Impact of prenatal exposure to delta-9-tetrahydrocannabinol on trajectories of brain development in mice.

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I dedicate this thesis to

Maile Cupo,

who taught me curiosity will take you farther than determination ever can,

Caroline Wright,

who can communicate more with a painting than I can with 50,000 words,

and **Daniel Guardia**,

who already knows the contents, without having to read a single page.

Contents

Abstract

Brain development, from conception to adulthood, is a carefully orchestrated process that can be sensitive to alterations in the fetal environment. One urgent environmental influence to study in the context of the developing brain is gestational cannabis exposure. The endocannabinoid system (ECS) is involved in many processes that regulate proper brain growth, organization, and maturation [Harkany et al., 2007], and exposure to external cannabinoids can interfere with typical development. Evidence from both the clinic and laboratory suggest prenatal cannabis exposure (PCE), especially exposure to the psychoactive component Δ^9-tetrahydrocannabinol (THC), can alter brain development in infants, children, and adolescents, reflected in changes to behavior, occupational outcomes, and brain organization. Of note, PCE and prenatal THC exposure (PTE) differ, and are not used as equivalent substitutes in this thesis, as PCE implies exposure to cannabis itself (including various endocannabinoids) and PTE implies exposure to THC alone.

It is especially urgent that potential consequences of PCE are identified because cannabis has been increasingly legalized and used among pregnant people [Young-Wolff et al., 2020, Young-Wolff et al., 2022]. There are many challenges to studying PCE in humans, therefore research using animal models has been employed to afford researchers greater experimental control in studying the effects of PCE. One major advantage of nonhuman animal studies is the ability to follow a specific animal from birth to adulthood within about three months. Such experiments provide an efficient means of demonstrating the lifetime effects of PCE or PTE. Despite initial investigations into growth and behavior following PCE in rodents, many of the underlying neurobiological changes remain unexplored, especially examining the brain as a whole without preselecting a region of interest, which may create blindspots in the understanding of how the brain is affected.

The content of this thesis seeks to address the gaps in the literature surrounding the impact of PTE on whole brain development across the lifespan using structural

magnetic resonance imaging (MRI) in the mouse as a model organism. **Chapters 1 and 2** provide an introduction to the thesis and topic with an overview of the current body of knowledge regarding cannabis use during pregnancy, the known effects on development, the importance of sex differences, and the benefits and drawbacks of using mice as a model system. **Chapter 2** further discusses the methodologies used in this thesis, specifically MRI and the statistical methods used to analyze results. **Chapter 3** presents an investigation of the impact of PTE on the whole brain and body of embryonic mice. The findings corroborated prior knowledge of intrauterine growth restriction (IUGR) following PCE, but expanded on the literature by highlighting volume differences in bilateral regions across the brain. **Chapter 4** investigates whether prenatal changes persist into the first weeks following birth, using longitudinal *in vivo* MRI. Findings highlighted catch up growth in the PTE pups, but a reduction in growth rate of brain regions across cortical and subcortical regions. Investigations in early life behaviors indicated a sex-dependent impact of PTE on social behavior of pups. **Chapter 5** investigates whether the early life changes persist through adolescence to adulthood with longitudinal *in vivo* MRI and two behavioral analyses in adolescence for anxiety-like behavior and sensorimotor gating. Weights were reduced in THC-exposed pups throughout the study. The main effect of THC exposure indicated reduced brain volumes with greater effects in females than males. Additionally, there was evidence of anxiety-like behavior in females, and trend-level impairments to sensorimotor gating. Finally, **Chapter 6** provides a summary and general discussion of all results, highlighting certain limitations of the work and potential future directions for the field. Overall, this thesis provides a thorough characterization of the impact of PTE on body growth and brain volume across the lifespan, highlighting novel regions of interest for future studies and exposing the importance of studying brain development as a dynamic process.

Abrégé

Le développement cérébral, de la conception à l'âge adulte, est un processus soigneusement orchestré qui peut être sensible aux altérations de l'environnement fœtal. Une influence environnementale extrêmement urgente à étudier dans le contexte du cerveau en développement est l'exposition gestationnelle au cannabis. Le système endocannabinoïde (SEC) est impliqué dans de nombreux processus différents qui régulent la croissance, l'organisation et la maturation normales du cerveau [Harkany et al., 2007], fournissant un mécanisme par lequel l'exposition aux cannabinoïdes externes peut interférer avec le développement typique. Des preuves provenant à la fois de la clinique et du laboratoire suggèrent que l'exposition prénatale au cannabis, en particulier à la composante psychoactive Δ^9-tetrahydrocannabinol (THC), peut altérer le développement cérébral chez les nourrissons, les enfants et les adolescents, se reflétant dans des changements de comportement, de résultats professionnels et d'organisation cérébrale [Goldschmidt et al., 2016].

Il est particulièrement urgent d'identifier les conséquences potentielles de l'exposition prénatale au cannabis car celui-ci est de plus en plus légalisé et utilisé parmi la population générale et les personnes enceintes [Young-Wolff et al., 2020, Young-Wolff et al., 2022]. De nombreux défis persistent dans l'étude de l'exposition prénatale au cannabis chez l'homme. La recherche utilisant des modèles animaux peut répondre à ces facteurs de confusion, offrant aux chercheurs un plus grand contrôle expérimental pour comprendre les effets causaux de l'exposition prénatale au cannabis sur le développement. Un avantage majeur des études sur les animaux non humains est la possibilité d'étudier un animal spécifique de la naissance à l'âge adulte en environ trois mois, tandis qu'une étude similaire chez les humains prendrait des décennies. Ainsi, de telles études offrent un moyen efficace de fournir des réponses concernant les effets à vie de l'exposition prénatale au cannabis malgré des études initiales qui examinent la croissance et le comportement après l'exposition au cannabis chez les rongeurs. De nombreux changements neurobiologiques sous-jacents restent non investigués, en

particulier l'étude du cerveau dans son ensemble sans présélectionner une région très spécifique d'intérêt, ce qui peut créer des angles morts dans la compréhension de la manière dont le cerveau est affecté.

Le contenu de cette thèse vise à combler les lacunes de la littérature concernant l'impact de l'exposition prénatale au cannabis sur le développement global du cerveau tout au long de la vie en utilisant l'Imagerie par Résonance Magnétique (IRM) structurelle chez la souris en tant qu'organisme modèle. **Les Chapitres 1 et 2** fournissent une introduction à la thèse et au sujet avec un aperçu du corpus actuel de connaissances sur l'utilisation du cannabis pendant la grossesse, les effets connus sur le développement, l'importance des différences de genre, et les avantages et les inconvénients de l'utilisation des souris comme système modèle. **Le Chapitre 2** discute davantage des méthodologies utilisées dans cette thèse, en particulier l'IRM et les méthodes statistiques utilisées pour analyser les résultats. **Le Chapitre 3** présente une enquête sur l'impact de l'exposition prénatale au cannabis sur l'ensemble du cerveau et du corps des souriceaux embryonnaires. Les résultats corroborent les connaissances antérieures de la intrauterine growth restriction (IUGR) après une exposition au cannabis, mais élargissent la littérature en mettant en évidence des différences de volume dans des régions bilatérales à travers le cerveau. Les différences de genre ont été étudiées, mais seulement trouvées dans des analyses exploratoires de différences de volume d'organes. **Le Chapitre 4** s'appuie sur les conclusions du **chapitre 3** en examinant si les changements prénataux persistent dans les premières semaines suivant la naissance, en utilisant l'*in vivo* IRM longitudinale. Les résultats mettent en évidence une croissance compensatoire chez les souriceaux exposés au cannabis, mais une réduction du taux de croissance des régions cérébrales à travers les régions corticales et sous-corticales. Les investigations sur les comportements de la petite enfance indiquent un impact dépendant du sexe de l'exposition au cannabis sur le comportement social des souriceaux, cependant, il n'y avait pas de différences de sexe dans le poids ou le volume cérébral. **Le Chapitre 5** élargit les conclusions des **Chapitres 3 et 4** en examinant si les changements de la petite enfance persistent à travers l'adolescence jusqu'à l'âge adulte avec l'*in vivo* IRM longitudinale et deux analyses comportementales à l'adolescence : l'open-field test (OFT) pour le comportement de type anxiété et le prepulse inhibition (PPI) pour le filtrage sensorimoteur. Les poids étaient réduits chez les souriceaux exposés au THC tout au long de l'étude. L'effet principal de l'exposition au THC indique que les volumes cérébraux réduits avant l'enfance persistent à l'âge adulte, cependant, les effets étaient plus importants chez les femelles que chez les mâles. De plus, il y avait des preuves de comportement de

type anxiété chez les femelles et une altération du filtrage sensorimoteur à un niveau de tendance. Enfin, **Le Chapitre 6** propose une discussion générale finale de tous les résultats, mettant en évidence certaines limites du travail et des orientations futures potentielles pour le domaine. Dans l'ensemble, cette thèse fournit une caractérisation approfondie de l'impact de l'exposition prénatale au cannabis sur la croissance corporelle et le volume cérébral tout au long de la vie, mettant en lumière des régions d'intérêt nouvelles pour des études futures et soulignant l'importance de l'étude du développement cérébral en tant que processus dynamique.

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List of Acronyms

11-OH-THC	11-Hydroxy-Δ^9-tetrahydrocannabinol
2-AG	2-Arachadonoyl glycerol
2-AGE	2-Arachidonyl glyceryl ether
ADHD	Attention Deficit Hyperactivity Disorder
AEA	N -arachidonoyl-ethanolamine [Anandamide]
ANTs	Advanced Normalization Tools
ASD	Autism Spectrum Disorder
ASL	Arterial Spin Labelling
BOLD	Blood Oxygen Level Dependent
C57	C57/Black6 J
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid-
CBD	Cannabidiol
CBDA	Cannabidiolic acid
ChAT	Choline acetyltransferase
CNR	Contrast-to-noise ratio
CNS	Central nervous system
cryo-probe	Cryogenically-cooled coil
CSF	Cerebral spinal fluid
CUD	Cannabis use disorder
DAGL	Diacylglycerol lipases
DBM	Deformation based morphometry
Dex	Dexmedetomidine
ECS	Endocannabinoid system
EM	Electron microscopy
EpCAM	Epithelial cell adhesion moleculen

FAAH	Fatty acid amide hydrolase
FC	Functional connectivity
FDR	False Discovery Rate
FGR	Fetal growth restriction
fMRI	Functional magnetic resonance imaging
FOV	Field-of-view
GABA	γ-aminobutyric acid
GAD	Glutamate decarboxylase
Gado	Gadoteridol
GAM	Generative additive models
GD	Gestational day
Gen R	Generation R
GFP	Green fluorescent protein
GPCRs	G-protein coupled receptors
HD	Harrell-Davis quantile estimator
HED	Human Equivalent Dosage
hIPSC	Human-induced pluripotent stem cells
IBA1	Ionized calcium-binding protein
ICC	Intraclass correlation
IHC	Immunohistochemistry
IP	Intraperitoneal
IRM	Imagerie par Résonance Magnétique
IUGR	Intrauterine growth restriction
LBW	Low birth weight
LMER	Linear mixed effects models
MAGL	Monoacylglycerol lipase
MHPCD	The Maternal Health Practices and Child Development Study
MIA	Maternal immune activation
MnCl	Manganese chloride 2
MR	Magnetic resonance
MRI	Magnetic resonance imaging
NAPE	N-arachidonoylphosphatidylethanolamine-specific phospholipase D
NMR	Nuclear magnetic resonance
OFT	Open-field test

OPPS	Ottawa Prenatal Perspective Study
OTO	Osmium-thiocarbohydrazide-osmium
PBS	Phosphate Buffered Saline
PCE	Prenatal cannabis exposure
PET	Positron emission tomography
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PND	Postnatal day
PPI	Prepulse inhibition
PTE	Prenatal THC exposure
QC	Quality control
RF	Radiofrequency
RNAseq	RNA-sequencing
ROI	Region of interest
rs-fMRI	Resting state functional magnetic resonance imaging
Sal	Saline
SM	Supplementary Methods
SNR	Signal-to-noise ratio
SR	Supplementary Results
SRI	Serotonin reuptake inhibitors
T	Tesla
T1w	T1-weighted
T2w	T2-weighted
TBV	Total brain volume
TE	Echo time
THC	Δ^9-tetrahydrocannabinol
THCA	Δ^9-tetrahydrocannabinolic acid
THCCOOH	11-Nor-9-carboxy-Δ^9-tetrahydrocannbinol
TR	Repetition Time
TRR	Test-retest reliability
USA	United States of America
USVs	Ultrasonic vocalizations
V1	Primary visual cortex
WIN	WIN 55,212-2

Acknowledgements

Even as my research investigates the impact of the environment on the development of an individual, I can clearly see reflected in my personal developmental trajectory the imprint of my mentors, friends, and family, my local environment, who I would like to acknowledge here. First, to Dr. Mallar Chakravarty, my PhD supervisor and mentor, thank you for taking a chance on me. Through your mentorship, supervision, and trust, I have transformed from an enthusiastic undergraduate student with a passion for the brain to a confident computational neuroscientist. I remember when I first came to visit the lab I asked you what you thought the attribute most determining success was in a PhD student, and you told me "being reflective". I have tried to embody that in my graduate school career, and I really feel I have thrived in the enriching environment you have provided. You have always been incredibly supportive in my scientific pursuits, encouraging me to think non-traditionally, question everything, and be open-minded and humble when I am questioned in return. These are invaluable skills, and I am very excited to see where they lead me next. Thank you, Mallar, for your continued support and encouragement, your insight in matters both within academia and without, and the trust that has been so critical in my development of confidence. I hope you also feel like the chance you took paid off.

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Contributions of Authors

Chapters 3, 4, and 5 of this thesis represent original work. For each of these projects, I led experimental design, data collection, processing, analysis, and interpretation, and drafting of the manuscripts under the supervision of Dr. M. Mallar Chakravarty. Nevertheless, each body of work incorporates intellectual and physical contributions from other co-authors, without whom the work would be impossbile. This section delineates their contributions:

Chapter 3: Lani Cupo, Elisa Guma, Annie Phan, Shoshana Spring, Brian Neiman, Gabriel A. Devenyi, M. Mallar Chakravarty. Impact of chronic prenatal exposure to THC early in gestation on brain volume of mouse embryos. *Unpublished.*

- Elisa Guma: taught me skills relevant to the project including animal handling and embryo extraction. Spearheaded THC work in the lab, providing preliminary research as we applied to obtain cannabis licenses. Established pipelines for embryo image preprocessing and advice on manual correction of masks.
- Annie Phan: provided support quality controlling images, manually correcting masks.
- Shoshana Spring: performed *ex vivo* scanning of embryos at the Mouse Imaging Centre in Toronto
- Brian Neiman: provided initial development of methods used for *ex vivo* imaging and assistance with the procedures
- Gabriel A. Devenyi: Provided technical support and expertise in embryo image preprocessing, quality assurance, registration, and analyses. Built in-house pipelines for deformation based morphometry analyses.
- M. Mallar Chakravarty: Provided significant oversight and advice on experimental design, analytic approach, and interpretation of results. Supplied in-depth feedback on manuscript.

Chapter 4: Lani Cupo, Annie Phan, Elisa Guma, Daniel Gallino, Jeremie Fouquet, Gabriel A. Devenyi, M. Mallar Chakravarty. Impact of chronic prenatal exposure to THC early in gestation on trajectories of development in neonatal mice. *Unpublished.*

- Annie Phan: Provided support collecting data for the experiment and quality controlling images, and provided expertise in assessing early life milestones in rodents.
- Elisa Guma: consulted on study design and methods development as well as analytic approach to study ultrasonic vocalization behavior and interpreting results.
- Daniel Gallino: Provided significant support scanning neonates.
- Jeremie Fouquet: Provided expertise in scan design and intellectual contributions to the design and interpretation of manganese assessment.
- Gabriel A. Devenyi: Provided advanced technical support and expertise in neonate image preprocessing and registration, including developments in registration pipelines to assure good quality within-subject neonatal registrations. Developed pipelines to examine voxel-wise changes over time and provided great support in interpretation of results.
- M. Mallar Chakravarty: Provided supervision throughout experiments, including in experimental design and follow-up experiments and interpretation of results. Supplied in-depth feedback on manuscript.

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- Elisa Guma: Provided data for normative models from prior experiments.
- Annie Phan: Provided data for normative models from prior experiments.

- Janice Park: Provided data for normative models from prior experiments.
- Lizette Herrera: Provided data for normative models from prior experiments.
- Marina Broomfield: Provided data for normative models from prior experiments.
- Mathilda Ryon: Provided data for normative models from prior experiments.
- Megan Park: Provided data for normative models from prior experiments.
- Gabriel A. Devenyi: Provided significant expertise in image preprocessing, registration, and analysis, as well as in interpretation of results.
- M. Mallar Chakravarty: Provided supervision throughout experiments, advice on additional analyses and support in interpreting results. Also provided in-depths review of manuscript.

Other related lead-author publications

Cupo, L., McIlwaine, S. V., Daneault, J.-G., Malla, A. K., Iyer, S. N., Joober, R., and Shah, J. L. (2021). Timing, Distribution, and Relationship Between Nonpsychotic and Subthreshold Psychotic Symptoms Prior to Emergence of a First Episode of Psychosis. Schizophrenia Bulletin, 47(3), 604–614.

Cupo, L., Plitman, E., Guma, E., and Chakravarty, M. M. (2021). A systematic review of neuroimaging and acute cannabis exposure in age-of-risk for psychosis. Translational Psychiatry, 11(1), 217.

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***Co-first authors**

Other related co-author publications

Bussy, A., Levy, J., Best, T., Patel, R., **Cupo, L.**, Van Langenhove, T., Nielsen, J., Pijnenburg, Y., Waldö, M. L., Remes, A., Schroeter, M. L., Santana, I., Pasquier, F., Otto, M., Danek, A., Levin, J., Le Ber, I., Vandenberghe, R., Synofzik, M., ... on behalf of the GENetic Frontotemporal dementia Initiative (GENFI). (2021). Cerebellar and subcortical atrophy contribute to psychiatric symptoms in frontotemporal dementia. In bioRxiv (p. 2021.11.12.468429). https://doi.org/10.1101/2021.11.12.468429

Guma, E., **Cupo, L.**, and Chakravarty, M. M. (2023). Chapter 34: Cannabis, neurodevelopment, and the "two-hit" hypothesis. In V. R. Preedy (Ed.), Cannabis Use, Neurobiology, Psychology, & Treatment. Academic Press.

Guma, E., **Cupo, L.**, Ma, W., Gallino, D., Moquin, L., Gratton, A., Devenyi, G. A., and Chakravarty, M. M. (2023). Investigating the "two-hit hypothesis": Effects of prenatal maternal immune activation and adolescent cannabis use on neurodevelopment in mice. Progress in Neuro-Psychopharmacology & Biological Psychiatry, 120, 110642.

Paquin, V., **Cupo, L.**, Malla, A. K., Iyer, S. N., Joober, R., and Shah, J. L. (2021). Dynamic association of the first identifiable symptom with rapidity of progression to first-episode psychosis. Psychological Medicine, 1–9.

Plitman, E., Bussy, A., Valiquette, V., Salaciak, A., Patel, R., **Cupo, L.**, Béland, M.-L., Tullo, S., Tardif, C. L., Rajah, M. N., Near, J., Devenyi, G. A., and Chakravarty, M. M. (2021). The impact of the Siemens Tim Trio to Prisma upgrade and the addition of volumetric navigators on cortical thickness, structure volume, and 1H-MRS indices: An MRI reliability study with implications for longitudinal study designs. NeuroImage, 238, 118172.

Original Contributions of Thesis

Chapter 3

- Novel investigation of the effects of prenatal exposure to THC chronically during early gestation on whole brain and body volume using *ex vivo* MRI
- Evidence for IUGR late in gestation following THC exposure
- Evidence for alterations in local brain volume including ventriculomegaly, larger volume in regions rich with CB_1 receptors, and smaller volume in the cerebellum and midbrain
- Using the mediation with residuals approach, not commonly used in neuroimaging, we demonstrate the impact of prenatal THC exposure (PTE) directly, accounting for IUGR as a covariate and potential mediator.
- Evidence for differences in local organ volume with exploratory whole-body MRI analyses
- A thorough investigation of potential sex-differences in early effects of PTE on brain and body volume with large sample size and interactions among variables.
- Inclusion of a null model to examine the potential effects of chronic injections on outcomes of interest, indicating potential IUGR following chronic injections, but little impact on brain volume

Chapter 4

- Building on Chapter 3, this project extends the investigation into early gestational THC exposure into early postnatal life with longitudinal *in vivo* MRI
- Provides evidence for catch-up growth in the two weeks of postnatal life

- Indicates reduction in brain growth in many cortical and subcortical regions following PTE
- Sex-specific alterations in ultrasonic vocalizations and social behavior in THC-exposed offspring
- Evidence for no difference between scanned offspring and unscanned littermates in body weight gain, but differences in ultrasonic vocalization behavior
- Provides additional evidence to literature about the impact of repeated exposure to manganese chloride 2 (MnCl) as a contrast agent in neonates
- Examines sex effects across outcome measures, detecting them only in behavior, not weight gain or brain volume
- Technical contributions include: longitudinal, *in vivo* scanning paradigm at the Douglas Cerebral Imaging Centre and registration pipeline for subject-wise neonatal registrations.

Chapter 5

- Expanding on Chapters 3 and 4, this project investigates the impact of early gestational THC exposure through adolescence and into adulthood using longitudinal *in vivo* MRI
- Provides evidence for persistent alterations in metabolism and weight following PTE, with reduced weights across all time points and both sexes
- Sex-dependent effects of PTE on offspring brain volume, with a main effect of THC exposure associated with smaller volume, especially in females
- Sex-dependent effects of PTE on behavior, with females especially showing increases in anxiety-like behavior during adolescence and trending impairments to sensorimotor gating
- No impact of prenatal exposure or multiple injections on dam nesting behavior, or time spent on nests over the first week of postnatal life
- Proof-of-concept for the use of normative models in developmental studies of mouse models including weight and total brain volume

Chapter 1

General Introduction

1.1 Motivation

It is commonly accepted that prenatal exposure to alcohol, nicotine, and addictive drugs of abuse, like heroin, are harmful to the developing fetus, however, the consequences of intrauterine exposure to cannabis are understudied. Despite significant evidence that prenatal cannabis exposure may impact the development of offspring [Nashed et al., 2021], there are also recent studies that dispute the clinical significance of the findings [Torres et al., 2020]. The lack of clear consensus among scientific studies leaves medical doctors cautioning patients against cannabis use during pregnancy, but without sufficient evidence to properly advise their patients on the benefits or harms of use during pregnancy. Even as medical and scientific professionals remain cautious, many lay resources, such as cannabis dispensaries in regions where access has been legalized [Dickson et al., 2018], encourage cannabis use as a means of managing nausea and vomiting associated with pregnancy. This mixed messaging can create confusion for pregnant people weighing the potential benefits of using cannabis (discussed further in Section 2.1.3) but concerned for the health of their fetus [Bayrampour et al., 2019]. Thus, to allow pregnant people to make better informed decisions about using or abstaining from cannabis, and to allow medical professionals to anticipate and mitigate any potential harm from exposure, it is critical for researchers to better establish the impact of prenatal exposure to cannabis on the developing brain and the mechanism(s) by which these alterations emerge.

Cannabis is considered the most widely used illicit drug (despite increased legalization), even during pregnancy [Mark et al., 2016, Wendell, 2013, Gray et al., 2010]. Cannabis comprises a host of compounds described fully in Section 2.2.1, but Δ^9-

tetrahydrocannabinol (THC) is the main psychoactive component, thought to be responsible for many of the psychiatric alterations following chronic, adolescent exposure [Luzi et al., 2008]. As the content of THC in widely-accessible cannabis has been increasing in recent decades, the effect of exposure to this compound in particular is urgent. Because it would be unethical to establish a causal link between prenatal THC exposure and offspring outcomes in humans, non-human animals, in this case mice, are used. Magnetic resonance imaging (MRI) provides a unique tool to noninvasively assess neuroanatomy multiple times within an individual's lifespan, allowing trajectories of development to be established. To our knowledge, these are the first studies that leverage the utility of MRI to causally investigate the impact of prenatal THC exposure on lifespan development.

1.2 Goals and Specific Questions

The goal of this thesis is to investigate how early chronic gestational exposure to THC impacts neurodevelopment in mice from the embryo to adulthood. Neurodevelopment is first operationalized as brain volume, which is assessed in embryos, neonates, and from weaning to adulthood with MRI. The volumetric analyses are further supported with gross measures of subject growth (embryo volume and subject weight) and with behavioral assessments. This comprehensive and integrative investigation allowed us to answer the following questions:

1. How does exposure to THC over the first week of gestation impact embryonic brain and total body volume?
2. How does exposure to THC over the first week of gestation impact the trajectories of brain volume during the first two weeks of life, subject growth through weight, and anxiety-like behavior of pups?
3. How does exposure to THC over the first week of gestation impact the trajectories of brain volume from weaning to adulthood as well as adolescent behavior?

1.3 Hypotheses

Corresponding with the questions stated in Section 1.2, the hypotheses of this thesis are as follows:

1. Compared to the saline (Sal) control group, brain volume of embryos exposed

prenatally to THC will be altered in regions rich in CB1-receptors, and total body volume will be smaller, consistent with prior evidence of fetal growth restriction.

2. The initial volumetric increase observed in embryos will be followed by a growth delay in the hippocampus, striatum, and cortex in neonatal mice exposed prenatally to THC compared to Sal controls. These delays will be associated with increased anxiety-like behaviours (increased calls to the dam to elicit maternal care).

3. Adolescents exposed to THC prenatally will demonstrate altered brain development trajectories in the hippocampus and cortex that will be associated with heightened anxiety-like behaviour and sensorimotor gating deficits compared to Sal controls, representing outcomes relevant to neurodevelopmental disorders.

Chapter 2

Background

2.1 Epidemiological evidence for cannabis use during pregnancy

2.1.1 Prevalence of use

As cannabis is increasingly legalized around the world, with current legalization status as of February 2023 seen in Figure 2.1, members of the medical community anticipate increased rates of use during pregnancy [Metz and Stickrath, 2015]. Regardless of legalization status, it is the most commonly used illicit (or recently-legalized) drug in Europe and North America [Metz and Stickrath, 2015]. The prevalence of cannabis use during pregnancy vary significantly, with reports from surveys ranging from 1.2 to 35% [Nashed et al., 2021, Metz and Stickrath, 2015]. The lowest rate is from a large cohort (>13,000) in metropolitan France, where use was assessed with an interview in the week following birth in 2010 [Saurel-Cubizolles et al., 2014]. The highest rate is from a relatively small sample (∼300) in Baltimore, Maryland, United States of America (USA) assessed through an anonymous survey between 2015 and 2016 [Mark et al., 2017]. A minority of studies supplement self-report data with maternal saliva [Gray et al., 2010], urine [Hurd et al., 2005], and neonatal meconium analyses [Gray et al., 2010, Hurd et al., 2005]. Assessing prevalence of use with a combination of self-report, maternal oral samples, and meconium analyses in a population of 86 pregnant people in Buffalo, New York, USA revealed 46.5% of participants used cannabis at some point during pregnancy [Gray et al., 2010], which exceeds the reports from surveys alone mentioned above [Mark et al., 2017].

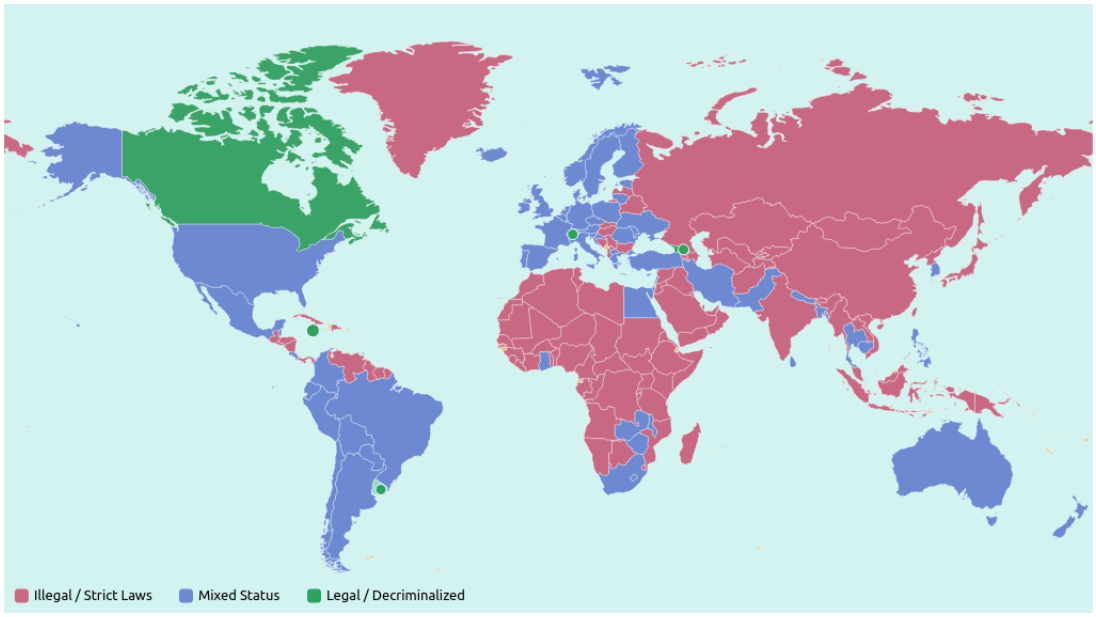

Figure 2.1: World map of cannabis legality (Feb 2023). Image created with information from [Allen, 2023]

2.1.2 Limitations to assessment

Accurately assessing prevalence of drug use during pregnancy is very difficult for several reasons. The most popular method, self report, has been demonstrated to underestimate drug use during pregnancy. One study in 83 pregnant people in New Mexico, USA compared routine urine drug screens to interview responses in the first trimester and after delivery [Garg et al., 2016]. The authors found significant under-reporting of drug use, with less than 60% of participants who used cannabis reporting use [Garg et al., 2016]. Pregnant people may choose not to report drug use because of the fear of stigmatization. Additionally, in many regions the use of drugs during pregnancy is a felonious offence, with parents facing increased surveillance, arrest, prosecution, and even child removal [Stone, 2015]. As legislators in the USA scramble to mitigate the impact of the growing opioid crisis, more states pass policies that are less supportive and more punitive of pregnant people who use substances during pregnancy [Thomas et al., 2018], despite a lack of evidence that these policies achieve the intended consequences [Atkins and Durrance, 2020].

While biological samples may be more reliable, each method has its own limitations. For maternal urine and serum, positive results last only 2-3 days in occasional users, or weeks in chronic users [Metz and Stickrath, 2015]. Serum collection is an invasive procedure, and Δ^9-tetrahydrocannabinol (THC) and metabolites have a shorter half-life

in blood serum [Metz and Stickrath, 2015]. Maternal hair samples are less accurate for cannabis than for other drugs, are costly to analyze, and may have false-positives due to passive second-hand exposure [Metz and Stickrath, 2015]. Fetal meconium analyses can only detect positives from second or third trimester exposure, have a high rate of false-positives, and are costly and impractical to analyze, as is true for neonatal hair samples, with the added disadvantage that these are less sensitive than meconium analyses [Metz and Stickrath, 2015]. Given that many using cannabis during the course of pregnancy fear stigma, there may be a bias in the pregnant individuals who do participate and are willing to disclose their use. While no studies to my knowledge explore this question, they may be more likely to have a medical cannabis prescription, or be more trusting of researchers.

2.1.3 Motivation for use

While many popular and medical sources caution pregnant people against drug use, including cannabis, there are several reasons why they may initiate or continue use. First, some pregnant people suffer from a cannabis use disorder (CUD), or co-morbid substance use disorders [Meinhofer et al., 2022]. Of 20,914,591 hospitalizations of pregnant people between 2010 and 2018 from 35 states, 1.19% involved a CUD. This small percentage actually represents 249,084 pregnancies, and this proportion increased from 0.008 in 2010 to 0.02 in 2018 [Meinhofer et al., 2022]. Of note, the increase of CUDs in recent years follow trends of cannabis legalization; the authors found that of the seven states with the highest rates of CUDs, five states had legalized recreational consumption [Meinhofer et al., 2022]. While cannabis is perceived as a low-risk substance, there is evidence that the three-stage framework for addiction applies to cannabis, like other drugs: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation [Zehra et al., 2018]. The first phase is characterized by striatal dopamine release (evidenced following acute exposure to THC as inhibitory interneurons are hyperpolarized promoting downstream dopamine release) [Zehra et al., 2018]. In turn, the second phase is characterized by a reduction in dopaminergic signalling in the nucleus accumbens and amotivation to non-drug rewards [Zehra et al., 2018]. Finally, the third phase is characterized by reduced executive control over craving, mediated by the prefrontal cortex in humans, with cognitive dysfunction observed after chronic exposure in nonhuman animals [Zehra et al., 2018]. This model of addiction may fuel continued cannabis use in pregnant people.

Some pregnant people use cannabis to help manage the symptoms associated with

morning sickness [Westfall et al., 2006]. Morning sickness generally occurs during the first trimester of pregnancy, beginning between weeks four to six of pregnancy, peaking between weeks 8-12, and usually diminishing by week 20 [Einarson et al., 2013]. A majority of pregnant people experience symptoms of morning sickness ranging from mild to extreme nausea and vomiting [Westfall et al., 2006]. One study surveying 79 participants found 65% (51) of participants reported using cannabis, with seven participants using it only therapeutically, 14 using it only recreationally, and the other 30 using it for both [Westfall et al., 2006]. Of the respondents who used cannabis as a treatment for morning sickness, 92% considered it extremely effective [Westfall et al., 2006]. Data from a recent publication supports these findings, with 35 of 103 participants indicating they had previously used cannabis during pregnancy, and 89% of these respondents indicating treatment for morning sickness as the primary reason for use [Daniels et al., 2022].

Pregnant people also report using cannabis to manage their moods and emotions. A Toronto-based focus group from a program for pregnant and parenting mothers with substance use disorders reports that participants discussed external stressors (such as financial and social stressors and concern about withdrawal from substances), internal stressors (guilt over substance use, leading to a vicious cycle), substance use as a coping strategy, and a misunderstanding of the potential consequences for their children [Latuskie et al., 2019]. Some pregnant people also believe cannabis is a safer alternative to pharmaceuticals that may be prescribed or recommended to treat their mood-disorders and morning sickness [Chang et al., 2019].

Finally, some pregnant people continue cannabis use recreationally, often downplaying the potential risks. Increased legalization may give the perception that cannabis can be consumed without risk, or more safely for everyone than other recreational drugs [LaSalle, 2021]. In one study from 2013-2017, the majority of pregnant women reporting past-month cannabis use were classified as using for non-medicinal purposes [Volkow et al., 2019]. Importantly, the classification was made based on the answer to the question "if any cannabis use was recommended by health care professionals" [Volkow et al., 2019]. This method would characterize self-medication for morning sickness as recreational use. As cannabis use is most frequent in the first trimester of pregnancy, it is likely that in many cases cessation of use succeeds knowledge of pregnancy, so early gestational use may reflect patterns of recreational use.

2.1.4 Patterns of use

Characterizations of patterns of cannabis use during pregnancy include frequency of use, drug dosage, and methods of use. As discussed in the previous section, there is ample evidence that cannabis use during pregnancy is most frequent during the first trimester, reduced in the second, and further reduced in the third [Volkow et al., 2019, Moore et al., 2010]. This pattern is characterized in one early study (The Maternal Health Practices and Child Development Study (MHPCD)) with 763 low-income parents from Pittsburgh, Pennsylvania. Here authors assessed cannabis use with an interview at the end of each trimester of pregnancy, finding 41.7% of their sample consumed cannabis in the first trimester, 23.2% continued to the second trimester, and 19.1% continued to the third [Goldschmidt et al., 2004].

Dosage is even more challenging to assess in pregnant populations than frequency of use. The MHPCD study characterized use as "light", meaning less than one "joint" (cannabis cigarettes) per day, or "heavy" meaning one or more joints per day [Goldschmidt et al., 2004]. While there is significant missing data for this variable, "light" usage declined from 27.4% of the total sample in the first trimester to 17.9% in the second trimester and 14.2% in the third. Meanwhile, "heavy" use declined from 14.4% in the first trimester to 5.3% in the second and 5% in the third. Importantly, the authors collected no information about the size of joints or potency of the cannabis consumed. Of note, this study employed a longitudinal design with up to 10 years follow up, and most of the data were acquired in the 1990's when cannabis was still illegal and heavily stigmatized. Cannabis would have been purchased from dealers without information (or with unreliable information) about the THC and cannabidiol (CBD) content of cannabis, which alters the balance of psychoactive and non-psychoactive constituents, explored more fully in Section 2.2.1. In short, psychoactive THC induces a host of motor and psychological effects unobserved after exposure to non-psychoactive CBD. These differential effects makes the THC:CBD balance important when assessing exposure. Additionally, the potency of cannabis (THC content) is estimated to have tripled from ∼4% in 1995 to ∼12% in 2014 [ElSohly et al., 2016], and almost ∼14% in 2019 [ElSohly et al., 2021]. The studies reporting this increase analyzed samples of cannabis seized by the USA's Drug Enforcement Agency, reflecting illicit cannabis and reported a decline in the content of CBD, the non-psychoactive component of cannabis. The preference for this increased THC:CBD ratio is reflected in legal cannabis on the market as well, where it is common to see preparations with high THC and almost no CBD. For example, cannabis advertised online in the USA, contained on

average 19.2% ±6.2 for medical cannabis and 21.5% ±6.0 for recreational cannabis [Cash et al., 2020]. Unless pregnant participants are actively recording and reporting the cannabis they purchase and consume, it is very challenging for studies to assess dosage. As a result, like in the MHPCD study [Goldschmidt et al., 2004], researchers often use frequency of use as a proxy for a dosing variable, such as in the Generation R (Gen R) study acquired in Rotterdam, the Netherlands between 2002 and 2006 [El Marroun et al., 2009]. In this study the participants reported whether cannabis use was daily, weekly, or monthly. In addition to the MHPCD study and the Gen R study, an older study published in 1980, the Ottawa Prenatal Perspective Study (OPPS), reported participants as non-users, irregular users (on average no more than one joint per week), moderate users (on average 2-5 joints per week), and heavy users (more than 5 joints per week) [Fried, 1980]. With the legalization of cannabis and increased access to information regarding potency of preparations, future epidemiological studies may be able to more accurately assess dosage during pregnancy to better characterize long-term impact on offspring.

A final variable impacting severity of fetal exposure is the method of use. As discussed in Section 2.2.2, route of administration can alter circulating metabolites of cannabinoids. The MHPCD, Gen R, and OPPS studies all characterize use in joints, however there are many alternative routes of administration, including oral administration through edibles, vaping, nasal spray, or topical administration. Historically, smoking cannabis through joints, blunts, pipes, or water pipes (bongs), has been the most popular route of administration and remains so today, however the other methods are also gaining popularity, and may be over-represented in pregnant women who want to avoid smoking [Spindle et al., 2019]. Vaporizers heat cannabis or extracts to sub-combustion temperatures aerosolizing the product for inhalation, and have become increasingly common in part because of a perceived reduction in the health risk [Spindle et al., 2019]. Cannabis concentrates (dabs, budder, wax, shatter–all different forms of concentrates derived from dry plant material) have also risen in popularity, but primarily due to their high potency as a party drug. In contrast, edibles are of special interest to the pregnancy literature as these preparations are more commonly used among women, older adults, and people who use cannabis for medicinal purposes [Spindle et al., 2019]. Again, there is a perception of reduced health risk, however edibles are often inaccurate as to reports of the potency of cannabis [Spindle et al., 2019]. Route of administration is important not only to accurately assess dosing of various cannabis metabolites, but also when translating to animal models, as discussed in later sections.

2.2 Cannabis and the Endocannabinoid System

2.2.1 THC, CBD, and Terpenes

Cannabis comprises over 550 chemical compounds with over 100 phytocannabinoids [Rock and Parker, 2021]. "Cannabinoids" refers to both chemical substances isolated from the cannabis plant and their derivatives, while "phytocannabinoids" refers to those compounds native to the plant. It has been speculated that cannabinoids originally served in the role of chemical defense for plants, especially as they express ultraviolet-absorbing and anti-herbivory properties (chemical defenses that dissuade consumption by animals) [Romero et al., 2020]. In addition to cannabinoids, which show variable affinities for endocannabinoid and other receptors, cannabis contains 120 identified terpenes, compounds in essential oils responsible for the plant's aroma. The number of identified cannabinoids and terpenes has risen over the past decades since THC was identified in 1964 [Maccarrone et al., 2015].

THC is one of the most researched cannabinoids and is thought to be responsible for the major psychoactive effects of cannabis, such as motor disturbances, reddened mucous membranes in the eyeball, increased aggression, lethargy, and reductions in spontaneous movement, euphoria, paranoia, and cognitive difficulties [Rock and Parker, 2021]. Interestingly, the effects are biphasic, with low doses decreasing anxiety and aggression, for example, and high dosages increasing them [Sharpe et al., 2020]. THC is a partial agonist at Cannabinoid- $(CB)_1$ and CB_2 receptors, meaning it binds and activates the receptors, although less effectively than a full agonist would [Rock and Parker, 2021].

CBD is the primary non-psychoactive cannabinoid, meaning ingestion does not elicit the host of symptoms observed after exposure to THC [Rock and Parker, 2021]. In contrast, there is evidence that CBD exposure has anxiolytic, anti-psychotic, and neuroprotective properties [Rock and Parker, 2021]. The difference of effect is likely explained by the fact that CBD does not activate CB_1 and CB_2 receptors, but rather is a noncompetitive CB_1 receptor antagonist, meaning it binds CB_1 receptors and reduces (but does not eliminate) the downstream effects [Rock and Parker, 2021]. Additionally, CBD is an inverse agonist at CB_2 receptors, meaning it binds the receptor but has the opposite effect of an agonist [Thomas et al., 2007]. The mechanisms of action of CBD are not limited to the endocannabinoid system (ECS), however. There is evidence that CBD is also an agonist at nuclear peroxisome proliferator-activated receptor-γ (PPAR-γ) receptors, the transient receptor potential of vanilloid types 1 (TRPV1) and 2 (TRPV2) channels, and at low concentrations, an antagonist at orphan G-

protein-coupled receptor GPR55, and the transient receptor potential of the melastatin type-8 (TRPM8) channel [Rock and Parker, 2021]. Thus, CBD does not lead to the psychoactive effects associated with activation at the CB receptors, but rather has varied mechanisms of action, potentially contributing to hypotheses regarding CBD's possible utility as an anti-psychotic and anxiolytic [Rock and Parker, 2021]

Other cannabinoids include δ-8-tetrahydrocannabinol, tetrahydrocannabivarin (THCV), and δ-9-tetrahydrocannabinol-Acid. These cannabinoids, however are less prevalent and therefore, less researched. As they are tangential to the topic of this thesis, they are excluded from further characterization here, but are thoroughly discussed elsewhere [Rock and Parker, 2021].

The combination of cannabis constituents may interact to alter the effects compared with each component individually, a proposed mechanism referred to as the "entourage effect". This hypothesis states that components of cannabis other than THC and CBD, including other phytocannabinoids as well as terpenes and terpenoids, contribute to the overall effect produced by cannabis [Ferber et al., 2020]. Terpenoids are naturally-occurring compounds, dozens of which are produced by the cannabis plant [Namdar et al., 2019]. They have not been as heavily researched as THC and CBD, yet there is preliminary evidence suggesting terpenoids may serve as potential instigators of biological activity [Namdar et al., 2019, Russo, 2011]. One study examined how the terpenoid assemblage from different strains of cannabis altered the effects of the associated phytocannabinoids and found combinations of phytocannabinoids and terpenoids were significantly more cytotoxic to human cancer cells compared with THC or terpenoids alone [Russo, 2011]. This holds potential for oncological therapeutics, however has yet to be deeply investigated in terms of the neurobiological effects, although cannabis and the entourage effect have been promoted as a potential antidepressant and anxiolytic [Ferber et al., 2020].

In contrast to terpenoids, a combination of THC and CBD may reduce the effect of THC alone [Rock and Parker, 2021]. Mechanistically, CBD may interfere with THC's activity at cannabinoid receptors in part accounting for reports of reduced psychoactive effects of THC when paired with CBD, however this interaction is still relatively understudied [Cupo et al., 2021]. For example, neuroimaging studies have reported opposite patterns of brain activity following exposure to THC or CBD, and pre-treatment with CBD prevents acute psychotomimetic effects of administered THC [Bhattacharyya et al., 2010]. Deeper examination of these reports, however, indicates that several papers claiming this opposition [Bhattacharyya et al., 2010, Borgwardt et al., 2008, Fusar-Poli et al., 2009] include participants from a single dataset

conducted on 15 males, which is a very small sample size for functional magnetic resonance imaging (fMRI). While it is important to appreciate that the combined effects of THC and CBD may differ from either individually, as discussed in Section 2.1.4, it is increasingly common to find cannabis preparations that include high rates of THC but almost no CBD. To better understand how these potent cannabis preparations may impact systems, it is important to generate a deep understanding of the compounds individually.

The remainder of this section will focus on THC as the main compound of interest to the thesis projects.

2.2.2 Bioavailability and metabolism of THC

THC is a viscous oil that is highly soluble in lipids, but not in aqueous solutions [Sharma et al., 2012]. When cannabis is smoked or cooked, heat-combustion decarboxylates Δ^9-tetrahydrocannabinolic acid (THCA) into THC (seen in Fig. 2.2A) and cannabidiolic acid (CBDA) into CBD [Romero et al., 2020]. Because THC is lipophilic, it is readily distributed in adipose tissue, the liver, lung, and spleen, (seen in Fig. 2.2B) and it is excreted relatively slowly, with an estimated half life between 20 hours and 12.6 days in humans [Sharma et al., 2012, Peng and Shahidi, 2021]. In the liver, lungs, gastrointestinal tract, and vasculature THC is subject to first-pass (phase I) metabolism [Herman and Santos, 2022]. In this process, drugs are metabolized at local sites, reducing the concentration of circulating active drug. First, THC is hydroxylated into the psychoactive compound 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) by the hepatic genes cytochrome P450 2C9, 2C19, and 3A4 (seen in Fig. 2.2C) and then further oxidized to the inactive 11-nor-9-carboxy-Δ^9-tetrahydrocannbinol (THCCOOH) (seen in Fig. 2.2D), which is excreted, and can be detected, in urine [Sharma et al., 2012, Peng and Shahidi, 2021]. Along with 11-OH-THC, there are over 100 primary metabolites, including di- and tri-hydroxy compounds, ketones, aldehydes, and carboxylic acids [Huestis, 2005]. Lower levels of 11-OH-THC are found after smoking THC than ingesting it orally [Huestis, 2005].

The effects of second-pass (phase II) metabolism are still being investigated. This step encompasses conjugation reactions, generally detoxifying drugs [Jancova et al., 2010]. There is evidence that cannabinoids are glucuronidated [Dinis-Oliveira, 2016]. This process of secondary metabolism attaches a glucuronide moiety to the substrates from first-pass metabolism, resulting in a highly hydrophilic, negatively-charged byproduct that cannot exit the cells without efflux transporters [Yang et al., 2017].

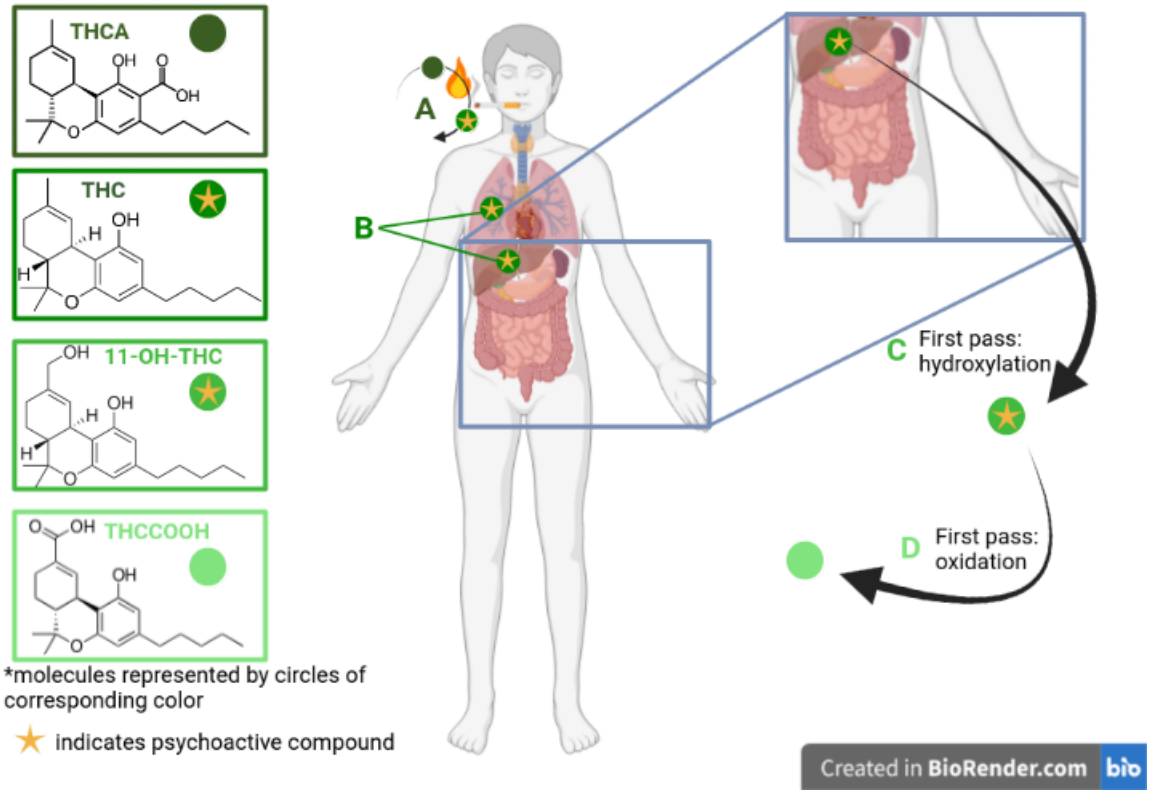

Figure 2.2: Metabolism of THC depicting A) decarboyxlation of THCA to THC, B) deposition of THC into organs C) Hydroxylation of THC to 11-OH-THC, and D) oxidation of 11-OH-THC to THCCOOH. Created with BioRender.com

Bioavailability of THC varies greatly. It is impacted by factors such as administration route, dose of THC in cannabis preparation. In smoked cannabis, inhalation variables such as depth of inhale, puff duration, and depth of breath-holding [Peng and Shahidi, 2021]. In humans, bioavailability following cannabis smoking has been reported to be 10-35%, with 30% lost to pyrolysis (burning) and the rest either unabsorbed or lost in side-smoke [Peng and Shahidi, 2021]. Following ingestion, the bioavailability of THC is lower than smoking, estimated to be 6-20% [Huestis, 2005]. THC levels peak higher and faster (6-10 minutes) after inhalation than ingestion (4-6 hours) [Huestis, 2005].

THC is distributed rapidly among highly perfused tissues including the lung, heart, brain, and liver. Estimates suggest only about 1% of peak dosage of THC is taken up into the brain [Huestis, 2005]. After the initial uptake, less perfused-tissues, such as fat accumulate the drug. Importantly, THC readily crosses the placenta, although

its metabolites cross less efficiently [Huestis, 2005]. A recent study examined the distribution of major metabolites in tissues from male rats 30 minutes following intraperitoneal (IP) injections of synthetic THC [Lust et al., 2022]. The highest rates of THC were found in the adipose and jejunum (small intestines). The highest rates of 11-OH-THC were found in the liver and jejunum. The highest rate of THCCOOH was found in the liver. The brain contained higher rates of 11-OH-THC than THC and no detectable THCCOOH [Lust et al., 2022]. In researching the impact of THC administration in isolation from other cannabinoids, it is important to consider the potential impact of the major metabolites of the compound itself.

In humans, THC achieves its terminal elimination phase (THCCOOH) and is distributed in fat about 10 hours after elimination [Huestis, 2005, Heuberger et al., 2015]. It is then slowly released, or reabsorbed from fatty tissue into the blood [Heuberger et al., 2015]. The elimination half-life of THC in humans has been estimated to be 21.5 hours, incorporating data from oral, intravenous, and inhaled administration [Heuberger et al., 2015].

2.2.3 Components of the Endocannabinoid System

The endocannabinoid system comprises receptors, endogenous ligands, known as endocannabinoids, synthesizing and degrading enzymes, and transporter proteins. Twenty-five years after THC was isolated, the ECS, comprising the CB_1 and CB_2 receptors and the two major endocannabinoids, N -arachidonoyl-ethanolamine [Anandamide] (AEA) and 2-arachadonoyl glycerol (2-AG), were identified [Mechoulam and Parker, 2013]. The CB_1 and CB_2 receptors are part of the superfamily of G-protein coupled receptors (GPCRs). The CB_1 receptor was first identified in the brain, where it is among the most prevalent GPCRs, however it has since been detected in many peripheral organs (sometimes at low levels) [Mechoulam and Parker, 2013]. In the rodent, CB_1 receptor densities are highest in the basal ganglia, substantia nigra, globus pallidum, cerebellum, hippocampus, and sensory and motor cortices [Mechoulam and Parker, 2013]. An early study in the primate brain confirmed the high density of CB_1 receptors in the hippocampus, cerebellum, and cerebral cortex, but also reported receptor staining in the amygdala, and light staining (reflective of fewer cells with CB_1 receptors) in the thalamus and basal ganglia (including the substantia nigra pars compacta, but not the substantia nigra pars reticulata) [Ong and Mackie, 1999]. A summary of areas expressing high levels of CB_1 receptors visualized on the human brain can be seen in Fig. 2.3. CB1 receptors are predominantly expressed in the presynaptic termi-

nuses of γ-aminobutyric acid (GABA) and glutamate-expressing neurons, suggesting cannabinoids play a role in both excitatory (glutamatergic) and inhibitory (GABAergic) neurotransmission [Hill et al., 2007]. Activation of CB_1 receptors inhibits neurotransmitter release at the synapse expressing it. CB_1 receptors couple G_i and G_o classes of G-protein, which results in reduced accumulation of cyclic adenosine monophosphate (cAMP)[Meyer et al., 2018]. The reduction of local cAMP decreases activity of A-type potassium channels and voltage-gated calcium channels [Meyer et al., 2018]. Additionally, it disrupts the fusion of neurotransmitter-filled vesicles with the presynaptic membrane, inhibiting neurotransmitter release [Meyer et al., 2018]. A cartoon of the process can be seen in Fig. 2.4

In contrast with CB_1, CB_2 receptors are largely present in immune cells in the peripheral nervous system, although they have also been identified in the central nervous system (CNS), particularly in microglia [Mechoulam and Parker, 2013]. While CB_1 agonists are associated with a host of psychoactive properties, CB_2 agonists like HU-308 do not induce these effects [Mechoulam and Parker, 2013]. In part because of the lack of psychotic effects, the CB_2 receptor has not been researched as extensively as the CB_1 receptor [Mechoulam and Parker, 2013]. Nevertheless, one way that CB_2 receptors play an important functional role is through microglia, the resident macrophages in the CNS [Cabral et al., 2008]. While the CB_1 receptor is expressed consistently at a low level in microglia, there is evidence suggesting that CB_2 receptor expression is dependent on the state of the microglia, not detected at baseline but increased as microglia become "responsive" (phagocytosis) and "primed" (presenting antigens) and again decreased when they are fully activated, visualized in Fig. 2.5 [Cabral et al., 2008]. Because of the presence of CB_2 receptors in the early phases of "activation," cannabinoids can modulate the chemotaxis of microglia [Cabral et al., 2008]. *In vivo* experiments have demonstrated increased microglia migration following exposure to 2-AG and auto-antibodies called anti-citrullinated protein antibodies [Komorowska-Müller and Schmöle, 2020]. Genetic deletion of CB_2 receptors results in decreased phagocytosis and arginase 1 (an antigen) [Komorowska-Müller and Schmöle, 2020].

In addition to the CB_1 and CB_2 receptors, 2-AG and AEA are important components of the ECS. The first endogenous ligand of cannabinoid receptors to be identified was AEA. AEA binds CB_1 more selectively than CB_2 receptors [Di Marzo et al., 2002]. Preliminary data suggests AEA has non-cannabinoid receptor binding sites [Di Marzo et al., 2002]. For example, AEA has been shown to activate TRPV1 channels, like phytocannabinoids. After being released post-synaptically, like 2-AG, AEA is hydrolyzed by fatty acid amide hydrolase (FAAH) [Di Marzo et al., 2002].

