up with male mice.
- Groups (**iii + iv**) and (**v + vi**) (*n* = 10 per group) were exposed to either normal water (**iii + v**) or cART (**iv + vi**) (refer to **Chapter 3** and **Addendum A** for details). Exposure in these mice began 21 days prior to mating. They continued to be exposed to said interventions throughout pregnancy and the subsequent nursing period. All dams as well as the juvenile, 3-week-old pups of groups (**iii + iv**) were euthanized, and the brains were sampled for further analysis. The pups of groups (**v + vi**) progressed to Phase 2.

1.5.2 Phase 2 – Investigation in adult offspring

To investigate the influence of perinatal cART exposure on the long-term biobehavioral profile of adult offspring, the 13-week old offspring of the group (**v + vi**) dams referred to above (*n* = 74 in total; 38 male, 36 female), were reared under standard laboratory conditions in same-litter, same-exposure, socially housed cages (refer to **Chapter 3** for a detailed explanation) while receiving only normal tap water and standard rodent chow until adulthood (age 13 weeks). At this point in time, they underwent the following procedures (**Figure 1-2**):

[1] combination antiretroviral treatment

- All offspring were separated into single-housed cages to assess nest building expression, which was conducted over 7 consecutive nights (**Chapter 3 and Addendum A**). Animals of each respective perinatal exposure cohort, i.e. offspring from the group (**v + vi**) dams respectively, were subsequently separated into normal nesting (NNB)[1] and LNB[2] cohorts so as to determine the respective intensities of nesting expression in the two adult subgroups.
- Animals selected for NNB and LNB expression were subsequently euthanized, and the whole brains were sampled for further analysis.

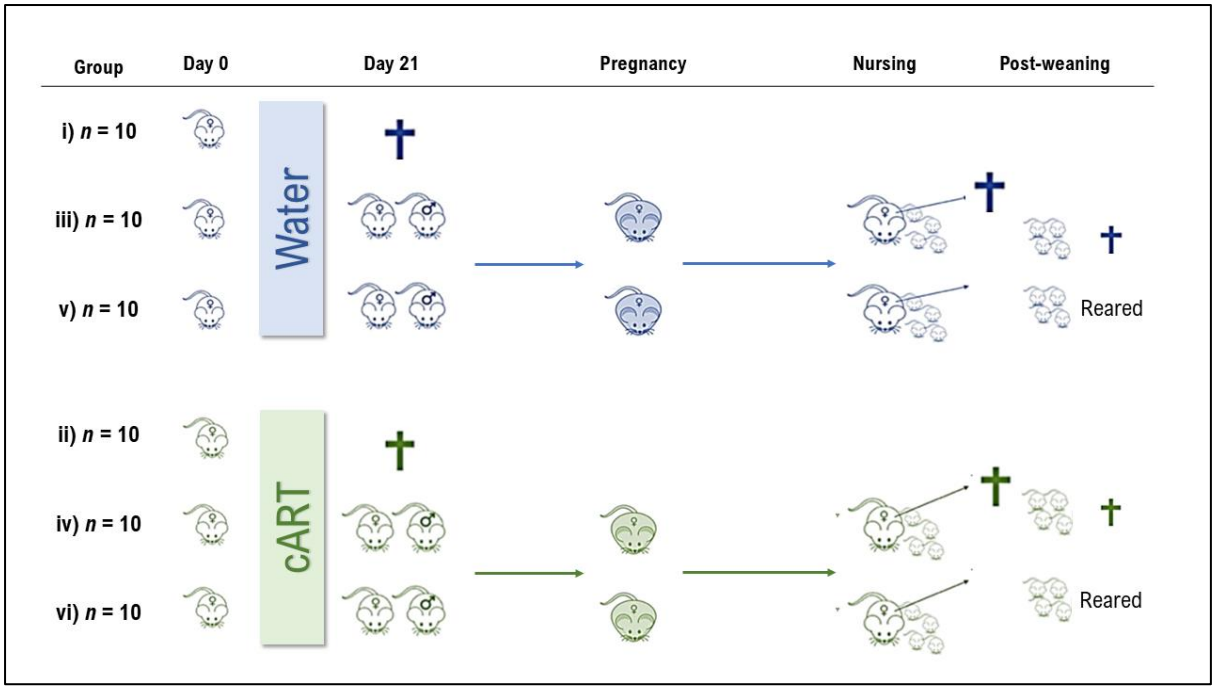

Figure 1-1 - Schematic layout of Phase 1

Groups i – vi are indicative of female deer mice, irrespective of nesting phenotype. Blue: water-treated trajectory. Green: cART-treated trajectory; crosses indicate euthanization. cART: combination antiretroviral treatment.

[1] normal nest building
[2] large nest building

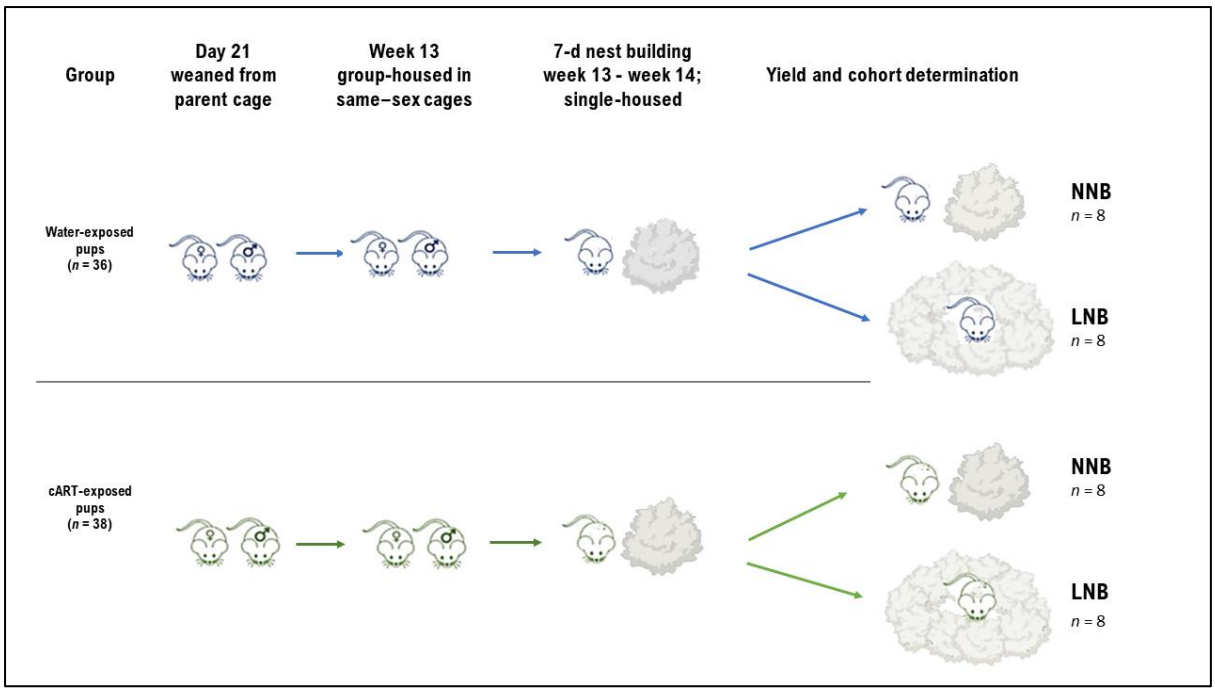

Figure 1-2 – Schematic layout of Phase 2

Blue: offspring from dams exposed to water during pregnancy and nursing. Green: offspring from dams exposed to cART during pregnancy and nursing. NNB: normal nest building; LNB: large nest building; cART: combination antiretroviral treatment

* * *

1.6 Ethical approval

This investigation has been approved by the North-West University AnimCare Research Ethics Committee (approval number: **NWU[1]-00524-20-A5**). Throughout the course of this investigation, the 3 R's[2] as they pertain to animal experimentation, were adhered to (Arora et al., 2011; Doke and Dhawale, 2015; Ferdowsian and Beck, 2011; Hooijmans et al., 2010; Ranganatha and Kuppast, 2012).

All procedures were performed in accordance with the code of ethics stipulated in the relevant national legislation (South African National Standard for the Care and Use of Animals for Scientific Purposes; SANS[3] 10386:2008).

* * *

[1] North-West University
[2] replacement, refinement and reduction
[3] South African National Standards

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2 Literature review

2.1 HIV and antiretroviral therapy

2.1.1 Introduction

Human immunodeficiency virus (HIV)[1] infection is a global plight. According to the Joint United Nations Programme on HIV/AIDS (UNAIDS)[2] (UNAIDS, 2019), approximately 40 million people globally were living with HIV at the end of 2018. Of these, 25 % reside in Eastern and Southern Africa. South Africa is home to the largest combined antiretroviral treatment (cART)[3] program in the world with 62 % of infected individuals receiving treatment at the end of 2018. However, cART, although successful in reducing the HIV viral load, is known to have HIV-unrelated adverse physiological effects, i.e. oxidative stress (M Gallego-Escuredo et al., 2010) and pro-inflammatory responses (Akay et al., 2014; Kaplan et al., 2015; Manda et al., 2011). In turn, oxidative stress and pro-inflammatory responses, either alone or presenting concurrently, have been associated with the manifestation, altered prognosis and poor treatment response of psychiatric conditions (de Sousa et al., 2014; Lindqvist et al., 2017; Ng et al., 2008). While lifelong cART for all HIV-positive pregnant women, or the so-called Option B+ era, has reduced mother-to-child, i.e. vertical, transmission rates to below 2 % (Chi et al., 2013), observational studies showed that infants born HIV-uninfected but that have been exposed to HIV/cART (iHEU)[4] *in-utero* and *perinatally*, i.e. the weeks immediately before and after birth, progress to present with increased morbidities, including neuropsychological difficulties (Cespedes and Aberg, 2006). However, the extent to which perinatal cART exposure results in long-term biobehavioral aberrations remains to be established, since the long-term study of children born from HIV-positive mothers remains problematic. Considering the long-lasting fundamental processes that transpire during the neurodevelopmental window (Nelson, 2002), e.g. neurulation, cell proliferation, axonal outgrowth and synaptogenesis (Lawson et al., 2016; Nelson, 2002), it is possible that newly born infants of mothers that have been treated with cART while pregnant and breast-feeding may be at risk to develop neuropsychiatric complications during the later stages of life due to such passive and involuntary drug exposure. In brief, this theme will form the core focus of this work.

[1] human immunodeficiency virus
[2] United Nations Programme on HIV/AIDS
[3] combination antiretroviral treatment
[4] HIV-uninfected, but HIV/cART exposed infants

2.1.2 HIV and psychiatry

While several studies putatively link the use of cART[1] with psychiatric symptomology, it remains overly difficult to disentangle the potential effect of HIV[2] on the one hand, and cART on the other, on neuropsychiatric symptom manifestation. That said, there are indications of direct relationships between HIV infection and the prevalence of psychiatric symptoms. First, it is well-reported that stigmatization of individuals living with HIV elicits strong emotional responses in patients that can be to the detriment of their mental health (Hyde, 2008; Travaglini et al., 2018). Second, HIV itself can directly affect central neurobiology, with studies showing behavioral anomalies, neurocognitive disturbances (Schouten et al., 2011; Sheppard et al., 2015), and even psychosis (Nakasujja et al., 2012; Nebhinani and Mattoo, 2013; Treisman and Angelino, 2004). Third, HIV patients are also more susceptible to opportunistic neurological as well as systemic infections, and the treatment of these infections can in some cases cause psychiatric symptomology (Matinella et al., 2015; Tan et al., 2012). Fourth, individuals suffering from HIV are at higher risk to suffer from substance abuse disorder, which in turn is significantly associated with other mental health comorbidities (Anand et al., 2010; Neaigus et al., 2017). Last, some treatment strategies used in highly active antiretroviral treatment (HAART[3]) regimens have been linked to psychiatric symptomology (Carrico et al., 2011; Palmer et al., 2010); this led to treatment nonadherence which further worsens the disease state (Campos et al., 2010; Mellins et al., 2011).

The prevalence of psychiatric comorbidities in HIV patients differs with respect to the specific comorbid condition studied. For example, Owe-Larsson et al. (2009) found that HIV patients are 2 – 7 times more likely to be diagnosed with major depressive disorder (MDD[4]), compared to healthy controls, with a prevalence rate of up to 50 % among HIV patients, having been reported. Hinkin et al. (2001) reported an increase in the prevalence of generalized anxiety disorder (GAD[5]) in HIV patients living with HIV. Interestingly, phobias and grief were also associated with HIV infection (Chandra et al., 2005). Further, cognitive dysfunction (Schouten et al., 2011; Sheppard et al., 2015) and acquired immunodeficiency syndrome (AIDS[6])-associated dementia (McArthur, 2004) are also often described.

With respect to children, it has been shown that HIV-infected infants as well as HIV-uninfected infants that were born from HIV-infected mothers, present with a higher risk for psychiatric illness compared

[1] combination antiretroviral treatment
[2] human immunodeficiency virus
[3] highly active antiretroviral treatment
[4] major depressive disorder
[5] general anxiety disorder
[6] acquired immunodeficiency syndrome

to the general age-matched population (Gaughan et al., 2004). In this study, most of the HIV[1]- or cART[2]-affected children were admitted for MDD[3] or another behavioral disorder, with 47 % of patients being hospitalized on multiple occasions. Affected children were between 4 and 7 years of age, with the median hospitalization age reported as 11 years (Gaughan et al., 2004).

2.1.3 Highly active antiretroviral treatment

The implementation of cART has played a crucial role in the fight against HIV and AIDS[4] (Feng et al., 2009) and has led to a reduction in the morbidity and mortality associated with HIV and AIDS (Feng et al., 2009). Combining drugs from the different classes have shown benefits in terms of efficacy, as nucleoside reverse transcriptase inhibitors (NRTI[5]) and non-nucleoside reverse transcriptase inhibitors (NNRTI[6]) act in a synergistic manner (Buckheit et al., 2001; King et al., 2002). Highly active antiretroviral treatment (HAART[7]) consists of NNRTI's such as nevirapine or efavirenz and two different NRTI's that form the backbone of the therapy, such as tenofovir, lamivudine, abacavir or emtricitabine (Gupta et al., 2009). NRTI's were some of the first antivirals found to inhibit reverse transcriptase, and later the nucleotide/nucleoside reverse transcriptase inhibitors (NtRTI's[8]) were added to the regimens. These drugs are analogs of the deoxynucleotides that are involved in the process of synthesizing viral DNA[9] that competes with the naturally occurring deoxynucleotides. They act as substrates that are added into a developing DNA chain and cause chain termination. Said chain termination takes place due to the substrate's shortage of a 3'-hydroxy group (Faltz et al., 2017; Sharma et al., 2004). NNRTI's were developed to inhibit HIV-1 replication via various mechanisms (Sluis-Cremer and Tachedjian, 2008), e.g. by directly binding to the HIV-1 reverse transcriptase enzyme at a different site to the active site, thereby inhibiting the enzyme's function (Faltz et al., 2017). This causes conformational changes within the enzyme and in doing so, decreases the rate of viral DNA synthesis while simultaneously leaving the binding affinity of natural deoxynucleoside triphosphate unaffected (Ai et al., 2012; Balzarini, 2004; Feng et al., 2009).

[1] human immunodeficiency virus
[2] combination antiretroviral treatment
[3] major depressive disorder
[4] acquired immunodeficiency syndrome
[5] nucleoside reverse transcriptase inhibitor
[6] non-nucleoside reverse transcriptase inhibitor
[7] highly active antiretroviral treatment
[8] nucleotide/nucleoside reverse transcriptase inhibitors
[9] deoxyribonucleic acid

2.1.4 Mother to child transmission and the antiretroviral treatment B+ option

Substantial advances have been made towards the struggle towards prevention of mother-to-child transmission (PMTCT[1]) of HIV[2] in sub-Saharan Africa (Chi et al., 2013). In 2010, the World Health Organization (WHO[3]) suggested two PMTCT prophylaxis options, i.e. Option A and Option B, for pregnant women that are not eligible to receive triple cART[4]. Option A suggests that maternal zidovudine (AZT[5]) be given prophylactically from 14 weeks of gestation until 7 days postpartum, and daily nevirapine (NVP[6]) for the infant until one week after cessation of breastfeeding. Option B refers to triple cART as prophylaxis starting from 14 weeks of gestation and continuing until 1 week after cessation of breastfeeding (World Health Organization, 2010a). In 2013 however, an innovative third option (Option B+) was introduced, offering cART to HIV-infected pregnant women regardless of their CD4[7] cell count (World Health Organization, 2010b). This option provides the same treatment regimen to women during pregnancy and throughout life. The Option B+ approach to life-long treatment presents multiple advantages for women that are not provided by the Option A and earlier Option B approaches. For example, PMTCT programs are simplified, since there is no need for CD4 testing to determine eligibility. Furthermore, continued treatment minimizes confusion as to when cART treatment should be stopped or continued after the risk for mother-to-child transmission (MTCT[8]) has been determined (World Health Organization, 2012).

The primary goal of PMTCT is to suppress the maternal plasma HIV viral load below the detectable limit. Earlier initiation of antiretroviral treatment significantly reduces the risk for such vertical viral transmission (Ioannidis et al., 2001; Study, 2007). Although the exact mechanisms underlying HIV MTCT have not yet been fully elucidated, they are likely multifactorial. For example, Kourtis and Bulterys (2010) note that MTCT can transpire via three processes. First, it can occur in utero via direct hematogenous transplacental circulation or via ascending infection of the amniotic fluid and membranes. Secondly, transmission can occur at the time of delivery via mucocutaneous contact between the infant and mother. Lastly, transmission can occur during breastfeeding. However, research is unclear as to what the contributions of the various transmission mechanisms are on the overall percentage of MTCT cases that are reported (Kourtis et al., 2006). Interestingly, approximately half of MTCT cases occur late in pregnancy, likely due to the separation of the placental lining with the uterine wall (Kourtis et al.,

[1] prevention of mother-to-child transmission
[2] human immunodeficiency virus
[3] World Health Organization
[4] combination antiretroviral treatment
[5] zidovudine
[6] nevirapine
[7] cluster of differentiation 4
[8] mother-to-child transmission

2001). Less than 4 % of MTCT[1] occurs in the first trimester, and by 36 weeks of pregnancy, this number is still below 20 %. Importantly, 18 % of infants born uninfected but exposed to HIV-1 (iHEU)[2] present with unintegrated virus in their peripheral blood mononuclear cells (Lee et al., 2004). While unintegrated viral intermediates are all biologically active, they cannot activate and hence degenerate over time. Hence, infantile viral entry does not necessarily equate to HIV[3]-1 transmission (Zack et al., 1992). Breastfeeding is also a potential risk for MTCT especially in cases when feeding substitutes are not freely available. By this mechanism, the postnatal transmission rate is approximately 15 % of mothers who breastfeed for the first 2 years (Fawzi et al., 2002; Kourtis et al., 2003; Miotti et al., 1999). Given the complexity of the factors contributing to MTCT, it is vital that strategies aimed at improving PMTCT[4] should target the early steps of viral entry and integration.

2.2 The effects of perinatal cART exposure framed against the background of neuropsychiatric health

As alluded to earlier in this literature review, cART[5] has been associated with significant neuropsychiatric symptomology, including mania, delusions, impulsivity, depression, post-traumatic stress disorder (PTSD)[6] and compulsive behavior (Cespedes and Aberg, 2006; Hauptman and Carchedi, 2013; Hawkins et al., 2005). Importantly, such symptoms are not only related to the use of efavirenz, which is well-known to cause neuropsychiatric symptoms (Read et al., 2011). The specific mechanisms underlying these associations are proposed to be related to neurotoxicity and neuroinflammatory damage caused by NRTI's[7]. Moreover, long-term cART can induce oxidative stress and modify central synaptophysin levels which can result in subsequent neuronal death (Manda et al., 2011). Previous investigations also showed cART-related changes in lipid and protein metabolism, oxidative stress, and mitochondrial damage, which collectively play a role in peripheral neurotoxicity (Akay et al., 2014). That said, the exact extent to which cART exposure can influence neurodevelopment across the lifespan in iHEU is yet unknown. It suffices to conclude that perinatal exposure to either HIV or cART may potentially induce long-lasting neuropsychiatric pathology (Kaplan et al., 2015). Since this possibility constitutes the major focus of this work, we will now afford attention to some of these potential levels of HIV-brain interactions.

[1] mother-to-child transmission
[2] HIV-uninfected, but HIV/cART exposed infants
[3] human immunodeficiency virus
[4] prevention of mother-to-child transmission
[5] combination antiretroviral treatment
[6] post-traumatic stress disorder
[7] nucleoside reverse transcriptase inhibitors

2.2.1 Immune system alterations

Cytokines are produced within the body as immunomodulatory molecules in response to infection, tissue injury, and other causes of immune reactions. Their expression is regulated by both transcriptional and posttranscriptional mechanisms. However, dysregulation of these mechanisms leads to disrupted cytokine secretion, which has been shown to contribute to the pathological effects of chronic inflammation and autoimmunity (Tanaka et al., 2014). Inflammation within the peripheral nervous system and the central nervous system (CNS[1]), i.e. neuroinflammation, is an underlying construct of many psychiatric disorders (Hong et al., 2016; Onore et al., 2012). With respect to the specific effects of cART on neuroinflammation, there is well-documented evidence of higher morbidity and mortality risks in iHEU[2] compared to unexposed and uninfected infants (iHU[3]) in sub-Saharan Africa (Epalza et al., 2010; Kuhn et al., 2005; Marinda et al., 2007; McNally et al., 2007; Shapiro et al., 2007; Slogrove et al., 2012). As cART-based prophylaxis acts on the placental environment and induces changes in cytokine expression (Abramczuk et al., 2011), the higher rates of mortality reported in iHEU can only partially be related to maternal disease stage or socioeconomic status (Marinda et al., 2007). Further, iHEU also display multiple other immune alterations (Abramczuk et al., 2011; Dirajlal-Fargo et al., 2019; Embree et al., 2001; Jones et al., 2011; Kidzeru et al., 2014; Mazzola et al., 2011; Nielsen et al., 2001; Reikie et al., 2013; Sanz-Ramos et al., 2013) including lower CD4[4] T cell counts and reduced thymic output (Nielsen et al., 2001; Reikie et al., 2013) as compared to iHU. Humoral vaccine responses of iHEU also differ from iHU (Dirajlal-Fargo et al., 2019; Sanz-Ramos et al., 2013). Overwhelming consensus points to iHEU presenting with elevated immune activation, i.e. heightened T cell and monocyte activation (Selvam et al., 2015), while elevated inflammatory cytokine responses to innate cell stimulation have also been shown in unstimulated iHEU peripheral blood (Lohman-Payne et al., 2018; Mazzola et al., 2011). That said, most studies assessing infant immunological and neurological development have been performed in iHEU born to mothers actively taking cART[5], making it difficult to distinguish between the effects of maternal cART and HIV[6] itself. With respect to its potential effects on central processes, alterations in neuroinflammatory processes can influence psychiatric health in various ways, which have been explained before.