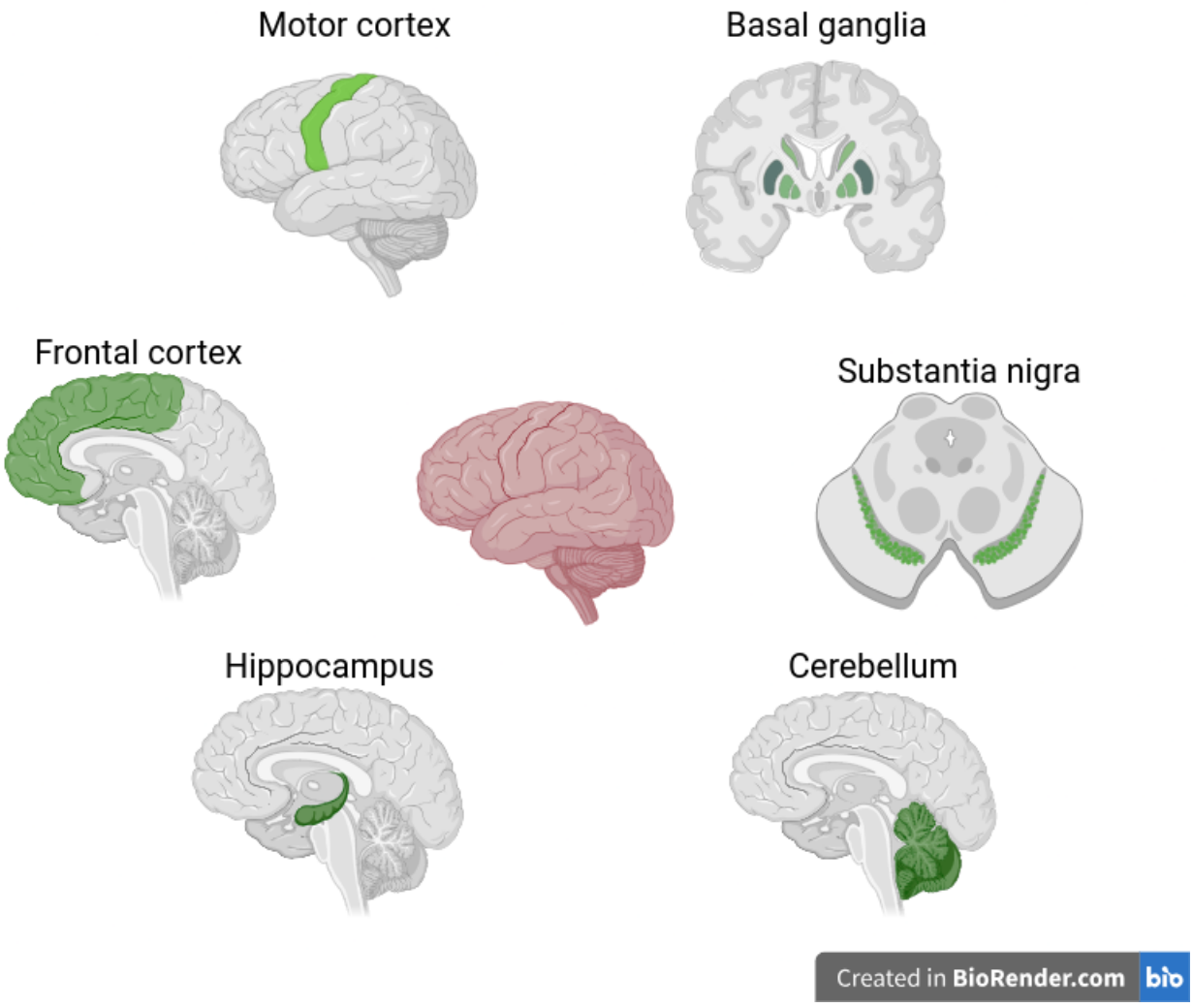

Figure 2.3: Visualization of regions with prominent CB_1 receptor distributions on the human brain, information from [Ong and Mackie, 1999, Moldrich and Wenger, 2000]. Created with BioRender.com

2-AG, the most abundant endocannabinoid found in the brain, is a receptor ligand that is thought to be synthesized from dyacylglycerol by diacylglycerol lipases (DAGL) on demand in postsynaptic neurons, unlike most neurotransmitters, which are stored in vesicles [Makara et al., 2005, Hermann et al., 2006, Shonesy et al., 2015]. Upon synthesis and release, 2-AG functions as a fast retrograde synaptic messenger, crossing the synapse and binding to presynaptic receptors, inhibiting excitation in that synapse [Mechoulam and Parker, 2013]. 2-AG is equally potent at both CB_1 and CB_2 receptors [Di Marzo et al., 2002]. It is then degraded by the enzyme monoacylglycerol lipase

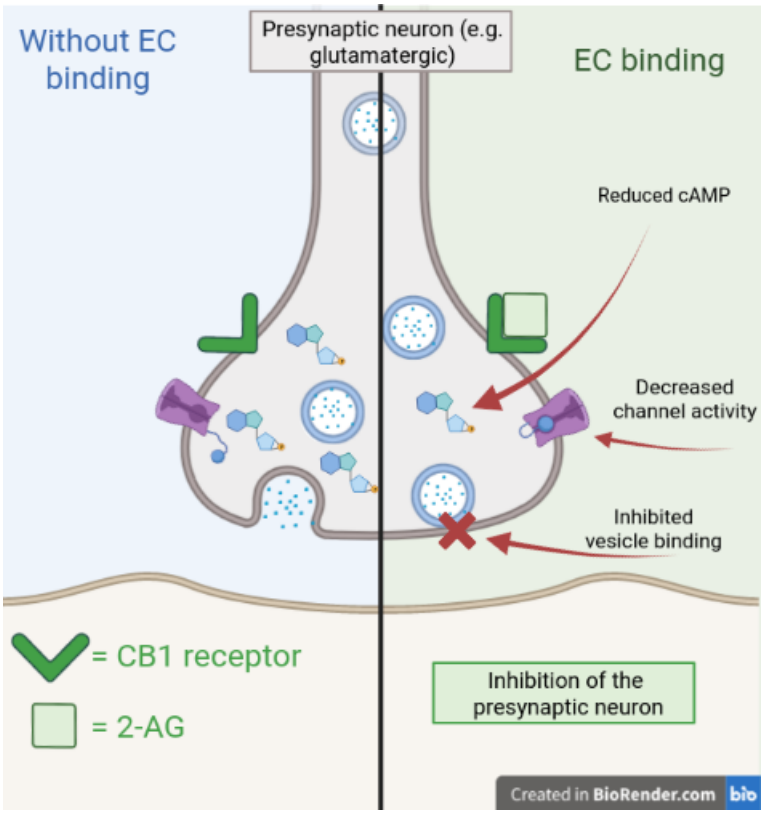

Figure 2.4: Cartoon of an example synapse without CB_1 binding (left) and with binding (right). Created with BioRender.com

(MAGL) [Petrosino and Di Marzo, 2010]. 2-AG plays a role in axonal growth and guidance during development [Mechoulam and Parker, 2013]. 2-arachidonyl glyceryl ether (2-AGE) has been proposed and researched as a third endocannabinoid, and some research supports its occurrence in the brain [Parkkari et al., 2006], nevertheless, the findings are contradictory and further research is required.

The ECS has been found in animals across the kingdom *animalia*, other than protozoa and insects [Silver, 2019]. In the mouse, as in the human, the ECS is a ubiquitous regulatory system in the CNS, recently investigated in the prefrontal cortex (PFC) [Lafourcade et al., 2007]. With a combination of confocal and electron microscopy, the authors found dense CB_1 immunoreactivity in layers II/III and V/VI of the cortex. In the deeper layers, the authors used double immunoelectron microscopy to investigate the localization of glutamatergic neurons (with mGluR5), CB_1 receptors, and 2-AG-producing DAGL [Lafourcade et al., 2007]. They found mGluR5 and DAGL colocalized in post-synaptic dendrites, but CB_1 was localized on presynaptic terminal membranes [Lafourcade et al., 2007]. These findings are consistent with the role the

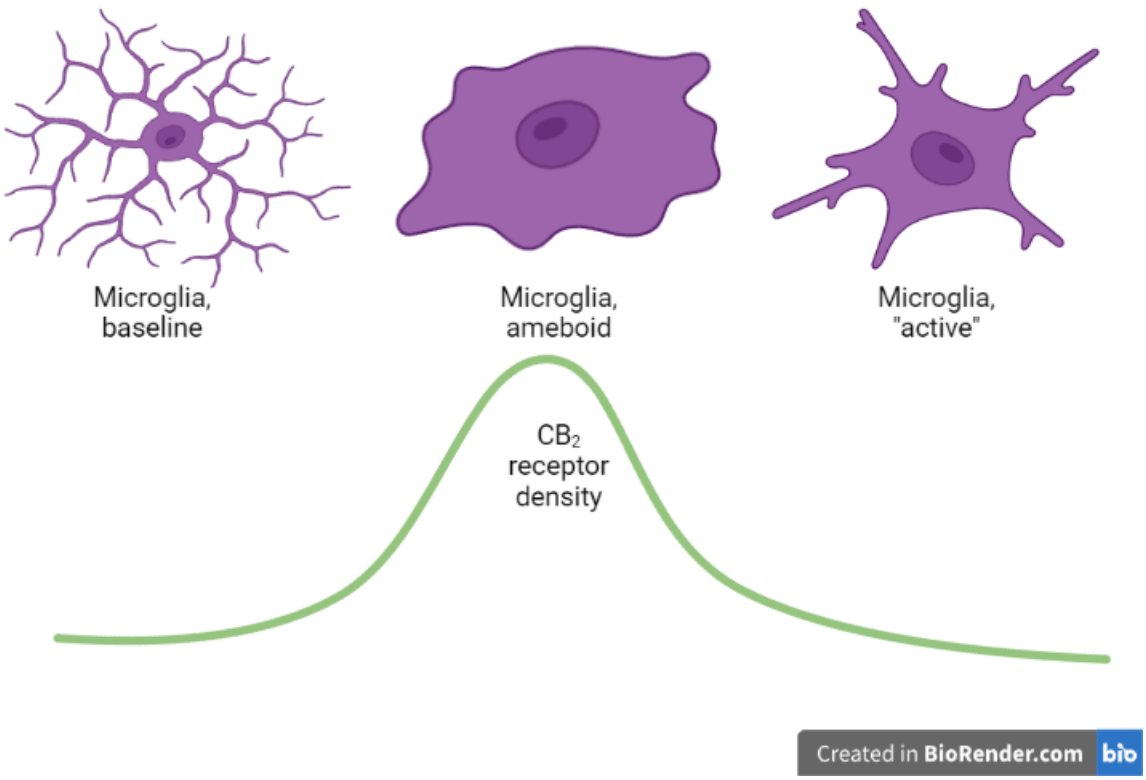

Figure 2.5: Visualization of a microglia in different stages from baseline on the far left, where CB_2 receptors are less expressed, through "responsive" and "primed" stages, where CB_2 receptors are most expressed, to a "fully activated" state, where CB_2 receptors are less expressed again. Created with BioRender.com

CB_1 receptor plays in humans.

2.2.4 Endocannabinoid System in Development

The ECS is actively present from the earliest stages of life and plays multiple roles throughout development. In the prenatal and early post-natal periods, the ECS is involved in embryo implantation, neural organization and development, and newborn suckling [Fride, 2008]. Prior to fertilization in mammals, AEA has been detected in the rodent and human uterus as well as rodent testis and human seminal fluid [Fride, 2008]. CB_1 and CB_2 receptors are present from pre-implantation stages in mice (gestational day (GD) 4), with concentrations of CB_1 even higher than those detected in the adult mouse brain [Yang et al., 1996, Fride, 2008]. For successful implantation, AEA levels

must be lowered on the day and site of implantation [Fride, 2008].

The placenta is a key organ involved in gestation, and the role of the ECS in its physiology and pathophysiology has been recently investigated [Maia et al., 2019]. One study examining mice without CB_1 receptors found reductions in placental weight associated with reductions in trophoblast cells (which form the outer layer of the blastocyte and provide nutrients to the developing embryo) [Maia et al., 2019]. Increased AEA has been associated with apoptosis in trophoblast cells [Costa et al., 2015], and 2-AG induces apoptosis with a CB_2-dependent mechanism [Costa et al., 2014].

After implantation, but during gestation, the ECS plays an important role in neurodevelopment. Levels of AEA in the brain slowly increase until adult levels are obtained, however levels of 2-AG in the fetal brain are similar to adulthood [Fride, 2008]. Thus, 2-AG levels are almost 1000 times higher than AEA [Fride, 2008]. CB_1 and CB_2 receptors are present in the brain during development, however the density of receptors shifts in distribution [Fride, 2008, Yang et al., 1996]. At an early age CB_1 and CB_2 receptors are abundant in white matter (making transient appearances on fibre tracts) and subventricular zones, however levels decrease before adulthood, obtaining adult distribution patterns late in the postnatal period [Basavarajappa et al., 2009, Mechoulam and Parker, 2013, Fride, 2008]. Additionally, CB_1 receptors are present at higher concentrations in the developing brain of rats and humans than of adults [Basavarajappa et al., 2009]. In the human fetal brain, the highest cannabinoid receptor binding densities are in the pyramidal tract, brachium conjunctivum and subventricular germinative zones, along with the corpus callosum, stria terminalis, anterior commissure, midbrain, and cerebral cortex [Fride, 2008]. Finally, GABAergic interneurons' axonal growth cones express CB_1 receptors in the rodent cortex [Fride, 2008]. Together, these data suggest a role for the ECS in neural differentiation (with a presence in proliferative zones) and axon guidance.

Finally in neonates, there is evidence that CB_1 receptor activation is critical in the initiation of feeding in pups [Fride, 2008]. In mouse pups where CB_1 receptor antagonists are injected in newborns, milk ingestion and pup growth is impaired in 75-100% of pups [Fride, 2008]. Additionally, pups deficient in CB_1 receptors fail to suckle sufficiently in the first days of life, and while this behavior develops by postnatal day (PND) 3, weight gain in these pups remain lower than C57BL/6 mice [Fride, 2008].

Changes to the ECS continue to underlie sensitive periods of development in adolescence. Rodents demonstrate peak CB_1 receptor expression around the onset of adolescence, around PND 30, with levels declining until around PND 70 [Meyer et al., 2018, Rodríguez de Fonseca et al., 1993]. This pattern is especially ob-

served in medial prefrontal and limbic/associative regions, while sensorimotor regions undergo changes around PND 40 [Heng et al., 2011]. In humans there is evidence that CB_1 receptors peak before 5 years of age and then gradually decrease until adult levels are reached [Meyer et al., 2018]. Regions in the cortex, hippocampus, amygdala, and hypothalamus demonstrate a trajectory with 2-AG levels high early and late in adolescence, but attenuated in mid-adolescence (roughly PND 35-45). In contrast, AEA levels in the same regions generally increase during adolescence, with dips around PND 25 and 45 [Meyer et al., 2018]. In contrast with the cortical regions, the nucleus accumbens and striatum exhibit separate trajectories. In these regions, there is a negative correlation between AEA and 2-AG, which contrasted with a positive correlation between the two endocannabinoids in the PFC [Ellgren et al., 2008]. During adolescence, FAAH peaks as well, potentially indicating tighter control of AEA levels, but the trajectories of MAGL are less-well characterized [Meyer et al., 2018].

During adolescence and adulthood, the ECS has been shown to play a role in a variety of functions. It has been well-established in stress, fear, and anxiety. Mice that lack CB_1 receptors express anxiogenic-like and depressive-like behavior, and blocking CB_1 receptors or inhibiting AEA metabolism in rats induces anxiety-like phenotypes [Viveros et al., 2005]. Additionally, the ECS is important to metabolism and the regulation of energy [Gatta-Cherifi and Cota, 2016]. CB_1 deficiency has been associated with depressive-like phenotypes such as anhedonia [Gallego-Landin et al., 2021]. Overactive CB_1 signalling contributes to the development of obesity, insulin resistance, and dyslipidemia [Gatta-Cherifi and Cota, 2016].

2.3 Mice as investigative models

While mice have proven to be indispensable laboratory animals over the history of neuroscience, they are, in essence a model. As George Box famously said: “all models are wrong, but some are useful” [Curchoe, 2020]. As such, it is important to acknowledge the innate limitations of modelling the human brain and human development with mice, and this section establishes some aspects of human biology that can accurately be represented by the mouse, and where these organisms diverge.

2.3.1 Comparative neuroanatomy

When considering mice as a model for human brain anatomy, the rodents have been assessed through the lens of gene expression, allometry (the study of the rela-

tive growth of various regions), connectivity, and cellular similarity [Strand et al., 2007, Bakken et al., 2021, Van Essen et al., 2019, Loomba et al., 2022, Defelipe, 2011]. Early in basic neuroscience, researchers investigated a plethora of model organisms including cats, pigs, nonhuman primates, birds, amphibians, reptiles, and fish [Manger et al., 2008]. However, in the 21^{st} century, rodents quickly rose in prominence, with mice predominating in the literature. This preference is largely driven by technological advancements that allow modification of the mouse genome with relative ease [Juntti, 2019]. Despite well-founded calls to diversify the model organisms employed in neuroscientific investigations, the mouse still holds value as a model organism.

Regional gene expression is well conserved between mice and humans. One study compared gene expression in three regions, the motor cortex, striatum, and cerebellum between mice and humans. Within the species, there were distinct patterns of gene expression among the regions, however the pattern of relative expression was conserved between the two species. For example, their results suggest that genes more highly expressed in the striatum of the mouse are also likely to be more highly expressed in the striatum of the human [Strand et al., 2007]. Using single-cell RNA-sequencing, the transcriptomic profile of the dorsal lateral geniculate nucleus of the thalamus has been explored in the mouse, macaque, and human [Bakken et al., 2021]. This area is of interest in translation because it receives visual information from the retina, and primates have high visual acuity compared with rodents. The authors found several notable differences across species, including increased diversity of GABAergic interneurons in the primate, and that in the primate, thalamocortical neurons could be grouped into distinct populations based on their gene transcriptomes, which could not be achieved in the mouse [Bakken et al., 2021]. Their findings also highlight similarities, such as a conservation of two major GABAergic cell-types across species. Another study quantified transcriptomic data from eleven regions in the mouse brain including the orbital, prelimbic, cingulate, motor, somatosensory, and entorhinal cortices, the caudate-putamen, the nucleus accumbens, the thalamus, the substantia nigra, and the ventral tegmental area [Brochier et al., 2008]. Of the 315 total genes they examined, the authors further compared 50 between mice and humans, finding that 30 genes showed conserved over-expression in humans. Of these, mice show over-expression of genes relating to membrane proteins, intracellular signalling cascades, and cellular lipid metabolism [Brochier et al., 2008]. The authors conclude that regional enrichment is conserved in humans in a majority of cases [Brochier et al., 2008].

In spite of the neuroanatomical similarities described in mice, there are still obvious differences which make the development of direct homologies difficult across the species.

Perhaps the most apparent difference is that human brains are gyrencephalic, while mice are lysencephalic, or smooth-brained. This reflects the disproportional increase of surface area moving up the evolutionary tree from mice to humans, with surface area increasing more quickly than cortical thickness and contributing to increased brain volume [Van Essen et al., 2019]. Additionally, humans are about 2500 times larger and 3800 times heavier than mice, and metabolic rates are inversely correlated with size [Perlman, 2016, Van Essen et al., 2019]. While the brain regions present in the human brain share anatomical and functional similarities with the mouse brain, there are some important differences. For example, humans and their closer relatives show an evolutionary prioritization of higher cognitive areas than auditory or visual areas, as evidenced by an overexpansion of frontal and parietal lobes in a comparative neuroanatomy study [Garin et al., 2022]. Additionally, some regions represent a single area in the mouse, such as the caudate-putamen, while they are two separate regions in the human (the caudate and the putamen) [Schröder et al., 2020a]. This highlights a challenge in interpreting the mouse brain in relation to the human: parcellation. There is variability in the human parcellation based on the modalities underlying the scheme, and there is little consensus on a murine parcellation either [Van Essen et al., 2019]. The authors present a new parcellation of flat maps based on multiple architectonic and immunocytochemical markers which highlights subtle but important differences between the mouse and primate areas, such as how primate somatosensory areas are arranged in a strip, but mice are more rounded, and the large primary visual cortex (V1) in primates is surrounded by the secondary visual cortex, whereas in the mouse V1, the surrounding areas form a variable mosaic [Van Essen et al., 2019]. While the fine structure of the hippocampus and its subfields are well-conserved from mice to humans, the orientation and location of the structure shows evolution. While the human hippocampus is situated in a basal position in the temporal lobe, the mouse hippocampus stretches from a cranial dorsal position to a lateroventral location in the caudal regions [Schröder et al., 2020b].

With the rise of rodent fMRI, studies of the similarity between rodent and human brain connectivity have become important for understanding translation from mice to humans. Similarity in network connectivity was initially assumed because of conserved action of ion channels, synaptic receptors, and molecular constituents of brain networks. In order to assess network similarity, however, a recent study quantitatively compared similarity in inter-species local network circuitry using three-dimensional electron microscopy [Loomba et al., 2022]. The first obvious difference in networks is that the human brain contains about 1000 times more neurons than the mouse brain, accompa-

nied by an increase in the proportion of inhibitory interneurons in the human brain [Loomba et al., 2022]. Reconstructing circuits from the human (temporal and frontal cortices) and macaque (temporal and parietal cortices) and comparing them to five mouse connectomes, the authors found that interestingly the proportion of inhibitory internueron to excitatory pyramidal neuron connections was not substantially impacted, but rather the human and macaque demonstrated an increase in bipolar interneurons that preferentially innervate other interneurons. This interneuron-interneuron network is almost completely absent in mice [Loomba et al., 2022]. Meanwhile, the synaptic input to pyramidal cells is almost constant from the mouse to the human. The maintenance of inhibitory and excitatory inputs to excitatory cells suggests a similar balance of excitation and inhibition across species, however the more complex inhibitory-inhibitory networks could indicate more opportunity for disinhibition in primates [Loomba et al., 2022].

The cellular architecture of the human brain is notably well-conserved in the mouse brain [Hodge et al., 2019]. For example, mammals share the columnar organization of the cortex established during development [Defelipe, 2011]. Additionally, most areas of both the mouse and human cortex comprise six layers, although the aggregates of neurons in layer IV of the mouse's barrel cortex represent one difference [Defelipe, 2011]. Nevertheless, there are marked differences in the proportions of these cells, their distribution among layers, their gene expression, and morphometry, investigated in both humans and mice in recent years. The proportion of synapse types per layer evidences the divergence between mice, rats, and humans. Synapses can be classified as either excitatory asymmetric synapses (glutamatergic), or inhibitory symmetric synapses (GABA-ergic) [Defelipe, 2011]. Examining the cortex in its entirety demonstrates consistent percentages of asymmetric (89%) and symmetric (11%) synapses, among human, rat, and mouse, however differences emerge when layers are examined individually. For instance, the mouse, compared to the human and rat, show a lower percentage of asymmetrical synapses in layers IIIb-VI [Defelipe, 2011]. These data are consistent with the reduced proportion of interneuron-interneuron connections reported at the network-level in the previous paragraph [Loomba et al., 2022].

From gross anatomy to network connectivity and cellular architecture, the convergence of brain structure across species is significant and motivates the use of mice as a model organism in neuroscience. Nevertheless, there are differences in the brain between mice and humans that must be accounted for in interpreting results. In the case of neurodevelopment, comparison cannot be assessed only at the adult timepoint, but must also be considered throughout gestation and early life, as explored in the

next section.

2.3.2 Comparative Development

In considering development of mice as a model organism, assessment must start with gestation. While human pregnancy is usually around 268 days (38 weeks and 2 days) [Jukic et al., 2013], gestation for C57/Black6 J (C57) mice is 18.5 days [JAX Laboratories, 2022]. A major concern in using mice as a model for human pregnancy is that mouse gestation is notably short, with much of organ development taking place post-natally [Carter, 2020]. Research from the mid-20th century assessing equivalence between mouse and human embryos place the end of mouse gestation around 13 weeks of human pregnancy (approx. end of the first trimester) [Otis and Brent, 1954]. Concerning a comparison of placenta development, gene expression patterns reveal that close similarity is observed when restricting analyses through the first 16 weeks of human pregnancy, suggesting mouse *in utero* development most closely resembles the first half of human gestation [Carter, 2020, Soncin et al., 2018]. Comparing hallmarks of intestinal development reveals that intestinal morphogenesis in mice is complete several weeks after birth, whereas it is completed several weeks *before* birth in humans [Lim et al., 2020]. In contrast, however, cardiac development follows similar sequences between mice and humans, with minor differences to vasculature morphology [Krishnan et al., 2014]. Unlike humans, mice are born hairless with eyes and ear canals closed. C57 mice become self-reliant and can be weaned at about PND 21, generally viewed as mid-childhood. Mice become sexually mature between 4 and 7 weeks (PND 28-35) [Ayadi et al., 2011]. Puberty in female humans can be assessed through thelarche (onset of secondary breast development) or menarche (first menstruation), and in recent decades has been dated on average around 10 years for the former and 13 for the latter in developed countries [Lee and Styne, 2013]. In human males, puberty can be assessed with genital maturation and is around 9-11 years in recent decades [Lee and Styne, 2013]. Because development of different organs follows divergent time courses when comparing mice and humans, it is difficult to identify age-equivalencies.

In addition to physical differences an inter-species comparison of neurodevelopment reveals important differences. Humans and rodents both undergo extensive neurological development postnatally. Human brain weight peaks at birth, and cortical neurogenesis and migration are thought to be complete by the first week of life [Watson et al., 2006]. There follows a rapid overproduction of synapses throughout

the first three months of post-natal life in the human, with myelination of the cortical hemispheres extending through the first 9 months of post-natal life into the early-20s [Watson et al., 2006]. Synapse formation peaks through 2-3 years of human life, after which selective pruning of neurons and elimination of synapses continues, evidenced in characteristic cortical thinning that underlies childhood and adolescence [Bethlehem et al., 2022, Watson et al., 2006]. In mice, brain weight increases sharply after birth until PND 14, after which rate of increase slows, peaking around PND 60 [Agrawal et al., 1968]. Additionally, myelin sheaths in mice are reportedly first seen at PND 11 [Sturrock, 1980], whereas they are first reported in the globus pallidus, internal capsule, and thalamus at 35 weeks gestation in humans [Hasegawa et al., 1992]. Examining four markers (1. synaptogenesis, 2. development of activity of the GABA synthesis enzyme, glutamate decarboxylase (GAD), 3. development of the acetylcholine synthesis enzyme choline acetyltransferase (ChAT), and 4. establishment of neurological electrical activity patterning active and quiet sleep), researchers established that the newborn human is roughly equivalent in neurodevelopment with PND 12-13 rat pup [Watson et al., 2006, Romijn et al., 1991]. More recently, examining rodents as a group, researchers suggest term infancy in humans is roughly equivalent to PND 7-10 in rodents based on the peak brain growth spurt, peak gliogenesis, increasing axonal and dendritic density, the maturation of oligodendrocytes, and consolidation of the immune system [Semple et al., 2013]. Nevertheless, when selecting an age-appropriate rodent model, expert recommendation is to select based on the specific process of neurodevelopment of interest [Semple et al., 2013]. While this presents a limitation in the longitudinal investigation of neurodevelopment at the level of an entire organism or system, it can be accounted for in interpreting the age of the effects.

Tracking the brain and behavior later in “childhood” and “adolescence”, reveals further similarities and differences between species. In humans, adolescence is a period of development that often co-occurs with puberty (which relies on physiological biomarkers) [Casey et al., 2008]. It is marked by behavioral and cognitive changes. Adolescence is accompanied by impulsive, risk-taking behavior, emotional reactivity, and independence-seeking [Casey et al., 2008]. Other species also undergo changes from childhood to adulthood where the necessary skills for independent survival must be developed. The development of certain behaviors are conserved across species [Spear, 2000]. The term “periadolescence” has been adopted to describe the period several days preceding puberty in rodents to several days after [Laviola et al., 2003]. While the psychological and social aspects of adolescence cannot be examined in mice, behavioral examinations during periadolescence compared to younger rodents reveal

increased exploratory behavior, improved performance at experiments that require locomotor activities to illicit a reward, and increased social play [Laviola et al., 2003].

In humans, along with the behavioral changes, the brain's anatomy continues to mature until adulthood. From birth to adolescence, human total cerebrum volume peaks around 6 years of age [Bethlehem et al., 2022]. In contrast, white matter increases continually throughout adolescence [Paus, 2005]. As in humans, mice exhibit overall cerebral growth from peripuberty to adulthood [Koshibu et al., 2004]. In mice, the striatum and hippocampus were disproportionately larger in adult mice compared to adolescent mice [Koshibu et al., 2004]. In humans, the relationships of age and volume of the hippocampus and amygdala have been examined from infancy to adulthood. One study in humans reports larger hippocampal volume in adults compared to adolescents only for males, and not for not females [Suzuki et al., 2005]. A more recent study examining both the hippocampus and amygdala of humans notes that female hippocampi and amygdalae peak in volume around 9-12 years, with some variation in the left and right structures [Uematsu et al., 2012]. The peak volumes for both structures is achieved slightly later in males, around 10-13 [Uematsu et al., 2012]. Sex differences are reported globally in humans as well. Male brain volume is about 10% larger than females, in humans [Lyall et al., 2016], however some magnetic resonance imaging (MRI) studies in mice have reported no differences between total brain volume based on sex [Koshibu et al., 2004]. More nuanced discussions of regional brain volume differences and their developmental trajectories report sex differences in adult mice, with females exhibiting larger volume of the anterior hippocampus, basolateral amygdalae, and lateral cerebellar cortex, while males displayed larger cerebral cortex, and medial amygdala and cerebellar cortex [Meyer et al., 2017]. These sex differences may be the result of chromosomal or hormonal differences [McCarthy et al., 2012] and can play a role underlying behavioral differences in healthy individuals, as well as serving as risk factors in childhood-onset developmental disorders (such as Autism Spectrum Disorder (ASD), Attention Deficit Hyperactivity Disorder (ADHD)) [Green et al., 2019], psychiatric disorders that emerge around adolescence (such as anxiety and depression) [Green et al., 2019], and neurodegenerative disorders (such as Alzheimer's Disease) [Schmidt et al., 2008].

A comparison of neurodevelopment in the mouse and human can be found in Fig. 2.6.

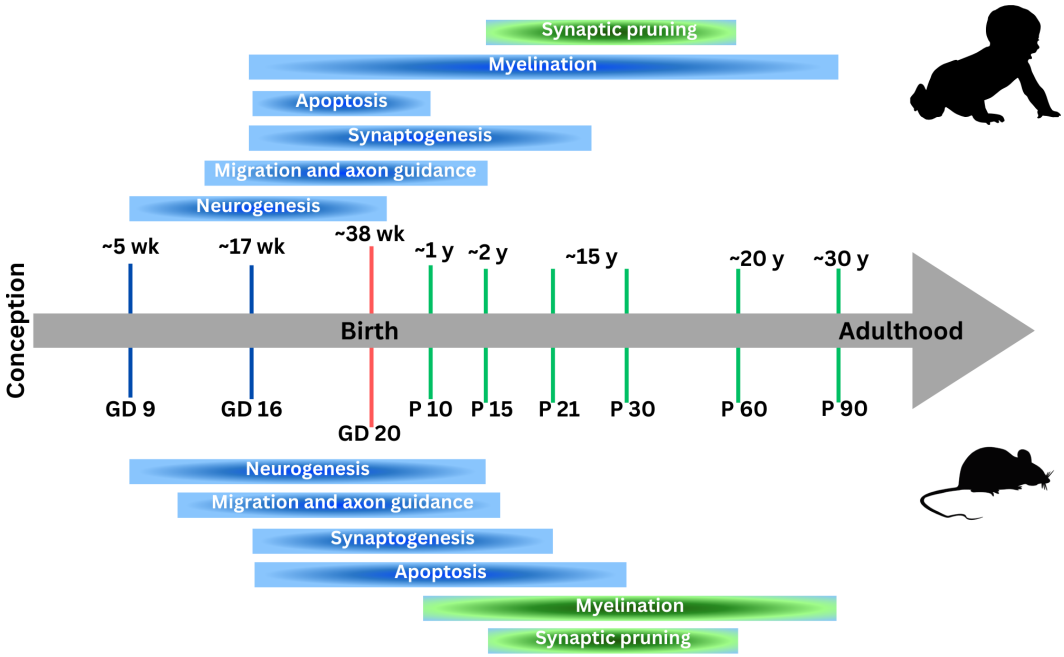

Figure 2.6: Comparative neurodevelopment *in utero* to adulthood between the human and mouse, with human timeline in gestational weeks and mouse timeline in gestational days, informed by [Gumusoglu and Stevens, 2019, Zeiss, 2021]. Processes that begin before birth are demarcated with blue bars, whereas processes that begin postnatally are demarcated with green.

2.3.3 Comparative ECS Development

While the ECS has been found to be well-preserved across adults of different mammalian species, little work has been done to establish cross-species similarities or differences in ECS development. The role of the ECS in development has been investigated in many species include zebra fish, chick, mouse, and human, with some consensus across species, despite no specific studies designed to compare or contrast [Bukiya, 2019]. For example, ECS components were detected in both mouse and chick embryos in pre-neuronal stages, GD 8.75 and stages 9-11, respectively, which are associated with GD 22-26 in human gestation [Bukiya, 2019]. Studies from both humans and non-human animals concur on the importance of ECS tone on embryo implantation, with elevated levels of AEA associated with interference with embryo

implantation and nonviable pregnancies across species [Bukiya, 2019]. Evidence from both rodents (mice and rats), and humans in mid-gestation suggest a role for the ECS in establishing concentration gradients key to proper axon-guidance and brain wiring [Bukiya, 2019]. Finally, in both rodents and humans, brain levels of CB_1 receptors are found to be higher than in adults of their species, especially in white matter, emphasizing the changing role of the ECS between juvenile periods and adulthood [Bukiya, 2019]. Future studies elucidating differences between the roles and timing of ECS events between species could aid translation of results from the laboratory to the clinic.

2.4 Outcomes of prenatal cannabis exposure

2.4.1 Gross Physiology and Obstetric Complications

Cannabis has few documented teratogenic effects (birth defects or organ malformation) [Orsolini et al., 2017]. Additionally, despite examinations, there is no evidence for increased risk of fetal mortality or perinatal death with moderate prenatal exposure to cannabis [Fergusson et al., 2002]. A recent meta-analysis found that cannabis use during pregnancy was associated with increased probability of preterm birth, an example of obstetric complications that may follow gestational cannabis use [Duko et al., 2022]. Mouse models with inactive CB_1 (but not CB_2) receptors reportedly demonstrate early onset of labor (GD 19.3-19.6 instead of GD 20) [Wang et al., 2008]. Few other studies report examining early onset of labor.

Gross morphological and physiological metrics are one of the most ubiquitous outcomes recorded in humans and experimental animals following prenatal exposure to cannabinoids. In neonates, one of the major questions is whether cannabis exposure leads to low birth weight or growth restriction. Low birth weight (LBW) has been associated with prenatal exposure to nicotine [Ko et al., 2014], but the literature is mixed on the association of prenatal cannabis exposure and LBW. One factor could be the difficulty in separating prenatal polydrug exposure [Nashed et al., 2021]. A number of studies investigate potential correlations between cannabis use during pregnancy and LBW [Gunn et al., 2016, Conner et al., 2016] or fetal growth restriction (FGR) [El Marroun et al., 2009]. While some studies report LBW and FGR [Gray et al., 2010, El Marroun et al., 2009], others find no difference [Fergusson et al., 2002, Conner et al., 2016], and yet others report increased birth weight following prenatal cannabis exposure [Day et al., 1991].

Animal studies control for factors like polydrug use, inaccurate self-report, and socioeconomic status, but the literature does not lend a clear answer to the question of whether prenatal cannabis exposure impacts weight at or before birth. Indeed, results paint as variable a picture as human studies, with some papers reporting subtle FGR or LBW [Benevenuto et al., 2017, Chang et al., 2017a, Gillies et al., 2020, Natale et al., 2020], and others reporting no difference [Newsom and Kelly, 2008, Silva et al., 2012, Breit et al., 2020]. Some papers that report initial LBW also report characteristic catch-up growth by PND 21 [Natale et al., 2020]. It has been suggested that variability in results could be due, in part, to route of administration, with LBW more often reported in studies administering cannabinoids with IP injection, null results reported after oral administration, and mixed results following vapor exposure [Nashed et al., 2021]. Dosage and species must also be considered, as well as age of assessment, as some studies examine fetuses [Natale et al., 2020, Chang et al., 2017a] and others birth weight [Gillies et al., 2020, Natale et al., 2020]. Longitudinal studies are particularly valuable as they would allow researchers to assess how weight might change over the course of the lifespan. In humans, high quality data has recently been made available, but such studies still struggle to delineate THC, CBD, or the ratio between them [Nashed et al., 2021].

2.4.2 Behavioral outcomes

Behavioral and cognitive outcomes have been a focus of prenatal cannabis exposure in the human literature. Measures related to behavior and cognition have been examined in children as young as 18 months through adolescents. Overall, findings are quite subtle and are somewhat varied. In 18 month old toddlers, there is evidence for increased risk of ASD, however the same study found no increased risk for other behavioral disorders, such as ADHD at 4 years of age [Corsi et al., 2020]. Prenatal cannabis exposure also increases anxiety, hyperactivity, and aggression (in girls) [Rompala et al., 2021]. Consistent with anxiety, the authors also found increased cortisol levels in the hair of the young children included in the experiment [Rompala et al., 2021]. Exposure to cannabis after knowledge of pregnancy (by the mother) was associated with adverse outcomes in 9-11 year old children, including psychotic-like experiences, externalizing, attention, thought, and social problems [Paul et al., 2021]. While the outcomes are examined at an early age for psychiatric disorders to first appear, they could indicate a risk for mood and psychotic disorders to emerge during adolescence. Ten-year-olds exposed to one or more joints per day in the first trimester of pregnancy show worse reading

and spelling scores, and worse teacher assessments in school [Goldschmidt et al., 2004]. Second trimester exposure was also associated with reduced reading comprehension and academic underachievement [Goldschmidt et al., 2004]. At age 14, prenatal cannabis exposure was associated with increased frequency and earlier of age of onset of cannabis consumption [Day et al., 2006].

There is some evidence for a catch-up effect and normalization in cognition or behavior by adolescence. Cognitive changes associated with prenatal cannabis exposure have been investigated during adolescence in participants aged 18-22 years. Despite early changes in academic performance, in adolescence a study found no differences in performance on a visuospatial 2-Back task, Go/NoGo task, Letter 2-Back task, or counting Stroop task [Smith et al., 2006, Smith et al., 2016].

As in the last section, animal studies provide a method for examining the prenatal exposure while controlling extraneous factors. Assessments for anxiety-like behaviors, motor behavior, and sensorimotor gating have been conducted in animal models from neonates to adulthood. In pups, anxiety-like behavior can be examined by recording ultrasonic vocalizations (USVs) when pups are separated from their dams. One study tested male offspring and found 2.5 and 5 mg/kg of THC administered at GD 15 to PND 9 in rats increased the number of USVs pups made, while 5 mg/kg alone reduced social play at PND 35 and decreased time in the open arm of the elevated plus maze at PND 80 (adulthood), all indicating anxiety-like phenotypes [Trezza et al., 2008]. Yet, another study found reductions of USVs in rat pups at PND 10 exposed to lower levels (0.5 mg/kg) of the cannabinoid-receptor agonist, WIN 55,212-2 (WIN), administered GD 5-20 [Antonelli et al., 2005]. The opposite directions of their findings could be related to differences in the methodology, associated either with alterations in the drug administered (THC or WIN), the differing dosages, that cannot be directly compared across drugs, or the different ages of administration (into postnatal life in the first study). In adolescence, one study examined the impact of oral exposure to THC (5 mg/kg) throughout pregnancy on a novelty suppressed feeding task, finding increased latency to approach food (representative of anxiety-like behavior) in males, but not females [Lallai et al., 2022]. Anxiety is commonly comorbid with other mood and psychiatric disorders such as depression [Pollack, 2005], bipolar disorder [Pavlova et al., 2015], psychosis [Dernovsek and Sprah, 2009], and ASD [Zaboski and Storch, 2018], and there is evidence in humans and animals to highlight the importance of considering levels of anxiety as an outcome of interest.

Adult spontaneous locomotion is frequently tested in offspring prenatally exposed to cannabinoids. While increased spontaneous locomotion can be interpreted as a

marker of increased exploration, and immobility is seen as another marker of anxiety or fear, many factors can impact locomotion, including sensory processes, environmental novelty, hunger/thirst, and time of day [Kelley, 1993]. Generally, increased spontaneous locomotion is still interpreted as exploratory, however the translation of "activity" to humans is nonspecific [Crusio, 2001], as it could implicate cognitive, motor, or sensory neural circuits and processes relating to fear, need, or "curiosity". Experiments have examined the impact of a range from 0.1 mg/kg to 5 mg/kg exposure to THC during the pre-and-peri-natal periods (GD5 to PND24) [Rubio et al., 1995, Navarro et al., 1994, Moreno et al., 2005]. The results are often sex-specific, with some studies finding prenatal exposure to 5 mg/kg THC increased locomotion in females only [Rubio et al., 1995], and others describing a timing effect, with increased locomotion in females at PND 70, but increased locomotion in males only at PND 20 [Navarro et al., 1994]. While THC at doses of 0.5 or 2 mg/kg decrease locomotion in males, in females there was evidence of a dose-effect, with levels of 0.1 or 0.5 mg/kg THC associated with decreased locomotion, but 2 mg/kg associated with increased locomotion [Moreno et al., 2005]. The mixed results necessitate further investigation of the impact of prenatal cannabis exposure on locomotion, however, they further highlight the importance of including female subjects, which several of the anxiety studies did not include [Trezza et al., 2008, Antonelli et al., 2005].

Assessments of sensorimotor gating with prepulse inhibition (PPI) provide one of the few tests that can be implemented in humans. Impairments in sensorimotor gating are associated with transdiagnostic disorders, including schizophrenia, obsessive compulsive disorder, ASD, Tourette syndrome, and Huntington's disease [Swerdlow et al., 2016]. Prenatal exposure to a cannabinoid-receptor agonist, WIN, did not affect sensorimotor gating, as measured with PPI, at PND 40, 60, or 80 in rats exposed from GD 5-20 to either of two doses of WIN (0.5 or 1 mg/kg) [Bortolato et al., 2006]. Rat pups exposed to 5 mg/kg THC orally throughout the course of pregnancy displayed sex-differential effects, with males only exhibiting deficits in sensorimotor gating [Lallai et al., 2022].

Finally, short-term memory has been explored following prenatal THC exposure. In both sexes, prenatal exposure to THC (oral exposure throughout pregnancy, 5 mg/kg) impaired short-term memory in rats [Lallai et al., 2022, Drazanova et al., 2019]. Impaired cognition could relate to the difficulty in school performance observed in humans, however further research would be required to confirm alterations in short-term memory and other aspects of cognition.

2.4.3 Neuroanatomical, functional, and molecular outcomes

Neuroanatomy *in vivo* can be examined non-invasively with MRI and *ex vivo* with cellular and molecular assays such as immunohistochemistry (IHC) and RNA-sequencing (RNAseq). While the scale of the assessments differ with MRI examining gross morphometry and IHC examining cellular composition, the techniques are complementary, as they both provide information about the structure of the brain and can be sensitive to insults, both genetic and environmental.

In children aged 6-8, increased cortical thickness in frontal regions was observed after prenatal exposure to cannabis [El Marroun et al., 2016]. Another study reports reduced cortical gray matter in children 10-14 prenatally exposed to cannabis, however these results must be considered carefully as the sample size was small and children were scanned in a polydrug study and only three of the eight participants were exposed to cannabis alone [Rivkin et al., 2008]. There is a clear lack of information on the structural impact of prenatal cannabis exposure in humans.

In nonhuman animals, unfortunately, the results are also scarce. One study used Arterial Spin Labelling (ASL), an MRI technique that noninvasively magnetically tags blood flowing into the brain to trace the path of cerebral blood flow. This study found perinatal exposure to THC in rats (5 mg/kg, GD 13 - PND 9) found no difference in ventricle size or regional blood perfusion when examining the Circle of Willis, hippocampus, sensorimotor cortex, PFC, or caudate putamen [Drazanova et al., 2019]. Importantly, with the authors' implementation of the technique, they pre-selected regions of interest from two coronal slices in the brain, limiting their sensitivity to whole-brain or volumetric changes.

Human brain function following prenatal cannabis exposure garnered more attention in early MRI work. The OPPS dataset included several batteries of cognitive tests during task-based fMRI paradigms, including visuospatial and letter 2-back tests for working memory, the go/no-go task for response inhibition, and a counting Stroop task for cognitive interference [Smith et al., 2016, Bush et al., 2006]. While the participants exposed prenatally to cannabis did not differ from the controls in performance on the tasks, there were significant changes in Blood Oxygen Level Dependent (BOLD) activity during tasks, such that cannabis-exposed adolescents required greater activation in posterior areas than controls across tasks [Smith et al., 2006, Smith et al., 2016]. Unfortunately, resting state functional magnetic resonance imaging (rs-fMRI) was not reported for these groups, potentially due to doubts about its validity in the early 21st century and subsequent technological developments increasing its reliability and

scientific interest [Cole et al., 2010]. The Gen R study collected rs-fMRI in children with reportedly good quality scans in 80% of participants [White et al., 2013]. Inter-regional functional connectivity (FC) was reported in these children, and the subjects exposed to substances, including cannabis along with selective serotonin reuptake inhibitors, nicotine, and low-folic acid levels were included in the sample, however no specific analysis was included to compare those with only prenatal cannabis exposure to those with other exposures or no prenatal insult [Langeslag et al., 2013]. A recent examination of fetal rs-fMRI following prenatal cannabis exposure provides insight into early changes in functional connectivity, finding increased resting-state connectivity between the hippocampus and medial prefrontal cortex, but reduced connectivity between the hippocampus and anterior insula and posterior cingulate cortex after corrections for multiple comparisons [Thomason et al., 2021]. While these results provide strong evidence that prenatal cannabis exposure alters brain connectivity from a very young age, the brain undergoes rewiring and strengthened inter-region connections throughout childhood and adolescence, highlighting the importance of rs-fMRI studies into these time points, which may represent a sensitive period [Langeslag et al., 2013].

In summary of the imaging findings, these early papers implicate several systems, including the limbic system, which canonically comprises the hippocampal formation, cingulate gyrus, amygdala, and hypothalamus [Rajmohan and Mohandas, 2007]. While the insular cortex is not typically considered part of the limbic circuit, it has been shown to share connections with limbic regions, such as the hippocampus and amygdala, as well as the prefrontal cortex, which has also been implicated in prenatal cannabis exposure [Uddin et al., 2017, Nagai et al., 2007]. Furthermore, the limbic circuit, insula, and prefrontal cortex have all been highlighted in studies examining neuropsychiatric disorders, emphasizing their potential importance regarding later life outcomes and neurodevelopment following prenatal cannabis exposure [Nagai et al., 2007, Xu et al., 2019].

In nonhuman animals, as well as human fetal tissue from elective abortions, invasive metrics can be used to develop a deeper understanding of changes induced by prenatal cannabis exposure. Evidence from both mouse and human fetuses suggests that prenatal THC exposure causes changes in brain connectivity. Prenatal THC exposure rerouted CB_1 neurons to the stratum radiatum of the cornu ammonus 1 of the hippocampus, increased the diameter of a corticofugal tract from the cortex [Tortoriello et al., 2014]. Assessments of hippocampal neurons from adult male rats prenatally exposed to WIN demonstrate reductions in extracellular glutamate, both basally and increases induced by potassium-chloride [Mereu et al., 2003]. This group

demonstrated a similar effect in adult male rats when examining the cortex, again reporting reductions in basal extracellular glutamate and reductions of the extracellular glutamate induced by potassium-chloride [Antonelli et al., 2004]. While their work indicated glutamate dysfunction as a contributing factor in the changes induced by prenatal cannabis exposure, dopamine may also play a role. Specifically, following oral gestational exposure to high-THC cannabis preparations (GD 5 to term, 20 mg/kg THC), rat pups, from just before birth to PND 10 showed alterations in dopaminergic indices, most prominently, a reduction in dopamine in males until PND 5 [Rodríguez de Fonseca et al., 1992]. In human infants, there is evidence of very slight increases of methylation in genes encoding for dopamine receptors at 8 weeks of age [Fransquet et al., 2017]. While, to date, no studies have examined the impact of prenatal cannabis exposure on microglia, some preliminary evidence suggests prenatal exposure to the synthetic cannabinoid HU-210 may impact the innate immune system of adult rats, reducing the number of immune cells in the spleen [del Arco et al., 2000].

The research highlighted here shows clear evidence of behavioral changes, however it also illuminates certain gaps in the literature examining the underlying neuroanatomical changes associated with prenatal cannabis exposure that I seek to address in future chapters.

2.4.4 Experimental confounds

Important to all studies examining development is the role maternal behavior and care can play in pup development. It is extremely difficult to separate what effects result from exposure to the cannabinoid itself and what results from changes in the dam. For example, cannabis is known to impact appetite and metabolism, therefore it could also impact feeding habits of dams during pregnancy [Cluny et al., 2015]. Changes at the level of the mother could be related, for example, to the placenta, milk content, or maternal behavior.

In recent years the placenta has seen increased attention as a focus for research on prenatal cannabis exposure. This historically-neglected organ holds a veritable treasure-trove of information for studying development and the impact of early life environmental exposures. The term placenta is a natural by-product of birth and often discarded, providing a wholly non-invasive sample rich with information for researchers [LaSalle, 2021]. It is also underused as an alternative specimen to identify late-gestation cannabis use in humans [Marchetti et al., 2017], and as the placenta is pivotal for fetal development, it can provide insight into various aspects of healthy

development, as well as responses to environmental insults. For example, it can be examined from a perspective of gross-morphometry by looking at the placental structure [Natale et al., 2020], at the cellular composition [Walker et al., 2020], or at a protein expression level with transcriptomics [Rompala et al., 2021]. In a rat model, following both inhalation and injection of THC, THC and its metabolites have been recorded in the dam's blood, the placenta, and the fetal brains of offspring [Baglot et al., 2022]. Furthermore, after exposure to 3 mg/kg THC from GD 6.5-22, there were structural changes in the rat placenta including a slightly increased labyrinth layer area (where trophoblasts proliferate), but a decrease in epithelial cell adhesion moleculen (EpCAM), a gene associated with trophoblast progenitor cells [Natale et al., 2020]. Additionally, the authors report placental vascular deficits as they examine the ratio of maternal-to-fetal blood space in the placenta [Natale et al., 2020]. These changes could relate to alterations seen in the gross morphometry of cannabis-exposed offspring, or subtler changes associated with nutrient access. A study in rhesus macaques exposed to THC edibles from 4 months before conception throughout pregnancy revealed a decrease in amniotic fluid in THC animals throughout pregnancy, as well as decreased placental perfusion and fetal oxygen availability throughout gestation [Roberts et al., 2022]. There were also increased microinfarctions (cell death following interrupted blood flow) and syncytial knots (aggregates of nuclei in placental villi caused by oxidative stress) in THC placentas, which may be further evidence for uteroplacental malperfusion [Roberts et al., 2022, Loukeris et al., 2010]. Finally, the authors also reported dysregulation in genes associated with vascular development, cell-adhesion, angiogenesis, and cytokine binding [Roberts et al., 2022]. This elegant set of analyses provide indications from multiple sources that blood and nutrient flow to the fetus may be disrupted. In vitro, human-derived placental cells exposed to THC display decreased syncytialization (the process by which cytotrophoblasts undergo cell fusion) [Walker et al., 2020, Mansilla et al., 2021], further highlighting this process in the placenta as potentially important to down-stream outcomes of prenatal THC exposure. Tissue samples derived from the placentas of non-cannabis-users were also used to demonstrate that the in-vitro application of THC impacted the expression of N-arachidonoylphosphatidylethanolamine-specific phospholipase D (NAPE) and FAAH [Maia et al., 2019]. Interestingly, NAPE, involved in the synthesis of the endocannabinoid AEA, was localized mainly in the syncytial layer [Maia et al., 2019]. Exposure to 10 or 40 micromolar of THC for 24 hours increased NAPE expression and decreased FAAH expression, but after 72 hours of THC exposure, NAPE expression decreased and FAAH expression increased [Maia et al., 2019]. While the extended

exposure to steady dosages of THC may have limited application to human and animal studies, these results do suggest a mechanistic link between exposure to exogenous cannabinoids and alterations in the syncytial zone of the placenta, as well as the downstream development of trophoblasts. One study linked placental transcriptomics with offspring outcomes in young children [Rompala et al., 2021]. While they found no differentially expressed genes that survived corrections for multiple comparisons, there were trending reductions in genes for pro-inflammatory cytokines and chemokines, as well as CB_1 receptors, in the placenta [Rompala et al., 2021]. The changes in placental morphology or function could contribute to downstream effects, making it difficult to distinguish between the direct effect of THC in the embryos.

In humans, the half-life of THC in breast milk was on average 17 days [Wymore et al., 2021]. While THC exposure during lactation has received attention as a potential source of offspring exposure [Metz and Stickrath, 2015], to my knowledge no studies have investigated the impact of THC on the quality of breast milk itself. One study did examine the impact of prenatal cannabis exposure on hormone production, finding THC exposure inhibited the production of prolactin, which could indicate an interference with the production of milk during lactation [Murphy et al., 1998]. Recent studies in rats have examined the impact of maternal immune activation on the nutritional content of milk, and similar studies within the model of prenatal cannabis exposure could lend insight into the origin of differences in offspring growth and weight [DeRosa et al., 2022].

Finally, changes to maternal care and behavior could contribute to the differences seen in offspring. To address this, some studies cross foster pups to dams not exposed to cannabinoids [DiNieri and Hurd, 2012]. Very early research conducted in the 1970s employing this technique found reductions in food and water intake associated with prenatal cannabis exposure [Abel et al., 1980]. This technique, however, has several drawbacks: it adds an additional stressor to the pups first days of life when they are separated from their dam and introduced to a foster-dam. Additionally, it necessitates a second pregnant dam on the same gestational timeline, which can be difficult to schedule. Finally, while cross fostering allows researchers to disentangle the impact of THC exposure from the impact of maternal behavior, it has little external validity in application to human studies, where it may be more appropriate to examine the combined impact of the substances direct effect on the pups and the indirect effects through the dam.

2.5 Sex differences in cannabis exposure

2.5.1 Sex differences following prenatal exposure

While sex differences have widely been documented in animal models following prenatal exposure to cannabinoids, few studies in humans either examine or report them. In humans, increased aggressivity was found in young girls compared to controls, while the same was not found in boys [Rompala et al., 2021]. In young adults, there is an association with prenatal cannabis exposure (PCE) and increased use of cannabis by the offspring themselves, which is more pronounced in men than women [Porath and Fried, 2005]. Behavioral results from nonhuman animals, however, provide many accounts of sex differences, although the direction of alterations (worse in males or females) is not always consistent. For example, the reported results from locomotion explored in the last section paint a particularly variable picture with differing doses of prenatal cannabinoids alternatively increasing or decreasing locomotion by sex [Rubio et al., 1995, Navarro et al., 1994, Moreno et al., 2005]. While the direction of results differs, they invariably report sex-effects within studies. These sex differences are also reflected in hormone levels, mostly examining stress hormones [Rubio et al., 1995, Navarro et al., 1994, Moreno et al., 2005]. It is unclear whether the variability in the sex-effects is due to differences in experimental design (administered substance, dosage, age of testing, method of statistical analysis), or other uncontrolled factors, such as social dominance hierarchy, which can account for variance seen in exploratory tests, glucocorticoid metabolites, and novel object discrimination [Varholick et al., 2019]. In models of maternal immune activation, another early life exposure, the importance of controlling (or at least reporting) factors such as the vendor of mice (or bred in-house), the style of cage ventilation, bedding provided, cage placement on rack, and the breeding paradigm, along with a host of other factors that could impact the outcome of either the main effect of interest (such as prenatal cannabis exposure), or additional outcomes, such as sex effects between studies [Kentner et al., 2019]. As the field of research examining the impact of prenatal cannabis exposure grows, clear and detailed reporting of such factors can help synthesize results among experiments. Unfortunately, however, many non-human animal studies reviewed here excluded female subjects entirely [Trezza et al., 2008, Antonelli et al., 2004, Drazanova et al., 2019, del Arco et al., 2000]. The importance of including animals of both sexes cannot be overstated, as there are clear indications that biological sex may impact the outcomes of prenatal cannabis exposure, and results

from only one sex may not be generalizable to others.

2.5.2 Potential mechanisms underlying sex-differences

Despite the limited evidence from prenatal cannabis exposure indicating sex differences, diverging results between sexes is unsurprising given differences in hypothesized mechanisms of cannabinoid metabolism and sex-effects of cannabis exposure in adolescence.

There are several potential mechanisms that could mediate the impact of cannabinoids between the sexes, including gonadal hormones or the pharmacokinetics and pharmacodynamics of THC [Cooper and Craft, 2018]. Related to gonadal hormones, it has been hypothesized that genes on the X or Y chromosome could control or moderate receptor expression [Cooper and Craft, 2018]. However, this has never been directly explored in experimentation. While it is also posited that gonadal hormones may impact cannabinoid sensitivity, sex-differences following cannabinoid exposure are not limited to peri- or post-pubertal timepoints in nonhuman animals, suggesting differential sensitization may be related to basal hormone levels [Borcel et al., 2004, Llorente-Berzal et al., 2011]. In gonadectomized adult rats, introduction of estradiol to female rats enhanced the antinociceptive effects of THC, while introduction of testosterone to male rats attenuated THC-induced hyperlocomotion [Craft and Leitl, 2008]. A further study from the same group found that administering estradiol and testosterone to gonadectomized adult rats of both sexes was not alone sufficient to consistently induce sensitization or desensitization to THC as expected, however [Craft et al., 2017]. This highlights the intermediary effects of THC metabolism [Craft et al., 2017]. The findings from Craft et al. (introducing estradiol or testosterone) point towards the potential of variation in the pharmacokinetics (how the body interacts with administered substances during exposure) and pharmacodynamics (the effect of the substance on the body) of cannabinoids between sexes due to hormone variation [Grogan and Preuss, 2022].