[1] central nervous system
[2] HIV-uninfected, but HIV/cART exposed infants
[3] HIV-unexposed infants
[4] cluster of differentiation 4
[5] combination anti-retroviral treatment
[6] human immunodeficiency virus

2.2.2 Oxidative stress

Oxidative stress can be defined as an imbalance between the production of reactive oxygen species (ROS[1]) and the body's innate defenses against such oxidative species (Betteridge, 2000). However, it is important to regard the term in a broad sense because its physiological sequelae depend in part on the cellular compartment in which ROS are produced. Generally viewed, ROS include both free radicals as well as their non-radical intermediates. Free radicals are species that have one or more unpaired electrons, which makes them highly chemically reactive. The body's response to ROS is facilitated by two pathways of antioxidant defense. The first is enzymatic, comprising of among others, superoxide dismutase and catalase that catalyze rate-limited anti-oxidant reactions (Fee and Bull, 1986). The second pathway is non-enzymatic, comprising the actions of molecular compounds such as reduced glutathione (GSH[2]) (Das and Roychoudhury, 2014). While ROS are normally involved in processes of intracellular messaging (Burton and Jauniaux, 2011), they can also contribute to protein and DNA[3] injury, tissue damage, inflammation, and cell death, if excessive (Marrocco et al., 2017; Negre-Salvayre et al., 2010; Roberts et al., 2010; Uttara et al., 2009).

Although an in-depth review of the origins of ROS formation falls beyond the scope of this review, a brief overview of aspects of relevance for the biomarker studied here, i.e. the GSH/oxidized glutathione (GSSG[4]) ratio, will be provided. ROS production occurs via various pathways, namely enzymatic activation to a free radical intermediate, disturbance of the mitochondrial electron transport chain and activation of enzyme systems such as nicotinamide adenine dinucleotide (NADH)[5] oxidase that produce superoxide and hydrogen peroxide. These processes cannot take place at the same time and the influence of one process on the oxidative state may vary with time (Wells et al., 1997; Wells et al., 2010). Under resting-state conditions, the main source of superoxide (O_2^-)[6] is the mitochondria (Manda et al., 2009; Phaniendra et al., 2015). Mitochondrial transfer of electrons along the energy chain is not always optimally efficient, as electrons leak and induce O_2^- formation. The rate of O_2^- formation is dependent on the number of electrons that are present in the chain, and therefore under certain conditions, e.g. hyperoxia, O_2^- can be elevated. Normally, 2 % of oxygen is converted to O_2^- and since the molecule is negatively charged, they are membrane-impermeable, hence remaining within the mitochondrial matrix. O_2^- can also be formed via leakage of electrons from the electron chain within the endoplasmic reticulum (Brown et al., 2010), while the enzymatic activation of nicotinamide adenine dinucleotide phosphate

[1] reactive oxygen species
[2] reduced glutathione
[3] deoxyribonucleic acid
[4] oxidized glutathione
[5] nicotinamide adenine dinucleotide
[6] superoxide

(NADPH)[1] oxidase, cytochrome P450 and various other oxidoreductases (Burton and Jauniaux, 2011; Pérez-Torres et al., 2017), also contributes to O_2^- formation. Careful regulation of the oxidant to antioxidant ratio is, therefore, necessary to maintain cell membrane integrity which in turn protects the functionality of cellular proteins and nucleic acid. To this end, the concept of equilibrium is of major importance because it allows us to understand that physiological responses to oxidative stress will be graded. In other words, whereas minor alterations in the balance will incur normal corrective homeostatic adaptions, major disturbances in the balance will lead to cellular damage and inevitably cell death (Burton and Jauniaux, 2011). In addition, it is important to note that oxidative stress-induced damage rarely transpires in isolation. Rather, complex interactions can occur between oxidative stress and other types of cell stress, while the resulting clinical presentation will largely depend on the balance between the endogenous metabolic activity within a cell or organ and the degree of homeostatic control over such activity (Burton and Jauniaux, 2011).

An important index of the oxidative balance is the rate of GSH[2] depletion (Mytilineou et al., 2002). Glutathione is synthesized in the brain by neurons as well as glial cells and is abundant in astrocytes. The brain is highly susceptible to oxidative stress due to its high energy demand and oxygen consumption as well as due to the presence of rich poly-unsaturated fatty acid content that is sensitive to lipid peroxidation. The antioxidant function of GSH stems from its ability to scavenge free radicals and prevent the formation of ROS[3] (Zitka et al., 2012). Under conditions of oxidative stress, GSH is oxidized to form GSSG[4], thereby altering the GSH/GSSG[5] ratio (Zitka et al., 2012), i.e. the higher the GSSG content, the higher the degree of oxidative stress will be.

With respect to cART[6], a body of clinical evidence points to significant and noteworthy alterations in the oxidative state of treated patients. Further, such findings were reported both on a genetic as well as a physiological level. For example, in iHEU[7] at birth, two genes related to the regulation of the oxidative balance were shown to be differentially expressed, i.e. those coding for arrestin domain-containing protein 4 (ARRDC4[8]) and thioredoxin-interacting protein (TXNIP[9]); being paralogs of each other, both ARRDC4 and TXNIP were increased in iHEU. ARRDC4 and TXNIP are modulated by several cellular stressors, including lactic acidosis (or low potential hydrogen; pH[10]) and endoplasmic-reticulum stress

[1] nicotinamide adenine dinucleotide phosphate
[2] reduced glutathione
[3] reactive oxygen species
[4] oxidized glutathione
[5] reduced to oxidized glutathione ratio
[6] combination antiretroviral treatment
[7] HIV-uninfected, but HIV/cART exposed infants
[8] arrestin domain containing protein 4
[9] thioredoxin-interacting protein
[10] potential hydrogen

(Nirogi et al., 2012; Schroder et al., 2010). Such alterations are intricately linked with exacerbated levels of oxidative damage, since both proteins are part of the α-arrestin family, which binds to thioredoxin (TRX[1]; an important redox protein and promoter of cell growth), thereby inhibiting the ability of TRX to reduce other proteins. Therefore, if increased, ARRDC4[2] and TXNIP[3] can increase redox stress. In turn, redox stress inhibits cell growth and accelerates apoptosis. TRX also controls the levels of ROS[4] in cells, playing a role in limiting oxidative stress damage, functions which are also inhibited by TXNIP (Chen et al., 2010; Lane et al., 2013; Patwari et al., 2006; Sikalidis et al., 2011; Zhou and Chng, 2013). Apart from genetic evidence, cART[5] exposure has also been linked directly with changes in markers of oxidative stress in adults. Studies have indicated that cART exposure influence the activity of glutathione peroxidase (GPx[6]) and glutathione reductase (GR[7]), two enzymes that play a vital role in the glutathione redox cycle (Sharma, 2014) Oxidative stress has also been correlated to NRTI[8]-induced peripheral toxicity, which can be caused by prolonged exposure to cART and in turn induce oxidative stress via sustained neuronal ROS accumulation (Akay et al., 2014). Further, studies also hint that prolonged cART exposure may compromise the actions of astrocytes that normally buffer ROS accumulation within the neurons.

In terms of psychiatric illness, dysfunctional defense against oxidative damage has been associated with among others Alzheimer's disease, Parkinson's disease, aging, and obsessive-compulsive disorder (OCD[9]) (Alici et al., 2016; Ozdemir et al., 2009). Here, we will attempt to shed more light on the potential effects of perinatal cART exposure on the long-term oxidative state in deer mice on one side, and its possible association with naturalistic behavioral persistence, i.e. compulsive-like behavior later in life, on the other side. Importantly, considering that a naturalistic model of compulsivity will form the primary research framework of this investigation, it would be valuable to extend findings showing that clinical OCD is linked to altered antioxidant status and increased lipid peroxidation (Kuloglu et al., 2002; Ozdemir et al., 2009; Selek et al., 2008).

[1] thioredoxin
[2] arrestin domain containing protein 4
[3] thioredoxin-interacting protein
[4] reactive oxygen species
[5] combination antiretroviral treatment
[6] glutathione peroxidase
[7] glutathione reductase
[8] nucleoside reverse transcriptase inhibitors
[9] obsessive-compulsive disorder

2.2.3 cART and its potential effects on human health: A collective summary

In summary, alterations in oxidative and immune processes are known to influence human health. Further, it is known that antiretrovirals (ARVs[1]), whether administered alone or as cART[2], can influence each of these constructs. Literature is also clear that while much remains unknown, functional interactions between these processes are likely the primary means by which our health is influenced. Also, considering the known contributions of oxidative processes (Wells et al., 2009) and the immune system (Marques et al., 2013) to our normal neurodevelopment, it is highly likely that functional changes in any of these two constructs from a young age may result in long-term health complications. However, since longitudinal studies in humans that aim to investigate this possibility are problematic, research must rely on preclinical models to broaden our understanding.

Therefore, and against the background of neuropsychiatric health, the present work will seek to explore the long-term effects of cART administration on the neurobiological underpinnings and manifestation of spontaneous compulsive-like large nest building (LNB[3]) in deer mice. Specifically, we will attempt to establish whether perinatally-induced short- and long-term alterations in oxidative and immune signalling processes are associated with changes in the well-known manifestation of adult LNB in the deer mouse (de Brouwer et al., 2020). To this end, we will now afford some attention to the research framework that will be used in this investigation.

2.3 A rapid introduction to obsessive-compulsive disorder

2.3.1 Epidemiology, diagnosis, and treatment

The Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-V)[4] defines a mental disorder as *"a syndrome characterized by clinically significant disturbances in an individual's cognition, emotion regulation, or behavior that reflects a dysfunction in the psychological, biological, or developmental processes underlying mental functioning"*. With respect to the current investigation in which a naturally occurring compulsive-like phenotype will be explored against the background of its potential modulation by peripheral processes, it is noteworthy that OCD[5] is a common and debilitating psychiatric disorder that is characterized by functional impairment and significant social and occupational disability (Markarian et al., 2010). The primary symptoms of the condition are obsessions and compulsions, with

[1] antiretrovirals
[2] combination antiretroviral treatment
[3] large nest building
[4] Diagnostic and Statistical Manual of Mental Disorders, 5th edition
[5] obsessive-compulsive disorder

either one constituting the basis of a valid diagnosis (DSM-V[1]). However, it is commonly accepted that patients engage in compulsive-like routines to mitigate the level of underlying anxiety caused by certain obsessive thoughts (APA, 2013). OCD[2], categorized as the archetype disorder within the 'Obsessive-Compulsive and Related Disorders' group of conditions in the DSM-V, is clinically heterogeneous (Markarian et al., 2010) in that patients present with distinct symptom phenotypes. The four most common obsession-compulsion (O/C)[3] themes are contamination/washing, covert thought processes relating to taboo themes (e.g. sex or religion), symmetry/ordering and safety/checking (Murphy et al., 2016).

Being broadly regarded as a condition of hyposerotonergic signaling, the first-line pharmacological treatment for OCD is high-dose, chronic treatment with selective serotonin uptake inhibitors (SSRIs)[4], e.g. escitalopram, fluoxetine and fluvoxamine (Atmaca, 2016; Sugimoto et al., 2007). However, the therapeutic response remains suboptimal as only 40 – 60 % of patients demonstrate a moderate to adequate response to treatment (Hudak et al., 2012). Treatment-resistant OCD is defined as the failure of at least two adequate therapeutic trials of serotonin reuptake inhibitors (SRIs[5])/SSRIs (Abudy et al., 2011). In such cases, augmentation strategies are followed. These include combinations of psychotherapy and pharmacotherapy (Albert et al., 2013; Atmaca, 2016) and/or augmenting current SSRI treatment with low-dose atypical, anti-dopaminergic drugs, e.g. risperidone (Albert et al., 2013). Interestingly, it should be highlighted that various factors, including exacerbated oxidative stress (Lindqvist et al., 2017) and systemic inflammation (Bot et al., 2011; Lindqvist et al., 2017) have previously been associated with worse treatment response.

2.3.2 The neurobiological basis of OCD

Since the present investigation mainly focuses on the influence of perinatal cART[6] administration on the long-term manifestation of LNB[7] in deer mice, the neuroanatomy and biology of OCD will only be discussed in brief.

OCD is characterized by both cognitive and biobehavioral abnormalities (Markarian et al., 2010). While different hypotheses attempt to explain the symptomology of OCD, it is evident that the brain areas implicated in OCD would be, among others those that translate cognitive planning and experiences into

[1] Diagnostic and Statistical Manual of Mental Disorders, 5th edition
[2] obsessive-compulsive disorder
[3] obsessive-compulsive
[4] selective serotonin reuptake Inhibitors
[5] serotonin reuptake inhibitors
[6] combination antiretroviral treatment
[7] large nest building

motor behavior, and subsequently mediate goal-directed behavior. These brain areas include the prefrontal cortex, striatum and thalamic nuclei that communicate with each other via different pathways (Evans et al., 2004; Van Den Heuvel and Pol, 2010). These structures are organized in such a manner that the cortex innervates the striatum, which subsequently influences other parts of the basal ganglia and ultimately exerts feedback via the thalamus to the cortex. Consisting of direct (behaviorally activating) and indirect (behaviorally inactivating) pathways, the cortico-striatal-thalamo-cortical (CSTC[1]) circuit is fundamental for the planning, execution and termination of complex motor behavior and reward-based learning – two major processes that are hypothesized to be dysfunctional in patients with OCD[2] (Stocco et al., 2010). Furthermore, it is believed that there is a bias in favor of the direct thalamus-activating pathway over the indirect thalamus-inhibiting pathway in OCD patients compared to healthy controls (De Brouwer et al., 2019; Saxena and Rauch, 2000). The subsequent hyperactivity in the CSTC circuit as a whole is believed to be central to the presentation of OCD (Ahmari et al., 2013; Bartz and Hollander, 2006; Saxena and Rauch, 2000).

The neurobiology of OCD can likely best be explained within a framework of reward processing. The term 'reward' denotes any form of satisfactory feedback, from the pleasant taste of foods to the successful completion of a certain task (Schultz, 2002). The anticipation and appraisal of reward are closely correlated with central dopaminergic signaling (Arias-Carrión et al., 2010; Cohen et al., 2012; Unoki et al., 2006). For example, during the initial anticipation of a novel reward, roughly 75 % of the dopaminergic neurons in the basal ganglia are activated (Arias-Carrión et al., 2010; Cohen et al., 2012). However, over time, following repetitive exposure to the same reward, we become conditioned to the reward via the complex associations thereof with different environmental, physiological, and circumstantial factors. Reward conditioning enables the brain to evaluate future confrontations with the same set of factors it was conditioned to, and to associate it with an applicable reward, even before the reward has been presented (Kirsch et al., 2003). Therefore, once the same reward is again presented, the subsequent neurobiological reaction can manifest as either one of the following: 1) if the reward is greater than that anticipated or unpredicted, dopaminergic signaling is elicited; 2) if the reward has been predicted in full, no dopamine response is elicited, and 3) if the anticipated reward was less than expected or omitted entirely, a suppression in dopaminergic signaling is induced (Clark et al., 2013; Redgrave et al., 1999). Thus, following such dopaminergic responses during reward appraisal, the basal ganglia code a 'reward prediction error' – a term used to represent the difference between the predicted and actual reward (Schultz, 2017). The reward prediction error is pivotal to the process of reward-based learning, as it is used to compute and plan any changes that must be implemented in future to either

[1] cortico-striatal-thalamo-cortical
[2] obsessive-compulsive disorder

keep experiencing the same reward (in the case of a positive error) or experiencing a better reward (in the case of a negative error). It can be postulated that the lack of a significant dopaminergic response after the manifestation of a fully predicted reward, may account in part for finding closure after task completion, as the lack of a dopamine response will not induce further reward-seeking behavior. Patients diagnosed with OCD[1] likely do not find closure after task completion. As they constantly exhibit repetitive compulsive behavior of some nature, it can be hypothesized that, concerning the processes of reward appraisal and reward-based learning, a dysfunctional reward system may be central to the pathology of OCD (Husted et al., 2006; Koch et al., 2018). The fact that behavioral inhibition is associated with serotonergic neurotransmission (Cools et al., 2008; Völlm et al., 2006; Völlm et al., 2010) provides a possible explanation for the ameliorative effects observed when treating OCD with drugs enhancing serotonergic functioning. Indeed, several authors propose a role for the serotonergic system to act as the behavioral opponent of dopamine (Cools et al., 2008; Dalley and Roiser, 2012; Daw et al., 2002). In his classic review, Daw et al. (2002) hypothesizes that if dopamine is responsible for approach behavior, motor excitement, and reward processing, serotonin will be associated with avoidance behavior, motor suppression, and processing of aversive or punishing stimuli. In different terms, it can be understood that the balance between reward-seeking behavior and aversion-related reactions is related to the balance between dopaminergic and serotonergic signaling respectively (Daw et al., 2002). Since an abnormal reward appraisal system may be central to the pathology of OCD and while OCD patients generally do not find closure after task completion, it is hypothesized that adequate reward-based learning does not occur. Moreover, an increase in serotonergic neurotransmission elicited by SSRIs may act as the behavioral opponent of dopaminergic transmission, resulting in the attenuation of OC[2] symptoms.

2.4 The deer mouse model of OCD

Several attempts have been made over the past 30 years to develop animal models of OCD, with the objective to extricate the underlying neurochemical, genetic and neuroanatomical substrates of this disorder. While the general purpose of pre-clinical research in animal models is to describe and find novel treatment targets for the human condition, animal models have contributed relatively little to this objective, likely since the cognitive underpinnings of the condition are overly difficult to explore in non-human animals (Alonso et al., 2015). That said, some significant advances have been made that improved our understanding of the genetics and neurobiology of compulsive-like behavioral persistence. Here, the present investigation will seek to further explore and apply the only naturalistic animal model of compulsive-like behavior, i.e. the deer mouse. As opposed to all other models that are induced by

[1] obsessive-compulsive disorder
[2] obsessive-compulsive

either behavioral training (Papakosta et al., 2013), genetic modification (Albelda and Joel, 2012; Alonso et al., 2015), inbreeding (de Haas et al., 2012), or pharmacological manipulation (Szechtman et al., 1998), the compulsive-like manifestations expressed by different sub-populations of deer mice, occur in an entirely spontaneous manner. This provides researchers with a unique opportunity to investigate and interrogate the psychobiological processes that may potentially play a role in the etiology and prognosis of OCD[1].

Apart from being naturalistic, deer mice also present with phenotypically heterogeneous compulsive-like behaviors (Scheepers et al., 2018), i.e. spontaneous stereotypy (observed in 45% of the population; Wolmarans de et al. (2013)), LNB (expressed by 30% of the population; Wolmarans, D. W. et al. (2016)) and high marble-burying (expressed by 11 – 15% of the population; Wolmarans, De Wet et al. (2016b)). Although (seemingly) differing in functional presentation, all three phenotypes develop in mice of both sexes by the age of 10 – 12 weeks. They are further equally persistent and repetitive and, while stereotypy and nest building respond to chronic, high-dose oral escitalopram treatment, high marble-burying remains treatment-refractory to such intervention (Scheepers et al., 2018); this phenotype responds to pro-dopaminergic exposure in the form of chronic rasagiline, a monoamine oxidase type B (MAO-B[2]) inhibitor.

With respect to the current investigation, some important aspects of the model and its application should be highlighted. In general, mice are an excellent tool to study long-term neurodevelopmental constructs as they can be followed from the developmental window before and after birth throughout adulthood. Deer mice reach adulthood by the age of 10 weeks and do not present with any significant age-related pathology until roughly 200 weeks of age (Chappell et al., 2003); this provides the researcher with a large investigation window. Furthermore, since the model is wholly naturalistic, spontaneous manifestations of aberrant behavior—most notably so LNB[3] as will be used here—can be studied with respect to the percentage yield, severity and treatment response following specific external interventions made either during the neurodevelopmental window or in adulthood. In fact, since accurate data exist on all three of these parameters, it is possible to discern the effects of any intervention on the naturalistic prognosis and treatment response of the observed LNB phenotype. Importantly, from an ethical perspective, the effects of HIV[4] and cART[5]-exposure can also not be separated in human infants. As such, animal models of cART exposure in the absence of HIV are crucial.

[1] obsessive-compulsive disorder
[2] monoamine oxidase type B
[3] large nest building
[4] human immunodeficiency virus
[5] combination antiretroviral treatment

2.4.1 Taking a closer look at large nest building

Nest building is a universal behavior expressed by a vast number of animals, including birds, rodents, fish, and even great apes (Gaskill et al., 2013; Hansell, 1984). This innate and highly goal-directed behavior has attracted the attention of researchers due to its potential utility for studies of neurological disorders characterized by diminished well-being and impaired daily activities (Hall et al., 2015; Heller et al., 2014; Palmiter, 2008). With respect to mice, it is important to note that both male and female mice build nests for various purposes. For example, nests are built for reproductive purposes, shelter, heat conservation, and to provide protection against predators and sudden environmental change (Neely et al., 2019). As alluded to above, nest building is innate in mice and will be expressed even within closely regulated laboratory settings (Deacon, 2006), i.e. in the absence of a need with regards to any of the abovementioned motivators. Hence, it stands to reason that any alterations or perturbations in mouse nesting behavior, as appraised against the background of the behavior expressed by the larger population of a laboratory-housed colony, can indicate a health or welfare alteration (Gaskill et al., 2013; Jirkof, 2014; Latham and Mason, 2004; Mason and Latham, 2004). This is also true for deer mice (*Peromyscus maniculatus bairdii*), which have been studied with respect to the compulsive-like expression of LNB[1] by approximately 30% of mice in the laboratory housed colony (de Brouwer et al., 2020; Wolmarans de et al., 2016; Wolmarans, De Wet et al., 2016a; Wolmarans, 2015).

Our assumption that LNB in deer mice represents a form of compulsive-like behavior, is based on findings that such behavior deviates significantly in size, repetition and persistence from the norm as expressed by the larger population of mice in our colony (Wolmarans, De Wet et al., 2016a). In fact, considering that LNB serves no apparent purpose since all animals are maintained and cared for under the same conditions as control animals, raises the question of why only some animals engage in such an inflated behavioral expression. Further, promising predictive validity has also been established by the demonstration that LNB in both house mice (Greene-Schloesser et al., 2011) and deer mice (Wolmarans, De Wet et al., 2016a) is attenuated by chronic, high dose SSRI[2]-treatment. That said, apart from its modest face and predictive validity, little has subsequently been done to determine the construct foundations of this behavior as a model of compulsivity. Here, we will apply LNB as expressed by deer mice to explore the potential long-term effects of perinatal cART[3] administration on the manifestation of said behavior in this species.

[1] large nest building
[2] selective serotonin reuptake inhibitors
[3] combination antiretroviral treatment

2.5 Conclusion

In this literature review, we afforded attention to the clinical dilemma of potential cART[1]-induced neurodevelopmental difficulties in perinatally exposed children. We also addressed the fact that longitudinal studies to elucidate our understanding of these potential associates are difficult. We subsequently highlighted that while much remains to be established in terms of the effects of perinatal cART exposure on the later life biobehavioral profile of children born from cART-exposed mothers, two potentially important constructs can be identified, i.e. oxidative stress and immune signaling. To this end, the present investigation aims to bridge the said difficulties and constructs, by investigating the effect of perinatal cART exposure on the immediate and long-term biobehavioral profile of deer mouse offspring. We believe that this investigation will shed valuable light on the manner in which perinatal cART exposure may contribute to later-life pathology, thereby highlighting potentially useful avenues for future investigation.