Sex differences in cannabinoid metabolism have been demonstrated across species, however there is more evidence in rodents than humans, which may reflect either cross-species sex differences or simply a bias in the underlying literature to study drug metabolism in animal models [Cooper and Craft, 2018]. Metabolism of THC in rats results in increased 11-OH-THC (psychoactive) and THCCOOH (inactive) in females than in males [Britch et al., 2017]. Furthermore, prior administration of CBD inhibited THC metabolism in females more than males [Britch et al., 2017].

It has been reported that the differences in THC metabolism between sexes could be due to alterations in enzymes produced in the liver [Cooper and Craft, 2018]. One early study found sex differences in the oxidative metabolism of THC both *in vivo* and *in vitro* [Narimatsu et al., 1991]. While the primary metabolite of THC in females was the psychoactive 11-OH-THC, in males THC was oxidized at various positions, producing a wider variety of inactive metabolites [Narimatsu et al., 1991]. This could, in part, explain increased sensitivity to THC in female rodents. Differences in cannabinoid receptors themselves could contribute to altered patterns of responses to THC between males and females. Sex differences in CB_1 receptor mRNA have been investigated in multiple brain regions (prefrontal cortex, hippocampus, amygdala, brainstem, hypothalamus, mesencephalon, pituitary, and cerebellum), and a review of the findings demonstrates inconsistent results of higher expression in males or females [Cooper and Craft, 2018]. Methodological variables described before, as well as the stage of estrus of female mice and circling testosterone levels of male mice could contribute to these differences. Nevertheless, the receptor availability could further contribute to observed behavioral changes.

Finally, it is possible that the sex of the embryo impacts blood and nutrient flow in utero, which could mediate the dosage of THC pups are exposed to. Across species there is evidence that early in gestation, male fetuses are larger than females, evidenced in cell number of mouse embryos (GD 3.5) and crown-rump length in human fetuses (weeks 8-12) [Alur, 2019]. Male fetuses, compared to females, generally face higher risk during adverse conditions, such as maternal malnutrition, with increased morbidity and mortality in male fetuses and newborns [Alur, 2019, Kalisch-Smith et al., 2017]. Nevertheless, female fetuses experience greater risk for outcomes such as intrauterine growth restriction and being small for gestational age, two outcomes that have been investigated in the context of prenatal cannabis exposure [Braun et al., 2022]. These differences have been investigated in the context of transcriptomic sex differences, identifying differentially expressed genes related to metabolic regulation, cell death, and cell-cell interaction, among a host of others [Braun et al., 2022]. Another potential factor associated with changes in fetal growth is placental blood circulation and vasculature [Widnes et al., 2017]. From 22 to 24 gestational weeks in humans, there is higher pulsatility index in female fetuses in the umbilical artery than male fetuses, which is often (although not always) correlated with increased resistance in the artery [Widnes et al., 2017]. Despite the implications of potential differences in placental vascular morphometry, few studies investigate structural or functional differences in the placenta between the sexes [Clifton, 2010]. It is possible that sex-specific

changes to placental morphometry or function could be responsible for differential exposure to cannabinoids introduced through the blood, which could contribute to downstream differences in outcomes between the sexes. Nevertheless, in investigations of cannabinoids and THC metabolites in the brain, there are no reported sex-differences [Baglot et al., 2022]. Altogether, there is reason to suggest cannabinoids (administered before birth or during adolescence) may impact male and female offspring differently, however the underlying mechanism (whether at the level of the placenta or the fetus) is yet unexplored.

2.6 Magnetic resonance imaging

2.6.1 Principles of nuclear magnetic resonance

MRI is a remarkable technology that can be leveraged to noninvasively generate images of the brain, assess the physical properties of unseen tissue types, and record dynamic changes during rest or a task. While acquisition sequences can be manipulated to change the information gathered, this thesis employs only contrast-based structural imaging, and the background will be limited to an exploration of this technique.

The principles of MRI are based on the physics of nuclear magnetic resonance (NMR) [Plewes and Kucharczyk, 2012]. Matter is composed of atoms, and atoms are composed of subatomic particles, such as electrons, protons, and neutrons, which are arranged with a positively-charged nucleus of protons and neutrons and a cloud of surrounding electrons [McGrayne et al., 2023]. Atomic nuclei with an uneven number of protons exhibit a property known as spin. While spin is intrinsically a quantum mechanical concept, it can be thought of as rotation of a proton around an axis [Plewes and Kucharczyk, 2012]. While many atoms exhibit spin, in MRI, the majority of techniques are based on the nucleus of hydrogen atoms (with a single proton) because of the abundance of hydrogen in water in the body (88M in human tissue) [Plewes and Kucharczyk, 2012]. The spin and charge of protons affords them properties similar to tiny bar magnets, useful to the conceptualization and visualization of the properties of MRI, as seen in Figure 2.7a) [Plewes and Kucharczyk, 2012]. In the absence of a magnet, these hydrogen protons are distributed between two states equally, cancelling out any net magnetic effect, however when a steady magnetic field (B0) is applied to the tissue, the protons tend to align either with or against the direction of the field Figure 2.7b) [Plewes and Kucharczyk, 2012]. The orientation aligned in parallel with B0 is slightly more common than the orientation aligned

against B0, creating a "bulk magnetization", which can be visualized as a single vector Figure 2.7c), pale purple arrow [Plewes and Kucharczyk, 2012]. The protons precess around the external magnetic field at a resonance frequency that scales with the strength of B0 called the Larmor frequency, which can be calculated with Equation 2.1 [Legchenko, 2013].

$$\omega = \gamma\beta \div 2\pi, \tag{2.1}$$

where ω is the Larmor frequency, γ is the gyromagnetic ratio (specific to the nuclei), and β is the B0 magnetic field strength [Legchenko, 2013].

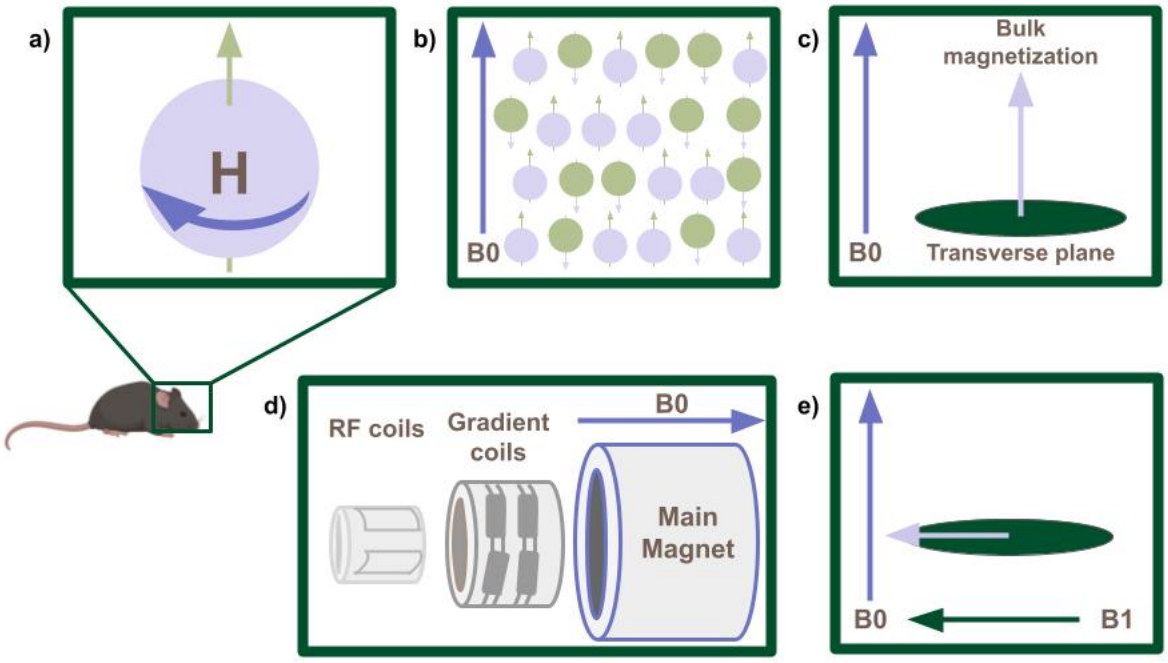

Figure 2.7: a) Representative hydrogen protons in tissue. b) Main magnetic field (B0) with protons aligned with and against. c) Vector representing bulk magnetization (light purple) and transverse plane (dark green). d) Main magnet with vector for B0, gradient coils within, and RF coils in interior e) Vector representing B1 and bulk magnetization "flipped" into transverse plane.

In order to create a magnetic resonance (MR) image, signal from the precessing nuclei must be detected. MRI technology relies on several subsystems of magnets to transmit, receive, and localize signal as seen in Figure 2.7d) [Gruber et al., 2018].

First, B0 is created by the main magnet, which aligns the spins of the hydrogen protons [Gruber et al., 2018]. Second, within the main electromagnet, gradient coils allow for encoding in each 3-dimensional direction (x, y, and z) for localization [Gruber et al., 2018]. Finally, radiofrequency (RF) coils transmit and receive signal to and from the tissue. Transmit coils generate an RF pulse which produces a small magnetic field oriented perpendicular to the main field B0 [Gruber et al., 2018]. This magnetic field, known as B1, has a frequency equal to the Larmor frequency and flips the nuclear spins perpendicular to B0 [Plewes and Kucharczyk, 2012]. These coils are called RF because the Larmor frequency is generally in the megahertz range corresponding with the RF section of the electromagnetic spectrum [Plewes and Kucharczyk, 2012]. The plane perpendicular to B0 is known as the transverse plane [Plewes and Kucharczyk, 2012]. When flipped into this plane, precessing nuclei can generate the electrical current that is the measurable MR signal, represented in Figure 2.7e) [Plewes and Kucharczyk, 2012]. The receive coil can then detect this signal [Gruber et al., 2018]. Transmit and receive can be achieved through a unique or separate coil sets, with a unique coil set useful for ultrahigh field strength MRI [Gruber et al., 2018]. With the spins precessing in the transverse plane, contrast between tissues can be generated based on the transverse or longitudinal relaxation time, explored in the following two sections.

2.6.2 Transverse relaxation

The relaxation mechanisms allow precessing hydrogen nuclei to generate different signal strength based on their environment. As mentioned before, the transverse plane is perpendicular to B0 [Plewes and Kucharczyk, 2012]. When the precessing bulk magnetization is flipped 90° into the transverse plane (Figure 2.8a), this rotating magnetization will create a very small signal in receiver coils, which are connected to an amplifier that is tuned to ω [Plewes and Kucharczyk, 2012]. The nuclei precesses around the Z-axis, however over time they move away from this axis [Plewes and Kucharczyk, 2012]. Importantly, unlike in a hypothetical ideal, nuclei in tissue will precess at slightly different values of ω [Plewes and Kucharczyk, 2012]. As a result, over time they "fan out" across the transverse plane, as some precess more quickly and others more slowly (Figure 2.8b). This behavior is known as "dephasing" [Plewes and Kucharczyk, 2012]. As more nuclei fall out of alignment, the signal generated from the transverse relaxation decreases [Plewes and Kucharczyk, 2012]. The process of dephasing and signal decay is also known as spin-spin relaxation, or T2 relaxation [Plewes and Kucharczyk, 2012].

In reality, however, inhomogeneities in the magnetic field impact the rate of signal decay, and the actual *observed* T2 is represented as T2*.

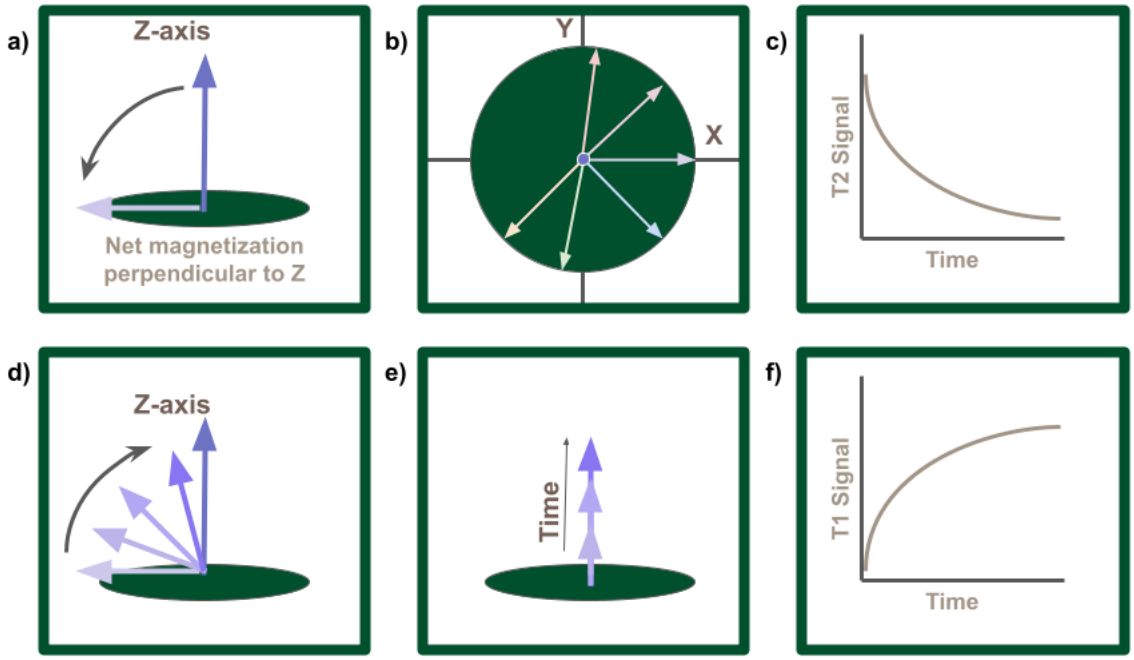

Figure 2.8: a) Representation of net magnetization flipped to transverse plane (pale purple) by excitation pulse b) Top-view of X-Y transverse plane with thin colored arrows representing dephasing nuclei c) Representative curve of T2 signal decreasing over time as spins dephase d) Representation of net magnetization returning to Z-axis over time. e) Net magnetization parallel to Z-axis returns to equilibrium over time. f) T1 signal increases over time

In a biological system, different tissue types have different magnetic properties which alter the relaxation time constants of the tissue [Plewes and Kucharczyk, 2012]. In the brain, cerebral spinal fluid (CSF) generally shows the longest relaxation time and white matter has the shortest relaxation time, with grey matter in the middle [Plewes and Kucharczyk, 2012]. The time between the "excitation pulse", in this case the 90° RF pulse pushing the magnetization into the transverse plane, and the time when the image is acquired is known as the echo time (TE) [Plewes and Kucharczyk, 2012]. With longer TE intervals, the signal from different tissue types diverge as nuclei in white matter dephase more quickly than those in

grey matter and CSF, creating a T2-weighted (T2w) image, discussed further in the following section [Plewes and Kucharczyk, 2012]. Images can also be generated from the proton density of tissue, however this technique was not used in the thesis, and is not discussed here.

2.6.3 Longitudinal relaxation

Along with signal from dephasing nuclei as they precess from the Z-axis, signal can also be generated as nuclei return to the parallel alignment with B0 (the Z-axis) (Figure 2.8d). After the excitation pulse, energy slowly leaves the spin system to the "lattice", the surrounding sample [Plewes and Kucharczyk, 2012]. As this energy is lost, the spinning nuclei slowly return to the state at equilibrium in line with the main magnetic field (Figure 2.8e). This process is known as longitudinal or "T1" relaxation (Figure 2.8f) [Plewes and Kucharczyk, 2012].

Similarly to T2 (transverse) relaxation, different tissue types have different T1 values, making it possible to distinguish in biological samples [Plewes and Kucharczyk, 2012]. Generally, T1 relaxation times are longer than T2 relaxation times. Otherwise, CSF still has the longest relaxation time, gray-matter the second longest, and white-matter the shortest [Plewes and Kucharczyk, 2012]. The following section explores how differences in T1 and T2 relaxation times can be leveraged to generate images differentiating tissue type in the brain.

2.6.4 Contrast-based MRI

The fact that nuclei in different chemical environments have different relaxation times can be harnessed to maximize contrast between gray and white matter and CSF. Contrast can reflect different relaxation mechanisms, including T1 and T2 [Plewes and Kucharczyk, 2012]. When an image's contrast is primarily defined by transverse relaxation, it is known as T2w, and when it is primarily defined by longitudinal relaxation, it is known as T1-weighted (T1w)[Plewes and Kucharczyk, 2012].

To generate a T2w image, if the time between the excitation pulse and acquisition (TE), is short, the difference between signal from tissue types will be slight (meaning contrast between tissue types will be less), as seen in Fig. 2.9a) [Plewes and Kucharczyk, 2012]. However, if the TE is longer, tissue types diverge in signal, as protons dephase at different rates in different tissue types [Plewes and Kucharczyk, 2012]. The resulting T2w image has bright CSF (high-

est signal/longest relaxation time), dark white matter, and medium gray matter [Plewes and Kucharczyk, 2012].

In generating T1w images, additional excitation pulses are required to re-excite the protons as they begin to relax back in the z-axis [Plewes and Kucharczyk, 2012]. As the protons begin to relax, the second excitation pulse pushes them back into the transverse plane, visualized in Fig. 2.9b). This time delay is known as Repetition Time (TR) [Plewes and Kucharczyk, 2012, Nitz and Reimer, 1999]. In T1w imaging, there is a very short TE with the image acquisition following quickly after the excitation pulse, to maximize tissue contrast [Nitz and Reimer, 1999]. After a short TR, tissues with a short T1 will have a greater signal in the Z-axis than tissues with a long T1, and so when these protons are flipped back into the transverse plane, if an image is acquired quickly after the excitation pulse, the difference between the tissue types is greater [Jung and Weigel, 2013]. The TE must also quickly follow the 90° excitation pulse to limit the effect of T2 relaxation (dephasing) on the signal [Plewes and Kucharczyk, 2012]. In the resulting T1w image, CSF, with the longer relaxation time, is dark, while tissue appears lighter [Plewes and Kucharczyk, 2012, Nitz and Reimer, 1999, Jung and Weigel, 2013].

While pulse sequences in real life vary and are more complex than the examples outlined here, the underlying principles apply to the T1- and T2-weighted imaging methods employed in this thesis.

2.6.5 Contrast Agents

Processing and analysis of contrast-based MRI depends on clear differences between tissue-types and brain structures. In cases such as neonatal imaging, water and fat content in the brain is different than in adults, altering T1 and T2 relaxation times of tissue [Dubois et al., 2021]. Importantly, the lipid myelin bilayer that wraps neuronal axons, a major source of fat in the brain, develops postnatally–in humans between the first 3-24 years of life [Deoni et al., 2011]. In fetal and neonatal MRI this results in a reduction of clear contrast between gray and white matter. To increase the contrast, paramagnetic particles can be introduced which shorten the T1 or T2 relaxation times in tissue, depending on the properties of the agent [Xiao et al., 2016]. For T1w imaging, this increases signal intensity, or for T2w imaging it decreases signal intensity [Xiao et al., 2016]. Importantly, contrast agents are not absorbed into tissue uniformly, but rather biodistribution differs based on the mechanism of action of the specific agent [Xiao et al., 2016]. While there are many contrast agents that differ in their

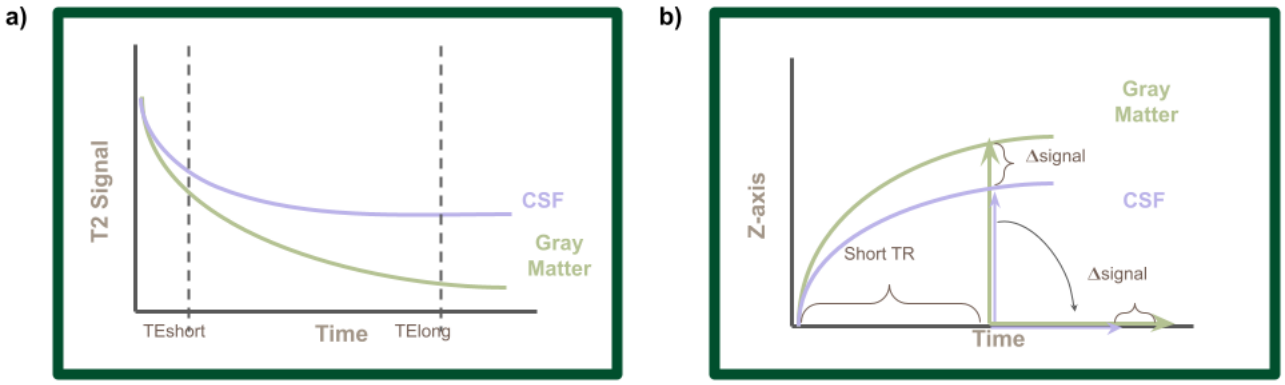

Figure 2.9: a) Simplified example of acquiring a T2w-image maximizing contrast between CSF and gray matter with a long echo time (TElong) b) A simplified example of acquiring a T1w image with a short TR

application, structure, mechanisms of action and biodeposition properties, here I discuss two specific agents used in the course of the thesis.

Gadoteridol (Gado) (ProHance) is a gadolinium-based chelate, meaning it is a compound comprising an organic ligand bonded to a metal atom (in this case gadolinium) [Kumar et al., 1996]. Gadolinium-based contrast agents are paramagnetic; they are attracted to a magnetic field [Xiao et al., 2016]. Exposing tissue to Gado reduces both T1 and T2 times, and as it is used as a positive agent enhanced areas appear brighter on T1w scans [Cleary et al., 2009]. While the effects are much smaller on T2-w images [Ibrahim et al., 2022], long scan times and fast spin-echo pulse sequences allow for T2-w acquisitions with high contrast [Nieman et al., 2005]. When administered intravenously *in vivo*, Gado is deposited in extracellular spaces and cannot cross into the cellular compartments [Koenig et al., 2013]. Immersing *ex vivo* samples in Gado solutions for more than 5 days also allows for permeation throughout the brain, reducing T1 relaxation time in the cortex, striatum, thalamus, hippocampus, and cerebellum [Huang et al., 2009].

Another contrast agent is manganese chloride 2 (MnCl). Like Gado, MnCl is paramagnetic, reducing T1 and T2 [Xiao et al., 2016]. In the MnCl chelate, manganese is the metal atom, rather than gadolinium [Xiao et al., 2016]. Importantly, because manganese is divalent, it can enter neurons through voltage-gated calcium channels when they fire, as depicted in Fig. 2.10, because calcium is also divalent [Lin and Koretsky, 1997]. *In vivo* in mice, MnCl-enhancement is maximized 24 hours after an injection, with marked increased intensity in regions of the olfactory bulbs, hippocampus, and cerebellum [Wadghiri et al., 2004].

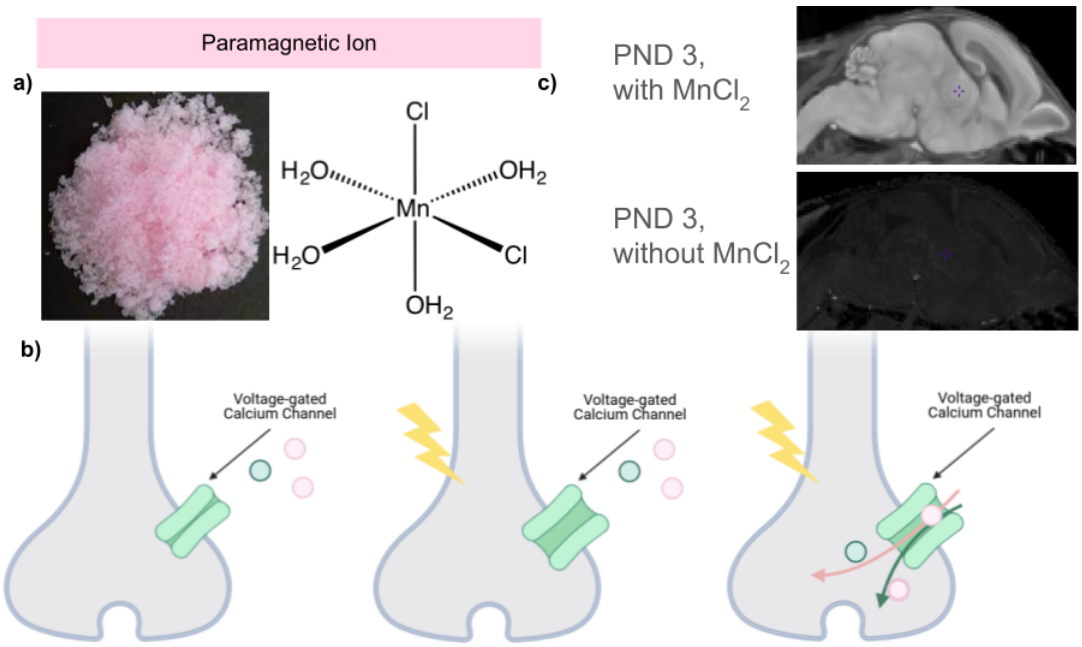

Figure 2.10: a) Depiction of manganese chloride. b) Illustrative example of manganese (pink) and calcium (green) entering a firing neuron. c) Difference between manganese-enhanced MRI (top) and non-enhanced MRI (bottom).

2.7 Small animal imaging

The first *in vivo* MRI was acquired in 1973 when Paul Lauterbur imaged a clam [Macchia et al., 2007]. Four years later, Raymond Damadian acquired the first image of a human body *in vivo* [Macchia et al., 2007]. In the following 50 years, MRI research has burgeoned into a rich field, expanding into multiple MR modalities, analysis techniques, age-ranges, organ systems, and species.

Small animal imaging differs from human imaging in several key ways. The adult mouse brain is on average about 3000 times smaller than the human brain [Badea et al., 2007, Ruigrok et al., 2014], and therefore images must be acquired at higher resolution than in humans. The smaller voxel (3 dimensional pixel) size of high resolution images allows MRIs to capture fine details of small structures in the rodent brain. Specifically, resolution must be increased approximately 15 times in each linear direction [Spencer Noakes et al., 2017]. To achieve this high resolution, however, MRI scanners require higher baseline magnetic fields, achieved by increasing the strength of the main magnet (for humans, commonly ranging from 1.5-3 Tesla (T) or rarely 7-10.5T) to 7T or greater for small animal imaging [Moser et al., 2017]. The strength of the baseline magnetic field (B0), known as field strength, is approximately proportional to the signal-to-noise ratio (SNR) achieved in a sample [Moser et al., 2017, Bernstein et al., 2006]. This means that when the field strength doubles, the SNR roughly doubles as well, allowing for the acquisition of higher resolution images [Bernstein,]. The SNR is further increased by using a cryogenically-cooled coil (cryo-probe) in order to reduce the thermal noise, increasing sensitivity without increasing field strength [Moser et al., 2017]. At the Douglas Mental Health University Institute Cerebral Imaging Centre where the *in vivo* imaging for the present experiments took place, both a cryo-probe and high field strength (7T) were employed to achieve voxel sizes of $(70\mu m)^3$ in anaesthetized mice.

2.7.1 *Ex vivo* embryo imaging

While *in vivo* imaging leverages the substantial benefits of MRI as a noninvasive technique, in special cases, such as small-animal embryo imaging, it can provide a unique set of metrics from *ex vivo* samples. When extracted from a pregnant mouse following timed mating to achieve a specified age, and properly prepared, multiple mouse embryos can be imaged simultaneously at ultra-high resolution with multi-hour scan times that could not be achieved *in vivo* [Nieman et al., 2005, Spencer Noakes et al., 2017, Guma et al., 2022]. Images acquired from the whole body of the embryo can be used not only to examine alterations in brain volume due to an intervention, but also changes in whole body and organ volume, allowing insight into global changes that may impact the brain and development [Guma et al., 2022].

At the Mouse Imaging Centre at SickKids Hospital at the University of Toronto, 16 samples can be imaged simultaneously, allowing for efficient through-put despite scan times of 14h for $40\mu m^3$ resolution scans [Spencer Noakes et al., 2017]. Samples are pre-

pared with Gado to reduce the global longitudinal relaxation time as discussed in Section 2.6.5 and the overall scan time [de Guzman et al., 2016, Spencer Noakes et al., 2017]. The 4-by-4 array for 16 sample acquisition introduces certain artefacts as the samples are placed at varying distances from the isocenter of the gradients, with the farthest samples from the isocenter most susceptible to artefacts [Spencer Noakes et al., 2017]. Therefore, certain corrections are applied during image reconstruction to assure high-quality images are obtained regardless of position in the array [Spencer Noakes et al., 2017]. Following image reconstruction, images undergo visual quality control to ensure none of the samples were squished in extractions, transit, or storage, and any bubbles in the solution can be masked-out. Next, preprocessing is applied. The automated preprocessing procedures include clamping out "hot", hyperintense voxels and the N4-bias field correction used in *in vivo* imaging as well and discussed in greater detail in the methods of Chapter 3 [Tustison et al., 2010], Section 3.4.4.1.

To analyze voxel-wise volume changes in the brain or organ mask, images are subject to a cross-sectional deformation based morphometry (DBM) analysis. In order to statistically compare groups or populations and identify macroscopic anatomical differences, all images must be in the same stereotactic space to achieve voxelwise correspondence across all images and map areas of relative volume difference [Ashburner et al., 1998]. As originally introduced, DBM maps a series of images to a template conforming to a standard anatomical space [Ashburner et al., 1998]. The images are warped to the template through a series of affine transformations (linear mappings that include translations, rotations, shears, and scales; Fig. 2.11) and local nonlinear transformations that achieve correspondence between the image and the target producing deformation fields from the nonlinear transformations (vector fields describing how much each voxel had to be warped to match the target) [Ashburner et al., 1998]. As originally used, DBM mapped changes from individual images to the template [Ashburner et al., 1998], however it is possible to instead first create an unbiased study-specific average of all images to be analyzed and map deformations from each image to this study average [Nieman et al., 2005]. This average serves as an intermediate between all images and reduces the need for a template that matches the strain, age, and imaging sequence of the current study [Nieman et al., 2005]. The Jacobian determinant (or Jacobian) can be calculated from the deformation field, deriving a voxelwise measurement of volume expansion or contraction [Friedel et al., 2014]. The Jacobians can be compared for group-wise differences based on experimental conditions as discussed further in Section 2.7.4.

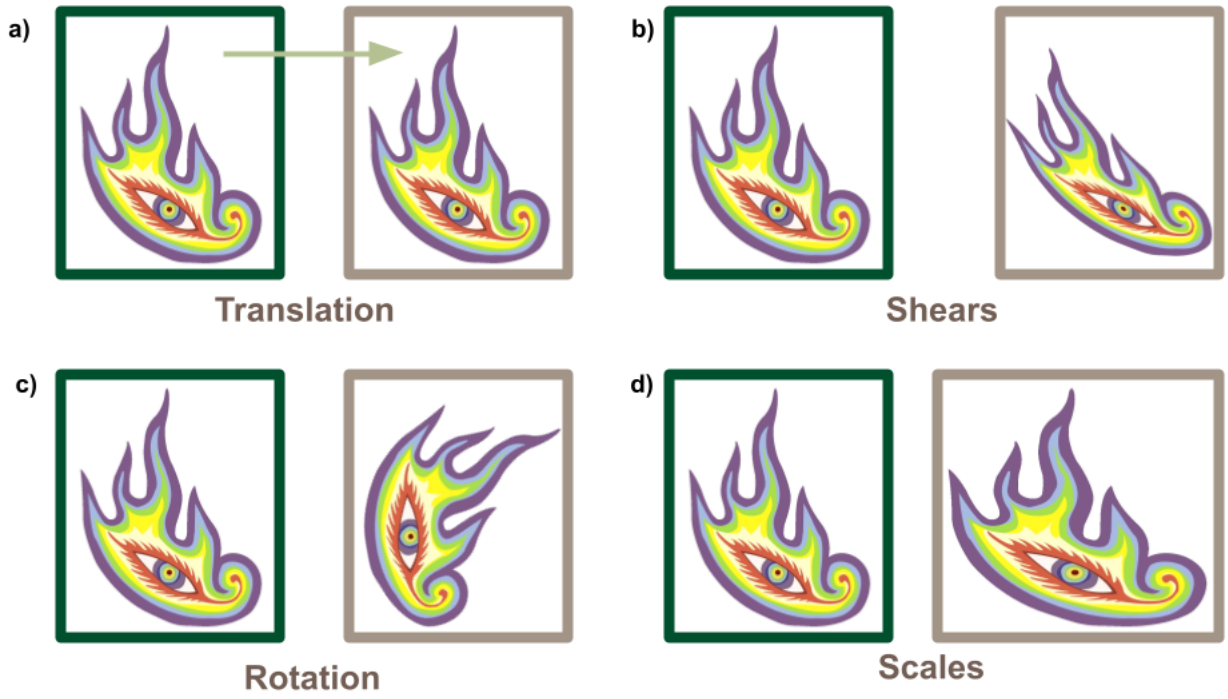

Figure 2.11: Visual representation of a) translation (shifting in the direction of the arrow) b) shear c) rotation d) scales

2.7.2 *In vivo* longitudinal imaging

Unlike most approaches that provide a window the brain, neuroimaging is non-invasive. Many procedures, such as immunohistochemistry are terminal. While a technique like calcium imaging allows for longitudinal assessments, it is invasive and has limitations on the field of view of the window [Rahn et al., 2022]. While some behavioral assessments such as the open-field test (OFT) for anxiety-like behaviors are noninvasive in the sense that they do not require long training periods or external stressors such as singly-housing mice, the test-retest reliability (TRR) may be difficult to assess as repeating a behavior could have habituation or carry-over effects, a topic that has been under-explored in mice [Shiguang et al., 2010].

In contrast, neuroimaging provides a unique approach to examine a specific subject's brain multiple times throughout its life. This allows us to calculate individual developmental trajectories and use statistical models that account for repeated measures to detect deviations from the control group's brain volume trajectories. One major advantage of this approach is that instead of preselecting cross-sectional timepoints to

examine a phenotype, we can select a range of ages and examine brain growth over time. Further, unlike calcium imaging, immunohistochemistry, or RNA-sequencing, neuroimaging allows for a whole-brain field-of-view (FOV), providing an excellent technique to identify region of interest (ROI)s that can be further explored with metrics sensitive to cellular changes with a clear MRI-informed hypothesis. Longitudinal assessments also allow for increased power compared to cross-sectional analyses, meaning with fewer animals, longitudinal MRI is sensitive to relatively subtle changes [Lerch et al., 2012]. This benefit facilitates the "3Rs" of humane animal research: refinement, reduction, and replacement [Hoyer et al., 2014]. Further while the TRR is dependent on scanner and protocol-specific factors, longitudinal structural MRI in rats has been assessed as being excellent—intraclass correlation (ICC) > 0.8—in all regions except for the retrosplenial granular cortex where TRR was estimated as "good" ($0.8 >$ ICC ≥ 0.6) [Jing et al., 2018]. This is especially important as the TRR of a technique determines the lower bound of an effect size that can reliably be detected between sessions (any effect smaller than inter-scan effects may just be an artefact). Finally, structural MRI is highly translatable as it can also be used longitudinally in humans, unlike positron emission tomography (PET), for example.

Nevertheless, there are some challenges to longitudinal small animal imaging. Unlike *ex vivo* imaging, costs and animal ethics preclude extremely long, overnight scanning times. Instead, scans are acquired at a lower resolution (70-100μm^3) for scan times less than 30 minutes. While protocols for awake rodent imaging have been adopted at different centers around the world, they typically require a week or more of intensive habituation to the apparatus and stressful stimuli [Yoshida et al., 2016]. It is still unclear how well they are adapted to mice at different ages (for example, the training would be impossible in neonates) or in longitudinal experiments. Instead, classical imaging protocols administer inhaled anaesthetics [Guma et al., 2023, Kong et al., 2018, Guma et al., 2021a]. The effect of repeated anaesthesia (such as isoflurane) exposure is often investigated at either a) higher doses, b) higher frequencies, or c) longer sessions than experimental protocols used for longitudinal MRI, which limits the applicability of these findings to mice in imaging experiments. Nevertheless, importantly, there are some effects of repeated anaesthesia exposure. For example, 6 sessions of exposure to isoflurane maintained at 1.75-2.5% for 45 minutes every 3-4 days in adult mice of both sexes was found to alter some behaviors compared with a single exposure, including burrowing behavior, food intake, free exploration, and mouse-grimace [Hohlbaum et al., 2017]. Nevertheless, nest building, home cage activity, body weight, and corticosterone were not effected in this paradigm [Hohlbaum et al., 2017]. Impor-

tantly, age plays a role in the effect of isoflurane on the brain. First, isoflurane acts on the GABA-ergic system, which is excitatory in mice until PND 9 [Valeeva et al., 2016]. This means younger mice are more resistant to the effects of isoflurane than older mice. Second, there is evidence that the impact of exposure to isoflurane early in life may be greater than adult exposure. For example, exposure to 1.2% isoflurane for 4 hours induced impairments to memory retention in young (3 month) but not middle-aged (12 month) male rats [Callaway et al., 2012].

Despite the limitations, *in vivo* MRI remains an unparalleled tool in developmental studies due to its longitudinal and translational advantages. Following acquisition of all timepoints, a voxelwise approach allows for the detection of subtle changes to brain volumes and developmental trajectories. As in embryo imaging, after quality control and preprocessing, DBM can be employed to compare the effects of exposures. Unlike cross-sectional analyses, a two-level approach is used to first register all images from a single animal across timepoints (level one) generating a subject-level average. Then, all subject-level averages are registered together to create the population average (level two) allowing differences to be examined among individuals and within an individual over time [Friedel et al., 2014, Devenyi, 2023]. In the longitudinal approach, Jacobians can be statistically compared for changes in trajectories or slopes over time, rather than volume changes at a single timepoint.

2.7.3 Differences between *in vivo* and *ex vivo* imaging

The differences between *in vivo* and *ex vivo* brain imaging have been assessed in small animals before, highlighting specific sensitivities and limitations of both methods. *Ex vivo* images can be acquired across longer scan times, since it precludes the necessity of anaesthetizing animals or monitoring physiological metrics [Lerch et al., 2012]. As discussed previously, this allows for higher resolution images to be acquired [Spencer Noakes et al., 2017]. Higher rates of contrast agents can be used in *ex vivo* scans, which contributes to a greater contrast-to-noise ratio (CNR) in *ex vivo* scans, however signal-to-noise ratio scales with voxel size, and voxel size is larger in *in vivo* images, contributing to a greater SNR in these images. Indeed, this was modelled in one study examining brains comparing mouse brains with tauopathies in both *in vivo* and *ex vivo* protocols [Holmes et al., 2017]. The authors found higher SNR in *in vivo* images for 7 of 9 investigated regions (but higher SNR in the cerebellum of *ex vivo* images) [Holmes et al., 2017]. Irrespective of differences in voxel size, the increase in SNR in the *in vivo* images compared to the *ex vivo* images was estimated to be an

increase by a factor of 53. A great benefit of the high resolution, however, involves the sensitivity of *ex vivo* imaging to group differences. Because of the small voxel size, *ex vivo* imaging can be more sensitive to group level differences than *in vivo* imaging [Holmes et al., 2017]. Nevertheless, there is a trade-off in sample size and power, as *in vivo* imaging can be conducted longitudinally, increasing the statistical power across the same number of animals [Lerch et al., 2012].

Additionally, formalin perfusion has been demonstrated to cause tissue shrinkage during fixation with an estimated 10% reduction in total brain volume comparing *in vivo* and *ex vivo* brains [Holmes et al., 2017, Lerch et al., 2012]. Importantly, this shrinkage is not homogeneous (across *in cranio* brains), with greater changes observed in the olfactory bulbs and brainstem/cerebellum [Holmes et al., 2017]. It is difficult to estimate whether volume reductions would be more symmetrical in embryo imaging, where the entire body is perfused, rather than the extracted brain, however it is possible that it would be so.

2.7.4 Statistical analyses

Analyzing voxelwise data from structural MRI bears several considerations. First, the Jacobian map, which represents the determinant of the Jacobian matrix of a deformation field, encodes volume differences between two images [Leow et al., 2007]. Importantly, a Jacobian map is lower-bounded by 0, but unbounded above. When comparing two images, a value between 0 and 1 represents smaller volume in a voxel in Image A compared with Image B, while a value between 1 and ∞ represents larger volume in the voxel of Image A compared with Image B [Leow et al., 2007]. More specifically, if the Jacobian determinant of a voxel is 0.9, it denotes 10% smaller volume in Image A compared with Image B, but if it is 1.1, it denotes a 10% larger volume in Image A compared with Image B [Leow et al., 2007]. Because of this asymmetry, the distribution of Jacobian determinants violates assumptions of most statistical models. Therefore, the Jacobians are log-transformed to fit a normal distribution.

Second, many animal experiments violate assumptions of expected variance. For example, with longitudinal experiments, repeated measurements from individuals over time means that measurements are not independent from one another; measurements from an individual over time might be more similar than measurements from different individuals [Harrison et al., 2018]. Even in cross-sectional studies, such as with the embryos, the data are often nested in a variable, such as litter, with up to four animals coming from a single dam. Litter-size, maternal behavior, maternal age,

shared genetics, paternity, and a host of other environmental factors may contribute to reduced variability within litter-mates compared to animals from other litters [Valiquette et al., 2023]. To account for both repeated measures and nested variables, linear mixed effects models (LMER) can be employed to correctly model the random effects structure, with variables such as subject ID and litter included as random effects [Harrison et al., 2018].

Finally, each image contains millions of voxels. When statistical tests are performed, a mask must be supplied to identify the extent of the brain where differences are meaningful. While this reduces the number of voxels compared to the entire cubic field of view, any test run on this high number of comparisons will produce false positives (type I errors) that must be accounted for. Family-wise error corrections for multiple comparisons aim to eliminate any type I errors, an approach which might be too strong for imaging data where the presence of a false positive need not render other positive inferences erroneous [Benjamini and Hochberg, 1995]. Instead, the False Discovery Rate (FDR) limits the proportion of positives likely to be false, providing a more reasonable correction in the field of imaging [Benjamini and Hochberg, 1995]. In interpretation, a heat map thresholded from 5% to 1% FDR means that up to 5% of the voxels that are statistically significant may be type I errors.

Together LMERs and FDR can be combined to provide extremely powerful and sensitive analyses of voxelwise structural MRI data.

2.8 Behavioral analyses in mice

While MRI allows for assessment of biologically-relevant information regarding brain volume and is translatable to humans, behavioral analyses are necessary to establish whether changes are potentially relevant to alterations observed in neuropsychiatric diseases in humans. It would be inappropriate to suggest that laboratory animals recapitulate the spectrum of symptoms representative of human neuropsychiatric conditions. Nevertheless, certain behavioral analyses have been shown to assess meaningful dimensions of psychopathology-like behavior. In this section, I focus on behavioral analyses employed in this thesis, including USVs in pups, the OFT, PPI for sensorimotor gating, and assessments of maternal behavior.

2.8.1 Ultrasonic vocalizations

Mice of all ages produce USVs, however the interpretation changes based on the frequency of the call and age of the animal [Portfors, 2007]. Unlike rats, adult mice do not produce USVs in aversive situations, however, when mouse pups are separated from their dams, they produce calls above 35 kHz [Portfors, 2007]. The function of these calls have been widely debated. They are most traditionally interpreted as a distress call for the dams to locate and retrieve pups, as mouse pups are born without fur and in danger of dying if they become too cold [Portfors, 2007]. In the early 21st century it was suggested that USVs may be incidental by-products of an abdominal compression reaction to stressful external stimuli, increasing cardiac blood flow [Blumberg and Sokoloff, 2001]. This position lead to a flurry of debate on whether USVs could be interpreted as a distress signal [Panksepp, 2003, Blumberg and Sokoloff, 2003]. While there is no conclusive empirical evidence ending this debate, USVs in rodent pups have still been used to assess distress [Scattoni et al., 2009] and social communication [Ferhat et al., 2016, Wöhr, 2014]. In mice, increased USVs have been associated with later anxiety-like phenotypes in the OFT, suggesting more calls may be associated with distress or anxiety-like behaviors in pups [Budylin et al., 2019]. Furthermore, benzodiazapines, a drug prescribed to treat anxiety disorders which work on the GABAergic system to induce calmness or drowsiness, have been shown to reduce USVs in mouse pups, providing further evidence of USVs as an anxiety-like phenotype [Takahashi et al., 2009].

In C57/BL6J mice, the in-bred strain used in these experiments, USVs tend to peak around PND 3, earlier than out-bred strains, which peak around 2-weeks of life [Scattoni et al., 2009]. Nevertheless, USVs have been demonstrated as late as PND 14 in C57/BL6J mice [Spencer et al., 2011]. As mouse pups are not ambulatory, tests such as the OFT are impossible for accurate behavioral assessments, making USVs an invaluable assessment for early-life behavior.

2.8.2 Open-field test

The OFT is routinely used in the laboratory to assess anxiety-like behaviors in mice [Carola et al., 2002]. Mice exhibit a natural aversion to open spaces where, in the wild, they can be vulnerable to predation [Carola et al., 2002]. A variety of metrics can be extracted from OFT. Commonly, the duration of time spent in the central area is calculated and used directly as a measure indicative of less anxiety-like behavior, or divided by the peripheral area to calculate a ratio [Brenes et al., 2009].

Typically, the total distance travelled is also assessed as a measure of general activity [Brenes et al., 2009, Lipkind et al., 2004]. Other metrics, such as time and frequency of exploratory rearing onto the hind legs or self-grooming can be recorded, however, these behaviors are much less frequently explored [Sturman et al., 2018]. They can be difficult to reliably track with automated software. Number of fecal boluses have also been tracked as a measure of anxiety in the OFT, however in one study examining TRR in the OFT, fecal bolus count did not display a high quality of reliability across trials [Shiguang et al., 2010]. In contrast both duration and percentage of time in central squares and rearing exhibited fair TRR, with an ICC between 0.5 and 0.6 [Shiguang et al., 2010]. This suggests the former metrics, rather than fecal boluses, are a better measure of anxiety-like behavior in the OFT.

Another common behavior assessed in the OFT and discussed in the context of prenatal cannabis exposure in Section 2.4.2 is locomotion. Locomotor activity is generally assumed to reveal "emotionality" in mice, however this interpretation has been heavily debated [Archer, 1973, Gray, 1979]. Decreased locomotion in a novel environment of the OFT is classically interpreted as anxiety, fearfulness, or timidity, while increased locomotion is interpreted as an exploratory drive [Archer, 1973]. Wild mice are naturally neophilic, allowing them to survive on a wide range of food sources and establishing that, after humans, they have the widest global distribution of any mammalian species [Singleton and Krebs, 2007]. Thus, "timidity" or reduced locomotion in mice is indicative of a maladaptive behavior in a novel territory. Recent studies compare overall distance travelled in the OFT as a method of assessing whether differences in locomotor activity might explain differences observed in time spent in the center of the arena [Eshaghi et al., 2019]. For example, if a mouse freezes when placed in the central zone of the OFT arena, it will have a high duration of time spent in the center zone, but low overall locomotion. Another mouse, however, may spend time openly exploring the central and outside zones, resulting in a fairly high amount of time spent in the central zone, but high overall locomotion. Mice that spend less time in the central zone of the OFT also show increased cortisol levels in serum, consistent with the interpretation of the behavior as reflecting a stress or anxiety-like response [Eshaghi et al., 2019]. Nevertheless, caution should be employed in interpreting behavioral test results, including OFT, to avoid anthropomorphizing the behavior of mice.

2.8.3 Prepulse inhibition

The PPI test provides an invaluable tool for rodent researchers for several reasons. First, unlike many other behavioral tests, it requires very minimal experimenter manipulation, other than placing the mouse in the apparatus. Additionally, processing the data requires no manual assessment. Furthermore, standardized hardware and software allow for identical procedures to be shared among laboratories [Instruments, 2018]. Although it is unclear whether the inter-rater reliability has been assessed and peer-reviewed in the test, because of the standardization of the procedure, it should be robust against certain experimenter factors that have been shown to impact other behaviors, such as the elevated plus maze [Bohlen et al., 2014].

Second, PPI assesses sensorimotor gating. Sensorimotor gating is the ability of a sensory stimulus to suppress a motor response. Specifically, an intense stimulus (pulse), such as an auditory stimulus in the form of a loud click, elicits a motor startle response in mice. A lower auditory stimulus (prepulse) presented before the pulse will typically attenuate the motor startle response [Powell et al., 2012]. Importantly, the PPI task can be conducted across species and even in humans where acoustic startles can be measured with eyeblinks [Swerdlow et al., 2016].

Third, disruptions to sensorimotor gating assessed with PPI have been proposed as an endophenotype of psychosis or schizophrenia in humans [Powell et al., 2012]. An endophenotype (a term from epidemiology) is a measurable behavioral biomarker that is associated with an illness due, in part, to underlying genetic causes [Turetsky et al., 2007, Glahn et al., 2016]. While deficits in sensorimotor gating were first tested in individuals suffering from schizophrenia, they have since been documented in other psychiatric disorders involving disruptions to sensory processing, such as schizotypal personality disorder, Obsessive Compulsive Disorder, Tourette's Syndrome, and Huntington's Disorder [Geyer, 2006]. Therefore, sensorimotor gating deficits cannot be extrapolated to a specific disorder in humans, and similar caution must be exhibited when interpreting laboratory results.

2.8.4 Maternal Behaviors

2.8.4.1 Nest building

Mice build nests in order to conserve heat and provide shelter. C57/BL6 mice are reliable nesters, and the quality of nests built by female mice is critical to success of their offspring early in life [Deacon, 2006]. Nest quality has been assessed

in other experimental manipulations including genetic backgrounds, infections, hormones, and brain lesions [Deacon, 2006], and the neuropsychology of nest building has been reviewed in maternal behavior [Numan and Sheehan, 1997, Gammie, 2005]. The quality of nests that mice build can be a sensitive marker to reflect their well-being [Jirkof, 2014]. Recent work has introduced thorough protocols to reliably assess the quality of nests built by dams using standardized nesting material [Deacon, 2006]. One major advantage of assessing nest building is that assessments can be minimally invasive and take place in the home cage [Jirkof, 2014]. While alterations in the quality of nests built could be impacted by many factors including stress, hormones, or the direct effect of THC exposure, it provides a minimally invasive method of assessing whether changes in offspring development are due to early life maternal distress during gestation or the THC directly.

2.8.4.2 Time on nest

Another factor that could impact development is maternal behavior during early life. The impact of prenatal cannabis use on maternal behavior is relatively under explored, however there is some evidence suggesting an impact of exposure to cannabinoids on nursing and pup retrieval [Navarro et al., 1994, Costa et al., 2014, Brancato and Cannizzaro, 2018]. Examining the potential impact of THC exposure on maternal care is important because poor maternal care can contribute to poor outcomes in developing pups, including anxiety-like behavior [Bailoo et al., 2014]. Many aspects of maternal behavior can be assessed, such as time spent nursing and the type of nursing (arched back vs passive nursing), licking, or grooming, and they have been explored extensively in other early life environmental exposures, such as maternal immune activation (MIA) [Potter et al., 2023]. In mice, however, the high walls of nests can make it challenging to capture video of maternal behavior noninvasively with cameras in the housing room aimed through the side of cages. Observation from such an angle may require reduction of nesting behavior, which is a known early life stressor [Walker et al., 2017]. Instead, in this thesis, we adapted previous protocols [Brummelte and Galea, 2010] to film dams from above in a behavioral room and record time spent on and off nest. While this is less sensitive and more invasive than protocols in rats that allow assessments of nursing and other behaviors, it allowed us to capture an aspect of maternal behavior regularly across multiple timepoints in both light and dark cycles to provide a gross measure of early life maternal care. It must be acknowledged that there are species specific variations that complicate

translating maternal behavior in mice to humans, such as the fact that mice, unlike other mammals, do not depend on gestational hormones to initiate maternal care when presented with neonates [Leckman and Herman, 2002], therefore indications of altered maternal care should not be directly extrapolated to humans, but rather studied in a species-specific manner.

2.9 Thesis outline

Chapter 1 provides a general introduction motivating the research question, outlining the objectives, and defining hypotheses. **Chapter 2** supplies relevant background information in interpretation of the research methods and findings. **Chapter 3** investigates the impact of prenatal THC exposure on late-gestation embryos. **Chapter 4** investigates the impact of the same exposure on neonatal pups over the first two-weeks of life. **Chapter 5** extends the research question from post-weaning to adulthood. **Chapter 6** provides a general discussion and **Chapter 7** concludes the thesis.

Chapter 3

Impact of chronic prenatal exposure to THC early in gestation on brain volume of mouse embryos

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3.1 Preface

The work presented in **Chapter 3** represents, to our knowledge, the first examination of the impact of prenatal THC exposure (PTE) on brain and body-wide volume of mouse pups exposed to Δ^9-tetrahydrocannabinol (THC) early in gestation. Evidence implicates prenatal cannabis exposure (PCE) in intrauterine growth restriction (IUGR) in both humans and nonhuman animal studies [El Marroun et al., 2009, Natale et al., 2020]. Structural magnetic resonance imaging (MRI)s of mouse embryos with PTE allows for assessments of not only total body volume, but also brain volume without limiting the regions of interest assessed. It has been used before to elucidate the early effects of prenatal exposures on brain development and identify regions of interest for future investigation [Guma et al., 2022]. This experiment also served to disentangle the direct effects of PTE on brain volume and the potentially mediating effects of IUGR. Further we provided evidence of the impact of chronic injections early in gestation on brain and body volume by comparing the saline (Sal) group with a noninjected control group. Finally, this chapter included exploratory analyses examining the effects of prenatal exposures on maternal and litter-level outcomes and the volume of organs between groups.

Our results revealed that chronic injections slightly reduced the volume of the total body of embryos, however THC further reduced embryo volume, consistent with previous findings of IUGR. While chronic injections barely impacted brain volume, THC, independent of the effects of body volume, induced a robust profile of brain volume alterations, including ventriculomegaly, regions of larger volume in regions across the brain, and regions of smaller volume in the brain stem and cerebellum. Our exploratory results showed volume differences in the liver and lungs, motivating future studies that investigate the brain as an organ embedded in other body systems. This chapter has been drafted for submission to a peer-reviewed journal.

3.2 Abstract

Despite warnings from government bodies and health professionals regarding the potential negative impact of cannabis exposure during gestation on child development, the prevalence of cannabis use during pregnancy is still increasing. A growing body of literature suggests negative neurodevelopmental outcomes for individuals exposed to cannabis before birth in both humans and non-human animal models, yet much is still unknown about the impact of THC, the main psycho-active component of

cannabis, on the developing brain. To address this gap, we investigated the impact of 5 mg/kg of THC injected in pregnant mice daily from gestational day (GD) 3-10 on the whole-body and voxelwise brain volume of GD 17 embryos with *ex-vivo* MRI. First, we found smaller body volume of THC-exposed pups. Second, we found bilateral ventriculomegaly, larger volume in white matter tracts like the corpus collosum, and smaller volume of the motor cortex, amygdala, and medulla in THC-exposed pups. Our findings suggest brain volume changes following prenatal THC exposure start early in development, affecting much of the brain. These changes could alter trajectories of neurodevelopment post-natally.

3.3 Introduction

As cannabis is legalized in countries across the world and the public views it as a safe drug, rates of cannabis use during pregnancy are increasing [Young-Wolff et al., 2022]. Importantly, the profile of cannabis as a readily-available substance is changing as well, with the concentration of THC, the main psychoactive component of cannabis increasing over the past decade from 10 to 14% [ElSohly et al., 2021], and preparations as high as 30% legally available for purchase [SQDC,]. These data highlight the urgency of understanding the consequences of PTE as more children are exposed to the drug before birth. As approximately 1/3 of consumed THC crosses the placenta to the fetal compartment [Huestis, 2005, Harkany et al., 2007], it is critical to understand the impact of this molecule specifically on the delicate processes of neurodevelopment.

Recently, studies have investigated the early-life effects of PCE, however few examine the brain at the level of the entire organ. Convergent evidence between human and rodent studies implicate PCE in contributing to IUGR [El Marroun et al., 2009, Natale et al., 2020]. In human studies it is very difficult to parse the impact of PCE and other confounders, including poly-drug use [Grewen et al., 2015], which laboratory studies overcome by causally modelling PTE in nonhuman animals. Vascular deficits at the level of the placenta have been identified in rat models of PTE, along with reductions in the ratio between the liver and bodyweight [Natale et al., 2020]. There is little evidence on the early life effects of PTE on the brain specifically. Notably, PTE has been shown to disrupt endocannabinoid signalling, rewiring fetal cortical circuitry such that in male embryos the diameter of fascicles comprising axons committed to the corticofugal tract was increased [Tortoriello et al., 2014]. This result suggests that the brain may be impacted at the level of networks, starting in early life.

Because of the wide range of mechanisms of actions and the ubiquity of the endocannabinoid system (ECS) across brain regions and developmental processes, a brain- and body-wide study in early life may provide insight into the most sensitive regions and networks impacted by PTE. Endocannabinoids contribute to a variety of processes including cell proliferation, migration, differentiation, and survival [Harkany et al., 2007]. Changes related to these processes can be identified as volume differences between groups with whole-brain neuroimaging [Zatorre et al., 2012].

Here we employ high-resolution MRI in extracted GD 17 mouse embryos to investigate the impact of early-gestation THC-exposure on local estimates of volume across the entire brain, as well as total-body volume as a proxy for intrauterine growth restriction, and exploratory organ-volume analyses. MRI provides not only a whole-brain field-of-view but also a technique with translatable potential into both later life stages as well as to humans. Together these results provide further evidence for the impact of PTE on body volume, but also a whole-brain characterization of the early effects of PTE, highlighting regions of interest for future work.