[1] combination antiretroviral treatment

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3 Journal article

The immediate and long-term effects of perinatal combined antiretroviral treatment (cART)-exposure on brain markers of oxidative stress and inflammation in the deer mouse model of compulsive-like behavioral persistence

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This article will be submitted to *'Pharmacology, Biochemistry and Behavior'* and was formatted according to the 'Guide for Authors' as prescribed by the journal. These can be found at the following link: Guide for authors - Pharmacology Biochemistry and Behavior - ISSN 0091-3057 (elsevier.com)

Important – Figures and tables are included at the end of the manuscript as per the guidelines to the authors. All supplementary data referred to in this manuscript, are included in Addendum A.

Author contributions

- JS designed the study under the supervision of DWW, AH and HBJ. She performed all the pharmacological and behavioral experiments and wrote the first and edited versions of this manuscript.
- AUH was a co-supervisor of this work and assisted with study design and cytokine analysis, and data interpretation.
- HBJ was a co-supervisor of this work and assisted with study design, funding, and data interpretation.
- DWW was the supervisor of this work. He conceptualized and designed the study in consultation with JS, HBJ, and AUH. He assisted with behavioral methodology, data interpretation and revision of the first and final versions of this article.

* * *

Abstract

Combination antiretroviral treatment (cART[1]), although successfully reducing the HIV[2] viral load, is associated with HIV-unrelated physiological effects, i.e. oxidative stress and proinflammatory responses, which can have long-term effects in the uninfected offspring. Therefore, we aimed to study the effect of perinatal cART exposure on markers of oxidative stress and cytokine expression in non-pregnant and pregnant deer mice *(Peromyscus maniculatus bairdii)* and their 3- and 14-week-old offspring. Three groups of 20 deer mouse dams were exposed to either normal water (control) or cART (tenofovir 60 mg/kg/day and emtricitabine 40 mg/kg/day, *n* = 10 per exposure, ethical approval nr.: **NWU[3]-00524-20-A5**) as follows: Group 1, all non-pregnant, was exposed to either intervention for 21 days and euthanized prior to pairing. Groups 2 and 3, all pregnant, were exposed from 21 days prior to mating until the end of nursing. Pups born from Group 2 were euthanized at 21 days (3-week-old group), while Group 3 pups were raised until adulthood when compulsivity testing, i.e. nesting assessment, was performed (14-week-old group). After collecting the whole-brain tissues of all animals, samples were analyzed for oxidative stress (GSH/GSSG[4] ratio) and cytokine profile (IL[5]-1β, IL-6, IL-10, IL-17, IFN-γ[6], TNF-α[7]). Our results show that perinatal cART exposure exacerbates oxidative stress and suppress immune signaling in pregnant vs. non-pregnant mice. Further, we show that cART-exposed NNB[8]- and LNB[9]-expressing offspring differ in terms of their immunological response to said exposure, with the long-term pro-inflammatory effects of cART exposure being masked in LNB-expressing offspring. Thus, we conclude that LNB uniquely associates with perturbed brain-immune interactions. Investigations using the deer mouse model of compulsive-like behavior and brain-immune interactions will need careful consideration in future work.

Keywords

combination antiretroviral treatment; perinatal exposure, oxidative stress, cytokine, inflammation, deer mouse, obsessive-compulsive disorder

[1] combination antiretroviral treatment
[2] human immunodeficiency virus
[3] North-West University
[4] reduced to oxidized glutathione ratio
[5] interleukin
[6] interferon-gamma
[7] tumor necrosis factor-alpha
[8] normal nest building
[9] large nest building

3.1 Introduction

South Africa has one of the largest combination antiretroviral treatment (cART[1]) programs in the world with an estimated 7.52 million individuals having lived with human immunodeficiency virus (HIV[2]) at the end of 2018 (Vagiri et al., 2018). In 2016, South Africa introduced a new treat-all approach, providing cART to all HIV-positive individuals, including pregnant women, regardless of CD4[3] count (Maughan-Brown et al., 2018). With this in mind, prevention of mother-to child transmission (PMTCT[4]) strategies reduced the mother-to-child—or vertical—HIV transmission rate to below two percent (Chi et al., 2013).

Although cART successfully reduces the HIV viral load, it is known to have HIV-unrelated adverse effects, i.e. oxidative stress (M Gallego-Escuredo et al., 2010) and proinflammatory responses (Akay et al., 2014; Kaplan et al., 2015; Manda et al., 2011). In turn, oxidative stress and central nervous system (CNS[5]) proinflammatory responses, either alone or presenting concurrently, have been associated with the manifestation, altered prognosis and poor treatment response of psychiatric conditions, e.g. bipolar disorder (BD[6]) and major depressive disorder (MDD[7]) (de Sousa et al., 2014; Lindqvist et al., 2017; Ng et al., 2008). A common mechanism underlying such associations may be related to neurotoxicity and neuroinflammation induced by the nucleoside reverse transcriptase inhibitors (NRTIs[8]); however, this still needs investigation. Moreover, long-term cART can induce oxidative stress and modify central synaptophysin levels which can result in subsequent neuronal death (Manda et al., 2011). These findings are informative, especially since the use of cART has been associated with neuropsychiatric symptomology, including mania, delusions, impulsivity, depression, post-traumatic stress disorder (PTSD)[9], and compulsivity (Cespedes and Aberg, 2006; Hauptman and Carchedi, 2013; Hawkins et al., 2005).

Against this background, infants born HIV-uninfected but that were exposed to both HIV and cART in-utero (iHEU)[10] and perinatally, i.e. the weeks immediately before and after birth, may be especially at risk. Observational studies indicate that some of these children progress to present with increased morbidities, including neuropsychological difficulties (Ajaykumar, 2019; Cespedes and Aberg, 2006). Indeed, considering the neurodevelopmental window, during which neurulation, cell proliferation, axonal

[1] combination antiretroviral treatment
[2] human immunodeficiency virus
[3] cluster of differentiation antigen 4
[4] prevention of mother-to-child transmission
[5] central nervous system
[6] bipolar disorder
[7] major depressive disorder
[8] nucleoside reverse transcriptase inhibitors
[9] post-traumatic stress disorder
[10] HIV-uninfected, but HIV/cART exposed infants

outgrowth and synaptogenesis occur (Lawson et al., 2016; Nelson, 2002), it is likely that infants of mothers that were treated with cART[1] throughout pregnancy and the breast-feeding period, may be at risk for developing biobehavioral complications during the later stages of life. However, the extent to which perinatal cART exposure may result in long-term biobehavioral aberrations remains to be established, since the long-term study of children born from HIV[2]-positive mothers, remains problematic.

Therefore, the broad aim of this work was to investigate potential cART (i.e. a combination of tenofovir and emtricitabine)-induced biobehavioral alterations, as it pertains to oxidative stress (as measured by reduced to oxidized glutathione ratio; GSH/GSSG[3]), immune signaling (as reflected by both pro-inflammatory and anti-inflammatory cytokines), and behavioral expression over the different developmental phases of life in an uninfected and validated naturalistic animal model of a psychiatric phenotype, i.e. compulsive-like behavior as expressed by deer mice *(Peromyscus maniculatus bairdii)*. This model system was chosen for this purpose, since a subpopulation, i.e. 30 – 35 %, of deer mice, irrespective of sex, naturalistically express persistent and repetitive large nest building (LNB[4]) behavior (Wolmarans de et al., 2016). Thus, by comparing the biobehavioral profile of normal nesting (NNB[5]) and LNB expressing animals that were perinatally exposed to either normal water or cART, the immediate and long-term effects of said exposure in a standardized normal and compulsive-like cohort, can be explored. The GSH/GSSG ratio was chosen as a marker of oxidative stress, since perturbations in this system has been shown in clinical obsessive-compulsive disorder (OCD[6]); (Mih et al., 2021) and in deer mice presenting with another compulsive-like phenotype, i.e. high motor stereotypy, before (Güldenpfennig et al., 2011). It was thus of value to expand on these findings in the present investigation. In terms of inflammatory profile, we highlighted interleukin (IL[7])-1β, IL-6, IL-17, IL-10, tumor necrosis factor-alpha (TNF-α[8]), and interferon-gamma (IFN-γ[9]) for investigation, as these have variably been implicated both in clinical OCD (Denys et al., 2004; Konuk et al., 2007; Rao et al., 2015; Rodríguez et al., 2017; Sivri et al., 2018) and in preclinical models of the disorder (Rodríguez et al., 2017; Sahbaz et al., 2020; Troyer et al., 2021; Turna et al., 2016). Briefly, we hypothesize that perinatal cART exposure will induce oxidative stress and pro-inflammatory signaling in female and pregnant deer mice, as well as in their respective 3- and 14-week-old offspring. Given the reported associations

[1] combination antiretroviral treatment
[2] human immunodeficiency virus
[3] reduced to oxidized glutathione ratio
[4] large nest building
[5] normal nest building
[6] obsessive-compulsive disorder
[7] interleukin
[8] tumor necrosis factor-alpha
[9] interferon-gamma

between such aberrations and neuropsychiatric symptomology as alluded to above, we further expect that perinatal cART[1] exposure would associate with an inflated and exacerbated degree of LNB[2] expression later in life as compared to the behavior of the control-exposed cohort.

Considering the overall shortage of data pertaining to the immediate and long-term effects of cART in iHEU[3] on the biobehavioral trajectory, this study may significantly contribute to our understanding of cART-related effects in exposed offspring—especially since a similar approach is difficult to implement in clinical studies. Also, attempts to accurately delineate the respective and potentially specific contributions of HIV[4] and cART on the long-term health outcomes in humans are generally unethical, as mothers diagnosed with HIV should receive cART treatment.

3.2 Materials and methods

3.2.1 Study layout

To investigate and elucidate the influence of cART exposure on markers of whole-brain oxidative stress and inflammation in both non-pregnant and pregnant adult female deer mice and their 3- and 14-week-old offspring, 60 female deer mice (age 13 weeks) were divided into 6 groups (n = 10 per group; **Fig 3-1A, i – vi**).

- Groups (**i**) and (**ii**) received either normal water (**i**; mock-exposed) or cART (**ii**) for 21 days without having been paired with male animals. These animals were not assessed for nesting expression and were euthanized at the age of 15 weeks after said exposure.
- Groups (**iii**) and (i**v**) were exposed to either normal water (**iii**; mock-exposed) or cART (i**v**). Exposure began 3 weeks prior to pairing and continued throughout pregnancy and nursing. Both dams and the 3-week-old pups were euthanized after the nursing period. Dams were approximately 21 weeks old when they were euthanized. These animals were not assessed for nesting expression.
- Groups (**v**) and (**vi**) were exposed to either normal water (**v**; mock-exposed) or cART (**vi**). Exposure began 3 weeks prior to pairing and continued throughout pregnancy and nursing. Dams (now approximately 21 weeks old) were euthanized after the nursing period. However. the pups borne from these groups (water-exposed: *n* = 36; cART-exposed: *n* = 38) were group-raised under standard laboratory conditions (4 – 6 same perinatal exposure, same-sex offspring

[1] combination antiretroviral treatment
[2] large nest building
[3] HIV-uninfected, but HIV/cART exposed infants
[4] human immunodeficiency virus

per cage) until the age of 13 weeks, at which time they were first assessed for nesting expression over one week (**Fig 3-1B**), and then euthanized at the age of 14 weeks.

3.2.2 Animals

One hundred (100) deer mice (*Peromyscus maniculatus bairdii*; 60 female; 40 male; aged 13 weeks at the onset of the experimentation) were randomly selected from the housing colony at the North-West University (NWU[1]) vivarium, Potchefstroom, South Africa (SAVC[2] registration number: FR15/13458; SANAS[3] GLP[4] compliance number: G0019; AAALAC[5] accreditation file: 1717), without litter or cage bias. All experimental procedures were carried out in the same facility (ethical approval number: **NWU-00524-20-A5**; AnimCare Research Ethics Committee, NWU) and complied with the South African National Standard (SANS[6]) for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008). Before experimentation commenced, all animals were group-housed in same sex cages [35 cm (l) x 20 cm (w) x 13 cm (h); Techniplast® S.P.A., Varese, Italy; 4 – 6 animals per cage]. Cages were automatically climate-controlled at a temperature of 23 °C and a relative humidity of 50 %. A normal 12-hour light/dark cycle (on at 06:00, off at 18:00) was used. Food and water (or cART[7] solutions when applicable) were provided ad libitum throughout the investigation, and cages were cleaned, and fresh corncob supplied weekly between 08:00 and 10:00 (Wolmarans et al., 2016), except if stated otherwise (e.g. during the nesting investigation). Environmental enrichment was provided in the form of paper towel and a small white polyvinyl chloride pipe [10 cm (l); Ø = 4 cm].

3.2.3 Drug exposure

As explained in paragraph 3.2.1, the 60 female deer mice were randomized into three groups (n = 20 per group). Each group was further subdivided into a normal water- (control) and cART-exposed group, each being euthanized at various time points (**Fig 3-1**).

Tenofovir disoproxil fumarate (TDF[8] 60 mg/kg/day) and emtricitabine (FTC[9] 40 mg/kg/day; both sourced from Aspen Pharmacare®, South Africa; Nair and Jacob (2016)) were prepared for simultaneous oral administration via the drinking water. The concentrations of the drugs in solution were calculated based

[1] North-west University
[2] South African Veterinary Council
[3] South African National Accreditation System
[4] Good Laboratory Practice
[5] Association for Accreditation of Laboratory Animal Care
[6] South African National Standard
[7] combination antiretroviral treatment
[8] tenofovir disoproxil fumarate
[9] emtricitabine

on the average fluid consumption of deer mice (0.25 mL/g/day; De Brouwer et al. (2020)) to deliver the desired daily dose. Further, the fluid consumption of both water- and drug-exposed deer mice were measured every day to confirm drug intake (supplementary data; **Addendum A**). Oral administration via drinking water, as opposed to intraperitoneal injection or oral gavage, is the preferred administration route in deer mice given the long duration of exposure. Importantly, unpaired female mice (Groups **i** & **ii**; **Fig 3-1**) were exposed for a 3-week period only (ages 13 – 15 weeks). All other females were exposed from 3 weeks prior to mating (weeks 13 – 15) and throughout pregnancy and nursing (weeks 16 – 21).

3.2.4 Nest building analysis and cohort selection

To explore the long-term effect of perinatal cART[1] exposure on the offspring of exposed dams, all adult, 13-week-old offspring of the Group **v** & **vi** dams (**Figs 3-1 & 3-2**; 74 in total) were screened for nesting behavior, and the NNB[2] and LNB[3] expressing animals selected (see below). Nesting expression varies between consecutive days and thus each animal underwent nesting assessment over a 7-day period; during this time, animals were single-housed until they were euthanized at the end of nesting assessment (De Brouwer et al., 2020). Each day, an excess of pre-weighed, unscented cosmetic cotton wool was supplied in the roof of each housing cage, the built nests were removed, and the remaining cotton wool weighed between 7:00 and 8:00. Thus, mice had access to the nesting material for more than 23 hours of each day. After completion of the 7-day nesting screen, the daily quantity of cotton wool that was utilised by each animal (in grams) was added and a 7-day total nesting score (also in grams) calculated for each subject (Wolmarans et al., 2016). Animals included in the LNB cohorts (**Figs 3-1B & 3-2**; n = 8 per perinatal exposure cohort) were those that expressed nesting behavior of which the total nesting score broadly clustered within the upper 75th percentile of the average total nesting score distribution. However, we also selected LNB mice based on the persistence with which they engaged in nesting behavior. Therefore, LNB animals also expressed daily nesting behavior of which the inter-day variance clustered within or as close as possible to the lower 25th percentile of the variance distribution. Conversely, animals selected for NNB behavior (n = 8 per perinatal exposure cohort; **Figs 3-1B & 3-2**), i.e. the behavioral control, were those that engaged in smaller nest building behavior, the scores of which clustered between the 25th and 50th percentile of the total nesting score distribution, but also with minimal variation. Animals not selected for inclusion in the group of 32 experimental adult offspring, i.e. the remaining 42 mice, were euthanized without being used for sample

[1] combination antiretroviral treatment
[2] normal nest building
[3] large nest building

collection. During periods of nest building analysis, food and water were available as normal, although animals were not provided with any additional form of nesting material.

3.2.5 Sample collection

Brain tissues were collected as follows: With respect to the adult non-pregnant and pregnant female mice, brain tissues were collected from all 40 animals in Groups **i** – **iv** and stored individually. With respect to the 3-week-old offspring, i.e. ten litters per exposure cohort (pups from **v** & **vi** dams; ten dams per cohort) were sacrificed. However, for these samples, two brains per litter, one male and one female, were pooled to yield a single sample, and as such, ten samples were collected per exposure cohort. This was necessary since the amount of tissue from a single 3-week-old pup was not sufficient to complete all analyses. With respect to the adult offspring, brain tissues were collected only from mice that were selected for NNB[1] and LNB[2] in the two respective exposure cohorts. Prior to euthanization, all animals were anesthetized by means of isoflurane inhalation and euthanized via rapid guillotine decapitation. Whole-brain tissues were immediately removed on an ice-cooled slab, halved lengthwise between the left and right hemispheres for GSH/GSSG[3] and cytokine analysis respectively, enclosed in 2 mL Eppendorf® tubes and snap-frozen in liquid nitrogen. Samples were stored at -80 ºC until analysis.

3.2.6 Chemicals and reagents

For the determination of the GSH/GSSG ratio, GSH[4], GSSG[5] and ethyl-4-hydroxy-2-quinolinecarboxylate (EHQC[6]; as internal standard) were obtained from Merck® (Johannesburg, South Africa). Reagents used for the mobile phase were liquid chromatography/ mass spectrometry (LC/MS[7]) grade deionized water, LC/MS grade methanol (MeOH)[8], and formic acid (99 %), while those used for the preparation of standards and samples were LC/MS grade MeOH, acetonitrile (ACN)[9], glacial acetic acid (99 %), and formic acid (99 %). Apart from the LC/MS grade water, all of these were also obtained from Merck®. For cytokine analysis, all reagents were included in the respective sample preparation and assay kits (Bio-Rad Laboratories® Inc., USA).

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[1] normal nest building
[2] large nest building
[3] reduced to oxidized glutathione ratio
[4] reduced glutathione
[5] oxidized glutathione
[6] ethyl-4-hydroxy-2-quinolinecarboxylate
[7] liquid chromatography/ mass spectrometry
[8] methanol
[9] acetonitrile

3.2.7 Oxidative stress: GSH/GSSG quantification

Redox status, as a measure of oxidative stress, was quantified from the GSH/GSSG[1] ratio. Briefly, we applied the method of Güldenpfennig et al. (2011) with slight modification. Immediately prior to analysis by means of a liquid chromatography/tandem mass spectrometry (LC/MS/MS[2]) method, brain tissue was allowed to thaw on ice, weighed and placed into a 1.5 mL Eppendorf tube. 1000 µL of the internal standard solution, i.e. 10 % EHQC[3] in a solvent mixture of 0.1 M formic acid in 100 % acetonitrile, was added to the samples. Subsequently, tissues were homogenized by means of sonification (twice for 12 sec, at an amplitude of 14 µ; MSE® ultrasonic disintegrator, Nuaillé, France). Mixtures were left on ice for 20 min to complete protein precipitation and centrifuged at 20 817 RCF for 20 min at 4 ºC. Chromatographic separation was achieved on a Venusil ASB C18 analytical column (Agela® Technologies, Torrance, CA, USA, 2.1 x 150 mm, particle Ø = 3 µm). Standards solutions were constituted by dissolving approximately 1 mg of each analyte separately in 10 mL of a 10 % methanol solution. From these stock solutions, serial dilution series were prepared to construct a standard calibration curve and determine the linear range of each metabolite. 1 µL of the standard dilutions and supernatants were injected onto the LC/MS/MS system (Ultivo® Triple Quadrupole LC/MS/MS system controlled by MassHunter™ software; Agilent Technologies, Inc., Santa Clara, CA, 95051 US) for analysis. Results were converted from ng/mL to a final result of ng/g of the wet brain tissue weight. Data were subsequently expressed as nmol/mg wet tissue, with the glutathione redox index calculated as [(GSH)+2(GSSG) / 2(GSSG) × 100] (Benzi and Moretti, 1995; Benzi et al., 1989).

3.2.8 Inflammatory signaling cytokine quantification

We analyzed both pro- (IL[4]-1β, IL-6, IL-17, IFN-γ[5] and TNF-α[6]) and anti-inflammatory (IL-10) cytokines via multiplex immunoassay (Bio-Plex Pro Mouse TH17 Cytokine Panel assayed on a Bio-Plex 200 Suspension Array System, Bio-Rad Laboratories® Inc., USA). Briefly, brain tissue samples were prepared using a Bio-Plex cell lysis kit (Bio-Rad Laboratories® Inc., USA), according to the manufacturer's instructions (supplementary methods; **Addendum A**) followed by total protein determination by means of Bradford (Güldenpfennig et al., 2011). A 5-parameter logistic regression formula was used to calculate the sample concentrations from the standard curves. The sensitivity of the panel was high (pg/mL). For any samples that had cytokine values below the limit of detection, half

[1] reduced to oxidized glutathione ratio
[2] liquid chromatography/tandem mass spectrometry
[3] ethyl-4-hydroxy-2-quinolinecarboxylate
[4] interleukin
[5] interferon-gamma
[6] tumor necrosis factor-alpha

of the lowest detectable value for that specific cytokine was assigned. For samples with values above the detection limit, the highest-detectable value was assigned. Six randomly selected samples were run across all the plates as inter-plate controls. Six more randomly selected samples were duplicated on each set of plates as intra-plate controls. A Spearman's rank order correlation was conducted to calculate the respective intra- and inter-assay correlation coefficients so as to confirm assay reliability and reproducibility.

3.2.9 Statistical analysis

All statistical analyses were performed with GraphPad® Prism® version 9.3 (GraphPad®, San Diego, California). Differences between brain markers of oxidative stress and inflammation in the respective water and cART[1]-exposed female and offspring cohorts were analyzed by means of ordinary two-way analysis of variance (2-way ANOVA[2]). The respective parameters measured were set dependent factors, while perinatal exposure, pregnancy state, age, and nesting cohort were set as independent variables, depending on the analysis performed. 2-way ANOVA analyses were followed up by Bonferroni's post-hoc pairwise comparisons, depending on the respective interaction and main effects. Pairwise differences were also explored for magnitude by means of Cohen's *d* effect size calculation and comparisons were informed by the 95% confidence interval of *d* (95CI). Only large effects sizes ($d \geq 0.8$) are shown. Spearman's rank-order correlations were used to assess the relationships between the individual total nesting scores generated by the 36 water- and 38 cART-exposed adult offspring, respectively, and the associated coefficients of variance calculated with respect to the daily nesting scores. Nesting cohort selection was broadly based on the 75th percentile of the individual total nesting score distribution (largest scores: LNB) and the lower 25th percentile of the coefficients of variance distribution (lower scores: applied to both cohorts). Last, within nesting cohort pairwise comparisons of the total nesting scores generated by NNB and LNB animals exposed to either normal water or cART, respectively, were done by means of Mann-Whitney *U*-tests. Statistical significance was set at $p < 0.05$ for all analyses.