3.4 Materials and Methods

3.4.1 Animals and timed mating

All animals were housed at the Douglas Mental Health University Institute Animal Facility, and all experiments were in compliance with Facility Animal Care Committee regulations. Female and male C57BL/6J mice of breeding age (8 to 12 weeks old) were subject to timed mating breeding procedures, as described in Supplementary Methods (SM) 3.7.1.1. All females were nulliparous. The day of an observed plug was considered GD 0.

3.4.2 Drug preparation and injection

The timeline of the experiment and workflow of samples is depicted in Fig 3.1.

Pregnant dams were randomly assigned to one of three groups: 1) a Null group where dams were restrained but not injected, 2) a vehicle group, heretofore called Sal, 3) the THC experimental group. The Null group was included to control for the potential effects of repeated injections. Intraperitoneal injections are not recommended for repeated administration in pregnant dams [DiNieri and Hurd, 2012], therefore subcutaneous injections were performed daily from GD 3-10 between 9:00 and 11:00.

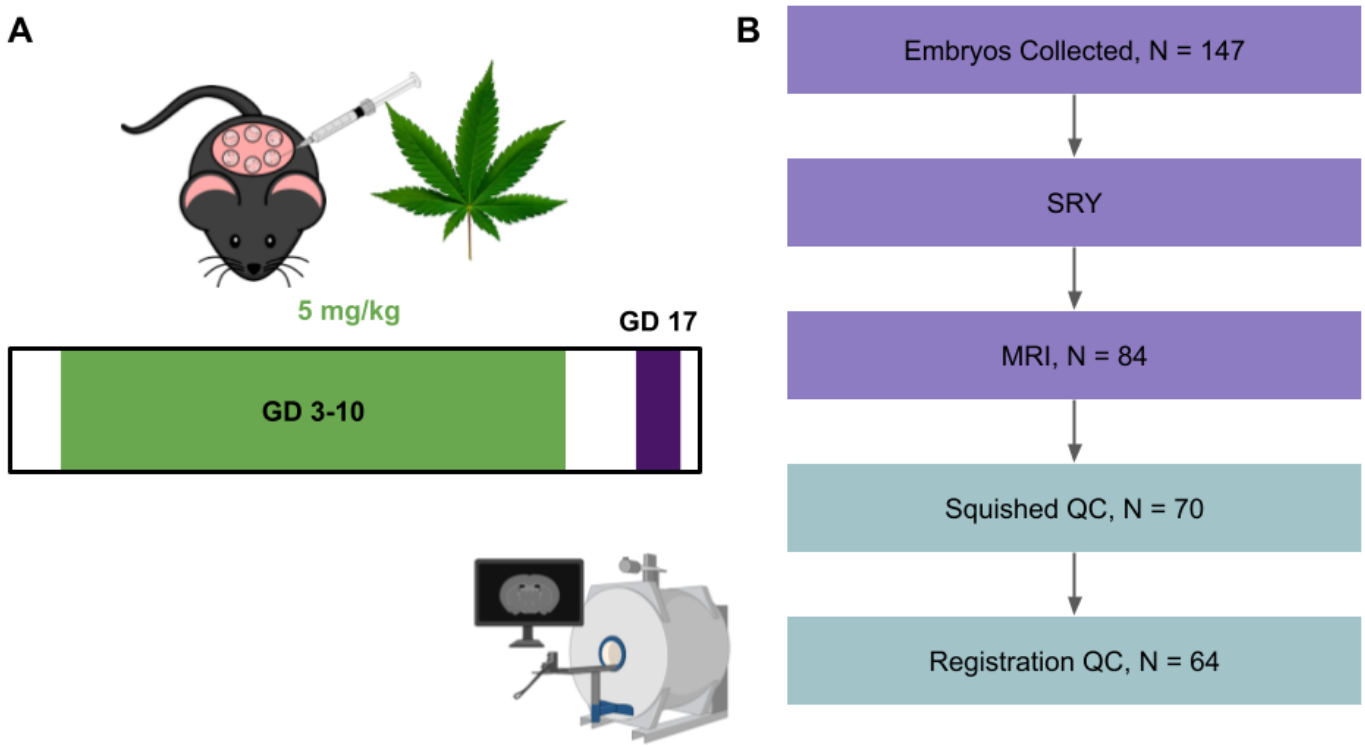


Figure 3.1: A) Timeline indicating days of chronic THC injections and day of extraction with MRI hardware (Biorender). B) Flow chart of processing steps with boxes indicating processing steps (purple = physical sample, teal = quality control). Sample size indicates count after that step. Gestational Day (GD); Quality control (QC).

In the Null group, the dam was restrained in a scruff hold and the injection area was touched with a finger tip.

A research license and import permit for THC were obtained from Health Canada, and 2g of THC were purchased from Cayman Chemical Company in a formulation of 10 mg/ml in ethanol. Ethanol was dehydrated, and THC was prepared in a solution of 1:18 cremophor:saline 3.7.1.2 as described previously [Guma et al., 2023]. The vehicle solution for the Sal group comprised a preparation of 1:18 cremophor:saline to serve as a control for the THC condition.

The dosage of THC injected was 5 mg/kg. This dosage has been characterized in both prenatal THC models [Rubio et al., 1995, Navarro et al., 1994, Lallai et al., 2022, Drazanova et al., 2019], as well as adolescent exposure models [Trezza et al., 2008,

Guma et al., 2023]. While it has been suggested that this dosage represents moderate human gestational exposure [Lallai et al., 2022], it can be challenging to accurately assess and model human cannabis use during pregnancy, as discussed further in Section 3.6.4. The dosage used here roughly corresponds to a dosage of 27.6 mg/kg in humans, which is likely a moderate-high dosage, as discussed in SM 3.7.1.2. The injection volume for both THC and Sal dams was 0.01 ml/g. Dams were weighed daily at the time of each injection and their weight recorded for later analysis, Section 3.7.2.1, SFig. 3.6.

3.4.3 Embryo extraction and sample preparation

Embryo extraction was conducted following previously published methods [Guma et al., 2022]. On GD 17, pregnant dams were euthanized by live cervical dislocation. The uterus was immediately dissected and placed into a petri dish of chilled Phosphate Buffered Saline (PBS). In turn, each embryo was removed to a dish of warm PBS to stimulate blood flow, uterine tissue and the yolk sac were carefully removed, and a small piece of the yolk sac was collected for genotyping the SRY gene to determine embryo sex (Transnetyx, Memphis, Tennessee, USA). The umbilical cord was cut to encourage blood drainage and mice were placed in a vial with warm PBS. The vial was placed alternately between a shaker to help exsanguination and an incubator to maintain PBS temperature until blood had cleared. After approximately 10 minutes, each embryo was checked and either returned to warm PBS for further drainage or transferred to a vial containing 4% paraformaldehyde (PFA) as a fixative and 2% Gadoteridol (Gado) (prohance; MRI contrast agent; Bracco Imaging S.p.A) for 1 week at 4 °C. After 1 week, embryos were transferred to a long-term storage solution of 2% Gado and 0.02% sodium azide 1x PBS solution at 4 °C until scanning. Gado was used as a contrast agent for *ex vivo* MRI, as it is absorbed into tissue and reduces the T1 and T2 relaxation times of the cortex, striatum, thalamus, hippocampus, and cerebellum [Huang et al., 2009], enhancing the contrast, especially with long scan times [Nieman et al., 2005]. Up to 2 male and 2 female pups from each litter were sent to Toronto (details for scanning in Methods 3.4.4.1 and SM 3.7.1.3) to minimize potential litter effects [Valiquette et al., 2023].

3.4.4 Image acquisition and processing

3.4.4.1 Magnetic resonance imaging

The samples were shipped to the Mouse Imaging Centre at the Hospital for Sick Children in Toronto, Canada in Styrofoam boxes packed with ice and ice packs. In advance of imaging, each sample was removed from the vial with contrast solution, blotted dry, and placed in 13-mm diameter plastic tubes filled with a proton-free susceptibility-matching fluid (Fluorinert FC-77, 3M Corp. St. Paul, MN), described previously in [Guma et al., 2022]. Images of the whole embryo body were acquired on a multichannel 7Tesla (T) MRI scanner with a 40-cm-diameter bore (Varian Inc.). A custom-built 16-coil solenoid array was used to acquire images of up to 16 samples simultaneously, facilitating long scan times (14 hours) overnight with an ultrahigh resolution voxelsize of $40\mu m^3$ isotropic [Spencer Noakes et al., 2017, Guma et al., 2022]. Further detail is provided in SM 3.7.1.3.

After image reconstruction, they were preprocessed to enable statistical analysis, inspired by previous work from our group [Guma et al., 2022, Devenyi, 2023]. First, a mask was created and corrected manually by an expert rater (L.C.) to exclude peripheral tissue and bubbles that were present in some images to enable extraction of the full embryo image. Each image was quality-controlled by two independent raters (L.C. and A.P.) to assess whether the sample was misoriented in comparison with a reference image and whether the sample had been distorted (e.g., compressed and therefore no longer morphologically or anatomically accurate) or damaged in transportation. Fourth, an in-house preprocessing pipeline was used to correct for B1 bias-field inhomogeneities and denoise the images. Further information about each step is included in SM 3.7.1.4. The volume of each corrected whole-body mask was examined with an in-house script (https://github.com/CoBrALab/minc-toolkit-extras/blob/master/collect_volumes.sh, Advanced Normalization Tools (ANTs): itk_label_stats), to collect pup total body volume.

3.4.4.2 Image processing

Following pre-processing, images were registered through an unbiased deformation based morphometry (DBM) pipeline built in-house with a Python wrapper around the antsMultivariateTemplateConstruction2.sh from the ANTs environment [Avants et al., 2011]. In brief, an unbiased modelbuild is performed on all images to create a study-specific average and ensure voxelwise correspondence across all images.

In the first stage, affine transformations are used to create a group average of all input images accounting for bulk changes in size and shape. In the second stage, nonlinear transformations are used to iteratively estimate the residual differences in contrast profiles among images and transform images to minimize these differences. Together, these affine and nonlinear transformations produce a minimal deformation field which maps each individual subject image to the average at the voxelwise level. The Jacobian determinant of the warp field can then be calculated to provide voxel-wise estimates volumetric difference relative to the average representation, with a value from from 0 to 1 indicating a volumetric compression and a value >1 indicating a higher volume relative to the anatomy of group [Chung et al., 2001, Friedel et al., 2014, Leow et al., 2007]. Statistical comparisons can be performed on a voxel-wise logarithmic transformation on the derived Jacobians as this has shown to better conform to assumptions required for the use of parametric statistical analyses. The relative Jacobians explicitly model only the volumetric differences modelled by the nonlinear part of the deformations and explicitly remove the contribution of the global linear differences (attributable to differences in total brain/body size) [Guma et al., 2022, Chung et al., 2001]. The relative Jacobians were smoothed with a Gaussian kernel at 80-μm full-width-at-half-maximum to further better conform to Gaussian assumptions for downstream statistical testing [Guma et al., 2022]. Following a first pass of the entire DBM pipeline, the overall registrations were quality control (QC)'d, failures were documented and the images were further manually registered before a second complete pass of the DBM. Precise details can be found in SM 3.7.1.5. While this DBM was used for organ results, the head was cropped in all images and head-only images were used for another DBM, thereby optimizing registration for the brain. Further detail on the DBM and QC can be found in SM 3.7.1.5. Brain regions impacted by PCE were identified by comparing to an atlas [Schambra, 2008].

3.4.5 Statistical analyses

3.4.5.1 Magnetic resonance imaging

All statistical tests were performed in the R programming language, version 3.5.1.

First we examined the impact of the interaction between condition and sex on total body volume. Including multiple pups from a single litter violates assumptions of statistical independence among individuals, and litter is considered a nesting variable [Harrison et al., 2018, Valiquette et al., 2023, Guma et al., 2021a]. Therefore, linear mixed effects models (LMER)s were employed to include a random effect of

litter, as well as coil position, since multiple samples were scanned on the same coil [Harrison et al., 2018] (lme4::lmer and lmerTest::summary). Instead of using coil number (1-16), coils were categorized by their position as either "corner" (4 coils), "edge" (8 coils), or center (4 coils). The fixed effects of the model included an interaction between condition and sex.

To examine the impact of condition and sex on local volume differences, we employed identical LMERs on the logged relative Jacobians from the DBM, resulting in a single model per voxel. Because IUGR has been shown to impact brain volume [Businelli et al., 2015], we sought to control for the effects in our model. As previous literature and evidence from this study suggest THC exposure impacts body volume [El Marroun et al., 2009, Natale et al., 2020], simply including volume in the model as a covariate could introduce co-linearity between condition and volume. Therefore, we employed a regression-with-residuals method to assess the mediating effect of body volume on the relationship between condition and brain volume [Zhou and Wodtke, 2019]. The residuals from the total body volume model were extracted and included as a covariate in the brain volume model. Thus, our fixed effects included the interaction between treatment and sex, and the body-volume residuals, and the random effects were litter size and coil category. To correct for multiple comparisons we used the False Discovery Rate (FDR), where a threshold from 5%-1% indicates up to 5% of significant voxels could be false positives.

As supplementary analyses to the main results, we examined the impact of treatment on total brain volume (TBV). Using the brain mask we calculated volume under the mask for each individual in R (RMINC::anatGetAll). We compared the conditions with an LMER, examining the impact of sex and treatment and including litter as a random effect.

3.4.5.2 Pregnancy outcomes following PTE

The impact of PTE on pregnancy outcomes was assessed in terms of dam weight gain, litter size, and sex ratio of the pups. First, we examined the impact of treatment on weight gain in the dams with an LMER, including a fixed effect of the interaction between treatment and GD, litter-size as a covariate, and dam ID as a random effect. The impact of treatment on both litter size and sex ratio (proportion of litter female) were assessed with linear models.

3.5 Results

3.5.1 Pregnancy outcomes

While all dams gained weight over time, Sal dams gained less weight than Null dams and THC dams gained less weight than Sal dams, with full results in Supplementary Results (SR) 3.7.2.1. There was no impact of condition on litter size or sex ratio.

3.5.2 Prenatal THC exposure impacts embryo volume

In total, 84 embryos (14 per sex per condition were scanned), however 14 were excluded for quality control, SR 3.7.2.3. Therefore, the body volume analysis included 70 embryos from the three groups (Null, N = 27 [14 female], Sal, N = 24 [10 female], THC, N = 19 [10 female]). The pups came from 6 Null dams, 7 Sal dams, and 6 THC dams.

Examining the impact of the interaction between treatment and sex on total body volume revealed a main effect of treatment, for both the Sal group compared to Null ($p = 0.009$) and Sal compared to THC ($p < 0.001$), with the Sal injection reducing body volume, but the THC injection further reducing it Fig 3.2. Surprisingly, neither the main effect of sex, nor the interaction with treatment were significant, full results in SR Tab. 3.4. There was an average percent change of 10% between Sal and Null, and -5% between Sal and THC.

Despite an impact of treatment on embryo volume, there was no impact (Null of THC) on TBV, Section SR 3.7.2.5.

3.5.3 Prenatal THC impacts local brain volume

Following registration QC, 5 additional images were excluded for a final sample size of 65: Null, N = 25 [14 female], Sal, N = 21 [8 female], THC, N = 19 [10 female]). Results of the initial LMER on the relative Jacobian determinants among the three groups revealed no interaction between treatment and sex, nor main effect of sex, but a main effect of treatment for the THC group, significant from 5%-1% FDR.

Compared to saline controls, THC exposure was associated most prominently with larger volume in the bilateral lateral ventricles (5% FDR), as well as white matter regions, like the corpus callosum, seen in Fig. 3.3.

There were several focal anterior, medial regions (near the preoptic area) that showed a smaller volume in the Null group compared to the Sal controls, seen in SFig.

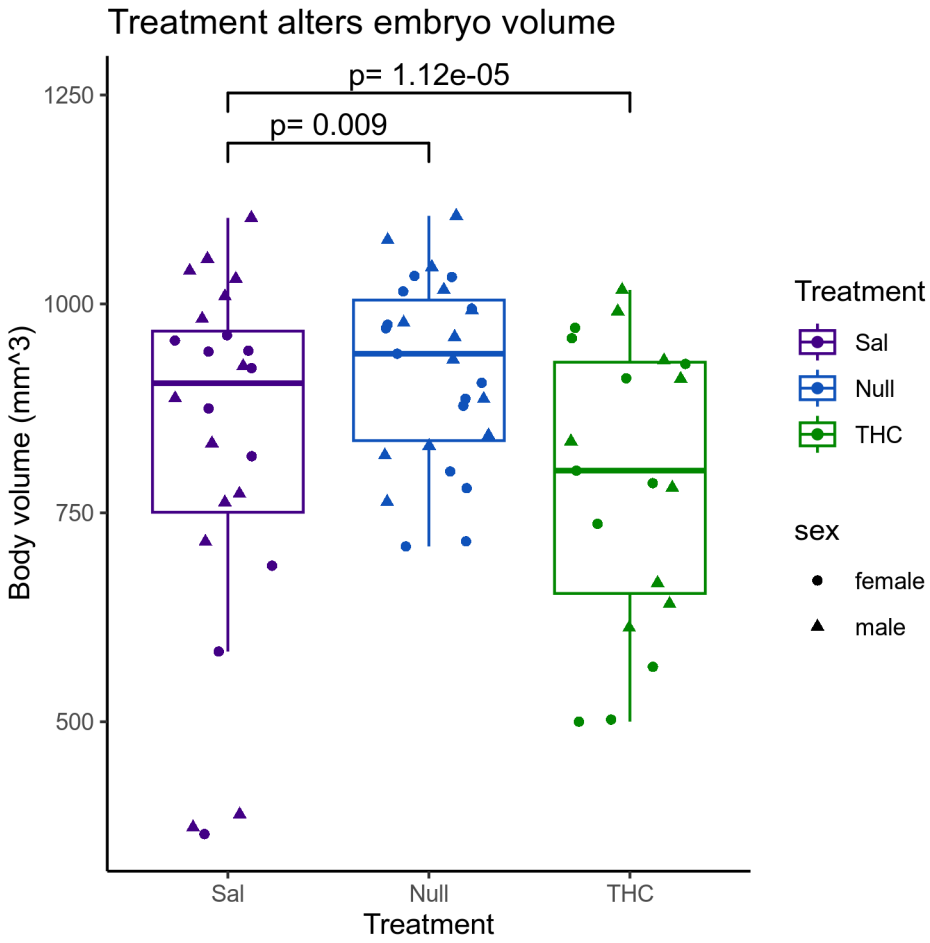


Figure 3.2: Both prenatal Sal and THC injections reduce the body volume of embryos at GD 17. A formal test for outliers was conducted with Rosner's test, however no data points were found to be significant outliers.

3.12.

There was also a main effect of the residuals of body volume from 5%-1%, Fig 3.4. Larger body volume was associated with smaller volume in bilateral regions throughout the brain, including the lateral ventricles, the medial preoptic area, the midbrain, and the visual cortices, and larger volume in the lateral hypothalami, amygdalae, and right hippocampus, seen in Fig. 3.4.

Similarly, there were focal regions of change in the organs for the THC group, as well as a sex-treatment interaction, but none comparing the Null to Sal group SR 3.7.2.6.

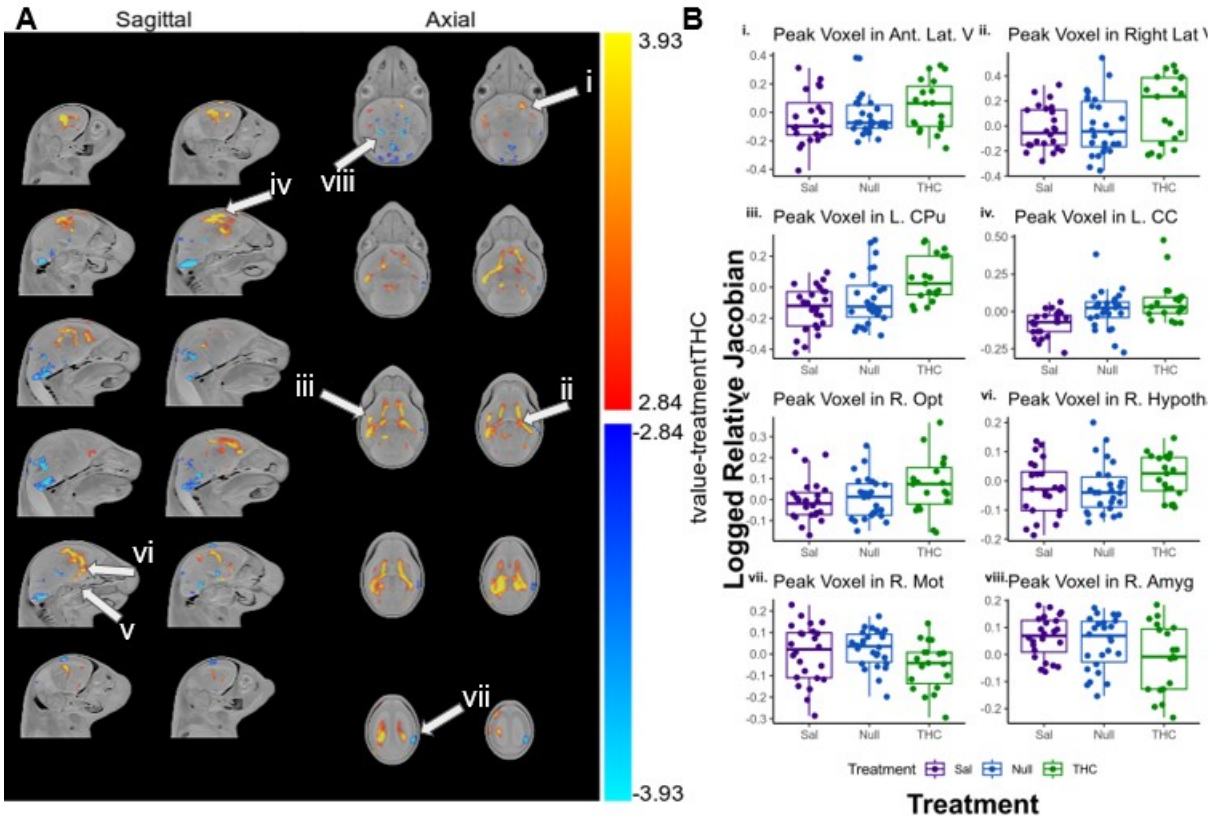


Figure 3.3: A) Heatmap of main effect of THC on embryo regional brain volume thresholded with FDR from 5%-1%. B) Box plots of peak voxels in i) right anterior lateral ventricle, ii) right lateral ventricle, iii) left caudate putamen, iv) left corpus collosum v) right optic tract, vi) right hypothalamic nuclei, vii) right motor cortex, and viii) right amygdala.

3.6 Discussion

There is significant need for studies investigating the impact of PCE on the brain. Studies have demonstrated PCE is associated with poor neuropsychiatric outcomes in humans [Rompala et al., 2021, Paul et al., 2021] and behavioral disruptions preclinically [Sarikahya et al., 2023, Rubio et al., 1995, Moreno et al., 2005]. Additionally, specific brain region of interest (ROI)s have been examined to investigate the mechanisms that underly the association between PCE and behavioral changes [Sarikahya et al., 2023, Sagheddu et al., 2021]. In humans, several studies have employed MRI to examine the impact of PCE with a whole-brain approach

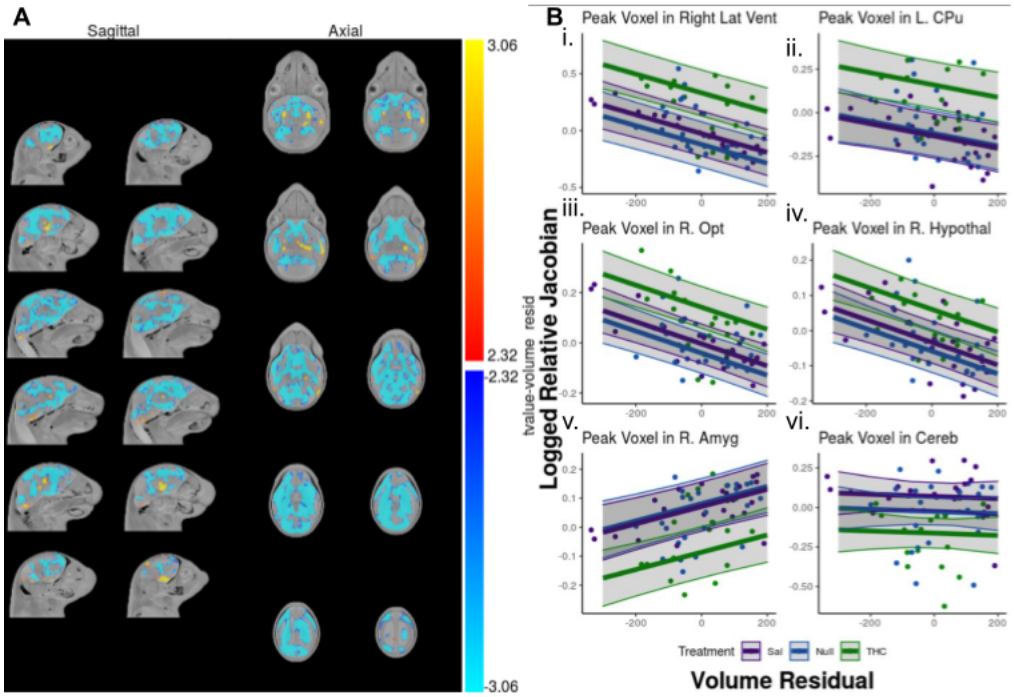


Figure 3.4: a) Heatmap displaying impact of whole body volume residuals, thresholded from 5-1%. Regions in blue indicate larger body volume is associated with smaller volume, and regions in warm colors indicate that larger volume is associated with larger volume. B) Peak voxels in the i) right lateral ventricle, ii) left caudate putamen, iii) right optic area, iv) right hypothalamic nuclei, v) right amygdala, and vi) cerebellum

[El Marroun et al., 2016, Rivkin et al., 2008], however these studies suffer from confounding factors that are impossible to eliminate in human studies and imprecise assessments of the timing and dosage of PCE. Preclinically, few studies have attempted to assess the impact of PCE on the whole brain. Leveraging MRI, we examined the impact of early gestational PCE on whole brain and body volume. Our findings suggest a phenotype of PCE with important implications for translational and clinical work.

3.6.1 IUGR following THC and Sal injections

First, our results suggest that PCE reduces overall volume of pups, consistent with prior literature from both humans and nonhuman animals. In the Generation R study, maternal cannabis use both early and throughout pregnancy was associated with reductions in fetal growth (measured with ultrasound) late in pregnancy, and

reductions in birth weight [El Marroun et al., 2009]. One study in mice exposed dams to vaporized cannabis (GD 5.5-17.5) and assessed intrauterine growth with ultrasound at GD 10.5 and 16.5, as well as the wet-weight of extracted fetuses at GD 18.5 [Benevenuto et al., 2017]. Interestingly, they found no evidence of growth restriction at GD 10.5 and 16.5, but did find reduced weight at GD 18.5, with weights differing almost 10% [Benevenuto et al., 2017]. Both of these findings are consistent with our results, despite differences in methodologies. Several direct causes and underlying mechanisms could be responsible for the observed growth restriction. Alterations in maternal weight gain or feeding could contribute to metabolic changes in the pups. We did not observe significant reductions in maternal weight gain early in pregnancy, although there is a non-significant reduction in the slope of increase in the THC group compared to the Sal group. The study by Benevenuto et al reports reduced maternal weight gain by the end of pregnancy [Benevenuto et al., 2017]. In humans, reduced maternal weight is associated with reduced offspring birthweight [Kirchengast and Hartmann, 1998]. The present study was not designed to examine maternal weight gain, and maternal weight was only tracked during the period of injections. A study investigating whether maternal THC exposure is associated with decreased weight gain, which could mediate the relationship between treatment and IUGR, should track weight throughout pregnancy. Along with maternal weight, the placenta may be altered following PCE, which could, in turn, impact fetal growth. For example, there is some evidence that the ECS may regulate nitric oxide production, an important component in facilitating fetal-placental circulation [Abán et al., 2018]. Prior results suggesting deficits in placental vasculature following PCE provide further evidence for the role of the placenta and circulation in growth restriction observed after prenatal THC exposure [Natale et al., 2020]. Nevertheless, THC could also act directly on the fetus to contribute to observed growth restriction. High levels of leptin, an adipocyte derived hormone, in newborns have been associated with IUGR [Gurugubelli Krishna and Vishnu Bhat, 2018], and previous evidence suggests chronic THC exposure in adolescent female rats has been associated with alterations in leptin levels, however the reports indicate decreased circulating leptin [Llorente-Berzal et al., 2011], and to our knowledge no study has specifically examined the impact of THC exposure in pregnancy on leptin levels of the offspring. Because IUGR is one of the most robust findings in the PCE literature, across methods and species, further investigation into the mechanism underlying this association could provide valuable information to researchers and clinicians.

One of the strengths of the present study is the inclusion of a non-injected control group, allowing us to compare the impact of chronic Sal injections. Interestingly, we

did find evidence of reduced embryo volume in the Sal-injected controls, although the THC-injected group showed further reduced volumes than the Sal-injected group. Maternal stress during gestation has been so robustly associated with IUGR that it is used as a model in the laboratory to induce fetal growth restriction [Vuguin, 2007]. The evidence presented here suggests that daily subcutaneous injections from GD 3-10 does constitute a meaningful stressor to the dam. When comparing the THC group to an injected Sal group, the injection is consistent across groups, nevertheless, the THC group could constitute a "double-hit", with one risk factor being the drug and another being the maternal stress.

3.6.2 Local brain volume

3.6.2.1 Impact of PCE

Despite the impact of both Sal and THC on the embryo volume, there was no impact of either on TBV of the embryos. This could potentially reflect the so-called "brain-sparing" effect, where, in IUGR, oxygen and nutrient supply is preferentially diverted to the brain [Cohen et al., 2015]. These results are consistent with studies in humans that find no differences in TBV following PCE [El Marroun et al., 2016]. Examining local volume changes with DBM, however, reveals striking alterations. In this analysis, we used a mediation with residuals approach to examine the effect while controlling for IUGR as a potential mediator. This allowed us to identify the effects of IUGR as well as the effects of condition, independent of IUGR. PCE was associated with larger bilateral lateral ventricles compared to the Sal controls. The hotspot includes both the anterior and posterior horn of the lateral ventricles, as visible in the axial view. In humans, ventricle size has been examined following PCE, but was not found to be altered [El Marroun et al., 2016]. One study in rats using arterial spin labelling to assess cerebral blood flow following PCE also reported visual enlargement of the ventricles in THC exposed adult offspring, however there were no significant differences between groups [Drazanova et al., 2019]. This imaging technique is not optimal for assessing volumetric changes, however, which could alter the authors' ability to detect a real effect.

Ventriculomegaly, or ventricle enlargement, is associated with neurodevelopmental disorders in humans [Lyall et al., 2012]. The ventricles of the brain are still very poorly understood, despite integral importance in development. Of note, cortical neurons do not develop in gray matter, but rather proliferate and migrate from zones adjacent to the lateral ventricle [Duy et al., 2022, Tabata and Nakajima, 2008]. Ventriculomegaly

or hydrocephaly in development is often associated with increased intracranial pressure associated with an imbalance of cerebral spinal fluid (CSF) production and reabsorption, and the symptoms can often be mitigated by neurosurgically diverting CSF, however other times there is no increase in intracranial pressure, and successfully diverting CSF does not ameliorate either ventricular volume or poor neuropsychiatric outcome in human patients [Duy et al., 2022]. In porcine models, enlarged ventricles were associated with reductions of the subventricular zone area, as well as increased cell death and neuroinflammation, and reductions in oligodendrocytes [Garcia-Bonilla et al., 2022]. Because we did not measure intracranial pressure, it is impossible to know whether an imbalance of CSF production:reabsorption underlies the increased ventricular volume. Importantly, no hydrocephalus was noticed during image QC, suggesting the ventriculomegaly was noticeable only through statistical analysis, and is unlikely to be an incidental finding in the PCE literature unless specifically tested for.

In addition to ventricular enlargement, there were larger volumes in the bilateral optic tract and hypothalamus, the right nucleus accumbens, left caudate putamen, corpus callosum, and motor cortex, and smaller volumes in the bilateral amygdala, posterior thalamic nuclei, medulla, bilateral cerebellar peduncle and cerebellum, and the right motor cortex. Many of these regions are notably rich in Cannabinoid- (CB)1 receptors [Kendall and Yudowski, 2016], including the cerebellum, basal ganglia and related nuclei (subthalamic nuclei, nucleus accumbens, caudate-putamen) [Lanciego et al., 2012]. While structural MRI cannot provide specific insight into the underlying cause of volumetric changes, studies demonstrate excess 2-arachadonoyl glycerol (2-AG) enlarges the corpus callosum due to misguidance of corticofugal axons, with increased fasciculation observed in the striatum as well [Alpár et al., 2014]. As corticofugal neurons project to thalamic, extrathalamic, and brainstem regions, it is possible that smaller volume in these regions is partially explained by neurons failing to reach their intended targets [Prasad et al., 2020]. In the cerebellum, neurons arise from the ventricular zone of the dorsal part of the anterior hindbrain, and neurons project through the cerebellar peduncle to the thalamus [Hashimoto and Hibi, 2012, Kim et al., 2021].

In contrast to the comparison between THC and Sal, the Null and Sal comparison revealed only focal regions with altered brain volume in the Null group. This indicates the injection alone is insufficient to produce a large effect in the brain.

3.6.2.2 Brain volume changes in the context of ECS and development

Early life brain volume changes are unsurprising following PTE, as neurodevelopment is a tightly orchestrated process, in which the ECS plays a major regulatory role. Exposure to external cannabinoids may impact many aspects of brain development. In humans, during the first trimester of pregnancy, central nervous system (CNS) development comprises neurulation, neurogenesis, the migration of immune cells and expansion of progenitor cells, and neuronal migration [Guma et al., 2019, Ouyang et al., 2019]. In human and animal models, there is evidence of ECS involvement at the earliest stages of conception [Harkany et al., 2007, Galve-Roperh et al., 2009]. Concentrations of the two major endocannabinoids, N -arachidonoyl-ethanolamine [Anandamide] (AEA) and 2-AG are tightly regulated over the course of gestation [Harkany et al., 2007]. For example, intrauterine AEA levels regulate the receptivity of the uterine lining for embryo implantation [Harkany et al., 2007]. The ECS continues its critical role in development through regulation of the CNS. It contributes to determining the fate of progenitor cells by regulating their proliferation, migration, and differentiation, as well as guiding axons and establishing synaptic connections [Harkany et al., 2007]. CB_1 receptors are a major component of the ECS, and their activation is associated with progenitor cell proliferation [Harkany et al., 2007]. The ECS is also important in determining cell-fate, with evidence of CB_1 receptor activation associated with a commitment to gliogenesis [Harkany et al., 2007, Aguado et al., 2006]. Once neurons are born, the ECS is thought to be involved in both the radial migration of pyramidal cells to appropriate cortical layers and also radial migration of neural precursors to locations in the neocortex and hippocampus, where they differentiate into γ-aminobutyric acid (GABA)-ergic interneurons [Zhou et al., 2014].

Volume differences in the embryonic brain at GD 17.5 could be due to several processes. In grey matter, especially in paraventricular zones, larger volumes could be related to increased proliferation of neural progenitors, increased synaptogenesis, or decreased apoptosis, as all of these processes occur by GD 17.5 in mice [Zeiss, 2021]. In white matter, such as the corpus callosum, larger volume could be related to increased axons committed to the pathway, and is highly unlikely to be related to oligodendrocytes or myelination as this occurs postnatally in the mouse [Zeiss, 2021, Alpár et al., 2014]. The ECS in late embryonic periods is involved in cell migration, morphogenesis, and axon migration, so it is possible that regions with smaller volumes (such as the cerebellum and brain stem) reflect either fewer cells (with cells failing to migrate), smaller cell morphology, or fewer synapses (with axons failing to properly synapse

in these locations) [Harkany et al., 2007]. Postmortem assays would be necessary to establish which of these underlying mechanisms contributes most heavily to regions with larger or smaller volumes.

3.6.2.3 Impact of IUGR

The effects of body volume on brain volume confirmed several previously reported associations with IUGR. There is a clear association between increased body volume and smaller volume in the lateral ventricle, in line with reports of ventriculomegaly following IUGR [Mallard et al., 1999]. Increased body volume was also associated with larger hippocampal volume, also consistent with previous reports of IUGR. Furthermore, larger body volume was associated with increased volume in the bilateral amygdaloid nuclei, also consistent with reports of IUGR and decreased amygdala volume in humans [Padilla et al., 2014]. The hypothalamus (including the medial preoptic area) has also previously been implicated with alterations in long-term changes in cellular metabolism [Pedroso et al., 2019]. While the goal of the present study was not to model IUGR, the results provide a potential validation for the methods by replicating previous studies and highlight the importance of considering the potential of PCE in inducing IUGR.

3.6.3 Impact of PCE on organ volume

Prior evidence suggests there is an interaction between PCE and offspring sex, however many of these effects are documented during adolescence or adulthood [Rubio et al., 1995, Navarro et al., 1994, Moreno et al., 2005, Lallai et al., 2022, Rompala et al., 2021], and it is still an open question whether these effects emerge *in utero*. One early study suggests sex-specific effects of PCE exist prior to birth, with male, but not female offspring showing increased dopamine content in the prosencephalic area at GD 18, however the authors did not explicitly test for an interaction between sex and treatment [Rodríguez de Fonseca et al., 1992]. Here, we found no evidence of sex effects (either main effects or interactions) in embryo volume, TBV, or regional brain volume, however we did see a sex-by-treatment interaction in regional organ volume. Most strikingly we observed smaller volume in the left lobe of the liver of male THC pups compared to female Sal pups. The liver is of particular interest because THC undergoes first-pass metabolism, in which the liver plays a critical role. While our exploratory analysis was conducted agnostic to region of interest, our findings complement a recent study that found decreased liver-to-body weight ratio in PCE rat offspring

[Oke et al., 2021]. Interestingly, this paper also found sex effects, with increased circulating triglyceride and cholesterol levels in adult male THC offspring [Oke et al., 2021]. These results are consistent with impairments in the hepatic lipogenic pathways that can contribute to the development of obesity or metabolic syndromes later in life [Oke et al., 2021]. Moreover, IUGR, as seen following PCE has been associated with dyslipidemia and future obesity [Gantenbein and Kanaka-Gantenbein, 2022]. Future research could investigate possible pathways associating the metabolic changes and the brain effects associated with PCE.

3.6.4 Limitations

Several limitations exist to the present study. First, in every laboratory study of cannabis exposure, a decision on route of administration must be made. Here we prioritized control of dose with injections, as opposed to a vaporization chamber, which has more external validity. Several studies have examined the impact of route of administration on THC-related outcome [Baglot et al., 2022, Wiley et al., 2021, Moore et al., 2021], however to our knowledge no study has compared whether the reliability of dosage among routes of administration. This could be an important question for the scientific community to answer, since passive vaporization exposure may result in variable drug inhalation based on factors such as animal breathing rates and depths. Second, while the dosage we used is common in laboratory rodents [Rubio et al., 1995, Navarro et al., 1994, Lallai et al., 2022, Drazanova et al., 2019, Trezza et al., 2008, Guma et al., 2023], it is high for a casual user of cannabis, as discussed in SM 3.7.1.2. The results from this study may not generalize to a lower dosage. Third, in this study we used pure THC, rather than a cannabis preparation that includes cannabidiol (CBD) or terpenes. While a preparation that includes other components of cannabis may have greater external validity, exploring their effects was outside the goals of the experiment, where we sought to characterize the impact of THC specifically. Further, cannabis preparations that contain high amounts of THC and almost no CBD are becoming increasingly common and widely available, although pregnant people aiming to use cannabis for medicinal purposes may avoid them. Further descriptive studies are needed to better understand the cannabis dosages and preparations used by pregnant people. Finally, the timing of THC exposure was chosen to mimic cannabis use during the equivalent of the first trimester of pregnancy when people may not know they are pregnant or may use to counteract the symptoms of morning sickness. However, it is unlikely that someone

would suddenly start using cannabis during the first trimester and is more common for use to start pre-conception. It is logistically complicated to start THC exposure pre-conception in timed mating procedures; for example, if a dam does not become pregnant she would be ineligible for a second attempt at breeding due to the variable timing of THC exposure. Additionally, high levels of endocannabinoids may disrupt embryo implantation, which could complicate generating successful pregnancies in experiments [Harkany et al., 2007]. These questions could be investigated in future studies.

3.6.5 Conclusions

This study offers several results of high interest for the scientific community. To our knowledge, it is the first to investigate the impact of prenatal THC exposure on mouse embryos with full-body MRI. First, we recapitulate findings of IUGR following PCE. Second, we find evidence of ventriculomegaly which could contribute to later neurodevelopmental abnormalities. Third, our exploratory analyses revealed alterations in organ volume outside of the brain, including in the liver and lungs. MRI could provide a powerful way to further examine the impact of PCE on the body systems. The fact that sex differences were present only in the organ analyses suggests that the sex differences documented in adolescence and adulthood likely emerge later, or as a result to changes in other systems. Together we characterize the phenotype associated with early PCE in an embryonic mouse model. Future research could extend similar methods into neurodevelopment through *in vivo* imaging and behavioral analyses.

3.7 Supplementary Information

3.7.1 Supplementary Methods and Materials

3.7.1.1 Animals

All C57BL/6J mice were housed in the animal facility in conventional ventilated cages with a 12-hour light cycle (8:00-20:00) with *ad libitum* access to food (standard rodent chow) and water. Female and male mice of breeding age (8-12 weeks) underwent timed mating. One male and one female were placed into a new cage in the late afternoon (15:00-17:00). The following morning (8:00-9:00), the male mouse was removed and the female was checked for the presence of a seminal plug and weighed on a scale with a readout to the tenths of grams. Mice were paired for one night only to

improve the accuracy of the timing of mating. When a seminal plug was observed, this was considered GD 0. If no plug was observed, a minimum of nine days elapsed before the female mouse was weighed again to check for pregnancy attributed weight gain (>2g), and if she was still of a breeding age, timed mating was attempted again. If the female was pregnant, she was sacrificed or her offspring used for other experiments/the next generation of parents. Males were used multiple times.

3.7.1.2 Drug preparation

To eliminate the potential influence of trace amounts of ethanol from the formulation, the solution of THC (Cayman Chemical Company, Annarbor, Michigan, USA) in ethanol was dehydrated in a Universal vacuum system UVS 400/supervac SCIIOA to obtain a highly viscous, dehydrated THC. Because THC is lipophillic, it must be dissolved in a 1:18 vehicle of cremophor (Sigma-Aldrich) and saline. A stock solution of 0.5 mg/mL of THC in solution was prepared to obtain an injection volume of 0.01 mL/g at a dosage of 5 mg/kg. The stock solution was then aliquoted to 1.5 ml Falcon tubes and frozen to prevent THC degradation in light and heat. The vehicle solution of 1:18 cremophor:saline was also prepared, aliquoted, and frozen, to avoid any confounding effects of cremophor or freezing.

Preparations of dosages for 2.5, 5, and 10 mg/kg of THC in stock solution were validated in a separate cohort of adult mice (both sexes) with gas chromotography mass spectometry to ensure that the expected dosage was being injected, and the confirmatory results have been previously published [Guma et al., 2023].

The injected dosage of THC was 5 mg/kg per day in dams, administered subcutaneously from GD 3-10. The Human Equivalent Dosage (HED) can be converted from the murine dosage with Equation 3.1.

$$HED(mg/kg) = AnimalDose(mg/kg) * \frac{AnimalK_m}{HumanK_m} \tag{3.1}$$

where K_m is a species-specific constant, which is a factor that normalizes by the body surface area [Reagan-Shaw et al., 2008]. In converting from mice to humans, this equation becomes:

$$HED(mg/kg) = 5 * \frac{3}{37} \tag{3.2}$$

which solves to 0.405 mg/kg for a human [Reagan-Shaw et al., 2008].

For a 68 kg person, this would result in a dosage of 27.54 mg of THC, which can

still be tricky to understand in the context of smoked cannabis or use during gestation. While the exact answer is not available, there are certain published statistics about cannabis preparations that can aid in the assessment of dosage. First, the average THC concentration of joints seized in Northern California in 2019 was 14%, which translates to 140 mg of THC [ElSohly et al., 2021, Devillers, 2019]. Second, donated joints from adult cannabis users in Barcelona contained a median of 6.56 mg of THC, but a range from approximately 3-75 mg of THC [Casajuana Kögel et al., 2017]. Third, among daily users, participants in the United Kingdom self-reported their cannabis use, estimating that they smoked about 1 gram each day, with a THC concentration of about 9%, using about 0.42 g of dried cannabis in each joint, which would result in joints containing roughly 37.8 mg of THC [Freeman et al., 2014]. These studies suggest great variability among cannabis users and much higher consumption of THC in preparations from daily users than from occasional recreational users. Together they provide evidence that our dosage is reasonable for a moderate-heavy dosage of THC for a daily user, although, to date, no study specifically assesses THC consumption in pregnant populations specifically.

3.7.1.3 MRI acquisition

The structural scan was a T2 weighted image acquired with a 3D fast spin echo sequence using a cylindrical k-space acquisition, with TR/TE=350/12 ms, echo train length=6, two averages, field-of-view 20 mm x 20 mm x 25 mm, matrix size=504 x 504 x 630 [Nieman et al., 2005, Spencer Noakes et al., 2017, Guma et al., 2022]. The 4-by-4 array for 16 sample acquisition introduces geometric distortions as the samples are placed at varying distances from the isocenter of the gradients in the magnet. Specifically, the farthest samples from the isocenter are most susceptible to artefacts [Spencer Noakes et al., 2017]. Therefore, corrections are applied during image reconstruction to assure high-quality images are obtained regardless of position in the array discussed in great detail in [Spencer Noakes et al., 2017]: 1) the amplitude of each echo is intensity corrected with a Wiener deconvolution, 2) each readout is aligned and phase-matched, 3) phase correction was predicted and removed from K-space prior to reconstruction, 4) where even and odd k-spaces were combined, they were aligned with translation and averaged prior to reconstruction [Spencer Noakes et al., 2017].

3.7.1.4 MRI preprocessing

In preprocessing the reconstructed image, the first step was mask creation. The ANTs ecosystem was used to first clamp negative intensities from the image (mincmath command with -clobber and -clamp flags) then to create an Otsu mask (ThresholdImage command). Full commands can be found in publicly available code. Once each mask was created, the Display command from the MINC Toolkit library was used to visualize each image with the mask as a label superimposed on it (https://bic-mni.github.io/). The masks were then corrected manually by either L.C. or A.P. to exclude any tissue outside of the bounds of the embryo body [CoBrALab, 2023]. Corrected masks were multiplied by the original image to result in an image with a clean boundary at the edge of the embryo body. Next, each image was quality controlled in comparison to a reference image, a GD 18 average from a previous experiment [Guma et al., 2022]. The command register from the MINC Toolkit was used to compare orientation, and images were assigned a value of 0 if they were misoriented or 1 if they were properly oriented. They were also assessed for whether they were squished (1) or not squished (0). If an image was noted as misregistered, "tags" were created indicating similar parts of the image and the reference (such as the mouse snout, back of head, and top of head). Then linear transformations were applied to the individual image to align it with the reference. Images that were noted as squished were removed from future analyses. In total, 14 images were removed for being squished, (Null, N = 1 [female = 0], Sal, N = 4 [female = 4], THC, N = 9 [female = 4]). Finally, an in-house pipeline used ANTs tools to preprocess the individual scans, including denoising with adaptive nonlocal means (minc_anlm) and N4 bias-field correction [Manjón et al., 2010, Tustison et al., 2010].

3.7.1.5 MRI processing

Images were aligned and averaged across the study with DBM. First, all images were aligned with affine transformations that preserved parallel lines in the images (translations, rotations, scales, and shears). Finally, iterative nonlinear transformations attained precise anatomical alignment between images in an automated fashion, similar to previous studies in adult animal brains [Guma et al., 2023], as well as embryo studies in our laboratory [Guma et al., 2022]. After the initial DBM, images representing the transforms from the individual scans to the study average were visually inspected, and if the registration failed, the original input image was visually inspected to identify the cause of the misregistration. One major cause of mis-registration was that some of the embryos are more curled, or c-shaped, from the tip of the nose to the tip of

the tail, as seen in SFig. 3.5. In order to assist the registration to the rest of the population, the c-shaped embryos were identified. Again, tags were created with the MINC tool "register" between the input image and the study-average produced from the DBM. The tags were converted to an LSQ12 transform file (tagtoxfm -lsq12) and the transformation was applied to the input image (mincresample). The LSQ12-transformed images was then re-registered to the study-average and tags between the two images were produced again. These tags were converted to a thin-plate spline non-linear transformation (tagtoxfm -tps), and the transformation was applied to the LSQ12 images. Another DBM was conducted, replacing the c-shaped images with the TPS-transformed images. This model-build resulted in 5 registration failures, which were excluded from the final analysis (Null, N = 2 [female = 0], Sal, N = 3 [female = 2], THC, N = 0).

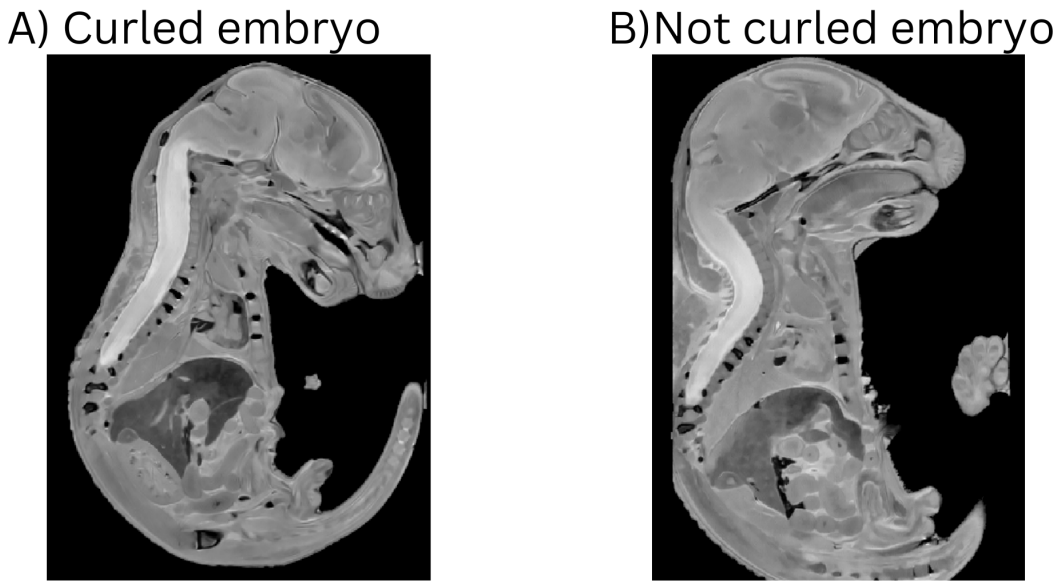


Figure 3.5: A) Example of curled embryo that required manual registration B) Example of embryo that did not exhibit the c-shaped curl and registered without additional manual registration.

Finally, to optimize registration for the brain, the head was cropped from each image and another DBM was run. A cubic mask was made with the SimpleITK package in the Python programming language identifying the head of the average

from the last DBM. This was then transformed to each of the input images using the transforms produced by the DBM (ANTS:antsApplyTransforms) and the bounding box was QC'd. Then, images were converted to mincs (nii2mnc) and the mask was applied as a bounding box to crop the images (minc-toolkit::autocrop –bbox). These cropped head images were used as inputs for a final DBM.

3.7.1.6 Statistical analyses

Statistical analyses were performed in R, version 3.5.1, with all packages and versions published with the code.

The voxelwise analysis of relative Jacobians from the DBM was performed twice, first for the brain, then for the organs. A brain mask was registered from a mask on the head of an average from a previously published study [Guma et al., 2022] using ANTs tools (https://github.com/CoBrALab/documentation/wiki/Making-a-stats-mask-for-2level-dbm-output). An LMER was run in each voxel included in the mask. The organ mask was manually created on the full body average.

3.7.2 Supplementary Results

3.7.2.1 PTE impacts dam weight gain

As expected, there was a main effect of GD ($p \leq 2e\text{-}16$), with all dams gaining weight over time. Interestingly, there was an interaction between treatment and GD for Null ($p = 0.0016$) compared to Sal and a trending effect of THC ($p = 0.073$) compared to Sal. Finally, there was also a trending main effect of treatment for Null ($p = 0.073$), SFig. 3.6A, full results in Tab. 3.1. There was no effect of litter size as a covariate. Because of the trending main effect, we examined the dams' weight at GD 0 with a linear model and found a significant difference for both Sal ($p = 0.028$) and THC ($p = 0.046$), SFig. 3.6B, full results in Tab. 3.2. The initial difference in weight was unexpected, and may reflect a slight difference in age of dams in the null group, despite efforts to randomize group assignment. The changes in weight gain across time have been documented in other studies [Rodríguez de Fonseca et al., 1992], and are sometimes attributable to differences in litter size, although this was not the cause in our study. Differences in litter-size are accounted for in the linear mixed effects models with the random effects, but incorporating another random effect of dam ID may introduce collinearity and add further complexity to the model, given the sample sizes.

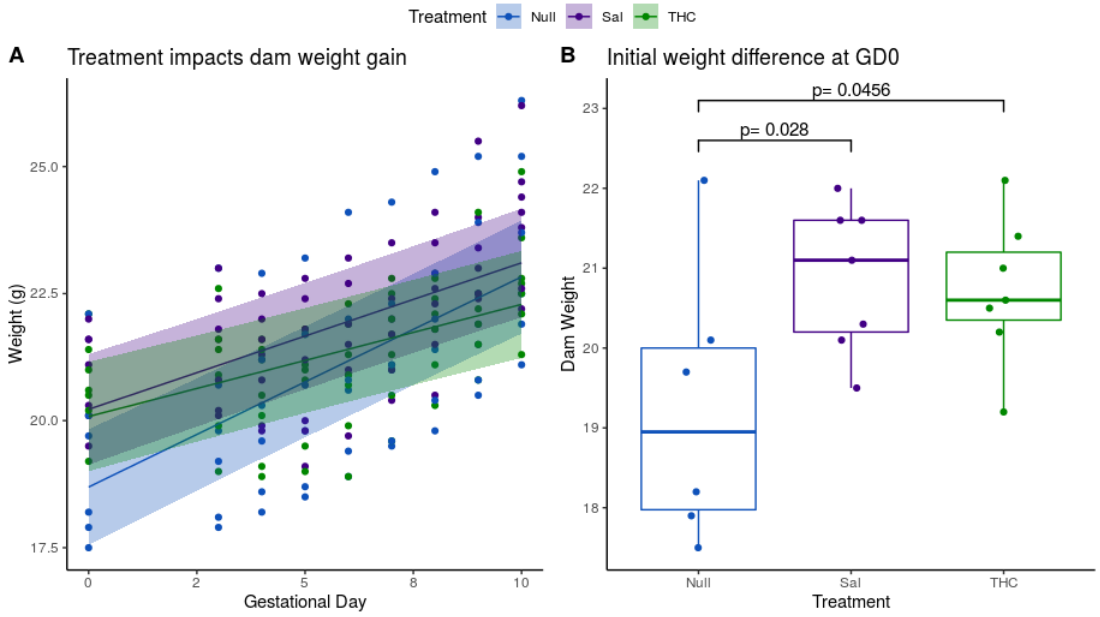


Figure 3.6: A) All dams gained weight over gestation, as expected, however there was a significant interaction between treatment and day, with Null dams exhibiting a steeper slope than Sal or THC, and THC dams exhibiting a reduced trajectory of weight gain. All dams ended at roughly equivalent weights, however B) Null dams were lighter at GD 0 than Sal and THC controls.

3.7.2.2 PTE does not impact litter size or sex of pups

Neither Sal, nor THC impacted either litter size or the sex ratio, SFig. 3.7.

3.7.2.3 PTE impacts QC score of image

Following QC of the raw images, we used an LMER to examine whether the interaction between treatment and sex could have impacted the quality of the image, including a random effect of dam_ID to account for the nesting effect in litter. There was a main effect of treatment such that Null embryos received better scores than Sal embryos ($p = 0.043$) and sex, such that males received better scores than females ($p < 0.005$), seen in SFig. 3.8. There were also interactions such that Null males received worse scores than Null females ($p = 0.01$) and THC males received worse scores than females ($p < 0.03$), full results in Tab. 3.3.

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	19.96	1.57	16.31	12.70	0.00
TreatmentNull	-1.53	0.80	18.84	-1.90	0.07
TreatmentTHC	-0.14	0.80	18.65	-0.18	0.86
Day	0.29	0.03	157.00	10.88	0.00
litter_size	0.04	0.18	16.00	0.19	0.85
TreatmentNull:Day	0.13	0.04	157.00	3.21	0.00
TreatmentTHC:Day	-0.07	0.04	157.00	-1.81	0.07

Table 3.1: Results from the LMER examining the interaction of treatment and day on weight gain.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	19.2500	0.4982	38.64	0.0000
TreatmentSal	1.6357	0.6789	2.41	0.0276
TreatmentTHC	1.4643	0.6789	2.16	0.0456

Table 3.2: Results from the LM examining dam weight before injections across the different groups.

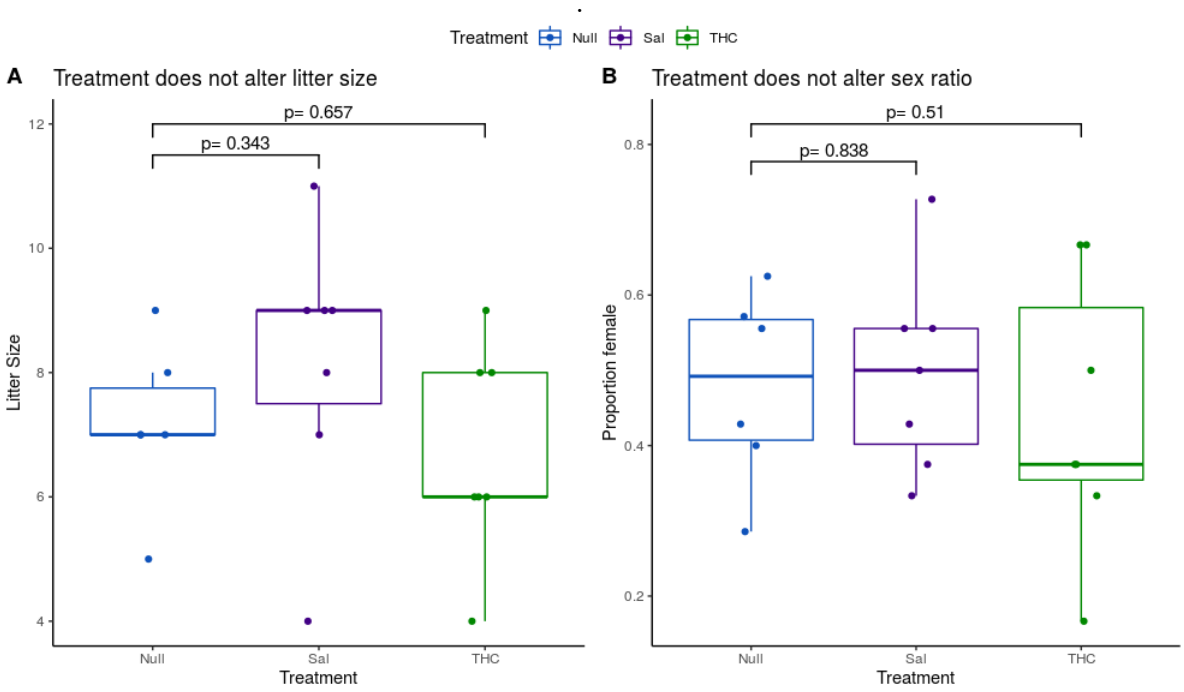


Figure 3.7: Neither prenatal Sal nor THC injections impacted the litter size (A) or ratio of females in the litter (B)

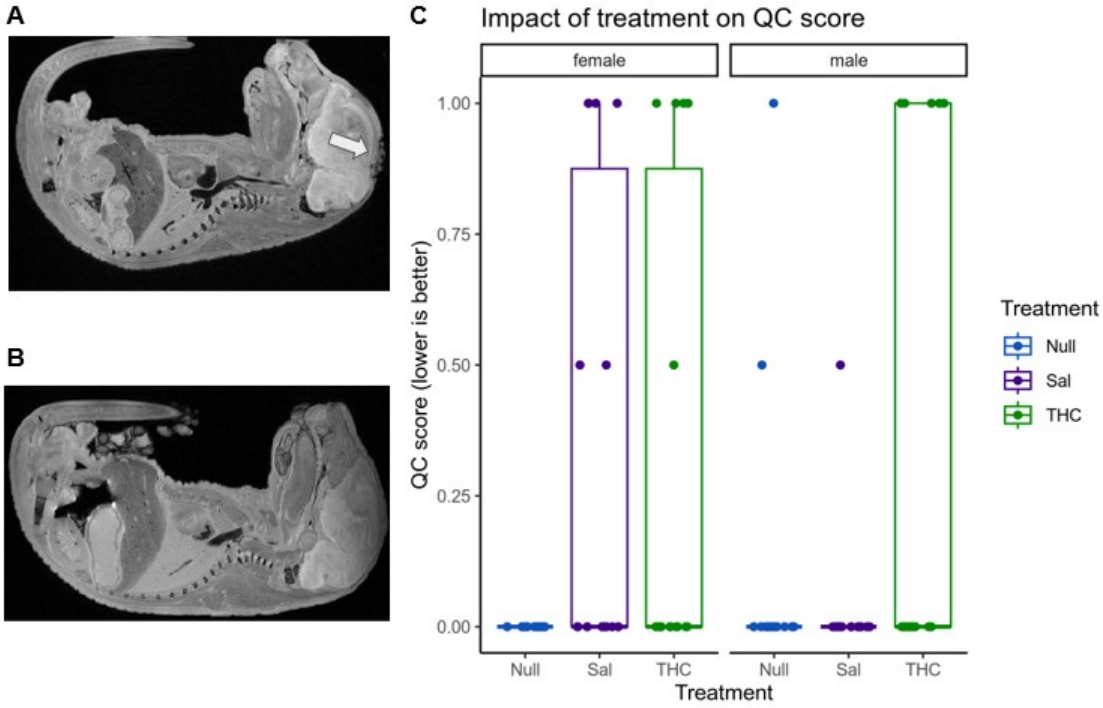


Figure 3.8: A) An example of an embryo that received a score of 1, indicating a QC failure with a white arrow indicating the squished region at the dorsal cortex B) An example of an image that received a score of 0, indicating passing QC C)Treatment and sex impacted scores received during QC. Overall, 14 images were excluded (excluded: Null, N = 1 [female = 0], Sal, N = 4 [female = 4], THC, N = 9 [female = 4]).

3.7.2.4 PTE and daily injections reduce body volume

The full output of the body volume model is found in Tab. 3.4, including larger volume in Null groups and smaller volume in THC group. No main effect of sex or interaction with either condition.

3.7.2.5 PTE does not impact TBV

Because of the marked decrease of total body volume, we examined whether the interaction between treatment and sex impacted TBV. There was no significant difference between groups for either the interaction, or the main effect of treatment or sex, Fig 3.9.

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	0.36	0.11	28.10	3.11	0.00
treatmentNull	-0.35	0.17	26.42	-2.12	0.04
treatmentTHC	-0.04	0.16	28.23	-0.23	0.82
sexmale	-0.32	0.11	60.75	-2.93	0.00
treatmentNull:sexmale	0.41	0.16	61.24	2.65	0.01
treatmentTHC:sexmale	0.36	0.16	62.15	2.28	0.03

Table 3.3: Results from the LMER examining QC score by treatment and sex.

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	804.50	75.30	11.06	10.68	0.00
treatmentNull	167.55	62.19	57.76	2.69	0.01
treatmentTHC	-156.53	67.69	59.22	-2.31	0.02
sexmale	55.15	58.65	57.23	0.94	0.35
treatmentNull:sexmale	4.24	79.69	56.90	0.05	0.96
treatmentTHC:sexmale	0.53	87.58	56.85	0.01	1.00

Table 3.4: Results from the LMER examining total body volume by treatment and sex, main effects and interaction.

3.7.2.6 PTE and sex impact organ volume

While there was no impact of the Null condition compared to Sal, there was both a main effect of THC and the interaction between condition and sex for THC significant from 10% to 5% FDR. The main effect of sex showed a trending effect from 15% to 10%. The main effect of THC showed clusters of voxels in the liver (medial and left lobes, larger in THC), and the lungs (mixed results) Fig 3.10. The interaction showed reduced volume in the right liver of THC males compared to Sal females, Fig 3.11.

3.7.2.7 Chronic injections slightly impact local brain volume

As reported in the main text, there were several focal anterior, medial regions (near the preoptic area) that showed a smaller volume in the Null group compared to the Sal controls, seen in SFig. 3.12.

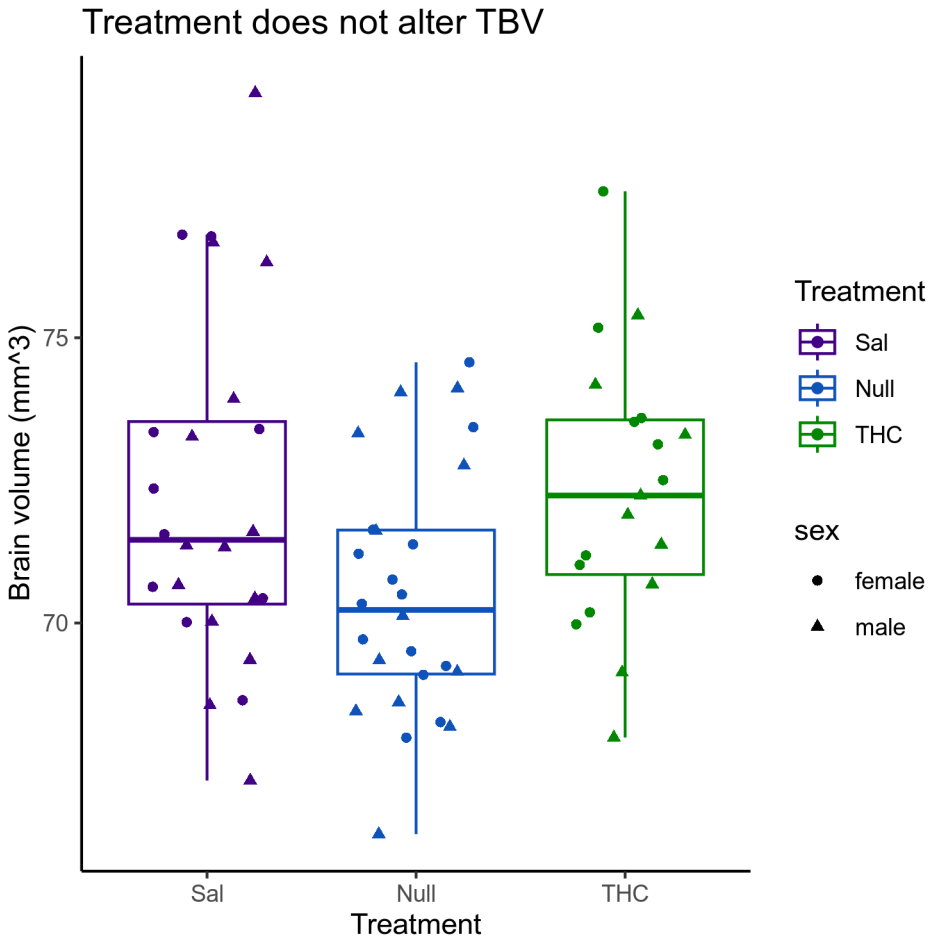


Figure 3.9: No main effect of treatment, sex, or interaction between the two on TBV

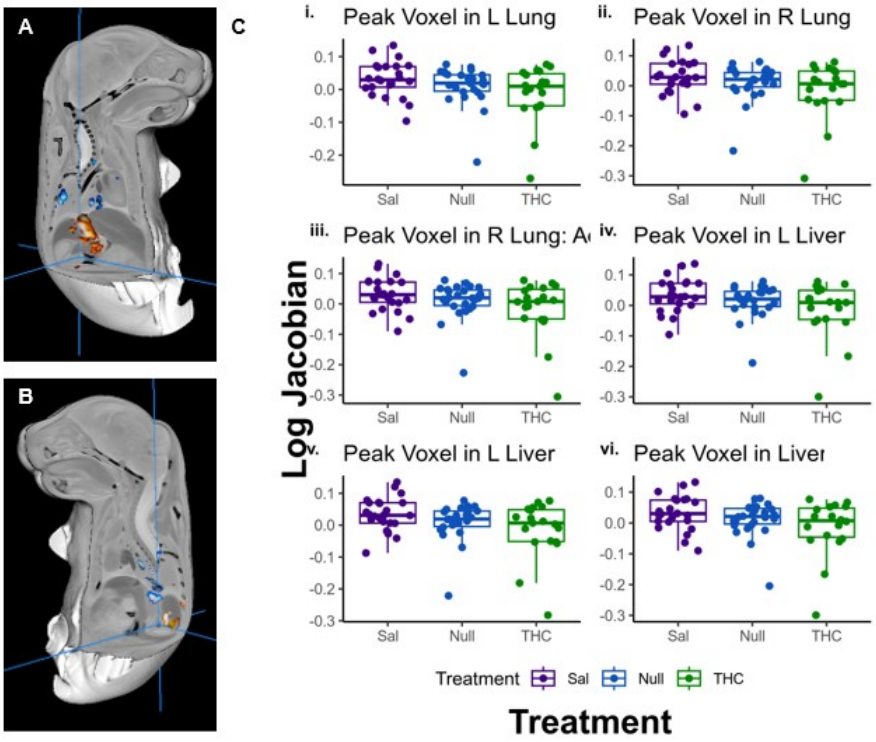


Figure 3.10: Heatmap of main effect of THC compared to Sal in organ volume, thresholded to 10%-5% FDR with cut outs showing right (A) and left (B). C) Boxplots showing peak voxels of interest in significant regions: i) left lung, ii) middle lobe of the right lung, iii) accessory lobe of right lung, iv and v) left liver, vi) medial lobe of liver

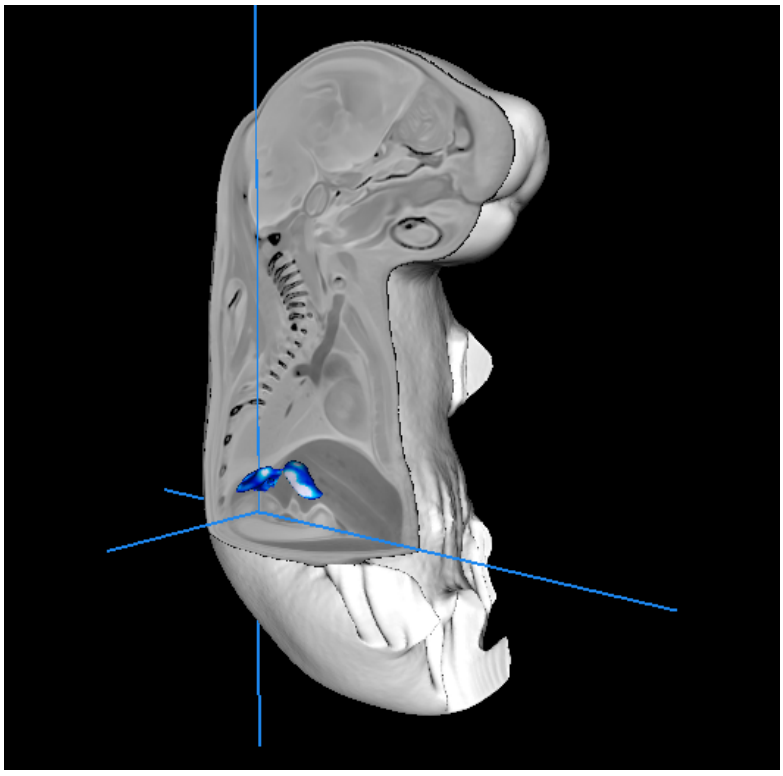


Figure 3.11: Heatmap of interaction between treatment and sex showing smaller volume in the liver of the THC male group (FDR 10%-5%).

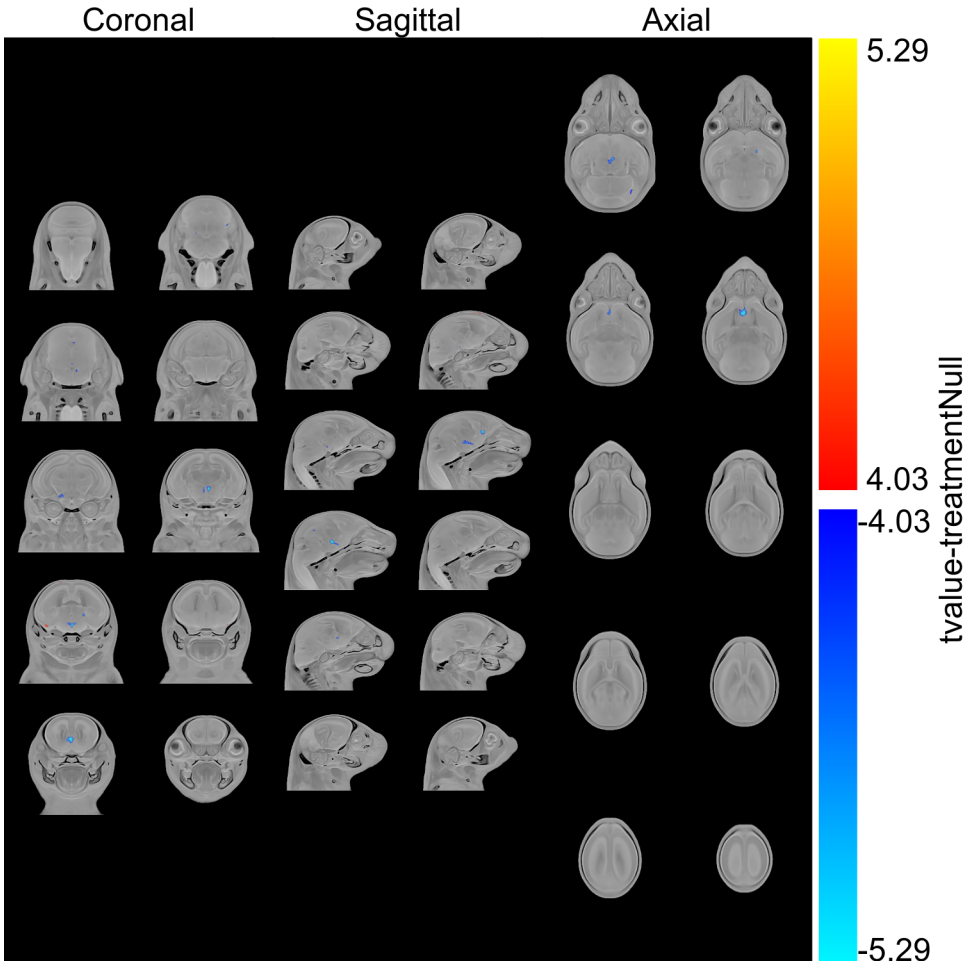


Figure 3.12: Heatmap displaying impact of Null compared to Sal, thresholded from 5-1%. Extremely focal regions of difference highlated in anterior, medial regions

Chapter 4

Impact of chronic prenatal exposure to THC early in gestation on trajectories of development in neonatal mice

Lani Cupo, Annie Phan, Elisa Guma, Daniel Gallino, Jeremie Fouquet, Gabriel A. Devenyi, M. Mallar Chakravarty

4.1 Preface

The work presented in **Chapter 4** built on the experiment and results from **Chapter 3** by examining an identical exposure to prenatal THC exposure (PTE) in neonatal mice. Having demonstrated minimal effects of chronic injections on the level of the brain specifically, we included only the saline (Sal) and Δ^9-tetrahydrocannabinol (THC) groups to reduce animal waste. In this chapter we expanded on results indicating intrauterine growth restriction (IUGR) in **Chapter 3** by examining the impact of PTE on weight gain. Further, we conducted longitudinal *in vivo* structural magnetic resonance imaging (MRI) between postnatal day (PND) 3 and 10 to establish whether volume changes detected in embryos persist into early postnatal life. To our knowledge, this is the first study to use longitudinal *in vivo* neonatal imaging in an experimental model. We also investigated the impact of PTE on ultrasonic vocalizations (USVs), a social behavior in mouse pups. Finally, we further explored the impact of the PTE on dam weight gain and pregnancy outcomes at the level of the litter.

First we found evidence for catch-up growth in the THC group. Scanning the neonates, however, did not impact their weight gain when compared with nonscanned littermates. Second, bilateral regions in the cortex, subcortical regions, and cerebellum showed reductions in growth rate over the first two weeks of life in THC pups compared to Sal controls. PTE had a sex-dependent effect on USVs, with males THC pups making more calls but female THC pups making fewer calls. Sex differences were not present in weight gain or brain volume. This chapter has been drafted for submission to a peer-reviewed journal.

4.2 Abstract

Since cannabis has been increasingly decriminalized and legalized around the world, the prevalence of pregnant people using it continues to increase. The effects of gestational exposure to cannabis on the developing brain have yet to be fully elucidated. To understand the impact of prenatal exposure to THC, the main psychoactive component of cannabis, on developmental trajectories of the brain in the earliest stages of life, we employ longitudinal *in vivo* structural MRI on PND 3, 5, 7 and 10 in mouse neonates following prenatal exposure to 5 mg/kg THC injected in the dam subcutaneously from gestational day (GD) 3-10. USVs pups make when separated from dams are assessed one day after the final scan timepoint. First, we observed that THC-exposed pups showed altered trajectories of brain development during the first

week of life. Specifically THC-exposure was related to a reduced rate of growth in bilateral regions throughout the brain compared to controls, including the ventral and dorsal hippocampi, motor cortices, thalami, and amygdalae. Second, we observed that THC-exposed pups, especially males, show increased medium-length calls compared to controls, potentially indicating anxiety-like behavior. Together these data suggest alterations to brain development that may have consequences for early psychiatric health that should be investigated early in life.

4.3 Introduction

Cannabis is increasingly viewed as safe and natural by the public [Khademi et al., 2023], however research on the potential consequences of exposure during pregnancy lags behind public opinion. While a host of articles have recently been published regarding the impact of prenatal cannabis exposure (PCE) both in human subjects and nonhuman animals [Sarikahya et al., 2023, Roberts et al., 2022, Lallai et al., 2022, Rompala et al., 2021], much is still unknown about how PCE affects early life development of the brain. Studies that examine the early postnatal period tend to assess a single early timepoint [Gunn et al., 2016, Fransquet et al., 2017, Day et al., 1991, Fergusson et al., 2002, Wang et al., 2008, Ko et al., 2014, Conner et al., 2016, Tortoriello et al., 2014, Gray et al., 2010, Thomason et al., 2021, Gillies et al., 2020, Antonelli et al., 2004, El Marroun et al., 2009, Benevenuto et al., 2017]. The main outcome measures of many of these experiments is by nature cross-sectional, such as birth weight [Gunn et al., 2016, Day et al., 1991, Fergusson et al., 2002, Wang et al., 2008, Ko et al., 2014, Conner et al., 2016, Gray et al., 2010, Benevenuto et al., 2017, Gillies et al., 2020], and other animal studies require a terminal procedure [Gillies et al., 2020, Benevenuto et al., 2017, Antonelli et al., 2005]. In human studies, resource limitations, or burden on participants can be factors limiting the number of assessments as well. Several studies do examine multiple timepoints in the post-natal life, however because of the same restraints outlined above, all of them use different subjects for each timepoint [Natale et al., 2020, Rodríguez de Fonseca et al., 1992, Mereu et al., 2003]. Brain development is inherently a dynamic process, however, that requires longitudinal studies in the same participant to properly assess. In humans, this process takes years, however the mouse pup undergoes rapid physiological changes, with their brain

roughly doubling in volume between PND 3 and 10. To properly elucidate the urgent question of how PTE may impact early life, longitudinal assessments can be leveraged in the mouse.

Several studies demonstrate that PCE has an impact on important aspects of brain development that occur during early infancy. Cumulatively, these studies provide key evidence indicating alterations in the brain. For example, At birth, mouse pups exposed to cannabis from GD 5.5-17.5 show reduced weight in the brain lungs, thymus, and liver [Benevenuto et al., 2017]. Rat pups exposed to cannabis from GD 5 to birth show sex-specific alterations in the activity of dopaminergic neurons in the prosencephalic area [Rodríguez de Fonseca et al., 1992]. One study in human neonates examined functional connectivity in neonates with PCE and polydrug exposure compared to controls with polydrug exposure but *not* PCE and neonates with no drug exposure, report hypoconnectivity in regions with high Cannabinoid- (CB)1-receptor expression [Grewen et al., 2015]. Nevertheless, there is a lack of comprehensive studies that investigate the brain as a developing system following PTE.

Recent studies in the mouse demonstrate how longitudinal, *in vivo* structural MRI can be used in a minimally-invasive manner to assay the dynamic brain remodelling that occurs at both the individual and group-level during the first week of life in the mouse [Qiu et al., 2018]. Effectively, this methodology allows us to image the brain in a single pup multiple times during a period of great change, with the ultimate goal of capturing subtle changes during a specific developmental window. Here, we employ this methodology to examine the impact PTE has in brain development. Further still, while this technique provides insight at the whole-brain technique, the regions highlighted in MRI analyses can be used as regions of interest for further analyses of the underlying mechanisms of volume change. In human studies, longitudinal structural MRI has been used in neonates to study both trajectories of healthy development [Giedd et al., 2015, Holland et al., 2014] as well as developmental disorders such as congenital heart disease [Claessens et al., 2019]. Such longitudinal neonatal MRI studies may be a crucial way forward to translate findings from small animal studies to humans, but the difficulty in acquiring high quality human neonate MRI, and the multi-year span of these studies highlights the promise of small animal imaging as a more immediate source of information.

Here, we study the impact of early gestational exposure to THC on trajectories of development in the early murine neonatal period. We exposed pregnant dams to THC during GD 3-10. Pups were scanned every other day during this period using structural MRI. On PND 12 we also collected USVs to determine if the prenatal

THC exposure impacted pup behaviour. We anticipated alterations in structural development, especially in regions rich with CB_1 receptors and indications of anxiety-like behavior in the pups.

4.4 Materials and Methods

4.4.1 Animals and timed mating

All experiments were conducted at the Douglas Mental Health University Institute Animal Facility in compliance with Facility Animal Care Committee regulations. Female and male C57BL/6J mice of breeding age (8 to 12 weeks old) were subject to timed mating breeding procedures, as described in previous experiments (Section 3.5.2) and with more detail in Supplementary Methods (SM) 4.7.1.1. All females were nulliparous, and GD 0 was determined as the day a plug was observed.

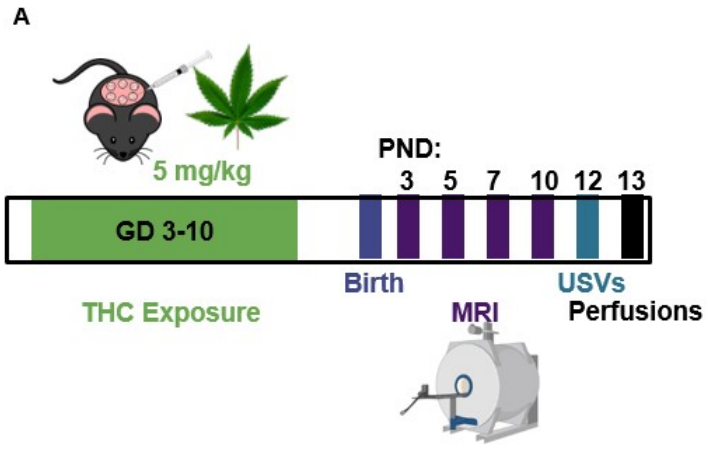
4.4.2 Drug preparation and injection

A representation of the timeline can be found in Fig 4.1.a.

Pregnant dams were randomly assigned to either the Sal vehicle group or the THC experimental group. As the Sal injections were found not to impact brain volume compared to an uninjected control group analyzed as embryos, we used only the Sal and THC groups, in accordance with the 3Rs of animal work, replacement, reduction, and refinement [Curzer et al., 2016]. Because intraperitoneal injections are not recommended for repeated administration in pregnant dams [DiNieri and Hurd, 2012], subcutaneous injections were performed from GD 3-10 between 9:00 and 11:00.

Two grams of THC were purchased from Cayman Chemical Company in a formulation of 10 mg/ml in ethanol under a research license and import permit for THC from Health Canada. Ethanol was dehydrated, and THC was prepared in a solution of 1:18 cremophor:saline (Section 4.7.1.2) as described previously in prenatal exposure (Section 3.7.1.2) and adolescent exposure [Guma et al., 2023]. The vehicle solution for the Sal group comprised a preparation of 1:18 cremophor:saline to serve as a control for the THC condition.

The dosage of THC injected was 5 mg/kg. This dosage has been characterized in both prenatal THC models [Rubio et al., 1995, Navarro et al., 1994, Lallai et al., 2022, Drazanova et al., 2019], as well as adolescent exposure models [Trezza et al., 2008, Guma et al., 2023]. It has been suggested that this dosage represents moderate human



B

$MnCl_2$-exposed pups (scanned)

	Female	Male
Sal	13 (10)	30 (17)
THC	19 (14)	18 (11)

C

$MnCl_2$-unexposed (scanned)

	Female	Male
Sal	5 (4)	6 (4)
THC	2 (2)	10 (6)

D

USVs collected (scanned)

	Female	Male
Sal	12 (9)	32 (19)
THC	17 (12)	20 (13)

Figure 4.1: a) Timeline of experiment including THC exposure, (green), scanning (purple), behavior (blue), and perfusions (black). Sample size, including total number of pups and scanned pups in parenthetical for a) the main experiment, $MnCl_2$-exposed offspring, b) the $MnCl_2$ investigation, c) a pooled sample for behavioral analysis

gestational exposure [Lallai et al., 2022], but it can be challenging to accurately assess and model human cannabis use during pregnancy, as discussed previously, Section 3.7.1.2. The dosage used here roughly corresponds to a dosage of 27.6 mg/kg in humans, which represents a moderately high dosage, Section 3.7.1.2. The injection volume for both THC and Sal dams was maintained at 0.01 ml/g. Dams were weighed daily at the time of each injection and their weight recorded for later analysis. The timeline and dosage of exposure was identical to that of previous experiments to allow interpretation across experimental outcomes, (Section 3.5.2).

4.4.3 Experimental timeline

On GD 1, litters were culled to 6 pups. On GD 2, 4, 6, and 9, 24 hours before each scan, dams were injected with 0.4 mmol/kg dose of 30 mM manganese chloride

2 (MnCl) solution, a contrast agent that pups ingest while nursing [Qiu et al., 2018]. MnCl shortens the T1 and T2 relaxation times, increasing the contrast of images [Wadghiri et al., 2004, Szulc et al., 2015]. Importantly, MnCl has been shown to impact development, transiently reducing weight gain of pups and brain volume (differences peaking about -10% before normalizing for both) discussed further in SM 4.7.1.7 [Szulc et al., 2015]. Nevertheless, the method has been used successfully in developmental studies before [Qiu et al., 2018]. On PND 3, 5, 7, and 10, four pups per litter, two males and two females where possible, were scanned. Before each scan, pups were examined for developmental milestones including physical (weight, incisor eruption, fur development, eye opening, pinnae detachment and auditory canal opening), sensorimotor (Vibrissa placing response, ear twitch, and auditory startle response), and motor (surface righting reflex and grasp reflex) metrics adapted from previous publications [Sarkar et al., 2020, Tamashiro et al., 2000, Anthwal and Thompson, 2016, Demaestri et al., 2020]. A full description of the tasks and timeline is presented in SM 4.7.1.3. On GD 12, pups were separated from their dams for 5 minutes and USVs they made during this time period were recorded to analyze the frequency and duration of calls, with further experimental detail available in SM 4.7.1.4. On GD 13, pups were weighed and sacrificed by transcardiac perfusion, and their brains were extracted for later postmortem analyses SM 4.7.1.5.

4.4.4 Imaging methods

4.4.4.1 Animal preparation

The day of the scan, pups were removed from the cage one at a time. They were weighed, assessed for age-appropriate milestones, and anaesthetized with isoflurane, induced at 5%, maintained at 2% during the scan. Pups were fitted into foam beds carved for each age and placed into the scan bed. A nose-cone was fitted over the animal's snout to deliver isoflurane, and the mouse was heated with warm air delivered to the bed, as to adult mice [Guma et al., 2023, Kong et al., 2018]. Following the first scan timepoint on PND 3, pups' paws were micro-tattooed with India ink for identification.

After the scan, pups were rubbed with bedding to assure their dam would accept them back into the nest and returned to their home-cage placed on a heating mat [Qiu et al., 2018, Szulc et al., 2015].

4.4.4.2 Scan details

The scanning protocol was adapted from previous neurodevelopmental studies in another center [Qiu et al., 2018]. All mice were scanned on a 7.0 Tesla (T) Bruker BioSpec with a 30cm bore magnet with AVANCE electronics, equipped with a cryogenically cooled surface coil. The T1-weighted scan protocol had the following parameters: 3D gradient echo sequence, TR = 26 ms, TE = 5.37 ms, flip angle = 37°, field-of-view = 17.1 × 14.8 × 11.1 mm, matrix size = 190 × 164 × 123, number of averages = 2, total acquisition time = 23 minutes, isotropic resolution = 90 mum.

4.4.4.3 Image processing

After images were reconstructed, they were converted to niftis, quality controlled, and preprocessed, with full details reported in SM 4.7.1.6. Images were processed with an in-house longitudinal two-level deformation based morphometry (DBM) pipeline using the ANTs toolkit (http://stnava.github.io/ANTs/) for linear and non-linear registration [Avants et al., 2011], which has been employed in later-life studies [Guma et al., 2023] and cross-sectionally in embryos (Section 3.4.4.1). Specific details can be found in SM 4.7.1.6, but, importantly, an unbiased average was first constructed for each subject overtime and then across subjects.

The final step of the DBM pipeline involves post-processing the consensus deformation fields to produce Jacobian determinants which encode the voxel-wise volumetric difference from each input scan to the consensus population average (ANTs toolkit). Registrations were quality controlled and, because of the great changes in brain size and morphometry between PND 3 to 10. We observed that there were many failed registration, especially in the cerebellum. We improved these registrations, that are critical to the performance of the pipeline by applying a manually developed brain mask on the initial unbiased average using the Display software available in minc-toolkit. This mask was transformed to the individual input images with ANTs tools (antsRegistration_affine_SyN.sh and antsApplyTransforms) [Avants et al., 2011]. Subject-level warp images were quality controlled and manually corrected, where necessary and then used to to extract the brain from the background of the image (ImageMath). The DBM pipeline was run again and results quality controlled to ensure all images were well-registered.

4.4.5 Statistical analyses

All statistical tests were performed in the R programming language, version 3.5.1.

4.4.5.1 Impact of THC on pregnancy outcomes

The impact of THC exposure on several pregnancy outcomes was assessed. First, linear mixed effects models (LMER)s were used to assess the impact of THC exposure on dam weight gain from GD 0-10. Fixed effects included the interaction between gestational day and condition with litter size as a covariate and random effects were dam_ID. Second, the litter size was assessed with linear models examining the impact of THC exposure on number of pups. Finally, the impact of condition on sex-ratio of pups were assessed with linear models. Importantly, litters were culled at PND 1, but sexed at PND 3. The litter size was counted before culling, however the sex ratio was only assessed after PND 3. In this study, pups were randomly selected for culling, so it is possible that some of the sex ratios do not accurately represent the sex ratio of the entire litter.

4.4.5.2 Pup weight

LMERs (lme4::lmer and lmerTest::summary) were used to assess the impact of treatment and scanning on pup weight. The weight model included an interaction between condition, age, and sex with scanning status (whether the pup was scanned or not) as a covariate, and littersize and animal ID as random effects.

4.4.5.3 Magnetic Resonance Imaging

To assess differences in local brain volume, mass univariate LMERs were run on the logged absolute Jacobian determinants produced by the DBM. Absolute Jacobians, as opposed to relative Jacobians, include affine components of registration, encoding changes in total brain volume. LMERs were used because including multiple pups from a single litter violates assumptions of statistical independence among individuals unless litter is modelled as nesting variable [Harrison et al., 2018]. Because our evidence suggests THC exposure impacts pup weight gain, and because weight could impact brain volume, we employed a regression-with-residuals method to control for the possible mediating effect of body weight on the relationship between condition and brain volume [Zhou et al., 2014]. The residuals from the pup weight model were extracted and included as a covariate in the brain volume model. Thus, the fixed

effects included the interaction between treatment, sex, and age, as well as the weight residuals, and the random effects were litter size and animal ID (lme4::lmer and lmerTest::summary). To correct for multiple comparisons we used the False Discovery Rate (FDR), where a threshold from 5%-1% indicates up to 5% of significant voxels could be false positives. Voxels were automatically labelled using RMINC tools (mincFindPeaks, mincLabelPeaks) from a PND 7 atlas adapted from an adult atlas and used in previous neonatal experiments [Guma et al., 2021b, Dorr et al., 2008], as PND 7 represents the mid-point between the included ages.

4.4.5.4 Ultrasonic vocalizations

First, to exclude background noise, calls were filtered for those between 5 and 300 ms [Guma et al., 2021b, Scattoni et al., 2009]. Then, to analyze differences in frequency of USVs of different lengths, we compared the distribution of calls, as previously [Guma et al., 2021b]. Descriptive measures, such as means or medians, may not accurately represent differences between groups, but a hierarchical shift function (rogme::shifthd_pbci) can be leveraged to compare distributions of data [Rousselet et al., 2017]. In this approach, the distribution of data are mapped between groups, with each distribution comprising the number of calls of different lengths (multiple calls per pup). The median and deciles of the distributions are calculated per condition (Sal and THC). Deciles are estimated with the Harrell-Davis quantile estimator (HD) [Rousselet et al., 2017, Harrell and Davis, 1982]. Then, the differences between the distributions are quantified; specifically, with the shift function it is estimated how and how much one distribution must be shifted to match another one [Rousselet et al., 2017]. Then, 95% confidence intervals per decile comparison are calculated with bootstrap resampling. To achieve this, the original data are resampled with replacement within condition 2,000 times. The number of resampled observations is determined by the number of iterations (in this case 2,000) multiplied by the number of observations per condition. For each iteration, the HD is calculated for the resampled distributions and the difference between deciles between the two resampled groups are calculated. After all resamples are complete, a p-value representing the significance of the difference between groups for the observed deciles is calculated by examining the proportion of resampled cases where the differences between groups is more extreme than the observed differences between groups. This value ranges from 0 to 1, with 0 indicating no resampled cases are more extreme than the observed difference (very significant observed difference) and 1 indicating all resampled

cases are more extreme than the observed different (not at all significant observed difference). The value is multiplied by 2 to generate a two-sided p-value. Finally, Hochberg's correction for multiple comparisons is applied [Hochberg, 1988]. Deciles where the confidence interval does not cross 0 indicate deciles that significantly differ between groups [Rousselet et al., 2017]. To examine the interaction between sex and treatment, data were separated into four groups: sal_female, sal_male, THC_female, THC_male. Comparisons in distributions were calculated between each of the groups. Supplementary examinations of the calls made by scanned vs unscanned ups were also included following the same statistical approach.

4.4.6 Manganese chloride investigation

In the course of the present study, the potential site-specific effects of postnatal MnCl exposure on Sal and THC pups was estimated. To this end, four additional litters (two Sal, two THC) were acquired unexposed to MnCl. Twenty-four hours prior to each scan, the dam was injected with Sal, but otherwise the experimental design was identical to the full experiment. Pups were compared in terms of growth rate with weight. Contrast was too poor for registration with DBM, however masks, produced during preprocessing, were manually corrected and used to assess total brain volume (TBV) (collect_volumes.sh). The impact of MnCl exposure on weight and TBV was assessed with LMERs.

4.5 Results

4.5.1 Pregnancy outcomes and pup milestones

Condition slightly reduced dam weight gain (SFig. 4.9), but not litter size or sex ratio of pups (SFig. 4.10), discussed further in Supplementary Results (SR) 4.7.2.1, with full results in Tab. 4.2. Condition did not impact the attainment of any milestones (not pictured).

4.5.2 PTE, but not scanning, impacts pup weight gain

At PND 1, litters were reduced to 6 pups to control the distribution of MnCl through the dam. While only four pups (two male and two female, where possible) were scanned per litter, all were included in the weight analysis to assess the impact of scanning. In total, there were 13 Sal females (2 not scanned), 30 Sal males (12 not

scanned), 19 THC females (5 not scanned), and 18 THC males (6 not scanned). There was a main effect of age ($p < 0.001$), and the interaction between condition and age ($p < 0.01$) 4.2, with full effects in SR Tab. 4.3. There was no effect of sex or scanning.

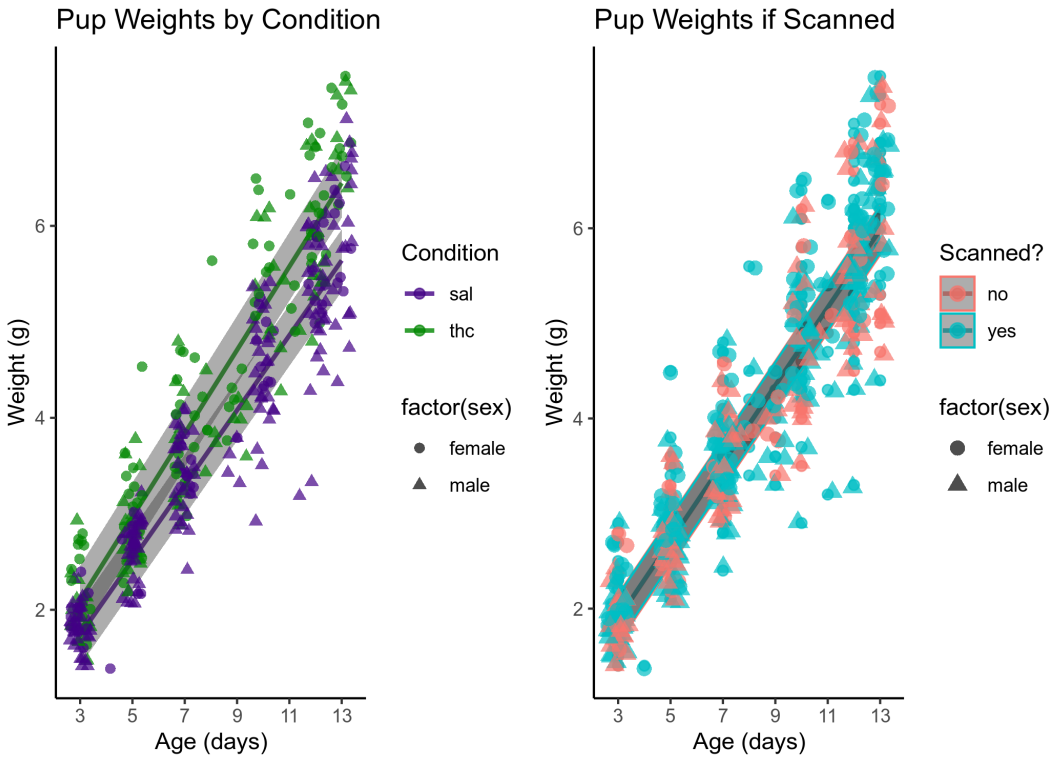


Figure 4.2: a) THC-exposed pups demonstrate a greater growth rate than Sal offspring. b) The covariate, scanned, was not significant for the included pups

4.5.3 Prenatal THC exposure reduces brain growth

After pups were scanned, quality controlled, and processed, the dataset was subset to only high quality scans and pups with at least 2 scans for the LMERs. 18 pups had four scans, 20 had three scans, and 11 had two scans. Neither sex nor condition impacted quality control (QC) scores, SR 4.7.2, SFig 4.11. A table with the number of samples per timepoint can be found in Tab. 4.1

First, results from the whole-brain analysis of absolute Jacobians with LMERs revealed a significant treatment by age interaction (significant from 5% to 1% FDR). The treatment and age interaction shows a very clear phenotype of an altered trajectory between THC and Sal pups, with the rate of growth reduced in regions including the bilateral hippocampus, hypothalamus, amygdala, basal forebrain, cerebellar cortex,

	PND 3		PND 5		PND 7		PND 10		
	Males	Females	Males	Females	Males	Females	Males	Females	Litters
Sal	16	8	13	4	16	9	12	9	8
THC	7	9	8	9	9	11	10	11	7

Table 4.1: Sample per timepoint following quality control. Postnatal day (PND); saline (Sal); delta-0-tetrahydrocannabinol (THC)

frontal lobe, parieto-temporal lobe, inferior and superior colliculi, corpus callosum, olfactory bulbs and tubercles, as well as the left striatum, lateral ventricle, medulla, midbrain, and pons, and the right fornix and thalamus, Fig 4.3. As expected, the main effect of age revealed that the entire brain, other than the lateral ventricles, increased linearly with age (FDR 5% to 1%), Fig 4.4.

Finally, there was a significant effect of pup weight, such that increased weight was associated with larger volume in regions across the brain, including the bilateral amygdala, basal forebrain, bed nucleus of the stria terminalis, cerebellar cortices, parieto-temporal lobe, superior colliculi, corpus callosum, hypothalamus, striatum, thalamus, right entorhinal cortex, and inferior colliculi, Fig 4.5.

The results of the relative Jacobians, which reflect local volume change without the affine component (which encodes gross brain volume changes) are included in SR 4.12.

4.5.4 Alterations in social behavior of THC-exposed pups

There was a sex-specific significant impact of condition as well as a main-effect of sex on duration of calls revealed with the shift function. Specifically, in female pups, THC pups make fewer medium and long calls than Sal pups, Fig. 4.6a. Comparing male and female Sal pups, however, males make fewer calls than females overall, Fig. 4.6b. In male pups, THC pups made more short and medium-length calls than Sal controls, Fig. 4.6c. Examining the difference between calls overall with a t-test does indicate a significant difference ($p<0.001$) however it is unclear where the difference originates, further motivating the shift function 4.6d. The distribution of calls made by Sal and THC pups of both sexes is represented in Fig 4.7, with distributions that significantly differ indicated by parenthetical bars on the right. Additional analyses examining differences in scanned vs. unscanned controls are presented in SR 4.7.2, SFig 4.15.

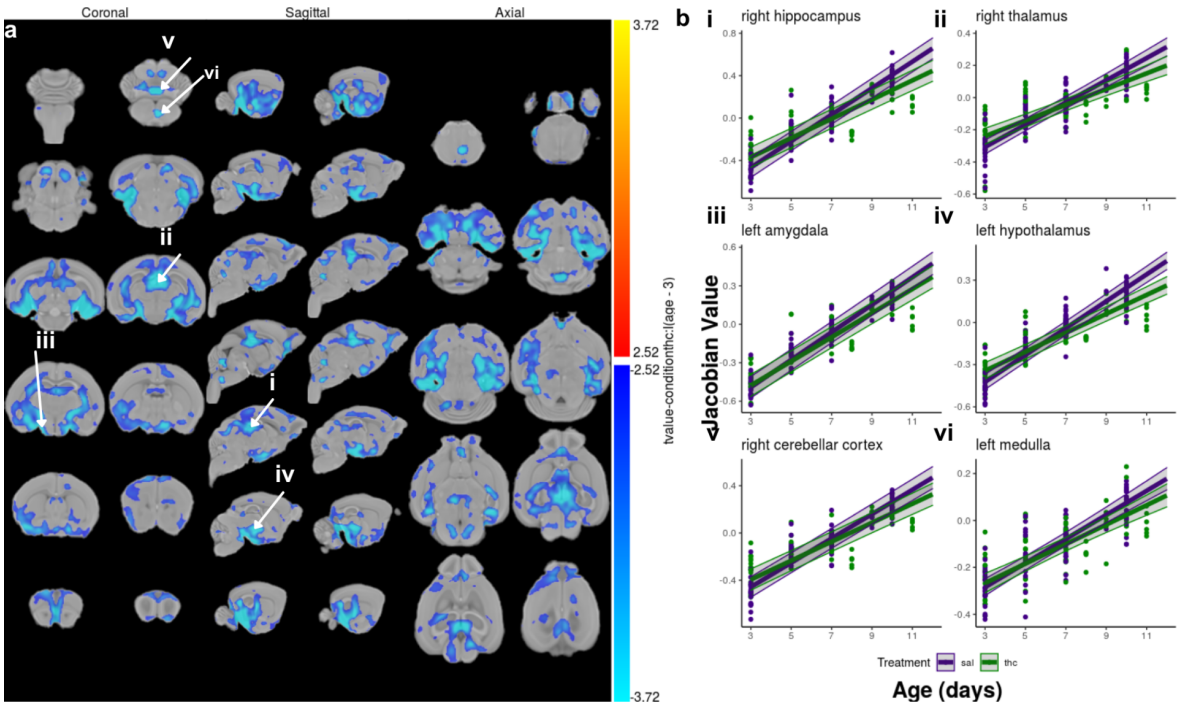


Figure 4.3: a) THC-exposed pups demonstrate a reduced growth rate in voxels (thresholded 5% to 1% FDR) across the brain (blue) than Sal offspring. b) Peak voxels plotted in i) the right hippocampus, ii) right thalamus, iii) left amygdala, iv) left hypothalamus v)right cerebellum vi) left medulla

4.5.4.1 $MnCl_2$ reduced TBV growth and weight gain

MnCl exposure impacted weight such that exposed pups showed reductions in growth rate compared to Sal controls ($p < 0.001$), Fig. 4.8a, full results reported in SR Tab. 4.4. Additionally, MnCl exposure impacted TBV such that exposed pups showed reductions in growth rate of TBV ($p < 0.001$), Fig. 4.8b, full results in SR Tab. 4.5. Further, conducting a mediation with residuals analysis, we investigated whether including the residuals from the weight model reduced the relationship between MnCl exposure and decreased TBV growth rate, but the manganese and age interaction remained significant suggesting the impact on TBV was not due solely to the impact of MnCl on weight.

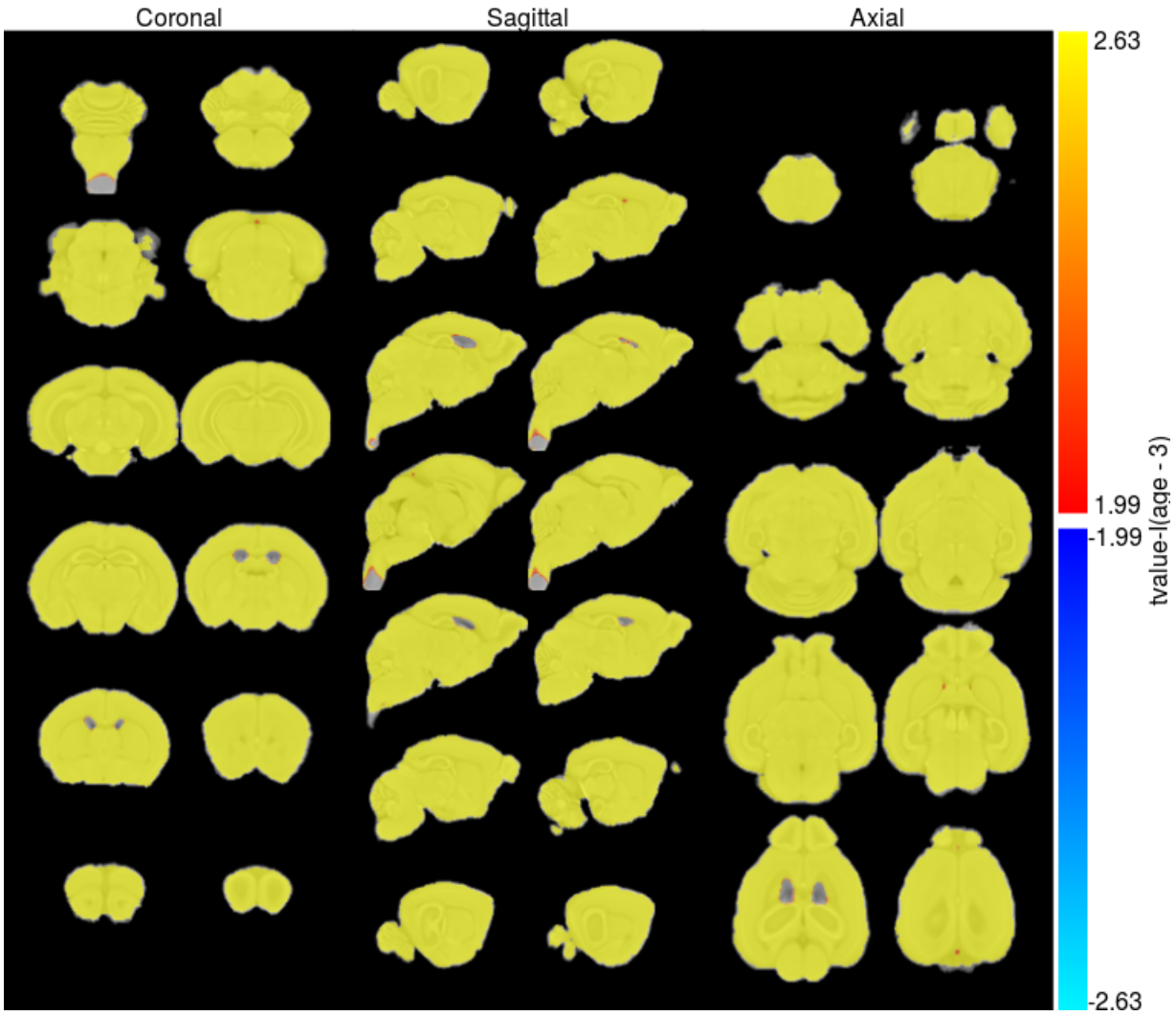


Figure 4.4: Heatmap thresholded from 5% to 1% FDR showing increased age associated with increased brain volume.

4.6 Discussion

The present study reveals increased weight gain in THC-exposed pups, contrasted with a decrease in brain growth rate in many regions, including key cortical and limbic regions. These are accompanied by alterations in USVs profiles of THC-exposed pups. The significance of the age by condition effect emphasizes the importance of longitudinal studies in revealing altered developmental trajectories that might be missed if studies examine single timepoints only.

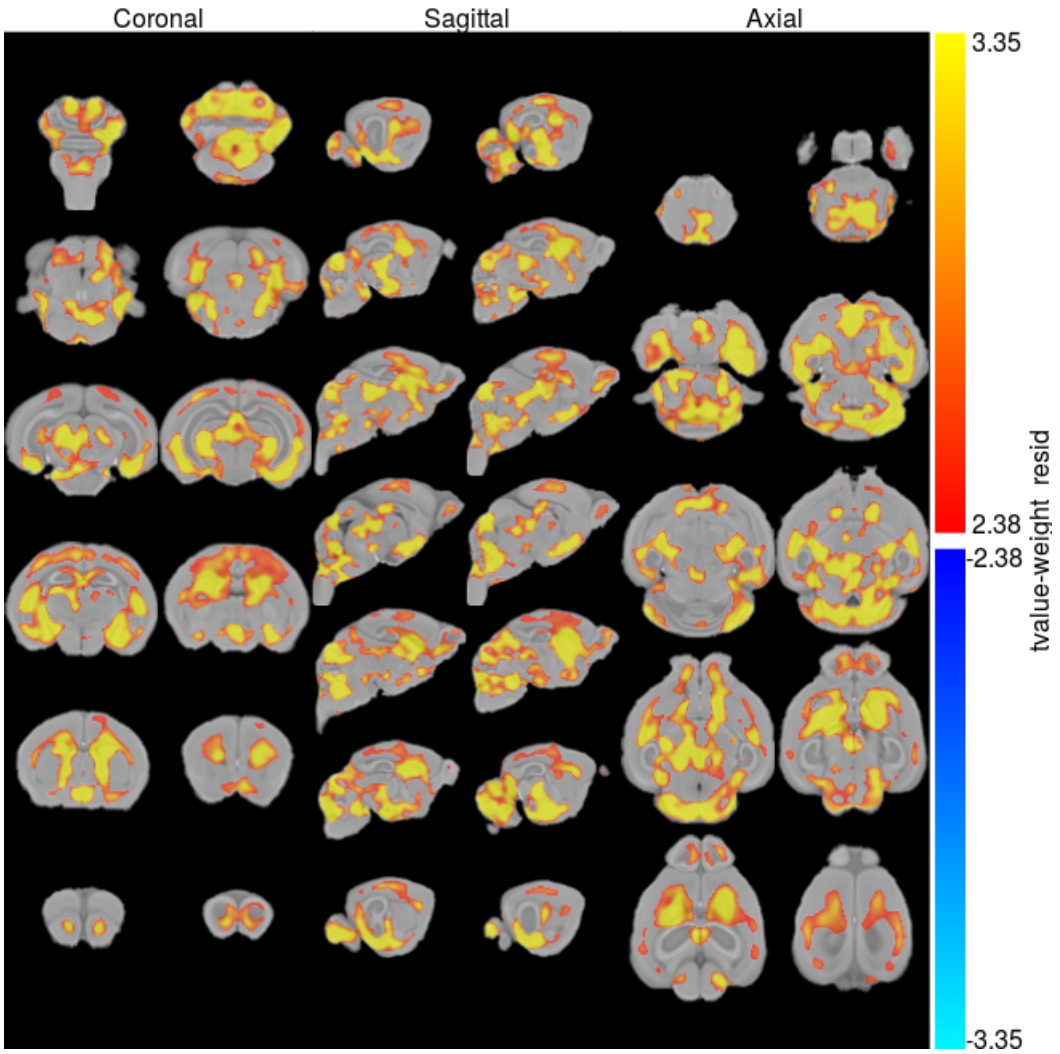


Figure 4.5: Heatmap thresholded from 5% to 1% FDR showing increased weight associated with increased brain volume.