3.3 Results

Due to the extent of the analyses performed, all descriptive data and statistical descriptors of the results pertaining to cytokine data are indicated in **Tables 3-1 & 3-2**. Corresponding figure numbers are indicated in bold. In all instances, *n* values are provided in the figure captions.

[1] combination antiretroviral treatment
[2] analysis of variance

3.3.1 Effect of cART exposure in non-pregnant and pregnant dams

3.3.1.1 GSH/GSSG: Figure 3-3A

Comparing the whole-brain GSH/GSSG[1] ratio of non-pregnant and pregnant dams, two-way ANOVA[2] did not reveal a significant drug-pregnancy interaction [$F(1,35) = 0.02$, $p = 0.898$] (**Fig 3-3A**). However, significant main effects of drug exposure [$F(1,35) = 9.01$, $p = 0.005$] and pregnancy state [$F(1,35) = 16.55$, $p = 0.0003$] were shown. Subsequent Bonferroni's multiple comparisons tests revealed the GSH/GSSG ratio of pregnant dams exposed to either control- or cART[3]-intervention to be significantly lower compared to their respective non-pregnant conspecifics (**control:** 0.052 ± 0.019 vs. 0.034 ± 0.012, 95CI: 0.0039 – 0.031, $p = 0.0097$, $d = 1.10$; **cART:** 0.039 ± 0.009 vs. 0.022 ± 0.009; 95CI: 0.0024 – 0.03, $p = 0.019$, $d = 1.83$). Further, although narrowly missing statistical significance (non-pregnant dams: $p = 0.062$; pregnant dams: $p = 0.106$), cART tended to decrease the GSH/GSSG ratio in both cohorts of dams to a greater extent, compared to pregnancy state alone (non-pregnant dams: $d = 0.88$; pregnant dams: $d = 1.13$).

3.3.1.2 Cytokine expression: Figure 3-4, Table 3-1(i)

Except for IL[4]-10 (**row C**), no significant two-way interactions between pregnancy state and drug exposure were shown for any of the cytokines measured. However, both pregnancy state and drug exposure had significant main effects on most of the cytokine levels measured, except for IL-6 (**row B**) and IL-10. With respect to IL-6, only drug exposure had a main effect on the result, while neither pregnancy state, nor drug exposure significantly affected the IL-10 concentrations measured.

Subsequent pairwise comparisons revealed significant reductions in the whole-brain IL-1β (**row A; Fig 3-3A**), IL-6 (**row B; Fig 3-3B**), and IL-10 (**row C; Fig 3-3C**) concentrations of pregnant cART- vs. control-exposed dams. Further, in non-pregnant dams, both IFN-γ[5] (**row E; Fig 3-3E**) and TNF-α (**row F; Fig 3-3F**), were lower in cART- vs. control-exposed dams. With respect to within-drug-exposure comparisons, pregnant, control-exposed dams presented with significantly lower IL-β and TNF-α[6], but higher IL-10 concentrations compared to their non-pregnant counterparts. IL- β were also significantly decreased in cART-exposed pregnant vs. non-pregnant dams.

[1] reduced to oxidized glutathione ratio
[2] analysis of variance
[3] combination antiretroviral treatment
[4] interleukin
[5] interferon-gamma
[6] tumor necrosis factor-alpha

3.3.2 Effect of perinatal cART exposure in 3- and 14-week-old offspring irrespective of nesting cohort

3.3.2.1 GSH/GSSG: 3- and 14-week-old offspring; Figure 3-3B

No significant two-way drug-age interaction [$F(1,46) = 0.34$, $p = 0.561$] (**Fig 3-3B**) was demonstrated for the whole-brain GSH/GSSG[1] ratios measured in 3- and 14-week old offspring. Further, neither drug exposure [$F(1,46) = 0.53$, $p = 0.471$], nor age [$F(1,46) = 2.32$, $p = 0.135$], significantly influenced the data reported here.

3.3.2.2 Cytokines: 3- and 14-week-old offspring; Figure 3-5; Table 3-1(ii)

With respect to the cytokines measured in 3- and 14-week-old offspring, no significant two-way drug-age interactions were shown for any of the analytes measured. However, age significantly impacted IL[2]-6 (**row B**), IL-10 (**row C**), IL-17 (**row D**), and IFN-γ[3] (**row E**) results, while drug exposure also had a significant main effect on IL-10 and IL-17 concentrations.

Bonferroni post-hoc comparisons revealed cART[4]-exposed 14-week-old offspring to present with significantly lower whole-brain IL-10 (**row C; Fig 3-5C**), but higher IL-17 (**row D, Fig 3-5D**) and IFN- γ (**row E; Fig 3-5E**) concentrations.

3.3.3 Effect of perinatal cART exposure in 14-week-old NNB and LNB-expressing offspring

3.3.3.1 Nesting expression; Figures 3-2 & 3-6

Nesting behavior was assessed in 36 control-exposed (**Fig 3-2A**) and 38 cART-exposed (**Fig 3-2B**) 13-week-old offspring, respectively. Significant negative linear relationships between the total nesting scores (in g) and the percentage coefficient of variance with respect to the daily nesting scores (in g) for both cohorts (**control:** $R^2 = 0.30$, $p = 0.0005$, regression equation: Y = -0.1426 (X) + 17.75; **cART:** $R^2 = 0.16$, $p = 0.013$, regression equation: Y = -0.1149 (X) + 15.73). The slopes that represent the behavioral expression of the two respective cohorts, did not differ significantly ($p = 0.65$). The upper 75th percentile of the total nesting score distribution was 13.57 g for control-exposed, and 13.32 g for

[1] reduced to oxidized glutathione ratio
[2] interleukin
[3] interferon-gamma
[4] combination antiretroviral treatment

cART[1]-exposed offspring. The lower 25th percentile of the percentage coefficient of variance was 39.37 % for control-exposed, and 36.41 % for cART-exposed offspring.

From this data, eight NNB[2] and LNB[3]-expressing mice from each exposure cohort were selected for further analysis of behavioral and biological data. Selections were made irrespective of sex, by considering only the total nesting score generated after one week of assessment, and the persistence of nesting behavior as determined by the percentage coefficient of variance (refer to **paragraphs 3.2.4, 3.2.5 and & 3.2.9** for detail). Final groups were constituted as follows: control-exposed NNB: 3 male, 5 female; control-exposed LNB: 6 male, 2 female; cART-exposed NNB: 5 male, 3 female; cART-exposed LNB: 6 male, 2 female.

Pairwise comparisons of the total nesting scores generated by control- and cART-exposed NNB (**Fig 3-6A**) and LNB-expressing offspring (**Fig 3-6B**) respectively, revealed a significant difference between the median nesting scores of water- and cART-exposed LNB- (U = 13, p = 0.0499), but not NNB-expressing animals (U = 24, p = 0.442).

3.3.3.2 GSH/GSSG: 14-week-old NNB vs. LNB offspring; Figure 3-3C

Two-way ANOVA[4] revealed a significant drug-phenotype interaction [$F(1,28) = 4.76$, $p = 0.038$] (**Fig 3-3C**) in terms of the whole-brain GSH/GSSG[5] ratios measured in the two nesting cohorts. However, neither drug exposure [$F(1,28) = 0.48$, $p = 0.494$], nor phenotype [$F(1,28) = 1.22$, $p = 0.28$], had a significant main effect on the reported ratio. Also, Bonferroni post-hoc tests did not reveal any significant pairwise differences with respect to either drug-exposure or phenotype comparisons.

3.3.3.3 Cytokines: 14-week-old NNB vs. LNB offspring; Figure 3-7; Table 3-2

Significant two-way interactions between drug exposure and nesting phenotype were shown for all the cytokines measured, except for IFN-γ[6] (**row E**). Further, both drug exposure and nesting phenotype significantly impacted whole-brain IL[7]-6 (**row B**), IL-10 (**row C**), whereas only drug exposure significantly impacted whole-brain IL-17 (**row D**) concentrations.

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[1] combination antiretroviral treatment
[2] normal nest building
[3] large nest building
[4] analysis of variance
[5] reduced to oxidized glutathione ratio
[6] interferon-gamma
[7] interleukin

With respect to pairwise comparisons, cART[1]-exposed NNB[2]-expressing offspring presented with significantly higher IL[3]-6 (**row B; Fig 3-7B**), IL-10 (**row C; Fig 3-7C**), and IL-17 (**row D; Fig 3-7D**) concentrations compared to their control-exposed conspecifics. Also, cART exposed LNB[4]-expressing offspring showed significantly lower IL-1β **(row A, Fig 3-7A**), IL-6, IL-10, IL-17, and TNF-α[5] (**row F; Fig 3-7F**) concentrations compared to cART-exposed NNB-expressing mice. No significant differences were shown with respect to any of the cytokines measured between control-exposed NNB- and LNB-expressing offspring, or between cART-exposed LNB-expressing offspring and their control-exposed counterparts of either nesting cohort.

3.4 Discussion

Considering that cART exposure is known to cause oxidative stress and immune aberrations in both HIV-positive mothers and their iHEU[6] children, and that such changes are often known to associate with neuropsychiatric illness, a better understanding of how such exposure can influence the biobehavioral development of children over the course of their developmental trajectory is necessary. However, such studies in human populations are difficult. Therefore, we aimed to explore the short- and long-term effects of perinatal cART exposure in a model of a naturalistically developing psychiatric-like phenotype, i.e. compulsive-like LNB expressed by deer mice. Our main findings are that 1) both pregnancy and cART significantly decreased the whole-brain GSH/GSSG[7] ratios in dams, 2) cART-, compared to control-exposure, varyingly decreased the concentrations of most cytokines measured in non-pregnant and pregnant dams, 3) differences in cytokine expression between control- and cART-exposed offspring only became apparent by the age of 14 weeks, in that cART-exposed offspring presented with significantly lower IL-10, but higher IL-17 and IFN-γ[8] concentrations than their age-matched control-exposed counterparts, 4) that cART-, compared to control-exposed LNB-expressing animals, generated lower total nesting scores, and 5) that the whole-brain concentrations of most cytokines were higher in cART-exposed 14-week-old NNB-, compared to LNB-expressing offspring.

[1] combination antiretroviral treatment
[2] normal nest building
[3] interleukin
[4] large nest building
[5] tumor necrosis factor-alpha
[6] HIV-uninfected, but HIV/cART exposed infants
[7] reduced to oxidized glutathione ratio
[8] interferon-gamma

3.4.1 Pregnancy, cART exposure and oxidative stress

The first finding of this investigation is that both pregnancy and cART[1] exposure, is associated with an imbalance in the glutathione system, as shown by a decreased GSH/GSSG[2] ratio, a relevant clinical indicator of cellular redox potential and oxidative stress (Owen and Butterfield, 2010). Further, although narrowly missing statistical significance (**Fig 3-3A**), cART exposure tended to have a greater impact on the pro-oxidant state of pregnant dams, compared to pregnancy alone. These data were expected, given similar results reported from clinical studies into the pro-oxidant effects of both pregnancy (Pereira and Martel, 2014; Samir et al., 2018; Toboła-Wróbel et al., 2020) and NRTI[3]- and protease inhibitor (PIs[4])-treatment (JareÑo et al., 2002; Sharma, 2014). Pregnancy itself contributes to oxidative stress (Casanueva and Viteri, 2003; Page, 1993; Sies, 2015), by increasing the basal rate of oxygen consumption and altering energy substrate use in the various organs, for example the fetoplacental unit (Casanueva and Viteri, 2003). Since the placenta is extensively perfused with blood and rich in mitochondria, it facilitates effective exchange of gases and metabolic products. As the pregnancy advances and the placenta matures, this oxygen- and mitochondria-rich environment becomes more mature, accelerating the production of reactive oxygen species (ROS[5]) (Liochev and Fridovich, 1997). In terms of the observed effect of cART exposure, it has been shown to influence the activity of glutathione peroxidase (GSH-Px[6]) and glutathione reductase (GSH -RD[7]), two enzymes that play a pivotal role in the glutathione redox cycle (Sharma, 2014); if not functioning optimally, increased concentrations of ROS, leading to higher levels of oxidative stress, are seen. Oxidative stress is a known correlate for NRTI-induced peripheral toxicity in that prolonged exposure to cART can induce oxidative stress via sustained neuronal ROS accumulation (Akay et al., 2014). Further, studies also hint that prolonged cART exposure may compromise the actions of astrocytes, cells that normally buffer ROS accumulation in the neurons of the CNS[8]. However, under conditions of high ROS generation, said protective mechanisms fail.

Remarkably, both 3- and 14-week-old offspring were protected from these influences, with pups presenting with similar GSH/GSSG ratios, irrespective of age or exposure cohort (**Fig 3-3B**). Nevertheless, an interesting observation of this work is that the mean GSH/GSSG scores shown by the offspring in this investigation, were similar to the ratios observed in pregnant and cART-exposed dams

[1] combination antiretroviral treatment
[2] reduced to oxidized glutathione ratio
[3] nucleoside reverse transcriptase inhibitors
[4] protease inhibitors
[5] reactive oxygen species
[6] glutathione peroxidase
[7] glutathione reductase
[8] central nervous system

(≈ 0.02) than to that of control-exposed non-pregnant dams (≈ 0.05), potentially pointing to a more effective glutathione redox system in adult female mice. This finding is interesting and warrants further investigation. Our finding that adult NNB[1]- and LNB[2]-expressing mice present with similar GSH/GSSG[3] ratios are vitally important, since we have demonstrated perturbations in this ratio in high stereotypical (H[4]) deer mice before (Güldenpfennig et al., 2011). H behavior is another proposed compulsive-like phenotype expressed by deer mice (De Brouwer et al., 2021). That LNB and H behavior differs in terms of its association with oxidative stress, is therefore informative for continuing efforts to explore unique neurobiological architectures of different clinical compulsive phenotypes.

3.4.2 Pregnancy, cART exposure and immune signaling

With respect to the pregnancy and cytokine expression, our results demonstrate that while pregnancy was associated with decreased concentrations of the proinflammatory cytokines, IL[5]-1β and TNF-α[6] and increased expression of anti-inflammatory IL-10, cART[7] broadly suppressed immune signaling, irrespective of pro- or anti-inflammatory molecule (**Fig 3-4A - F**). Although this effect was differentially observed in the pregnant and non-pregnant cohorts, it suffices to conclude that cART exposure had immunosuppressive effects in this model system. Optimal regulation of the innate and acquired immune responses is a vital component of a normal pregnancy, since it not only contributes to maintaining the pregnancy (Challis et al., 2009), but also protects the fetus from the effects of potential pathogens (Hoo et al., 2020; Semmes and Coyne, 2022). One mechanism by which the immune system is regulated, is by means of cytokine production. Cytokines differ in terms of their origin and functional roles, in that IL-1β, IL-6, IL-17, IFN-γ[8] and TNF-α, which are mainly produced by T-helper cell type 1 (Th1[9]) cell clones, promote inflammation and T-cell-mediated responses (Orsi and Tribe, 2008). Conversely, IL-10, which is produced by T-helper cell type 2 (Th2[10]), which modulates humoral, i.e. antibody, responses (Komai et al., 2018) and exerts potent anti-inflammatory action (Bazzoni et al., 2010; de Vries, 1995). During pregnancy, the activity equilibrium between Th1 and Th2 shifts in favor of Th2 activity, i.e. the so-called 'Th2 phenomenon' (Wegmann et al., 1993). This is important for the fetal-maternal relationship, as a shift towards Th1 can trigger inflammatory cytokine production that can be to the detriment of both

[1] normal nest building
[2] large nest building
[3] reduced to oxidized glutathione ratio
[4] high stereotypical
[5] interleukin
[6] tumor necrosis factor-alpha
[7] combination antiretroviral treatment
[8] interferon-gamma
[9] T helper cell type 1
[10] T-helper cell type 2

mother and fetus (Wegmann et al., 1993). This phenomenon explains the pattern of findings observed in non-pregnant vs. pregnant deer mice (**Fig 3-4A, C, &E**).

Our finding with respect to the broad immunosuppressive effect of cART[1] exposure in female and pregnant deer mice is highly informative. The overall reduction in cytokine expression in cART-exposed mice can possibly be explained by the immune modulatory properties of cART treatment, especially regimens that include tenofovir (Melchjorsen et al., 2011), which has been shown to negatively regulate inflammatory cytokines. However, in this work, we also highlight that cART exposure prevented the anti-inflammatory, i.e. IL[2]-10-related, response seen in pregnant control-exposed deer mice (**Fig 3-4C**). TDF[3] and FTC[4], both NRTIs[5], are included in HIV[6] pre-exposure prophylaxis (PrEP[7]) regimens, due to their three-tiered ability to prevent infection (Castillo-Mancilla et al., 2015), one mechanism of which is to prevent T-cell activation (which decreases the number of HIV-susceptible target cells; Card et al. (2013)) and the subsequent synthesis and release of pro-inflammatory cytokines. While our findings therefore corroborate such actions of TDF/FTC, further investigation is needed to explore the actions of said intervention on the expression of pro-inflammatory cytokines. In fact, the long-term effect of perinatal cART exposure (see below), may potentially rest on the manner in which cART dismantles the orthogonal relationship between pro- and anti-inflammatory signaling which is normally seen in pregnancy and that has been shown here.

Again, the effects of cART on immune signaling as observed in adult female and pregnant deer mice, were not observed in the 3-week-old offspring (**Fig 3-5A – F**), indicating that pups were largely isolated from the immunosuppressive effects of perinatal cART exposure. Nevertheless, our data revealed an interesting age-related phenomenon, whereby perinatal cART exposure associated with decreased IL-10 (**Fig 3-5C**) and increased IL-17 (**Fig 3-5D**) expression in 14-week-old vs. 3-week-old offspring. Further, control-exposed 14-week-old offspring presented with significantly increased IFN-γ[8] concentrations, compared to their younger counterparts. While perinatal cART exposure in iHEU[9] may therefore bolster pro-inflammatory immune signaling during the later stages of life, the age-related maturation and functioning of the immune system in iHEU, vs. non-exposed children, will require further elucidation. This is important, since a study by Afran et al. (2014) showed iHEU to present with

[1] combination antiretroviral treatment
[2] interleukin
[3] tenofovir disoproxil fumarate
[4] emtricitabine
[5] nucleoside reverse transcriptase inhibitor
[6] human immunodeficiency virus
[7] pre-exposure prophylaxis
[8] interferon-gamma
[9] HIV-uninfected, but HIV/cART exposed infants

significantly higher levels of IL[1]-17 at both a young and early age compared to the uninfected control groups. This is especially interesting with respect to the present model system (see below), since altered IL-17 concentrations have been associated with obsessive-compulsive disorder before (Özkan et al., 2021; Rodríguez et al., 2019; Şimşek et al., 2016).

In terms of the cytokine expression in 14-week-old NNB[2]- and LNB[3]-expressing offspring, our data revealed several important and valuable findings. First, control-exposed animals of the two cohorts showed similar cytokine concentrations across all measures (**Fig 3-7**), except with respect to IL-6 (**Fig 3-7B**), which although narrowly missing statistical significance (**Table 2**), trended towards being increased in LNB-, compared to NNB-expressing mice. Since this is, according to our knowledge, the first work to explore immune signaling in an entirely naturalistic model of compulsive-like behavior, the potential role of pro-inflammatory IL-6 in the manifestation of LNB, will need further investigation. Interestingly, we have previously shown that drug-naïve LNB-expressing animals present with a gut microbiota profile with a higher loading of potentially pro-inflammatory genera, i.e. *Peptococcus, Aestuariispira,* and *Desulfovermiculus.* Regarding the present and our earlier findings as a collective, a potential role for inflammatory processes in LNB is highlighted.

The present data with respect to perinatal cART[4] exposure on the later life biobehavioral profile of deer mice, are striking. Following from our hypothesis that said early-life exposure would fundamentally and permanently alter neurodevelopmental processes due to its effects on oxidative stress and proinflammatory responses, our findings reveal that NNB- and LNB-expressing animals were indeed uniquely affected by said intervention, a finding that is masked by the overall lack of effect of cART on the collapsed cohort of control- and cART-exposed 14-week-old offspring (**Fig 3-5**). First, cART exposure *blunted* the compulsive-like, but not normal behavioral expression of deer mice (**Fig 3-6B**), thus contradicting our hypothesis. Such attenuation of nesting scores was shown in parallel to a significant and robust reduction in most of the cytokines measured in cART-exposed LNB-, vs. NNB-expressing animals. We considered this finding to be representative of immune suppression in LNB, but not NNB animals. However, that the mean cytokine concentrations of cART-exposed animals did not differ from the collapsed control-exposed cohort, indicates that such a conclusion is invalid. Rather, it is possible that 'normal' deer mice (as defined against the background of behavioral expression), are more affected by cART, than the compulsive-like cohort. Thus, while it seems that LNB-expressing animals are not affected by cART as considered against the cytokine profiles of control-exposed animals, our conclusion is that perinatal cART exposure has pro-inflammatory effects in a generalized population,

[1] interleukin
[2] normal nest building
[3] large nest building
[4] combination antiretroviral treatment

which is masked in LNB[1] animals, due to potentially innate, naturalistic altered immune processes in LNB, compared to NNB[2] animals. It can therefore be argued that perinatal cART[3] exposure, albeit showing immunosuppressive properties in adult female mice, have long-lasting pro-inflammatory effects in iHEU[4] offspring, which is not observed in LNB animals due to a unique profile of brain-immune interactions underlying such behavior.

Another important aspect of this model system and the data reported here, needs highlighting. While the nesting scores of cART-exposed NNB-expressing animals did not show a significant increase compared to the water-exposed NNB-expressing cohort, an upwards trend with a medium effect size was observed (**Fig 3-6A;** $d = 0.44$). This is noteworthy, since robust and large increases in IL[5]-6, IL-10, and IL-17, all varyingly implicated in clinical OCD (GÖNENİR ERBAY et al., 2018), were seen in cART- vs. water -exposed NNB animals. It thus stands to reason that if nesting expression is regarded on a scale from very small to very large, as is the basis for the classification of NNB and LNB, a direct relationship between nesting intensity and cART-related pro-inflammatory immune aberrations may exist that is potentially masked in naturalistically expressing compulsive-like LNB animals. Unfortunately, the numbers of mice that could ultimately be included in the NNB and LNB groups respectively, were not sufficient to perform a correlational analysis to confirm this possibility. Further research is therefore imperative to explore and elucidate the present findings.