4.6.1 Potential catch-up growth following PCE

From PND3-13, THC-exposed pups exhibit a greater weight gain than controls. While our measurements started at PND3 to reduce stress to the dams as much as possible preceding the scanning period, evidence from our lab and others suggest PCE reduced intrauterine fetus volume [El Marroun et al., 2009, Benevenuto et al., 2017]. In humans, IUGR has been associated with postnatal "catch-up growth", where periods of growth retardation are followed by an increased growth weight [Wit and Boersma, 2002]. Mouse models of IUGR have also observed evidence of catch-up growth in pups when dams are returned from restricted caloric intake to induce IUGR to normal caloric

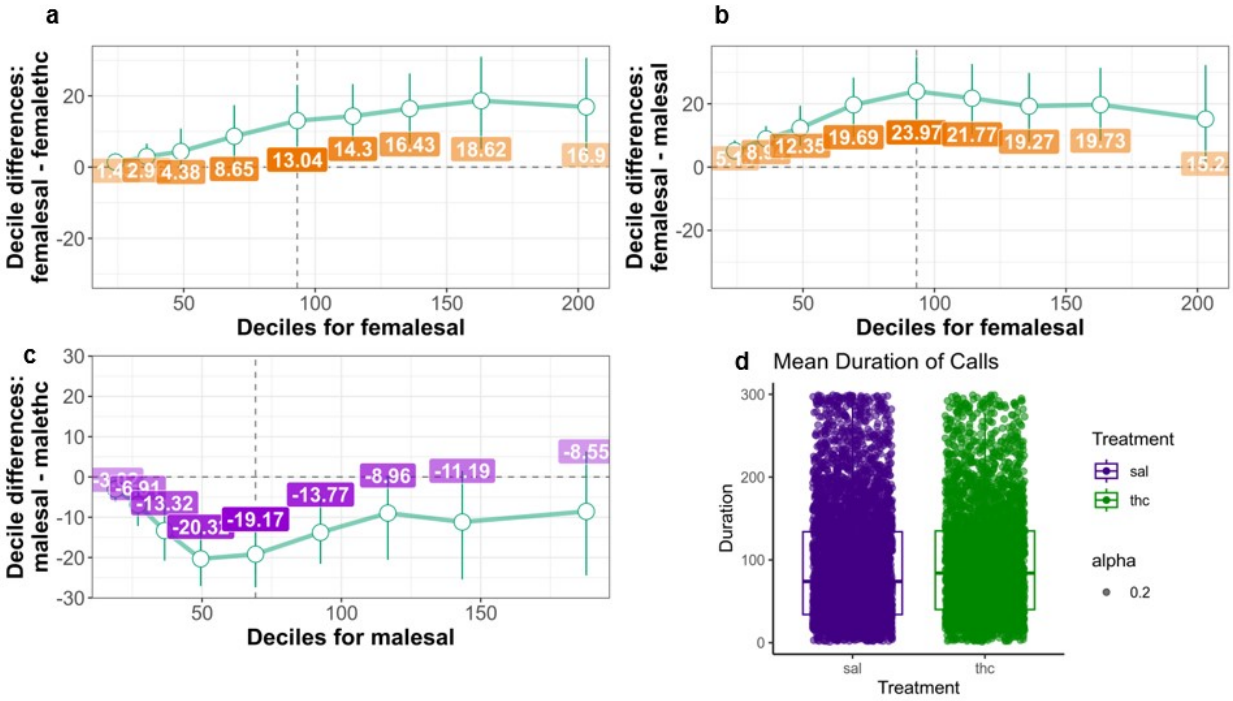


Figure 4.6: Shift function comparing deciles between sex∗treatment groups: female_sal, male_sal, female_thc, male_thc. X-axis indicates the decile values for one group and y-axis indicates the difference between that value and the decile value for the compared group. Negative values indicate the first group's decile is smaller (shorter duration) than the comparison group's value. Confidence intervals were derived from bootstrapping, and where the confidence interval does not cross 0, the decile difference is significant. a) Displays comparison between female_sal and female_thc groups. Deciles for female_thc group are lower (shorter duration) than female_sal group, indicated by positive differences. b) Differences between female_sal and male_sal. c) Differences between male_sal and male_thc. d) Boxplot of calls overall, significant ($p<0.001$) with t-test, but uninformative compared to distributional analyses.

intake post-partum [Duran Fernandez-Feijoo et al., 2017]. Because there was an interaction between age and THC-exposure and the growth curves continued to separate over the observed period, it is possible that pups were born at a lower weight than controls and overtook the controls while nursing. It is yet unclear whether increases in weight gain are associated with alterations in metabolic function of the THC-exposed pups, or alterations in maternal behavior in the THC-exposed dams, a question outside

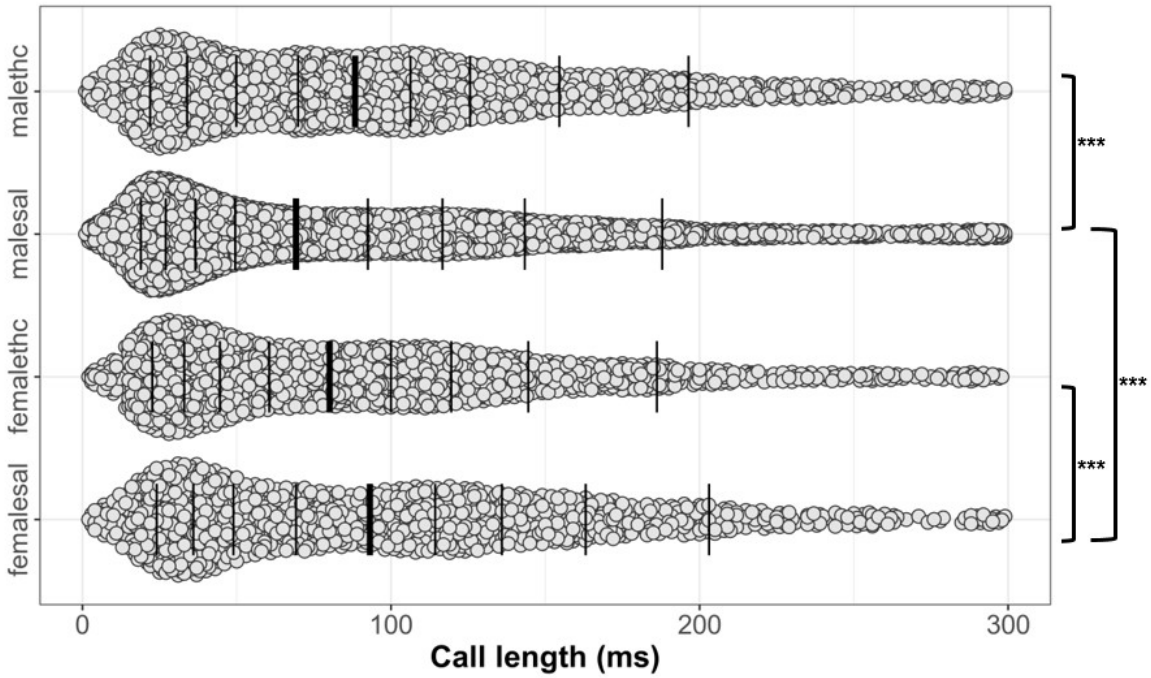


Figure 4.7: Distribution of calls made by male_thc, male_sal, female_thc, and female_sal (top to bottom) groups. Lines indicate decile values and bold line indicates the median. Distributions clarify directionality of significant differences from shift function.

of the scope of the present study which future studies should consider. Importantly, scanning did not impact the weight gain of pups, suggesting time separated from the dam did not alter the growth rate, either because of access to dam or dam rejection.

Because catch-up-like growth curves growth might have followed IUGR prenatally, this value might influence brain volume over development, however because of the impact of THC on weight gain, simply including weight as a covariate in the model might have introduced colinearity into it. Therefore we chose to take a regression-with-residuals approach by extracting the residuals from the THC-on-weight model and including them as a covariate in the model of prenatal condition on brain volume. This approach ensures that the condition-by-age interaction effect reflects the impact of PTE over time rather than a confounding effect of weight.

In general catch-up growth has been implicated in later development of obesity and cardiovascular disease [Claris et al., 2010]. These effects have not, to my knowledge, been demonstrated following PTE. Instead, PTE has been associated with sustained

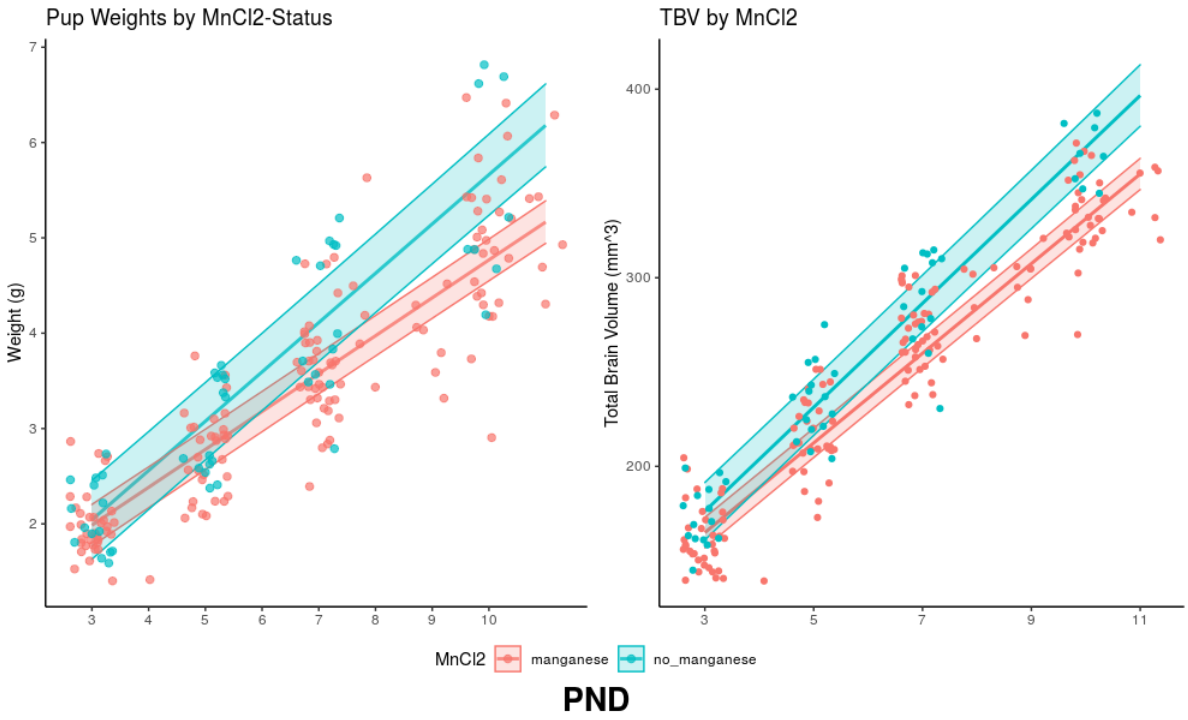


Figure 4.8: a) MnCl impacts weight gain such that exposed pups gain less weight than unexposed pups. b) MnCl impact TBV such that exposed pups show decreased growth rate in TBV compared to unexposed pups

reductions in growth weight and weight gain [Hume et al., 2023]. The underlying cause of these metabolic alterations are unknown and somewhat at odds with our early findings of increased growth rate, however it is possible that these metabolic changes consistent with decreased weight emerge later in life, as in the cited study weight is measured in adolescence and adulthood [Hume et al., 2023]. Still, it would be worthwhile to examine the potential emergence of cardiovascular changes in later life following PTE given the occurrence of both IUGR, the potential brain-sparing, and catch-up growth.

4.6.2 PCE is associated with whole-brain growth delay

Examining the impact of PCE on absolute Jacobians across the brain reveals a signature of growth retardation in regions including the dorsal and ventral hippocampi, cortical regions, basal ganglia, amygdala, and cerebellum. While volumes at PND 3 start at the same volume, or even a little larger in THC offspring compared to

Sal controls, they grow at a different trajectory, and between days 8 and 11 show smaller volumes than controls. Larger volumes are not always better, however reduced growth rates could implicate several underlying mechanisms responsible for lasting brain alterations. For example, increased levels of endocannabinoids early in life have been associated with increased microglial phagocytosis of astrocytes demonstrated in the amygdala [VanRyzin et al., 2019]. Phagocytosis or apoptosis of neural and glial cells could underly smaller increases in brain volume. Additionally, evidence suggests prenatal THC exposure contributes to misguiding axons, reducing SGC10, a protein responsible for microtubule integrity [Tortoriello et al., 2014]. This could alter the morphology of white matter tracts and contribute to the ectopic formation of filopodia [Tortoriello et al., 2014]. In turn, axons that terminate off-target may fail to form active synapses, resulting in greater synapse elimination during circuit refinement [Yasuda et al., 2021]. The present study cannot answer whether smaller cell or synapse counts are responsible for the decreased growth rate, but the whole-brain MRI approach highlights regions for future investigation with transcriptomic or immunohistochemical approaches.

In addition to the whole-brain growth retardation, examination of relative Jacobians that do not include gross brain changes indicate regions that are relatively decreased or preserved. Notably, many regions show decreased growth rate in the relative Jacobians as well as the absolute Jacobians, such as the bilateral hippocampi, and frontal cortical regions.

4.6.3 Value for human studies

Early neonatal brain development in mice more closely resembles third trimester brain development in humans [Zeiss, 2021]. While results cannot be directly transferred from mice to humans, it is possible that these results highlight the most vulnerable regions and brain networks susceptible to PTE. MRI results suggest that longitudinal neonatal studies may be able to establish whether trajectories reflecting reduced growth rate are present in human neonates as well. Further research involving environmental interventions in nonhuman animals may also highlight nonpharmacological interventions that could be useful to human infants with known PCE, such as environmental enrichment, which has shown promise in other prenatal environmental exposures [Núñez Estevez et al., 2020]. Longitudinal MRI may be able to provide a bridge between nonhuman animal and human studies, or could, in time, provide evidence for the efficacy of various interventions.

4.6.4 PCE induces a sex-specific anxiety-like phenotype in neonates

While the whole-brain voxelwise analysis reveals significant differences between the Sal and THC groups, the relevance to human development, especially potential neurodevelopmental disorders can only be assessed with behavioral analyses. We chose to assess maternal separation-induced USVs to examine whether there was a difference in pup behavior. The interpretation of increased or decreased number and duration of USVs in neonatal rodents has been debated. It has been suggested that USVs are a by-product of increased heart rate in neonates [Blumberg and Sokoloff, 2001], but increased USVs have been associated with later-life anxiety-like phenotypes [Budylin et al., 2019], and benzodiazapenes are effective in reducing USVs in mouse pups [Takahashi et al., 2009]. The approach we take, examining the distribution of call frequency based on deciles of call duration allows us to identify a sex-specific difference in the impact of THC exposure. In females, THC-exposed pups make fewer medium- and long-duration calls than Sal controls, whereas in males, THC pups make *more* short- and medium-length calls than Sal controls. In-line with prior literature, we interpret this as an anxiety-like phenotype in the THC-exposed male pups [Budylin et al., 2019, Caruso et al., 2020]. There was also an overall sex effect in the Sal group, such that female pups made more calls overall. This makes it difficult to assess whether the reduced calls in the female THC group compared to the female Sal group indicates the lack of an adaptive social behavior (calling for the dam) in THC-exposed females [Chang et al., 2017b], or whether THC was protective against other factors contributing to anxiety in the female Sal group.

Additionally, there was a significant difference between scanned and un-scanned pups, such that pups that were not scanned made more medium-length calls than the scanned pups. It is possible that the separation from the dam increased their anxiety-like behavior, or that scanned pups were habituated towards the separation because of scanning separations on previous days. It is also possible that isoflurane exposure may have increased the anxiety-like pheontoype of these pups. To our knowledge, the impact of repeated exposure to isoflurane in neonates has not been assessed in terms of USVs, however it does increase susceptibility to chronic variable stress, potentially indicating priming for anxiety-like behavior [Peng et al., 2021]. Because both Sal and THC pups are exposed to the stressors of scanning and isoflurane, Sal mice should present an effective control, however it is possible that the gestational exposure could make THC dams more sensitive to postnatal stressors, and it cannot be ruled out

entirely that this added stressor contributes to THC results. To answer this question, future studies focused on the impact of PTE on maternal behavior could assess whether dams respond differently to early life stressors, however to our knowledge, this has not been done.

While we are interpreting increased call frequency as anxiety-like behavior, it is important to consider how calls may either reflect or cause differences in maternal behavior. For example, pups that call more, may receive greater maternal attention. In turn, pups that are accustomed to receiving more maternal attention may call more during periods of separation. This could further motivate future studies to include investigations of maternal behavior to see whether or not it is altered in the THC condition.

4.6.5 Potential impact of MnCl exposure

We believe it is important to consider the potential effects of MnCl on growth trajectories of pups, especially including potential interactions with prenatal THC exposure. In the MnCl investigation, we identified several effects, potentially of interest. First, we replicated previously-documented effects of reduced weight in pups exposed to MnCl [Szulc et al., 2015]. Second, we found MnCl reduced the growth rate of TBV, however our data suggest mouse weight is an incomplete mediator of the relationship between MnCl exposure and TBV. While the analysis of TBV is not as nuanced as local volume changes, which were precluded by reduced quality in the non-MnCl exposed group, the data suggest MnCl impacts overall development and may also have a specific effect on the brain.

4.6.6 Limitations

There are several important limitations to the present study. First, as mentioned before, all pups were necessarily exposed to MnCl to facilitate scanning. While both the Sal and THC pups were exposed to the contrast agent, it is possible that there could be an interaction between prenatal THC exposure and post-natal THC exposure. The contrast agent cannot be disregarded when comparing our results to other studies that do not need exposure to MnCl.

Second, while we compared scanned pups to un-scanned controls on several outcomes, the sample size was very different, since for every litter of 6 pups, 4 pups were scanned leaving only 2 un-scanned controls. Results from these comparisons should be

viewed with caution given the discrepancy in sample size.

Third, the behavior test for USVs was conducted after scanning was completed to avoid extra stress on pups and dams. As a result, conducting the test on PND 12 means vocalizations were conducted later than days corresponding with peak vocalizations in C57/Bl6 mice, where USVs peak around PND 3 [Scattoni et al., 2009]. Additionally, the equipment and software used to collect and analyse the USVs data did not facilitate analysis of more complex aspects of the calls, such as the spectographic shape.

Fourth, while the phenotype of reduced brain growth is striking, without cellular or molecular investigations into the underlying effects, the utility of the finding in identifying potential mechanisms is limited. Nevertheless, this falls outside of the scope of the present study. Results are translatable to human early life imaging studies and assessments of anxiety in infants, but a further limitation in mouse-human translation is the choices of administration route, dosage, and timing of the experimental design.

4.6.7 Contributions

The present study offers several scientific contributions. Using MRI and behavioral analyses, we offer a characterization of the early life effects of moderate-high, early-gestational exposure to THC. We establish a pattern of weight catch-up, accompanied by a reduction in growth rate in regions across the brain. Further, we show increased anxiety-like behavior in male THC pups and females. Future research could investigate regions of interest identified in the study with cellular and molecular techniques, examine similar phenotypes in human neonates with known cannabis exposure, or follow THC-exposed offspring later in life to establish whether the altered developmental trajectories continue.

4.7 Supplementary Information

4.7.1 Supplementary Methods and Materials

4.7.1.1 Animals

All C57BL/6J mice were housed within the animal facility under controlled environmental conditions, residing in conventionally ventilated cages. A 12-hour light-dark cycle (8:00 AM - 8:00 PM) was maintained throughout the study, and the mice were provided with unrestricted access to standard rodent chow and water (*ad libitum*).

Female and male mice of reproductive maturity, aged between 8 to 12 weeks, were

selected for timed mating procedures. The mating process commenced in the late afternoon, specifically between 3:00 PM and 5:00 PM. During this period, one male and one female mouse were introduced into a fresh cage. The following morning, between 8:00 AM and 9:00 AM, the male mouse was removed from the cage. Subsequently, the female mouse underwent examination for the presence of a seminal plug and was weighed on a scale calibrated to record measurements to the tenths of grams.

To enhance the precision of timing with respect to mating, mice were allowed to pair for a single night. The observation of a seminal plug marked the initiation of the gestation period, designated as GD 0. In instances where no seminal plug was detected, a minimum interval of nine days was permitted before reevaluating the female mouse's weight to ascertain any pregnancy-related weight gain exceeding 2 grams. If the female remained within the reproductive age range, another attempt at timed mating was undertaken.

Should the female mouse exhibit signs of pregnancy, she was either humanely sacrificed or utilized for further experiments, or as part of the next generation of breeding parents. Male mice were subjected to multiple mating sessions as needed for the research.

4.7.1.2 Drug preparation

In order to eliminate any potential impact of residual ethanol within the formulation, the solution containing THC (obtained from Cayman Chemical Company, Ann Arbor, Michigan, USA) in ethanol was subjected to dehydration using a Universal vacuum system (UVS 400/supervac SCIIOA). This process resulted in the production of a highly viscous, dehydrated form of THC.

Due to the lipophilic nature of THC, it necessitated dissolution in a vehicle comprising a 1:18 ratio of cremophor (obtained from Sigma-Aldrich) and saline. A stock solution of THC with a concentration of 0.5 mg/mL was prepared to achieve an injection volume of 0.01 mL per gram of body weight at a dosage of 5 mg/kg. Subsequently, this stock solution was distributed into 1.5 mL Falcon tubes and promptly frozen to prevent degradation of THC due to exposure to light and heat.

Simultaneously, a vehicle solution containing a 1:18 mixture of cremophor and saline was also prepared, aliquoted, and frozen to ensure that neither cremophor nor freezing introduced any confounding factors in the experiments.

The validation of the dosage in the preparation and the justification for the human equivalent dose have been thoroughly discussed in previous work [Guma et al., 2023].

4.7.1.3 Pup milestones

Pup milestones were adapted from previous research. They were designed to be as minimally invasive as possible and, therefore, assessed only on days when the pups were separated from their dams for scanning or behavioural assessment. First, pup weight was assessed at PND 3, 5, 7, 10, 12, and 13. The anogenital distance was assessed at PND 3, when pups were sexed. Incisor eruption was assessed at PND 10 and 12. Fur development was assessed on PND 3 and 5. Eye opening was assessed on PND 12 and 13. Pinnae detachment was assessed at PND 3 and 5. Auditory canal opening was assessed at PND 12 and 13. Vibrissa placing response was assessed by tickling the whiskers with a Q-tip and marked as present if the mouse turned its head towards the Q-tip. It was assessed on PND 5, 7, 10, 12, and 13. Ear twitch was assessed by tickling the pinnae of the ear on PND 10 and marked as present if the ear twitched. Auditory startle response was assessed on PND 7, 10, 12, and 13, by clapping the hands and assessing whether the mouse flinched in response. Surface righting was assessed on PND 7. Mice were held supine and, when released, a stop watch was started. If the mouse turned prone in less than 1 second, surface righting was considered present. Grasp reflex was assessed on PND 5, 7, 10, 12, and 13. The middle section of a Q-tip was brushed against the forepaws of the pup, and if it curled its paws around the bar and exerted some force when the bar was drawn away, grasp was assessed as present. For all behaviors and physical milestones, the pup was assessed for first appearance of the milestone, with the exception of weight which was assessed on all specified days.

4.7.1.4 Pup USVs

USVs were assessed on PND 12, 48 hours after the final scan. All mice from the litter were assessed, regardless of whether they were scanned or not. Additionally, due to technical malfunctions, data was available from a restricted sample. For all sex and condition analyses, data from pups unexposed to MnCl were included in the analysis, see Section 4.7.1.7 for details. The sample size is reported as follows, with MnCl-exposed pups in parentheticals: Sal female - 12 (7), THC female - 17 (15), Sal male - 32 (26), THC male - 20 (12). USVs were assessed with procedures that have been previously reported [Guma et al., 2021b, Baharnoori et al., 2012, Fernández de Cossío et al., 2017]. The dam was removed to a clean cage and pups were tested one-by-one. The home cage was placed on a heating pad and in turn each pup was isolated in the behavioral testing room, placed in the apparatus of the Noldus UltraVox^{TM} system (Noldus Information Technology, Leesburg, VA). The Plexiglas box was also placed on a heating pad. After

USVs were collected, the mouse was weighed and any milestones collected before it was placed back in the home cage. Pups were rubbed with bedding material following the final pup's testing and the dam was replaced in the cage.

4.7.1.5 Perfusion and brain extraction

One day after the behavioral tests, on PND 13, pups were given an interperitoneal injection of ketamine, xylazine, and acepromazine at a dose of 0.1 mL/100 g before being transcardially perfused. Perfusion was performed using 1x Phosphate Buffered Saline (PBS) solution with heparin, followed by 4% PFA. Brains were extracted from the skull and post-fixed in the same paraformaldehyde (PFA) solution for 24 hours. After 24 hours, the brains were transferred from the PFA-PBS solution to a solution of PBS and 30% sucrose for cryoprotection.

4.7.1.6 Image processing

First, images were converted to niftis with the BrkRaw module tool, brkraw_tonii [Lee, 2023]. Next, they were quality controlled by two independent raters (L.C. and A.P.). Each scan was examined for motion and artefacts and given a score from 1 to 5, with 1 being the best and 5 being unusable. Scans that received a 1 or 2 from both raters were included, those that received a 4 or 5 were excluded, and scans that received a 3 or where there was disagreement were examined again for a consensus to be reached. Next, all scans were pre-processed with an in-house pipeline using Advanced Normalization Tools (ANTs) tools to denoise each image with adaptive nonlocal means (minc_anlm) and apply an N4 bias-field correction [Manjón et al., 2010, Tustison et al., 2010].

Following preprocessing, in-house software was used to conduct a twolevel model-build, first registering all images within a subject, and then across subjects to create a population average [Devenyi, 2023]. In each level of the model build, all images were aligned with affine transformations that preserved parallel lines in the images (translations, rotations, scales, and shears). Then, iterative nonlinear transformations attained precise anatomical alignment between images in an automated fashion, similar to previous studies in adult animal brains [Guma et al., 2023], as well as embryo studies in our laboratory [Guma et al., 2022] and *ex vivo* neonatal studies [Guma et al., 2021b].

4.7.1.7 Manganese Chloride Investigation

In order to achieve sufficient contrast for accurate registration of structural images in neonatal mice with MRI, manganese chloride must be administered to the

dam and absorbed by the pup through milk [Qiu et al., 2018, Wadghiri et al., 2004, Szulc et al., 2015]. Nevertheless, despite the necessity of the contrast agent, there is evidence of its adverse impact on development. Studies examining models for environmental exposure to manganese report alterations in locomotion, novelty seeking, and hyperactive behaviors in juvenile male mice, as well as increases in striatal dopamine levels [Moreno et al., 2009]. Exposed neonatal rodents also display reduced body weight gain and increased startle response [Dorman et al., 2000]. Importantly, however, the dosing regimens and administration routes differ in studies modelling environmental exposure to manganese and those investigating the impact of MnCl as it is administered for manganese-enhanced MRI. Studies that specifically look at early MnCl exposure as a contrast agent and compare the impact on young mice better reveal the potential impacts. Such studies re-iterate the impact of MnCl on body weight: scanned pups exposed to MnCl show decreases in weight, peaking at 20% at PND 6-10 and recovering to 7% at PND 20 [Szulc et al., 2015]. Comparing brain volume in neonates that received MnCl every other day to those who received a single dose before the scan, it was found that the brains of pups with repeated exposure to MnCl were 10% smaller than controls at PND 11, but only 6% smaller at PND 21 [Szulc et al., 2015].

4.7.2 Supplementary Results

4.7.2.1 Impact of THC on pregnancy outcomes

THC dams showed a slightly altered trajectory of weight gain during the course of the injection, such that THC-exposed dams gain weight more slowly than controls ($p = 0.049$), Fig. 4.9a. Importantly, there is no difference in the initial weight of dams before being assigned to either condition, Fig. 4.9b.

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	24.11	1.17	12.37	20.63	0.00
TreatmentTHC	1.33	0.72	14.35	1.85	0.08
Day	0.23	0.02	118.00	9.05	0.00
litter_size	-0.15	0.15	12.00	-1.02	0.33
TreatmentTHC:Day	-0.07	0.04	118.00	-1.99	0.05

Table 4.2: Results from the LMER examining the interaction of treatment and day on weight gain.

Treatment did not impact the size of litters, Fig 4.10a or the ratio of female pups,

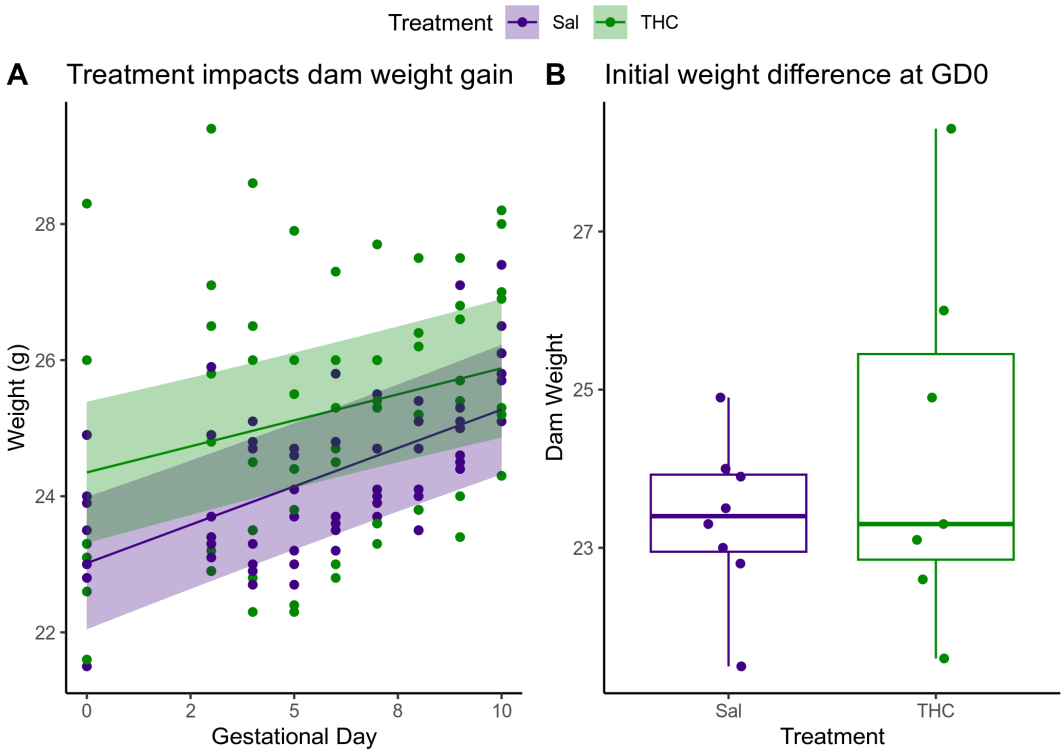


Figure 4.9: a) Trajectories of weight gain in THC and Sal dams. b) Initial weight before onset of treatment

Fig 4.10b.

4.7.2.2 No impact of treatment on quality control

Quality control of images was assessed with an LMER in case treatment impacted the amount of movement or the effect of isoflurane on the pups, however, there was no difference between groups, seen in SFig. 4.11.

4.7.2.3 Impact of PTE on weight gain

Age and the interaction between age and THC impact weight gain, with full results from the model in Tab. 4.3.

4.7.2.4 Whole brain, voxelwise analysis with relative Jacobians

The relative Jacobians encode local volume changes without the affine component that represent global changes. First, there was a significant interaction of condition and age (5% to 1% FDR) 4.12, demonstrating relatively reduced volume growth comparing

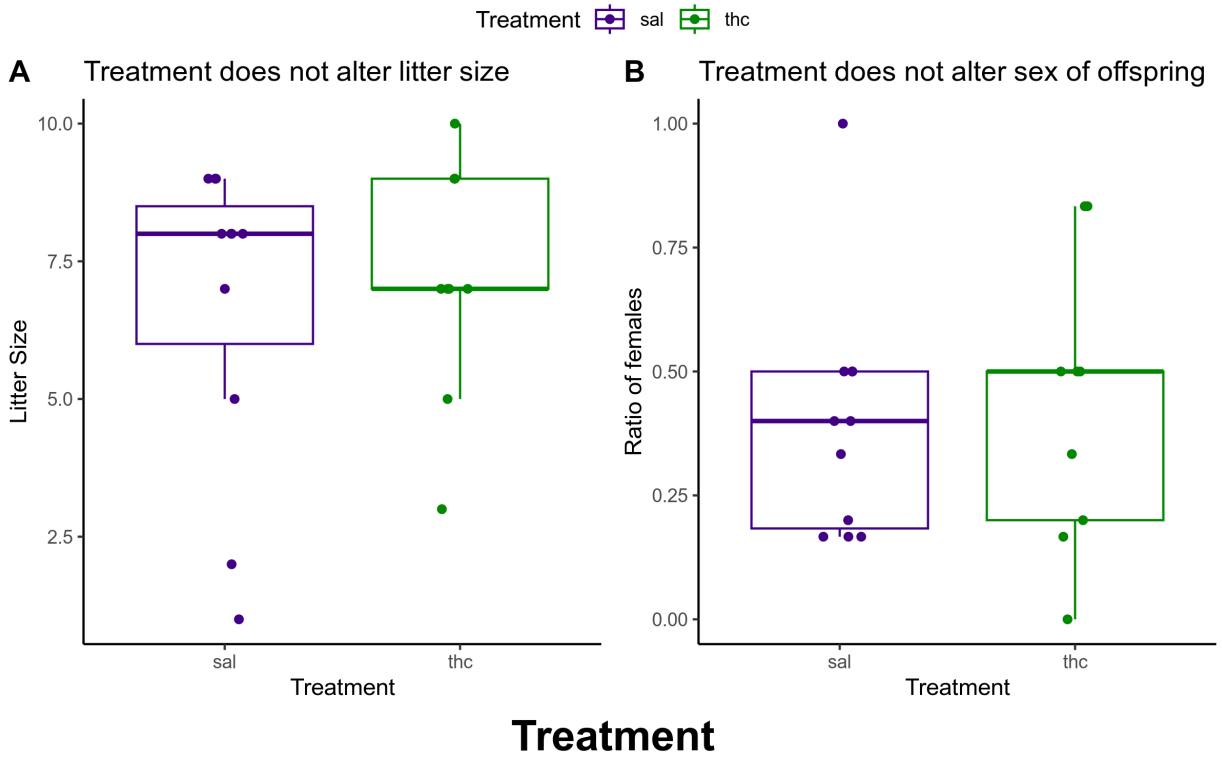


Figure 4.10: a) No difference in size of litter between conditions, as counted pre-culling b) No difference in sex ratio of pups, as counted at PND 3

the THC to Sal pups in the bilateral hippocampi, frontal cortices, and olfactory bulbs, as well as the left parieto-temporal lobe and cerebellar cortex, and the right stria medullaris and corpus callosum. The results also indicated increased volume over time comparing the THC to Sal pups in the bilateral thalamus and nucleus accumbens, as well as the left inferior colliculus, hypothalamus, striatum, medulla, midbrain, and pons.

Second, there was a main effect of THC (significant at 10% FDR indicating a larger volume in the bilateral hippocampus, as well as the right stria medullaris, SFig. 4.13.

The age effect (5%-1% FDR) was interesting insofar that it indicated regions that increased in volume over time including most of the cortex, limbic system, and basal ganglia, but also indicated regions that decreased over time, such as in the midbrain, ventricles, and posterior cortex 4.14. Importantly, the absolute Jacobians indicate these regions increase overtime, but the relative Jacobians suggest that they decrease relative to the whole brain.

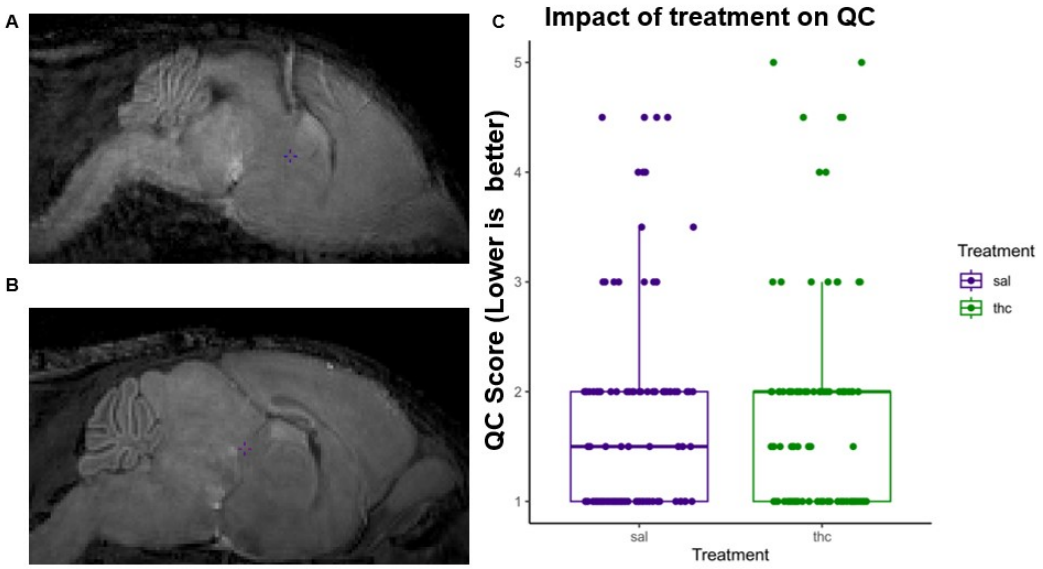


Figure 4.11: a) Unused scan from PND 10, low quality due to motion b) Acceptable scan from PND 10 c) Distribution of scan quality based on condition, where 1 was a perfect scan, 2-3 had minor artefacts or motion, and 4-5 were unusable. Analyses were repeated with high quality scans (1 or 2) without changing results.

4.7.2.5 Impact of scanning status on anxiety-like behavior

A final comparison between scanned and un-scanned pups, regardless of condition reveals un-scanned pups make more medium-length calls than scanned pups, SFig. 4.15

4.7.2.6 $MnCl_2$ impact on TBV and weight

As reported in the main body, MnCl exposure reduced the growth of TBV and weight gain. Full results from the LMERs are reported below with the weight model in Tab. 4.4 and the TBV volume in Tab. 4.5.

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	0.62	0.20	28.56	3.19	0.00
conditionthc	0.31	0.27	20.61	1.14	0.27
age	0.39	0.01	332.29	42.90	0.00
sexmale	-0.09	0.13	160.49	-0.72	0.47
total_scannedyes	0.00	0.07	59.08	0.06	0.95
conditionthc:age	0.04	0.01	329.75	3.00	0.00
conditionthc:sexmale	-0.13	0.19	168.10	-0.69	0.49
age:sexmale	-0.00	0.01	330.73	-0.42	0.67
conditionthc:age:sexmale	0.01	0.02	330.50	0.83	0.41

Table 4.3: Results from the LMER examining the interaction of treatment, PND, and sex on pup weight gain.

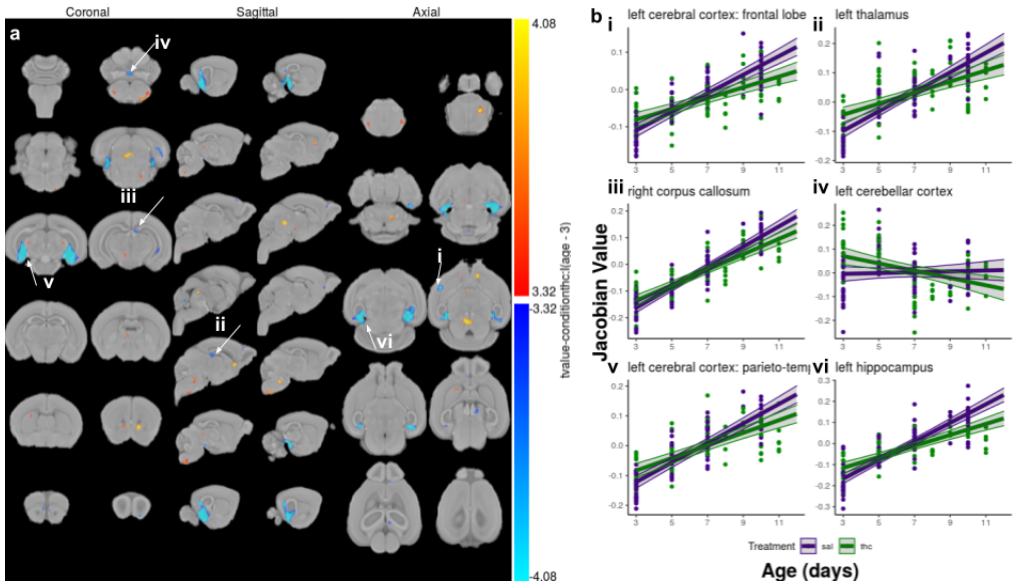


Figure 4.12: a) In relative Jacobians, THC-exposed pups demonstrate a reduced growth rate in voxels (thresholded 5% to 1% FDR) across the brain (blue) than Sal offspring, but an increased rate in other areas (warm colors). b) Peak voxels plotted in blue regions in i) left frontal lobe ii) left thalamus, iii) right corpus callosum, iv) left cerebellar cortex, v) left parieto-temporal lobe, and vi) left hippocampus

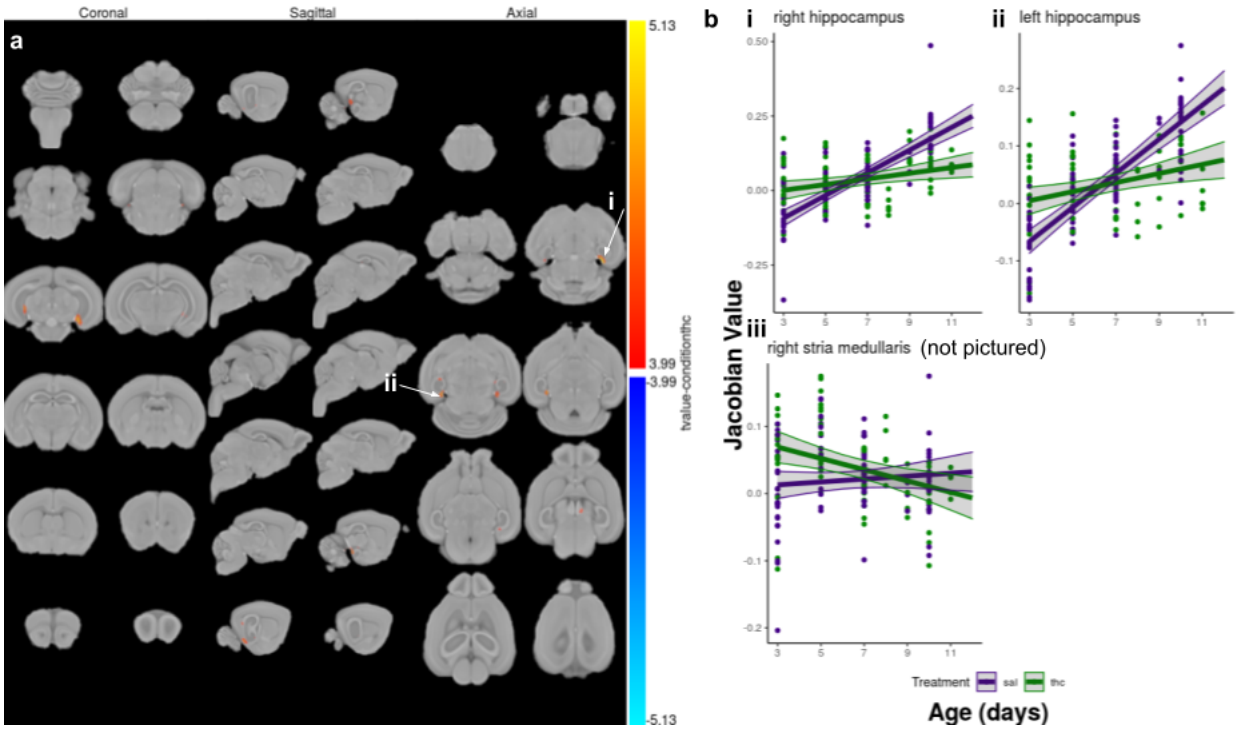


Figure 4.13: a) Heatmap thresholded from 5% FDR to maximum indicating regions that have a main effect of condition. b) Peak voxels plotted in the i) right and ii) left hippocampi, as well as the right stria medullaris

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	0.72	0.16	22.67	4.56	0.00
MnCl2no_manganese	-0.12	0.35	18.12	-0.33	0.75
age	0.37	0.01	134.91	35.59	0.00
conditionthc	0.15	0.24	22.68	0.63	0.54
MnCl2no_manganese:age	0.20	0.02	132.27	8.86	0.00
MnCl2no_manganese:conditionthc	-0.41	0.50	18.56	-0.81	0.43
age:conditionthc	0.05	0.02	138.09	3.20	0.00
MnCl2no_manganese:age:conditionthc	-0.18	0.03	133.46	-5.41	0.00

Table 4.4: Results from the LMER examining the interaction of treatment, age, and manganese status on pup weight gain.

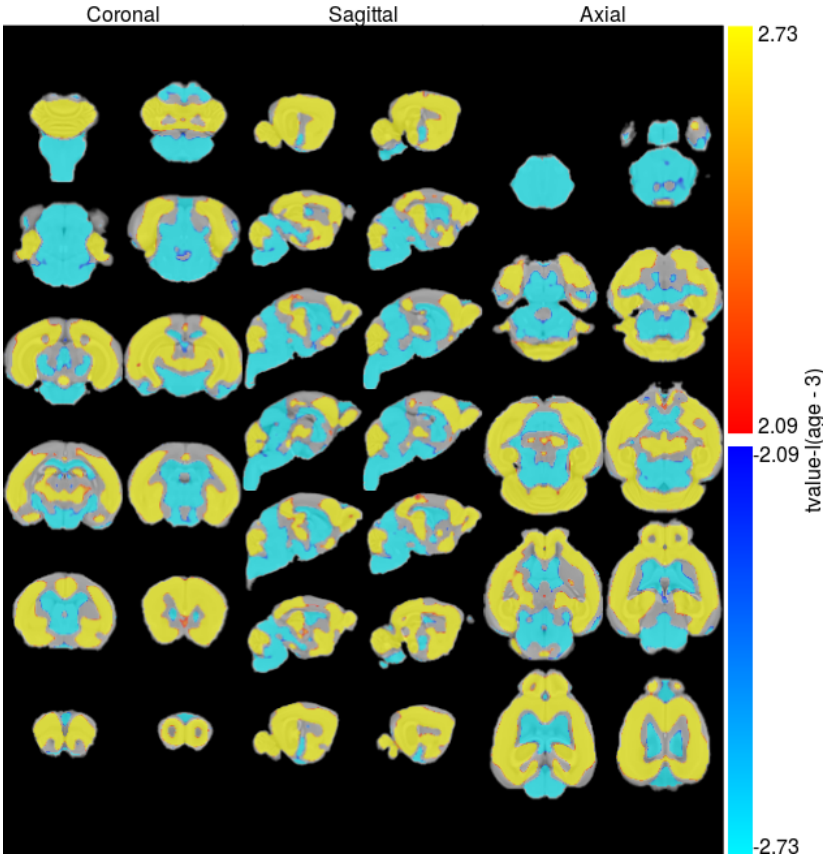


Figure 4.14: Heatmap thresholded from 5% FDR to 1% indicating change in volume overtime, irrespective of the overall increase in brain size.

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	84.17	5.93	24.75	14.19	0.00
MnCl2no_manganese	17.30	13.08	19.80	1.32	0.20
age	24.10	0.44	137.23	54.41	0.00
conditionthc	20.42	9.00	25.14	2.27	0.03
MnCl2no_manganese:age	4.38	0.97	133.05	4.51	0.00
MnCl2no_manganese:conditionthc	-37.73	18.89	20.44	-2.00	0.06
age:conditionthc	-0.73	0.68	141.56	-1.07	0.29
MnCl2no_manganese:age:conditionthc	-1.29	1.42	134.95	-0.91	0.36

Table 4.5: Results from the LMER examining the interaction of treatment, age, and manganese status on pup TBV growth.

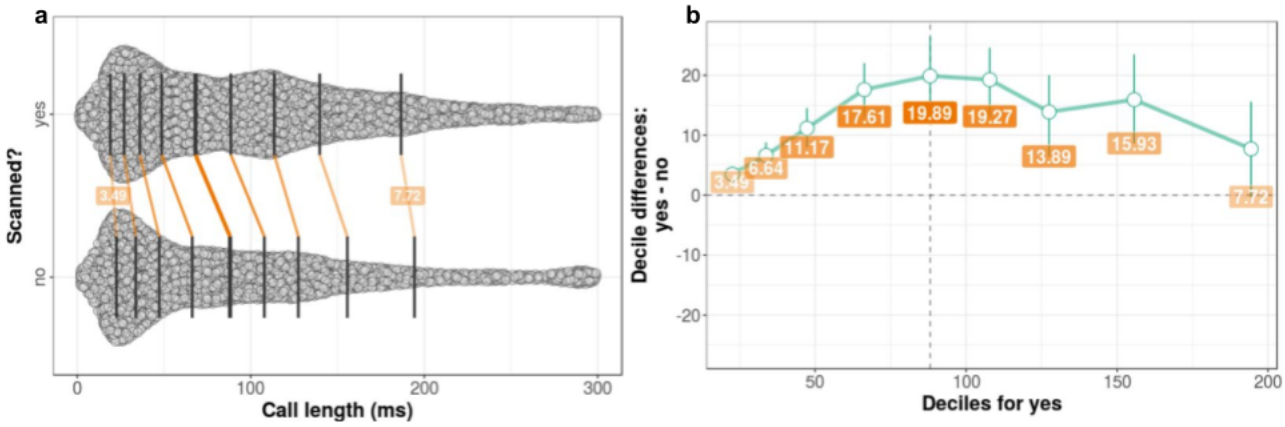


Figure 4.15: a) Distribution of calls made by scanned and un-scanned pups. Lines indicate decile values and bold line indicates the median. Orange lines connect the plots indicating un-scanned decile is at a higher duration than scanned decile. b) Shift function comparing deciles from scanned and un-scanned pups. X-axis indicates the decile values for the scanned group and y-axis indicates the difference between that value and the decile value for the un-scanned group, where positive values indicate the scanned decile is smaller (shorter duration) than the unscanned value. Exact difference is indicated in the orange boxes. Confidence intervals were derived from bootstrapping, and where the confidence interval does not cross 0, the decile difference is significant

Chapter 5

Impact of chronic prenatal exposure to THC early in gestation on trajectories of murine development in adolescence and adulthood

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5.1 Preface

The work presented in **Chapter 5** expands on the work presented in both **Chapter 3** and **Chapter 4** by investigating whether changes following prenatal THC exposure (PTE) early in life persist through adolescence into adulthood. Using an identical administration as previous chapters, we employed *in vivo*, longitudinal whole-brain structural magnetic resonance imaging (MRI) at postnatal day (PND) 25, 35, 60, and 90. After the second scan, we examined anxiety-like and sensorimotor behavior with the open-field test and prepulse inhibition test respectively, allowing two days for rest between tasks. By weighing subjects carefully at each timepoint, we extended the analysis of embryonic intrauterine growth restriction (IUGR) and early postnatal catch up growth into adulthood. Finally, in addition to litter-level outcomes, we incorporated two metrics of maternal behavior: nest-building before and after the onset of prenatal injections and recording of time spent on nest for ten minutes at 9:00 and 21:00 on PND 2, 4, 6, and 8.

Our results highlighted reduced weight gain in male and female Δ^9-tetrahydrocannabinol (THC)-exposed offspring persistent into adulthood. Second, we identified brain regions that showed smaller volume in THC offspring regardless of age, with a sex and treatment interaction indicating more extreme volume reductions in females than males. Third, we found evidence for anxiety-like behavior, especially in THC females. There were only trending impairments to sensorimotor gating, however. Finally, results indicated no differences in maternal behavior, suggesting the changes observed in offspring are unlikely to be due to maternal behavior, rather than the effects of PTE on the offspring directly. This chapter has been drafted for submission to a peer-reviewed journal.

5.2 Abstract

As the prevalence of cannabis use during pregnancy increases, it is urgent that scientists and clinicians understand the potential consequences on neurodevelopment in order to better inform policy and the public. Few longitudinal assessments in humans or nonhuman animals follow offspring into adulthood to establish the impact of prenatal cannabis exposure (PCE) on trajectories of development. To understand the impact of prenatal exposure to THC, the main psycho-active component of cannabis, on developmental trajectories of the brain from adolescence to adulthood, we used longitudinal *in vivo* structural MRI on PND 25, 35, 60, and 90 following prenatal

exposure to 5 mg/kg THC injected in the dam subcutaneously from gestational day (GD) 3-10. During adolescence, PND 37-41, we assessed anxiety-like behavior with the open-field test (OFT) and impairments to sensorimotor gating with prepulse inhibition (PPI). Finally, we incorporated assessments of maternal behavior, including nest-quality assessments before and after the injections and assessments of time spent on nest on PND 2, 4, 6, and 8 in the light and dark cycle to establish whether alterations in neurodevelopment are partially attributable to changes in maternal behavior. First, we find alterations in the growth rate of pups, with THC-exposed offspring showing a reduced overall growth rate in weight trajectories. Second, we find that in females, but not males, THC-exposed offspring show a main effect of treatment, which relates to a smaller volume in the caudate putamen, hippocampus, cerebellum, and olfactory bulbs. Interestingly, there was no interaction between condition and age, suggesting these changes emerge in females early in life and persist into adulthood. Third, we observe a significant anxiety-like phenotype in THC-exposed female pups as indexed by decreased locomotion and decreased passes through and duration in the center of the open field test. We see trending impairments to sensorimotor gating in females only in the PPI test, which has been canonically associated with psychiatric disorders including psychosis in humans. The present study suggests that early life impairments may normalize in male mice, but they persist in females, including both brain changes and behavior. These results highlight that it is crucial to examine sex-specific effects in humans and nonhuman animals.

5.3 Introduction

The prevalence of cannabis use during pregnancy has increased over the past decade [Young-Wolff et al., 2022]. Simultaneously, the prevalence of exposure to cannabis with another drug remains the same, suggesting pregnant people may preferentially be using cannabis [Young-Wolff et al., 2022]. Given the increase in PCE, it is urgent that the scientific and medical communities understand what potential consequences there may be for the development at different stages of the developmental trajectory.

In humans, several studies have followed participants prenatally exposed to cannabis into adulthood, finding evidence for increased psychosis proneness [Day et al., 2015] and poorer educational and work attainment [Goldschmidt et al., 2016]. In the Ottawa Prenatal Perspective Study (OPPS), task-based functional MRI studies reveal no differences in performance on short-term memory tasks (visuospatial or letter n-Back),

and attention, inhibition, cognitive flexibility, or processing speed (Counting Stroop task) [Smith et al., 2006, Smith et al., 2016]. There were, however subtle differences in brain activity, including increased activity during task in posterior regions and decreased activity in the right pre-central gyrus during the visuospatial n-back task [Smith et al., 2006, Smith et al., 2016]. Because the participants are adults at the time of study, actual PCE occurred, by necessity, several decades prior. These types of studies, while critical to our understanding of the neurodevelopmental consequences of PCE, may not reflect the current realities of cannabis use as the potency of readily available cannabis has increased in the years since [ElSohly et al., 2016]. Whether or not this increase in THC potency may increase behavioural and psychopathological impairment following PCE is yet unclear. In humans, studies of adulthood outcomes of prenatal exposures take decades, however in nonhuman animals, adulthood outcomes can be assessed in a matter of months. For example, it takes a mouse approximately 90 days postnatally to reach adulthood. Further, animal models are advantageous in the context of examining prenatal exposures (such as PCE) given the control that experimenter has on timing, dose, the ability to examine other confounding factors (e.g., maternal care), and ability to design the experimental manipulation to be investigated. The adult impact of PCE has been studied in animal models, with reports of anxiety-like behavior in females [Navarro et al., 1994], and reduced baseline cortical glutamate (only tested in males) [Antonelli et al., 2004]. Further, there is evidence for alterations in social behavior, exploratory behavior, and preference for morphine in adult rats prenatally exposed to 5 mg/kg THC administered orally to dams [Rubio et al., 1995]. Despite indications that PCE impacts many domains of behavior, there are limited studies that examine the impact of gestational cannabis exposure on brain development as a dynamic process using longitudinal assays. Studies that do examine the neurobiology underlying behavioral changes conduct cross-sectional analyses that force the researchers to choose a single timepoint and preclude examining subject-level changes [Smith et al., 2006, Smith et al., 2016, Rubio et al., 1995, Drazanova et al., 2019, Antonelli et al., 2004, Moreno et al., 2005, del Arco et al., 2000].

Interactions between sex and prenatal exposure may occur on different time courses, highlighting the importance in establishing trajectories of development and deviation from typical development. The current literature on PCE suggests sex-dependent and sex-specific effects in offspring behavior. In humans, men exposed to cannabis before birth show evidence for increased risk of cannabis use as young adults [Porath and Fried, 2005, Tirado-Muñoz et al., 2020]. Adult female mice show in-

creased risk to self-administer morphine [Vela et al., 1998, Tirado-Muñoz et al., 2020]. Females also show evidence of altered basal opioid activity in the caudate putamen [Corchero et al., 1998, Tirado-Muñoz et al., 2020]. These results could implicate changes in reward pathways or risk-taking behavior. Male mice show impairments to sensorimotor gating [Frau et al., 2019, Tirado-Muñoz et al., 2020], consistent with a potential increased psychosis-like symptomatology in males. Male pups make fewer ultrasonic vocalizations (USVs) early in life [Manduca et al., 2020] and show impairments in social behavior in adulthood [Bara et al., 2018], implicating alterations in sociability that persist. Females show increased corticosterone [Rubio et al., 1995] and anxiety-like behaviours as indexed in the marble burying tasks [Maciel et al., 2022]. Taken together, these data data implicate many aspects of cognition and behavior, however much of the underlying neurobiology remains unexplored for sex-differences or using sex-specific analyses.

In humans, atypical development across the early lifespan has been identified in a sex-specific manner with normative models representing growth curves, first by weight and length/height, used commonly in pediatrics, and, more recently, neuroimaging data [Bethlehem et al., 2022, Ge et al., 2023]. To our knowledge, this has not been attempted with mouse data, however the PTE model could be of interest as a proof-of-concept comparison between controls and THC-exposed pups due to established differences in weight gain [Hume et al., 2023, El Marroun et al., 2009, Oke et al., 2021] and potential changes to total brain volume (TBV). Such an approach could establish sensitive periods of alterations following PTE from embryos to adulthood and establish an approach to motivate pooling data and creating a more generalizable resource, a C57/BL6 normative model, for future studies to compare different populations to.

In this study we sought to investigate trajectories of neurodevelopment from post-weaning to adulthood in mice prenatally exposed to THC. In employing a longitudinal design, we are able to capture windows of development roughly equivalent to human childhood, adolescence, and adulthood, allowing us to examine whether sex differences occur on different timecourses. Using *in vivo* longitudinal MRI we take a whole-brain approach to assessing regions of alteration. Additionally, we employ two behavioural analyses: open field test for anxiety-like behaviors and prepulse inhibition for sensorimotor gating in adolescence to see if brain volume changes are associated with changes in behavior. We examine potential alterations in maternal behavior to assess whether any changes in the offspring can be partially attributable to changes in the dam. Finally, we present sex-specific normative models for weight and TBV, pooling control data across our laboratory, and compare trajectories in control mice

to THC offspring. Based on prior evidence from our studies, we expected to observe sustained alterations in regions rich with Cannabinoid- (CB)−1 receptors, including the hippocampus, cerebellum, sensorimotor cortex, frontal cortex, and the caudate putamen.

5.4 Materials and Methods

5.4.1 Animals and timed mating

A timeline of the experimental design can be seen in Fig. 5.1.

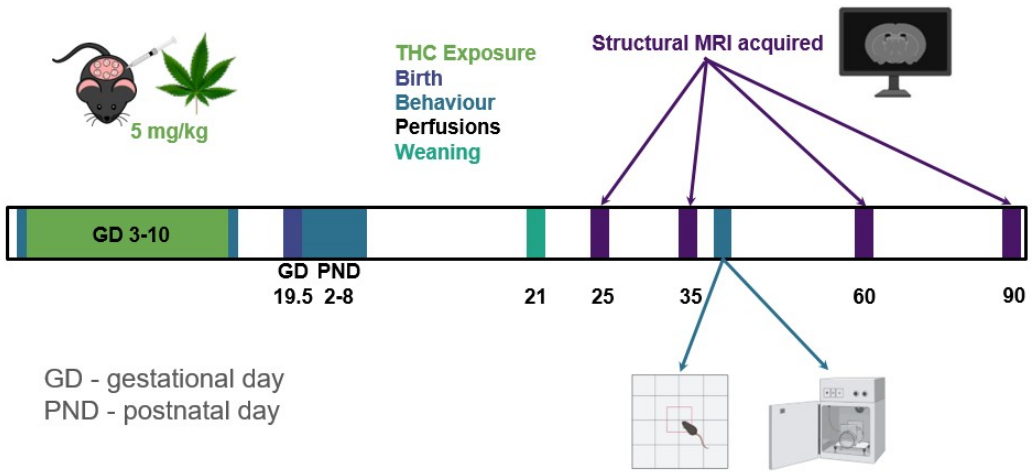

Figure 5.1: a) Timeline of experiment including THC exposure, (green), weaning (turquoise), scanning (purple), and behavior (blue). Maternal behavior includes nest quality (before and after THC administration), and observations on PND 2, 4, 6, and 8. Offspring behavior include OFT and PPI.

All experiments were conducted at the Douglas Mental Health University Institute Animal Facility in compliance with Facility Animal Care Committee regulations. Female and male C57BL/6J mice of breeding age (8 to 12 weeks old) were subject to timed mating breeding procedures, as previously described (Sections 3.7.1.1 and 4.4.1). All females were nulliparous, and GD 0 was determined as the day a plug was observed. Timed mating procedures are described in detail in previous sections (Sections 3.7.1.1 and 4.4.1).

5.4.2 Drug preparation and injection

To increase the interpretability of results in the context of previous experiments, an identical administration paradigm of THC was used as previously reported. Specifically, dams were randomly assigned to the vehicle (saline (Sal)) or THC groups. Two grams of THC in ethanol was purchased from Cayman Chemical Company, under a research license and import permit from Health Canada. Ethanol was dehydrated and THC prepared in a 1:18 cremaphor:saline solution, previously described in publications [Guma et al., 2023].

For the THC dams, 5 mg/kg of THC was administered to the dam by subcutaneous injections on GD 3-10 between 9:00 and 11:00. For the Sal dams, the vehicle solution (1:18 cremaphor:saline) was administered. Both injection volumes were maintained at 0.01 ml/g. The dosage of THC roughly corresponds to a dosage of 27.6 mg/kg in humans, which represents a moderately high dosage, with preparation and dosage previously discussed in detail [Guma et al., 2023].

5.4.3 Experimental timeline

First, the quality of nests dams built during the dark period was assessed at two timepoints, the night before the first injection (night of GD 2-3) and the night after the last injection (night of GD 10-11). On PND 1, all pups were weighed and litters were culled to six. Observations of maternal behavior were made on PND 2, 4, 6, and 8 at 9:00 (one hour after lights on) and 21:00 (one hour after lights off). On PND 21, pups were sexed and weaned. On the two days prior to the first scan (PND 25), pups were handled for a minute to habituate them to handling. OFT was acquired on PND 38, and PPI was acquired on PND 41. Finally, the third scan was acquired on PND 60, and the fourth on PND 90. Pups were sacrificed on PND 91.

5.4.4 Maternal outcomes

5.4.4.1 Nest quality

Nest quality was assessed with a previously published protocol [Deacon, 2006]. Specifically, at 19:00 on GD 2 and 10, 3.0g of Nestlet material was placed into a fresh cage with corncob bedding but no standard paper enrichment material. The next morning (GD 3 or 11) at 9:00, the quality of the nest was visually assessed and recorded. Additionally, any unshredded material was weighed. Paper enrichment material was added back to the cage.

5.4.4.2 Maternal observations

Maternal behavior was recorded on video during both the light and dark cycle on PND 2, 4, 6, and 8. Specifically, at 8:30 and 20:30 on these days, the dams were moved to the behavioral testing room in their home cage, the opaque lid and food grate were removed, and a clear Plexiglas cover was placed over the home cage to ensure the dam could not escape. Dams were allowed to habituate for 30 minutes. At 9:00 and 21:00, the camera was turned on and recorded 10 minutes of free maternal behavior in the cage, after which the dam was returned to the housing rooms.

Videos were manually assessed with Ethovision XT12 software (Noldus, Leesburg, VA, USA) to record duration of time and frequency of passes in the nest as opposed to the rest of the cage.

5.4.4.3 Pregnancy outcomes

Dam weight gain was examined with a linear mixed effects models (LMER) (fixed effects: condition and gestational day interaction, random effects: dam_ID), and litter size and sex ratio (proportion female) were assessed with linear models. Importantly, litters were culled at PND 1, so litter size was compared before culling, but pups were sexed at PND 21 during weaning, so it is possible that the sex ratio does not accurately reflect the sex ratio of the entire litter.

5.4.5 Imaging methods

5.4.5.1 Animal preparation

Before each scan, mouse was weighed and anaesthesia was induced with 3.5% isoflurane. Pups were transferred to the scanner, where anaesthesia was maintained at 1.5% isoflurane, administered in an 80% air, 20% oxygen mixture. Pups were restrained, and warmed by hot air to maintain body temperature. On PND 25, 35, 60, and 90, structural MRI was collected for 30 minutes.

After the first scan, mice were identified with an ear notch, and after each scan they were allowed to recover for 5 minutes on a heating pad under the home cage set to “medium”.

5.4.5.2 Scan details

All mice were scanned on a 7.0 Tesla (T) Bruker BioSpec with a 30cm bore with AVANCE electronics, equipped with a cryogenically cooled surface coil. The

T1-weighted structural scan was a fast low-angle shot sequence with the following parameters: 3D gradient echo sequence, TR = 20 ms, TE = 4.5 ms, flip angle = 10°, field-of-view = 18.0 × 16.0 × 9.0 mm, matrix size = 180 × 160 × 90, number of averages = 2, total acquisition time = 10 minutes 48 seconds, isotropic resolution = 100 μm.

5.4.5.3 Image processing

As in previous studies [Guma et al., 2023], after images were reconstructed, they were converted to niftis, quality controlled, and preprocessed, with further details reported in Supplementary Methods (SM) 5.7.1.1. Following preprocessing, we employed an in-house, longitudinal, two-level deformation based morphometry (DBM) pipeline, including registration tools from ANTs (http://stnava.github.io/ANTs/) to register all images, first within subject, then across subjects to create a population average, as reported in other studies [Guma et al., 2023]. Registration steps include both affine and non-linear transformations to obtain final alignment, and specific details can be found in SM 4.7.1.6. The final outcome of the processing includes a population average among all images and transformations for each image to the population average.

Jacobian determinants [Chung et al., 2001] were calculated from the deformation field across subject-wise registrations and resampled into the population average space to allow for voxelwise comparisons.

5.4.6 Behavioral assessments

5.4.6.1 Open field test

On the day the OFT was conducted, mice were brought to the testing room at 8:30 and allowed to habituate for 30 minutes. Up to four opaque, grey OFT boxes ($45cm^3$) were placed in the field of view of an overhead camera. A mouse was gently placed in each open field box and allowed to explore for 15 minutes while video was recorded. Mice of the same sex were tested together.

To assess behavior, videos were analyzed with the software Ethovision XT12 (Noldus Information Tech Inc., Leesburg, VA). The bottom of the open field captured on video was conceptually divided into zones including a center zone (40% of the area) and the outer edge and corners. The mouse was tracked automatically, and the total distance travelled was analyzed, as well as the duration of time in and the passes through the center zone.

5.4.6.2 Prepulse inhibition

As with OFT, on the day PPI was conducted, mice were habituated in the testing room from 8:30 to 9:00. PPI to acoustic startle was measured with commercially produced startle chambers (San Diego Instruments, San Diego, CA). Details of the chambers have been previously reported [Guma et al., 2021a] and are available in the supplement SM 5.7.1.2. Mice were gently placed in the holder in each PPI chamber and the pre-programmed experiment (described in SM 5.7.1.2) was run.

5.4.7 Statistical analyses

All statistical analyses were conducted in the R programming language, version 3.5.1.

5.4.7.1 Pup weights

Weights of the developing mice, acquired on scan days (PND 25, 30-35, 60, and 90) and the day they were sacrificed (PND 91). Trajectories of development were assessed with LMERs (lme4::lmer and lmerTest::summary), with fixed effects including the interaction between treatment, sex, and the quadratic effect of age, and random effects of subject ID and litter.

5.4.7.2 Magnetic resonance imaging

A mass univariate, whole-brain voxelwise analysis was conducted with tools from the RMINC package on logged absolute Jacobian determinants produced by the DBM. Absolute Jacobians, as opposed to relative Jacobians, include affine components of registration, encoding changes in total brain volume. As in previous studies, our evidence suggests THC exposure impacts offspring weight gain, and weight could impact brain volume, therefore we employed a regression-with-residuals method to control for how body weight might mediate the relationship between condition and brain volume. The residuals from the pup weight model were extracted from that model and included as a covariate in the brain volume model. Therefore, fixed effects included the interaction between treatment, sex, and the quadratic effect of age, and the weight residual, and random effects of subject ID and dam_ID. Multiple comparison corrections were conducted with the False Discovery Rate (FDR).

5.4.7.3 Open field test

Total distance moved, duration of time in the center zone, and frequency of passes through the center zone were compared with LMERs. Fixed effects included the interaction between treatment and sex, with litter as a random effect.

5.4.7.4 Prepulse inhibition

Percent PPI was calculated averaged across trials for each prepulse intensity with the following equation:

$$(startle\ response - prepulse\ response)/startle\ response * 100$$

Percent PPI was assessed with LMERs, with fixed effects including the interaction between sex, treatment, and PPI level, and random effects included subject_ID and mom_ID.

5.4.7.5 Normative models

Normative models have been used to categorize alterations from trajectories of normative development in humans [Bethlehem et al., 2022]. To our knowledge, the same has never been done in mice. We sought to develop normative trajectories of development in weight and TBV. Using found data previously acquired in our laboratory, we compiled weight from control C57/Bl6 J mice, including weight from 251 females and 235 males between 2 days before birth and PND 115. While we had a slightly reduced sample size for TBV, it included 112 females and 119 males. For the THC sample, we pooled weight from 51 females and 59 males, and for TBV, volumes from 38 females and 41 males, including embryonic and neonatal data from previous studies (Chapters 3 and 4).

We used the mgcv package in R to create generative additive models (GAM)s for TBV and weight in the sexes separately, creating one model for the control data and one for the THC data. Plotting the curves with confidence intervals highlights time periods of deviation from normal growth in the males and females at different timecourses.

5.5 Results

A table of all results summarizing all results can be found in Tab. 5.1.

Test	Results	Fig. #
Maternal nest building	No difference between groups or timepoints	SFig. 5.9
Maternal behavior	No difference between groups	SFig. 5.10
Offspring weight	Main effect of THC	Fig. 5.2
Brain volume	Main effect of THC, sex-THC ixn, sex-age ixn	Fig 5.3-5.5
OFT	Main effect of THC	Fig. 5.6
PPI	Trend-level effect of THC	Fig. 5.7
Normative model: weight	Sex-dependent effects	Fig. 5.8a,b
Normative mode: TBV	Sex-dependent alterations	Fig. 5.8c,d

Table 5.1: Table of results with major effects reported and figures for specific results listed. ixn = interaction

5.5.1 Prenatal THC exposure and maternal outcomes

Neither timepoint, nor condition impacted the quality of nests built by dams, seen in SFig. 5.9. While condition did not impact time spent on nest, there was an effect of day, such that dams spent more time on nests at later PNDs, seen in SFig. 5.10, and discussed in Supplementary Results (SR) 5.7.2.1. THC exposure slightly reduced dam weight gain (full results in SR Tab. 5.3) and litter size (full results in SR Tab. 5.4), but did not alter sex ratio of pups, reported in Section 5.7.2.1.

5.5.2 Prenatal THC exposure impacts pup weight into adulthood

At PND 1, litters were culled to 6 pups, and 4 pups (2 males, 2 females where possible) were included for scanning. The sample included 26 Sal pups from 6 litters (12 females) and 24 THC pups (13 females).

Examining the interaction between sex, treatment and the quadratic effect of age, there was an overall effect of treatment, such that THC pups weighed less than Sal controls across the course of the experiment ($p = 0.016$), seen in Fig. 5.2. There was also, as expected, a main effect of sex, such that females weighed less than males ($p < 0.001$), and both the linear and quadratic age term ($p < 0.001$) such that mice gained weight over time. Finally, there was an interaction between the linear term of age and sex, such that males gained more weight over time than females did ($p < 0.001$). There was no interaction between sex and treatment or time and treatment, and the three-way interaction was not significant, with full results in SR 5.7.2.2 Tab. 5.5.

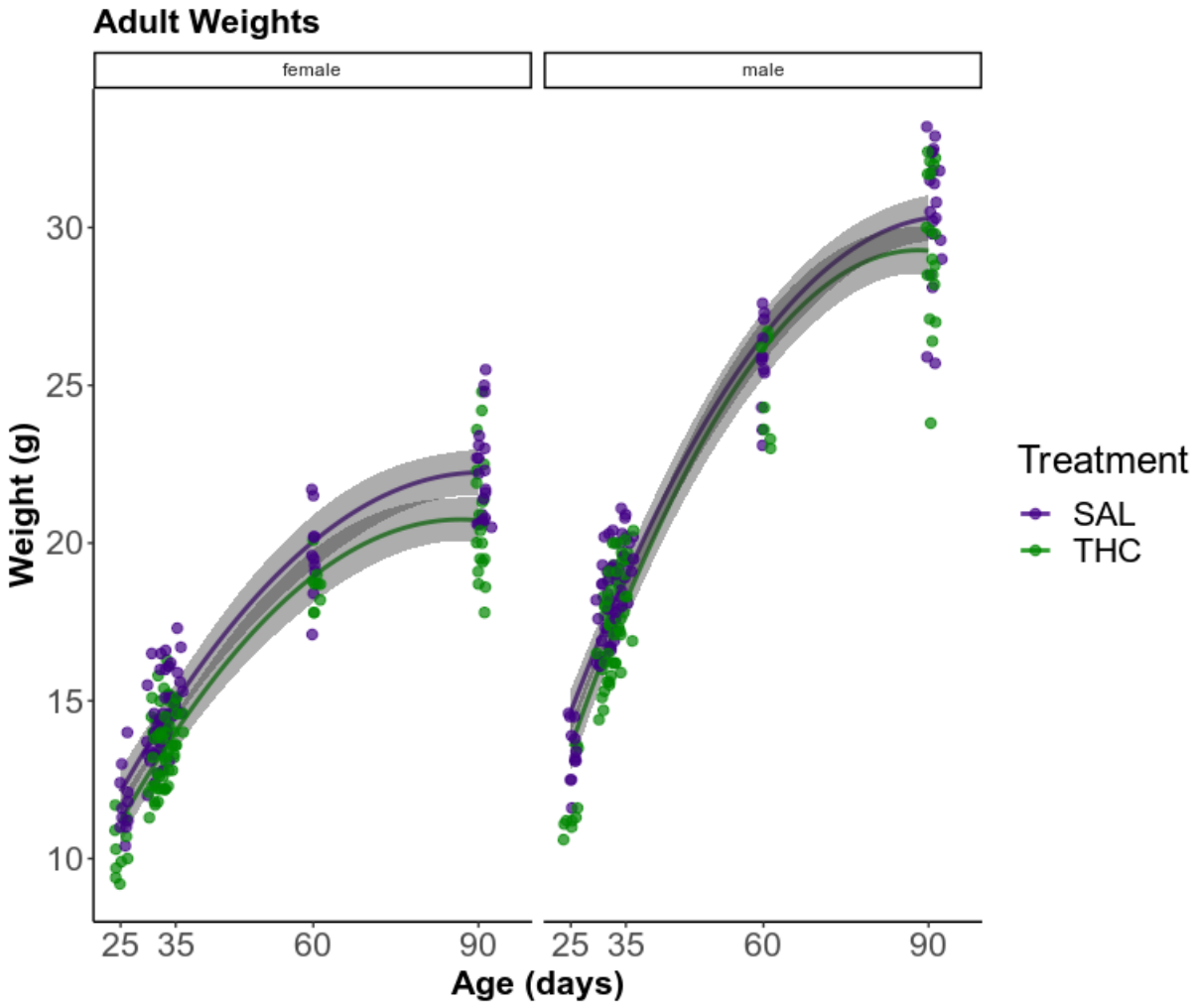

Figure 5.2: a) THC-exposed pups are lighter than Sal offspring, regardless of sex and age.

5.5.3 Prenatal THC exposure impacts brain volume in females

After pups were scanned, quality controlled, and processed, the dataset was subset to only high quality scans and pups with at least 2 scans for the LMERs. 22 mice had four scans, 21 had three scans, and 7 had two scans. A table with the full sample per timepoint is reported below in Tab. 5.2.

First, there was a main effect of condition, significant from 10% - 5% FDR such that THC mice showed a lower volume, regardless of age in regions such as the bilateral striatum, thalamus, olfactory bulb, piriform cortex, corpus callosum, visual cortex, cerebellum, left cingulate, inferior colliculus, hypothalamus, medulla, somatosensory

	PND 25		PND 35		PND 60		PND 90		
	Males	Females	Males	Females	Males	Females	Males	Females	Litters
Sal	12	12	14	12	14	10	8	8	6
THC	9	9	10	13	7	9	9	9	6

Table 5.2: Sample per timepoint (approx. PND) following quality control. Postnatal day (PND); saline (Sal); delta-0-tetrahydrocannabinol (THC)

and visual cortices, and the right amygdala, hippocampus, and motor cortex, Fig. 5.3.

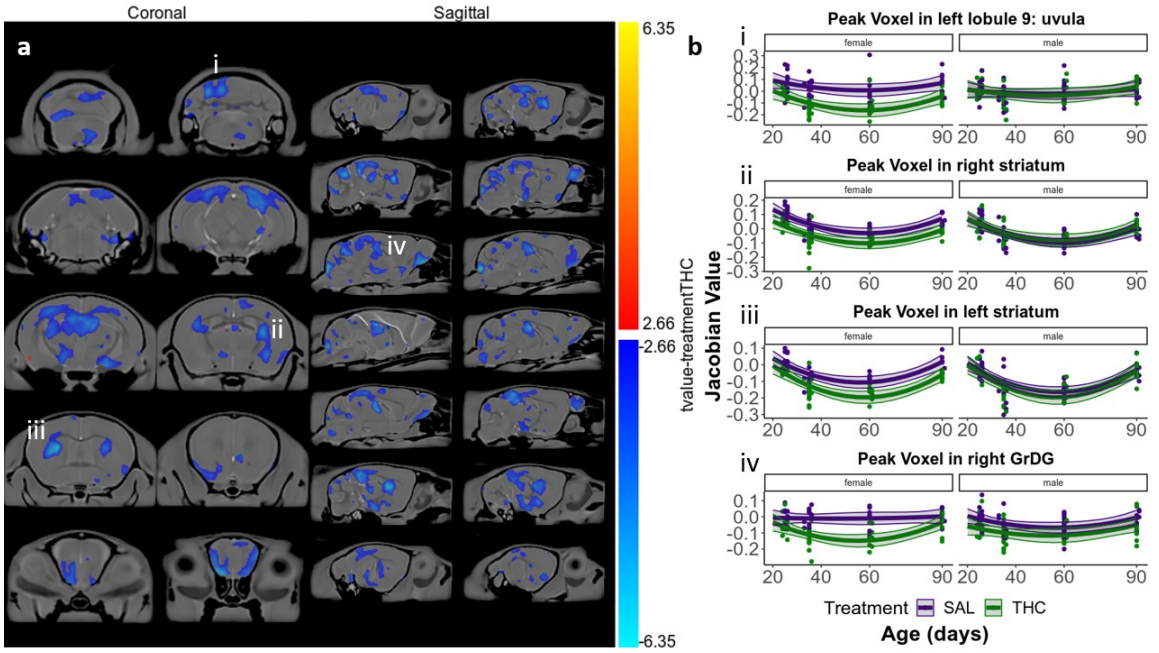

Figure 5.3: a) heatmap showing regions with a smaller volume, regardless of age in cool colors. b) Peak voxels plotted in i) the cerebellum, ii) the right striatum, iii) the left striatum, iv) the cingulate cortex

There was also a significant condition by sex interaction in many of the same regions in the opposite direction THC exposure reduced volume in these regions overall, male THC pups showed a positive effect. As evidence by the peak voxel plots, the reduced volume is present in female THC pups, but not males. Regions that show both the main negative effect of THC as well as the positive effect for male THC animals include the bilateral olfactory bulbs, left cerebellum, medulla, piriform cortex, and arbor vita, as well as the right flocculus, striatum, and thalamus. Regions that showed the overall effect of THC but not the sex and treatment interaction included the left superior and

right inferior colliculi, the left pons, and the right cingulate cortex.

Interestingly, there was no age by treatment interaction, which implies that any alterations that occur as a result of THC exposure occur before PND 25.

There was a main effect of sex (FDR 5%-1%), such that males showed larger volume in regions including the cingulate cortex, frontal association cortex, motor, visual, somatosensory, entorhinal and ectorhinal, and orbital cortices, hippocampus, striatum, thalamus, and amygdala, midbrain, hypothalamus, and olfactory bulbs, but smaller volume in voxels in the colliculi, and some regions of the striatum, Fig. 5.4.

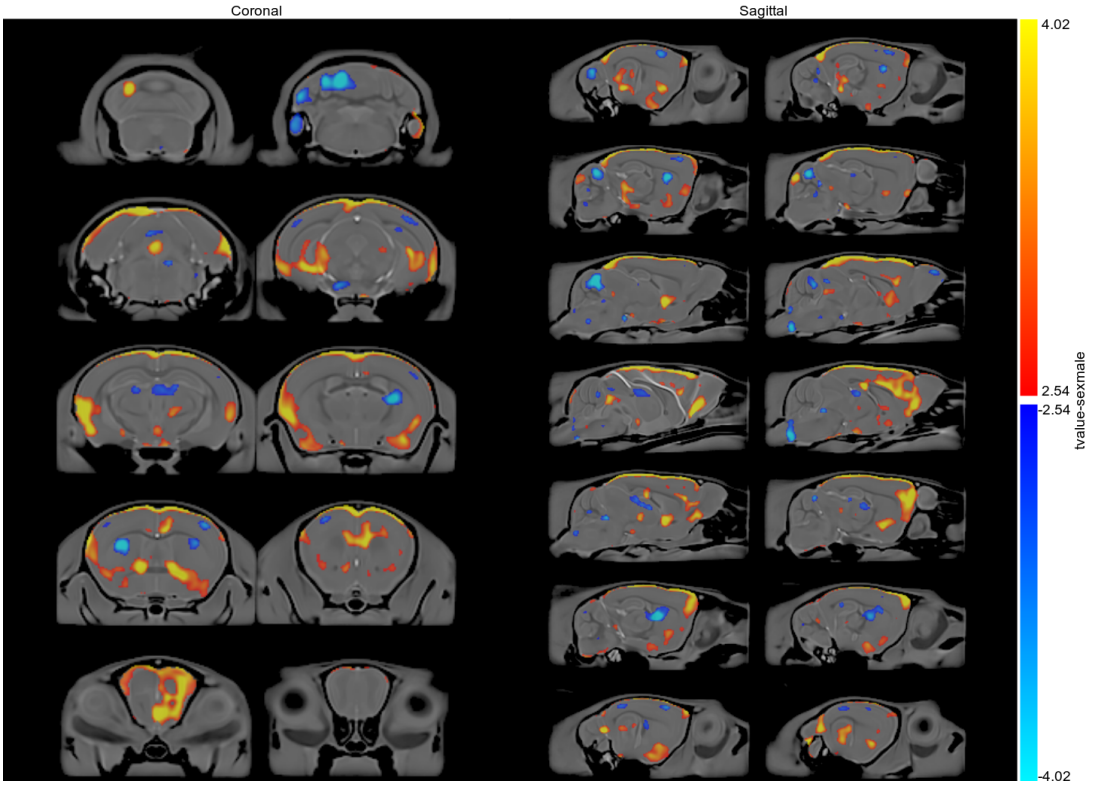

Figure 5.4: In warm colors, regions where males show a larger volume, regardless of condition or age, than females. In cool colors, females show a larger volume than males

A significant sex by quadratic age interaction (FDR 5%-1%) showed altered trajectories of brain development between the sexes, regardless of THC exposure, with males generally showing negative slopes (an inverted quadratic trajectory) compared to females including in cortical regions, the hippocampus, the striatum, hypothalamus, and amygdala, Fig. 5.5.

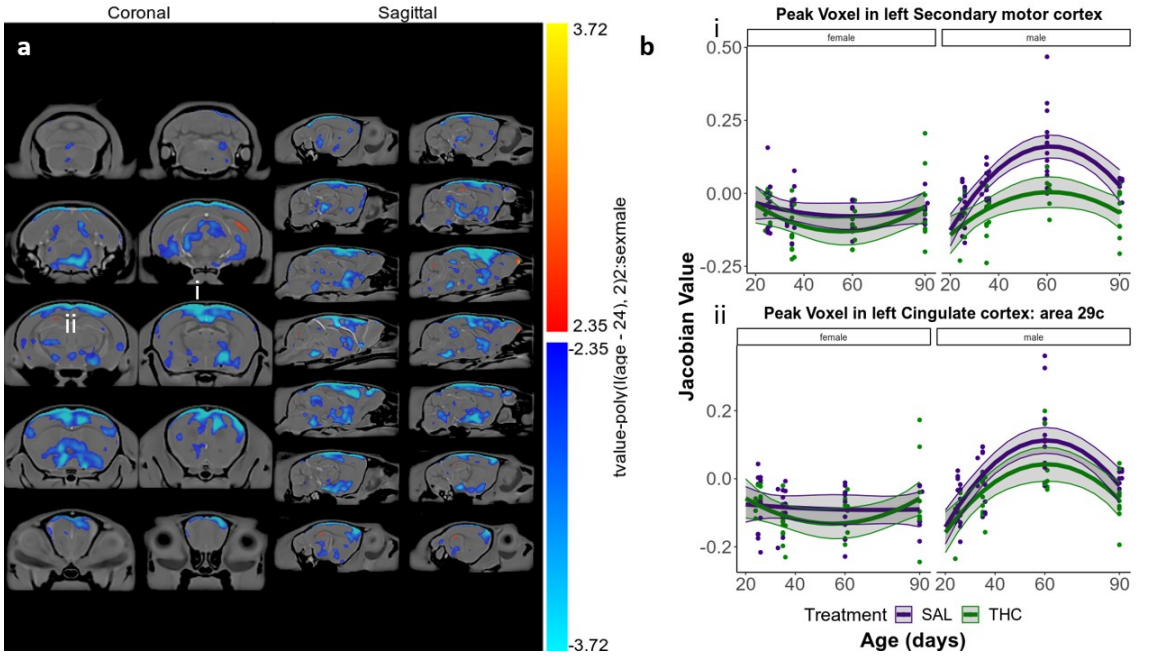

Figure 5.5: a) In warm colors, regions where the males show a more positive slope (positive quadratic) compared to females. In cool colors, regions where males show a more negative slope (inverted quadratic) compared to females. b) Peak voxels with slopes plotted in i) the motor cortex, and ii) the cingulate cortex

5.5.4 PTE induces anxiety-like behavior in adolescent females

Examining the impact of treatment and sex on total distance moved revealed a main effect of treatment ($p < 0.05$), a main effect of sex ($p < 0.001$), and an interaction between treatment and sex ($p < 0.001$). Specifically, THC offspring moved less overall, and males moved less than females, but THC-exposed females moved less than Sal females, whereas there was no obvious difference between Sal and THC males, as seen in Fig. 5.6a. Additionally, THC-exposed pups spent less time in the center of the open field compared with controls, indicated in the main effect of treatment ($p < 0.01$), and there was an interaction between treatment and sex, such that female THC-exposed pups spent less time in the center than female controls, although there was no obvious difference for males, Fig. 5.6b. Finally, there was a main effect of condition, such that THC-exposed pups made fewer passes through the center than Sal pups ($p < 0.001$), but again there was an interaction, such that female THC-exposed pups made fewer passes through the center than Sal females, while the relationship was not apparent for males. Full results are recorded in SR 5.7.2.3, Tabs. 5.6–Tabs. 5.8.

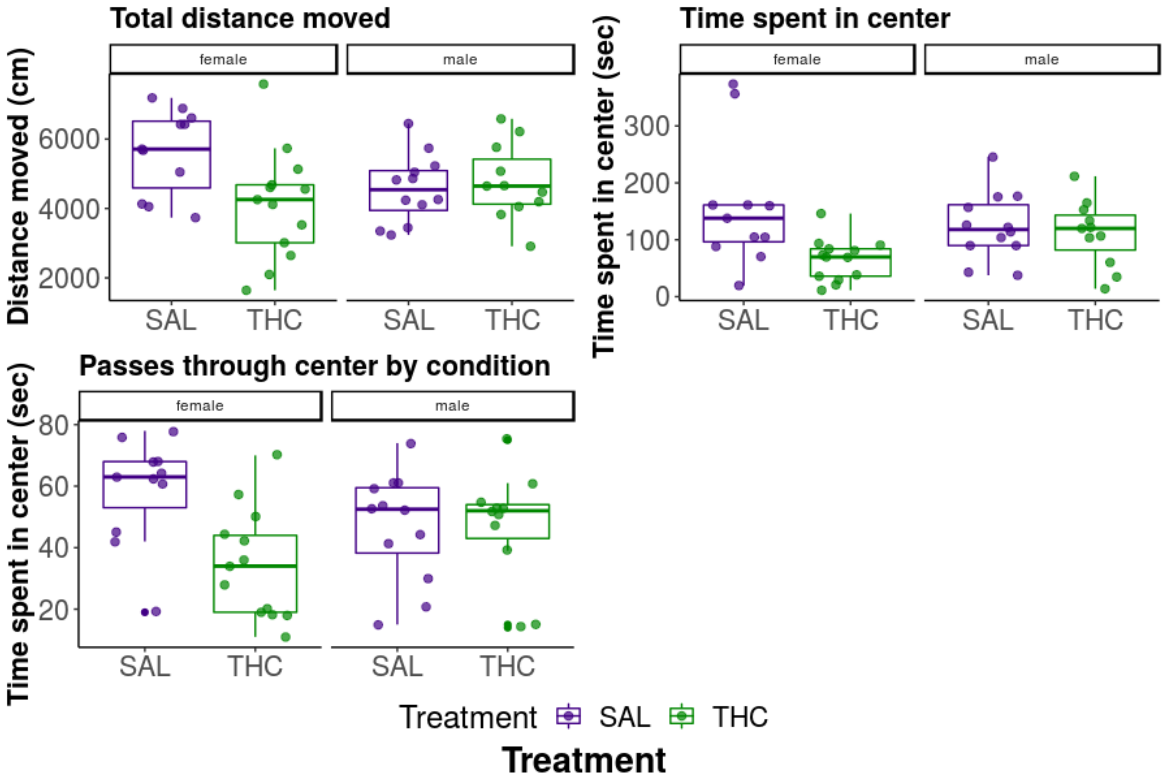

Figure 5.6: a) THC-exposed females move less than Sal-exposed females, while there is no obvious difference between males, b) THC-exposed females spend less time in the center than Sal-exposed females, while there is no obvious difference between males, c) THC-exposed females make fewer passes through the center than Sal-exposed females, while there is no obvious difference between males.

5.5.5 No effect of prenatal THC exposure on sensorimotor gating

As expected, with increasing prepulse level percent PPI increased as well ($p < 0.001$). There was a trending effect ($p < 0.07$) of THC exposure such that THC exposed mice exhibited decreased percent PPI, indicative of impaired sensorimotor gating, Fig. 5.7.

5.5.6 Normative models

For the weight models, female THC pups show some overgrowth between birth and PND 25, volume then falls off, however, and remains reduced, consistent with our

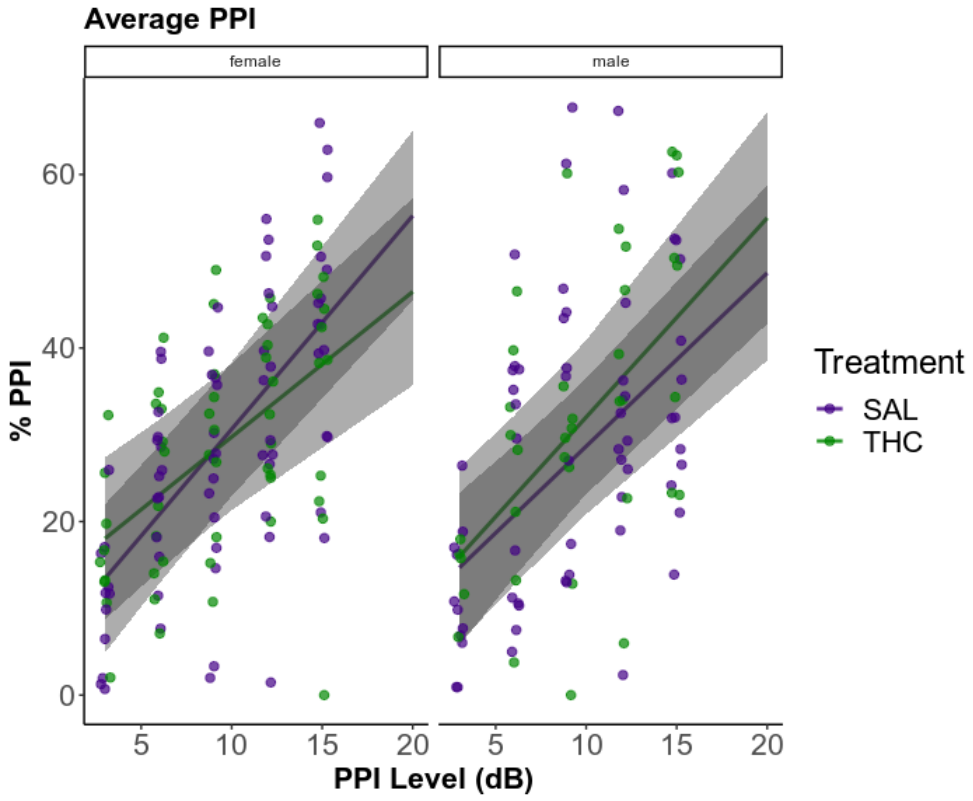

Figure 5.7: Expected increase in % PPI with increased prepulse level. Trendlevel impairment to PPI in THC-exposed pups regardless of prepulse level.

linear models, seen in Fig. 5.8a. In males, the overgrowth is more pronounced than in females, and despite early fall-off in growth in early adolescence, the males seem to normalize, seen in Fig. 5.8b.

For the TBV model, in females volume trajectories diverge between PND 10 and 20 and remain lower than controls, Fig. 5.8c. In males, however, there is an initial undershoot in early adolescence that normalized by PND 30, seen in Fig. 5.8d.

5.6 Discussion

The present study reveals decreased weight and weight gain in THC-exposed pups in both sexes, as well as a smaller brain volume, especially in females, in both cortical and subcortical regions. Additionally, these brain volume differences are accompanied by anxiety-like behavior in the females during adolescence, and trending impairments to sensorimotor gating.

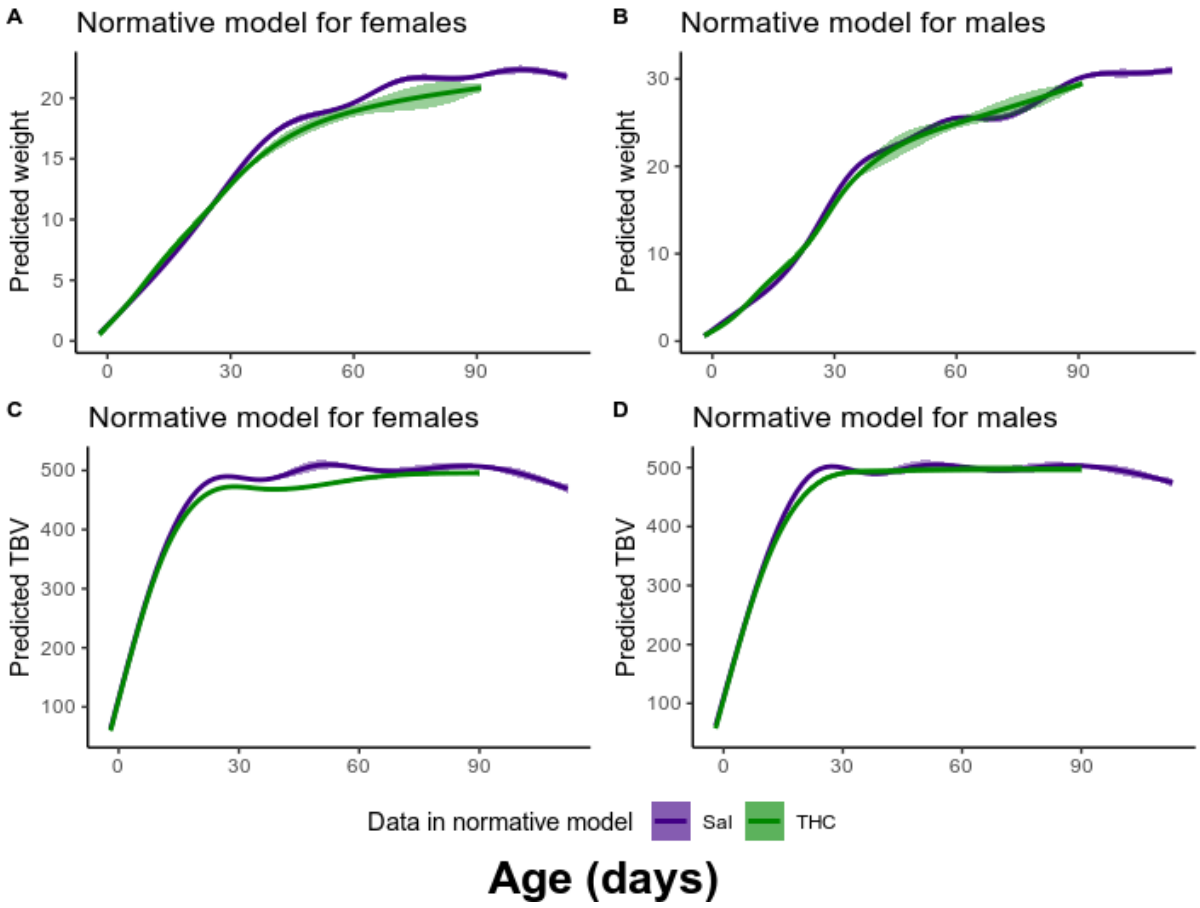

Figure 5.8: Normative models constructed with GAMs for control mice (purple) and THC offspring (green) built separately for weight in females (A) and males (B), and for TBV in females (C) and males (D).

5.6.1 Metabolic consequences following PTE

Examining the weight of THC and Sal offspring revealed that, regardless of sex, THC pups weighed less between PND 25 and 90 than Sal controls. The fact that there was no interaction between condition and time suggests that the changes occurred early in life and were maintained into adulthood. The potential metabolic consequences of PCE exposure have been highlighted previously. One of the first indications is the robust finding of fetal growth restriction in both humans [El Marroun et al., 2009] and our nonhuman animal studies (Section 3.5.2). PCE and PTE have been recently reviewed in the context of postnatal metabolism, including hepatic function, lipid metabolsim, and glucose homeostasis [Lee and Hardy, 2021]. This review importantly highlights various methods by which metabolism could be altered following PTE. First,

for example, IUGR is known to contribute to catch-up growth and increased risk factors for obesity in later life. Second, THC undergoes first-pass metabolism in the liver, which expresses CB1 receptors early in gestation, and PTE has been associated with deficits in liver growth [Natale et al., 2020, Lee and Hardy, 2021]. In addition, prenatal exposure to THC vapor has recently been associated with reduced rat pup weight as well as reduced weight gain when placed on low or high-fat diet, accompanied by sex and diet dependent alterations in glucose metabolism [Hume et al., 2023]. Our results in this context provide evidence for the role of prenatal THC specifically in modulating metabolic function into adulthood. Because we did not monitor feeding behavior or exercise, future studies would have to examine whether behavioral or metabolic changes underlie reported decreases in weight gain.

5.6.2 Sex dependent reductions in brain volume

The significance of a main effect of PTE but no condition by age interaction in brain volume highlights the early life changes that are present by PND 25 and persist into adulthood. The sex by treatment interaction indicates that in many regions the effect of THC exposure is mainly driven by females, whereas males experience a normalization in brain volume. Many of the regions that show this pattern are known to be rich in CB_1 receptors, including the cerebellum [Moldrich and Wenger, 2000], striatum [Ong and Mackie, 1999], the piriform cortex [Terral et al., 2020], and the olfactory bulbs [Hutch et al., 2015]. Specifically, the endocannabinoid system (ECS) has been investigated in the context of food intake and feeding behavior, with mice lacking CB1 receptors in glutamatergic neurons of the olfactory bulbs [Terral et al., 2020, Bellocchio et al., 2010] and the hypothalamus [Cardinal et al., 2012] showing reductions in fasting-induced food intake [Bellocchio et al., 2010] and lack of weight gain [Cardinal et al., 2012] respectively. This context is especially interesting given the simultaneous reductions in volume of the olfactory bulb and reductions in weight observed in our study. Other regions with observed brain volume changes have little presence of CB_1 receptors, such as the thalamus, colliculi, and medulla [Miederer et al., 2020]. Volume reductions in these regions may not be associated with the direct effect of PTE in these regions, but rather the effect on other regions, with indirect effects on these, such as the misguidance of neurons and failure to synapse on targets in these regions.

Several regions do not show the sex-effect, but only the main effect of PTE, such as the colliculi, pons, cingulate cortex, and hippocampus. Sex differences in trajectories of brain growth in these regions have been thoroughly investigated, and indicate some

interesting patterns [Qiu et al., 2018]. Of the regions that do *not* show the sex-effect of PTE, the pons and hippocampus have been described as showing few differences in early postnatal life, but become relatively larger in females during puberty in mice [Qiu et al., 2018]. Other regions of the hippocampus and cingulate are relatively larger in males throughout development. Of the regions that show sex differences in PTE exposure, the striatum, thalamus, olfactory bulbs, sensory cortices, and amygdala show sex differences in development [Qiu et al., 2018]. In general, this previous work highlights that regions that are larger in males emerge earlier in life, whereas regions that are relatively larger in females emerge post-puberty [Qiu et al., 2018].

5.6.3 Anxiety-like behavior in females following PTE

In the OFT, we observed reduced total distance moved as well as time in and passes through the center zone in females. These results suggest an anxiety-like phenotype in female THC-exposed offspring during adolescence. While many studies have examined anxiety previously, findings are equivocal. In children exposed prenatally to cannabis, there is evidence for increased anxiety at age 3-6 [Rompala et al., 2021]. Additionally, in adolescent rats prenatally exposed to THC (oral exposure) throughout gestation, there was a modest increase of anxiety-like behavior in males only [Lallai et al., 2022]. In contrast, in rats exposed to 5 mg/kg THC during gestation and lactation, anxiety-like behavior was observed in adult female, but not males [Navarro et al., 1994]. Following prenatal exposure to various dosages of THC, decreased plasma corticosterone was observed in male rats in adulthood, while increased levels were found in females [Moreno et al., 2005].

5.6.4 Trending effects in PPI

Sensorimotor gating has been investigated in laboratory rodents following prenatal exposure to cannabinoids with PPI, providing mixed results. Following prenatal exposure to THC orally throughout gestation, adolescent male rats, but not females, showed deficits in sensorimotor gating in early adolescence [Lallai et al., 2022]. Another study examining the effects of a CB_1 receptor agonist (WIN 55,212-2) from GD 5-20 in rats did not find an impact on sensorimotor gating in adolescence and adulthood. In our study, there was a trend-level effect of THC consistent with impairments to sensorimotor gating. While there was no clear sex by treatment interaction, the plotted data indicate the impairment is more pronounced in females than males, consistent with

our other data. It is possible that the administered compound (THC vs. WIN) and method of exposure (injected vs. oral) impacts the mechanism of action of alterations specifically related to sensorimotor gating.

5.6.5 Considerations in sex differences

There are many possible mechanisms underlying sex differences, and several factors must be considered, especially when generalizing and synthesizing among studies. The timing of exposure may enhance differences in one sex over another, for example. Additionally, the timing of outcomes and the tested domains may account for some studies finding sex differences, while others do not. While these data seem to suggest a more pronounced effect in females, rather than males, this outcome is certainly not universal. Interestingly, however, our previous studies with the identical administration paradigm examining outcomes in embryos and neonates did not find sex differences, suggesting these effects may emerge in puberty in this model of PTE. It is also important to note that simply including animals of both sexes in an experiment is not sufficient to discovering sex differences; the analytic approach is also important [Galea et al., 2020]. Interactions must be examined between the main effect of interest and sex for sex-dependent or sex-specific effects to emerge. Merely including sex as a covariate can only reveal main effects of sex in the outcome of interest. Future studies must continue including sex-balanced samples and properly modelling sex and treatment interactions, rather than merely including sex as a covariate, so that findings can be properly documented and synthesized with increasing availability of literature and results.

5.6.6 Normative Model

Normative models have increasingly been used in brain imaging to understand normal variation in populations and normative trajectories of development, as has been used in weight and height to track children's growth [Bethlehem et al., 2022, Ge et al., 2023]. To our knowledge, this has not been undertaken in the C57/Bl6 J mouse, but developing normative models would allow future studies to compare values such as weight and TBV against a wider population of controls, potentially indicating time periods where trajectories of development differ. We chose to examine TBV in addition to weight in this proof-of-concept design because it was widely available for control mice acquired at our center with minimal processing and can provide a

gross value associated with brain growth that can be extracted from different scanning sequences and does not require an atlas with correspondence across all developmental periods from embryos to adulthood. Future studies could expand upon this work by a) increasing the sample size, b) investigating more nuanced values (such as regional brain volume with a consistent atlas) or c) pooling data from different scanners and centers in order to create a more generalizable model verified across research groups and methodologies. Other studies could also benefit from normative models by comparing various developmental models to an accepted model across centers.

5.6.7 Limitations

Several limitations exist in this study. Here we investigate THC in absence of other phytocannabinoids or components of cannabis that may impact the mechanism of action or long-term consequences. Further, we investigate a fairly high injected dosage, present only in the first trimester of pregnancy, not before conception or during lactation, so it may have reduced external validity compared to a model with a wider window, lower dosage of administration, or vaporized cannabis model. Additionally, we investigate a limited set of behaviors during adolescence only - it is possible that males may display more alterations in a different domain or at a different timepoint that we do not capture with the present approach. One benefit to acquiring both imaging and behavioral data from the same animals is relating the two statistically, such as with multivariate analyses, as has been done before [Guma et al., 2023, Ge et al., 2023]. Nevertheless, these multivariate techniques often require much larger datasets to generate generalizable patterns of brain-behavior relationships. While the present sample size was powered to examine longitudinal imaging differences, it would be small for an approach that often requires sample sizes closer to 100 (based on number of parameters) [Willaby et al., 2015]. Finally, it was outside of the scope of the present study to investigate the mechanisms of action of the impact of PCE on body and brain growth and behavioral outcomes. Future studies could identify regions of interest with our results and further investigate potential cellular and molecular changes in these regions throughout development.

5.6.8 Conclusions

This study provides findings that expand the current body of knowledge around PTE. First, indicate alterations in growth curves into adulthood across both sexes,

motivating future research into feeding behavior and metabolism. Second, we find evidence for brain volume changes, notably reduced volume in females that emerge before the onset of puberty. Third, we observe patterns of altered behavior, specifically anxiety-like behavior, in females. Finally, we examine trajectories of growth in both total weight and brain volume in the context of normative models, identifying windows of change between groups and providing a proof-of-concept for use of normative models in mouse developmental studies. These results suggest a phenotype of persistent alterations following PTE, highlighting that human studies should carefully investigate adolescent and adult outcomes fully and paving the way for future studies to more specifically examine regions of interest highlighted in the present work.

5.7 Supplementary Information

5.7.1 Supplementary Methods and Materials

5.7.1.1 Image processing

First, images were converted to niftis with the BrkRaw module tool, bids_helper [Lee, 2023]. Next, they were quality controlled by two independent raters (L.C. and K.B.). Each scan was examined for motion and artefacts and given a score from 1 to 5, with 1 being the best and 5 being unusable. Scans that received a 1 or 2 from both raters were included, those that received a 4 or 5 were excluded, and scans that received a 3 or where there was disagreement were examined again for a consensus to be reached. Next, all scans were pre-processed with an in-house pipeline using Advanced Normalization Tools (ANTs) tools to denoise each image with adaptive nonlocal means (minc_anlm) and apply an N4 bias-field correction [Manjón et al., 2010, Tustison et al., 2010].

Following preprocessing, in-house software was used to conduct a twolevel model-build, first registering all images within a subject, and then across subjects to create a population average [Devenyi, 2023]. In each level of the model build, all images were aligned with affine transformations that preserved parallel lines in the images (translations, rotations, scales, and shears). Then, iterative nonlinear transformations attained precise anatomical alignment between images in an automated fashion, similar to previous studies in adult animal brains [Guma et al., 2023], as well as embryo studies in our laboratory [Guma et al., 2022].

5.7.1.2 Prepulse Inhibition

Acoustic startle chambers from San Diego Instruments consist of a Plexiglas, cylindrical chamber (8cm diameter, 16 cm long) set on a Plexiglas base in a sound-attenuating chamber. A speaker is located in the ceiling 24 cm above the animal which provides background noise (70 dB) as well as acoustic stimuli. Beneath the cylindrical chamber where the mouse is placed is a piezoelectric accelerometer which detects and transducts motion accompanying acoustic startle. A microcomputer using commercial software package from SR-LAB was used to control pulse parameters, digitize, and record the stabilimeter readings.

After animals were placed in the cylindrical chambers, they underwent 5 minutes of habituation to the background noise before undergoing a total of 50 trials (5-30 s intertrial duration). The first 8 and final 7 trials measured startle magnitude to a stimulus (50 ms 120 dB) without prepulse, and the middle 35 trials the startle tone was presented alone or preceded by a 30 ms prepulse ranging from 3-15 dB above the tone (3 dB increments: 73-85 dB). The prepulse varied randomly between trials with 5 trials presented per prepulse stimulus. The average startle response was derived from the 100 1 ms readings taken after onset of the startle stimulus.

5.7.2 Supplementary Results

5.7.2.1 Maternal and pregnancy outcomes

Nest quality was assessed pre-injection (GD 2-3) and post-injection (GD 10-11) (x-axis). Manual assessments were log-transformed (y-axis) and compared with LMERs to examine effect of timepoint and treatment on outcomes. There was no difference in the quality of nests built by Sal or THC dams, nor difference in nest quality before or after injections, Fig. 5.9

There was no difference in either the amount of time spent on nest nor the number of passes on nest for Sal and THC dams. Over the days, dams spent increasing time and made increased passes on the nests. There was also notable variability in dam behavior, seen in Fig. 5.10. Because of the outlier behavior of one dam in number of passes in the nest, her offspring were checked to see whether they were outliers in the behavior, but they were not.

There were slight reductions in dam weight gain in THC exposed dams compared to Sal controls (full results, Tab. 5.3), however there was no initial difference in weight before the onset of injections, seen in SFig. 5.11.

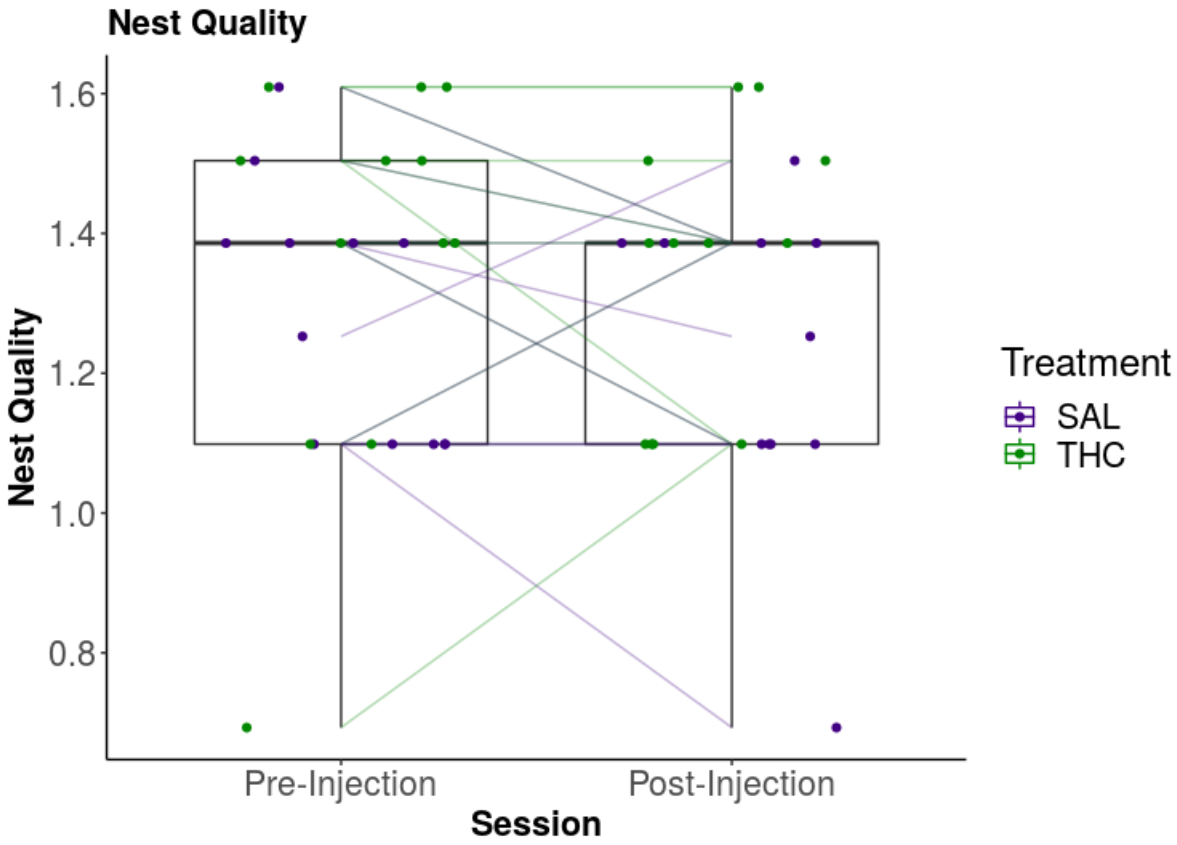

Figure 5.9: No difference in quality of nests between Sal (purple) and THC (green) dams. No difference between timepoints either before injections (GD 2-3m left boxplot) or after injections (GD 10-11, right boxplot). Thin lines connecting points represent values for a single dam across both timepoints.

THC dams had smaller litters than Sal dams (full results in Tab. 5.4, however there was no difference in sex ratio of pups, seen in SFig 5.12.