This work was not without some noteworthy shortcomings. First, an early-life measure of compulsive-like behavioral expression would be useful to link distinct patterns of early- and later-life cytokine expression to behavioral manifestation across both age groups. Second, it would have been highly informative if adult LNB-expressing were to be exposed to pro-inflammatory interventions, so as to determine whether a reversal in the cytokine profile measured, would result in inflated nesting expression to a level akin to that which was observed in the control-exposed LNB-expressing cohort. Future studies with this aim are being conceptualized. Third, since our NNB and LNB group sizes ($n = 8$ each) were not sufficient to draw direct correlations between cytokine concentrations and behavioral expression, this would have been useful. Last, it would have been informative if the female mice and dams have also been selected for inclusion in this work-based nesting expression. Such approach would have enabled both prospective and retrospective analyses of the relationship between oxidative stress, cytokine concentrations, and behavioral data. Given that the present model system is entirely naturalistic, such investigations will become increasingly important to expand our understanding of how

[1] large nest building
[2] normal nest building
[3] combination antiretroviral treatment
[4] HIV-uninfected, but HIV/cART exposed infants
[5] interleukin

paternal and maternal factors contribute to the biobehavioral profile of offspring. Sadly, this could not have been accomplished in the time that was available.

3.5 Conclusion

As it is difficult to distinguish between the effects of maternal cART[1] administration and the effects of HIV itself on the long-term development of affected children in a human cohort, this investigation was designed as a longitudinal exploration of the effects of cART on oxidative stress, immune signaling and behavioral expression in a naturalistic model of compulsive-like behavioral expression. We showed that perinatal cART exposure exacerbates oxidative stress and suppress immune signaling in pregnant vs non-pregnant mice. We further showed that cART-exposed NNB[2]- and LNB[3]-expressing offspring differ in terms of their immunological response to said exposure, with the long-term pro-inflammatory effects of cART exposure being masked in LNB-expressing offspring. Thus, we conclude that LNB uniquely associates with perturbed brain-immune cross-talk. Investigations using the deer mouse model of compulsive-like behavior and brain-immune interactions that will need careful consideration in future work.

3.5.1 Funding and disclosure

This investigation was jointly funded by institutional research funding awarded to DWW and HBJ.

[1] combination antiretroviral treatment
[2] normal nest building
[3] large nest building

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Figures

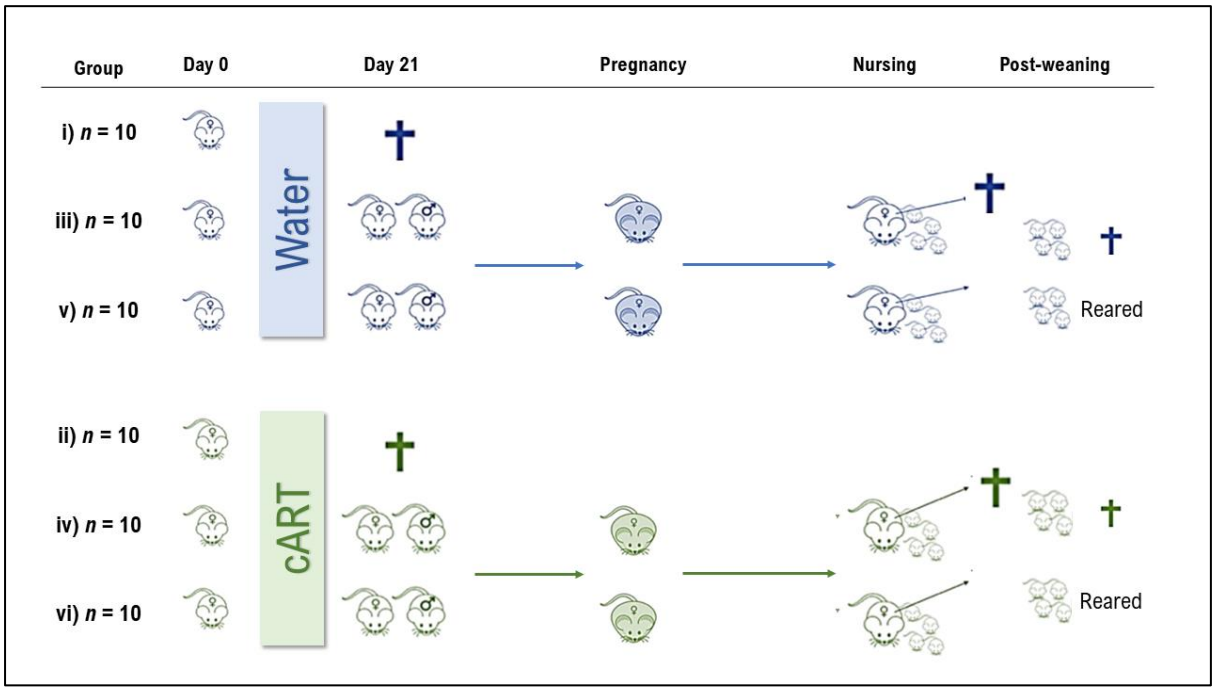

Figure 3-1A – Schematic layout of experimental design pertaining to female mice, dams, and 3-week-old offspring.
Groups i – vi are indicative of female deer mice, irrespective of nesting phenotype; Blue: water-exposed trajectory; Green: cART-exposed trajectory; crosses indicate rounds of decapitation; cART: combination antiretroviral treatment.

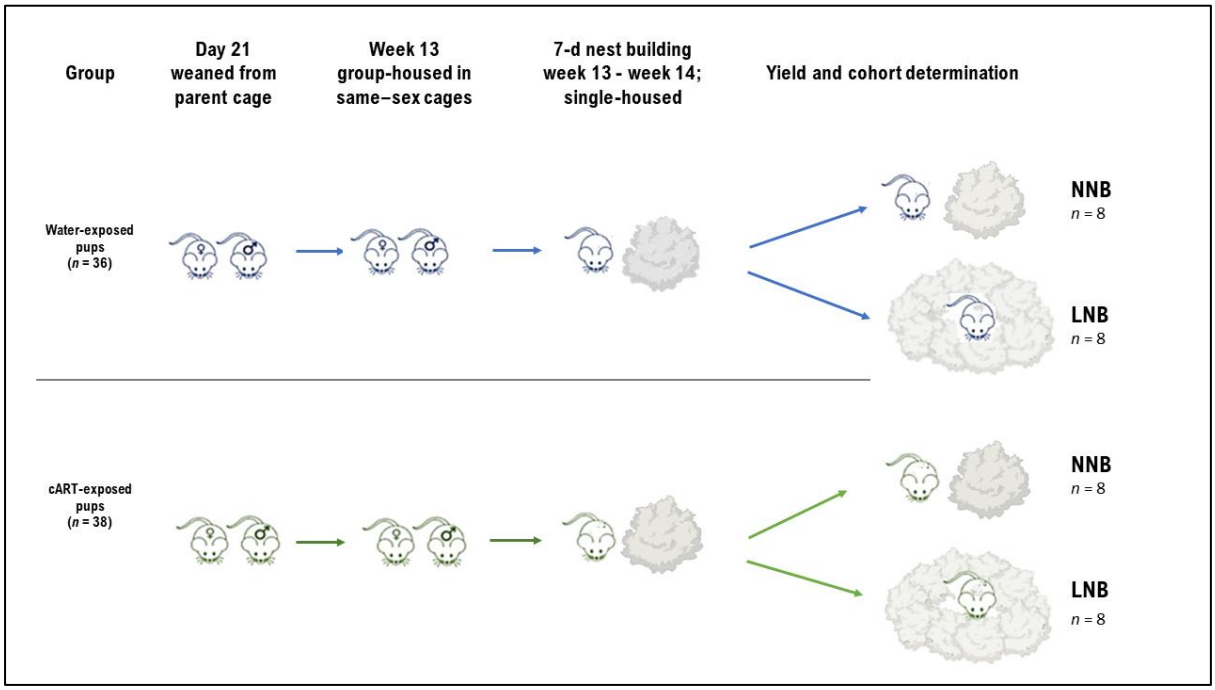

Figure 3-1B – Schematic layout of experimental design pertaining to reared, 14-week-old offspring.
Blue: offspring from dams perinatally exposed to water; Green: offspring from dams perinatally exposed to cART; NNB: normal nest building; LNB: large nest building; cART: combination antiretroviral treatment.

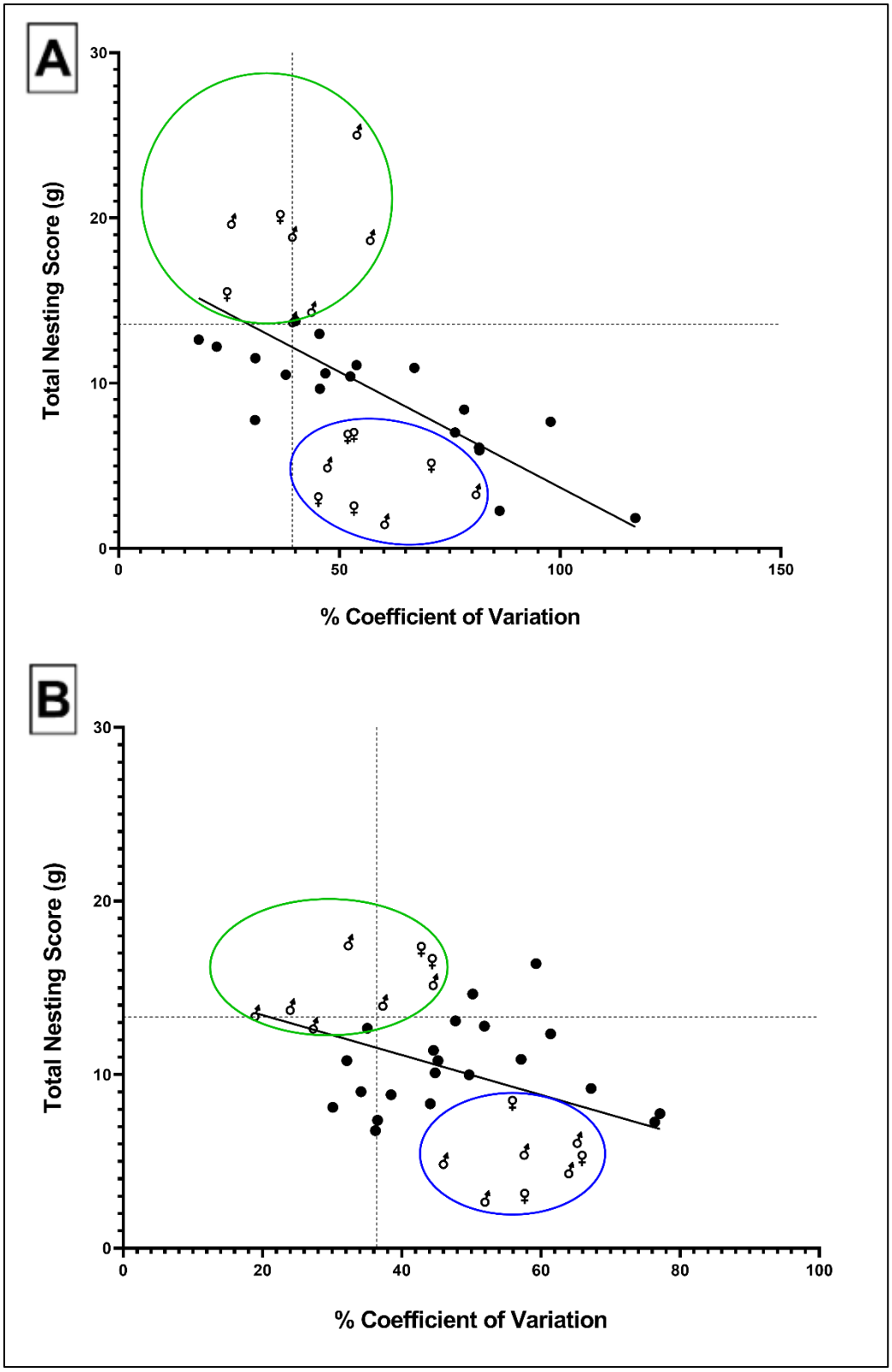

Figure 3-2 – NNB and LNB cohort selection in water- (A) and cART-exposed (B) adult offspring.
Blue ovals: animals selected for NNB; Green ovals: animals selected for LNB (n = 8 per exposure group and nesting cohort); Regression equations: (A) Y = -0.1426 × X + 17.75, p = 0.0005, R^2 = 0.303; (B) Y = -0.1149 × X + 15.73, p = 0.0133, R^2 = 0.163.

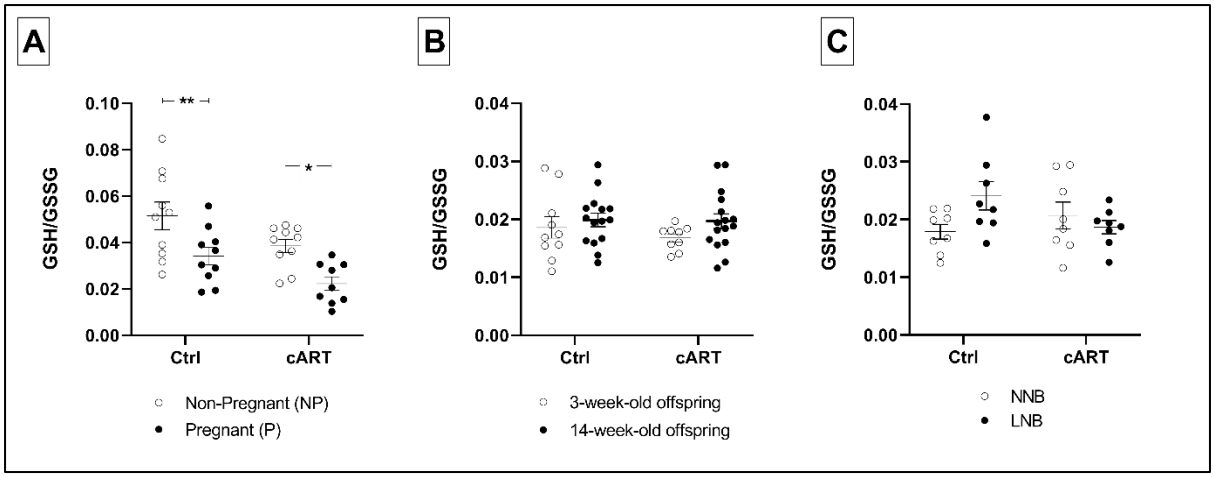

Figure 3-3 – Whole-brain GSH/GSSG ratios in (A) non-pregnant and pregnant deer mouse females, (B) 3- and 14-week-old offspring irrespective of nesting phenotype and (C) 14-week-old NNB- and LNB-expressing mice, that were perinatally exposed to either normal water or cART.

*2-way ANOVA followed by Bonferroni's multiple comparisons tests. Data represent mean ± 95CI. *p < 0.05; **p < 0.01; Ctrl: control / normal water; cART: combination antiretroviral treatment; NNB: normal nest building; LNB: large nest building; GSH/GSSG: reduced to oxidized glutathione ratio*

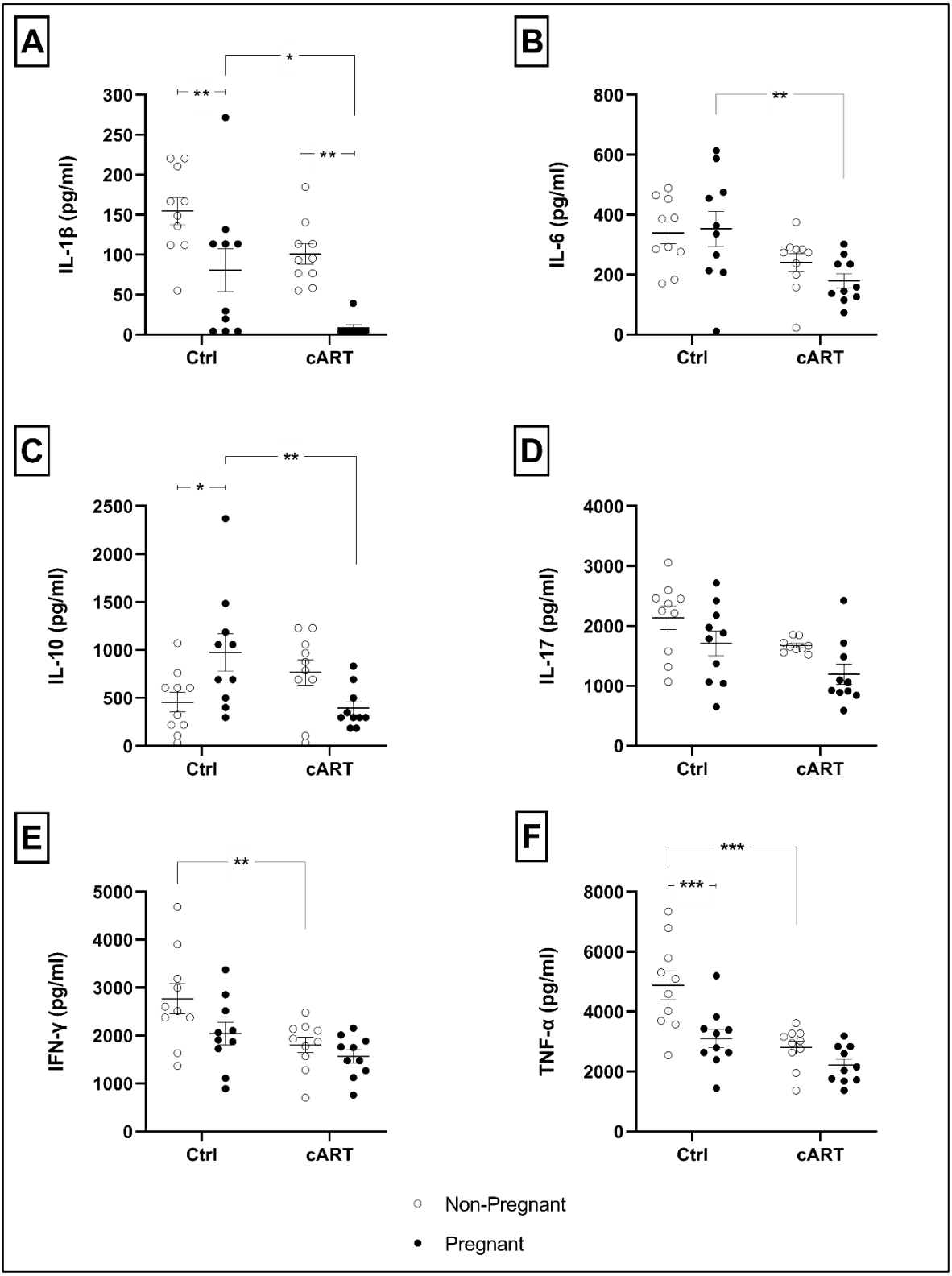

Figure 3-4 – Whole-brain (A) IL-1β, (B) IL-6, (C) IL-10, (D) IL-17, (E) IFN-γ, and (F) TNF-α in non-pregnant and pregnant deer mouse females that were perinatally exposed to either normal water or cART.

*Outliers removed by means of Grubb's. All groups are n = 10, except (A) cART-P (n = 9), (D) cART-NP (n = 9); 2-way ANOVA followed by Bonferroni's multiple comparisons tests. Data represent mean ± 95CI. *p < 0.05; **p < 0.01;***p < 0.001; Ctrl: control / normal water; cART: combination antiretroviral treatment, P: pregnant; NP: non-pregnant.*

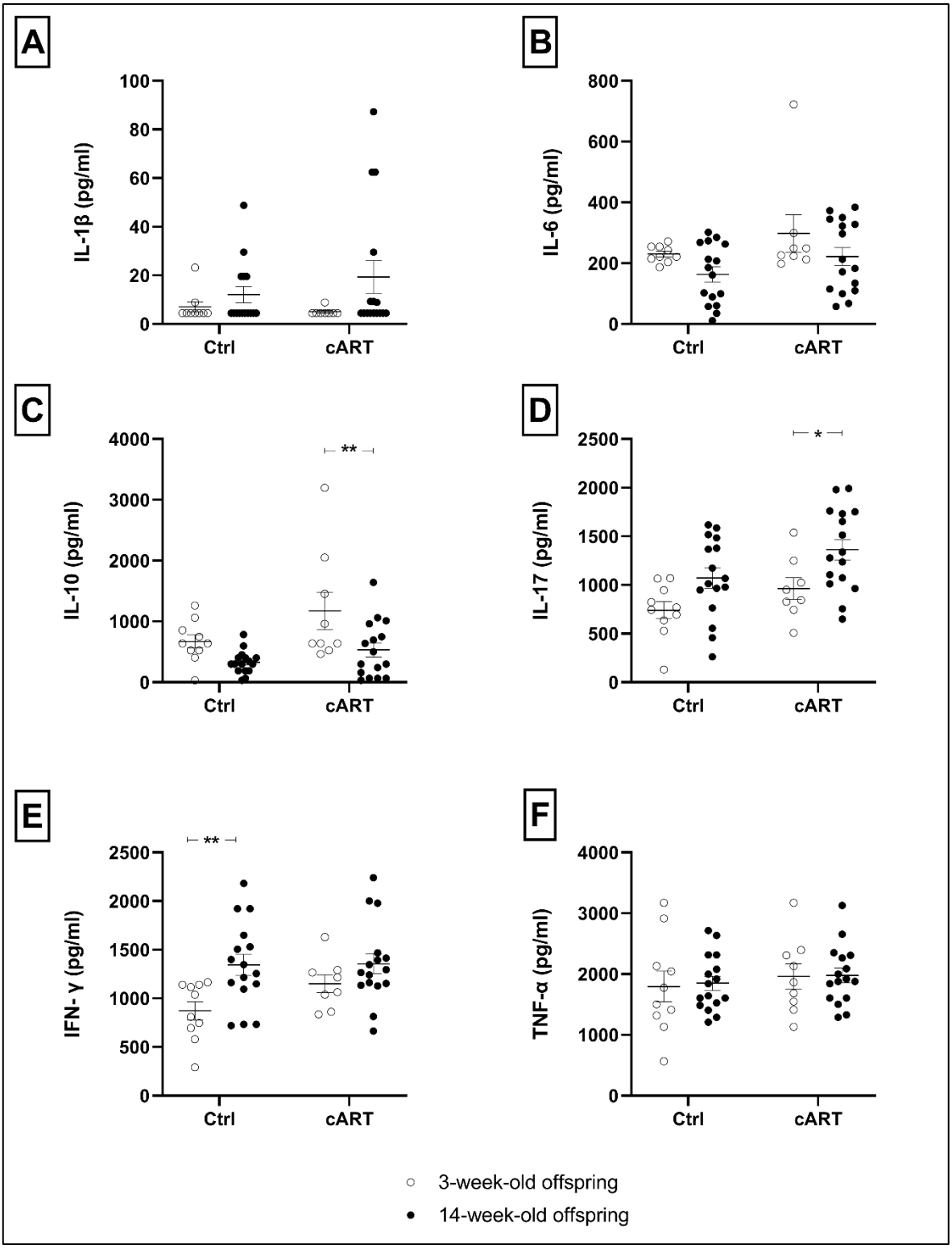

Figure 3-5 – Whole-brain (A) IL-1β, (B) IL-6, (C) IL-10, (D) IL-17, (E) IFN-γ, and (F) TNF-α in 3- and 14-week-old offspring that were perinatally exposed to either normal water or cART.