5.7.2.2 Impact of THC on offspring weight gain

PTE reduced weight gain across sexes, with full effects of the LMER reported in Tab. 5.5

5.7.2.3 Impact of THC on offspring OFT behavior

PTE impacted anxiety-like behavior displayed on the OFT, with full results reported for total distance moved (5.6), time spent in the center (5.7), and frequency of passes

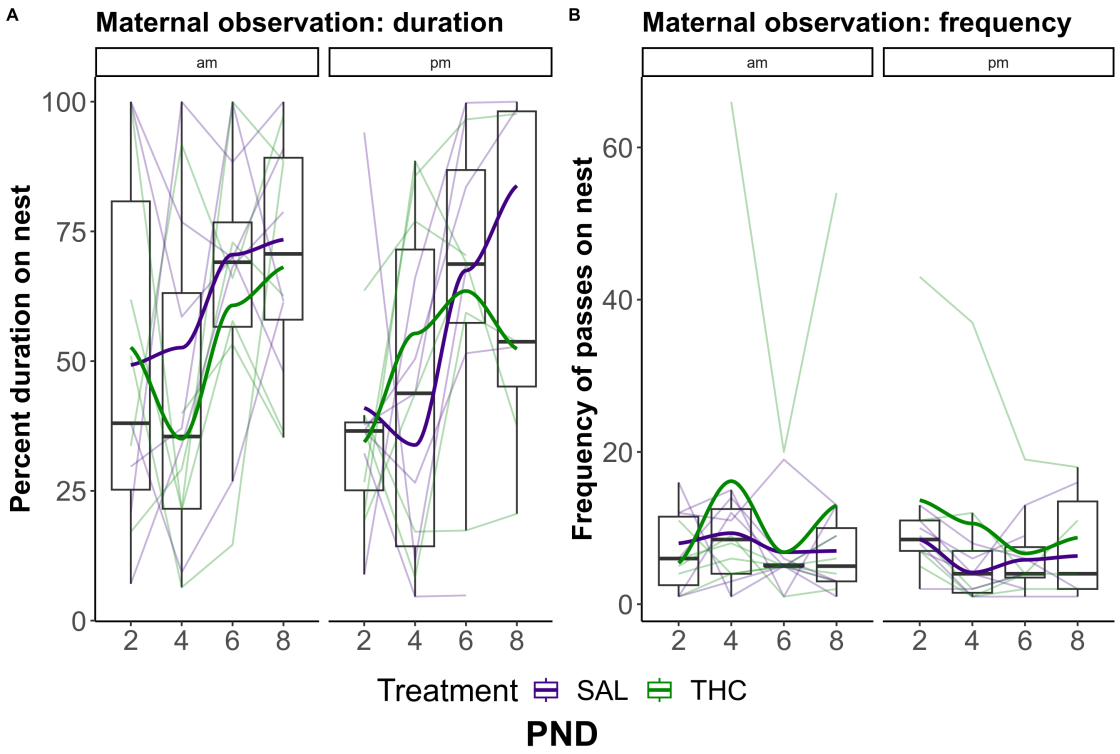

Figure 5.10: In time spent on nests (A), there was no difference between 9:00 or 21:00, nor THC or Sal dams, however dams did spend more time on nests over postnatal days. The same patterns were observed in frequency of passes (B), although this metric also highlighted the outlier behavior of one THC dam. Thin lines represent values for a single dam and thick lines represent values for the group.

through the center (5.8).

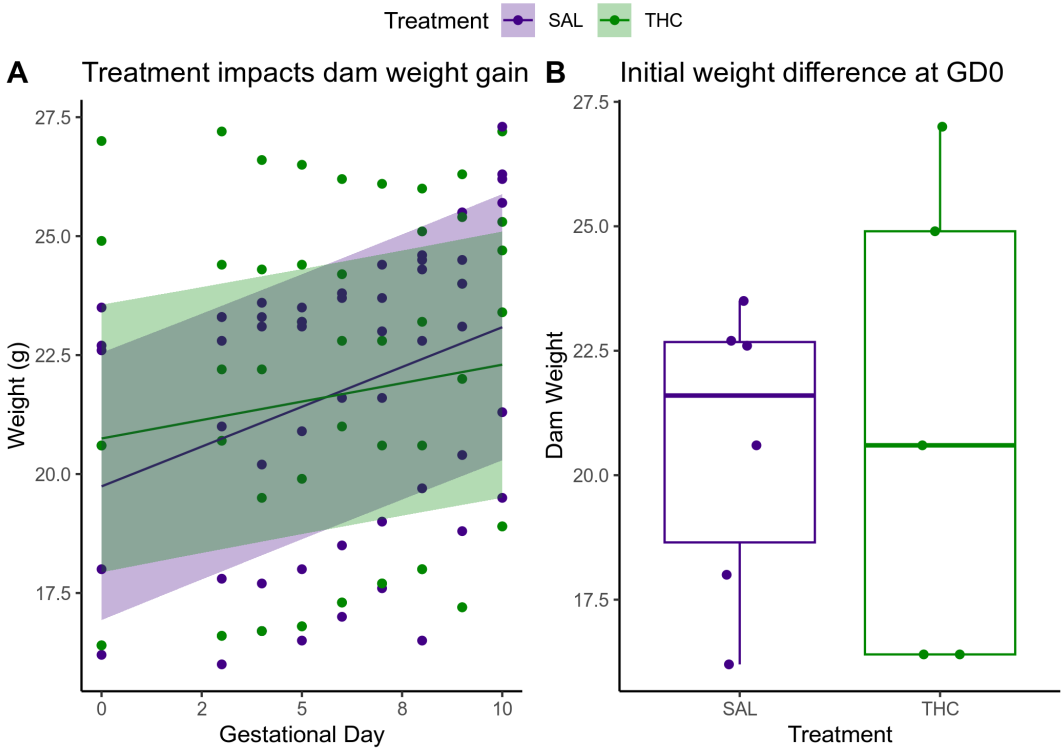

Figure 5.11: a) Trajectories of weight gain in THC and Sal dams. b) Initial weight before onset of treatment

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	19.74	1.42	10.38	13.94	0.00
treatmentTHC	1.01	2.00	10.41	0.50	0.63
GD	0.33	0.03	91.00	10.02	0.00
treatmentTHC:GD	-0.18	0.05	91.01	-3.72	0.00

Table 5.3: Results from the LMER examining the interaction of treatment and day on weight gain.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	8.4000	0.5000	16.80	0.0000
conditionTHC	-2.2000	0.7071	-3.11	0.0144

Table 5.4: Results from the LM examining the impact of THC on litter size.

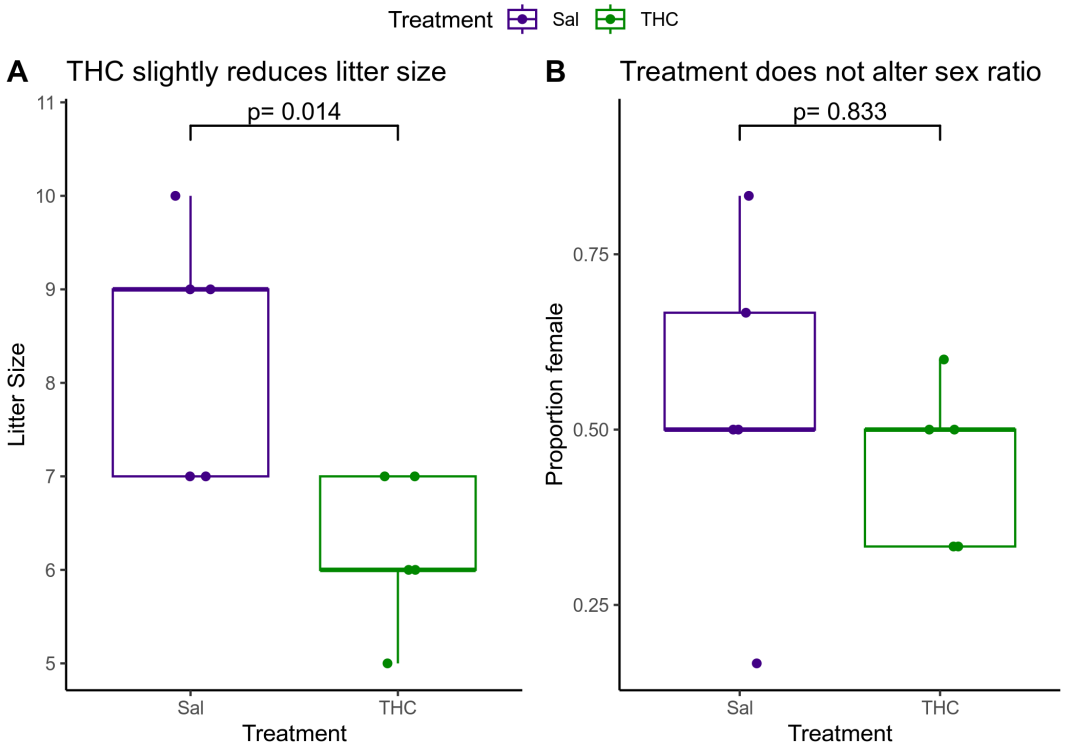

Figure 5.12: a) Number of pups before culling reduced in THC litters. b) No difference in sex ratio (proportion female)

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	16.48	0.30	24.69	55.20	0.00
treatmentTHC	-1.07	0.42	23.94	-2.58	0.02
poly(age, 2)1	73.84	2.63	332.16	28.03	0.00
poly(age, 2)2	-17.06	2.91	332.84	-5.86	0.00
sexmale	4.78	0.39	38.63	12.34	0.00
treatmentTHC:poly(age, 2)1	-4.95	3.67	331.78	-1.35	0.18
treatmentTHC:poly(age, 2)2	-1.06	4.15	333.89	-0.26	0.80
treatmentTHC:sexmale	0.26	0.55	37.97	0.47	0.64
poly(age, 2)1:sexmale	41.26	3.71	332.43	11.13	0.00
poly(age, 2)2:sexmale	-6.85	3.96	332.09	-1.73	0.08
treatmentTHC:poly(age, 2)1:sexmale	3.78	5.29	331.78	0.71	0.48
treatmentTHC:poly(age, 2)2:sexmale	-3.53	5.98	334.69	-0.59	0.55

Table 5.5: Results from the LMER examining the impact of THC on offspring weight gain.

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	5596.48	401.53	25.06	13.94	0.00
treatmentTHC	-1442.81	550.21	22.98	-2.62	0.02
sexmale	-1018.64	497.21	35.39	-2.05	0.05
treatmentTHC:sexmale	1593.93	695.51	34.99	2.29	0.03

Table 5.6: Results from the LMER examining the impact of THC and sex on total distance moved in the OFT.

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	5596.48	401.53	25.06	13.94	0.00
treatmentTHC	-1442.81	550.21	22.98	-2.62	0.02
sexmale	-1018.64	497.21	35.39	-2.05	0.05
treatmentTHC:sexmale	1593.93	695.51	34.99	2.29	0.03

Table 5.7: Results from the LMER examining the impact of THC and sex on time spent in the center of the open field.

	Estimate	Std. Error	df	t value	Pr(>|t|)
(Intercept)	58.33	5.74	23.59	10.16	0.00
treatmentTHC	-23.61	7.89	21.66	-2.99	0.01
sexmale	-11.26	6.89	35.14	-1.63	0.11
treatmentTHC:sexmale	22.95	9.63	34.76	2.38	0.02

Table 5.8: Results from the LMER examining the impact of THC and sex on frequency of passes through the the center of the open field.

Chapter 6

Overall Discussion

6.1 Summary of results

Recent years have brought increased attention to the urgency of establishing the lifetime consequences of prenatal cannabis exposure (PCE) and prenatal THC exposure (PTE). Despite clinical and preclinical studies that highlight potential impacts on behavior and specific brain processes, relatively little is known about the impact of PTE on trajectories of whole brain development from the fetus to adulthood.

Chapter 3 presents an investigation of the impact of prenatal exposure to 5 mg/kg Δ^9-tetrahydrocannabinol (THC) injected into the dam subcutaneously from gestational day (GD) 3-10 on brain and body volume of embryos extracted and imaged ex-vivo with magnetic resonance imaging (MRI) at GD 17. At the level of the dam, we observed THC exposure reduced weight gain during early gestation, but did not impact the litter size or ratio of female pups. In the embryos, we identified slight intrauterine growth restriction (IUGR) associated with injections (Sal compared to non-injected controls), but further IUGR comparing THC and saline (Sal) offspring, consistent with prior literature. Building on prior studies, we found ventriculomegaly in THC offspring, as well as larger volume in regions rich in Cannabinoid- $(CB)_1$ receptors (such as the caudate putamen) and white matter regions such as the corpus callosum. In contrast there were smaller volumes in regions such as the cerebellum and mid-brain. In an exploratory organ analyses, we found smaller volume in regions of the liver and bidirectional effects in the lungs, along with some potential sex-differences that were not present at the level of the brain. This experiment not only provided further confirmation of the IUGR effect following specifically PTE early in gestation, but also expanded known results, highlighting regions of interest that may be first

impacted following PTE.

The initial characterization established in **Chapter 3** was extended in **Chapter 4** into a longitudinal, in-vivo examination of brain volume trajectories following PTE at an identical dosage and route of administration. Additionally, we tracked the impact on weight gain and behavior in pups. Again, at the level of the dam we found evidence of decreased weight gain in THC dams during early gestation, without changes in litter size or number of females. Over the course of the experiment, there was evidence of catch-up growth in the THC pups, which displayed an increased growth-rate compared to Sal controls. This catch-up growth has been reported following other models of IUGR, but is rarely examined in the PCE literature. In contrast, scanning pups did not seem to impact weight gain. At the level of the brain, many regions that initially showed alterations in embryos showed decreased growth rates post-natally, including the caudate putamen, corpus callosum, and cerebellum, with additional regions, such as the hippocampus emerging with a decreased growth rate. Finally, there was evidence for sex-dependent effects of THC on social behavior in pups, with female THC pups calling less than Sal controls, but male THC pups calling more than Sal controls. The results from this study highlight the persistent metabolic impact of PTE and indicate that postnatal development is impaired in critical early stages of development, not only at the level of brain volume, but also in social communication. It highlights the emergence of sex effects in behavior, that seem not to reach significance in the brain.

Early alterations in trajectories of development reported in **Chapter 4** examined through adolescence and adulthood in **Chapter 5**. At the level of the dam, we not only examined the result of PTE on weight gain (decreased weight gain), litter size (decrease in THC litters) and proportion of female pups (no change), but also on maternal behavior, finding no difference in nest building before or after injections or in postnatal time spent on nest. These results suggest any alterations reported are unlikely to be due to changes in maternal behavior. Alterations in weight of THC offspring persist, with reduced weight present in both females and males across the experiment. There are also reductions in brain volume in many similar regions such as the striatum, thalamus, and hippocampus, but, interestingly, this volume reduction seems driven by females, is present by postnatal day (PND) 25, and persists into adulthood. Reduced weight and brain volume is also accompanied by alterations in behavior during adolescence - specifically anxiety-like behavior in females and trending impairments in sensorimotor gating. These results strongly suggest lasting alterations in metabolism, brain volume, and behavior following early gestational PTE. Of great importance, they illuminate the emergence of sex differences around puberty that do

not seem present earlier in life.

In summary, this thesis presents a thorough characterization of changes following PTE to neurodevelopmental trajectories from in utero to adulthood, captured by brain volume. In doing so, we draw attention to regions that may be most impacted by PTE, such as the basal ganglia, subcortical regions like the thalamus, and regions rich in CB_1 receptors, like the cerebellum and hippocampus. Further, we highlight windows in which different regions may show alterations (the ventricles in early life and the hippocampus post-natally), as well as different timecourses when sex-differences may emerge in different domains (organs in embryos, behavior in neonates, and brain volume during adolescence). By examining the impact of PTE on maternal behavior, we draw further attention to the direct impact of THC on the fetus (or placenta), and we highlight the importance of considering models of IUGR in interpreting results following PTE. These findings set the stage for future investigations into cellular and molecular mechanisms, the impact of PTE on various behavioral domains as well as metabolism and feeding behavior, and increased attention to properly examining and modelling sex differences in PTE or PCE studies.

6.2 Consistently affected regions

Over the course of the experiments, several regions were identified as being consistently affected. Importantly, we did not use a consistent atlas in identifying regions across the ages as brain regions change drastically between GD 17.5 and PND 90. Nevertheless, regions that showed volume differences across all ages included the caudate putamen, thalamus, olfactory bulbs, corpus callosum, amygdala, medulla, and hypothalamus. The ventricles were altered in the first two experiments (early life) and the hippocampus was altered in the last two experiments (postnatal life).

Of these regions, some have been demonstrated to be rich in CB_1 receptors including the basal ganglia, cerebellum, and hippocampus [Ong and Mackie, 1999]. These regions may be more obvious targets for studies examining PCE due to the overt presence of the endocannabinoid system (ECS). In contrast, other affected regions have relatively low levels of CB_1 receptors, such as the thalamus, hypothalamus, and medulla [Miederer et al., 2020]. Changes in volume in these regions suggest possible down-stream effects, rather than alterations directly resulting from THC's activation of CB_1 receptors. This is unsurprising given the complex roles and mechanisms of action of the ECS on brain organization, development, and homeostasis, which are

still being investigated today.

It is also worth noting that some regions, such as the thalamus, may be CB_1 receptor poor, but are known to be closely connected to other regions rich in CB_1 receptors, such as the cortex, substantia nigra, and, thereby, caudate putamen through the cortical-basal ganglia-thalamic network [Foster et al., 2021]. The ventricles must also be affected by mechanisms beyond direct activation of CB_1 receptors. While the ventricles are often neglected in studies of the developing brain, they provide key regions in the birth of new neurons, and it is possible that the ventriculomegaly is also surrounded by increased volume in paraventricular regions in the embryos. It is also of interest that the ventricles are one of few regions that show increased growth rate in the relative Jacobians of THC neonates compared to controls, potentially indicating persistent ventriculomegaly in postnatal life.

Finally, initially large volume of the corpus callosum of THC-exposed embryos, followed by a decreased growth rate in neonates is of interest because it is consistent with evidence suggesting the role of increased endocannabinoids in miswiring the brain [Alpár et al., 2014]. While this thesis does not offer evidence into the mechanisms underlying volume change, the future direction below (6.7.1) proposes several lines of investigation that could seek to address this very question.

6.3 Limitations

Many different models of both PCE and PTE have been used in the laboratory, varying on administered substances, routes of administration, dosages, and timelines. There are notable benefits of this model including precise dosage control, low overhead, and reduced stress for injections. Further, observed effects following the exposure can be attributable to THC rather than other substances, serving to disentangle the various effects. Nevertheless, there are limitations that must be considered as well.

First of all, the model has low construct validity, in the sense that few, if any, people inject cannabis [Russell et al., 2018]. Oral or vaporized administration doubtlessly provide advantages in comparison when considering how the route of administration mimics human use. Oral exposure, however, represents a very different mechanism of action than inhaled cannabis, as bioavailability and metabolism of THC differ between these routes of administration [Heuberger et al., 2015]. As vaporization chambers become increasingly available for preclinical cannabis studies, comparisons have been made between inhaled and injected cannabinoid exposure before birth, suggesting

exposure following injected THC may be higher in the fetal brain (roughly equivalent to that in the maternal blood) than that following inhaled cannabis (1/3 of that in the maternal blood) [Baglot et al., 2021]. These results suggest the dosage of fetal-exposure may be higher than that regularly observed in humans, although to our knowledge, no study has specifically estimated THC exposure during pregnancy. Given the potential of increased THC exposure in the fetus following injections, as well as the fact that 5 mg/kg already represents a high dosage of exposure, as discussed in Section 3.7.1.2, the present studies may reflect an extreme exposure to THC during pregnancy, and future research could examine whether the results remain true for reduced dosages.

Second, the timing of exposure presents is both advantageous and a limitation. Unlike many previous studies which continue exposure to cannabinoids throughout pregnancy [Rubio et al., 1995, Drazanova et al., 2019, Gillies et al., 2020, Moreno et al., 2005, del Arco et al., 2000, Roberts et al., 2022, Navarro et al., 1994], we chose to limit the dosage to a timepoint roughly equivalent to the first trimester of human pregnancy. We made this decision for two reasons: first, this is the period of highest observed cannabis use in humans either to alleviate morning sickness, or because pregnancy is unknown [Volkow et al., 2019, Moore et al., 2010]. Second, limiting the window of exposure allows us to interpret results in relation to a specific window of development since in our model the THC exposure is early (GD 3-10), primarily underlying neurogenesis in the developing mouse brain [Zeiss, 2021]. Nevertheless, it is still possible that THC exposure during this time period impacts other aspects of the developing ECS, which can cause down-stream changes. A drawback to this window of exposure, however, is that it is very unlikely pregnant people would suddenly start using cannabis. A more realistic model might begin exposure before conception, as has been modelled in the macaque [Roberts et al., 2022], however in a mouse model this could lead to significant waste if, for example, a dam was exposed to THC for several days, timed mating was attempted for one night, but unsuccessful and the dam could not be used again.

Third, in this approach we specifically examined the impact of PTE, however cannabis is a complex substance containing many phytocannabinoids (in addition to cannabidiol (CBD)) and terpenes. As discussed in the Background, Section 2.2.1, these constituent components likely have entourage effects which might impact the mechanisms of action or consequences of exposure. As levels of THC in available and regularly-used cannabis are increasing [Young-Wolff et al., 2019], it is essential to understand the impact of prenatal exposure to this substance, however it must be

acknowledged that results may differ if exposure included CBD and other cannabinoids. One study in adult cannabis users collected donated joints which participants prepared to reflect their use habits and then analyzed the THC content in the joints [Casajuana Kögel et al., 2017]; a similar study in pregnant people could greatly inform laboratory studies seeking to closely mimic patterns of actual cannabis use in humans, although significant care would have to be taken on the part of the researchers to ensure participants are comfortable presenting them with accurate data without fear of stigmatization.

In addition to limitations surrounding the cannabinoid exposure itself, there are further limitations to investigating an animal model. While a similar causative study would be entirely unethical in humans, animal models can never recapitulate the full range of human experience and or possible symptoms. For example, evidence suggests that humans with PCE experience increased self-reported, sub-threshold psychotic symptoms [Day et al., 2015], which cannot be tested for in mice. Some recently developed methods purport to examine hallucination-like perceptions in rodents [Schmack et al., 2021], however this paradigm requires extensive training, making it untenable in an adolescent timepoint or in the middle of a longitudinal experiment. Further, animal studies are unable to accurately capture the potential additive effects of early-life consequences. For example, PCE has been associated with decreased academic performance early in childhood [Goldschmidt et al., 2004], which could further contribute to early-life challenges and disadvantages to PCE youth, potentially isolating them from peers or increasing other symptoms, like anxiety. In a mouse model, impairments in cognition early in childhood, may not adversely effect development in other domains to a similar extent.

Another limitation of using mice as a model for a gestational exposure is that stages of brain development *in utero* and after birth differ between mice and humans. Studies aligning neurodevelopmental milestones across species place birth in mice roughly at the beginning of the human third trimester [Gumusoglu and Stevens, 2019]. In contrast, rabbit models more closely resemble the timecourse of human gestation in terms of embryo and feto-placental development [Fischer et al., 2012]. Some neural processes, such as myelination and synaptogenesis, begin well before birth in the humans but partially or fully postnatally in the mouse. This lack of alignment between major neurobioligical milestones poses considerable limitations in translating findings [Zeiss, 2021]. Nonetheless, it has been suggested that relying on neurobiological milestones during development rather than chronological age may allow for better cross-species comparisons [Semple et al., 2013, Guma et al., 2019].

Finally, while structural MRI presents a very powerful method of establishing developmental growth trajectories in mice, there are certain disadvantages to the approach. In longitudinal *in vivo* imaging, it is impossible to entirely disambiguate the impact of repeated exposure to the scanning process and age. Not only is scanning stressful to mice, but they are also exposed to anaesthetics like isoflurane which have been demonstrated to impact animal behavior as previously discussed in Section 2.7.2. Finally, in our work the neonates are exposed not only to stress and isoflurane, but also manganese chloride 2 (MnCl) as a contrast agent. While the control and experimental groups are equally exposed, a case could be made for this PTE model representing a multi-hit model where the THC is paired with early life stress or postnatal compounds as well. In general the interaction effects between exposures necessary to conduct the experiment and the treatment effect itself requires more study.

6.4 The need for standardization in reporting PCE methods

While the PCE literature is still relatively new, recent decades have seen the number of publications about PCE skyrocket, as evidenced in a PubMed search, Fig. 6.1.

Studies conducted by different laboratories and researchers vary in many factors, including species tested, substance, preparation of substance, route of administration, dosage, timing of administration, housing conditions of animals, methods of assessment, timing of assessment, sample size, and statistical analyses. One example of variability is whether THC, purchased in ethanol, is dehydrated before combining with the vehicle, as we and others undertake [Rubio et al., 1995, Navarro et al., 1994], or whether it is combined directly in ethanol, as has been reported in other studies [Baglot et al., 2021]. This variability can improve the literature by establishing the robustness of certain results (e.g. is anxiety-like behavior reported regardless of administration route?), however it can also make it extremely challenging to synthesize findings across the literature. In recent years, experts in the field of maternal immune activation (MIA) collaborated to design and publish reporting guidelines for studies investigating MIA in order to improve the rigor, reproducibility, and transparency of studies [Kentner et al., 2019]. As part of these guidelines, the authors also published a downloadable checklist that can be published alongside any papers reporting results of this model to increase transparency of some methods that are not always published

(such as the ventilation type of the home cage). As PCE models increase in use, it could be possible to adopt a similar reporting style in order to improve the transparency and reproducibility of our results and ease interpretation across studies. As an example, an adapted reporting table (edited to replace maternal infection with cannabis exposure), as that from [Kentner et al., 2019], can be found below: Table 6.4.

Prenatal cannabis exposure

~~Maternal Immune Activation~~ Model Reporting Guidelines Checklist

PCE

ARRIVE Reporting Guideline & Recommendation	Arrive Item	~~MIA~~ Model Specific Reporting Recommendation *Please complete this chart for each point outlined below. If not applicable, write N/A*
Study design cannabis exposure ➢Overview of ~~immune activation~~ issues For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimize the effects of subjective bias when allocating animals to treatment (e.g. randomization procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	PCE 6	~~MIA~~ Specific Reporting: PCE a. General need for improved reporting in ~~MIA~~ model methods + reporting pilot data ○ Details on pilot data: 1. PTE in embryos a. > 19 embryos per group, including THC, Sal, and Null, half female, half male b. dams randomized per group. experimentors blinded during scanning, QC, analysis c. animal as experimental unit, controlling for dam 2. PTE in neonates a. > 25 neonates per group, THC or Sal, half female, half male b. dams randomized per group. experimentors blinded during scanning, QC, analysis c. animal as experimental unit, controlling for dam 3. PTE in later-life a. > 23 animals per group, THC or Sal, half female, half male b. dams randomized per group. experimentors blinded during scanning, QC, analysis c. animal as experimental unit, controlling for dam
Experimental procedures ➢Compounds ➢Validation measures For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	7	Provide details of: a. Compounds – source, vehicle, preparation/storage, administration route, volume administered, whether anesthetics were used at time of ~~immune challenge~~. cannabis exposure ○ Name of compound: Delta-9-THC ○ Catalogue number: Cayman Chemicals, Q453003 ○ Lot number: NA ○ Vehicle control used: 1:18 kremophor:Saline ○ Route of administration: S.C. injections ○ Volume administered: 0.1 ml/g ○ Storage conditions: frozen ○ Anesthetic (type, dose, duration) used: isoflurane in experiment 2 and isoflurane + dexmedetomidine in exp 3. b. Housing variables at injection - temperature of room at injection time, cage change at time of injection or not ○ Light cycle of animal housing room: 8a-8p light, 8p-8a dark ○ Time of day of injection: 10-12am ○ Room temperature at injection time: 22 c ○ Did a cage change occur at time of injection: NO

cannabis exposure

		c. Validation of ~~immune activation~~ – behavior, physiological indices and/or cytokine data, including pilot dosing data cannabis exposure ○ Method used to verify ~~immune activation~~: Validation of dosages performed in previous experiments (Guma, Cupo et al., 2023). THC and metabolites examined in blood 1 hour following injection. d. Validation of gestational timing – vaginal plug, estrous cycle, weight gain ○ Method of validating gestational timing: Presence of vaginal plug the morning following male and female pairing. Additional comments:
Experimental animals ➢Species/strain/vendor a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	8	Provide details of: a. Species – considerations for appropriate species (mouse, rat, non human primate, other) ○ Species: mouse b. Strain – variability in strain can influence model ○ Strain: C57/BL6J cannabis exposure c. Maternal/Offspring Physiological Variables at time of ~~immune challenge~~ – age, body weight ○ Maternal Age at challenge: 8-12 weeks of age ○ Maternal Body weight: ○ Offspring Age at challenge: GD 3-10 ○ Offspring Sex: Both males and females tested ○ Offspring Body weight: d. Vendor – even within the same strain, vendor can influence endpoints ○ Vendor: Parents of dams/males purchased from Jackson Laboratories ○ Location of Vendor: Canada ○ Room/area where animals originated from: NA

		Additional Comments: C57BL6/J mice were ordered from the Jackson Laboratory to serve as " grandparent" breeders. All experimental animals (including parents) were bred in house to ensure that they were exposed to the same environment throughout development.
Housing and husbandry ➢Cage, ventilation, bedding, enrichment Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding program, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	9	Provide details of: a. Caging systems ○ *At breeding* Material of cage: Sawdust bedding plus nesting material Cage dimensions: 8.75 x 12.125 x 6.395 (Inches) 22.2 x 30.80 x 16.2 ○ *After parturition* Material of cage: Sawdust bedding plus nesting material Cage dimensions: 8.75 x 12.125 x 6.395 (Inches) 22.2 x 30.80 x 16.2 ○ *At weaning* Material of cage: Sawdust bedding plus nesting material (Cage dimensions: 8.75 x 12.125 x 6.395 (Inches) 22.2 x 30.80 x 16.2 b. Animal Holding room ○ Temperature in room: 22 C ○ Humidity in room: 50% ○ Ventilation system: Ventilated cages ○ Specific pathogen free [SPF]: NO ○ Are males & females housed in the same or separate rooms: Housed in same room after weaning c. Bedding exchanges/bedding type ○ Type of cage bedding used: corncob ○ Frequency of cage changes per week *during gestation:* 1 *during neonatal period:* 1 *following weaning:* 1 d. Breeding - bred on site or timed pregnant, how many different sires (are the same fathers breeding with both experimental and control dams) Breeding location: In house at Douglas Animal Facility

		○ Gestational age at shipping: NA ○ Biological age of dams (if not listed in Section 8c): 8-12 weeks ○ Number of Dams bred: 46 ○ How many times have dams been mated previously: 0 ○ How many times did the dams mate and not become pregnant: ~33 ○ Are the dams primiparous or multiparous? All dams are primiparous ○ What was the frequency of maternal handling during the gestational/neonatal period (e.g. cage cleanings, weighing, blood collection manipulations): 8-16 (experiment dependent) ○ Biological age of sires: 8-16 weeks ○ Number of sires bred: ~30 ○ How many times have sires been mated previously: <2 ○ How many times did the sires mate successfully (e.g. mating resulted in pregnancy, full term birth): 30% ○ If bred previously, what was the interval between mating times: ○ Are sires matched to experimental and control dams: YES ○ Describe the mating design (1:1, 1:2 etc): 1:1 e. Social enrichment – number of cage companions ○ Number of cage companions prior to breeding: 3-4 ○ Gestational age when dam separated for parturition: GD0 ○ Number of cage companions at weaning: 2-4 f. Physical enrichment – describe enrichment devices, and when enrichment is in the cage (removed when pups born? Or present throughout study), does the enrichment type change? How frequently? ○ Describe what type of enrichment devices (and how many) are included in cage/housing room: Nesting material including shredded brown paper and nestlets.

		○ Does enrichment type/access change across study? YES ○ If so, when does enrichment type/access change (e.g. enrichment removed prior to parturition and replaced in late neonatal period): It did not change excpet in dams where nest-building was assessed. For 2 nights (GD 2-3) and GD 10-11, these dams nesting materials were removed and replaced with 3g of Nestlet material only. The next morning paper bedding was added back in. Additional Comments:
Sample size ➢Litter versus offspring a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	10	Provide details of: a. Maternal N vs offspring N ○ What is the total number of dams/litters included in the study: 46 ○ What is the total number of offspring per litter included the study: 4 b. Litter size and sex distribution ○ What size was each litter maintained at: 6 ○ What age did culling take place at: P1 ○ How many males and females were maintained in each litter: where possible, 3 males and 3 females c. Cross fostering ○ Did cross fostering occur: NO ○ If so, at what age did cross fostering occur: Additional Comments:

Allocating animals to experimental groups a. Give full details of how animals were allocated to experimental groups, including randomization or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	11	a. How many offspring per litter were used in each measure: 2 males and 2 females b. Randomization/Matching procedures ○ What procedures were used to assign animals to groups: Dams were randomly assigned to condition. c. Sex as a biological variable (behavioral and physiological outcomes) ○ Were both males and females evaluated in each behavioral and physiological outcome: YES Additional Comments:
Experimental outcomes ➢Behavioral testing ➢Physiological endpoints Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioral changes).	12	a. Maternal behavior and pup interactions ○ If maternal care was evaluated, were there differences following cannabis exposure ~~immunogen challenge~~ (if so, please briefly describe): No differences were observed in nest building or maternal time on nest. b. Age(s) of offspring at behavioral testing/physiological evaluation endpoints: GD 17.5, PND 3, 5, 7, 10, 12, 25, 35, 60, 90. c. Order of testing (e.g. behavioral test order) ○ Were animals evaluated in a counter-balanced order in terms of: *presentation of tests to each animal:* NO *order of experimental/control groups run through each test:* YE ○ What was the inter-test interval if a single animal underwent a battery of tests: 2 days

		Additional Comments:
Statistical methods a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	13	a. Unit of analysis for each data set ○ Is the unit (n) of each analysis based on number of litters, or number of animals used per group: Using linear mixed effects analyses, we examined pups controlling for inter-subject change (random effect of subject) and inter-litter difference (random effect of litter).
Other Disclosures		Please make note of any other extraneous variables that you would like to report (e.g. fire alarms, construction, temporary relocations, other variables that you think we should be considering in our studies etc.):

The recommended use of this reporting form is to fill it out and include it as supplemental material for each of your laboratory's research publications. If there are difficulties utilizing/adapting this fillable form, please contact one of the corresponding authors to request a copy. The authors give permission for this table to be edited for use in reporting on other animal models (e.g. postnatal immune challenge models, early life stress models) as appropriate.

Kentner AC, Bilbo AD, Brown AS, Hsiao EY, McAllister AK, Meyer U, Pearce BD, Pletnikov MV, Yolken RH, Bauman MD. (2018). Maternal immune activation: reporting guidelines to improve the rigor, reproducibility, and transparency of the model. Neuropsychopharmacology, https://doi.org/10.1038/s41386-018-0185-7.

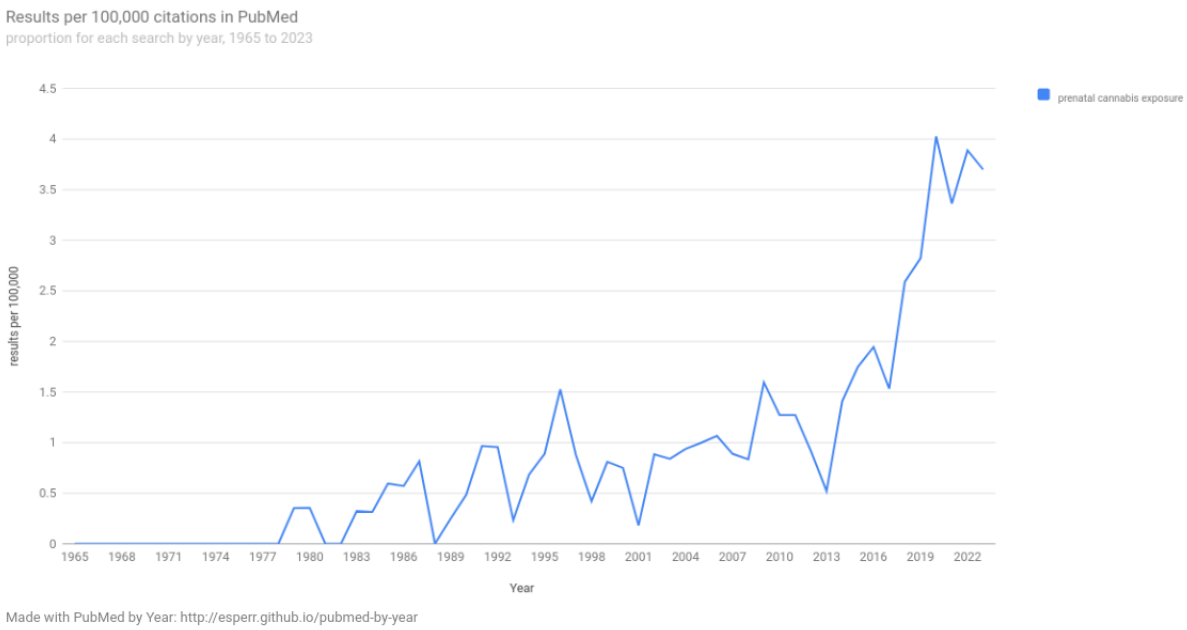

Figure 6.1: Graph created with PubMed by Year for the search term "prenatal cannabis exposure" in November, 2023

6.5 Translation from clinic to the laboratory and back again

While not unique to the study of PCE, the need for strengthened communication among researchers, medical professionals, policy makers, and the public is critical in this field. Cannabis legalization may increase the quality of available data, government oversight, and access to care for users, however preliminary data from regions with legalized cannabis also suggest an increase in use among adults (but potentially not youth), reduction of the price, and increase in potency [Hall and Lynskey, 2020]. There is significant evidence that pregnant people want more concrete information about the potential harms of cannabis use during pregnancy [Young-Wolff et al., 2020]. While national guidelines in the United States and Canada suggest pregnant people should refrain from cannabis use, both health-care providers and budtenders (cannabis store retailers) are consulted for information regarding perinatal cannabis use, and there is evidence that they perceive an insufficient body of knowledge and training to provide counseling [Barbosa-Leiker et al., 2022]. Together these evidence highlight the need for increased lines of communication between laboratory studies, where evidence about the

potential harms of PTE are accumulating, and health care providers, budtenders, and the general public. While care must be taken to ensure results are not over-generalizaed and are properly contetualized, as with any science communication, there are several ways that connections could be forged among these groups. Lay abstracts could be published alongside papers, openly accessible to the public. Increased contact could also be established between laboratory researchers and health care providers/budtenders in the form of information sessions or discussion groups. Finally, public forums and conferences could be arranged to increase the accessibility of scientists to the general public.

Importantly, the benefit of open lines of communication is not unidirectional; laboratory researchers have as much to learn from health care providers and social science researchers as vice versa. For example, the more information experimental scientists have about patterns of human cannabis use, the better they can design experiments that reflect real world use cases with results that could better generalize to the public and inform clinicians. As highlighted above in Section 6.3, one way laboratory experiments would improve from population studies would be a more accurate representation of the dosages of cannabinoids pregnant people are exposed to. While there is evidence that THC potency in cannabis is increasing in general [Young-Wolff et al., 2019], it is possible that pregnant people who are using cannabis with knowledge of their pregnancy are selecting CBD-rich preparations, perceiving the effects as more beneficial or the cannabinoid as "safer" than THC-potent preparations [Spinella et al., 2023]. Only studies in real-world populations will afford a better understanding of what questions are the most pressing in the field of PCE for laboratory researchers to experimentally model.

One critical finding emergent from studies that investigate the motivation of cannabis use in pregnant people is that many take cannabis for therapeutic reasons and because they think it is more natural and therefore safer than prescription pills [Barbosa-Leiker et al., 2022]. For example, pregnant people suffering from anxiety have reported concern about pharmocological interventions and low willingness to take prescription drugs during pregnancy [Lemon et al., 2020]. These concerns need to be carefully considered, not dismissed as ignorance, especially as there are some studies of prenatal exposure to pharmacotherapies such as serotonin reuptake inhibitors (SRI)s on fetal development that suggest adverse fetal and neonatal events [Creeley and Denton, 2019]. Much of this literature, however, suffers from similar issues as the PCE literature, such that few studies follow the early life of prenatally exposed children longitudinally. Further research is certainly necessary to further

establish comprehensive guidelines for the prescription of pharmaceuticals during gestation and the proper communication of potential risks to pregnant patients. It must also be communicated that just because cannabis is a natural substance does not mean it is inherently safer than the alternatives. Simultaneously, the needs of the pregnant person cannot be disregarded either. First of all, the pregnant person is a patient deserving of state-of-the-art care, necessitating effective strategies to ameliorate the symptoms of mental health disorders such as anxiety and depression. Second, maternal anxiety in-and-of-itself has been demonstrated to impact pregnancy and child development [Correia and Linhares, 2007]. There is some evidence that cognitive behavioral therapy and interpersonal psychotherapy may afford significant benefit to pregnant patients with psychiatric disorders and may be as effective as SRIs, without negatively impacting child development, however further research is required [Nillni et al., 2018]. Pairing such psychotherapies with information on potential consequences of prenatal cannabis exposure is critical for pregnant people who are unwilling to treat their symptoms with pharmaceuticals while pregnant.

6.6 The need for more CBD research

As mentioned in the last section, CBD has been widely accepted as communicating therapeutic benefits without the harmful consequences of THC. CBD has been investigated as a potential treatment for epilepsy, substance abuse and dependence, schizophrenia, social phobia, post-traumatic stress, depression, bipolar disorder, sleep disorders, and Parkinson's disorder [Crippa et al., 2018]. In part because of the attention on therapeutic effects of CBD exposure, "light" cannabis, with a high CBD:THC ratio is largely seen as harmless [Gabaglio et al., 2021]. Recent studies following adolescent exposure to either pure THC, potent cannabis with a high THC:CBD ratio, or "light" cannabis in female rats demonstrated that comparing the potent cannabis to the pure THC groups, the inclusion of CBD did somewhat reduce negative behavioral effects, however there were also long-term negative effects of chronic exposure to the "light" cannabis, with impairments to short-term memory and increased anhedonia [Gabaglio et al., 2021]. While this study highlights the need for further recent into "light" cannabis and chronic CBD exposure. Recently, several studies have undertaken to examine the effects of prenatal CBD exposure [Lunardi Baccetto, 2023, Iezzi et al., 2022]. Following prenatal exposure to CBD (3 mg/kg GD 5-18), sex-dependent effects were observed in pup ultrasonic vocalizations

(USVs), such that female CBD pups made more high-frequency calls than controls, but male CBD pups made fewer calls than controls [Iezzi et al., 2022]. The sex-dependent effect is present in this study, as it was in our neonates, however the direction of effects is opposite. In rats, exposure to very high dosages of CBD (5 mg/kg or 10 mg/kg) from GD 6-20 were associated with reduced body weight and a delay in the development of reflexes after birth [Lunardi Baccetto, 2023]. These results, while preliminary, suggest CBD may not be the safe alternative to THC that it is often considered, especially when administered prenatally, and highlights the desperate need for more studies considering the impact of CBD or different ratios of the two.

6.7 Future directions

6.7.1 Mechanisms underlying volume changes

One of the primary questions raised but not answered by this thesis is what cellular changes may underlie observed volume differences. Many different mechanisms can contribute to changes in volume detected with MRI. In gray matter regions, volume change could comprise changes in neurons (more neurons, increased dendritic arborization, or synapotogenesis), changes in glia (increased number of astrocytes or microglia, or shape changes in these cells), or changes in vasculature (such as angiogenesis) [Zatorre et al., 2012]. In white matter, changes in volume could reflect changes in glia (such as increased myelination from oligodendrocytes) [Zatorre et al., 2012], or, in early stages, axon misguidance. The lack of specificity of structural MRI affords some benefit, along with challenges in interpretation. Because it is nonspecific, this allows us to detect changes that may be related to multiple mechanisms, especially across the whole brain. For example, it is unlikely that identical mechanisms contribute to large volumes in the ventricles, but small volumes in the cerebellum, therefore these changes could be missed if an experiment was designed to look solely at increased cerebral spinal fluid (CSF) pressure (potentially contributing to ventriculomegaly) or decreased cerebellar neuronal proliferation (a potential mechanism contributing to small volumes in the cerebellum). Naturally, however, this nonspecificity also affords challenges in interpreting what it "means" that one region may have volume increase or decrease. While these questions open entirely new lines of research that were outside the scope of this thesis, there are many potential follow-up experiments that could begin to answer these important questions.

One approach that could mimic elements of the present design could involve devel-

oping and implementing forms of MRI that encode brain changes with higher specificity. For example, quantitative MRI can reveal alterations in brain microstructure correlated with changes in myelination and iron content. While work has been done to implement these imaging methodologies in small animals, it has mostly been conducted in postmortem samples, or longitudinally later in life, and further development of these scanning techniques for *in vivo* analyses early in life could expand the understanding of processes underlying volume changes [Cao et al., 2014, Argyridis et al., 2014, Thiessen et al., 2013].

Postmortem assessments are another way to gain specificity underlying volumetric information gained from MRI. These approaches obviously cannot harness longitudinal metrics either, however researchers can acquire samples from different animals at each age in the study. One approach that could preserve the 3-dimensional information beneficially derived from neuroimaging is clearing the tissue of the brain with an approach like CLARITY before staining for relevant cellular properties [Zhang et al., 2018]. 3D histological images have then been registered to *ex vivo* MRI images to examine how microstructural imaging properties relate to underlying cell nuclei, astrocytes, parvalbumin-expressing interneurons, and blood vessels [Stolp et al., 2018]. Such innovative, multi-modal techniques could be developed and applied to assay the developing brain, as well as the effect of various developmental insults such as PTE to provide further information what cellular changes may contribute to volume changes. Other techniques, such as spatial RNA-sequencing (RNAseq) can be applied to entire brain slices and registered to slices extracted from MRIs in order to unify gene expression and volumetric information and offer insights into potential transcriptomic differences underlying volumentric differences [Pak et al., 2023]. Notably, all of these techniques require intensive development and validation, but could provide invaluable information to increase our understanding of the impact of PTE.

This thesis also serves to generate some hypotheses about the potential mechanisms underlying long-term changes of PTE to brain and behavior. For example, evidence of increased volume in paraventricular regions of embryos could be consistent with *in situ* evidence suggestive of increase neuronal differentiation [Miranda et al., 2020]. Increased volume in the corpus callosum at this stage is also consistent with evidence that increased levels of 2-AG increases the fascicle spread of the corpus callosum in mice [Alpár et al., 2014]. Since midline fusion only occurs between GD 14 and 15 in mice , and axons only start crossing the midline at GD 15.5 [Richards et al., 2004], it is unlikely that the THC exposure, which ended at GD 10 in our study, contributed to axon misguidance, however it is possible that it caused downstream effects on the ECS

that contributed to increased neuronal proliferation and axon misguidance. Exposure to external cannabinoids is also associated with increased apoptosis and failure to flourish in neurons [Miranda et al., 2020]. It is possible that misguided axons fail to form synapses, which could contribute to their selective pruning in postnatal life and reduced integrity of connectivity between regions, as evidenced in human neonates [Grewen et al., 2015]. Many of the regions that demonstrate hypoconnectivity with fMRI in human neonates also show reduced volume in our model, so future studies could examine the connectivity profile of these regions following PTE with postmortem assessments or fMRI.

6.7.2 Disentangling PTE and IUGR; long-term metabolic consequences of PCE

Another direction for future examinations is the impact of PTE and PCE on metabolism and feeding behavior of offspring. One of the most robust findings in the PCE literature is the impact of PCE on fetal growth and birthweight, as has been discussed throughout this thesis. Of note, while some studies are designed to assess the impact of PCE on birth weight, very few that report behavioral or neurological consequences are able to control for or disentangle the potential impact of PCE and initial IUGR, which may be colinear. This is important because there are known cognitive and developmental effects of IUGR, such as reduced cognitive scores in school-age children [Chen et al., 2016]. This is also a finding that has been reported in children following PCE, however [Goldschmidt et al., 2004]. Early postnatal life catch-up growth, as observed in the THC group in our study, has also been associated with IUGR [van Wyk et al., 2013]. IUGR is also known to be a risk factor for altered adult metabolism and obesity however [Longo et al., 2013], while we observed reduced weight gain into adulthood. In the embryo study we have a measure of full body volume that allows us to account for potential IUGR in our model of brain volume using the mediation with residuals approach. In later studies, however, like many other studies not specifically designed to examine the impact of PCE on IUGR, we do not have an estimate of fetal volume and use current weight instead. There is some evidence of an impact of IUGR on adolescent brain volumes, with small for gestational age term infants displaying reduced volumes in the thalamus and cerebellar white matter volumes at age 15 [Rogne et al., 2015]. Interestingly, these results are in line with regions and directions where we see volume differences in **Chapter 5**, so it is possible that some of the effects could be attributable to IUGR resulting from PTE

rather than the direct effect of PTE. If PCE is always accompanied by IUGR in humans, it may be less pressing to differentiate the direct effects of the PCE compared to the indirect effects of IUGR, however it may be beneficial for clinicians to highlight the potentially worsened effects of PCE in fetuses and babies that show evidence for IUGR as well.

Beyond the initial IUGR there is growing evidence for the potential consequences of PCE on weight gain, feeding behavior, and metabolism. A recent preprint investigated the impact of prenatal exposure to vaporized THC in rats during the entire gestational period on adiposity, glucose metabolism, and feeding behavior of offspring [Hume et al., 2023]. The authors found when rats were exposed to either high fat or control diet PTE rat bodyweight gain was decreased potentially related to increased energy expenditures, such as increased locomotion, thermogenesis, or respiration [Hume et al., 2023]. Studies such as this can further uncover mechanisms altering persistant changes in weight and weight gain in PCE models, potentially assisting early detection of health consequences in the clinic.

6.7.3 Sex differences

While the PCE literature firmly indicates the presence and importance of sex differences [Fine et al., 2019, Porath and Fried, 2005, Bara et al., 2018, Manduca et al., 2020, Frau et al., 2019, Lallai et al., 2022, Benevenuto et al., 2017, Rubio et al., 1995, Rompala et al., 2021, Maciel et al., 2022, Vela et al., 1998, Corchero et al., 1998], and they are further supported in our work, there are many potential causes underlying sex differences that need to be investigated. Sex differences could emerge on different time courses across development, or in different domains. One sex could be more sensitive to an insult, such as PTE at a specific window of development. Sex hormones could be differentially affected, or could moderate the effect of PTE. The sexes may show opposite effects in a metric (e.g., female THC pups making fewer calls while male THC pups make more calls than controls), or the differences may only be in one group (e.g., female THC pups show increased anxiety-like behaviors, while males show no differences between groups). Such variability in the possible causes of discovering (or not discovering) sex differences can make generalization across studies a challenge and render it nearly impossible (and perhaps oversimplified past the point of utility) to declaim that one sex of the other is more sensitive to PCE. Instead, because sex has emerged as an important biological factor in this field, researchers, reviewers, and publishers should take care

that rigorous methodologies are employed to properly account for sex as a biological variable in humans and nonhuman animals.

Sex differences could emerge from several different factors, considering the ECS and THC exposure, or a combination of them. First, sex differences could arise from underlying differences in the ECS between the sexes. For example, males have higher levels of CB_1 receptors overall than females [Tirado-Muñoz et al., 2020]. Perhaps for this reason, chronic exposure to cannabinoids has been found to induce tolerance in females more quickly than males, reducing CB_1 expression [Tirado-Muñoz et al., 2020]. Pharmacokinetic factors may also alter the impact of the PTE, even in utero. The primary metabolite of THC in females is psychoactive 11-OH-THC, whereas in males it is more rapidly converted to other inactive metabolites [Tirado-Muñoz et al., 2020]. Finally, in the adolescent/adult experiment, the effects of gonadal hormones could impact normalization in males but not females. Specifically, there is evidence that estradiol and progesterone promote tolerance in females (reduced expression in CB_1 receptors), which could be related to why changes are sustained in females but not males [Tirado-Muñoz et al., 2020].

To properly account for the potential impact of sex or sex-dependent effects, studies should strive to attain several goals. First, they should always include both male and female animals in their studies regardless of if they think one sex will be more effected than others, as has been cited as a reason for excluding females in the PTE literature before [Tortoriello et al., 2014]. While many recent studies do examine both sexes in nonhuman animals, the field is still rife with studies that examine only males [Tortoriello et al., 2014, Drazanova et al., 2019, Antonelli et al., 2004, Mereu et al., 2003, Antonelli et al., 2005, del Arco et al., 2000, Trezza et al., 2008]. In general, rigorous experimental design should include subjects of both sexes. Second, while it is good practice to include both sexes in an experiment, sample size should also be increased to properly allow for assessments of sex differences. In 2020, it was noted that while the National Institutes of Health and Canadian Institute of Health Research began mandating inclusion of both sexes in funded studies, they do not increase the funding available [Galea et al., 2020], therefore researchers may be hesitant to increase their sample sizes. While the funding agencies maintain that sample size does not need to be doubled necessarily when both sexes are included [Government of Canada et al., 2018], compared to when only a single sex is included, it is often extremely difficult to accurately anticipate effect sizes of novel studies, making it nigh impossible to accurately assess how much a sample size needs to increase to be properly powered to examine effects of treatment and sex on the outcomes of

interest [Galea et al., 2020]. Third, the question of appropriate sample size is especially important in the decision of how to model sex in statistical analyses. While studies that *do* include subjects of both sexes frequently merely include sex as a covariate to control for the statistical effect of sex in the main effect of the intervention, rather than incorporating an optimal design for the discovery of sex effects, where sex is interacted with the treatment. Importantly, these interactions do require greater sample sizes than main effects [Galea et al., 2020, Cohen, 2013]. One review comparing sex differences examined in neuroscientific and psychiatric studies between 2009 and 2019 found an increase in studies that use both sexes from 38% to 68%, however barely noticeable increases in papers that use an "optimal design" (balanced groups across sexes) from 14% to 19%, or "optimal analyses" (interactions between sex and treatments) from 2% to 5% [Rechlin et al., 2022]. Of studies that include only a single sex, nine times as many look only at males than those that look only at females [Rechlin et al., 2022]. Reporting guidelines in the PCE literature could increase transparency regarding the sample size of both sexes and the analytic approaches to modelling sex.

6.7.4 Normative Modelling

Finally, our study presents a proof-of-concept for using normative models in C57/Bl6 mice to track growth curves and brain volume during development. The normative model confirms findings from the individual studies, identifying catch up growth and fall-off in brain volume and weight trajectories, however it also highlights certain findings, such as the catch-up growth mainly present in males and normalization of their weights and brain volume in adulthood. Future studies could build on the normative models in several key ways. First, increasing the sample size would improve the accuracy of estimates, especially at ages that are not represented in the current sample, such as between PND 13 and PND 25. Second, including samples from different laboratories and centers could improve the generalizability of the normative model, making it more relevant as a resource for other investigators. Third, rather than modelling brain volume as a single metric representing the total brain volume (TBV), which does not provide the greatest sensitivity, future studies could implement a common atlas across all ages, or even develop voxel-wise models, registering brains from the earliest to the latest timepoints in order to provide region or voxel-wise trajectories of development. Together these improvements could provide a rigorous and valuable asset to developmental studies in mice and the field of small animal MRI, allowing for comparison across models, scanning sequences, and laboratories.

6.7.5 Beyond this thesis

In time this work could contribute to a deeper understanding of how PCE in humans impacts the development of children and what interventions are effective at ameliorating any negative consequences, however certain experiments in both preclinical models and humans would be necessary to establish this knowledge. First of all, human studies implementing fetal and neonatal imaging would be useful to demonstrate parallels between the brain changes observed here and those found in humans, especially paired with markers of behavioral alterations in children early in life to understand which brain networks and systems are most impacted. The time course of emerging alterations may differ as well, since the early postnatal life in these models most resembles the third trimester of pregnancy. Second, informed by findings from the proposed experiments, early life animal models could again examine the efficacy in certain interventions in ameliorating changes to brain development and behavior. For example, one approach that has shown some promise in both rodent models of maternal immune activation (a risk factor for neurodevelopmental disorders) [Zhao et al., 2020] and early human studies of neurodevelopmental disorders, is environmental enrichment [Ball et al., 2019]. A therapeutic, non-pharmacological approach like this may also be neuroprotective in known cases of PCE, where outcomes are often fairly nonspecific – PCE has not been unequivocally associated as a risk factor for a certain disorder, but rather is a risk factor for worsened outcomes across a variety of domains. Once these interventions have been established in animal models, they could be employed in the clinic in randomized control trials to examine whether they afford protections for developing children.

Chapter 7

Main conclusions of thesis

Several consistent themes emerged across the three studies included in this thesis. First, irrespective of sex, PTE impacted weight across the lifespan, with decreased embryo volume in **Chapter 3**, catch-up growth in **Chapter 4**, and persistent decreased weight in **Chapter 5**. Second, brain volume was impacted by PTE across the ages. Many regions showed larger volumes in embryos exposed to THC in **Chapter 3**, however many of the same regions showed a reduction of growth rate between GD 3 and 10 in **Chapter 4**. Interestingly, while there were regions that showed reduced brain volume, especially in females between adolescence and adulthood in **Chapter 5**, there was no age by treatment interaction, implying most changes appeared before PND 25. Sex-dependent effects in behavior were discovered in sociability early in life in **Chapter 4** and anxiety-like behavior in **Chapter 5**. Because very few changes were seen at the level of the dam in litter size, sex-ratio of pups, or behavior (in **Chapter 5**) it is unlikely observed effects are due to maternal behavior, however there was persistent evidence for decreased weight gain in THC-exposed dams, of note for future experiments and clinical observation. The original work presented in this thesis characterizes the impact of a high dosage of PTE early in gestation on whole brain and body development from embryonic stages to adulthood. It contextualizes volumetric changes with alterations to body growth and behavior, providing further evidence for certain observed outcomes while also generating hypotheses and contributing technical developments for future studies, both in this field and in other investiations of environmental effects in the prenatal period. The results can inform, not only future PTE studies in nonhuman animals, but also investigation of PCE in humans, contributing to the general knowledge of consequences of PTE exposure across the lifespan.

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