*Outliers removed by means of Grubb's. All 3-week-old groups are n = 10, except (A) Ctrl-3wk n = 9, cART-3wk n = 8, (B) Ctrl-3wk n = 9, cART-3wk n = 8, (C) cART-3wk n = 9, (D) cART-3wk n = 8, (E) cART-3wk n = 8, (F) cART-3wk n = 9. All 14-week-old groups are n = 16, except (A) cART-14wk n = 15; 2-way ANOVA followed by Bonferroni's multiple comparisons tests. Data represent mean ± 95CI. *p < 0.05; **p < 0.01; Ctrl: control / normal water; cART: combination antiretroviral treatment; 3wk: 3-week-old offspring; 14wk: 14-week-old offspring.*

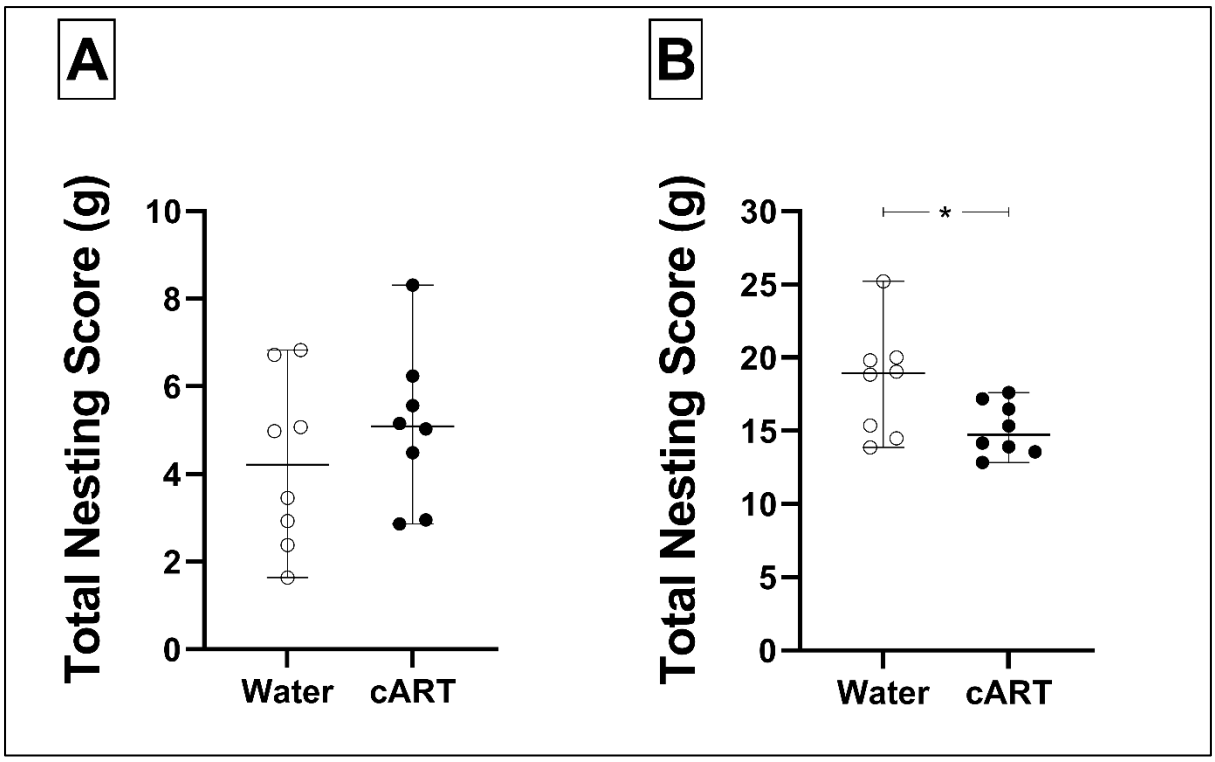

Figure 3-6 – Pairwise comparisons of the median nesting scores generated by (A) NNB- and (B) LNB-expressing 14-week-old offspring that were perinatally exposed to either normal water or cART.

Mann-Whitney U-test; $p < 0.05$. $n = 8$ for all groups.

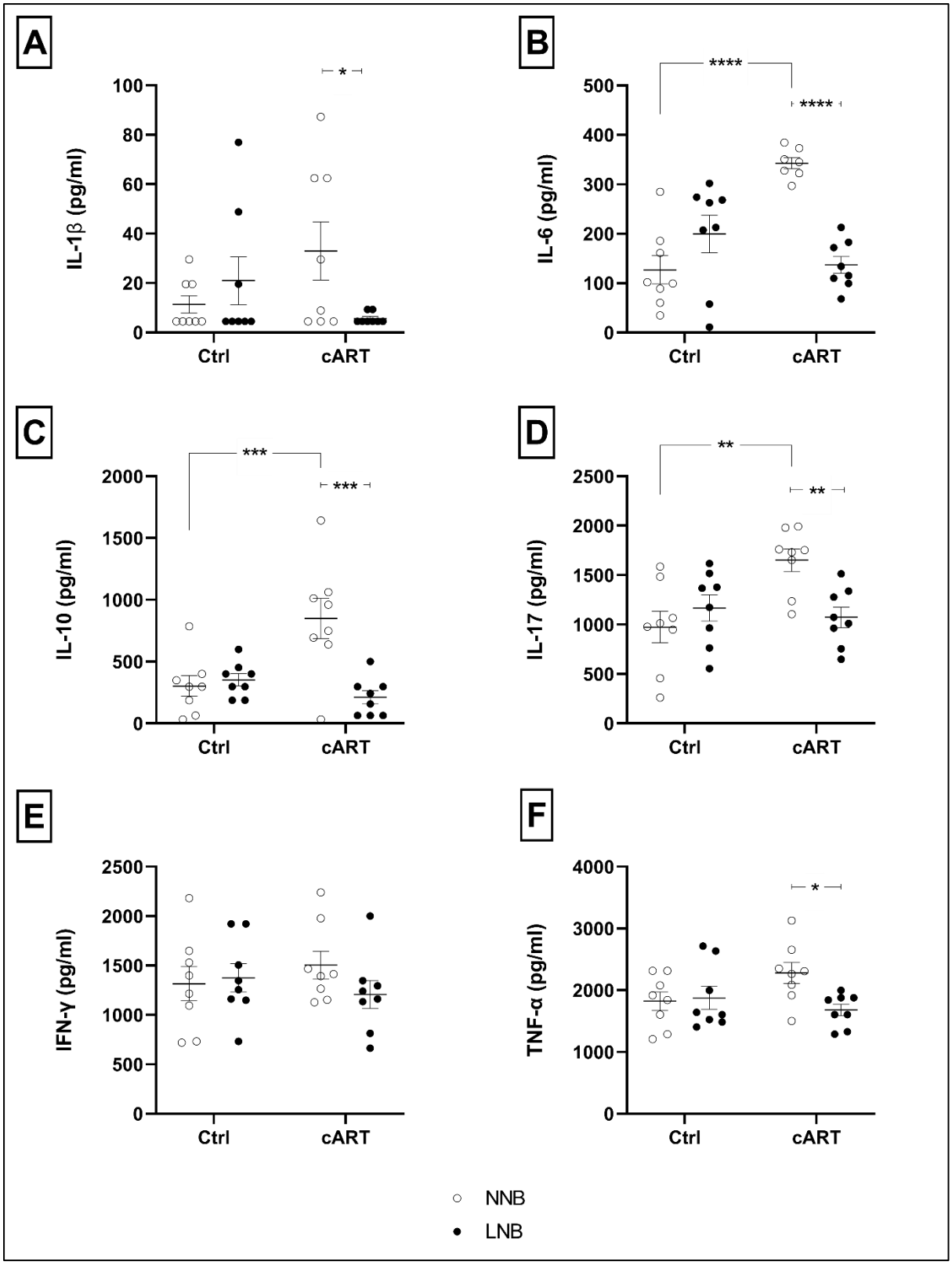

Figure 3-7 – Whole-brain (A) IL-1β, (B) IL-6, (C) IL-10, (D) IL-17, (E) IFN-γ, and (F) TNF-α in 14-week-old NNB- and LNB-expressing offspring that were perinatally exposed to either normal water or cART.

Outliers removed by means of Grubb's. All groups are n = 8, except (B) cART-exposed NNB n = 7; 2-way ANOVA followed by Bonferroni's multiple comparisons tests. Data represent mean ± 95CI. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; Ctrl: control / normal water; cART: combination antiretroviral treatment.

Tables

Table 3-1: Descriptive statistics – comparisons between the cytokine profiles of pregnant vs. non-pregnant dams (i) and 3- and 14-week-old offspring (ii)

		(i) Non-pregnant vs. pregnant dams					*(ii) 3- vs. 14-week-old offspring*				
		Mean ± SD (pg/mL)	***F***	***p***	***d***	**CI*d***	**Mean ± SD (pg/mL)**	***F***	***p***	***d***	**CI*d***
(A) IL-1β		***Interaction***					***Interaction***				
	Interaction		(1,35) = 0.26	0.6108				(1,44) = 0.76	0.3869		
		Main Effects					***Main Effects***				
	Drug exposure		(1,35) = 12.67	**0.0011****				(1,44) = 0.24	0.6253		
	(i) Pregnancy state (ii) Age		(1,35) = 22.07	**0.0001*****				(1,44) = 3.36	0.0734		
		Pairwise Comparisons					***Pairwise Comparisons***				
	(i) Non-pregnant vs. Pregnant ***(ii) 3- vs. 14-week-old***										
	Ctrl	154.93 ± 53.87 vs. 80.64 ± 85.70		**0.0099****	**1.04**	-1.96 — -0.09	7.02 ± 6.24 vs. 12.10 ± 13.10		0.9900	0.46	-0.39 — 1.29
	cART	100.85 ± 39.57 vs. 8.31 ± 11.60		**0.0018****	**3.10**	-4.45 — -1.71	5.00 ± 1.57 vs. 19.32 ± 26.75		0.1308	0.65	-0.23 — 1.51
	Ctrl vs. cART										
	(i) Non-pregnant (ii) 3-week-old	154.93 ± 53.87 vs. 100.85 ± 39.57		0.0716	**1.14**	-2.08 — -0.18	7.02 ± 6.24 vs. 5.00 ± 1.57		>0.9999	0.43	-1.39 — 0.54
	(i) Pregnant (ii) 14-week-old	80.64 ± 85.70 vs. 8.31 ± 11.60		**0.0149***	**1.15**	-2.12 — -0.16	12.10 ± 13.10 vs 19.32 ± 26.75		0.5140	0.34	-0.37 — 1.05
(B) IL-6		***Interaction***					***Interaction***				
	Interaction		(1,36) = 0.88	0.3552				(1,45) = 0.016	0.8990		
		Main Effects					***Main Effects***				
	Drug exposure		(1,36) = 11.81	**0.0015****				(1,45) = 3.45	0.0700		
	(i) Pregnancy state (ii) Age		(1,36) = 0.35	0.5571				(1,45) = 4.40	**0.0416***		
		Pairwise Comparisons					***Pairwise Comparisons***				
	(i) Non-pregnant vs. Pregnant ***(ii) 3- vs. 14-week-old***										
	Ctrl	339.13 ± 114.42 vs. 352.76 ± 186.83		>0.9999	0.09	-0.80 — 0.96	230.16 ± 27.36 vs. 163.26 ± 98.95		0.3247	**0.82**	-1.66 — 0.04
	cART	240.03 ± 96.02 vs. 179.41 ± 75.11		0.5735	0.70	-1.60 — 0.21	297.52 ± 174.31 vs. 221.96 ± 118.12		0.2592	0.55	-1.40 — 0.32
	Ctrl vs. cART										
	(i) Non-pregnant (ii) 3-week-old	339.13 ± 114.42 vs. 240.03 ± 96.02		0.1712	**0.94**	-1.85 — 0.001	230.16 ± 27.36 vs. 297.52 ± 174.31		0.4527	0.56	-0.42 — 1.52
	(i) Pregnant (ii) 14-week-old	352.76 ± 186.83 vs. 179.41 ± 75.11		**0.0077****	**1.22**	-2.17 — -0.24	163.26 ± 98.95 vs. 221.96 ± 118.12		0.2976	0.54	-0.17 — 1.24

		(i) Non-pregnant vs. pregnant dams					*(ii) 3- vs. 14-week-old offspring*				
		Mean ± SD (pg/mL)	***F***	***p***	***d***	**CId**	**Mean ± SD (pg/mL)**	***F***	***p***	***d***	**CId**
(C) IL-10		***Interaction***					***Interaction***				
	Interaction		(1.36) = 11.22	**0.0019****				(1.47) = 1.10	0.3001		
		Main Effects					***Main Effects***				
	Drug exposure		(1.36) = 1.02	0.3191				(1.47) = 6.03	**0.0178***		
	(i) Pregnancy state (ii) Age		(1.36) = 0.30	0.5907				(1.47) = 11.71	**0.0013****		
		Pairwise Comparisons					***Pairwise Comparisons***				
	(i) Non-pregnant vs. Pregnant ***(ii) 3- vs. 12-week-old***										
	Ctrl	456.17 ± 328.45 vs 974.61 ± 618.70		**0.0184***	**1.05**	0.09 — 1.97	669.59 ± 344.25 vs 327.27 ± 188.93		0.0640	**1.33**	-2.19 — -0.44
	cART	767.76 ± 415.37 vs 393.89 ± 215.72		0.1097	**1.13**	-2.07 — -0.17	1174.84 ± 920.22 vs 530.32 ± 465.83		0.5082	**0.98**	-1.83 — -0.10
	Ctrl vs. cART										
	(i) Non-pregnant (ii) 3-week-old	456.17 ± 328.45 vs 767.76 ± 415.37		0.2136	**0.83**	-0.10 — 1.73	669.59 ± 344.25 vs 1174.84 ± 920.22		0.1888	0.74	-0.20 — 1.67
	(i) Pregnant (ii) 14-week-old	974.61 ± 618.70 vs 393.89 ± 215.72		**0.0078****	**1.25**	-2.21 — -0.27	327.27 ± 188.93 vs 530.32 ± 465.83		**0.0064****	0.57	-0.14 — 1.27
(D) IL-17		***Interaction***					***Interaction***				
	Interaction		(1.35) = 0.02	0.8842				(1.46) = 0.10	0.7513		
		Main Effects					***Main Effects***				
	Drug exposure		(1.35) = 8.07	**0.0075****				(1.46) = 5.19	**0.0274***		
	(i) Pregnancy state (ii) Age		(1.35) = 6.91	**0.0127***				(1.46) = 10.59	**0.0021****		
		Pairwise Comparisons					***Pairwise Comparisons***				
	(i) Non-pregnant vs. Pregnant ***(ii) 3- vs. 14-week-old***										
	Ctrl	2138.04 ± 620.20 vs 1709.87 ± 662.26		0.1680	0.67	-1.56 — 0.24	741.35 ± 277.59 vs 1071.00 ± 412.98		0.0729	**0.90**	0.058 — 1.72
	cART	1673.17 ± 116.51 vs 1194.38 ± 539.99		0.1220	**1.19**	-2.16 — -0.20	961.32 ± 317.56 vs 1362.56 ± 421.82		**0.0371***	**1.03**	0.11 — 1.92
	Ctrl vs. cART										
	(i) Non-pregnant (ii) 3-week-old	2138.04 ± 620.20 vs 1673.17 ± 116.51		0.1370	**1.014**	-1.96 — -0.04	741.35 ± 277.59 vs 961.32 ± 317.56		0.4560	0.74	-0.23 — 1.70
	(i) Pregnant (ii) 14-week-old	1709.87 ± 662.26vs 1194.38 ± 539.99		0.0786	**0.853**	-1.76 — 0.08	1071.00 ± 412.98 vs 1362.56 ± 421.82		0.0700	0.70	-0.02 — 1.41

		(i) Non-pregnant vs. pregnant dams					*(ii) 3- vs. 14-week-old offspring*				
		Mean ± SD (pg/mL)	***F***	***p***	***d***	**CI*d***	**Mean ± SD (pg/mL)**	***F***	***p***	***d***	**CI*d***
(E) IFN-γ		***Interaction***					***Interaction***				
	Interaction		(1.36) = 1.19	0.2822				(1.46) = 1.41	0.2414		
		Main Effects					***Main Effects***				
	Drug exposure		(1.36) = 10.38	**0.0027****				(1.46) = 1.65	0.2051		
	(i) Pregnancy state (ii) Age		(1.36) = 4.65	**0.0379***				(1.46) = 9.07	**0.0042****		
		Pairwise Comparisons					***Pairwise Comparisons***				
	(i) Non-pregnant vs. Pregnant (ii) 3- vs. 14-week-old										
	Ctrl	2765.88 ± 990.85 vs 2042.44 ± 744.98		0.0552	**0.83**	-1.73 — 0.10	872.02 ± 296.72 vs 1344.85 ± 434.11		**0.0070****	**1.22**	0.35 — 2.07
	cART	1804.98 ± 512.24 vs 1568.03 ± 431.71		0.9139	0.50	-1.39 — 0.40	1150.45 ± 259.11 vs 1355.93 ± 414.67		0.4379	0.55	-0.32 — 1.41
	Ctrl vs. cART										
	(i) Non-pregnant (ii) 3-week-old	2765.88 ± 990.85 vs 1804.98 ± 512.24		**0.0086****	**1.22**	-2.17 — -0.24	872.02 ± 296.72 vs 1150.45 ± 259.11		0.2600	**0.99**	-0.012 — 1.97
	(i) Pregnant (ii) 14-week-old	2042.44 ± 744.98vs 1568.03 ± 431.71		0.2818	0.78	-1.68 — 0.14	1344.85 ± 434.11vs 1355.93 ± 414.67		>0.9999	0.026	-0.67 — 0.72
(F) TNF-α		***Interaction***					***Interaction***				
	Interaction		(1.36) = 3.47	0.0705				(1.47) = 0.011	0.9156		
		Main Effects					***Main Effects***				
	Drug exposure		(1.36) = 21.54	**<0.0001****				(1.47) = 0.77	0.3836		
	(i) Pregnancy state (ii) Age		(1.36) = 13.66	**0.0007*****				(1.47) = 0.043	0.8373		
		Pairwise Comparisons					***Pairwise Comparisons***				
	(i) Non-pregnant vs. Pregnant (ii) 3- vs. 14-week-old										
	Ctrl	4875.30 ± 1496.96 vs 3103.36 ± 996.28		**0.0007*****	**1.39**	-2.36 — -0.39	1796.57 ± 798.35 vs 1848.77 ± 461.95		>0.9999	0.09	-0.71 — 0.88
	cART	2802.11 ± 675.61 vs 2218.20 ± 610.20		0.4067	**0.91**	-1.82 — 0.03	1961.03 ± 617.44 vs 1977.69 ± 488.12		>0.9999	0.03	-0.79 — 0.85
	Ctrl vs. cART										
	(i) Non-pregnant (ii) 3-week-old	4875.30 ± 1496.96 vs 2802.11 ± 675.61		**0.0001*****	**1.79**	-2.82 — -0.72	1796.57 ± 798.35 vs 1961.03 ± 617.44		>0.9999	0.23	-0.68 — 1.13
	(i) Pregnant (ii) 14-week-old	3103.36 ± 996.28 vs 2218.20 ± 610.20		0.1146	**1.07**	-2.00 — -0.12	1848.77 ± 461.95 vs 1977.69 ± 488.12		>0.9999	0.27	-0.43 — 0.97

Table 3-2: Descriptive statistics – comparisons between the cytokine profiles of normal vs. large nest-building 14-week-old offspring

		Normal vs. large nest-building offspring				
		Mean ± SD (pg/mL)	***F***	***p***	***d***	**CI*d***
(A) IL-1β		***Interaction***				
	Interaction		(1.28) = 5.56	**0.0256***		
		Main Effects				
	Drug exposure		(1.28) = 0.16	0.6890		
	Phenotype		(1.28) = 1.28	0.2671		
		Pairwise Comparisons				
	NNB vs LNB					
	Ctrl	11.36 ± 10.02 vs 20.95 ± 27.46		0.7875	0.46	-0.54 — 1.45
	cART	32.98 ± 33.20 vs 5.66 ± 2.24		**0.0400***	**1.16**	-2.21 — -0.08
	Ctrl vs cART					
	NNB	11.36 ± 10.02 vs 32.98 ± 33.20		0.1218	**0.88**	-0.16 — 1.90
	LNB	20.95 ± 27.46 vs 5.66 ± 2.24		0.3565	0.79	-1.79 — 0.25
(B) IL-6		***Interaction***				
	Interaction		(1.27) = 27.34	**<0.0001****		
		Main Effects				
	Drug exposure		(1.27) = 8.26	**0.0078****		
	Phenotype		(1.27) = 6.30	**0.0184***		
		Pairwise Comparisons				
	NNB vs LNB					
	Ctrl	127.08 ± 80.49 vs 199.45 ± 107.27		0.1217	0.76	-0.27 — 1.77
	cART	342.81 ± 30.25 vs 136.76 ± 48.50		**<0.0001****	**5.01**	-7.15 — -2.84
	Ctrl vs cART					
	NNB	127.08 ± 80.49 vs 342.81 ± 30.25		**<0.0001****	**3.45**	1.77 — 5.08
	LNB	199.45 ± 107.27 vs 136.76 ± 48.50		0.2034	0.75	-1.76 — 0.28
(C) IL-10		***Interaction***				
	Interaction		(1.28) = 12.33	**0.0015****		
		Main Effects				
	Drug exposure		(1.28) = 4.29	**0.0476***		
	Phenotype		(1.28) = 8.97	**0.0057****		
		Pairwise Comparisons				
	NNB vs LNB					
	Ctrl	301.96 ± 235.75 vs 352.57 ± 139.46		>0.9999	0.26	-0.73 — 1.24
	cART	849.27 ± 456.76 vs 211.38 ± 154.44		**0.0002*****	**1.87**	-3.05 — -0.65
	Ctrl vs cART					
	NNB	301.96 ± 235.75 vs 849.27 ± 456.76		**0.0010*****	**1.51**	0.36 — 2.61
	LNB	352.57 ± 139.46 vs 211.38 ± 154.44		0.6344	**0.96**	-1.99 — 0.10

		Normal vs. large nest-building offspring				
		Mean ± SD	***F***	***p***	***d***	**CI*d***
(D) IL-17		***Interaction***				
	Interaction		(1.28) = 8.92	**0.0058****		
		Main Effects				
	Drug exposure		(1.28) = 5.10	**0.0320***		
	Phenotype		(1.28) = 2.24	0.1456		
		Pairwise Comparisons				
	NNB vs LNB					
	Ctrl	974.78 ± 450.73 vs 1167.21 ± 375.72		0.6023	0.46	-0.54 — 1.45
	cART	1652.15 ± 321.45 vs 1072.97 ± 293.70		**0.0073****	**1.88**	-3.06 — -0.66
	Ctrl vs cART					
	NNB	974.78 ± 450.73 vs 1652.15 ± 321.45		**0.0018****	**1.73**	0.54 — 2.88
	LNB	1167.21 ± 375.72 vs 1072.97 ± 293.70		>0.9999	0.28	-1.26 — 0.71
(E) IFN-γ		***Interaction***				
	Interaction		(1.28) = 1.41	0.2449		
		Main Effects				
	Drug exposure		(1.28) = 0.0055	0.9416		
	Phenotype		(1.28) = 0.63	0.4332		
		Pairwise Comparisons				
	NNB vs LNB					
	Ctrl	1315.40 ± 488.52 vs 1374.30 ± 403.96		>0.9999	0.13	-0.85 — 1.11
	cART	1504.63 ± 398.34 vs 1207.22 ± 399.06		0.3437	0.75	-1.75 — 0.28
	Ctrl vs cART					
	NNB	1315.40 ± 488.52 vs 1504.63 ± 398.34		0.7598	0.43	-0.58 — 1.41
	LNB	1374.30 ± 403.96 vs 1207.22 ± 399.06		0.8750	0.42	-1.40 — 0.58
(F) TNF-α		***Interaction***				
	Interaction		(1.28) = 4.51	**0.0427***		
		Main Effects				
	Drug exposure		(1.28) = 0.70	0.4101		
	Phenotype		(1.28) = 3.13	0.0878		
		Pairwise Comparisons				
	NNB vs LNB					
	Ctrl	1821.44 ± 426.15 vs 1876.11 ± 523.42			0.12	-087 — 1.09
	cART	2277.67 ± 483.66 vs 1677.71 ± 266.37			**1.54**	-2.65 — -0.39
	Ctrl vs cART					
	NNB	1821.44 ± 426.15 vs 2277.67 ± 483.66			**1.00**	-0.06 — 2.03
	LNB	1876.11 ± 523.42 vs 1677.71 ± 266.37			0.48	-1.47 — 0.53

4 Conclusion

In this work, we aimed to explore the short- and long-term effects of perinatal combination antiretroviral treatment (cART[1]) exposure in a naturalistic model of compulsivity. Our main findings are that 1) both pregnancy and cART significantly decreased the whole-brain reduced to oxidized glutathione (GSH/GSSG[2]) ratios in dams, 2) cART-, compared to control-exposure, varyingly decreased the concentrations of most cytokines measured in non-pregnant and pregnant dams, 3) differences in cytokine expression between control- and cART-exposed offspring only became apparent by the age of 14 weeks, in that cART-exposed offspring presented with significantly lower interleukin (IL[3])-10, but higher IL-17 and interferon-gamma (IFN-γ[4]) concentrations than their age-matched control-exposed counterparts, 4) that cART-, compared to control-exposed large nest building (LNB[5])-expressing animals, generated lower total nesting scores, and 5) that the whole-brain concentrations of most cytokines were higher in cART-exposed 14-week-old normal (NNB[6])-, compared to LNB-expressing offspring.

* * *

The first finding of this investigation is that both pregnancy and cART exposure, is associated with an imbalance in the glutathione system, as shown by a decreased GSH/GSSG ratio (**Fig 3-3A**). This was expected, given similar results reported from clinical studies (Casanueva and Viteri, 2003; Page, 1993; Sharma, 2014; Sies, 2015). Our finding that adult NNB- and LNB-expressing mice present with similar GSH/GSSG ratios is vitally important, since we have demonstrated perturbations in this ratio in high stereotypical (H[7]) deer mice before (Güldenpfennig et al., 2011). H behavior is another proposed compulsive-like phenotype expressed by deer mice (De Brouwer et al., 2021). That LNB and H behavior differs in terms of its association with oxidative stress, is therefore informative for continuing efforts to explore unique neurobiological architectures of different clinical compulsive phenotypes. Collectively, our data indicate that while water- and cART exposed, non-pregnant and pregnant dams may differentially present with varying degrees of oxidative stress, offspring are protected from such variance well into adulthood.

* * *

[1] combination antiretroviral treatment
[2] reduced to oxidized glutathione ratio
[3] interleukin
[4] interferon-gamma
[5] large nest building
[6] normal nest building
[7] high stereotypical

With respect to the pregnancy and cytokine expression, we show that while pregnancy was associated with decreased concentrations of the proinflammatory cytokines, IL[1]-1β and TNF-α[2] and increased expression of anti-inflammatory IL-10, cART[3] broadly suppressed immune signaling (**Fig 3-4A - F**). The overall reduction in cytokine expression in cART-exposed mice can possibly be explained by the immune modulatory properties of cART treatment, especially regimens that include tenofovir (Melchjorsen et al., 2011), which has been shown to negatively regulate inflammatory cytokines. However, in this work, we also highlight that cART exposure prevented the anti-inflammatory, i.e. IL-10-related, response seen in pregnant control-exposed deer mice (**Fig 3-4C**). While our findings confirm the pharmacological actions of TDF[4]/FTC[5], further investigation is needed to explore the actions of said intervention on the expression of pro-inflammatory cytokines.

* * *

The effects of cART on immune signaling as observed in adult female and pregnant deer mice, were not observed in the 3-week-old offspring (**Fig 3-5A – F**), indicating that pups were largely isolated from the immunosuppressive effects of perinatal cART exposure. Nevertheless, we show that perinatal cART exposure associated with decreased IL-10 (**Fig 3-5C**) and increased IL-17 (**Fig 3-5D**) expression in 14-week-old vs 3-week-old offspring. Further, control-exposed 14-week-old offspring presented with significantly increased IFN-γ[6] concentrations, compared to their younger counterparts. It is possible that cART exposure in iHEU[7] may bolster pro-inflammatory immune signaling during the later stages of life and thus, the age-related maturation and functioning of the immune system in iHEU, vs. non-exposed children, will require further elucidation.

* * *

With respect to the normal vs. psychiatric phenotype used here, our data revealed several important and valuable findings. First, control-exposed animals of the two nesting cohorts showed similar cytokine concentrations across all measures (**Fig 3-7**), except with respect to IL-6 (**Fig 3-7B**). Since this was, according to our knowledge, the first work to explore immune signaling in an entirely naturalistic model of compulsive-like behavior, the potential role of pro-inflammatory IL-6 in the manifestation of LNB[8], will need further investigation. Still, our data with respect to perinatal cART exposure on the later life

[1] interleukin
[2] tumor necrosis factor-alpha
[3] combination antiretroviral treatment
[4] tenofovir disoproxil fumarate
[5] emtricitabine
[6] interferon-gamma
[7] HIV-uninfected, but HIV/cART exposed infants
[8] large nest building

biobehavioral profile of deer mice, are striking. NNB[1]- and LNB[2]-expressing animals were indeed uniquely affected by said intervention. cART[3] exposure also *blunted* the compulsive-like, but not normal behavioral expression of deer mice (**Fig 3-6B**). Such attenuation of nesting scores was shown in parallel to a significant and robust reduction in most of the cytokines measured in cART-exposed LNB-, vs. NNB-expressing animals. We conclude that it is possible that 'normal' deer mice (as defined against the background of behavioral expression), are more affected by cART, than the compulsive-like cohort. While it seems that LNB-expressing animals are not affected by cART as considered against the cytokine profiles of control-exposed animals, our conclusion is that perinatal cART exposure has pro-inflammatory effects in a generalized population, which is masked in LNB animals, due to potentially innate, naturalistic altered immune processes in LNB, compared to NNB animals. It can therefore be argued that perinatal cART exposure, albeit showing immunosuppressive properties in adult female mice, have long-lasting pro-inflammatory effects in iHEU[4] offspring, which is not observed in LNB animals due to a unique profile of brain-immune interactions underlying such behavior.

* * *

[1] normal nest building
[2] large nest building
[3] combination antiretroviral treatment
[4] HIV-uninfected, but HIV/cART exposed infants

4.1 Final summary of study aims and outcomes

The present work had two broad aims, i.e.:

i) To investigate the influence of cART[1] exposure on brain markers of oxidative stress, i.e. the glutathione (GSH[2]) redox index, and inflammatory profile, i.e. interleukin (IL[3])-1β, IL-6, IL-10, IL-17, interferon-gamma (IFN-γ[4]), and tumor necrosis factor-alpha (TNF-α[5]) in both non-pregnant and pregnant adult (13 weeks) female deer mice, as well as in their respective 3- and 14-week-old offspring, and

ii) Exploring the influence of perinatal cART exposure on the long-term behavioral nesting expression of the adult offspring of both water- and cART-exposed dams.

In Table 4-1, we summarize the main hypothesized outcomes of this work as stated in Chapter 1, and the associated final outcomes.

Table 4-1: Summary of hypothesized outcomes and final outcomes

Expected Outcome	Actual Outcome
cART exposure in non-pregnant and pregnant females will be associated with increased levels of central oxidative stress.	✓ cART exposure, as well as pregnancy, induced higher levels of oxidative stress in female deer mice.
Perinatal cART exposure will be associated with increased levels of central oxidative stress in 3- and 14-week-old offspring.	✗ This was not shown by the data presented here. In fact, both pups and adult offspring were fully protected against showing oxidative stress.
cART exposure in non-pregnant and pregnant females will be associated with increased markers of central inflammation evinced by alterations in the pro- and anti-inflammatory cytokine levels.	✗ ✓ cART exposure as well as pregnancy differentially affected cytokine markers, with pregnancy in general having an anti-inflammatory effect. However, cART exposure also exerted broad immunosuppressive effects.

[1] combination antiretroviral treatment
[2] glutathione
[3] interleukin
[4] interferon-gamma
[5] tumor necrosis factor-alpha

Perinatal cART[1] exposure will be associated with increased markers of central inflammation in 3- and 14-week-old offspring, respectively.	✗ Compared to water-exposed 3- and 14-week-old offspring, perinatal cART-exposure did not have a significant pro-inflammatory effect.
Perinatal cART exposure will result in exacerbated nesting expression in LNB[2]-animals, compared to the behavior of water-exposed LNB animals.	✗ Perinatal cART blunted the expression of LNB, likely due to perturbed brain-immune crosstalk systems naturalistically inherent to LNB animals.
Perinatal cART exposure will associate with unique inflammatory profiles in NNB[3] and LNB expressing animals, respectively.	✓ Perinatal cART exposure had a pro-inflammatory effect in NNB-, compared to LNB expressing animals, that trended to associate with inflated nesting expression in the former cohort. Further work is needed to elucidate this finding.

4.2 Study shortcomings and future directions

This work was not without some noteworthy shortcomings that need mentioning.

First, an early-life measure of compulsive-like behavioral expression would be useful to link distinct patterns of early- and later-life cytokine expression to behavioral manifestation across both age groups. Second, it would have been highly informative if adult LNB-expressing were to be exposed to pro-inflammatory interventions, so as to determine whether a reversal in the cytokine profile measured, would result in inflated nesting expression to a level akin to that which was observed in the control-exposed LNB-expressing cohort. Future studies with this aim are being conceptualized. Third, since our NNB and LNB group sizes ($n = 8$ each) were not sufficient to draw direct correlations between cytokine concentrations and behavioral expression, this would have been useful. Last, it would have been informative if the female mice and dams have also been selected for inclusion in this work based nesting expression. Such approach would have enabled both prospective and retrospective analyses of the relationship between oxidative stress, cytokine concentrations, and behavioral data. Given that the present model system is entirely naturalistic, such investigations will become increasingly important to

[1] combination antiretroviral treatment
[2] large nest building
[3] normal nest building

expand our understanding of how paternal and maternal factors contribute to the biobehavioral profile of offspring. Sadly, this could not have been accomplished in the time that was available.

4.3 References

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Addendum A

Additional details relating to the methodology employed in this investigation

This addendum includes supplementary details regarding the methodology followed throughout this investigation and should be read along Chapter 3.

Additional information pertaining to animal husbandry, housing, and care

- Prior to the onset of this investigation, deer mice were bred in the North-West University (NWU[1]) vivarium, Potchefstroom (SAVC[2] registration number: FR15/13458; SANAS[3] GLP[4] compliance number: G0019; AAALAC[5] accreditation file: 1717).
- Deer mouse births were monitored and noted in separate pairs of animals prior to the experimental onset, so as to 1) identify suitable breeding pairs, 2) determine the average time until conception, 3) establish the average litter size.
- Once the experimental phase of this work commenced, all deer mouse pups were weaned on postnatal day 21 and group housed (4 - 6 same sex animals per cage). The home cage specifications were as follows: 35 cm (l) x 20 cm (w) and 13 cm (h). Cages were acquired from Techniplast® S.P.A., Varese, Italy.
- From the onset of nest building screening at the age of 13 weeks, deer mice were single housed in identical cages until they were euthanized for further investigations.
- All food (mouse pellets; Labchel® Nutrionhub® (Pty) Ltd.; Reg nr. 2012/141960/07) was autoclaved at 90 ºC for 15 min before use.

Daily routine during the behavioral investigation

- On each morning between 08:00 and 10:00 throughout this investigation, the weights of water bottles were noted to monitor fluid intake. Water (or drug solutions where applicable) were replaced every day by adding 35 mL of drug or water to the water bottles.
- Each morning within the same window, the weights of the female animals were noted to track pregnancy and monitor the health of animals during pregnancy.
- Every day, the humidity and temperatures of the housing and experimental rooms were recorded. In the event that discrepancies were noted, the relevant stakeholders were informed, and the problem identified and resolved.
- Once a week, all cages were cleaned by replacing the corncob bedding (in addition to the daily change of water or drug solutions, where applicable). Paper towel was provided as a form of nesting substrate, but not during times when nesting assessment was performed. A small piece of polyvinyl chloride pipe (100 mm x 40 mm) was provided as a form of environmental

[1] North-West University
[2] South African Veterinary Council
[3] South African National Accreditation
[4] Good laboratory Practice
[5] Association for Assessment and Accreditation of Laboratory Animal Care

enrichment. Cages were cleaned with F10® veterinary disinfectant cleaning solution (Reg No Act 29, GNR 529/29990/040/150; DAFF[1] Registration Number: G3073).

Information pertaining to nest building screening

- Each day, an excess of pre-weighed, unscented cosmetic cotton wool was supplied in the roof of each housing cage, the built nests were removed, and the remaining cotton wool weighed between 7:00 and 8:00
- Mice had access to the nesting material for more than 23 hours of each day
- After completion of the 7-day nesting screen, the daily quantity of cotton wool that was utilized by each animal (in grams) was added and a 7-day total nesting score (also in grams) calculated for each subject (Wolmarans et al., 2016)
- Animals selected for inclusion in the LNB[2] cohorts (n = 8 per perinatal exposure cohort) were those that expressed nesting behavior of which the total nesting score broadly clustered within the upper 75th percentile of the average total nesting score distribution. We also selected LNB mice based on the persistence with which they engaged in nesting behavior.
- Conversely, animals selected for NNB[3] behavior (n = 8 per perinatal exposure cohort), i.e. the behavioral control, were those that engaged in smaller nest building behavior, the scores of which clustered between the 25th and 50th percentile of the total nesting score distribution, but also with minimal variation
- Animals not selected for inclusion in the group of 32 experimental adult offspring, were euthanized without being used for sample collection.
- During periods of nest building analysis, food and water were available as normal, although animals were not provided with any additional form of nesting material

Information pertaining to drug administration

- Fresh solutions have been constituted and replaced every day for the duration of the drug administration phase.
- Tenofovir disoproxil fumarate (TDF[4]) (60 mg/kg/day) and emtricitabine (FTC[5]) (40 mg/kg/day) (Nair and Jacob, 2016) were prepared daily for oral administration in normal water.

[1] Department Agriculture Forestry and Fisheries
[2] large nest building
[3] normal nest building
[4] tenofovir disoproxil fumarate
[5] emtricitabine

- The final concentrations of the drug solutions were 16 mg TDF and 24 mg FTC per 100 mL vehicle. These doses were deemed sufficient in mice as they were translated from the 200 mg and 300 mg clinically used daily doses in adults.
- Drugs were constituted so that the required daily dose would be provided at a rate of 0.25 mL/g mouse weight/day, as this is the average rate of fluid consumption by mice (Aschhoff et al., 2000; Wolmarans de et al., 2013). Also, the addition of drugs to the drinking water was not associated with any changes in fluid consumption (**Fig A-1**), as has been confirmed by the daily records pertaining to fluid intake (**Fig A-2**).

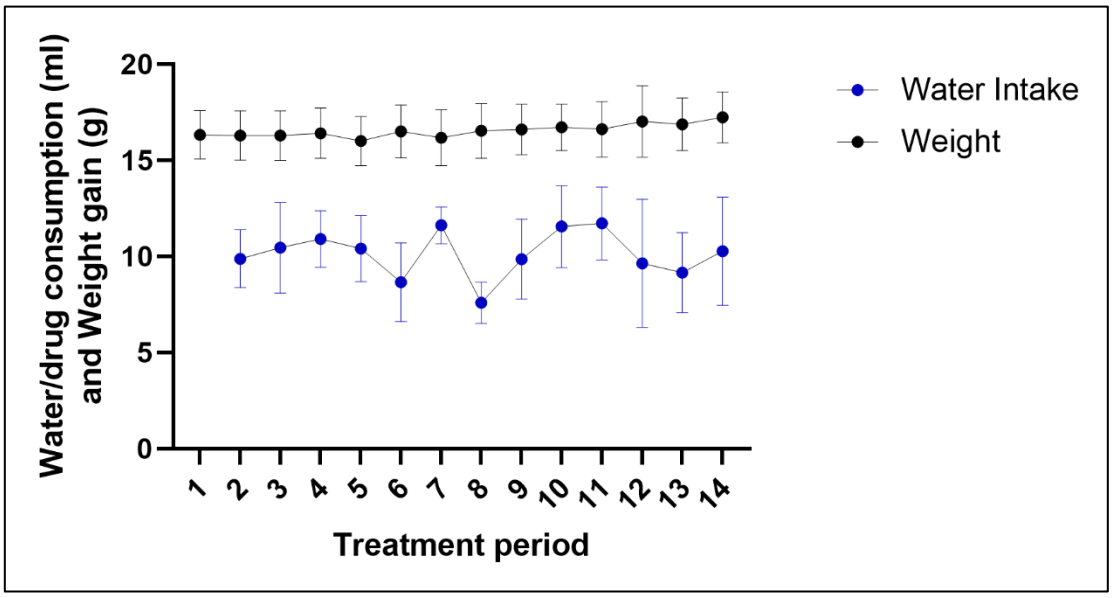

Figure A-1 – Graphical representation of body weight and fluid intake in the transitional period when water (until day 7) was switched to cART solutions (from day 8).

Data are mean ± SEM of 10 animals exposed to cART and water in the pilot investigation. cART: combination antiretroviral treatment.

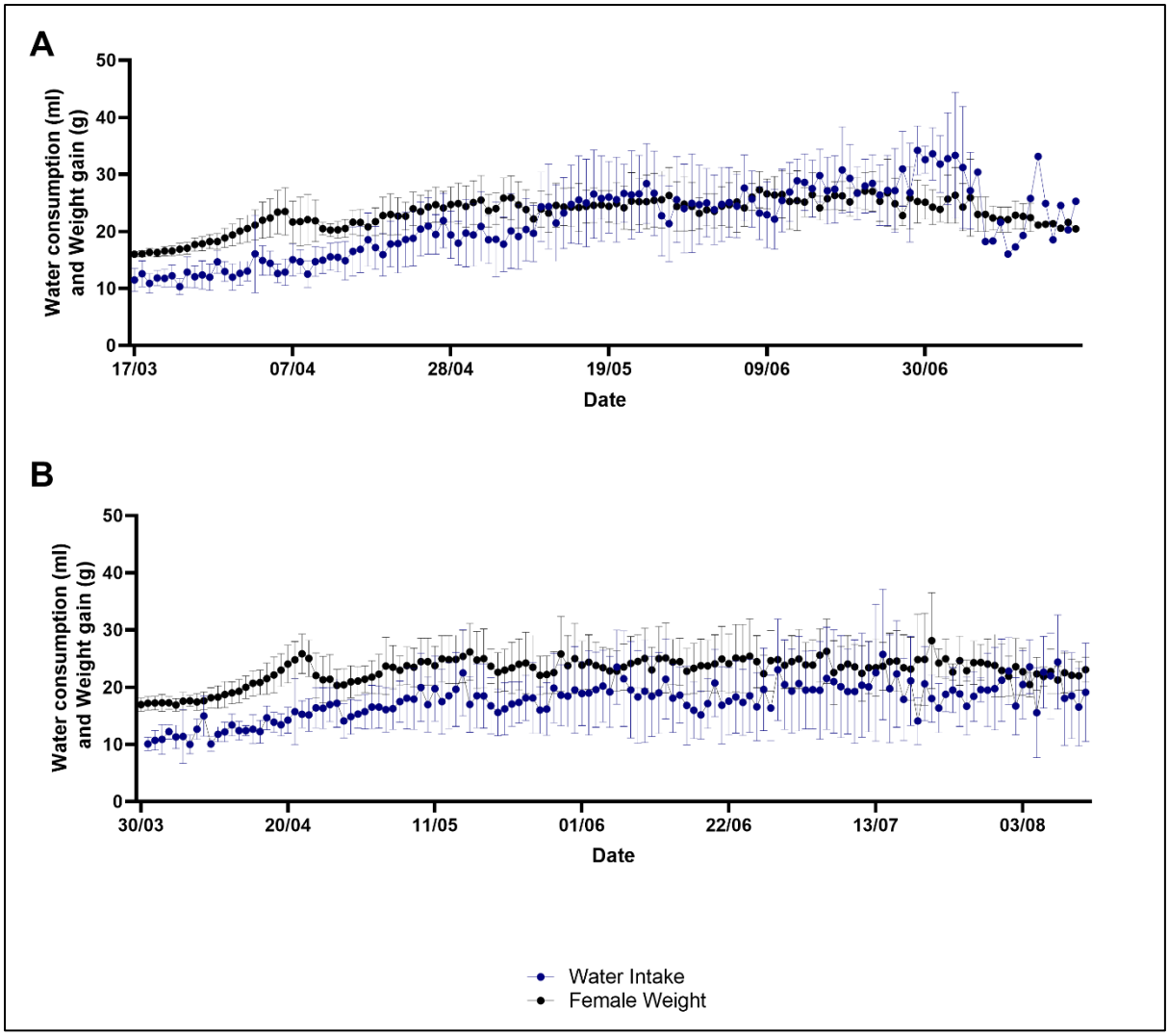

Figure A-2 -Graphical representation of body weight and fluid intake in animals receiving either cART solutions (A) or normal water (B)

Data are mean ± SEM of 10 animals receiving cART (A) and 8 animals receiving normal water (B). cART: combination antiretroviral treatment.

Methodology

Cytokine analysis

Tissue preparation: cell lysis

The cytokine determination required the brain tissue samples to be prepared in lysate form. Lysates were prepared using a Bio-Plex Cell Lysis Kit (Bio-Rad® Laboratories Inc., USA). The samples were prepared according to the manufacturer's instructions, which are attached below this section. Briefly, the process was as follows:

1) Immediately prior to cell lysis preparation, tissues were allowed to thaw on ice and weighed.
2) The brain tissue (one half-brain per sample, except for the 3-week-old pups, of which 2 half brain samples were chosen at random from the same litter, one male and one female, and pooled to represent a single sample) was homogenized in Eppendorf® Safe-Lock™ tubes by sonication (twice for 10 sec, at an amplitude of 14 μ; MSE® ultrasonic disintegrator, Nuaillé, France) and left on ice.
3) We then prepared 500 mM phenylmethylsulphonyl fluoride (PMSF[1]) by dissolving 0.436 g PMSF in 5 mL dimethyl sulfoxide (DMSO[2]), which was kept on ice for the duration of the procedure).
4) Lysing solution was prepared as follows: 10 mL lysing solution = 40 μL of Factor 1 (provided in the kit) and 20 μL of Factor 2 (provided in the kit) added to 9.9 mL of lysis buffer (provided in the kit). 250 μL of lysing solution was then prepared for each sample to ensure that enough sample was prepared for all the cytokine determinations.
5) The solutions were then vortexed and set aside on ice.

Subsequently, the following steps took place:

6) 40 μL of the 500 mM PMSF solutions was added to each sample.
7) 250 μL of the lysing solution was added to each sample and sonicated further.
8) The mixtures were transferred into clean Eppendorf® Safe-Lock tubes and frozen at -80°C.
9) Samples were then thawed again and sonicated on ice.
10) Samples were centrifuged at 4500 × *g* for 4 min.
11) Supernatants were collected without disturbing the pellet and transferred to clean Eppendorf® Safe-Lock tubes.

[1] phenylmethylsulfonyl fluoride
[2] dimethyl sulfoxide

12) 5 µL of the supernatant was extracted from the samples in (11) and further constituted in 95 µL distilled water. These were allocated for Bradford protein analysis.
13) The remaining supernatants (cell lysates) were then frozen at -80°C and stored until the analysis.

Bradford method: protein determination

Protein determination of the brain homogenates was carried out according to the Bradford method (Bradford, 1976; Kruger, 2009).

1) Bradford reagents were gently shaken in the reagent container and a sufficient amount extracted and allowed to reach room temperature in a dark environment.
2) Protein standards were prepared from a 2 mg/mL bovine serum albumin (BSA[1]) solution as follows:

Protein concentration (mg/mL)	Volume of 2mg/mL BSA solution (µl)
0	0
0.25	2.5
0.5	5
0.75	7.5
1.0	10
1.5	15
2.0	20

3) 3 x 5 µL of the diluted supernatant (cell lysate) and 3 x 5 µL of each dilution was added to separate wells on a 96-well plate in triplicate.
4) 250 µL of the Bradford reagent was then added to each individual well and immediately shaken using the mixing facility of the plate reader for 30 sec.
5) The plate was then left to incubate for 15 min at room temperature.
6) The absorbance of each well was determined using a 560 nm filter.
7) Protein concentrations of the cell lysates were calculated from the straight line calculated from the standards.
8) Concentrations were analyzed and samples that did not fall within the 200 - 900 µg/mL protein range, were diluted with phosphate-buffered saline (PBS[2]) until the allotted concentrations were met.

[1] bovine serum albumin
[2] Phosphate

Cytokine Assay

Cytokine analysis was subsequently performed according to the below-included instruction manual. The additional information regarding this process is briefly summarized here.

1) The concentrations of 6 cytokines (IL[1]-1β, IL-6, IL-10, IL-17, IFN-γ[2], TNF-α[3]) were assayed.
2) Multiplex analyses of lysates were done by means of a Luminex Milliplex® assay using the Bio-Plex Pro Mouse TH17 Cytokine panel (Bio-Rad Laboratories® Inc., USA).
3) The assay plates were read by means of a Bio-Plex® Suspension Array Reader and analyzed by means of the associated Bio-Plex Manager software version 4 (Bio-Rad Laboratories® Inc., USA).
4) A 5-parameter logistic regression formula was used to calculate the sample concentrations from the standard curves.
5) The sensitivity of the panel was high (pg/mL).
6) For any samples that had cytokine values below the limit of detection, half of the lowest detectable value for that specific cytokine was assigned.
7) For the samples that had values that were above the detection limit, the highest-detectable value was assigned.
8) Six randomly selected specimens were run across all the plates as interplate controls. Another six randomly selected specimens were duplicated on each set of plates as intraplate control as a measure of quality control.
9) A Spearman's rank order correlation was conducted to confirm intra-assay and inter-assay reliability and reproducibility.

[1] interleukin
[2] interferon-gamma
[3] tumor necrosis factor-alpha

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Introduction

The Bio-Plex cell lysis kit has been developed specifically to prepare cell culture and tissue lysate samples for analysis with Bio-Plex phosphoprotein and total target assays. The cell lysates can be tested for the presence of phosphorylated proteins using Bio-Plex phosphoprotein assays or for the abundance of target proteins using Bio-Plex total target assays. This cell lysis kit can also be used to prepare cell lysates for western blot analysis (request bulletin 3033).

Product Description

The following components are provided with the Bio-Plex cell lysis kit:
Cell wash buffer
Cell lysis buffer
Cell lysis buffer, factor 1 (250x
Cell lysis buffer, factor 2 (500x

2. Prepare 500 mM PMSF by dissolving 0.436 g PMSF in 5 ml DMSO. Store as 0.5 ml aliquots at –20ºC. Aliquots can be frozen and thawed up to 5 times.

3. Prepare an adequate volume of lysing solution (refer to the table on the left). For 10 ml of lysing solution, add 40 µl of *factor 1* and 20 µl of *factor 2* to 9.9 ml of *cell lysis buffer*. Vortex gently to mix and set aside on ice. Then add 40 µl of 500 mM PMSF.

Lysing Solution Volume Guide

Culture vessel	Culture medium volume	Lysing solution volume	Notes
96-well plate	100 µl/well	75 µl/well	Grow cells to 80–85% confluence Recommend leaving external wells empty due to edge effect
10 cm culture dish	10 ml	2–3 ml	Grow cells to 80–90% confluence

4. Lyse the samples:

Adherent and Suspension Cells

a) Immediately add the lysing solution to the cells. The amount of lysing solution needed depends on the cell

b) Agitate the cells as follows:

Culture Plate — For suspension cells, place the plate on ice and pipet the contents of the wells up and down 5 times. For adherent cells, scrape the cells with a cell scraper. For both, agitate the plate on a microplate shaker at 300 rpm for 20 min at 4ºC.

Other Culture Vessel — Transfer the cell lysate to a centrifuge tube and rotate for 20 min at 4ºC.

HINT: Freeze-thawing the lysate once using dry ice or a –20ºC freezer may increase the extent of the lysis. Alternatively, briefly sonicate (eg., with a Sonifier 450 as follows: Duty cycle = 40, Output = 1, Pulse sonicating = 18 times).

c) Centrifuge the samples at 4,500 g for 20 min at 4ºC.

Storage and Stability

The cell wash buffer and cell lysis buffer should be stored at 4ºC. Factors 1 and 2 should be stored at –20ºC and can be frozen and thawed up to 5 times. All components are guaranteed for 6 months from the date of purchase when stored as specified.

Materials Required but Not Supplied

Sigma catalog #P7626

Sigma catalog #D2650

Laemmli sample buffer,
Bio-Rad catalog #161-0737

2-Mercaptoethanol,
Bio-Rad catalog #161-0710

Lysate Preparation

Adherent and Suspension Cell Preparation

1. Rinse the samples with *cell wash buffer* as follows:

Adherent Cells — Stop the treatment reaction by aspirating the culture medium and quickly rinsing the cells with ice-cold cell wash buffer. The volume of cell wash buffer required is the same as the volume of aspirated cell culture medium. Keep the cells on ice.

Suspension Cells — Stop the treatment reaction by adding ice-cold wash buffer to the cells. The volume of cell wash buffer required is twice that of the culture medium. Centrifuge the cells at 1,000 rpm for 5 min at 4ºC. Aspirate the supernatant.

Tissue Samples — Rinse the tissue sample with cell wash buffer once. Cut the tissue into 3 x 3 mm pieces and transfer them to a 2 ml tissue grinder.

Tissue Samples

a) Immediately add 500 µl of lysing solution to the tissue grinder and grind the tissue sample on ice using about 20 strokes.

b) Transfer the ground tissue to a clean microcentrifuge tube and freeze the sample at –70ºC.

c) Thaw the samples, then sonicate on ice as suggested above.

d) Centrifuge the samples at 4,500 g for 4 min.

5. Collect the supernatant without disturbing the pellet.

Suggested Protocol for Lysate Preparation of Histone H3 Assay:

1. Follow steps 1-3 in lysate preparation.

4. Lyse the samples:

a) Immediately add the lysing solution to the cells. The amount of lysing solution needed depends on the cell

b) Briefly sonicate (e.g., with a Sonifer 450 as follows: Duty cycle = 40, Output = 1, Pulse sonicating = two 10 minute pulses with a 1 minute break in between).

c) Agitate the cells. Transfer the cell lysate to a centrifuge tube and rotate for 20 min at 4ºC.

d) Centrifuge the samples at 4,500 g for 20 min at 4ºC.

5. Collect the supernatant without disturbing the pellet.

For Bio-Plex Phosphoprotein Assays and Bio-Plex Total Target Assays

1. Determine the lysate protein concentration. The protein concentration should be 200–900 µg/ml. It may be necessary to test-lyse your samples with different volumes of lysing solution to obtain the specified protein concentration range.

2. Add an equal volume of *assay buffer* to the lysate.

3. If the lysate is not tested immediately, store at –20ºC. The lysate is stable for up to 5 freeze-thaw cycles. For Bio-Plex Histone H3 assay, freeze lysate (overnight) at –20ºC and thaw before testing.

4. For further assay instructions refer to the Bio-Plex phosphoprotein detection instruction manual.

For Western Blot Analysis

1. Determine the lysate protein concentration. The protein concentration should be 200–900 µg/ml. It may be necessary to test-lyse your samples with different volumes of lysing solution to obtain the specified protein concentration range.

2. If the lysate is not tested immediately, store at –20ºC. The lysate is stable for up to 5 freeze-thaw cycles.

3. Prepare fresh sample loading buffer using a 1:20 dilution of 2-mercaptoethanol and Laemmli sample buffer. Alternatively, another sample loading buffer can be used.

4. Dilute 1 part sample with 2 parts sample loading buffer.

5. For further instructions, refer to Bio-Rad's Laemmli sample buffer manual.

Safety Considerations

Eye protection and gloves are recommended while using this product. Consult the MSDS for additional information.

BIO-RAD

Bio-Plex Pro Mouse Th17 Cytokine Assays

Quick Guide

For Use with	Instruction Manual #
Bio-Plex Pro Cytokine, Chemokine, and Growth Factor Assays	10000111560

This guide can be used to prepare and run a full 1 x 96-well assay plate. New users should download the complete manual, which includes detailed instructions and a list of kit components, at bio-rad.com/bio-plex.

Initial Preparation

1. Plan the plate layout.
2. Start up/warm up the Bio-Plex Multiplex Immunoassay System (**30 min**).
   - Bring diluents, including wash buffer, assay buffer, standard diluent, detection antibody diluent HP, and sample diluent, to room temperature (RT). Keep the other items on ice until needed
     - Mix by inversion to ensure all salts are in solution
     - Prepare 1x wash buffer: dilute **1 part** 10x wash buffer (60 ml) with **9 parts** distilled water (540 ml)
   - Begin to thaw the frozen samples
3. Prepare the sample dilution according to the guidelines provided in the following table. It is important to centrifuge serum or plasma samples at **1,000 x g** for **15 min** at **4°C** to remove particulates from all samples prior to use.

	Serum and Plasma		Culture Supernatant and Other Fluids		Cell and Tissue Lysate	
Assay	Dilution	Diluent	Dilution	Diluent	Dilution	Diluent
Mouse and rat cytokines	1:4	Bio-Plex Sample Diluent	User optimized	Cell culture medium or buffer similar to sample*	User optimized (1:2 of lysates at 200–900 µg/ml protein)	Bio-Plex Sample Diluent
Mouse ICAM-1	1:100	Bio-Plex Standard and Sample Diluents	User optimized			

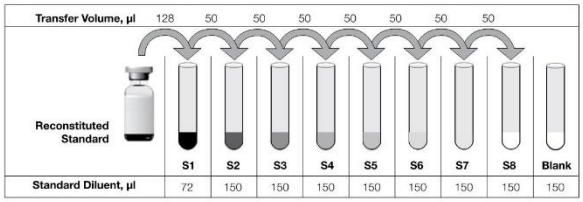
* If samples are serum-free, add bovine serum albumin (BSA) to 0.5% final w/v.

For example:

- For serum or plasma cytokine assays, dilute samples 1:4 by adding 40 µl sample + 120 µl Bio-Plex Sample Diluent
- For serum or plasma ICAM-1 assays, dilute samples 1:100
  - First dilution (1:4): 10 µl sample + 30 µl Bio-Plex **Sample Diluent**
  - Second dilution (1:25): 5 µl from the first dilution + 120 µl Bio-Plex **Standard Diluent**

4. Calibrate the Bio-Plex System in Bio-Plex Manager Software.
5. Reconstitute the standards and control by adding **500 µl** of standard diluent to each. **Vortex** at medium speed for **5 sec** and incubate all vials on ice for precisely **30 min**.
6. Prepare a fourfold standard dilution series and blank as shown. **Vortex** at medium speed for **5 sec** between liquid transfers.

   Note: The control is ready to use after reconstitution. Controls are included with the fixed panel only.



7. **Vortex** the coupled beads at medium speed for **30 sec** and **dilute to 1x** in Bio-Plex Assay Buffer as shown. Protect from light.

Premixed Panels

Number of Wells	10x Beads, µl	Assay Buffer, µl	Total Volume, µl
96	570	5,130	5,700

Singleplex Assays

	Singleplex #1	Singleplex #2		
Number of Wells	10x Beads, µl	10x Beads, µl	Assay Buffer, µl	Total Volume, µl
96	570	570	4,560	5,700

Note: 10x singleplex beads allow multiplexing up to ten analytes.

Running the Assay

1. **Vortex** the diluted (1x) beads. **Add 50 µl** to each well of the assay plate.
2. **Wash the plate two times** with **100 µl** Bio-Plex Wash Buffer.
3. **Vortex** the samples, standards, blank, and control. **Add 50 µl** to each well.
4. Cover the plate with sealing tape. Incubate on shaker at **850 ± 50 rpm** at RT for **30 min**.
5. With 10 min left in the incubation, **vortex** the detection antibodies for **5 sec** and quick-spin to collect liquid. **Dilute to 1x** as shown.

Premixed Panels

Number of Wells	10x Detection Antibodies, µl	Detection Antibody Diluent HP, µl	Total Volume, µl
96	300	2,700	3,000

Singleplex Assays

	Singleplex #1	Singleplex #2		
Number of Wells	10x Detection Antibodies, µl	10x Detection Antibodies, µl	Detection Antibody Diluent HP, µl	Total Volume, µl
96	300	300	2,400	3,000

Note: 10x singleplex beads allow multiplexing up to ten analytes.

6. **Wash the plate three times** with **100 µl** wash buffer.
7. **Vortex** the diluted (1x) detection antibodies. **Add 25 µl** to each well.
8. Cover the plate with sealing tape and incubate at **850 ± 50 rpm** for **30 min** at RT. Meanwhile, prepare the Bio-Plex Manager Software protocol; enter standard S1 values and units provided in the assay kit.
9. With 10 min left in the incubation, **vortex** 100x streptavidin-phycoerythrin (SA-PE) for **5 sec** and quick-spin to collect liquid. **Dilute to 1x** as shown and protect from light.

Number of Wells	100x SA-PE, µl	Assay Buffer, µl	Total Volume, µl
96	60	5,940	6,000

10. Wash the plate three times with **100 µl** wash buffer.

11. Vortex the diluted (1x) SA-PE. **Add 50 µl** to each well.

12. Cover the plate with sealing tape and incubate at **850 ± 50 rpm** for **10 min** at RT.

13. Wash the plate three times with **100 µl** wash buffer.

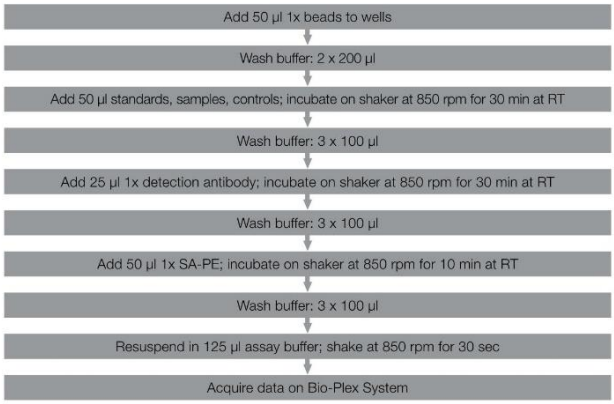
14. Resuspend the beads in **125 µl** assay buffer. Cover and shake at **850 ± 50 rpm** for **30 sec**.

15. Remove the sealing tape and **read plate** using the following settings:

Instrument	RP1 (PMT)	DD Gates	Bead Events
Bio-Plex 3D*	Standard	Select MagPlex Beads	50
Bio-Plex 100, 200*	Low	5,000 (low); 25,000 (high)	50
Luminex MAGPIX	N/A, use default instrument settings		

* Or similar Luminex System.

Assay Workflow



Liquid chromatography mass spectrometric method used for the quantification of GSH and GSSG in whole brain samples

The reduced glutathione GSH[1] and oxidized glutathione GSSG[2] analyses were conducted according to Güldenpfennig et al. (2011), with slight modification. Here, only additional detail to that already provided in Chapter 3, is provided.

Standards, mobile phase, instrument setup, and sample preparation

Standard solutions

- Approximately 1 mg of each of the analytes was dissolved separately in 10 mL of a 10 % methanol solution in amber volumetric flasks.
- From the stock solutions of each analyte, a combined serial dilution series consisting of six concentrations were prepared to construct a standard calibration curve and determine the linear range of each metabolite.
- The straight-line equation of each analyte was used to determine each analyte's concentration in each of the test serum samples.

Internal standard solutions

- A stock solution of the internal standard, ethyl-4-hydroxy-2-quinolinecarboxylate (EHQC[3]), was prepared at a concentration of 100 µg/mL using a solvent mixture of 0.1 M formic acid in 100 % acetonitrile.
- Subsequently, a working internal standard solution with a final concentration of 250 ng/mL was prepared from the stock solution using the same solvent mixture.
- The working solution was also used for the preparation of the different biological sample matrices.

Mobile phases

- A gradient mobile phase consisting of (A) 0.1 % formic acid/LC/MS[4] grade water and (B) 0.1 % formic acid/MeOH[5] was prepared.

[1] reduced glutathione
[2] oxidized glutathione
[3] ethyl-4-hydroxy-2-quinolinecarboxylate
[4] liquid chromatography/mass spectrometry
[5] methanol

Sample preparation

- Brain tissues were allowed to thaw on ice and was individually weighed prior to preparation.
- 1000 µL of the internal standard solution was added to the brain samples
- Tissues were homogenized by sonication (twice for 10 sec, at an amplitude of 14 µ; MSE® ultrasonic disintegrator, Nuaillé, France).
- Mixtures were left on ice for 20 min to complete protein precipitation and subsequently centrifuged at 20 817 RCF for 20 min at 4 ºC.
- 1 µL of the supernatants were injected onto the LC/MS[1] system.
- The results were converted from ng/mL to a final result of ng/g of the wet weight of brain tissue.

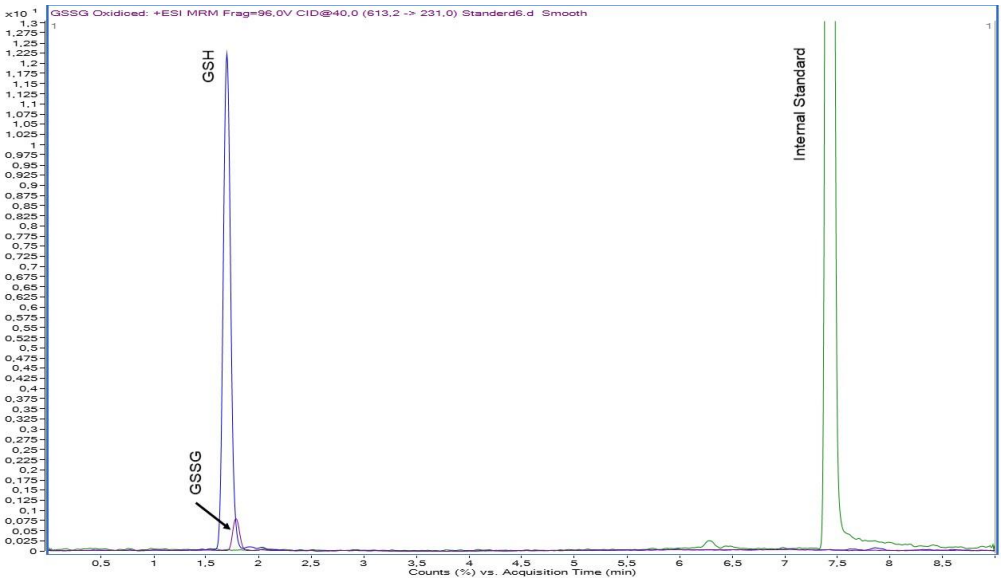
Linearity and calibration curve of the standards

Analyte	**Concentration range (ng/mL)**	**y = mx + c (determined by MassHunter™; line forced through origin)**	***R*²**
GSH[2]	20,0590; 40,1190; 80,2380; 160,4760; 320,9530; 641,0	y = 42,852998* x – 1150,389	0.9960
GSSG[3]	26,3930; 52,7870; 105,5740; 211,1480; 422,2960; 844,0	y = 0,189172 * x – 1,861589	0.9941

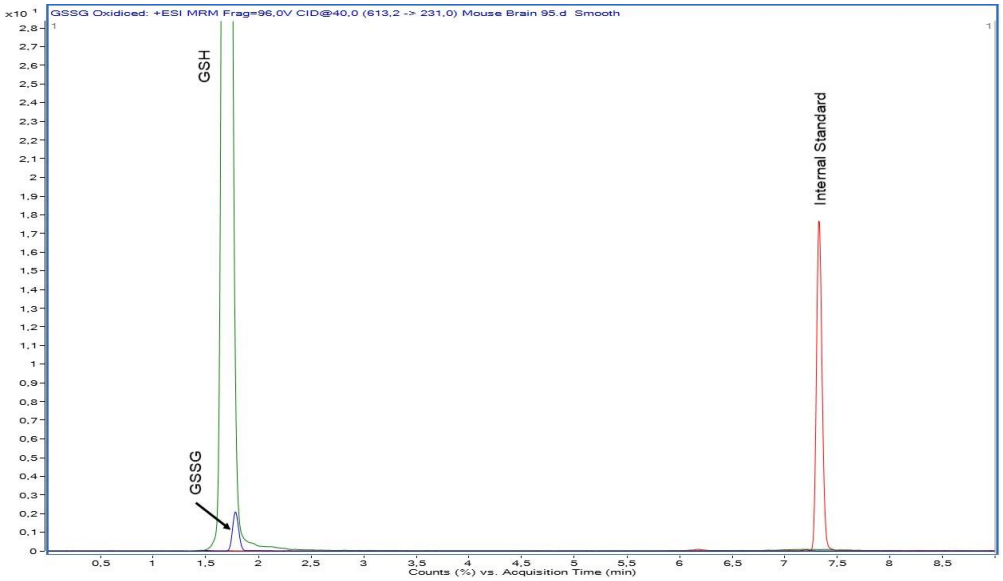
[1] liquid chromatography – mass spectrometry
[2] reduced glutathione
[3] oxidized glutathione

MRM (multiple-reaction monitoring) chromatograms

Standards



Brain samples



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